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Inverse association between serum endocan levels and small LDL and HDL particles in patients with type 2 diabetes mellitus

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Abstract. – OBJECTIVE: Determination of lipoprotein size and subclasses distribution can provide more significant information on cardiovascular disease risk than measurement of traditional lipid parameters alone. Accordingly, we aimed to examine their potential relationship with the novel biomarker of endothelial dysfunction, such as endocan in patients with type 2 diabetes mellitus (T2D), since there are no studies concerning this issue.

PATIENTS AND METHODS: This case-control study included a total of 42 individuals with T2D and 64 diabetes-free participants. Serum endocan, lipid parameters, and lipoprotein subclasses were measured.

RESULTS: Patients with T2D exhibited higher proportion of the smallest high-density lipoprotein (HDL) particles HDL 3c, as compared with diabetes-free participants (p=0.047). Higher serum endocan levels in T2D patients with low small dense low-density lipoprotein (LDL) particles (sdLDL) %, as compared with corresponding group of diabetes-free subjects was shown (p<0.01). Univariate binary logistic analysis revealed significant positive association of endocan and LDL diameter (OR=1.686, p=0.004), and negative associations of endocan with proportions of sdLDL (OR=0.928, p=0.007) and HDL3b (OR=0.789, p=0.009) particles. In a multivariate analysis, LDL diameter and proportions of sdLDL and HDL3b subclasses remained independent predictors of endocan levels in tested population.

CONCLUSIONS: The results of our study showed that larger LDL diameters, but lower sdLDL and HDL3b proportions were associated with higher endocan levels in population with T2D. More studies in the future are needed to confirm the observed relationship and to examine its causal nature.

Key Words:
Diabetes, Endocan, Endothelial dysfunction, Lipid subclasses.

Introduction

Cardiovascular disease (CVD) still remains the leading cause of death in individuals with type 2 diabetes mellitus (T2D). The same pathophysiological mechanisms, such as predominance of reactive oxygen species relative to antioxidants, increased inflammation and dysfunction of endothelium underlie both T2D and CVD[1].

Insulin resistance (IR) promotes increased lipolysis of triglycerides (TG) in adipose tissue, with excess of free fatty acids (FFA) that reach the liver. Consequently, increased lipogenesis leads to increased synthesis of TG-rich very-low density lipoproteins (VLDL), higher concentrations of small dense low-density lipoproteins (sdLDL), change in high-density lipoproteins (HDL) composition, and concomitantly its increased clearance[2].

The vicious circle of IR-dyslipidemia leads to further increase in sdLDL and TG levels and reduction in HDL. This so-called "atherogenic dyslipidemia", along with diminished synthesis of nitric oxide, compromised vasodilatation, and higher inflammation precede initiation and/or progression of atherosclerosis and CVD[1,2].

A remarkable heterogeneity among lipoprotein subclasses is reported[3,4]. HDL subclasses are generally classified in HDL2 (larger and less dense, which exert favourable effect on insulin sensitivity) and HDL3 (smaller and denser, which

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are regarded to have proatherogenic properties)[3-5].

Similarly, several LDL subclasses were identified[4]. Among them, LDL III and LDL IV subclasses are referred to sdLDL particles, exerting the most atherogenic properties[4]. The sdLDL particles carry less antioxidants, which make them susceptible to oxidation, and thus enhance their atherogenicity. The sdLDL also have the potential for binding for arterial proteoglycans, as well as for penetration through the endothelial barrier due to its small size[6-8].

Endocan is emerging as a novel parameter that reflects the dysfunction of endothelium[9]. The major site of its secretion are endothelial cells, stimulated by variety of pro-inflammatory factors, such as vascular endothelial growth factor (VEGF) and cytokines (interleukin-1, tumor necrosis factor-alpha)[9]. Several important actions on endothelium are attributed to this proteoglycan, such as regulation of cell adhesion, migration, proliferation, and neo-angiogenesis[9]. Some studies have reported its higher levels in cardiometabolic disorders tightly related to IR, such as obesity[10], T2D[11,12], liver steatosis[13], polycystic ovary syndrome[14], hypertension[15]. Moreover, its relationship with carotid intima-media thickness (cIMT), as surrogate marker of endothelial dysfunction was also shown[16]. Additionally, it was reported that its lowering could reduce the endothelial cells activation, which may postpone the progression of atherosclerosis[17]. As well, significant decrease of this inflammation marker was observed after coronary artery by-pass intervention in those patients following acute coronary syndrome[18]. Taken all these data together, it is assumed that endocan might be a promising marker for assessing cardiometabolic risk.

Since determination of lipoprotein subclasses can provide additional information on CVD risk than measurement of traditional lipid parameters alone[8] and having in mind that there are no studies that examined their potential association with endocan, we wanted to explore this relationship in patients with T2D. This might provide better understanding of the different atherosclerotic pathophysiological mechanisms in this population group.

Patients and Methods

Subjects

The current case-control study derived from our previous research[11] that examined endocan levels, markers of oxidative stress, inflammation and dyslipidemia, in relation to glycoregulation in patients with prediabetes and T2D. In order to gain deeper insight into the relationship between endocan and lipid profile, we have determined lipoprotein particle sizes and subclasses distribution in 64 diabetes-free participants and 42 individuals with T2D. Namely, we excluded from previous group participants that used lipid lowering therapy in order to reduce potential bias of such therapy on lipoprotein particle size and distribution[4,19]. The recruitment of participants was explained in detail previously[11]. Briefly, the data about somatic illnesses, lifestyle habits (e.g., medication use, cigarette smoking, alcohol consumption, data about duration of T2D) and demographic data were collected by a questionnaire that each participant had filled in. All examinees signed informed consent after the study was approved by the Ethics Committee of the Primary Health Care Center in Podgorica, Montenegro.

Systolic (SBP) and diastolic blood pressure (DBP), as well as anthropometric parameters were measured as described previously[11].

Examinees were diagnosed with T2D if reported previously known T2D or if fulfilled some of the following criteria on two different measurements: fasting glucose levels $\geq$ 7.0 mmol/L, glycated haemoglobin (HbA1c) level $\geq$ 6.5%, random glucose level of $\geq$ 11.1 mmol/L, or with glucose level $\geq$ 11.1 mmol/L measured two hours after an oral glucose tolerance test (OGTT)[20]. Diabetes-free group (T2D-) consisted of participants who were not using any antihyperglycemic medications, with HbA1c level $\leq$ 6.4% and fasting glucose < 7.0 mmol/L, or < 11.1 mmol/L after performing OGTT.

In addition to participants on hypolipidemic therapy, those with a history of acute myocardial infarction or stroke in the last 6 months, type 1 diabetes mellitus, pregnancy, severe anaemia, hepatic disease other than steatosis, thyroid dysfunction, renal disease other than diabetic nephropathy, patients with ethanol consumption >20 g/day and with high sensitivity C-reactive protein (hsCRP) > 10 mg/L, were excluded from the study.

A total of 24% of T2D participants reported that they used insulin therapy, whereas 81% of them reported oral antihyperglycemic medications use [of them metformin, sulfonylureas, inhibitors of dipeptidyl peptidase 4 (DPP-4 inhibitors) were reported to be used by 88%, 18%,

and 21% patients, respectively]. Antihypertensive drugs were used by 45% and 76% participants in diabetes-free and T2D group, respectively.

Methods

The blood samples were taken after an overnight fast of at least 8 hours, as previously reported[11] in tubes containing K_2EDTA, as well as in tubes containing serum separator and clot activator. Samples in tubes with K_2EDTA were used for immunoturbidimetric determination of HbA1c levels. Samples in tubes with serum separator, after being left to clot for 30 minutes were centrifuged for 10 minutes at 3000xg. The obtained sera were used for measurement of lipid parameters [i.e., total cholesterol (TC), TG, high density lipoprotein cholesterol (HDL-c), low density lipoprotein cholesterol (LDL-c)] and fasting glucose. All these measurements were done on Roche Cobas c501 chemistry analyzer (Roche Diagnostics GmbH, Mannheim, Germany). Another aliquot of sera was stored at -80°C for later determination of hsCRP, endocan and lipoprotein subclasses. Determination of hsCRP levels was done nephelometrically (Behring Nephelometer Analyzer, Marburg, Germany). The measurement of endocan levels were performed by using an Enzyme-Linked Immunosorbent Commercial Assay (ab213776; Human ESM1 ELISA Kit, Abcam, Cambridge, UK).

To separate LDL and HDL subclasses, polyacrylamide gradient gel electrophoresis at 8°C in a Hoefer SE 600 Ruby unit (Amersham Pharmacia Biotech, Vienna, Austria) was used, as described previously[21]. Carboxylated polystyrene microspheres (Duke Scientific, Palo Alto, CA, USA), high molecular weight protein standards (Amersham Pharmacia Biotech, Vienna, Austria), and standardized human samples were used for calibration of gels. The SBB dye was used for staining the gels for lipids (Sigma-Aldrich, St. Louis, MO, USA), whereas the CBB G-250 dye was used for staining proteins (Sigma-Aldrich, St. Louis, MO, USA). Image Scanner (Amersham Pharmacia Biotech, Vienna, Austria) with Image Quant software (version 5.2; 1999; Molecular Dynamics) was used for the analysis of the gels. The relative proportions of HDL and LDL subclasses were assessed in line with the areas of densitometric scans that corresponded to each subclass. The most prominent peaks in HDL and LDL lipoprotein regions, with its calculated diameters were defined as dominant HDL and LDL particle diameters.

Statistical Analysis

Depending on the number of participants in each group, distribution of data was tested with Kolmogorov-Smirnov or Shapiro-Wilk tests. Normally, continuously distributed data were shown as mean ± standard deviation (SD). Variables with non-Gaussian distribution were *log*-transformed to achieve normality and those data were given as geometrical mean and 95% confidence interval (CI). Normally and *log*-transformed distributed data were compared by Student *t*-test. Continuous variables which did not achieve Gaussian distribution even after logarithmic transformation were given as median (interquartile range) and compared by Mann-Whitney test. All biochemical markers that were significantly different between tested groups were adjusted for age and body mass index (BMI) using analysis of covariance (ANCOVA) and rank analysis of covariance (Quade's test). Variables were examined by the Chi-square test for contingency tables and were given as absolute frequencies. Associations between endocan and biochemical markers and lipoprotein diameters and subclasses proportions were tested by Spearman's correlation analysis and univariate binary logistic regression analysis. Multivariate binary regression analysis was used to identify possible independent associations between size and proportions of LDL and HDL particles and endocan levels in tested population. A concentration which corresponded to the 75th percentile of endocan distribution in the diabetes-free group (457.76 pg/mL) was used as a cut-off and coded as 1 (dependent variable). Continuous variables which correlated with endocan in Spearman's correlation analysis and categorical variables significantly different between tested groups were entered into the binary logistic model. Data from Spearman's correlation analysis were presented as coefficient correlation (ρ) and data from univariate and multivariate binary regression analyses were presented as odds ratio (OR) and 95% CI for odds. The explained variation in endocan levels was given by Nagelkerke R^2 value. Statistical tests were performed using SPSS version 22.0 for Windows (SPSS Inc., Armonk, NY, USA). Statistical significance was set at $p<0.05$.

Results

As given in Table I, unequal distribution of gender was found in tested groups. There were more females in the diabetes-free group as com-

Table I. Demographic characteristics of examined groups.

	T2D - group	T2D + group	p
N (male/female)	64 (22/42)	42 (25/17)	**0.008**
Age, years	59 ± 10	66 ± 10	**0.001**
BMI, kg/m^{2a}	27.5 (24.9-30.2)	30.8 (26.8-32.6)	**0.005**
SBP, mmHg	138 ± 21.6	135 ± 14.0	0.460
DBP, mmHg	87 ± 11.8	83 ± 9.4	0.126
Smoking habits, (Smoker/Non-smoker)	18/46	5/37	**< 0.001**
Antihypertensives (Yes/No)	29/35	32/10	**0.001**
Antihyperglycemics (Yes/No)	0/64	34/8	**< 0.001**
Insulin (Yes/No)	0/64	10/32	**< 0.001**
Duration of diabetes, years[a]	–	4.0 (1.0-10.0)	–

Data are presented as arithmetic mean ± standard deviation and compared by Student's *t*-test. [a]Skewed distributed data are presented as median (interquartile range) and compared by Mann-Whitney test. Categorical variables are presented as absolute frequencies and compared by Chi-square test for contingency table.

pared with T2D group. Also, more smokers were found in the diabetes-free group. Opposite to that, more participants using antihypertensive, antihyperglycemic and insulin therapy were found in diabetic group than in diabetes-free group.

The patients with diabetes had higher glucose, HbA1c, HDL3c proportion and endocan levels but lower TC and LDL-c levels (Table II).

As demonstrated by Spearman's correlation analysis, serum endocan level correlated positively with age, BMI, TG and hsCRP levels and LDL size and negatively with HDL-c level, sdLDL, LDL IIIB, LDL IVA and HDL3b proportion (Table III).

In order to further explore the relationship between serum endocan levels and sdLDL particles, we divided T2D- and T2D+ groups according to sdLDL% [i.e., low sdLDL% (<50%) *vs.* high sdLDL (≥50%)], (Table IV). Accordingly, we

Table II. Biochemical, lipid and lipoprotein data in examined groups.

	T2D - group	T2D + group	p
Glucose, mmol/L	5.8±1.5	8.0±1.6	**< 0.001**
HbA1c, %[a]	5.7 (5.3-6.0)	7.0 (6.4-8.7)	**< 0.001**
TC, mmol/L[b]	6.04 (5.70-6.40)	5.28 (4.92-5.68)	**0.007**
HDL-c, mmol/L[a]	1.38 (1.11-1.70)	1.21 (1.00-1.43)	0.051
LDL-c, mmol/L[a]	3.71 (2.93-4.31)	3.11 (2.77-3.68)	**0.006**
TG, mmol/L[b]	1.57 (1.39-1.77)	1.88 (1.62-2.19)	0.057
LDL diameter, nm[b]	26.16 (24.73-26.95)	25.89 (25.51-26.67)	0.909
sdLDL, %[a]	53.1 (47.2-57.6)	53.3 (47.8-57.1)	0.882
LDL I, %[a]	20.6 (17.9-23.5)	20.7 (18.3-22.9)	0.777
LDL IIA, %[a]	11.4 (9.9-13.6)	11.9 (10.8-13.4)	0.293
LDL IIB, %[a]	14.2 (12.8-16.6)	14.2 (12.7-16.6)	0.716
LDL IIIA, %[a]	14.2 (12.6-15.1)	13.7 (12.3-14.9)	0.492
LDL IIIB, %[a]	7.5 (6.6-8.6)	7.2 (6.3-8.3)	0.467
LDL IVA, %[a]	12.9 (11.6-14.3)	12.5 (11.2-14.6)	0.767
LDL IVB, %[a]	16.8 (13.7-20.4)	18.3 (14.7-20.2)	0.522
HDL diameter, nm[a]	9.33 (8.93-10.38)	9.46 (9.18-9.90)	0.661
HDL 2b, %[a]	39.4 (35.2-47.6)	38.3 (34.3-44.5)	0.657
HDL 2a, %[a]	20.2 (19.4-21.6)	19.6 (18.2-21.2)	0.153
HDL 3a, %[a]	14.8 (12.9-16.5)	14.4 (13.3-15.2)	0.356
HDL 3b, %[a]	10.2 (8.3-12.3)	9.8 (8.5-11.0)	0.590
HDL 3c, %[a]	13.0 (9.5-17.3)	16.8 (11.1-20.4)	**0.047**
hsCRP, mg/L[b]	1.18 (0.93-1.51)	1.38 (1.02-1.87)	0.452
Endocan, pg/mL[a]	228.7 (150.4-456.8)	491.8 (320.2-985.7)	**0.005**

Data are presented as arithmetic mean ± SD. [a]Skewed distributed data are presented as median (interquartile range). [b]Log-normal distributed data are presented as geometric mean (95% CI).

Table III. Correlations between endocan, demographic and laboratory data in all participants.

Endocan	ρ	*p*
Age, years	0.242	0.014
BMI, kg/m^2	0.244	0.013
Glucose, mmol/L	0.492	< 0.001
HBA1c, %	0.505	< 0.001
TC, mmol/L	-0.057	0.569
HDL-c, mmol/L	-0.263	**0.016**
LDL-c, mmol/L	-0.025	0.803
TG, mmol/L	0.252	**0.010**
LDL diameter, nm	0.223	**0.024**
sdLDL, %	-0.207	**0.036**
LDL I, %	0.182	0.065
LDL IIA, %	0.187	0.058
LDL IIB, %	0.158	0.110
LDL IIIA, %	-0.091	0.362
LDL IIIB, %	-0.206	**0.037**
LDL IVA, %	-0.202	**0.041**
LDL IVB, %	-0.149	0.134
HDL diameter, nm	-0.034	0.735
HDL2b, %	0.120	0.228
HDL2a, %	0.056	0.574
HDL3a, %	-0.187	0.059
HDL3b, %	-0.213	**0.031**
HDL3c, %	0.001	0.991
hsCRP, mg/L	0.257	**0.009**

Data are presented as correlation coefficient Rho (ρ). *p < 0.05; **p < 0.01.

demonstrated higher levels of serum endocan in T2D patients with low sdLDL% as compared with diabetes-free subjects with low sdLDL% (p<0.01).

We further subdivided diabetes-free participants according to sdLDL% and glycoregulation (Table V). In line with this, we demonstrated higher levels of serum endocan in low sdLDL% and impaired glycoregulation, as compared with corresponding subgroup with good glycoregulation (p<0.001).

Similarly, we subdivided T2D participants according to sdLDL% and glycoregulation (Table VI). However, we observed no difference in serum endocan levels in low sdLDL% and impaired glycoregulation, as compared with corresponding subgroup with good glycoregulation.

At the end, to examine further in depth associations of endocan with LDL and HDL particle sizes and subclasses distributions, binary logistic analysis was performed (Table VII). Univariate analysis revealed significant positive association of endocan and LDL diameter (OR=1.686, p=0.004), negative associations of endocan with proportions of sdLDL (OR=0.928, p=0.007), and HDL3b (OR=0.789, p=0.009) particles. Only these significant predictors were tested in multivariate analysis. Covariates included in the models were age, BMI, HDL-c, TG, hsCRP levels, gender, smoking status, antihyperglycemic therapy and antihypertensive therapy and each of the above-mentioned predictors. The results indicated LDL diameter and proportions of sdLDL and HDL3b particles are the independent predictors of endocan levels in tested population and each model included could be able to explain variation in endocan levels by 31.8%, 28.1% and 28.9%, respectively (Table VII).

Discussion

As far as we are aware, this is the first study that evaluated relationship between serum endocan levels, lipoprotein size and subclasses in patients with T2D. According to our data, higher endocan levels were associated with higher LDL diameters, but lower proportions of sdLDL and HDL3b particles in patients with T2D.

Only a few studies examined the association between endocan and traditional lipid parameters and showed no correlation at all[14] or positive association with TC and LDL-c, as well as negative association with HDL-c levels[22]. Given the urgent need for overcoming the knowledge gap in pathophysiological processes that lead to increased mortality due to CVD in T2D patients, we aimed

Table IV. Serum endocal levels according to proportion of sdLDL particles in examined groups.

	T2D -		T2D +	
	Low sdLDL % (< 50%)	High sdLDL % (≥ 50%)	Low sdLDL % (< 50%)	High sdLDL % (≥ 50%)
N	26	38	16	26
Endocan, pg/mL	382 (173-848)	192 (136-338)	560 (291-1046)*	496 (335-931)**

**p<0.01-the difference in serum endocan levels between high sdLDL % in T2D – *vs*. T2D+; *p < 0.05-the difference in serum endocan levels between low sdLDL % in T2D – vs. T2D+.

Table V. Serum endocal levels according to proportion of sdLDL particles and glycoregulation in diabetes-free group.

	Low sdLDL % (< 50%) and good glycoregulation (HbA1c≤ 5.7%)	Low sdLDL % (< 50%) and impaired glycoregulation (5.7% ≤ HbA1c ≤ 6.4%)	High sdLDL % (≥ 50%) and good glycoregulation (HbA1c < 5.7%)	High sdLDL % (≥ 50%) and impaired glycoregulation (5.7% ≤ HbA1c ≤ 6.4%)
N	6	19	17	22
Endocan, pg/mL	149 (129-242)	583 (284-901)***	142 (125-177)	281 (196-457)**

**p<0.001-the difference in serum endocan levels between low sdLDL % and good glycoregulation and low sdLDL % and impaired glycoregulation in diabetes-free group; **p<0.01-the difference in serum endocan levels between high sdLDL % and good glycoregulation and high sdLDL % impaired glycoregulation in diabetes-free group.

to further explore the relationship of endocan and non-traditional lipoprotein parameters that can provide more information on CVD risk above routinely measured ones.

Our results show that patients with T2D exhibited higher proportion of HDL3c as compared with diabetes-free individuals (Table II), supporting previous data that HDL particles distribution in subjects with T2D is shifted toward smaller HDL3, triglyceride-rich particles due to impaired HDL maturation[23]. In contrast to HDL, LDL subclasses distributions in diabetes-free and T2D groups were similar (Table II). This finding might be explained by the fact that diabetes-free group in not comprised of exclusively healthy individuals, since some of them have already impaired glucose tolerance, although not T2D. It cannot be excluded that some of them even exhibit metabolic syndrome, since they used antihypertensive medications, as well.

Small HDL3 particles exert dual effect in atherogenesis, since the functionality of HDL subclasses in T2D individuals is impaired[23]. Decreased lecithin-cholesterol acyltransferase (LCAT) and increased cholesteryl-ester transfer protein (CETP) activities are typical finding in T2D[23]. Increased CETP activity favours HDL cores enrichment in TG on the account of cholesterol esters depletion, enabling them to be a good substrate for lipolysis by hepatic lipase[24]. Furthermore, decreased LCAT diminishes esterification of cholesterol, consequently decreasing the maturation of HDL3 to HDL2[24]. Additionally, smaller HDL3 are prone to oxidative modifications, thus exerting decreased apolipoprotein A-I (apoA-I) stability and resulting in diminished removal of oxidized lipids from LDL[23]. Even more, decreased apoA-I also results in inhibition of reverse cholesterol efflux, promoting the formation of foam cells and fatty streaks in arterial vessels[5].

We have shown the negative association between endocan and HDL3b proportion (Table III). This inverse relationship can be explained by the assumption that HDL3b has lost its anti-inflammatory properties[5], and due to its dysfunctionality, the expression of endocan might be increased.

It is generally assumed that changes in the proportion of large HDL2 particles are strongly associated with CVD risk, as compared to HDL3[25]. However, there are discrepant results in some previous studies that showed no correlation between HDL subclasses distribution and cIMT[26] or even an inverse association between, both HDL2 and HDL3 subfractions and cIMT[24,27]. Our results are in line with the latter ones since endocan and cIMT are highly correlated[16] and both might be regarded as surrogate markers of endothelial dysfunction.

Table VI. Serum endocal levels according to proportion of sdLDL particles and glycoregulation in T2D group.

	Low sdLDL % (< 50%) and good glycoregulation (HbA1c < 7.0%)	Low sdLDL % (< 50%) and impaired glycoregulation (HbA1c > 7.0%)	High sdLDL % (≥ 50%) and good glycoregulation (HbA1c ≤ 7.0%)	High sdLDL % (≥ 50%) and impaired glycoregulation (HbA1c > 7.0%)
N	6	10	12	14
Endocan, pg/mL	807 (344-1051)	348 (290-903)	394 (282-1040)	524 (350-931)

Table VII. Univariate and multivariate binary logistic regression for the associations between endocan and LDL and HDL subfractions.

Predictors	Unadjusted OR (95% CI)	p	Nagelkerke R^2
LDL diameter, nm	1.686 (1.177- 2.414)	**0.004**	0.119
sdLDL, %	0.928 (0.879- 0.980)	**0.007**	0.105
HDL3b, %	0.789 (0.660-0.944)	**0.009**	0.097
Models	**Adjusted OR (95% CI)**	**p**	**Nagelkerke R^2**
LDL diameter, nm	2.093 (1.260-3.297)	**0.004**	0.318
sdLDL, %	0.926 (0.869-0.985)	**0.016**	0.281
HDL3b, %	0.751 (0.604-0.934)	**0.010**	0.289

Models: each consisted of LDL or HDL particle characteristics and age, BMI, HDL-c, TG, hsCRP (continuous variables); gender, smoking status, antihyperglycemic therapy and antihypertensive therapy (categorical variables).

The results of our study also reported positive association between endocan level and LDL diameter and its inverse relationship with the proportion of sdLDL particles in serum (Table III). This result might be explained by increased permeability of the endothelium in T2D[28], which might enhance the penetration of sdLDL in subendothelial space, due to its small size[4,8]. This might result in lower levels of sdLDL in circulation. It has been shown that distribution of cells in glycocalyx (i.e., a surface cover of endothelium layer consisted of glycoproteins and proteoglycans) is diminished in patients with T2D compared to diabetes-free subjects, which causes the dysfunction of endothelium[28,29]. Hypeglycemia has been shown to have an adverse effect on dynamics of the endothelial glycocalyx which favours vascular difficulties[30], and consequently leads to increase in endocan expression. Thus, high endocan expression could possibly enhance redistribution of LDL particles across the endothelium, and accumulation of sdLDL in the subendothelial space, which should be confirmed by future experimental data.

Previous studies have demonstrated significantly smaller LDL size in patients with coronary artery disease, compared with controls[31], as well as its importance as significant predictor of disease progression[7]. Oxidative modifications of sdLDL particles in subendothelial space promote the onset of atherosclerosis[8]. In line with this, it has been reported that sdLDL was predictor of the increase of cIMT and IR in patients with prediabetes and T2D[8]. Even more, it was shown that sdLDL was better predictor of carotid atherosclerosis than HDL subclasses[26].

In order to further explore the relationship between serum endocan levels and sdLDL particles, we divided participants according to sdLDL% (i.e., low sdLDL% *vs.* high sdLDL). In line with this, we have found higher serum endocan levels in T2D patients with low sdLDL% as compared with corresponding group of diabetes-free subjects (Table IV). These findings also support the assumption of the association between higher expression of endocan and decreased sdLDL particles in circulation due to its redistribution in the subendothelium[28].

Moreover, when subdivided diabetes-free participants according to sdLDL% and glycoregulation, we reported higher levels of serum endocan in patients with low sdLDL% and impaired glycoregulation, as compared with corresponding group with good glycoregulation (Table V). This further supports the influence of IR on the higher serum endocan levels[11]. On the other hand, no such difference was obtained in T2D group (Table VI), which might be attributed to the effect of therapy. Namely, although we excluded participants with lipid-lowering therapy, we were not able to exclude those on oral antihyperglycemic medications and insulin regimen, which might represent a source of bias considering these results. Therefore, the results of the present study could be assigned, at least in part, to the insulin effects application in some patients with T2D. It was previously shown that insulin therapy was related to larger LDL diameter, but also to decreased proportion of sdLDL and small HDL subclasses[32].

In addition to this, it was shown that the administration of metformin increased LDL particle size[33]. As well, sdLDL level was reported to be decreased after the metformin treatment in patients with polycystic ovary syndrome[34]. It is regarded that metformin might enhance the activation of lipoprotein lipase by targeting mus-

cle cells and/or adipocytes. Increased activity of this enzyme by metformin therapy can enhance the catabolism of lipoproteins enriched with TG, thus leading to increase of LDL particle size[33]. Above all, Zolali et al[28] have shown that hyperglycemia decreased the ability of endothelial cells (i.e. human umbilical vein endothelial cells) to accumulate endocan into the cells, whereas its release to the supernatant medium was enhanced. They reported the beneficial effect of metformin on increase the endothelial cells proliferation in hypeglycemic millieu, by the modulation of endocan. They have also shown the ability of metformin to increase endocan levels in kidneys of diabetic animal models[30].

All these questions about the complex effects of therapy for T2D on these examined biomarkers is one of the limitations of the current study. Another one is its cross-sectional design which does not enable us to confirm causality, but only associations between mentioned parameters. The results of our study might be attributed to several factors other than medication treatment. Namely, the differences in demographic characteristics of examinees (age, variety of participants with obesity), as well as different methods for separation lipoprotein subclasses and lack of standardization among them[5,6,25] might, in part, explain such discordances in previous studies concerning the relationship between lipoprotein subclasses and endothelial dysfunction markers. However, this study is the first one that has demonstrated the independent association of serum endocan levels (as marker of endothelial dysfunction) with LDL size and proportions of sdLDL and HDL3b particles, in patients with T2D. More studies in the future are needed to confirm the observed relationship and to examine its causal nature.

Conclusions

To our knowledge, this is the first research that investigated the association between serum endocan levels and lipoprotein size and subclasses. Higher serum endocan levels are inversely associated with small LDL and HDL particles in patients with T2D. Longitudinal studies are necessary to enlighten the distribution of lipoprotein particles and their associations with endocan levels in order to reveal better target therapy for such cardiometabolic disturbances and prevent and/or delay the atherosclerotic process in this vulnerable population group.

Conflict of Interest

The Authors declare that they have no conflict of interests.

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