Oxidation of sitosterol and transport of its 7-oxygenated products from different tissues in humans and ApoE knockout mice

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Abstract

The most common phytosterols in the human diet are sitosterol and campesterol, which originate exclusively from plant derived food. These phytosterols are taken up by NPC1L1 transport from the intestine into the enterocytes together with cholesterol and other xenosterols. Phytosterols are selectively pumped back from the enterocytes into the intestinal lumen and on the liver site from hepatocytes into bile by heterodimeric ABCG5/G8 transporters. Like cholesterol, both phytosterols are prone to ring and side chain oxidation. It could be shown that oxyphytosterols, found in atherosclerotic tissue, are most likely of *in situ* oxidation (Schött et al.; Biochem Biophys Res Commun. 2014 Apr 11;446(3):805-10). However, up to now, the entire mechanism of phytosterol oxidation is not clearly understood. Here, we provide further information about the oxidation of sitosterol and the transport of its oxidation products out of tissue. Our survey includes data of 104 severe aortic stenosis patients that underwent an elective aortic valve cusp replacement. We studied their phytosterol concentrations, as well as absolute and substrate corrected oxyphytosterol levels in plasma and valve cusp tissue. In addition, we also examined phytosterol and oxyphytosterol concentrations in plasma and tissues (from brain and liver) of 10 male ApoE knockout mice. The ratio of 7-oxygenated-sitosterol-to-sitosterol exceeds the ratio for 7-oxygenated-campesterol-to-campesterol in plasma and tissue of both humans and mice. This finding indicates that sitosterol is oxidized to a higher amount than campesterol and that a selective oxidative mechanism might exist which can differentiate between certain phytosterols. Secondly, the concentrations of oxyphytosterols found in plasma and tissue support the idea that oxysitosterols are preferably transported out of individual tissues. Selective oxidation of sitosterol and preferred transport of sitosterol oxidation products out of tissue seem to be a metabolic pathway of forced sitosterol clearance from tissue compartments.

Key words: sitosterol, phytosterol, oxyphytosterol, oxidation, transport, metabolism

Introduction

Phytosterols are neutral sterols which are structurally similar to cholesterol but exclusively synthesized by plants [1]. Like cholesterol in animals, phytosterols take over the role of integral membrane components in plants [2]. The most common phytosterols are sitosterol and campesterol that differ from cholesterol in the modification of the side chain [3, 4]. In sitosterol and campesterol a hydrogen atom at position C24 is substituted by an additional ethyl- or methyl-group. Phytosterols determined in animal tissue and body fluids can exclusively originate from absorbed plant diet, whereby sitosterol is the phytosterol that occurs in highest concentrations in the human and animal diet, followed by campesterol. However, conditions are different in blood and normally the concentration of campesterol exceeds the sitosterol concentration. The fractional absorption rate of campesterol in the intestine is threefold higher compared with sitosterol [5, 6]. Similar to cholesterol, phytosterols are prone to autoxidation by the presence of oxygen, heat, light and transition metals. Under these conditions phytosterols form autoxidative products such as 7α- or 7β-diols, 5α, 6α-epoxids; 5β, 6β-epoxids; and cholestane-triols; as well as 7-keto-oxyphytosterols [7-9]. Until the present day it is understood that oxyphytosterols can be formed by a radical mechanism at the ring structure or at the side chain and that some side chain oxyphytosterols might to be formed enzymatically [10, 11].

The main pathway of cholesterol metabolism is the degradation to primary bile acids and excretion via the bile. Contrary to cholesterol, phytosterols cannot follow this excretion and detoxification pathway because of a different substitution at position C24 which prevents side chain shortening [12]. Only little information with regard to the phytosterol and oxyphytosterol metabolism is available and still basic questions have to be answered: 1) How are the absorbed and endogenously formed oxyphytosterols transported within or into the blood circulation? 2) How is the transport mechanism designed that transfers oxyphytosterols between extracellular and intracellular compartments? 3) What is the mechanism to form oxyphytosterols? 4) Do oxyphytosterols play a role in the metabolism of phytosterols? Additionally, data on the origin of oxyphytosterols in the human circulation, their distribution in different body compartments, and their intestinal absorption rates from the diet are

rare. Among available models, the apolipoprotein E- deficient (apoE$^{-/-}$) mouse is particularly popular because of its propensity to spontaneously develop atherosclerotic lesions on a standard chow diet. On western type diet the apoE$^{-/-}$ mouse demonstrates very high levels of circulating cholesterol and plant sterol levels. Development of atherosclerosis and oxidative processes in normal mice would take longer periods of treatment with western type diets [13]. Another option would have been to take LDL-R k.o. mice. Fortunately, data and material from oxyphytosterol ingestion studies in ApoE k.o. mice were available [14, 15]. The article on hand will give further descriptive insights in the oxidation of phytosterols and transport of its products in humans and ApoE$^{-/-}$ mice. As result of the evaluation in humans and mice, selective oxidation of sitosterol in peripheral tissue could be a protective measure to control local plant sitosterol concentrations.

Material and Methods

This article summarizes the results of our survey on absolute phytosterol and oxyphytosterol (OPS) concentrations and the ratio of oxyphytosterol-to-substrate (OPS-to-substrate) concentrations of plasma and aortic valve tissue of human patients that underwent an aortic valve cusp replacement due to severe aortic stenosis. Selective phytosterol oxidation and transport of its oxidation products from the aortic valve cusps tissue into the circulation are investigated. Correlations of phytosterols and oxyphytosterols in plasma and tissue and between plasma and tissue, as well as the relationship of phytosterols and oxyphytosterols in patients with coronary artery diseases (CAD) found in the very same data set have already been recently published [16, 17]. In a follow-up survey, the assumptions encountered on the basis of the human data were also tested in an ApoE $^{-/-}$ knockout mouse model.

Human study

In this study 104 patients (36 females/68 males) between 40 to 87 years of age (BMI 28.4 ± 6.2 kg/m^2) were included. They were admitted to the hospital for elective aortic valve replacement due to severe aortic stenosis. At admission, a detailed medical history was taken with special attention to established cardiovascular risk factors and statin treatment. Table 1A and 1B refer to the individual absolute plant sterol and oxyphytosterol levels in human serum and human aortic valve cusp tissues comparing gender specific differences and patients with or without diabetes type 2 (Fig. 1A), statin versus non-statin users, as well as smokers and non-smokers (Fig. 1B).

Venous blood samples were obtained the day before the scheduled aortic valve replacement. Blood samples were centrifuged immediately after blood drawing for 5 min at 4000 rpm. Per milliliter plasma 0.25 mg butylated hydroxytoluene (BHT) was added as an antioxidant to avoid autoxidation during sample storage. The plasma samples were stored at -20 °C until analysis. Aortic cusps were removed from aortic rings during the operation and kept frozen at -80 °C until sample work-up. Excised valve cusps were dried in a Savant™ SpeedVac™ concentrator (Thermo Fisher Scientific, Schwerte, Germany) for 24 h and the calcified parts mechanically sorted from the valve cusps tissue as described in detail by our group [17]. The study

protocol was approved by the institutional and governmental guidelines of the University Hospital of Saarland, Homburg, Germany.

Animal study

Male apoE-/-(C57/Bl6 genetic background) mice, 8-12 weeks of age, weighing 20-25 g were purchased from Charles River, Sulzfeld, Germany. The 10 apoE-/- mice were fed a "Western-type" diet (40 kcal% butterfat, 0.15% (w/w) cholesterol, 0.054 +/- 0.006 µg/mg campesterol and 0.084 +/- 0.009 µg/mg sitosterol.). Diets were prepared by the SNIFF Company (Soest, Germany). Over the 4 week study period mice had ad libitum access to water and chow. Each day of the study period the mice were intraperitoneally injected with 250 µL of 30% cyclodextrin dissolved in 0.9% sodium chloride solution. After 4 weeks the animals were sacrificed by intravenous injection of a mixture of ketamin (1 g/kg body weight)/xylazin (0.1 g/kg body weight). Venous blood samples were drawn from the abdominal vena cava with EDTA blood plasma monovettes (Sarstedt, Nümbrecht, Germany). Plasma was separated by centrifugation at 3500 rpm for 10 min at 18 °C (Heraues multifuge 4KR centrifuge, Osterode, Germany). To avoid autoxidation 250 µg butylated hydroxytoluene (solved in methanol) were added to 1 mL plasma. Livers, brains and descending aorta were excised and washed in an ice-cold phosphate buffer solution. Plasma and organs were stored at -70 °C. Aliquots of the organs were dried in a Savant™ SpeedVac™ concentrator (Thermo Fisher Scientific, Schwerte, Germany) for 12 h at 4 °C. Animal experiments were performed in accordance with the German animal protection law

Phytosterol and oxyphytosterol extraction and GC-MS determination

The phytosterols campesterol and sitosterol and the oxyphytosterols (7α-OH-, 7β-OH- and 7keto-campesterol/sitosterol) were extracted from tissue aliquots (dry weight) with 10 mL Folch-reagent (chloroform/methanol; 3:1; v:v) per mg tissue. Butylated hydroxytoluene (BHT) (0.25 mg added per mL solvent) was used as antioxidant and added prior to extraction [14, 16, 17]. Extraction was performed for 48 h at 4 °C in a dark cold room. The extracts were kept at -20 °C until analysis. One mL plasma and two mL of the Folch extract of valve cusps were used for sterol and oxyphytosterol determination in the human experiment. Fifty microliters of mouse plasma and two mL of the Folch extract of liver and brain tissue was used for sterol and oxyphytosterol determination in the human experiment. After alkaline hydrolysis

phytosterols were extracted by cyclohexane and OPS using dichloromethane in two different work-up processes. Phytosterols and oxyphytosterols in both, the human and animal study, were derivatized to the corresponding trimethylsilyl- (TMSi-) ethers and determined by gas chromatography - mass selective detection (using epicoprostanol for phytosterols and using the corresponding deuterium labeled oxyphytosterols as internal standards for oxyphytosterols) as described in detail previously [18, 19].

Statistical analyses

Data were tested for normal and Gaussian distribution. Differences for phytosterols and oxyphytosterols between gender, statin and non-statin users, patients with and without diabetes mellitus type 2, as well as smokers and non-smokers were tested by unpaired, two-tailed Student´s t-test. Differences of the mean of phytosterols, oxyphytosterols and the ratio of oxyphytosterol-to-substrate in plasma and tissue of the human population and ApoE knockout mice were tested by unpaired, two-tailed Student´s t-test. The data are reported as mean ± SD. All statistical tests were performed with SPSS 21 (Chicago, Illinois, U.S.A.) software.

Results

In the whole patient population there was no influence of gender, diabetes mellitus, smoking habits, and statin treatment on phytosterol and oxyphytosterol concentrations in the plasma and tissue observed (see baseline characteristics of the patient population listed in Table 1A and 1B).

Preferential oxidation of sitosterol in the circulation of humans and ApoE knockout mice

The average sitosterol concentrations in human plasma and aortic valve tissue were determined with 68.8% and 77.8% of the concentrations of campesterol. Contrary to the findings for phytosterols, the ratios of the total oxidized-sitosterol-to-sitosterol compared to the ratio of total oxidized-campesterol-to-campesterol were 3.27-fold and 1.42-fold higher in plasma and tissue in humans (see also Table 2 and Table 3). Based on previous findings in this patient collective oxyphytosterols appear to be formed *in situ* [17]. As less sitosterol will reach the tissue we would expect lower oxysitosterol serum and tissue levels if there is no differentiation between the oxidation rates of campesterol to sitosterol. However, serum and tissue levels of absolute oxysitosterol levels were 2.4 and 1.12-fold higher than the oxycampesterol levels ($p<0.001$ for both). This indicates a preferred oxidation for sitosterol. Corrected levels of oxysitosterol appear even more significantly higher. Oxyphytosterol concentrations correct for the substrate are used as a marker for the degree of oxidation in dependency of the present substrate level.

To test this assumption based on findings in the human population and to achieve information about other tissues, we evaluated phytosterol and oxyphytosterol concentrations in plasma, brain and liver tissue of ApoE knockout mice (Table 2 and 3) Like in the human study, sitosterol concentrations in plasma, liver and brain are lower than campesterol concentrations in ApoE knockout mice. The ratio of total 7-oxygenated-sitosterol-to-sitosterol, compared to the ratio of total 7-oxygenated-campesterol-to-campesterol were 4.10-, 2.03-, 3.70-fold higher in plasma, liver and brain, respectively. In addition, the animal data also support the assumption that in particular sitosterol is more suspended to oxidative processes than campesterol in humans and ApoE (-/-) mice.

Preferential transport of sitosterol oxidation products from tissue into the circulation

Absolute oxyphytosterol concentrations and the ratio of oxyphytosterol-to-substrate were determined in plasma and tissue samples from the human patient collective (Table 2 and Table 3). In aortic valve cusps tissue, the absolute 7α-OH-, and 7β-OH-campesterol concentrations and their ratio OPS-to-substrate are significantly higher compared to the concentrations of the corresponding sitosterol oxidation products. Contrary to the human aortic valve cusps tissue, the absolute concentrations and the ratios of OPS-to-substrate of 7β-OH-sitosterol in plasma are significantly higher than that of 7β-OH-campesterol.

However, considering the mainly local origin of 7β-OH-sitosterol in the valve cusps tissue, our results could still indicate that a preferable transport of 7β-OH-sitosterol from the aortic valve cusp tissue into the blood circulation could exist; and that this causes the variations of both 7β-oxyphytosterol concentrations between plasma and tissue. In addition, the individual ratios of 7α-OH-, 7β-OH-, 7keto-sitosterol to their corresponding campesterol oxidation products in human plasma are significantly higher than those found in aortic valve cusp tissue (Table 3).

To validate these results found in the human patient collective, absolute phytosterol concentrations and the ratios phytosterol-to-substrate in plasma, liver and brain of 10 ApoE knockout mice were examined (see Table 2 and Table 3). In ApoE knockout mice, we found absolute brain and liver tissue concentrations of 7α-OH- and 7β-OH-campesterol significantly higher than those of the corresponding 7-oxygenated-sitosterols. In brain tissue, we found that 7keto-sitosterol is 1.39-fold increased. Considering the relatively high standard deviation of the 7keto-phytosterols in the brain and the fact that the sum of oxy-campesterol are not significantly different from the oxy-sitosterol levels these data in total should not be overinterpretated.

Discussion

In plasma and tissue of both, humans and ApoE (-/-) mice, we found the same pattern of the different 7-oxygenated phytosterols. Our results give evidence that on one hand, sitosterol is selectively oxidized and on the other hand, its oxidation products (especially 7β-OH-sitosterol) are preferably transported from tissue into the circulation in human and mice. Summarized, our findings allow the hypothesis that selective oxidation of sitosterol and preferred transport of its oxidation products from peripheral tissue could be considered as pathway mechanism to reduce sitosterol concentrations in peripheral body tissues.

The transport and metabolism of phytosterols within the human body is not yet completely understood. A selective mechanism that is preferentially oxidizing individual phytosterols at position C7 is not described until the present day. The results of our human and animal studies show, despite the fact of significant lower sitosterol concentrations in plasma and tissues, that absolute oxysitosterol concentrations and the sum of the ratio of oxysitosterols-to-sitosterol are higher than the corresponding values of campesterol, its oxidation products, and the ratios of the oxygenated campesterol to campesterol. This finding allows the conclusion that any kind of selective or preferred oxidation especially of sitosterol might exist. However, our data give no insight in which way this oxidation process could be designed. Several mechanisms that can cause a selective oxidation of individual phytosterols can be discussed: 1) By now it has not been documented if oxygenases, which metabolize cholesterol, reveal additional activity for phytosterols. 2) Additionally, it has not been tested yet if other known enzymes, involved in metabolic processes, dispose distinct activity for a ring oxidation of sitosterol. However, regarding the concentration pattern of 7α-OH-, 7β-OH- and 7keto-phytosterols, which is similar to the cholesterol autoxidation pattern, we have to assume that most of the oxyphytosterols in the human circulation originate from an endogenic oxidation process triggered by radicals [20]. This circumstance would strengthen the idea of a non-enzymatic oxidation process. 3) Therefore, a non-enzymatic oxidation process could be discussed. It is generally assumed that phytosterols, like other Δ5-unsaturated sterols are prone to autoxidation by reactive oxygen species (ROS) at

the allylic C7 position [9, 21-23]. Additionally, it is assumed that oxidation by ROS, e. g. superoxide or hydroperoxide, is neither a structure selective nor a stereo- selective process, because ROS lacking any structure that could determine region specificity. Phytosterols from cell plasma membrane areas and internal cell membranes areas could be transported to cell compartments with oxidative activity within one of the organelles of the cytoplasm. However, the cell compartments in which oxidation of phytosterols occur are not know yet and must be identified. In these compartments, possibly microsomes or peroxisomes, phytosterols could undergo oxidative processes. Proteins that selectively enrich sitosterol in cell compartments could exist and lead to locally higher concentrations of sitosterol within this specific cell compartments. In these compartments a non-enzymatic oxidation of all sterols could take place which would result in higher oxidation rates for sitosterol than for campesterol. Reproducible determinations of sterol and oxysterol concentrations in different cell compartments are very difficult. To our knowledge, no comprehensive studies have been performed so far to study the subcellular distribution of phytosterols and oxyphytosterols. Therefore, a selective enrichment of individual phytosterols in different cell compartments and the design of this enrichment mechanism must be demonstrated to support this assumption. 4) Finally, beside other possible oxidation mechanism, it is conceivable that phytosterols absorbed from lipoproteins are directly oxidized after uptake by the cell. However, only more careful research on oxidation processes of phytosterols will give clear insight on the selective sitosterol oxidation process.

Previously, our group could demonstrate that the oxyphytosterols in the aortic valve originate from local oxidation processes in the tissue [17]. In the human study the average aortic valve cusp tissue concentrations of 7β-OH-sitosterol are lower than concentrations of 7β-OH-campesterol (0.019 ± 0.010 ng/mg vs. 0.048 ± 0.030 ng/mg). The animal study supports our finding with higher 7β-OH-campesterol concentrations compared to 7β-OH-sitosterol concentrations in brain (0.14 ± 0.07 ng/mg vs. 0.06 ± 0.01 ng/mg) and liver tissue (0.91 ± 0.79 ng/mg vs. 0.44 ± 0.22 ng/mg), respectively. Considering that 7β-OH-sitosterol concentrations in human and mouse plasma are higher than 7β-OH-campesterol concentrations, these differences suggest that in this tissues explicit transport mechanisms exist that force an efflux of 7β-OH-sitosterol out of the cell. We were able to show that 7β-OH-sitosterol

generates significant oxidative stress in the arterial wall of ApoE (-/-) mice which could reason a selective transport out of the cell as protective mechanism [15].

Despite the fact, that the human diet contains between about 150-450 mg/day phytosterols the physiological phytosterol concentrations are a hundred times lower than that of cholesterol in the human circulation [24]. This circumstance is caused by the low (only 0.5-2.0%) absolute absorption rate of phytosterols from the diet [25]. In addition, Lütjohann et al. showed that the fractional absorption of oxidized campesterols is three times higher than that of oxidized sitosterols [5, 6].

Several physiological mechanisms involved in sterol absorption are identified which collectively collaborate to regulate phytosterol, in particular sitosterol, concentrations in low concentrations ranges in the human circulation: 1) It could been shown that the sterol uptake transporter Niemann-Pick 1 like 1 reveals lower affinity to sitosterol than to cholesterol; 2) the ATP binding cassette transporter ABCG5/G8 at the intestine pumps back absorbed phytosterols into the intestinal lumen [26, 27]. In the liver the same transporter facilitates the transport of sterols into the bile [26, 27]. Generally, this ABCG5/G8 transporter reveals higher affinity to sitosterol than to campesterol which leads to a higher excretion of sitosterol; 3) Sitosterol is only a poor substrate for acyl-CoA:cholesterol acyltransferase 1 which results in lower esterification rates than that of cholesterol [26, 27]. However, despite these effective mechanisms which control phytosterol concentrations in the human circulation, the remaining amounts of the absorbed sitosterol and campesterol are transported within the blood stream to different organs and will be distributed in all kind of different tissues of the organism. Up to now, only little information about the phytosterol storage in peripheral tissue compartments and the return transport of phytosterols for its elimination is known. Oxidation of cholesterol and transport in form of oxy(chole)sterols is a fundamental mechanism for cholesterol homeostasis in the brain and of the reverse cholesterol transport from peripheral tissue [28-31].

Our findings allow the hypothesis that oxidation of sitosterol and especially its transport from peripheral tissues into the blood stream and eventually to the liver could represent a metabolic mechanism that allow the reduction of tissue sitosterol concentrations and return transport for excretion in form of oxysitosterols. Our survey strongly supports this hypothesis by showing selective oxidation of sitosterol and selective transport of its oxidation products from different tissues in ApoE (-/-) mice

and humans. For the first time we can give information about a putative excretion and transport mechanism which, additionally to the already described phytosterol absorption rate lowering mechanism, allows the control of phytosterol concentrations in peripheral tissue.. In conclusion, only more carefully performed experiments on transport, oxidation and intracellular distribution of phytosterols and oxyphytosterols can support our results and confirm the hypothesis of oxidative phytosterol metabolism in peripheral tissue.

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Table

Table 1 A. Absolute and cholesterol corrected plant sterol and absolute oxyphytosterol levels in human serum, stratified for differences by gender and in patients with diabetes mellitus type 2

		gender			diabetes mellitus type 2		
		female (n = 36)	male (n =n 68)	*p*- value[a)]	no typ 2 DM (n = 76)	typ 2 DM (n = 26)	*p*- value[a)]
human serum							
campesterol	[mg/dL]	0.378 ± 0.206	0.360 ± 0.230	*0.692*	0.373 ± 0.242	0.348 ± 0.158	*0.630*
sitosterol	[mg/dL]	0.270 ± 0.145	0.243 ± 0.153	*0.379*	0.259 ± 0.166	0.230 ± 0.094	*0.386*
7α-OH-campesterol	[ng/mL]	0.146 ± 0.259	0.137 ± 0.129	*0.806*	0.134 ± 0.125	0.157 ± 0.301	*0.596*
7α-OH-sitosterol	[ng/mL]	0.062 ± 0.025	0.068 ± 0.052	*0.524*	0.067 ± 0.050	0.065 ± 0.026	*0.907*
7β-OH-campesterol	[ng/mL]	0.204 ± 0.075	0.229 ± 0.106	*0.164*	0.226 ± 0.100	0.207 ± 0.088	*0.389*
7β-OH-sitosterol	[ng/mL]	0.507 ± 0.152	0.518 ± 0.209	*0.768*	0.517 ± 0.206	0.508 ± 0.142	*0.846*
7keto-campesterol	[ng/mL]	0.885 ± 0.315	0.939 ± 0.352	*0.438*	0.943 ± 0.378	0.872 ± 0.190	*0.360*
7keto-sitosterol	[ng/mL]	2.552 ± 0.963	2.451 ± 0.810	*0.575*	2.518 ± 0.922	2.417 ± 0.686	*0.609*
∑ oxy-campesterol	[ng/mL]	1.235 ± 0.430	1.305 ± 0.513	*0.485*	1.303 ± 0.524	1.236 ± 0.368	*0.544*
∑ oxy-sitosterol	[ng/mL]	3.121 ± 1.097	3.037 ± 1.021	*0.702*	3.101 ± 1.119	2.990 ± 0.815	*0.644*
∑ oxyphytosterols	[ng/mL]	4.355 ± 1.390	4.343 ± 1.467	*0.966*	4.405 ± 1.568	4.226 ± 0.995	*0.588*
human aortic valve cusp tissue							
campesterol	[ng/mg]	57.61 ± 42.87	53.41 ± 34.98	*0.591*	52.15 ± 35.52	64.72 ± 43.57	*0.145*
sitosterol	[ng/mg]	45.95 ± 32.74	40.79 ± 25.88	*0.381*	40.76 ± 26.37	49.29 ± 33.80	*0.189*
7α-OH-campesterol	[ng/mg]	0.052 ± 0.031	0.061 ± 0.036	*0.203*	0.058 ± 0.036	0.061 ± 0.032	*0.701*
7α-OH-sitosterol	[ng/mg]	0.011 ± 0.005	0.012 ± 0.006	*0.535*	0.011 ± 0.006	0.012 ± 0.005	*0.814*
7β-OH-campesterol	[ng/mg]	0.043 ± 0.028	0.050 ± 0.031	*0.254*	0.048 ± 0.031	0.050 ± 0.028	*0.762*
7β-OH-sitosterol	[ng/mg]	0.018 ± 0.009	0.019 ± 0.010	*0.503*	0.018 ± 0.010	0.020 ± 0.009	*0.414*
7keto-campesterol	[ng/mg]	0.275 ± 0.116	0.268 ± 0.088	*0.749*	0.266 ± 0.084	0.289 ± 0.131	*0.306*
7keto-sitosterol	[ng/mg]	0.396 ± 0.155	0.390 ± 0.108	*0.812*	0.387 ± 0.104	0.415 ± 0.176	*0.341*
∑ oxy-campesterol	[ng/mg]	0.370 ± 0.156	0.380 ± 0.138	*0.745*	0.372 ± 0.134	0.400 ± 0.170	*0.392*
∑ oxy-sitosterol	[ng/mg]	0.425 ± 0.165	0.421 ± 0.117	*0.883*	0.417 ± 0.112	0.446 ± 0.186	*0.338*
∑ oxyphytosterols	[ng/mg]	0.795 ± 0.313	0.800 ± 0.230	*0.917*	0.789 ± 0.221	0.846 ± 0.349	*0.331*

[a)]Student´s t-test unpaired

Table

Table 1 B. Absolute and cholesterol corrected plant sterol and absolute oxyphytosterol levels in human serum, stratified for statin-users versus non-users and smoker versus non-smoker

		statin treatment			smoking		
		no statin (n = 36)	statin (n = 68)	*p*- value[a)]	non smoker (n = 56)	smoker (n = 48)	*p*- value[a)]
human serum							
campesterol	[mg/dL]	0.396 ± 0.275	0.350 ± 0.188	*0.374*	0.352 ± 0.219	0.382 ± 0.225	*0.494*
sitosterol	[mg/dL]	0.295 ± 0.207	0.229 ± 0.103	***0.081***	0.246 ± 0.141	0.259 ± 0.161	*0.668*
7α-OH-campesterol	[ng/mL]	0.185 ± 0.277	0.118 ± 0.104	*0.175*	0.112 ± 0.086	0.175 ± 0.253	*0.109*
7α-OH-sitosterol	[ng/mL]	0.070 ± 0.048	0.064 ± 0.043	*0.482*	0.062 ± 0.031	0.070 ± 0.056	*0.339*
7β-OH-campesterol	[ng/mL]	0.239 ± 0.098	0.210 ± 0.095	*0.155*	0.189 ± 0.075	0.257 ± 0.107	***0.000***
7β-OH-sitosterol	[ng/mL]	0.525 ± 0.149	0.509 ± 0.209	*0.681*	0.483 ± 0.155	0.552 ± 0.221	*0.064*
7keto-campesterol	[ng/mL]	0.976 ± 0.394	0.892 ± 0.305	*0.234*	0.862 ± 0.286	0.989 ± 0.385	*0.058*
7keto-sitosterol	[ng/mL]	2.632 ± 0.947	2.411 ± 0.814	*0.220*	2.368 ± 0.775	2.627 ± 0.947	*0.130*
∑ oxy-campesterol	[ng/mL]	1.399 ± 0.584	1.220 ± 0.417	*0.111*	1.163 ± 0.349	1.421 ± 0.582	***0.010***
∑ oxy-sitosterol	[ng/mL]	3.228 ± 1.100	2.984 ± 1.012	*0.263*	2.913 ± 0.914	3.250 ± 1.163	*0.103*
∑ oxyphytosterols	[ng/mL]	4.627 ± 1.527	4.203 ± 1.372	*0.156*	4.076 ± 1.212	4.670 ± 1.614	***0.035***
human aortic valve cusp tissue							
campesterol	[ng/mg]	54.43 ± 35.72	55.09 ± 39.03	*0.932*	54.36 ± 39.16	55.44 ± 36.42	*0.885*
sitosterol	[ng/mg]	45.37 ± 26.78	41.09 ± 29.29	*0.467*	41.32 ± 28.37	44.03 ± 28.64	*0.630*
7α-OH-campesterol	[ng/mg]	0.052 ± 0.036	0.061 ± 0.034	*0.198*	0.053 ± 0.032	0.064 ± 0.038	*0.115*
7α-OH-sitosterol	[ng/mg]	0.011 ± 0.005	0.012 ± 0.006	*0.813*	0.011 ± 0.004	0.012 ± 0.007	*0.253*
7β-OH-campesterol	[ng/mg]	0.042 ± 0.029	0.051 ± 0.031	*0.187*	0.044 ± 0.027	0.052 ± 0.033	*0.151*
7β-OH-sitosterol	[ng/mg]	0.017 ± 0.009	0.019 ± 0.010	*0.338*	0.018 ± 0.008	0.019 ± 0.011	*0.347*
7keto-campesterol	[ng/mg]	0.280 ± 0.096	0.265 ± 0.099	*0.461*	0.261 ± 0.106	0.282 ± 0.088	*0.286*
7keto-sitosterol	[ng/mg]	0.401 ± 0.082	0.388 ± 0.144	*0.620*	0.380 ± 0.134	0.406 ± 0.115	*0.297*
∑ oxy-campesterol	[ng/mg]	0.375 ± 0.156	0.377 ± 0.138	*0.934*	0.358 ± 0.147	0.398 ± 0.138	*0.158*
∑ oxy-sitosterol	[ng/mg]	0.429 ± 0.094	0.418 ± 0.153	*0.703*	0.409 ± 0.142	0.438 ± 0.126	*0.274*
∑ oxyphytosterols	[ng/mg]	0.804 ± 0.241	0.796 ± 0.272	*0.879*	0.767 ± 0.280	0.836 ± 0.233	*0.178*

[a)] Student´s t-test, unpaired

Table

Table 2) Absolute phytosterol and oxyphytosterol concentrations in plasma and tissue of humans (n=104) and ApoE knockout mice (n=10).

	campesterol	sitosterol	ratio[a)]	*p*- value[c)]	7α-OH-campesterol	7α-OH-sitosterol	ratio[b)]	*p*- value[c)]	7β-OH-campesterol	7β-OH-sitosterol	ratio[b)]	*p*- value[c)]	7keto-campesterol	7keto-sitosterol	ratio[b)]	*p*- value[c)]	Σ oxy-campesterol	Σ oxy-sitosterol	ratio[b)]	*p*- value[c)]
	[mg/dL]	*[mg/dL]*			*[ng/mL]*	*[ng/mL]*			*[ng/mL]*	*[ng/mL]*			*[ng/mL]*	*[ng/mL]*			*[ng/mL]*	*[ng/mL]*		
human plasma	0.37 ± 0.22	0.25 ± 0.15	**0.68**	*< 0.001*	0.14 ± 0.18	0.07 ± 0.04	**0.46**	*< 0.001*	0.22 ± 0.10	0.51 ± 0.19	**2.32**	*< 0.001*	0.92 ± 0.34	2.49 ± 0.86	**2.71**	*< 0.001*	1.28 ± 0.48	3.07 ± 1.04	**2.40**	*< 0.001*
ApoE (-/-) mice plasma	11.31 ± 2.71	3.39 ± 0.96	**0.30**	*< 0.001*	84.71 ± 49.39	47.72 ± 21.48	**0.56**	*0.005*	273.7 ± 158.1	405.1 ± 151.8	**1.48**	*0.010*	264.9 ± 137.0	294.8 ± 206.9	**1.11**	*0.392*	614.9 ± 284.6	742.8 ± 322.1	**1.21**	*0.133*
	[ng/mg]	*[ng/mg]*			*[ng/mg]*	*[ng/mg]*			*[ng/mg]*	*[ng/mg]*			*[ng/mg]*	*[ng/mg]*			*[ng/mg]*	*[ng/mg]*		
human aortic valve tissue	54.86 ± 37.74	42.57 ± 28.39	**0.78**	*< 0.001*	0.06 ± 0.03	0.01 ± 0.01	**0.20**	*< 0.001*	0.05 ± 0.03	0.02 ± 0.01	**0.39**	*< 0.001*	0.27 ± 0.10	0.39 ± 0.13	**1.45**	*< 0.001*	0.38 ± 0.14	0.42 ± 0.14	**1.12**	*< 0.001*
ApoE (-/-) mice liver	0.21 ± 0.05	0.07 ± 0.02	**0.33**	*< 0.001*	0.77 ± 0.76	0.09 ± 0.06	**0.11**	*0.013*	0.91 ± 0.79	0.44 ± 0.22	**0.48**	*0.036*	1.88 ± 1.10	1.76 ± 0.70	**0.94**	*0.445*	3.56 ± 2.61	2.29 ± 0.91	**0.64**	*0.047*
ApoE (-/-) mice brain	0.07 ± 0.01	0.02 ± 0.00	**0.33**	*< 0.001*	0.13 ± 0.08	0.02 ± 0.01	**0.16**	*0.003*	0.14 ± 0.07	0.06 ± 0.01	**0.44**	*0.009*	1.08 ± 0.22	1.50 ± 0.69	**1.39**	*0.027*	1.33 ± 0.28	1.58 ± 0.70	**1.18**	*0.211*

[a)] ratio of sitosterol to campesterol

[b)] ratio of 7-oxidized sitosterol to corresponding 7-oxidized campesterol

[c)] *P* -values calculated with Student´s t-test, two tailed, unpaired

Table

Table 3) Ratio of 7-oxygenated phytosterol concentrations to the corresponding substrate concentration in plasma and tissue of humans (n=104) and ApoE knockout mice (n=10).

	r_7α-OH-campesterol	r_7α-OH-sitosterol	ratio[a)]	p-value[b)]	r_7β-OH-campesterol	r_7β-OH-sitosterol	ratio[a)]	p-value[b)]	r_7keto-campesterol	r_7keto-sitosterol	p-value[b)]	p-value	r_Σ oxy-campesterol	r_Σ oxy-sitosterol	ratio[a)]	p-value[b)]
human plasma	0.04 ± 0.05	0.03 ± 0.02	**0.69**	*0.001*	0.07 ± 0.04	0.24 ± 0.11	**3.26**	*<0.001*	0.32 ± 0.19	1.16 ± 0.52	**3.63**	*<0.001*	0.44 ± 0.24	1.43 ± 0.62	**3.27**	*< 0.001*
ApoE (-/-) mice plasma	0.78 ± 0.45	1.58 ± 0.96	**2.02**	*0.002*	2.46 ± 1.48	12.65 ± 5.92	**5.15**	*< 0.001*	2.33 ± 1.17	8.43 ± 5.41	**3.62**	*0.002*	5.49 ± 2.56	22.51 ± 9.50	**4.10**	*< 0.001*
human aortic valve tissue	1.27 ± 0.74	0.33 ± 0.17	**0.26**	*< 0.001*	1.01 ± 0.55	0.50 ± 0.23	**0.49**	*< 0.001*	6.99 ± 4.50	12.35 ± 6.73	**1.77**	*<0.001*	9.26 ± 5.13	13.18 ± 6.93	**1.42**	*< 0.001*
ApoE (-/-) mice liver	3.39 ± 3.08	1.18 ± 0.65	**0.35**	*0.019*	4.06 ± 2.74	6.33 ± 2.06	**1.56**	*0.008*	8.89 ± 4.51	25.64 ± 8.77	**2.88**	*< 0.001*	16.34 ± 10.00	33.15 ± 10.13	**2.03**	*< 0.001*
ApoE (-/-) mice brain	1.96 ± 1.19	1.02 ± 0.45	**0.52**	*0.046*	2.18 ± 1.10	2.93 ± 0.92	**1.35**	*0.195*	16.83 ± 4.50	73.00 ± 47.58	**4.34**	*0.003*	20.77 ± 5.34	76.84 ± 48.65	**3.70**	*0.004*

[a)] ratio of 7-oxidized sitosterol-to-substrate to corresponding 7-oxidized campesterol-to-substrate

[b)] p-values calculated with Student´s T-test, two tailed, paired