Serum bilirubin levels in familial hypercholesterolemia: A new risk marker for cardiovascular disease?

**Abbreviated title:** Serum bilirubin levels in familial hypercholesterolemia

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**ABBREVIATIONS:** CVD cardiovascular disease; FH familial hypercholesterolemia; LDL-C low-density lipoprotein cholesterol; MI myocardial infarction; ECG electrocardiogram; HDL-C high-density lipoprotein cholesterol; TG triglycerides; BMI body mass index; ALAT alanine-amino transferase; ASAT aspartate-amino transferase; HO-1 heme oxygenase 1; CAD coronary artery disease.
ABSTRACT

Objective: Low concentrations of bilirubin are associated with an increased risk for cardiovascular disease (CVD). Possibly, bilirubin exerts its effect through the protection of LDL from oxidation. Therefore, we examined whether low bilirubin might also be a risk marker for CVD in patients with familial hypercholesterolemia (FH) and whether statins influence serum bilirubin levels.

Methods: Patients with FH were recruited from 37 Lipid Clinics. After a washout period of 6 weeks, all patients were started on monotherapy with simvastatin 80 mg for a period of two years.

Results: A total of 514 patients were enrolled. Bilirubin at baseline was inversely associated with the presence of CVD, also after adjustment for age, gender, presence of hypertension and high-density lipoprotein cholesterol levels. Moreover, bilirubin levels were significantly raised by 7% from 10.0 to 10.8 μmol/L after treatment with simvastatin 80 mg.

Conclusion: We hypothesize first that high bilirubin levels might protect patients with FH from CVD. Furthermore, bilirubin levels were significantly increased after treatment with simvastatin 80 mg, independent from changes in liver enzymes, which might confer additional protection against CVD. Whether this is also true for lower doses of simvastatin or for other statins remains to be investigated.

Keywords: Atherosclerosis, Drug therapy, LDL, Oxidized lipids, Statins, Cardiovascular disease
INTRODUCTION

Serum total bilirubin concentrations have been shown to be inversely associated with the risk for cardiovascular disease (CVD) (1-10). The explanation for this association is not fully understood. In contrast, bilirubin was for a long time regarded as cytotoxic, in particular for its role in neonatal jaundice. It is only since the end of the 1980s that a physiological role for bilirubin functioning has emerged as a potent antioxidant (11,12). In fact, in vitro evidence suggests that low-density lipoprotein (LDL) can be protected from oxidation by bilirubin (13). Therefore, low bilirubin concentrations could be a reflection of a heightened oxidative state and increased consumption of bilirubin. Furthermore, bilirubin has been shown to have anti-inflammatory properties (14). Taken together, these results point to potential beneficial effects of bilirubin towards the chronic inflammatory state we currently associate with atherosclerosis.

Familial hypercholesterolemia (FH) is an autosomal dominant disorder of lipoprotein metabolism and affects approximately 1 in 400 people in the Netherlands (15). The underlying defect consists of mutations in the gene encoding for the low-density lipoprotein (LDL) receptor protein or in its ligand, apolipoprotein B100. These mutations result in markedly elevated plasma cholesterol levels, predisposing FH patients to premature atherosclerosis and CVD (16). Statins – or HMG-CoA reductase inhibitors – are currently considered the preferred lipid-lowering agents in these patients since they have been proven safe and well-tolerated agents that reduce LDL-cholesterol (LDL-C) levels as well as the incidence of coronary artery disease.

To the best of our knowledge, it has not been examined before whether low bilirubin is associated with the presence of CVD in FH patients and whether statins can raise serum bilirubin levels. We therefore set out to study the role of bilirubin in these patients and the effect of simvastatin therapy on bilirubin levels. Here we present our results.
METHODS

Study design and subjects

Data for the present analysis were derived from the database of the ExPRESS FH (Examination of Proband and Relatives in Statin Studies with Familial Hypercholesterolemia) study, in which the two-year efficacy and safety of simvastatin 80mg were evaluated in 526 heterozygous FH patients (17). For this open-label study, subjects were recruited from 37 Lipid Clinics in the Netherlands. Patients were included if they met the following criteria: all patients had to have either a molecular diagnosis for FH or were diagnosed with definite FH and had to have six or more points, according to an algorithm (to allow standardization of the diagnosis of FH based on clinical findings, personal and familial clinical history and biochemical parameters) (18); at least 18 years of age; patients with a history of myocardial infarction (MI), coronary artery bypass graft or percutaneous transluminal coronary angioplasty could be included if the physician thought it was medically allowed for the patient to have a washout period. Patients were excluded if they: were pregnant or nursing women, or pre-menopausal women not using adequate contraceptives; had acute liver disease, hepatic dysfunction, or persistent elevations of serum transaminases; had hypersensitivity or intolerance to simvastatin or any of its components; had hyperlipidemia type I, III, IV or V or homozygous FH; had a recent history of alcohol or drug abuse; had secondary hypercholesterolemia due to any cause; had inadequately controlled diabetes, unstable angina or intermediate coronary syndrome or clinically significant ventricular arrhythmia at study entry or MI within the past 3 months; were on concurrent use of erythromycin and similar drugs affecting the cytochrome P450 enzyme or had a history of cancer.
The Ethics Institutional Review Boards Committees of all the 37 centers approved the protocol and written informed consent was obtained from all participants. The investigation was conformed according to the principles outlined in the Declaration of Helsinki.

After a six-week washout period, patients started monotherapy with simvastatin 80 mg for the duration of two years. No other lipid-lowering medication was allowed. Medical history, physical examination and additional risk factors for CVD as well as laboratory analysis of lipid and lipoprotein levels and routine safety parameters were obtained in all patients.

**Cardiovascular disease**

CVD was considered to be present if subjects met one of the following criteria: subjects who had 1) a myocardial infarction, proven by electrocardiogram (ECG) abnormalities and enzyme changes; 2) an ischemic stroke; 3) a diagnosis of clinically documented angina pectoris; 4) a history of intermittent claudication documented by ultrasound; 5) coronary bypass surgery or percutaneous coronary interventions; 6) a clinically significant stenosis on coronary angiogram; or 7) an unequivocally positive exercise ECG.

**Biochemical analysis**

Blood samples were taken in the morning after an overnight fast. Total plasma cholesterol (TC), high-density lipoprotein cholesterol (HDL-C) and triglycerides (TG) were routinely determined in the different laboratories and standardised by a virtual central laboratory. LDL-C was calculated using the Friedewald formula (19). Total serum bilirubin was routinely measured in the different laboratories by spectrophotometry. All results were harmonized to one level according to the standardised Jendrassik-Grof method by the virtual central laboratory (20).
Statistical analysis

Mean values in lipids between subgroups were compared using the independent sample-t-test. Other parameters (TG and bilirubin) were compared by the non-parametric Mann-Whitney U test, because they had a skewed distribution. Chi-square tests were applied for comparing distributions of dichotomous data (gender, smokers, presence of hypertension or diabetes and bilirubin levels (>17 µmol/L versus ≤ 17 µmol/L). The association between presence of CVD and bilirubin levels at baseline was evaluated using a logistic regression model. We adjusted for potential confounders, i.e. age, gender, hypertension, diabetes, body mass index (BMI), HDL-C and TG by means of stepwise backward elimination. Mean values in lipids before and after treatment were compared using the paired sample t-test. TG, bilirubin, alanine-amino transferase (ALAT) and aspartate-amino transferase (ASAT) levels were compared by the non-parametric Wilcoxon test, because they had a skewed distribution. Pearson correlations were applied to evaluate the correlation between absolute changes in bilirubin, ASAT and ALAT. All statistical analyses were performed using the SPSS package (version 15.0 Chicago, Illinois). A p-value of less than 0.05 was considered to be statistically significant.
RESULTS

Study population

Among the 526 FH patients who participated in the ExPRESS FH study, baseline total bilirubin levels were available for 514 patients and these patients comprised our study population. Age ranged from 18 to 80 years with a mean age of 47.4 years (standard deviation (SD) ± 13.2) and 188 (37%) patients were known to have CVD. Baseline demographic and clinical characteristics of patients with and without CVD are summarized in table 1. Patients with CVD were older and had on average higher values of BMI, in addition to a higher prevalence of hypertension and diabetes compared to those without CVD. Less current smokers were seen in the CVD group. Furthermore, mean HDL-C was lower in patients with CVD, whereas the median TG level was significantly higher.

FH patients and bilirubin levels

Median baseline serum bilirubin level in all FH patients was 10.0 µmol/L (interquartile range (IQ): 7.8 to 12.8). In patients with CVD, the median bilirubin level was significantly lower compared to patients without CVD (9.7 [IQ: 7.3-11.7] versus 10.5 [7.8-13.5] µmol/L, respectively; p= 0.006). A significant lower proportion of patients with elevated bilirubin levels (i.e. bilirubin >17 µmol/L) was observed in those with CVD compared to patients without CVD (3.7% versus 9.8%, respectively; p=0.01).

Association between CVD and bilirubin

We evaluated the association between bilirubin levels and CVD in a logistic regression model with CVD as the response variable and bilirubin as the explanatory variable. Levels of bilirubin were negatively associated with CVD (OR 0.94; 95% CI 0.90-0.98; p=0.005). By means of multiple regression models we further explored the role of potential confounders.
Backward hierarchical elimination strategy was used to identify the final model and subsequently TG (OR 0.96; 95% CI 0.79-11.79; p=0.719), presence of diabetes (OR 6.49; 95% CI 0.68-61.96, p=0.104) and BMI (OR 1.06; 95% CI 0.97-1.14, p=0.062) dropped out of the model. In the final model adjusted for age (OR 1.11; 95% CI 1.08-1.13; p<0.0001), male gender (OR 1.85; 95% CI 1.14-3.01; p=0.01), presence of hypertension (OR 1.96; 95% CI 1.11-3.46; p=0.02), and HDL-C (OR 0.33; 95% CI 0.16-0.65; p=0.002), bilirubin (OR 0.92; 95% CI 0.88-0.97; p=0.004) remained significantly associated with CVD. Additionally, we performed the same analyses with bilirubin as a dichotomous variable (cut-off at 17 µmol/L). In the univariate analysis as well as in the multiple analyses adjusted for age, gender, hypertension and HDL-C, bilirubin was significantly related with CVD (OR 0.36; 95% CI 0.15-0.82; p=0.02 and OR 0.27; 95% CI 0.08-0.73; p=0.01, respectively).

Treatment with simvastatin and bilirubin

In table 2 treatment effects of simvastatin 80 mg on lipids, bilirubin and liver enzymes are given. TC, LDL-C and TG levels were reduced by 39.2%, 48.0% and 26.3%, respectively, whereas HDL-C levels were elevated by 12.7%. Median bilirubin levels were significantly raised after treatment with 80 mg of simvastatin with 7% from 10.0 [IQR: 7.8; 12.8] to 10.8 [IQR: 7.8; 13.7] µmol/L. This increase was more pronounced in the patients with CVD compared to those without CVD (1.40 [IQR: -0.6; 3.1] µmol/L versus 0.40 [IQR: -2.0; 2.5] µmol/L; p=0.008). Notably, no correlation was observed between change in bilirubin and change in ASAT (r=0.05; p=0.26), ALAT (r=-0.02; p=0.63) or in BMI (r=0.032, p=0.51).
DISCUSSION

Baseline bilirubin and CVD

The current study is the first to show that serum bilirubin levels in patients with FH were independently and inversely associated with the presence of CVD. These findings indicate that bilirubin levels at the low end of the normal range are related with a significantly higher prevalence of CVD and suggest cardiovascular protection from elevated serum bilirubin levels in these high risk patients.

Our results are consistent with findings of both previous retrospective (2-4) and prospective studies (5-9). In these studies, similar inverse associations have been shown not only between serum bilirubin concentrations and CVD, but also between bilirubin and peripheral vascular disease, carotid-intima-media thickness and stroke (21,22). Furthermore, a meta-analysis by Novotny et al. of eleven studies has shown an inverse and dose-dependent relationship between serum bilirubin and different types and severities of CVD. It was found that a serum bilirubin level of 10 µmol/L is the cut-point for discrimination of cardiovascular risk (10). Accordingly, in our study with a much smaller study cohort, the median level of bilirubin levels in patients with and without CVD was just below and above 10 µmol/L, respectively. However, women were not included in this meta-analysis and it is therefore unclear whether these findings can be extrapolated to our study population. As for the strength of the found association, Hopkins et al. found that bilirubin levels were comparable to HDL-C in terms of CVD protection (2). Similarly in our study, bilirubin levels above 17.0 µmol/L were associated with comparable CVD protection as 1 mmol/L increase in HDL-C levels in FH patients.

Some methodological aspects of our study merit discussion. First of all, because of the cross-sectional design we could not establish a causal relation between serum bilirubin levels and occurrence of CVD, i.e. that high levels of bilirubin prevent future CVD. However, a
considerable number of prospective cohort studies that have shown an inverse relationship between bilirubin and CVD provide direct support that bilirubin may have a causal role in atherosclerotic vascular disease (5-9). Moreover, several studies have shown that bilirubin acts as an antioxidant and suppresses lipid oxidation,(12,23,24) which is known to prevent plaque formation and atherosclerosis.

Due to the observational nature of our data, the association between CVD and bilirubin could possibly be explained by undiscovered confounding. Aspirin use could be a potential confounder since it has been suggested that aspirin raises bilirubin levels through heme oxygenase 1 (HO-1) activity induction whereas it is also associated with CVD (25,26). However, despite the use of aspirin in most of the CVD patients we found that bilirubin levels were significantly lower in these patients, which actually suggest an underestimation of the found association. Furthermore, when we adjust for use of aspirin in a stepwise multiple regression analyses to evaluate the relation between bilirubin levels and CVD, aspirin use did not emerge as a significant confounder. Another potential confounder could be smoking. Not only is smoking a well known risk factor for CVD, it is also inversely associated with bilirubin concentrations (27). Strikingly, in our study cohort with FH patients fewer smokers were seen in the group of patients with CVD. This discrepancy may be explained by a high number of former smokers in the CVD group, who stopped smoking after their first cardiovascular event. Unfortunately, we did not collect any data on previous smoking habits. Since current smoking would not accurately reflect previous exposure to smoking, we have chosen not to include this variable in the multiple regression analyses. Seasonal variation in bilirubin could also be of influence (28), however subjects were recruited for the study throughout the whole year, so we assume that this phenomenon played a minor role. Lastly, the association between CVD and bilirubin could possibly be confounded by patients with concomitant elevated liver enzymes (5,10). In our study, one of the exclusion criteria was acute liver disease, hepatic dysfunction or persistent elevations of serum transaminases. In
order to avoid confounding we also performed all analyses in patients in which liver enzymes were below certain strict limits (ASAT<40 U/L and ALAT< 45 U/L). This yielded 458 patients at baseline and 335 patients after two years of therapy. However, results in this group subgroup were similar to the results obtained in the whole study cohort (data not shown).

Effect of simvastatin on bilirubin

We observed that bilirubin levels were increased by 7% from 10.0 to 10.8 µmol/L in patients with heterozygous FH, after two years of treatment with simvastatin 80 mg. This increase was more pronounced in the patients with CVD as compared to those without CVD. Although liver enzymes were also slightly increased, changes in ASAT or ALAT levels were not correlated with change in bilirubin.

No other reports are available with regards to the effects of statins on bilirubin or HO-1 levels in clinical studies. The effect of statins on bilirubin levels and HO-1 protein levels were measured in vitro and in vivo studies (29-32) that showed a raise of bilirubin levels by statins. These results may further explain the pleiotropic, antioxidant, anti-inflammatory and antiatherogenic actions of statins. Unfortunately, we did not measure HO-1 activity and development of a serial monitoring system of HO-1 activity for use in clinical setting is desirable. At present, there is no specific and sensitive marker for HO-1 activity in vivo.

An increase of 0.8 µmol/L of bilirubin levels is modest but in the multiple regression analysis an increase of 1 µmol/L was associated with an 8% decrease in CVD. This is in line with Hunt who found in 328 patients with early coronary artery disease (CAD) a 7% increase of CAD prevalence for each 1 µmol/L decrease in serum bilirubin (33). Whether this bilirubin increase by simvastatin confers additional benefit over and above cholesterol reduction cannot be answered by our study, both because of small numbers of events and the likely overwhelming effect of LDL-C lowering.
The observation that the bilirubin increase was more pronounced in the patients with CVD as compared to those without CVD, can possibly be explained by the fact that the groups with and without CVD were moderately heterogeneous with regard to factors such as sex, age and smoking status. In fact, we did observe a trend towards a greater increase in bilirubin in males and in subjects >50 years, which are both more represented in the CVD group. For the subjects who did not smoke, a significant greater increase in bilirubin was seen as compared to the subjects who did smoke (1.1 ± 4.4 vs -0.2 ± 3.1 μmol/L, respectively; p=0.007). As there were more non-smokers in the CVD group, this could have contributed to the observed difference in bilirubin increase as well. Nevertheless, more studies will have to confirm our results and delineate the role of bilirubin and HO-1 in atherogenesis and CVD.

In summary, we found significant lower serum total bilirubin levels in FH patients known to have CVD as compared to FH patients without CVD. Based on these findings, we hypothesize that high bilirubin levels might protect FH patients from CVD. Furthermore, bilirubin levels were significantly increased after two years of treatment with simvastatin 80 mg, independent from changes in liver enzymes, which might confer additional protection against CVD. Whether this is also true for lower doses of simvastatin or for other statins remains to be investigated.
Reference List


Table 1.Baseline characteristics of FH patients with and without CVD

<table>
<thead>
<tr>
<th></th>
<th>FH with CVD</th>
<th>FH without CVD</th>
<th>p-value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Age (years)</td>
<td>55.5 ± 9.8</td>
<td>42.7 ± 12.6</td>
<td>&lt;0.0001</td>
</tr>
<tr>
<td>Male gender, n (%)</td>
<td>110 (58.5)</td>
<td>177 (54.3)</td>
<td>0.36</td>
</tr>
<tr>
<td>Current smoking, n (%)</td>
<td>34 (18.0)</td>
<td>102 (31.3)</td>
<td>0.001</td>
</tr>
<tr>
<td>Hypertension, n (%)</td>
<td>51 (27.0)</td>
<td>29 (8.9)</td>
<td>&lt;0.0001</td>
</tr>
<tr>
<td>Diabetes, n (%)</td>
<td>9 (4.8)</td>
<td>1 (0.3)</td>
<td>&lt;0.0001</td>
</tr>
<tr>
<td>Body mass index (kg/m²)</td>
<td>26.7 ± 3.4</td>
<td>25.4 ± 3.5</td>
<td>&lt;0.0001</td>
</tr>
<tr>
<td>Total cholesterol (mmol/L)</td>
<td>10.65 ± 2.36</td>
<td>10.36 ± 2.05</td>
<td>0.16</td>
</tr>
<tr>
<td>LDL cholesterol (mmol/L)</td>
<td>8.45 ± 2.31</td>
<td>8.28 ± 2.02</td>
<td>0.40</td>
</tr>
<tr>
<td>HDL cholesterol (mmol/L)</td>
<td>1.18 ± 0.31</td>
<td>1.25 ± 0.36</td>
<td>0.02</td>
</tr>
<tr>
<td>TG (mmol/L)</td>
<td>1.90 (1.40-2.78)</td>
<td>1.60 (1.10-2.30)</td>
<td>&lt;0.0001</td>
</tr>
<tr>
<td>Total bilirubin (µmol/L)</td>
<td>9.7 (7.2-11.7)</td>
<td>10.5 (7.8-13.5)</td>
<td>0.006</td>
</tr>
<tr>
<td>Bilirubin &gt;17 µmol/L</td>
<td>7 (3.7)</td>
<td>32 (9.8)</td>
<td>0.01</td>
</tr>
</tbody>
</table>

Except where given as percentages, all values are given as mean levels ± standard deviation. Only triglycerides and bilirubin are given as median with interquartile range between brackets. FH, familial hypercholesterolemia; CVD, cardiovascular disease; LDL, low-density lipoprotein; HDL, high-density lipoprotein; TG, triglycerides.
<table>
<thead>
<tr>
<th></th>
<th>Baseline n = 514</th>
<th>After two Years n = 436</th>
<th>%change</th>
<th>p-value*</th>
</tr>
</thead>
<tbody>
<tr>
<td>Total cholesterol (mmol/L)</td>
<td>10.52 ± 2.17</td>
<td>6.30 ± 1.41</td>
<td>-39.2 ± 11.8</td>
<td>&lt;0.0001</td>
</tr>
<tr>
<td>LDL cholesterol (mmol/L)</td>
<td>8.38 ± 2.14</td>
<td>4.29 ± 1.31</td>
<td>-48.0 ± 13.5</td>
<td>&lt;0.0001</td>
</tr>
<tr>
<td>HDL cholesterol (mmol/L)</td>
<td>1.23 ± 0.35</td>
<td>1.36 ± 0.36</td>
<td>12.7 ± 21.8</td>
<td>&lt;0.0001</td>
</tr>
<tr>
<td>Triglycerides (mmol/L)</td>
<td>1.80 (1.20 to 2.40)</td>
<td>1.20 (0.90 to 1.70)</td>
<td>-26.3 (-46.2 to -5.6)</td>
<td>&lt;0.0001</td>
</tr>
<tr>
<td>Total bilirubin (μmol/L)</td>
<td>10.0 (7.8 to 12.8)</td>
<td>10.8 (7.8 to 13.7)</td>
<td>7.0 (-14.1 to 31.1)</td>
<td>&lt;0.0001</td>
</tr>
<tr>
<td>ASAT (U/L)</td>
<td>20 (17 to 24)</td>
<td>23 (19 to 27)</td>
<td>14 (0 to 32)</td>
<td>&lt;0.0001</td>
</tr>
</tbody>
</table>

*Baseline versus after two years. All values are given as median with interquartile range between brackets, only total cholesterol, LDL- and HDL-cholesterol levels are given as mean levels ± standard deviation. FH, familial hypercholesterolemia; LDL, low-density lipoprotein; HDL, high-density lipoprotein; ASAT, aspartate-amino transferase; ALAT, alanine-amino transferase