

Vistafin as an Independent Predictor for Declined Numerous of Non-Classical Endothelial Progenitor Cells in Patients with Type 2 Diabetes Mellitus

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Abstract: *Background: The decreased number and impaired functions of endothelial progenitor cells (EPCs) may associate with cardiovascular disease including atherosclerosis. The role of vistafin in regulation of angiogenic EPC subset maturation in T2DM patients without known atherosclerosis is still not fully understood*

The aim of the study: *to investigate an association of serum vistafin level and number of circulating EPCs in T2DM patients beyond known CV disease.*

Methods: *This case-control observational investigation was retrospectively evolved 54 subjects with T2DM and 35 healthy volunteers. The criteria for exclusion were as follows: typical anginal symptoms, evidences of preexisting coronary artery disease, i.e. myocardial infarction / acute coronary syndrome, angiographic evidence of asymptomatic coronary atherosclerosis obtained by multi spiral contrast-enhanced tomography angiography provided prior study entry, heart failure, declined glomerular filtration rate < 60 mL/min/1.73 m², candidates for insulin therapy. The flow cytometry was used for predictably distinguishing cell subsets, which depend on expression of CD45, CD34, CD14, Tie-2, and VEGFR2. Biomarkers were measured at baseline of the study.*

Results: *All T2DM patients were divided depending median of vistafin level (5.88 ng/mL) in to two cohorts with low vistafin level (< 5.88 ng/mL; n=29) and high vistafin level (≥ 5.88 ng/mL; n=25) respectively. Multivariate regression analysis we found that serum vistafin level associated significantly with BMI, age, hs-CRP level, waist circumference, numbers of CD14⁺CD309⁺ EPCs, and numbers of CD14⁺CD309⁺Tie²⁺ EPCs. Logistic regression analysis has shown that vistafin, hs-CRP, age and BMI were the best variables in the prediction of EPC number labeled as CD14⁺CD309⁺ and CD14⁺CD309⁺Tie²⁺ cells. After adjustment of the model to age and BMI elevated vistafin level remained the best predictor for both CD14⁺CD309⁺ and CD14⁺CD309⁺Tie²⁺ EPCs (OR 0.92, 95% CI: 0.88–0.95; P=0.001 and OR 0.90, 95% CI: 0.87–0.96; P=0.001 respectively).*

In conclusion, *we found that elevated level of vistafin was an independent predictor for declined numerous of non-classical EPCs labeled as CD14⁺CD309⁺ and CD14⁺CD309⁺Tie²⁺, whereas CD34⁺ subsets of EPCs did not associate with vistafin level in T2DM individuals.*

Keywords: *Diabetes mellitus; vistafin; endothelial-derived progenitor cells; cardiovascular risk factors.*

1. INTRODUCTION

The prevalence of type 2 diabetes mellitus (T2DM) has increased over recent decades, reaching worldwide epidemic [1]. T2DM exhibits substantial impact on global health and requires increased expenditures of economic resources and medical care, particularly when the disease is associated with microvascular and macrovascular complications [2]. Recent studies have revealed that increased cardiovascular (CV) risk in T2DM is linked to impaired endothelial function, which accompanies to insulin resistance, inflammatory activation, dyslipidemia, glucose and lipid cytotoxicity even beyond asymptomatic atherosclerosis and known CV disease [3-5]. Moreover, vascular complications are leading causes of CV morbidity and mortality in diabetes [5]. However, the metabolic crosstalk between endothelium integrity, vascular function and the endothelial repair systems is largely unknown and innate molecular mechanisms regarding cooperation of above-mentioned factors remain incompletely understood [6].

Visceral adipose tissue (VAT) communicates with other organs by the synthesis and release of wide spectrum of adipocytokines that have a critical role in inflammation, insulin resistance, obesity, T2DM and CV disease [7, 8]. Visfatin (also known as nicotinamide phosphorybosil transferase) is an adipocytokine, which widely expressed in liver, skeletal muscles, bone marrow and predominantly secreted by white VAT [9]. Recently, elevated level of visfatin was found in overweight/obesity, T2DM, metabolic syndrome and CV disease [10, 11]. Circulating level of visfatin has revealed closely relation rather VAT mass and low-grade systemic inflammation than insulin resistance [12, 13]. It has been reported that visfatin might mimic the effects of insulin, mediates cell growth and differentiation, and exhibit a vascular protective capacities [14-16]. Indeed, visfatin is thought to regulate the process of differentiation bone marrow mononuclear and endothelial progenitor cells and thereby it contributes in endothelium reparation and tissue protection [17].

Endothelial progenitor cells (EPCs) is a heterogenic population of mononuclear cells expressing endothelial and progenitor markers i.e. CD34+ antigen and VEGFR-2+ vascular growth ligands (Vascular Endothelial Growth Factor Receptor-2), CD133+, as well as CD14+, and Tie2+ (tyrosine kinase ligand) [18]. EPCs contribute in tissue repair, angiogenesis, and revascularization [19]. EPCs are mobilized from bone marrow and probably from peripheral tissues upon injury in result in growth factors and inflammatory cytokines [20]. Decreased numerous of circulating EPCs was found a marker of impaired endothelial function and early atherosclerosis [21]. Therefore, increased production of reactive oxygen species, insulin resistance, reduced NO bioavailability have been identified as mediators of tissue injury leading to lowered EPC functionality and reduced angiogenic capacity *in vivo* [22-24]. Although decreased number and impaired functions of EPCs may associate with CV disease including atherosclerosis, the role of visfatin in regulation of angiogenic EPC subset maturation in T2DM patients without known atherosclerosis is still not fully understood. **The aim** of the study: to investigate an association of serum visfatin level and number of circulating EPCs in T2DM patients beyond known CV disease.

2. METHODS

2.1. Study Population

This case-control investigation was retrospectively evolved 54 subjects with T2DM and 35 healthy volunteers. The criteria for exclusion were as follows: [a] typical anginal symptoms, [b] evidences of preexisting coronary artery disease, i.e. myocardial infarction / acute coronary syndrome, [c] angiographic evidence of asymptomatic coronary atherosclerosis obtained by multi spiral contrast-enhanced tomography angiography provided prior study entry, [d] heart failure, [e] declined glomerular filtration rate $< 60 \text{ mL/min/1.73 m}^2$, [f] candidates for insulin therapy. All the patients have given their informed written consent for participation in the study. The study was approved by the local ethics committee of State Medical University, Zaporozhye, Ukraine. The study was performed in conformity with the Declaration of Helsinki.

T2DM was diagnosed with revised criteria provided by American Diabetes Association when source documents were reviewed [25]. When one or more of the following components were found (glycated hemoglobin [HbA1c] $\geq 6.5\%$; fasting plasma glucose $\geq 7 \text{ mmol/L}$; 2-h plasma glucose $\geq 11.1 \text{ mmol/L}$ during an oral glucose tolerance test; a random plasma glucose $\geq 11.1 \text{ mmol/L}$; exposure of insulin or oral antidiabetic drugs; a previous diagnosis of T2DM) T2DM was determined.

2.2. Smoking Status

Current smoking was defined as consumption of one cigarette daily for three months.

2.3. Anthropometric Measurements

Anthropometric measurements (body mass, waist and hip circumference) were made using standard procedures at baseline of the study.

2.4. Image of Coronary Arteries' Analysis

All scans of coronary arteries were interpreted by highly trained radiologists and one of sub-investigator independently each other before study entry. The identification of plaque and stenosis, as well as the amount of plaques per segment, was determined according conventional recommendation [26]. Plaques were recognized as abnormal structures in the artery wall that were within 1 mm² of the vessel lumen. Asymptomatic CAD was defined as the presence of any types of plaque (calcified, non-calcified, and mixed) in coronary arteries.

2.5. Cardiovascular Risk Calculation

A 10-year cardiovascular risk for study patients was calculated using the Framingham General Cardiovascular Risk Score (2008) by on-line calculator and interpreted using contemporary approaches [27].

2.6. Calculation of Glomerular Filtration Rate

Glomerular filtration rate (GFR) was calculated with CKD-EPI formula [28].

2.7. Blood Sampling

To determine circulating biomarkers, blood samples were collected at baseline in the morning (at 7-8 a.m.) into cooled silicone test tubes wherein 2 mL of 5% Trilon B solution were added. Then they were centrifuged upon permanent cooling at 6,000 rpm for 3 minutes. Plasma was collected and refrigerated immediately to be stored at a temperature -70°C.

2.8. Measurement of Circulating Biomarkers

Serum high-sensitive C-reactive protein (hs-CRP), adiponectin and vistafin were measured by high-sensitive enzyme-linked immunosorbent assays using commercial available kits (R&D Systems GmbH, Wiesbaden-Nordenstadt, Germany) according to the manufacturers' recommendations. The inter-assay coefficients of variation were as follows: hs-CRP: 4%, adiponectin: 5%, vistafin: 4.2%.

Fasting insulin level was measured by a double-antibody sandwich immunoassay (Elecys 1010 analyzer, F. Hoffmann-La Roche Diagnostics, Mannheim, Germany). The intra-assay and inter-assay coefficients of variation were <5%. The lower detection limit of insulin level was 1.39 pmol/L.

Concentrations of total cholesterol (TC), cholesterol of high-density lipoproteins (LDL-C), and cholesterol of high-density lipoproteins (HDL-C) were measured by enzymatic colorimetric method according standardized methodology on Beckman Synchron LX20 chemistry analyzer.

Direct Enzymatic HbA1c Assay was used for glycated hemoglobin A1c (HbA1c) measurements on Beckman Synchron LX20 chemistry analyzer.

2.9. Insulin Resistance Determination

Insulin resistance was assessed by the homeostasis model assessment for insulin resistance (HOMA-IR) [29] using the following formula:

$$\text{HOMA-IR} (\text{mmol/L} \times \mu\text{U/mL}) = \text{fasting glucose} (\text{mmol/L}) \times \text{fasting insulin} (\mu\text{U/mL}) / 22.5$$

IR was arbitrarily defined as a HOMA-IR value above the 75th percentile of normal glucose tolerance equal 2.45 mmol/L × μU/mL.

2.10. Blood Sampling for Measurement of Circulating Endothelial Progenitor Cells and Mononuclear Progenitor Cells

Blood samples were received from peripheral vein in blood collection tubes. Each sample contains 75 μL into 1mL PBS containing 5 μM EDTA (10 μL of 0.5 M stock). To prevent clotting samples were mixed immediately. Peripheral blood mononuclear cells were removed using density gradient centrifugation with Ficoll-Paque (Miltenyi Biotec Inc., Germany). After layer 35 mL of diluted cell suspension over 15 mL of Ficoll-Paque in a 50 mL conical tub all blood samples with anticoagulants

(EDTA) have centrifuged at $400\times g$ for 30–40 minutes at 20°C in a swinging-bucket rotor without brake. The upper layer leaving the mononuclear cell layer was aspirated to prevent a contamination of samples before measurement of real EPCs.

2.11. Assay of Circulating Endothelial Progenitor Cell and Mononuclear Progenitor Cell Subsets

The flow cytometric technique (FCT) was used for predictably distinguishing circulating cell subsets, which depend on expression of CD45, CD34, CD14, Tie-2, and CD309 (VEGFR2), using High-Definition Fluorescence Activated Cell Sorter (HD-FACS) methodology [30]. Accordingly, the cells were labeled on the basis of their forward scatter characteristic (FSC) and side scatter characteristic (SSC) profiles, and standardize and calibrate instruments, fluorescence and light scatter resolution, and sensitivity were determined accordingly standard protocol.

The cells were directly stained and analyzed for phenotypic expression of surface proteins using anti-human monoclonal antibodies, including anti-CD45 FITS (BD Biosciences, San Jose, CA, USA), anti-CD34 FITS (BD Biosciences), anti-VEGFR-2 known as anti-CD309 (BD Biosciences), anti-Tie2 (BD Biosciences) and anti-CD14 (BD Biosciences). The fluorescence minus one technique was used to provide negative controls and establish positive stain boundaries. After lysis of erythrocytes with UTILIZE wash solution, the samples were centrifuged at $200\times g$ for 15 min. Then the samples were washed twice with PBS and fixed immediately. Double- or triple-positive events were determined using Boolean principles (“and”, “not”, “or”, etc.).

2.12. Determination of Circulating Endothelial Progenitor Cells

Circulating EPCs were defined as CD34/ CD309 (VEGFR2) positive cells with lack of CD45 expression. From each tube 500,000 events were analyzed. For CD14+ populations, co-expression with Tie-2– and/or VEGFR-2– was determined using quadrant analysis. Standardized cell counts were presented as a percentage of the total of the white blood cells count, identified as the total number of all CD45+ cells. The FITC-labeled isotype control was analyzed with the same gate and window settings. The Fluorescence Minus One Control (FMO control) was used to properly interpret flow cytometry data. Figure 1 represents a sample of FACS results for CD45-/CD34+, CD309+/antiTie2-, CD14+/CD309+, CD14+/antiTie2- EPCs. FMO controls for CD14+/CD309+ is reported Figure 2.

Pro-angiogenic phenotype for EPCs was determined as CD14⁺CD309⁺ (VEGFR2) Tie-2⁺ antigen presentation. The reproducibility of EPC measurements using the standard protocol was 3.5%.

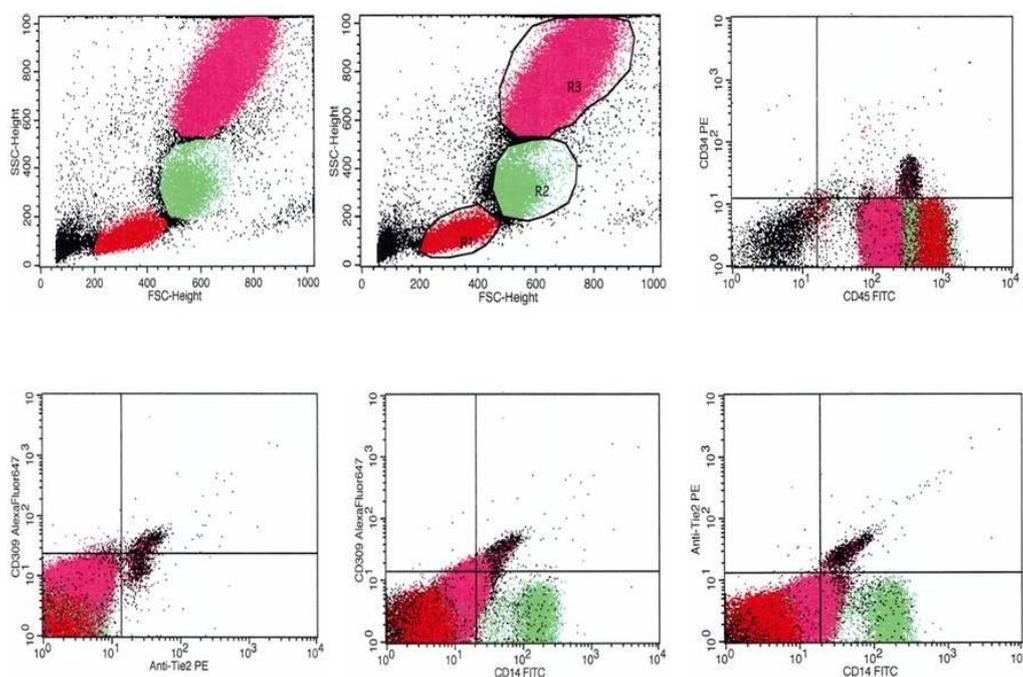


Figure1. The FACS Plots Present Results for CD45-/CD34+, CD309+/AntiTie2-, CD14+/CD309+, CD14+/AntiTie2- EPC Measurements

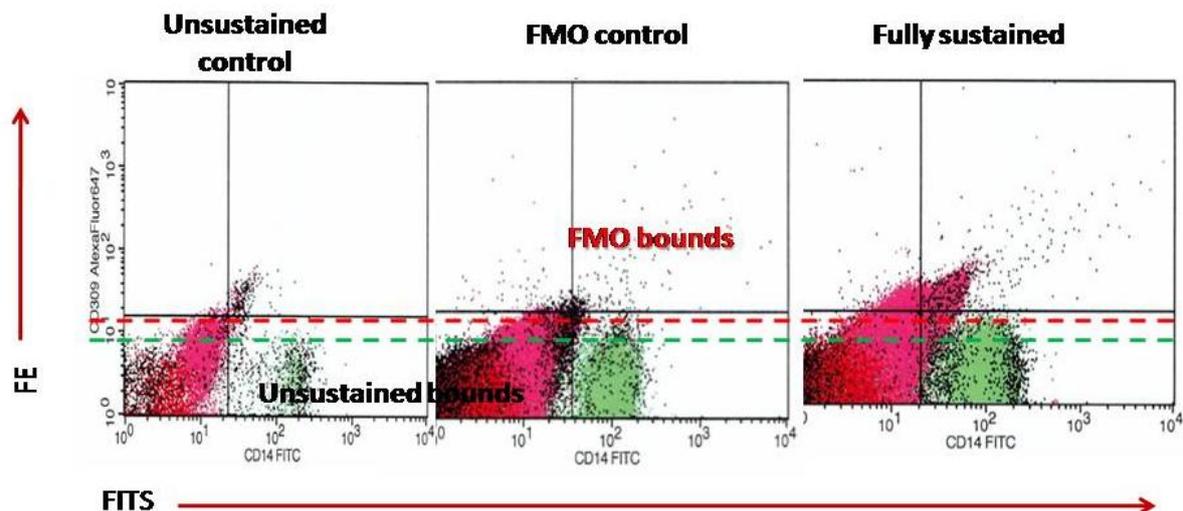


Figure2. FMO Controls for CD14+/CD309+ Measurement

3. STATISTICAL ANALYSIS

Statistical analysis of the results obtained was performed in SPSS system for Windows, Version 22 (SPSS Inc, Chicago, IL, USA). The data were presented as mean (M) and standard deviation (\pm SD) or 95% confidence interval (CI); as well as median (Me) and 25%-75% interquartile range (IQR). To compare the main parameters of patient cohorts, two-tailed Student t-test or Mann-Whitney U-test were used. To compare categorical variables between groups, Chi2 test (χ^2) and Fisher F exact test were used. The association between circulating visfatin and age, BMI, waist circumference, metabolic parameters, adiponectin, hs-CRP was done with univariate and multivariate regression analysis. β -coefficient and 95% CI were calculated for each variable. Goodness of fit for obtained regression models was assessed with the determination coefficient R^2 . Predictors of depleted EPCs in patients were examined in multivariate regression analysis. A two-tailed probability value of <0.05 was considered as significant.

4. RESULTS

General characteristic of patients participating in the study was reported in Table 1. The mean age for patients with T2DM and healthy volunteers was 48.50 years and 46.12 years ($P=0.68$). Therefore 63.0% of T2DM patients and 65.7% of healthy volunteers were men ($P=0.86$). There was a significant difference between healthy volunteers and entire cohort of T2DM patients in BMI, waist circumference, CV risk factors (hypertension, dyslipidemia, adherence to smoking), Framingham risk score, HOMA-IR, and lipid abnormalities. There were higher levels of HbA1c, fasting blood glucose, insulin, hs-CRP, TG, and vistafin in T2DM patient compared with healthy volunteers. Contrary, adiponectin level has defined lower in T2DM than in healthy volunteers. There was no significant difference in number of CD34+ subset cells (CD45+CD34+ and CD45-CD34+) between T2DM patients and healthy volunteers. However, decreased numerous of non-classical EPCs labeled as CD14+ subsets (CD14+CD309+ and CD14+CD309+Tie2+) were found in T2DM compared with healthy volunteers ($P=0.001$ for all cases).

Table1. General characteristic of patients participating in the study

	Healthy volunteers (n=35)	Entire cohort of T2DM patients (n=54)	P value
Age, years	46.12 \pm 4.22	48.50 \pm 6.60	0.68
Males, n (%)	23 (65.7%)	34 (63.0%)	0.86
BMI, kg/m ²	21.5 (16.1-23.5)	28.5 (16.8-32.1)	0.001
Waist circumference, sm	78 (63-89)	98 (89-110)	0.001
Hip circumference (cm)	87 (83- 91)	106 (102-113)	0.001
Hypertension, n (%)	-	36 (66.7%)	0.001
Dyslipidemia, n (%)	-	33 (61.1%)	0.001
Adherence to smoking, n (%)	6 (17.1%)	15 (27.7%)	0.001

Framingham risk score	2.55±1.05	8.18 ± 2.32	0.001
Systolic BP, mm Hg	122±5	136±5	0.001
Diastolic BP, mm Hg	72±4	86±4	0.001
Heart rate, beats per 1 min.	66±6	72±5	0.01
GFR, mL/min/1.73 m ²	102.1 (91.4–113.2)	93.8 (80.4–106.8)	0.12
HbA1c, %	4.75 (4.36–5.12)	7.3 (4.3–9.1)	0.001
Fasting blood glucose, mmol/L	4.52 (4.43–4.76)	5.54 (4.49–9.0)	0.01
Insulin, μU/mL	4.98 (1.5–14.1)	15.6 (12.9–16.8)	0.001
HOMA-IR, mmol/L × μU/mL	1.01 (0.91–1.07)	3.86 (3.41–4.10)	0.001
Creatinine, μmol/L	62.1 (55.7–82.4)	71.2 (59.9–87.2)	0.24
Total cholesterol, mmol/L	4.76 (4.21–5.05)	5.4 (4.8–5.8)	0.001
LDL-C, mmol/L	3.10 (2.78–3.21)	3.80 (3.20–4.20)	0.001
HDL-C, mmol/L	1.13 (1.05–1.17)	0.94 (0.88–1.04)	0.001
TG, mmol/L	1.18 (1.07–1.30)	1.45 (1.42–1.51)	0.001
hs-CRP, mg / L	4.11 (0.97 – 5.03)	8.10 (4.80 – 9.54)	0.001
Adiponectin, mg / L	13.65 (10.12–24.93)	6.17 (3.44–10.15)	0.001
Vistafin, ng/mL	3.67 (1.85–4.50)	5.88 (3.15 – 8.15)	0.001
CD45 ⁺ CD34 ⁺ , cells × 10 ³ /μL	0.114 (0.095–0.120)	0.112 (0.090–0.121)	0.76
CD45 ⁻ CD34 ⁺ , cells × 10 ⁻¹ /μL	0.06 (0.05–0.07)	0.057 (0.053–0.067)	0.86
CD14 ⁺ CD309 ⁺ , cells × 10 ⁻¹ /μL	4.26 (3.70–5.74)	2.96 (2.25–4.21)	0.001
CD14 ⁺ CD309 ⁺ Tie ²⁺ , cells × 10 ⁻¹ /μL	0.465 (0.253–0.710)	0.270 (0.241–0.411)	0.001

Note. Data are presented as mean and ±SE or 95% CI; median and 25-75% IQR. Categorical variables are expressed as numerous (n) and percentages (%). P-value is a comparison of mean or median variables between both cohorts (ANOVA test).

Abbreviations. CI – confidence interval; IQR – inter quartile range; BMI - Body mass index, T2DM – type two diabetes mellitus, TG – triglycerides, BP – blood pressure, BMI - Body mass index, GFR - glomerular filtration rate, HDL-C - high-density lipoprotein cholesterol, LDL-C - Low-density lipoprotein cholesterol, hs-CRP – high sensitive C reactive protein.

Patients with T2DM were treated with life-style modification, diet and orally taken antidiabetic drugs except sulfonylurea derivates and glitazones. Metformin in monotherapy or in combination with dipeptidyl peptidase-4 inhibitors or glucagon-like peptide-1 receptor agonists was given in individually optimized daily doses to be achieving full or partly full control for T2DM. Therefore, insulin was not used in T2DM patients.

All T2DM patients were divided depending median of vistafin level (5.88 ng/mL) in to two cohorts with low vistafin level (< 5.88 ng/mL; n=29) and high vistafin level (≥ 5.88 ng/mL; n=25) respectively. Figure 3 is reported the circulating vistafin level in both T2DM cohorts in comparison with healthy volunteers.

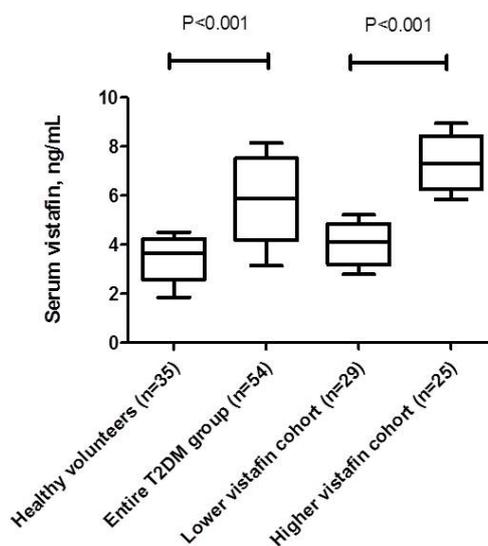


Figure3. Circulating Vistafin Level in Both T2DM Cohorts in Comparison with Healthy Volunteers

Values are reported as median and IQR, and were compared using ANOVA.

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The line within the box represents the median value; the top and bottom lines of the box reflect the 25th and 75th percentile respectively; the top and bottom vertical lines outside of the boxes represent 10th and 90th percentile respectively.

Abbreviations: ANOVA – analysis of variance, IQR - interquartile range

There were not significant differences between both cohorts of T2DM subjects with low and high vistafin levels in age, sex, hip circumference, CV risk factors (incidence of dyslipidemia, hypertension, and adherence to smoking), Framingham risk score, blood pressure, HbA1c, fasting blood glucose, insulin, HOMA-IR, creatinine, adiponectin, total cholesterol, HDL-C and LDL-C (Table 2). Increased BMI, waist circumference, triglyceride and hs-CRP concentrations were found in T2DM patients with high vistafin level than in subjects with low vistafin level.

Table 2. Demographic, Risk Factors, Blood Pressure, Circulating Biomarkers, and Number of Epcs in T2DM Patients Depending Vistafin Level

Variables	Low vistafin level (n=29)	High vistafin level (n=25)	P value
Age, years	49.10±5.90	48.30±5.70	0.92
Males, n (%)	18 (62.0%)	16 (64.0%)	0.98
BMI, kg/m ²	24.3 (16.9–28.6)	29.1 (25.3–32.5)	0.042
Waist circumference, sm	100 (90–108)	106 (97–115)	0.046
Hip circumference (cm)	106 (103–110)	107 (102–114)	0.72
Hypertension, n (%)	19 (65.5%)	17 (68.0%)	0.78
Dyslipidemia, n (%)	16 (55.2%)	17 (68.0%)	0.84
Adherence to smoking, n (%)	8 (27.5%)	7 (28.0%)	0.82
Framingham risk score	8.10± 2.14	8.25 ± 2.15	0.80
Systolic BP, mm Hg	135±5	137±6	0.94
Diastolic BP, mm Hg	87±4	88±5	0.96
Heart rate, beats per 1 min.	73±5	74±4	0.96
GFR, mL/min/1.73 m ²	94.2 (85.1–106.1)	92.7 (83.5–107.4)	0.66
HbA1c, %	7.2 (4.4–8.8)	7.4 (4.5–9.3)	0.46
Fasting blood glucose, mmol/L	5.49 (4.29–8.11)	5.57 (4.50–8.6)	0.48
Insulin, µU/mL	15.4 (12.6–15.9)	15.6 (12.9–17.1)	0.88
HOMA-IR, mmol/L × µU/mL	3.78 (3.39–4.03)	3.89 (3.40–4.15)	0.62
Creatinine, µmol/L	70.6 (57.2–88.5)	72.1 (58.3–90.6)	0.88
Total cholesterol, mmol/L	5.3 (4.6–5.9)	5.4 (4.8–5.8)	0.86
LDL-C, mmol/L	3.78 (3.20–4.15)	3.82 (3.30–4.20)	0.72
HDL-C, mmol/L	0.95 (0.90–1.13)	0.94 (0.87–1.11)	0.14
TG, mmol/L	1.25 (1.15–1.32)	1.41 (1.32–1.50)	0.044
hs-CRP, mg / L	7.20 (3.96 – 9.80)	9.04 (5.30 – 11.24)	0.024
Adiponectin, mg / L	6.19 (3.37–10.12)	6.14 (3.49–10.23)	0.82
Vistafin, ng/mL	4.12 (2.85 – 5.21)	7.30 (5.90 – 8.50)	0.001
CD45 ⁺ CD34 ⁺ , cells × 10 ³ /µL	0.114 (0.090–0.117)	0.110 (0.087–0.122)	0.26
CD45 ⁺ CD34 ⁺ , cells × 10 ⁻¹ /µL	0.06 (0.055–0.069)	0.055 (0.051–0.065)	0.16
CD14 ⁺ CD309 ⁺ , cells × 10 ⁻¹ /µL	3.10 (2.31–4.33)	2.89 (2.21–4.03)	0.001
CD14 ⁺ CD309 ⁺ Tie ²⁺ , cells × 10 ⁻¹ /µL	0.290 (0.250–0.410)	0.259 (0.236–0.388)	0.012

Note. Data are presented as mean and ±SE or 95% CI; median and 25–75% IQR. Categorical variables are expressed as numerous (n) and percentages (%). P-value is a comparison of mean or median variables between both cohorts (ANOVA test).

Abbreviations. CI – confidence interval; IQR – inter quartile range; BMI - Body mass index, T2DM – type two diabetes mellitus, BP – blood pressure, BMI - Body mass index, GFR - glomerular filtration rate, EPCs – endothelial progenitor cells; TG – triglycerides, HDL-C - high-density lipoprotein cholesterol, LDL-C - Low-density lipoprotein cholesterol, hs-CRP – high sensitive C reactive protein.

Similarities in numerous of CD34⁺ EPCs were determined in both T2DM cohorts. Contrary, CD14⁺CD309⁺ and CD14⁺CD309⁺Tie²⁺ subsets of circulating EPCs were determined in higher concentration among T2DM subjects with low vistafin level versus individuals with high vistafin level (P=0.001 and P=0.012 respectively).

Univariate and Multivariate Regression Analysis

In entire T2DM group univariate regression analysis has shown statistically significant associations between serum visfatin level and BMI ($\beta = 2.36$, 95% CI = 1.92 to 2.96; $p = 0.001$), waist circumference ($\beta = 1.98$, 95% CI = 1.06 to 2.78; $p = 0.001$), hs-CRP level ($\beta = 3.22$, 95% CI = 1.92 to 5.16; $p = 0.001$), HOMA-IR ($\beta = 8.22$, 95% CI = 7.12 to 10.12; $p = 0.001$), numbers of CD14⁺CD309⁺ EPCs ($\beta = -3.15$, 95% CI = -5.17 to -1.75; $p = 0.001$), adiponectin ($\beta = -1.90$, 95% CI = -2.55 to -1.70; $p = 0.001$), numbers of CD14⁺CD309⁺Tie²⁺ ($\beta = -2.96$, 95% CI = -4.33 to -1.47; $p = 0.001$), and age ($\beta = -1.27$, 95% CI = -1.72 to -0.88; $p = 0.001$).

In T2DM cohort with low visfatin level there were significant associations between serum visfatin level and BMI ($\beta = 3.06$, 95% CI = 2.10 to 4.20; $p = 0.001$), waist circumference ($\beta = 2.15$, 95% CI = 1.44 to 2.80; $p = 0.001$), hip circumference ($\beta = 1.76$, 95% CI = 1.30 to 2.25; $p = 0.001$), hs-CRP level ($\beta = 2.65$, 95% CI = 1.88 to 3.70; $p = 0.001$), HOMA-IR ($\beta = 12.50$, 95% CI = 6.90 to 19.50; $p = 0.001$), adiponectin ($\beta = -2.38$, 95% CI = -2.90 to -1.86; $p = 0.001$), age ($\beta = -2.36$, 95% CI = -3.55 to -1.13; $p = 0.001$), numbers of CD14⁺CD309⁺ EPCs ($\beta = -1.03$, 95% CI = -1.22 to -0.16; $p = 0.001$), and numbers of CD14⁺CD309⁺Tie²⁺ ($\beta = -2.15$, 95% CI = -2.67 to -1.62; $p = 0.001$).

In high visfatin level individuals with T2DM we found significant association with serum visfatin level and BMI ($\beta = 3.19$, 95% CI = 2.23 to 4.34; $p = 0.001$), hs-CRP level ($\beta = 4.12$, 95% CI = 2.15 to 6.55; $p = 0.001$), waist circumference ($\beta = 2.37$, 95% CI = 1.90 to 3.05; $p = 0.001$), numbers of CD14⁺CD309⁺ EPCs ($\beta = -2.11$, 95% CI = -3.15 to -1.21; $p = 0.001$), and numbers of CD14⁺CD309⁺Tie²⁺ ($\beta = -2.17$, 95% CI = -3.80 to -0.86; $p = 0.001$).

Multivariate regression analysis we found that serum visfatin level associated significantly with BMI, age, hs-CRP level, waist circumference, numbers of CD14⁺CD309⁺ EPCs, and numbers of CD14⁺CD309⁺Tie²⁺ EPCs (Table 3).

Table 3. Association between Serum Visfatin Level and Age, BMI, Metabolic Parameters, Numbers of Epc in Entire Group of T2DM and Separated Cohorts. The Results of Multivariable Regression Analysis

Variables	Entire T2DM group (n=54)			Low visfatin level cohort (n=29)			High visfatin level cohort (n=25)		
	β	95% CI	P value	β	95% CI	P value	β	95% CI	P value
BMI, kg/m ²	2.12	1.88 to 2.58	0.001	2.15	1.72 to 2.44	0.003	2.21	1.65 to 2.70	0.001
Waist circumference, sm	2.21	1.59 to 3.31	0.001	2.25	1.53 to 3.37	0.002	2.28	1.82 to 3.23	0.001
Age, years	-1.30	-1.66 to -1.02	0.001	-1.18	-1.46 to -0.85	0.001	-1.25	-1.51 to -1.10	0.001
hs-CRP level, mg / L	2.90	1.94 to 3.96	0.001	2.33	1.58 to 3.12	0.002	2.86	1.60 to 4.10	0.001
CD14 ⁺ CD309 ⁺ , cells $\times 10^{-1}/\mu\text{L}$	-2.56	-4.20 to -1.15	0.001	-2.40	-4.12 to -1.08	0.001	-2.62	-4.30 to -1.10	0.001
CD14 ⁺ CD309 ⁺ Tie ²⁺ , cells $\times 10^{-1}/\mu\text{L}$	-2.60	-4.15 to -0.33	0.001	-2.23	-3.80 to -0.66	0.001	-2.57	-3.70 to -0.90	0.001

Abbreviations. T2DM, Type 2 Diabetes Mellitus; CI, 95% Confidence Interval; BMI, Body Mass Index

The age- and BMI-adjusted correlation coefficients of serum visfatin and hs-CRP with other studied variables in T2DM patients are reported in Table 4. Visfatin correlates significantly with CD14⁺CD309⁺ cells, CD14⁺CD309⁺Tie²⁺ cells, hs-CRP and adiponectin. Therefore, there is weak correlation between hs-CRP and numerous of CD14⁺CD309⁺ and CD14⁺CD309⁺Tie²⁺ cells, adiponectin, HOMA-IR, and hip circumference.

Table 4. The Age- and BMI-Adjusted Correlation Coefficients of Serum Visfatin and Hs-CRP with other Studied Variables in T2DM Patients

Variables	Visfatin		hs-CRP	
	R ²	P Value	R ²	P Value
Waist circumference, sm	0.11	0.015	0.10	0.046
Hip circumference, sm	0.10	0.012	0.11	0.044

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hs-CRP, mg/L	0.16	0.012	1.00	0.904
Vistafin, ng/mL	1.00	0.892	0.16	0.012
Adiponectin, mg / L	0.12	0.046	0.12	0.05
HOMA-IR, mmol/L × μU/mL	0.10	0.044	0.11	0.042
CD14 ⁺ CD309 ⁺ , cells × 10 ⁻¹ /μL	0.17	0.001	0.12	0.044
CD14 ⁺ CD309 ⁺ Tie ²⁺ , cells × 10 ⁻¹ /μL	0.18	0.001	0.13	0.010

Abbreviations. *CI*, Confidence Interval; *BMI*, Body Mass Index; *Epcs*, Endothelial Progenitor Cells; *Hs-CRP*, High Sensitive C Reactive Protein

Logistic regression analysis was employed to predictors of circulating numbers of CD14⁺ EPCs (Table 5). Vistafin, hs-CRP, age and BMI were the best variables in the prediction of EPC number labeled as CD14⁺CD309⁺ and CD14⁺CD309⁺Tie²⁺ cells. After adjustment of the model to age and BMI elevated vistafin level remained the best predictor for both CD14⁺CD309⁺ and CD14⁺CD309⁺Tie²⁺ EPCs (OR 0.92, 95% CI: 0.88–0.95; P=0.001 and OR 0.90, 95% CI: 0.87–0.96; P=0.001 respectively).

Table 5. Logistic Regression Analysis Demonstrating the Predictors Of EPC Numbers in T2DM Patients

Factors	CD14 ⁺ CD309 ⁺ EPCs		CD14 ⁺ CD309 ⁺ Tie ²⁺ EPCs	
	OR (95% CI)	P Value	OR (95% CI)	P Value
BMI per 5.0 kg/m ²	0.95 (0.92–0.98)	0.012	0.96 (0.90–0.99)	0.014
Waist circumference per 5.5 sm	0.99 (0.92–1.04)	0.564	0.97 (0.94–1.02)	0.626
Hip circumference per 5.0 sm	0.96 (0.90–1.00)	0.142	0.96 (0.91–1.01)	0.448
Age per 5 years	0.95 (0.92–0.97)	0.016	0.96 (0.90–0.98)	0.044
hs-CRP per 4.50 mg/L	0.95 (0.93–0.98)	0.001	0.95 (0.91–0.99)	0.028
Vistafin per 2.5 ng/mL	0.94 (0.90–0.97)	0.001	0.91 (0.87–0.98)	0.012
Adiponectin per 4.5 mg / L	0.96 (0.94–1.00)	0.246	0.96 (0.92–1.01)	0.559
HOMA-IR per 0.65 mmol/L × μU/mL	0.97 (0.92–1.02)	0.489	0.98 (0.95–1.02)	0.632
Constant	0.09	0.012	0.08	0.001

Abbreviations. *CI*, confidence interval; *BMI*, Body mass index; *EPCs*, endothelial progenitor cells; *hs-CRP*, high sensitive C reactive protein.

5. DISCUSSION

The results of the study exhibit that elevated level of vistafin in T2DM individuals predicts decreased number of non-classic angiopoetic EPCs labeled as CD14⁺CD309⁺ and CD14⁺CD309⁺Tie²⁺ beyond conventional CV risk factors. We confirmed that increased BMI, waist circumference, triglyceride, age, adiponectin, and hs-CRP were the variables that appeared to be significantly associated with vistafin level in T2DM. Although both biomarkers vistafin and hs-CRP correlated well with number of CD14⁺ EPCs, in age- and BMI-adjusted model vistafin has exhibited the best predictive discriminant for decreased angiopoetic EPCs.

Diabetes-induced EPC dysfunction is a critical factor in microvascular and macrovascular complications [31–33]. Glucose toxicity and lipid toxicity impair endothelial nitric oxide synthase activity as well as enhances the production of reactive oxygen species, leading to endothelial dysfunction and pro-atherogenetic alterations [32, 33]. Angiopoetic EPCs were found as component of endogenous repair system that plays a pivotal role in endothelial function restore, inducing angiogenesis, and neovascularization [33].

Recent studies have demonstrated controversial findings regarding an association of vistafin level with BMI, insulin, glucose, hs-CRP, or HOMA-IR [15, 34, 35]. Because serum level of vistafin in non-CV complicated T2DM patients was higher than in diabetics with known CV disease [36], it has been suggested that vistafin might have a positive effect of repair system and prevent of microvascular and macrovascular complications [17]. Indeed, vistafin has antiapoptotic activity and has a regulatory role in inflammation, regulation of glucose homeostasis, lipid profile and immunity [35, 37, 38]. Probably, vistafin via insulin-mimicking mechanism might play a positive role in attenuating insulin resistance [39]. It can be expected that vistafin would be attenuated number and functionality of angiopoetic EPCs in T2DM without known CV disease. Unfortunately, the results of the study did not confirm the suggestion. As an explanation of the finding it has deemed that decreased number of non-classical EPCs with angiopoetic activity might be an effect of inflammatory cytokines, such as CRP. Recently it has been found a closely association between hs-CRP levels,

oxidative stress and insulin resistance in obese individuals and patients with T2DM beyond CV disease [40, 41]. The data obtained in the study suggested a negative correlation of the hs-CRP level with numerous of EPCs and positive correlation with vistafin.

It could be questioned why there is a duality in expected effect of visfatin in T2DM? Despite elevated vistafin might positively modulate EPC differentiation via its proliferative capacity leading to increased endogenous repair ability of endothelium [42], the elevation of inflammatory cytokines, oxidative stress and insulin resistance as systemic factors could contribute in functionality and ability to differentiation of progenitor cells differenced origin including EPCs [43]. Therefore, it cannot exclude that vistafin is able to directly induce apoptotic effect and the cellular expression of inflammatory cytokines such as TNF-alpha, IL-1beta, and IL-6 in target cells [44, 45].

Nevertheless, vistafin expression is up-regulated in the persistence of inflammation that is suitable for older age, T2DM and obesity [46, 47]. Through regulation of cellular levels of NAD vistafin impacts not only cellular energetics but also NAD-dependent enzymes, such as sirtuins, which may attenuate cell survival via NF- κ B-dependent transcription [44, 47]. Obviously, vistafin has functional importance for EPCs and probably its cellular microenvironment as a growth factor mediating proliferation and differentiation [48]. Finally, vistafin might be found as adipokine expressed antagonistic effect on angiopoietic EPCs independently insulin resistance, glycemic control, metabolic factors, as it has been elucidated in the study [49].

Taken together, it has been suggested that vistafin could contribute to impaired diabetic reparation including impaired neovascularization and deficient EPC recruitment. Most importantly, vistafin leads to diabetes-induced EPC dysfunction in closely relation to systemic inflammation, although the underlying molecular mechanisms of vistafin might be contribute in increase of repair ability of EPCs. Further investigations are suggested on visfatin functions, signaling, and clinical significance, as well as determinants of serum visfatin level to elucidate whether this molecule can be a candidate in biomarker of T2DM progression and CV event development.

6. STUDY LIMITATIONS

This study has some limitations. The distribution of body fat and its visceral deposits were not directly assessed. Whether is interrelation between VAT, vistafin and deficiency of EPCs with angiopoietic activity is not defined and it requires further investigations. Therefore, the causal relationship between vistafin and functionality of EPCs could not be determined because we investigated number of circulating EPCs. Yet, an effect of glycemic control on predictive value of vistafin on decreased EPC count needs to be determined in future studies. Finally, authors believe that a greater cohort of patients with more incidences detected is desirable to improve the credibility of the study.

7. CONCLUSION

we found that elevated level of vistafin was an independent predictor for declined numerous of non-classical EPCs labeled as CD14⁺CD309⁺ and CD14⁺CD309⁺Tie²⁺, whereas CD34⁺ subsets of EPCs did not associate with vistafin level in T2DM individuals.

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AUTHORS' CONTRIBUTIONS

Alexander E Berezin initiated the hypothesis and designed the study protocol, contributed to collect, analyze and interpret the data, performed statistical analysis, wrote the manuscript and approved final version of the paper. Tatyana A Samura collected, analyzed and interpreted the image scans, performed statistical analysis. Alexander A. Kremzer contributed to enroll the patients; collected and analyzed the data reviewed the source documents. Tatyana A. Berezina contributed to enroll the patients in the study and collect the data. Martovitskaya YuV and Elena A Gromenko contributed in blood sampling, performing of biomarker measurements with ELISA and cytofluometry, and interpreted of the obtained results.

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