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RELATIONSHIP BETWEEN CARDIOVASCULAR RISK SCORE AND TRADITIONAL AND NONTRADITIONAL CARDIOMETABOLIC PARAMETERS IN OBESE ADOLESCENT GIRLS

POVEZANOST KARDIOVASKULARNOG RIZIKA SA TRADICIONALNIM I NETRADICIONALNIM KARDIOMETABOLIČKIM PARAMETRIMA KOD GOJAZNIH ADOLESCENTKINJA

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Summary

Background: Since the cardiovascular (CV) risk score in the young population, children and adolescents, is underestimated, especially in developing countries such as Montenegro, where a strong interaction exists between the genetically conditioned CV risk and environmental factors, the purpose of this study was to estimate CV risk in apparently healthy adolescent girls. Moreover, we aimed to test some new, emerging CV risk factors and their interaction with the traditional ones, such as obesity. Precisely, we aimed to assess the impact of low bilirubin levels, as a routine biochemical parameter, as an additional risk factor for atherosclerotic disease in the adult phase.
Methods: Forty-five obese adolescent girls (mean age 17.8±1.22 years) and forty-five age- and sex-matched normal weight controls, all nonsmokers, were included. Anthropometric and biochemical parameters were measured. Cardiovascular Risk Score (CVRS) was calculated by adding the points for each risk factor (e.g. sex, HDL-c, non-HDL-c, blood pressure and fasting glycemia).
Results: A significant positive relationship between CVRS and ALT, hsCRP and TG/HDL-c, but an opposite relationship between CVRS and total bilirubin were found (P<0.001). Multiple linear regression analysis showed that

Kratak sadržaj

Uvod: Kako je skor kardiovaskularnog (KV) rizika često potcenjen kod dece i adolescenata, naročito u zemljama u razvoju, kakva je Crna Gora, gde postoji jaka interakcija između genetski uslovljenog KV rizika i sredine, cilj ove studije je bio da se proceni KV rizik kod zdravih adolescentkinja. Takođe, cilj je bio i da se ispitaju neki novi KV faktori rizika u interakciji sa tradicionalnim, kao što je gojaznost. Preciznije, imali smo za cilj da ispitamo uticaj niskih vrednosti bilirubina, rutinskog biohemijskog parametra, kao dodatnog faktora rizika za aterosklerozu u adultnom dobu.
Metode: Ukupno 45 gojaznih adolescentkinja, srednje starosne dobi 17,8±1,22 godina, i 45 normalno uhranjenih adolescentkinja, uparenih po godinama starosti, nepušača, uključeno je u studiju. Mereni su antropometrijski i biohemijski parametri. Skor kardiovaskularnog rizika (CVRS) računat je dodavanjem poena za svaki faktor rizika (pol, HDL-holesterol, non-HDL-holesterol, krvni pritisak i glikemija našte).
Rezultati: Utvrđena je statistički značajna pozitivna povezanost između CVRS i ALT, hsCRP i TG/HDL-holesterola (P<0,001). Višestruka linearna regresiona analiza je pokazala da su veći obim struka i viši LDL-holesterol, a niži HDL-holesterol nezavisni prediktori nižih vrednosti bilirubina (R^2= 0,603, P<0,001).

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higher waist circumference (WC) and LDL-c, but lower HDL-c were independent predictors of lower bilirubin values (adjusted $R^2=0.603$, $P<0.001$).
Conclusions: Obese adolescent girls are at an increased risk of cardiovascular disease late in life. In addition to the traditional risk factors, total bilirubin may have the potential to discriminate between low and higher risk for cardiovascular disturbances in healthy adolescent girls.

Keywords: adolescents, cardiovascular risk, inflammation, obesity, total bilirubin

Zaključak: Gojazne adolescentkinje imaju povećan rizik za KV bolesti u kasnijem dobu. Pored tradicionalnih faktora rizika, ukupni bilirubin može imati potencijal da diskriminiše između niskog i većeg rizika za KV promene kod zdravih adolescentkinja.

Ključne reči: adolescenti, kardiovaskularni rizik, inflamacija, gojaznost, ukupni bilirubin

Introduction

Traditional coronary heart disease risk factors are recognized and should be quantified even in children and adolescents in order to detect young individuals with a high probability of having increased risk for cardiovascular disease late in life (1).

The effect of obesity on cardiovascular disease (CVD) risk often tracks from childhood and adolescence, even if frank heart disease rarely presents before adulthood (2, 3, 4).

Oxidative stress has appeared in recent years as a hallmark of the obese state, intrinsically linked to chronic low-grade inflammation (5). Hypertrophied adipocytes have been reported as a significant source of reactive oxygen species (ROS), promoting adipose tissue dysfunction by increased expression of adipokines and macrophage infiltration in the adipose tissue, thus further leading to overproduction of ROS and inflammatory cytokines, and altogether establishing a systematic feedback-loop between inflammation and oxidative stress in obese adipose tissue (5, 6).

High levels of ROS generated by hypertrophied adipocytes impact many metabolic signaling pathways (e.g., insulin sensitivity, promote inflammation, and alter lipid metabolism or endothelial dysfunction). Conversely, vascular damage and inflammation participate actively in ROS generation, therefore entertaining a vicious circle and maintaining high levels of oxidative stress, as an important contributor to obesity-related disorders such as atherosclerosis (5).

Although considered as a harmful metabolite of heme catabolism, recent findings indicate that serum bilirubin has antioxidant and antiinflammatory properties (6). The ability of bilirubin to scavenge reactive oxygen species (ROS) and to suppress the oxidation of lipids and lipoproteins, especially low density lipoprotein (LDL) particles, may be of significant importance in the pathogenesis of many cardiovascular (CV) disorders (7, 8). In addition, bilirubin inhibits tumor necrosis factor-alpha (TNF-α)–induced upregulation of E-selectin, vascular cell adhesion molecule 1 (VCAM-1), and intercellular adhesion molecule 1 (ICAM-1) *in vitro* (8), thus also showing antiinflammatory properties.

Since cardiovascular risk score in the young population, children and adolescents is underestimated, especially in developing countries such as Montenegro, where strong interaction exists between the genetically conditioned CV risk and environmental factors, the purpose of this study was to estimate CV risk in apparently healthy adolescent girls. Moreover, we wanted to test some new, emerging CV risk factors and their interaction with the traditional ones, such as obesity. Precisely, we aimed to assess the impact of a low bilirubin level, as a routine biochemical parameter, as an additional risk factor for atherosclerotic disease in the adult phase.

Materials and Methods

Study population

A total of 45 obese adolescent girls, mean age 17.8±1.22 years, and 45 age- and sex-matched normal weight controls participated in this cross-sectional study. Participants were selected from two suburban secondary schools in Podgorica, Montenegro and were recruited in the Primary Health Care Center at their regular check-up, in the period from December 2012 to March 2013. All the participants completed a questionnaire including demographic characteristics, somatic illnesses, medication use, and lifestyle habits. Medical history and clinical examinations were carried out on the same day.

Inclusion criteria were normal weight and obese, otherwise healthy adolescent girls between the ages of 16 and 19. Girls younger than 16 years and older than 19 years, as well as participants with known liver dysfunction, diabetes mellitus, renal dysfunction, thyroid dysfunction, cardiovascular disorders, with signs and symptoms of acute inflammatory disease and/or high sensitivity CRP > 10 mg/L (9), with a history of alcohol consumption and smoking, and those who used any medications, were excluded from the study. Participants were instructed not to perform any vigorous physical activity on the day before the blood was drawn. All the participants provided written informed consent, and for those younger than 18 years parents' written approval was also obtained. The study protocol was approved by the Ethical Committee of Primary Health Care Center in Podgorica and the research was carried out in compliance with the Declaration of Helsinki (10).

Anthropometric measurements

Basic anthropometric measurements: body height (cm), body weight (kg) and waist circumference (WC) (cm) were obtained in the morning. Weight was measured to the nearest 0.1 kg on a balance beam scale, with the subjects barefoot and with light clothing. Height was measured to the nearest 0.1 cm using a wall-mounted stadiometer, without shoes. Waist circumference was measured with the nonstretchable tape, over the unclothed abdomen at the midpoint between the lowest rib and the iliac crest, and at the end of normal expiration. The tape was parallel to the floor and did not compress the skin. Body mass index (BMI) was calculated as weight in kilograms divided by height in meters squared (kg/m^2). BMI z-score was calculated, also. Using the World Health Organization (WHO) growth reference, 5–19 years (11) old girls were categorized as normal weight (-2SD ≤ BMI z-score ≤ +1SD), and overweight (+1SD < BMI z-score < +2SD) or obese (with BMI z-score ≥ +2 SD). Blood pressure was measured with a sphygmomanometer. The average of three measurements taken on the right arm was recorded. All measurements were taken by the same trained evaluator.

Although our obese group consisted of slightly obese (overweight, +1SD < BMI z-score < +2SD) and moderately obese (with BMI z-score ≥ +2 SD) girls, for simplicity reasons, we referred to them as the obese participants.

Table I Modified Risk Scores for predicting atherosclerotic lesions for adolescent girls ages between 16–19 years.

Risk factor	Points
Female sex	–1
Non-HDL-c (mmol/L)	
<3.4	0
3.4–4.0	2
4.1–4.8	4
4.9–5.6	6
>5.7	8
HDL-c (mmol/L)	
<1.04	1
1.04–1.55	0
>1.55	–1
Smoking	+1
Blood pressure (mm Hg)	
Normal	0
High SBP≥130 mm Hg and/or DBP≥85 mm Hg	4
Hyperglycemia (fasting glucose ≥ 5.6 mmol/L)	5

The risk for CVD was estimated using modified Risk Score for identifying young individuals with a high probability of having advanced atherosclerotic lesions, reported by McMahan et al. (1). Cardiovascular Risk Score (CVRS) was calculated by adding the points for each risk factor (e.g., female sex, HDL-c, non-HDL-c, smoking, blood pressure and fasting glycemia) (*Table I*).

Comparing with the Framingham Risk Score in adult population, beside the modifiable risk factors such as blood pressure and HDL-c, we also included non-HDL-c and fasting glycemia parameters which can add significant contribution to CV risk assessment. Hypertension was defined as systolic blood pressure (SBP) or diastolic blood pressure (DBP) ≥ 130/85 mm Hg, and hyperglycemia was defined as fasting glucose ≥ 5.6 mmol/L (12).

Biochemical analyses

The blood samples were taken between 7 and 9 a.m., after 12–14 hours of overnight fasting. Serum levels of total bilirubin, fasting glucose, alanine aminotransferase (ALT), total cholesterol (TC), high density lipoprotein cholesterol (HDL-c), low density lipoprotein cholesterol (LDL-c) and triglycerides (TG) were measured spectrophotometrically (Roche Cobas 400, Mannheim, Germany) using standardized enzymatic procedures. High sensitivity C-reactive protein (hsCRP) levels were determined using a nephelometric assay (Behring Nephelometer Analyzer, Marburg, Germany).

Statistical analysis

Statistical analysis was performed using SPSS statistical package (version 15.0 for Windows, SPSS, Chicago, IL, USA). Data are presented as mean ± standard deviation, median (inter-quartile range), or counts and percentages. Differences between groups were evaluated with a Student's t test for normally, Mann-Whitney test for non-normally distributed parameters, or one-way ANOVA, and Kruskal–Wallis nonparametric analysis of variance where appropriate. Chi squared test was used to analyze the differences between categorical data. A correlation analysis, Spearman's (ρ) correlation coefficient was used to determine the relationships between total bilirubin and other variables. Multiple linear regression analysis (MLR) was performed to identify independent factors affecting serum total bilirubin and to estimate the final predictors of its variability. Receiver Operating Characteristic (ROC) curve analysis was used with the purpose of testing the discriminatory potential of a group of parameters selected in MLR analysis, with Risk Score as dependent variable. In all analyses P value < 0.05 was considered as statistically significant.

Results

Table II shows the general clinical and biochemical characteristics of the adolescent girls involved in this study, divided into normal weight and obese. Compared with normal weight, obese girls had higher anthropometric measures (P<0.001) and lower HDL-c (P=0.046), as well as total bilirubin level (P=0.002). They also exhibited higher fasting glucose (P=0.006), non-HDL-c (P=0.014), ALT activity, hsCRP level (P<0.001, respectively) and blood pressure (P<0.001). No significant difference was found with respect to age, TC, LDL-c and TG, between these two groups.

Table II General characteristics of studied adolescent girls according to their obesity status.

Characteristics	Normal weight (n=45)	Obese (n=45)	P
Age (years)	18.0±0.99	17.7±1.21	0.219
BMI (kg/m^2)	21.3±1.84	28.7±3.00	<0.001
BMI z-score	–0.03±0.60	1.49±0.33	<0.001
WC (cm)	77.2±5.14	95.6±11.54	<0.001
Fasting glucose (mmol/L)	5.00±0.38	5.25±0.42	0.006
TC (mmol/L)	4.13±0.59	4.33±0.69	0.245
LDL-c (mmol/L)	2.33±0.54	2.48±0.53	0.199
HDL-c (mmol/L)	1.49±0.37	1.35±0.35	0.046
TG (mmol/L)#	0.77 (0.58–1.01)	0.87 (0.64–1.23)	0.117
TG/HDL ratio#	0.50 (0.35–0.81)	0.65 (0.43–1.11)	0.063
Non-HDL-c (mmol/L)	2.64±0.60	2.98±0.67	0.014
ALT (U/L)#	11.00 (8.00–14.00)	14.00 (12.00–22.25)	<0.001
hsCRP (mg/L)#	0.35 (0.23–0.63)	0.89 (0.56–2.02)	<0.001
Total bilirubin (μmol/L)#	8.30 (5.37–14.00)	6.50 (4.42–8.25)	0.002
SBP (mm Hg)	103±13.2	116±18.4	<0.001
DBP (mm Hg)	68.5±8.70	76.0±11.01	<0.001

Data are presented as mean ± standard deviation or #– data with non-Gaussian distribution are shown as median values (interquartile range), or counts and percentages; BMI – body mass index; WC – waist circumference; TC – total cholesterol; HDL-c – high density lipoprotein cholesterol; LDL-c – low density lipoprotein cholesterol; TG – triglycerides; ALT – alanine aminotransferase; hsCRP – high sensitivity C–reactive protein; SBP – systolic blood pressure; DBP – diastolic blood pressure.

In the current study, we also aimed to test the association of obesity status, as well as cardiometabolic parameters with the risk for CVD (as estimated using the modified Risk Score). Therefore, we have calculated this cardiovascular Risk Score (CVRS) for every adolescent girl included in the study, and compared the results according to their obesity status. Obese girls exhibited a significantly higher Risk Score level than their normal weight counterparts (P<0.001) (*Figure 1*).

Additionally, regarding their risk status level, we divided girls into low, medium and higher risk groups (-2 ≤ Risk Score ≤ 1, 2 ≤ Risk Score ≤ 4, Risk Score ≥ 5, respectively). We found a significantly higher number of obese girls as compared with normal weight counterparts in the higher risk group, compared with low and medium risk groups (χ^2=15.4, P<0.001). Moreover, we also found significant difference in several parameters, which are independent of the Risk Score calculation, i.e., significantly higher ALT activity (P<0.01), hsCRP level (P<0.001), TG/HDL-c ratio (P<0.01), but lower total bilirubin level in the high risk group (P<0.001). This part of results is shown in *Table III*.

Thereafter, we performed Spearman's nonparametric correlation in order to examine the potential relationship between cardiovascular risk score and cardiometabolic parameters independent of the Risk Score calculation (total bilirubin concentration, ALT activity, hsCRP level and TG/HDL-c ratio in the group of adolescent girls).

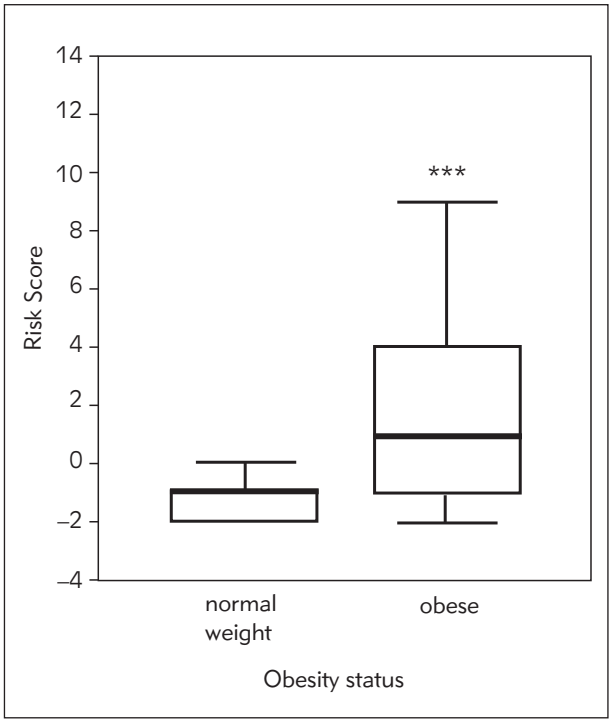

Figure 1 Risk score level in obesity status subgroups.

Table III Cardiometabolic parameters independent of Risk Score calculation, in subgroups according to cardiovascular risk level.

Parameter	Low Risk Score (-2 to 1) n=63	Medium Risk Score (2 to 4) n=14	Higher Risk Score (≥5) n=13	P*
ALT (U/L)	12.1±4.7	20.4±15.1^{aa}	24.8±16.6^{aaa}	<0.01
Bilirubin (μmol/L)#	8.20 (6.42–13.27)	5.25 (4.09–7.82)^{aa}	4.20 (3.70–7.13)^{aa}	<0.001
hsCRP (mg/L)#	0.47 (0.30–0.71)	1.04 (0.74–3.39)^{aa}	1.55 (0.67–2.30)^{aa}	<0.001
TG/HDL-c ratio#	0.50 (0.34–0.80)	0.67 (0.46–1.24)^{a}	0.77 (0.54–1.19)^{a}	<0.01
Risk Score Value (points) #	-1 (-2– -1)	3.0 (2.9–4.0)^{aaa}	8.0 (5.0–8.0)$^{aaa, bbb}$	<0.001
Obese/normal weight girls, n (%)	23/40 (51/89)	11/3 (24.5/7)	11/2 (24.5/4)	χ^2=15.4 <0.001

a – P<0.05, aa – P<0.01, aaa – P<0.001 vs. Low Risk Score, bbb – P<0.001 vs. Medium Risk Score.

– data with non-Gaussian distribution are shown as median values (interquartile range) *P value from one-way ANOVA or Kruskal–Wallis nonparametric analysis of variance, followed by nonparametric Mann–Whitney U test, where appropriate.

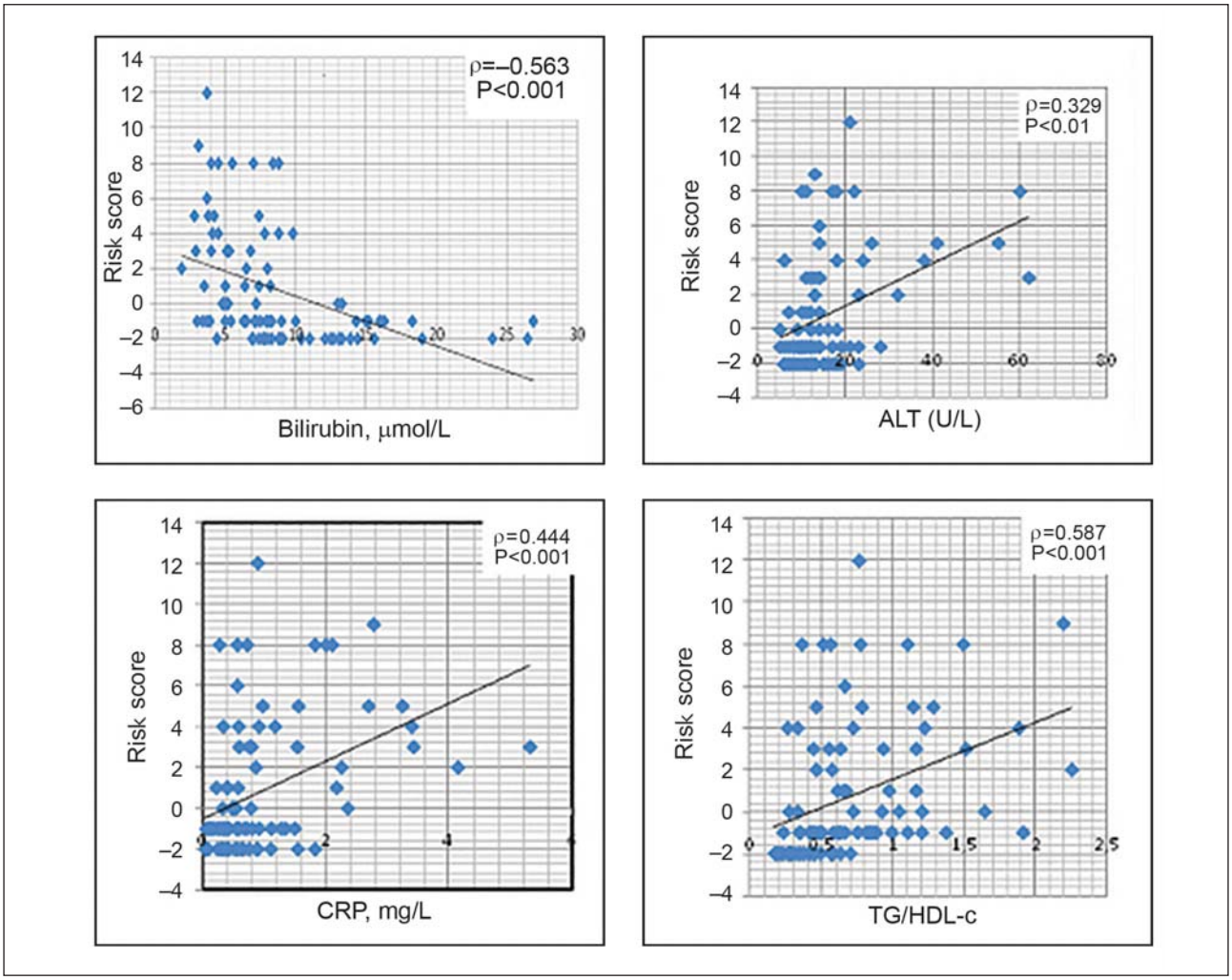

Figure 2 Correlation between cardiovascular risk score and total bilirubin, ALT activity, hsCRP level and TG/HDL-c concentration in a group of adolescent girls.

Spearman's nonparametric correlation revealed a significant opposite relationship between CVRS and bilirubin concentration in the group of adolescent girls (ρ=-0.563, P<0.001), and also a positive correlation of ALT activity (ρ=0.329, P<0.01), hsCRP level (ρ=0.444, P<0.001) and TG/HDL-c ratio (ρ=0.587, P<0.001) value with CVRS (*Figure 2*).

We conducted a receiver operating characteristic (ROC) analysis of the selected parameters to test their discriminatory ability regarding Risk Score status (low *vs.* higher risk). Additionally, we constructed a Model consisting of 4 parameters (total bilirubin, ALT, hsCRP and TG/HDL-c ratio) by using logistic regression analysis generated predictive probabilities. *Figure 3* shows the ROC curve graph and *Table IV* shows the most important ROC parameters: area under the curve (AUC) with the 95% confidence interval (CI) of selected parameters and the Model. *Table IV* also shows sensitivities, specificities and cut-off values for the selected parameters.

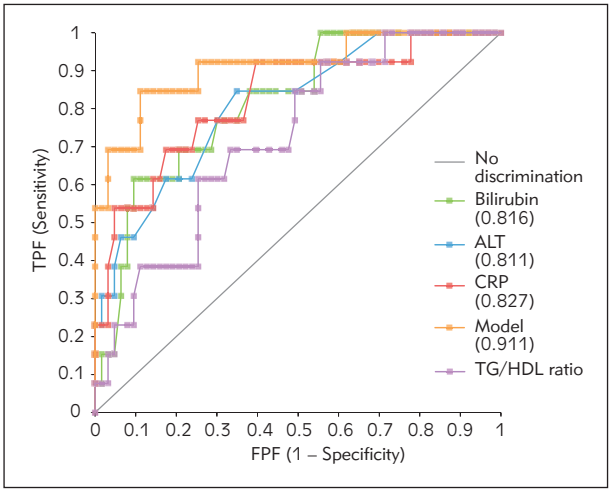

Figure 3 ROC curves of selected parameters' discriminatory ability regarding cardiovascular Risk Score status (low *vs.* higher risk).

ROC curves comparison showed that all separate curves have comparable discriminatory capability towards risk level status. Construction of a model consisting of 4 Risk Score formula independent parameters (bilirubin, ALT, hsCRP and TG/HDL-c ratio) by using logistic regression analysis showed that the new ROC curve had outstanding discriminatory capability (AUC>0.900, according to Hosmer and Lemeshow's rules) (13). Model ROC curve has significantly larger AUC than the ROC curve for TG/HDL-c ratio, hsCRP and ALT alone, respectively. Only total bilirubin alone has comparable AUC for cardiovascular discrimination compared to Model ROC (P=0.055) (*Table IV*). Because of this potential of total bilirubin as a routine biochemical parameter to discriminate between low and higher risk for cardiovascular disturbances in an apparently healthy adolescent female population, further analyses were performed focusing on total bilirubin level.

In order to confirm the association of decreased total bilirubin values with obesity status, as well as with cardiometabolic risk factors, we divided the patients according to the tertile values of total bilirubin concentration and counted the number and percentage of obese subjects. The data are shown in *Table V*.

Chi-square analysis confirmed that a significantly lower number of obese subjects, as well as a significantly higher number of normal weight counterparts were in the group with the highest total bilirubin values (20%, compared to 40% in the first and second tertile group, and 44% compared to 27% in the first and 29% in the second tertile group, respectively, -χ^2=6.2, P=0.046). Moreover, with increasing tertiles of serum total bilirubin levels, significant decreases in fasting glucose, lipid parameters (e.g., LDL-c, TG, non-HDL-c), ALT activity, hsCRP and blood pressure, as well as an increase in HDL-c were found.

The possible relationship between serum total bilirubin and the clinical and biochemical characteris-

Table IV Area under the curve, 95% Confidence Interval and Standard Error for the parameters of selected parameters' discriminatory ability regarding Risk Score status (low vs. higher risk); pairwise comparison of the areas under ROC curves (AUCs) for Model and separate parameters.

Parameter	AUC	95% CI	SE	Sensitivity (%)	Specificity (%)	Cut-off value (%)	AUC difference*	P**
Model (4 parameters)	0.911	0.811–1.010	0.051	85	89	/	/	/
TG/HDL-c ratio	0.722	0.581–0.864	0.072	92	45	0.45	0.189	0.003
hsCRP	0.827	0.695–0.959	0.067	92	60	0.56	0.084	0.018
ALT	0.811	0.683–0.938	0.065	85	65	12.50	0.100	0.049
Bilirubin	0.816	0.696–0.935	0.061	90	62	4.65	0.095	0.055

AUC – area under ROC curve; CI – confidence interval; SE – standard error; Model: bilirubin, ALT, hsCRP and TG/HDL-c ratio.
* – difference between Model ROC and separate parameters.
** – P from pairwise comparison for AUC differences between Model and separate parameters.

Table V Obesity distribution in total bilirubin tertile values subgroups.

Obesity status	Total bilirubin tertile values			
	I tertile (n=30) ≤5.8 μmol/L	II tertile (n=28) 5.9–8.7 μmol/L	III tertile (n=32) ≥8.8 μmol/L	P*
Age (years)	17.8±1.15	18.2±0.97	17.6±1.13	0.069
BMI (kg/m^2)	26.3±4.48^{aaa}	26.2±4.51^{aaa}	22.7±3.40	0.001
BMI z-score	1.01±0.82^{aaa}	0.95±0.81^{aaa}	0.29±0.90	0.002
WC (cm)	92.3±13.88^{aaa}	88.8±12.64^{aaa}	78.8±7.38	<0.001
Glucose (mmol/L)	5.28±0.46^{aa}	5.16±0.33^{a}	4.95±0.40	0.007
TC (mmol/L)	4.31±0.66	4.22±0.62	4.17±0.67	0.685
HDL-c (mmol/L)	1.17±0.18^{aaa,bbb}	1.43±0.28^{a}	1.65±0.41	<0.001
LDL-c (mmol/L)	2.62±0.53^{aa}	2.41±0.43	2.20±0.56	0.007
TG (mmol/L) #	1.07(0.81–1.34)^{aaa,bb}	0.79 (0.54–0.98)	0.64 (0.56–0.84)	<0.001
TG/HDL-c ratio #	0.92 (0.72–1.16)^{aaa}	0.49 (0.39–0.64)	0.39 (0.28–0.63)	0.140
Non-HDL-c (mmol/L)	3.14±0.62^{aaa,b}	2.79±0.64	2.51±0.56	0.001
ALT (U/L) #	14.0 (12.0–21.0)^{aa}	12.5 (8.5–17.0)	11.0 (8.5–14.5)	0.012
hsCRP (mg/L) #	0.76 (0.52–2.00)^{aa}	0.67 (0.44–1.30)^{aa}	0.39 (0.18–0.65)	0.002
SBP (mm Hg)	118±19^{aaa}	110±17^{a}	102±12	0.002
DBP (mm Hg)	76.8±12.07^{aa}	71.8±9.35	68.4±8.47	0.006
Obese, n (%)	18 (40)	18 (40)	9 (20)	χ^2=6.2 P=0.046
Normal weight, n (%)	12 (27)	13 (29)	20 (44)	

^{aaa} – p<0.001, ^{aa} – p<0.01, ^{a} – p<0.05 vs. third bilirubin tertile; ^{bbb} – p<0.001, ^{bb} – p<0.01, ^{b} – p<0.05 vs. second bilirubin tertile.

Data are presented as mean ± standard deviation or # – data with non-Gaussian distribution are shown as median values (interquartile range), or counts and percentages; *P value from one-way ANOVA or Kruskal–Wallis nonparametric analysis of variance, followed by nonparametric Mann-Whitney U test, where appropriate; BMI – body mass index; WC – waist circumference; TC – total cholesterol; HDL-c – high density lipoprotein cholesterol; LDL-c – low density lipoprotein cholesterol; TG – triglycerides; ALT – alanine aminotransferase; hsCRP – high sensitivity C–reactive protein; SBP – systolic blood pressure; DBP – diastolic blood pressure.

tics in the group of obese girls was tested with Spearman's nonparametric correlation analysis (*Table VI*).

Serum total bilirubin correlated negatively with anthropometric measures, glucose, lipid parameters (e.g., LDL-c, TG, TG/HDL-c ratio), ALT, hsCRP, blood pressure, and positively with HDL-c (*Table VI*).

Multiple linear regression (MLR) analysis was performed to identify which of the measured markers have the best association with serum total bilirubin level. Namely, all variables found to have a significant predictive value in Spearman's nonparametric correlation (eg., BMI z-score, WC, glucose, HDL-c, LDL-c, TG, TG/HDL-c ratio, ALT, hsCRP, SBP) were further

Table VI Spearman's nonparametric correlation between serum total bilirubin concentration and clinical variables in obese adolescent girls.

Variable	ρ	P
Age (years)	–0.069	0.648
BMI (kg/m^2)	–0.357	0.018
BMI z-score	–0.352	0.019
WC (cm)	–0.601	<0.001
Fasting glucose (mmol/L)	–0.458	0.002
TC (mmol/L)	–0.016	0.916
HDL-c (mmol/L)	0.530	<0.001
LDL-c (mmol/L)	–0.328	0.029
TG (mmol/L)	–0.495	0.001
ALT (U/L)	–0.383	0.011
hsCRP (mg/L)	–0.498	0.001
TG/HDL-c ratio	–0.556	<0.001
Non-HDL-c (mmol/L)	–0.258	0.087
SBP (mm Hg)	–0.417	0.006
DBP (mm Hg)	–0.385	0.011

BMI – Body mass index; WC – waist circumference; TC – total cholesterol; HDL-c – high density lipoprotein cholesterol; LDL-c – low density lipoprotein cholesterol; TG – triglycerides; ALT – alanine aminotransferase; hsCRP – high sensitivity C – reactive protein; TG/HDL-c – triglycerides – high density lipoprotein cholesterol ratio; non-HDL-c–non – high density lipoprotein cholesterol.

Table VII Multiple linear regression standardized β coefficients, 95% Confidence Interval and P values for the parameters in the best-fit model for the association of several parameters with serum total bilirubin levels, backward selection.

Coefficients	Standardized Coefficients	95% Confidence Interval for β	P
WC	–0.4	–0.192–0.064	<0.001
HDL-c	0.4	2.186–6.432	<0.001
LDL-c	–0.3	–3.033–0.434	<0.010

WC – waist circumference; HDL-c – high density lipoprotein cholesterol; LDL-c – low density lipoprotein cholesterol.

analyzed in MLR analysis for total bilirubin values prediction. The backward selection enabled us to find the best model consisting of 3 parameters (e.g., WC ($p<0.001$), HDL-c ($p<0.001$) and LDL-c ($p<0.010$)) which are shown in *Table VII*. Adjusted R^2 for the best model was 0.603, which means that 60% of variation in the total bilirubin level could be explained with this model.

Discussion

In our study, the risk for CVD was estimated using modified Risk Score (see *Table I*) for identifying young individuals with a high probability of having advanced atherosclerotic lesions, reported by McMahan et al. (1). Compared with the Framingham Risk Score in the adult population, beside modifiable risk factors such as blood pressure and HDL-c, we also included non-HDL-c and fasting glycemia, parameters which can add a significant contribution to the assessment of CV risk. We found a significantly higher number of obese girls as compared with the normal weight counterparts in the higher risk group, compared with low and medium risk groups.

Beside the expected positive relationship between cardiovascular risk score and ALT activity, hsCRP level and TG/HDL-c ratio, which are known as established CV risk factors (9, 14, 15), we also revealed a significant opposite relationship between cardiovascular risk score and bilirubin concentrations in the group of adolescent girls (*Figure 2*). Therefore, we conducted a ROC analysis of selected parameters to test their discriminatory ability regarding Risk Score status (low vs. higher risk). Additionally, construction of a model consisting of 4 Risk Score formula independent parameters (bilirubin, ALT, hsCRP and TG/HDL-c ratio) by using logistic regression analysis showed that the new comprehensive ROC curve (*Figure 3*) has outstanding discriminatory capability (11). We showed that Model ROC curve had significantly larger AUC than ROC for TG/HDL-c ratio, hsCRP and ALT alone, respectively, and only total bilirubin alone had a comparable AUC for cardiovascular discrimination compared to Model ROC (*Table IV*).

This potential of total bilirubin as a routine biochemical parameter to discriminate between low and higher risk for cardiovascular disturbances in an apparently healthy adolescent female population is the novel finding in our study.

The results of studying individuals with benign hyperbilirubinemia (Gilbert's syndrome – GS) have greatly added to the exploration of the physiological protective effects of bilirubin, showing that GS individuals are at decreased risk of CVD (7).

Since only girls were included in our study, and all were nonsmokers, we reported lower score values than the previous study conducted by McMahan et al.

(1). Moreover, they emphasized that although the girls reported to have increased cardiovascular risk relative to their low risk counterparts, the probability of atherosclerotic lesions in young persons (aged 16–19 years) is certainly low at present, but in older persons, when contributions of age are included with risk due to similar values of the modifiable risk factors as in the period of adolescence, the probability of vessel wall lesions may be greatly increased (1).

Tatami et al. (16) demonstrated an independent association of low serum bilirubin levels with severe carotid atherosclerosis in coronary artery disease (CAD) patients. In line with these results, Song et al. (17) in a prospective Korean cohort during a 4-year follow-up showed that the lowest serum total bilirubin level category (bilirubin $\leq$ 5.47 μmol/L) was an independent risk factor for future CAD events.

On the contrary, Jørgensen et al. (18), in a study including almost 10.000 overweight/obese patients with high cardiovascular risk, showed that low bilirubin was not a risk factor independent of other traditional cardiovascular risk factors. Even though they showed a trend towards lower risks associated with increased bilirubin levels, after adjustment for classical cardiovascular risk factors (e.g., blood pressure, lipids, BMI, smoking, microalbuminuria), the lower risk of CVD in subjects with bilirubin values higher than 8 μmol/L compared to bilirubin levels $\leq$ 8 μmol/L was no longer statistically significant. Additional adjustment for liver function indicators did not alter the results, suggesting that low bilirubin may not be an independent risk marker for CVD in this cohort of patients with high baseline risk of adverse cardiovascular outcomes.

In our study, lower serum bilirubin levels were related to higher anthropometric indices and unfavorable cardiometabolic markers in obese adolescent girls. Moreover, multiple linear regression analysis showed that higher WC and LDL-c, but lower HDL-c, the well-known, established cardiovascular risk factors were found to be independent predictors of lower total bilirubin levels. This may be explained by the notion that bilirubin has antioxidant and antiinflammatory properties (7). Under normal concentrations, unbound bilirubin has scavenging properties to singlet oxygen. In the presence of hydrogen peroxide bilirubin acts as a reducing agent (19). Namely, under oxidative stress (which plays an important role in atherosclerosis development) one of the protective agents is the hemeoxygenase (HO) which reduces cytotoxic effects and degrades atherogenic heme into biliverdin, carbon monoxide (CO), and Fe^{+2} (20). The former is then converted into bilirubin by the activity of bilirubin reductase on biliverdin. Substrate heme is highly reactive and cytotoxic in its unbound form, whereas CO and bilirubin are recognized as mediators that, in the case of CO, inhibit inflammatory processes, and, in the case of bilirubin, scavenge free radicals by inhibiting NADPH oxidase (21).

Here, obese adolescent girls exhibited lower serum total bilirubin levels as compared with the normal weight counterparts. This is in accordance with the results by Jenko-Pražnikar et al. (22) who reported lower total bilirubin levels in healthy overweight nonsmoking middle-aged adults of both sexes. Belo et al. (23) reported an inverse correlation between body fat percentage and total bilirubin in obese children and adolescents. We also confirmed the inverse relationship between serum total bilirubin level and unfavorable lipid profile including higher LDL-c and TG, but a positive relationship with HDL-c. This is in accordance with previous results (22, 24), thus suggesting that bilirubin may act as a physiological hypolipidemic agent that protects from CVD (25). However, only one study reported decreasing HDL-c concentrations with an increasing bilirubin level (26). Further, we reported a significant inverse correlation between total bilirubin and the proatherogenic, comprehensive markers of lipid status, such as non-HDL-c and TG/HDL-c ratio. Inverse relationship between bilirubin and non-HDL cholesterol was recently shown in a study by McArdle et al. (27). Moreover, to our knowledge, this is the first study examining the potential relationship between total bilirubin and TG/HDL-c ratio. Namely, TG/HDL-c ratio, an estimate of small, dense low-density lipoprotein particles, is an independent determinant of arterial stiffness in adolescents and young adults, especially in obese youth, suggesting that the use of TG/HDL-c ratio may be helpful in identifying young adults requiring aggressive intervention to prevent atherosclerotic CVD (15). This could be another confirmation of the close relationship between total bilirubin level and CV risk in the adolescent population.

Furthermore, in agreement with the notion that higher bilirubin levels may contribute to reduced systemic low-grade inflammation, some studies (23, 28) have shown an inverse relationship of hsCRP with serum total bilirubin. Our results also confirmed the negative correlation between total bilirubin level and hsCRP, but it was not independent of other traditional CV factors.

The ability of bilirubin to scavenge ROS and to inhibit low density lipoprotein (LDL) oxidation (7, 8) may be of significant importance in the pathogenesis of many cardiovascular disorders characterized by enhanced systemic low-grade inflammation and/or increased oxidative stress. Limitations of the present study must be considered. Due to its cross-sectional design, a causal relationship between total bilirubin, anthropometric and cardiometabolic markers could not be established. It should also be emphasized that the small number of participants is a limitation for a study dealing with cardiovascular risk estimation. However, to get better insight into the association

between total bilirubin and obesity-related disorders, we examined a broad spectrum of biochemical parameters that may be important determinants of cardiometabolic disturbances. Thus, despite the limited size of cohorts, the findings of the present study support the hypothesis that lower circulating total bilirubin could reflect an unfavorable cardiometabolic profile in obese adolescent girls.

Conclusion

Obese adolescent females are at increased risk of cardiovascular disease. In addition to traditional risk factors, total bilirubin level may have the potential to discriminate between low and higher risk for cardiovascular disturbances in an apparently healthy adolescent female population. Furthermore, serum total bilirubin level is significantly decreased in obese adolescent girls and the constellation of increased WC and LDL-c and decreased HDL-c was an independent predictor of its lower values. Prospective studies are needed to clarify the potential role of total bilirubin in obesity-related disorders which may lead to the discovery of new target therapy used to induce the protective effects of bilirubin in individuals at increased risk of CVD.

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Conflict of interest statement

The authors stated that they have no conflicts of interest regarding the publication of this article.

References

1. McMahan CA, Gidding SS, Fayad ZA, Zieske AW, Malcom GT, Tracy RE, et al. Risk scores predict atherosclerotic lesions in young people. Arch Intern Med 2005; 165: 883–90.
2. Herouvi D, Karanasios E, Karayianni C, Karavanaki K. Cardiovascular disease in childhood: the role of obesity. Eur J Pediatr 2013; 172(6): 721–32.
3. Kocova M, Sukarova-Angelovska E, Tanaskoska M, Palcevska-Kocevska S, Krstevska M. Metabolic setup and risks in obese children. J Med Biochem 2015; 34: 31–7.
4. Zdravković V, Sajić S, Mitrović J, Stefanović I, Pavićević P, Nikolić D, Dimić J, Lalić MN.The diagnosis of prediabetes in adolescents. J Med Biochem 2015; 34: 38–45.
5. Le Lay S, Simard G, Martinez MC, Andriantsitohaina R. Oxidative stress and metabolic pathologies: from an adipocentric point of view. Oxid Med Cell Longev 2014; 2014: 908539.
6. Savini I, Catani MV, Evangelista D, Gasperi V, Avigliano L. Obesity-associated oxidative stress: strategies finalized to improve redox state. Int J Mol Sci 2013; 14(5): 10497–538.
7. Vítek L. The role of bilirubin in diabetes, metabolic syndrome, and cardiovascular diseases. Front Pharmacol 2012; 3: 55.
8. Lin JP, Vítek L, Schwertner HA. Serum bilirubin and genes controlling bilirubin concentrations as biomarkers for cardiovascular disease. Clin Chem 2010; 56(10): 1535–43.
9. Ridker PM. Clinical application of C-reactive protein for cardiovascular disease detection and prevention. Circulation 2003; 107: 363–9.
10. World Medical Association Declaration of Helsinki: Recommendations guiding physicians in biomedical research involving human subjects. JAMA 1997; 277: 925–6.
11. World Health Organization (WHO), Growth reference 5–19 years, 2007, http: //www.who.int/growthref/who2007_bmi_for_ age/en/.
12. Zimmet P, Alberti K, George MM, Kaufman F, Tajima N, Silink M, et al. The metabolic syndrome in children and adolescents – an IDF consensus report. Pediatric Diabetes 2007: 8; 299–306.
13. Hosmer D, Lemeshow S, Sturdivant RX. Applied logistic regression. 3rd ed. New York, NY: John Wiley & Sons Inc, 2013.
14. Schindhelm RK, Dekker JM, Nijpels G, Bouter LM, Stehouwer CD, Heine RJ, Diamant M. Alanine aminotransferase predicts coronary heart disease events: a 10-year follow-up of the Hoorn study. Atherosclerosis 2007; 191: 391–6.
15. Urbina EM, Khoury PR, McCoy CE, Dolan LM, Daniels SR, Kimball TR. Triglyceride to HDL-C ratio and increased arterial stiffness in children, adolescents, and young adults. Pediatrics 2013; 131: 1–9.
16. Tatami Y, Suzuki S, Ishii H, Shibata Y, Osugi N, Ota T, et al. Impact of serum bilirubin levels on carotid atherosclerosis in patients with coronary artery disease. IJC Metab Endocr 2014; 5: 24–7.
17. Song YS, Koo BK, Cho NH, Moon MK. Effect of low serum total bilirubin levels (≤0.32 mg/dl) on risk of coronary artery disease in patients with metabolic syndrome. Am J Cardiol 2014; 114(11): 1695–700.
18. Jørgensen ME, Torp-Pedersen C, Finer N, Caterson I, James WP, Legler UF, et al. Association between serum bilirubin and cardiovascular disease in an overweight high risk population from the SCOUT trial. Nutr Metab Cardiovasc Dis 2014; 24(6): 656–62.
19. Abraham NG, Kappas A. Pharmacological and clinical aspects of hemeoxygenase. Pharmacol Rev 2008; 60: 79–127.

20. Morita T. Hemeoxygenase and atherosclerosis. Arterioscler Thromb Vasc Biol 2005; 25: 1786–95.

21. Gul M, Kalkan AK, Uyarel H. Serum bilirubin: a friend or an enemy against cardiovascular diseases? J Crit Care 2014; 29(2): 305–6.

22. Jenko-Pražnikar Z, Petelin A, Jurdana M, Žiberna L. Serum bilirubin levels are lower in overweight asymptomatic middle-aged adults: An early indicator of metabolic syndrome? Metabolism 2013; 62(7): 976–85.

23. Belo L, Nascimento H, Kohlova M, Bronze-da-Rocha E, Fernandes J, Costa E, et al. Body fat percentage is a major determinant of total bilirubin independently of UGT1A1*28polymorphism in young obese. PLoS One 2014; 9: e98467.

24. Oda E. Cross-sectional and longitudinal associations between serum bilirubin and dyslipidemia in a health screening population. Atherosclerosis 2015; 239(1): 31–7.

25. Bulmer AC, Verkade J, Wagner KH. Bilirubin and beyond: a review of lipid status in Gilbert's syndrome and its relevance to cardiovascular disease protection. Prog Lipid Res 2012; 52: 193–205.

26. Hwang HJ, Kim SH. Inverse relationship between fasting direct bilirubin and metabolic syndrome in Korean adults. Clin Chim Acta 2010; 411(19–20): 1496–501.

27. McArdle PF, Whitcomb BW, Tanner K, Mitchell BD, Shuldiner AR, Parsa A. Association between bilirubin and cardiovascular disease risk factors: Using Mendelian randomization to assess causal inference. BMC Cardiovasc Disord 2012; 12: 16.

28. Deetman PE, Bakker SJL, Dullaart RPF. High sensitive C-reactive protein and serum amyloid A are inversely related to serum bilirubin: effect-modification by metabolic syndrome. Cardiovasc Diabetol 2013; 12: 166.

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