Plasma oxysterols and angiographically determined coronary atherosclerosis: a case-control study

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Several *in vitro* and *in vivo* experiments have implicated oxysterols in the aetiology and progression of atherosclerosis. Oxysterols may be formed endogenously by oxidation of cholesterol and thus may form a marker of LDL oxidation. They may also be obtained exogenously through dietary intake. We investigated the association of oxysterols with the degree of coronary stenosis in patients undergoing coronary angiography. Cases with severe coronary atherosclerosis (>80% stenosis in one of the major coronary vessels, *n*=80) were compared with controls with no or minor stenosis (<50% stenosis in all three major coronary vessels, *n*=79). Cases and controls were prestratified on age, gender and smoking habits. Evaluated were plasma levels of unesterified 7α-hydroxycholesterol, 7β-hydroxycholesterol, 25-hydroxycholesterol, 7-ketocholesterol, cholestane-triol and 5,6α-epoxycholestanol. 7α-Hydroxycholesterol made up 67% of the total amount of plasma oxysterol concentration and was the only one significantly higher in cases (1.53 μg per 100 ml vs 1.27 μg per 100 ml, $p < 0.05$). Further, cases had somewhat higher LDL cholesterol levels and significantly lower HDL cholesterol levels than controls. After multivariate adjustment to account for this difference in lipid levels and for the prestratification factors the mean difference between cases and controls for 7α-hydroxycholesterol (0.14 μg per 100 ml) was no longer significant. Also the other oxysterols showed no significant association with the degree of coronary stenosis. Multiple logistic regression analyses showed an adjusted odds ratio of 1.07 (95% CI, 0.45–2.59) in the highest tertile of total plasma oxysterol level. We conclude, that this study does not support the hypothesis that plasma oxysterols form an additional risk factor for coronary atherosclerosis.

Keywords: *oxysterols, hydroxycholesterol, coronary stenosis, angiography.*

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Introduction

Two lines of evidence have implicated oxidation of cholesterol in the pathogenesis of atherosclerosis. Firstly, Peng *et al.* (1978, 1979) hypothesized that dietary intake of oxidation products of cholesterol may play an important role in the pathogenesis of atherosclerosis, since oxysterols have been proven to be cytotoxic and atherogenic in several *in vitro* and *in vivo* models (Imai *et al.* 1976, Kandutsch *et al.* 1978, Peng and Taylor 1984, Peng *et al.* 1985, Sevanian and Peterson 1986, Hubbard *et al.* 1989, Morin and Peng 1989, Smith and Johnson 1989). Cholesterol in food products can rapidly oxidize during processing and storage. Indeed, high dietary intake of oxysterols through ghee, clarified butter oil, has been suggested to explain the high rate of cardiovascular disease (CVD) in Indian immigrants in the United Kingdom, despite low levels of other risk factors (Jacobson 1987). Plasma levels of oxysterols may thus be associated with CVD since they may reflect dietary intake of exogenous oxysterols. The second hypothesis linking cholesterol oxidation to atherosclerosis is through oxidation of LDL (Steinberg *et al.* 1989). Native LDL cholesterol becomes more atherogenic after it has been oxidized by free radicals. By oxidative modification the uptake of LDL by macrophages is accelerated, which is the beginning of the fatty streak. Oxysterols may be a marker of this endogenous oxidation of LDL cholesterol.

Several oxysterols have been identified in atherosclerotic lesions (Teng and Smith 1975), but there have been no epidemiological studies on the association between CVD and plasma oxysterols. Epidemiological studies may so far have been hampered by the rapid auto-oxidation of plasma cholesterol during sampling and analysis. In studies in myocardial infarction patients, the acute event may influence oxysterol levels and hamper interpretation. We now report a study in which we measured oxysterol concentrations in patients with either high or low degree of angiographically defined coronary atherosclerosis.

METHODS

Study population

The study was conducted in 1991 and 1992 in several hospitals in Rotterdam, The Netherlands. The study was approved by an ethical committee on human research and all participants gave their informed consent. In this period 1467 patients had coronary angiography for suspected CVD. Ineligible were subjects: over 68 years of age ($n = 352$); with a previous bypass surgery ($n = 110$); with a myocardial infarction in the 12 months prior to the study period ($n = 143$); under cardiac care for more than 2.5 years ($n = 295$);

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elapsed between angiography and case selection (n = 117); who had diabetes mellitus, liver, kidney or thyroid disease, or showed evidence of alcohol or drug abuse (n = 63). Of the remaining 387 patients, 51 refused to participate, 22 could not be contacted or were otherwise indisposed and 7 had died. Of the remaining 307 patients, cases were selected on the basis of having more than 80% stenosis in at least one of the three major coronary vessels and controls having less than 50% stenosis in all three major coronary vessels. The percentage stenosis were scored by the cardiologist performing the angiography. Cases and controls were prestratified on age, gender and smoking habits. For seven participants, plasma samples were stored for more than three months and not analysed for oxysterols. This left 159 subjects; 80 cases and 79 controls, for data analysis.

Data collection

Information on medical history was obtained from the medical status and through a questionnaire within 2 months after angiography. Also, information on the use of medication, dietary-, smoking- and drinking habits, occupation and occurrence of CVD in parents and siblings was obtained. In addition anthropometric data were gathered and blood pressure was measured. Fasted venous blood samples (10 ml) were collected into EDTA Vacutainer tubes to which the antioxidant glutathione (10 mg) had been added. The blood samples were immediately placed on ice and within 30 mins the plasma was isolated by centrifugation and 50 $\mu g\ ml^{-1}$ of the antioxidant butylated hydroxytoluene (BHT) was added to the plasma. All samples were kept at 4°C during processing and within 2 hours after venapunction, the isolated plasma was stored at –80°C.

Preparation and isolation of cholesterol and oxysterols

After a maximum 3 months storage period plasma (2 g) was extracted with 40 ml Folch mixture. After filtering, washing and addition of betulin as internal standard the extract was concentrated in 1 ml chloroform.

Preliminary fractionation of the lipid extract, necessary prior to HPLC, was conducted using successively two solid phase extraction columns. A silica column (Mega Bond Elut, 2 g packing, Analytichem International, Cambridge, UK) was conditioned with 6 ml chloroform. After the lipid extract was applied to the column, a chloroform (8 ml) wash step was performed. Elution of analytes was accomplished with 14 ml acetone. The acetone was evaporated and the residue was redissolved in 1 ml HPLC mobile phase (acetonitrile:isopropanol:water 51:45:4 v/v/v). A C18 column (500 mg, Analytichem International) was washed with 3 ml methanol and 3 ml HPLC solvent before applying the oxysterol fraction. Elution was performed with 7 ml HPLC mobile phase. Again this fraction was evaporated and redissolved in 0.265 ml mobile phase solvent. Removal of cholesterol was carried out by HPLC. A 0.2 ml sample fraction from column II was injected and the eluate (approximate 4 ml) was collected until cholesterol passed the UV-detector (202 nm). This fraction was evaporated and dissolved in 0.5 ml dry pyridine. A Sperisorb 5-ODS glass (i.d. 3 mm, length 200 mm) column (Chrompack, Bergen op Zoom, The Netherlands) operating at a flow of 0.4 ml min^{-1} was utilized. The preparation of trimethyl silyl esters has been described previously (Van de Boventamp *et al.* 1988). The volume of hexane addition was changed to 0.1 ml.

A 2 μ sample aliquot was splitless injected into a Hewlett Packard 5890 series II gas chromatograph (Avondale, PA19311 USA) with an HP 7673A autosampler. GC conditions were: splitless column fused silica, CP-Sil5/CB (Chrompack), 0.25 mm i.d. x 25 m length, film thickness 0.12 μm; oven programme: 2.5 min at 70°C, followed by a rise to 220°C at a rate of 40°C min^{-1} and subsequently at a rate of 1°C min^{-1} to 250°C, this temperature was held for 1 min, then the oven was programmed at 40°C min^{-1} to a final temperature of 285°C for 25 min; carrier gas hydrogen at 100 kPa; injection temperature 300°C; detector, flame ionization, 325°C. Data acquisition and quantificantion were carried out with HP 3365 series II Chemstation version A03-01 software on an HP Vectra 386/25 computer.

Recoveries for the oxysterol standards ranged from 89% for 25-hydroxycholesterol to 110% for 7α-hydroxycholesterol. Replicate spiked control samples included in duplicate in each run showed that between run variations were comparable to within run variations. The overall coefficient of variation for the sum of all cholesterol oxides was 14.7%. To ensure that storage of blood samples would not influence plasma oxysterol levels we conducted a test in which spiked and control samples stored at –80°C were analysed weekly over a 6 month period. No changes in total plasma oxysterol concentration were observed.

Analytical measurements

HDL cholesterol was determined as described by Warnick *et al.* (1982), triglycerides were analysed according to Sullivan *et al.* (1985). Total plasma cholesterol was determined by use of a Spectrum analyser (Abbot Laboratories, North Chicago, IL 60064, USA) with CHOD-PAP reagent (Boehringer, Mannheim, Germany). LDL cholesterol concentrations were calculated using the forumula of Friedewald *et al.* (1972). For all laboratory analysis, cases and controls were divided in equal numbers over each laboratory run.

Data analysis

First, basic characteristics, lipids and plasma oxysterol concentrations between cases and controls were compared by Student's t-test for unpaired samples. The association between oxysterols and continuous variables were quantified by Pearson's correlation coefficient. Multiple linear regression analysis was used to adjust differences in oxysterols between cases and controls for age, gender, smoking habits and serum HDL- and LDL cholesterol. Stratified analyses were performed to identify confounders or effect modifiers. In order to quantify the association of oxysterols with coronary stenosis, patients were divided into tertiles based on oxysterol concentrations and for each tertile the odds ratio was calculated. Multiple logistic regression analysis was used to adjust odds ratios for age, gender, smoking habits and HDL- and LDL cholesterol. All statistics were calculated using the BMDP package (Dixon 1992).

Results

Basic characteristics and medical history of the 159 patients are presented in table 1. HDL cholesterol was significantly higher in controls, whereas LDL cholesterol was somewhat, though not significantly lower (p=0.14). Cases more frequently reported the use of cholesterol restricted diet, history of myocardial infarction (MI) or percutane transluminal coronary angioplasty (PTCA) and past smoking. Several medications were used more frequently by cases, i.e. β-blockers, calcium-antagonists, nitrates, salicylic acid and HMG-CoA reductase inhibitors. For all other medications no significant difference between cases and controls was observed (results not shown). The 7α-hydroxycholesterol was the only one of the oxysterols which was initially significantly higher in cases. However, after multivariate adjustment this mean difference between cases and controls was no longer significant (Table 2). The 7α-hydroxycholesterol also made up the bulk of the total plasma oxysterol concentration, approximately 67%. The remaining five oxysterols, neither separately nor pooled, showed a

	Cases (n=80)[a]	Controls (n=79)[a]
	Mean ± SD	
Age (years)	53.7 ± 8.7	52.7 ± 9.3
Body mass index (kg m^{-2})	26.2 ± 3.1	25.4 ± 3.0
Systolic blood pressure (mmHg)	132.9 ± 17.4	131.8 ± 16.4
Diastolic blood pressure (mmHg)	83.1 ± 10.1	83.3 ± 10.1
Under cardiac care (years)	0.84 ± 0.69	0.69 ± 0.49
Years stopped smoking	6.5 ± 8.8*	10.8 ± 11.3
Plasma HDL cholesterol (mmol l^{-1})[b]	1.08 ± 0.31*	1.35 ± 0.42
Plasma LDL cholesterol (mmol l^{-1})[b]	4.17 ± 0.89	3.95 ± 0.99
Plasma total cholesterol (mmol l^{-1})[b]	6.21 ± 0.90	5.99 ± 1.08
Cigarettes per day	11.3 ± 9.0	9.0 ± 7.7
	Proportion (%) of:	
Males	66	65
Cholesterol lowering diet	50*	23
Vitamin C or E supplement	5	8
Current smokers	23	28
Ex-smokers	68*	49
Alcohol drinkers	70	77
History of hypertension	44	38
History of MI	18*	3
History of PTCA	11*	3
Family history of CVD	70	66

Table 1. Basic characteristics and medical history of patients with different degrees of coronary stenosis.

[a] Cases >80% stenosis in at least one coronary vessel; controls <50% stenosis in all three coronary vessels.

[b] One value missing.

* $p < 0.05$.

difference in concentration between cases and controls.

HDL cholesterol was negatively associated with total plasma oxysterol concentration (r=–0.152, p=0.057) while LDL cholesterol showed a slight positive association (r=0.142, p=0.075). Age was positively associated (r=0.166, p=0.036) with total plasma oxysterol concentration. 7α-Hydroxycholesterol showed a positive correlation with LDL cholesterol (r=0.163, p=0.04) and with age (r=0.171, p=0.03) as well as a slight negative correlation with HDL cholesterol (r=–0.142, p=0.075).

In the controls, we observed a significant difference in total plasma oxysterol concentration between men and women (1.77 μg per 100 ml vs 2.19 μg per 100 ml) as well as a difference in HDL- and LDL cholesterol. Even after adjustment for these lipid levels, age and smoking habits, the difference between genders (0.57 μg per 100 ml, 95% CI; 0.09–0.91 μg per 100 ml) was still statistically significant. There was no significant difference in total oxysterol concentration between controls on a cholesterol restricted diet compared with those who were not on such a diet (1.78 μg per 100 ml vs 1.96 μg per 100 ml), between

Oxysterols μg per 100ml, mean ± SD	Cases[a] (n = 80)	Controls[a] (n = 79)	Adjusted case-control difference (95% CI)[b]
7α-Hydroxycholesterol	1.53 ± 0.92[c]	1.27 ± 0.67	0.14 (–0.13–0.41)
7β-Hydroxycholesterol	0.15 ± 0.06	0.14 ± 0.06	0.00 (–0.02–0.02)
7-Ketocholesterol	0.13 ± 0.05	0.13 ± 0.07	0.00 (–0.02–0.02)
5,6α-Epoxycholestanol	0.15 ± 0.09	0.15 ± 0.07	0.00 (–0.02–0.02)
Cholestane-triol	0.15 ± 0.17	0.14 ± 0.14	–0.01 (–0.07–0.05)
25-Hydroxycholesterol	0.07 ± 0.12	0.06 ± 0.03	0.00 (–0.04–0.04)
Sum of five oxysterols[d]	0.65 ± 0.37	0.62 ± 0.27	–0.01 (–0.13–0.11)
Total plasma oxysterols	2.18 ± 1.04	1.92 ± 0.77	0.13 (–0.42–0.16)

Table 2. Plasma oxysterol concentrations in patients with different degrees of coronary stenosis.

[a] Cases >80% stenosis in at least one coronary vessel; controls <50% stenosis in all three coronary vessels.

[b] Mean difference in oxysterol concentrations between cases and controls after adjustment for age, gender, smoking habits and HDL- and LDL cholesterol.

[c] Student's t-test for unpaired samples (p <0.05).

[d] All oxysterols except 7α-hydroxycholesterol.

current smokers and non-smokers (1.90 μg per 100 ml vs 1.93 μg per ml) nor between non-smokers who had never smoked and ex-smokers (1.89 μg per 100 ml vs 2.07 μg per 100 ml). After multivariate adjustment, mean differences in total oxysterol (95% CI) for these groups were; 0.18 μg per 100 ml (–0.23 – 0.38 μg per 100 ml), –0.01 μg per 100 ml (–0.40 – 0.38 μg per 100 ml) and –0.01 μg per 100 ml (–0.46 – 0.44 μg per 100 ml) respectively. None of the other dichotomous variables in table 1 nor any of the medications used showed an association with the oxysterols. The number of controls with a history of MI or PTCA was too small to investigate the association with plasma oxysterols. However, analysis in which these patients were omitted showed similar results as those in table 2 (results not shown). Likewise, similar results were obtained for separate analysis of case-control differences for current smokers, non-smokers, ex-smokers and subjects who were not on a cholesterol restricted diet (results not shown).

Table 3 shows the odds ratios for coronary stenosis in the different tertiles of plasma oxysterol concentrations. In the crude analysis, there appears to be a somewhat higher odds ratio in the highest tertile. The difference with the lowest tertile however is not significant nor is there a tendency for an increasing trend over the tertiles. When adjusted for lipids and prestratification factors the odds ratios are essentially similar over the tertiles.

Discussion

The purpose of this case-control study was to test the hypothesis that oxysterols form

Tertiles	Low	Medium	High
Crude OR (95% CI)			
Total oxysterols	1.00	0.86 (0.39–1.86)	1.71 (0.79–3.71)
7α-Hydroxycholesterol	1.00	0.85 (0.38–1.79)	1.76 (0.81–3.82)
Sum of five oxysterols[b]	1.00	1.12 (0.52–2.41)	1.31 (0.60–2.83)
Adjusted OR[c] (95% CI)			
Total oxysterols	1.00	0.60 (0.25–1.45)	1.07 (0.45–2.59)
7α-Hydroxycholesterol	1.00	0.85 (0.34–1.93)	1.14 (0.49–2.68)
Sum of five oxysterols[b]	1.00	0.99 (0.42–2.39)	0.92 (0.39–2.18)

Table 3. Crude and adjusted odds ratios for severe coronary atherosclerosis in tertiles[a] of plasma oxysterol concentration.

[a] The patients are divided into tertiles on the basis of oxysterol concentrations.
[b] All oxysterols except 7α-hydroxycholesterol.
[c] Adjusted for age, gender, smoking habits and HDL- and LDL cholesterol.

factor for coronary atherosclerosis. Following this hypothesis, one would expect to find higher levels of oxysterols among cases. Of the six oxysterols investigated, 7α-hydroxycholesterol made up the major part of the total plasma oxysterol concentrations. It also was the only one initially significantly higher in the cases. This difference however disappeared after multivariate adjustment. The other five oxysterols, neither separate nor pooled, showed any association with the degree of coronary stenosis, neither before nor after multivariate adjustment.

It appears unlikely that the observation of no association as found in this study has been biased by flaws in the study design. We took special care to prevent auto-oxidation of cholesterol in the blood samples by adding the glutathione and BHT antioxidants and by rapidly processing the samples. In addition blood samples were analysed within 3 months, whereas we have observed that the total plasma oxysterol concentration did not increase over a 6 month storage period.

Both study groups were under cardiological treatment for vascular complaints and can thus be considered to be equally prone to dietary changes. Nevertheless, significantly more cases were on a cholesterol restricted diet. However, we observed no association between such a diet and plasma oxysterol levels. To reduce the effect of possible dietary or life-style changes as a response to the cardiology report, blood samples were taken within 2 months after angiography. A change in diet after angiography was reported in six cases and four controls, of whom two subjects in both groups reported use of a cholesterol- or fat-restricted diet. Exclusion of all subjects on a cholesterol-restricted diet yielded essentially similar results.

The results could have been biased by smoking habits, since more cases were ex-smokers. As smoking causes oxidative stress (Pryor *et al.* 1983) this may increase endogenous oxysterol formation. However, no differences in plasma oxysterol levels were seen between current and ex-smokers, nor did we find a correlation between the number of cigarettes smoked and oxysterol levels. Moreover, analysis for non-smokers and smokers separately yielded similar results.

Several medications were more frequently used by cases, of which HMG-CoA reductase inhibitors, being cholesterol lowering drugs, are of particular interest. By lowering the cholesterol level they could also lower oxysterol concentrations. However, we observed no association of plasma oxysterol levels and use of HMG-CoA reductase inhibitors nor with use of any of the other medications.

Of quite a different order is the possible bias caused by subjects with a history of PTCA (percutane transluminal coronary angioplasty). Subjects could have been misclassified as controls due to previous PTCA. However, only three controls had undergone PTCA, and an analysis omitting PTCA subjects yielded similar results as those in table 2 (results not shown).

In evaluating the possible biological significance of our results, it should be realized that our study group may not be representative of the general population. Both cases and controls are selected from individuals referred because of manifest clinical symptoms of CVD and the controls include patients with known minor coronary stenosis. However, in the control group 59% were scored as having no stenosis and the mean percentage stenosis over the three coronary vessels did not exceed 30%. Coronary arteriography allows for an improved diagnosis of coronary artery disease, for an objective identification of controls and cases and, most importantly, for potential atherogenic agents to be directly associated with coronary stenosis (Pearson 1984). Since associations of risk factors with stenosis vary by definition of control group (Pearson 1984, Reed and Yano 1991), we also examined the patients with more extreme degrees of coronary stenosis, i.e. controls with <10% stenosis (n=52) and cases with >90% stenosis (n=64). This would allow stronger inference to be made about the association of oxysterols with coronary stenosis. This analysis gave the same results as those already obtained from the less stringent data analysis.

We had hypothesized that plasma levels of oxysterols might be implicated in atherosclerosis as a reflection of exogenous intake since these may reflect exogenous intake. Experimental evidence indicates that dietary oxysterols are absorbed from the

gastrointestinal tract and are distributed among the lipid fractions, in particular VLDL and LDL (Morin and Peng 1989). In a mixed Dutch diet the total amount of oxysterols ranges from 3.6 to 6.2 $\mu g\,g^{-1}$ dry weight (van de Bovenkamp *et al.* 1988). At a daily food intake of about 500 g the average mixed Dutch diet would lead to the ingestion of a few milligrams of oxysterols. If plasma oxysterols are primarily of dietary origin, concentration profiles in diet and plasma would be expected to be similar. However, oxysterols in food show a totally different concentration profile (Jacobson 1987, Nourooz-Zadeh and Apelqvist 1988, van de Bovenkamp *et al.* 1988). In particular, in food the 7α-hydroxycholesterol does not predominate which indicates that plasma 7α-hydroxycholesterol is of endogenous origin. The fact that 7α-hydroxycholesterol forms an intermediate in the biosynthesis of bile acids from cholesterol (Björkhem *et al.* 1987) may explain its relatively high plasma concentration and its correlation with the total cholesterol level. Indeed, plasma levels of 7α-hydroxycholesterol have been suggested to be useful as a marker for cholesterol 7α-hydroxylase activity, the rate-limiting enzyme in bile acid biosynthesis (Björkhem *et al.* 1987).

Mean plasma level of free (unesterified) 7α-OH cholesterol observed in our cases (1.53 μg per 100 ml) is in the same order of magnitude as levels reported in cholelithiasis patients (1.94 μg per 100ml) and in gastric cancer patients without hepatobiliary disease (2.25 μg per 100 ml) (Oda *et al.* 1990). This study also indicated that about 20–25% of the total 7α-hydroxycholesterol in plasma is free (unesterified). Likewise Björkhem *et al.* (1987) reported levels of 3.0 μg per 100 ml (unesterified), in patients with gallstone disease. Koopman *et al.* (1987) reported levels of 1.96–16.4 μg per 100 ml in healthy volunteers using an assay for the sum of esterified and free 7α-hydroxycholesterol. The somewhat lower levels for free 7α-hydroxycholesterol in our study may be explained by our efforts to prevent auto-oxidation of cholesterol. Thus, the total plasma oxysterol concentration as found in the cases was a mere 2.18 μg per 100ml and even less when 7α-hydroxycholesterol is omitted (0.65 μg per 100 ml). This is far less than the minimum 5–10 μg per ml oxysterol concentration required in the culture medium to induce aortic smooth-muscle cell death in *in vitro* experiments (Peng *et al.* 1979). Moreover, our results do not indicate an association of plasma oxysterols in the observed range with coronary atherosclerosis and thus suggest that intake of oxysterols from an average mixed Dutch diet does not pose a major health risk with regard to atherosclerosis. This observation clearly cannot readily be extrapolated to other populations having other dietary habits. For instance in ghee oxysterols make up 12.3% of the sterols (258 μg per g dry weight) (Jacobson 1987). High dietary intake of ghee could thus lead to far higher plasma oxysterol levels which could indeed pose a risk. In our study we did not have data on subjects with high ghee intake.

The second part of our hypothesis linking oxysterols to coronary atherosclerosis was that plasma oxysterols may be a marker of LDL oxidation. We measured unesterified oxysterols, which can be assumed to be located among the unesterified cholesterol on the surface of the lipoprotein, the location most prone to oxidation. Total plasma oxysterol levels measured are a mere fraction ($<1.10^{-4}$%) of total cholesterol level. This may be interpreted as indicating that not much oxidation of cholesterol occurs *in vivo*. Moreover, we observed no association of plasma oxysterols with coronary stenosis. These observations seem to plea against a role of oxidation of LDL cholesterol in the aetiology of coronary atherosclerosis. However, it should be realized that oxidation of LDL may only occur extravascularly, i.e. in the arterial wall. In the microenvironment of the vascular intima, water and lipid-soluble antioxidant that are readily available in the circulation may become sufficiently depleted to allow the oxidation of LDL and formation of oxysterols (Steinberg *et al.* 1989). This oxidation may not have an impact on plasma oxysterol levels since the oxidized LDL is rapidly taken up by the resident macrophages. Moreover, any oxidized LDL that might leak away into the circulation will rapidly be cleared by the liver (Steinberg *et al.* 1989). Our results therefore may better be interpreted as indicating that plasma oxysterols are a poor marker of extravascular LDL oxidation. A possible better marker to study the association of LDL oxidation with coronary atherosclerosis may be the resistance of LDL against *ex-vivo* induced chemical oxidation (Esterbauer *et al.* 1989, Princen *et al.* 1992), which has been reported to be decreased in patients with severe coronary atherosclerosis (Regnström *et al.* 1992). Our results therefore may not be applicable to the LDL oxidation hypothesis since plasma oxysterols may be a poor marker of LDL oxidation *in vivo*.

We conclude that this study provides evidence that oxysterols measured in plasma are not a risk factor for coronary atherosclerosis in Dutch subjects. Dietary intake of oxysterols at levels from an average Dutch diet may therefore not pose a major health risk with regard to coronary atherosclerosis.

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