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*Research paper*

# Endothelial progenitor cells as a cardiometabolic risk factor marker in prediabetes

Angeliki Angelidi,[1] Andreas Melidonis,[1] Ioannis Protopsaltis,[1] Theodoros N. Sergentanis,[2] Angelos Gritzapis,[3] Ioannis Kornezos,[4] Theofanis Economopoulos,[5] George Dimitriadis,[5] Eleni Boutati,[5] Sotirios A. Raptis[5,6]

*[1]Diabetes Center, "Tzaneio" General Hospital of Piraeus; [2]Department of Epidemiology and Biostatistics, Athens University Medical School, Athens; [3]Department of Pathology, Locus-Medicus SA, Athens; [4]Department of Radiology, "Tzaneio" General Hospital of Piraeus; [5]2nd Department of Internal Medicine, Research Institute and Diabetes Center, "Attikon" University Hospital, Athens University Medical School, Athens; [6]Hellenic National Center for Research, Prevention and Treatment of Diabetes Mellitus and its Complications (H.N.D.C.), Athens; Greece*

## ABSTRACT

**OBJECTIVE: Endothelial progenitor cells (EPCs) have recently been considered as a potential novel marker of vascular integrity, atherosclerosis and cardiovascular risk. This study was performed to investigate the main determinants of EPC levels in individuals with prediabetes. DESIGN: Thirty-nine participants with newly diagnosed prediabetes were enrolled. Flow cytometric analysis was used to quantify EPCs (CD34+CD133+VEGFR-2+). Traditional risk factors, high-sensitivity C-reactive protein (hs-CRP), homeostasis model assessment of insulin resistance (HOMA-IR) and anthropometric parameters, including ultrasonographic-determined visceral and subcutaneous fat, were recorded. RESULTS: In univariate analysis, EPC levels significantly correlated with waist circumference ($p=0.017$), mean arterial pressure ($p=0.009$), total cholesterol ($p=0.003$), hs-CRP ($p=0.006$), HOMA-IR ($p=0.031$) and visceral fat ($p=0.040$). However, in stepwise multivariate ordinal logistic regression analysis, only visceral fat retained its statistical significance (OR=0.79, 95%Cl:0.64-0.98, p=0.032). CONCLUSIONS: Visceral fat seems to be the main determinant of EPC levels in individuals with prediabetes and to form a plausible link between mild metabolic abnormalities, cardiovascular risk and vascular homeostasis process.**

**Key words:** Endothelial progenitor cells, Insulin resistance, Prediabetes, Visceral fat

*Address for correspondence:*
Angeliki Angelidi, Diabetes Center, Tzaneio General Hospital of Piraeus, Greece
14 Talantou Str., 18755 Keratsini, Greece,
Tel.: +30 6974512771, Fax: +30 2104015636,
E-mail: angieang9@gmail.com

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## INTRODUCTION

Prediabetes is defined as an elevation of plasma glucose values above the normal range but below the diagnostic criteria for diabetes.[1] The prediabetic state is characterized by insulin resistance and is often associated with inflammation and the presence

of additional atherosclerotic risk factors linked to impaired endothelial function.[2] Endothelial damage and dysfunction are the early signs in the development of atherosclerosis and predictors of cardiovascular events.[3]

During the last two decades, several studies have focused on circulating endothelial progenitor cells (EPCs) as a novel biomarker of endothelial function. EPCs originate primarily from bone marrow and were first identified in peripheral blood by Asahara and colleagues in 1997.[4] EPCs play a prominent role in neovascularization, incorporate into sites of endothelial damage, help to maintain endothelial integrity and contribute to accelerated reendothelization and vascular homeostasis.[5] Decreased amounts of EPCs, as a possible biological marker, are correlated with atherosclerotic disease progression and increased cardiovascular risk.[6,7]

To date, studies have focused on the associations between EPCs and sociodemographic, anthropometric as well as cardiometabolic risk factors either in healthy populations or in patients with diabetes.[8,9] Age, sex, exercise, smoking, lipid profile, insulin resistance, inflammation, hypertension and genetic background have emerged as meaningful factors correlated with EPCs.[8,9] It has been shown that the prediabetic state is negatively associated with EPCs.[10] Nevertheless, examination of the aforementioned risk factors, especially in patients with prediabetes, remains an unexplored field. The present study aims to investigate and elucidate possible associations between cardiometabolic parameters of EPC levels in individuals with prediabetes in the absence of cardiovascular disease.

## MATERIALS AND METHODS

### *Study design and population*

The study population of this cross-sectional study consisted of general population subjects recruited in the outpatient diabetes clinic and internal medicine departments of Tzanio General Hospital, Piraeus, Greece. The study was approved by the Local Ethics Committee and performed in accordance with the Declaration of Helsinki. Written informed consent was obtained from all participants. The sample population consisted of 39 voluntary adults with prediabetes free of overt cardiovascular disease at study initiation. None of the participants had been diagnosed with alterations in carbohydrate metabolism prior to enrollment. Cardiovascular disease was excluded via a complete medical history, comprehensive physical examination, electrocardiogram, echocardiogram, treadmill exercise test and thallium 201 cardiac scintigraphy when applicable. Prediabetes was defined after performing 75g OGTT according to the 2011 ADA position statement.[1] Individuals were classified as having impaired fasting glucose (IFG) (fasting plasma glucose levels: 5.6 mmol/l to 6.9 mmol/L) and/or impaired glucose tolerance (IGT) (2-h glucose levels in the oral glucose tolerance test: 7.8 mmol/l to 11 mmol/L). The newly adopted criterion for defining prediabetes by using HbA1c (5.7% to 6.4%) could not be used in our study since the HbA1c measurements in our laboratory were not in accordance with the National Glycohemoglobin Standardization Program (NGSP). All participants were free of overt renal and hematologic disease. Exclusion criteria also included: history of stroke, peripheral arterial disease, chronic heart failure (NYHA class III and IV), secondary hypertension, history of malignancy, history of infection in the last month, autoimmune disease, hyper- or hypothyroidism, excess alcohol intake (>14 drinks/wk for women and >21 drinks/wk for men), hormone replacement therapy and intake of oral contraceptives. Participants were classified as physically active if they were engaged in moderate physical activity (a minimum of 150 minutes of moderate aerobic activity per week) or otherwise sedentary. Vigorous exercise was not reported by any participant.

### *Measurements*

Medical history and anthropometric and biochemical parameters were obtained from all participants. Waist circumference was measured midway between the lowest border of the ribs and the iliac crest in a horizontal plane and hip circumference at the widest level over the greater trochanters. Visceral and subcutaneous abdominal fat thickness were assessed according to an ultrasonographic protocol.[11] Subcutaneous fat was defined as the depth between the cutaneous boundary and the linea alba. Visceral fat was defined as the depth between the peritoneum and the lumbar spine at the end of a quiet expiration without distortion of the abdominal cavity. In addition,

fasting venous blood samples were collected from all participants for the determination of EPCs, lipid profile, glucose, insulin and high sensitive C-reactive protein (hs-CRP). The levels of hs-CRP in the serum samples were measured with high-sensitivity methods using nephelometry (BN II Nephelometer-Siemens). Insulin resistance was estimated by the homeostasis model assessment of insulin resistance (HOMA-IR) as described by Matthews et al.[12]

### *Quantification of circulating EPCs*

EPCs were detected with flow cytometry and defined by the surface expression of CD34, CD133 and VEGFR-2. The antibody cocktail consisted of CD34+FITC (Beckman Coulter, Fullerton, CA, USA), anti-CD133PerCPeFluor 710 (eBioscience, San Diego, CA, USA) and anti-VEGFR-2 (R&D Systems). As the CD34FITC antibody concentration was not specified in the respective leaflet, they were mixed and titrated in umbilical cord blood in order to define the dilutions that minimized non-specific staining. Following these preliminary experiments, 5ul of anti-CD34FITC, 3ul of anti-CD133PerCPeFluor 710 and 3ul of anti-VEGFR-2-PE were mixed and added to 50 ul of whole blood. The fixed amount of blood was added to BD Trucount tubes™ (BD Biosciences, San Jose, CA, USA), which contain known numbers of microbeads, allowing us to measure the absolute number of EPCs, as described in the literature.[13] A blood specimen was added to the antibody mix by reverse pipetting to improve accuracy and reproducibility. Following incubation for 15 min at room temperature in the dark, 450 ul of NH4Cl were added and incubated for 15min at room temperature in the dark. Each specimen was stained in duplicate, 8000 beads of each sample were acquired on FacsCan (BD Biosciences) and control samples were analyzed using BD Cell-Quest™ software. Control samples stained with isotype-matched antibodies, were used to exclude unspecific staining. The gated CD34+ cells, based on ISHAGE protocol,[14] were examined for the dual expression of CD133 and VEGFR-2 antigens. The absolute number of EPCs was expressed as the number of EPCs per mL of blood and calculated according to the formula: number of events in region containing cell population/number of events in absolute count bead region X number of beads per test/test volume.

The most frequently used and satisfactory method to define EPCs is by determination of co-expression of CD34+CD133+VEGFR-2+, or even dual expression CD34+VEGFR-2+. In our study sample, CD34+CD133+VEGFR-2+ and CD34+VEGFR-2+ concentrations were closely associated with each other (Spearman's rho=0.905, $p<0.0001$); given their close collinearity, CD34+CD133+VEGFR-2+ were only examined as the dependent variable so as to avoid unnecessary duplication of findings.

### *Statistical analysis*

Descriptive statistics were performed; categorical variables were presented as frequency (%), normally distributed variables as mean±SD, whereas non-normally distributed variables were summarized as per median and range. The Shapiro-Wilk test was performed for the evaluation of deviation from normality. Subsequently, for the evaluation of the factors associated with EPCs concentration, a standard two-step approach was followed: univariate and multivariate analysis. In univariate analysis, non-parametric tests were appropriately implemented given that EPCs concentration significantly deviated from normality. Specifically, Spearman's rank correlation coefficient (SR) was calculated when the independent variable was continuous or ordinal; the Mann-Whitney-Wilcoxon test for independent samples (MWW) was conducted when the independent variable was binary, whereas the Kruskal-Wallis test (KW) was performed when the independent variable was categorical with three or more subgroups.

In multivariate analysis, ordinal logistic regression was performed with backward selection of variables. The concentration of EPCs (dependent variable) was converted to an ordinal variable corresponding to the four levels/quartiles. The four levels were as follows: 1: minimum value (0 cells/mL) to 25th percentile (51 cells/mL); 2: 25th percentile (51 cells/mL) to median (158 cells/mL); 3: median (158 cells/mL) to 75th percentile (363 cells/mL); 4: 75th percentile (363 cells/mL) to maximum value (1133 cells/mL). As appropriate, factors proven significant in univariate analysis were tested in the stepwise multivariate model as independent variables. The level of statistical significance was set at 0.05. Statistical analysis was performed using STATA 8.0 statistical software (Stata Corporation, College Station, TX, USA).

## RESULTS

The study population consisted of 17 males and 22 females, aged 49.5±11.4 years (median: 49, range: 32-67). 28.2% of the study sample were current smokers (n=11). In relation to exercise status, 18 subjects (46.2%) declared physical exercise, whereas 21 subjects (53.8%) did not. The sample consisted mainly of patients with IFG (69.2%) and secondarily of patients with IFG and IGT (20.5%), as well as subjects with IGT (10.3%). HbA1c ranged between 5.5 and 6.3% (mean±SD: 5.93±0.21, median: 5.9%). EPCs ranged between 0 and 1133cells/mL (mean±SD: 247±273, median: 158 cells/mL). The baseline clinical and metabolic characteristics of the 39 participants are summarized in Table 1.

Univariate association analyses were performed to determine which variables were most closely associated with EPCs levels. Prediabetic status (p=0.910, KW), age (p=0.270, SR), sex (p=0.842, MWW), waist to hip ratio (p=0.744, SR), subcutaneous fat (p=0.497, SR), exercise (p=0.380, MWW), smoking status (p=0.490, MWW), statin use (p=0.078, MWW), renin-angiotensin-aldosterone system inhibitors (p=0.497, MWW), fasting glucose (p=0.560, SR), insulin (p=0.465, SR), HbA1c (p=0.093, SR), HDL-cholesterol (p=0.228, SR), triglycerides (p=0.257, SR) were not significantly associated with EPC levels. It should be noted that 40% of the participants in the quartile with the highest levels of EPCs were receiving statins; accordingly, a marginal trend towards an association between statin consumption and higher EPC levels was observed (322±154 EPCs/mL vs. 233±289 for participants not receiving statins; p=0.078, MWW). In contrast, EPC levels were significantly inversely correlated with BMI (rho=-0.660, p<0.001), waist circumference (rho=-0.407, p=0.017), visceral fat, (rho=-0.339, p=0.041), HOMA-IR (rho=-0.400, p=0.031), systolic (rho=-0.400, p=0.019) and mean blood pressure (rho=-0.440, p=0.009), total cholesterol (rho=-0.471, p=0.003), LDL cholesterol (rho=-0.373, p=0.023) and hs-CRP (rho=-0.442, p=0.006). Variables shown to be statistically associated with EPC levels after performing univariate analyses are summarized in Table 2.

Continuous variables have been presented as <median and >median for purely descriptive reasons; their continuous nature has been appropriately taken into account in the univariate tests.

**Table 1.** Main characteristics of the study population (total number of participants=39)

| **Categorical variables** | **Frequency (%)** |
|---|---|
| Prediabetes subgroup | |
| *IFG* | 27 (69.2) |
| *IGT* | 4 (10.3) |
| *IFG and IGT* | 8 (20.5) |
| Male | 17 (43.6) |
| Current smoking | 11 (28.2) |
| Exercise | 18 (46.2) |
| Hypertension | 12 (30.8) |
| Statins | 6 (15.4) |
| Renin-Angiotensin system inhibitor | 10 (25.6) |
| **Normally distributed continuous variables** | **Mean±SD** |
| Age (years) | 49.5±11.4 |
| Waist to Hip Ratio | 0.93±0.08 |
| Fasting glucose (mmol/L) | 6.01±0.54 |
| HbA1c | |
| (%) | 5.93±0.21 |
| (mmol/mol) | 41.3 ±2.3 |
| Diastolic blood pressure (mmHg) | 86.5±10.1 |
| Total cholesterol (mmol/l) | 5.16±1.07 |
| LDL-cholesterol (mmol/l) | 3.37±0.89 |
| **Non-normally distributed continuous variables** | **Median (range)** |
| EPCs (cells/mL) | 158 (0-1133) |
| Weight (kg) | 88.9 (58.9-159) |
| BMI (kg/m$^2$) | 32.5 (23.5-43.5) |
| Waist circumference (cm) | 107 (85-148) |
| Subcutaneous fat (cm) | 1.76 (0.81-4.68) |
| Visceral fat (cm) | 8.28 (5.65-18.31) |
| Insulin (pmol/L) | 125.7 (69.5-451.4) |
| HOMA-IR | 4.4 (2.47-15.73) |
| Systolic blood pressure (mmHg) | 141 (112-174) |
| Mean blood pressure (mmHg) | 124 (95.3-147.7) |
| HDL-cholesterol (mmol/l) | 42 (30-57) |
| Triglycerides (mmol/l) | 124 (46-592) |
| hs-CRP (mg/L) | 2.59 (0.17-21.5) |

After stepwise multivariate ordinal logistic regression analysis, only visceral fat remained significantly associated with the EPC levels (OR 0.79 [95% CI 0.64, 0.98] $p$=0.032), whereas the remaining parameters

**Table 2.** Variables significantly associated with EPCs; results of the univariate analyses

| Variables | EPCs (cells/mL, median: 25th-75th percentile) | *p value* (Spearman's rho) |
|---|---|---|
| BMI (kg/m$^2$) | | |
| <32.5 | 292 (158-431) | <0.0001 (-0.660) |
| ≥32.5 | 84 (0-234) | |
| Waist Circumference (cm) | | |
| <107 | 263 (111-374) | 0.017 (-0.407) |
| ≥107 | 69 (0-133) | |
| Visceral fat (cm) | | |
| <8.28 | 305 (69-470) | 0.040 (-0.339) |
| ≥8.28 | 84 (0-234) | |
| HOMA-IR | | |
| <4.4 | 258 (0-470) | 0.031 (-0.400) |
| ≥4.4 | 77 (26-184) | |
| Systolic Blood Pressure (mmHg) | | |
| <141 | 196 (74-374) | 0.019 (-0.400) |
| ≥141 | 69 (0-292) | |
| Mean Blood Pressure (mmHg) | | |
| <124 | 196 (74-374) | 0.009 (-0.440) |
| ≥124 | 69 (0-292) | |
| Total cholesterol (mmol/L) | | |
| <5.10 | 272 (69-431) | 0.003 (-0.471) |
| ≥5.10 | 84 (0-158) | |
| LDL-cholesterol (mmol/L) | | |
| <3.49 | 318 (69-431) | 0.023 (-0.373) |
| ≥3.49 | 84 (0-158) | |
| hs-CRP(mg/L) | | |
| <2.59 | 282 (158-363) | 0.006 (-0.442) |
| ≥2.59 | 69 (0-133) | |

(BMI, waist circumference, HOMA-IR, systolic and mean blood pressure, total cholesterol, LDL cholesterol and hs-CRP) lost their statistical significance (Table 3).

Interestingly, as a *post hoc* analysis showed, the majority of the variables which lost their statistical significance were closely associated with visceral fat ($p<0.0001$, SR, for BMI; $p<0.0001$, SR, for waist circumference; $p=0.001$, SR, for HOMA-IR; $p=0.001$, SR, for Systolic Blood Pressure; $p=0.0006$, SR, for Mean Blood Pressure; $p=0.0001$, SR, for hs-CRP; no significant associations for total cholesterol or LDL); this may possibly provide an explanation for their observed loss of significance.

**Table 3.** Variables significantly associated with EPCs; multivariate analysis ordinal logistic regression with backward selection of variables.

| Variable | OR (95% CI) | *p value* |
|---|---|---|
| Visceral fat (cm) | 0.79 (0.64 – 0.98) | 0.032 |

The remaining parameters (BMI, waist circumference, HOMA-IR, systolic and mean blood pressure, total cholesterol, LDL cholesterol and hs-CRP) lost their statistical significance

## DISCUSSION

To the best of our knowledge, the present report is the first to extrapolate from prediabetes a host

of previous findings in healthy controls or individuals either with diabetes or cardiovascular disease. Taking into account that duration of diabetes and $HbA_1c$ level may be associated with reduced EPCs,[8,15] we sought to identify the main contributing factors of EPC levels in individuals with newly diagnosed prediabetes. In our study, EPC levels were inversely correlated with atherosclerotic risk factors such as obesity, insulin resistance, hypertension, dyslipidemia and inflammation.

In addition, the statin-induced increase in EPC levels[9,16] was also observed in our study sample. However, when all risk factors were jointly examined, only visceral fat remained independently associated with a significant reduction in EPC levels. Regarding the subgroups of prediabetes, no statistical association was documented, although a numerical trend pointing to lower EPC levels in individuals with IGT and those with IGT and IFG was noted (IFG: 266 ± 296 EPCs/mL, IGT: 230 ± 129 EPCs/mL, IFG and IGT: 191 ± 210 EPCs/mL). The former pattern is in accordance with that described by Fadini et al.[10]

Among the null associations that were observed in our sample, the neutral effect of age and gender should be noted. Although estrogen appears to regulate EPC number and function, our neutral results may be attributed to the fact that both pre- and postmenopausal women were enrolled in our study.

There is a clear connection between visceral adiposity with insulin resistance and inflammatory mediators. Insulin resistance may be associated with selective abnormalities of the phosphatidylinositol 3-kinase (PI3K)/protein kinase B (AKT) pathway, which has been implicated in the mobilization and differentiation of EPCs, as well in the inhibition of EPC apoptosis.[17] Meanwhile, the mitogen-activated protein kinase (MAPK) signaling, which has been reported to play a critical role in the downregulation of EPCs,[18] is maintained.

Inflammatory stimuli are likely to disrupt insulin-stimulated AKT phosphorylation. According to animal models, the coexistence of insulin resistance and inflammatory cytokines such as TNF-a is linked with insulin signaling defect and increased senescence of EPCs.[17] Moreover, a deleterious effect of hs-CRP on EPC differentiation, function and survival, in part via interfering with eNOS expression, has been also suggested.[19] Adipose tissue not only produces and releases a number of (pro)inflammatory cytokines, such as TNF-a, but also secretes adipokines, a variety of factors, including adiponectin, which may also play a role in EPC levels. Recent studies have documented the suppressive effect of adiponectin on high glucose-induced reactive oxygen species production and on the activation of p38 MAPK, leading to increased EPC levels.[20]

Our findings are in contrast to those of Biasucci et al[21] who reported a paradoxical positive correlation between obesity and EPC levels. It should be noted that the aforementioned results were obtained from healthy, insulin-sensitive individuals. Therefore, we hypothesize that worsening glucose metabolism combined with other disturbances (such as insulin resistance, inflammation) may progressively induce a possible exhaustion or failure of the EPC repair mechanisms, in which obesity seems to play a prominent role.

It has been firmly established that common complications of obesity such as insulin resistance, metabolic abnormalities and diabetes are more closely related to fat distribution than the absolute degree of fatness per se. Taking also into account that the number of EPCs may be an indicator of endothelial dysfunction and a possible biological marker of cardiovascular outcomes, our data suggest that visceral fat may actually be a causal link between EPC levels, vascular impairment, endothelial (dys)function and multiple cardiometabolic disorders, including cardiovascular disease and diabetes. Our findings strongly support the importance of identifying suitable therapeutic targets, lifestyle modifications and, particularly, weight reduction in subjects with prediabetes.

Concerning the limitations of the present report, the relatively small sample size may diminish the statistical power, while the cross-sectional design does not allow the substantiation of an etiological or pathophysiological hypothesis. Larger study samples seem to be required in order to establish any heterogeneity within prediabetes. The abovementioned fact that the small sample size may have substantially limited the statistical power could have led to the retention of the sole, most meaningful variable with

the multivariate approach. Indeed, it should not be precluded that in larger studies, additional, secondary covariates may also prove significant.

More importantly, the notion of "control participants" was not deemed necessary, since our study was focused on a specific subpopulation (prediabetes) in which the major determinants of EPC levels were sought. Numerous studies have shown that states of impaired glucose metabolism such as prediabetes and diabetes mellitus are directly related to reduced levels of EPCs.[10,22] As a result, the validation and replication of previously documented differences (such as comparisons of individuals with prediabetes vs controls)[10] was considered redundant and was not performed. However, the lack of an age-matched control group in order to validate the aforementioned findings may be considered a major limitation of the present study.

Finally, it should be mentioned that magnetic resonance imaging (MRI) and computed tomography (CT) are the gold standard methods for the accurate quantification of visceral fat. However, MRI and CT are relatively impractical and expensive, while the risk of CT radiation exposure should also be considered. In addition, since the validity of ultrasound to estimate visceral and subcutaneous fat has been examined in several studies,[11,23,24] in our study visceral fat was estimated by ultrasound technique, in accordance with others.[11,23]

## CONCLUSIONS

In conclusion, visceral fat seems to be a major determinant of EPC levels in individuals with prediabetes. This association might be the missing link between vascular homeostasis, cardiovascular risk and mild metabolic disturbances.

## CONFLICT OF INTEREST

The authors disclose no actual or potential conflicts of interests.

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