Studies on the pathophysiological aspects of the metabolic syndrome in transgenic mice

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Chapter 6

Decreased circulating endothelial progenitor cell counts accompany elevated CRP levels in subjects with the metabolic syndrome without overt cardiovascular disease

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Abstract

To assess the influence of inflammation on circulating endothelial progenitor cells (EPC) and haematopoietic stem cells (HSC) in relation to carotid atherosclerosis in non-diabetic patients with metabolic syndrome (MS) without manifest cardiovascular disease (CVD). Seventy three male non-diabetic patients with MS without manifest CVD were included. A threshold of 1.8mg/L was used to define elevated (CRP+) and low (CRP-) CRP levels. Number of HSCs and EPC were quantified by fluorescence-activated cell sorter analysis. Cytokine and adhesion molecule profiles were assessed. Carotid atherosclerosis and adipose tissue distribution was assessed by MRI. Significantly lower number of EPC (40%, p = 0.001) and HSC (61%, p = 0.006) were found in CRP+. Patients with atherosclerotic plaques and CRP+ had lower HSC (p = 0.005) and EPC (p = 0.07) compared to CRP-. Subcutaneous adipose tissue area was higher in CRP+ at the waist and the hip (25%; p = 0.002 and 21%; p = 0.005 respectively). In a multiple linear regression analysis TNF-α (ß:-0.02; SE: 0.009; p = 0.03) and waist circumference (ß:-0.016; SE: 0.008; p = 0.049) were found as explanatory variables for EPC counts; P-selectin (ß:-0.007; SE: 0.002: p < 0.001) and TNF-α (ß: -0.024; SE: 0.008; p = 0.025) for HSCs.

In conclusion, decreased CEPC and HSC numbers were shown to accompany elevated CRP levels in non-diabetic patients with MS without manifest cardiovascular disease. This relation was observed in subjects with atherosclerotic plaques and elevated CRP levels but not in the absence of plaques.

Introduction

Circulating endothelial progenitor cells (EPC) are vasculogenic cell populations present in the mononuclear fraction of peripheral blood, which are thought to be derived from hemangioblastic cell that reside in the bone marrow. EPC can incorporate into the endothelial monolayer, stimulate proliferation of neighbouring endothelial cells and induce the formation of new blood vessels.[1,2] Significantly lower numbers of EPC have been shown in subjects with a high risk for cardiovascular disease[3] and in subjects with overt cardiovascular disease[4], especially in combination with the metabolic syndrome.[5] In addition, EPC may have prognostic[6] and therapeutic potential.[7,8] The relation between EPC and the extent of atherosclerotic disease burden is not yet clearly established.

The metabolic syndrome (MS) is associated with increased cardiovascular morbidity and mortality[9,10]. MS and visceral obesity are also associated with increased plasma C-reactive protein (CRP) levels[11-13]. CRP is a marker of the systemic inflammatory state and is shown to be associated with endothelial dysfunction[14], atherosclerosis[15,16], and future cardiovascular events[17]. CRP attenuates EPC survival, differentiation and function *in vitro*[18]. Furthermore, CRP levels have been related to circulating cytokine levels such as TNFα and IL6 in patients with characteristics of the metabolic syndrome. These key inflammatory cytokines have previously been linked to endothelial function and the regulation of bone marrow derived vasculogenic cells[19-21]. The influence of systemic inflammation on these cellular markers of endothelial dysfunction has not been studied *in vivo* in patients with the metabolic syndrome. We hypothesized that patients with MS and elevated systemic inflammation would have decreased EPC counts compared to MS patients with low systemic inflammation. Therefore, we assessed the influence of systemic inflammation on EPC and HSC counts in relation to the extent of carotid atherosclerosis in subjects with the metabolic syndrome without diabetes or overt cardiovascular disease. In addition, we explored prevailing cytokine and adhesion molecule levels. MRI assessments of the carotid artery and adipose tissue distribution were used to obtain measures of obesity and atherosclerotic disease burden.

Methods

Study design & Subjects

We included 73 male subjects above 50 years of age with visceral obesity and the metabolic syndrome according to the International Diabetes Federation criteria.[22] Exclusion criteria were type 2 diabetes mellitus, manifest cardiovascular disease, use of statins or non-steroidal anti-inflammatory drugs, current smoking, familial history of premature cardiovascular disease, severe obesity(BMI>40 kg/m^2), and contraindications for MRI. The subjects were divided into two groups with varying inflammatory states defined by the previously

published mean of CRP in male subjects as threshold (i.e. 1.8 mg/L)[23]. The study complies with the Declaration of Helsinki and was approved by the institutional review committee and all subjects gave informed consent. HdB was supported by the Dutch Heart Foundation (Grant NHS2006B106)

Anthropometric and laboratory assessments

Screening of potential candidates was performed by medical history, anthropometry and selected laboratory values. Extensive laboratory assessments were performed in all included subjects within two weeks of MRI assessments. These values were used in the statistical analysis. Blood pressure was assessed using an automatic blood pressure monitor (Omron 705IT, Hoofddorp, The Netherlands). Three measurements were averaged for use in the analysis. Blood samples were collected after a 12 hour overnight fast for chemical and haematological laboratory assessments. The high sensitive CRP assay was performed with the Tina Quant C-reactive protein (latex) high sensitive assay (Roche, Basel, Switzerland). Participants with CRP levels above 15mg/L were regarded as having an intercurrent infection and were not included in the assessments. Insulin was determined in heparinised plasma using a solid-phase, two-site chemiluminescent immunometric assay carried out on an Immulite 2500(DPC, Los Angeles, USA).

Cytokines and adhesion molecules

The serum levels of cytokines and adhesion molecules were measured using a Randox Evidence Investigator and the Cytokine & Growth Factors Biochip Array and Adhesion Molecules Biochip Array. The cytokine array contains discrete test regions of immobilized antibodies specific to IL-1α, IL-1β, Il-2, IL-4, IL-6, IL-8, IL-10, IFN-γ, TNF-α. The adhesion molecule array contains antibodies specific to soluble E-, L- and P-selectin, ICAM-1 and VCAM-1. The light signal generated from each test region on the Biochip with antibodies labelled with Horse Radish Peroxidase is detected using a super cooled charge coupled device camera and compared to that from a stored calibration curve. Sample preparation in short: the samples were diluted with assay buffer or diluent and applied to a biochip (well). The biochip (carrier) was incubated at 37 °C and shaken at 370 rpm at a thermoshaker for 60 min. After washing the conjugate (HRM labelled antibodies) was added and again incubated at 37°C and shaken at 370 rpm at a thermoshaker for 60 min. After washing 250 µl of a 1:1 mix of luminol and peroxide was added and incubated for 2 minutes. Finally the carrier is imaged using an Investigator System conform the manufacturers instruction.

3T Magnetic Resonance Imaging Protocol

Carotid Imaging: Magnetic resonance imaging was performed on a 3T scanner (Philips, Achieva, Best, The Netherlands) as previously described and validated[24]. In short, after performing the preparatory scan sequences, a dual inversion recovery (black-blood), spoiled segmented k-space fast gradient echo sequence with spectral selective fat suppression

was used to obtain ten contiguous transverse slices covering 2 cm of the carotid bulb and the common carotid artery. In all subjects, images were acquired from the left carotid artery. Images were analysed using the Vessel MASS software package developed at our institution, as described previously[25]. Atherosclerotic disease burden was assessed by the total vessel wall area of the scanned vessel segment (Total VWA). All scans were also visually scored for the presence of atherosclerotic plaques as follows: 0: no plaques, 1: focal asymmetrical vessel wall thickening < 2x the opposite vessel thickness, 2: focal asymmetrical vessel wall thickening ≥ 2x & < 3x the opposite vessel thickness. 3: focal asymmetrical vessel wall thickening ≥ 3x the opposite vessel thickness. When more than one plaque was present, the largest was used for classification.

Adipose tissue imaging: Subjects were positioned in the magnet in a supine position. The body coil was used for obtaining the images. A sagittal single shot gradient echo sequence survey scan was used for the imaging of the vertebral column in the lumbar region. Subsequently, a second single shot gradient echo sequence in the transversal plane was used for obtaining three contiguous slices of 10mm without angulations. The slices were centred at the intervertebral disk level between the 4^{th} and 5^{th} lumbar vertebra. The following parameters were used for image acquisition: echo time 3.7 ms (TE), repetition time7.5 (TR), pulse angle 45 degrees, and 2 signal averages were performed. The images were obtained with 3 breath holds of 6 seconds. The field of view was 500mm. A voxel size of 1mm x 1.3mm x 10mm was obtained. Images were assessed using the MASS software package developed at our institution allowing a semi-automated detection of subcutaneous (SAT) and visceral adipose tissue (VAT) area.

Enumeration of EPC and HSCs

Enumeration of circulating hematopoietic stem cells (HSCs) and circulating endothelial progenitor cells (EPC) was performed as recently described.[26] This method uses Trucount tubes that contain a defined number of brightly fluorescent microbeads, permitting the acquisition of absolute counts of cells, even at very low numbers. Circulating HSCs are defined as cells with low-expression of CD45, positive for CD34, and located in the lympho-gate on a side- and forward-scatter plot. This gating strategy was extended by calculating the number of $CD34^+$ cells that also express vascular endothelial growth factor receptor-2 (VEGFR-2) to define the number of EPC. This strategy avoids inclusion of mature endothelial cells, which are also positive for CD34 and VEGFR-2, since they are located outside the lympho-gate.

Statistical analysis

Continuous variables are presented as mean values ± standard error or as medians and interquartile ranges if the assumption of normality was not met. Comparisons between continuous variables were performed with independent samples *t* tests or Mann-Whitney *U* tests when not normally distributed. Correlations were analyzed with bivariate correlation analysis (Pear-

son's or Spearman's correlation depending on distribution). Logarithmically transformed HSCs and EPC were used to compare the influence of CRP levels in groups with and without atherosclerotic plaques and in multiple linear regression analyses. Multiple linear regression analysis was performed to evaluate the determinants of precursor cell counts.. Variables were selected from all determined anthropometric, laboratory, cytokine profiles, and MRI determined adipose tissue distribution if the obtained p-value in a univariate linear regression analysis was lower than 0.1. The selected variables were used in a multiple regression analysis with forward selection. Analyses were performed using SPSS version 12.01 (SPSS, Chicago, Illinois, USA). All analyses were two-sided, with a level of significance of α=0.05.

Results

Patient characteristics

Of the 73 included participants in the study, 51 subjects had elevated CRP levels (CRP+), and 22 subjects had low CRP levels (CRP-). Patient characteristics are shown in **table 1**. There were no differences between both groups in age, systolic, and diastolic blood pressures, and total cholesterol levels. Subjects with elevated CRP levels had significantly higher waist circumferences (p = 0.01), body weight (p = 0.005), HDL cholesterol levels (p = 0.028), and LDL cholesterol levels (p = 0.02) in comparison to subjects with low CRP levels.

Table 1 Patient characteristics

	CRP- n = 22	CRP+ n = 51	*p*
Age (years)*	58.9 (1.14)	59.2 (0.68)	ns
Waist circumference (cm)	103.5 (99.8-106.9)	108 (102-116)	*0.01*
Body weight (kg)	87.6 (81.9-94.2)	97.1 (87.9-107.3)	*0.005*
Systolic BP (mmHg)*	151.8 (4.0)	151.1 (2.5)	ns
Diastolic BP (mmHg)*	88.5 (1.9)	89.1 (1.1)	ns
TC (mmol/L)*	5.6 (0.2)	5.9 (0.13)	ns
HDL (mmol/L)	1.11 (1.02-1.29)	1.31 (1.11-1.54)	*0.028*
TG (mmol/L)	2.30 (1.49-3.49)	1.68 (1.24-2.13)	ns
LDL (mmol/L)	3.32 (2.64-3.75)	3.70 (3.22-4.30)	*0.02*
FBG (mmol/L)	5.2 (4.7-5.4)	5.1 (4.9-5.7)	ns
HbA1c*	4.9 (0.1)	5.1 (0.1)	ns
Insulin (mU/L)	8.5 (6.0-12.4)	10.0 (7.0-15.0)	ns
HOMA	1.1 (0.7-1.7)	1.4 (0.9-2.0)	ns
CRP (mg/L)	0.94 (0.56-1.47)	2.46 (1.59-3.29)	*0.000*
Framingham*	14.15 (1.34)	13.02 (0.60)	ns

CRP- and CRP+ data are presented as median (interquatile range).*Data are presented as mean (SE). BP: blood pressure, TC: total cholesterol, HDL: high-density lipoprotein, TG: triglycride, LDL low density lipoprotein, FBG: fasting blood glucose, HOMA: HOMA insulin resistance index, CRP: C-reactive protein

EPC and HSCs (Table 2)

EPC and HSCs were significantly decreased in CRP+ compared to CRP- (**Figure 1**). Furthermore, the EPC/HSC ratio was observed to be significantly lower in CRP+ in comparison to CRP- ($p = 0.022$).

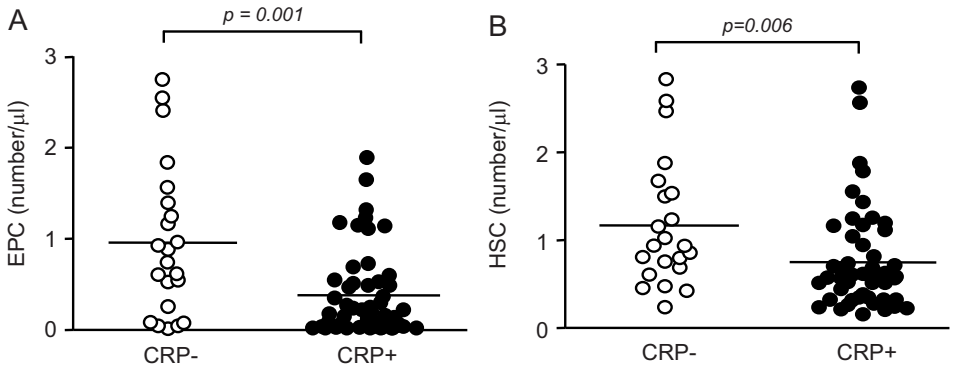

Figure **EPC (A) and HSC (B) numbers in patients with elevated CRP (CRP ≥ 1.8 mg/L, CRP+) and low LRP (< 1.8 mg/L, CRP-) levels.**

Table 2 **Circulating endothelial progenitor cell and haematopoietic stem cell numbers.**

	CRP- (n = 22)	CRP+ (n = 51)	*p*
EPCs (number/µl)	0.81 (0.21-1.43)	0.18 (0.02-0.51)	0.001
HSCs (number/µl)	0.93 (0.66-1.57)	0.59 (0.34-1.04)	0.006
CEPC/HSC ratio	0.90 (0.39-0.95)	0.47 (0.03-0.92)	0.022

Data are presented as median (interquartile range). EPC: circulating endothelial progenitor, HSC: haematopoietic stem cells

Cytokine and adhesion molecule profiles (Table 3)

Plasma concentrations of IL-6 and TNFα were significantly higher in CRP+ vs CRP-, 2.4 pg/ml [1.90-5.40] vs 1.20 pg/ml [0.88-2.28] and 7.80 pg/ml [0-10.30] vs 6.40 pg/ml [0-7.73] respectively. Plasma levels of other cytokines and adhesion molecules did not differ significantly between the two groups. Significant correlations were observed between EPC and IL-6 (r:-0.32, $p = 0.007$) and TNF-α (r:-0.26, $p = 0.029$). HSCs correlated significantly with CRP (r:-0.27, $p = 0.02$), IL-6 (r:-0.27, $p = 0.02$), TNFα (r:-0.26, $p = 0.026$), ICAM-1 (r:-0.33, $p = 0.004$) and P-selectin (r:0.39, $p = 0.001$).

Table 3 The cytokine and adhesion molecule profiles

	CRP- (n = 20)	CRP+ (n = 51)	*p*
IL-1α (pg/ml)	0.50 (0-0.85)	0 (0-0.80)	ns
IL-1β (pg/ml)	0.85 (0-1.73)	0 (0-1.8)	ns
IL-2 (pg/ml)	0 (0-6.05)	0 (0-9.70)	ns
IL-4 (pg/ml)	4.80 (3.18-5.10)	5.2 (4.80-6.50)	ns
IL-6 (pg/ml)	1.20 (0.88-2.28)	2.4 (1.90-5.40)	*0.01*
IL-8 (pg/ml)	9.40 (6.28-11.53)	9.5 (7.20-11.80)	ns
IL-10 (pg/ml)	0 (0-0)	0 (0-0)	ns
INF-γ (pg/ml)	0 (0-6.18)	0 (0-6.70)	ns
TNF-α (pg/ml)	6.40 (0-7.73)	7.80 (0-10.30)	*0.024*
VCAM-1 (ng/ml)	509.80 (468.15-630.95)	490.40 (415.80-564.90)	ns
ICAM-1 (ng/ml)	305.70 (258.48-372.55)	303.10 (274.60-359.00)	ns
E-Selectin (ng/ml)	16.50 (12.88-20.55)	16.60 (12.50-20.90)	ns
P-Selectin (ng/ml)*	154.01 (8.54)	152.74 (5.66)	ns
L-Selectin (ng/ml)	988.25 (892.15-1110.28)	994.50 (901.10-1097.50)	ns

Data are presented as median (interquartile range). *Data are presented as mean (SE)

Table 4 MRI assessed atherosclerotic and adipose tissue distribution

	CRP- (n = 22)	CRP+ (n = 51)	*p*
Total VWA (cm²)	4.40 (3.85-5.46)	4.53 (4.07-5.30)	ns
Plaque 0 no. (%)	7 (31.8)	8 (15.7)	ns
Plaque 1 no. (%)	8 (36.4)	25 (49.0)	ns
Plaque 2 no. (%)	5 (22.7)	12 (23.5)	ns
Plaque 3 no. (%)	2 (9.1)	6 (11.8)	ns
VAT (cm²)	388.0 (300.6-515.4)	448.9 (348.8-563.8)	ns
Waist SAT (cm²)	649.4 (512.3-722.1)	761.1 (671.8-983.8)	*0.001*
Hip SAT (cm²)	475.8 (391.5-574.4)	582.1 (510.1-707.9)	*0.002*

Data are presented as median (interquartile range). VWA: vessel wall area, VAT: visceral adipose tissue, SAT: subcutaneous adipose tissue

3T MRI assessments (Table 4)

The extent of carotid atherosclerotic disease burden did not significantly differ between the two groups. No difference was observed in the number of atherosclerotic plaques between CRP+ and CRP-. Subjects with atherosclerotic plaques and elevated CRP levels had statistically significant lower HSCs [0.59/μl (1.87) vs 1.07/μl (1.91), $p = 0.005$] and non-significantly lower EPC [0.22/μl (4.69) vs 0.55/μl (5.24), $p = 0.07$] compared to in CRP-. No difference was seen in HSC and EPC counts in subjects without atherosclerotic plaques. Increased deposits of subcutaneous adipose tissue were observed in CRP+ both at the waist ($p = 0.001$) and the hip ($p = 0.002$). Visceral adipose tissue did not vary between the two groups.

Multiple regression analysis

Multiple linear regression analysis was performed to adjust for possible confounders influencing the observed differences in EPC and HSC counts as described in *Methods*. For EPC, waist circumference, CRP, hip SAT, and TNFα, had p-values <0.1 in the univariate linear regression analyses and were used as independent variables in the multiple linear regression analysis with forward selection. The obtained model consisted of TNFα (β:-0.02; SE:0.009; $p = 0.03$) and waist circumference (β:-0.016; SE:0.008; $p = 0.049$) as explanatory variables. For the multiple linear regression analysis regarding HSC counts, the following parameters were used as independent variables: CRP, TNFα, ICAM, and P-Selectin. The final model obtained consisted of P-selectin (β:-0.007; SE:0.002; $p < 0.001$) and TNFα (β:-0.024; SE:0.008; $p = 0.025$) as explanatory variables.

Discussion

This study demonstrates that elevated CRP levels are accompanied by decreased counts of EPC, HSCs and EPC/HSC ratio in subjects with the metabolic syndrome without diabetes and without overt cardiovascular disease. Subjects with atherosclerotic plaque on MRI have lower HCS and EPC counts when low grade inflammation is present while this effect was not observed in the absence of plaques. Further exploration related the low cell counts to prevailing TNFα and p-Selectin concentrations.

Significantly lower EPC counts have previously been demonstrated in subjects with insulin resistance[27], and in subjects with high risk for cardiovascular disease[28], especially in combination with the metabolic syndrome[29]. We now studied subjects with the metabolic syndrome without DM2 or overt cardiovascular disease to test the hypothesis that systemic inflammation is the driver of low cell counts in these patients. Low grade systemic inflammation was indeed accompanied by significantly lower EPC and HSC counts in metabolic syndrome. This is in line with studies reporting low EPC in other chronic inflammatory disease states such as rheumatoid arthritis[30]. The populations compared in our study were significantly different in body weight, HDL-c, LDL-c, and CRP levels. To adjust for the possible effects of adipose tissue, we repeated the analysis after matching CRP- for body weight to a subset of CRP+. Metabolic, anthropometric profile and adipose tissue distribution were not different between CRP+ versus the CRP-. Also in this sub-analysis we observed significantly lower EPC and HSC counts CRP+ (Data not shown).

EPC are thought to be related to the atherosclerotic process.[31] Patients with proven coronary artery atherosclerosis have low EPC counts.[32] Similarly, low EPC counts have been reported in subjects with an increased risk of accelerated progression of atherosclerosis.[33] Furthermore, decreased EPC counts have been described in other conditions known to be

accompanied by an increased burden of atherosclerosis such as type 2 diabetes mellitus[34] and chronic kidney disease[35]. The exact relation between EPC and HSCs and atherosclerosis is not clearly established. We used carotid artery MRI to assess atherosclerotic burden, as previously described.[36] No statistically significant difference in atherosclerotic disease burden was observed between CRP+ and CRP-. The influence of CRP on HSC and EPC counts was also observed in patients with atherosclerotic plaques and not in the absence of plaques. We propose that EPC contribute to the maintenance of the structural stability of atherosclerotic lesions. Consequently, loss of EPC would result in increased plaque vulnerability. Lower EPC counts have been shown to be associated with higher future cardiovascular events.[37] Our study extends these finding by adding the effect of systemic inflammation on precursor cell counts. Thus inflammation, precursor cells, plaque vulnerability and events could be regarded as mutually dependent, causally linked phenomena.

We explored the intermediate metabolic and inflammatory pathways related to EPC and HSCs. TNFα and IL-6 levels were significantly different between the groups with varying CRP levels. TNFα and IL-6 also correlated with EPC and HSC counts. In multiple linear regression analysis, TNFα and waist circumference significantly determined EPC counts, whereas HSC counts were associated with serum P-selectin and TNFα concentrations. The role of IL-6 in the multiple linear regression analysis may have been underestimated in our study. The study population consisted of viscerally obese subjects only and visceral obesity (waist circumference) was shown to play an important role in determining EPC numbers. On the other hand, IL-6 is one of the cytokines most strongly related to visceral adiposity. This may have confounded the relation between IL6 and EPC counts. The pronounced effect of visceral obesity (waist circumference) as explanatory variable for EPC suggests other adipocytokines may affect precursor cell status in addition to IL-6 in these patients. TNFα and IL-6 have previously been linked to endothelial function [38,39]. TNFα has also been shown to inhibit EPC proliferation and differentiation *in vitro* in a dose dependent manner.[40] P-selectin contributes to the adhesive surface of activated platelets adhered to inflamed endothelial cells or extracellular matrix.[41,42] This surface is regarded to serve as the anchor place for circulating cells. Our *in vivo* observations relating cytokines and adhesion molecules to EPC and HSC counts are inline with these previous *in vitro* studies.

Our study has limitations. It was designed as a cross-sectional observational study only exploring circulating inflammatory cytokines and adhesion molecules. In addition, no in depth differentiation analysis of other bone marrow derived circulating cells was performed. However, our observations point to the relevance of EPC and HSCs in this patient category, and show for the first time the significance of low grade inflammation on fate of bone marrow derived cells in these patients. Future research may focus on the exact differentiation routes *in vivo,* including the effect of therapeutic manipulation of the systemic inflammation by for instance salicylates or thiazolidinediones.

In conclusion, lower counts of EPC and HSCs were shown to accompany elevated CRP levels in subjects with the metabolic syndrome without diabetes or overt cardiovascular disease. Interestingly, this was also observed in subjects with atherosclerotic plaques and not in the absence of plaques. If EPC are important for the vascular response to injury, systemic inflammation may profoundly affect this process.

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