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Association of growth-differentiation factor-15 with the number of circulating proangiogenic endothelial progenitor cells in patients with type 2 diabetes mellitus

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Abstract

The objective: to investigate the relationship between levels of growth differentiation factor-15 (GDF-15) and circulating number of endothelial progenitor cells (EPCs) with angiopoietin phenotypes: CD34+CD14+CD309+, and CD34+CD14+CD309+Tie2+ in patients with type 2 DM. **Methods:** The study was retrospectively involved 76 patients with type 2 DM aged 38 to 55 years and 30 healthy volunteers. Data collection included demographic and anthropometric information, hemodynamic performances and biomarkers of the diseases. Flow cytometry was used to determine EPCs' populations. **Results**: The levels of GDF-15 in peripheral blood of diabetics associated with age (r = 0.31, P = 0.044), high-sensitive C-reactive protein [hs-CRP] (r = 0.40, P = 0.001), smoking (r = 0.38, P = 0.001), body mass index [BMI] (r = 0.34, P = 0.001), LDL cholesterol (r = 0.28, P = 0.001), glycated hemoglobin [HbA1c] (r = -0.28, P = 0.001), number of CV risk factors (r = 0.26, P = 0.001). In univariate logistic regression analysis we found that level of GDF-15 ≥ 618 pg/mL, hs-CRP ≥7.12 mg/L, HbA1c ≥6.4%, fasting glucose ≥6.7 mmol/L, and BMI ≥27.3 kg/m2 predicted deficiency of both angiopoetic phenotypes of EPCs. In multivariate logistic regression model GDF-15 ≥618 pg/mL demonstrated the best odds ratio values for declining of EPCs in diabetics in comparison with other predictors including BMI, HbA1c and hs-CRP. **Conclusion**: GDF-15 was remarkably evaluated in type 2 DM population to healthy volunteers, and it was an independent factor that contributes to mobilization and probably proliferation of endothelial precursors with high angiopoetic activity.

1. Introduction

Prevalence of diabetes mellitus (DM) increases rapidly worldwide reaching pandemic proportion in developing and developed countries [1–3]. The impact of DM on the public health strong associated with vascular complications, which predominantly determine morbidity and mortality from the disease [4]. Indeed, DM regardless of phenotypes exhibit close link with atherosclerosis, stable ischemic heart disease, myocardial infarction / acute coronary syndrome, peripheral artery disease (PAD), retinopathy, stroke, heart failure chronic kidney disease and occur death prematurely due to cardiovascular (CV) causes [5,6]. Despite being well-designed programs for lifestyle modification, glycemic, lipids and blood pressure control patients with type 2 DM (T2DM) have demonstrated 2-8-fold higher CV mortality rate to those who did not have T2DM [7,8]. In this context, early stratification of people with diabetes at high risk of vascular complication based on biomarker assay was recognized as promising [9]. Because developing of T2DM associated with glycaemia, insulin resistance, overweight / abdominal obesity, adipocytokine dysfunction, and other co-existing abnormalities (mitochondrial energy deficiency, hypercoagulation, oxidative stress, protein glycation, immune responses, inflammatory activity, some hormonal changes and metabolic memory phenomenon) [10,11], there needs a biological marker reflecting several faces of pathophysiological process and corresponding to metabolic syndrome and T2DM.

Growth differentiation factor-15 (GDF-15) belongs to the transforming growth factor-beta/bone morphogenetic protein superfamily that involves in the pathogenesis of several diseases (CV disease, infective and inflammatory bowel diseases, metabolic diseases, rheumatic and autoimmune diseases) and it was recently found an independent predictor of all-cause and CV mortality in general population and T2DM patients [12–14]. The levels of circulating GDF-15 were positively related to traditional CV risk factors (age, smoking, hypertension, DM, body mass index), non-traditional CV risk factors (uric acid, pro-inflammatory cytokines, oxidative stress biomarkers, telomere length) [15]. GDF-15 releases from broad spectrum of the cells (cardiomyocytes, endothelial cells, adipocytes, macrophages / mononuclear, vascular smooth muscle cells, astrocytes) due to direct pro-inflammatory cytokine stimulation and after tissue injury and hypoxia [16]. Most of the investigators reported that GDF-15 plays a pivotal role in the protection of tissue from different injuries, although the innate molecular mechanisms of the effect remain unclear. The hypothesis of the study based on the assumption that GDF-15 could regulate vascular function and restores endothelial integrity through mobilization of endothelial progenitor cells (EPCs) with angiopoietin phenotypes and thereby mediate tissue repair activity.

According to molecular characteristics, EPCs may express appropriate surface antigens, such as CD31, CD 144, CD309 (vascular endothelial growth factor receptor-2), and CD133, but in the absence of CD45. The CD45(-) cells, which are detected and isolated based on single or combined expression of CD34, CD133 and CD309, referred to as EPCs [17], while after differentiation EPCs lose CD133 antigen and begin to be positively on CD31, vascular endothelial cadherin, endothelial NO synthase (eNOS) and von Willebrand factor [18]. Depending on ability to appear in the fibronectin-coated dish all EPCs were divided into early outgrowth or late outgrowth endothelial cells. Interestingly, the late outgrowth precursors originated from peripheral blood, and ex vivo demonstrated appropriate immune phenotype CD31+CD146+CD105+CD309+ and Tei2 and functional properties suitable mature endothelial cells. There are two distinct populations of late outgrowth progenitor cells based on differential expression of the cell surface marker CD34. The population of EPCs with co-expression of CD34 antigen additionally to CD31(+), CD146(+), CD105(+), and CD309(+) exhibited higher proliferative capability and angiopoietin activity to CD34(-) EPCs [19]. There is evidence regarding that the absence of CD34(+) EPC in the colony led to cultures collapsed within one or two passages that confirm an idea of strong hierarchy in self-renewal EPCs may be an essential functional feature of precursors. Finally, late outgrowth precursors may differentiate into functionally mature endothelial cells and progenitor-like angiogenesis-promoting cells (CD34+ EPCs).

Numerous clinical studies have shown the lowered number and weak function of CD34+ EPCs in patients with pre-diabetes and T2DM [20–22]. Moreover, vascular complications of DM including retinopathy and atherosclerosis, muscular related to declined circulating the number of CD34+ EPCs with high proliferative capacity and ability to restore vascular integrity and function [23]. Thus, GDF-15 and CD34+EPCs could coordinate vascular repair and restore endothelial function in diabetics. This study aimed to investigate the relationship between levels of GDF-15 and circulating number of EPCs with proliferative and angiopoietin phenotypes: $CD34^{+}CD14^{+}CD309^{+}$, and $CD34+CD14^{+}CD309^{+}Tie2^{+}$ in patients with T2DM without known CV diseases.

2. Methods

The study cohort consisted of 76 patients with type 2 DM (41 males) aged 38 to 55 years who were retrospectively involved between March 2014 and July 2017. All DM patients included in the study have no known CV diseases including angina pectoris, previous myocardial infarction/stroke, heart failure, and asymptomatic atherosclerosis (defined by the negative result of the contrast-enhanced multiple spiral tomography angiography). Apart from established CV disease, the criteria of non-inclusion were acute infections; active inflammation; pulmonary edema; tachyarrhythmia; valvular heart disease; thyrotoxicosis; ischemic stroke; intracranial hemorrhage; surgery; trauma, autoimmune disease, malignancy, before the study entry. As a control cohort, we enrolled 30 healthy volunteers matched for age and sex with type 2 DM patients.

(a) Ethical declaration

All the patients have given their written informed consent for participation in the study. The study was approved by the Local Ethical Committee. The investigators followed strictly all the requirements to clinical trials in conformity with the World Medical Association Declaration of Helsinki, 1964, good clinical practice provided by International Conference on Harmonization, Council of Europe Convention for the Protection of Human Rights and Dignity of the Human Being in view of using achievements in biology and medicine, convention on Human Rights and Biomedicine, including Additional Protocol to the Convention on Human Rights and Biomedicine, concerning Biomedical Research, and legislation of Ukraine.

Diagnosis of T2DM was checked and confirmed according to the current recommendation of ADA (2018) [24]. Patients with type 2 DM were treated according to current clinical guideline [6]. All DM patients were treated with metformin in individually adjusted daily doses under continuous control of fasting glycemia, the daily profile of glucose concentration and glycated hemoglobin level (HbAc1). No insulin given patients with T2DM were selected in the study. Twenty-five T2DM individuals with mild-to-moderate arterial hypertension were treated with angiotensin-II receptor antagonist valsartan in daily doses 80 mg to 160 mg depending on office systolic and diastolic BP values. Dyslipidemic T2DM patients have treated with statins predominantly atorvastatin in averaged doses 40-80 mg/daily.

(b) Demographic data, smoking status, and anthropometric measurements

Demographic factors such as age, gender, height, weight, body mass, body mass index, waist circumference, and waist-to-hip ratio past medical and medication history were collected at baseline. Current smoking was defined as consumption of one cigarette daily for three months [25].

Anthropometric data were measured by professional health attendants with the participants standing without shoes and heavy outer garments with a wall-mounted stadiometer (OMRON, Japan). Body mass index (BMI) was calculated by the staff person as weight (kg) divided by height

squared (m^2). Waist and hip circumference were measured in a standing position per protocol [26, 27].

(c) Cardiac ultrasound and Doppler procedures

Transthoracic echocardiography was performed on ACUSON ultrasound system (Siemens, Germany) in B-mode regimen and Tissue Doppler Imaging (TDI) regimen from parasternal, subcostal, and apical positions over the short and long axis using 5 MHz phased transducers. Left ventricular ejection fraction (LVEF) was measured by the modified Simpson's method [28]. Left ventricular (LV) mass was estimated using the formula recommended by American Society of Echocardiography's Guidelines and Standards Committee and the Chamber Quantification Writing Group [28]. LV hypertrophy (LVH) was defined as an LV mass/body surface area (BSA) $\geq$ 96 g/m^2, for women, and $\geq$ 116 g/m^2, for men [29]. To measure peak systolic (Sm), early diastolic (Em), and late diastolic (Am) myocardial velocities TDI was carried out according to the American Society of Echocardiography [29].

(d) Calculation of glomerular filtration rate

CKD-EPI formula was used to calculate Glomerular Filtration Rate (GFR) [30].

(e) Blood sampling

Blood samples were drawn in the morning following overnight fasting (at 7-8 a.m.) into barcoded silicone test tubes (Thermo Fisher Scientific, Waltham, MA, USA) wherein two mL of 5% Trilon B solution were added. Then samples were centrifuged upon permanent cooling at 6,000 rpm for 3 minutes, and then plasma was collected to be immediately refrigerated. Each aliquot was stored at a temperature -70^{o}C.

The GDF-15 level was measured by ELISA assay using commercial kit manufacturing by LifeSpan BioSciences (USA) on Elecsys 1010 analyzer (Roche, Mannheim, Germany). Detection range was 31.2 to 2,000 pg/mL.

High-sensitivity C-reactive protein (C-RP) levels were measured by the nephelometric technique with the commercial kit (Eagle Biosciences, Nashua, NH, USA) and obtained with "AU640 Analyzer" (Olympus Diagnostic Systems Group, Japan).

High-performance liquid chromatography method was performed to determine hemoglobin A1c (HbA1c) in 5% Trilon B anticoagulated blood samples.

Concentrations of total cholesterol (TC), cholesterol of high-density lipoproteins (HDL-C), low-density lipoproteins (LDL-C) and triglycerides (TG) were measured by the direct enzymatic method with commercial kits (DIALAB, Neudorf, Austria) using automatic analyzer Roche P800 (F. Hoffmann-La Roche AG, Basel, Switzerland).

(f) Sample preparation for isolating peripheral blood mononuclear cells

We used a standard method for isolating of peripheral blood mononuclear cells from collected blood samples by means density gradient centrifugation using "Lympholyte" solution (Cedarlane Laboratories, Burlington, ON, Canada). Each prepared sample contained 5 mL of peripheral blood were previously exposed to 1:2 dilution with Phosphate-Buffered Saline (PBS, Gibco^{TM} PBS buffers, Thermo Fisher Scientific, Waltham, MA, USA) and stratified onto 5 mL of "Lympholyte" solution. All received samples were centrifuged 30 min at room temperature at 3,000 rpm without a brake. After separation, white blood cells were recollected, diluted with PBS+0.02% Tween and centrifuged at 1,200 rpm for 5 min at room temperature. For erythrocytes removal pellet was treated with RBC lysis buffer and washed 6X with 300μL in PBS before analysis.

(g) Determining endothelial progenitor cells

Cell populations were phenotyped by multicolor flow cytometry in a single-tube panel consisting of CD45, CD34, CD14, CD309(VEGFR2) and Tie-2 antigens as per HD-FACS (High-Definition Fluorescence Activated Cell Sorter) methodology according to gating strategy of International Society of Hematotherapy and Graft Engineering sequential (ISHAGE protocol of gating strategy) [31]. To determine appropriate antigens on the surfaces of EPCs we used FITC-CD34 (BD Biosciences, Franklin Lakes, NJ, USA), FITC-CD14 (BD Biosciences, Franklin Lakes, NJ, USA),APC-CDTie2 (Miltenyi Biotec, Bergisch Gladbach, Germany), PE-CD45 (R&D Systems, Minneapolis, MN, USA), and PE-CD309 (R&D Systems, Minneapolis, MN, USA). Angiopoietin immune phenotypes of EPC have been identified as $CD45^{-}CD34^{+}CD14^{+}CD309^{+}$ and $CD45^{-}CD34{+}CD14^{+}CD309^{+}Tie2^{+}$. For each sample, 500 thousand events have been analyzed. As evident from Figure, representative dot-plots reported coherent EPC phenotyping.

(h) Statistical Analysis

Statistical analysis of the results obtained was carried out in SPSS system for Windows, Version 20 (SPSS Inc, Chicago, IL, USA). The data were expressed as mean (M) and error of the mean (±m) or a 95% confidence interval (CI); the median (Me) and the interquartile range (IQR). Categorical variables were reported as numerous (n) and percentages (%). Shapiro–Wilk test and Kolmogorov-Smirnov test were used to assay the normality of continuous variables. To compare the main parameters of patients' groups (subject to the type of distribution of the parameters analyzed), one-tailed Student *t*-test or Mann-Whitney U-test were used. The two-tailed version of the Wilcoxon test was used for paired comparison of parameter values inside the group. Categorical variables between groups were compared with Chi^2test ($\chi 2$) and Fisher F exact test. The factors, which could be associated potentially with the number of circulating EPCs, were determined by means of univariate analysis of variance (ANOVA); and then, the identified factors with $P< 0.1$ also were studied by means of multivariate analysis of variance (MANOVA). The odds ratio (OR), Wald $x^2$and 95% CI were calculated for all the independent predictors of declining of the circulating number of EPCs with angiogenic phenotypes. The calculated difference of $P<0.05$ was considered statistically significant.

3. Results

Table 1 shows a general characteristic of the individuals included in the study. Patients with type 2 DM and healthy volunteers were matched for age, sex, smoking status, heart rate, left ventricular ejection fraction (LVEF) and frequency of left ventricular hypertrophy. However, at least 32% of all patients with established type 2 DM had hypertension, 36.8% of individuals had dyslipidemia, and 31.5% / 27.6% demonstrated overweight / obesity. Expectedly, body mass index and weight to hip ratio were significantly lower in healthy volunteers when compared with people with diabetes. Systolic and diastolic blood pressure levels in people with diabetes were slightly higher for healthy individuals.

Biological markers in individuals participating in the study are reported in **Table 2**. There were no significant differences between both cohorts in estimated GFR as well as in levels of creatinine. Fasting glucose, HbA1c, hs-CRP, and GDF-15 in blood were sufficiently higher in people with diabetes to healthy volunteers. Additionally, lipid profile abnormality was found in people with diabetes rather than in healthy volunteers. Total cholesterol (TC), cholesterol of low-density lipoproteins (LDL) and triglycerides (TG) were higher, but cholesterol of high-density lipoproteins (HDL) was lower in people with diabetes in comparison with healthy individuals.

The number of EPCs with angiopoietin phenotypes in individuals participating in the study presented in **Table 3**. There was significant difference (P=0.001) between cohorts in the number of circulating EPCs labeled $CD45^{-}CD34^{+}CD14^{+}CD309^{+}$ and $CD45^{-}CD34^{+}CD14^{+}CD309^{+}Tie^{2+}$.

Table 1. General characteristic of individuals participating in the study

Parameters	Healthy volunteers (n=30)	Entire group of T2DM patients (n=76)	P value
Age, years	45.30±5.30	47.90±5.10	0.66
male, n (%)	16 (53.3%)	41 (53.9%)	0.82
Adherence to smoking, n (%)	6 (20.0%)	18 (23.7%)	0.16
Hypertension, n (%)	-	25 (32.9%)	0.001
Dyslipidemia, n (%)	-	28 (36.8%)	0.001
Overweight, n (%)	-	24 (31.5%)	0.001
Obesity, n (%)	-	21 (27.6%)	0.001
BMI, kg/m2	23.2 (21.7–25.2)	27.3 (24.3–29.5)	0.04
WHR, units	0.85 (0.82 – 0.87)	1.02 (0.96 – 1.10)	0.001
Systolic BP, mm Hg	121±4	132±7	0.044
Diastolic BP, mm Hg	68±4	78±5	0.042
Heart rate, beat per min.	65.25±4.18	70.15±5.20	0.12
LVEF, %	67.2 (61.9 – 72.8)	60.3 (53.1 – 67.2)	0.22
E/Am, U	8.6±0.54	11.1±1.61	0.12
E/Em, U	7.6±0.70	11.0±1.68	0.12
LVH, %	-	31 (40.8%)	0.001

Notes: Data are expressed as mean (M) and standarddeviation (±SD), median (Me)and interquartile range (IQR), numerous (n)and frequencies (%). Abbreviations: **T2DM** - Type two diabetes mellitus, **LVEF**– left ventricular ejection fraction; **LVH** – LV hypertrophy; **WHR**, weight to hipratio; **U** – unit, **Em** - early diastolic myocardial velocity, **Am** - late diastolic myocardial velocity, **E** – peakvelocity of early diastolic left ventricular filling.

Table 2. Biological markers in individuals participating in the study

Parameters	Healthy volunteers (n=30)	Entire group of T2DM patients (n=76)	P value
GFR, mL/min/1.73 m2	95.7 (80.3–114.5)	82.8 (71.5–103.1)	0.14
creatinine, μmol/L	74.9 (65.1–86.3)	87.6 (79.3–95.1)	0.06
fasting blood glucose, mmol/L	4.9 (4.6-5.5)	6.7 (5.8-7.7)	0.001
HbA1c, %	5.1 (4.6-5.8)	6.4 (5.9-7.0)	0.012
Total cholesterol, mmol/L	4.9 (4.4-5.3)	5.8 (5.0-6.6)	0.001
LDL-C, mmol/L	3.00 (2.8–3.7)	3.7 (3.2–4.0)	0.001
HDL-C, mmol/L	0.9 (0.8–1.1)	0.8 (0.7–1.1)	0.04
TG, mmol/L	2.2 (2.1-2.4)	2.8 (2.1-3.1)	0.001
GDF-15, pg /mL	288 (254–323)	618 (410–825)	0.001
hs-CRP, mg/L	3.10 (1.03-4.90)	7.12 (5.30-9.10)	0.001

Abbreviations: **CI** – confidence interval; **T2DM** – typetwo diabetes mellitus, **GFR** - glomerularfiltration rate, **HbA1c**- glycated hemoglobin, HDL-C - high-density lipoprotein cholesterol, **LDL-C** - low-density lipoprotein cholesterol. *Note*: Categorical variables are expressed as numerous (n) and percentages (%).

The deficiency of these EPCs was found in people with diabetes in comparison with healthy volunteers.

Table 3. Cell phenotypes in individuals participating in the study

Cell phenotypes	Healthy volunteers (n=30)	Entire group of T2DM patients (n=76)	P value
CD45- CD34+CD14+CD309+, cells/μL	10.54 (6.33-18.12)	6.20 (4.30-9.15)	0.001
CD45- CD34+CD14+CD309+Tie2+, cells/μL	7.23 (5.10-10.20)	1.85 (1.01-2.95)	0.001

Abbreviations: **T2DM**, type 2 diabetes mellitus. *Note*: The values are presented as the median and the interquartile range (IQR), thedifferences validity values obtained by means of two-tailed Mann-Whitney test.

The univariate linear regression analysis has shown an association between number of EPCs with immune phenotypes determined $CD45^-CD34^+CD14^+CD309^+$ and $CD45^-CD34^+CD14^+CD309^+Tie^{2+}$ and CV risk factors, hemodynamic performances, and various biomarkers. In diabetics the number of $CD45^-CD34^+CD14^+CD309^+$ cells in peripheral blood inversely related to GDF-15 (r = -0.42, P = 0.001), BMI (r = -0.40, P = 0.001), hs-CRP (r = -0.38, P = 0.001), LV hypertrophy(r = -0.36, P = 0.012), fasting glucose (r = -0.36, P = 0.001), number of CV risk factors (r = -0.38, P = 0.001), LDL cholesterol (r = -0.30, P = 0.002), TG (r = -0.30, P = 0.001), age (r = -0.24, P = 0.014). Number of EPCs with immune phenotype $CD45^-CD34^+CD14^+CD309^+Tie^{2+}$ related inversely to GDF-15 (r = -0.44, P = 0.001), BMI (r = -0.44, P = 0.001), hs-CRP (r = -0.42, P = 0.003), LV hypertrophy(r = -0.36, P = 0.012), fasting glucose (r = -0.38, P = 0.001), HbA1c (r = -0.34, P = 0.001), TG (r = -0.32, P = 0.001), number of CV risk factors (r = -0.36, P = 0.002), smoking (r = -0.32, P = 0.003), LDL cholesterol (r = -0.26, P = 0.002) and age (r = -0.24, P = 0.014).

Therefore, levels of GDF-15 in peripheral blood of diabetics associated with age (r = 0.31, P = 0.044), hs-CRP (r = 0.40, P = 0.001), smoking (r = 0.38, P = 0.001), BMI (r = 0.34, P = 0.001), LDL cholesterol (r = 0.28, P = 0.001), HbA1c (r = -0.28, P = 0.001), number of CV risk factors (r = 0.26, P = 0.001), whereas in healthy individuals concentrations of GDF-15 were not associated with age, but it related to adherence to smoke (r = 0.32, P = 0.001).

Multivariate linear regression analysis has shown that the number of $CD45^-CD34^+CD14^+CD309^+$ and $CD45^-CD34^+CD14^+CD309^+Tie^{2+}$ EPCs in peripheral blood related to BMI (r = -0.42, P = 0.001 and r = -0.44, P = 0.002 respectively), concentration of GDF-15 (r = -0.40, P = 0.001 and r = -0.42, P = 0.001 respectively), hs-CRP (r = -0.34, P = 0.001 and r = -0.36, P = 0.001 respectively), fasting glucose (r = -0.32, P = 0.002 and r = -0.34, P = 0.002 respectively), HbA1c (r = -0.24, P = 0.001 and r = -0.30, P = 0.001 respectively), LDL cholesterol (r = -0.26, P = 0.002 and r = -0.30, P = 0.001 respectively). After BMI and LDL cholesterol adjusting multivariate linear regression analysis has demonstrated that the number of $CD45^-CD34^+CD14^+CD309^+$ and $CD45^-CD34^+CD14^+CD309^+Tie^{2+}$ EPCs significantly related to concentration of GDF-15 (r = -0.38, P = 0.001 and r = -0.40, P = 0.001 respectively), hs-CRP (r = -0.32, P = 0.001 and r = -0.36, P = 0.001 respectively), HbA1c (r = -0.28, P = 0.001 and r = -0.30, P = 0.001 respectively) and fasting glucose (r = -0.32, P = 0.001 and r = -0.33, P = 0.003 respectively).

In univariate logistic regression analysis we found that level of GDF-15 above the median of plasma concentration ($\geq$ 618 pg/mL), hs-CRP $\geq$7.12 mg/L, HbA1c $\geq$6.4%, fasting glucose $\geq$6.7 mmol/L, and BMI $\geq$27.3 kg/m^{2}predicted deficiency of both angiopoietin phenotypes of circulating EPCs. In multivariate logistic regression model GDF-15 $\geq$ 618 pg/mL demonstrated the best odds ratio (OR) values for declining of EPCs in people with diabetes in comparison with other predictors including BMI, HbA1c, and hs-CRP **Table 4**. Thus, our hypothesis regarding that

level of GDF-15 could relate to the circulating number of EPCs with angiopoietin phenotypes was found confirmation in the results of the study.

Table 4. Univariate and multivariate predictors for declined number of angiopoetic EPCs in diabetics

Variables	Univariate OR (95% CI)	Wald x2	P-value	Multivariate OR (95% CI)	Wald x2	P-value
Dependent variable: number of CD45- CD34+CD14+CD309+ EPCs						
GDF-15 (≥ 618 pg / mL versus < 618 pg / mL)	1.12 (1.02-1.28)	18.2	0.001	1.10 (1.04-1.19)	12.3	0.001
hs-CRP (≥ 7.12 mg / L versus < 7.12 mg / L)	1.04 (1.02-1.07)	9.3	0.048	1.02 (1.00-1.04)	8.3	0.06
Fasting glucose ≥ 6.7 mmol/L versus <6.7 mmol/L	1.03 (1.00-1.10)	4.4	0.062	-	-	-
HbA1c ≥ 6.4% versus <6.4%	1.08 (1.02-1.16)	8.5	0.001	1.03 (1.01-1.07)	7.9	0.05
BMI ≥ 27.3 kg/m2 versus <27.3 kg/m2	1.14 (1.05-1.23)	19.1	0.003	1.08 (1.02-1.17)	9.4	0.014
Dependent variable: CD45- CD34+CD14+CD309+Tie2+ EPCs						
GDF-15 (≥ 618 pg / mL versus < 618 pg / mL)	1.16 (1.06-1.31)	19.5	0.001	1.12 (1.03-1.26)	15.1	0.001
hs-CRP (≥ 7.12 mg / L versus < 7.12 mg / L)	1.08 (1.04-1.16)	11.6	0.001	1.04 (1.00-1.10)	10.4	0.052
Fasting glucose ≥ 6.7 mmol/L versus <6.7 mmol/L	1.04 (1.02-1.09)	4.1	0.012	1.03 (1.00-1.06)	3.8	0.058
HbA1c ≥ 6.4% versus <6.4%	1.09 (1.04-1.18)	9.4	0.001	1.05 (1.02-1.09)	6.6	0.048
BMI ≥ 27.3 kg/m2 versus <27.3 kg/m2	1.12 (1.01-1.22)	16.4	0.001	1.10 (1.02-1.23)	14.4	0.001

Abbreviations: **OR** – Odds ratio, **CI** – confidenceinterval, **EPCs** – endothelial progenitor cells; **GDF-15** – growth differentialfactor-15; **VRP** – C-reactive protein; **HbA1c** – glycated hemoglobin; **BMI**- bodymass index.

4. Discussion

This is the first study that determines the inverse association between levels of GDF-15 in peripheral blood and circulating number of EPCs with angiopoietin capacity labeled $CD45^{-}CD34^{+}CD14^{+}CD309^{+}$ and $CD45^{-}CD34^{+}CD14^{+}CD309^{+}Tie^{2+}$ in type 2 DM patients without established CV diseases. We confirmed that elevated levels of GDF-15 in diabetic population positively related to age, BMI, smoking, and hs-CRP and that circulating number of EPCs in people with diabetes was significantly lower to healthy volunteers.

In this study, we have been hypothesized that deficiency of EPCs with high proliferative and angiopoietin activity in patients with type 2 DM associating with altered vascular repair could be compensated with the protective capability of GDF-15. Indeed, previous studies have revealed that elevated GDF-15 level could be a protector from injury of numerous tissues, such as heart, adipose tissue, and endothelium, by inhibiting c-Jun N-terminal kinase, Bcl-2-associated death promoter, and epidermal growth factor receptor and activating Smad, eNOS, PI3K, and AKT signaling pathways [32–34]. However, there are severe controversies in this issue, because few reports in opposite revealed that lowered GDF-15 levels are beneficial against endothelial injury

and microvascular inflammation [35,36]. In contrast, there is evidence that successful glycemic control and treatment of dyslipidemia with statins in moderate-to-high doses did not cause changes in plasma levels of GDF-15 [37,38]. Although traditionally achieving of full control for hyperglycemia and hyperlipidemia is considered a predictor of better clinical outcomes in people with diabetes, lack of GDF-15 dynamic requires to be clarified. We suggest that GDF-15 as stress-induced cytokine interplays PI3K / AKT signaling in EPCs supporting their proliferative capacity. On the other hand, inhibiting c-Jun N-terminal kinase and Bcl-2-associated death promoter with GDF-15 could protect EPCs epigenetically modified by LDL cholesterol, TG, pro-inflammatory cytokines, phospholipases, reactive oxygen species and improve survival of proangiogenic EPCs. Therefore, activating Smad / eNOS in early growth EPCs may accompany by eliminating apoptotic EPCs from circulation that prevent vascular injury [39]. Nevertheless, stimulating of Tie2/PI3K/Akt/eNOS signaling in late growth EPCs supports their re-endothelialization capacity and survival [40]. The final effect of GDF-15 on EPCs is high likely positive and associate with vascular reparation and protection of resident cells from "metabolic memory" phenomenon [41].

Additionally, there is a large body of evidence regarding that other CV risk factors corresponding to DM development and progression could directly influence on vascular wall and endothelium. Indeed, hypertension, dyslipidemia, hyperglycemia, and inflammation are established risk factors for multiple focus atherosclerosis and vascular calcification [6]. Whether GDF-15 could be involved as the protector from atherosclerotic injury with similar molecular mechanisms as mentioned above is not sufficiently clear. Although some scientists reported that levels of GDF-15 in people with diabetes demonstrated the close positive association with traditional CV risk factors, such as LDL cholesterol, BMI, age, inflammatory activity, but not with TG [14,15]. The results of our study well correspond with these findings, and we yielded that GDF-15 could be a component of the endogenous vascular repair system that maintains vascular integrity and function. In fact, the role of GDF-15 in DM is not entirely understood and requires to be investigated in the large clinical trials focusing on protective mechanisms of GDF-15 on EPCs and other components of vascular repair system.

(a) Study limitations

There were several limitations of the study predominantly relating to a small number of patients and no randomized design. Although it was a small sample size in this study, statistical power was adequate. To note, the duration of T2DM for each patient included in the study was not known and hence. It can be significant because there was a strong negative correlation between the number of circulating angiogenic EPCs and severity of the disease, as well as fluctuation of fasting glucose and the level of HbAc1. Therefore, in the study, we included T2DM patients without established CV disease including asymptomatic atherosclerosis. Probably, an association between GDF-15 and circulating number of angiogenic EPCs requires to be compared in people with diabetes with known atherosclerosis and/or CV disease. Additionally, our findings regarding protective role of GDF-15 remains to be confirmed via validation in external cohorts, particularly in large clinical trials.

5. Conclusions

The results of the study clarified that GDF-15 was an independent factor influenced on number of endothelial precursors with high angiopoetic activity in T2DM patients. These findings could take into consideration in risk stratification and individualized care of the T2DM patients.

6. Open Access

7. List of abbreviations

Am: late diastolic myocardial velocityl; **BMI**: body mass index; **BP**: blood pressure; **CI**: confidence interval; **CV**: cardiovascular; **DM**: diabetes mellitus; **E**: peak velocity of early diastolic left ventricular filling; **Em**: early diastolic myocardial velocity; **EPCs**: endothelial progenitor cells; **GDF-15**: growth / differential factor-15; **GFR**: glomerular filtration rate; **HD-FACS**: High-Definition Fluorescence Activated Cell Sorter; **HDL-C**: high-density lipoprotein cholesterol; **hs-CRP**: high sensitive C-reactive protein; **LDL-C**: low-density lipoprotein cholesterol; **LV**: left ventricular; **LVEF**: left ventricular ejection fraction; **LVH**: LV hypertrophy; **OR**: odds ratio; **SSC**: side scatter characteristic; **WHR**: weight to hip ratio

8. Ethics approval and consent to participate

All the patients have given their written informed consent for participation in the study. The study was approved by the Local Ethical Committee. The investigators followed strictly all the requirements to clinical trials in conformity with the World Medical Association Declaration of Helsinki, 1964, good clinical practice provided by International Conference on Harmonization, Council of Europe Convention for the Protection of Human Rights and Dignity of the Human Being in view of using achievements in biology and medicine, convention on Human Rights and Biomedicine, including Additional Protocol to the Convention on Human Rights and Biomedicine, concerning Biomedical Research, and legislation of Ukraine.

9. Competing interests

The authors declare that they have no conflicts of interest.

10. Authors' contributions

The authors have contributed equally to this paper.

11. Acknowledgments

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