Diagnostic value of bilirubin concentrations compared with novel and traditional biomarkers in atherosclerosis with coronary artery disease

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Reasons behind the development of coronary artery disease (CAD) and their elimination are vitally important for life. A large number of risk factors have been described to be involved in the process of atherogenesis. In this respect, bilirubin seems to represent an important endogenous agent with cytoprotective activity against oxidative stress due to its potent antioxidant properties, which were demonstrated by in vitro, animal and human studies. In addition to being a potent antioxidant, bilirubin is considered to play a role in tissue protection against inflammatory damage by its anticomplement action. Coronary artery disease often occurs in the absence of traditional risk factors. Natural antioxidant defenses have evolved to protect humans against deleterious effects of free radicals. The primary enzymatic defenses are intracellular, but other antioxidant defenses are largely extracellular, including antioxidative substrates such as uric acid and unconjugated bilirubin, the predominant bile pigment in the intravascular compartment. For many years, the bile pigment was considered as a toxic waste product formed during heme catabolism. However, more recent evidence suggests that bilirubin is a potent physiological antioxidant that may provide important protection against atherosclerosis, coronary artery disease and inflammation. In 1994, Schwertner et al[1] were the first to observe a significant inverse correlation between total bilirubin plasma concentrations and the prevalence of CAD. Subsequently, Hunt et al[2] noted that patients with early familial CAD have an average total serum bilirubin of 8.9 ± 6.1 μmol/L, compared with 12.4 ± 8.1 μmol/L in healthy control subjects. Low serum bilirubin concentrations have been shown to be independently and inversely associated with an increased risk for CAD. The strength of the association between bilirubin and CAD appears to be similar to that of high-density lipoprotein-cholesterol (HDL-C). The antioxidant capacity of bilirubin and its ability to provide potent scavenging of peroxyl radicals have led to suggestions that mildly increased circulatory bilirubin may have a physiologic function to protect against disease processes that involve oxygen and peroxyl radicals. Antioxidant activity and cardioprotective potential may be attributable to any of the bilirubin forms, including 3 unconjugated bilirubin, protein-bound unconjugated bilirubin, delta bilirubin or mono-/diconjugated bilirubin. Under physiological conditions, the predominant circulatory form of bilirubin is the unconjugated, albumin-bound form.[2] In recent years, "nontraditional factors"(Novel) such as high sensitive C-reactive protein, total-homocysteine, as well as oxidative stress have been proposed as risk factors for the development and progression of atherosclerosis and atherothrombotic cardiovascular disease. The purpose of this study was to examine the relationship between traditional concentrations and nontraditional biomarkers of CAD in coronary angiography patients and in apparently healthy control subjects. All patients referred to the Department of Cardiology, University of Gaziantep, between March and August 2003 for which clinical data were available were included in our study. Included were 319 subjects who were admitted to the hospital with chest pain and who underwent coronary angiography. The CAD group consisted of 262 patients (63 females and 199 males) with stenosis of the coronary arteries. The apparently healthy control group consisted of 50 subjects (17 females and 23 males). Coronary artery disease was divided into groups according to the maximum coronary stenosis at angiography: 0-20% (no detectable CAD), 20-49% (mild disease), 50-70% (moderate disease), and 70-100% (severe disease). Other classification of severity of the disease was assessed by counting both the number of diseased vessels (0 to 3). All subjects were questioned for established cardiovascular risk factors, including diabetes, smoking, medication and hypertension. The study was approved by the local Ethics Committee, and the individuals participating in the study gave their informed consent. Although the healthy subjects did not have coronary angiograms they underwent comprehensive physical examination by a physician, completed the World Health Organization standard Rose questionnaire on chest pain, and answered other questions regarding their past medical history. None of the individuals in the healthy group had angina or a prior history of CAD. All of them had normal electrocardiograms according to Minnesota Coding Criteria. All patients were monitored for somatic illness throughout the investigation period and were

excluded if symptoms of infection or systemic illness were present (acute or chronic liver disease, cancer, renal disorder, rheumatic disease, and others). Patients diagnosed with acute coronary syndrome 6 months prior to the study were excluded. Additional exclusion criteria included the use of aspirin, S-adenosyl-methionine, vitamin intake, alcohol intake anticonvulsants, estrogen, lipid-lowering therapy and other medications that might affect bilirubin, C-reactive protein and homocysteine metabolism. We measured serum total bilirubin by a diazo method with a detergent to accelerate azo-coupling and to prevent the precipitation of protein. The test was performed by means of an autoanalyzer (Hitachi Modular DP Systems, Roche Diagnostics, and Mannheim, Germany). Total bilirubin levels below 1.1 mg/dl are normal for adults. Measurement is linear from 0.1-30 mg/dl. The intra-assay imprecision coefficient variation (CV) was 1.3% and inter-assay imprecision CV was 1.9% at a bilirubin concentration of 2.1 mg/dl. Serum hs-CRP and t-Hcy concentrations were determined with the Immulite® one analyzer and Immulite® reagent (DPC, Los Angeles, CA, USA) according to the manufacturer's instructions. The assays were linear from 2 - 50 mmol/L (t-Hcy) and 10 - 160 mg/L (hs-CRP), calibrators and controls were supplied by the manufacturers. Specifications of intra-assay co-efficients of variation (CV) of hs-CRP (6%) and t-Hcy (9.9%) and inter-assay CV of hs-CRP (5.3%) and t-Hcy (9.1%) were assessed from quality control data of the laboratory. Total homocysteine in the patient serum or plasma sample is released from its binding proteins and converted to S-adenosyl-homocysteine (SAH) by an off-line 30-minute incubation at 37ºC in the presence of S-adenosyl-L-homocysteine hydrolyses and dithiothreitol (DTT). Two-tailed $p<0.05$ values were taken into consideration. All statistical analyses and illustrations were obtained with SPSS® and MedCalc® statistical software. There was no significant difference between the groups in body mass index, waist/hip ratio and age. Serum bilirubin levels were significantly higher in apparently healthy subjects (0.81 ± 0.32 mg/dl) than the patients with CAD (0.55 ± 0.39) group who underwent coronary angiography ($p<0.01$). However, t-Hcy (10.7 mmol/L) and hs-CRP (0.43 ± 0.61 mg/dl) levels were significantly lower in the apparently healthy subjects group than the patients with CAD (t-Hcy = 19.4 ± 8.73 mmol/L; hs-CRP = 1.54 ± 0.87mg/dl). Serum levels of hs-CRP (1.7 ± 2.1 mg/dl) and t-Hcy (19.8 ± 9.6 mmol/L) were highest in the patients who smoked. The predicting variables obtained by regression analysis for the number of stenotic vessels in CAD patients are given in text; gender (male, $p=0.001$), age ($p=0.004$) and t-Hcy ($p=0.0001$) strongly related and HT ($p=0.019$) moderately and HDL-C ($p=0.022$), glucose ($p=0.028$), TG ($p=0.047$) weakly related with the number of stenotic vessels (severity of disease). Bilirubin, hs-CRP, and other parameters were not related to the number of diseased vessels and the degree of occlusion ($p>0.05$). Optimal cut-off levels and the associated diagnostic performances (sensitivity, specificity and diagnostic value) of serum bilirubin, hs-CRP, t-Hcy, based on ROC analysis, are given in **Table 1**. Optimal cut-off levels of bilirubin (0.59 mg/dl), hs-CRP (1.09 mg/dl) and t-Hcy (12.1 mmol/L) providing the maximum efficiency were found in patients (n=319) with CAD. The ROC curve-based sensitivity of bilirubin was

Table 1 - Optimal cut-off levels and associated specificity, sensitivity and diagnostic value of concentrations of biomarkers for the diagnosis of angiographically documented CAD.

Biomarkers	Cut-off level	Sensitivity (%)	Specificity (%)	Diagnostic value (AUC)	+ LR	- LR
Bilirubin (mg/dl)	0.59	70.9	40.4	0.507	1.19	0.72
WBC(103/m l)	6700	70	38.8	0.535	1.14	0.77
UA (mg/dl)	4.5	88.7	28.1	0.578	1.23	0.45
HDL-C(mg/dl)(female)	31	64.9	47.1	0.598	1.22	0.75
HDL-C(mg/dl) (male)	25	28.5	95	0.599	5.7	0.75
TC (mg/dl)	184	52.6	73.6	0.630	1.99	0.64
Lp(a) (g/L)	0.24	59.5	64.9	0.630	1.7	0.62
TG(mg/dl)	144	65.4	58.2	0.631	1.56	0.59
hs-CRP(mg/dl)	1.09	50	80.7	0.648	2.59	0.62
t-Hcy(mmol/L)	12.1	76.8	70.2	0.781	2.67	0.29

found to be 70.9%, while hs-CRP to be 50% and t-Hcy levels 76.8%. The specificity of bilirubin was 40.4%, 80.7% for hs-CRP and 70.2% for t-Hcy. Furthermore, the present study demonstrated that patients with angiographically confirmed CAD had significantly higher serum hs-CRP and t-Hcy levels than the apparently healthy control group. These data strongly suggest that serum t-Hcy helps to identify individuals at risk of atherosclerosis (AUC value 0.781), especially among those with elevated hs-CRP and decreased bilirubin levels. The t-Hcy showed the highest AUC value (0.781) compared to hs-CRP (AUC 0.648) and bilirubin (AUC = 0.507). The risk factors of atherosclerosis are summarized in **Table 1**. In agreement with previous reports, we found that the bilirubin levels in serum were significantly lower in the patients with CAD than in age and gender matched controls. We found that a serum bilirubin concentration of 10.0 μmol/L (0.58 mg/dl) discriminated between high and low cardiovascular risks. This association was independent from the extent of CAD, BMI, diabetes, hypertension, and smoking. In 1995, Breimer et al[3] performed a prospective study of 7685 middle-aged men enrolled in the British Regional Heart Study and found that both low and high bilirubin concentrations were associated with an increased risk of CAD. More recently, Vitek et al[4] reported on the prevalence of CAD in individuals with Gilbert's syndrome who were found to have a CAD prevalence rate of 2% compared with 12.1% in the general population. Whereas, we did not find that association of serum bilirubin concentration and severity of atherosclerosis, neither in men nor in women. However, the number of stenotic coronary arteries was significantly associated with elevated serum t-Hcy and hs-CRP concentration.[5] An involvement of bilirubin in immune reactions and inflammatory processes has also been documented. The levels of bilirubin may be related to an inflammatory condition in patients with CAD. Another possibility is that low bilirubin concentration is not per se a major causative factor in the development of CAD, but rather a reflection of the presence of this ailment. According to this view, low bilirubin is a result of increased oxidative activity in CAD-prone individuals, leading to consumption of a natural antioxidant such as bilirubin. Our data suggest that serum bilirubin concentration is more closely associated with the oxidative stress marker such as serum uric acid level (0.267 ± 0.014, ß ± SEM; p<0.0001), than smoking. Problems in risk assessment also arise from overlapping properties (shared pathophysiological pathway) of traditional risk factors such as hypertension, obesity, age, gender, smoking and diabetes. In summary, we found only little evidence of an association between the serum concentration of bilirubin and atherosclerosis. In contrast, the concentration of novel (t-Hcy and hs-CRP) and traditional risk markers may be stronger markers for atherosclerosis in CAD patients. Finally, in the future, specific drugs directed against the oxidant and inflammatory process in the atherosclerotic plaque should be developed and tested in outcome trials.

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References

1. Schwertner HA, Jackson WG, Tolan G. Association of low serum concentration of bilirubin with increased risk of coronary artery disease. ***Clin Chem*** 1994; 40: 18- 23.
2. Hunt SC, Kronenberg F, Eckfeldt JH, Hopkins PN, Myers RH, Heiss G. Association of plasma bilirubin with coronary heart disease and segregation of bilirubin as a major gene trait: the NHLBI family heart study. ***Atherosclerosis*** 2001; 154: 747-754.
3. Breimer LH, Wannamethee G, Ebrahim S, Shaper AG.Serum bilirubin and risk of ischemic heart disease in middle-aged British men. ***Clin Chem*** 1995; 41: 1504-1508.
4. Vítek L, Jirsa Jr. M, Brodanová M, Kaláb M, Mare ek Z, Danzig V, et al. Gilbert syndrome and ischemic heart disease: a protective effect of elevated bilirubin levels. Atherosclerosis 2002; 160: 449-456.
5. Eren E, Yilmaz N, Pence S, Kocoglu H, Göksu S, Kocabas R, Kadayıfcı S. Diagnostic value of C-reactive protein in patients with angiographically documented coronary heart disease. ***Acta Medica*** 2002; 45: 155-160.