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Total bilirubin in young men and women: Association with risk markers for cardiovascular diseases

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ABSTRACT

Objective: The aim of this study was to investigate whether high bilirubin concentration is a protective factor in cardiovascular disease (CAD) and how it correlates with parameters of oxidative stress in young males and females.

Methods: The study comprised 628 healthy subjects of both genders, 18–22 years of age. In fasting sera the concentration of total bilirubin (Tbil), parameters of cardiovascular risk and oxidative stress were determined. The results were analyzed by appropriate statistical methods.

Results: We found no gender differences in body mass index (BMI), blood pressure and lipid profile between subjects with low and high Tbil level. Men with high Tbil had higher concentrations of albumin and uric acid ($p < 0.001$) and lower of oxLDL (<0.05), while women had higher albumin ($p < 0.05$) and lower TBARS ($p < 0.05$). Significant positive correlation in men was found between Tbil, uric acid and albumin, while for glucose and TBARS this association was negative. In female significant positive correlation was between Tbil, HDL-C, fibrinogen, albumin and uric acid and negative between Tbil and TBARS. The high concentration of Tbil in men was independently associated with uric acid ($p < 0.05$) and oxLDL ($p < 0.001$), while in women it was independently associated with TBARS ($p < 0.05$). After adjustment for traditional lipid parameters the predictive power of high bilirubin in men remained for uric acid ($p < 0.001$) and TBARS in women ($p < 0.05$).

Conclusion: These findings jointly support the concept that bilirubin via its antioxidant potential has a protective effect against cardiovascular disease in young male and female.

Introduction

Bilirubin acts as a potent physiological antioxidant that may provide important protection against atherosclerosis, CAD and inflammation [1–3]. Human studies guide to conclusions that bilirubin production is involved in antioxidant defense mechanisms and those higher bilirubin concentrations are associated with a lower incidence of oxygen radical-mediated injury [4–6]. The antioxidant capacity of bilirubin and its potent ability to scavenge peroxyl radicals led to the concept that mildly increased circulatory bilirubin may have a physiologic function to protect against disease processes that involve oxygen and peroxyl radicals [7]. Inverse correlation between the presence of CAD and total bilirubin in circulation was reported in several independent studies [8,9]. Furthermore, bilirubin correlates inversely with several established factors for CAD, including increased LDL-cholesterol, diabetes, and obesity, but is directly proportional to the protective factor HDL-cholesterol [8]. Oxidized low-density lipoproteins (oxLDL), a recognized oxidative stress marker, was positively associated with central obesity, metabolic syndrome manifestation and atherosclerosis [10,11].

The effect of bilirubin on cardiovascular disease biomarkers was investigated in middle-aged population [12,13] and predominantly in men [14,15]. We did not find a comprehensive study on parameters of cardiovascular risk and oxidative stress in young male and female. Therefore, in the present study, we examined the association of total bilirubin with oxidative stress markers as well as anthropometric and metabolic parameters in young healthy men and age-matched women. We also wanted to establish which parameters are predictors of high bilirubin concentration and whether gender differences exist.

In the present study, we therefore examined the potential association of total bilirubin with oxLDL concentrations, oxidative stress markers as well as anthropometric and metabolic (glucose and lipid profiles) data in young healthy subjects of both genders.

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Materials and methods

Study protocol

The study group consisted of 628 healthy volunteers (442 men and 186 women aged 18 to 22 years). Inclusion criteria were that participants were normotensive, normocholesterolemic, nondiabetic and were receiving no medication (including vitamin supplements). Subjects with any of the following were excluded: chronic liver disease, CVD, anemia, abnormal liver function, defined as an elevation of aspartate aminotransferase (AST) or alanine aminotransferase (ALT) three times more than the upper normal limits or Tbil above 34.2 μmol/L. Also excluded were individuals with a history of alcohol-related liver disease and alcoholism by an interview with the participants and by the baseline liver function test. Furthermore, to exclude the acute effects of alcohol intake on lipid peroxidation, taking alcoholic drinks 24 h before the study was prohibited to the participants. The women reported regular menstrual cycles (every 26 to 36 days) before the study, and none of them were taking oral contraceptives. All participants were nonsmokers. The study protocol included height and weight measurement for body mass index (BMI) calculation.

All subjects were categorized into two groups according to Tbil values. In the first group were participants with Tbil values below the upper limit of reference values for our population ($\leq$24 μmol/L for men and $\leq$16.3 μmol/L for women). In the further text they were marked as "low bilirubin". The second group comprised those with Tbil above the upper limit of reference values (>24 μmol/L for men and >16.3 μmol/L for women), furthermore marked as "high bilirubin".

The study was planned according to the ethical guidelines following the Declaration of Helsinki. All subjects involved in the study gave written consent. The study was approved by the Ethics Committee of the Institute for Health Care of the Ministry of Internal Affairs.

Biochemical measurements

Laboratory tests were performed after the subjects had fasted for 12 h. Since it is known that bilirubin concentration tends to be higher in fasting individuals or when the caloric intake is reduced [16], participants were asked to return after eating, and Tbil reassayed.

Plasma and serum were separated and multiple aliquots of each sample stored at −80 °C, were protected from light, until the analyses.

Total bilirubin was determined in serum by a commercial test (Instrumental Laboratory, Milano, Italy), based on the modified Jendrassik and Grof method [17]. The test was run on ILab 600 analyzer (Instrumental Laboratory, Milano, Italy).

The concentration of hsCRP was measured by latex-enhanced immunoturbidimetric method (Quantex hsCRP kit, Biokit, Barcelona, Spain). Serum albumin was measured by dye-binding using bromcresol green reagent on ILab 600 analyzer [18]. Fibrinogen was assayed in citrate plasma using Clauss method [19] on an ACL 200 Instrumental Laboratory Analyzer with supplied reagents. Serum uric acid, lipids and other biochemical blood measurements were determined by automatic colorimetric methods with appropriate DIALAB tests (DIALAB® GmbH, Wiener Neudorf, Austria). The concentration of LDL-C was calculated by the Friedwald formula. The thiobarbituric acid-reacting substances (TBARS) concentration was measured as described previously by Girotti [20]. Briefly, 0.4 mL samples were taken and mixed with 0.4 mL of 1% thiobarbituric acid in 50 mmol/L NaOH, 0.2 mL de 20% of H_3PO_4 and 40 mL of 10 N NaOH. The mixture was heated to 100 °C for 15 min. The mixture was shaken and subsequently centrifuged at 2000 *g* during 5 min. The optical density was measured at 535 nm. The molar extinction coefficient used to calculate TBARS concentration was $1.56 \times 10^6\ M^{-1}\ cm^{-1}$.

An OxiSelect™ Human Oxidized LDL ELISA kit (MDA-LDL Quantification) by Cell Biolabs, Inc. (San Diego, CA, USA), with an intra-assay and inter-assay coefficients of variation of 4.0% and 7.5%, respectively, was used to determine oxLDL concentrations in serum.

Statistical analysis

Data are shown as mean ± standard deviation for normally distributed variables and as relative or absolute frequencies for categorical variables. Comparisons of continuous variables were performed using the Student's *t*-test. Analyses of categorical variables used the Chi-square test for contingency tables. A logarithmic transformation of TG levels was performed due to the skewed distribution in analysis using the Student's *t*-test analysis [21]. Spearman's nonparametric correlation analysis was employed to determine possible correlation between bilirubin concentration and biochemical as well as oxidative stress parameters in men and women. We used multiple regression analysis to estimate the independent contribution of predictors to the variance in bilirubin levels. Spearman's rho correlation test was used for screening the independent variables. If P-values were <0.10, the variables were included in further regression analysis. The tolerance option was used to prevent multicollinearity among the independent variables [22].

Binary logistic regression was used to seek possible independent association between high total bilirubin concentration and lipid profile, inflammation and oxidative stress parameters. The lower bilirubin concentration group in men and women was used as the reference group and was coded 0, while the higher bilirubin concentration group was coded 1. Firstly, we applied univariate logistic regression analysis. Adjustment was performed to correct the influence of lipid profile parameters on parameters that showed independent association with high total bilirubin concentration in univariate analysis. Confounding variables were entered as continuous. For each odds ratio (OR) we estimated two-tailed probability values and the 95% confidence interval (95% CI).

All statistical analyses were performed using STATGRAPHIC Plus (version 4.2), CBstat (version 4.3.2) and Medcalc software's. All statistical tests were considered significant at the 0.05 probability level.

Results

Anthropometric, clinical and biochemical data (mean ± SD) categorized by Tbil value into two groups are presented in Table 1. For both genders no differences were found in BMI, blood pressures and lipid profile parameters between individuals with lower and higher Tbil concentrations. Men with high Tbil concentration (>24 μmol/L) showed significantly higher concentrations of albumin and uric acid ($p < 0.001$), and lower of oxLDL ($p < 0.05$). In female with high Tbil (>16.3 μmol/L) albumin was significantly higher ($p < 0.05$) and TBARS significantly lower ($p < 0.05$).

For better understanding of associations between Tbil concentrations and some variables of interest, Spearman's coefficient correlation analysis was performed (Table 2). In men we found significant positive correlation of Tbil with albumin ($\rho = 0.215$, $p < 0.001$), uric acid ($\rho = 0.179$, $p < 0.001$), and TBARS ($\rho = 0.231$, $p < 0.05$), while for glucose this association was the inverse ($\rho = -0.159$, $p < 0.001$).

In the female there was significant positive correlation between Tbil concentration and HDL-C ($\rho = 0.162$, $p < 0.05$), fibrinogen ($\rho = 0.164$, $p < 0.05$), albumin ($\rho = 0.264$, $p < 0.001$) and uric acid ($\rho = 0.242$, $p < 0.001$) concentrations. A significant negative association was between Tbil and TBARS ($\rho = -0.255$, $p < 0.05$). Data are presented in Table 2.

We performed additional statistical analysis in order to find possible factors associated with total bilirubin concentration. The multiple linear regression analysis was applied to identify the determinants of Tbil concentration among the independent variables. BMI, glucose, albumin, uric acid, TBARS and oxLDL were included in the model as

Table 1
General, biochemical, lipid profile parameters, oxidative stress and inflammatory markers according to the values of total bilirubin (μmol/L) in men and women.

Total bilirubin (μmol/L)	Men			Women		
	Total bilirubin (≤24 μmol/L) n = 352	Total bilirubin (>24 μmol/L) n = 90	p[1]	Total bilirubin (≤16.3 μmol/L) n = 146	Total bilirubin (>16.3 μmol/L) n = 40	p[1]
Age, years	19.8 ± 2.27	19.6 ± 1.28	0.247	19.3 ± 1.06	19.7 ± 1.62	0.237
BMI, kg/m^2	23.5 ± 2.27	23.6 ± 2.11	0.653	21.2 ± 1.81	20.8 ± 1.65	0.181
Smoking, (%)	13.1	3.3	0.115	12.3	5	0.399
SBP, mmHg	120.1 ± 6.5	120.6 ± 6.4	0.645	115.6 ± 6.84	115.0 ± 6.64	0.721
DBP, mmHg	73.5 ± 5.51	74.2 ± 5.32	0.365	71.5 ± 4.92	71.9 ± 5.49	0.781
Glucose, mmol/L	5.1 ± 0.41	5.1 ± 0.37	0.587	4.9 ± 0.38	4.7 ± 0.44	0.844
Total cholesterol, mmol/L	4.4 ± 0.72	4.3 ± 0.86	0.237	4.6 ± 0.79	4.4 ± 0.82	0.246
Triglycerides, mmol/L[2]	0.82 (0.79–0.86)	0.77 (0.71–0.83)	0.072	0.8 ± 0.27	0.79 ± 0.29	0.824
LDL-cholesterol, mmol/L	2.6 ± 0.62	2.5 ± 0.71	0.228	2.6 ± 0.64	2.5 ± 0.65	0.466
HDL-cholesterol, mmol/L	1.4 ± 0.35	1.4 ± 0.29	0.820	1.6 ± 0.34	1.7 ± 0.35	0.200
Albumin, g/L	50.6 ± 4.39	51.5 ± 2.36	<**0.001**	49.2 ± 2.33	50.1 ± 2.02	<**0.05**
Fibrinogen, g/L	2,5 ± 0.22	2.5 ± 0.21	0.391	2.82 ± 0.41	2.9 ± 0.48	<**0.05**
hsCRP, mg/L[3]	0.61 (0.56–0.61)	0.61 (0.56–0.66)	0.403	0.24 (0.22–0.26)	0.22 (0.14–0.285)	0.495
Hemoglobin, g/L	158.1 ± 11.79	160.3 ± 9.01	<**0.05**	135.1 ± 11.87	136.8 ± 10.4	0.376
Uric acid, μmol/L	326.4 ± 63.2	353.6 ± 50.86	<**0.001**	226.5 ± 54.32	236.9 ± 54.17	0.287
oxLDL, ng/ml	64.3 ± 18.43	52.9 ± 18.35	<**0.05**	67.7 ± 18.28	58.9 ± 32.4	0.547
TBARS, μmol/L	3.5 ± 0.75	3.1 ± 0.59	0.473	2.8 ± 1.28	2.2 ± 0.82	<**0.05**

[1] Compared by Student-*t* test.
[2] Presented as geometric mean and 95 CI.
[3] Presented as mediana (25th–75th).

independent variables. Uric acid and oxLDL concentrations were independently associated with increased Tbil concentrations in men. In women TBARS was independently associated with increased Tbil (Table 3).

Results of binary logistic regression indicate that level of oxLDL and uric acid concentration associates independently with high bilirubin concentration in men while TBARS concentration independently associates with high bilirubin concentration in women (Table 4).

Thereafter, we constructed a new logistic regression model to further test the potential independent association of the oxLDL and uric acid concentration in men and TBARS concentration in women with high bilirubin concentration. The model incorporated adjustments for traditional lipid profile parameters. After taking into account lipid profile parameters the predictive power of oxLDL was lost in men. In contrast, the association between the level of uric acid in men and TBARS in women with high bilirubin concentration remained strong, regardless of the confounding variable (Table 5).

Discussion

The present study demonstrated that subjects with lower bilurubin concentration also have lower albumin concentration. This is in accordance with published data on the association between lower albumin concentrations with a higher risk of myocardial infarction [23]. Albumin is a negative acute-phase protein and its concentration falls during the inflammatory process. In the inflammatory state, the activity of macrophages and other cells of the immune system is enhanced, and macrophages show an increased free radical production which is implicated in CAD development. Albumin may act as an indirect and sacrificial antioxidant and inhibits peroxidase free radical generation [24]. Our

Table 2
Spearman's correlation analysis between total bilirubin concentration and biochemical and oxidative status parameters in men and women.

Bilirubin (μmol/L)	Men		Women	
	ρ	p	ρ	p
BMI, kg/m^2	0.080	0.094	−0.057	0.442
SBP, mmHg	−0.04	0.953	−0.088	0.435
DBP, mmHg	0.050	0.430	−0.031	0.786
Glucose, mmol/L	−0.159	<**0.001**	−0.030	0.687
hsCRP, mg/L	0.002	0.959	−0.038	0.611
Uric acid, μmol/L	0.179	<**0.001**	0.129	0.079
T-C, mmol/L	−0.062	0.193	0.006	0.930
TG, mmol/L[#]	0.032	0.509	0.162	<**0.05**
HDL-C, mmol/L	0.069	0.149	−0.065	0.375
LDL-C, mmol/L	−0.049	0.308	0.264	<**0.001**
Albumin, g/L	0.215	<**0.001**	0.242	<**0.001**
Fibrinogen, g/L	−0.014	0.776	0.164	<**0.05**
oxLDL, ng/mL	−0.061	0.497	−0.094	0.608
TBARS, μmol/L	−0.231	<**0.05**	−0.255	<**0.05**

Table 3
Multiple regression analysis for the association of investigated parameters with the total bilirubin concentration in men and women.

Men				Women			
R^2 = 0.614				R^2 = 0.470			
Adjusted R^2 = 0.377				Adjusted R^2 = 0.220			
Parameter	β	SE (β)	*P*	Parameter	β	SE (β)	*P*
BMI, kg/m^2	0.204	0.913	0.458	Uric acid, μmol/L	0.236	0.020	0.114
Glucose, mmol/L	−0.136	5.284	0.580	T-C, mmol/L	−0.225	1.790	0.213
Albumin, g/L	0.430	0.826	0.145	HDL-C, mmol/L	0.027	4.212	0.896
Uric acid, μmol/L	0.476	0.024	<**0.05**	Albumin, g/L	0.207	0.520	0.182
TBARS, μmol/L	−0.347	0.195	0.113	Fibrinogen, g/L	0.105	2.386	0.438
oxLDL, ng/mL	−0.467	0.101	**0.051**	TBARS, μmol/L	−0.254	0.926	<**0.05**

Table 4
Predictors of high bilirubin concentration in men and women: univariate logistic regression analysis (enter method).

	Men		Women	
Variables	OR (95% CI)[a]	p	OR (95% CI)[a]	p
TC, mmol/L	0.807 (0.586–1.111)	0.188	0.758 (0.480–1.195)	0.232
TG, mmol/L	0.590 (0.308–1.132)	0.113	0.855 (0.236–3.096)	0.811
LDL-C, mmol/l	0.778 (0.534–1.133)	0.191	0.811 (0.464–1.417)	0.462
HDL-C, mmol/L	1.072 (0.551–2.085)	0.838	0.507 (0.177–1.451)	0.205
oxLDL, ng/L	0.981 (0.962–1.001)	<**0.05**	0.980 (0.939–1.023)	0.362
TBARS, μmol/L	0.967 (0.821–1.139)	0.689	0.524 (0.288–0.956)	<**0.05**
UA, μmol/L	1.006 (1.002–1.009)	<**0.001**	1.04 (0.288–0.956)	0.284
hsCRP, mg/L	1.258 (0.805–1.965)	0.313	1.483 (0.790–2.782)	0.220

[a] OR, odds ratios; 95% CI, 95% confidence interval.

results on negative correlation between higher Tbil levels and glucose stand to reason that higher Tbil levels may be protective against the autoimmune, inflammation-related pathology and oxidative stress associated with development of type 2 diabetes [25]. Recent research suggests that physiological levels of Tbil block the production of various free radicals that might hinder the inhibitory responses of the cell to take up the high glucose [26], and to prevent the vascular endothelial activation from the oxidative stress in the vessels [27,28]. The positive correlation of higher bilirubin concentrations with uric acid is in coordinance with the results of both *in vitro* and *in vivo* studies showing that uric acid is a powerful free radical scavenger in humans and these antioxidant properties can offer a number of benefits within the cardiovascular system [29].

We found that uric acid and oxLDL concentrations are independently associated with Tbil in men and that 37.7% of Tbil increase could be explained by uric acid and oxLDL concentrations. In the female our results indicate that TBARS concentration is independently associated with Tbil, meaning that 22% of the total bilirubin increase can be explained by TBARS concentrations. The present data demonstrated that biomarkers of oxidative stress are higher in healthy young men than in age-matched women. Greater oxidative stress in men is due to an increased generation of ROS and/or reduced activity of antioxidants. Under healthy conditions, cellular respiration in mitochondria is the dominant source of ROS. Therefore, a higher baseline metabolic rate in men than in women might contribute to a higher level of oxidative stress in men [30]. However, after adjustment for lipid parameters in men it turned out that the only predicative parameter of high total bilirubin in men is uric acid, while in women TBARS. High bilirubin concentration decreased TBARS concentration by 53.1%.

From all that have been presented it can be concluded that in both young male and female population high bilirubin concentration acts as protective factor of CAD. The gender differences in independent predictors of high bilirubin are of great importance for further follow-up of CAD risk during aging.

Table 5
Predictors of high bilirubin concentration in men and women: multivariate logistic regression analysis.

Bilirubin, μmol/L					
Men			Women		
Variables	OR (95% CI)[a]	p[b]	Variables	OR (95% CI)[a]	p[b]
oxLDL, ng/mL	0.976 (0.955–0.998)	0.176	TBARS, μmol/L	0.531 (0.287–0.983)	<**0.05**
UA, μmol/L	1.005 (1.003–1.010)	<**0.001**			

[a] OR, odds ratios; 95% CI, 95% confidence interval.
[b] Values are adjusted for lipid profile parameters (T-C, TG, LDL-C and HDL-C).

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