Increased Plasma 7β-Hydroxycholesterol Concentrations in a Population With a High Risk for Cardiovascular Disease

Bo Ziedén, Arvydas Kaminskas, Margareta Kristenson, Zita Kucinskiene, Bengt Vessby, Anders G. Olsson, Ulf Diczfalusy

Abstract—The mortality in coronary heart disease among 50- to 54-year-old men is 4 times higher in Lithuania than in Sweden. It was recently suggested that traditional risk factors could not explain this mortality difference. LDL of Lithuanian men showed, however, a lower resistance to oxidation than that of Swedish men. In addition, the plasma concentration of γ-tocopherol, lycopene, and β-carotene were lower in Lithuanian men. In the present investigation, we determined plasma oxysterols in men from Lithuania and Sweden and found that the plasma concentration of 7β-hydroxycholesterol was higher in Lithuanian men, 12±5 versus 9±8 (SD) ng/mL (P=0.0011). This oxysterol is a cholesterol autoxidation product and there is no indication that it should have an enzymatic origin. Mean LDL oxidation lag time was shorter in Lithuanian men (75±14 versus 90±13 minutes, P<0.0001) and the concentration of LDL linoleic acid was lower (249±56 versus 292±54 μg/mg of LDL protein, P<0.0001). Lipid corrected γ-tocopherol was 0.07±0.02 mg/mL in Vilnius men and 0.12±0.04 mg/mL (P<0.0001) in Linköping men. There was a negative correlation between the concentration of 7β-hydroxycholesterol and lag time (R=−0.31, P=0.0023). It is suggested that the higher 7β-hydroxycholesterol concentration in Lithuanian men is an indication of an increased in vivo lipid peroxidation. (Arterioscler Thromb Vasc Biol. 1999;19:967-971.)

Key Words: oxysterols ■ LDL oxidation ■ vitamins ■ fatty acids ■ cross-sectional study

Coronary heart disease (CHD) incidence and mortality is rising in the East European countries, in contrast to the decreasing trend in Western Europe and in the United States.1–3 For example, the mortality in CHD among 50- to 54-year-old men was 4 times higher in Lithuania than in Sweden in 1994. In LiVicordia I, a cross-sectional population study, we investigated the prevalence of ultrasound-detected subclinical atherosclerosis and possible causes for these diverging coronary mortality trends in the 2 countries.4 Lithuanian men had more carotid atherosclerosis. Although traditional risk factors could not explain the difference in CHD mortality, we found that Lithuanian men had a higher LDL susceptibility to oxidation and lower concentrations of the plasma antioxidant vitamins γ-tocopherol, β-carotene, and lycopene than Swedish men. There is a high correlation between the plasma concentrations of these vitamins and the corresponding vitamin content in LDL.5 The increased susceptibility to oxidation and lower concentrations of LDL-associated vitamins in the Lithuanian population may be caused by dietary factors or an increased in vivo oxidation of LDL. We have recently shown that cholesterol oxidation products (oxysterols) are good markers of LDL oxidation in vitro.6 In addition, the oxysterol 7β-hydroxycholesterol, together with an increased oxidation susceptibility of VLDL+LDL, were the strongest predictors of progression of carotid atherosclerosis in Finnish men.7 We therefore decided to determine oxysterols in plasma from the 2 populations of 50-year-old men from Lithuania and Sweden.

Methods

Subjects

LiVicordia II is a cross-sectional study conducted in Vilnius and Linköping from December 1994 to May 1995. A list of 200 randomly selected men, born between July 1, 1943, and June 30, 1944, was obtained from the census register in each city. Of these, 120 men were invited to LiVicordia I and the remainder were invited to LiVicordia II.

Exclusion criteria for LiVicordia II were the same as for LiVicordia I, ie, serious acute or chronic diseases that could influence the results of the investigation or make participation impossible.4

Sampling

The volunteers came to the hospital between 7:30 and 9:30 AM after an overnight fast. Smoking was not allowed during the morning before blood sampling. The prescribed dose of morning drugs was taken. Body weight, sagittal diameter of the abdomen, height, and blood pressure were measured and blood was collected into pre-

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cooled blood collection tubes (Vacutainer, 7 mL) containing 0.12 mL of 0.34 mol/L EDTA. Plasma was prepared by centrifugation (1700g) at 4°C and stored in the dark or frozen at −20°C until transport and analysis. Fresh plasma samples for lipid analysis, LDL oxidation (lag time), and LDL fatty acid analyses were sent from Vilnius to Linköping as express packages cooled to 4°C. Samples from both Linköping and Vilnius were kept in the dark and handled in the same way with regard to temperature and time to analysis. Samples for analyses of tocopherols, oxysterols, and malondialdehyde (MDA) were frozen at −70°C and analysis was performed within 5 months. The storage time for plasma, until analysis of lag time, never exceeded 8 days.

Fatty acid analysis was performed in 1 laboratory consecutively and in random order. The time from blood sampling to analysis was the same for samples from both cities.

**Plasma Lipoproteins**

All plasma lipoproteins were analyzed in Linköping. Plasma concentrations of cholesterol and triglycerides were analyzed by enzymatic colorimetric methods (Monotest cholesterol CHOD-PAP and Triglycerides GPO-PAP, Boehringer Mannheim GmbH).

ApoA1 and B were determined with a commercial immunochromatographic kit (Turbiquant, Behring). ApoB-containing lipoproteins were precipitated with phosphotungstic acid and magnesium ions and the cholesterol in the remaining supernatant was defined as HDL cholesterol. LDL cholesterol was calculated according to Friedewald et al. 9

**LDL Oxidation**

LDL was isolated and tested for oxidation susceptibility as described by Kleinveld et al. 10 In brief, saline was layered on top of plasma in a centrifuge tube and ultracentrifuged at 120 000g for 4 hours at 4°C (Koma KTSF 45.6 fixed-angle rotor and Centriflux T-2070 ultracentrifuge). The LDL-containing fraction was collected and ultracentrifuged for another 18 hours, and LDL protein was determined the same day according to the method of Lowry et al 11 with BSA as the standard. The following day, 1 mL of the LDL layer was dialysed in the dark for 20 hours against 4 L of PBS containing 10 μmol/L EDTA and 0.1 mg/mL chloramphenicol, and filtered through a 0.45-μm filter.

The LDL was diluted with EDTA-free PBS to a final concentration of 25 μg/mL protein and 1 μmol/L of EDTA and oxidation was initiated with CuSO4 (final concentration, 5 μmol/L). LDL oxidation kinetics were monitored spectrophotometrically by the change in absorbance at 234 nm at 30°C. The absorption was recorded every 2 minutes for 4 hours. Oxidation rate and diene production were calculated by using a molar extinction coefficient for conjugated dienes of 29 500 L·mol⁻¹·cm⁻¹, and the oxidation indices were expressed as nmol·min⁻¹·mg⁻¹ of LDL protein and nmol/mg of LDL protein, respectively. The interassay variation was ±5.1% for all oxidation indices.

**Fatty Acids in LDL**

LDL was isolated as described above. Lipids from LDL were extracted according to Folch et al. 12 These samples were transesterified with HCl/methanol for 2 hours at 100°C. For quantification of fatty acids in LDL, heptadecanoic acid was used as internal standard. Fatty acid methyl esters were chromatographed on a 50 m×0.25-mm CP-SIL 88 glass capillary column (Chrompack). Analysis was performed on a Hewlett-Packard 5890 Series II Plus GC equipped with a flame ionization detector. Helium was used as the carrier gas at a constant flow rate of 1.18 mL/min with electronic pressure control. The injector temperature was 250°C and the detector temperature was 300°C. The oven temperature program was as follows: 140°C to 190°C (18°C/min); 190°C for 5 minutes; 190°C to 205°C (1°C/min); 205°C for 10 minutes; 205°C to 210°C (18°C/min); and 210°C for 10 minutes. The volume of injected sample was 1 μL, split ratio was 1:50. The fatty acids were identified by using standard mixtures of known fatty acids (Sigma Chemicals).

**Serum Tocopherols and MDA**

Three tocopherols, α-, β-, and γ-tocopherol, were analyzed, according to Öhrvall et al. 13 In brief, 500 μL of ethanol with 0.005% butylated hydroxytoluene (BHT) was added to 500 μL of serum. After adding 2 mL of hexane, the 2 phases were separated and the supernatant was analyzed by HPLC, using a Merck Hitachi pump and a LiChrospher 100 NH2, 250×4 mm (Kebo) column. The fluorescence detector had an excitation wavelength of 295 nm and an emission wavelength of 327 nm. The injected sample was 20 μL. The serum tocopherol concentration was divided by the sum of plasma cholesterol and triglyceride concentration. 14

MDA was determined as described by Öhrvall et al. 13 In summary, 750 μL of 0.15 mol/L phosphoric acid, 300 μL of water and 250 μL of thioarbituric acid (TBA) were added to 200 μL of plasma. After boiling for 60 minutes, the mixture was cooled on ice. Methanol was added to the MDA–TBA mixture and 20 μL of the mixture was measured on an HPLC system by using a fluorescence detector with an excitation wavelength of 532 nm and an emission wavelength of 553 nm. Calibration was made against standard solutions of 1,1,3,3-tetraethoxyxopropane (Sigma).

**Plasma Oxysterols**

Plasma oxysterols were determined by isotope dilution mass spectrometry as described earlier. 16 In brief, deuterium-labeled internal standards were added to 1 mL of plasma and the sample was subjected to mild alkaline hydrolysis under an argon atmosphere (0.35 mol/L KOH, 22°C, 2 hours). The reaction mixture was taken to neutral pH and extracted with chloroform/ethanol. The extract was applied to solid-phase extraction, to separate oxysterols from cholesterol. The oxysterol fraction was treated with pyridine/hexamethyldisiloxane/trimethylchlorosilane (3:2:1, by volume) to convert alcohol groups to trimethylsilyl ethers and was finally analyzed by gas chromatography mass spectrometry. The oxysterols determined were 7α- and 7β-hydroxycholesterol, 7-oxocholesterol, cholesterol-5α, 6α-epoxide, cholesterol-5β,6β-epoxide, and 24-, 25-, and 27-hydroxycholesterol. The coefficient of variation for 7α-hydroxycholesterol, 7β-hydroxycholesterol, 7-oxocholesterol, 24-hydroxycholesterol, and 27-hydroxycholesterol was between 2% and 8%.

**Statistical Methods**

Results are presented as mean±standard deviation (SD). Mann-Whitney U test was used for testing differences between groups. Correlation coefficients were calculated with the Spearman rank correlation test. Multiple linear regression analysis was calculated with the LDL lag time as the dependent variable. All statistics were calculated using StatView 4.5 for Macintosh.

Values of P<0.05 were considered to be statistically significant.

**Results**

The participation rate in Linköping was 79%; 63 were invited, 11 refused, and 2 did not answer. The corresponding figures for Vilnius were 64% participation rate, 78 invited, 8 refused, and 20 did not answer. The numbers of participants (Vilnius/Linköping) with myocardial infarction or stroke were 7/2 and 2/0, respectively. Four men from Vilnius were receiving medical treatment for hypertension compared with 6 men from Linköping. Of the men from Vilnius, 16 were current smokers versus 17 from Linköping.

Men from Vilnius had a higher body mass index (BMI) than men from Linköping but abdominal diameter did not differ. There was no difference in systolic or diastolic blood pressure. Plasma LDL cholesterol, HDL cholesterol, and triglycerides were similar in the 2 groups and apoA1 and apoB did not differ (Table 1).

Lipid-corrected γ-tocopherol in plasma was found in significantly lower concentrations among men from Vilnius. The corresponding α-tocopherol concentration did not differ al-
though men from Vilnius showed a tendency to lower concentrations of this vitamin. The concentration of $\beta$-tocopherol in plasma was lower in the Linköping group (Table 2).

Mean lag time after in vitro oxidation of LDL in the Vilnius group was 75±14 minutes compared with 90±13 minutes in the Linköping group ($P<0.0001$). There were no differences in the rate of LDL oxidation or in diene production. Serum MDA did not differ between men from the 2 cities (Table 2).

Men from Vilnius showed lower concentrations of palmitic (16:0), palmitoleic (16:1), and linoleic (18:2n6) acids in LDL than men from Linköping (Table 3). The sum of all fatty acids in LDL was also lower in the Vilnius group.

Plasma 7$\beta$-hydroxycholesterol was found in higher concentrations among men from Vilnius than among men from Linköping (12±5 and 9±8 mg/mL, respectively, $P=0.0011$), whereas other oxysterols did not differ significantly (Table 4).

A multiple regression analysis with 7$\beta$-hydroxycholesterol as the dependent variable, with the country as the first variable entered, showed that LDL oxidation rate ($r=-0.28$, $P=0.0084$) and total plasma cholesterol ($r=0.83$, $P=0.007$) contributed significantly to the concentration of 7$\beta$-hydroxycholesterol, but the $R^2$ value was only 0.15.

Spearman correlation analysis for the 2 groups pooled showed strong negative correlations between lag time and MDA ($r=-0.36$, $P=0.0008$), lag time and 7$\beta$-hydroxycholesterol ($r=-0.31$, $P=0.0023$), and lag time and 7$\alpha$-hydroxycholesterol ($r=-0.23$, $P=0.022$). Lag time was also positively correlated to lipid-corrected $\alpha$-tocopherol ($r=0.34$, $P=0.0006$) and $\gamma$-tocopherol ($r=0.32$, $P=0.0014$) and linoleic acid ($r=0.26$, $P=0.0091$); but when the groups were analyzed separately, only linoleic acid and $\alpha$-tocopherol were significantly correlated to lag time in the Vilnius group.

### TABLE 1. Clinical Characteristics (Mean±SD) Among Men From Vilnius and Linköping

<table>
<thead>
<tr>
<th></th>
<th>Vilnius (n=50)</th>
<th>Linköping (n=50)</th>
<th>$P$</th>
</tr>
</thead>
<tbody>
<tr>
<td>BMI (kg/m²)</td>
<td>27.0±2.9</td>
<td>25.0±2.8</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>Sagittal diameter (cm)</td>
<td>21.8±2.5</td>
<td>21.0±2.6</td>
<td>0.09</td>
</tr>
<tr>
<td>Systolic BP (mmHg)</td>
<td>137±17</td>
<td>134±17</td>
<td>0.12</td>
</tr>
<tr>
<td>Diastolic BP (mmHg)</td>
<td>86±11</td>
<td>85±12</td>
<td>0.28</td>
</tr>
<tr>
<td>Total chl (mmol/L)</td>
<td>5.44±1.05</td>
<td>5.48±0.97</td>
<td>0.63</td>
</tr>
<tr>
<td>LDL chl (mmol/L)</td>
<td>3.64±0.95</td>
<td>3.70±0.87</td>
<td>0.59</td>
</tr>
<tr>
<td>HDL chl (mmol/L)</td>
<td>1.18±0.26</td>
<td>1.14±0.34</td>
<td>0.26</td>
</tr>
<tr>
<td>Total TG (mmol/L)</td>
<td>1.55±1.6</td>
<td>1.45±0.89</td>
<td>0.28</td>
</tr>
<tr>
<td>LDL/HDL ratio</td>
<td>3.17±0.98</td>
<td>3.52±1.27</td>
<td>0.17</td>
</tr>
<tr>
<td>ApoA1 (g/L)</td>
<td>1.21±0.18</td>
<td>1.18±0.23</td>
<td>0.23</td>
</tr>
<tr>
<td>ApoB (g/L)</td>
<td>1.22±0.29</td>
<td>1.25±0.27</td>
<td>0.56</td>
</tr>
</tbody>
</table>

BP indicates blood pressure; chl, cholesterol; TG, triglycerides; and Apo, apolipoprotein.

### TABLE 2. Lipid-Corrected Vitamins and MDA- and LDL-Oxidation Indices (Mean±SD) Among Men From Linköping and Vilnius

<table>
<thead>
<tr>
<th></th>
<th>Vilnius (n=50)</th>
<th>Linköping (n=50)</th>
<th>$P$</th>
</tr>
</thead>
<tbody>
<tr>
<td>$\alpha$-tocopherol* (µg/mL)</td>
<td>1.49±0.30</td>
<td>1.54±0.19</td>
<td>0.11</td>
</tr>
<tr>
<td>$\beta$-tocopherol* (µg/mL)</td>
<td>0.032±0.01</td>
<td>0.027±0.01</td>
<td>0.0024</td>
</tr>
<tr>
<td>$\gamma$-tocopherol* (µg/mL)</td>
<td>0.07±0.02</td>
<td>0.12±0.04</td>
<td>&lt;0.0001</td>
</tr>
<tr>
<td>MDA in serum (µmol/L)</td>
<td>0.68±0.16</td>
<td>0.68±0.13</td>
<td>0.87</td>
</tr>
<tr>
<td>Lag time (minutes)</td>
<td>75.4±13.6</td>
<td>89.5±13.1</td>
<td>&lt;0.0001</td>
</tr>
<tr>
<td>Rate (mmol · min⁻¹ · mg⁻¹)</td>
<td>4.91±0.79</td>
<td>5.16±0.77</td>
<td>0.08</td>
</tr>
<tr>
<td>Diene production (nmol/mg)</td>
<td>379±56.9</td>
<td>374±50.8</td>
<td>0.8</td>
</tr>
</tbody>
</table>

*Vitamin concentration divided by the sum of plasma cholesterol concentration and triglyceride concentration.

### Discussion

In a previous study (LiVicordia I), 2 populations of 50-year-old men from Vilnius (Lithuania) and Linköping (Sweden) were compared with respect to serum lipids, serum antioxidants, and other factors that could be expected to explain the marked difference in CHD risk between the groups.4 There were, however, no differences in risk factors such as smoking, HDL cholesterol, triglycerides, Lp(a), apoA1, or apoB100. However, total and LDL cholesterol were lower in Vilnius men. It was found that LDL from Lithuanian men was significantly more susceptible to oxidation than the LDL from Swedish men.

In the present study, LiVicordia II, it is shown that this difference is accompanied by a slightly higher mean concentration of the oxysterol 7$\beta$-hydroxycholesterol in plasma from Lithuanian men than from Swedish men. This oxysterol has been shown to be a good marker of lipid peroxidation in vitro6 and a potential predictor of progression of carotid atherosclerosis in vivo.7 The higher concentration of 7$\beta$-hydroxycholesterol in Lithuanians is interpreted as an indication of an increased in vivo lipid peroxidation. This oxysterol is a cholesterol autoxidation product and there is no indication that it should have an enzymatic origin.17 If this oxysterol were of dietary origin, it would be expected that other cholesterol autoxidation products such as the cholesterol epoxides and 7-oxocholesterol would also differ between the 2 groups of men. It seems likely that plasma 7$\beta$-hydroxycholesterol is a more sensitive marker for in vivo lipid peroxidation than other markers such as TBA-reactive products (TBARS).7

Oxysterols are present in low concentrations in plasma and careful sample handling is important, to avoid artificial oxysterol formation. Especially the cholesterol epoxides are formed very easily. In the present study the plasma concentrations of the cholesterol epoxides were low and did not differ between the groups of men, indicating that samples had been handled properly previous to analysis.

The relative amounts of cholesterol autoxidation products in plasma from the volunteers in this study were in decreasing order, as follows: 7-oxocholesterol>cholesterol-5α,6α-epoxide>7$\beta$-hydroxycholesterol>25-hydroxycholesterol (Table 4). Cholesterol-5β,6β-epoxide was found in higher concentrations than 7-oxocholesterol, but this oxysterol is easily formed during sample handling and workup and was probably formed during the analytical procedure.18 Copper oxidation of isolated LDL gave the corresponding order, as follows: 7-oxocholesterol>7-hydroxycholesterol>cholesterol-5,6-epoxide>25-hydroxycholesterol, and the same order was found in copper-oxidized plasma.19 Although 7-oxocholesterol is the cholesterol autoxidation product formed in highest concentration after oxidation of LDL or plasma,
7β-hydroxycholesterol was the most sensitive marker of oxidation in the samples from the volunteers. The findings that LDL from Lithuanian men contains less linoleic acid, and LDL-associated antioxidants (γ-tocopherol, β-carotene, and lycopene) than LDL from Swedish men, may be due to different dietary habits of the 2 populations.

In the LiVicordia I study, it was shown that the 2 populations had similar plasma concentrations of the antioxidant α-tocopherol. This is interesting, as it has recently been reported that the plasma α-tocopherol concentration does not correlate to the LDL α-tocopherol concentration whereas plasma γ-tocopherol, β-carotene, and lycopene concentrations are strongly correlated with the corresponding concentrations in LDL.5 The present study confirmed that there was no difference in plasma α-tocopherol concentration between the study populations, but showed a significantly lower plasma concentration of γ-tocopherol in the Lithuanian population. In a recent report, smokers were reported to have lower plasma antioxidant concentrations than nonsmokers, and plasma γ-tocopherol levels were significantly lower than in nonsmokers.20 Furthermore, smoking cessation resulted in significant increases in total plasma vitamin C levels and in LDL α- and β-carotene content. Although no change was observed in plasma α-tocopherol after smoking cessation, there was a significant increase (34%) in plasma γ-tocopherol.20 In the present study we also observed lower plasma γ-tocopherol levels in smokers than in nonsmokers with the 2 groups pooled (0.10 ± 0.05 mg/mmol in nonsmokers versus 0.08 ± 0.06 mg/mmol in smokers, P = 0.04), but this was not significant when the 2 cities were analyzed separately.

Smoking does not seem to explain the difference in plasma γ-tocopherol concentration in the 2 populations studied, as the number of smokers was similar in the 2 populations (16 Vilnius, 17 Linköping).

It has been reported that vitamin E supplementation as well as alcohol intake decrease plasma γ-tocopherol.21 If anything, vitamin E supplementation seems to have a beneficial effect on CHD risk,22,23 and is unlikely to be the cause of the decreased γ-tocopherol levels in the Lithuanian population. The alcohol intake was similar in the 2 study populations.4 The finding that the Lithuanian population with a high risk for cardiovascular disease has a lower plasma γ-tocopherol level is also interesting in relation to a recent report that CHD patients have reduced serum levels of γ- but not α-tocopherol.24 Although γ-tocopherol is less efficient as an antioxidant compared with α-tocopherol, it has been shown to be superior in detoxifying nitrogen dioxide.25

Similar results were reported by Christen et al.,26 and they also found that γ-tocopherol was more efficient than α-tocopherol to inhibit peroxynitrite-induced lipid peroxidation in phosphatidylcholine liposomes but not in LDL.

One could speculate that the lower γ-tocopherol concentration in the Lithuanians could be caused by consumption in connection with scavenging of NOx either from environmental sources or formed endogenously, eg, during decomposition of peroxynitrite.

In conclusion, the higher plasma concentration of 7β-hydroxycholesterol in the Lithuanian group suggests the possibility of an increased in vivo lipid peroxidation. Whether the lower linoleic acid and γ-tocopherol contents in LDL from Lithuanians is a consequence of dietary habits or of an increased lipid peroxidation cannot be answered by the present study.

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References

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