Plasma 7β-Hydroxycholesterol as a Possible Predictor of Lung Cancer Risk[1]

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Abstract

Epidemiological data suggests a role of dietary cholesterol in the etiology of lung cancer without having a clear biological hypothesis. Although smoking as the outstanding risk factor for lung cancer may enhance lipid peroxidation reactions, this study was planned to assess smoking-independent associations between the extent of cholesterol oxidation and the risk for lung cancer. In the frame of a nested case-control study in European Prospective Investigation on Cancer-Heidelberg, six cholesterol oxidation products (COPs) were determined in plasma samples of 20 incident lung cancer patients obtained 1.9 ± 0.6 years before diagnosis and in 40 matched (including smoking habits) controls. Odds ratios (ORs) and 95% confidence intervals (CIs) were calculated by conditional logistic regression. Among all COP compounds tested, plasma 7β-hydroxycholesterol was associated with lung cancer risk. The high crude risk estimate (OR ∼ 5) became significant after adjustment for sports activity (OR = 6.83, CI = 1.08–43.01, 3rd *versus* 1st tertile). For the independent effect of 7β-hydroxycholesterol, *i.e.,* adjusted for other COP compounds, an OR of 8.08 (CI = 1.12–58.54, 3rd *versus* 1st tertile) was calculated (*P* = 0.04 for trend). Lung cancer risk adjusted for sports activity significantly increased by 26% (CI = 1.050–1.506, *P* = 0.01) per unit (1 nmol/mmol plasma cholesterol) of 7β-hydroxycholesterol. No dietary factor had a significant effect in the regression model, but the dietary intake of meat, eggs, animal fat, cholesterol, and fruits (inversely) correlated with plasma COP concentrations. In this small study, plasma 7β-hydroxycholesterol appeared to be a smoking-independent predictor of lung cancer risk and might therefore be used as a biomarker. Because of the

rather high-risk estimate, research on possible intrinsic biological effects of this compound should be encouraged.

Introduction

Worldwide, lung cancer reflects a major public health problem because of high incidence rates combined with high fatality rates (1). Although cigarette smoking is the leading cause of lung cancer with an attributable risk of ∼90% in men, only 15% of smokers are eventually diagnosed with lung cancer (2). This indicates that other factors such as dietary factors or inherited differences in metabolic activities may determine lung cancer risk.

According to World Cancer Research Fund/American Institute for Cancer Research (2), diets high in cholesterol possibly increase the risk of lung cancer. Additionally, there is also a weak but consistent association between intake of food of animal origin (total meat intake, animal fat intake) and lung cancer risk (2, 3). Some new studies highlighted special dietary aspects that might modulate lung cancer risk such as the role of food preparation methods applied to meat (4) or the intake of phytosterols, which are generally known as inhibitors of cholesterol absorption (5). On the other hand, the evidence that vegetable and fruit intake decreases lung cancer risk is judged as convincing (2).

Dietary cholesterol as well as endogenous cholesterol is prone to oxidation, and COPs[3] have been known to have a role in the etiology of atherosclerosis for some time (6–9). The bioavailability of dietary COPs in humans was already demonstrated (10). Besides the enzymatic oxidation of cholesterol in mammals (*e.g.,* during synthesis of bile acids), autoxidation of cholesterol occurs, which might be increased in an antioxidant-depleted environment (11). Considering the epidemiological data, a link between cholesterol intake (probably as a proxy for COP intake) and antioxidant intake, or the intake of food of animal origin *versus* fruits and vegetables, might exist in the form of COP concentrations in the body. If this hypothesis holds true, it would be an important finding because valid biomarkers for lung cancer risk have not yet been identified.

One of the most important problems in the search for causal factors in lung cancer development other than smoking is the most appropriate adjustment for smoking. Either overadjustment or incomplete adjustment for smoking make a valid risk assessment for weaker risk factors, such as dietary factors, difficult; however, such risk factors may have an important attributable risk (12). Considering the present study hypothesis, smoking is an important factor that may enhance oxidation processes (13) and increase the nonenzymic oxidation of cholesterol as well (14). Therefore, the control subjects should be

[3] The abbreviations used are: COP, cholesterol oxidation product; CI, confidence interval; OR, odds ratio; ROS, reactive oxygen species; HST, human sulfotransferase.

Table 1 Sample characteristics (Mean $\pm$ SD or percentage)

Matching criteria[a]		
Sex (women/men, %)		31/69
Smoking (never/ever/current, %)		5/20/75
Amount currently smoked:		
6–19 cigarettes		47%
20–30 cigarettes		33%
$\geq$40 cigarettes		20%

	Cases ($n = 20$)	Controls ($n = 40$)
Age (yr)	54.9 ± 8.9	54.0 ± 8.3
Body Mass Index (kg/m^2)	26.7 ± 4.0	26.9 ± 4.0
WHR	0.90 ± 0.10	0.90 ± 0.10
Total yr smoked[b]	30.2 ± 10.0	31.8 ± 10.3
Total amount smoked (pack years)	30.5 ± 21.9	33.9 ± 28.9
No. of cigarettes currently smoked	22.1 ± 11.8	23.3 ± 16.4
Sports activity (yes/no, %)	20/80	48/52
Hours of sports/week	0.7 ± 2.7	1.1 ± 1.8
Observational time (yr)[c]	1.9 ± 0.6	2.3 ± 0.6
Blood storage period (yr)	4.1 ± 1.2	4.3 ± 1.2

	Smokers		Ex-smokers	
	Cases ($n = 15$)	Controls ($n = 30$)	Cases ($n = 4$)	Controls ($n = 8$)
Age at start of smoking	20.9 ± 6.5	19.1 ± 3.7	20.0 ± 2.2	17.9 ± 2.5
Age at stop of smoking			40.3 ± 1.3	42.3 ± 13.7
No. of cigarettes/day	22.1 ± 11.8	23.3 ± 16.4	15.8 ± 8.5	19.3 ± 16.0
Total years smoked[b]	32.9 ± 9.5	33.7 ± 8.7	20.3 ± 3.2	24.4 ± 12.9
Total amount smoked (pack years)	36.2 ± 21.7	39.1 ± 29.6	16.4 ± 9.6	23.0 ± 21.2
Hours of sports/week	1.0 ± 3.1	1.2 ± 1.9	0.0 ± 0.0	0.9 ± 1.6

[a] Additional matching criteria: age group (5-year interval), duration of sample storage (1-year interval).
[b] Ex-smoker: begin smoking until end of smoking; current smoker: begin smoking until day of recruitment.
[c] Recruitment date until date of first follow-up or date of death.

carefully matched to the smoking habits of the cases. The consequent results may give an answer to whether plasma COP concentrations, largely independent of smoking, are associated with lung cancer risk.

Materials and Methods

Study Subjects. All subjects enrolled in the present investigation were participants of the European Prospective Investigation into Cancer and Nutrition-Heidelberg cohort study (15). For this nested case-control study, all incident lung cancer cases until September 2000 from whom blood samples were obtained during recruitment were included in this study. The recruitment period started in June 1994 and ended in October 1998, the first active follow-up to identify newly diagnosed cases ran between January 1998 and November 1999. This resulted in a mean observation time of ~2 years (Table 1). All lung cancer cases were verified by a trained physician (ICD-O2 C34.1-C34.9). Most histological subtypes were adenocarcinoma ($n = 9$), followed by small-cell carcinoma ($n = 5$), squamous cell carcinoma ($n = 4$), and others ($n = 2$). Because of the low number of cases, histological subtypes were not considered for statistical evaluation. For each case, two matched control subjects were randomly selected. The matching criteria referred to sex, 5-year age group, smoking (never, ever, current), number of cigarettes currently smoked (5 groups), and duration of blood sample storage (1-year interval; Table 1). No prevalent or incident case of any cancer site or those with a history of myocardial infarction or stroke was allowed to become a control subject. Overall, 20 lung cancer cases and 40 matched controls were included in the evaluation.

At the beginning of the study, 4 cases identified by death certificates only had been included for which 8 matched controls were selected. By means of the verification process, these 4 cases turned out to be prevalent lung cancer cases and were therefore excluded from statistical analyses. Because of the intensive matching, the 8 controls could not serve as controls for other cases and were also excluded, except for the calculation of correlations between plasma COP concentration and dietary intake data.

Sample Collection and Laboratory Measurements. At recruitment, blood samples were obtained according to a standard protocol. For practical reasons, it was not possible to ensure that the participants were in a (12-h) fasting state. All subjects were asked for the time point of their last eating and drinking before blood sampling; the types of food were not recorded. After centrifugation of the blood samples, plasma samples were stored in liquid nitrogen until the start of the laboratory analysis. For each person, an aliquot of 0.5-ml plasma was used. Total plasma cholesterol concentrations were determined enzymatically by a test combination (Monotest Cholesterin, Boehringer Mannheim, Germany). Enzymatic hydrolysis of plasma cholesterol esters by cholesterol esterase (EC 1.1.1.13; Serva Electrophoresis GmbH, Heidelberg, Germany) preceded the COP determination to get total (free and esterified) COP concentrations. Plasma samples were incubated for 1 h at pH 7.0 and 37°C with the enzyme buffered in 10 mM Na-cholate in phosphate buffer. Enzyme activity added was ~12.5 units/2 mM cholesteryl ester. A detailed description of the methodology of COP determination is given elsewhere (11). Briefly, after lipid extraction, COPs were isolated by solid-phase extraction using a Sep-Pak silica cartridge (Waters Corporation, Milford, MA). The eluted acetone fraction containing all COPs was

evaporated to dryness and redissolved in 60 μl of Deriva Sil (Chrompack GmbH, Frankfurt, Germany) to get trimethylsilyl ethers of the COPs. For quantification of COPs, a Hewlett Packard 5890 gas chromatograph with a mass selective detector MSD 5971A installed with a HP1-MS column (0.25-mm i.d. × 30 m; Hewlett-Packard GmbH, Waldbronn, Germany) was used. Carrier gas (helium) was delivered at a flow rate of 1 ml/min with a split ratio of 1:25. Oven temperature was 290°C for 12 min and decreased with a rate of 10°C/min to 280°C and held isothermally for 7 min. Injector port and detector temperatures were 290 and 300°C, respectively.

To avoid additional oxidation of cholesterol during analytical procedures measures like addition of butylated hydroxytoluene to solvents, N_2 in headspaces and darkness during latency steps were taken. Because of technical problems during analysis, the results of one control subject could not be included in the evaluation.

Six COPs were determined (trivial name in parentheses): 5-cholestene-3β,7α-diol (7α-hydroxycholesterol); 5-cholestene-3β,7β-diol (7β-hydroxy-cholesterol); 5,6α-epoxy-5α-cholestan-3β-ol (cholesterol-α-epoxide); 5,6β-epoxy-5β-cholestan-3β-ol (cholesterol-β-epoxide); 5α-cholestan-3β,5α,6β-triol (cholestanetriol); and 5-cholesten-3β-ol-7-one (7-ketocholesterol). 5-Pregnen-3β-ol-7,20-dione (7-keto-pregnenolone) was used as the internal standard. All standard substances were delivered by Steraloids, Inc. (Wilton, NH), and Sigma (Deisenhofen, Germany). Identification of the sample peaks was made by retention times of the standard substances and the characteristic ion fragmentation. For quantification, a selected ion monitoring program was used recording the abundance of two main and/or characteristic ions/substance as follows: ions m/z 456 and 457 for 7α- and 7β-hydroxycholesterol; ions m/z 120 and 474 for cholesterol-α-epoxide; ions m/z 135 and 474 for cholesterol-β-epoxide; ions m/z 403 and 456 for cholestanetriol; ions m/z 367 and 472 for 7-ketocholesterol; and ions m/z 317 and 402 for 7-ketopregnenolone. Calibration was done within each series of samples injected into the gas chromatography. Recovery (percentage of added COP standard substances) and reproducibility (coefficient of variation) were 91.5 and 1.4% for 7α-hydroxycholesterol, 93.3 and 1.7% 7β-hydroxycholesterol, 98.3 and 3.1% for cholesterol-β-epoxide, 87.7 and 4.3% for cholesterol-α-epoxide, 106.8 and 7.3% for cholestanetriol, 94.4 and 3.5% for 7-ketocholesterol, and 95.1 and 5.0% for the sum of COPs, respectively.

The intrapersonal variation in plasma COP concentrations was estimated in 4 healthy subjects (2 females, 2 males) from whom a blood sample was drawn once monthly over a time period of 4 months. The results indicate a variation (mean variation coefficient) of 16.8% for 7α-hydroxycholesterol, 18.1% for 7β-hydroxycholesterol, 37.7% for cholesterol-β-epoxide, 77.5% for cholesterol-α-epoxide, 34.7% for choletanetriol, 14.8% for 7-ketocholesterol, and 12.5% for the sum of COPs.

During recruitment, several assessment tools were applied to get detailed information on lifestyle factors, medical history, and dietary habits (16). Anthropometric data were measured during the visit of each participant in the study center according to standard procedures. Of the lifestyle data obtained by questionnaire and face-to-face interview, data on smoking habits and sports activity were used in the present evaluation. Regular dietary intake was assessed using a validated food frequency questionnaire (17, 18). After calculation of the intake estimates of the 149 food items by the indicated frequency of consumption and portion size, the food items were aggregated to food groups. Nutrient intake was calculated on the basis of the German food composition table BLS II.3 (BgVV, Berlin, Germany).

Statistical Analysis. Descriptive data for cases and controls are presented as arithmetic mean, SD of the mean, and 95% CI. Partial correlation coefficients were calculated for the relationship of food group and nutrient intake data and plasma COP concentrations. Risk evaluation was performed by conditional logistic regression using categorized plasma COP concentrations (tertiles) as well as the continuous variables. The results are given as ORs and 95% CIs. Variables describing smoking habits in more detail (besides the matching variables), as well as data on sports activity, dietary intake (tertiles), anthropometry, and socioeconomic status were tested for their modulating effect on the relationship between single COP compounds and lung cancer risk. An additional model also considered simultaneously all analyzed COP compounds. The software packages SPSS 10.0.5 (SPSS, Inc., Chicago, IL) and SAS 8.01 (SAS Institute, Inc., Cary, NC) were used for descriptive analysis and for risk evaluation, respectively.

Results

The results of the COP determination in plasma samples of 20 lung cancer cases and 39 matched controls were included in the present risk evaluation. Plasma total cholesterol concentrations amounted to 4.20 ± 0.63 mM in cases and 4.32 ± 0.78 mM in controls (means ± SD). Although the difference in mean plasma cholesterol values between groups was small, COP concentrations were expressed/unit plasma cholesterol. The resulting mean plasma COP concentrations as well as the corresponding CIs were (slightly) higher in cases as compared with controls (Table 2), except for 7α-hydroxycholesterol and cholesterol-β-epoxide.

In the unadjusted risk analysis, high but nonsignificant ORs were found for 7β-hydroxycholesterol and lung cancer risk (OR ~ 5; Table 3). Crude risk estimates were above unity also for cholesterol-α-epoxide, cholestanetriol, 7-ketocholesterol, the sum of 7β-hydroxy- and 7-keto-cholesterol, as well as the sum of all COPs but did not reach statistical significance. Additional adjustment for smoking variables gave hardly any visible modification of the results, indicating an almost complete matching for smoking. Of the various other lifestyle factors tested, only sports activity (dichotomous variable) showed a significant protective effect on lung cancer risk. Adjustment for sports activity modified the results; the suggested association between plasma 7β-hydroxycholesterol concentration and lung cancer became statistically significant for the third tertile (OR = 6.83, CI = 1.08–43.01, *versus* 1st tertile; P = 0.043 for trend; Table 3). To determine the independent effect of each COP compound on lung cancer risk, all six COPs were simultaneously included in the analysis. The results demonstrate that the association between plasma 7β-hydroxycholesterol and lung cancer were again statistically significant (OR = 8.08; CI = 1.12–58.54, for comparison of the highest *versus* lowest tertile, P = 0.041 for trend; Table 3). The risk estimates for cholesterol-α-epoxide, cholestanetriol, the sum of 7-hydroxy and 7-ketocholesterol, as well as the sum of all COP compounds did not reach statistical significance.

Introducing plasma COP concentrations as continuous variables to the regression model (Table 4) confirmed the observed significant association of 7β-hydroxycholesterol with lung cancer risk. The relative risk of lung cancer significantly increased by 25.7%/unit plasma 7β-hydroxycholesterol (CI = 1.050–1.506; adjusted for sports activity); the risk estimate was about twice as high (OR = 1.524) when further adjusted for

Table 2   Plasma total COPs (nmol/l plasma; nmol/mmol total plasma cholesterol) in incident lung cancer cases and in matched controls

	Cases (*n* = 20)		Controls (*n* = 39)	
	Mean ± SD	95% CI	Mean ± SD	95% CI
	nmol/l plasma			
7α-Hydroxycholesterol	163.4 ± 107.9	112.9–213.8	185.5 ± 181.7	125.8–245.3
7β-Hydroxycholesterol	56.3 ± 15.2	49.2–63.3	50.2 ± 13.3	45.9–54.6
Cholesterol-β-epoxide	215.9 ± 165.2	138.6–293.2	232.2 ± 158.0	180.3–284.1
Cholesterol-α-epoxide	273.5 ± 314.9	126.1–420.8	209.5 ± 307.1	108.6–310.5
Cholestanetriol	164.6 ± 59.1	136.9–192.3	162.8 ± 75.4	138.0–187.6
7-Ketocholesterol	99.0 ± 43.4	78.7–119.3	99.3 ± 38.7	86.5–112.0
7β-Hydroxy- + 7-ketocholesterol	155.3 ± 53.8	130.1–180.4	149.5 ± 48.8	133.5–165.5
Σ COPs	972.6 ± 282.5	840.3–1104.8	966.3 ± 305.1	837.5–1095.2
	nmol/mmol total plasma cholesterol			
7α-Hydroxycholesterol	39.6 ± 28.0	26.4–52.7	43.2 ± 37.6	30.8–55.5
7β-Hydroxycholesterol	13.7 ± 4.5	11.6–15.9	11.9 ± 3.7	10.7–13.2
Cholesterol-β-epoxide	52.1 ± 39.2	33.8–70.5	53.6 ± 32.2	43.0–64.2
Cholesterol-α-epoxide	67.7 ± 83.9	28.5–107.0	51.9 ± 84.4	24.2–79.7
Cholestanetriol	40.7 ± 17.1	32.7–48.7	38.4 ± 17.7	32.5–44.2
7-Ketocholesterol	24.3 ± 11.5	18.9–29.6	23.4 ± 9.8	20.2–26.6
7β-Hydroxy- + 7-ketocholesterol	38.0 ± 15.0	31.0–45.0	35.3 ± 12.7	31.2–39.5
Σ COPs	238.1 ± 85.9	197.8–278.3	222.4 ± 107.0	187.2–257.5

other COP compounds. In contrast, the risk estimates for all other COP compounds were near unity; for sports activity, an OR of 0.041 (CI = 0.030–0.513) was calculated in this model.

Repeating the analysis with the COP concentrations expressed in nmol/l plasma very similar results were obtained. Of course, the higher absolute values of plasma 7β-hydroxycholesterol resulted in a smaller increase in risk/unit (nmol/l) 7β-hydroxycholesterol.

As can be expected from the number of cases and controls included in this study, no food group or nutrient was significantly associated with lung cancer risk. Introduction of selected dietary parameters into the model was tested but gave no significant results. Instead, partial correlation coefficients were computed for the whole sample (including 8 additional controls). Food groups and nutrients showing a statistically significant linear correlation with plasma COP concentrations were given in Table 5. Intake of food (food groups) of animal origin is associated with plasma COP concentrations. Especially, 7β-hydroxycholesterol and 7-ketocholesterol plasma values were positively correlated with the intake of meat, eggs, animal fats, and cholesterol, whereas there was an inverse association with fruit intake.

Discussion

According to the results of the present pilot study, plasma 7β-hydroxycholesterol is significantly associated with lung cancer risk revealing quite high risk estimates, regardless of the type of statistical analysis (categorical or continuous model) applied. For none of the other COP compounds, a significant association could be established. Because there is an urgent need for valid biomarkers for lung cancer (19), this finding might be important. However, it has to be reminded that the samples size is small, and the possibility of chance findings cannot be excluded (no correction for multiple hypothesis testing was performed). Because COP determination was performed in plasma samples of subjects who were not necessarily in a fasted state (see "Materials and "Methods"), a small contribution of exogenous (dietary) cholesterol and COP intake by recent meals to the observed values is possible.

Several open questions still have to be addressed: the two most important questions arising from these results refer to (*a*) the reasons for the increased plasma 7β-hydroxycholesterol concentrations in incident lung cancer cases ~2 years before onset of the disease and (*b*) whether the elevated 7β-hydroxycholesterol levels itself may have pathophysiological consequences (*e.g.,* for lung tissue).

In the context of atherogenesis, Jialal *et al.* (20) were among the first who proposed to use COP measurement as a reliable index of endogenous nonenzymic oxidation processes affecting low-density lipoproteins. Especially, 7-oxygenated cholesterol derivatives seem to reflect autoxidation processes *in vivo, i.e.,* 7α-hydroxycholesterol, 7β-hydroxycholesterol, and 7-ketocholesterol; 7α-hydroxycholesterol, however, is also formed enzymatically during bile acid synthesis (6–9, 21). Because both 7α- and 7β-hydroxycholesterol may react to form 7-ketocholesterol, the latter compound may not be the best indicator of autoxidation processes because of the considerably high contribution of 7α-hydroxycholesterol. Also the sum of 7β- and 7-ketocholesterol was not significantly associated with lung cancer risk even after adjustment for the other COP compounds (Tables 3 and 4). This would imply an outstanding and very specific role of 7β-hydroxycholesterol as marker of endogenous lipid peroxidation probably originating from lung tissues and reflecting enhanced inflammatory processes.

In a study on the role of endogenous lipid oxidation in patients with coronary heart disease, a very strong relationship between serum 7β-hydroxycholesterol concentrations and the rapid progression of atherosclerotic changes was found. Salonen *et al.* (22) selected 20 patients with fast progression and 20 patients with no progression of carotid atherosclerosis within a 3-year period from participants of the Kuopio Atherosclerosis Prevention Study. Serum 7β-hydroxycholesterol was found to be the strongest predictor of the progression of carotid wall thickening (followed by smoking, low-density lipoprotein-thiobarbituric acid-reactive substances, and lag time). The risk of experiencing a rapid progression increased by 80%/unit (μg/liter) of serum 7β-hydroxycholesterol. The authors interpreted 7β-hydroxycholesterol largely as an index of *in vivo* lipid oxidation but also acknowledged the known cytotoxic activity of 7β-hydroperoxycholesterol in endothelial cells. Another

Table 3 Crude and adjusted[a] odds ratios and confidence intervals (95% CI) for lung cancer risk by tertiles (I, II, III) of COP concentrations in plasma samples before diagnosis of lung cancer ($n = 20$) *versus* matched controls ($n = 39$)

Tertiles	Crude			Adjusted for sports activity			Adjusted for COPs and sports activity		
	I	II	III	I	II	III	I	II	III
7α-Hydroxycholesterol									
Conc. (nmol/mmol chol.)[c]				<27.4	27.4–<43.9	≥43.9			
Cases/controls (n)				9/13	5/13	6/13			
OR	1	0.54	0.62	1	0.65	0.72	1	1.05	0.97
(CI)		(0.13–2.30)	(0.14–2.80)		(0.14–3.10)	(0.15–3.54)		(0.17–6.43)	(0.16–5.75)
P for trend			0.500			0.647			0.932
7β-Hydroxycholesterol									
Conc. (nmol/mmol chol.)[c]				<9.8	9.8–<13.5	≥13.5			
Cases/controls (n)				2/13	9/13	9/13			
OR	1	4.98	4.86	1	3.15	**6.83**	1	3.48	**8.08**
(CI)		(0.91–27.2)	(0.92–25.8)		(0.57–17.35)	**(1.08–43.01)**		(0.59–20.58)	**(1.12–58.54)**
P for trend			0.118			**0.043**			**0.041**
Cholesterol-β-epoxide									
Conc. (nmol/mmol chol.)[c]				<35.2	35.2–<59.9	≥59.9			
Cases/controls (n)				10/13	3/13	7/13			
OR	1	0.24	0.71	1	0.15	0.67	1	0.16	0.39
(CI)		(0.04–1.46)	(0.21–2.45)		(0.02–1.29)	(0.18–2.56)		(0.01–1.86)	(0.07–2.16)
P for trend			0.544			0.632			0.287
Cholesterol-α-epoxide									
Conc. (nmol/mmol chol.)[c]				<11.3	11.3–<31.0	≥31.0			
Cases/controls (n)				4/13	7/13	9/13			
OR	1	2.03	2.70	1	1.69	2.11	1	1.46	2.77
(CI)		(0.47–8.84)	(0.59–12.37)		(0.35–8.08)	(0.49–8.98)		(0.25–8.38)	(0.56–13.69)
P for trend			0.270			0.349			0.215
Cholestanetriol									
Conc. (nmol/mmol chol.)[c]				<28.5	28.5–<39.4	≥39.4			
Cases/controls (n)				5/13	4/13	11/13			
OR	1	0.72	2.58	1	1.03	3.61	1	0.84	2.52
(CI)		(0.14–3.76)	(0.67–9.97)		(0.15–7.03)	(0.73–17.82)		(0.10–7.22)	(0.48–13.26)
P for trend			0.205			0.105			0.256
7-Ketocholesterol									
Conc. (nmol/mmol chol.)[c]				<18.7	18.7–<27.5	≥26.1			
Cases/controls (n)				6/13	5/13	9/13			
OR	1	0.91	1.58	1	0.90	1.75	1	1.28	1.31
(CI)		(0.22–3.74)	(0.44–5.73)		(0.21–3.89)	(0.41–7.58)		(0.20–8.11)	(0.25–6.98)
P for trend			0.546			0.443			0.776
7β-Hydroxycholesterol + 7-ketocholesterol									
Conc. (nmol/mmol chol.)[c]				<28.6	28.6–<38.9	≥38.9			
Cases/controls (n)				5/13	4/13	11/13			
OR	1	0.84	3.13	1	1.02	4.10	1	1.15	3.45
(CI)		(0.19–3.68)	(0.70–13.97)		(0.20–5.13)	(0.71–23.52)		(0.20–6.60)	(0.55–21.71)
P for trend			0.186			0.121			0.192
Σ COPs									
Conc. (nmol/mmol chol.)[c]				<158.6	158.6–<222.4	≥222.4			
Cases/controls (n)				5/12	6/13	9/13			
OR	1	1.43	2.26	1	1.16	2.51			
(CI)		(0.36–5.72)	(0.54–9.40)		(0.24–5.63)	(0.53–11.87)			
P for trend			0.336			0.283			

[a] Adjusted for sports activity (yes/no).
[b] Matched for sex, age, smoking (never/ever/current), amount currently smoked (5 groups), blood storage period.
[c] conc., concentration; chol., total plasma cholesterol.

study compared established and putative risk factors for heart disease between Swedish and Lithuanian men because the latter had a 4-fold higher risk (23). Despite similar results for traditional risk factors (including plasma cholesterol), they found higher plasma levels of 7β-hydroxycholesterol in Lithuanians. Furthermore, a Japanese group reported significantly elevated serum concentrations of 7β-hydroxycholesterol in patients with coronary artery stenosis (especially in the subgroup with unstable angina) as compared with angiographically normal subjects (24). Thus, there is evidence that increased plasma levels of 7β-hydroxycholesterol, possibly as a marker for elevated *in*

vivo autoxidation processes, are associated with an increased risk of atherosclerosis.

Similar studies on COPs in cancer patients are not available. However, oxidative DNA damage (etheno DNA base adducts) via lipid peroxidation and oxidative stress is suggested to be involved in the development of several malignancies such as breast cancer, colon cancer, and liver cancer (25). Unless DNA adducts are restored by DNA repair enzymes, etheno-modified DNA bases like carcinogen-derived DNA adducts may represent promutagenic lesions (26). The possible link between 7β-hydroxycholesterol measurement in plasma and

Table 4 Crude and adjusted maximum likelihood estimates for lung cancer risk by plasma concentrations (continuous) of COPs (nmol/mmol plasma cholesterol) before diagnosis of lung cancer ($n = 20$) *versus* matched[a] controls ($n = 39$)

	χ^{2b}	OR[b]	95% CI[b]
Crude			
7α-Hydroxycholesterol	0.759	0.997	0.980–1.015
7β-Hydroxycholesterol	0.158	1.102	0.963–1.262
Cholesterol-β-epoxide	0.968	1.000	0.984–1.015
Cholesterol-α-epoxide	0.476	1.002	0.996–1.008
Cholestanetriol	0.700	1.007	0.976–1.039
7-Ketocholesterol	0.731	1.009	0.958–1.063
7β-Hydroxy- + 7-ketocholesterol	0.462	1.015	0.975–1.056
COPs	0.522	1.002	0.996–1.007
Adjusted for sports activity (yes/no)			
7α-Hydroxycholesterol	0.993	1.000	0.983–1.017
7β-Hydroxycholesterol	**0.013**	**1.257**	**1.050–1.506**
Cholesterol-β-epoxide	0.722	1.003	0.986–1.020
Cholesterol-α-epoxide	0.562	1.002	0.996–1.008
Cholestanetriol	0.455	1.014	0.9781–1.051
7-Ketocholesterol	0.333	1.032	0.969–1.098
7β-Hydroxy- + 7-ketocholesterol	0.108	1.042	0.991–1.096
COPs	0.339	1.003	0.997–1.009
Adjusted for sports activity and other COPs			
7α-Hydroxycholesterol	0.942	1.001	0.978–1.024
7β-Hydroxycholesterol	**0.012**	**1.524**	**1.097–2.118**
Cholesterol-β-epoxide	0.874	1.002	0.975–1.031
Cholesterol-α-epoxide	0.349	1.004	0.995–1.013
Cholestanetriol	0.193	0.966	0.918–1.017
7-Ketocholesterol	0.342	0.950	0.856–1.055

[a] Matched for sex, age, smoking (never/ever/current), amount currently smoked (5 groups), blood storage period.
[b] χ^2, P of χ^2 test.

the extent of etheno DNA base adduct formation has not yet been addressed. If it would hold true that both reflect *in vivo* peroxidation reactions, COP measurement would be the faster and cheaper alternative of both biomarker candidates.

Smoking contributes to the amount of ROS, which in turn may induce radical chain reactions (13). The double bonds of polyunsaturated fatty acids are among the most sensitive compounds for ROS-induced damage, leading also to the formation of COPs (-acyl esters; 9). Because smoking is well established as the dominant cause of lung cancer, a quite intensive matching for smoking has to be applied to investigate smoking-independent associations in such a small study. As indicated by the missing effects of introducing additional smoking variables in the statistical analysis, the applied way of matching worked quite well. The results, therefore, must be regarded as largely independent of smoking and of smoking-associated ROS production.

Besides the endogenous formation, dietary intake of COP-rich foodstuff may contribute to 7β-hydroxycholesterol plasma concentrations. The literature provides a lot of diverging data on the COP content of food and some enhancing conditions for COP formation during packaging, storage, and preparation of food are described as well (27). It seems plausible that dietary intake of COP-rich food may specifically enhance 7β-hydroxycholesterol plasma concentrations because of the known differences in the bioavailability of the diverse COP compounds both in rats and humans (10, 28). Additional support for an effect of dietary intake (reflecting mean dietary intake over the past year) on plasma COP concentrations is provided by the results of the correlation analysis (Table 5) conducted in this study. The results hint toward a link between the intake of foods of animal

origin and cholesterol and plasma COP concentrations, whereas fruit consumption showed an inverse association. It seems surprising that in this small sample, a link between diet and plasma COP concentrations is suggested. Epidemiological data still do not allow a final conclusion on the effect of meat and cholesterol intake in lung cancer etiology (2, 3). 7β-Hydroxycholesterol was the only COP significantly associated with meat intake, which would be in line with a lung cancer risk increasing effect of high meat intake. Possibly, hazardous effects of meat intake are exerted by some meat subgroups such as meat that was preserved or prepared by certain methods; thus far such aspects have rarely been considered in epidemiological studies (4). In contrast, the evidence for a protective effect of fruit intake on lung cancer risk is convincing (2, 3). Among the suggested mechanisms by which fruit intake exerts health-relevant effects, the antioxidant hypothesis is one of the most striking hypotheses and would best fit to the suggestion that 7β-hydroxycholesterol is an indicator of endogenous nonenzymic reactions (lipid peroxidation). It has been demonstrated that dietary supplementation with antioxidants (vitamins C and E) resulted in reduced serum 7β-hydroxycholesterol concentrations in humans (14, 29).

Not only endogenous production and dietary intake but also an attenuated metabolism of 7β-hydroxycholesterol could contribute to the elevated plasma concentrations in lung cancer patients. In general, COPs undergo a rapid clearance by the liver with biliary secretion and fecal excretion (7–9). However, COPs are distributed by the blood stream (lipoproteins), and certain COPs have been reported to accumulate in several tissues, including malignant tissues of the breast and prostate (30). Thus far, no indication for COP involvement in lung cancer development can be found in the literature. In contrast, it was demonstrated > 10 years ago that several COPs inhibit tumor cell growth (hepatoma and lymphoma cells) in culture at micromolar concentrations (31). More recent data indicate that the cytotoxic potential of COP compounds, especially those oxidized at C7 (including 7β-hydro(per)oxycholesterol), is mediated by induction of apoptosis in several normal cell lines and in tumor cells (lymphocytes; Ref. 32). Therefore, they also have been suggested as chemotherapeutic agents (33). The physiological role of COPs as signaling molecules that regulate a number of transcription factors (nuclear hormone receptors liver X receptor (LXR):retinoid X receptor and farnesol X receptor (FXR):retinoid X receptor heterodimers, sterol regulatory element binding proteins (SREBP) became apparent in the last years (34, 35). By transcriptional control they (co)regulate the expression of genes that participate in cholesterol, lipid, and glucose metabolism. Furthermore, they are mediators of reverse sterol transport (from periphery to liver) and may be substrates for steroid hormone synthesis. 7α-Hydroxylation of the sterol nucleus is the key regulatory step in bile acid synthesis and is demonstrated in cell culture for a series of COP compounds (36). Especially, the human lung (alveolar macrophages) is described as the organ with the highest activity of 27-hydroxylase (CYP27), converting cholesterol to 27-hydroxycholesterol and cholestenoic acid (37). C27-hydroxylation of other sterol substrates (including COP) is catalyzed as well, *e.g.,* during bile acid synthesis (36). Babiker *et al.* (37) concluded from their experiments that the sterol 27-hydroxylase is important for cholesterol homeostasis in the lung. Furthermore, the recently described isoforms of HST2a and HST2b reveal substrate specificity for cholesterol and COPs (38). Therefore, only few weak suggestions for an attenuated metabolism of 7β-hydroxycholesterol can be provided, *i.e.,* by a decreased activity of sterol 27-hydroxylase and HST2 isoforms

Table 5   Partial correlation coefficients[a] for the relationship between plasma COPs (nmol/mmol plasma cholesterol) and food group or nutrient intake (g/day) as assessed by means of a food frequency questionnaire in incident lung cancer cases before diagnosis ($n = 20$) and matched controls ($n = 47$[b]; pooled analysis)

COPs	Food groups				Nutrient
	Meat[c]	Eggs	Animal fats	Fruits	Cholesterol
7α-Hydroxycholesterol	0.059	0.118	**0.328**	−0.195	0.171
	(0.634)	(0.338)	**(0.006)**	(0.111)	(0.164)
7β-Hydroxycholesterol	**0.255**	0.186	**0.237**	−0.177	**0.267**
	(0.036)	(0.128)	**(0.052)**	(0.150)	**(0.028)**
Cholesterol-β-epoxide	0.137	−0.045	0.089	**−0.204**	−0.010
	(0.264)	(0.714)	(0.472)	**(0.095)**	(0.938)
Cholesterol-α-epoxide	−0.195	−0.178	−0.183	−0.050	**−0.286**
	(0.110)	(0.146)	(0.135)	(0.689)	**(0.018)**
Cholestanetriol	0.120	0.179	**0.226**	**−0.292**	0.172
	(0.331)	(0.145)	**(0.064)**	**(0.016)**	(0.161)
7-Ketocholesterol	0.101	**0.229**	0.3026	**−0.217**	**0.230**
	(0.414)	**(0.060)**	**(0.012)**	**(0.076)**	**(0.060)**
7β-Hydroxy- + 7-ketocholesterol	0.154	**0.233**	**0.304**	**−0.220**	**0.257**
	(0.212)	**(0.056)**	**(0.012)**	**(0.072)**	**(0.034)**
Σ COPs	−0.059	−0.065	0.067	**−0.265**	−0.127
	(0.635)	(0.600)	(0.585)	**(0.029)**	(0.304)

[a] Controlled for sports activity; *P* for two-tailed significance in brackets; bold figures for $P < 0.10$.

[b] Including 8 additional controls (the corresponding 4 cases have been verified as being prevalent and were therefore excluded from analyses).

[c] Without meat products.

(*e.g.,* by functionally relevant polymorphisms of the encoding genes).

Among the tested confounders (and confounder interactions), sports activity was the only one with a significant effect on lung cancer risk. It seems likely that sports activity reflects a more health-conscious behavior and is possibly linked to socioeconomic characteristics as well.

In conclusion, the results of this pilot study indicate that plasma 7β-hydroxycholesterol is associated with lung cancer risk and thus might possibly be used as a biomarker. This parameter is likely to reflect the extent of endogenous (lipid) peroxidation reactions and the amount of dietary COP intake and is possibly also affected by interindividual differences in the metabolism of this compound. The validity of this concept has to be confirmed in additional studies comprising a larger cohort. It remains unknown whether 7β-hydroxycholesterol itself might be actively involved in the etiology of lung cancer.

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