Carotid intima-media thickness, 25-OH vitamin D, homocysteine and subclinical coronary artery atherosclerosis in patients with type 1 diabetes mellitus

Gordana Milić[1,*], Jasna Gačić[2], Ana Mladenović-Marković[3], Ivan Soldatović[4], Dragan Matić[5], Srđan Popović[1], Željko Marković[3] and Svetozar Damjanović[1]

[1]*Clinic for Endocrinology, Diabetes and Metabolic Diseases, Clinical Center of Serbia*, Belgrade, Serbia
[2]*Clinical Hospital Center "Bežanijska kosa"*, Belgrade, Serbia
[3]*Center for Radiology and MR, Clinical Center of Serbia*, Belgrade, Serbia
[4]*Institute for Medical Statistics and Informatics, Faculty of Medicine, University of Belgrade*, Serbia
[5]*Clinic for Cardiology, Clinical Center of Serbia*, Belgrade, Serbia

***Corresponding author**: dr.milicgoga@gmail.com

Abstract: Individuals with type 1 diabetes have an increased risk of premature atherosclerosis. The aim of this study was to evaluate the possible predictive significance of elevated plasma total homocysteine (tHcy), lower serum 25-hydroxy vitamin D (25(OH)D) concentrations and increased carotid intima-media thickness (CIMT) for the development of coronary atherosclerosis in patients with type 1 diabetes mellitus (T1D) and no previous history of ischemic heart disease. The study included 73 patients previously diagnosed with T1D. The patients were divided into groups with and without non-obstructive moderate coronary artery stenosis. Coronary artery stenosis was examined using coronary multidetector computed tomographic angiography (MDCTA); CIMT was measured by B-mode ultrasound. The patients with moderate stenosis had significantly higher HbA1c (p<0.001), elevated tHcy (p<0.001), increased CIMTmax. (p<0.001) but lower 25(OH)D (p<0.001) in comparison to patients without detectable coronary atherosclerosis. Homocysteine (AUCHcy=0.955; p<0.001), vitamin D (AUCvit D=0.792; p<0.001) and CIMT max (AUCCIMT=0.743; p<0.001) (AUC or area under the curve) appear to be adequate markers for detecting stenosis of coronary arteries using receiver operating characteristic (ROC) curve analysis. Multivariate logistic regression analysis showed that serum homocysteine was the only significant predictor of moderate coronary artery stenosis. Our study implies that tHcy can be used as a reliable predictor of coronary artery atherosclerosis in patients with T1D. 25(OH)D and CIMT can also be used, but with lower diagnostic accuracy.

Keywords: type 1 diabetes mellitus; atherosclerosis; multidetector computed tomography coronary angiography; carotid artery; coronary artery

INTRODUCTION

Preclinical coronary artery disease (CAD) is more often observed in patients with T1D, even at an early age. Furthermore, altered endothelial function is also noticed at an extremely early stage of T1D [1]. It is of interest to note that the extent of endothelial dysfunction is in significant correlation with blood glucose concentration and is conversely related to the duration of diabetes mellitus. The Pittsburgh Epidemiology of Diabetes Complications (EDC) study showed that adults with endothelial dysfunction symptoms were at a greater risk of developing CAD [2].

Previous studies analyzed endothelial function, arterial stiffness, CIMT, autonomic neuropathy and left ventricular (LV) function in T1D-associated hyperglycemia with preclinical atherosclerosis [3-6]. After an 18-year follow-up period, the Oslo study confirmed correlation between HbA1c and the degree of atherosclerosis examined by intravascular ultrasound. More precisely, a 1% increase of mean HbA1c was associated with a 6.4% increase in coronary vessel stenosis [7].

Carotid intima-media thickness (CIMT) is a valuable indicator of subclinical cardiovascular disease, owing to a positive correlation between CIMT and cardio-

vascular risk factors [8]. In children, adolescents and adults with T1D, CIMT values were higher than in the control group without diabetes [1,9-11]. The Diabetes Control and Complications Trial/Epidemiology of Diabetes Interventions and Complications (DCCT/EDIC) study showed that CIMT progression was more rapid in subjects with T1D within 6 years of follow-up [9].

Hyperhomocysteinemia is considered to be an independent risk factor for coronary atherosclerotic vascular disease [12]. Homocysteine is an amino acid, which is highly reactive. It is synthesized from another amino acid, methionine. It is worth pointing out that there are a number of enzymes that have a role in its metabolic pathway. Gene mutations involved in methionine metabolism, such as methylene tetrahydrofolate reductase and methionine synthase reductase and homocysteine (e.g., cystathionine β synthase (CBS)) underlie hyperhomocysteinemia [13]. Additionally, serum folate and vitamin B12 deficiencies are among common nutritional causes of hyperhomocysteinemia [14]. Since McCully [15] found the association between tHcy and atherosclerosis for the first time in 1969, there has been ample evidence confirming that elevated tHcy levels represent a cardiovascular risk factor in patients diagnosed with T1D. It is thought that the role of tHcy in endothelial dysfunction is mediated by mechanisms that include oxidative stress, nuclear factor κB (NF-κB) activation, inflammation and inhibition of endothelial nitric oxide synthase (eNOS) [16]. Cardiovascular disease has more frequently been associated with excessive levels of tHcy in patients diagnosed with T2D in comparison to a nondiabetic control group [17]. Thus far, several cross-sectional studies assessing the relationship between tHcy and CAD in T1D patients have confirmed such a positive association [18-22].

Several studies have investigated vitamin D deficiency in relation to CAD [23-25]. According to Jain and Micinski [26], vitamin D contributes to oxidative stress inhibition and monocyte adhesion by mediating the upregulation of glutamate cysteine ligase (GCL) and glutathione (GSH) in endothelial cells. Cell culture studies have showed that high glucose and/or acetoacetate are likely to trigger an increase in reactive oxygen species (ROS) and reduce cellular cystathionine-γ-lyase (CSE) expression at mRNA and protein levels, thus leading to impaired cellular

H_2S homeostasis [26]. Moreover, Li et al. [27] state that vitamin D is a negative regulator of the renin angiotensin aldosterone system (RAA system). Another study reported that the prevalence of 25(OH)D deficiency was twice as high in the group of patients with T1D in comparison to a control group of healthy individuals [28,29]. The correlation between Vitamin D deficiency and risk of developing diabetes mellitus and other diabetes-related complications has been confirmed by other recent studies [29-34].

Studies in T1D have confirmed a positive correlation between tHcy and CIMT (EDIC DCCT study) [35] and a negative correlation between vitamin D concentrations and CIMT in the Young Finns Study [36]. Furthermore, an increase in plasma homocysteine concentration has been linked to a decrease in 25(OH)D levels in asymptomatic adults [37] and patients with T2D [38].

The aim of the present study was to evaluate the possible predictive significance of hyperhomocysteinemia, lower serum 25(OH)D concentrations and increased CIMT for the development of coronary atherosclerosis in asymptomatic patients with T1D.

MATERIALS AND METHODS

Patient selection and data sampling

The study protocol was reviewed and approved by the Ethical Committee of the Faculty of Medicine, University of Belgrade, Serbia (Protocol No. 29/V-15). The study cohort consisted of 73 patients with type 1 diabetes mellitus, aged 19-40 years, with a duration of diabetes over 5 years, who were referred to the Center of Radiology and the Clinic of Endocrinology, Diabetes and Metabolic Diseases of the Clinical Center of Serbia, from 2014 to 2016. The conducted study was a cross-sectional study. All patients had no history of diabetic nephropathy, cardiac rhythm disorders or serious cardiovascular presence and diseases (acute myocardial infarction, unstable angina, heart failure, acute myocarditis, acute pericarditis, arterial hypertension), iodine allergy, malignancies, acute infectious diseases, pregnancy. All patients were treated with intensive insulin therapy. The electromyoneurography (EMNG) test was used to diagnose

diabetic neuropathy. All patients were examined by an ophthalmologist, i.e. a retina specialist, in order to diagnose diabetic retinopathy.

Trained clinical staff obtained information on the demographic characteristics, medical history, family history and lifestyle via questionnaires and interviews. All subjects underwent detailed clinical examinations, including measuring blood pressure twice in a supine position and measuring their height and weight to calculate the body mass index (BMI). The BMI was calculated by dividing the weight in kg with the height in meters squared. Laboratory analyses were conducted in the morning after at least 12 h of fasting. Lipids, cholesterol, HDL cholesterol and triglycerides were measured by a spectrophotometric method, while low density lipoprotein (LDL) was calculated indirectly using the following formula: LDL=Cholesterol-(TG/2.2+HDL) [39]. C reactive protein (CRP) was quantified by latex-enhanced nephelometry using a Behring Nephelometer II analyzer [40]. Glycated hemoglobin (HbA1c) was measured (immune-inhibition method) as a parameter of long-term glucoregulation (3 months average amount of glucose); normal levels 4.0-6.0% [41]. The degree of albuminuria was assessed by the nephelometric method (Siemens Health Care Diagnostics Inc, Newark, USA). Serum homocysteine was measured using an Abbott Homocysteine assay on the Abbot AxSym analyzer, a fully automated fluorescence polarization immunoassay method from Abbott Diagnostics, USA. Vitamin D was measured by the chemiluminescent micro particle assay (CMIA) method (Siemens Health Care Diagnostics, USA) with a precision of 2.3%-3.9% within run and linearity>37-916 ng/mL 25(OH)D deficiency was defined for values <75nmol/l. Elevated serum homocysteine was defined for values >12 μmol/L. MDCT coronarography and ultrasound measurements of CIMT were performed in all patients on two separate days.

Multi-slice computer tomography (MSCT) protocol and coronary artery calcium score

All examinations were performed by a 64-slice CT Scanner (Light Speed GE, USA), in the CardIQX-press Proprogram (MIP, Angiographic View, Tree VR Vessel Analysis, VR Analysis, Cardiac Transparency, Cardiac Reformat, EF and other tools). First, a non-contrast-enhanced prospective ECG-triggered CT was performed. This CT was used to calculate the Agatston coronary artery calcium score (CACS) [42]. Next, MSCT-CA was performed after an intravenous injection of a bolus (80-100 mL at 4-6 mL/s) of non-ionic iodinated contrast agent (iomeprol 400 mg/mL, Iomeron, Bracco, Italy), followed by a saline chaser (50 mL at 4-6 mL/s). If the heart rate was >65 bpm, additional intravenous beta-blockers (5-10 mg atenolol) were provided when tolerated.

Multidetector computer tomography (MDCT) data analysis

The overall CACS was recorded for each patient using dedicated software (CardiIQXpress Pro program (MIP, Angiographic View, Tree VR Vessel Analysis, VR Analysis, Cardiac Transparency, Cardiac Reformat, EF and other tools)). The quantification of the overall CACS was based on the scoring algorithm of Agatston et al. [42], where coronary artery calcium was identified as a dense area located in the coronary artery and greater than 1 mm², exceeding the threshold of 130 Hounsfield units. All 16 coronary segments, as established in the American Heart Association classification, were taken into consideration. Subsequently, the lesion was classified visually as obstructive (>50% luminal narrowing) or non-obstructive (≤50% luminal narrowing). In the present study, we classified non-obstructive stenosis of coronary arteries as follows: minimal stenosis (<25% luminal narrowing) and moderate stenosis (25-50% luminal narrowing).

Assessment of carotid IMT and carotid ultrasonography

IMT measurements were obtained by B-mode ultrasound (Siemens Accuson Antares device, USA). A single radiologist, who was blinded to the participants, took all CIMT measurements and assessed the presence of plaque. This was performed using a linear array probe (10 MHz and 42 mm) with the patients in the supine position. Thereby, left and right common carotid arteries were examined. IMT was measured in the far wall of the common carotid artery, 1 cm proximal to the carotid bulb, in the area free of plaques and at the optimum angle for measuring the CIMT of the proximal and distal walls.

We defined plaque as a focal wall thickening 50% greater than the surrounding wall thickness and con-

Table 1. Clinical, anthropometric and biochemical characteristics of patients and CIMT.

	Patient with moderate stenosis		p value
	No (n=45)	Yes (n=28)	
Age (yrs.)	30.06±6.29	31.39±6.23	0.32
Duration of diabetes (yrs.)	8.52±2.64	8.46±3.00	0.85
BMI (kg/m^2)	23.14±2.91	22.44±1.67	0.87
Waist circumference (cm)	83.96±10.18	78.57±8.72	0.61
Hip circumference (cm)	96.02±5.82	93.54±6.12	0.14
Waist to hip ratio	0.87±0.69	0.84±0.05	0.71
HbA1c(%)	7.75±1.24	9.19±1.62	<0.001
Total cholesterol(mmol/L)	5.52±0.77	5.68±0.78	0.36
HDL cholesterol (mmol/L)	1.17±0.24	1.20±0.27	0.57
LDL cholesterol(mmol/L)	3.36±0.72	3.52±0.73	0.27
Triglycerides (mmol/L)	1.57±0.54	1.72±0.96	0.74
Systolic BP (mmHg)	121.35±11.30	120.36±10.97	0.72
Diastolic BP (mmHg)	77.76±8.74	77.14±6.30	0.75
CRP (mg/L)	2.20 (0.90-2.80)	1.74(0.85-2.70)	0.50
Homocysteine (μmol/L)	7.42(6.30-8.40)	14.29(13.30-15.20)	<0.001
Vitamin D(nmol/L)	52.66(22.87-87.00)	23.79(13.95-28.00)	<0.001
CIMT max. (mm)	0.70(0.60-0.72)	0.97(0.72-1.15)	<0.001

BMI – body mass index; BP – blood pressure;
CRP – C Reactive Protein, CIMT – carotid intima-media thickness;

Table 2. CIMT≥0.9mm as a screening test for detecting coronary artery stenosis.

	Parameter (95% CI)
Sensitivity	0.714 (0.511-0.860)
Specificity	0.956 (0.836-0.992)
Positive predictive value	0.909 (0.693-0.984)
Negative predictive value	0.843 (0.708-0.925)
Likelihood ratio of a positive test	16.07 (4.064-63.555)
Likelihood ratio of a negative test	0.299 (0.166-0.538)

CIMT – carotid intima-media thickness

firmed by electronic calipers. Left and right carotid bifurcations as well as internal and common carotid arteries were examined for the presence of plaque. Subclinical atherosclerosis was defined as IMT≥0.9mm and/or the presence of ≥1cm carotid plaque.

Statistical analyses

Results are presented as counts (%), means±standard deviation (SD) or median (25th-75th percentile) depending on the data type and distribution. Groups were compared using parametric (t-test) and nonparametric (chi-square, Mann-Whitney U test) tests. Areas under the curve (AUC) and diagnostic accuracy measure-

ments were used (sensitivity, specificity, positive and negative predictive value, likelihood ratios) to assess significant markers of coronary artery atherosclerosis. Pearson's correlation was used to assess the correlations between the variables. Logistic regression was performed to evaluate the relationship between dependent variable and independent variables. All p values less than 0.05 were considered significant. All data was analyzed using SPSS 20.0 (IBM Corp. Released 2011. IBM SPSS Statistics for Windows, Version 20.0. Armonk, NY: IBM Corp. USA).

RESULTS

The study included 73 patients with or without coronary artery stenosis. Out of the 73 patients, 45 patients had no signs of stenosis (61.64%) while 28 patients (38.36%) were diagnosed with moderate non-obstructive stenosis. The distribution of coronary artery stenosis (not patients) are shown in Supplementary Table S1. Coronary atherosclerosis was observed in 28 (38.36%) asymptomatic patients by coronary MDCTA. In most cases, moderate stenosis was detected in the right coronary artery (RCA).

The clinical, anthropometric and biochemical characteristics of patients and CIMT are presented in Table 1. The average age and duration of diabetes was similar in both groups of patients with moderate stenosis and without coronary artery stenosis. HbA1c was significantly higher in patients with stenosis. Patients diagnosed with stenosis had higher serum homocysteine concentrations and lower serum vitamin D concentrations than the controls. We found a positive association between increased CIMT max and coronary stenosis.

CIMT measurements were obtained using diagnostic accuracy measurements, i.e. by ultrasound, which is perceived as a highly specific test for detecting carotid artery stenosis in patients with type 1 diabetes (Table 2).

Hyperhomocysteinemia, lower serum concentration 25(OH)D and increased CIMT max appear to be sensitive and specific markers for detecting coronary
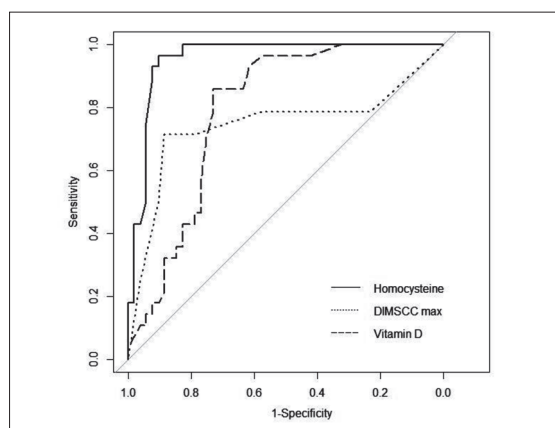
Fig. 1. ROC analysis of investigated parameters.
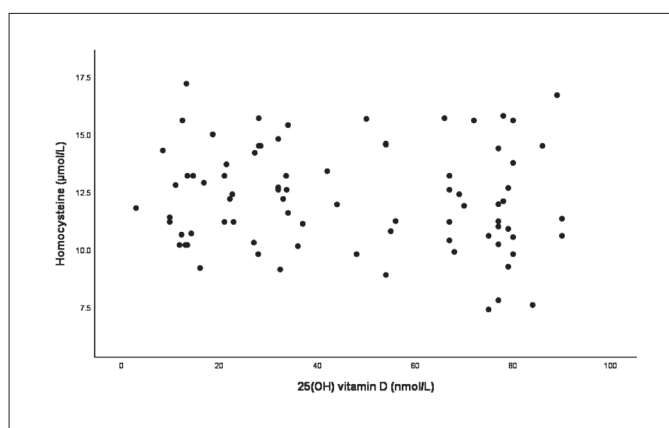
Fig. 2. Linear regression model analysis of relationship between homocysteine and serum vitamin D levels.

Table 3. Multivariate logistic regression analysis of moderate coronary artery stenosis as a dependent variable.

Variable	p value	OR (95% CI)
BMI	0.219	1.597 (0.757-3.370)
Homocysteine	0.008	4.427 (1.471-13.319)
Vitamin D	0.460	0.961 (0.865-1.067)

BMI – body mass index; OD – odds ratio; CI – confidence interval

artery atherosclerosis using ROC analysis (AUCHcy=0.955; (95%CI 0.990-1.000); p<0.001), vitamin D (AUCvit D=0,792; (95% CI 0.657-0.873); p<0.001) and CIMT max (AUCCIMT=0.743; (95%CI 0.643-0.910); p<0.001). Homocysteine proved to be the most reliable diagnostic marker of these three markers followed by the other two, respectively. The results of ROC analysis are presented in Fig. 1.

Pearson's correlation analysis revealed a negative statistically significant correlation between serum homocysteine concentrations and serum vitamin D concentrations (r=-0.242; p=0.030), while there was a positive and statistically significant correlation between homocysteine and carotid IMT (r=0.456; p<0.001). However, the linear regression model revealed no significant relationship between homocysteine and serum vitamin D concentrations (b=-0.009 (95% CI -0.027 to 0.009); p=0.335) (Fig. 2). In addition, no significant correlation was observed regarding CIMT and Vitamin D (r=0.091; p=0.422).

Multivariate logistic regression analysis of moderate coronary artery stenosis as a dependent variable is shown in Table 3. The analysis excluded CIMT because this predictor was insignificant and the confi-

dence interval was extremely high when entered in multivariate analysis. The analysis showed that serum homocysteine concentration was the most reliable predictor of moderate coronary artery stenosis.

DISSCUSION

This study confirmed a positive correlation between increased HbA1c concentrations, increased plasma homocysteine concentrations, lower serum Vitamin D concentrations and elevated carotid IMT with coronary artery stenosis in asymptomatic patients with T1D. Coronary atherosclerosis was observed in one third of asymptomatic diabetic patients by coronary MDCTA. In most cases moderate RCA stenosis was detected. The diagnostic potential of noninvasive coronary MDCTA has been appreciated as it may help identify early atherosclerosis in asymptomatic diabetic patients [43]. In other words, not only can it be used to detect atherosclerosis in order to contribute to the primary prevention of CAD, but it can also be applied to atherosclerotic plaques in patients undergoing statin treatment [44].

We confirmed that excessive levels of HbA1c and increased tHcy represent traditional risk factors for the development of coronary atherosclerosis in patients with T1D. Our findings are in agreement with the results of previous studies, since we have been able to identify poor glycemic control as a predictor of CAD in these patients. This is in line with previous research indicating a strong association between

HbA1c variability and the number of patients diagnosed with coronary artery stenosis [7]. Additionally, our study shows that coronary atherosclerosis is associated with elevated tHcy, although this association has more frequently been observed in patients with type 2 diabetes [17]. A plethora of previous cross-sectional studies have reported a positive association between tHcy and diabetic microvascular complications, whereas several studies have reported a correlation between hyperhomocysteinemia and CAD [18-22]. Nevertheless, the biological mechanism responsible for vascular complications that occur as a consequence of the interaction between diabetes and elevated tHcy has not been fully elucidated. Hyperhomocysteinemia associated with monocyte chemoattractant protein 1 (MCP-1) expression in the kidney via nuclear factor kappa-light-chain-enhancer of activated B cells (NF-κB) activation may have contributed to renal injury in a hyperhomocysteinemic rat [21]. However, another study reported that the development of retinal vascular disease occurred due to the fact that tHcy superoxide from nicotinamide adenine dinucleotide phosphate (NADPH) oxidase led to impaired endothelium-dependent nitrogen oxide (NO)-mediated dilation in the retinal arterioles [22].

The present study adds to the evidence that reduced serum vitamin D levels may have a role in the development of coronary artery stenosis in patients with T1D. The findings of the Framingham Offspring study [23], the Health Professionals Follow-Up study [24] and a cross sectional study by Dobing [25] showed that low serum vitamin D was a risk factor for cardiovascular mortality in healthy subjects. The role of vitamin D in the pathophysiology of T1D has been explored in many studies including ones that investigated its effects on immune-mediated destruction [30]. A study assessing the impact of specific vitamin D receptor polymorphisms on HLA class II histocompatibility antigen DRB1 beta chain (HLADRB1) alleles did not identify it as a major risk factor for T1D [31]. The Finnish Birth Cohort Study reported that children who had been taking the recommended dose of 2000 IU/day of vitamin D were at a substantially lower risk of developing diabetes as opposed to those who had been receiving inadequate dosage [28]. Some studies have confirmed that severe vitamin D deficiency may lead to the development of diabetic neuropathy, retinopathy and nephropathy in patients with T1D [32-34]. An experimental study reported that vitamin D/vitamin D receptor signaling in podocytes might help protect the kidney from diabetic injury [34]. Also, the association between vitamin D deficiency and age-related macular degeneration should be considered [32]. Another experimental study examined the link between vitamin D and the regulation of neurotrophins such as nerve growth factor (NGF) and neuronal Ca^{2+} homeostasis, along with its neuroprotective effect on peripheral nerves, respectively [33]. However, Sachs et al. [45] did not find evidence linking impaired vitamin D metabolism with increased risk of subclinical CVD in T1D. In addition, Serra-Planas et al. [29] evaluated the relationship between the concentrations of 25(OH)D and the presence of early atherosclerosis in asymptomatic T1D patients with no previous history of ischemic heart disease. Although T1D patients have lower concentrations and a 2-fold-higher prevalence of 25(OH)D deficiency than control individuals, they did not reveal an association between 25(OH)D concentrations and subclinical CAD [29].

Moreover, we have also established a positive association between elevated CIMT and both the number of cases diagnosed with coronary artery stenosis and elevated plasma homocysteine levels in T1D patients. The progression of CIMT caused by cardiovascular risk factors can predict further cardiovascular events [8]. In addition, the association between cardiovascular risk and increased CIMT in patients with T1D has been observed in some studies [1,9-11]. In a study of young people with T1D who had been diagnosed with macrovascular disease or microalbuminuria, CIMT was increased by 25% (p<0.001) as compared to the control group of healthy individuals [11]. A significant correlation between CIMT and the percentage of the area affected by coronary vessel stenosis, which was measured by intravascular ultrasound, was reported in female patients [10]. Some studies on T1D have detected a positive correlation between CIMT and HbA1c [3] and CIMT and tHcy, respectively; [35] as well as a negative association between vitamin D concentrations and carotid IMT [38].

The present study has determined a negative correlation between serum homocysteine levels and serum concentrations of vitamin D. A substantial increase in plasma homocysteine was associated with a reduction in 25(OH)D levels [37, 38]. It seems that

supplementation may help reduce homocysteine, thereby fostering the prevention of atherosclerotic vascular disease [37]. Homocysteine is metabolized by transsulfuration and remethylation (folate-dependent) pathways. The transsulfuration pathway represents a key route for homocysteine disposal and its conversion into cystathionine in the presence of the enzyme cystathionine-β-synthase (CBS) and cofactor vitamin B6. Hyperhomocysteinemia has been linked to CBS enzyme deficiency [13]. A significant increase in lower basal CBS mRNA levels was detected in murine preosteoblasts after incubation with activated vitamin D. This suggests that CBS is a target gene of VDR and that vitamin D may modulate homocysteine metabolism while affecting its serum concentration [46]. However, one of the limitations of this study is that it is a cross-sectional study and that data could provide potential correlations between homocysteine levels and the development of atherosclerosis in patients with T1D while further clinical investigations are still necessary.

CONCLUSION

Our study indicates that patients with T1D and hyperhomocysteinemia, lower 25(OH)D serum concentrations and increased CIMT have a higher risk of developing coronary artery atherosclerosis. The presented data also suggest that tHcy can be used as a reliable predictor for the development of coronary artery atherosclerosis in asymptomatic patients with T1D.

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Supplementary Material

The Supplementary Material is available at: http://serbiosoc.org.rs/NewUploads/Uploads/Milic%20et%20al_4462_Supplementary%20Material.pdf