Serum Bilirubin Concentration in Patients With an Established Coronary Artery Disease

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Summary

Bilirubin has been considered an antioxidant, with capacity to remove reactive species of oxygen. Studies have suggested that an increased bilirubin level promotes protection against atherosclerosis.

The case group was composed of 100 patients with coronary artery disease and the control group 100 patients with normal coronaries. Blood samples were collected to determine bilirubin concentrations. Bivariate analysis, multiple logistic regression models, and Spearman's correlation index were performed. A P value < 0.05 was considered to be significant.

The case group was predominantly composed of men and the control group of women, with a mean age of 60 ± 8.8 versus 56 ± 10.9 (P = 0.015). The total bilirubin average was significantly higher in the control group than in the case group (0.76 mg/dL versus 0.39 mg/dL, P < 0.001). The level of ultrasensitive C reactive protein (us-CRP) was increased in the case group (3.63 mg/L versus 0.93 mg/L, P < 0.001). Although the correlation index for this inverse association has been weak, both are independently associated with a higher prevalence of coronary artery disease, total bilirubin ≤ 0.56 mg/dL (OR: 10.04; IC: 3.48-28.90; P < 0.001), and ultrasensitive C reactive protein > 3 mg/L (OR: 1.17; IC: 1.04-1.33; P = 0.009).

Reduced serum levels of bilirubin were shown to be associated with a higher prevalence of coronary artery disease emerging as a new potential risk factor marker. Additional studies are still necessary to confirm and demonstrate the association of these findings with clinical outcomes. (Int Heart J 2010; 51: 86-91)

Key words: Coronary artery disease, Bilirubin, Atherosclerosis

The adaptive and protective responses of arterial vasculature against oxidative stress are important in the prevention of atherosclerosis. The heme oxygenase (HO) responsible for the degradation of heme grouping of hemoglobin is a stress inducible enzyme with antioxidative properties. There are 3 identified isoforms: HO-1, HO-2, and HO-3, with the HO-1 isoform being the most important one in the vascular system.[1)] The products of its reaction (bilirubin, carbon monoxide and iron) develop a potential protective role against atherosclerosis.[2)] Studies have suggested that different circulating forms of bilirubin and its precursor biliverdine have the capacity to remove a variety of reactive species of oxygen, and inhibit the oxidation of LDL particles and the chemotaxis of monocytes, all of which are crucial steps in atherogenesis.[3-7)] Besides its antioxidative capacity, bilirubin can inhibit vascular cell adhesion molecule-1 (VCAM-1), hindering the transendothelial migration of *in vitro* leukocytes,[7-9)] and block the proliferation of smooth muscular cells.[10)]

Considering that there already is some clinical evidence of protection against the atherosclerotic process[11-15)] with increased bilirubin levels, the aim of this study was to evaluate the association between variations in its serum levels and the presence of coronary artery disease (CAD).

Methods

Study design: Case-control study with the aim of comparing the levels of serum bilirubin in patients with an established CAD diagnosis, defined as the case group, with those in patients without an established diagnosis, defined as the control group.

Selection of patients: During the period from January 2006 to February 2007, patients from a cardiology reference hospital in the south of Brazil were analyzed. The inclusion criteria for patients in the case group was an established angiographic CAD diagnosis, characterized by the accomplishment of elective myocardial revascularization procedures, either surgical or percutaneous, as decided by an assisting physician. The inclusion criteria for the control group were patients who underwent normal coronary angiograms which revealed the complete absence of evidence of CAD. Coronary angiography is the gold standard diagnostic tool for the detection and diagnosis of the presence of coronary artery disease. The selection of patients for the case and control groups was conducted after the successful completion of a coronary angiogram and its interpretation when it respectively revealed significant presence or complete absence of evidence of CAD. The authors did not interfere in

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the angiogram indication. The assisting physician, based on a clinical judgment or other findings, determined the coronary angiogram indication. The exclusion criteria for both groups were the need for emergency coronary angiograms, absence of previous 12-hour fasting, and/or the presence of hepatic, hematological, or inflammatory diseases. All participants gave their informed consent and the local ethics committee approved this study. Investigations were conducted in accordance with the Declaration of Helsinki.

Biochemical dosages: The blood samples were obtained through venous peripheral puncture after a 12-hour fast and then sent for laboratory analysis. Total and direct bilirubin (colorimetric method) were analyzed, as were cholesterol and triglycerides (enzymatic-Trinder method), HDL-cholesterol (detergent-selective accelerator method), serum transaminases SGOT and SGPT (cinetic method), and glucose (GOD-Trinder method) through the Bayer RAXT 1000®. Ultra-sensitive C reactive protein (us-CRP) was measured by immunoturbodimetry using a Cobas Mira® (Roche). Indirect bilirubin (IB) was calculated using the formula: IB = Total Bilirubin (TB) – Direct bilirubin (DB). LDL was calculated using the Friedwald equation.[16]

Analysis of the risk factors for CAD: The subjects were asked to fill out a questionnaire so we could analyze the following cardiovascular risk factors: age, gender, family history (considered positive before 55 years of age for men and before 65 years of age for women), history of dyslipidemia, obesity (BMI > 25 kg/m^2), hypertension, history of diabetes and/or smoking (categorizing active smokers as those patients who reported being users of cigarettes, pipes or cheroots during the period of this study, ex-smokers as those who had quit smoking for at least one year, and non-smokers as those who had never smoked).

Information concerning the degree of physical activity of the subjects was obtained through the use of the International Physical Activity Questionnaire (IPAQ)[17] analyzed according to the IPAQ protocol, which classifies the population into three categories: insufficiently active, sufficiently active, and very active.[18]

Coronary plaque burden angiographic classification: Physicians from the interventional cardiology department analyzed all coronary angiograms. For the analysis of CAD severity, the Humphries score system was used,[19] and the groups were classified into minimum coronary atherosclerosis load (1 to 4 points), moderate load (5 to 9 points), and severe load (10 to 15 points).

Statistical analysis: Total sample size was estimated in 200 patients using the 5.6 version WinPepi program, considering an alpha error of 0.05 and a power of 90%.

The statistical analysis was performed using the Statistical Package for Social Sciences (SPSS) 15.0 version. Numerical variables are described as the average and standard deviation, or median and interquartile intervals (25%-75%), and categorical variables are described as proportions. The chi-square test was used for categorical variables and the Student *t* test or Mann Whitney test for numerical variables. The comparison of measures with the Kruskal-Wallis test was used to evaluate the association between total bilirubin levels and CAD severity, and correlations were analyzed using Pearson's rank correlation coefficient (*r*) or Spearman's rank correlation coefficient (rho) for the assessment of non-parametric correlations. The receiver operator characteristic (ROC) curve was used to determine the total bilirubin cut-off level capable for CAD potential prediction.

In order to assess factors related to major and minor outcomes, logistic regression models (backward stepwise) were used, in which variables were included according to theoretical assumptions ($P < 0.15$) in the bivariate analysis and clinically significant variables. A significance level of 5% was adopted for all comparisons.

Results

Two hundred patients were assessed, 100 of whom had a proven angiographic characterization of CAD (case group) and 100 without any coronary lesions according the coronary angiograms (control group). The case group was mainly composed of men and the control group of women (69/31 versus 22/78, $P < 0.001$), with a mean age of 60 ± 8 years versus 56 ± 10 ($P = 0.015$). The clinical and laboratory characteristics are presented in Table I.

The TB, DB, and IB mg/dL average measures were significantly lower in the case group than in the control group patients. TB was 0.39 mg/dL versus 0.76 mg/dL ($P < 0.001$), BD 0.08 mg/dL versus 0.15 mg/dL ($P < 0.001$), and BI 0.29 mg/dL versus 0.55 mg/dL ($P < 0.001$) (Figure 1).

The same can be observed for the dosage of HDL-c between the groups; its average was increased in the control group compared to the case group (44.88 ± 12.26 versus 38.38 ± 8.60, $P < 0.001$). In an inverse and expected relation, the us-CRP average levels were significantly increased in the case group compared to the control group (3.63 mg/L versus 0.93 mg/L, $P < 0.001$), although the Spearman's rank correlation coefficient between TB and us-CRP was weak in the control group (rho = -0.221, $P = 0.027$), while in the case group no significant correlation was evident (rho = -0.350, $P = 0.734$). We observed a direct correlation between TB and TG in the control group ($r = 0.197$, $P = 0.049$) and an inverse correlation between TB and G in the case group (rho = -0.209, $P = 0.038$) and control group (rho = -0.202, $P = 0.044$).

Due to technical and logistic issues, only 81 patients in the case group were eligible for the severity plaque burden CAD analyses. Of these, 8.6% (7) presented a mild atherosclerotic load, 49.4% (40) presented a moderate atherosclerotic load, and 42% (34) presented a severe atherosclerotic load. The bilirubin concentration measure was 0.36 mg/dL in the minimum atherosclerotic load group, 0.33 mg/dL in the moderate load group, and 0.42 mg/dL in the severe load group, and the difference was not statistically significant ($P = 0.316$) (Table II).

Through the ROC curve it was possible to ascertain that using the cut-off value of 0.56 mg/dL for the total serum bilirubin, the optimum capability in the potential CAD prediction was reached with an area under the curve of 0.735 (IC95% 0.664-0.805; $P < 0.001$), accuracy of 70.5%, sensitivity of 71%, and specificity of 70% (Figure 2).

To avoid bias and the influence of other risk factors, multiple logistic regression analysis was performed with the following 11 variables: age, gender, family history, systolic and diastolic arterial blood pressure, history of diabetes,

Table I. Clinical and Laboratory Characteristics of the Study Population

Characteristic	CASE (n = 100)	CONTROL (n = 100)	P
Age (years)	60.21 ± 8.85	56.76 ± 10.94	0.015
Male/female	69 (69%)/31 (31%)	22 (22%)/78 (78%)	< 0.001
BMI (kg/m^2)	27.62 ± 4.27	28.18 ± 5.59	0.431
Smoking status			
Active smokers	17	14	
Ex-smokers	20	13	0.285
Non-smokers	63	73	
History of dyslipidemia	56	55	0.937
Family history of CAD	52	68	0.030
Systolic BP (mmHg)	135.30 ± 23.12	127.12 ± 20.00	0.009
Diastolic BP (mmHg)	85.10 ± 13.94	81.47 ± 10.71	0.044
History of diabetes	30	15	0.018
Physical activity			
Insufficiently active	49	14	
Sufficiently active	39	61	< 0.001
Very active	12	25	
TB (mg/dL)	0.39 (0.26-0.65)	0.76 (0.48-1.15)	< 0.001
DB (mg/dL)	0.08 (0.03-0.12)	0.15 (0.03-0.32)	< 0.001
IB (mg/dL)	0.29 (0.18-0.50)	0.55 (0.25-0.89)	< 0.001
Total cholesterol (mg/dL)	189.34 ± 47.48	199.37 ± 47.44	0.138
HDL-choletserol (mg/dL)	38.38 ± 8.60	44.88 ± 12.26	< 0.001
LDL-cholesterol (mg/dL)	114.18 ± 38.66	121.20 ± 40.30	0.211
Triglycerides (mg/dL)	183.90 ± 83.27	166.43 ± 92.51	0.163
Glucose (mg/dL)	100 (89-120)	95 (85.25-109.75)	0.177
SGOT (mg/dL)	10 (7-14)	13 (10-17)	0.001
SGPT (mg/dL)	6 (4-9)	7 (5-14)	0.001
us-CRP (mg/L)	3.63 (1.65-7.9)	0.93 (0.66-2.29)	< 0.001

Comparison between the groups using the Student t test, chi-square test, and Mann-Whitney test. BP indicates blood pressure; TB, total bilirubin; DB, direct bilirubin; IB, indirect bilirubin; and us-CRP, ultrasensitive C reactive protein.

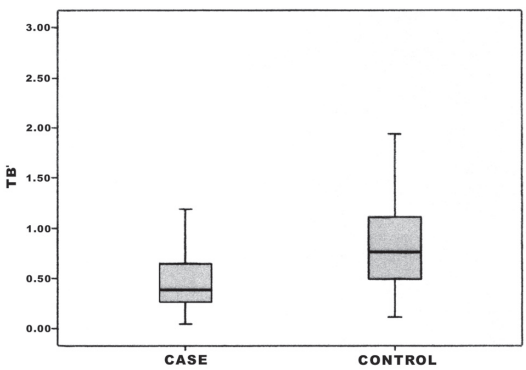

Figure 1. Concentration of TB between the groups.

Table II. Classification of Severity of CAD and Bilirubin Concentration

Lesion	n	TB (mg/dL)	P
Minimum load	7	0.36 (0.27-0.96)	0.316
Moderate load	40	0.33 (0.23-0.46)	
Severe load	34	0.42 (0.28-0.67)	

Kruskal-Wallis.

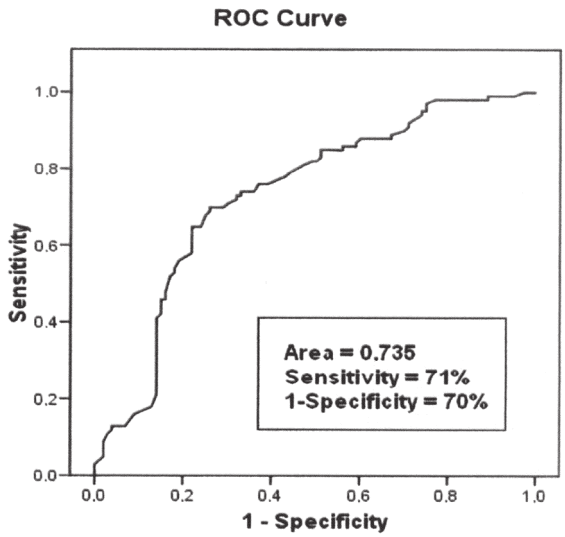

Figure 2. ROC curve for the concentration of TB and CAD.

IPAQ, TB, HDL-c, SGOT, and SGPT e us-CRP. The DB and IB were not included in the model due to collinearity with the TB. The independent variables of gender (OR: 12.34; IC: 3.91-38.98), diastolic blood pressure (OR: 1.06; IC: 1.01-1.11), insufficiently active (OR: 12.03; IC: 2.56-56.43), BT ≤ 0.56 mg/dL (OR: 10.04; IC: 3.48-28.90), and

Table III. Multiple Logistic Regression for CAD

Variable	Odds Ratio	CI 95%	*P*
Age	1.04	0.99-1.09	0.060
Gender (male)	12.34	3.91-38.98	< 0.001
Diastolic blood pressure	1.06	1.01-1.11	0.014
IPAQ			
Insufficiently active*	12.03	2.56-56.46	0.002
Sufficiently active	1		
Very active*	1.20	0.37-3.86	0.758
TB ≤ 0.56 mg/dL	10.04	3.48-28.90	< 0.001
HDL-c	0.95	0.90-1.00	0.059
SGPT	0.82	0.72-0.92	0.001
us-CRP	1.17	1.04-1.32	0.009

CI indicates confidence interval; us-CRP, ultrasensitive C reactive protein; and * compared to the group in which physical activity was sufficient.

us-CRP (OR: 1.17; IC: 1.04-1.32) were significantly associated with the prevalence of the CAD, while HDL-c (OR: 0.95; IC: 0.90-1.00) and SGPT (OR: 0.82; IC: 0.72-0.92) became protection factors (Table III).

Discussion

We decided to use a case-control study with the purpose of comparing the serum bilirubin levels in patients with an established CAD diagnosis (case group) and those with a proved absence of angiographic signs of coronary atherosclerosis (control group). This criterion was very strict considering that this was the main criterion for study entry. We also attempted to match all baseline characteristics between patients. However, CAD was much more prevalent in certain subgroups of patients and clinical conditions.

The male gender is probably one of the most important risk factors for CAD. This is most likely why our case group population was mainly composed of men and the control group population consisted mostly of women. Unfortunately, we were unable to match the case and control groups according to gender. Interestingly, the same phenomenon occurred in the case-control study conducted by Hopkins and coworkers[12] that accessed the risk for early familial coronary artery disease. Their findings agreed with ours. In their study, male gender was almost 20% more prevalent in the case group. They had lower levels of bilirubin and were at higher risk for CAD development, supporting the potential antioxidant protective effect of bilirubin. Coincidently all other important and classical risk factors (old age, smoking, hypertension, diabetes, and dyslipidemia) were also more common in the case group. Due to this difficulty in matching gender and other risk factors, a previous cross-sectional study from Schwertner, *et al*,[11] which corroborates the idea that high bilirubin levels could be protective, may have allocated only men for this analysis. This was also the case in The Prospective Epidemiological Study of Myocardial Infarction (PRIME), conducted by Troughton and coworkers,[15] whose aim was to investigate novel risk factors and included a population of 10,593 patients composed of only men. In this very large study, the differences between cases and controls regarding body mass index, waist-to-hip ratio, blood pressure, and cholesterol and glucose levels were statistically significantly, but were probably attenuated by the single gender selection. In the current paper, we also found some differences when comparing the cases and controls with regards to age, family history of CAD, blood pressure, diabetes, physical activity, HDL cholesterol, and us-CRP.

In spite of these restrictions and difficulties in matching the populations, imposed by the selected study design, a very interesting question regarding gender and bilirubuin levels is raised. According to the Third National Health and Nutrition Examination Survey, a comprehensive assessment of health and nutrition performed in the United States between 1988 and 1994,[20] serum bilirubin levels were significantly higher in men (0.72 ± 0.004 mg/dL) than in women (0.52 ± 0.003 mg/dL).

Considering this, if the presumed presented inverse association between bilirubin levels and coronary artery disease was not valid, our results should be different. In this scenario, the bilirubin levels in the case group (composed mainly of men) should be higher than in the control group (composed mainly of women). In fact, we observed exactly the opposite. This interpretation and extrapolation reinforces the significance of male gender as a key CAD risk factor and the idea that reduced serum levels of bilirubin could be potentially associated with a higher prevalence of CAD.

In this study we verified an inverse association between the total bilirubin concentrations levels and the CAD prevalence, in other words, the bilirubin diminished in patients with CAD when compared to healthy individuals. Schwertner and coworkers had already described a similar finding and were the first to observe an inverse correlation between total circulating bilirubin and CAD occurrence.[11] Hopkins, *et al* [12] and Troughton, *et al* [15] also observed this same association with TB concentration levels in the case group and control group of (0.51 ± 0.35 mg/dL versus 0.72 ± 0.47 mg/dL) and the geometric average of 0.46 mg/dL versus 0.52 mg/dL.

Our data are in agreement with previous studies, suggesting that most likely patients with CAD presented a diminished concentration of TB. In the present study, we did not observe a significant difference between the concentration of total bilirubin and lesion severity or atherosclerotic load as described by Schwertner and coworkers, where the groups with nonsignificant, moderate, and serious CAD had the following bilirubin concentrations levels: 0.85 ± 0.41 mg/dL, 0.76 ± 0.28 mg/dL, and 0.72 ± 0.34 mg/dL.[11] A possible explanation for such different findings might be due to the fact that our sample size was smaller. Unexpectedly, the concentration of total bilirubin was not significant, but was slightly higher in the group with severe atherosclerotic load than in the group with moderate load.

Total bilirubin levels under the cut-off point of 0.56 mg/dL behaved as an independent risk factor for CAD, with the higher concentration being associated with the protection against atherosclerosis. Although bilirubin, by itself, does not have the capacity for predicting CAD, the search for new markers and findings that can be used together with classical risk factors has great importance since these can

contribute to the prevention and treatment of coronary artery disease.

Our findings cannot be extrapolated to other clinical situations, such as coronary syndrome X, in which the presentation could be confused with CAD. In general, it is a diagnosis of exclusion and the pathogenesis, despite not being well or completely understood yet, seems to be completely different than the one responsible for CAD. It is mainly related to female gender, oxidative stress, estrogen, and other factors. However, there is a consensus[21] that microvascular abnormalities, caused by endothelial dysfunction, play a major role and appear to be responsible for myocardial ischemia in patients with cardiac syndrome X. Endothelial dysfunction is likely to be multifactorial in these patients and it is conceivable that risk factors such as hypertension, hypercholesterolemia, diabetes mellitus, and smoking can contribute to its development. This manuscript was neither designed nor powered to address this issue and even if syndrome X was present in some patients in the control group, it would probably not interfere with our clinical findings.

The main limitation of the present study resides in the fact it is a case-control study in which its selection bias is inherent to its design and could lead to misinterpretation of the results, thus, caution in extrapolation to its potential clinical implications is needed. In addition, the patients in the case group had gender, age group, and risk factors different to those of the controls, and the collection of blood samples was performed at different moments for the case and control groups. Another limiting factor is that the size and power of our study was not suitable for the analysis of hard clinical outcomes.

A deeper understanding of the molecular basis of cardiovascular diseases is being sought with the aim of identifying subclinical atherosclerosis, ultimately ending with an early diagnosis and the establishment of preventive or therapeutic measures, with the objective of improving the prognosis of CAD. This is very well exemplified in studies on polymorphism in the promoter of the HO-1 gene, which might be associated with an increased risk of cardiovascular disease[22,23] and restenosis.[24] Present efforts are aimed at therapeutic agents such as statins and rapamicin, which have the capacity of inducing the HO-1, resulting in a higher expression of this enzyme and, consequently an increase in the bilirubin concentration, offering a promising path for the diagnosis and treatment of CAD.[25,26]

In the present study, reduced serum levels of bilirubin (< 0.56 mg/dL) were associated with a higher prevalence of CAD, emerging as a new and potential risk marker that can contribute to the diagnosis of CAD. Additional studies are still necessary to confirm and demonstrate the association of these findings with clinical outcomes.

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