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Association between proprotein convertase subtilisin/kexin 9 (PCSK9) and lipoprotein subclasses in children with type 1 diabetes mellitus: Effects of glycemic control

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HIGHLIGHTS

- Plasma PCSK9 level was increased in patients with poor/suboptimal glucoregulation.
- Patients with poor glucoregulation had the highest levels of small, dense LDL (sdLDL) .
- High PCSK9 correlated with small HDL particles in poor/suboptimal controlled type 1 diabetes mellitus (T1DM).
- In well-controlled T1DM, PCSK9 level was inversely associated with sdLDL particles.
- Metabolic control modifies association of PCSK9 and lipoprotein subclasses in T1DM.

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ABSTRACT

Background and aims: Dyslipidemia in type 1 diabetes mellitus (T1DM) is characterised by altered distributions of low-density lipoprotein (LDL) and high-density lipoprotein (HDL) subclasses. Recent studies suggested that proprotein convertase subtilisin/kexin 9 (PCSK9) may contribute to the development of dyslipidemia in T1DM. In this cross-sectional study, we investigated the association between PCSK9 and lipoprotein subclasses in young T1DM patients, with respect to glycemic control.

Methods: Plasma PCSK9 and lipoprotein subclasses were determined in 207 patients with T1DM (106 boys and 101 girls), aged 13.9 ± 3.0 years and treated by intensive insulin therapy.

Results: Plasma PCSK9 levels significantly increased with worsening of glycemic control ($p < 0.001$). T1DM patients with poor glucoregulation had the highest proportion of small, dense LDL (sdLDL) and smaller HDL particles, as well. PCSK9 was positively associated with markers of glucose homeostasis and serum lipid parameters only in patients with suboptimal/poor glucoregulation. In well-controlled T1DM, plasma PCSK9 level was inversely associated with a relative proportion of sdLDL particles ($p < 0.01$) and this association remained significant in multivariate analysis. In T1DM patients with suboptimal/poor glycemic control, PCSK9 was positively associated with the proportion of the smallest HDL3c particles ($p < 0.001$), but negatively with HDL size ($p < 0.05$).

Conclusions: The extent of achieved metabolic control modifies the association between PCSK9 and lipoprotein subclasses in T1DM. Further investigations are needed to reveal whether the observed effects of glycemic control on PCSK9 and sdLDL levels have causal consequences on CVD risk in young patients with T1DM.

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1. Introduction

The worsening of metabolic control in T1DM may trigger secondary disorders of lipid metabolism [1]. In patients with poor and suboptimal glucoregulation, the level of glycated hemoglobin (HbA_{1c}) was found to be positively associated with triglycerides (TG), low-density lipoprotein (LDL) and non-high-density lipoprotein cholesterol (non-HDL-C) levels [1]. Yet, T1DM patients with well-controlled glycemia tend to have lipid levels within recommended values, or even may have increased HDL-C, particularly, if they were treated by intensive insulin therapy [2,3]. Dyslipidemia in T1DM is also characterised by qualitative changes of plasma lipoproteins, the most prominent being alterations of LDL particles [2].

In recent years, novel biomarkers for evaluation of dyslipidemia have been identified, with potential for therapeutic targeting [4]. Proprotein convertase subtilisin/kexin 9 (PCSK9) is a serin-protease that plays a pivotal role in LDL metabolism. PCSK9 binds to LDL receptors (LDL-R) and promotes their intracellular degradation, resulting in increased plasma LDL-C levels [4]. In addition to the previously observed relationship with serum lipid parameters, PCSK9 also positively correlates with markers of glucose homeostasis [5]. Similar results have also been found in a large study carried out in healthy children and adolescents [6]. Results of recent studies in patients with metabolic syndrome and type 2 diabetes suggested that PCSK9 might be one of the factors responsible for the development of diabetic dyslipidemia and increased CVD risk [7]. More recently, a first report has been published on increased PCSK9 in patients with T1DM [8].

So far, a link between PCSK9 and sdLDL particles has been scarcely investigated. A positive correlation was reported in patients with CVD [9,10], whereas no association was found in healthy subjects [11,12]. Hence, it is possible that the relationship between circulating PCSK9 and sdLDL particles varies in health and diseases. Despite the growing body of knowledge on the link between PCSK9 and plasma lipoproteins, such association in pediatric patients with T1DM has not been explored and identified so far. We hypothesized that the link between PCSK9 and lipoprotein subclasses distributions in T1DM depends on the achieved metabolic control, which could be related to insulin action/resistance and plasma TG levels. Therefore, the aim of the present study was to investigate the effect of glycemic control on the association between PCSK9 and LDL and HDL subclasses in young T1DM patients.

2. Materials and methods

2.1. Study group

This cross-sectional study included 207 patients with T1DM, aged 13.9 ± 3.0 years (106 boys and 101 girls), recruited at the Mother and Child Health Care Institute of Serbia “Dr Vukan Čupić" in Belgrade. The diagnosis of T1DM was established following national and international guidelines [13,14]. Mean duration of diabetes in the studied group was 6.5 years (median: 7 years; interquartile range: 4–8 years). The patients were treated by intensive insulin regimen (mostly basal-bolus regimen) in the form of multiple daily insulin injections (n = 193) or by continuous subcutaneous insulin infusion, i.e. insulin pump (n = 14). None of the patients received lipid-lowering therapy. In all subjects, detailed clinical examination and laboratory evaluation were performed, showing no evidence of any diabetic complications. Patient's height, weight, waist and hip circumferences were measured and pubertal development stage was assessed (Tanner method). Body mass index (BMI) was calculated and converted to z-score for the assessment of nourishment status (z-scores between the 5th and 85th percentile were criteria for normal weight, the scores ≥85th, but < 95th for overweight and ≥95th percentile for obesity). Written, informed consent was obtained from all children and their parents or guardians. Study protocol was approved by the Ethics Committee of the Mother and Child Health Care Institute of Serbia „ Dr Vukan Čupić” and conducted in accordance with the Declaration of Helsinki.

2.2. Laboratory analyses

Blood samples were obtained after an overnight fast. Serum levels of glucose, total cholesterol (TC), HDL-C and TG were determined by standard enzymatic methods. The level of LDL-C was calculated by the Friedwald equation. A competitive turbidimetric inhibition immunoassay was used for HbA_{1c} determination and estimated average glucose (eAG) level was also calculated. All assays were performed using Roche/Hitachi c501 automated analyser (Roche, Mannheim, Germany). Achieved glycemic control was assessed based on HbA_{1c} levels, as follows: $HbA_{1c} < 7.5\%$ was a criterion for good glycemic control, the level of $HbA_{1c} \geq 7.5\%$, but < 9% for suboptimal and the level ≥9% was an indicator of poor glycemic control [15]. Urinary albumin concentration was measured by immunonefelometric method in the timed overnight sample with Siemens nefelometer BN ProSpec® System (Siemens, Erlangen, Germany). Early elevation of albumin excretion rate was considered as ≥ 7.5 μg/min [16]. Insulin resistance in T1DM patients was assessed by estimated glucose disposal rate (eGDR) [17]. The lower eGDR level is an indicator of greater insulin resistance [17].

Concentration of PCSK9 was measured in plasma by quantitative sandwich enzyme immunoassay (Quantikine ELISA, R&D Systems Europe Ltd). Plasma LDL and HDL particles were separated by gradient gel electrophoresis method in Hoefer SE 600 Ruby electrophoresis unit (Amersham Pharmacia Biotech, Vienna, Austria), as previously described [18]. The gels were analysed by Image Scanner (Amersham Pharmacia Biotech, Vienna, Austria) using the Image Quant software (version 5.2; 1999; Molecular Dynamics, Sunnyvale, CA, USA). Diameters of the most prominent peaks in the LDL and HDL regions were calculated from the calibration curve and designed as LDL and HDL sizes. The relative proportions of four LDL and five HDL subclasses were determined by estimating the areas under the peaks of densitometric scans corresponding to particular subclass. The proportion of sdLDL particles was determined as the area of the densitometric scan at or below 25.5 nm [19].

2.3. Statistical analysis

Normally distributed continuous variables were presented as mean ± standard deviation and compared by ANOVA with the Tukey's *post hoc* test or Student-t test. Skewed variables were presented as median (interquartile range) and compared by Kruskal-Wallis test or Mann-Whitney *U* test. All significant differences were further adjusted for daily insulin dose and nourishment status (0-normal weight; 1-overweight and obese), using parametric and non-parametric ANCOVA tests. Categorical variables were presented as relative or absolute frequencies and analysed by Chi-square tests for contingency tables. Associations between PCSK9 levels and investigated clinical and laboratory variables were assessed by Pearson's correlation analysis. Multiple linear regression analysis was used to seek possible independent association between PCSK9, lipoprotein subclasses, clinical and laboratory variables. Lipoprotein subclasses data and laboratory variables which correlated with PCSK9 in univariate analysis were included in the models for multiple testing. Lipoprotein particle sizes and subclasses proportions were put in the separate models and adjusted for gender, nourishment status and other variables whose *p* values in

Table 1
Demographic and clinical data of T1DM patients according to glycemic control.

Parameter	Glucoregulation			*p* value
	Good	Suboptimal	Poor	
	$HbA_{1c} < 7.5\%$ (n = 87)	$7.5\% \leq HbA_{1c} < 9\%$ (n = 83)	$HbA_{1c} \geq 9\%$ (n = 37)	
Age (years)	14.1 ± 3.4	13.9 ± 2.7	13.8 ± 3.0	0.818
Gender (boys/girls), %	51.7/48.3	51.8/48.2	48.6/51.4	0.943
Pubertal status, %				0.469
Prepubertal	20.7	21.7	18.9	
Pubertal	23.0	33.7	35.1	
Postpubertal	56.3	44.6	45.9	
Diabetes duration (years)[a]	6.0 (4.0–8.0)	7.0 (5.0–8.0)	7.0 (4.0–9.0)	0.345
Age at diabetes onset (years)[a]	8.0 (5.0–11.0)	6.0 (4.0–11.0)	6.5 (4.0–9.0)	0.288
BMI (kg/m^2)	20.1 ± 3.3	20.2 ± 3.2	20.8 ± 3.7	0.564
BMI z-score[a]	0.25 (−0.38–0.68)	0.30 (−0.40–0.80)	0.40 (−0.58–1.08)	0.517
Nourishment status, %				0.100
Normal weight	88.4	86.7	70.3	
Overweight	8.1	10.8	21.6	
Obese	3.5	2.4	8.1	
Waist circumference (cm)	73.8 ± 9.0	73.8 ± 8.6	75.4 ± 8.4	0.642
Hip circumference (cm)	91.4 ± 12.3	91.5 ± 10.8	93.3 ± 9.9	0.746
Insulin dose (U/kg/day)[a]	0.97 (0.71–1.14)	1.05 (0.89–1.24) [bb]	1.07 (0.89–1.31)[b]	< 0.01
Glucose (mmol/L)	10.32 ± 4.24	11.86 ± 4.41	12.79 ± 5.54 [b]	< 0.05
HbA_{1c} (%)	6.8 ± 0.5	8.1 ± 0.4 [bb]	10.4 ± 1.9 [bb, cc]	< 0.001
eAG (mmol/L)	8.21 ± 0.90	11.00 ± 5.96 [bb]	14.07 ± 3.32 [bb, cc]	< 0.001
Urinary albumin excretion rate (μg/min)[a]	4.3 (2.9–6.8)	4.4 (2.6–7.5)	5.2 (3.2–8.0)	0.848
Elevated albumin excretion rate, %	22.5	25.0	32.1	0.611

HbA_{1c}: glycated hemoglobin; BMI: body mass index; eAG: estimated average glucose.
[a] Data are compared by Kruskal-Wallis test and presented as median (interquartile range).
[b] Significantly different from group with good glucoregulation (b: $p < 0.05$; bb: $p < 0.01$).
[c] Significantly different from group with suboptimal glucoregulation (c: $p < 0.05$; cc: $p < 0.01$).

univariate analysis were ≤0.1. All variables were entered in the models as continuous, except gender and nourishment status. Differences with $p < 0.05$ were considered as statistically significant.

3. Results

3.1. Clinical and laboratory variables according to glycemic control

Demographic and clinical data of T1DM patients with respect to glycemic control are shown in Table 1. The groups of good, suboptimal and poor glucoregulation had similar age and gender distribution and showed no difference in diabetes duration. Also, we found no significant differences in anthropometric variables between groups. The patients with suboptimal and poor glycemic control received a higher dose of insulin per day.

Serum lipid and lipoprotein subclasses profile of T1DM patients are presented in Table 2. The groups of suboptimal and poor glucoregulation had significantly higher TC, TG and LDL-C levels than the patients with good glycemic control. The level of HDL-C did not differ between groups. The differences in TC, LDL-C and TG levels remained significant after adjustment for daily insulin dose ($p < 0.01$ for each comparison) and after adjustment for nourishment status ($p < 0.01$ for each comparison). Analysis of LDL subclasses revealed a gradual rearrangement toward smaller particles with deterioration of glycemic control, as evidenced by a significant reduction of LDL I ($p < 0.05$). In addition, T1DM patients with poor glycemic control had the smallest HDL particle size, significantly less HDL2b, but more HDL 3c particles than the groups with good and suboptimal glucoregulation. Group differences in HDL size ($p < 0.01$) and relative proportions of LDL I, HDL 2b and HDL 3c subclasses persisted after adjustment for nourishment status ($p < 0.01$, $p < 0.05$ and $p < 0.01$, respectively), as well as after adjustment for daily insulin dose ($p < 0.05$ for each comparison).

3.2. PCSK9 levels according to glycemic control

No difference in PCSK9 levels was found between boys (median: 210.81 ng/mL; interquartile range: 182.81–259.00 ng/mL) and girls (median: 235.56 ng/mL; interquartile range: 188.61–277.22 ng/mL; $p = 0.226$). Next, we analysed gender-related differences in metabolic syndrome traits and PCSK9 levels with respect to glycemic control. The level of TG gradually increased with worsening of glycemic control in both genders. In addition, boys with poor glucoregulation had significantly higher level of glucose than boys with good glucoregulation. We found no differences in metabolic syndrome traits and PCSK9 levels between boys and girls with the same degree of glycemic control (Supplementary Table 1).

Fig. 1 presents circulating PCSK9 levels in T1DM patients with respect to glycemic control. We found that the level of PCSK9 significantly increase as glycemic control worsens. In detail, PCSK9 level in the group with good glucoregulation (median: 206.13 ng/mL; interquartile range: 170.35–244.75 ng/mL) was significantly lower than the level in T1DM patients with suboptimal (median: 221.78 ng/mL; interquartile range: 193.00–264.57 ng/mL) and poor glucoregulation (median: 293.00 ng/mL; interquartile range: 237.74–338.43 ng/mL). The difference in PCSK9 levels between the groups with suboptimal and poor glycemic control was also significant ($p < 0.001$). Group

Table 2
Lipid profile of T1DM patients according to glycaemic control.

Parameter	Glucoregulation			*p* value
	Good	Suboptimal	Poor	
	HbA_{1c} < 7.5% (n = 87)	7.5% ≤ HbA_{1c} < 9% (n = 83)	HbA_{1c} ≥ 9% (n = 37)	
TC (mmol/L)	3.89 ± 0.60	4.25 ± 0.88 [b]	4.94 ± 1.15 [bb, cc]	< 0.001
TG (mmol/L)[a]	0.61 (0.52–0.78)	0.79 (0.65–0.93) [bb]	0.93 (0.66–1.32) [bb,c]	< 0.001
LDL-C (mmol/L)	1.94 ± 0.55	2.20 ± 0.75 [b]	2.77 ± 1.02 [bb, cc]	< 0.001
HDL-C (mmol/L)	1.65 ± 0.36	1.66 ± 0.36	1.59 ± 0.49	0.639
LDL size (nm)	26.29 ± 1.17	26.43 ± 1.30	25.95 ± 1.25	0.147
LDL size ≤ 25.5 nm, %	25.0	27.7	37.8	0.347
LDL I (%)	22.4 ± 5.8	24.9 ± 8.2	20.5 ± 7.4[c*]	< 0.05
LDL II (%)	27.2 ± 5.2	27.6 ± 5.2	28.5 ± 7.6	0.527
LDL III (%)	21.3 ± 3.7	20.6 ± 4.5	22.2 ± 6.2	0.227
LDL IV (%)	29.2 ± 5.8	26.8 ± 6.4	28.8 ± 12.2	0.122
sdLDL (%)	50.4 ± 8.0	47.5 ± 9.3	51.0 ± 11.8	0.063
HDL size (nm)	10.17 ± 0.79	10.06 ± 0.92	9.35 ± 1.00 [bb, cc]	< 0.001
HDL size ≤ 8.8 nm, %	11.9	20.5	45.9	< 0.001
HDL 2b (%)	42.9 ± 8.8	41.9 ± 7.5	38.0 ± 7.4 [bb,c]	< 0.01
HDL 2a (%)	21.5 ± 4.4	20.4 ± 3.0	21.1 ± 3.8	0.164
HDL 3a (%)	16.8 ± 3.6	17.6 ± 5.0	18.2 ± 5.4	0.239
HDL 3b (%)	10.4 ± 4.5	11.1 ± 5.2	11.4 ± 3.9	0.430
HDL 3c (%)	8.4 ± 4.4	8.9 ± 3.9	11.2 ± 4.3 [bb,c]	< 0.01

HbA_{1c}: glycated hemoglobin; TC: total cholesterol; TG: triglycerides; LDL-C: low-density lipoprotein cholesterol; HDL-C: high-density lipoprotein cholesterol; sdLDL: small, dense low-density lipoprotein.
[a] Data are compared by Kruskal-Wallis test and presented as median (interquartile range).
[b] Significantly different from group with good glucoregulation (b: $p < 0.05$; bb: $p < 0.01$).
[c] Significantly different from group with suboptimal glucoregulation (c: $p < 0.05$; cc: $p < 0.01$).

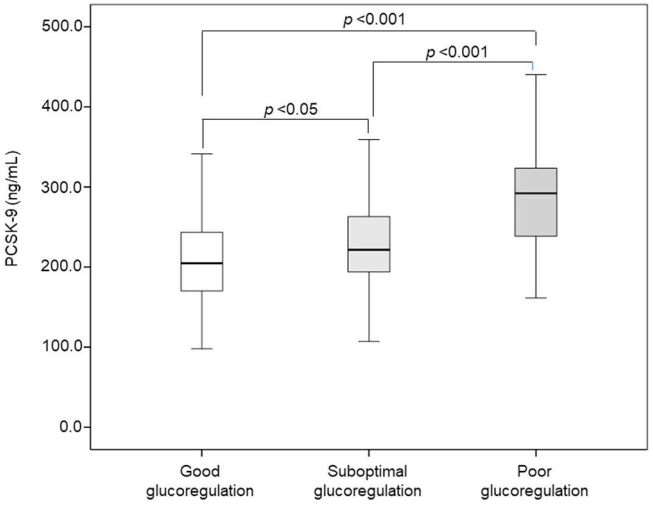

Fig. 1. Circulating PCSK9 levels in T1DM patients according to glycemic control.

differences remained significant after adjustment for daily insulin dose ($p < 0.001$) and nourishment status ($p < 0.001$). Similar trends were found for the effect of glucoregulation when boys and girls were analysed separately (Supplementary Table 1).

3.3. Correlations of PCSK9 with lipoprotein subclasses

Univariate and multivariate associations of circulating PCSK9 levels with lipoprotein subclasses and clinical and laboratory variables, with respect to the achieved glycemic control, are shown in Table 3. Simple regression analysis in the subgroup with good glucoregulation showed that PCSK9 correlated positively with LDL size, but negatively with relative proportion of sdLDL particles, while the association with HDL size and the levels of HbA_{1c} and TG did not reach statistical significance. In patients with suboptimal/poor glycemic control, concentration of PCSK9 was positively associated with daily insulin dose, HbA_{1c}, TC, LDL-C and TG concentrations. In this group of T1DM patients PCSK9 levels were inversely associated with HDL size and positively with proportions of HDL 3c subclasses. To further explore the relationship between PCSK9 and lipoprotein subclasses characteristics, two linear regression models were generated. In T1DM patients with good glucoregulation, the following variables were included in the model 1: LDL size, gender, nourishment status, HDL size, HbA_{1c} and TG levels. In model 2, the proportion of sdLDL particles was entered instead of LDL size, while other variables were same as in the model 1. Following multivariate analysis, PCSK9 remained significantly associated with LDL size ($\beta = 0.303$; $p < 0.01$; model 1), as well as with the relative proportion of sdLDL particles ($\beta = -0.304$; $p < 0.01$; model 2). In T1DM patients with suboptimal/poor glucoregulation, model 1 included HDL size, gender, nourishment status, insulin dose, HbA_{1c}, TC, LDL-C and TG levels. In model 2, the proportion of HDL 3c particles was included instead of HDL size. In multivariate analysis, the association between PCSK9 and proportion of HDL 3c particles remained significant ($\beta = 0.245$; $p < 0.01$; model 2), while the association between PCSK9 and HDL size was lost ($\beta = -0.002$; $p = 0.983$; model 1).

3.4. Correlations of PCSK9 and sdLDL with eGDR, an index of insulin resistance in T1DM

A correlation of PCSK9 level and sdLDL proportion with eGDR, an index of insulin resistance in T1DM, was also analysed. In T1DM patients with good glucoregulation, we found an inverse association between eGDR and PCSK9 levels ($\beta = -0.233$; $p < 0.05$), while the association between eGDR and proportion sdLDL particles was not significant ($\beta = 0.184$; $p = 0.111$). Similarly, in T1DM patients with suboptimal/poor glucoregulation, eGDR showed a negative correlation with the levels of PCSK9 ($\beta = -0.276$; $p < 0.01$) and no association with sdLDL proportions ($\beta = -0.089$; $p = 0.378$). Finally, we found an

Table 3
Univariate and multivariate linear regression analyses of the association between PCSK9 level and lipoprotein subclasses in T1DM patients stratified by glucoregulation.

Patients with good glucoregulation (n = 87)				Patients with suboptimal and poor glucoregulation (n = 120)		
	Variable	β	*p* value	Variable	β	*p* value
Unadjusted	LDL size (nm)	0.215	< 0.05	HDL size (nm)	−0.184	< 0.01
	sdLDL (%)	−0.312	< 0.01	HDL 3c (%)	0.318	< 0.001
	HDL size (nm)	−0.207	0.058	Insulin dose (U/kg/day)	0.200	< 0.05
	HbA_{1c} (%)	0.202	0.062	HbA_{1c} (%)	0.332	< 0.001
	TG (mmol/L)	0.197	0.069	TC (mmol/L)	0.321	< 0.001
				TG (mmol/L)	0.345	< 0.001
				LDL-C (mmol/L)	0.283	< 0.01
Model 1[a]	LDL size (nm)	0.303	< 0.01	HDL size (nm)	−0.002	0.983
	HbA_{1c} (%)	0.199	0.068	Insulin dose (U/kg/day)	0.193	< 0.05
	TG (mmol/L)	0.112	0.318	HbA_{1c} (%)	0.132	0.187
	HDL size (nm)	−0.301	< 0.01	TC (mmol/L)	0.250	0.243
				TG (mmol/L)	0.290	< 0.001
				LDL-C (mmol/L)	−0.097	0.646
Model 2[a]	sdLDL (%)	−0.304	< 0.01	HDL 3c (%)	0.245	< 0.01
	HbA_{1c} (%)	0.171	0.116	Insulin dose (U/kg/day)	0.157	0.074
	TG (mmol/L)	0.061	0.588	HbA_{1c} (%)	0.133	0.164
	HDL size (nm)	−0.233	< 0.01	TC (mmol/L)	0.338	0.080
				TG (mmol/L)	0.219	< 0.001
				LDL-C (mmol/L)	−0.194	0.311

LDL: low-density lipoprotein; sdLDL: small, dense low-density lipoprotein; HDL: high-density lipoprotein; HbA_{1c}: glycated hemoglobin; TG: triglycerides; TC: total cholesterol.

[a] Categorical variables included in Models 1 and 2: gender (0-girl; 1-boy) and nourishment status (0- normal weight; 1- overweight and obese).

inverse association between sdLDL proportion and insulin dose in T1DM patients with good glucoregulation ($\beta = -0.240$; $p < 0.05$), whereas insulin dose was not associated with the proportion of sdLDL particles in the T1DM group with suboptimal/poor glucoregulation ($\beta = -0.065$; $p = 0.493$).

4. Discussion

In the present study, we investigated the association between circulating PCSK9 levels and lipoprotein size heterogeneity in pediatric patients with T1DM, who were treated with intensive insulin therapy and were free of any diabetic complications. To the best of our knowledge, this is the first investigation addressing the relationship between PCSK9 and lipoprotein subclasses in pediatric population with T1DM, with respect to achieved metabolic control.

The main finding of the current study is that the extent of achieved metabolic control could modify associations between PCSK9 and lipoprotein subclasses in T1DM. We found that pediatric T1DM patients with poor glucoregulation had increased levels of both PCSK9 and sdLDL particles, similarly as other high-risk populations and patients with CVD [7–9]. The main reason for accumulation of sdLDL particles in the plasma of T1DM patients with deteriorated glycemic control is overproduction and/or delayed catabolism of TG-rich lipoproteins [3]. A positive association between PCSK9 and TG levels has been previously described [20] and it is also documented in the current study (Table 3). It has been demonstrated that PCSK9 mediates synthesis of apoB-100 and assembly of TG-rich lipoproteins [21], but also that it may delay the uptake of TG-rich lipoproteins via LDL-R [11]. All of the above-mentioned mechanisms promote hypertriglyceridemia and accumulation of sdLDL particles. Notably, PCSK9 promotes LDL-R degradation [4] and sdLDL particles are cleared less efficiently via LDL-R [22].

Adverse changes in HDL subclasses distribution, structure and functionality have been well characterised in patients with diabetes [3,23]. On the other hand, limited data regarding the link between PCSK9 and HDL subclasses has been reported and no previous study investigated this association in diabetes. Apparently, higher PCSK9 is associated with smaller HDL subclasses in patients with CVD [24] and PCSK9 inhibitor therapy predominantly increases large HDL particles [25]. In the current study, higher PCSK9 levels were positively associated with smaller HDL 3c subclasses in patients with suboptimal and poor glycemic control (Table 3). This finding completes previously reported data on distinguishing effects of glucoregulation on PCSK9-mediated dyslipidemia in T1DM [26].

The most intriguing finding of the present study is the inverse association of PCSK9 and sdLDL particles in patients with optimal glycemic control. This association remained significant in a multivariate analysis (Table 3). Available literature data indicate that plasma sdLDL particles are positively related to the levels of PCSK9, at least in patients with CVD [9,10]. The observed reciprocal association between PCSK9 and sdLDL in our study could be assigned to the effects of insulin administration, since insulin increases PCSK9, but lowers sdLDL [27]. In line with previous, insulin dose was inversely associated with the proportion of sdLDL in our patients with well-controlled T1DM. Another possible explanation relies on the data from *in vitro* studies, which provided evidence that a great portion of plasma PCSK9 is actually bound to LDL particles [28]. It has been demonstrated that upon attachment to LDL, PCSK9 has delayed plasma clearance and, more importantly, loses its ability to mediate LDL-R degradation [29]. Our findings fit the conclusion of Glerup and colleagues [30] that plasma LDL-C level (or LDL particles number) may serve as a feedback mechanism that regulates PCSK9 activity. Conversely, there is also the possibility that LDL-bound PCSK9 form has residual activity toward LDL-R [31]. Namely, PCSK9 can be found in two main forms in plasma, as an LDL-bound form and as a free, mature PCSK9 [31]. Based on the findings that cleavage of free PCSK9 by plasma protease furin reduces

its activity, whereas PCSK9 bound to LDL remains intact, it is considered that LDL-bound form of PCSK9 has biological activity [32]. The observed inconsistency between the results mainly originates from the different methodology and experimental models. Whether measurements of LDL-bound PCSK9 and/or PCSK9 activity might be a more accurate marker of PCSK9 function and physiological effects remains to be further established.

Expression of circulating PCSK9 is regulated by sterol response binding element protein-2 (SREBP-2) and has marked diurnal rhythm, parallel to cholesterol synthesis [33]. In addition, multiple genetic, hormonal and metabolic factors may influence plasma PCSK9 level [30]. Regulation of PCSK9 production is different in insulin deficiency and insulin resistance state. Namely, experimental model of T1DM demonstrated that insulin deficiency reduce hepatic PCSK9 gene expression [34]. Based on the data from *in vitro* studies, synthesis of PCSK9 in hepatic cells increases in the presence of insulin [35,36]. On the other hand, in the clinical setting of insulin resistance, acute hyperinsulinemia (induced by euglycemic clamp) significantly decreased circulating PCSK9 levels. Therefore, a hypothesis has been raised that plasma PCSK9 level is inversely related to hepatic insulin sensitivity in the case of acute induced hyperinsulinemia [5]. In our study, higher PCSK9 levels were associated with greater insulin resistance (as indicated by lower eGDR), regardless of glycemic control. Greater insulin resistance is associated with higher risk of micro- and macrovascular complications in T1DM, including dyslipidemia [37]. These data indicate that insulin resistance could be a link between PCSK9 and lipoprotein subclasses distribution in T1DM, although underlying mechanisms behind these associations are yet to be established.

Intensive insulin therapy delays the onset and slows the progression of complications in T1DM [15]. Data from the study involving participants of the Diabetes Control and Complications Trial showed that the patients treated by intensive insulin therapy had less sdLDL and smaller HDL particles than those on conventional insulin regimen [27]. However, to the best of our knowledge, no clinical trial investigated effects of intensive insulin treatment on PCSK9 levels so far. Having in mind recent achievements on LDL reduction with novel PCSK9 inhibitor therapy [38], further studies on PCSK9 in T1DM patients are warranted.

The cross-sectional design of our study allowed us to demonstrate associations, while conclusions regarding causality cannot be drawn. Future studies with isolated lipoprotein subclasses would be required to give an insight into mechanisms behind the observed associations. Also, data on disposition index would additionally support the potential role of insulin resistance on PCSK9 level and qualitative changes in lipoprotein distribution in T1DM. Since currently available ELISA tests are not able to distinguish various forms of PCSK9, but measure its total plasma level, development of the tests to quantify both PCSK9 forms and/or PCSK9 activity would enable further insight into role of PCSK9 in T1DM.

In conclusion, the results of our study show that association of PCSK9 level with LDL and HDL subclasses in T1DM is influenced by the extent of achieved metabolic control. In well-controlled T1DM, plasma PCSK9 level was independently and inversely related to sdLDL particles, whereas such association was lost with worsening of glucoregulation. Further investigations are needed to reveal whether the observed effects of glycemic control on PCSK9 and sdLDL levels have causal consequences on CVD risk in young patients with T1DM.

Conflicts of interest

The authors declared they do not have anything to disclose regarding conflict of interest with respect to this manuscript.

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CRediT authorship contribution statement

Dragana Bojanin: Methodology. **Jelena Vekic:** Conceptualization, Methodology, Writing – review & editing. **Tatjana Milenkovic:** Conceptualization, Writing – review & editing. **Rade Vukovic:** Methodology, Writing – review & editing. **Aleksandra Zeljkovic:** Methodology, Writing – review & editing. **Aleksandra Stefanovic:** Methodology, Writing – review & editing. **Jelena Janac:** Methodology. **Jasmina Ivanisevic:** Methodology. **Katarina Mitrovic:** Methodology, Writing – review & editing. **Milica Miljkovic:** Methodology. **Vesna Spasojevic-Kalimanovska:** Conceptualization, Writing – review & editing.

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.atherosclerosis.2018.11.020.

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