Association between the UGT1A1 TA-Repeat Polymorphism and Bilirubin Concentration in Patients with Intermittent Claudication: Results from the CAVASIC Study

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BACKGROUND: Bilirubin has antioxidative and cytoprotective properties. Low plasma concentrations of bilirubin are reportedly associated with the development of coronary and cerebrovascular disease, and bilirubin concentrations are strongly correlated with the enzyme activity of the hepatic uridine diphosphate glucuronosyltransferase (UGT1A1). The activity of UGT1A1 is influenced by a TA-repeat polymorphism in the promoter of the UGT1A1 gene (UDP glucuronosyltransferase 1 family, polypeptide A1). In a case-control study, we investigated the association between the UGT1A1 polymorphism, bilirubin concentration, and intermittent claudication.

METHODS: We included 255 consecutive male patients presenting with intermittent claudication in the investigation and matched the patients by age and diabetes mellitus with 255 control individuals.

RESULTS: Plasma bilirubin concentrations were significantly lower in patients than in controls [mean (SD), 7.3 (3.1) mg/L vs 9.0 (4.6) mg/L; P < 0.001]. We found a clear association between the number of TA repeats and plasma bilirubin concentration. Considering the 6/6 TA-repeat genotype as the wild type, we observed a slight increase in bilirubin concentration individuals with the heterozygous 6/7 genotype and pronounced increases for those with the homozygous 7/7 genotype. This association occurred in both controls and patients; however, patients and controls were not significantly different with respect to UGT1A1 TA-repeat genotype frequencies.

CONCLUSIONS: Our study of a well-phenotyped group of patients with intermittent claudication and control individuals revealed a clear association between low bilirubin concentrations and peripheral arterial disease but no association between the UGT1A1 polymorphism and the disease.

The heme degradation product bilirubin has potent antioxidative capacities that play a protective role in various diseases, including coronary artery and vascular disease (1, 2). Studies on the relationship between bilirubin concentration and cardiovascular disease have shown an inverse relationship between bilirubin concentration and atherosclerosis (3–13). Low bilirubin concentrations have also been independently and inversely related to impaired flow-mediated vasodilation and increased carotid intima-media thickness in clinically healthy subjects (14, 15). These in vivo observations and experimental studies (16) suggest a protective effect of high physiological bilirubin concentrations on early steps in atherogenesis.

By linkage analysis we recently identified a region on chromosome 2q significantly linked to bilirubin concentration (17), a finding that was subsequently confirmed by investigators for the Framingham Heart Study (18). The identified chromosomal region harbors the gene for the uridine diphosphate glucuronosyltransferase (UGT1A1, UDP glucuronosyltransferase 1 family, polypeptide A1), the major enzyme of...
bilirubin glucuronidation, which mainly determines bilirubin elimination in humans. The activity of \textit{UGT1A1} is substantially influenced by a TA-repeat polymorphism in the \textit{UGT1A1} promoter (19). Individuals homozygous for 7 TA repeats (7/7) have lower promoter activity and higher subsequent bilirubin concentrations than heterozygous (6/7) or wild-type homozygous (6/6) individuals (19–21). In an investigation of the correlation between the TA-repeat polymorphism and the incidence of cardiovascular and coronary heart disease in the Framingham Offspring Study, we observed a statistically significant decreased risk for individuals with the 7/7 genotype (8).

Previous studies of bilirubin have primarily focused on patients with coronary artery disease. To our knowledge, the present study is the first to investigate an association between plasma bilirubin concentration and the \textit{UGT1A1} TA-repeat polymorphism in a male cohort with symptomatic peripheral arterial disease (PAD) and an age- and diabetes-matched group of control individuals.

Materials and Methods

STUDY PARTICIPANTS AND STUDY DESIGN

The CAVASIC (Cardiovascular Disease in Intermittent Claudication) Study is a prospective case-control study initiated in 2002 to identify cardiovascular risk factors in patients with intermittent claudication. Patient and controls were enrolled in 2 clinical centers, the Department of Vascular Surgery, Medical University Innsbruck, and the 3rd Medical Department of Metabolic Diseases and Nephrology, Hietzing Hospital, Vienna, Austria. Participants are to be followed for at least 6 years. We describe our study of the association of bilirubin concentration and the \textit{UGT1A1} polymorphism with the presence of symptomatic PAD at baseline.

Patients (n = 255) were consecutively included in the study when they presented with or had a history of intermittent claudication (PAD IIa or IIb, according to the criteria of Fontaine), regardless of whether they had already undergone a treatment procedure (bypass surgery or intervention). Patients were excluded from the study for any of the following reasons: presence of acute or critical limb ischemia (Fontaine III or IV), impaired liver function with increased enzyme concentrations (aspartate aminotransferase >50 U/L, alanine amino transferase >25 U/L, \( \gamma \)-glutamyltransferase >60 U/L), impaired kidney function with serum creatinine >133 \( \mu \)mol/L, malignancy, past organ transplantation, and therapy with nicotinic acid or corticosteroids.

We recruited 255 control individuals from the same geographic region and matched them with the patients with respect to age and presence of type 2 diabetes mellitus (T2DM). All members of the control group had volunteered to participate in the study after the publication of an invitation in newspapers. We applied the same exclusion criteria to the control group as we used for the patients. Control individuals with symptomatic PAD were excluded, but those with a history of cardiovascular disease were allowed to participate. Eighteen of the 255 controls either had a positive cardiovascular history for angina pectoris according to their responses on the Rose questionnaire (22) or had documented cardiovascular events or procedures, such as myocardial infarction, aortocoronary bypass surgery, percutaneous transluminal coronary angioplasty, and/or coronary angiography or stroke.

Neither the patients nor the controls had acute illnesses or clinically detectable inflammatory processes at the time of enrollment. All study participants provided written informed consent, and the ethics committee of the participating study centers approved the examination protocol.

To minimize interobserver bias, we had a single physician at each of the 2 clinical centers conduct all of the interviews and examinations; these physicians were specially trained in vascular examinations and echocardiography.

BASELINE VASCULAR EXAMINATION

For the measurement of the ankle-brachial index (ABI), the systolic brachial blood pressure was initially measured once on both arms, and 2 additional blood pressure measurements were made on the arm with the higher systolic value. We used the mean of these 2 additional measurements on the arm with the higher systolic value for further calculations. Systolic blood pressures on the lower extremity were measured 3 times for each artery (dorsalis pedis artery and tibialis posterior artery, on both the left and right ankles). The mean of the second and third measurements for each site was used to calculate the ABI for each of the 4 lower-extremity sites. The ABI was calculated as the ratio of the systolic blood pressure (mean of second and third measurements) of each of the 4 sites to the mean systolic blood pressure of the arm. We used the lowest ABI value from the 4 sites for further data analysis to yield a higher sensitivity of the ABI (23) and to be in line with the Reduction of Atherothrombosis for Continued Health (REACH) Registry (24).

The Edinburgh questionnaire was used to identify symptomatic intermittent claudication (25). Pa-
tients who presented with symptoms of intermittent claudication and had an ABI <0.90 were considered to have PAD. Furthermore, we performed a pulse volume recording and evaluated walking distance with a standardized constant-load treadmill examination (12% acceleration and 3.0 km/h). If any further therapy was planned, additional ultrasound scanning or magnetic resonance imaging of the arteries of the lower extremity was done. Conventional angiography was performed in cases requiring further endovascular treatment.

**OTHER PHENOTYPIC CHARACTERIZATION**

Demographic data, clinical history, smoking status, alcohol consumption, diet, amount of leisure, physical activity during work, and atherosclerosis risk profile were recorded via a standardized interview. Current medications were recorded at the baseline exam.

All participants underwent a clinical examination that was focused on the heart and arteries and included electrocardiographic and echocardiographic evaluations.

Participants received a diagnosis of diabetes mellitus if their fasting plasma glucose concentration was >6.99 mmol/L and/or they were being treated with antidiabetes drugs. Participants were considered hypertensive when the systolic blood pressure was ≥140 mmHg, if the diastolic blood pressure was ≥90 mmHg, and/or if they were being treated with antihypertension drugs.

**LABORATORY MEASUREMENTS**

Plasma and serum samples treated with EDTA and citrate were obtained after an overnight fasting period of 10–14 h, immediately processed, centrifuged, and stored in aliquots at −80 °C until laboratory measurements were made. Bilirubin was measured immediately after blood collection via a colorimetric method (diazotized sulfanilic acid reaction; Roche Diagnostics reagent sets) in a Modular P800 analyzer.

**GENOTYPING**

DNA was extracted from whole-blood samples by a salting-out method (Invisorb Blood Universal Kit; Invitae). The UGT1A1 promoter polymorphism was analyzed as recently described (8). Samples were genotyped within the Genotyping Unit of the Gene Discovery Core Facility at the Innsbruck Medical University, Innsbruck, Austria.

**STATISTICAL ANALYSIS**

Variables for the patient and control groups were evaluated statistically by means of unpaired Student t-tests, nonparametric Wilcoxon rank sum tests, and the Pearson χ² test. Besides the 6 and 7 TA-repeat alleles for the UGT1A1 polymorphism, we also observed 6 individuals with a 5 TA-repeat allele and 2 persons with an 8 TA-repeat allele. Because of the low frequencies of these alleles and functional studies that revealed decreasing promoter activity with increasing numbers of TA repeats, we combined the 5 and 6 TA-repeat alleles and the 7 and 8 TA-repeat alleles for statistical analyses. Variables contributing to PAD status were identified by logistic regression analysis, and an adjusted general linear regression model was used to estimate the proportion of the variation in bilirubin concentration explained by different variables. The full model included the UGT1A1 polymorphism (coded as a recessive model for the 7 TA repeats), age, LDL cholesterol, smoking status, T2DM, and glucose. A submodel included these variables without the UGT1A1 polymorphism. The difference in r² between the 2 models was considered to be the amount of the variation in bilirubin concentration explained by the UGT1A1 polymorphism.

Statistical analyses were performed with the Statistical Package for the Social Sciences for Windows (SPSS) version 15.0 and SAS version 9.1 (SAS Institute).

**Results**

**BASELINE CHARACTERISTICS**

Between July 2002 and July 2006, we included 255 unrelated male patients (age range, 35–70 years) who presented with intermittent claudication graded as stage IIa or IIb according to the Fontaine classification. Thirty-eight of these patients had T2DM. The patients and the age- and T2DM-matched controls had similar T2DM durations [mean (SD), 11.7 (7.4) years vs 10.7 (8.4) years] and similar glycosylated hemoglobin (HbA1c) values [7.5% (1.4%) vs 7.4% (1.1%)]. These differences were not statistically significant.

Table 1 summarizes the characteristics of the patients and control individuals. The patient group had a significantly higher frequency of smokers and significantly higher triglyceride and creatinine concentrations and lower HDL cholesterol and albumin concentrations than the control group. Hypertension frequency and blood pressures were markedly higher in the patient group.

**BILIRUBIN CONCENTRATIONS AND THE UGT1A1 POLYMORPHISM IN PATIENTS AND CONTROLS**

Patients with PAD had significantly lower bilirubin concentrations than the controls [mean (SD), 12 (5.3) μmol/L vs 15 (7.9) μmol/L; P < 0.001] (Table 2). Because the control group included 18 individuals with a history of cardiovascular disease, we performed a sensitivity analysis and excluded these 18 control individuals; nevertheless, the mean bilirubin concentration...
for the control group remained unchanged [15 (7.7) μmol/L].

There was a clear association between the TA-repeat polymorphism in the UGT1A1 promoter and bilirubin concentration. Considering the 6/6 TA-repeat genotype as the wild type, we observed a slight increase in bilirubin concentration in individuals with the heterozygous 6/7 genotype and a pronounced increase for the homozygous 7/7 genotype. This association was apparent in both the controls (12 μmol/L, 15 μmol/L, and 27 μmol/L, respectively; \( P < 0.001 \)) and the patients (11 μmol/L, 12 μmol/L, and 17 μmol/L, respectively; \( P < 0.001 \)) (Fig. 1). The proportion of the total variation in bilirubin concentration explained by the TA-repeat polymorphism was 12.3% in the patients after adjusting for covariates, compared with 27.9% in the control group (Table 3).

A comparison of the bilirubin concentrations for the patient and control groups by genotype revealed lower bilirubin concentrations in the patients than in the controls for each genotype (Fig. 1). The difference was most pronounced for individuals with the 7/7 TA-repeat genotype (17 μmol/L vs 27 μmol/L; \( P < 0.001 \)). The patients and control individuals showed no differences with respect to genotype frequencies, however, regardless of whether the 18 control individuals with a history of cardiovascular disease were included in the analysis (Table 2) or not (data not shown).

### Table 1. Baseline clinical and laboratory data for patients with peripheral arterial occlusive disease and for control individuals matched by age and diabetes mellitus.\(^a\)

<table>
<thead>
<tr>
<th></th>
<th>Controls (n = 255)</th>
<th>Patients (n = 255)</th>
<th>( P )</th>
</tr>
</thead>
<tbody>
<tr>
<td>Age, years</td>
<td>57.2 (9.2)</td>
<td>58.0 (7.0)</td>
<td>NS</td>
</tr>
<tr>
<td>Body mass index, kg/m</td>
<td>26.8 (5.7)</td>
<td>26.8 (4.0)</td>
<td>NS</td>
</tr>
<tr>
<td>Smoking (nonsmokers/former smokers/current smokers), n (%)</td>
<td>114/99/42 (44.7/38.8/16.5)</td>
<td>19/109/124 (7.5/43.3/49.2)</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>Total cholesterol, mmol/L</td>
<td>5.39 (0.906)</td>
<td>5.34 (1.06)</td>
<td>NS</td>
</tr>
<tr>
<td>LDL cholesterol, mmol/L</td>
<td>3.52 (0.855)</td>
<td>3.44 (0.958)</td>
<td>NS</td>
</tr>
<tr>
<td>HDL cholesterol, mmol/L</td>
<td>1.54 (0.451)</td>
<td>1.28 (0.321)</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>Triglycerides, mmol/L(^b)</td>
<td>1.51 (0.915)</td>
<td>1.94 (1.40)</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>[0.904, 1.29, 1.75]</td>
<td>[1.06, 1.51, 2.41]</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Glucose, mmol/L</td>
<td>5.79 (1.71)</td>
<td>5.95 (1.60)</td>
<td>0.006</td>
</tr>
<tr>
<td>Creatinine, μmol/L</td>
<td>90 (12)</td>
<td>88 (17)</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>Albumin, g/L</td>
<td>45.5 (4)</td>
<td>44.6 (5)</td>
<td>0.014</td>
</tr>
<tr>
<td>Systolic blood pressure, mmHg</td>
<td>140 (17)</td>
<td>150 (20)</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>Diastolic blood pressure, mmHg</td>
<td>82 (9)</td>
<td>83 (10)</td>
<td>0.180</td>
</tr>
<tr>
<td>Hypertension, n (%)(^c)</td>
<td>153 (60.5)</td>
<td>214 (84.9)</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>Ankle-brachial index(^d)</td>
<td>1.04 (0.15)</td>
<td>0.73 (0.24)</td>
<td>&lt;0.001</td>
</tr>
</tbody>
</table>

\( ^a \) Data are expressed as the mean (SD) except where noted.
\( ^b \) Data are expressed as the mean (SD) [25th, 50th, 75th percentiles].
\( ^c \) Hypertension was defined as a systolic blood pressure \( \geq 140 \) mmHg, a diastolic blood pressure \( \geq 90 \) mmHg, and/or receiving antihypertension treatment.
\( ^d \) The lowest ABI value from the 4 sites was used for data analysis (see Materials and Methods). Individuals with ABI values \( \geq 1.30 \) were excluded from this analysis.

### Table 2. Bilirubin concentrations and the UGT1A1 TA-repeat polymorphism in controls and patients with peripheral arterial occlusive disease.

<table>
<thead>
<tr>
<th></th>
<th>Controls (n = 255)</th>
<th>Patients (n = 255)</th>
<th>( P )</th>
</tr>
</thead>
<tbody>
<tr>
<td>Bilirubin, μmol/L(^e)</td>
<td>15 (7.9)</td>
<td>12 (5.3)</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>[9.9, 14, 18]</td>
<td>[9.1, 11, 15]</td>
<td></td>
<td></td>
</tr>
<tr>
<td>UGT1A1 TA-repeat genotype frequency, n (%)</td>
<td>6/6</td>
<td>94 (37.0)</td>
<td>91 (36.8)</td>
</tr>
<tr>
<td>6/7</td>
<td>127 (50.0)</td>
<td>119 (48.2)</td>
<td></td>
</tr>
<tr>
<td>7/7</td>
<td>33 (13.0)</td>
<td>37 (15.0)</td>
<td></td>
</tr>
</tbody>
</table>

\( ^e \) Data are expressed as the mean (SD) [25th, 50th, 75th percentiles].

### Table 3. Multivariate logistic regression analysis

Finally, we investigated whether bilirubin concentration was associated with the case-control status. After adjustment for age, we found a 1.7-μmol/L increase in bilirubin concentration decreased the probability of being a patient by 11.5% (Table 4, model 1). This asso-
Association was still significant after we adjusted for other risk factors, such as smoking status, HDL cholesterol, glucose, creatinine, and hypertension (Table 4).

Discussion

To our knowledge, this study is the first to investigate plasma bilirubin concentration and the UGT1A1 promoter TA-repeat polymorphism in a cohort of patients with PAD and an age- and diabetes-matched control group. We observed significantly lower bilirubin concentrations in patients than in controls, and the UGT1A1 polymorphism was strongly associated with bilirubin concentration in both patients and controls. Interestingly, the polymorphism was not associated with PAD.

Activation of heme oxygenase and the heme catabolic pathway are both strongly influenced by genetics and have been proposed to have beneficial effects on diseases by protecting against oxidative stress, possibly through the action of bilirubin or in conjunction with bilirubin metabolism (2, 26). Several studies have described an association between low bilirubin concentrations and coronary heart disease (3–13). The association of bilirubin with atherosclerosis might be explained by in vitro findings that have shown bilirubin to have antioxidative and cytoprotective properties (27, 28). Bilirubin scavenges peroxyl radicals and suppresses oxidation in liposomes more efficiently than α-tocopherol (27, 28). It also has antithrombotic properties (29) and modulates macrophage activation within atherosclerotic lesions (30). Because the enzyme activity of uridine diphosphate glucuronosyltransferase is strongly influenced by the investigated TA-repeat polymorphism (19–21), the hypothesis of an association between this polymorphism and atherosclerotic outcome is attractive.

The highly significant association between bilirubin concentration and PAD noted in our study is convincing and in line with the majority of the other studies on coronary heart disease (3–13). Furthermore, we found a strong association between the UGT1A1 polymorphism and bilirubin concentration; however, we were surprised that we could find no association between the UGT1A1 polymorphism and PAD. In a recent Framingham Heart Study investigation, a strong association was observed between the 7/7 TA-repeat genotype and a lower risk of cardiovascular disease. During a 24-year follow-up, carriers of this genotype had about one third the risk for cardiovascular disease and coronary heart disease as carriers of the 6 allele (8). This finding is in contrast to the longitudinal population-based Rotterdam Study of elderly individuals, which found no association of either the TA-repeat
polymorphism or bilirubin concentration with coronary heart disease (31). Similarly, the ECTIM case-control study of myocardial infarction found no association of the UGT1A1 7 TA-repeat allele with coronary heart disease (32).

The differences between these studies and our study of PAD patients in comparison with the Framingham Heart Study are obvious: the Framingham Heart Study is a population-based prospective cohort study of individuals free of cardiovascular disease at baseline. Although the Rotterdam Study was a prospective study, participants had a mean age of nearly 70 years at baseline, which makes a survival bias highly likely. We cannot exclude a survival bias in our study because concomitant PAD and cardiovascular disease is the rule rather than the exception.

In this context we consider the following observation highly interesting: Although we observed an association between the TA-repeat polymorphism and bilirubin concentration in both the controls and the cases, the relative and absolute differences in bilirubin concentration between individuals with the 6/6 and 7/7 TA-repeat genotypes was much more pronounced in the controls than in the patients. Control individuals with the 7/7 TA-repeat genotype had bilirubin concentrations that were about 120% higher than controls with the 6/6 TA-repeat genotype. This difference in PAD patients was only about 52%, however (Fig. 1). We speculate that the lower relative difference in bilirubin concentration in PAD patients with the 7/7 genotype could be explained by a selection bias between the patients and controls within the 7/7 genotype group. If this speculation is accurate, we expect the bilirubin concentrations of patients with the 7/7 genotype to fall in the lower range of expected values for this genotype, whereas the bilirubin concentrations in the control group would fall more toward the center of the expected range. Such a phenomenon might account for the observation that the TA-repeat polymorphism explained only about half of the bilirubin variance in PAD patients compared with controls (12.6% vs 25.6%, Table 3). If we consider that only 12.6% of the variance in bilirubin concentration is explained by the TA-repeat polymorphism, it is no longer surprising that we cannot detect an association of this polymorphism with PAD outcome, although the association of bilirubin concentration with PAD is relatively strong. It is possible that thousands of patients and controls would be needed in a case-control study to detect such an association, if it exists. The clear association observed in the Framingham Heart Study might be explained by its having a prospective study design that is less confounded by most kinds of selection and survival bias than case-control designs.

In summary, our study of a well-phenotyped group of PAD patients and control individuals shows a clear association between low bilirubin concentrations and peripheral arterial occlusive disease.
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