


Is Low Serum Bilirubin an Independent Risk Factor for Coronary Artery Disease in Men but Not in Women? Georg Endler,1,2 Ahmad Hannu,1,2 Raute Sunder-Plassmann,1 Markus Exner,1 Thomas Vukovich,3 Christine Mannhalter,1 Johann Wojta,4 Kurt Huber,4 and Oswald Wagner1† (1 Clinical Institute of Medical and Chemical Laboratory Diagnostics and 2 Department of Internal Medicine II, Division of Cardiology, University of Vienna Medical School, Waehringer Gürtel 18-20, A-1090 Vienna, Austria; * these authors contributed equally to this work; † author for correspondence: fax 43-1-40400-5389, e-mail Oswald.Wagner@univie.ac.at)

For many years, the bile pigment bilirubin was considered to be only a toxic waste product formed during heme catabolism. Recent evidence, however, suggests that bilirubin acts as a potent physiologic antioxidant that may provide important protection against arteriosclerosis, coronary artery disease (CAD), and inflammation (1–3). The antioxidant capacity of bilirubin and its potent ability to scavenge peroxyl radicals have 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 (4). Indeed, inverse correlations between the presence of CAD and total bilirubin concentrations in the circulation were reported recently in several independent studies (5, 6). Additionally, plasma bilirubin correlates inversely with several established risk factors for CAD, including smoking, increased LDL-cholesterol, diabetes, and obesity, but is directly proportional to the protective factor HDL-cholesterol (5, 7). The effect of bilirubin on the risk of cardiovascular disease is apparent in men (8) but is less clear in women (6, 9, 10). In the present study, we therefore examined the influence of gender on total bilirubin concentrations.

All patients referred to the Department of Cardiology, University of Vienna, between August 1999 and September 2001 for whom clinical data were available were included in our study. Patients were divided in a CAD and a non-CAD group. The CAD group consisted of 544 patients (157 females and 387 males) with clinically relevant CAD. Clinically relevant CAD was defined as an exercise-induced ischemic ST-segment depression >0.1 mV (12%) (11) and/or a history of myocardial infarction (53%) or coronary intervention [coronary artery bypass (8%) or percutaneous transluminal coronary angioplasty (27%)]. In the non-CAD group (359 patients; 186 females and 173 males), the presence of CAD was excluded by objective tests that indicate the absence of clinically relevant coronary ischemia (exercise testing and/or thallium-persantin scintigraphy). These patients (non-CAD group, controls) had various diseases, including nonischemic chest pain (23%), valvular disease (13%), nonischemic cardiomyopathy (18%), and nonischemic arrhythmias (38%), and served as controls.

All patients were questioned for established cardiovascular risk factors, including diabetes, smoking (>20 cigarettes/day for more than 5 years), hypertension (systolic blood pressure >140 mmHg or diastolic blood pressure >80 mmHg at repeated measurements or a known history of hypertension and treatment with antihypertensive drugs), body mass index (BMI), and family history of cardiovascular disease. Diabetes mellitus was considered present in patients with a known history of diabetes and in patients with a fasting glucose >7 mmol/L (126 mg/dL) according to American Diabetes Association criteria (12). All blood samples were taken at the time of admission between 0800 and 1000 after an overnight fast. The study was approved by the local ethics committee, and all individuals participating in the study gave informed consent.

We measured serum bilirubin by a diazo method with a detergent to accelerate theazo-coupling and to prevent the precipitation of protein (13). The test was run on a Hitachi 747 (Roche) with a measurement range of 1.0–
 Median serum bilirubin was lower in the females. These differences were statistically significant in both the control group \((P < 0.001)\) and the CAD group \((P = 0.002)\) and when comparing all males and females: median for all males, 7.1 mg/L (25th–75th percentiles, 5.1–9.6 mg/L); median for all females, 6.0 mg/L (25th–75th percentiles, 4.4–7.8 mg/L); \(P < 0.001\). We obtained similar results when we compared male controls (median, 7.5 mg/L; 25th–75th percentiles, 5.1–1.1 mg/L) with female controls (median, 6.0 mg/L; 25th–75th percentiles, 4.4–8.0 mg/L; \(P < 0.001\)). Thus, for all further analyses the groups were stratified by sex.

The baseline characteristics for controls and CAD patients dependent on their gender are presented in Table 1. In men, median serum bilirubin was lower in CAD patients than in controls \((P < 0.001; \text{Fig. } 1)\). Interestingly, we found no such difference among females. After correction for age in a multivariate analysis, the results did not change significantly.

In controls, total cholesterol was slightly higher than in CAD patients, possibly because of ongoing lipid-lowering medication. To our knowledge, no evidence has been reported that lipid-lowering therapy might influence bilirubin. Additionally, in the multivariate analysis the effect of bilirubin was independent of serum cholesterol. Thus a possibly confounding effect of lipid-lowering therapy seems unlikely.

More than 60% of the male patients with CAD had bilirubin <8.0 mg/L. To estimate the effect of bilirubin concentrations on the risk for CAD, we calculated the odds ratio in individuals with values >8.0 mg/L vs those with values <8.0 mg/L after adjustment for the confounding factors (age, smoking, diabetes, hypertension, HDL, triglycerides, and BMI) in a multivariate logistic regression model. We obtained an odds ratio of 0.6 (95% confidence interval, 0.4–0.9; \(P = 0.02\)), which indicates a 40% reduction in prevalence odds for patients with higher serum bilirubin concentrations (>8.0 mg/L), whereas we found no significant differences in females (odds ratio, 0.8; 95% confidence interval, 0.5–1.5; \(P = 0.5\)).

To estimate the relationship between serum bilirubin and other risk factors for CAD, we compared bilirubin concentrations in patients with and without diabetes, in patients with and without hypertension, and in smokers vs nonsmokers. Bilirubin concentrations were significantly lower in smokers than in nonsmokers after adjustment for sex, age, and disease/control status in a multivariate analysis, whereas we found no association between bilirubin and the risk factors diabetes and hypertension. Interestingly, we observed a moderate but statistically significant correlation between HDL and bilirubin.

### Table 1. Clinical and laboratory characteristics of the patients in this study.

<table>
<thead>
<tr>
<th></th>
<th>Non-CAD</th>
<th>CAD</th>
<th>(P^a)</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Males</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>n</td>
<td>173</td>
<td>385</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>Median age,(^b) years</td>
<td>57 (45–63)</td>
<td>61 (55–71)</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>Smokers, n (%)</td>
<td>46 (26.6%)</td>
<td>199 (51.4%)</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>Diabetes, n (%)</td>
<td>23 (13.3%)</td>
<td>107 (27.6%)</td>
<td>&lt;0.04</td>
</tr>
<tr>
<td>Hypertension, n (%)</td>
<td>63 (36.4%)</td>
<td>246 (63.6%)</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>Median triglycerides,(^b) g/L</td>
<td>1.07 (0.82–1.72)</td>
<td>1.37 (0.97–1.98)</td>
<td>0.01</td>
</tr>
<tr>
<td>Median cholesterol,(^b) g/L</td>
<td>2.03 (1.73–2.24)</td>
<td>1.98 (1.69–2.28)</td>
<td>0.4</td>
</tr>
<tr>
<td>Median HDL,(^b) mg/L</td>
<td>460 (380–570)</td>
<td>420 (340–510)</td>
<td>0.1</td>
</tr>
<tr>
<td>Median bilirubin,(^b) mg/L</td>
<td>7.5 (5.1–11.1)</td>
<td>7.0 (5.0–9.2)</td>
<td>&lt;0.01</td>
</tr>
<tr>
<td>Bilirubin &gt;8.0 mg/L, n (%)</td>
<td>81 (46.8%)</td>
<td>147 (38.0%)</td>
<td>&lt;0.01</td>
</tr>
<tr>
<td>Median BMI,(^b) kg/m(^2)</td>
<td>26.7 (23.8–29.4)</td>
<td>27.1 (25.1–29.5)</td>
<td>0.7</td>
</tr>
<tr>
<td><strong>Females</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>n</td>
<td>186</td>
<td>157</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>Median age,(^b) years</td>
<td>59 (49.5–73)</td>
<td>70 (59.5–75.5)</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>Smokers, n (%)</td>
<td>38 (20.4%)</td>
<td>44 (28.0%)</td>
<td>0.004</td>
</tr>
<tr>
<td>Diabetes, n (%)</td>
<td>16 (8.6%)</td>
<td>46 (29.3%)</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>Hypertension, n (%)</td>
<td>76 (40.9%)</td>
<td>114 (72.6%)</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>Median triglycerides,(^b) g/L</td>
<td>1.13 (0.80–1.60)</td>
<td>1.24 (0.93–1.71)</td>
<td>0.3</td>
</tr>
<tr>
<td>Median cholesterol,(^b) g/L</td>
<td>2.14 (1.88–2.38)</td>
<td>2.02 (1.67–2.35)</td>
<td>0.03</td>
</tr>
<tr>
<td>Median HDL,(^b) mg/L</td>
<td>550 (440–680)</td>
<td>490 (410–590)</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>Median bilirubin,(^b) mg/L</td>
<td>6.0 (4.4–8.0)</td>
<td>6.0 (4.5–7.7)</td>
<td>0.3</td>
</tr>
<tr>
<td>Bilirubin &gt;8.0 mg/L, n (%)</td>
<td>48 (25.8%)</td>
<td>33 (21.0%)</td>
<td>0.3</td>
</tr>
<tr>
<td>Median BMI,(^b) kg/m(^2)</td>
<td>25.3 (22.4–28.6)</td>
<td>25.6 (23.1–29.1)</td>
<td>0.2</td>
</tr>
</tbody>
</table>

\(^a\) Multivariate adjusted \(P\) values were calculated with a binary logistic regression model including age, smoking, diabetes, hypertension, triglycerides, total cholesterol, bilirubin, and BMI.

\(^b\) Values for age, triglycerides, cholesterol, bilirubin, and BMI are given as the unadjusted median (25th–75th percentiles).
in males ($r = 0.14; P = 0.001$) but not females ($r = -0.05; P = 0.4$).

We found no significant associations between serum bilirubin and either the number of diseased coronary arteries or a history of myocardial infarction (data not shown).

The lower serum bilirubin in women may reflect the influence of estrogens (14), possibly related to increased bilirubin excretion by induction of UDP-glucuronyltransferase in the liver, as suggested in animal studies (15). Estrogens also decrease LDL and increase HDL, reduce the oxidation of LDL, and increase the local production of nitric oxide in the vascular wall (16). Thus, the potential proatherogenic effect of female sexual steroids via a decrease in serum bilirubin seems to be outweighed by the beneficial effects of estrogens. In our study, information concerning menopausal status, hormone replacement therapy, and oral contraception was not available. Thus, we currently cannot confirm this hypothesis.

Another explanation for the gender-related differences in total bilirubin concentrations could be the different risk profiles found in males and females: 28% of the female CAD patients were smokers, whereas 51% of the male CAD patients smoked. Bilirubin concentrations were significantly lower in smokers than in nonsmokers independent of sex, whereas we found no association between bilirubin and the risk factors diabetes and hypertension. This was also reported by Schwertner (17), who reported an inverse association between smoking and serum total bilirubin concentrations in individuals with and without CAD. These findings are in accordance with our data and support the hypothesis that cigarette smoking may also increase the risk for CAD by increasing the consumption of the endogenous antioxidant bilirubin. Nevertheless, the gender-related differences in serum bilirubin concentrations remained statistically significant in a multivariate analysis after adjustment for smoking and other environmental risk factors, indicating that these findings are not exclusively determined by environmental cardiovascular risk factors.

The observations in our study confirm similar results in the Framingham Offspring Study cohort, reported previously by Djousse et al. (9) and Hunt et al. (10), who also reported an association of bilirubin and CAD in men but not in women. Their results and ours contrast, however, with results reported by Hopkins et al. (6).

We observed a 40% reduction in prevalence odds for CAD in males with bilirubin values $>8.0 \text{ mg/L}$ independent of other risk factors. Similarly, Schwertner et al. (5) reported a comparable risk reduction resulting from a 50% increase in total bilirubin.

The inclusion of bilirubin, alone or in combination with cholesterol/HDL ratios, as a potential predictive risk marker could help to identify individuals at risk for CAD as suggested by Mayer (4) and Schwertner and Fischer (18). However, all observed changes in median serum bilirubin were within the very narrow reference interval of total serum bilirubin (0–10 mg/L), and our data indicate that the association between serum bilirubin and CAD is restricted to males.

References


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**Generation, Characterization, and Use of Monoclonal Antibodies against Parathyroid Hypertensive Factor, Svetlana Krylova,1 Teresa Labedz,2 Richard Z. Lewanczuk,2,3 and Christina G. Benishin4**

Parathyroid hypertensive factor (PHF) may be useful as a diagnostic marker of salt-sensitive, low-renin hypertension. PHF was discovered in the plasma of salt-sensitive hypertensive humans (1) and was also found to be increased in spontaneously hypertensive rats, DOCA-salt hypertensive rats, and Dahl-salt-sensitive rats, but not in two-kidney one-clip rats or in Dahl-salt-insensitive rats (2, 3). Studies on the mechanism of PHF action indicate that this substance acts directly on vascular smooth muscle cells to enhance Ca2+ influx (4), likely associated with depolarization of the plasma membrane by inhibition of voltage-gated K+ channels (5). Together these actions will sensitize vascular tissues to other vasoconstrictors, such as norpinephrine and angiotensin II (6). PHF may therefore be a causative factor in the development of hypertension in some individuals. It was found that PHF-positive (salt-sensitive) patients respond best to calcium channel blockers and diuretics and that PHF-negative patients respond better to angiotensin-converting enzyme inhibitors and beta blockers (7). Recently, an enzyme immunoassay for detection of PHF in human plasma has been reported that uses anti-PHF oligoclonal antibodies (8). The present study describes the further development, characterization, and clinical application of monoclonal antibodies (MAbs) against PHF.

Male BALB/c mice were immunized with partially purified PHF (9) prepared from medium harvested from cultured parathyroid glands of spontaneously hypertensive rats, as was described previously (10). Splenocytes were harvested and fused with SP/0-2 myeloma cells by standard procedures (11). The recloning procedure involved limiting dilution conditions in 96-well plates (0.3 cells/well).

The bovine serum albumin (BSA) conjugate of PHF was synthesized by standard procedures (12) and used in a direct ELISA for anti-PHF antibodies. Microtitre plates were coated with BSA-PHF (100 μL/well; 1:2000 dilution). After the plates were washed with phosphate-buffered saline (PBS) containing 5 mL/L Tween 20, supernatants of hybridoma cell cultures were added (100 μL/well) and incubated for 2 h at 37 °C. The bound antibodies were detected with peroxidase-conjugated goat anti-mouse polyvalent immunoglobulins (100 μL/well; 1:2000 dilution), with 3,3′,5,5′-tetramethylbenzidine as the substrate.

Two cell lines, C40D21 B12 P4 and C40D21 B22 P6 (designated MAb B12 and MAb B22, respectively), as well as their parent cell line, C40D21 B2 (designated MAb B2), had 100% recloning efficiency and the best anti-PHF activity. These were chosen for the large-scale production of MAbs in ascites. All antibodies were shown to be of the IgM isotype. Hybridoma cell lines (1–5 × 10⁵ cells/mL) were injected into pristane-primed BALB/c mice. Anti-PHF antibodies were purified from ascites on Superdex G-200 and eluted with PBS. All three cell lines were tested by bioassay for their ability to inactivate PHF mixed with antibody before injection into normotensive rats. The blood pressure bioassay for PHF (13) was adapted for bioassay of anti-PHF antibodies. MAbs produced by all three cell lines inactivated PHF mixed with antibody in concentrations of 300–400 mg/L.

PHF was measured in plasma samples by competitive ELISA as described previously (8). Briefly, microtitre plates were coated with 2 mg/L purified anti-PHF antibodies (100 μL/well) in phosphate buffer. The plates were washed, and triplicates of controls, unknown samples, or each PHF calibrator in plasma (50 μL/well; 1:20 dilution with PBS) were added, followed by PHF-horseradish peroxidase conjugate (50 μL/well; 1:2000 dilution with PBS containing 10 g/L BSA). Bound PHF-horseradish peroxidase was measured with 3,3′,5,5′-tetramethylbenzidine as the peroxidase substrate.

The calibration curves for the ELISAs using any of the three MAbs were linear in the range 0.03–1 unit/mL when plotted on a log-linear basis. The limit of detection for PHF (the smallest single value that could be distinguished from zero) was calculated to be 0.02 units/mL (the mean + 2 SD from 20 determinations of the zero calibrator). The precision of the ELISA using MAb B2 was estimated with three different plasma pools of samples containing PHF. The intraassay imprecision (as CV), determined from the mean of triplicates measured 18 times in the same assay,