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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