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Endothelial progenitor cell levels in obese men with the metabolic syndrome and the effect of simvastatin monotherapy vs. simvastatin/ezetimibe combination therapy†

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Aims Endothelial progenitor cells (EPCs) contribute to endothelial regeneration and thereby protect against cardiovascular disease (CVD). Patients with manifest CVD have reduced EPC levels, but it is not clear if this also occurs in subjects at high CVD risk without manifest atherosclerotic disease. Therefore, we aimed to first, measure circulating levels of EPCs in subjects without manifest CVD but at high cardiovascular risk due to obesity and presence of the metabolic syndrome. Second, we evaluated the effect on EPC levels of two lipid-lowering treatments.

Methods and results Circulating CD34+KDR+ EPC levels were reduced by nearly 40% in obese men with the metabolic syndrome compared to non-obese healthy controls (331 ± 193 vs. 543 ± 164 EPC/mL, $P = 0.006$). In a randomized double-blind cross-over study comparing intensive lipid-lowering treatment using 80 mg simvastatin mono-treatment with combination treatment of 10 mg simvastatin and 10 mg ezetimibe, we found a similar treatment effect on EPC levels. Secondary analyses of these data suggested that both treatment regimens had increased circulating EPCs to control levels (626 ± 428 after combination treatment, $P < 0.01$; 524 ± 372 EPC/mL after monotherapy, $P < 0.05$). Serum levels of EPC-mobilizing factor SCF-sR correlated with reduced EPC levels and normalized concurrently with treatment.

Conclusion EPC levels are reduced in apparently healthy men with abdominal obesity and the metabolic syndrome, even in the absence of manifest CVD. This is important as EPCs contribute to endothelial regeneration and thereby protect against CVD. SCF-sR may be a candidate serum marker of circulating EPC levels. Treatment with low-dose statin with ezetimibe combination therapy or high-dose statin monotherapy has similar effects on the reduced EPC levels.

Keywords Endothelial progenitor cells • Obesity • Metabolic syndrome • Lipids • Statin therapy

Introduction

The intact endothelium and maintenance of endothelial integrity play a central role in protecting against the development of atherosclerotic vascular disease.[1] Cardiovascular risk factors cause loss of endothelial cells or impair endothelial cell physiology. In recent years, it has become clear that bone marrow-derived endothelial progenitor cells (EPCs) may replace damaged or lost endothelial cells.[2] However, the presence of cardiovascular disease (CVD) is associated with lower numbers of circulating EPCs.[3,4] Interestingly, the reduced EPC numbers correlated with the impairment of endothelial function.[5] Reduced EPC levels quantified as

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CD34+KDR+ cells on flowcytometry were also found to be an independent predictor of cardiovascular events in patients with coronary artery disease in two prospective cohort studies.[6,7] These observations are consistent with a pathogenic role of decreased endothelial protection due to reduced levels of circulating EPCs in atherosclerotic disease progression. However, these findings are mostly based on studies in patients with manifest CVD. Whether the presence of CVD risk factors affects circulating CD34+KDR+ EPC levels in subjects in the general population without manifest CVD and thus in an earlier pathophysiological state is not as clear. We hypothesized that reduced EPC levels are present in apparently healthy subjects without manifest atherosclerotic disease but at high CVD risk. We, therefore, studied EPC levels in obese men recruited from the general population with metabolic abnormalities consistent with the metabolic syndrome, which is a highly prevalent condition, and in age-matched healthy volunteers. Furthermore, as restoring reduced EPC levels may improve endothelial protective capacity and attenuate the development of CVD, we evaluated the effect of lipid-lowering therapy on circulating EPC levels in these men. Statins have previously been shown to increase EPC levels in mice[8–10] and patients with manifest CVD.[11,12] It is not clear if the increase in EPC levels observed with statin treatment is fully mediated by the LDL-lowering effect. In support of a pivotal role of LDL lowering are the inverse correlation between EPC numbers and LDL plasma levels in hypercholesterolemic patients[13] and the observation that non-pharmacological cholesterol reduction by changing dietary habits increases EPC levels.[14] However, a previous study in chronic heart failure patients showed that lipid-lowering therapy with 10 mg simvastatin but not 10 mg ezetimibe monotherapy-enhanced EPC levels,[12] which may point at pleiotropic actions of statins. Although ezetimibe monotherapy is not commonly used clinically, combination therapy of ezetimibe and a statin are an effective LDL-lowering strategy and becoming regular practice. However, there may be uncovered disadvantages of this combination therapy because of the decreased statin dose. Less effective stimulation of circulating EPC levels may be such a disadvantage. To date, this has not been studied. We performed a double-blind randomized cross-over trial to compare the effects on EPC levels of high-dose HMG-CoA-reductase inhibitor simvastatin (80 mg) monotherapy with combination therapy of low dose simvastatin (10 mg) and the cholesterol-absorption inhibitor ezetimibe (10 mg).

Methods

Subjects

The Institutional Review Board of the University Medical Center Utrecht approved the study protocol and the study was conducted in accordance with the Declaration of Helsinki. All participants in the study gave their written informed consent. Study subjects were recruited from the general population by posted advertisements and advertisement in the local newspaper asking obese men with a waist circumference of >102 cm to come to our clinic for further screening. Thirty-two subjects were screened for the inclusion and exclusion criteria. We included 20 male subjects between 18 and 70 years of age with a body mass index (BMI) of >25 but <35 kg/m^2 who also met the ATP III criteria for the metabolic syndrome, i.e. three or more of the following criteria: waist circumference >102 cm; blood pressure ≥ 130 mmHg systolic or ≥ 85 mmHg diastolic; serum triglycerides ≥ 1.70 mmol/L (150 mg/dL); low high-density lipoprotein (HDL) cholesterol <1.04 mmol/L (40 mg/dL); fasting glucose ≥ 6.1 mmol/L (110 mg/dL). Glucose level ≥ 7.8 mmol/L after a standardized oral glucose tolerance test was also regarded as fulfilling the glucose criterion. Ten age-matched healthy male controls were recruited by posted advertisements, who were allowed to meet no more than two of the ATP-III criteria for the metabolic syndrome.

Exclusion criteria were smoking, thyroid disease (TSH >5 mU/L with clinical symptoms of hypothyroidism), hepatic disease (ASAT or ALAT >2 times upper limit of normal), renal disease (serum creatinine >1.7 times the upper limit of normal), a history of CVD (coronary heart disease, cerebrovascular disease or peripheral arterial disease), use of drugs for the primary prevention of CVD including lipid lowering and antihypertensive medication, blood pressure $\geq 180/110$ mmHg, BMI >35 kg/m^2, HbA1c $>6.5\%$ or triglycerides >8.0 mmol/L. All participants in this study were assessed on an individual basis by trained research physicians or research nurses during a screening visit to our hospital using a standard score sheet and identical calibrated machines to perform anthropometric measurements. Routine physical examination was performed prior to entering the study protocol. Flowcytometric analysis of EPCs was performed on an individual basis immediately after blood collection. Blood plasma was isolated using a standard protocol and stored at -80°C until further analysis, which was performed in single batches including samples from both controls and study subjects before and after the treatment.

Subjects meeting inclusion and exclusion criteria were enrolled in the study without further selection based on the screening order until the target sample size was achieved. The pre-specified target sample size of 10 controls and 20 obese subjects with the metabolic syndrome was based on previous studies at our laboratory involving EPC quantification in populations at increased CVD risk.[15,16] Obese subjects with the metabolic syndrome were entered in a randomized, cross-over, double-blind trial. Randomization was performed at the pharmacy department with the use of sub-blocks, with four subjects per sub-block. A number was assigned to each participant, which was used as a code for the data collection and analysis period of the study. After a baseline visit, subjects were randomized to a treatment order of receiving either low dose (10 mg) simvastatin combined with 10 mg cholesterol-absorption inhibitor ezetimibe or high-dose (80 mg) simvastatin monotherapy first. Subjects were asked not to make changes in regular physical activity during the study or to try to lose weight. The effect of treatment was assessed after 6 weeks drug-use. Following a 4 week washout period, subjects received the alternative treatment for again 6 weeks. Randomization was blinded to the study subjects, participating research physicians and nurses and other members of the study group. The pills for the two treatments were specially prepared for our study by the pharmacy department at our institution and carried no label. There were no visual differences between the pills containing the simvastatin monotherapy and those containing the simvastatin/ezetimibe combination therapy. Data collection on analysis was performed using coded samples. Laboratory analyses were performed in batches including all samples from all subjects at all time points where possible. The code for treatment order was broken only after all of the data collection and analysis had been completed. Study enrolment and completion of patients and controls, as well as schematic overview of the study design is shown in a patient flow diagram in *Figure 1*.

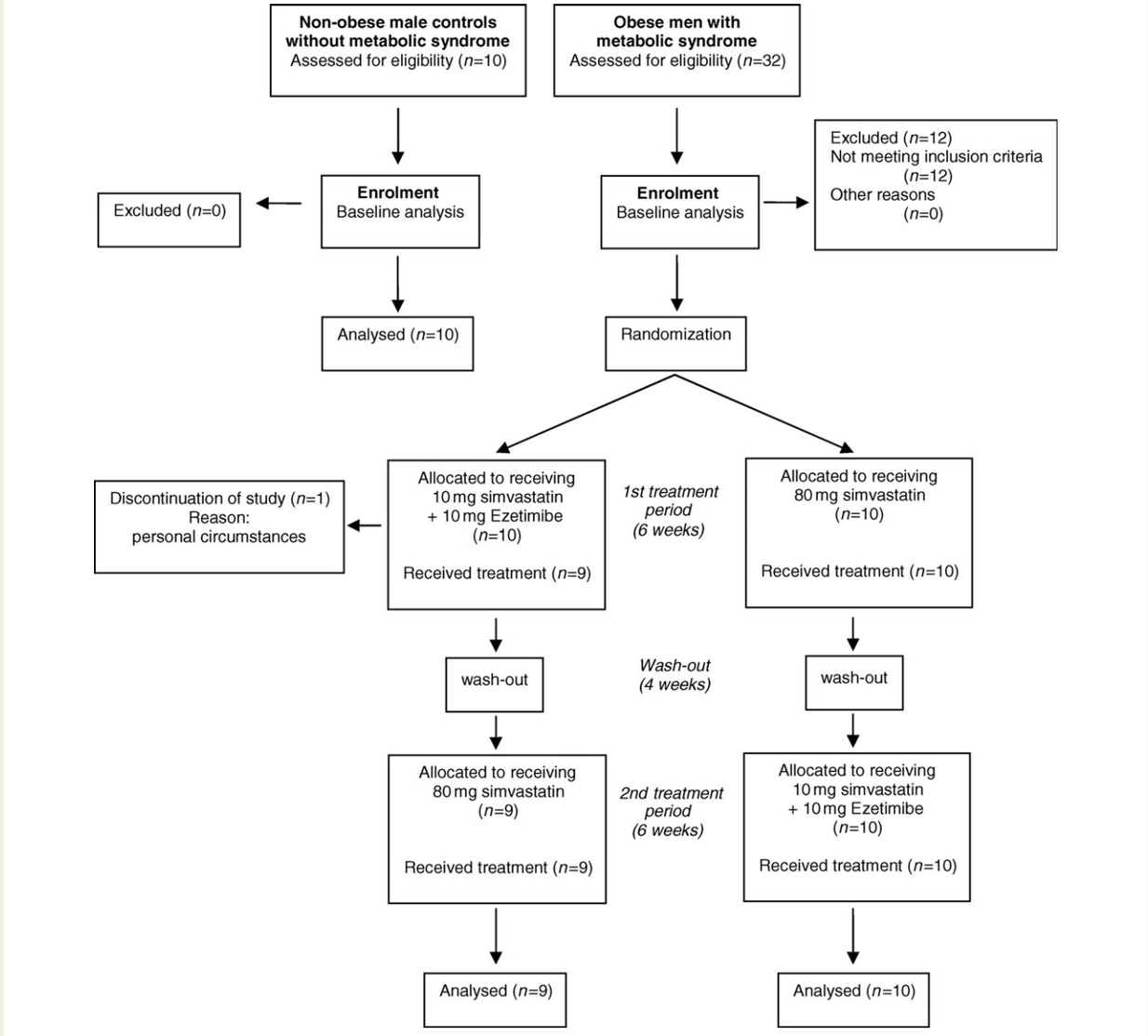

Figure 1 Patient flow diagram.

Lipid profile, hs-C-reactive protein, vascular endothelial growth factor, SCF, and SCF-sR measurements

Fasting blood was sampled for all measurements. Total cholesterol, HDL cholesterol, and LDL-cholesterol were analysed using commercially available assays (Wako, Osaka, Japan) with a Cobas Mira auto analyzer (Roche, Basal, Switzerland). VLDL-cholesterol was calculated (VLDL-cholesterol is equal to total cholesterol minus LDL-cholesterol and minus HDL-cholesterol). Plasma triglycerides were measured using a commercially available assay (Unimate, Roche), as were apolipoprotein A and B levels (Wako). hs-C-reactive protein, vascular endothelial growth factor (VEGF), SCF, and SCF-sR levels were measured by commercially available ELISA according to the manufacturer's instructions (R&D systems, Minneapolis, MN, USA).

Anthropometric measurements

Weight and height were measured and BMI was calculated as weight to height squared. Waist circumference was measured halfway between the lower rib and the iliac crest. Body fat percentage was estimated by using Omron body fat monitor BF306 (Omron, Matsusaka, Japan). Blood pressure was measured using a semiautomatic oscillometric device (Omega 1400, Invivo research laboratories Inc., Oklahoma, USA).

Flow cytometry for circulating endothelial progenitor cells and haematopoietic stem cells

EDTA blood was incubated with anti-CD34-FITC (BD Pharmingen, San Diego, USA), anti-KDR-PE (R&D Systems), and anti-CD45-PE-Cy7 (BD Pharmingen) antibodies. Erythrocytes were lysed in an

ammonium chloride buffer. CD34+ haematopoietic stem cells (HSCs) and CD34+KDR+ EPCs were quantified in duplicate relative to the number of CD45+$FS_{high}SS_{high}$ granulocytes in the sample using a flow cytometer (Beckman Coulter, Fullerton, USA). HSC and EPC numbers per millilitre blood were subsequently estimated based on a full blood granulocyte count made using a haematocytometer. Isotype-stained samples served as negative controls.

Statistical analysis

Data are expressed as mean ± SD and were analysed using SPSS version 12.0 or Graphpad Prism version 4.0 software. Normal distribution of data and equality of variances were tested where needed. The population of interest for this study were obese men with metabolic syndrome in the general population. During data collection and analysis, all samples were coded and investigators were blinded to treatment order designation in the randomized controlled trial. Primary data analyses were: (i) a comparison between EPC levels in obese men with the metabolic syndrome and healthy controls using a student's *t*-test and (ii) a comparison between the treatment effect of simvastatin monotherapy and simvastatin/ezetimibe combination therapy using a Student's *t*-test using data from the randomized controlled cross-over trial included in this study. For this analysis of the results of the cross-over trial, the treatment contrasts (difference in achieved EPC levels after the first vs. the second treatment) were compared using a two-sample *t*-test between the results from the AB-group (combination treatment first) vs. the BA-group (high dose simvastatin therapy first), thereby adjusting for a possible period effect. The data for the cross-over trial were analysed on a per protocol basis. Pre-specified exclusion criteria for the per protocol analysis were failure to complete both endpoint measurements in the cross-over trial or failure to take at least 90% of the pills containing the treatments, which was checked by questionnaires and counting of the returned pills.

Secondary analyses were: (i) a comparison between baseline and post-treatment EPC levels for the obese men with the metabolic syndrome using a repeated-measurements ANOVA based on the multivariate ANOVA (MANOVA) approach and (ii) a comparison between post-treatment EPC levels for the obese men with the metabolic syndrome and control levels using regular ANOVA. The Newman–Keuls *post hoc* test was used to compare between data sets after ANOVA. Additional, hypotheses-generating, analyses have included similar comparisons between control and obese men with the metabolic syndrome at baseline and after the two treatments, for measurements other than EPC levels. Here, Bonferroni correction for multiple testing was not included. Regression analysis was performed using linear univariate models. A *P*-value of <0.05 in two-sided analysis was considered statistically significant.

Results

Subject characteristics

Besides abdominal obesity, which was the primary screening criterion, the majority of subjects had hypertension and evidence of insulin resistance with high fasting blood glucose levels and/or impaired oral glucose tolerance test. Overall, HDL levels were non-significantly lower compared to control subjects and triglyceride levels were not different from controls. hs-C-reactive protein levels were higher in the obese men with the metabolic syndrome. The estimated 10 years risk for coronary heart disease based on the Framingham risk score was nearly two-fold on average in the obese men with metabolic syndrome compared to controls (11.1 ± 5.6 vs. 6.4 ± 3.1%, $P = 0.023$). None of the subjects used any medication, except two subjects who used ranitidine and terazosin, respectively, which were considered unlikely to interfere with the study. Further details on study subject characteristics are included in *Table 1*. Body weight remained constant during the trial, indicating that subjects adhered to the request not to change diet or exercise patterns. One patient was excluded from the per protocol analysis based on the failure to complete the trial. All remaining subjects adhered well to the treatments with at most missing a single dose during the treatment period, based on questionnaires and pill counts.

Endothelial progenitor cell levels are reduced in obese men with the metabolic syndrome

Circulating EPC levels were 39% lower in obese men with the metabolic syndrome than in controls (331 ± 193 vs. 543 ± 164/mL blood, $P = 0.006$, *Figure 2*). The number of CD34+ haematopoietic stem cells was 18% lower, but this was not statistically significant (2509 ± 1117 vs. 3065 ± 1167 /mL blood, $P = 0.220$).

Univariate analysis of obese men with the metabolic syndrome at baseline and controls combined did not show significant correlations between EPC levels and any of the individual components of the metabolic syndrome, although trends towards inverse relationships were observed for EPC levels and waist circumference as well as systolic blood pressure (*Table 2*). The number of components of the metabolic syndrome according to the ATP III criteria present in the individuals was significantly associated with lower circulating EPC levels (standardized regression coefficient $\beta = -0.517$, $P = 0.004$). Significant correlations were observed for reduced EPC levels and the BMI ($\beta = -0.387$, $P = 0.038$) and the body fat percentage ($\beta = -0.389$, $P = 0.037$). Other parameters, such as age ($\beta = -0.222$, $P = 0.246$) and hs-C-reactive protein levels ($\beta = -0.138$, $P = 0.475$) did not show a significant correlation (*Table 2*).

Intensive lipid-lowering therapy with both treatment regimens increases endothelial progenitor cell levels

After treatment with both intensive lipid-lowering treatment regimens, plasma LDL-cholesterol and triglycerides levels were lower than at baseline, while HDL cholesterol levels were similar (*Table 1*). LDL levels after combination treatment of 10 mg ezetimibe and low dose (10 mg) simvastatin therapy were 2.08 ± 0.45 vs. 2.10 ± 0.53 mmol/L after high dose (80 mg) simvastatin monotherapy, vs. 3.73 ± 0.67 mmol/L at baseline. Although the study was not designed to compare effectiveness of LDL-reduction, these results are consistent with a successful choice of dosages for achieving comparable LDL-levels after treatment. Surprisingly and not in line with previous studies,[17] hs-C-reactive protein-levels increased after treatment. As this was also not statistically significant, this is most likely attributable to chance.

Table 1 Baseline subject characteristics and effect of treatment on general parameters

	Controls ($n = 10$)	MetS baseline ($n = 19$)	MetS after 6 weeks 10 mg simvastatin +10 mg ezetimibe	MetS after 6 weeks 80 mg simvastatin
Metabolic syndrome components				
Triglycerides (mmol/L)	1.70 ± 0.51	1.67 ± 0.56	1.22 ± 0.39#	1.15 ± 0.49#
HDL-cholesterol (mmol/L)	1.27 ± 0.28	1.14 ± 0.26	1.12 ± 0.26	1.14 ± 0.31
Glucose (mmol/L)	5.2 ± 0.7	6.2 ± 0.7*	6.1 ± 0.8	6.1 ± 0.6
Waist circumference (cm)	96 ± 6	111 ± 7*	110 ± 7	111 ± 6
Systolic blood pressure (mmHg)	124 ± 8	138 ± 13*	131 ± 8	135 ± 13
Diastolic pressure (mmHg)	87 ± 8	89 ± 6	87 ± 5	86 ± 6
Total number of MetS components according to ATP III criteria	0 (0–2)	3 (3–5)	–	–
Other parameters				
Age (years)	46 ± 6	52 ± 8	–	–
Height (m)	1.85 ± 0.06	1.83 ± 0.06	–	–
Weight (kg)	86.9 ± 8.7	100.3 ± 11.5*	100.3 ± 11.3	100.9 ± 11.2
Body mass index (kg/m^2)	25.5 ± 2.3	30.2 ± 2.5*	30.1 ± 2.6	30.2 ± 2.5
Body fat (%)	26 ± 4	31 ± 3*	30 ± 3#	31 ± 3‡
Haemoglobin (mmol/L)	9.7 ± 0.6	9.1 ± 1.0	9.2 ± 0.7	9.4 ± 0.7
Platelets (exp^9/L)	246 ± 71	221 ± 47	214 ± 35	193 ± 39#
Leucocytes (exp^9/L)	5.5 ± 0.7	5.6 ± 1.1	5.6 ± 1.2	5.5 ± 1.1
Total cholesterol (mmol/L)	5.38 ± 1.09	5.58 ± 0.92	3.75 ± 0.85#	3.71 ± 0.86#
LDL-cholesterol (mmol/L)	3.53 ± 0.94	3.73 ± 0.67	2.08 ± 0.45#	2.10 ± 0.53#
VLDL-cholesterol (mmol/L)	0.58 ± 0.27	0.71 ± 0.25	0.56 ± 0.37#	0.46 ± 0.25#
Apolipoproteïn A (g/L)	123 ± 15	114 ± 15	109 ± 18	112 ± 17
Apolipoproteïn B (g/L)	85 ± 20	98 ± 16	71 ± 18#	70 ± 17#
hs-C-reactive protein (mg/L)	0.8 ± 0.7	2.9 ± 1.8*	3.7 ± 3.1	3.9 ± 3.9
10-years risk for coronary heart disease based on FRS charts**	6.4 ± 3.1	11.1 ± 5.6*	–	–

Clinical characteristics are shown of controls and obese men with the metabolic syndrome both at baseline and after the two treatment regimens.
*$P < 0.05$ compared to controls.
#$P < 0.05$ vs. baseline.
Values represent mean ± SD or median (range), *$P < 0.05$ compared to controls, #$P < 0.05$ compared to baseline, ‡$P < 0.05$ compared to alternative treatment group, **based on Framingham Risk Score (FRS) sheet: http://www.nhlbi.nih.gov/about/framingham/riskmen.pdf.

In our primary analysis of the effect of the two treatment regimens, we found that there was no statistically significant difference in treatment effect (calculated as mean difference in achieved EPC levels, which was −103 EPC/mL blood with a 95% confidence interval of −363 to 157 for the overall data set; $P = 0.48$). In a secondary analysis of the data, including also the individual baseline EPC levels, we found that EPC levels increased during both treatment regimens. During 10 mg simvastatin/10 mg ezetimibe combination therapy, EPC levels increased to 626 ± 428/mL blood (89% increase, $P < 0.05$ compared to baseline, *Figure 2*). Simvastatin monotherapy (80 mg) resulted in 524 ± 372 EPC/mL blood (58% increase, $P < 0.05$ compared to baseline, *Figure 2*). The difference between the achieved EPC levels was no longer reduced compared to controls.

The individual increase in the number in circulating EPCs did not correlate with the individual reduction in LDL ($r = 0.268$, $P = 0.267$ for simvastatin/ezetimibe combination therapy and $r = 0.295$, $P = 0.221$ for simvastatin monotherapy), although it must be noted that this analysis was insensitive for detecting a correlation at this level as the heterogeneity in cholesterol-lowering was small between subjects.

Serum vascular endothelial growth factor and SCF-sR correlate with progenitor cell levels at baseline and are modulated by lipid-lowering treatment

Serum levels of endogenous EPC-mobilizing cytokine VEGF correlated positively with EPC levels (*Table 3*) in the overall dataset of control subjects and subjects with the metabolic syndrome at baseline. VEGF levels in men with the metabolic syndrome were lower than in controls, although not statistically significant (55.1 ± 51.8 vs. 66.6 ± 53.9, $P = 0.58$, *Figure 3*). Serum levels of progenitor-cell mobilizing SCF were significantly reduced in the metabolic syndrome (1013 ± 151 vs. 1162 ± 210 pg/mL; $P = 0.04$, *Figure 3*), but did not correlate with circulating progenitor cell levels (*Table 3*). However, serum levels of the soluble form of SCF receptor c-kit, soluble SCF-receptor (SCF-sR), were both

significantly reduced in subjects with the metabolic syndrome compared to controls (53 ± 8 vs. 63 ± 9; $P = 0.003$, *Figure 3*) and correlated with HSC and EPC levels (*Table 3*).

After lipid lowering treatment with both 10 mg simvastatin/10 mg ezetimibe combination therapy and 80 mg simvastatin monotherapy, VEGF levels were substantially reduced to 38.7 ± 35.8 pg/mL ($P < 0.01$, *Figure 3*) and 36.3 ± 32.9 pg/mL ($P < 0.01$, *Figure 3*), respectively. Thus, EPC levels increased while VEGF levels decreased despite a positive correlation between VEGF and EPC levels at baseline. Serum SCF-sR, however, increased to control levels in conjunction with the observed normalization of circulating EPC levels after treatment with both simvastatin monotherapy (to 68 ± 21 ng/mL; $P < 0.05$, *Figure 3*) and 10 mg simvastatin/10 mg ezetimibe combination therapy (to 68 ± 19 ng/mL; $P < 0.05$, *Figure 3*). Of note, the changes in VEGF and SCF-sR levels were statistically significant in a secondary analysis of the data and the study was not primarily designed to investigate modulation of cytokine levels.

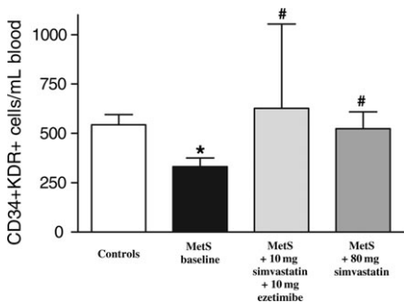

Figure 2 Endothelial progenitor cell (EPC) levels are reduced in subjects with the metabolic syndrome. Both lipid-lowering treatments increased EPC levels. Circulating levels of CD34+KDR+ EPCs quantified per mL blood were reduced in obese men with the metabolic syndrome (MetS) when compared to matched controls. Combination treatment of 10 mg simvastatin with 10 mg ezetimibe lowered LDL-cholesterol similarly as monotherapy with 80 mg simvastatin. There were no significant differences between the two treatment arms. Mean ± SD cells/mL. *$P < 0.05$.

Discussion

Here we report that obese men with the metabolic syndrome but without diabetes or manifest CVD have low levels of circulating EPCs; approximately 40% lower than in age-matched controls without the metabolic syndrome. Obesity-associated parameters BMI and body fat percentage were significant clinical predictors of low EPC levels in univariate analysis. The number of ATP III criteria for the metabolic syndrome correlated inversely with the level of circulating EPCs. In addition, our finding that serum levels of the soluble form of SCF receptor c-kit, soluble SCF-receptor (SCF-sR) correlated with HSC and EPC levels, were significantly reduced in subjects with the metabolic syndrome, and increased to control levels after lipid lowering treatment in conjunction with changes in circulating EPC levels, suggests the potential use of SCF-sR as a serum marker of circulating progenitor cell levels.

To the best of our knowledge, our study is the first to report an effect of the presence of common CVD risk factors on levels of circulating CD34+KDR+ EPCs determined by flow cytometry in obese subjects at high CVD risk due to the presence of the metabolic syndrome recruited from the general population without manifest CVD. Metabolic syndrome is a highly prevalent condition that severely impacts the occurrence of CVD in our society. The metabolic syndrome is a clustering of cardiovascular risk factors including abdominal obesity, dyslipidaemia, hyperglycaemia, and hypertension.[18] The age-adjusted prevalence is 20–25% in seemingly healthy subjects.[19] People with the metabolic

Table 2 Regression analysis for endothelial progenitor cell levels in healthy controls and obese men with the metabolic syndrome at baseline combined

	Standardized β-coefficient	*P*-value	Non-standardized β-coefficient (95% CI)
Metabolic syndrome components			
Triglycerides (mmol/L)	−0.164	0.396	−64 (−215 to 88)
HDL-cholesterol (mmol/L)	0.155	0.422	120 (−181 to 421)
Glucose (mmol/L)	−0.298	0.117	−74 (−168 to 20)
Waist circumference (cm)	−0.301	0.113	−7 (−15 to 2)
Systolic blood pressure (mmHg)	−0.361	0.055	−6 (−12 to 0)
Diastolic pressure (mmHg)	−0.060	0.757	−2 (−14 to 10)
Total number of MetS components according to ATP III criteria	−0.517	0.004*	−72 (−118 to −25)
Other parameters (sign. only)			
Body mass index (kg/m^2)	−0.386	0.038*	−24 (−47 to −1)
Body fat (%)	−0.389	0.037*	−20 (−39 to −1)

Univariate linear regression analysis of associations between subject characteristics and circulating EPC levels are shown.
*$P < 0.05$, without correction for multiple testing.

Table 3 Regression analysis of progenitor cell mobilizing cytokines with progenitor cell levels

	CD34+ HSC			CD34+KDR+ EPCs		
	Standardized β-coefficient	*P*-value	Non-Standardized β-coefficient (95% CI)	Standardized β-coefficient	*P*-value	Non-Standardized β-coefficient (95% CI)
VEGF	0.411	0.027*	9.1 (1.1 to 17.2)	0.433	0.019*	1.7 (0.3 to 3.2)
SCF	0.007	0.970	0.1 (−2.4 to 2.5)	0.275	0.149	0.3 (−0.2 to 0.7)
SCF-sR	0.431	0.019*	46.6 (8.1 to 85.1)	0.493	0.007*	9.7 (2.9 to 16.4)

Univariate linear regression analysis of associations between serum levels of progenitor cell mobilizing cytokines VEGF, SCF, and SCF-sR and circulating levels of CD34+ haematopoietic stem cells (HSCs) and CD34+KDR+ endothelial progenitor cells (EPCs). * $P < 0.05$, without correction for multiple testing.

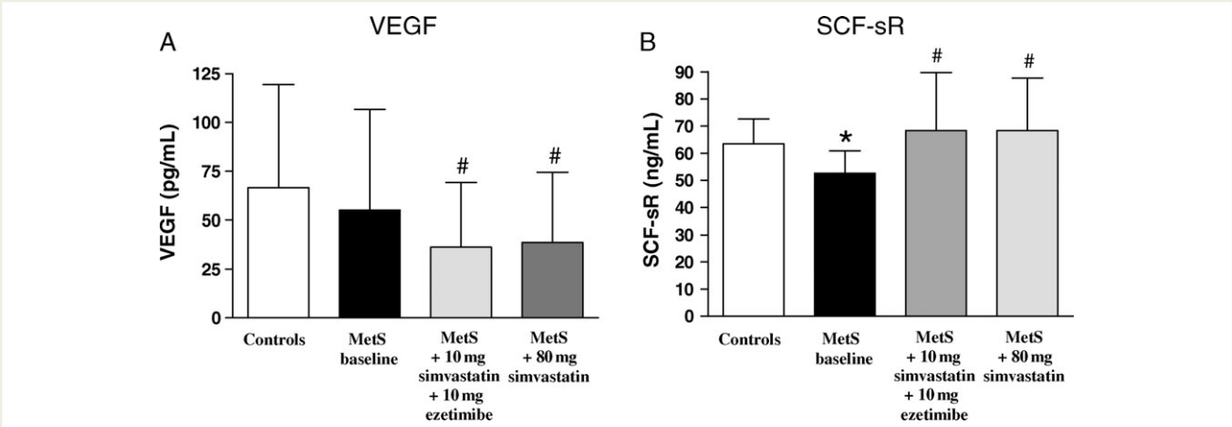

Figure 3 Serum levels of progenitor cell mobilizing cytokines vascular endothelial growth factor (VEGF) and SCF-sR before and after treatment. At baseline, serum VEGF levels were lower in subjects with the metabolic syndrome, although this was not statistically significant (*A*). After both lipid-lowering treatment regimens, VEGF levels further decreased (A). Serum SCF-sR levels were significantly reduced in subjects with the metabolic syndrome at baseline compared to controls and increased to control levels after both treatment regimens (*B*). Mean ± SD pg or ng/mL. *$P < 0.05$ vs. controls. #$P < 0.05$ vs. baseline.

syndrome have a three- to four-fold increased risk to develop type II diabetes[20] and a two- to three-fold increased risk for future morbidity and mortality of CVD.[20–22] Therefore, the subjects included in our study have a substantially increased risk of developing CVD and may have pre-atherosclerotic vascular changes or subclinical atherosclerosis.[23] Reduced EPC levels preceding the development of manifest CVD is consistent with an early pathophysiological role, which might relate to a decreased capacity to replace damaged or lost endothelial cells. Consistently, in a population of middle-aged subjects from the general population, reduced EPC levels were an independent predictor of an increased intima-media thickness of the carotid artery, which is a strong indicator of subclinical atherosclerotic disease.[24]

We identified circulating EPCs using flow cytometry for CD34+ haematopoietic stem cells (HSCs) that co-express the endothelial marker KDR. CD34+KDR+ EPCs are present in the circulation in low numbers and represent a defined subset of true progenitor cells. Importantly, prospective data showed an independent association of lower CD34+KDR+ EPC levels with increased rates of CVD events.[6,7] In cohort studies of patients with coronary artery disease, decreased levels of circulating CD34+KDR+ EPCs were associated with increased age, higher (systolic) blood pressure, smoking, LDL-cholesterol, and the presence of coronary artery disease *per se*.[4,6,7] However, these study populations consisted of patients with manifest CVD. Other studies reported on numbers of EPCs identified by *in vitro* culture of peripheral blood mononuclear cells under conditions facilitating outgrowth of angiogenic cells with an endothelial phenotype. Associations have been reported between decreased EPC outgrowth in culture and impaired endothelial function, Framingham risk score, Type I and II diabetes, hypertension, renal insufficiency, and increased age, in part also in subjects without manifest CVD.[5,15,25,26] However, these cultured EPCs constitute a different cell population and are mostly monocyte-derived cells.[27,28] In apparently healthy subjects with occasional presence of cardiovascular risk factors, numeric outgrowth of cultured EPCs does not correlate with the number of circulating CD34+KDR+ cells.[29] Our current study shows that subjects from the general population at increased CVD risk but without manifest CVD do indeed have reduced CD34+KDR+ EPC levels.

In our double-blind randomized cross-over trial with previously untreated obese men with the metabolic syndrome, we did not

observe an inferior effect on EPC levels of simvastatin/ezetimibe combination treatment compared to a high dose statin treatment achieving an equal reduction in LDL-cholesterol. Secondary analysis of the treatment effects compared to baseline showed that intensive lipid-lowering therapy can normalize decreased CD34+KDR+ EPC levels, even when average LDL levels were not significantly above control values. Statins have previously been shown to increase EPC levels in mice,[8–10] in patients with coronary artery disease,[11] and in patients with chronic heart failure.[12] In *in vivo* experimental injury models, statin therapy increased EPC levels while restoring age-related impaired neovascularization in mice[30] and augmented EPC-mediated re-endothelialization of denuded arterial segments in rats.[31] *In vitro*, statins exerted direct effects on EPCs with enhanced EPC differentiation,[8,32] enhanced EPC proliferation,[9,33] inhibited oxidative-stress-induced EPC apoptosis,[34] reduced the rate of senescence,[33,35] and enhanced migratory[9] and angiogenic function.[36] These *in vitro* observations are made in the absence of cholesterol products in their milieu, suggesting that these are LDL-lowering independent effects of statins. Notably, a previous study showed that lipid-lowering therapy with 10 mg simvastatin but not 10 mg ezetimibe enhanced EPC levels in chronic heart failure patients,[12] also consistent with a pleiotropic cholesterol-independent effect of statins on EPC levels. This might possibly also imply that low-dose statin/ezetimibe combination treatment may be less effective in restoring reduced EPC levels than high-dose statin monotherapy. However, in our population, high-dose statin and low-dose statin in combination with ezetimibe resulting in similar LDL-reduction were equally effective in enhancing EPC levels. This suggests a more important role for LDL-reduction in contrast to potential pleiotropic effects of statin therapy for the observed EPCs increase in our patients. In line with this, non-pharmacological cholesterol reduction by changing dietary habits also increased EPC levels.[14] In addition, in hypercholesterolemic patients, EPC function was impaired and EPC numbers were reduced and inversely correlated with LDL plasma levels.[13] However, an alternative explanation could be that the maximal effect of simvastatin for increasing EPC levels in our study had already been achieved at a dose of 10 mg.

The mechanism through which lipid-lowering therapy restored reduced EPC levels in our study population remains speculative. *In vitro*, oxidized LDL directly inhibits EPC differentiation and accelerates the rate of senescence through reducing telomerase activity.[37–39] Statin therapy increased the activity of the NO-producing enzyme eNOS in rat bone marrow[40] and experimental evidence shows that bone marrow NO-production is pivotal in EPC mobilization from the bone marrow.[10,41] eNOS activation is also thought to mediate VEGF-induced EPC mobilization.[42] We observed a baseline correlation between serum VEGF levels and circulating EPC numbers, but while EPC levels increased, serum levels of VEGF decreased after lipid-lowering treatment, consistent with previous observations.[43–45] This supports an effect of statins (or lipid-lowering therapy in general) on an intermediate in the signalling pathway of VEGF, such as eNOS. Lower VEGF levels without reducing EPC levels may be beneficial for (sub)clinical atherosclerosis, since plasma VEGF stimulates plaque neovascularization,[46] which in turn causes plaque progression[46] and destabilization.[47] On the other hand, VEGF is also capable to provide a vascular cytoprotective activity through the release of NO and PGI2 which can prevent the recruitment, adhesion and transmigration of proinflammatory cells.[48] Furthermore, VEGF promotes endothelial cell migration and proliferation.[48] Thus, based on the knowledge available at this time, a reduction of VEGF serum level must be considered with caution.

Interestingly, serum levels of SCF-sR were significantly reduced in subjects with the metabolic syndrome compared to controls and increased to control levels after lipid lowering therapy, concurrently with the restoration of EPC levels. SCF-sR is produced by various tissues including haematopoietic cells and vascular endothelium, and induces mobilization of haematopoietic progenitor cells from the bone marrow to the circulation.[49] How lipid-lowering therapy affects SCF-sR production and if this represents a mechanistic link remains to be established. However, our data indicate that SCF-sR might serve as a surrogate indicator of circulating progenitor levels that adequately modulate during EPC-mobilizing therapy.

Whether SCF-sR and/or CD34+KDR+ EPC levels are useful for cardiovascular risk assessment in clinical practice cannot be concluded from our study. EPC levels are increasingly recognized as novel intermediate cardiovascular endpoint that independently correlates with cardiovascular outcome. Various studies in recent years have underscored the pathophysiological role of EPCs and their potential as novel therapeutic target. In contrast to some other intermediate endpoints, changes in EPC levels can be assessed on a short-term, which allows monitoring for effect of treatment. Although it is feasible to quantify EPCs from a small volume of peripheral blood in routine clinical practice, this is a costly procedure. A plasma measurement of e.g. SCF-sR may therefore represent a superior candidate for use in clinical practice. Further studies are required to assess if including quantification of EPCs or related factors provide sufficient additional information over the current risk indicators to be effectively implemented in clinical practice.

Our study has several limitations. The treatments were given during a short period with intensive patient monitoring. Also, we have analysed our data on a per protocol basis (and not intention-to-treat) and we have indeed excluded a patient that dropped out during treatment. This experimental setting of treatment and the per protocol analysis were chosen as the aim of this trial was primarily to assess the mechanism of effects on EPC levels by lipid-lowering treatment. Consequentially, direct extrapolation to clinical practice is somewhat limited as e.g. differences in patient compliance between treatments are relatively unlikely to have occurred in our study. We did not investigate if the elevation of EPC levels observed with lipid-lowering therapy after 6 weeks in our population extends beyond that time period. This may be important to evaluate in future trials as a recent non-randomized observational cohort study of patients with coronary artery disease long-term statin therapy was associated with *decreased* rather than *increased* EPC levels.[35] Also, we did not investigate if cessation of lipid-lowering treatment was associated with a decline in EPC levels and with this did not verify if EPC levels had returned to baseline levels at the end of the washout

period. Another limitation of our study is that we have assessed circulating CD34+KDR+ EPCs only and not *ex vivo* cultured monocytic EPCs. In contrast to the monocytic EPCs, CD34+KDR+ EPCs cannot be obtained in sufficient quantities to allow assessment of functional characteristics.

In conclusion, EPC levels are significantly lower in obese men with the metabolic syndrome, even without manifest vascular disease. This suggests that reductions in EPC levels are a relatively early and potentially etiological pathophysiological phenomenon in the development of atherosclerosis, potentially contributing to an increased risk for future cardiovascular events in these patients. Intensive lipid-lowering therapy using either high-dose simvastatin or a combination of low-dose simvastatin with ezetimibe is equally effective in modulating EPC levels, and secondary analysis of our data suggests that such treatment may even fully normalize reduced EPC levels. If the EPC mobilizing effect of lipid-lowering (statin) therapy proves structural on the long-term, this may contribute to reducing CVD risk through restoring the endothelial regenerative capacity in subjects with metabolic syndrome.

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