Genetics and therapy of familial hypercholesterolemia

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

Submitted for publication

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Chapter 3

ABSTRACT

BACKGROUND
Low concentrations of bilirubin are associated with an increased risk for coronary artery disease both in retrospective and prospective studies. Possibly, bilirubin exerts its effect through the protection of LDL from oxidation. We examined whether low bilirubin might also be a risk marker for cardiovascular disease (CVD) in patients with familial hypercholesterolemia (FH).

METHODS AND RESULTS
A total of 507 FH patients were included. After a washout period of 6 weeks, all patients started monotherapy with simvastatin 80 mg for two years. Median baseline bilirubin levels were significantly lower in male patients with CVD compared to those without (9.8 μmol/L versus 11.6 μmol/L; p=0.001). In particular, bilirubin was inversely related to the presence of CVD, both in univariate and multivariate analyses after adjustment for age, gender, presence of hypertension and high-density lipoprotein cholesterol levels. Moreover, treatment with simvastatin 80 mg increased bilirubin levels by 7% from 10.0 to 10.8 μmol/L independent from changes in liver enzymes.

CONCLUSIONS
We hypothesise that high bilirubin levels might protect FH patients from CVD. Furthermore, treatment with simvastatin 80 mg significantly increases bilirubin levels, 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.
INTRODUCTION

Low concentrations of serum bilirubin have been shown to be independently associated with an increased risk for coronary artery disease (CAD). This association has been observed both retrospectively in patients with CAD\textsuperscript{14} and prospectively in 7685 middle-aged British men\textsuperscript{5} and in the Framingham Offspring Study.\textsuperscript{6} The explanation for this association is not fully understood. Since long, bilirubin was regarded as cytotoxic, in particular because of its role in neonatal jaundice. It is only since the early 1990s that a more physiological role for bilirubin as a potent antioxidant has emerged. In vitro evidence suggests that low-density lipoprotein (LDL) can be protected from oxidation by bilirubin.\textsuperscript{7,8} Therefore, low bilirubin concentrations could be a reflection of a heightened oxidative state and the resulting increased consumption of bilirubin. Apart from the in vitro studies suggesting an antioxidant role of bilirubin, other studies have reported that smoking may lower bilirubin levels\textsuperscript{9,10} and that high bilirubin levels might be associated with protection against cancer.\textsuperscript{11}

Taken together, these results point to potential beneficial effects of the antioxidant properties of bilirubin.

Familial hypercholesterolemia (FH) is an autosomal dominant disorder of lipoprotein metabolism and affects approximately 1 in 400 people in the Netherlands.\textsuperscript{12} Mutations in the LDL-receptor gene, located on the short arm of chromosome 19, cause a reduction in the clearance of LDL cholesterol (LDL-C) in these patients, which consequently leads to a rise in LDL-C levels and predisposes to the development of atherosclerosis.\textsuperscript{13} Typically, approximately 45\% of male and 20\% of female patients have documented CAD by the age of 50.\textsuperscript{14} Since long, we are involved in the elucidation of novel risk markers in these FH patients.\textsuperscript{15,17}

To the best of our knowledge, it has not been examined before whether low bilirubin might also be a risk marker for cardiovascular disease (CVD) in FH patients. We therefore set out to study the role of bilirubin in these patients. Here we present the results of our studies.

METHODS

Study design and subjects

For a multicenter FH study (ExPRESS: Examination of Proband and Relatives in Statin Studies with Familial Hypercholesterolemia) FH patients 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 6 or more points, according to an algorithm (to allow standardisation of the diagnosis of FH based on clinical findings, personal and familial clinical history and biochemical parameters); one of the following criteria had to be met: patients required a change in therapy because LDL-C was greater than goals published in nationally recognised guidelines while on simvastatin 40 mg treatment or an equipotent dose of another statin or baseline LDL-C levels required a reduction of at least 40% to achieve the goal or patients were already on treatment with simvastatin 80 mg; 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 centres approved the protocol and written informed consent was obtained from all participants. The investigation conformed to the principles outlined in the Declaration of Helsinki. After a six-week washout period, patients started monotherapy with 80 mg simvastatin. No other lipid lowering medication was allowed. Medical history, physical examination and additional risk factors for cardiovascular disease 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 intervention; 6. A clinically significant stenosis on the coronary angiogram; 7. An unequivocally positive exercise ECG.

**Biochemical analysis**

Blood samples were taken in the morning after an overnight fast. Total plasma cholesterol (TC), HDL-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. Apolipoprotein A-I (apoA-I) and apolipoprotein B (apoB) were determined by an immunological rate-nephelometric procedure using a polyclonal goat anti-human antibody (Array protein system, Beckman Coulter, Netherlands). Lipoprotein (a) (Lp(a)) samples were measured by an immunological rate-nephelometric procedure using a polyclonal rabbit antibody directed against Lp(a) (Array protein system, Beckman Coulter, Netherlands). Total serum bilirubin was routinely measured in the different laboratories by spectrophotometry. All results were harmonised to one level according to the standardised Jendrassik-Groff method by the virtual central laboratory. In order to determine plasma total homocysteine (tHcy), samples were pre-treated with tri-n-butylphosphine to release homocysteine from plasma proteins and tHcy was measured by a sensitive and selective HPLC method using pre-column derivatization with 7-fluoro-2-oxa-1,3-diazole-4-sulfonate.

**Statistical analysis**

Mean values in lipids and lipoproteins between subgroups were compared using the independent sample t-test. Other parameters (TG, Lp(a), tHcy 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). Baseline bilirubin levels, for men and women separately, were divided into three groups based on the 33.3 and 66.6 percentile and presence of CVD in the different tertiles was compared by the Chi-square test.

The relation between cardiovascular disease and baseline variables was explored using logistic regression analysis. Mean values in lipids and lipoproteins before and after treatment were compared using the paired sample t-test and the statistical significance
of the relative change (for those patients with levels at both baseline and two years of treatment) as compared to baseline, was tested using the one 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 10.1, Chicago, Illinois). A p-value of less than 0.05 was considered to be statistically significant.

RESULTS

Baseline characteristics
From the 527 FH patients total bilirubin levels were measured at baseline in 507 patients. In these 507 patients, age ranged from 18 to 80 years. Mean age was 47.3 years (standard deviation (SD) ± 13.2). Slightly more males (55.6%) than females (44.4%) were included. More than 36% of all patients had a history of CVD with a mean age of onset of 46.7 years (SD ± 9.8). Risk factors such as diabetes, hypertension and current smoking were present in respectively 2.0%, 15.4% and 26.2% of all patients. Mean body mass index (BMI) was 25.9 ± 3.5 kg/m² and 44% of all patients had xanthomas. Mean TC (10.49 ± 2.18 mmol/L) and LDL-C (8.36 ± 2.14 mmol/L) levels were, as can be expected in FH patients, severely elevated.

FH patients and cardiovascular disease
Baseline characteristics of patients with and without CVD are summarised in table 1. Mean age in patients with CVD was significantly higher and in the group of CVD patients significantly more patients had hypertension, diabetes or had a higher BMI compared to those without CVD. Strikingly, less current smokers were seen in the CVD group than in the group without CVD. Furthermore, mean HDL-C was significantly lower in patients with CVD (1.18 versus 1.25 mmol/L; p=0.02), median TG levels were significantly higher (2.00 versus 1.60 mmol/L; p<0.0001) and median tHcy levels (12.60 versus 11.80 µmol/L; p=0.03) were also higher.

FH patients and bilirubin levels
Median baseline serum bilirubin levels in all FH patients were 10.0 µmol/L (interquartile range: 7.8 - 12.8). In FH patients with CVD, median bilirubin levels were significantly
Table 1. Baseline characteristics of FH patients with and without CVD.

<table>
<thead>
<tr>
<th>Variable</th>
<th>FH with CVD (n = 184)</th>
<th>FH without CVD (n = 323)</th>
<th>p-value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Age (years)</td>
<td>55.5 ± 9.9</td>
<td>42.6 ± 12.5</td>
<td>&lt;0.0001</td>
</tr>
<tr>
<td>Male gender (n)</td>
<td>107 (58.2%)</td>
<td>175 (54.2%)</td>
<td>0.39</td>
</tr>
<tr>
<td>Current smoking (n)</td>
<td>33 (17.9%)</td>
<td>100 (31.0%)</td>
<td>0.001</td>
</tr>
<tr>
<td>Hypertension (n)</td>
<td>50 (27.2%)</td>
<td>28 (8.7%)</td>
<td>&lt;0.0001</td>
</tr>
<tr>
<td>Diabetes (n)</td>
<td>9 (4.9%)</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>10.71 ± 2.36</td>
<td>10.37 ± 2.06</td>
<td>0.09</td>
</tr>
<tr>
<td>LDL cholesterol (mmol/L)</td>
<td>8.50 ± 2.31</td>
<td>8.29 ± 2.03</td>
<td>0.29</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>Triglycerides (mmol/L)</td>
<td>2.00 (1.40-2.80)</td>
<td>1.60 (1.10-2.30)</td>
<td>&lt;0.0001</td>
</tr>
<tr>
<td>Apolipoprotein A1 (g/L)</td>
<td>1.20 ± 0.19</td>
<td>1.22 ± 0.22</td>
<td>0.24</td>
</tr>
<tr>
<td>Apolipoprotein B (g/L)</td>
<td>2.02 ± 0.48</td>
<td>1.94 ± 0.42</td>
<td>0.08</td>
</tr>
<tr>
<td>Lp(a) (mg/L)</td>
<td>150 (54-537)</td>
<td>129 (41-348)</td>
<td>0.10</td>
</tr>
<tr>
<td>Homocysteine (μmol/L)</td>
<td>12.60 (10.40-15.20)</td>
<td>11.80 (10.20-13.83)</td>
<td>0.03</td>
</tr>
<tr>
<td>Total bilirubin (μmol/L)</td>
<td>9.70 (7.30-11.70)</td>
<td>10.50 (7.80-13.60)</td>
<td>0.005</td>
</tr>
</tbody>
</table>

Except where given as percentages, all values are given as mean levels ± standard deviation only triglycerides, Lp(a), homocysteine and bilirubin levels are given as median with the interquartile range between brackets. FH, familial hypercholesterolemia; CVD, cardiovascular disease; LDL, low-density lipoprotein; HDL, high-density lipoprotein; Lp(a), lipoprotein (a).

Lower compared to patients without CVD (9.7 versus 10.5 μmol/L; p=0.005). Total bilirubin levels above 17 μmol/L are considered to be elevated. Significantly fewer patients with bilirubin levels above 17.0 μmol/L were present in those with CVD compared to patients without CVD (3.8% versus 9.9%; p=0.01). Moreover, median bilirubin levels were higher in male compared to female patients (10.5 versus 9.5 μmol/L; p<0.0001). Male FH patients with CVD had statistically significant lower bilirubin levels compared to those without CVD (9.8 versus 11.6 μmol/L; p=0.001) whereas this was not significant in female patients (9.5 versus 9.6 μmol/L; p=0.35). Subsequently, baseline bilirubin levels were divided into three groups (tertiles). Figure 1A illustrates that male patients with CVD were significantly less often found in the highest tertile compared to the lower 2 tertiles (p=0.004), but this was not significant for women (p=0.60) (figure 1B).
Independent role of bilirubin and CVD

All risk markers, including bilirubin, were first evaluated in a univariate logistic regression model with cardiovascular disease as the outcome variable. Odds ratios (OR) and 95% confidence intervals (CI) for significant variables are presented in table 2. High levels of bilirubin were negatively associated with CVD as a continuous

Table 2. Odds ratios and 95% confidence intervals for CVD risk markers in FH patients.

<table>
<thead>
<tr>
<th>Variable</th>
<th>Univariate</th>
<th></th>
<th></th>
<th></th>
<th>Multivariate</th>
<th></th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>OR</td>
<td>95% CI</td>
<td>p-value</td>
<td>OR</td>
<td>95% CI</td>
<td>p-value</td>
<td></td>
</tr>
<tr>
<td>Age</td>
<td>1.10</td>
<td>1.08 - 1.12</td>
<td>&lt;0.0001</td>
<td>1.11</td>
<td>1.09 - 1.14</td>
<td>&lt;0.0001</td>
<td></td>
</tr>
<tr>
<td>Male gender</td>
<td>1.18</td>
<td>0.82 - 1.69</td>
<td>0.39</td>
<td>1.86</td>
<td>1.14 - 3.03</td>
<td>0.013</td>
<td></td>
</tr>
<tr>
<td>Hypertension</td>
<td>3.93</td>
<td>2.37 - 6.52</td>
<td>&lt;0.0001</td>
<td>2.08</td>
<td>1.18 - 3.69</td>
<td>0.012</td>
<td></td>
</tr>
<tr>
<td>Diabetes</td>
<td>16.48</td>
<td>2.08 - 130.7</td>
<td>0.008</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td></td>
</tr>
<tr>
<td>Body mass index (kg/m²)</td>
<td>1.11</td>
<td>1.05 - 1.17</td>
<td>&lt;0.0001</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td></td>
</tr>
<tr>
<td>HDL cholesterol (mmol/L)</td>
<td>0.54</td>
<td>0.31 - 0.93</td>
<td>0.03</td>
<td>0.36</td>
<td>0.18 - 0.72</td>
<td>0.004</td>
<td></td>
</tr>
<tr>
<td>Triglycerides (mmol/L)</td>
<td>1.19</td>
<td>1.04 - 1.36</td>
<td>0.01</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td></td>
</tr>
<tr>
<td>Total bilirubin (μmol/L)</td>
<td>0.94</td>
<td>0.90 - 0.98</td>
<td>0.004</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td></td>
</tr>
<tr>
<td>Bilirubin &gt;17 μmol/L</td>
<td>0.36</td>
<td>0.16 - 0.83</td>
<td>0.02</td>
<td>0.26</td>
<td>0.10 - 0.72</td>
<td>0.009</td>
<td></td>
</tr>
</tbody>
</table>

CVD, cardiovascular disease; FH, familial hypercholesterolemia; OR, odds ratio; CI, confidence interval; HDL, high-density lipoprotein
Bilirubin, a new risk marker for CVD?

variable (OR 0.94; 95% CI 0.90-0.98; p=0.004) and similarly as a dichotomous variable with a cut-off at 17 µmol/L (OR 0.36; 95% CI 0.16-0.83; p=0.02). In the multivariate analysis, age (OR 1.11; 95% CI 1.09-1.14; p<0.001), presence of hypertension (OR 2.08; 95% CI 1.17-3.69; p=0.01), male gender (OR 1.86; 95% CI 1.14-3.03; p=0.01), low HDL-C (OR 0.36; 95% CI 0.18-0.72; p=0.004) and bilirubin >17 µmol/L (OR 0.26; 95% CI 0.10-0.72; p=0.009) were identified as significant risk markers for CVD. Bilirubin as continuous variable in the same model did not change the model except for bilirubin (OR 0.92; CI 0.87-0.97; p=0.003). Diabetes, TG levels and BMI were not significantly related in these multivariate analyses.

Treatment with simvastatin 80 mg and bilirubin

In table 3, treatment effects of simvastatin 80 mg on lipids, lipoproteins, liver enzymes and bilirubin are given. Mean TC, LDL-C and median TG levels were reduced by -39.2%, -48.0% and -26.3%, respectively. Mean HDL-C levels were elevated by 12.7%. Bilirubin levels were significantly raised by simvastatin with 7.0% from 10.0 to 10.8 µmol/L and especially in the patients with CVD bilirubin levels were increased more compared to those without CVD (1.39 µmol/L versus 0.41 µmol/L; p=0.02). Notably, no correlation was observed between change in bilirubin and change in ASAT (r=0.05; p=0.34) or in ALAT (r=-0.03; p=0.61).

Table 3. Treatment effects of simvastatin 80 mg on lipid, lipoprotein and bilirubin levels in FH patients.

<table>
<thead>
<tr>
<th>Variable</th>
<th>Baseline (n = 507)</th>
<th>Year 2 (n = 430)</th>
<th>% change</th>
<th>p-value*</th>
</tr>
</thead>
<tbody>
<tr>
<td>Total cholesterol (mmol/L)</td>
<td>10.49 ± 2.18</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>8.36 ± 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>
<tr>
<td>ALAT (U/L)</td>
<td>23 (17 to 30)</td>
<td>29 (20 to 40)</td>
<td>+18 (-4 to +54)</td>
<td>&lt;0.0001</td>
</tr>
</tbody>
</table>

*P-values are the same for absolute and % change. All values are given as mean levels ± standard deviation only triglycerides and bilirubin levels are given as median with the interquartile range between brackets. FH, familial hypercholesterolemia; LDL, low-density lipoprotein; HDL, high-density lipoprotein; ASAT, aspartate-amino transferase; ALAT, alanine-amino transferase.
DISCUSSION

Baseline bilirubin and CVD

Our results indicate that bilirubin levels at the high end of the normal range are associated with a significantly lower prevalence of CVD.\textsuperscript{3,6} In addition, and to the best of our knowledge, this study is the first to show that high bilirubin levels in FH patients are associated with protection against CVD. In particular, bilirubin was inversely related to the presence of CVD, both in univariate and multivariate analyses, even after adjustment for age, gender, presence of hypertension and HDL-C levels. As for the strength of this relationship, Hopkins et al found that bilirubin levels were comparable to HDL-C in terms of CVD protection.\textsuperscript{1} Similarly in our study, bilirubin levels above 17.0 µmol/L were associated with comparable CVD protection as a 1 mmol/L increase in HDL-C levels in FH patients. Our results are also in line with the recently published results in Gilbert’s syndrome where bilirubin levels in the range of 20-70 µmol/L were associated with a low prevalence of CAD.\textsuperscript{24}

Also in line with previous results, bilirubin levels were higher in FH males when compared to females.\textsuperscript{1,4,25} Indeed, the association between bilirubin levels and CVD was present in male but not in female FH patients. Interestingly, some studies that have investigated bilirubin and CVD have included only men.\textsuperscript{2,3,5} Studies that included both men and women found conflicting results.\textsuperscript{1,6,25} The fact that bilirubin levels were higher in males compared to females while males in general have higher risk for CVD than females may be the consequence of complicated interactions in atherogenesis, as for example, women may be less susceptible to low levels of bilirubin than men, as a consequence of their hormonal status.

The association between CVD and bilirubin could possibly be confounded by patients with concomitant elevated liver enzymes.\textsuperscript{3} 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 2 years of therapy. However, results were similar to the results obtained in the whole study cohort (data not shown).

Effect of simvastatin on bilirubin

No other reports are available with regards to the effects of statins on bilirubin levels. We observed that simvastatin 80 mg increased bilirubin levels by 7% from
10.0 to 10.8 µmol/L. Although liver enzymes were also slightly increased, changes in ASAT or ALAT levels were not correlated with change in bilirubin. This effect of simvastatin might be the result of LDL-C lowering and subsequent lower consumption of bilirubin as antioxidant. An increase of 0.8 µmol/L of bilirubin levels is modest but in multivariate analysis an increase of 1 mmol/L has an OR of 0.92 for CVD (95% CI 0.87-0.97; p=0.003). 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 affect of LDL-C lowering. Nevertheless, more studies will have to confirm our results and delineate the role of bilirubin in atherogenesis and CVD.

In summary, we hypothesise that high bilirubin levels might protect male FH patients from CVD. Furthermore, treatment with simvastatin 80 mg significantly increases bilirubin levels, especially in patients with CVD. Whether this is also true for lower doses of simvastatin or for other statins remains to be investigated.

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REFERENCES