Brief Communication
Higher fasting glucose levels are associated with reduced circulating angiogenic cell migratory capacity among healthy individuals

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Abstract: Background: Chronic or severe acute elevations in plasma glucose are associated with decreases in the number and function of circulating angiogenic cells (CACs). However, less is known about whether fasting plasma glucose levels (FPG) within the normal or pre-diabetic range among healthy individuals are associated with decreased CAC function. Establishing this relationship is an important step in developing a line of research that may ultimately lead to preventative lifestyle interventions intended to maximize endogenous CAC function and reduce cardiometabolic disease risk. Objectives: 1) To examine whether increases in FPG are associated with decreases in CAC migration among healthy individuals with FPG levels below the threshold for hyperglycemia, and 2) to contrast effect of FPG on CAC migration toward a pro-angiogenic stimulus (vascular endothelial growth factor; VEGF) with effect on intrinsic cell migratory capacity (i.e., random migration with no stimulus). Methods: 28 men and women ranging from 20-57 years of age and free of cardiovascular disease participated in a pilot study, involving a fasting blood draw for FPG and isolation of peripheral blood mononuclear cells. CAC migration toward VEGF and random cell migration (control) were assessed *in vitro*. VEGF-induced migration that was normalized to control migration, representing the VEGF-response component of chemotaxis independent of motility, was calculated to determine whether any impairment in migration to VEGF was due to lower specific response to VEGF or to lower non-specific migratory capacity. Results: Increased levels of FPG were associated in a dose-response fashion with a significantly lower random migration under control conditions (CTRL: r= -.408, p=.031), no differences in migration to VEGF (r= -.039, p=.842) and a borderline association with VEGF-induced migration normalized to control migration (VEGF/CTRL: r=.349, p=.069). The relationship between FPG and random migration under control conditions remained significant when controlling for gender and body mass index (p's<.05), and became borderline significant when controlling for age (p=.062). Conclusions: Among healthy individuals, higher fasting glucose levels, despite falling below the diabetic range, are associated with decreased random CAC migration. These findings suggest a need for further studies investigating the effects of lifestyle or dietary interventions on glucose regulation and CAC function.

Keywords: Impaired fasting glucose, metabolism, endothelial progenitor cells (EPCs), circulating angiogenic cells (CACs), chemotaxis, chemokinesis, motility, migration, angiogenesis, cardiovascular, pre-diabetic

Introduction

Among non-diabetic individuals, a number of epidemiological studies have found that higher fasting plasma glucose (FPG) levels are associated with a significantly greater risk of cardiovascular and coronary heart disease events [1-3], although the association remains controversial [4, 5]. In addition, one study found that increasing concentrations of fasting plasma glucose (FPG) were associated with increasing arterial stiffness [6]. The mechanisms that may underlie this association are currently unknown, but some evidence suggests that impaired function of circulating angiogenic cells (CACs, sometimes called endothelial progenitor cells in the literature [7]) could potentially play a role.

CACs are thought to provide an important mechanism of endothelial repair and rejuvena-

tion, which is important, even in healthy individuals, for countering daily wear and tear [7, 8]. A number of studies have shown that patients with diabetes mellitus have impaired CAC function, as assessed by proliferation, adhesion, and tubule incorporation [9]. One study also found a progressive decline in the number of circulating CD34/KDR cells (a phenotype commonly used to assess the number of circulating angiogenic cells) across categorically defined patient groups ranging from healthy to diabetic, representing increasing impairment of glucose metabolism [10]. As a complement to these *in vivo* findings, several studies in which high levels of glucose were applied *in vitro* have reported impairment of CAC migration and other functional measures using cells derived from both diabetic patients [11] and healthy individuals [12, 13]. In sum, ample evidence supports the notion that high glucose can impair CAC function; however, it is not known whether impaired CAC function is similarly associated with *in vivo* FPG levels within the pre-diabetic range.

In order for CACs to facilitate endothelial repair, they must successfully home to sites of damage, which attract CACs by local release of cytokines such as vascular endothelial growth factor (VEGF) or stromal cell-derived factor (SDF-1). Hence, one frequently-used index of CAC function is the capacity to migrate toward a chemotactic gradient of VEGF. However, previous research suggests that the total migration has two components: directional migration towards a chemotactic gradient and non-directional chemokinesis, or random migration cell movement [14]. Hence, this study investigates both components of migration by assessing the potential association of FPG both with decreased migration to VEGF and also with decreases in random migration (i.e., to control media), as well as the ratio of the two.

This study sought to investigate whether fasting plasma glucose (FPG) levels below the threshold for diabetes (126 mg/dL) [15] are associated with lower indices of CAC function among healthy, non-diabetic individuals. We hypothesized that higher fasting glucose levels, even when below the threshold of hyperglycemia, would be associated in a dose-response fashion with decreased migration of CACs. As an exploratory aim, we additionally examined whether fasting glucose differentially affected random migration capacity versus migration toward a chemotactic gradient of VEGF.

Materials and methods

Participants

Thirty healthy individuals were recruited as part of a larger study at the University of California, San Francisco (UCSF) investigating specific mechanisms of CAC dysfunction. Informed consent was obtained in accordance with UCSF's Institutional Review Board, the Committee on Human Research, and the Declaration of Helsinki. The original study recruited 10 participants in each of the following categories: healthy men <45 years, healthy women <45 years, healthy men and women ≥ 45 years, and men and women with coronary artery disease (CAD) ≥ 45 years. For the purposes of this secondary data analysis, only the participants who did not have a CAD diagnosis were included. Inclusion criteria for healthy participants in the parent study included being 18 years or older as well as the absence of hypertension (blood pressure >140/90 mmHg), dyslipidemia (LDL >160 mg/dL), diabetes mellitus (FPG >126 mg/dL), cigarette smoking, evidence of coronary or peripheral artery disease, malignancies, terminal renal failure, acute inflammation, pregnancy, or medication with statins, estrogen replacement, hormonal birth control or erectile dysfunction medication. Health behaviors and psychological status were not assessed in the current study.

Sample characteristics on the final sample of 28 healthy participants with complete data on FPG and CAC function are described in **Table 1**. Of the original 30 participants, one male participant <45 years who was thought to be healthy had a FPG level of 136 and was therefore excluded from all analyses. One female participant <45 had no FPG data available due to problems with the plasma sample. In addition, data reported for CAC counts (**Tables 1** and **2**) were not available for one additional male participant <45 years due to laboratory error during processing.

Fasting glucose assessment

Blood samples were collected after a 12-hour overnight fast. Fasting glucose levels were analyzed using a glucose assay kit (Synchron CX System, Beckman Coulter, Inc).

Isolation of CACs

Blood samples were collected by venipuncture.

Table 1. Sample characteristics

Variable	Statistic	N
Age, M (SD), years	36.57 (12.40)	28
Male, n (%)	15 (53.6%)	28
Caucasian, n (%)	17 (60.7%)	28
Body mass index, M (SD)	23.88 (3.70)	28
Systolic blood pressure, M (SD)	109.71 (13.39)	28
Diastolic blood pressure, M (SD)	66.36 (9.23)	28
Fasting plasma glucose, M (SD)	93.36 (9.15)	28
Migration to VEGF, M (SD)	19.50 (9.87)	28
Migration to control, M (SD)	11.46 (4.72)	28
CD34/KDR cells, median (IQR)	0.12 (.09-.37)	27
CD133/KDR cells, median (IQR)	0.07 (.03-.11)	27
Medication use, n (%)	3 (10%)	28

*Claritin & Flonase (n=1), Anti-anxiety medications: Klonopin, Celexa, Neurontin (n=1), Differin & Clindamycin (n=1). M(SD)=mean (standard deviation of the mean); IQR=interquartile range.

Peripheral blood mononuclear cells (PBMCs) were isolated from whole blood by density centrifugation in Accuspin™ System-Histopaque-1077 (Sigma) and plated on fibronectin-coated dishes in endothelial cell basal medium (EBM-2; Lonza) supplemented with EBM-2MV SingleQuot and 20% FBS. After three hours, non-adherent cells were collected by pipetting and frozen at a concentration of 1×10^7 cells/mL in EBM-2 with EBM-2MV SingleQuot, 20% FBS and 10% DMSO. Initially adherent cells were discarded to exclude circulating endothelial cells.

PBMC thawing and CAC culture

CACs were thawed and plated on day one on fibronectin-coated 6-well plates at a density of 1×10^7 cells/well with 2 ml basal medium with EBM-2MV SingleQuot and 20% FBS. Cells were maintained by adding 1 mL media every other day. At day seven, CACs were harvested for the migration assay.

Migration assay

Migration of CACs was quantified by a transwell chemotaxis assay using a modified Boyden chamber [16]. Per our previously published protocol [17], 600 μL of EBM-2 media with or without 100 ng/mL VEGF were added to the bottom of a 24-well transwell chamber plate (Corning). 2×10^4 CACs were resuspended in 100 μL EBM-2 supplemented with 0.5% BSA, added to each migration insert (8 μm, Corning) and placed in the companion 24-well tissue culture plate. Each sample was loaded in triplicate inserts. Cell migration occurred during a six-hour incubation at 37°C. Plates were removed from the incubator, cells attached to the underside of the membrane were fixed in 4% formaldehyde and cells attached to the top-side of the insert membrane were removed with a cotton swab (Q-tip). The membrane was removed, mounted on a glass slide, and stained using Hoechst 33342. Fluorescence microscopy was used to capture 5 random fields (10× objective) per membrane and results were expressed as the average of the number of cells visualized per field.

CAC count in whole blood by fluorescence-activated sorting (FACS)

3 mL of whole blood was collected at the same time as above in an evacuated tube containing heparin. After centrifugation at 300 rcf 4°C for 7 min, the packed cells were Fc-blocked by treatment with human IgG (Invitrogen) on ice for 15 min. 100 μL whole blood was staining with anti-human VEGFR2 (KDR)-allophycocyanin (APC) (R&D Systems), CD34-PE (R-phycoerythrin) (Becton Dickinson), and CD133-PE (Miltenyi Biotec), followed by lyses of RBCs. As a control, cells in a separate tube were treated with mouse IgG1-APC and mouse IgG1-PE (BD Biosciences). The CAC number was measured by FACS cytometry as CD34/KDR and

CD133/KDR double-positive cells in the lympho-mononuclear cell gate.

Data analyses

All analyses were conducted using IBM SPSS Statistics 19 for Macintosh. All variables were inspected for deviations from normality using Q-Q plots. The associations among FPG, age, BMI, migration to VEGF, control (CTRL), the VEGF/CTRL ratio were assessed using Pearson correlation coefficients with two-tailed significance tests using a critical alpha of .05. Associations with CD34/KDR and CD133/KDR were conducted as Spearman rank order correlations due to their skewed distributions. Regression analyses were used to confirm whether associations between FPG and CAC outcomes remained significant when controlling for relevant covariates (e.g., age, body mass index, medication use), entered one at a time into sequential regressions to avoid over-fitting [18].

Because the relationships among FPG, age and CAC function are potentially complex, additional analyses were conducted to elucidate them. In previous studies, increasing age has been associated with an increasing risk of having high FPG levels (i.e., between 110-125 mg/dL [19]) and greater impairments in CAC migration [20]. Although statistical dichotomization of FPG is useful in epidemiological or clinical research, it is less appropriate for basic research questions in small samples, because it can substantially reduce power to find significant effects and bias results [21]. Furthermore, the utility of one cut-off versus another for predicting CVD events is still highly controversial [3, 5, 15]. Therefore, this study focused on continuous relationships between FPG and CAC outcomes.

Results

Increases in FPG were associated in a dose-response fashion with significantly lower migration under control conditions (CTRL; i.e., non-specific cell migratory capacity) (r=-.408, p=.031; **Figure 1**). No significant association between FPG and migration to VEGF (r=-.039, p=.842) was identified. However, FPG exhibited a borderline positive association with the normalized ratio of VEGF to CTRL migration (VEGF/CTRL; r= .349, p=.069; **Table 2**), which suggests that the sensing of the VEGF signal or the signal transduction leading to cell migration is intact,
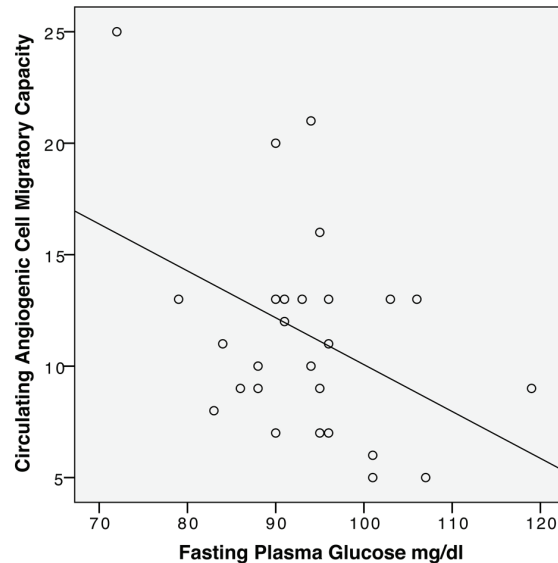

Figure 1. Scatterplot of the association between fasting plasma glucose and CAC migration to a control stimulus among healthy individuals. This relationship was significant (r= -.408, p=.031) among 28 healthy individuals free of cardiovascular disease with fasting glucose levels below the threshold for hyperglycemia, using a critical alpha of .05.

despite possible impairments in general cell motility. The relationship between FPG and the CTRL migration remained significant when controlling for gender, body mass index, being a former smoker, systolic or diastolic blood pressure, and medication use (p's≤.05 for all). As three participants had taken medications that could potentially impact CACs in the previous month (Claritin & Flonase for allergic rhinitis (n=1), anti-anxiety medications: Klonopin, Celexa, Neurontin (n=1), Differin & Clindamycin (n=1)), we verified that the association between FPG and CTRL remained significant (p<.05), when these three individuals were removed from the sample. FPG was not associated with CAC number, quantified as cells double-positive for CD34/KDR or CD133/KDR.

As previous literature suggests that the relationships among age, FPG, and CAC migration may be complex, a more in-depth examination was conducted. In this sample, increasing age was not significantly associated with impaired migration to VEGF (r=-.291, p=.132), CTRL (r=-.224, p=.252), or their ratio (r=-.022, p=.913). This suggests that age is not driving the relationship

Table 2. Raw correlations among fasting plasma glucose, demographic factors and circulating angiogenic cell outcomes

	FPG	Age	BMI	VEGF	CTRL	VEGF/CTRL	CD34/KDR	CD133/KDR
FPG	-	.318‡	.241	-.039	-.408*	.349†	.032	.164
Age	.318‡	-	.570**	-.291	-.224	-.022	-.202	-.191
BMI	.241	.570**	-	-.306	-.090	-.161	.239	.133
VEGF	-.039	-.291	-.306	-	.621**	.602**	.182	.226
CTRL	-.408*	-.224	-.090	621**	-	-.127	.177	.234
VEGF/CTRL	.349†	-.022	-.161	602**	-.127	-	-.130	-.002
CD34/KDR	.032	-.202	.239	.182	.177	-.130	-	.428*
CD133/KDR	.164	-.191	.133	.226	.234	-.002	.428*	-

**p ≤.01, *p ≤.05, †p ≤.07, ‡p ≤.10. Critical alpha = 0.05. FPG = fasting plasma glucose; BMI = body mass index; VEGF = vascular endothelial growth factor; CTRL = Control (random) migration. Pearson correlations were conducted for all associations except those involving CD34/KDR and CD133/KDR, for which Spearman correlations were conducted to accommodate their skewed distributions. N=28 for all correlations except those involving CD34/KDR and CD133/KDR, for which n=27.

between FPG and CTRL migration. Nonetheless, FPG and age are borderline correlated with a small-to-moderate effect size (r=.318, p<.10; **Table 2**); therefore, it is expected that the p-values for both factors will increase when both are entered as independent predictors in a regression equation. Indeed, when age was entered into the regression of FPG predicting CTRL migration, the effect of FPG became borderline significant (p=.062), whereas age remained a non-significant predictor of CTRL. To further eliminate the possibility that age might be the potential underlying causal factor driving the FPG-CTRL relationship, we additionally examined the association in the subsample of 18 individuals under 45 years of age (this cut-off was defined as part of the recruitment strategy in the parent study). In that subsample, the effect size of the relationship between FPG and CTRL actually increased, and was borderline significant (r=-.457, p=.057). Similarly, a nearly significant effect was found for FPG and VEGF/CTRL (r=.466, p=.052). This post-hoc analysis suggests that age does not drive the relationship between FPG and CTRL; rather, the effect sizes increase in the younger subsample.

Discussion

These data suggest that among healthy individuals, pre-diabetic elevations in FPG are associated with decreased random CAC migratory capacity. Interestingly, no associations were found between FPG and the functional response of CACs to VEGF or the number of CD34/KDR or CD133/KDR cells. If confirmed, these data suggest the possibility that elevated but pre-diabetic levels of glucose might divergently affect specific chemotaxis and general cell motility, at least among healthy individuals. Thus, it is possible that in pre-diabetics, moderately elevated glucose levels impair the basic mechanisms of cell motility, but these relatively non-motile cells are still able to exhibit an intact molecular response to VEGF, which eventually becomes impaired under more extreme diabetic conditions. This study reports findings of statistical significance, but future research is needed to establish whether or not they are clinically meaningful. Nonetheless, these data raise the possibility that impaired CAC function may be a mechanism that helps explain the epidemiological literature linking impaired fasting glucose and increased cardiovascular disease risk [1-3].

These observations underscore the distinction between the inductive and mechanical aspects of response to a chemotactic signal, and how they may be differentially influenced by the physiological processes being studied. They also shed new light on the existing literature, raising questions about the need to distinguish migration to VEGF from response to VEGF. Migration to VEGF will reflect the combination of CAC responses to VEGF specifically (chemotaxis or directed migration) and random cell migration. Most previously published studies using migration to VEGF as an outcome have not assessed or controlled for random migration. Hence, the current study suggests the possibility that previ-

ous research linking CAC migration with disease outcomes cannot definitively conclude that the specific VEGF response was impaired unless random migratory capacity or other indicators of specific responses were also assessed.

Multiple pathways are involved in mediating the damaging effects of elevated glucose on the vasculature, but it has been suggested that activation of oxidative stress or mitochondrial overproduction of superoxide may be the key common underlying process [22, 23]. Reactive oxygen species are key regulators of actin reorganization, which plays an important role in cell migration [24]. Moreover, many of the transcriptional factors that mediate CAC responses to hyperglycemia regulate signaling pathways involved in oxidative stress sensing and protection, metabolic control, cell cycle and apoptosis [25, 26]. Specifically, the forkhead box O (Foxo) subclass of transcription factors are critical mediators of hyperglycemia-induced CAC functional impairment [25], and silencing of Foxo1 and Foxo3 gene expression increased the migratory capacity of human umbilical vein endothelial cells [26]. Hence, future studies seeking to replicate and extend the current findings might explore the potential role of the aforementioned mechanisms.

As this study was a secondary analysis, a limitation is that the study was not explicitly designed to compare individuals above and below established thresholds of FPG [15], provide comprehensive measurement of glucose metabolism, or identify the precise mechanisms of the effects on CACs. Future research extending these findings should investigate both glucose and insulin, including fasting levels and the increase in response to challenge tests (e.g., the oral glucose tolerance test) in a larger sample. Given that age was not associated with migration in this sample, it is unlikely that association between FPG and random migration was attributable to age. Nonetheless, given the magnitude and consistent direction of the correlation coefficients between age and migration to both VEGF and CTRL (Table 2), it is possible that these relationships were non-significant because they were underpowered. Statistically controlling for age, as we have done herein, assumes that its effects on migration are completely independent of FPG. However, it is plausible that the effects of age on migration would be partially mediated by age-related metabolic changes. This could not be tested herein due to sample size limitations. Future studies replicating this finding in either a larger, age-stratified sample or a sample with a more limited age-range might help address this question without relying on statistical controls.

Reduced CAC number and function are now recognized markers for future cardiovascular disease risk [27, 28]. Moreover, a growing body of research suggests that modifiable health behaviors such as diet [29-32], exercise [33-35] and smoking [36, 37] have an important impact on CAC number and/or function. A few studies have demonstrated that increasing consumption of specific dietary compounds (e.g., flavanols found in cacao, green tea and wine) has beneficial effects on CAC function [30] or levels of circulating CD34/KDR cells [31, 32], although not all reports are positive [38]. One study reported that, among healthy young women, increasing vegetable intake increased the number of PBMC-derived CACs, which were counted *in vitro* after 7-day culture [29]. An important contribution of the current study is that it underscores the need to increase our understanding of how eating behaviors, metabolic indicators such as FPG, and CAC function are interconnected among healthy individuals, particularly those at increased risk for CVD or diabetes.

To our knowledge, this study is the first to demonstrate a relationship between FPG and CAC function among healthy, non-diabetic individuals. If confirmed and extended, these data contribute to a body of evidence suggesting that CAC function may be a useful outcome measure to optimize the efficacy of CVD prevention research promoting lifestyle changes.

Acknowledgements

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References

[1] Coutinho M, Gerstein HC, Wang Y and Yusuf S. The relationship between glucose and incident cardiovascular events. A metaregression analysis of published data from 20 studies of 95,783 individuals followed for 12.4 years. Diabetes Care 1999; 22: 233-240.

[2] Levitzky YS, Pencina MJ, D'Agostino RB, Meigs JB, Murabito JM, Vasan RS and Fox CS. Impact of impaired fasting glucose on cardiovascular disease: the Framingham Heart Study. J Am Coll Cardiol 2008; 51: 264-270.

[3] Wen CP, Cheng TY, Tsai SP, Hsu HL and Wang SL. Increased mortality risks of pre-diabetes (impaired fasting glucose) in Taiwan. Diabetes Care 2005; 28: 2756-2761.

[4] Tai ES, Goh SY, Lee JJ, Wong MS, Heng D, Hughes K, Chew SK, Cutter J, Chew W, Gu K, Chia KS and Tan CE. Lowering the criterion for impaired fasting glucose: impact on disease prevalence and associated risk of diabetes and ischemic heart disease. Diabetes Care 2004; 27: 1728-1734.

[5] Dekker JM, Balkau B. Counterpoint: impaired fasting glucose: The case against the new American Diabetes Association guidelines. Diabetes Care 2006; 29: 1173-1175.

[6] Salomaa V, Riley W, Kark JD, Nardo C and Folsom AR. Non-insulin-dependent diabetes mellitus and fasting glucose and insulin concentrations are associated with arterial stiffness indexes. The ARIC Study. Atherosclerosis Risk in Communities Study. Circulation 1995; 91: 1432-1443.

[7] Steinmetz M, Nickenig G and Werner N. Endothelial-regenerating cells: an expanding universe. Hypertension 2010; 55: 593-599.

[8] Shantsila E, Watson T and Lip GY. Endothelial progenitor cells in cardiovascular disorders. J Am Coll Cardiol 2007; 49: 741-752.

[9] Tepper OM, Galiano RD, Capla JM, Kalka C, Gagne PJ, Jacobowitz GR, Levine JP and Gurtner GC. Human endothelial progenitor cells from type II diabetics exhibit impaired proliferation, adhesion, and incorporation into vascular structures. Circulation 2002; 106: 2781-2786.

[10] Fadini GP, Pucci L, Vanacore R, Baesso I, Penno G, Balbarini A, Di Stefano R, Miccoli R, de Kreutzenberg S, Coracina A, Tiengo A, Agostini C, Del Prato S and Avogaro A. Glucose tolerance is negatively associated with circulating progenitor cell levels. Diabetologia 2007; 50: 2156-2163.

[11] Thum T, Fraccarollo D, Schultheiss M, Froese S, Galuppo P, Widder JD, Tsikas D, Ertl G and Bauersachs J. Endothelial nitric oxide synthase uncoupling impairs endothelial progenitor cell mobilization and function in diabetes. Diabetes 2007; 56: 666-674.

[12] Krankel N, Adams V, Linke A, Gielen S, Erbs S, Lenk K, Schuler G and Hambrecht R. Hyperglycemia reduces survival and impairs function of circulating blood-derived progenitor cells. Arterioscler Thromb Vasc Biol 2005; 25: 698-703.

[13] Chen YH, Lin SJ, Lin FY, Wu TC, Tsao CR, Huang PH, Liu PL, Chen YL and Chen JW. High glucose impairs early and late endothelial progenitor cells by modifying nitric oxide-related but not oxidative stress-mediated mechanisms. Diabetes 2007; 56: 1559-1568.

[14] Heiss C, Schanz A, Amabile N, Jahn S, Chen Q, Wong ML, Rassaf T, Heinen Y, Cortese-Krott M, Grossman W, Yeghiazarians Y and Springer ML. Nitric oxide synthase expression and functional response to nitric oxide are both important modulators of circulating angiogenic cell response to angiogenic stimuli. Arterioscler Thromb Vasc Biol 2010; 30: 2212-2218.

[15] Genuth S, Alberti KG, Bennett P, Buse J, Defronzo R, Kahn R, Kitzmiller J, Knowler WC, Lebovitz H, Lernmark A, Nathan D, Palmer J, Rizza R, Saudek C, Shaw J, Steffes M, Stern M, Tuomilehto J and Zimmet P. Follow-up report on the diagnosis of diabetes mellitus. Diabetes Care 2003; 26: 3160-3167.

[16] Falk W, Goodwin RH Jr and Leonard EJ. A 48-well micro chemotaxis assembly for rapid and accurate measurement of leukocyte migration. J Immunol Methods 1980; 33: 239-247.

[17] Heiss C, Wong ML, Block VI, Lao D, Real WM, Yeghiazarians Y, Lee RJ and Springer ML. Pleiotrophin induces nitric oxide dependent migration of endothelial progenitor cells. J Cell Physiol 2008; 215: 366-373.

[18] Babyak MA. What you see may not be what you get: a brief, nontechnical introduction to overfitting in regression-type models. Psychosomatic Medicine 2004; 66: 411-421.

[19] Harris MI, Flegal KM, Cowie CC, Eberhardt MS, Goldstein DE, Little RR, Wiedmeyer HM and Byrd-Holt DD. Prevalence of diabetes, impaired fasting glucose, and impaired glucose tolerance in U.S. adults. The Third National Health and Nutrition Examination Survey, 1988-1994. Diabetes Care 1998; 21: 518-524.

[20] Heiss C, Keymel S, Niesler U, Ziemann J, Kelm M and Kalka C. Impaired progenitor cell activity in age-related endothelial dysfunction. J Am Coll Cardiol 2005; 45: 1441-1448.

[21] MacCallum RC, Zhang SB, Preacher KJ and Rucker DD. On the practice of dichotomization of quantitative variables. Psychological Methods 2002; 7: 19-40.

[22] Giugliano D, Ceriello A and Esposito K. Glucose metabolism and hyperglycemia. Am J Clin Nutr 2008; 87: 217S-222S.

[23] Brownlee M. Biochemistry and molecular cell biology of diabetic complications. Nature 2001; 414: 813-820.

[24] Moldovan L, Mythreye K, Goldschmidt-Clermont PJ and Satterwhite LL. Reactive oxygen species in vascular endothelial cell motility. Roles of NAD(P)H oxidase and Rac1. Cardiovasc Res 2006; 71: 236-246.

[25] Marchetti V, Menghini R, Rizza S, Vivanti A, Feccia T, Lauro D, Fukamizu A, Lauro R and Federici M. Benfotiamine counteracts glucose toxicity effects on endothelial progenitor cell differentiation via Akt/FoxO signaling. Diabetes 2006; 55: 2231-2237.

[26] Potente M, Urbich C, Sasaki K, Hofmann WK, Heeschen C, Aicher A, Kollipara R, DePinho RA, Zeiher AM and Dimmeler S. Involvement of Foxo transcription factors in angiogenesis and postnatal neovascularization. J Clin Invest 2005; 115: 2382-2392.

[27] Keymel S, Kalka C, Rassaf T, Yeghiazarians Y, Kelm M and Heiss C. Impaired endothelial progenitor cell function predicts age-dependent carotid intimal thickening. Basic Res Cardiol 2008; 103: 582-586.

[28] Schmidt-Lucke C, Rossig L, Fichtlscherer S, Vasa M, Britten M, Kamper U, Dimmeler S and Zeiher AM. Reduced number of circulating endothelial progenitor cells predicts future cardiovascular events: proof of concept for the clinical importance of endogenous vascular repair. Circulation 2005; 111: 2981-2987.

[29] Mano R, Ishida A, Ohya Y, Todoriki H and Takishita S. Dietary intervention with Okinawan vegetables increased circulating endothelial progenitor cells in healthy young women. Atherosclerosis 2009; 204: 544-548.

[30] Heiss C, Jahn S, Taylor M, Real WM, Angeli FS, Wong ML, Amabile N, Prasad M, Rassaf T, Ottaviani JI, Mihardja S, Keen CL, Springer ML, Boyle A, Grossman W, Glantz SA, Schroeter H and Yeghiazarians Y. Improvement of endothelial function with dietary flavanols is associated with mobilization of circulating angiogenic cells in patients with coronary artery disease. J Am Coll Cardiol 2010; 56: 218-224.

[31] Kim W, Jeong MH, Cho SH, Yun JH, Chae HJ, Ahn YK, Lee MC, Cheng X, Kondo T, Murohara T and Kang JC. Effect of green tea consumption on endothelial function and circulating endothelial progenitor cells in chronic smokers. Circ J 2006; 70: 1052-1057.

[32] Huang PH, Chen YH, Tsai HY, Chen JS, Wu TC, Lin FY, Sata M, Chen JW and Lin SJ. Intake of red wine increases the number and functional capacity of circulating endothelial progenitor cells by enhancing nitric oxide bioavailability. Arterioscler Thromb Vasc Biol 2010; 30: 869-877.

[33] Hoetzer GL, Van Guilder GP, Irmiger HM, Keith RS, Stauffer BL and DeSouza CA. Aging, exercise, and endothelial progenitor cell clonogenic and migratory capacity in men. J Appl Physiol 2007; 102: 847-852.

[34] Sonnenschein K, Horvath T, Mueller M, Markowski A, Siegmund T, Jacob C, Drexler H and Landmesser U. Exercise training improves in vivo endothelial repair capacity of early endothelial progenitor cells in subjects with metabolic syndrome. Eur J Cardiovasc Prev Rehabil 2011; 18: 406-414.

[35] Lenk K, Uhlemann M, Schuler G and Adams V. Role of endothelial progenitor cells in the beneficial effects of physical exercise on atherosclerosis and coronary artery disease. J Appl Physiol 2011; 111: 321-328.

[36] Heiss C, Amabile N, Lee AC, Real WM, Schick SF, Lao D, Wong ML, Jahn S, Angeli FS, Minasi P, Springer ML, Hammond SK, Glantz SA, Grossman W, Balmes JR and Yeghiazarians Y. Brief secondhand smoke exposure depresses endothelial progenitor cells activity and endothelial function: sustained vascular injury and blunted nitric oxide production. J Am Coll Cardiol 2008; 51: 1760-1771.

[37] Kondo T, Hayashi M, Takeshita K, Numaguchi Y, Kobayashi K, Iino S, Inden Y and Murohara T. Smoking cessation rapidly increases circulating progenitor cells in peripheral blood in chronic smokers. Arterioscler Thromb Vasc Biol 2004; 24: 1442-1447.

[38] Park CS, Kim W, Woo JS, Ha SJ, Kang WY, Hwang SH, Park YW, Kim YS, Ahn YK and Jeong MH. Green tea consumption improves endothelial function but not circulating endothelial progenitor cells in patients with chronic renal failure. Int J Cardiol 2010; 145: 261-262.