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The Relationships of Plasma Plant Sterols and Cholesterol Metabolism with Coronary Artery Disease and Mortality (The LUdwigshafen RIsk and Cardiovascular Health Study)

Doctoral Thesis

submitted in partial fulfilment of the requirements for the degree of

Doctor medicinae universae et scientiae medicae
Dr. med. univ. et scient. med.

of the Medical University of Graz, Austria

by

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2009

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I hereby declare that the work presented in this Doctoral thesis, entitled "The Relationships of Plasma Plant Sterols and Cholesterol Metabolism with Coronary Artery Disease and Mortality (The LUdwigshafen RIsk and Cardiovascular health study)", is entirely my own and that I did not use any sources or auxiliary means other than those referenced.

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List of Abbreviations

ACAT	acyl-CoA cholesterol acyltransferase
ABC	ATP binding cassette transporter
ANOVA	analysis of variance
BHT	butylated hydroxytoluene
CAD	coronary artery disease
CoA	coenzyme A
CRP	C-reactive protein
EMM	estimated marginal mean
FDA	Food and Drug Administration
FS	Friesinger Score
GC	gas chromatograph
GCMS	gas chromatography and mass spectrometry
GLM	general linear model
HDL	high density lipoprotein
HMG	hydroxymethyl-glutaryl
HR	hazard ratio
IDL	intermediate density lipoprotein
IPP	inositol-pyrophosphat
MSTFA	N-methyl-N-(trimethylsilyl)-trifluoroacetamide
LDL	low density lipoprotein
LURIC	Ludwigshafen Risk and Cardiovascular health
LXR	liver X receptor
MS	mass spectrometer
mRNA	messenger ribonucleic acid
NCEP	National Cholesterol Education Program
NPC1L1	Niemann-Pick C1 like 1

OR	odds ratio
SD	standard deviation
SEM	standard error of the mean
SIM	single ion monitoring mode
SRBI	scavenger receptor of class B type 1
SREBP	sterol regulatory element binding protein
VLDL	very low density lipoprotein
TMCS	trimethylchlorosilane

1. Introduction

1.1. Coronary artery disease, lipid lowering and the use of plant sterols

Coronary artery disease (CAD) is the main cause of premature death in industrialized countries (1) with prevalence still rising. For Germany an annual increase of 280 000 cases is being predicted (2).

Elevated total- and low density lipoprotein- (LDL)-cholesterol represent major cardiovascular risk factors (3). There is broad evidence that lowering of total and LDL-cholesterol is beneficial to cardiovascular health (4). Hence, research based guidelines for primary and secondary prevention of CAD by cholesterol lowering were published in the United States (National Cholesterol Education program, (NCEP)) (5, 6).

For more than fifty years phytosterols (7), plant-derived cholesterol analogues, have been known as cholesterol lowering agents (8, 9). Today, phytosterols are increasingly used as pharmaceutically active ingredients in functional foods (10).

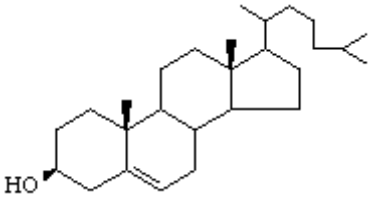
Support is provided by a health claim of the Food and Drug Administration, US Department of Health Health and Human Services (FDA) (11).

1.2. Sterols, structure and nomenclature

Sterols are substances containing a fused cyclopentanophenanthrine ring plus an alcohol moiety on position three of the sterol ring. Position three can be glycosilated, acylated, and esterificated. Cholesterol is the most important sterol (*Figure 1*). Non-cholesterol sterols are divided into metabolites of cholesterol synthesis, degradation products of cholesterol, and plant sterols. Plant sterols are structurally similar to cholesterol only differing in the side chain. They have either an additional methyl or ethyl group at the carbon-24 position or an additional double bond in the side chain. Campesterol and sitosterol are the most abundant phytosterols (12) (*Figure 1*). Lathosterol, an important metabolite of cholesterol synthesis, has a double bond in position 7-8 in place of the 5-6 double bond of cholesterol (13) (*Figure 1*). Cholestanol, a faecal steroid, is the 5-alpha reduced correlative of cholesterol (14). Campestanol and sitostanol represent the 5-alpha reduced homologues of campesterol and sitosterol, respectively (12). Epicoprostanol, which is a faecal steroid such as cholestanol, is the structural epimer of cholestanol (15).

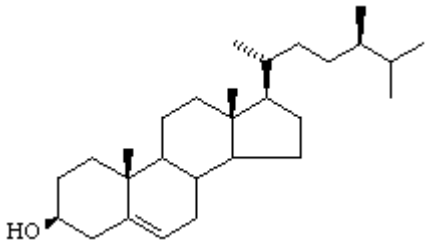
Figure 1: Structure and nomenclature of important sterols

CHOLESTEROL/ 5-CHOLESTEN-3β-OL/ CHOLESTERIN/ 3β-HYDROXY-5-CHOLESTENE



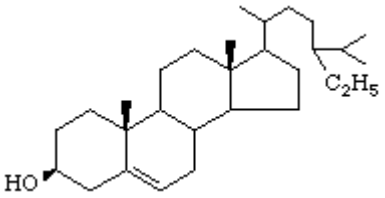
Formula C_{27} H_{46} O
Molecular Weight 386.65

CAMPESTEROL/ 5-CHOLESTEN-24α-METHYL 3β-OL/ ERGOST-5-EN-3β-OL



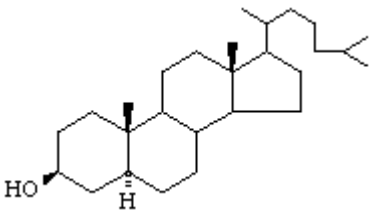
Formula C_{28} H_{42} O
Molecular Weight 394.63

SITOSTEROL/ 5-CHOLESTEN-24β-ETHYL-3β-OL / BETASITOSTEROL/ CINCHOL /24α-ETHYLCHOLESTEROL/ β-SITOSTEROL



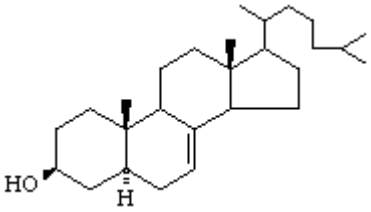
Formula C_{29} H_{50} O
Molecular Weight 414.71

CHOLESTANOL/ β-CHOLESTANOL/ 5α-CHOLESTAN-3β-OL/ DIHYDROCHOLESTEROL/ 3β-HYDROXY-5α-CHOLESTANE



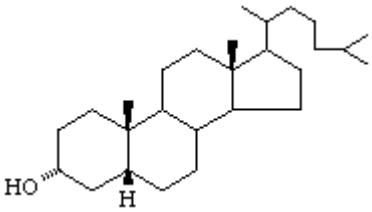
Formula C_{27} H_{48} O
Molecular Weight 388.67

LATHOSTEROL/ γ-CHOLESTENOL/ 3β-HYDROXY-5α,7-CHOLESTENE



Formula C_{27} H_{46} O
Molecular Weight 386.65

EPICOPROSTANOL/ EPICOPROSTEROL/ 5β-CHOLESTAN-3α-OL



Formula C_{27} H_{48} O
Molecular Weight 388.67

Modified according to (16)

1.3. Cholesterol and plant sterol metabolism

1.3.1. Lipoproteins

Sterols are transported in the blood in the form of various lipoproteins (chylomicrons, chylomicron remnants, very low density lipoproteins (VLDL)s, VLDL remnants/intermediate density lipoproteins (IDL)s, LDLs, high density lipoproteins (HDL)s). Dietary sterols enter the blood stream via the intestine as a part of chylomicrons. The chylomicrons are hydrolyzed by the endothelial-bound lipoprotein lipase and the resulting chylomicron remnants are taken up into the liver. Contrary, sterols are released into the blood by the liver as VLDLs. These VLDLs are again hydrolyzed by the lipoprotein lipase to VLDL remnants/IDLs. VLDL remnants are either taken up into the liver or are further metabolized to LDLs. Cholesterol is delivered to all body cells in the form of LDLs. Via HDLs cholesterol is carried from all body cells to the liver (17).

1.3.2. Cholesterol synthesis

Contrary to plant sterols cholesterol can be synthesized in the human body (18). The liver considered to be the primary organ for cholesterol homeostasis is only responsible for about 15 % of de novo cholesterol synthesis. About 85 % of cholesterol synthesis occurs in extrahepatic organs (19).

The process has five major steps:

1. Acetyl-CoenzymeAs (CoA)s are converted to 3-hydroxy-3-methylglutaryl-CoA (HMG-CoA)

2. HMG-CoA is converted to mevalonate

3. Mevalonate is converted to the isoprene based molecule, isopentenyl-pyrophosphate (IPP), with the concomitant loss of CO_2

4. IPP is converted to squalene

5. Squalene is converted to cholesterol.

The rate limiting step is the conversion of HMG-CoA into mevalonate by the enzyme HMG-CoA-reductase (20). In the conversion from squalene to cholesterol, a very important metabolite is lathosterol. Plasma concentrations of cholesterol precursors indicate cholesterol biosynthesis (13, 21). Newly synthesized cholesterol is released into the circulation incorporated in VLDLs (17).

1.3.3. Cholesterol and plant sterol absorption

Sterols are absorbed in the small intestine (22). Bile acids are important for the emulsification of dietary and biliary sterols forming sterol-laden lipid micelles. Solubilized in micellar form and interacting with the enterocyte brush border sterols are absorbed with active and passive processes being involved (23). The detailed mechanisms of cholesterol absorption are not fully understood. Scavenger receptors of class B type I (SRBI) (24) and the Niemann-Pick C1-like 1 protein (NPC1L1) (25) seem to play a crucial role in the intestinal sterol uptake.
Within the enterocyte sterols are esterified by the acyl-CoA-cholesterol acyltransferase (ACAT) and integrated into chylomicrons at the basolateral membrane. As part of chylomicrons sterols enter the circulatory system and reach the liver and other tissues (23).

Unesterified sterols are resecreted into the intestinal lumen by heterodimer members of the ATP-binding cassette transporter (ABC) family, ABCG5 and ABCG8 (26-28).
About 75 % of cholesterol in the intestinal lumen is derived from the bile and only 25 % from the diet. Approximately 50 % of intestinal cholesterol and most of intestinal bile acids are absorbed (29). In contrast, less than one % of dietary plant sterols is absorbed and retained (30, 31).
Cholestanol and plant sterols, such as campesterol and sitosterol, are used as surrogate markers of cholesterol absorption (13, 14, 32).

1.3.4. Cholesterol and plant sterol elimination

Binding lipoproteins via LDL-receptors and SRBIs, the liver removes excessive sterols from the blood (17). In the liver surplus cholesterol is either transformed into bile acids or excreted directly into bile for faecal elimination (23).
Contrary to cholesterol plant sterols are not metabolized to bile acids (33). Since the sterol transporter proteins ABCG5 and ABCG5 are not only expressed in the intestine but also in the liver plant sterols are secreted much faster into bile than cholesterol (34).

1.4. Plasma concentrations of sterols and stanols

Average serum total cholesterol concentration is about 200 mg/dl (35, 36). In comparison, serum total plant sterol concentration in healthy individuals is less than one mg/dl (37-44). The serum lathosterol and cholestanol concentrations are similar to the serum campesterol and sitosterol levels (38-40, 42-44) (*Table 1*). Both, serum campestanol and sitostanol concentrations are about 10 µg/dl (43). Epicoprostanol is not found in the circulation.

Table 1: Serum concentrations of campesterol and sitosterol in different studies

	Total sterols	Campesterol	Sitosterol
Glueck et al. (40) [1]	260 (56)	0.21 (0.16)	0.30 (0.16)
Sutherland et al. (41) [1]	265 (41)	0.46 (0.36)	0.34 (0.35)
Rajaratnam et al. (39) [2]			
cases	230 (5)	0.61 (0.04)	0.33 (0.02)
controls	220 (5)	0.49 (0.02)	0.27 (0.01)
Sudhop et al. (40) [1]			
cases	242 (31)	0.50 (0.17)	0.40 (0.11)
controls	242 (46)	0.38 (0.16)	0.31 (0.11)
Wilund et al. (41) [2]			
cases	185 (2)	0.28 (0.09)	0.16 (0.06)
controls	181 (1)	0.28 (0.09)	0.17 (0.04)
Assmann et al. (42) [1]			
cases	255 (46)	0.40 (0.28)	0.20 (0.14)
controls	228 (39)	0.36 (0.21)	0.18 (0.09)
Fassbender et al. (43) [1]			
cases	235 (43)	0.32 (0.22)	0.30 (0.18)
controls	234 (46)	0.36 (0.20)	0.34 (0.17)
Pinedo et al. (44) [1, 3]			
cases	253 (48)	0.31 (0.21 - 0.44)	0.21 (0.15 - 0.28)
controls	243 (47)	0.32 (0.23 - 0.44)	0.21 (0.17 - 0.29)

Values are means (mg/dl) and standard deviations (SD)[1], standard error of the means (SEM) [2], or medians and 95 % confidence intervals [3]; cases: subjects are symptomatic of CAD (clinical diagnosis, coronary calcium) or have a family history of CAD; controls: subjects do not have CAD.

1.5. Occurrence of plant sterols and stanols in foods

Plant sterols occur in the food in approximately the same quantity as cholesterol. Daily consumption, similarly to cholesterol, is assumed at about 170 to 440 mg (45). The main source of dietary plant sterols is vegetable oil. In less concentrated form plant sterols are found in nuts, breads, and whole vegetables (46) (*Table 2*). Plant stanols have been found in hydrogenated vegetable oils and cereals (47, 48).

Table 2: Phytosterol contents of foods

Food	Phytosterols (mg/100g)
Corn oil	952
Sunflower oil	725
Safflower oil	444
Soybean oil	221
Olive oil	176
Almonds	143
Beans	76
Corn	70
Wheat	69
Palm oil	49
Lettuce	38
Banana	16
Apple	12
Tomato	7

modified according to (46).

1.6. Determinants of cholesterol absorption and synthesis

1.6.1. Interrelations of cholesterol and glucose metabolism

Glucose and cholesterol metabolism are closely interrelated. Insulin upregulates lipogenesis via stimulation of liver X receptors (LXR)s. These LXRs represent transcription factors of the nuclear receptor superfamily (49). Sterol regulatory element binding proteins (SREBP)s are involved in the control of lipogenesis and cholesterol synthesis via the insulin and LXR pathway (50). Of note, insulin was shown to induce the expression of the *ABCG5* and *ABCG8* genes in the intestine and in the liver resulting in decreased intestinal cholesterol absorption and increased cholesterol excretion into bile (51). Furthermore, dietary carbohydrate modification was demonstrated to have an impact on cholesterol homeostasis via enhanced insulin secretion (52). Interestingly, changes in cholesterol metabolism also affect glucose homeostasis. Statins for example were described to improve insulin action (53, 54). Moreover, polymorphisms in *ABCG5* were found to be associated with insulin resistance (55).

1.6.2. Cholesterol metabolism in the metabolic syndrome and type 2 diabetes

Prospective data have revealed that central and total obesity, high triglycerides and low HDL-cholesterol, hypertension, and impaired glucose tolerance predict the development of type 2 diabetes and CAD (56-59). Due to the clustered incidence of these risk factors the “metabolic syndrome” was defined as a discrete disorder (60). Individuals with the metabolic syndrome or type 2 diabetes were found to have an altered cholesterol homeostasis. Patients with the metabolic syndrome show increased cholesterol synthesis (61). Obesity is characterized by low cholesterol absorption and increased cholesterol synthesis (62). In particular, visceral obesity strongly affects cholesterol metabolism (63). In individuals with type 2 diabetes increased body weight is associated with low cholesterol absorption and high cholesterol synthesis (64). Weight reduction in subjects with type 2 diabetes induces increased cholesterol absorption (65). In non-diabetics high normal blood glucose correlates with low intestinal absorption and high synthesis of cholesterol (66). Insulin resistance is associated with increased cholesterol synthesis and decreased cholesterol absorption (67). Subjects with type 2 diabetes compared to healthy controls display decreased cholesterol absorption and increased cholesterol synthesis (68, 69).

1.6.3. Cholesterol metabolism in type 1 diabetes

Subjects with type 1 diabetes were demonstrated to have an increased absorption and a decreased synthesis of cholesterol compared with type 2 diabetics (70) or with non-diabetic controls (71, 72). These findings were supported by a study in children with type 1 diabetes and healthy controls (73). Furthermore, the expression of hepatic and intestinal messenger ribonucleic acid (mRNA) of *ABCG5* and *ABCG8* was found to be decreased in rats with streptozotocin induced diabetes compared to controls resulting in an increased cholesterol absorption rate (74)

1.6.4. Cholesterol metabolism and the use of statins

Under statin treatment the synthesis of cholesterol is suppressed and the absorption of cholesterol is increased (75). Baseline cholesterol metabolism predicts the effectiveness of simvastatin to decrease the concentration of serum cholesterol. Synthesis is suppressed more effectively in subjects with high endogenous synthesis of cholesterol compared to individuals with low synthesis of cholesterol (76). Thus, subjects with high synthesis and low absorption of cholesterol are responders to statin treatment whereas those with low

synthesis and high absorption are non-responders. In agreement, an elevated baseline serum cholestanol concentration was found to predict an increased risk of recurrent coronary events under simvastatin treatment in a subgroup of Scandinavian Simvastatin Survival Study (77).

1.6.5. Cholesterol metabolism and systemic inflammation

High C-reactive protein (CRP) is correlated with low plasma plant sterol levels and an increased plasma lathosterol concentration suggesting that cholesterol metabolism and systemic inflammation are interrelated (78). In addition, plant sterol containing functional foods were demonstrated to decrease serum CRP (79).

1.7. Plant sterols and stanols as lipid lowering drugs

1.7.1. Background

Phytosterols were first discovered to reduce serum cholesterol in the 1950s (8, 80). It was not until 1995 that esterificated plant stanols were introduced as cholesterol lowering agents in Benecol™ margarine by the Finnish company Raisio. Unesterified plant sterols and stanols have low lipid solubility. Esterification of plant sterols or stanols increases their lipid solubility so that they can be incorporated into foods (80). Since 1995 many plant sterol and stanol containing products have been brought onto the market and this process is ongoing. Becel pro-activ™ margarine was launched by Unilever, Heart Wise™ orange juice by Coca Cola, Healthy Heart Nature Valley™ granola bars and Healthy Heart™ yoplait yogurts by General Mills and the Cocoa Via™ chocolate bar by Master Foods (80). The distribution of plant sterol or stanol containing nutriceuticals is supported by a Health Claim of the FDA (11).

1.7.2. Mechanism of action

Plant sterols and cholesterol are incorporated into mixed micelles in the intestinal tract. Competitively inhibiting the incorporation of cholesterol into the mixed micelles plant sterols interfere with the intestinal absorption of cholesterol (81, 82). Of note, plant sterols do not necessarily need to be consumed with every meal to achieve maximum cholesterol lowering. Therefore, dietary intake of plant sterols is speculated to induce the expression of the intestinal cholesterol efflux transporters *ABCG5* and *ABCG8* (83).

1.7.3. Dosage

A daily intake of 2 to 2.5 grams is recommended (9).

1.7.4. Effects

With two grams of plant sterols or stanols consumed per day an average LDL-cholesterol reduction of 21 mg/dl, 17 mg/dl, and 13 mg/dl can be achieved in people aged 50-59, 40-49, and 30-39, respectively (9, 84, 85). Furthermore, plasma cholesterol is more efficiently lowered by a combined plant sterol or stanol and statin regimen compared to a single statin therapy (86). In apolipoprotein E or LDL receptor deficient mice antiatherogenic effects of phytosterol treatment have repeatedly been demonstrated (87-89). Furthermore, evidence suggests that plant stanol treatment improves carotid artery compliance (90). However, to this date, prospective clinical data with hard cardiovascular endpoints investigating if the use of plant sterols will reduce cardiovascular risk are not available.

1.7.5. The use of plant sterol margarines and plasma plant sterol concentrations

Due to the consumption of plant sterol and stanol containing margarines serum plant sterol and plant stanol concentrations are forced up, respectively. In long term users of about 1 gram of plant sterols per day the ratios of campesterol and sitosterol to cholesterol significantly increased by 103 % and 22 %, respectively. Similarly, serum sitostanol and campestanol significantly increased by 197 % and 196 %, respectively, in long term users of plant stanols (91). In accordance, plasma sitosterol and campesterol levels were reported to be elevated in users of soybean oil sterol margarine and significantly decreased in users of sitostanol ester containing margarine (85).

1.8. Sitosterolaemia

1.8.1. Epidemiology

Since the discovery of Phytosterolaemia/Sitosterolaemia in 1974 (92) not more than 100 cases have been reported. A cumulated incidence of this rare disease is observed in the population of Amish Mennonites (93).

1.8.2. Genetics, pathomechanism, and plasma sterol concentrations

Sitosterolaemia is an autosomal recessive disorder (93). Causative mutations occur in either the *ABCG5* or *ABCG8* gene each of which encodes a sterol half-transporter expressed in the intestine and in the liver (26-28). Due to their genetic defect sitosterolaemic patients display an increased absorption of plant sterols and are unable to sufficiently excrete the plant sterols into bile (94-96). Furthermore, sitosterolaemic patients have decreased hepatic HMG-CoA reductase activity (97) and increased hepatic LDL-receptor binding (98). In addition, the activities of two enzymes important for the synthesis of bile acids, the sterol 27-hydroxylase and the cholesterol-7-alpha-hydroxylase, are decreased in patients with homozygous sitosterolaemia (97, 99). Because of the homozygote mutation causing a loss of function in *ABCG5* or *ABCG8* sitosterolaemic patients show more than 50-fold elevated plasma plant sterol levels compared to healthy subjects (92). According to a multicenter study average serum/plasma levels of sitosterol, campesterol, and cholesterol are 20.26 mg/dl, 10.36 mg/dl, and 215 mg/dl, respectively (100). However, data on serum cholesterol concentrations in sitosterolaemic patients are controversial. Actually, serum cholesterol concentration is assumed not to be affected by this genetic defect (92). Yet, about 50 % of individuals with sitosterolaemia were reported to also have increased plasma cholesterol concentrations (101). Furthermore, there are case reports about children with sitosterolaemia suffering from severe hypercholesterolemia (102, 103) (*Table 3*).

Table 3: Plant sterol and total sterol concentrations in subjects with sitosterolemia

	Sitosterol (mg/dl)	Campesterol (mg/dl)	Total sterols (mg/dl)
Bhattacharyya et al. (92)	27.1	9.7	193
	17.7	8.2	206
Salen et al. (109)	16.8	10.7	251
Lütjohann et al. (111)	42.8	34.7	
	20.5	10.9	
Stalenhoef AF (112)	39.4	13.1	
Wang et al. (113)	28.2		418
	47.7		456
	18.4		357
	22.3		278
Solca et al. (114)	26.4		166

1.8.3. Phenotypic heterogeneity

The main clinical features of sitosterolaemia are tissue and tendon xanthomas (92, 102-115), especially on the Achilles tendon and on the extensor tendons of the hands. These xanthomas contain plant sterols and can even be found in children who are younger than ten years (92). Xanthelasmas are reported as well (113). Sitosterolaemic patients often develop serious atherosclerotic lesions leading to premature CAD (105, 109, 110, 113) suggesting that high plasma levels of plant sterols might be harmful. Of note, lethal atherosclerosis at the age of 18 has been reported in an individual suffering from sitosterolaemia (109). Moreover, cerebrovascular disease, arthritis, and arthralgias have been associated with sitosterolaemia (113, 114). There are several case reports about sitosterolaemic patients showing hypochromic anaemia with red cell abnormalities, thrombocytopenia (110, 113), and hypersplenism (105). In another case report sitosterolaemia was speculated to play a causal role in the pathogenesis of Mediterranean stomatocytosis or Mediterranean macrothrombocytopenia. This is an idiopathic haematological condition characterized by stomatocytic haemolysis combined with the presence of very large platelets (116). Interestingly, similar haematological abnormalities have been observed in children under soya-based intravenous feeding regimes which are rich in phytosterols (117). Moreover, sitosterolaemia might cause chronic active liver disease (118).

To sum up, in addition to xanthomas and premature CAD, subjects with sitosterolaemia may show hypochromic anaemia, thrombocytopenia, hypersplensim, chronic active liver disease, cerebrovascular disease, xanthelasmas, arthritis, and arthralgia.

1.8.4. Treatment

The basic therapy of sitosterolaemia is a diet low in (plant-) sterols (margarines/nuts/ chocolate/seeds) (102, 119, 120). Bile acid resins, such as cholestyramine or colestipol, are effective in the reduction of plasma sterol concentrations in patients with sitosterolaemia. Studies indicate that with 8-15 grams of cholestyramine per day alone or combined with a diet low in sterols plasma plant sterol levels can be reduced by up to 50 % (102, 119, 120). Statins are not useful in the treatment of sitosterolaemia (119). The cholesterol absorption inhibitor ezetimibe has been shown to be most effective to achieve a reduction of plasma plant sterol concentrations in sitosterolaemic patients. In a placebo controlled clinical trial sitosterolaemic patients either were treated with ezetimibe (10 mg per day) or received placebo. Whereas plasma sitosterol was significantly decreased by 24 % in the ezetimibe group no significant changes in the plasma sitosterol concentration were observed in the placebo group (100). The effectiveness of ezetimibe in the treatment of patients with sitosterolaemia is supported by a case report. An eleven year old sitosterolaemic female

presenting prominent xanthomas in the subcutaneous tissue of both elbows, bilateral carotid bruits, systolic murmur at the left upper sternal border, and a platelet count of 111000/µl was treated with ezetimibe (10 mg per day) in addition to an ongoing cholestyramine regimen. Within one year plasma sitosterol and campesterol levels decreased by approximately 50 %, carotid bruits completely resolved, systolic murmur diminished, platelet count rose to 268000/µl, and the tuberous xanthomas on the elbows completely disappeared (121). Another therapeutic option to treat sitosterolaemia is surgery. Partial ileal bypass surgery interrupts the enterohepatic circulation of bile acids and interferes with cholesterol absorption (122). However, since with ezetimibe a potent cholesterol absorption inhibitor is available partial ileal bypass surgery cannot be recommended. In severe chronic active liver disease caused by mutations in the *ABCG5* or *ABCG8* gene orthotopic liver transplantation is indicated (118).

1.9. Plasma plant sterols, cholesterol metabolism and CAD

1.9.1. Background

Because patients with sitosterolaemia often develop severe premature atherosclerosis plant sterols are suspected to be atherogenic (105, 109, 110, 113). Importantly, plasma plant sterols are forced up by the consumption of plant sterol margarines (86). Therefore, there are concerns if the pharmaceutical use of plant sterols in the prevention of coronary artery disease is safe.

1.9.2. Pathomechanisms by which plant sterols might promote atherogenesis

First, lipoproteins rich in phytosterols are suggested to be exceedingly susceptible to tissue uptake (92, 105). Thus, high plasma phytosterols might account for increased plaque formation. Second, plant sterols, are less resistant to oxidation than cholesterol (123). Of note, oxidized lipoproteins are regarded as mediators of the atherosclerotic inflammatory process (124).

1.9.3. Clinical studies on plasma plant sterols and CAD

1.9.3.1. Clinical studies revealing associations of high plasma plant sterols with CAD

In the first study investigating the role of plant sterols in plaque formation, plant sterol deposits have been found in aortic tissue of postmortem adults and infants. Of note, plant sterol levels were about ten times higher in tissue samples of mature atheromatous plaques compared to normal aortic tissue samples (125).

More exactly, the relationships between the concentrations of plant sterols in serum and atherosclerotic plaques have been described in a study involving 25 patients undergoing carotid endarterectomy. The ratios of plant sterols to cholesterol in serum were positively correlated with those in tissue samples of atherosclerotic plaques (126).

These findings have been extended and confirmed by another clinical trial in 82 subjects who underwent aortic valve replacement surgery. The plasma plant sterol to cholesterol ratios were significantly associated with the tissue plant sterol to cholesterol ratios of the aortic valves. In addition, subjects who had consumed plant sterol margarines before aortic valve replacement had increased plasma levels of plant sterols. As a result of high plasma plant sterol concentrations, plant sterol content of the aortic valves was also increased in frequent users of sterol margarines. Of interest, in a subgroup of the study collective high plasma plant sterols were associated with a family history of CAD (127).

In a collective of 595 subjects with hypercholesterolaemia, a subgroup of 506 study participants answered the question on personnel or family history of premature CAD. The plasma campesterol concentration was marginally higher in the subgroup with a personnel or family history of premature CAD compared to the subgroup without CAD (37).

The relationships between non-cholesterol sterols and the severity of coronary atherosclerosis have been investigated in a study involving 44 patients with ischemic heart disease. The plasma lathosterol to sitosterol ratio was inversely related with the number of lesions ≥ 25 % stenosis and the number of lesions ≥ 50 % stenosis. Plasma sitosterol and the sitosterol to cholesterol ratio were significantly associated with an increased number of lesions ≥ 50 % stenosis (38).

Independent associations of plasma non-cholesterol sterol concentrations with CAD were described in a study involving 48 subjects with angiographically verified CAD and 61 age-

matched, healthy controls. The individuals with CAD had increased plasma levels of plant sterols but a decreased plasma concentration of lathosterol compared to the healthy control subjects (39).

An association of elevated plasma plant sterols with a family history of CAD was found in a collective of 53 subjects, who all underwent coronary artery bypass graft surgery. The cohort was divided into two subgroups, one subgroup with a family history of CAD and one subgroup without family history of CAD. The patients with a family history of CAD had significantly higher plasma concentrations of campesterol and sitosterol compared to patients without a family history of CAD (40).

In a subgroup of the Prospective Cardiovascular Münster study high plasma sitosterol was correlated with an increased occurrence of major coronary events in a ten year follow up. A total of 477 study participants comprising 159 subjects being symptomatic of myocardial infarction or sudden coronary death in a ten year follow up and 318 healthy controls were included in the analysis (42).

In participants of The Framingham Offspring Study-Cycle 6 increased plasma plant sterol to cholesterol ratios were associated with the presence of CAD. In contrast, coronary patients had decreased plasma lathosterol to cholesterol ratios. A total of 122 individuals with CAD and 301 healthy matched controls were studied (128).

Moreover, high plasma plant sterol to cholesterol ratios and a low lathosterol to cholesterol ratio were predictive of an increased mortality and recurrence of cardiovascular events in 376 participants of the Drugs and Evidence-BAsed medicine in The Elderly study. The subjects investigated were aged 75 years or older (129).

1.9.3.2. Clinical studies arguing against associations of plasma plant sterols with CAD

The study with the greatest number of participants does not support an association between plasma plant sterol levels and atherosclerosis. In 3252 participants of the Dallas Heart Study plasma plant sterol levels were measured. A total of 2542 subjects who answered the question if they had a family history of CAD obtained an electronic beam computer tomography (EBCT) scan of the heart to quantify the coronary calcium deposits. Campesterol and sitosterol were not associated with a family history of CAD or the EBCT-score (41)

In a subgroup of the European Prospective Investigation of Cancer Norfolk study, a collective of individuals who were healthy at the baseline examination, plasma plant sterol levels were not associated with future CAD. A total of 1131 subjects were studied, 373 subjects being symptomatic of CAD in a six year follow-up and 758 controls who did not develop CAD during the follow-up (44).

1.9.3.3. Clinical studies revealing associations of low plasma plant sterols with CAD

Moderate elevation of serum plant sterol levels was associated with reduced cardiovascular risk in the Longitudinal Aging Study Amsterdam, a prospective study with 1242 participants. The serum sitosterol to cholesterol ratio was significantly increased in subjects without CAD compared to those with CAD (43).

1.9.3.4. Evaluation of clinical data on plant sterols as cardiovascular risk factors

Summing up, clinical evidence on the relationships of serum plant sterol concentrations with CAD is controversial. Nevertheless, most studies indicate a positive association of elevated plasma plant sterol levels with CAD (personal/family history of CAD, incidence of future coronary events, positive coronary angiogram). (37-40, 42, 125-129).

1.9.3.5. The relationships of cholesterol absorption and synthesis with CAD

Plasma plant sterol concentrations are surrogate markers of the intestinal cholesterol uptake (13, 14). Glueck *et al*. and Sutherland *et al.* (37, 38) were the first to suggest atherogenic effects of an increased absorption of sterols. Of note, the hypothesis that high absorption and low synthesis of cholesterol might be a cardiovascular risk factor, has been supported by further studies (39, 128, 129).

The mechanisms by which an increased absorption of sterols might cause atherosclerosis remain unclear. It seems rather improbable that an increased absorption in particular of plant sterols promotes atherogenesis. More likely, a higher cholesterol lifetime burden, as suggested previously (129) might link detrimental changes in cholesterol homeostasis to CAD. In support of this hypothesis, polymorphisms in the *ABCG5* gene, which plays an important role in intestinal cholesterol uptake, were associated with the response to dietary cholesterol (130).

1.9.3.6. Cholesterol homeostasis, plasma plant sterols, and CAD

Efforts to examine a possible causal relationship of plasma plant sterol concentrations with the pathogenesis of CAD have to consider that plasma plant sterols are surrogate markers of cholesterol absorption (13, 14). Of note, changes in cholesterol homeostasis are suggested to be associated with CAD and mortality on their own. Thus, it has to be differentiated between detrimental effects of high plasma plant sterols and adverse effects induced by an unfavourable balance between cholesterol absorption and synthesis.

1.10. Objective

Plant sterols are increasingly used as lipid lowering agents in functional foods (9, 10). Due to the consumption of plant sterol containing margarines plasma plant sterol concentrations are forced up (85, 91). Of note, patients suffering from phytosterolaemia, a rare genetic disorder characterized by up to 100-fold elevated plasma plant sterol levels, often develop severe premature atherosclerosis (105, 109, 110, 113). Therefore, high plasma plant sterols are speculated to be atherogenic and there are concerns if the use of plant sterol containing margarines is safe. Furthermore, evidence suggests that an increased intestinal uptake of sterols might represent a cardiovascular risk factor (37-39, 128, 129).

There is a lack of large epidemiologic studies investigating if moderately elevated plasma plant sterols or an unfavourable balance of cholesterol absorption and synthesis are associated with CAD in individuals without sitosterolaemia. Moreover, previous reports on these issues have been controversial.

Our aim was to study the relationships of plasma plant sterols and cholesterol metabolism with CAD in participants of the Ludwigshafen Risk and Cardiovascular health (LURIC) study (131). Thus, we planned to measure the plasma concentrations of cholestanol (not a plant sterol, marker of cholesterol absorption) (14), campesterol, sitosterol (plant sterols, markers of cholesterol absorption), and lathosterol (marker of cholesterol synthesis) (13) in a subgroup of this large collective. First, we were interested in the associations of plasma plant sterols and cholesterol metabolism with the grade of coronary stenosis determined by angiography. Second, we wanted to find out if plasma plant sterols and cholesterol metabolism predict all-cause- and cardiovascular mortality during a follow up of eight years (131). In order to quantify plasma non-cholesterol sterol levels we had to introduce and validate a novel gas chromatography and mass spectrometry (GCMS) based method.

Our hypothesis was that an increased absorption and a decreased synthesis of cholesterol is an independent cardiovascular risk factor. We assumed that elevated plasma plant sterol levels indicate an increased risk for atherosclerosis. Yet, we thought that a possible positive correlation of plasma plant sterols with CAD was caused by atherogenic effects of high intestinal cholesterol uptake.

2. Methods

2.1. Study design

We studied participants of the LURIC study (131). The LURIC study is a cross-sectional and prospective clinical trial of individuals with and without cardiovascular disease at baseline. The study was approved by the ethics committee of the “Ärztekammer Rheinland-Pfalz” and was conducted in accordance with the “Declaration of Helsinki”. Informed written consent was obtained from all participants.

Study participants were recruited between July 1997 and January 2000 and were numbered by the order of recruitment. They were patients scheduled for coronary angiography at the Herzzentrum Ludwigshafen, a cardiology unit in a tertiary care medical centre in South-West Germany. Subjects in whom diabetes had not been diagnosed previously underwent oral glucose tolerance testing.

In addition to the cross-sectional design there was a follow up of 8.01 years on the vital status. Furthermore, the causes of death were classified.

Plasma non-cholesterol sterol measurement was performed in a randomly selected subgroup of the LURIC cohort at the Clinical Institute of Medical and Chemical Laboratory Diagnostics, Medical University of Graz, Austria between 2005 and 2007.

2.2. Participants

2.2.1 Inclusion and exclusion criteria

2.2.1.1. Inclusion criteria

Individuals had to be Caucasian and of German ancestry living in the South-West of Germany. Except for acute coronary syndromes, the subjects were required to be in a stable clinical condition (i.e. no concomitant acute illness such as infection or recent accident/surgery). The availability of a coronary angiogram was postulated. Indications for angiography in individuals in clinically stable condition were chest pain and/or noninvasive test results consistent with myocardial ischemia. Both, patients with and without statin treatment were included in the study.

2.2.1.2. Exclusion criteria

Subjects suffering from any acute illness other than acute coronary syndrome (other acute cardiac disease, such as decompensated heart failure or decompensated valvular disease; or acute non-cardiac disease, such as infection, endocrine disease or any type of surgery within the previous three months) were excluded from the study. Furthermore, patients with chronic polymorbid disease in which the non-cardiac disease predominated (i.e. chronic renal failure and haemodialysis, severe rheumatoid arthritis, persistent incapacitation after accident/trauma) or with a history of malignant disease within the previous five years and those subjects incapable of understanding the purpose of the study were ruled out. Seven subjects suffering from type 1 diabetes were eliminated from the present cross-sectional and prospective investigations. Individuals who received statin treatment were excluded from the prospective analysis.

2.2.2. Subjects

2.2.2.1. Cross-sectional analysis

Cross-sectional analyses were performed in 2440 of 3316 individuals with coronary angiograms. Preliminary analyses revealed that the relationships of cholesterol homeostasis and the severity of CAD were similar in users of statins and subjects who did not take lipid lowering drugs.

2.2.2.2. Prospective analysis

Prospective analyses were performed in a subgroup of 1257 individuals who did not receive lipid lowering drugs because the associations of cholesterol metabolism with all-cause- and cardiovascular mortality were blunted in users of statins.

2.3. Clinical Characterization

2.3.1. The Friesinger Score

CAD was diagnosed by angiography with maximum luminal narrowing estimated by visual analysis. The severity of CAD was quantified with the Friesinger Score (FS) (132). The FS ranges from 0 to 15. Each of the three main coronary arteries is scored separately from zero to five. The scores are: 0, no angiographic abnormalities; 1, trivial luminal narrowing < 29 %; 2, localized 30-68 % luminal narrowing; 3, multiple 30-68 % luminal narrowing; 4, 69-100 %

luminal narrowing without 100 % occlusion of proximal segments; 5, total obstruction of a proximal segment.

2.3.2. Type 2 diabetes

Diabetes mellitus was diagnosed according to the criteria of the American Diabetes Association (133). Furthermore, individuals with a history of diabetes or treatment with oral antidiabetics or insulin were considered diabetic.

2.3.3. Hypertension

Hypertension was diagnosed if the systolic and/or diastolic blood pressure exceeded 140 mmHg and/or 90 mmHg or if there was a history of hypertension, evident through the use of antihypertensive drugs.

2.3.4. Follow up

The median time of follow up was 8.01 years. Information on vital status was obtained from local person registries. Medical records of local hospitals and death certificates were used for the classification of the causes of death. The deceased were classified into those who died from cardiovascular and non-cardiovascular causes. Three experienced clinicians who were blinded to any data of the study participants independently classified the causes of death. In cases of a disagreement or uncertainty concerning the coding of a specific cause of death, classification was made by one of the principal investigators of LURIC (131).

2.4. Laboratory procedures

2.4.1. Sample collection and storing

All analyses were performed in plasma obtained from fasting blood samples collected before angiography. Plasma was kept frozen at -80 °C between the date of blood withdrawal and the day of the laboratory analyses.

2.4.2. Standard laboratory methods

Total cholesterol and triglycerides were measured enzymatically with reagents from Roche, Mannheim, Germany on a Hitachi 717 analyzer. Plasma glucose was determined using the

enzymatic hexokinase/glucose 6-posphate dehydrogenase method again with reagents from Roche, Mannheim, Germany on a Hitachi 717 analyzer. Ultrasensitive CRP was quantified with an mAb/nephelometric assay on an N Latex CRP mono/Behring nephelometer II obtained from Dade Behring GmbH, Marburg, Germany (131).

2.4.3. Non-cholesterol-sterol measurement

2.4.3.1. Introduction of a new GCMS based method

In order to perform non-cholesterol sterol measurement we introduced and validated a new GCMS based method (134).

2.4.3.2. Target compounds

The target compounds for the present analysis were cholestanol, campesterol, sitosterol, and lathosterol.

2.4.3.3. Materials

Cholestanol, campesterol, and lathosterol were obtained from Steraloids, United States of America and sitosterol from Sigma, Germany. Epicoprostanol and butylated hydroxytoluene (BHT) from Sigma, Germany were used as internal standard and antioxidant, respectively. N-methyl-N-(trimethylsilyl)-trifluoroacetamide (MSTFA) was purchased from ABCR, Germany, trimethylchlorosilane (TMCS) and pyridine from Pierce, United States of America. Silica gel and all solvents and reagents of analytical grade were obtained from Merck, Germany.

2.4.3.4. Instruments and analytical method

A Thermo Trace 2000 gas chromatograph (GC) coupled to a Fisons MD 800 quadrupole mass spectrometer (MS) was used. The column was directly connected to the ion source of the MS. The GC was equipped with a HT5 fused silica capillary column (25 m x 0.22 mm i.d., 0.1 µm film thickness) from SGE. The splitless Grob-injector was kept at 220 °C. Helium was used as carrier gas with a constant flow of 1 mL/min. The initial column temperature was 200 °C for 1 minute, followed by an increase of 15 °C per min to 300 °C, and an isothermal hold of seven minutes. The transfer line between GC and MS was kept at 310 °C. The ion source temperature was 150 °C. Electron impact spectra were recorded with an electron energy of 70 eV and an emission current of 150 µA. Non-cholesterol sterols were analyzed applying electron ionization GCMS, single-ion-monitoring (SIM) mode. The significant ions were m/z

445.4 for cholestanol, m/z 382.4 for campesterol, m/z 357.3 for sitosterol, m/z 458.5 for lathosterol, and m/z 370.4 for epicoprostanol.

2.4.3.5. Sample preparation

A 100 µl portion of the internal standard solution was mixed with 100 µL of plasma. The methanolic internal standard solution contained two nmol of epicoprostanol and 20 µg/100µL of BHT. 250 µL of a KOH-solution (50 % in water) and 800 µL of ethanol absolute were added. The sealed vial was kept at 75 °C for one hour, cooled to room temperature and after addition of 1 mL of water and 2 mL of hexane the content was extracted (10 min, 360 ° - shaker) and centrifuged (3 min, 3000 r/min). The supernatant was decanted and applied to a silica gel column, which had been prewashed with 4 mL of hexane. After preelution using 2 mL of hexane, the target compounds were eluted with 4 mL of hexane/iso-propanol (70/30). The solvent was evaporated under a stream of nitrogen. 50 µL of an MSTFA-solution (MSTFA, containing 1 % TMCS, in pyridine (2/1, v/v)) were added, shaken, and incubated at room temperature for 30 minutes. Subsequently the samples were dried under nitrogen, redissolved in 100 µL hexane, transferred to autosampler vials, sealed, and an aliquot of 2 µL was subjected to GCMS analysis.

2.4.3.6. Validation

All target compounds were reliably quantified in a range from 0.012 to 3.125 nMol per vial (100 µL of plasma). The samples stored below -80 ^{o}C were proved to be stable. No degradation occurred during freezing and thawing. The target compounds could be measured in 50 % sample aliquots.

2.5. Statistical analysis

2.5.1. Cross-sectional analysis

The FS was broken down to four (A-D) categories of the severity of CAD (A = FS 0-1, B = FS 2-4, C = FS 5-8, D = FS 9-15). The clinical and biochemical characteristics of the study participants subdivided according to the four FS categories were expressed as numbers and percentages for categorical variables and as means with standard deviations (SD)s or as medians with interquartile-ranges for continuous variables. Comparisons among the four groups of the FS were made using the χ^2-test or univariate analysis of variance (ANOVA). Adjusted p-values were calculated with logistic regression or the general linear model (GLM)

with covariates as indicated. Ratios of non-cholesterol sterols to cholesterol were calculated to standardize for the variation in the plasma cholesterol concentration. Additionally, ratios of absorption sterols to lathosterol were computed. The distributions of continuous variables were examined for skewness and kurtosis. Crude non-cholesterol sterols, the ratios of non-cholesterol sterols to cholesterol, and the ratios of the absorption sterols to lathosterol were transformed logarithmically. Pearson correlations between non-cholesterol sterols and cholesterol were computed. In addition, Pearson correlations among the non-cholesterol sterol to cholesterol ratios were calculated. Using GLMs in which those factors not under examination were included as covariates the effects of sex, age, BMI, diabetes, hypertension, and statin use on the non-cholesterol sterol to cholesterol ratios were studied. Similarly, GLMs adjusting for sex, age, BMI, diabetes, hypertension, smoking, and statin use were generated to examine the relationships of the FS with the non-cholesterol sterol to cholesterol ratios and the absorption marker to lathosterol ratios. All statistical tests were 2-sided and $p < 0.05$ was considered significant. The SPSS 15.0 statistical package (SPSS Inc., Chicago, United States of America) was used.

2.5.2. Prospective analysis

Tertiles of the non-cholesterol sterol to cholesterol ratios were calculated in the subgroup of the study participants who did not receive statins. The clinical and biochemical characteristics of the study participants subdivided according to the cholestanol to cholesterol tertiles were expressed as numbers and percentages for categorical variables and as means (SD)s or medians with interquartile ranges for continuous variables. Comparisons among the three groups were made with the χ^2 test for categorical data and with ANOVA for continuous variables. Adjusted p-values were calculated with logistic regression or the GLM with covariates as indicated. Survival analyses were performed using the Kaplan-Meier method followed by a log-rank test. To further examine the impact of the non-cholesterol sterol to cholesterol ratios on all-cause- and cardiovascular mortality hazard ratios (HR)s with 95% confidence intervals (CI)s were calculated using the Cox proportional hazards model. In each case the first non-cholesterol sterol to cholesterol tertile was used as the reference. Two models of adjustment were applied, model 1 with the covariates sex and age and model 2 with the covariates sex, age, BMI, diabetes, hypertension, smoking, and CRP. The backward stepwise LR selection method was used and the results of the final step were shown. All statistical tests were 2-sided; $P < 0.05$ was considered significant. The SPSS 15.0 statistical package (SPSS Inc. Chicago, United States of America) was used.

3. Results

3.1. Cross sectional analysis

3.1.1. Baseline characteristics of the cross-sectional analysis

Study participants with FS > 1 were significantly older and rather male than female than those with FS 0-1. The percentage of subjects receiving statins increased in parallel with the FS. Current or past smoking, type 2 diabetes, and hypertension were more prevalent in subjects with high FS. Elevated triglycerides, CRP, and blood glucose and decreased HDL cholesterol were associated with an increased severity of CAD. Due to their more frequent use of statins subjects with high FS had lower total- and LDL-cholesterol compared to individuals without CAD. There were modest differences in BMI among the four FS categories (*Table 4*).

Table 4: The clinical and biochemical parameters of the study participants by the four Friesinger Score categories

Friesinger Score (4 categories)	A (0-1)	B (2-4)	C (5-8)	D (9-15)	P*
N	502	553	793	592	
Age, years	58.1 (11.6)	64.1 (9.8)	63.6 (10.1)	64.9 (9.3)	<0.001[a]
Male sex	255 (50.8)	340 (61.5)	590 (74.4)	491 (82.9)	<0.001[b]
Statin use	97 (19.3)	212 (38.3)	483 (60.9)	391 (66.0)	<0.001
BMI, kg/m2	27.1 (4.2)	27.8 (4.5)	27.5 (4.2)	27.3 (3.5)	0.014
Type 2 diabetes					<0.001
No	418 (83.3)	392 (70.9)	518 (65.3)	358 (60.5)	
Yes, no insulintreatment	77 (15.3)	135 (24.4)	236 (29.8)	181 (30.6)	
Yes, insulintreatment	7 (1.4)	26 (4.7)	39 (4.9)	53 (9.0)	
Fasting blood glucose, mg/dl	104 (30)	111 (33)	115 (34)	119 (40)	<0.001
Hypertension	310 (61.8)	402 (72.8)	606 (76.4)	452 (76.4)	0.001
Systolic blood pressure, mmHg	136 (22)	143 (23)	144 (24)	142 (24)	0.012[c]
Diastolic blood pressure, mmHg	80 (11)	82 (11)	82 (11)	80 (11)	0.003[c]
Smoking					<0.001
Never	267 (53.2)	234 (42.3)	250 (31.5)	154 (26.0)	
Past	152 (30.3)	208 (37.6)	388 (48.9)	334 (56.4)	
Current	83 (16.5)	111 (20.1)	155 (19.5)	104 (17.6)	
Total-Cholesterol, mg/dl	199 (37)	195 (36)	192 (41)	186 (38)	0.132[d]
LDL-Cholesterol, mg/dl	118 (32)	117 (32)	114 (37)	111 (33)	0.028[d]
HDL-Cholesterol, mg/dl	43 (12)	40 (11)	38 (10)	36 (10)	<0.001[d]
Triglycerides, mg/dl	142	151	157	157	0.001[d,e]
Percentile 25, mg/dl	98	107	115	114	
Percentile 75, mg/dl	194	208	202	203	
C-reactive protein, mg/l	2.40	3.50	4.09	4.36	<0.001[d,e]
Percentile 25, mg/l	1.00	1.49	1.46	1.58	
Percentile 75, mg/l	5.87	8.21	9.54	9.93	

Values are means (SD) or medians with inter-quartile ranges in the cases of continuous variables

Values represent numbers (percentages) of subjects in the cases of categorical variables

*GLM or logistic regression, respectively, adjusted for age and gender.

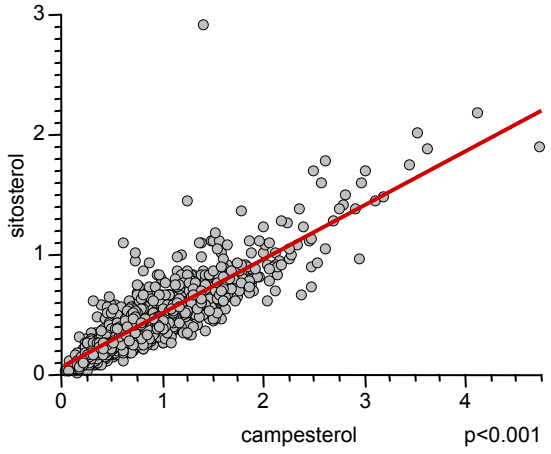
[a]GLM, adjusted for gender only; [b]Logistic regression, adjusted for age only; [c]Additionally adjusted for the use of beta blockers, ACE inhibitors, AT 1 receptor antagonists, calcium channel blockers, diuretics, and statins; [d]Additionally adjusted for the use of lipid-lowering agents; [e]GLM of logarithmically transformed values.

3.1.2. Plasma non-cholesterol sterol concentrations

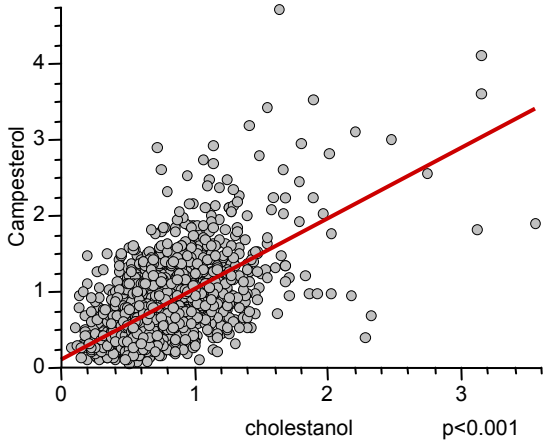
The absolute mean plasma concentrations ± SD of campesterol, sitosterol, cholestanol, and lathosterol were 7.57 ± 4.50 µMol/l, 3.87 ± 2.33 µMol/l, 7.22 ± 3.01 µMol/l, and 7.00 ± 4.81 µMol/l, respectively. All non-cholesterol sterols were positively correlated with total cholesterol ($r = 0.360$, $p < 0.001$; $r = 0.350$, $p < 0.001$; $r = 0.507$, $p < 0.001$; and $r = 0.436$, $p < 0.001$ for campesterol, sitosterol, cholestanol, and lathosterol, respectively). There was a strong positive correlation between the ratios of campesterol and sitosterol to cholesterol ($r = 0.879$, $p < 0.001$) (*Figure 2*). An increase in the ratio of cholestanol to cholesterol ratio went in parallel with high plant sterol to cholesterol ratios ($r = 0.475$, $p < 0.001$ and $r = 0.454$, $p < 0.001$ for campesterol and sitosterol, respectively) (*Figure 2*). The lathosterol to cholesterol ratio was inversely correlated with the ratios of campesterol, sitosterol, and cholestanol to cholesterol ($r = -0.306$, $p < 0.001$; $r = -0.247$, $p < 0.001$; $r = -0.350$, $p < 0.001$, respectively), revealing the expected reciprocal relationship between cholesterol absorption and synthesis.

Figure 2: Bivariate correlations among non-cholesterol sterols

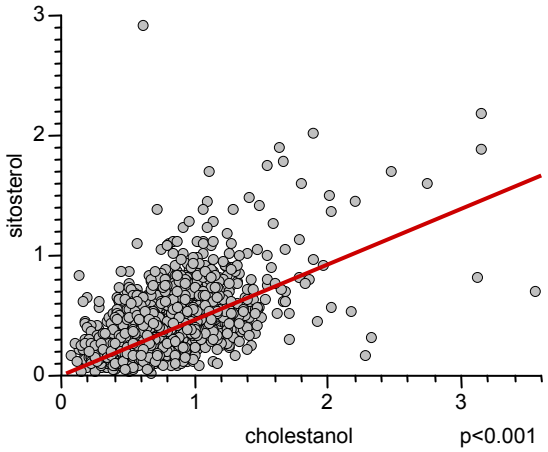
(a) Bivariate correlation between sitosterol and campesterol (arbitrary units)



(b) Bivariate correlation between campesterol and cholestanol (arbitrary units)



(c) Bivariate correlation between sitosterol and cholestanol (arbitrary units)



3.1.3. The associations of non-cholesterol sterol to cholesterol ratios with clinical parameters and statin use

We examined the relationships of non-cholesterol sterol to cholesterol ratios with sex, age, components of the metabolic syndrome, and the use of statins. BMI and statin use were strongly correlated with the non-cholesterol sterol to cholesterol ratios. In the subgroup with high BMI (> 26 kg/m2 and > 27 kg/m2 in males and females, respectively) absorption marker ratios were decreased ($p < 0.001$) and the lathosterol to cholesterol ratio was increased ($p < 0.001$) (*Figure 3*). Statin users had higher plant sterol to cholesterol ratios than non-users ($p < 0.001$) whereas the cholesterol biosynthesis was suppressed, as expected ($p < 0.001$)

(*Figure 4*). The cholestanol to cholesterol ratio was slightly elevated in subjects taking statins (p = 0.105) (*Table 5*).

Figure 3: The relationship of cholesterol absorption and synthesis with BMI

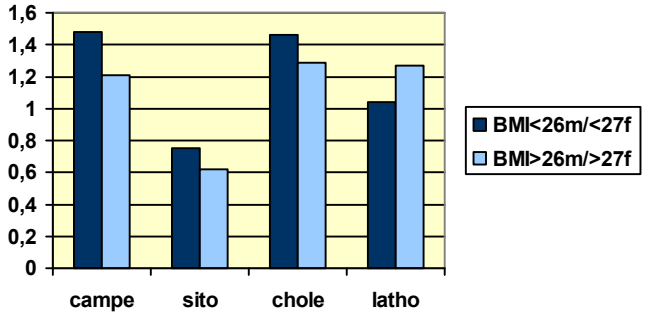
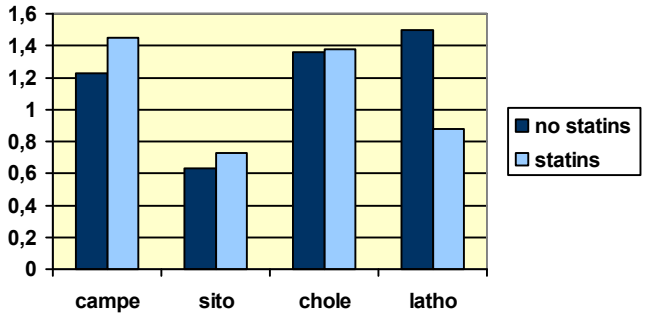


Figure 4: The relationship of cholesterol absorption and synthesis with the use of statins



Provided that patients did not receive insulin treatment, type 2 diabetes was significantly associated with high synthesis and low absorption of cholesterol. Individuals older than 60 years had decreased non-cholesterol sterol to cholesterol ratios compared with those younger than 60 years. Hypertension was associated with increased lathosterol to cholesterol ratio ($p < 0.01$) and male gender was correlated with elevated campesterol to cholesterol ratio (p = 0.006) (*Table 5*).

Table 5: The associations of the non-cholesterol sterol to cholesterol ratios with clinical parameters and statin use

	N	EMM	Diff	P	EMM	Diff	P	EMM	Diff	P	EMM	Diff	P
		Campesterol:Cholesterol			Sitosterol:Cholesterol			Cholestanol:Cholesterol			Lathosterol:Cholesterol		
Gender													
Men	1676	1.36			0.68			1.37			1.17		
Women	764	1.28	-5.8	†	0.67	-1.6	0.462	1.37	-0.2	0.889	1.14	-1.9	0.412
Age, (years)													
<60	874	1.41			0.70			1.39			1.25		
60-70	890	1.30	-8.1	‡	0.68	-2.6	0.284	1.34	-3.9	*	1.15	-7.8	†
>70	676	1.27	-10.1	‡	0.67	-4.1	0.12	1.38	-1.0	0.566	1.06	-14.9	‡
Body mass index, (kg/m2)													
<26♂or27♀	1161	1.49			0.76			1.45			1.05		
>26♂or27♀	1279	1.20	-19.7	‡	0.62	-17.9	‡	1.30	-10.6	‡	1.27	20.4	‡
Diabetes mellitus													
No	1686	1.36			0.70			1.38			1.15		
Yes, no insulin treatment	629	1.24	-9.1	‡	0.64	-9.1	‡	1.32	-4.3	†	1.20	4.6	0.092
Yes, insulin treatment	125	1.40	3.1	0.502	0.68	-2.7	0.566	1.43	3.1	0.321	1.01	-12.6	†
Hypertension													
No	670	1.36			0.71			1.39			1.09		
Yes	1770	1.32	-3.3	0.159	0.67	-5.2	*	1.36	-2.2	0.175	1.19	8.8	†
Statins													
No	1257	1.23			0.64			1.36			1.50		
Yes	1183	1.44	17.0	‡	0.73	15.0	‡	1.39	2.2	0.105	0.88	-41.6	‡

GLM adjusted for each of the other variables; EMM are estimated marginal means, (mmol/mol); Diff indicates the percent change between the respective categories; *P<0.05; † P<0.01; ‡ p<0.001

3.1.4. Non-cholesterol sterol to cholesterol ratios, absorption sterol to lathosterol ratios, and the severity of CAD

High FS went in parallel with increased plasma plant sterol to cholesterol ratios. Compared to FS category A, the campesterol and sitosterol to cholesterol ratios were elevated in the FS category D (+7.8 %, p = 0.026 and +5.7 %, p = 0.110, respectively). The cholestanol to cholesterol ratio was also increased in the FS category D in comparison with the FS category A (+6.6 %, p = 0.006). In contrast, the lathosterol to cholesterol ratio was decreased in the group of patients with a high grade of angiographic CAD versus the group of patients in whom CAD was ruled out (-13.6 %, p = 0.001) (*Table 6*).

Table 6: The associations of the non-cholesterol sterol to cholesterol ratios with the severity of CAD

Friesinger Score (4 categories)	N	EMM (95 % CI)	Difference (%)	P
		Campesterol:Cholesterol		
A (0-1)	502	1.28 (1.22-1.34)		
B (2-4)	553	1.32 (1.27-1.38)	3.6	0.260
C (5-8)	793	1.34 (1.30-1.39)	5.4	0.087
D (9-15)	592	1.37 (1.32-1.43)	7.8	0.026
		Sitosterol:Cholesterol		
A (0-1)	502	0.66 (0.63-0.69)		
B (2-4)	553	0.68 (0.65-0.71)	3.7	0.260
C (5-8)	793	0.69 (0.66-0.71)	3.9	0.232
D (9-15)	592	0.70 (0.67-0.73)	5.7	0.110
		Cholestano:Cholesterol		
A (0-1)	502	1.31 (1.26-1.35)		
B (2-4)	553	1.37 (1.34-1.41)	5.2	0.018
C (5-8)	793	1.39 (1.36-1.42)	6.4	0.004
D (9-15)	592	1.39 (1.35-1.43)	6.6	0.006
		Lathosterol:Cholesterol		
A (0-1)	502	1.28 (1.22-1.35)		
B (2-4)	553	1.15 (1.10-1.20)	-10.5	0.001
C (5-8)	793	1.13 (1.09-1.17)	-12.0	<0.001
D (9-15)	592	1.11 (1.06-1.16)	-13.6	<0.001

GLM, adjusted for gender, age, BMI, type 2 diabetes with and without insulin treatment, hypertension, smoking, and the use of statins; A-D FS categories; N number of patients; EMM are estimated marginal means for the respective non-cholesterol sterol to cholesterol ratios (mmol/mol); CI confidence interval; Difference indicates the percent difference in the non-cholesterol sterol to cholesterol ratios between the FS categories B-D and FS category A.

The ratios of the absorption sterols to lathosterol increased in parallel with the FS. Versus the FS category A, the ratios of campesterol, sitosterol, and cholestanol to lathosterol were increased in the FS category D (+25.0 %, $p < 0.001$; +22.4 %, $p < 0.001$; +13.5 %, $p < 0.001$, respectively) (*Table 7*).

Table 7: The associations of the absorption sterol to lathosterol ratios with the severity of CAD

Friesinger Score (4 categories)	N	EMM (95 % CI)	Difference (%)	P
		Campesterol:Lathosterol		
A (0-1)	502	0.99 (0.92-1.07)		
B (2-4)	553	1.15 (1.07-1.23)	15.8	0.004
C (5-8)	793	1.19 (1.12-1.26)	20.0	<0.001
D (9-15)	592	1.24 (1.16-1.33)	25.0	<0.001
		Sitosterol:Lathosterol		
A (0-1)	502	0.51(0.48-0.55)		
B (2-4)	553	0.59 (0.56-0.63)	15.8	0.003
C (5-8)	793	0.61 (0.57-0.64)	18.1	0.001
D (9-15)	592	0.63 (0.59-0.67)	22.4	<0.001
		Cholestanol:Lathosterol		
A (0-1)	502	1.02 (0.95-1.09)		
B (2-4)	553	1.20 (1.13-1.27)	17.6	<0.001
C (5-8)	793	1.23 (1.17-1.29)	20.9	<0.001
D (9-15)	592	1.26 (1.18-1.33)	23.5	<0.001

GLM, adjusted for gender, age, BMI, type 2 diabetes with and without insulin treatment, hypertension, smoking, and the use of statins; A-D FS categories; N number of patients; EMM are estimated marginal means for the respective absorption sterol to lathosterol ratios (dimensionless). CI Confidence Interval; Difference indicates the percent difference in the absorption sterol to lathosterol ratios between the FS categories B-D and FS category A.

3.2. Prospective analysis

3.2.1. Baseline characteristics of the prospective analysis

Measurement of cholestanol, campesterol, sitosterol, lathosterol, and cholesterol was complete in 1257 of 1706 individuals who did not receive lipid lowering drugs. The campesterol and sitosterol to cholesterol ratios increased in parallel with the cholestanol to cholesterol tertiles. In contrast, the lathosterol to cholesterol ratio was inversely related to the tertiles of the cholestanol to cholesterol ratio. There were no differences in sex and age among the tertiles of the cholestanol to cholesterol ratio. Subjects in the third tertile of the cholestanol to cholesterol ratio had decreased BMI compared with subjects in the first tertile. Type 2 diabetes was most frequent in the tertile with a low cholestanol to cholesterol ratio. The prevalence of systemic hypertension was not associated with the tertiles of the cholestanol to cholesterol ratio. Systolic and diastolic blood pressure, and fasting triglycerides were decreased in third compared to the first tertile of the cholestanol to cholesterol ratio. Individuals in the highest tertile of the cholestanol to cholesterol ratio were more likely to be current smokers. Fasting blood glucose and CRP were positively correlated with the cholestanol to cholesterol tertiles. Plasma total cholesterol, LDL-cholesterol and HDL-cholesterol were not significantly associated with the tertiles of cholestanol to cholesterol (*Table 8*).

Table 8: Baseline characteristics of the study participants without statins by cholestanol to cholesterol tertiles

	Cholestanol to cholesterol tertiles			
	1st tertile	**2nd terile**	**3rd tertile**	**P***
Intervals, mmol/mol	<1.20	1.202-1.545	>1.545	
Number	419	419	419	
Campesterol:Cholesterol, mmol/mol	1.05 (0.49)	1.29 (0.55)	1.92 (1.03)	<0.001
Sitosterol:Cholesterol, mmol/mol	0.55 (0.24)	0.67 (0.28)	0.99 (0.64)	<0.001
Lathosterol:Cholesterol, mmol/mol	2.06 (0.92)	1.78 (1.05)	1.32 (0.78)	<0.001
Age, years	63.0 (10.6)	62.7 (10.8)	62.7 (11.5)	0.907*a*
Male sex	273 (65.2)	269 (64.2)	289 (69.0)	0.300*b*
BMI, kg/m2	28.3 (4.2)	27.1 (4.0)	26.5 (4.1)	<0.001
Type 2 diabetes				0.213
No	273 (65.2)	309 (73.7)	305 (72.8)	
Yes, no insulintreatment	131 (31.3)	87 (20.8)	90 (21.5)	
Yes, insulintreatment	15 (3.6)	23 (5.5)	24 (5.7)	
Fasting blood glucose, mg/dl	104 (30)	111 (33)	115 (34)	0.002
Systemic hypertension	306 (73.0)	310 (74.0)	288 (68.7)	0.208
Systolic blood pressure, mmHg	144 (23)	144 (23)	140 (23)	0.027*c*
Diatolic blood pressure, mmHg	83 (11)	82 (11)	81 (11)	0.031*c*
Smoking				0.197
Never	176 (42.0)	175 (41.8)	162 (38.7)	
Past	177 (42.2)	179 (42.7)	165 (39.4)	
Current	66 (15.8)	65 (15.5)	92 (22.0)	
Total-Cholesterol, mg/dl	199 (35)	203 (35)	201 (38)	0.411
LDL-Cholesterol, mg/dl	120 (31)	124 (30)	125 (34)	0.087
HDL-Cholesterol, mg/dl	40 (11)	41 (11)	40 (12)	0.815
Triglycerides, mg/dl	155	146	125	<0.001*d*
Perzentile 25, mg/dl	113	112	95	
Perzentile 75, mg/dl	210	205	167	
C-reactive protein, mg/l	3.01	3.23	3.59	0.128*d*
Perzentile 25, mg/l	1.24	1.26	1.26	
Perzentile 75, mg/l	7.36	7.83	9.24	

Values are means (SD) or medians with inter-quartile ranges in the cases of continuous variables

Values represent numbers (percentages) of subjects in the cases of categorical variables

*GLM or logistic regression, respectively, adjusted for age and gender.

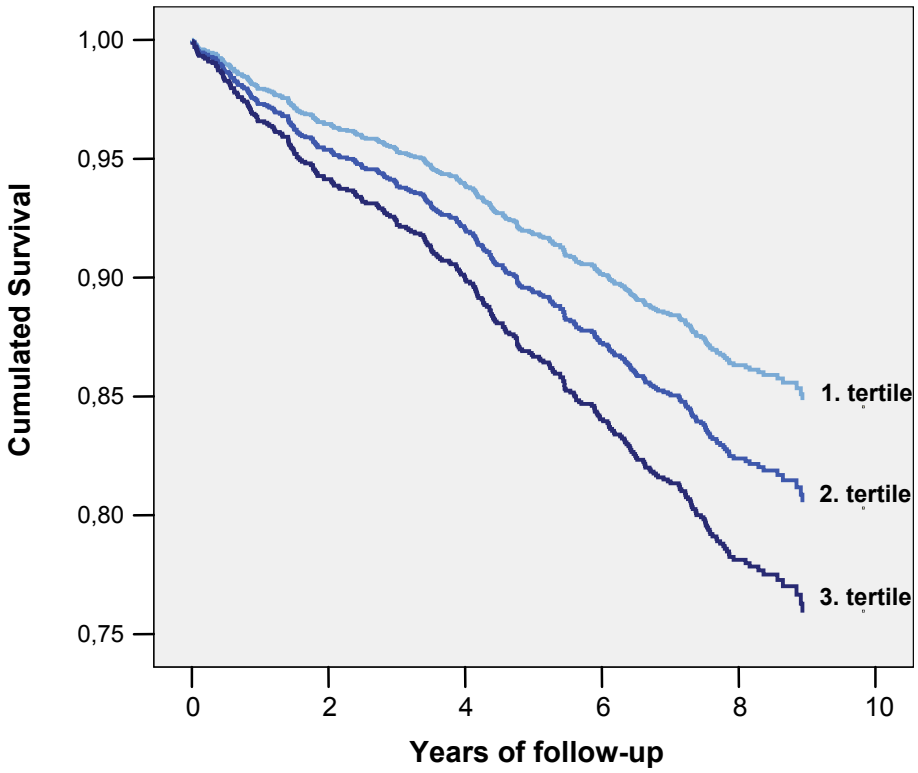
aGLM, adjusted for gender only; ***b***Logistic regression, adjusted for age only;
cAdditionally adjusted for the use of beta blockers, ACE inhibitors, AT 1 receptor antagonists, calcium channel blockers, diuretics, and statins; ***d***GLM of logarithmically transformed values.

3.2.2. Non-cholesterol sterol to cholesterol tertiles and mortality

During a median follow-up time of 8.01 years, 304 (24.2 %) of 1257 LURIC participants who did not take statins and in whom non-cholesterol sterol measurement was complete have died. Of 304 deaths, 192 (63.2 %) were accounted for by cardiovascular events and 112 (36.8 %) deaths were due to non-cardiovascular disease. The Kaplan-Meier curves for all-cause- and cardiovascular mortality by the tertiles of the cholestanol and lathosterol to cholesterol ratios are depicted in the *Figures 5-8*.

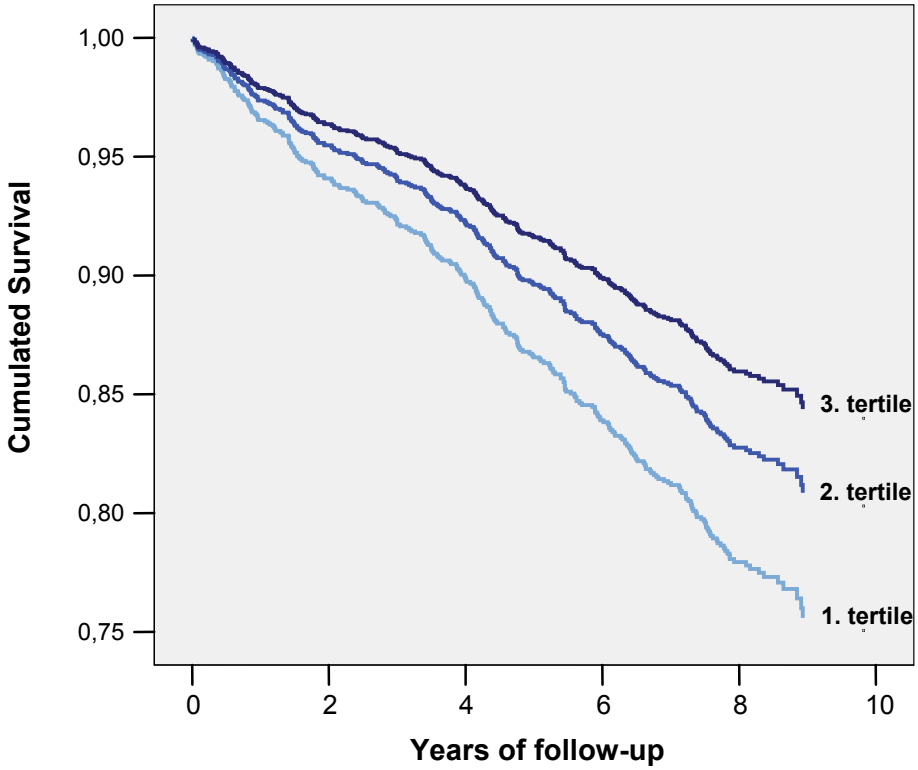
There was a significant association between the tertiles of the cholestanol, lathosterol, and campesterol to cholesterol ratios and the risk of all-cause mortality (*Table 9*, *Figure 5*, *Figure 6*). All-cause mortality increased in parallel with the cholestanol and campesterol to cholesterol tertiles (*Table 9*, *Figure 5*).

Figure 5: All-cause mortality by cholestanol to cholesterol tertiles



In contrast, all-cause mortality was inversely related to the tertiles of the lathosterol to cholesterol ratio (*Table 9*, *Figure 6*). The survival rate was not correlated with the tertiles of the sitosterol to cholesterol ratio.

Figure 6: All-cause mortality by lathosterol to cholesterol tertiles



Comparing the third tertiles of the cholestanol, lathosterol, campesterol, and sitosterol to cholesterol ratios to the respective first tertiles the HRs (CI 95 %) for all-cause mortality adjusted for sex and age were 1.88 (1.41-2.50, $p < 0.001$), 0.54 (0.41-0.73, $p < 0.001$), 1.27 (0.97-1.66, $p = 0.080$), and 0.960 (0.73-1.26, $p = 0.770$), respectively (*Table 9*). After additional adjustment for established cardiovascular risk factors the relationships of the cholestanol and lathosterol to cholesterol tertiles with all-cause mortality remained statistically significant ($p = 0.001$) (*Table 9*). In addition, the associaton of the campesterol to cholesterol tertiles with all-cause moratilty reached statistical significance applying the multivariate model ($p = 0.025$) (*Table 9*).

Table 9: Associations of non-cholesterol sterol to cholesterol ratios with all-cause mortality

		Model 1		Model 2	
	N	HR	P	HR	P
Cholestanol:Cholesterol					
1st quartile	419	1.0 reference		1.0 reference	
2nd quartile	419	1.46 (1.09-1.97)	0.012	1.32 (0.97-1.78)	0.077
3rd quartile	419	1.88 (1.41-2.50)	<0.001	1.68 (1.25-2.25)	0.001
Lathosterol:Cholesterol					
1st quartile	419	1.0 reference		1.0 reference	
2nd quartile	419	0.71 (0.54-0.92)	0.009	0.76 (0.58-0.99)	0.044
3rd quartile	419	0.54 (0.41-0.73)	<0.001	0.61 (0.45-0.82)	0.001
Campesterol:Cholesterol					
1st quartile	419	1.0 reference		1.0 reference	
2nd quartile	419	0.92 (0.69-1.22)	0.561	0.94 (0.71-1.26)	0.696
3rd quartile	419	1.27 (0.97-1.66)	0.080	1.38 (1.04-1.82)	0.025
Sitosterol:Cholesterol					
1st quartile	419	1.0 reference		1.0 reference	
2nd quartile	419	0.85 (0.64-1.11)	0.236	0.92 (0.70-1.21)	0.550
3rd quartile	419	0.96 (0.73-1.26)	0.77	1.06 (0.80-1.41)	0.669

Model 1: Adjusted for gender and age;
Model 2: Adjusted for gender, age, BMI, type 2 diabetes with and without insulin treatment, hypertension, smoking, and CRP;
HR Hazard ratio calculated by Cox regression

In accordance, the cholestanol and lathosterol to cholesterol tertiles were also predictive of cardiovascular mortality (*Table 10*, *Figure 7*, *Figure 8*). The campesterol and sitosterol to cholesterol tertiles were not significantly associated with cardiovascular mortality (*Table 10*).

Figure 7: Cardiovascular mortality by cholestanol to cholesterol tertiles

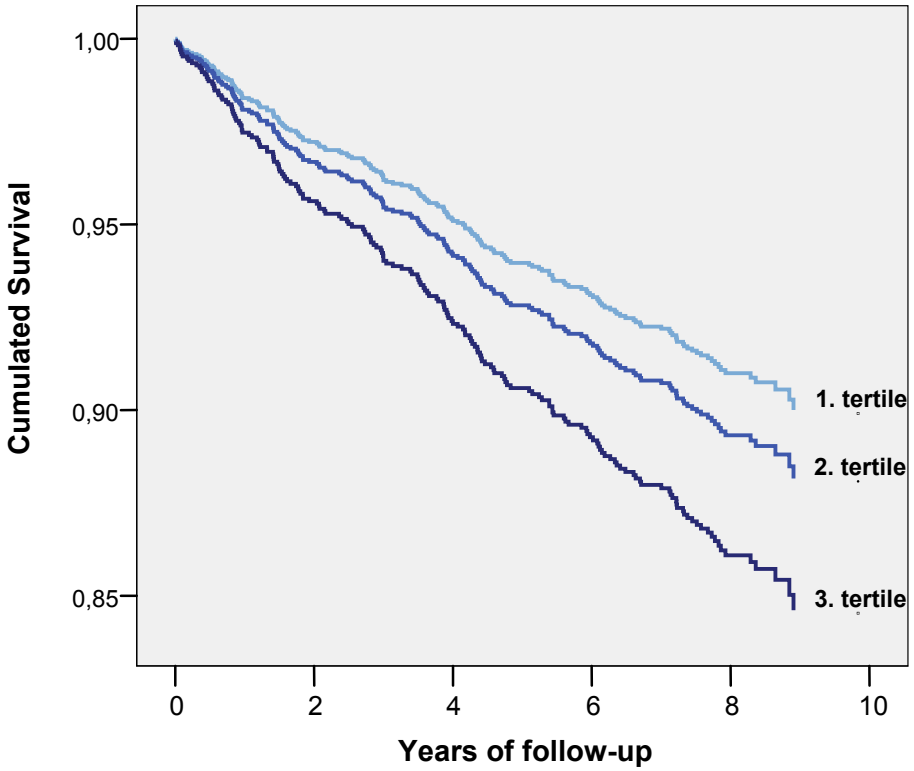
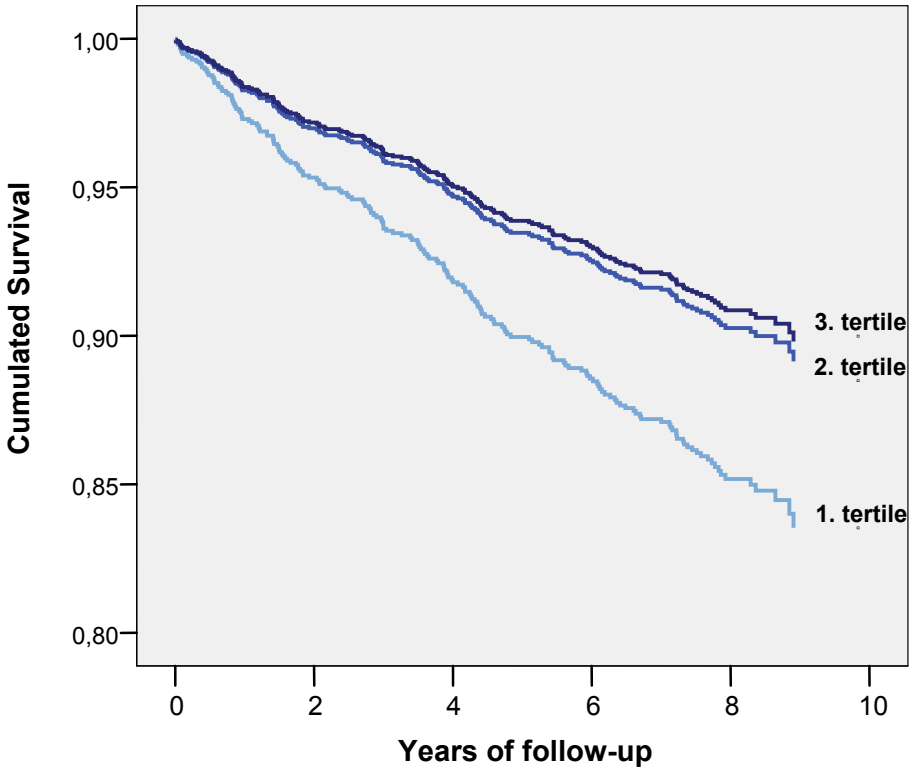


Figure 8: Cardiovascular mortality by lathosterol to cholesterol



Comparing the third tertiles of the cholestanol, lathosterol, campesterol, and sitosterol to cholesterol ratios to the respective first tertiles the HRs (CI 95 %) for cardiovascular mortality adjusted for sex and age were 1.72 (1.21-2.44, p = 0.003), 0.57 (0.40-0.80, p = 0.002), 1.14 (0.81-1.59, p = 0.462) and 0.94 (0.67-1.32, p = 0.728), respectively (*Table 10*). Multivariate adjustment for established cardiovascular risk factors confirmed the significant relationships between the tertiles of the cholestanol and lathosterol to cholesterol ratios and cardiovascular mortality (p = 0.012 and p = 0.006, respectively) (*Table 10*).

Table 10: Associations of non-cholesterol sterol to cholesterol ratios with cardiovascular mortality

		Model 1		Model 2	
	N	HR	P	HR	P
Cholestanol:Cholesterol					
1st quartile	419	1.0 reference		1.0 reference	
2nd quartile	419	1.28 (0.88-1.86)	0.193	1.20 (0.82-1.75)	0.354
3rd quartile	419	1.72 (1.21-2.44)	0.003	1.59 (1.11-2.27)	0.012
Lathosterol:Cholesterol					
1st quartile	419	1.0 reference		1.0 reference	
2nd quartile	419	0.71 (0.43-0.86)	0.004	0.64 (0.45-0.90)	0.011
3rd quartile	419	0.57(0.40-0.80)	0.002	0.60 (0.41-0.87)	0.006
Campesterol:Cholesterol					
1st quartile	419	1.0 reference		1.0 reference	
2nd quartile	419	0.86 (0.60-1.23)	0.408	0.91 (0.64-1.31)	0.617
3rd quartile	419	1.14 (0.81-1.59)	0.462	1.29 (0.90-1.83)	0.162
Sitosterol:Cholesterol					
1st quartile	419	1.0 reference		1.0 reference	
2nd quartile	419	0.82 (0.58-1.16)	0.253	0.91 (0.64-1.29)	0.588
3rd quartile	419	0.94 (0.67-1.32)	0.728	1.06 (0.77-1.56)	0.609

Model 1: Adjusted for gender and age;
Model 2: Adjusted for gender, age, BMI, type 2 diabetes with and without insulin treatment, hypertension, smoking, and CRP;
HR Hazard ratio calculated by Cox regression

4. Discussion

4.1. Concept

The aim of this research was to carefully investigate the relationships of the plasma plant sterol concentrations and cholesterol metabolism with the severity of CAD determined by coronary angiography. Furthermore, it was our objective to study the associations of plasma plant sterols and cholesterol metabolism with all-cause- and cardiovascular mortality. For this purpose, the plasma concentrations of the non-cholesterol sterols were measured in participants of the LURIC study, a cross-sectional and prospective clinical trial (131). To quantify plasma non-cholesterol sterol levels a new analytical method was developed, validated, and applied (134).

4.2. Non-cholesterol sterol measurement

The measurement of the plasma non-cholesterol sterols including the quantification of plasma plant sterols is the only accepted and applicable method to assess cholesterol absorption and synthesis in large clinical studies (13, 14, 135).
To analyze the plasma non-cholesterol sterol concentrations we introduced a new GCMS-based method (134). The validation of the method revealed that the intra-day and inter-day variations of the analytes were within the twenty % range. Thus, plasma non-cholesterol sterols could reliably be quantified. However, applying our newly introduced method the quantification of the plasma non-cholesterol sterol concentrations was extremely time-consuming. Pretreatment of 70 plasma samples cost about ten hours. In addition, the manual integration of the MS peaks took about four hours for 70 samples.
We measured the plasma concentrations of campesterol and sitosterol, which are the most abundant plant sterols. Furthermore, we analyzed the plasma levels of cholestanol and lathosterol. Whereas the plasma levels of plant sterols and cholestanol indicate cholesterol absorption, the plasma lathosterol concentration is a surrogate marker of cholesterol synthesis (13, 14).

4.3. Study collective

The LURIC study is a large cross-sectional and prospective clinical trial. A total of 3316 individuals with and without CAD were recruited. In all participants the coronary status was

determined by angiography. Moreover, there was a follow-up on all-cause- and cardiovascular mortality of 8.01 years (131). The plasma concentrations of the non-cholesterol sterols were quantified in 2440 participants of the LURIC study that were randomly selected.

Cross-sectional analyses were performed in all 2440 individuals with complete non-cholesterol sterol profile comprising 1181 users of statins and 1257 subjects without statin medication. The subgroup of 1181 individuals who took lipid lowering drugs was ruled out from the prospective analysis because the relationships of cholesterol homeostasis and plasma plant sterols with all-cause- and cardiovascular mortality were blunted in users of statins.

The strong points of the LURIC study are the large number and the exact clinical as well as biochemical characterization of the participants. Most precisely, the coronary status was determined by angiography (131). Of note, the present survey on the relationships of plasma plant sterols and cholesterol metabolism with CAD being diagnosed on the basis of coronary angiography and with all-cause- and cardiovascular mortality is the largest so far.

In contrast to the LURIC study, CAD was estimated by EBCT scans in 2542 participants of Dallas Heart Study (41). All other cross-sectional clinical trials on the relationships of non-cholesterol sterols with CAD except from the Longitudinal Aging Study Amsterdam have a sample size of considerably less than one thousand (43). Up to now the major prospective study investigating the impact of cholesterol metabolism and plasma plant sterols on future cardiovascular events and mortality is the European Prospective Investigation of Cancer Norfolk Study comprising 1131 individuals (44).

Some limitations pertain to the LURIC study. The control individuals were recruited at a tertiary referral center, underwent coronary angiography, and therefore may not be representative of a random population sample. However, this may also be regarded as a strength of the study. Importantly, the prevalence of clinically asymptomatic coronary atherosclerosis has been very high in subjects at or older than 50 years of age (136). Hence, angiography based recruitment of controls prevents that individuals with significant, yet clinically unapparent, CAD are incorrectly allocated to the control group. Furthermore, the major cardiovascular risk factors occur at a similar frequency in LURIC controls compared with the general population. The prevalence of hypertension is close to that found in a random probability sample from Germany (137). In contrast, type 2 diabetes appears 2-3 times more frequently in LURIC participants than in the German population (138). However,

this is most likely due to the fact that diabetes was not only diagnosed on the basis of self-reports. In addition, fasting glucose was measured and an oral glucose tolerance test was performed in individuals not previously known to have type 2 diabetes (139). Considering clinical history or fasting glucose measurements, the National Health and Nutrition Examination Surveys 1999–2000 reports prevalence rates of type 2 diabetes mellitus of 9.2 % and 19.3 % in adults aged 40–59 years or older than 60 years, respectively (140). In the current study, 11.4 % of the controls had type 2 diabetes according to this criterion, while another 5.3 % were detected by elevated post-challenge glucose only.

4.4. Plasma non-cholesterol sterol concentrations

The plasma concentrations of the non-cholesterol sterols were in agreement with the data previously shown (41, 43). In accordance with earlier studies the average total plasma plant sterol concentration was about 0.5 mg/dl (41, 43).

All plasma non-cholesterol sterols were positively correlated with the plasma cholesterol concentration. As expected, there was a strong positive association between the ratios of campesterol and sitosterol to cholesterol. Furthermore, high plant sterol to cholesterol ratios went in parallel with increased cholestanol to cholesterol ratio. The lathosterol to cholesterol ratio was inversely related to the ratios of the absorption sterols to cholesterol revealing the well-established reciprocal relationship between cholesterol absorption and synthesis.

Confounding due to the consumption of plant sterol or stanol ester margarines can be ruled out because the LURIC participants were recruited before plant sterol or stanol containing functional foods were brought onto the market in Germany.

Taken together, these findings argue for the validity of our data.

4.5. The relationships of cholesterol metabolism with distinct parameters

We were able to confirm the reported associations of the plasma non-cholesterol sterol to cholesterol ratios with the components of the metabolic syndrome (62-69) and the use of statins (75, 76).

The greatest effects were seen for BMI and statin use. In the subgroup with high BMI the absorption marker to cholesterol ratios were decreased, whereas the lathosterol to cholesterol ratio was increased. Users of statins had low cholesterol synthesis evident through a markedly decreased plasma lathosterol to cholesterol ratio. In contrast, the ratios of campesterol and sitosterol to cholesterol were increased in individuals who received statin medication. These results confirm that fractional cholesterol absorption is increased by the use of statins. Unlike the plant sterol to cholesterol ratios the cholestanol to cholesterol ratio was only slightly elevated in users of statins compared to subjects without statin therapy. However, this has also been observed in a subgroup of the Scandinavian Simvastatin Survival Study (75, 76). Provided that the individulas did not receive insulin treatment, type 2 diabetes was significantly associated with high synthesis and low absorption of cholesterol. Both, the fractional absorption and synthesis of cholesterol, were inversely related to age. This is again in line with previous work (44). Furthermore, male gender was correlated with an increased campesterol to cholesterol ratio in an earlier and in our study (44).

These results also verify the accuracy of the plasma non-cholesterol sterol measurement in the LURIC cohort.

4.6. The relationships of plasma plant sterols and cholesterol metabolism with the severity of CAD, all-cause mortality, and cardiovascular mortality

In the LURIC cohort, high absorption and low synthesis of cholesterol are associated with a more severe coronary atherosclerosis which was determined by angiography. The positive association of the plasma campesterol to cholesterol ratio with CAD rather reflects an increased absorption of sterols than a specific atherogenic role of plant sterols. In agreement with the cross-sectional data, high absorption and low synthesis of cholesterol predict an increased all-cause- and cardiovascular mortality in participants of the LURIC study. However, there is no increase in the mortality which is specifically attributable to high plasma plant sterols.

Up to now, no large study has been published investigating the relationships between cholesterol homeostasis and CAD being diagnosed on the basis of coronary angiography. Furthermore, the impact of cholesterol metabolism on mortality has not been sufficiently clarified. Nonetheless, these questions seem to be of interest since not only the synthesis but also the absorption of cholesterol is amenable to pharmacological manipulation.

Some previous surveys have proposed atherogenic effects of high cholesterol absorption. However, due to a low number of study participants, sometimes restriction of the findings to certain subgroups or an imprecise classification of the coronary status, the results of these studies have not been generally accepted (37-40, 42, 125-129).

Our study provides a sufficiently large and reliable body of data on the relationships of plasma plant sterols and cholesterol homeostasis with CAD and all-cause- and cardiovascular mortality. Coronary angiography was used to diagnose or rule out CAD in a total of 2440 LURIC participants and there was an exact follow-up of 8.01 years on all-cause- and cardiovascular mortality (131).

We attempted to investigate whether the repeatedly observed positive association between plasma plant sterols and CAD reflects atherogenic effects of an increased absorption of sterols or is accounted for by special perils of high plasma phytosterol concentrations. For that purpose, in addition to campesterol and sitosterol, we measured plasma cholestanol which is a surrogate marker of cholesterol absorption but not a plant sterol (14). Both, the plasma plant sterol and the plasma cholestanol to cholesterol ratios increased in parallel to the severity of coronary atherosclerosis. There was an even more pronounced association between cholesterol homeostasis and the severity of CAD when cholesterol absorption and synthesis were estimated by the absorption marker to lathosterol ratios. The relationship of the sitosterol to cholesterol ratio with the FS did not reach statistical significance, which argues against specific atherogenic effects of high plasma plant sterol levels.

In accordance with the cross-sectional analysis, a high plasma cholestanol to cholesterol ratio was associated with a markedly increased all-cause- and cardiovascular mortality. In contrast, a high plasma lathosterol to cholesterol ratio predicted a decreased mortality. Subjects in the third campesterol to cholesterol tertile had an increased all-cause mortality compared to individuals in the first tertile. The ratio of the plasma sitosterol concentration to cholesterol did not significantly correlate with all-cause- or cardiovascular mortality. Hence, we were able to conclude that the relationships of the non-cholesterol sterol to cholesterol ratios with mortality were accounted for by changes in cholesterol homeostasis. Special perils of high plasma plant sterols could be ruled out.

The fact that plant sterols were detected in atherosclerotic plaques does not conflict with our findings because they obviously do not accumulate in the plaque disproportionately to cholesterol (125, 126). Indeed, plant sterols may exert harmful effects when their plasma levels are up to 100-fold elevated (105, 109) but this apparently does not apply to variation

within or close to their physiological range. The existence of very effective secretion pumps for plant sterols in the intestine and the liver underlines that these compounds are unwanted by the human body (26, 27). However, because of this very efficient protective mechanism plant sterols are probably not able to promote atherogenesis in non-sitosterolaemic subjects.

Most clinical trials report positive associations between plasma plant sterols and CAD or mortality (37-40, 42, 125-129). Some authors have also suggested an increased absorption and a decreased synthesis of cholesterol to be an independent cardiovascular risk factor (37-39, 128, 129). In the Dallas Heart Study (41) and European Prospective Investigation of Cancer-Norfolk Study (44) plasma plant sterols were not adversely related to CAD or mortality. The results of the Longitudinal Aging Amsterdam Study arguing for beneficial effects of high plasma plant sterol concentrations were not adjusted for BMI (43).

Our findings are in support of previous studies reporting high absorption and low synthesis of cholesterol to be associated with CAD or mortality. We think the repeatedly observed positive correlation of plasma plant sterols with CAD reflects atherogenic effects of an increased intestinal cholesterol uptake.

The pathophysiological mechanisms underlying the relationships of cholesterol homeostasis with CAD are not fully understood yet. With the use of statins a reduction in coronary events can be achieved in subjects with hypercholesterolaemia (3). It is well established that the effectiveness of statin treatment in lowering the plasma total- and LDL-cholesterol concentrations depends on the baseline cholesterol metabolism. Statins suppress the synthesis of cholesterol considerably less efficiently in subjects with a low synthesis and a high absorption of cholesterol (76). In consequence, coronary events have not been reduced by the use of statins in those patients with high cholesterol absorption (77).

However, cholesterol metabolism not only predicts the risk of future coronary events in patients under statin regimen but also in those who do not receive lipid lowering drugs. It seems rather improbable that an increased absorption in particular of plant sterols promotes atherogenesis. More likely, a higher cholesterol lifetime burden, as suggested previously (129), might link detrimental changes in cholesterol homeostasis to CAD. In support of this hypothesis, polymorphisms in the *ABCG5* gene, which plays an important role in intestinal cholesterol uptake, were associated with the response to dietary cholesterol (130).

The impact of cholesterol absorption and synthesis on the plasma cholesterol concentration and CAD has also been demonstrated in an animal model. Deficiency of the NPC1L1 protein

resulted in a decreased plasma cholesterol concentration and prevented atherosclerosis in *NPC1L1* knock-out mice (141). In contrast, the malfunction or the lack of the ABCG5 or the ABCG8 protein is associated with an increased absorption of sterols and premature atherosclerosis in knock-out mice (87-89) and individuals suffering from sitosterolaemia (105, 109, 110, 113).

4.7. The use of plant sterol and stanol ester margarines and ezetimibe in the prevention and treatment of CAD

Evidence is growing that high cholesterol absorption increases the risk of coronary atherosclerosis. Thus, the use of drugs interfering with cholesterol absorption in addition to statins might be favourable in the prevention and treatment of CAD. Particularly those patients who do not reach total- and LDL-cholesterol goals under a statin regimen alone are expected to potentially profit from cholesterol absorption inhibitors. These are Ezetimibe (142, 143) and plant stanol or sterol margarines (83-85). However, definite recommendations for the use of cholesterol absorption inhibitors in addition to statins will require large prospective studies with hard cardiovascular end points. To this date these data are not available but clinical trials are ongoing. In a recently published study however, carotid intima-media thickness was surprisingly not reduced by an ezetimibe plus simvastatin regimen compared to simvastatin alone despite a more pronounced reduction of LDL-cholesterol in the ezetimibe plus simvastatin group (143). Yet, ezetimibe was found to inhibit the development of atherosclerosis in mice (144). Concerns about the safety of plant sterol margarines with regard to atherogenesis are not warranted (145). The physiological plasma plant sterol concentration is usually less than one mg/dl (41, 44). Due to the regular intake of plant sterol containing functional foods the plasma plant sterol concentration is forced up by about the factor two (91). However, patients with sitosterolaemia, who develop severe premature atherosclerosis, have up to hundred-fold elevated plasma plant sterols (105, 109, 110, 113). In addition, the administration of plant sterols was shown to be effective in the prevention of coronary atherosclerosis in animal models (87-89) and to be associated with increased carotid artery compliance in a recent clinical trial (146). Prospective data investigating the effects of plant sterol or stanol margarine consumption on the cardiovascular risk are still lacking. Nevertheless, hitherto existing data argue against concerns about the safety of plant sterol or stanol margarine consumption with regard to atherogenesis. Of note, a reduction in LDL-cholesterol of about ten percent can be achieved by the regular intake of plant sterol or stanol containing functional foods (83-85). Thus,

considering pharmacoeconomic aspects particularly plant sterol or stanol margarines seem to be suitable for the long term prevention of CAD (147-149).

4.8. Conclusion

In LURIC participants, high absorption and low synthesis of cholesterol are associated with a more severe CAD and increased all-cause- and cardiovascular mortality. However, there is no increase in the severity of coronary atherosclerosis or in mortality which is definitely and specifically attributable to high plasma plant sterol concentrations. Hence, the use of ezetimibe and plant sterol or stanol margarines in addition to a statin regimen might be favourable for the treatment of elevated plasma total- and LDL-cholesterol concentrations. Especially those patients with icreased total and LDL-cholesterol in spite of an ongoing statin regimen might potentially profit from cholesterol absorption inhibitors. Furthermore, plant sterol and stanol ester margarines appear to be particularly suitable for the long term prevention of CAD. According to our findings their use is safe. However, to give definite recommendations for a treatment with cholesterol absorption inhibitors, large prospective clinical trials testing their effects on hard cardiovascular end points are required. To this date, these data are not available. Thus, prospective clinical studies investigating if the use of cholesterol absorption inhibitors will reduce cardiovascular risk are needed.

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6. Supplemental Material

Supplement 1: Health claim for phytosterols

FDA
TALK PAPER

Food and Drug Administration
U.S. Department of Health and Human Services
5600 Fishers Lane Rockville, MD 20857

FDA Talk Papers are prepared by the Press Office to guide FDA personnel in responding with consistency and accuracy to questions from the public on subjects of current interest. Talk Papers are subject to change as more information becomes available.

T00-40 Print Media: Ruth Welch, 202-205-4144
September 5, 2000

Consumer Inquiries 888-INFOFDA

FDA AUTHORIZES NEW CORONARY HEART DISEASE HEALTH CLAIM FOR PLANT STEROL AND PLANT STANOL ESTERS

The FDA has authorized use of labeling health claims about the role of plant sterol or plant stanol esters in reducing the risk of coronary heart disease (CHD) for foods containing these substances. This interim final rule is based on FDA's conclusion that plant sterol esters and plant stanol esters may reduce the risk of CHD by lowering blood cholesterol levels.

Coronary heart disease, one of the most common and serious forms of cardiovascular disease, causes more deaths in the U.S. than any other disease. Risk factors for CHD include high total cholesterol levels and high levels of low density lipoprotein (LDL) cholesterol.

This new health claim is based on evidence that plant sterol or plant stanol esters may help to reduce the risk of CHD. Plant sterols are present in small quantities in many fruits, vegetables, nuts, seeds, cereals, legumes, and other plant sources. Plant stanols occur naturally in even smaller quantities from some of the same sources. For example, both plant sterols and stanols are found in vegetable oils.

Foods that may qualify for the health claim based on plant sterol ester content include spreads and salad dressings. Among the foods that may qualify for claims based on plant stanol ester content are spreads, salad dressings, snack bars, and dietary supplements in softgel form.

Foods that carry the claim must also meet the requirements for low saturated fat and low cholesterol, and must also contain no more than 13 grams of total fat per serving and per 50 grams. However, spreads and salad dressings are not required to meet the limit for total fat per 50 grams if the label of the food bears a disclosure statement referring consumers to the Nutrition Facts section of the label for information about fat content. In addition, except for salad dressing and dietary supplements, the food must contain at least 10% of the Reference

Daily Intake (RDI) or Daily Reference Value (DRV) for vitamin A, vitamin C, iron, calcium, protein, or fiber. FDA is also requiring, consistent with other health claims to reduce the risk of CHD, that the claim state that plant sterol and plant stanol esters should be consumed as part of a diet low in saturated fat and cholesterol.

Scientific studies show that 1.3 grams per day of plant sterol esters or 3.4 grams per day of plant stanol esters in the diet are needed to show a significant cholesterol lowering effect. In order to qualify for this health claim, a food must contain at least 0.65 grams of plant sterol esters per serving or at least 1.7 grams of plant stanol esters per serving. The claim must specify that the daily dietary intake of plant sterol esters or plant stanol esters should be consumed in two servings eaten at different times of the day with other foods.

An example of a health claim about the relationship between plant sterol esters and reduced risk of heart disease is:

Foods containing at least 0.65 grams per serving of plant sterol esters, eaten twice a day with meals for a daily total intake of at least 1.3 grams, as part of a diet low in saturated fat and cholesterol, may reduce the risk of heart disease. A serving of [name of the food] supplies ____ grams of plant sterol esters.

An example of a health claim about the relationship between plant stanol esters and reduced risk of heart disease is:

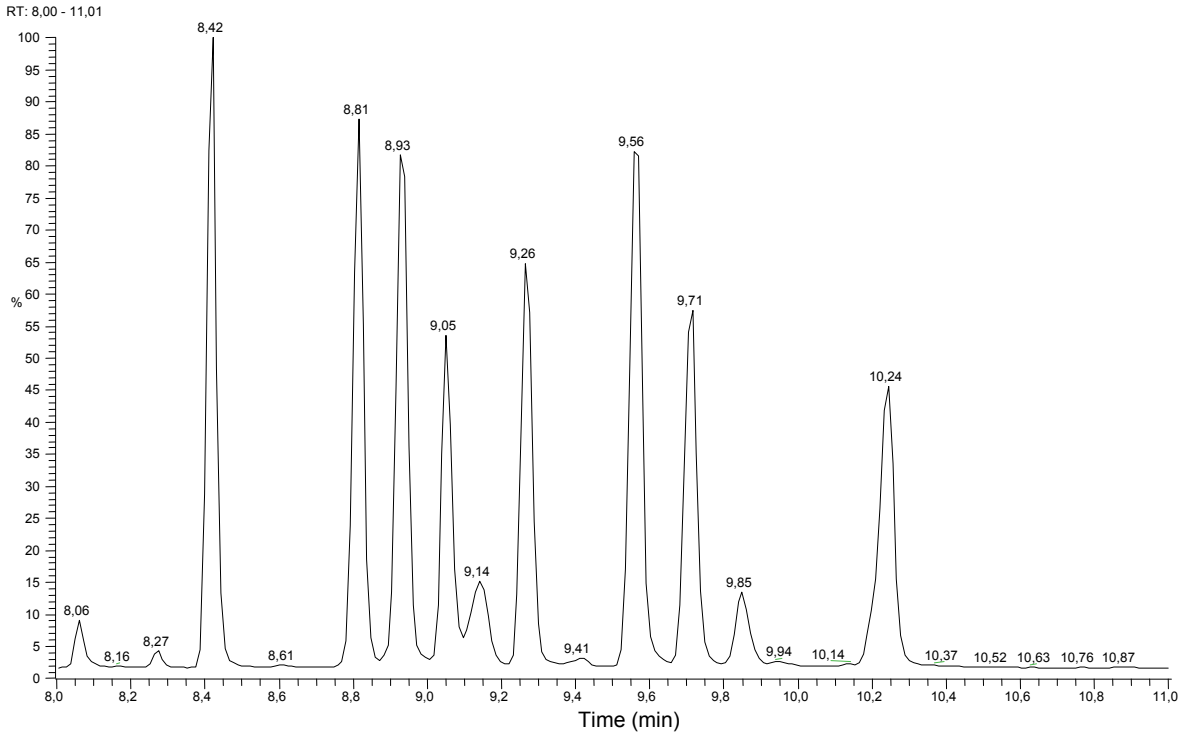
Diets low in saturated fat and cholesterol that include two servings of foods that provide a daily total of at least 3.4 grams of plant stanol esters in two meals may reduce the risk of heart disease. A serving of [name of the food] supplies ___ grams of plant stanol esters.

This new health claim interim final rule responds to petitions submitted to the FDA by Lipton (plant sterol esters) and McNeil Consumer Healthcare (plant stanol esters). The FDA is issuing this rule as an interim final rule. It is effective immediately with an opportunity for the public to comment. The final rule on this health claim may differ from this interim rule, and manufacturers would be required to revise their labeling to conform to any changes adopted in the final rule.

Written comments will be received until 75 days after date of publication in the Federal Register and may be addressed to: Dockets Management Branch (HFA-305), Food and Drug Administration, 5630 Fishers Lane, Rm. 1061, Rockville, MD 20852.

Supplement 2: Chromatogram of sterols, full scan

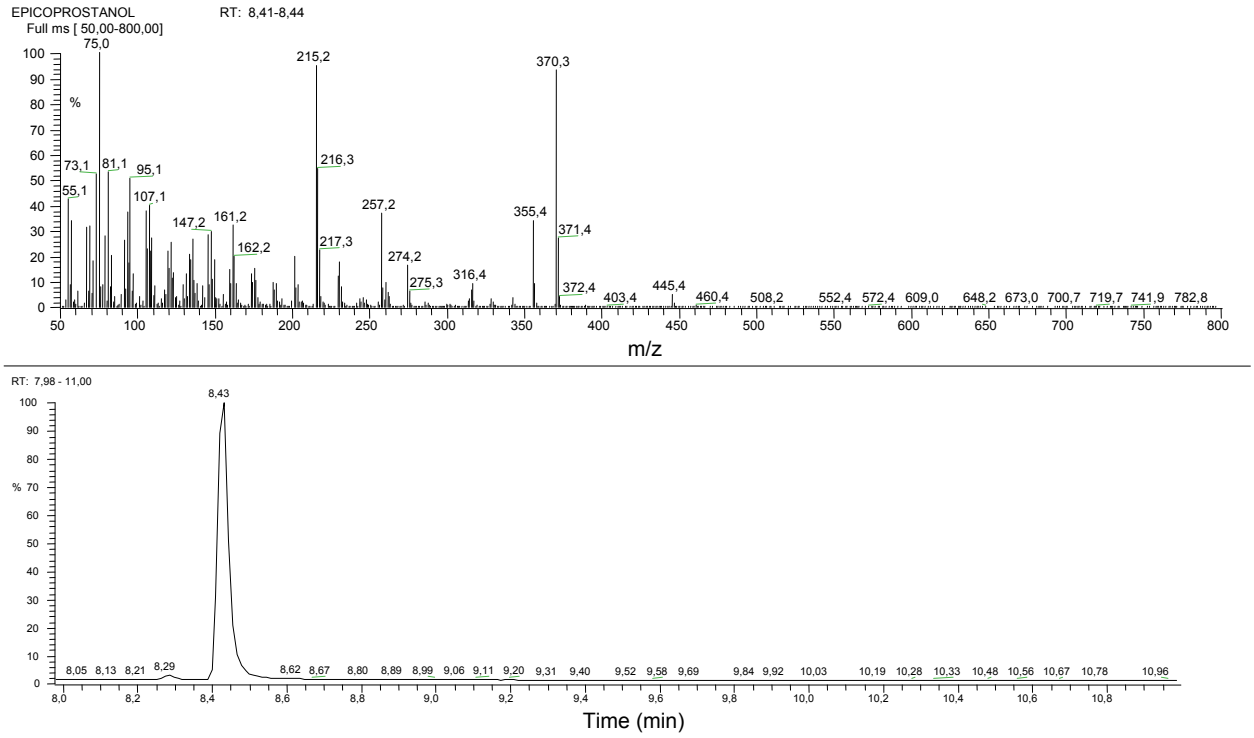
MIX



Supplement 3: Chromatogram of epicoprostanol, SIM

Epicoprostanol: Retention time 8,43**;** molecule peak 460,4**;** significant ion 370,4

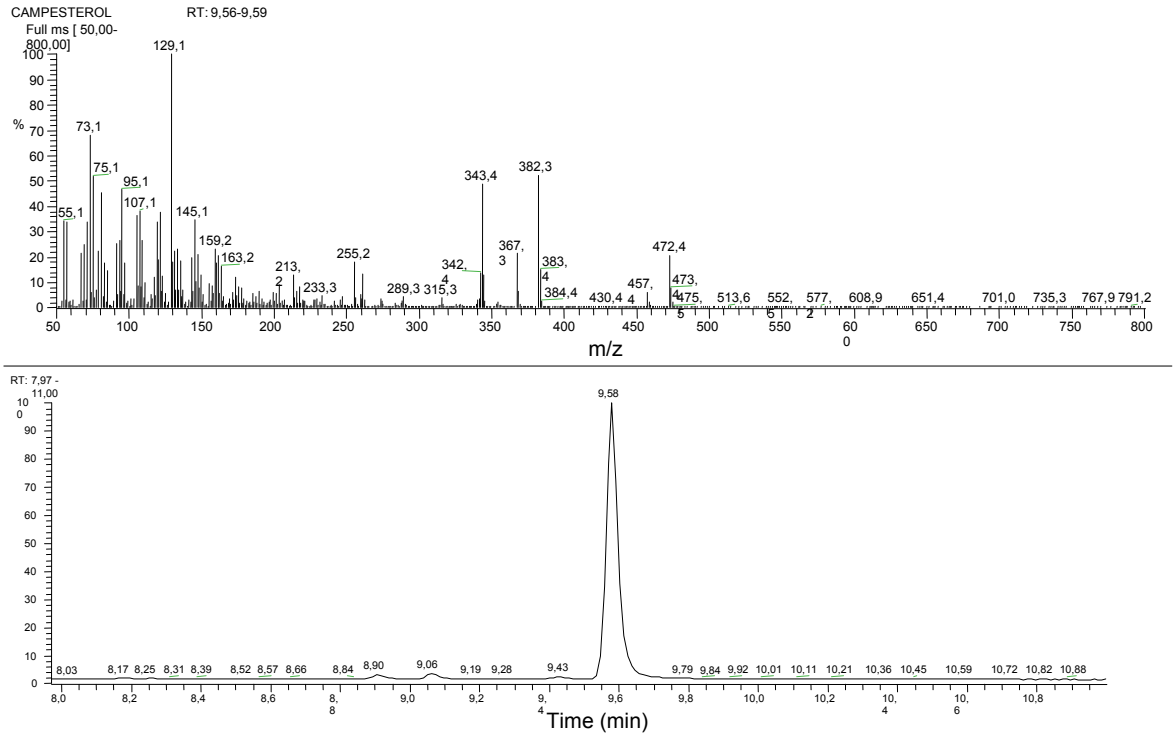
EPICOPROSTANOL



Supplement 4: Chromatogram of campesterol, SIM

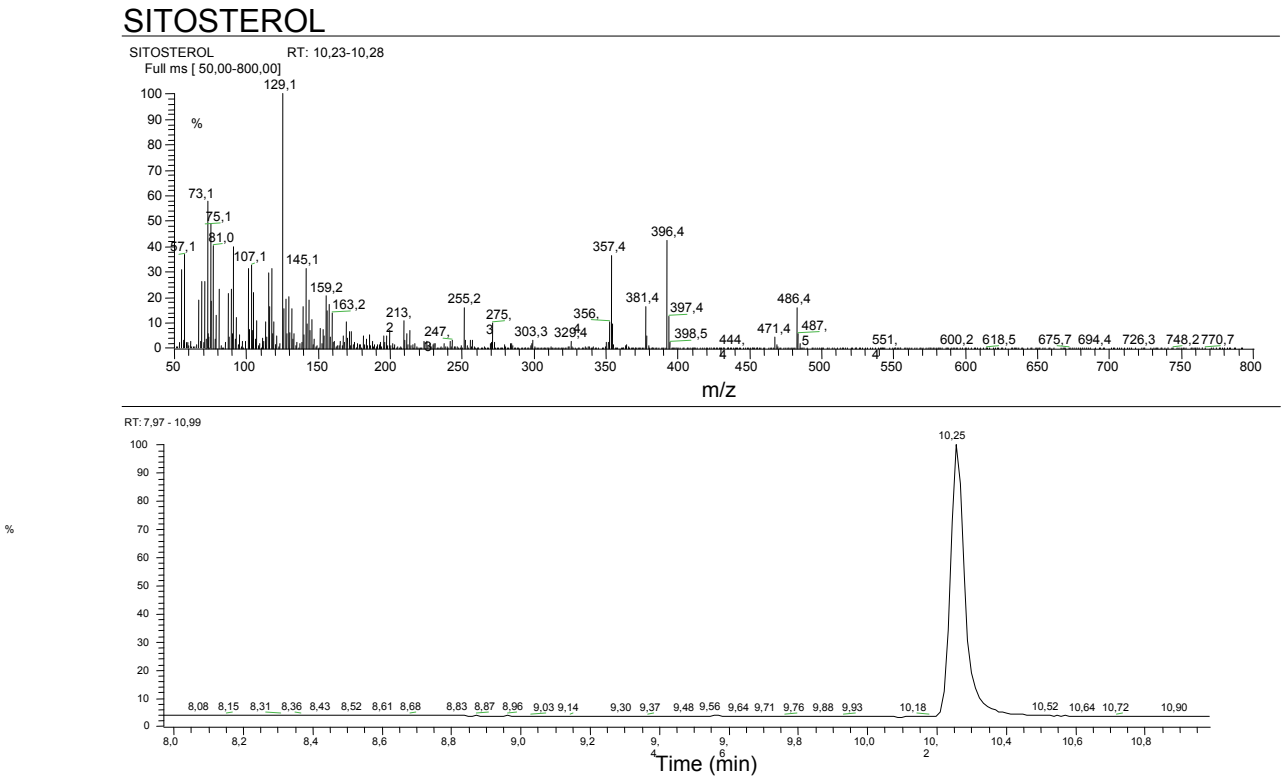
Campesterol: Retention time: 9,58; molecule peak 472,4; significant ion 382,4

CAMPESTEROL



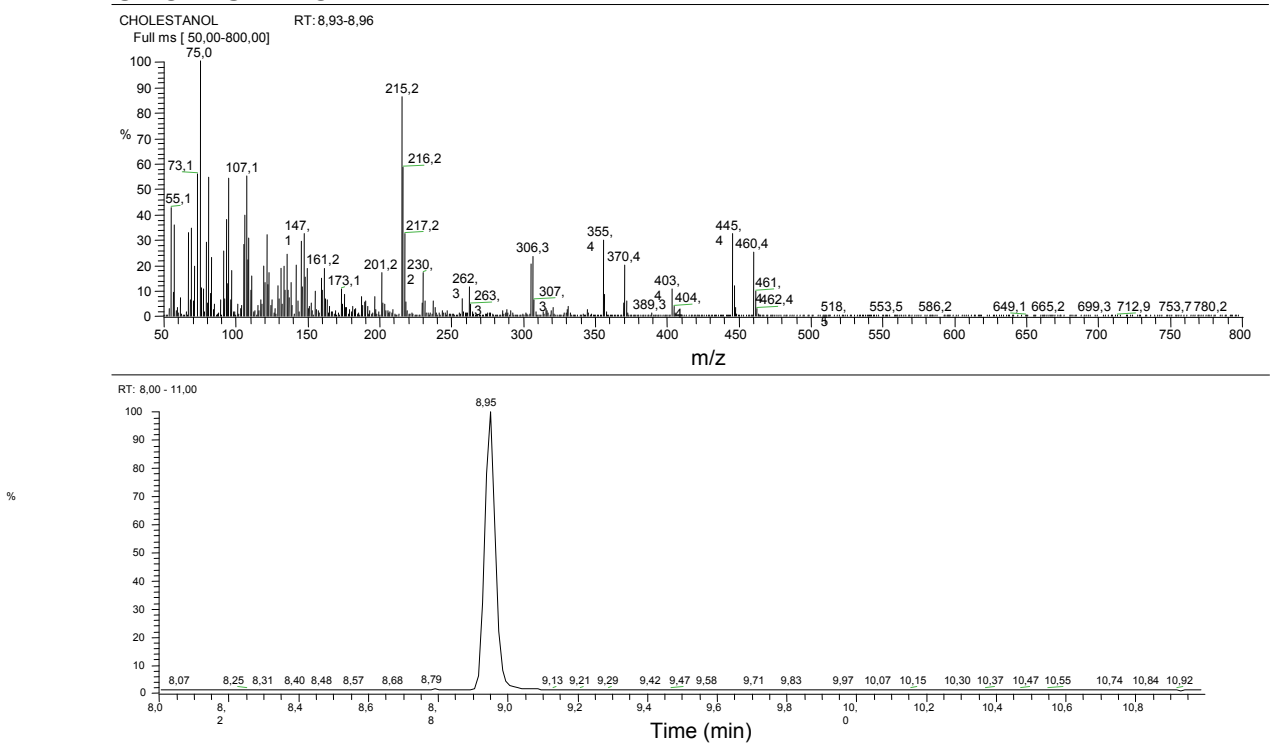
Supplement 5: Chromatogram of sitosterol, SIM

Sitosterol: Retention time 10,25; molecule peak 486,4; significant ion 357,3



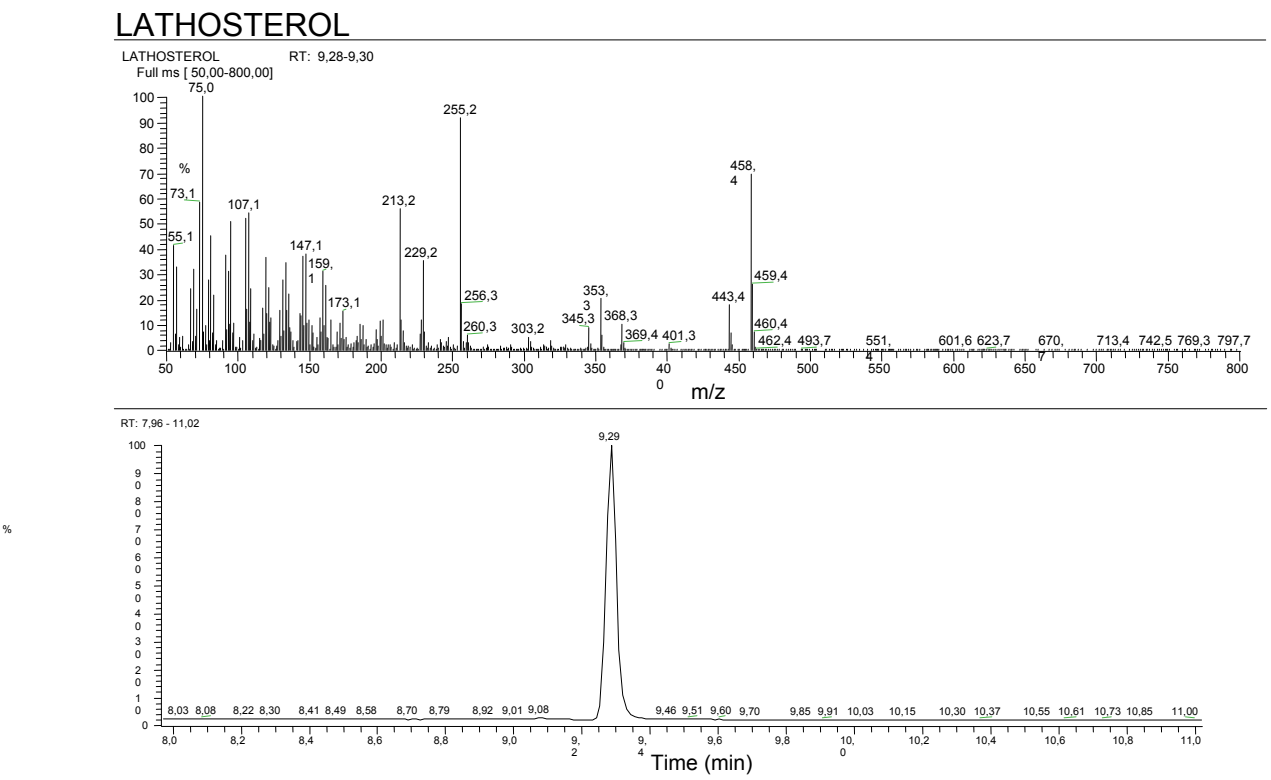
Supplement 6: Chromatogram of cholestanol, SIM

Cholestanol: Retention time 8,95; molecule peak 460,4; significant ion 445,4



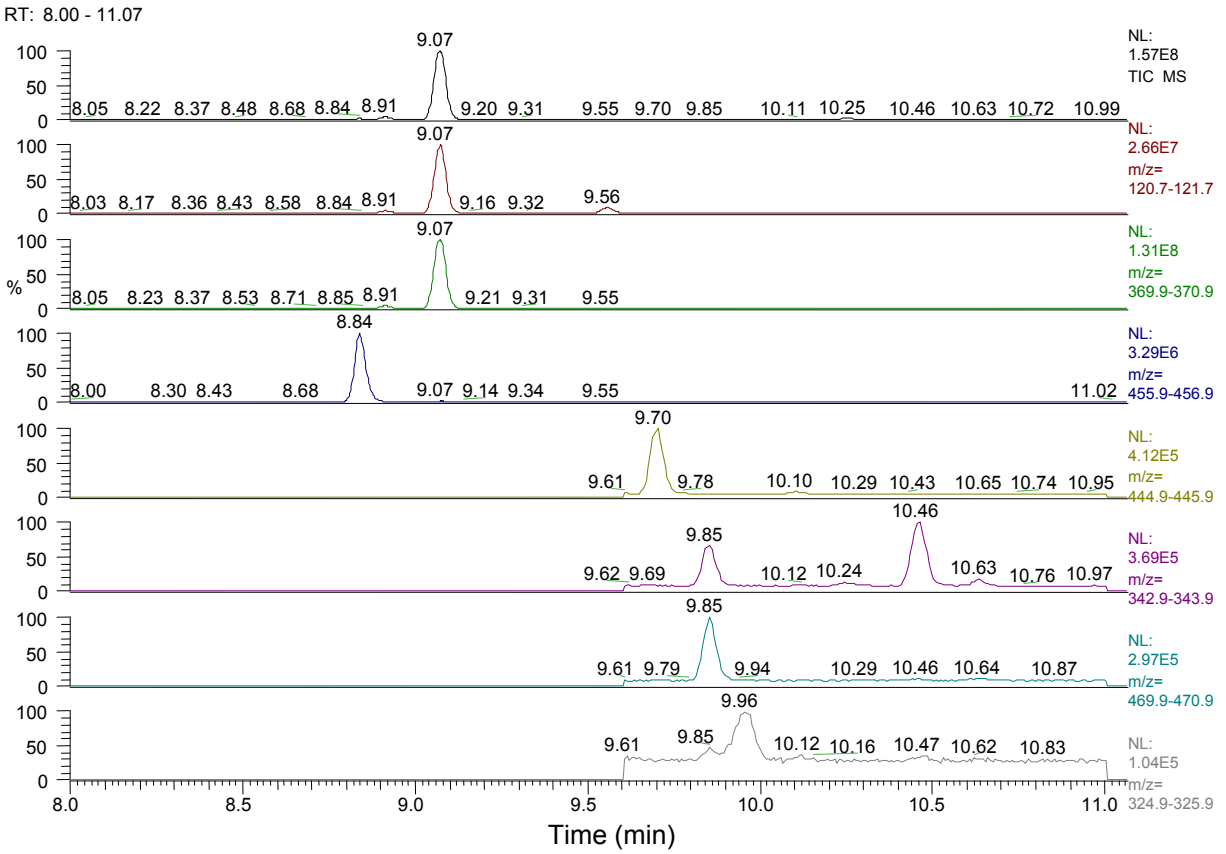
Supplement 7: Chromatogram of lathosterol, SIM

Lathosterol: Retention time 9,29; molecule peak 458,4; significant ion 458,5

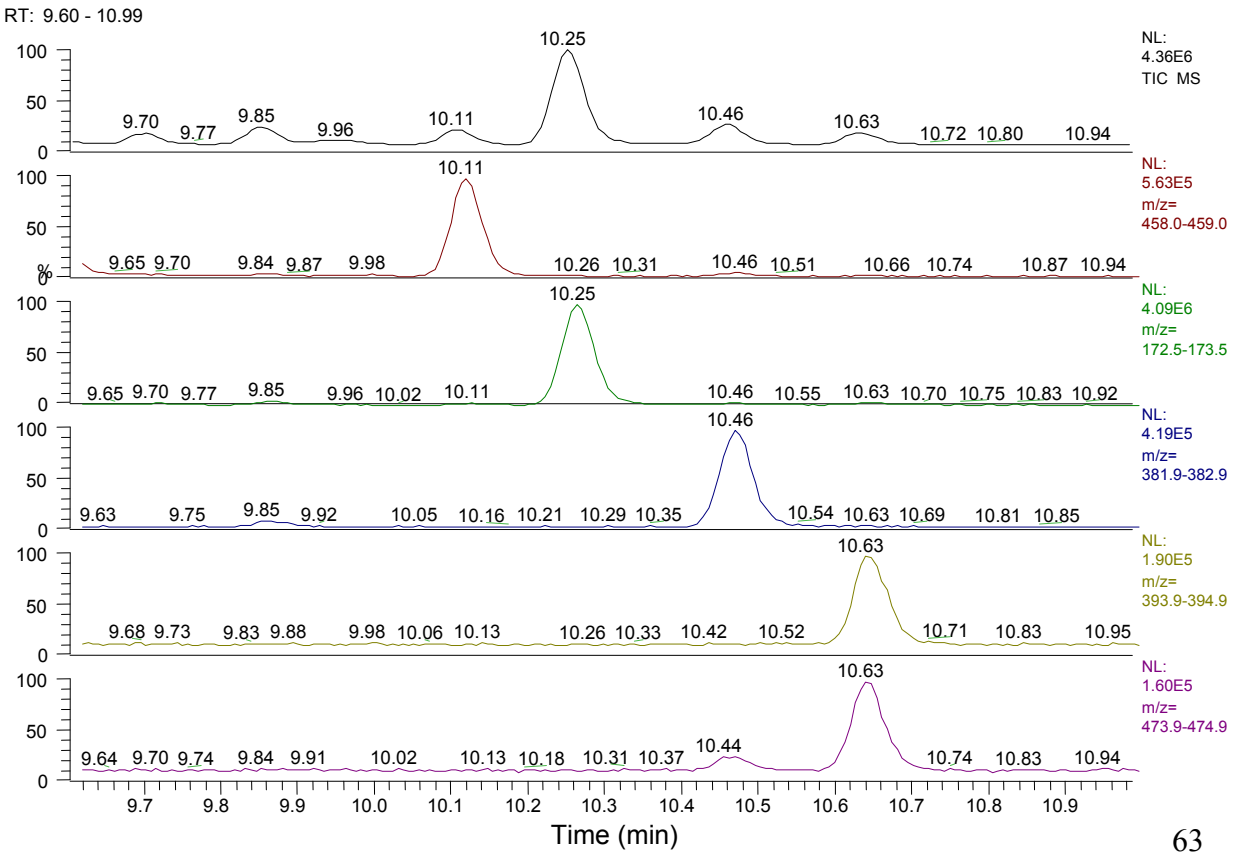


Supplement 8: Sample Analysis, chromatogram, SIM

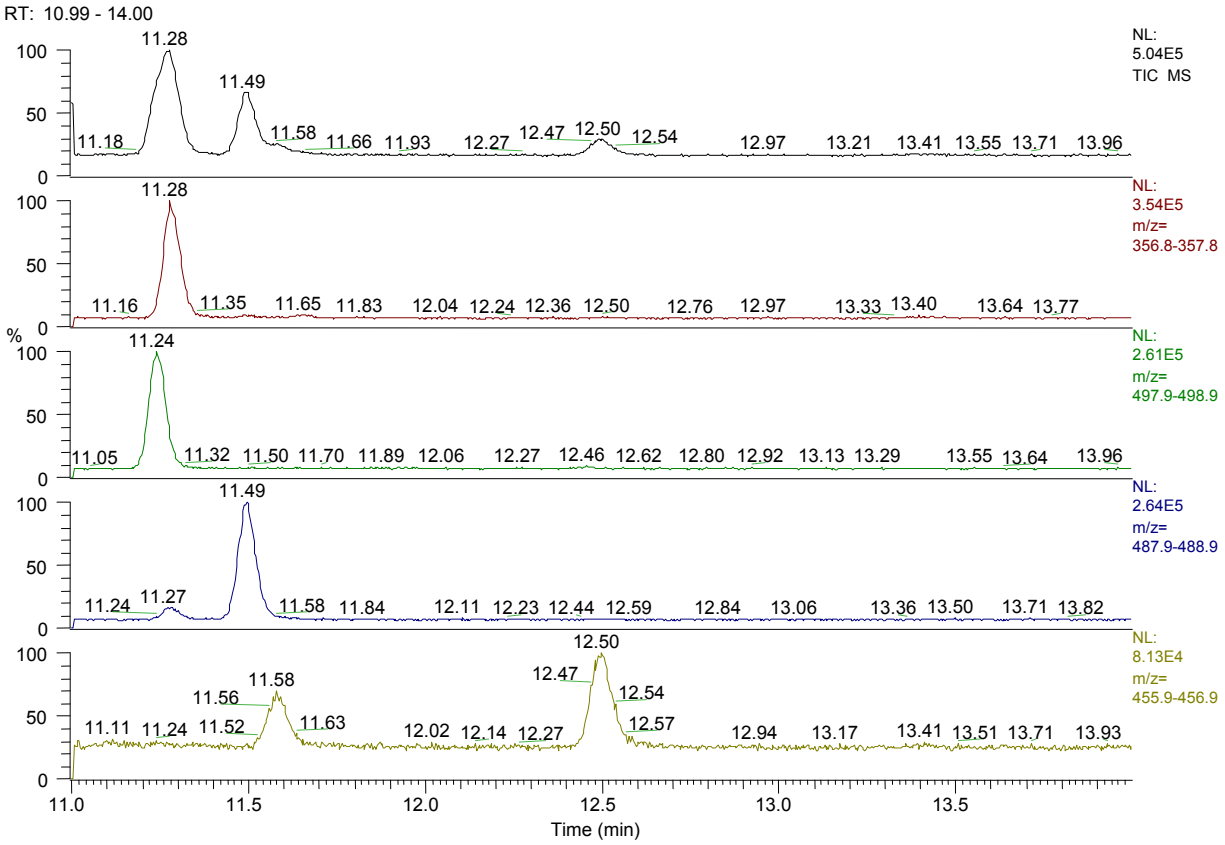
Sample analysis SIM Qual-Browser Part 2



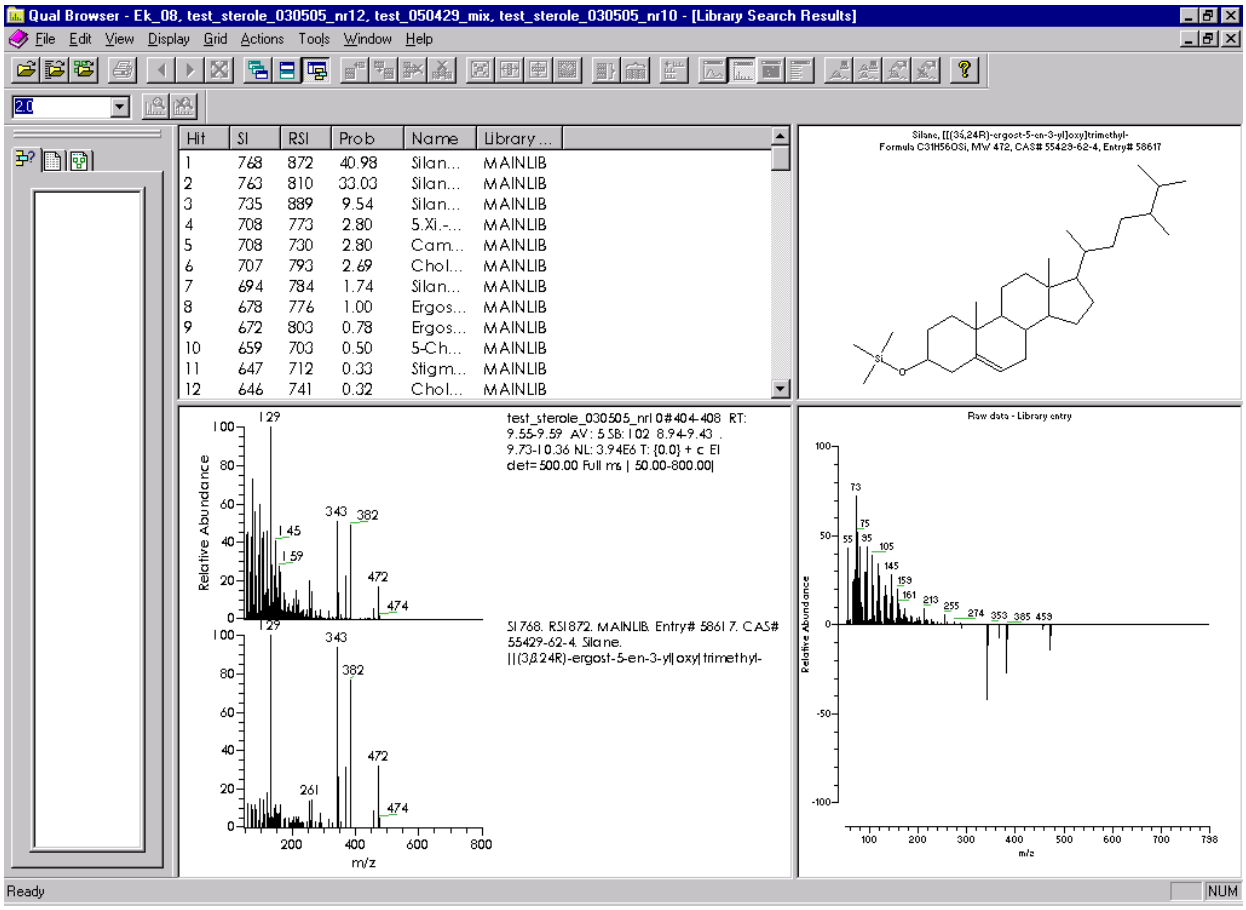
Sample analysis SIM Qual-Browser Part 2



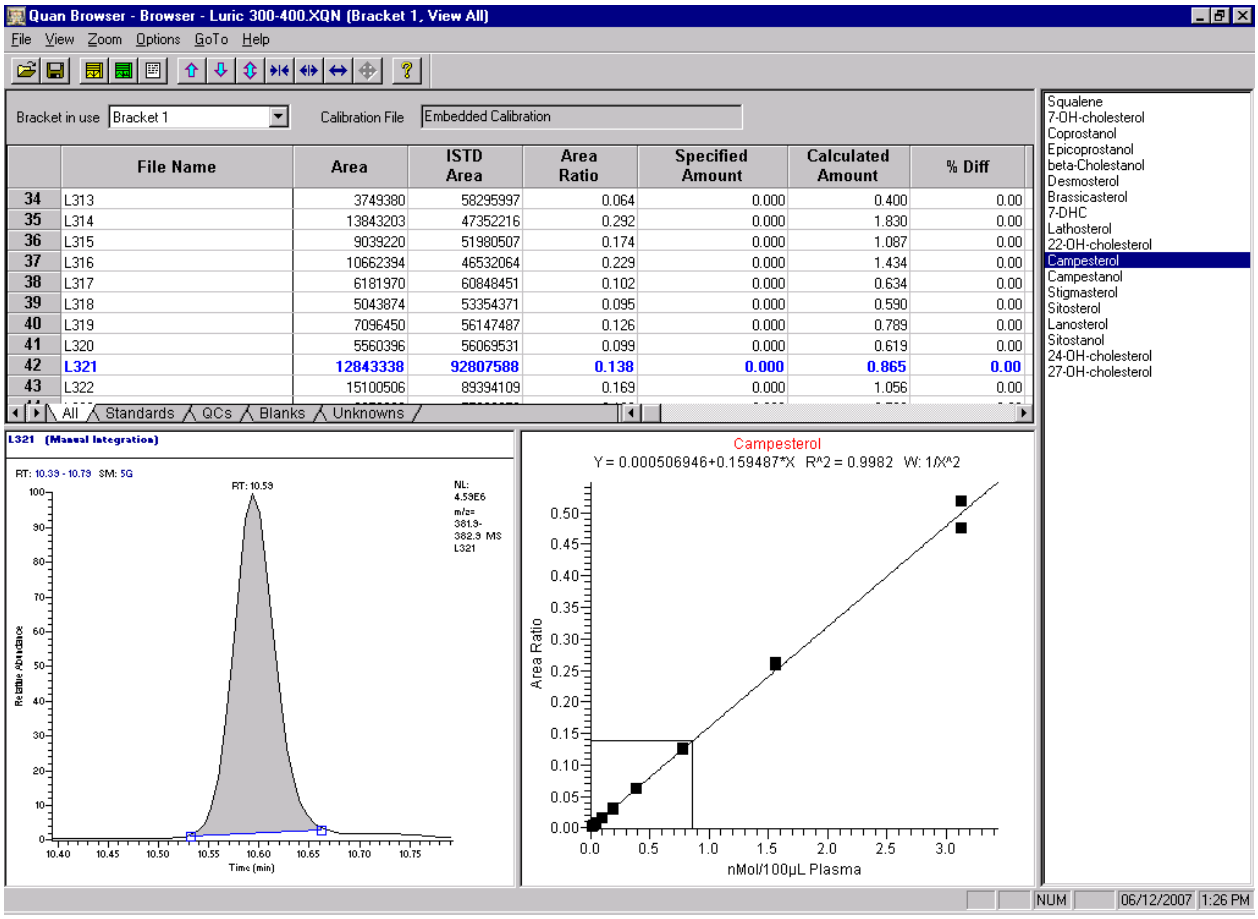
Sample analysis SIM Qual-Browser Part 3



Supplement 9: Qualitative analysis: Silane-campesterol



Supplement 10: Quantitative analysis: Manual integration



7. Summary

Background: There is a discussion if an unfavourable balance of intestinal cholesterol absorption and endogenous cholesterol synthesis or moderately elevated plasma plant sterol concentrations are atherogenic.

Aims: Our aim was to elucidate the relationships of cholesterol metabolism and plasma plant sterols with the severity of coronary artery disease (CAD) and mortality.

Methods: We studied individuals of the LUdwigshafen RIsk and Cardiovascular health (LURIC) cohort. LURIC is a large cross-sectional and prospective clinical trial involving 3316 subjects who were admitted to the Ludwigshafen General Hospital for coronary angiography. The severity of coronary atherosclerosis was determined by the Friesinger Score (FS). There was a median follow-up on all-cause- and cardiovascular mortality of 8.01 years. A gas chromatography and mass spectrometry based analytical method for measurement of non-cholesterol sterols was developed and validated. We quantified the plasma concentrations of campesterol and sitosterol (pant sterols, markers of cholesterol absorption), cholestanol (not a plant sterol, marker of cholesterol absorption), and lathosterol (cholesterol precursor, marker of cholesterol synthesis) in a subgroup of 2440 participants of the LURIC study. These individuals were included in the cross-sectional analysis. The prospective analysis comprised a total of 1257 subjects, who did not take statins.

Results: Increased plasma cholestanol and campesterol to cholesterol ratios and decreased plasma lathosterol to cholesterol ratio were significantly associated with a more severe coronary atherosclerosis. The plasma sitosterol to cholesterol ratio did not significantly correlate with the FS. In accordance with the cross-sectional analysis high plasma cholestanol to cholesterol ratio and low plasma lathosterol to cholesterol ratio were predictive of increased all-cause- and cardiovascular mortality. High plasma campesterol to cholesterol ratio was associated with modestly increased all-cause mortality. The plasma sitosterol to cholesterol ratio was neither significantly correlated with all-cause mortality nor with cardiovascular mortality.

Conclusion: High intestinal absorption and low endogenous synthesis of cholesterol go along with a more severe coronary atherosclerosis and increased all-cause- as well as cardiovascular mortality in participants of the LURIC study. An atherogenic role of moderately elevated plasma plant sterols, however, seems unlikely.

8. Acknowledgement

I am most grateful to Prof. Dr. med. Winfried März for the support, the expert advice, and for making it possible to perform this work at the Clinical Institute of Medical and Chemical Laboratory Diagnostics, Medical University of Graz, Austria. Furthermore, I wish to thank PD Dr. rer. nat. Günter Fauler for the sucessful cooperation and Prof. Dr. rer. nat. Gerhard Kostner for reviewing the doctoral thesis. I am also thankful to Ass. Prof. Dr. med. Wolfgang Erwa and PD Dr. rer. nat. Wilfried Renner for their help. Moreover, I would like to express my appreciation to the participants of the LURIC study without their collaboration this work would not have been written. Finally, I would like acknowledge the LURIC study team either temporarily or permanently involved in patient recruitment and sample and data handling, the laboratory staff at the Ludwigshafen General Hospital and at the Clinical Institute of Medical and Chemical Laboratory Diagnostics, Medical University of Graz, and the Universities of Graz, Freiburg, Ulm, and Tübingen.

9. Curriculum vitae

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Studies

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