Analysis of Plant Sterols and Oxysterols in the Serum of Patients with Sitosterolemia under different Drug Treatments

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To my parents

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List of Abbreviations

ABCG5	ATP-binding cassette transporter subfamily (G5)
ABCG8	ATP-binding cassette transporter subfamily (G8)
ACAT	Acyl coenzyme A: cholesterol acyltransferase
Apo B-100	Apolipoprotein B-100
Apo E	Apolipoprotein E
ARH	Autosomal recessive hypercholesterolemia
AST	Aspartate aminotransferase enzyme
ATP	Adenosine triphosphate
BHT	Butylated hydroxytoluene
˚C	Degree Celsius
CHD	Congestive heart disease
CI	Chemical ionization
CM	Chylomicrons
CO2	Carbon dioxide
COPs	Cholesterol oxidation products
CTX	Cerebrotendinous xanthomatosis
CVD	Cardiovascular disease
CYP450	Cytochrome P450 monooxygenase
DB-XLB	(14%-Diphenyl-methyl polysiloxane) capillary column
EI	Electron impact
EDTA	Ethylenediaminetetraacetic acid
FDB	Familial defective apo B-100
FH	Familial hypercholesterolemia
FID	Flame ionization detection
GC	Gas chromatography
GC-MS	Gas chromatography-mass spectrometry
HDL	High density lipoproteins
HMG-CoA	3-hydroxy-3-methylglutaryl-coenzyme A
HMG-CoAR	3-hydroxy-3-methylglutaryl-coenzyme A reductase
H_2O	Water

ISTD	Internal standard
KOH	Potassium hydroxide
LDL	Low density lipoprotein
LDL-C	Low density lipoprotein cholesterol
LDL-R	Low density lipoprotein receptor
LXR	Liver X receptor
M	Molarity
M^+	Molecule ion
mmol	Millimole
MS	Mass spectroscopy
m/z	Mass to charge ratio
NaCl	Sodium chloride
NADPH	Nicotinamide adenine dinucleotide phosphate (reduced form)
NPC1L1	Niemann-pick-C1-Like 1 protein
pH	Molar concentration of hydrogen ions
POPs	Phytosterol oxidation products
ROS	Reactive oxygen species
Rt	Retention time
SIM	Single ion monitoring
SR-BI	Scavenger receptor class B
SD	Standard deviation
TMS	Trimethylsilyl
v/v	Volume/Volume
VLDL	Very low density lipoprotein

1 Introduction

Plant sterols (phytosterols) are natural compounds from plants, found in low concentration in blood and tissues of humans. There exists a wide variety of phytosterol structures, but the most frequent phytosterols in nature are sitosterol, campesterol and stigmasterol. The major plant sterol is sitosterol (approximately 80%). These compounds are not synthesized in the human body, but rather are derived entirely from the diet. The average Western diet contains between 160 to 360 mg/day of both sitosterol and campesterol and 20 to 50 mg/day of sitostanol (1).

Phytosterols are structurally similar to cholesterol except for substitutions at the C24 position of the side chain. Compared with cholesterol, campesterol has a methyl group at the C24 position of the side chain, whereas sitosterol has an additional ethyl group. Stigmasterol differs from sitosterol because of its additional double bond at the C22 position. Saturation of the delta 5 double bond of campesterol and sitosterol leads to the formation of campestanol and sitostanol, respectively.

Cholesterol and plant sterols can be oxidized to produce oxycholesterols and oxyphytosterols, respectively. Several oxidation products of cholesterol possess a number of biological effects and have been implicated in the etiology of atherosclerosis and cytotoxicity (2). Because of the structural similarity between plant sterols and cholesterol, analogous oxidation products might be formed by oxidation of the plant sterols. Thus, oxyphytosterols may have effects similar to those of oxycholesterols and therefore implications for human health.

7-hydroperoxycholesterol and its degradation products 7α- and 7β- hydroxycholesterol and 7-ketocholesterol have been found in LDL, whereas 7-hydroperoxycholesterol appears to be the most cytotoxic oxygenated lipid in LDL (3). An alternative pathway involved in the elimination of excess cholesterol involves side chain-oxidized oxycholesterol with an oxygen function at C27 position by sterol 27-hydroxylase, due to its ability to pass cell membranes at a much faster rate than cholesterol (4).

Sitosterolemia (also called phytosterolemia) is a rare autosomal recessively inherited disease. Only 40 patients have been identified worldwide so far (5). The disease is characterized by the accumulation of plant sterols and 5α-saturated stanols in plasma and tissues and clinically by tendon xanthomatosis and premature coronary artery atherosclerosis. The major biochemical

feature of sitosterolemia is hyperabsorption of sterols and reduction of biliary excretion of sterols (6). Since sitosterol is usually most abundant plant sterol in the diet, the disease has been named sitosterolemia.

In most Western countries, atherosclerosis is the leading cause of death. In the United States alone, it causes almost one million deaths every year, twice as many as from cancer (7). Despite significant medical advances, coronary artery disease and atherosclerotic stroke are responsible for more deaths than all other causes combined. The risk of premature atherosclerosis was observed in several subjects with sitosterolemia who died due to acute myocardial infarctions caused by extensive coronary and aortic atherosclerosis (8).

Recently, it was found that oxyphytosterols are present in elevated concentration in serum from sitosterolemic patients, while the concentration of serum oxyphytosterols in healthy control subjects was below the limit of detection (9). However, the biological effects of phytosterol oxidation products are not well known. Patients with sitosterolemia hyperabsorb and retain all sterols, including plant sterols and shellfish sterols. This is caused by mutations in one of the two tandem ATP-binding cassette transporter (ABCG5 or ABCG8) genes. These ABC transporters pump plant sterols out of the mucosa cells into the gut lumen and out of the liver into bile; therefore mutations in these transporters, resulting in hyperabsorption and diminished biliary excretion of plant sterols, is followed by markedly elevated concentrations of plant sterols in serum (10).

A low-sterol diet is one treatment option intended to reduce high plasma sterol levels in sitosterolemic patients. These dietary changes are, however, not very efficient. Bile acid binding resins (e.g. cholestyramine) or ileal bypass operations can effectively reduce the plasma sterol concentrations and the xanthomas (6) in patients with sitosterolemia.
HMG-CoA reductase inhibitors (lovastatin, simvastatin, pravastatin, atorvastatin and fluvastatin), which are widely used in the treatment of hypercholesterolemia, have also been tried in treatment of patients with sitosterolemia, but were not effective.
Ezetimibe is the first agent in a new class of drugs known as selective cholesterol absorption inhibitors, which have been found to be useful for lowering total cholesterol and low density lipoprotein levels (11). Research examining the effect of ezetimibe on serum plant sterol

concentrations in sitosterolemic patients found that ezetimibe produces significant and progressive reductions in the plant sterol levels in those patients (12).

2 *Aim of the study*

Sitosterolemia is a very rare inherited disease characterized biochemically by increased serum levels of plant sterols and stanols and clinically by premature coronary artery artheriosclerosis and xanthomatos. This disease can be treated by illeal pass operation, by using bile acids binding resins like cholestyramine or, more recently, by treatment with ezetimibe, the first agent in the new class of drugs called cholesterol absorption inhibitors.
Several oxidation products of cholesterol have been implicated in the etiology of atherosclerosis and cytotoxicity. Because plant sterols are structurally similar to cholesterol, oxidation products corresponding to the oxycholesterols might be formed by oxidation. Whether these oxidized compounds of plant sterols have biological or pathological effects (as has been suggested for oxycholesterols) is still unknown.

The aim of the present two studies can be explained as follows:
The first study examined whether oxidized plant sterols and oxycholesterols could be identified in human serum of sitosterolemic patients and in two frequently used soy-based lipid emulsions. Since ezetimibe reduces not only cholesterol absorption (11) but also serum plant sterol concentrations (12), we investigated whether ezetimibe also reduces serum concentration of oxycholesterols and oxyphytosterols in sitosterolemic patients during different drug treatments. In addition, serum samples from patients receiving intravenous infusions of lipid emulsion were analyzed for the presence of oxycholesterols and oxyphytosterols.
The aim of the second study was to investigate which drug could best be used in the treatment of sitosterolemia before ezetimibe was available.
To pursue these investigations, we employed a sensitive gas chromatography-mass spectrometry method for the first study, using deuterium labelled for oxycholesterols and oxyphytosterols as internal standards, whereas in the second study we used a gas chromatography method with 5α-cholestane as an internal standard.

3 General background

3.1 Cholesterol

Cholesterol is an extremely important biological molecule that plays a central role in the membrane of cells, as well as being a precursor for the synthesis of a number of equally important steroids, including bile acids, adrenocortical hormones, sex hormones, and vitamin D. Like all steroids, the cholesterol molecule consists of two parts, a nucleus and a side chain. The nucleus is based on phenanthrene (rings A, B, and C) to which a cyclopentane ring (D) is attached (Figure 1).

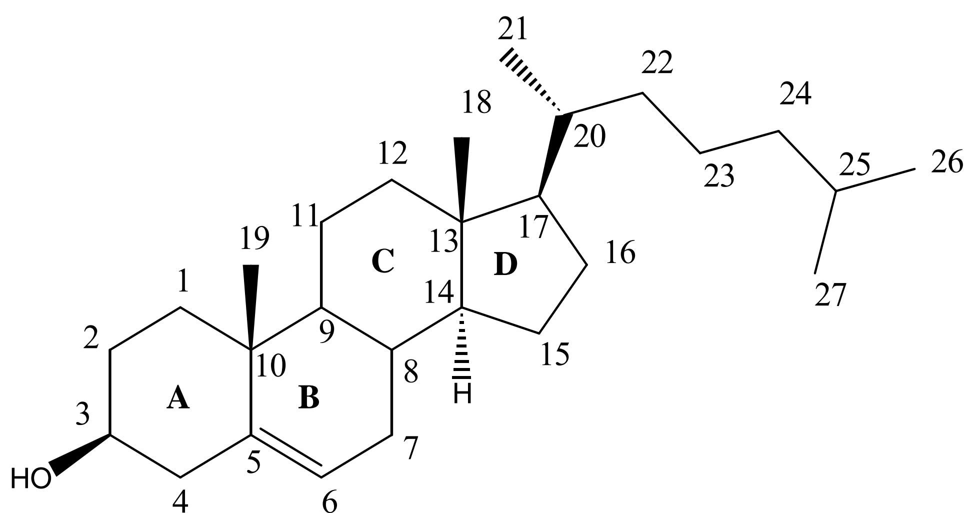


Figure 1 The chemical structure of cholesterol

3.1.1 Cholesterol absorption

The human body fulfills its requirement for cholesterol in two ways: it is synthesized in the body itself (mainly in the liver) and it is absorbed through the intestine during digestion of foods from animal sources (including egg yolks, meat and whole milk products) (13). Cholesterol and plant sterols are insoluble in the aqueous environment of the intestine. The solubilization of dietary sterols takes place in micelles containing conjugated bile acids, hydrolytic products of triglycerides, lecithin and fatty acids (14). The molecular mechanism of the dietary cholesterol absorption process from the intestine is only poorly understood. Dietary cholesterol, which is mostly unesterified, is solubilized in micelles and reaches the intestinal mucosa, where it passes

through the brush border. It is partially esterified by intestinal acyl CoA: cholesterol acyltransferase (ACAT) packaged into chylomicrons (CM) and secreted into the lymphatic system. As chylomicrons circulate, the core triglycerides are hydrolyzed by lipoprotein lipase, resulting in the formation of chylomicron remnants, which are rapidly removed by the liver (Figure 2) (15).

More recently, Altmann et al. (16) identified Niemann-Pick C1 Like1 (NPC1L1) as a critical protein (expressed at the apical surface of enterocytes) involved in the intestinal absorption of both dietary and biliary cholesterol. Furthermore, adenosine triphosphate (ATP)-binding cassette (ABC) transporters ABCG5 and ABCG8 represent apical sterol export pumps that promote active efflux of cholesterol and plant sterols from enterocytes back into the intestinal lumen for excretion. This provides an explantion why cholesterol absorption is a selective process, with plant sterols and other noncholesterol sterols being absorbed poorly. The ABCG5 and ABCG8 transporters are also expressed at the level of the canalicular membrane in the liver. They work as efflux pumps and transport free sterols into the bile, thereby regulating the absorption as well as the biliary secretion of cholesterol and plant sterols (15, 17). This explains the increase in intestinal sterol absorption and decrease in biliary sterol elimination in patients with sitosterolemia.

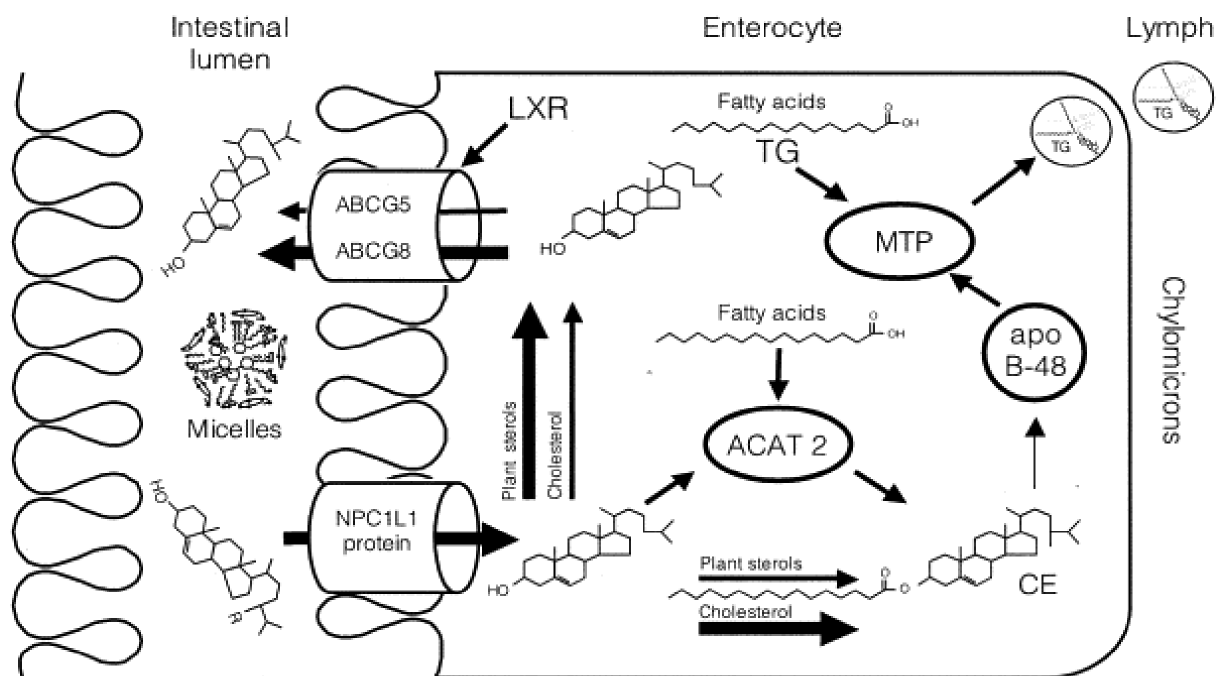


Figure 2 Cholesterol intestinal absorption (15)

3.1.2 Cholesterol biosynthesis

Cholesterol synthesis occurs in the cytoplasm and microsomes from the two-carbon acetate groups of acetyl-CoA. The acetyl-CoA utilized for cholesterol biosynthesis is derived from an oxidation reaction (e.g. fatty acids or pyruvate) in the mitochondria and is transported to the cytoplasm. All reduction reactions of cholesterol biosynthesis use NADPH as a cofactor. Acetyl CoA units are converted to mevalonate by a series of reactions that begins with the formation of HMG-CoA. Two moles of acetyl-CoA are condensed in a reversal of the thiolase reaction, forming acetoacetyl-CoA. Acetoacetyl-CoA and a third mole of acetyl-CoA are converted to HMG-CoA by the action of HMG-CoA synthetase. HMG-CoA is converted to mevalonate by HMG-CoA reductase (HMG-CoAR). HMG-CoAR absolutely requires NADPH as a cofactor and two moles of NADPH are consumed during the conversion of HMG-CoA to mevalonate. The reaction catalyzed by HMG-CoAR is the rate limiting step of cholesterol biosynthesis (Figure 3).

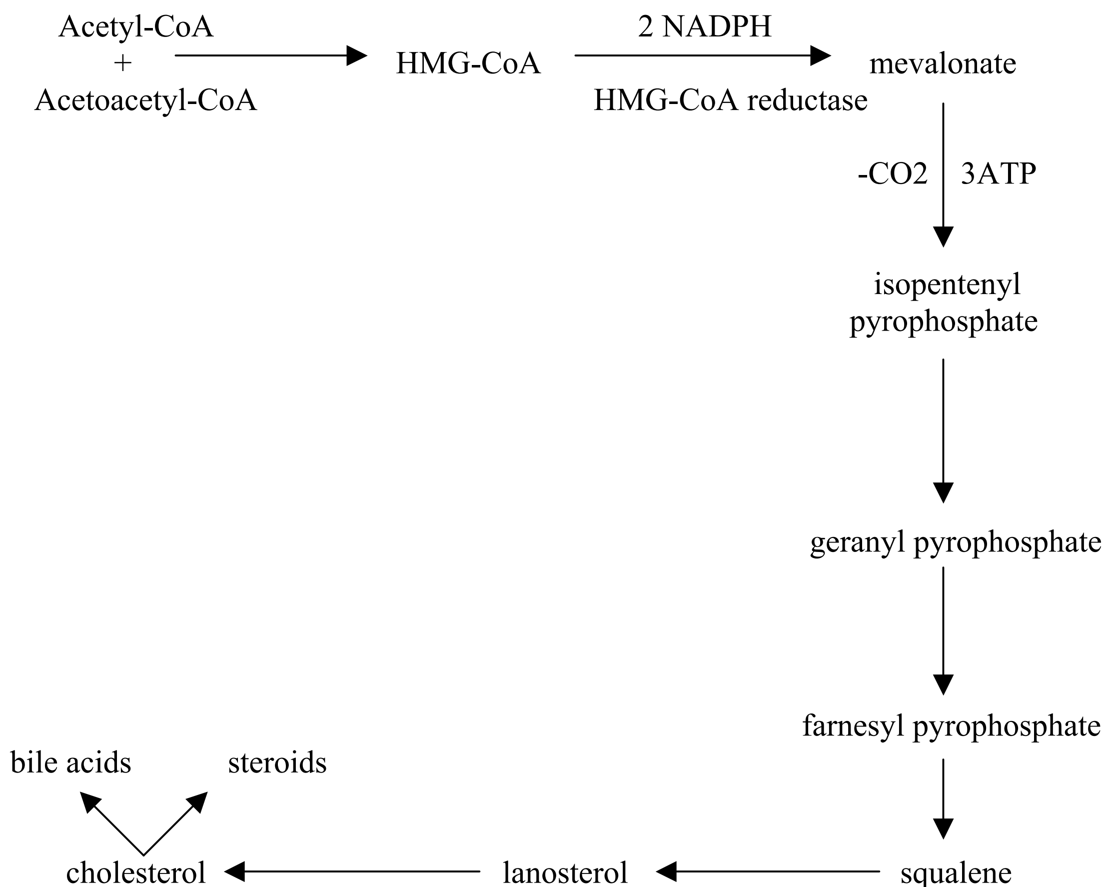


Figure 3 Cholesterol biosynthesis

3.1.3 Cholesterol distribution

Intestinal cholesterol and triglycerides are packaged with apolipoproteins in enterocytes of the small intestine to be secreted into the lymphatic system as chylomicrons. Intestinal cholesterol has four possible fates once it reaches the liver: it can be esterified and stored as cholesteryl esters in hepatocytes, packaged into very low density lipoprotein (VLDL) particles and secreted into the plasma, secreted into the bile, or converted into bile acids and secreted into the bile (18). Cholesterol in the blood is transported to and from cells by lipid-protein complexes called lipoproteins. They are of several kinds: Chylomicrons, intermediate lipoproteins (IDL), very low density lipoproteins (VLDL), low densitiy lipoproteins (LDL) and high density lipoproteins (HDL), Approximately 70% of circulating cholesterol is transported in LDL (18,19). These lipid-protein complexes contain triglycerid droplets and cholesterol esters surrounded by polar phospholipids and proteins identified as apolipoproteins.

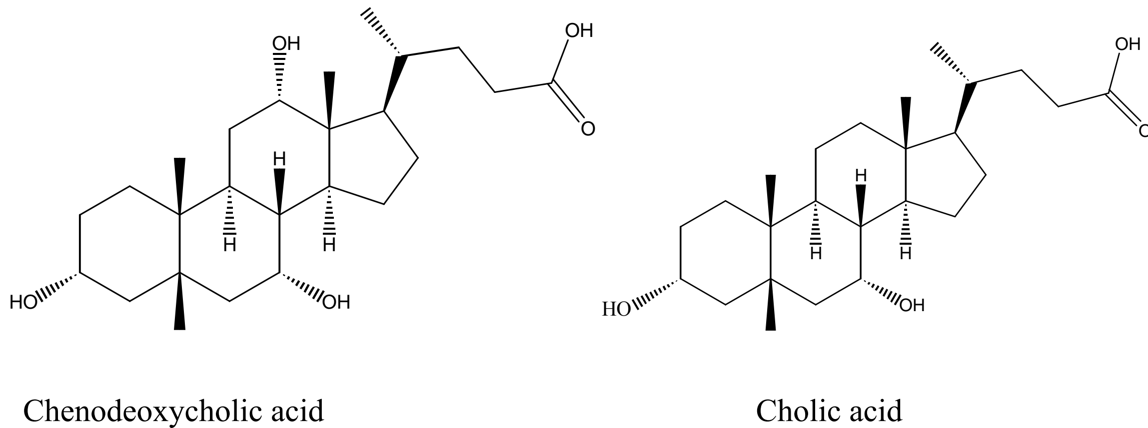
3.1.4 Low Density Lipoproteins (LDL)

Cholesterol can be transported to extra-hepatic tissues by VLDL which, is synthesized in the liver. In the circulation VLDL is converted to LDL through the action of endothelial cell-associated lipoprotein lipase enzyme. The almost exclusive apolipoprotein of LDL is apoB-100. The cellular uptake of cholesterol from LDL occurs following the interaction of LDL with the LDL receptors (also called apoB-100/ apoE receptors). The LDL receptor is a polypeptide of 839 amino acids that spans the plasma membrane. An extracellular domain is responsible for apoB-100/apoE binding, once LDL binds to the receptor, complexes are rapidly endocytosed. ATP dependent proton pumps lower the pH in the endosomes which results in dissociation of LDL from the receptor.

3.1.5 Cholesterol excretion

Hepatic conversion of cholesterol to bile acids followed by biliary secretion and subsequent fecal loss is a major metabolic step for the elimination of cholesterol from the body (20) but the excretion of cholesterol in this form is insufficient to compensate an excess dietary intake of cholesterol. The most abundant bile acids in human bile are chenodeoxycholic acid and cholic acid (Figure 4), referred to as the primary bile acids. Within the intestine the primary bile acids

are converted to the secondary bile acids by bacteria to deoxycholate (from cholate) and lithocholate (from chenodeoxycholate). Both primary and secondary bile acids are reabsorbed by the intestine and delivered back to the liver via the portal circulation. However, the absorption of lithocholates acid is much less than of all other bile acids.



Chenodeoxycholic acid Cholic acid

Figure 4 The chemical structure of bile acids

3.1.6 Cholesterol and atherosclerosis

While cholesterol is an essential part of a healthy body, high level of cholesterol (in particular high level of LDL-C) in the blood known as hypercholesterolemia, is a well established risk factor for development of atherosclerosis and cardiovascular disease.

During the last decade, several lines of evidence have suggested that oxidative modification of LDL plays a key role in this respect. Oxidation of LDL has been found to increase its uptake in macrophages leading to formation of macrophage foam cells which are the basis of fatty streaks, which are the first morphological stage of atherosclerosis (21). Other studies have indicated that oxidized LDL may induce vascular inflammation and even give rise to autoimmune reactions in the vascular wall (22). Familial hypercholesterolemia (FH) is the most common and most severe form of monogenic hypercholesterolemia. FH is caused by mutation in the LDL-R gene; patients with two mutated LDL-R alleles (FH homozygotes) are much more severely affected than those with one mutated allele (FH heterozygotes) (18). FH homozygotes develop cutaneous xanthomas

and coronary atherosclerosis in childhood (23). Atherosclerosis is characterized by thickening of arterial walls through accumulation of lipids in the inner layers (intima) of the arteries. The development of atherosclerosis has been shown to be positively correlated to the level of cholesterol in plasma. Atherosclerotic plaques consist primarily of lipids such as cholesterol, cholesterol esters and phospholipids (24). The severity of atherosclerosis is proportional to the extent and duration of elevated plasma LDL-C (25, 26).

Some individuals with a clinical presentation similar to FH and reduced rates of LDL catabolism were found to have normal LDL-R activity. When LDL from these patients was infused into normocholesterolemic subjects, the heterologous LDL was cleared at a reduced rate compared with normal autologous LDL (27). The disease, familial defective apo B-100 (FDB) results from a missense mutation in the LDL-R- binding domain of apo B-100.

Large scale clinical trials provide convincing evidence that substantial LDL lowering reduces cardiovascular morbidity and mortality, even in subjects who do not have elevated LDL-C levels (28, 29).

3.2 Oxycholesterols

3.2.1 Formation and sources of oxycholesterols

The oxygenation of cholesterol results in compounds known as oxycholesterols or cholesterol oxidation products (COPs), which can be defined as oxygenated derivatives of cholesterol that are intermediates or even end products in cholesterol excretion pathways. They may be formed directly by physical processes such as heating and radiation (30), by non-enzymatic processes involving reactive oxygen and free radical species or enzymatically by specific cytochrome P450 (CYP450) monooxygenases (30, 31).

7α-hydroxycholesterol, 7β-hydroxycholesterol, 7-ketocholesterol, 5α,6α-epoxycholesterol, 5β, 6β-epoxycholesterol and 3β, 5α, 6β-trihydroxycholestane can be formed either by enzymatic pathways or by autoxidation at ring B (Figure 5). On the other hand, the side chain is mainly attacked by CYP450 specific enzymes, resulting in various hydroxy derivatives of cholesterol like 24S-hydroxycholesterol and 27- hydroxycholesterol (2, 4).

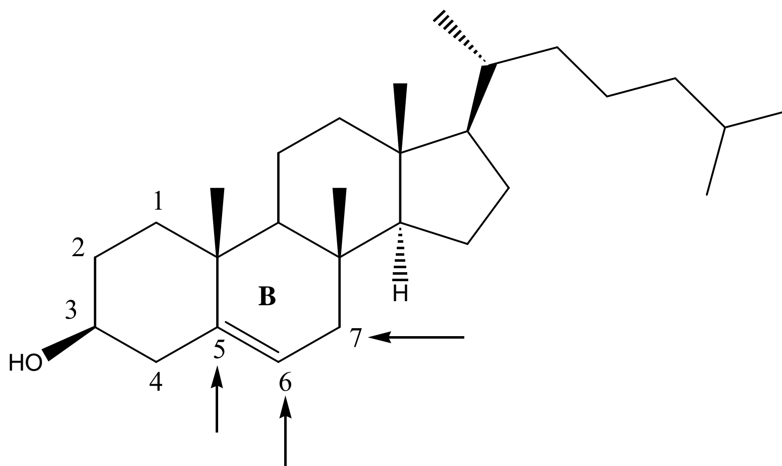


Figure 5 Autoxidation at ring B of the cholesterol nucleus

While all cells may have at least some ability to oxygenate cholesterol, the liver and endocrine organs have the highest capacity for such reaction. The physical properties of oxycholesterols allow them to pass lipophilic membranes and to be redistributed in the cells at a much faster rate than cholesterol itself, resulting in rapid degradation and excretion (32). The major oxycholesterols in the circulation are transported by lipoproteins, and their distribution between different lipoprotein fractions is almost similar to that of cholesterol (33).

According to in vitro studies, oxycholesterols play a number of important roles in cholesterol

turnover, atherosclerosis, apoptosis, necrosis, inflammation and development of gallstones (2, 34). These studies also demonstrate the cytotoxic effects on the cultured endothelial cells and arterial smooth muscle cells, suggesting that oxycholesterols have an atherogenic role (4, 35). The structures of some important oxycholesterols are depicted in Figure 6.

7-hydroperoxycholesterol seems to be the most cytotoxic oxygenated lipid present in oxidized LDL (36). This oxycholesterol is rapidly decomposed into 7α- and 7β-hydroxycholesterol and 7-ketocholesterol which may be found in relatively high concentration in foam cells and fatty streaks.

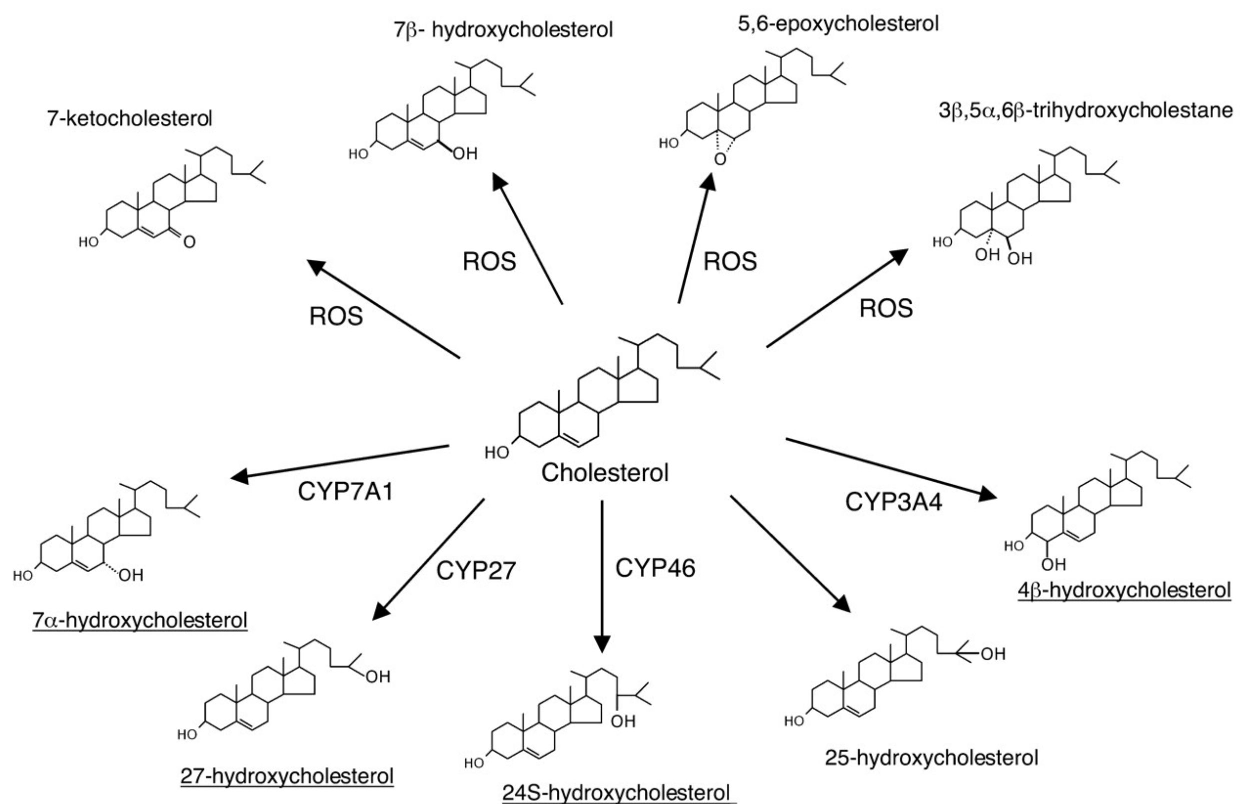


Figure 6 Primary cholesterol oxygenation reaction mediated by different cytochrom P-450 species or occuring non-enzymatically in the presence of reactive oxygen species (ROS). 25–hydroxycholesterol is formed by the enzyme cholesterol-25-hydroxylase (4).

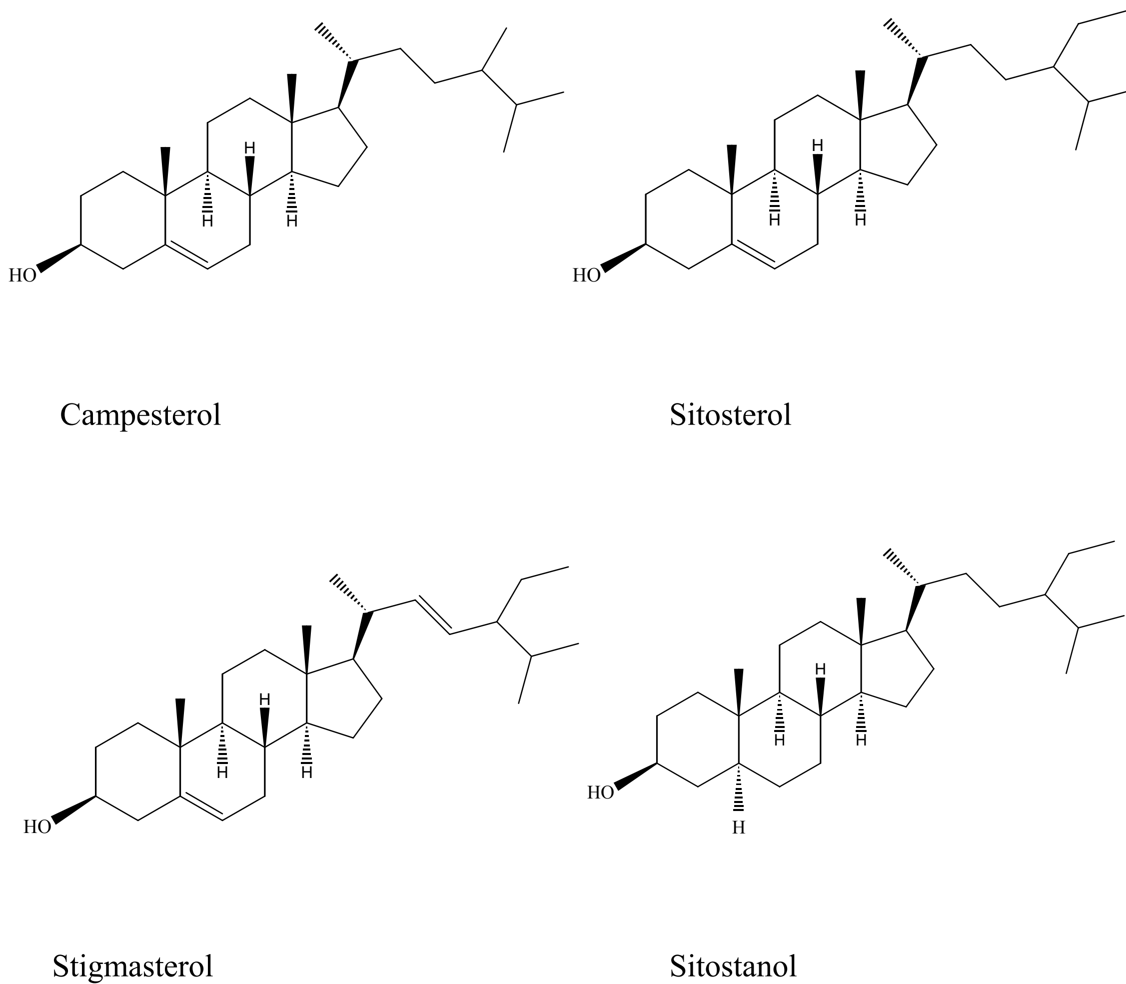
Many oxycholesterols have been found in human tissue and fluids such as human plasma, brain, atherogenic lipoproteins and atherosclerotic plaque. How these oxycholesterols originate in vivo is not fully understood. Some researchers suggest that they come from dietary sources, while others believe that they may be generated by non-enzymatic oxidation (2). Dietary sources of oxycholesterols are cholesterol-rich foods, especially those products which are heated in the presence of oxygen or are stored for long periods. The most commonly detected oxycholesterols in foods are the major products of cholesterol autoxidation: 7-keto-, 7β-hydroxy-, 5,6α/β-epoxy-cholesterol. The presence of dietary oxycholesterols in the circulation or in a specific tissue depends on the rate of absorption from the gut, transport to the specific tissue, and elimination of the COPs from the tissue by transport or metabolism. It has been shown that intestinal cholesterol absorption is mediated by NPC1L1 and the ATP-binding cassette tranporters ABCG5 and ABCG8, but it is yet not known whether these transporters are also involved in COPs absorption.

3.2.2 Oxycholesterols and atherosclerosis

It has been suggested, based on in vitro experiments, that cholesterol oxidation compounds are atherogenic, while oxycholesterols demonstrate cytotoxic effects on cultured endothelial cells. The highly nonphysiological conditions used in most experiments, however, preclude drawing strong conclusions. Numerous studies have been published about the effects of dietary oxycholesterols on experimental animals (2). Out of thirteen of these studies, six indicate a proatherogenic effect and four an antiatherogenic effect, while three show no significant effect. To date there is no direct evidence that dietary oxycholesterols contribute to atherosclerosis in humans (4). On the other hand, many studies have detected oxycholesterols in human atherosclerotic plaques (37, 38, 39), although relatively few have presented quantitative data (2). Oxidative modified LDL, which has proatherogenic effects, appears to be the most cytotoxic lipid. It contains 7-hydroperoxycholesterol and its degradation products 7α-hydroxycholesterol, 7β-hydroxycholesterol and 7-ketocholesterol.

3.3 Phytosterols

Plant sterols, also referred to as phytosterols, are non-nutritive compounds that have a chemical structure related to that of cholesterol. They differ in their side chain configuration only by an additional ethyl (β-sitosterol) or methyl (campesterol) group at the 24 carbon atom (39, 40) (Figure 7). Dehydrogenation of the carbon 22-23 bond of sitosterol leads to formation of stigmasterol, another common plant sterol. Phytostanols are saturated phytosterols where the delta 5 double bond is saturated and the hydrogen atom is positioned at the 5α position.



Campesterol Sitosterol

Stigmasterol Sitostanol

Figure 7 The chemical structure of plant sterols

3.3.1 Metabolism of plant sterols

Phytosterols are not endogenously synthesized in the body. In healthy humans, the absorption rate of plant sterols depends on their side chain length and is less than of cholesterol (41). Recent studies have indicated that plant sterols and stanols are taken up by the mucous cells almost to the same extent as cholesterol (42). However, they are rapidly resecreted into the intestinal lumen by the ABCG5 and/or ABCG8 co-transporters. The resecretion into the intestine may be due to the fact that the enzyme acyl CoA: cholesterol acyltransferase 2 (ACAT 2) does not esterify plant sterol. Research by Davis et al. indicates that NPC1L1 is important for the uptake of both cholesterol and structurally related phytosterols. The plasma levels of plant sterols were found to be undetectable in NPC1L1 null mice and were reduced by more than 90% compared to wild-type mice (43).

Intestinal phytosterol absorption is a selective process, with campesterol absorbed at a higher rate than sitosterol. This difference in intestinal absorption may be due to a variation in the side chain, where the phytosterol uptake decreases with an increasing number of carbon atoms at C24 of the sterol chain (41). A small percentage of plant sterols is absorbed and packaged with chylomicrons for transport to the systemic circulation. From the liver, plant sterols are rapidly re-excreted into bile, and only small amounts are transported to other peripheral tissues by VLDL and LDL. Non-absorbed plant sterols are metabolized by intestinal microflora in the same way as cholesterol, where metabolites corresponding to coprostanone and coprostanol are formed in the intestine (44).

Phytosterol elimination takes place via the biliary route and appears to be more rapid than that of cholesterol (45). Accordingly, due to poor absorption in the intestine and faster excretion via bile, the pool of endogenous phytosterols is small compared with cholesterol.

3.3.2 Oxidized plant sterols and their cytotoxic effect

Based on a limited number of studies on phytosterol oxidation processes, it has been concluded that the main oxidation route is generally the same as for cholesterol, resulting in the so-called oxyphytosterols or phytosterol oxidized products (POPs). Similarly, the analysis of oxyphytosterols follows the same procedures as in the case of COPs. Sitosterol oxidizes to 7α- and 7β-hydroperoxysitosterol, which is reduced to 7α-hydroxysitosterol and

7β- hydroxysitosterol; and dehydration of hydroperoxysitosterol leads to the formation of 7-ketositosterol. Epoxidation of the double bond between C5 and C6 atoms of sitosterol results in 5α,6α- and 5β,6β-epoxysitosterol, which could be converted to sitostantriol (Figure 8). Campesterol and other plant sterols are converted to the corresponding products (46, 47).

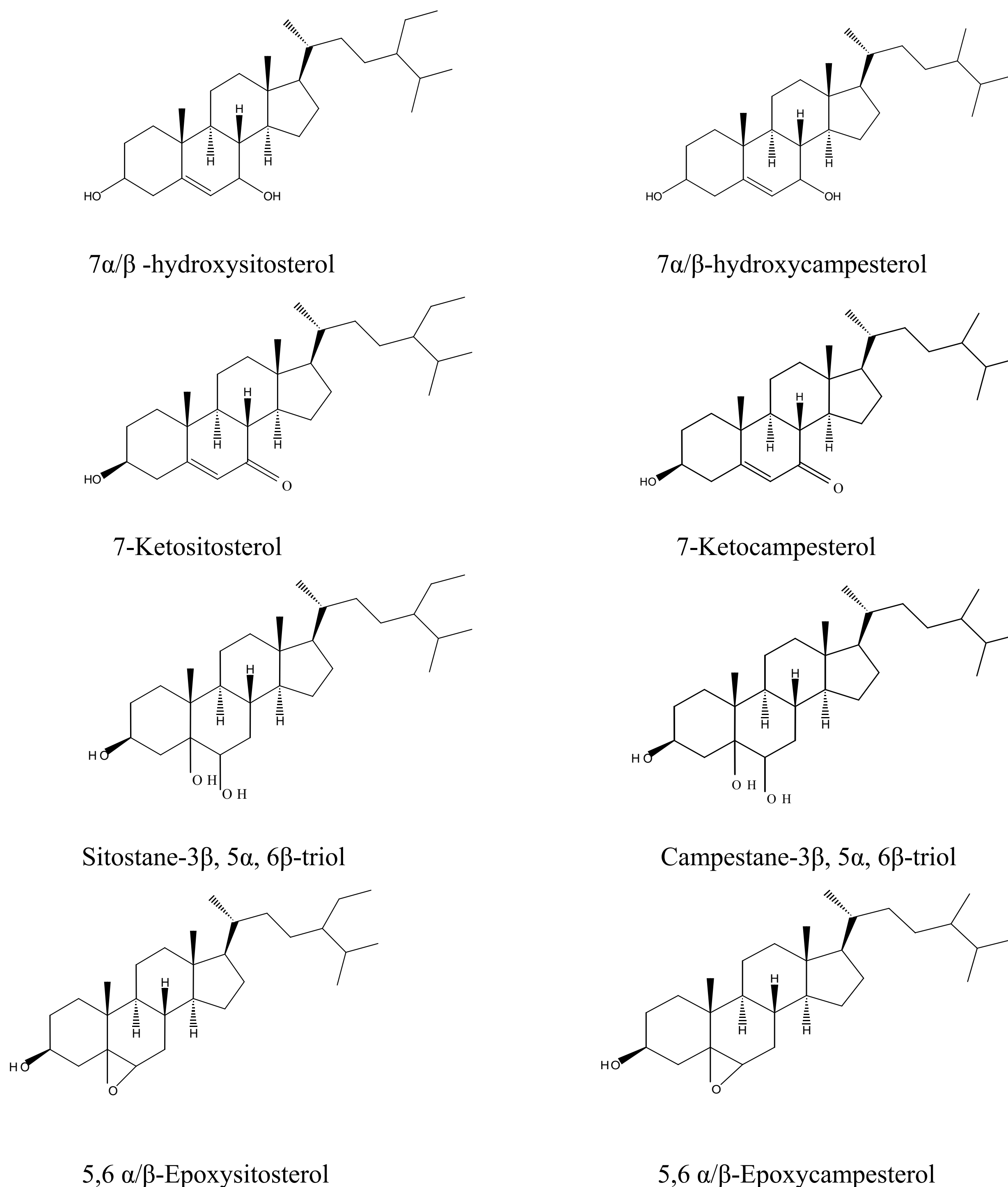


Figure 8 The chemical structure of some oxyphytosterols

Despite its similarity in chemical structure to cholesterol, Boberg et al. found that there is no significant conversion of C^{14} sitosterol into C24-bile acids in humans (48). By contrast, female Wistar rats can convert sitosterol into highly polar trihydroxylated C21-bile acids (49).
In theory, the presence of an ethyl or methyl group at C24 should prevent or at least reduce side-chain shortening after β-oxidation and thus inhibit conversion of plant sterol into bile acids by the same mechanism as that utilized for conversion of cholesterol into C24-bile acids. Furthermore, sitosterol is a poor substrate for 7α-hydroxylation, the initial and rate-limiting reaction in bile acid biosynthesis from cholesterol. Plat et al. (9) have pointed out the presence of oxysterols in serum from sitosterolemic patients and in two lipid emulsions, but it is still unknown whether these compounds affect health, as has been suggested for oxycholesterols. Whereas a study mentioned that α-and β-epoxides of sitosterol were observed in the plasma of a patient with Waldenström's macroglobulinaemia (50), the oxidized derivatives of phytosterols were also identified in plasma samples from thirteen healthy human volunteers (51). Grandgirard et al. (52) have shown in experimental animals that two of the main classes of oxyphytosterols (7-keto and epoxides) were absorbed at low rates, 4.7% of the given dose for epoxy derivatives and 1.5% for 7-keto compounds. He also found that campesterol oxides are absorbed better than sitosterol oxides, confirming that the length of the side chain is important for movement through the intestinal barrier. More recently, when hamsters were fed 100 µg/g oxyphytosterols in their diet, no POPs were recovered from plasma or tissues; however, significant amounts of various POPs were found after the feeding level was increased above 500 µg/g, thus indicating that the incorporation process is dose-dependent (53).
The cytotoxicity of oxyphytosterols has been studied in a culture-derived macrophage cell line, where the results showed that the oxides of sitosterol and campesterol have similar patterns of toxicity compared with oxycholesterols (54, 55). Adcox et al. (54) demonstrated that a mixture of sitosterol/campesterol oxides produces cell damage (which was monitored by lactate dehydrogenase leakage, cell viability and mitochondrial dehydrogenase activity) and that it may be due to hydroxy compounds identified in the mixtures of oxidized sterols. The damage caused by cholesterol oxides was greater than that caused by phytosterol oxides. In another study, Maguire et al. (55) demonstrated that sitosterol oxides exhibit patterns of toxicity similar to those of oxycholesterols on human monocytic U937 cells, which are a human cell line used as an in vitro model for monocyte/macrophage differentiation.

3.4 Sitosterolemia

3.4.1 Definition

Sitosterolemia, also known as phytosterolemia, is a rare autosomal recessively inherited sterol storage disorder. It was first recorded in 1974 (56) with a description of two normocholesterolemic siblings with large xanthomas and elevated plasma levels of the major plant sterols (27.1 and 17.7 mg/dl; where the normal range is < 1 mg/dl). This disease is characterized by the presence of tendon and tuberous xanthomas and by a strong predisposition to premature coronary artery disease, but with normal to moderately elevated plasma cholesterol levels (6, 57). Clinically it differs from familial hypercholesterolemia by its pattern of inheritance and by the diagnostic hallmark of elevated plasma phytosterol levels. Hemolysis, arthralgias and arthritis are also frequently associated with this disorder (6).

Studies have demonstrated that the absorption of plant sterols in sitosterolemic patients is higher than in healthy subjects (58). The plant sterol concentrations in plasma from sitosterolemic patients vary between 16-27 % of total sterol content (58, 59 and60). Studies have also shown a significantly reduced rate of biliary phytosterol excretion (59). A lack of 7α-hydroxylation (6, 48) results in markedly expanded plant sterol pools.

Biochemically, it was found that, the rate-limiting enzyme 7α-hydroxylase (which converts cholesterol into bile acids) is also reduced, due to competitive inhibition of this enzyme by sitosterol (61). Recently, it has been shown that the genetic defect in patients with sitosterolemia is due to mutations in either one of the two ATP-binding cassette (ABC) half-transporters, ABCG5 or ABCG8 (10). These two genes are expressed almost exclusively in the intestine and liver and are co-regulated by the nuclear hormone receptor, liver X receptor (LXR) (62).

3.4.2 Genetic mutations in sitosterolemia

No obvious differences in disease manifestation are apparent between patients with mutations in ABCG5 or in ABCG8. Interestingly, most Caucasian patients have mutations in ABCG8 whereas all Japanese individuals with sitosterolemia identified have mutations on ABCG5 (18, 63). Berge et al. (10) concluded that ABCG5 and ABCG8 normally cooperate to limit intestinal absorption and promote biliary excretion of sterols, and the mutated forms of these transporters predispose to sterol accumulation and atherosclerosis. Immediately following Berge’s report, Patel’s group

found that ABCG5 was mutated in nine unrelated sitosterolemic patients and also described mutations in ABCG8 in other patients (64). The identification of these genes should lead to a better understanding of the molecular mechanism(s) governing the highly selective absorption and retention of cholesterol by the body.

3.4.3 Sitosterolemia and atherosclerosis

The youngest sitosterolemic patient reported to have died due to CHD was a five-year-old girl with severe atheromatous narrowing of both coronary ostia (65). The association of high plant sterols levels in sitosterolemic subjects and atherosclerosis is documented in the work of Salen et al. (66). These authors confirmed the correlation between the plant sterols and atherosclerosis in an 18-year-old male with sitosterolemia who died suddenly of an acute myocardial infarction. Their findings indicate that atherosclerosis occurs prematurely in sitosterolemia and probably results from accelerated plasma sterol levels. Moreover, postmortem examination of the coronary and aortic vessels and myocardium revealed extensive atherosclerosis and microscopic infarction. Additionally, Hidaka (67) showed clinical signs of atherosclerosis obliterans, i.e., arterial bruit in femoral arteries and decreased ankle blood pressure, in two cases of four sitosterolemic patients. A case report refers to a 19-year-old man who presented with acute myocardial infarction with obstruction of one coronary artery. He proved to have sitosterolemia, which had resulted in juvenile coronary atherosclerotic lesions (68). A study by Sudhop et al. (69) raised questions regarding the role of plant sterols as a CHD risk factor as presumed by Glueck et al. in nonsterolemic subjects (70). It is not clear whether plant sterols are a risk factor in patients without sitosterolemia.

3.4.4 Laboratory investigations

Sitosterolemia must be considered in all patients with xanthomatosis and hypercholesterolemia, especially if there is a family history of premature cardiovascular disease. The diagnosis of sitosterolemia is established by demonstrating increased amounts of plant sterols and 5α-stanols in plasma and tissues. The usual enzymatic method does not distinguish sitosterol and other plant sterols from cholesterol. Therefore, gas-liquid chromatography needs to be used in addition to gas chromatography/mass spectroscopy or high-pressure liquid chromatography.

3.4.5 Treatment of sitosterolemia

Dietary control is the first attempt in the treatment of sitosterolemia, with a diet containing a minimal amount of plant sterols recommended. When dietary treatment alone is insufficient, the following kinds of drugs may be considered:

1 Bile acids binding resins (such as cholestyramine and colestipol)

2 Cholesterol absorption inhibitor (ezetimibe)

3.4.5.1 Bile acids binding resins

Bile acids binding resins, also known as bile acid sequestrants, are a special class of cholesterol-lowering agents. Resins exert their effect in the intestine, where they bind bile acids, preventing their reabsorption into the circulation and facilitating their subsequent loss in the feces. This interruption in the enterohepatic circulation of bile acids enhances the conversion of cholesterol to bile acids in the liver and results in a reduction of plasma cholesterol levels. This mechanism was also found to be effective in the treatment of sitosterolemia and reduction of plasma plant sterol concentrations (71,59), where the percent reduction in plasma sterol concentrations obtained with these drugs is greater than in similarly treated hypercholesterolemic subjects (72). Clinical improvement including disappearance of xanthomas, elimination of aortic murmur and decreased frequency of angina pectoris have been noted in several patients treated with cholestyramine (71). Bhattacharya et al. (73) showed that cholestyramine treatment of sitosterolemic patients (up to 12 g per day) reduces circulating levels of cholesterol, plant sterols and their 5α-analogues by about 50%. Lütjohann et al. (74) reported that the interruption of treatment with cholestyramine for six weeks resulted in an increase of circulating cholesterol by 85%, campesterol by 57% and sitosterol by 40% compared to treatment with resins.

3.4.5.2 Ezetimibe

Ezetimibe, the first member of a new class of lipid-lowering drugs known as cholesterol absorption inhibitors, has been found to be useful in the treatment of primary hypercholesterolemia, due to its reducing effect on LDL-C, total cholesterol and apo B. It can also be used in combination therapy with statins for the treatment of familial hypercholesterolemia. The drug is absorbed into the intestinal epithelial cells and remains

associated with the apical cell membrane, where it is believed to interfere with the putative sterol transport system. This would prevent both free cholesterol and phytosterols from being transported into the cell from the intestinal lumen.

Ezetimibe is rapidly absorbed; its time of maximum concentration is about one hour (75). Once absorbed, ezetimibe undergoes phase II metabolism in the intestine and liver to form an active glucuronide metabolite (76). Both ezetimibe and its active metabolite are highly bound to plasma proteins, and the extensive enterohepatic circulation allows for recycling of the drug and prolongs the effective half-life (22 hr) (77). Ezetimibe impairs the intestinal absorption of dietary cholesterol as well as biliary cholesterol.

The precise mechanism involved in the action of this drug remains unclear; however, several studies and experiments have attempted to identify a particular target or mechanism by which ezetimibe exerts its sterol-lowering effect. The activity of ACAT is not inhibited by ezetimibe (78), and other studies indicate that SR-BI is not the site of ezetimibe action (79). Ezetimibe also has no effect on ABCG5, ABCG8 and various intestinal adenosine triphosphate (ATP) binding cassette (ABC) transporters (10). More recently, Altmann and Davis (16, 43) found that the profile of cholesterol absorption in NPC1L1 null mice closely resembles the profile for an ezetimibe-treated animal, suggesting that ezetimibe acts through the pathway that contains NPC1L1 protein. Sudhop et al. (11) have shown that treatment with ezetimibe for two weeks reduces cholesterol absorption by 54% compared with the placebo group, with the concentrations of both LDL and total cholesterol being reduced by 20.4% and 15%, respectively. However, campesterol and sitosterol concentrations were also reduced by 48% and 41%, respectively. The effect of ezetimibe on the plant sterols in plasma was studied by Salen et al. in patients with sitosterolemia (12). This work has clearly indicated that a dose of 10 mg ezetimibe per day for eight weeks leads to a reduction in sitosterol concentration of 21%, compared with a nonsignificant rise of 4% in the group receiving a placebo. Campesterol concentration was also significantly decreased 24% by ezetimibe treatment, whereas with administration of a placebo it slightly increased 3% . It is interesting to note the reduction in xanthomas in the ezetimibe-treated group relative to the placebo group after eight weeks of treatment (12). Whether this decrease in xanthomas indicates that other complications including atherosclerosis and hemolysis may be reduced with ezetimibe remains to be elucidated.

4 *Intravenous lipid emulsion as parenteral nutrition*

The essential components of parenteral nutrition are fluids, carbohydrates, electrolytes, proteins, lipids, vitamins and minerals. Intravenous lipid emulsions are a major source of energy; they provide the required essential fatty acids and help to improve nitrogen balance. There are two types of lipid emulsions currently used in parenteral nutrition: 1) lipid emulsions prepared from soybean oil, which are composed of long-chain triacylglycerols and 2) lipid emulsions composed of half medium-chain triacylglycerols and half long triacylglycerols from soybean oil; both of these components may contain phytosterols. The cholesterol and phytosterol content of some lipid emulsions has been determined by gas chromatography-mass spectroscopy, and the results are shown in Table 1 (80).

Table 1 Concentrations (mg/dl) of plant sterols in samples of four commercially available lipid emulsions (80).

Lipid emulsion	Ivelip 20%	Lipofundin 20%	Lipofundin 20%*	Intralipid 20%
Cholesterol	27.0	0.0	75.3	44.8
Campesterol	22.8	31.2	20.8	8.4
Stigmasterol	14.8	27.2	11.5	7.4
Sitosterol	42.2	77.0	45.1	26.9
Sitostanol	1.7	0.0	0.0	1.7
Total sterols	108.5	135.4	152.7	89.2

* MCT/LCT medium chain triglycerols / long chain triglycerols.

A new intravenous lipid emulsion consisting of a mixture of soybean and olive oils has been studied to assess its safety and efficacy for children (81). The use of lipid emulsions has been associated with certain complications in neonates, including hyperlipidemia, hyperbilirubinemia and changes in pulmonary function when the rate of administration is too high (82). Cholestasis is the most serious metabolic complication seen with long-term parenteral nutrition in neonates (83) and may result in biliary cirrhosis or end-stage liver disease. Parenteral nutrition-associated cholestasis is particularly common in small premature infants due to reduced bile acid synthesis rates and pool sizes and an immature bile secretory system. One important factor affecting the incidence of parenteral nutrition-associated cholestasis is the duration of intravenous feeding

(84). The observation that children who were receiving parenteral lipid emulsion and showed high plasma phytosterol concentration developed cholestasis led to the hypothesis that accumulation of these plant sterols contributes to cholestasis (84). It was hypothesized that the liver has a limited capacity for handling plant sterols. Under normal circumstances this limited capacity is not important, since plant sterols are poorly absorbed from the diet. In patients receiving parenteral nutrition, however, the plant sterols given intravenously must be metabolized or excreted. Conversion of the plant sterols to bile acids is inefficient, and they may inhibit the conversion of cholesterol to bile acids (85, 86). Plant sterols secreted into bile are likely to be less soluble than cholesterol and may precipitate if bile salt concentrations are reduced. Children requiring parenteral nutrition often have a reduced bile salt pool in their enterohepatic circulation; if precipitation of sterols occurs, it contributes to biliary sludge.

It has been proposed that sterol accumulation in the liver and/or precipitation in bile play a significant role in the pathogenesis of parenteral nutrition-associated liver disease (80, 84). Clayton et al. (84) studied 29 children aged two months to nine years who were receiving parenteral nutrition and 29 matched controls with ages ranging from three months to fourteen years. The study showed that five children with severe parenteral nutrition-associated liver disease had plasma concentrations of phytosterols and sitostanol that were as high as those seen in patients with sitosterolemia (see Table 2). A reduction in lipid emulsion intake was associated with a decrease in plasma phytosterol concentration and an improvement in liver function and platelet counts.

Table 2 Sterol and stanol concentrations in patients receiving parenteral nutrition and in control subjects (82).

	Concentration in plasma (mg/dl)		
	Control	Patients receiving total PN	
Analyte	Normal liver function	Mild liver dysfunction	Severe liver dysfunction
Campesterol	1.0 ± 0.2	2.3 ± 0.5	10.6 ± 1.1
Stigmasterol	0	1.1 ± 0.3	6.5 ± 1.2
Sitosterol	0.6 ± 0.1	6.3 ± 1.7	39.3 ± 3.8
Sitostanol	0	0.6 ± 0.2	3.7 ± 0.8
Cholesterol	176.1 ± 17.7	182.8 ± 14.7	185.8 ± 13.9
Cholestanol	0.03 ± 0.01	0.2 ± 0.1	0.8 ± 0.5

Plat et al. (9) showed the presence of oxyphytosterols in two frequently used soybeans oil-based lipid emulsions in total parenteral nutrition protocols, where emulsion A contained lower levels of plant sterols and more cholesterol than emulsion B (Table 3). Oxidized plant sterol concentrations were somewhat higher in emulsion B. The ratios of oxidized sitosterol to sitosterol, however, were comparable: 0.038 in emulsion A and 0.041 in emulsion B. This suggests that the higher oxyphytosterol concentrations in emulsion B were simply due to higher plant sterol concentrations.

Table 3 Plant sterol and oxidized sitosterol concentrations in lipid emulsions (9).

	Emulsion A	Emulsion B
Cholesterol (mg/dl)	29.8	17.8
Plant sterols (mg/dl)[a]	11.3	27.7
Sitosterol (mg/dl)	8.6	20.1
Campesterol (mg/dl)	2.7	7.6
7-ketositosterol (µg/ml)	0.70	1.26
7β-hydroxysitosterol (µg/ml)	0.65	0.37
5α,6α-Epoxysitosterol (µg/ml)	2.73	9.24
3β,5α,6β-tri-hydroxysitosterol (µg/ml)	0.16	0.49
7α-hydroxycholesterol (ng/ml)	19.50	9.70

a Plant sterols are calculated as the sum of sitosterol and campesterol

Because these emulsions are currently used in neonatal parenteral nutrition, their direct venous introduction might be potentially dangerous because of the possible atherogenic role of cholesterol and plant sterol oxidation products.

5 Methods

A - First study

5.1 Chemicals

1. Solvents:

   Cyclohexane, dichloromethane, phosphoric acid, ethanol, toluene, propanol and hexane were purchased from Merck-Darmstadt (Germany). All were of analytical grade.
2. Potassium hydroxide and sodium chloride (analytical grade) were obtained from Merck-Darmstadt (Germany).
3. Silylation reagent: dry pyridine, hexamethyldisilazane, trimethylchlorosilane (TMS) 3:2:1 (v/v/v).
4. Oxycholesterols:

   Cholesterol, 7β-hydroxycholesterol and 7-ketocholesterol were from Sigma-Aldrich (Germany), while 7α-hydroxycholesterol was from Steroloids (USA).

   3β, 5α, 6β-cholestantriol and epoxycholesterol were synthesized in the lab of clinical pharmacology department in Bonn university (87).
5. All deuterated oxycholesterol compounds (26,26,26,27,27,27-d6-cholesterol, 26,26,26,27, 27,27-d6-7α-hydroxycholesterol, 26,26,26,27,27,27-d6-7β-hydroxycholesterol, 26,26,26, 27,27,27-d6-3β,5α,6β-cholestantriol, 26,26,26,27,27,27-d6-7-ketocholesterol, 26,26,26,27, 27,27-d6-5α,6α-epoxycholesterol and 26,26,26,27,27,27-d6-5β,6β-epoxy-cholesterol) as internal standards (ISTD) were prepared according to Plat et al. (9).
6. Oxyphytosterols:

   Sitosterol was from Sigma-Aldrich (Germany) and deuterated sitosterol (2,2,4,4,6-d5-sitosterol/campesterol) was from Sugaris GmbH (Germany).

   All other deuterated oxyphytosterol compounds (2,2,4,4,6-d5-7α-hydroxysitosterol/ campesterol, 2,2,4,4,6-d5-7β-hydroxysitosterol/campesterol, 2,2,4,4,6-d5-3β,5α,6β–sitostantriol/campestantriol, 2,2,4,4,6-d5-7-ketositosterol/campesterol, 2,2,4,4,6-d5-5α, 6α-epoxysitosterol/campesterol and 2,2,4,4,6-d5-5β,6β-epoxysitosterol/campesterol) as internal standards (ISTD) and non-deuterated oxyphytosterols were prepared according to Plat et al. (9).

5.2 Sample collection

Before and after eight weeks of treatment with ezetimibe (10 mg/day), blood samples were taken from 13 sitosterolemic patients, ranging in age from 12 to 57 years (their weight ranged between 39 and 98 kg). Four of the patients were being treated with statins (two of them with simvastatin 40 and 20 mg once a day and the other two were with atrovastatin in a dose of 20 mg daily in a tablet form) and three patients were taking bile acid binding resins (one of them was with cholestyramine powder in a dose of 8 gm daily and the other two patients were with cholestipol tablets in a dose of 16 gm daily) , whereas the other six patients were not being treated with drugs.

Soybean oil-lipid emulsion (lipofundin^{R} 10% and lipovenöse^{R}) used in total parenteral nutrition protocols was analyzed for oxyphytosterols and oxycholesterols by the same procedures as described for serum samples.

The following measurements of oxysterols were carried out:

1 Oxyphytosterols and oxycholesterols in the serum from patients with sitosterolemia as compared with serum samples from normolipemic controls
2 The effect of ezetimibe on oxyphytosterol and oxycholesterol concentrations in the three different groups of patients (those being treated with statins, with bile acid binding resins and without drugs)
3 Oxyphytosterols and oxycholesterols in two samples of commercially available lipid emulsions used as intravenous nutrition
4 Oxyphytosterols and oxycholesterols in serum from nine patients receiving intravenous lipid emulsions.

5.3 GC-MS analysis

5.3.1 Sample preparation

5.3.1.1 Alkaline hydrolysis and extraction

The serum samples from patients were kept at -80 °C with 10 µl BHT (25 mg/ml in methanol). By modifying the method used by Dzeletovic et al. (88), one ml of serum was taken in a 50 ml test tube and 100 µl of internal standard (ISTD) were added to the serum sample. Before saponification and extraction, the serum sample was extensively saturated with argon to minimize autoxidation. Saponification was carried out by adding 10 ml of 0.35 M ethanolic KOH in a

closed tube under argon for 2 hours at room temperature. 130 µl of phosphoric acid (50% , v/v in H_2O) was added after saponification to neutralize the solution, followed by addition of 6 ml NaCl solution in H_2O (9 mg/ml). The unsaponifiable part was extracted by liquid-liquid partitioning into dichloromethane (20 ml). The lower layer was transferred into a conical flask and dried by evaporation under vacuum. The residue was dissolved in 2 ml ethanol and again evaporated under vacuum to remove all traces of water. The residue was dissolved in 1 ml toluene.

5.3.1.2 Solid-phase extraction

Silica cartridges (Bond Elut, bonded phase SI, 100 mg, 1 ml; Varian, Harbor City, CA 90710, USA) were eluted with 2 ml hexane before the toluene fraction was loaded. Neutral sterols including cholesterol, sitosterol and campesterol were eluted from the column with 10 ml 0.25% isopropanol in cyclohexane (v/v), whereas the absorbed oxysterols were eluted with 8 ml of 30% isopropanol in cyclohexane (v/v).

The oxysterols fraction was dried under vacuum. Finally, the oxidized sterols were dissolved in 500 µl cyclohexane and transferred to an injection vial, dried with nitrogen and silylated by addition of 100 µl of TMS. Then the vial was heated for 1 hr at 90 °C , after which the sample was diluted with 200 µl cyclohexane for GC-MS measurement.

To minimize the autoxidation of both cholesterol and plant sterols, the samples were kept at -80 ºC with BHT (which acts as an antioxidant). In addition, all the processing was done under cold conditions. Samples were saturated with argon, thus creating an inert atmosphere. Isotope-labelled sterols were added to trace the loss of oxysterols during sample processing (88, 89).

5.3.2 Chromatographic conditions

Oxyphytosterols and oxycholesterols were analyzed by GC-MS as TMS derivatives, where 2 µl of the TMS derivatives in cyclohexane were injected via an AS2000 autosampler (Thermoquest CE Instruments, Egelsbach, Germany) on a Trace GC2000 (Thermoquest CE Instruments) gas chromatograph equipped with a DB-XLB column (30 m x 0.25 mm internal diameter, 0.25 µm film thickness) coupled to a trace MS (Thermoquest CE Instruments). The analysis was carried out in single ion monitoring (SIM) mode, making *m/z* the primary resolving parameter other than only retention time. The injector temperature was set at 280 °C. Helium was used as carrier gas (constant flow 1 ml/min). The oven temperature gradient was programmed for 150 s at 150 °C, then increased by 10 °C/min toward 290 °C, and then increased by 7 °C/min toward 320 °C and

kept there for 20 minutes. Thus, one analytical run lasted approximately 42 minutes. The ions and retention times of all individual compounds are given in Table 4. In addition to plant sterols and oxyphytosterols, also retention times and *m/z* values of cholesterol oxidation products (and also of 26,26,26,27,27,27-d6-cholesterol oxidation products) are shown, which illustrates that interference of oxycholesterols and oxyphytosterols in GC-MS identification is not a problem.

Table 4 Retention times and m/z values of oxycholesterols and oxyphytosterols.

	D0		D5/6	
Compound TMS	Retention Time	m/z	Retention Time	m/z
Cholesterol	18.24	458	18.15	464
7α-hydroxycholesterol	17.39	456	17.17	462
7β-hydroxycholesterol	18.64	456	18.57	462
3β,5α,6β-cholestantriol	20.22	456	20.14	462
5α,6α-epoxycholesterol	19.95	384	19.75	390
5β,6β-epoxycholesterol	19.54	384	19.38	390
7-Ketocholesterol	21.50	472	21.41	478
Sitosterol	19.98	486	19.88	492
7α-hydroxysitosterol	18.87	484	18.87	488
7β-hydroxysitosterol	20.27	484	20.24	488
3β,5α,6β-sitostantriol	22.15	484	21.93	488
5α,6α-epoxysitosterol	21.62	412	21.54	416
5β,6β-epoxysitosterol	21.33	412	21.27	416
7-ketositosterol	23.48	500	23.41	504
Campesterol	19.13	472	19.05	478
7α-hydroxycampesterol	18.11	470	18.10	474
7β-hydroxycampesterol	19.51	470	19.49	474
3β,5α,6β-campestantriol	21.24	470	21.09	474
5α,6α-epoxycampesterol	20.73	398	20.68	402
5β,6β-epoxycampesterol	20.45	398	20.42	402
7-ketocampesterol	22.45	486	22.39	490

5.3.3 Derivatization

Oxyphytosterols and oxycholesterols were analyzed as TMS derivatives by silylating them using dry pyridine, hexamethyldisilazane, trimethylchlorosilane (TMS) 3:2:1 (v/v/v). by silylation, active hydrogen is replaced by an alkylsilyl group.

100 µl of TMS were added to the sample in GC vials and heated at 90° C for one hour. Compared

with their parent compounds, silyl derivatives are more volatile, less polar and more thermally stable. As a result, GC separation is improved and detection is enhanced. The silylation is usually conducted in pyridine medium, which must be anhydrous─since water competes with TMS, moisture in tubes and reagents could lead to incomplete silylation.

5.3.4 Selected ion monitoring

The unlabelled and labelled oxidized sterol derivatives were analyzed by SIM mode using specific masses counting. Characteristic ions for oxycholesterols and oxyphytosterols were monitored (see Table 4).

5.3.5 Calibration

Calibration curves for all individual compounds were calculated after preparing a standard mixture of all compounds by dissolving crystals of synthesized standards in cyclohexane (except trihydroxy-sterols, which were dissolved in dichloromethane) to a final concentration of 1 mg/10 ml. 100 µl of deuterated solutions of both oxycholesterols and oxyphytosterols were added to the vials labelled as follows: 0, 10, 20, 50, 80 and 100. Non-deuterated solutions were added in volumes of 0, 10, 20, 50, 80 and 100 µl. The vials were evaporated by nitrogen, then silylated with 100 µl TMS and heated at 90 ˚C for one hour and diluted with 100 µl cyclohexane for GC-MS measurement.

5.3.6 Quantification

Quantification of oxyphytosterols and oxycholesterols was based on the ratio between the calculated peak area from selected ions of corresponding different oxidized sterols and their corresponding labelled internal standards. The concentration of different oxidized sterols was then calculated using the linear regression of the calibration curve.

5.3.7 Method validation

5.3.7.1 Specificity

Cholesterol, sitosterol, campesterol, oxycholesterols and oxyphytosterols were identified on the basis of retention time, the appearance of labelled analogous and the specific m/z of both labelled and unlabelled compounds.

5.3.7.2 Linearity

The correlation coefficient, y-intercept and slope of the regression line of 7α- hydroxycholesterol as example for linearity are shown in Figure 10. In order to show the deviations from linearity, the response factor (m_i) was calculated using the following formula:

$$m_i = (y-b)/x$$

Where;

b: Y-intercept of calibration curve

y: ratio between nondeuterated and deuterated compound

x: amount of the compound

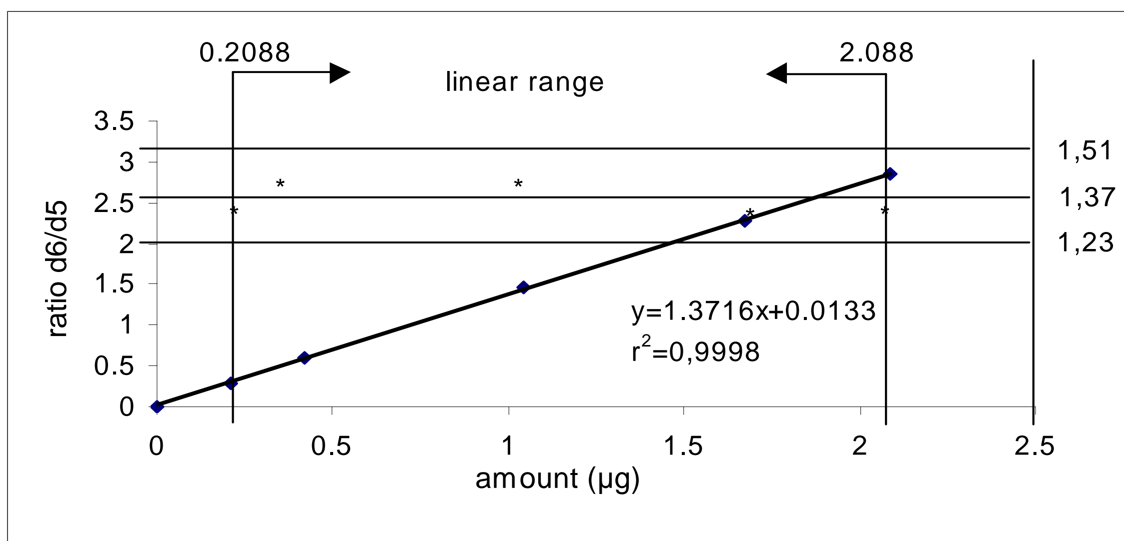
Table 5 Determination of linear range of 7α- hydroxycholesterol.

Amount (µg) (x)	D6	D0	D0/D6 (y)	Regression line	Response factor (m_i)
0	63456857	166623	0.002626		0
0.2088	70723207	21006062	0.297018	m=1.3716	1.330067
0.4176	70277274	42409735	0.603463	b= 0.0133	1.398858
1.044	41245720	60495716	1.466715	r^2= 0.9998	1.386413
1.6704	41384223	94571463	2.285206		1.356505
2.088	50000713	142855832	2.857076		1.359088

In addition to the correlation coefficient and y-intercept, 10% more or less than the response factor of the regression line (m) was used as a range to accept and evaluate the linearity data, which was in the range of 1.37±0.14 (1.23-1.51) (Figure 10).

For 7α- hydroxycholesterol, all response factors (m) were in the range between 1.23 and 1.51; therefore the linear range of the regression line was between 0.2088 and 2.088 µg (Figure 9).

A linear relationship was observed over the range shown in Table 6 for all oxycholesterols and oxyphytosterols except for 7-ketositosterol and 7-ketocampesterol, which were calculated by using a polynomic curve.



* Response factor

Figure 9 Calibration curve and linear range of 7α- hydroxycholesterol

Table 6 Linear range for quantification of oxycholesterols and oxyphytosterols.

Oxysterols	Linear range (µg)
7α-hydroxycholesterol	0.208-2.08
7β-hydroxycholesterol	0.112-1.12
7-ketocholesterol	0.329-3.29
3β,5α,6β-cholestantriol	0.140-1.40
7α-hydroxysitosterol	0.039-0.392
7β-hydroxysitosterol	0.067-0.674
3β,5α,6β-sitostantriol	0.098-0.981
7α-hydroxycampesterol	0.020-0.206
7β-hydroxycampesterol	0.046-0.463
3β,5α,6β-campestantriol	0.062-0.629

5.3.7.3 Analytical recovery

Tables 7a, b and c show the results of several experiments which were performed to determine the percentage recovery of oxycholesterols and oxyphytosterols from spiked serum samples (88, 90).

Serum samples were analyzed before and after addition of 10, 20 and 100 µl of standard mixtures of oxycholesterols and oxyphytosterols. Tables 7a, 7b and 7c show that when the amount of standard mixture of oxysterols added to the serum decreases, the percentage recovery decreases. As in case of 10 µl, the percentage recovery was between 55% and 76%, whereas in case of 100 µl, the percentage recovery was between 82% and 123%.

Table 7a Recovery of oxycholesterols and oxyphytosterols from spiked serum (10 µl).

Oxysterols	Unspiked samples (µg/ml) [a]	Spiked samples (µg/ml) [b]	Amount (µg) added in 10 µl	Recovery (%)
7α-hydroxycholesterol	0.12 ± 0.0009	0.23 ± 0.010	0.21	55
7β-hydroxycholesterol	0.06 ± 0.002	0.13 ± 0.001	0.11	62
3β,5α,6β-cholestantriol	0.06 ± 0.002	0.14 ± 0.003	0.14	60
7-ketocholesterol	0.13 ± 0.009	0.36 ± 0.070	0.33	69
7α-hydroxysitosterol	0.001 ± 0.0007	0.03 ± 0.001	0.04	69
7β-hydroxysitosterol	0.007 ± 0.0015	0.05 ± 0.003	0.07	66
3β,5α,6β-sitostantriol	0.005 ± 0.0016	0.06 ± 0.003	0.10	61
7-ketositosterol	0.012 ± 0.002	0.01 ± 0.002	0.04	60
7α-hydroxycampesterol	0.001 ± 0.0001	0.02 ± 0.001	0.21	76
7β-hydroxycampesterol	0.002 ± 0.0001	0.04 ± 0.003	0.05	70
3β,5α,6β-campestantriol	0.001 ± 0.0001	0.04 ± 0.002	0.07	63
7-ketocampesterol	0.006 ± 0.001	0.03 ± 0.003	0.03	68

a amount in µg ; mean ± SD (n=3); Sample volume 1.0 ml

b amount in µg ; mean ± SD (n=6); Sample volume 1.0 ml

Table 7b Recovery of oxycholesterols and oxyphytosterols from spiked serum (20 µl).

Oxysterols	Unspiked samples (µg/ml) [a]	Spiked samples (µg/ml) [b]	Amount (µg) added in 20 µl	Recovery (%)
7α-hydroxycholesterol	0.12 ± 0.0009	0.42 ± 0.010	0.42	71
7β-hydroxycholesterol	0.06 ± 0.002	0.26 ± 0.006	0.22	86
3β,5α,6β-cholestantriol	0.06 ± 0.002	0.25 ± 0.023	0.28	70
7-ketocholesterol	0.13 ± 0.009	0.60 ± 0.028	0.66	71
7α-hydroxysitosterol	0.001 ± 0.0007	0.06 ± 0.005	0.08	76
7β-hydroxysitosterol	0.007 ± 0.0015	0.11 ± 0.001	0.13	76
3β,5α,6β-sitostantriol	0.005 ± 0.0016	0.15 ± 0.005	0.20	74
7-ketositosterol	0.012 ± 0.002	0.09 ± 0.007	0.09	82
7α-hydroxycampesterol	0.001 ± 0.0001	0.03 ± 0.001	0.04	81
7β-hydroxycampesterol	0.002 ± 0.0001	0.08 ± 0.005	0.09	78
3β,5α,6β-campestantriol	0.001 ± 0.0001	0.96 ± 0.003	0.13	76
7-ketocampesterol	0.006 ± 0.001	0.06 ± 0.005	0.06	92

a amount in µg ; mean ± SD (n=3); Sample volume 1.0 ml

b amount in µg ; mean ± SD (n=3); Sample volume 1.0 ml

Table 7c Recovery of oxycholesterols and oxyphytosterols from spiked serum (100 µl).

Oxysterols	Unspiked samples (µg/ml) [a]	Spiked samples (µg/ml) [b]	Amount (µg) added in 100 µl	Recovery (%)
7α-hydroxycholesterol	0.12 ± 0.0009	2.20 ± 0.07	2.09	100
7β-hydroxycholesterol	0.06 ± 0.002	1.10 ± 0.03	1.12	92
3β,5α,6β-cholestantriol	0.06 ± 0.002	1.20 ± 0.02	1.40	82
7-ketocholesterol	0.13 ± 0.009	3.19 ± 0.07	3.29	93
7α-hydroxysitosterol	0.001 ± 0.0007	0.41 ± 0.004	0.39	92
7β-hydroxysitosterol	0.007 ± 0.0015	0.75 ± 0.008	0.67	111
3β,5α,6β-sitostantriol	0.005 ± 0.0016	1.01 ± 0.018	0.98	100
7-ketositosterol	0.012 ± 0.002	0.55 ± 0.011	0.45	123
7α-hydroxycampesterol	0.001 ± 0.0001	0.21 ± 0.006	0.02	103
7β-hydroxycampesterol	0.002 ± 0.0001	0.46 ± 0.005	0.46	102
3β,5α,6β-campestantriol	0.001 ± 0.0001	0.64 ± 0.012	0.63	99
7-ketocampesterol	0.006 ± 0.001	0.38 ± 0.010	0.32	121

a amount in µg ; mean ± SD (n=3); Sample volume 1.0 ml

b amount in µg ; mean ± SD (n=3); Sample volume 1.0 ml

5.3.7.4 Accuracy

The accuracy of an analytical method describes the closeness of the mean test results obtained by the method to the true value (concentration) of the analyte. In this work accuracy was determined by replicate analysis of samples containing 3 different known amounts of the analyte using ten determinations per concentration. The mean values were within 15% of the actual value.

5.3.7.5 Precision

The repeatability of the method was evaluated using six spiked serum samples, each of which was analyzed after the addition of 100 µl of oxysterols standard mixture following the procedure

of extraction, derivatization and GC-MS analysis. Table 8 shows the means, SD and variation coefficients of all compounds.

Table 8 Repeatability for the determination of oxysterols by GC-MS.

Sample no.	1	2	3	4	5	6	Mean	SD	CV
				Oxysterols (µg/ml)					
7α-hydroxycholesterol	2.11	2.31	2.29	2.14	2.17	2.21	2.20	0.07	3.3
7β-hydroxycholesterol	1.14	1.07	1.16	1.13	1.07	1.07	1.11	0.04	3.3
3β,5α,6β-cholestantriol	1.21	1.25	1.18	1.19	1.21	1.19	1.21	0.02	1.8
7-ketocholesterol	3.11	3.30	3.26	3.16	3.11	3.24	3.20	0.07	2.3
7α-hydroxysitosterol	0.40	0.40	0.41	0.42	0.40	0.41	0.41	0.01	1.1
7β-hydroxysitosterol	0.75	0.76	0.74	0.76	0.75	0.75	0.75	0.01	1.1
3β,5α,6β-sitostantriol	1.00	0.98	1.00	1.00	1.03	1.03	1.01	0.02	1.9
7-ketositosterol	0.54	0.53	0.55	0.56	0.55	0.56	0.55	0.01	2.2
7α-hydroxycampesterol	0.22	0.21	0.20	0.22	0.21	0.22	0.21	0.01	3.2
7β-hydroxycampesterol	0.47	0.46	0.46	0.47	0.46	0.47	0.46	0.01	1.2
3β,5α,6β-campestantriol	0.63	0.63	0.65	0.65	0.64	0.65	0.64	0.01	2.0
7-ketocampesterol	0.37	0.37	0.38	0.39	0.39	0.39	0.38	0.01	2.6

5.3.7.6 Limit of detection and limit of quantification

The detection limit of the assay was estimated by determining the signal-to-noise ratios of the peaks of the unlabelled compounds using the software (Xcalibur™ Version 1,2. Finnigan Corp. 1998-2000) supplied with the mass spectrometer controlling program. The detection limit can be defined as the smallest concentration of analyte that can be reliably detected by the instrumental method. This smallest concentration was estimated by using the following formula:

Limit of detection = amount of analyte . 3/signal: ratio recorded (90).

The limit of quantification is the smallest amount of analyte that can be quantified. It can be

calculated as 3 times the limit of detection:

Limit of quantification = 3 . limit of detection.

Table 9 shows the detection limit and quantification limit of all oxysterols.

Table 9 Detection limit and quantification limit of oxysterols.

Oxysterols	Detection limit (ng/ml)	Quantification limit (ng/ml)
7α-hydroxycholesterol	0.15	0.44
7β-hydroxycholesterol	0.13	0.40
3β,5α,6β-cholestantriol	0.89	2.67
7-ketocholesterol	0.98	2.95
7α-hydroxysitosterol	0.26	0.77
7β-hydroxysitosterol	0.45	1.36
3β,5α,6β-sitostantriol	0.81	2.42
7-ketositosterol	5.91	17.72
7α-hydroxycampesterol	0.15	0.46
7β-hydroxycampesterol	0.42	1.25
3β,5α,6β-campestantriol	0.59	1.77
7-ketocampesterol	2.22	6.66

B - second study

5.4 Chemicals

1. Sodium hydroxide, ethanol, cyclopean, pyridine and silylation reagent (dry pyridine, hexamethyldisilazane, trimethylchlorosilane (TMS) 3:2:1 (v/v/v)) were purchased from Merck, Darmstadt.
2. 5α-cholestane as internal standard was purchased from Serva Electrophoresis GmbH, Heidelberg. All the chemicals were of analytical grade.

5.5 Sample collection

The blood samples were collected from sitosterolemic patients and the serum was obtained by centrifugation. The serum samples were kept at -20 °C after addition of 10 µl butylated hydroxytoluene (BHT) (25 mg/ml in ethanol).

The patients were between 9 and 72 years old (11 males and 20 females and their weight ranged between 37 to 103 Kg). Ten of these patients were being treated with bile acid binding resins (five patients were with cholestyramine powder in a dose ranged between 4 to 16 gm daily, where the other five patients were with cholestipol in a dose of 6 to 20 gm daily), seven with statins (two patients were with simvastatin 20 and 40 mg daily, two patients were with cholestin tablets in a dose of 600 mg daily, where the other two patients were with atrovastatin 20 mg per day and the last patient was with fluvastatin 60 mg daily) and four with a combined therapy of statins and resins (atrovastatin 10-40 mg plus cholestyramine in a dose of 4-16 gm).

In order to see the effect of different drug treatment on the level of sterols, stanols and cholesterol precursors like lathosterol, ten patients who were receiving no drug treatment for sitosterolemia were also included.

5.6 GC analysis

5.6.1 Sample preparation

50 µl of 5α-cholestane (mg/ml) and 10 µl of epicoprostanol (100 µg/ml) were added to 100 µl of the serum sample as internal and external standards, respectively. Alkaline hydrolysis was performed at 68 °C for 1 hour by addition of 1 ml 1 M ethanolic sodium hydroxide. The solution cooled at room temperature and 500 µl of distilled water were added. The unsaponified lipid was extracted three times into 3 ml of cyclohexane. The combined organic phases were dried under nitrogen at 65 °C. 500 µl of the n-decane were added to the residue and transferred into micro-vials for GC-analysis after derivatization process.

5.6.2 Derivatization

Sterols and stanols were analyzed as TMS derivatives by silylating the hydroxyl groups of these compounds using dry pyridine, hexamethyldisilazane, trimethylchlorosilane (TMS) 3:2:1 (v/v/v). by silylation, active hydrogen is replaced by an alkylsilyl group.

5.6.3 Chromatographic conditions

Sterols and stanols were analyzed by GC as TMS derivatives, where 2 µl of TMS derivatives were injected automatically via an HP 7683 injector at 280 °C. Hydrogen was used as carrier gas (constant flow 1.1 ml/min) with an inlet pressure of 9.97 psi. The oven temperature was kept at 150 °C for 3 min. and raised at a rate of 30 °C/min to a final temperature of 290 °C, which was maintained for 22.33 min. The different compounds of sterols and stanols were separated on a dimethylsilicone capillary column (J and W, Folsom, USA) (30 m x 0.25 mm internal diameter, 0.25 µm film thickness) in a Hewlett Packard (HP) GC 5890.

The entire GC was controlled and the total chromatogram was integrated automatically by the computer software (Rev. A. 08.03 [847], Agilent Technologies 1990-2000). The exact retention times as gained from a serum standard chromatogram could be taken from a calibration table.

The calibration table included information on the sterol retention times, internal standard qualification and amount. The information about the usual integration parameters was saved in an integration event table. After an automatic inspection of basic integration, the integration of each peak was inspected again. If the peak was not integrated correctly, the integration was performed manually. The final integration results including the raw data and a chromatogram of the serum

sample were printed out and saved in a file. The raw data and the concentrations for the different sterols were converted from the HP data-file to an EXCEL spreadsheet.

5.6.3 Method Validation

The gas chromatographic method used in this study to measure the concentration of cholesterol and non-cholesterol sterols in plasma was developed and validated at the Department of Clinical Pharmacology, University of Bonn (VALGCSTER 1.1- Validation of analytical procedures: methodology validated by Dr. Dr. D. Lütjohann) using its associated SOP (Standard Operating Procedure Serum Sterols GC SOP-KP-3.5, dated 12.08.2002; Dr.Dr. D Lütjohann).

5.6.5 Statistical analysis

The one-way analysis of variance (ANOVA) test was used to see whether there is a significant change between the different groups when the samples distributed normally. When the samples were not in a normal distribution, non-parametric tests (such as the Kruskal-Wallis and Mann-Whitney tests) were used. The Kruskal-Wallis test was used to determine whether there was a significant change between different drug treatments, whereas the Mann-Whitney test was used to find out between which two drug treatments this significant change exists.

The Anderson-Darling test was used to determine whether the samples distributed normally or not, where $P = 0.05$ was used as a significant level.

6 Results

A. Results of the first study

6.1 Cholesterol oxidation products (COPs)

6.1.1 COPs in lipid emulsions as parenteral nutrition

The aim of this preliminary study was to assess the possible presence of cholesterol oxidation products (COPs) and plant sterol oxidation products (POPs) in two intravenous lipid emulsions with different fatty acid compositions (lipofundin^R 10% and lipovenöse^R). The emulsions were analyzed when the bottles were opened (i.e., under normal condition of administration).

Table 10 shows the contents of COPs in lipofundin^R 10% and lipovenöse^R emulsions. In lipofundin^R 10% emulsion, there was only a slight difference between the concentrations of different COPs; however, in lipovenöse^R emulsion , the content of cholestantriol was lower.

Table 10 Concentrations of cholesterol oxidation products in lipid emulsions (ng/ml).

Cholesterol oxidation products	Lipofundin^R 10%	Lipovenöse^R
7α-hydroxycholestrol	216.9	186.6
7β-hydroxycholestrol	207.5	180.4
7-ketocholesterol	214.7	123.7
3β,5α,6β-cholestantriol	195.3	53.9

6.1.2 COPs in control subjects and in patients receiving lipofundin^R 10%

Table 11 summarizes the concentrations of COPs in the serum of six healthy volunteers (two males and four females), as well as in patients receiving parenteral nutrition with lipid emulsion (n=9). As can be seen, a wide range of COP concentrations was recorded, especially for the typical non-enzymatic COPs (although the concentrations of 7α-hydroxycholesterol were

expected to vary widely because they reflect the cholesterol 7α-hydroxylase activity in the liver). Cholesterol non-enzymatic oxidation products, such as 7β-hydroxycholesterol and 7-ketocholesterol were not expected to show such great variation.

Table 11 Concentrations of COPs in serum from control subjects and patients receiving lipofundin^R 10% emulsion (ng/ml).

	7α-hydroxy-cholesterol		7β-hydroxy-cholesterol		7-Keto-cholesterol		3β,5α,6β-cholestantriol	
Sample no.	A	B	A	B	A	B	A	B
1	69.8	16.9	20.7	9.0	165.3	30.8	91.8	18.7
2	28.5	12.5	4.3	6.5	17.4	22.9	5.6	10.4
3	23.0	12.4	5.5	5.3	18.6	19.7	4.1	6.1
4	46.0	5.6	24.5	6.3	52.0	17.1	35.8	1.8
5	39.3	13.3	3.5	14.8	10.4	42.0	2.6	8.5
6	19.0	13.9	36.4	8.4	84.9	16.1	11.6	5.3
7		29.0		8.8		21.0		4.6
8		7.3		6.3		22.5		1.9
9		9.5		8.5		19.8		8.1
Mean	37.6	13.4	15.8	8.2	58.1	23.5	25.2	7.3
SD	18.7	6.8	13.5	2.8	59.6	8.1	34.9	5.2

A = healthy volunteer; B = patients under lipid emulsion infusion

6.1.3 COPs in the serum from sitosterolemic patients

6.1.3.1 COPs in the serum from sitosterolemic patients treated with resins and resins plus ezetimibe

Three patients were treated with bile acid binding resins and for eight weeks with ezetimibe (see Table 12). The patients showed no significant difference in the concentrations of oxycholesterols before and after ezetimibe treatment. As expected, in patients treated with bile acid binding resins, 7α-hydroxycholesterol and other oxycholesterols were higher compared with those treated with statins. The addition of ezetimibe to bile acid binding resins had no significant effect on the concentrations of oxycholesterols as the *P* values of t test were > 0.05 .

Table 12 Concentrations of COPs in serum of 3 patients with sitosterolemia treated with resins and resins plus ezetimibe (ng/ml).

	7α-hydroxy-cholesterol		7β-hydroxy-cholesterol		7-Keto-cholesterol		3β,5α,6β-cholestantriol	
Patient	A	B	A	B	A	B	A	B
1	253.9	87.1	158.1	107.8	361.9	263.9	100.8	85.1
2	494.1	400.7	399.8	436.5	607.7	758.4	164.6	82.5
3	173.3	134.8	38.0	25.8	39.8	14.0	10.5	26.5
Mean	307.1	207.5	198.6	190.1	336.5	345.4	92.0	64.7
SD	166.9	169.0	184.3	217.4	284.8	378.9	77.5	33.1
P value	0.508		0.959		0.974		0.608	

A = bile acid binding resins; B = bile acid binding resins plus ezetimibe

6.1.3.2 COPs in the serum from sitosterolemic patients treated with statins and statins plus ezetimibe

Combined treatment with statins and ezetimibe for eight weeks in patients with sitosterolemia (n=4) produced no difference in the concentration of COPs, indicating that ezetimibe has no effect. These results can be seen in Table 13. T test was used, where the statistical *P* values of the different oxycholesterols were > 0.05.

Table 13 Concentrations of COPs in serum of 4 patients with sitosterolemia treated with statins and statins plus ezetimibe (ng/ml).

	7α-hydroxy-cholesterol		7β-hydroxy-cholesterol		7-Keto-cholesterol		3β,5α,6β-cholestantriol	
Patient	A	B	A	B	A	B	A	B
1	68.8	82.1	35.2	131.0	31.5	110.0	33.0	36.2
2	53.5	47.5	49.1	42.8	39.9	37.1	17.7	20.8
3	146.7	175.7	61.6	107.0	79.8	106.1	20.6	26.2
4	128.6	85.9	147.9	70.5	179.8	103.3	49.7	46.7
Mean	99.4	97.8	73.5	87.8	82.7	89.1	30.2	32.5
SD	45.3	54.7	50.8	39.0	68.0	34.8	14.6	11.5
P value	0.968		0.666		0.861		0.816	

A = statins; B = statins plus ezetimibe

6.1.3.3 COPs in sitosterolemic patients treated with ezetimibe only

Although ezetimibe showed a significant cholesterol-reducing effect in hypercholesterolemic patients (11), ezetimibe did not produce any effect on the COP concentrations in sitosterolemic patients (see Table 14), as the calculated *P* values of t test for oxycholesterols showed no significant difference ($P > 0.05$).

Table 14 Concentrations of COPs in serum of 6 sittosterolemic patients treated with ezetimibe only (ng/ml).

	7α-hydroxy-cholesterol		7β-hydroxy-cholesterol		7-Keto-cholesterol		3β,5α,6β-cholestantriol	
Patient	A	B	A	B	A	B	A	B
1	56.8	57.1	20.5	32.6	22.0	27.9	33.0	29.8
2	53.7	55.4	29.9	132.0	41.7	212.4	66.7	88.6
3	90.8	43.9	126.6	54.1	119.6	47.9	50.7	39.7
4	62.6	64.1	64.8	95.5	144.1	138.4	66.5	46.9
5	264.8	354.6	39.3	62.2	53.3	113.9	17.5	23.0
6	137.8	76.3	19.4	54.3	20.5	63.0	17.5	22.1
Mean	108.1	108.0	50.1	71.8	66.9	100.6	42.0	41.7

SD	81.6	121.0	41.0	35.9	52.4	68.7	22.7	24.9
P value	0.972		0.348		0.365		0.981	

A = patients without treatment; B = patients treated only with ezetimibe

6.2 Phytosterol oxidation products (POPs)

6.2.1 POPs in lipid emulsions used as parenteral nutrition

The presence of POPs in lipid emulsions used for intravenous nutrition was first confirmed by Plat et al. (9). In the present study, the contents of oxidized sitosterol and campesterol are summarized in Tables 15a and 15b, where it can be seen that 7-ketositosterol and 7-ketocampesterol are present in both emulsions in higher concentrations than the other sitosterol and campesterol oxidation products. However, the concentrations of all campesterol oxidation products are lower than those of sitosterol oxidation products, except in the case of campestantriol.

Table 15a Concentrations of sitosterol oxidation products in lipid emulsions (ng/ml).

Sitosterol oxidation products	lipofundin^{R} 10%	Lipovenöse^{R}
7α-Hydroxysitosterol	14.5	10.3
7β-Hydroxysitosterol	28.0	21.7
7-Ketositosterol	184.5	177.5
3β,5α,6β-sitostantriol	20.8	13.2

Table 15b Concentraions of campesterol oxidation products in lipid emulsions (ng/ml).

Camptosterol oxidation products	lipofundin^{R} 10%	Lipovenöse^{R}
7α-Hydroxycampesterol	9.8	6.4
7β-Hydroxycampesterol	16.3	13.3
7-Ketocampesterol	75.5	52.7
3β,5α,6β-campestantriol	22.3	16.1

6.2.2 POPs in control subjects and in patients receiving lipofundin^R 10%

Concentration of oxyphytosterols ranged from 8.2 to 92.3 ng/ml serum for oxidized sitosterol (see Table 16a) and between 1.8 and 60.2 ng/ml serum for oxycampesterol (see Table 16b). The concentrations of oxyphytosterols in patients receiving lipid emulsions were not significantly different from the concentrations in normal subjects. The highst concentrations were found for 7-ketositosterol and 7-ketocampesterol in both healthy subjects and patients receiving lipid infusion.

Table 16a Concentrations of oxysitosterols in Serum from control subjects and patients receiving lipofundin^R 10% (ng/ml).

	7α-hydroxy-sitosterol		7β-hydroxy-sitosterol		7-Keto-sitosterol		3β,5α,6β-sitostantriol	
Sample no.	A	B	A	B	A	B	A	B
1	9.3	8.7	20.7	20.6	75.9	69.8	16.8	17.5
2	8.3	8.7	21.7	19.9	76.8	75.6	13.8	13.8
3	8.2	8.3	20.4	19.4	73.2	77.7	12.0	13.1
4	15.1	8.5	30.9	18.8	92.3	77.0	24.5	14.2
5	8.3	9.9	24.1	22.3	69.1	78.6	14.9	16.6
6	9.3	8.4	21.8	20.6	76.9	82.2	17.0	16.4
7		9.7		21.0		83.8		15.1
8		8.2		21.9		69.6		14.2
9		9.1		23.1		79.9		15.5
Mean	9.8	8.8	23.3	21.0	77.4	77.1	16.5	15.2
SD	2.7	0.6	4.0	1.3	7.9	4.9	4.3	1.5

A = healthy volunteers; B = patients under lipid emulsion infusion

Table 16b Concentrations of oxycampesterols in serum from control subjects and patients receiving lipofundin[R] 10% (ng/ml).

	7α-hydroxy-campesterol		7β-hydroxy-campesterol		7-Keto-campesterol		3β,5α,6β-campestantriol	
Sample no.	A	B	A	B	A	B	A	B
1	1.9	2.8	10.4	13.2	48.6	44.4	10.7	10.7
2	2.4	2.8	13.6	13.9	49.6	48.8	9.1	9.1
3	3.2	2.9	12.6	13.4	48.1	50.8	9.0	8.2
4	6.3	2.5	19.0	12.7	60.2	50.3	15.3	9.4
5	3.0	2.5	13.4	13.2	44.5	50.2	6.7	10.4
6	1.8	2.7	11.9	13.1	48.9	52.8	9.4	21.0
7		3.3		14.6		55.2		9.9
8		2.4		11.2		46.3		6.8
9		2.5		13.3		52.4		9.8
Mean	3.1	2.7	13.5	13.2	50.0	50.1	10.0	10.6
SD	1.6	0.3	2.9	0.9	5.3	3.3	6.7	4.1

A = healthy volunteers; B = patients under lipid emulsion infusion

6.2.3 POPs in the serum from sitosterolemic patients

6.2.3.1 POPs in the serum from sitosterolemic patients treated with resins and resins plus ezetimibe

Table 17a shows that there was no significant difference in the concentration of oxysitosterols before and after the addition of ezetimibe. The same result was found with the concentration of oxycampesterols (see Table 17b) as t test was calculated where the *P* values were > 0.05.

Table 17a Serum concentrations of oxysitosterols in patients with phytosterolemia treated with resins and resins plus ezetimibe (ng/ml).

	7α-hydroxy-sitosterol		7β-hydroxy-sitosterol		7-Keto-sitosterol		3β,5α,6β-sitostantriol	
Patient	A	B	A	B	A	B	A	B
1	21.8	15.6	48.6	37.2	153.4	121.5	17.4	15.6
2	10.2	11.4	31.5	42.0	110.7	108.4	14.8	13.2
3	12.1	9.8	28.7	24.4	70.8	69.5	14.7	14.2
Mean	14.7	12.3	36.3	34.5	111.6	99.8	15.6	14.3
SD	6.3	2.9	10.7	9.1	41.3	27.0	1.5	1.2
P value	0.523		0.879		0.704		0.435	

Table 17b Serum concentrations of oxycampesterols in patients with phytosterolemia treated with resins and resins plus ezetimibe (ng/ml).

	7α-hydroxy-campesterol		7β-hydroxy-campesterol		7-Keto-campesterol		3β,5α,6β-campestantriol	
Patient	A	B	A	B	A	B	A	B
1	4.1	1.6	17.7	14.7	79.8	67.7	15.7	12.7
2	3.6	3.4	13.1	11.3	64.1	65.2	13.1	10.7
3	3.2	3.4	14.0	13.4	44.2	41.5	11.0	11.6
Mean	3.6	2.8	14.9	13.1	62.7	58.1	13.3	11.7
SD	0.4	1.0	2.5	1.7	17.8	14.5	2.3	1.0
P value	0.251		0.250		0.740		0.196	

For Tables 17a and 17b; A = bile acid binding resins; B = bile acid binding resins plus ezetimibe

6.2.3.2 POPs in the serum from sitosterolemic patients treated with statins and statins plus ezetimibe

Addition of ezetimibe to statin treatment did not result in any significant effect on the concentrations of oxysitosterols in sitosterolemic patients (see Table 18a). There is also no difference between the concentrations of oxycampesterol before and after ezetimibe treatment (see Table 18b) as the *P* values of t test were > 0.05.

Table 18a Serum concentrations of oxysitosterols in sitosterolemic patients treated with statins and statins plus ezeimibe (ng/ml).

	7α-hydroxy-sitosterol		7β-hydroxy-sitosterol		7-Keto-sitosterol		3β,5α,6β-sitostantriol	
Patient	A	B	A	B	A	B	A	B
1	14.6	22.4	42.4	57.3	86.2	92.1	19.4	20.0
2	12.7	13.6	31.8	29.8	71.1	70.5	14.4	17.5
3	15.6	20.4	37.6	43.1	85.4	88.5	16.5	17.0
4	24.6	12.6	51.6	34.9	92.2	82.3	18.9	16.4
Mean	16.9	17.3	40.9	41.3	83.7	83.4	17.3	17.7
SD	5.3	4.9	8.4	12.0	9.0	9.5	2.3	1.6
P value	0.895		0.949		0.942		0.613	

Table 18b serum concentrations of oxycampesterols in sitosterolemic patients treated with statins and with statins plus ezetimibe (ng/ml).

	7α-hydroxy-campesterol		7β-hydroxy-campesterol		7-Keto-campesterol		3β,5α,6β-campestantriol	
Patient	A	B	A	B	A	B	A	B
1	4.4	8.5	19.1	28.5	54.3	60.9	18.7	17.4
2	4.7	4.1	17.1	17.5	45.3	44.2	8.9	11.6
3	5.4	8.6	20.8	27.0	54.1	54.0	11.8	12.5
4	6.5	2.0	25.4	16.9	58.7	50.9	12.9	11.1
Mean	5.2	5.8	20.6	22.5	53.1	52.5	13.1	13.2
SD	0.9	3.3	3.5	6.1	5.6	6.9	4.1	2.9
P value	0.651		0.645		0.869		0.851	

For Tables 18a and 18b: A = statins; B = statins plus ezetimibe

6.2.3.3 POPs in the serum from sitosterolemic patients treated with ezetimibe only

Statistical analysis by t-test for dependent variables showed no significant difference in the concentration of either oxysitosterol or oxycampesterol in sitosterolemic patients before and after treatment with ezetimibe, as $P > 0.05$ (see Tables 19a and 19b).

Table 19a Serum concentrations of oxysitosterols in sitosterolemic patients treated with ezetimibe only (ng/ml).

	7α-hydroxy-sitosterol		7β-hydroxy-sitosterol		7-Keto-sitosterol		3β,5α,6β-sitostantriol	
Patient	A	B	A	B	A	B	A	B
1	11.4	10.9	26.7	24.8	76.2	72.3	17.2	14.6
2	11.9	13.6	30.2	29.8	75.4	84.3	16.5	15.8
3	18.1	11.2	42.1	24.0	86.4	76.7	15.9	15.3
4	11.3	10.9	25.9	25.6	70.7	73.6	15.7	14.9
5	10.4	10.5	24.6	24.6	68.2	73.3	15.5	16.1
6	11.1	14.4	29.0	40.5	79.4	81.6	15.5	15.6
Mean	11.9	11.9	29.7	28.2	76.1	77.0	16.1	15.4
SD	2.9	1.7	6.4	6.4	6.5	5.0	0.7	0.5
P value	0.646		0.666		0.807		0.177	

Table 19b Serum concentrations of oxycampesterols in sitosterolemic patients treated with ezetimibe only (ng/ml).

	7α-hydroxy-campesterol		7β-hydroxy-campesterol		7-Keto-campesterol		3β,5α,6β-campestantriol	
Patient	A	B	A	B	A	B	A	B
1	2.5	3.5	13.6	14.1	44.3	44.7	11.4	11.6
2	3.7	17.3	14.9	15.1	47.8	54.6	15.2	13.0
3	4.9	2.5	20.3	13.8	51.1	49.6	11.8	9.9
4	2.8	2.0	14.0	15.2	41.8	47.4	10.7	9.7
5	2.4	1.4	12.9	13.2	44.9	48.3	11.8	11.3
6	4.8	5.0	14.7	19.2	49.6	52.0	14.7	14.4
Mean	3.5	5.3	15.1	15.1	46.6	49.4	12.6	11.7

SD	1.1	6.0	2.7	2.2	3.5	3.5	1.9	1.9
P value	0.406		0.825		0.183		0.491	

For Tables 19a and 19b; A= patients without treatment; B = patients treated only with ezetimibe

B. Results of the second study

6.3 Cholesterol

Table 20 show the concentrations of cholesterol in the serum of sitosterolemic patients receiving different drug treatments. The Anderson-Darling test reveals that the data samples were normally distributed. By using the ANOVA test, it could be concluded that the different drug treatments have no significant effect, as $P= 0.209$.

Table 20 Cholesterol concentrations in the serum of sitosterolemic patients under different drug treatments (mg/dl).

	Patients without treatment	Patients with statins	Patients with resins	Patients with statins + resins
1	122	238	141	117
2	140	147	162	210
3	171	195	177	96
4	339	182	123	161
5	168	143	171	
6	176	145	96	
7	244	238	98	
8	113		158	
9	92		134	
10	205		103	
Mean	177	184	136	146
SD	72.5	41.9	30.5	50.5

6.4 Lathosterol

Since lathosterol is a precursor for cholesterol biosynthesis, the level of this parameter in the serum of patients with sitosterolemia could be used as an index for the effect(s) of different drug

treatments on cholesterol biosynthesis. The data samples shown in Table 21 were not normally distributed; therefore non-parametric tests were used.

The Kruskal-Wallis test revealed the significant difference between the control group and the other groups under different drug treatments as $P = 0.006$. By using the Mann-Whitney test, we could determine where these significant changes exist, as $P \leq 0.05$. These changes were between 1) the group treated with resins and the control group; 2) the group undergoing combined therapy (statins plus resins) and the control group; 3) the statins group and the resins group; and 4) the group undergoing combined therapy and the resins group.

Table 21 Lathosterol concentrations in the serum of sitosterolemic patients under different drug treatments (mg/dl).

	Patients without treatment	Patients with statins	Patients with resins	Patients with statins + resins
1	0.087	0.112	0.422	0.056
2	0.194	0.092	0.259	0.070
3	0.159	0.154	0.230	0.088
4	0.279	0.352	0.714	0.098
5	0.178	0.170	1.410	
6	0.09	0.060	0.318	
7	0.105	0.106	0.331	
8	0.947		0.067	
9	0.218		0.249	
10	0.244		1.352	
Mean	0.25	0.15	0.535	0.078
SD	0.25	0.10	0.53	0.02

6.5 Plant sterols

The following tables (Tables 22, 23, 24 and 25) show the concentrations of plant sterols in the serum of sitosterolemic patients treated with different drugs. The Anderson-Darling test showed that the data samples for campesterol, sitosterol and sitostanol were normally distributed, as $P > 0.05$. The data samples were analyzed parametrically using the ANOVA test, but did not reveal any significant difference among the various groups for any of the above-mentioned plant sterols, as P values were more than 0.05.

In the case of campestanol, the *P* value of the Anderson–Darling test was less than 0.05, meaning that the sample data was not normally distributed. It could be analyzed non-parametrically by using the Mann-Whitney test, which indicated that a difference existed between the group treated with statins and the group treated with resins.

Table 22 Campesterol concentrations in the serum of sitosterolemic patients under different drug treatment (mg/dl).

	Patients without treatment	Patients with statins	Patients with resins	Patients with statins + resins
1	2.286	12.267	6.912	8.401
2	13.049	9.141	7.055	13.230
3	11.934	12.436	14.523	12.259
4	14.898	12.891	13.211	9.574
5	9.354	15.433	5.956	
6	5.905	12.348	16.497	
7	20.956	28.503	4.267	
8	3.642		11.086	
9	6.185		8.227	
10	9.534		3.831	
Mean	9.774	14.717	9.156	10.866
SD	5.66	6.34	4.41	2.25

Table 23 Campestanol concentrations in the serum of sitosterolemic patients under different drug treatment (mg/dl).

	Patients without treatment	Patients with statins	Patients with resins	Patients with statins + resins
1	0.725	4.035	0.914	3.044
2	2.746	2.182	1.870	1.764
3	2.587	2.782	3.364	2.876
4	4.155	2.756	2.438	2.597
5	3.324	3.717	1.172	
6	2.006	3.458	2.144	

7	4.872	8.525	1.197	
8	0.818		3.450	
9	1.997		2.155	
10	2.237		0.994	
Mean	2.547	3.922	1.97	2.57
SD	1.32	2.13	0.93	0.57

Table 24 Sitosterol concentrations in the serum of sitosterolemic patients under different drug treatment (mg/dl).

	Patients without treatment	Patients with statins	Patients with resins	Patients with statins + resins
1	5.629	23.466	14.576	18.802
2	27.591	18.102	18.294	26.541
3	31.230	20.771	25.103	16.622
4	34.729	20.698	28.619	19.519
5	19.708	28.293	11.182	
6	15.216	20.309	24.189	
7	28.797	43.511	15.364	
8	9.783		21.064	
9	12.007		15.303	
10	21.795		11.449	
Mean	20.649	25.021	18.514	20.371
SD	9.86	8.77	5.99	4.29

Table 25 Sitostanol concentrations in the serum of sitosterolemic patients under different drug treatment (mg/dl).

	Patients without treatment	Patients with statins	Patients with resins	Patients with statins + resins
1	1.294	8.691	2.520	7.833
2	7.173	5.736	5.193	5.734
3	7.055	6.123	7.298	6.584
4	10.064	6.596	7.028	6.852

5	7.804	9.347	3.600	
6	5.328	7.343	4.355	
7	8.502	15.464	5.234	
8	2.976		6.713	
9	5.021		5.408	
10	5.918		3.773	
Mean	6.114	8.471	5.112	6.751
SD	2.60	3.35	1.58	0.86

6.6 Lathosterol/cholesterol ratio

The ratio of lathosterol to cholesterol can be used as an index of cholesterol biosynthesis (see also 6.4). Table 26 showed that the highest ratio was in the patient group treated with resins only (4.19), and the lowest ratio was with the patients under statins plus resins treatment (0.58).

Table 26 The ratio of lathosterol to cholesterol concentrations in the serum of sitosterolemic patients under different drug treatments (µg/mg).

	Patients without treatment	Patients with statins	Patients with resins	Patients with statins + resins
1	0.711	0.471	2.988	0.478
2	1.388	0.629	1.600	0.333
3	0.932	0.789	1.306	0.920
4	0.821	1.929	5.790	0.607
5	1.061	1.185	8.228	
6	0.512	0.417	3.306	
7	0.430	0.445	3.362	
8	8.405		0.427	
9	2.372		1.852	
10	1.190		13.122	
Mean	1.782	0.838	4.198	0.584
SD	2.39	0.55	3.89	0.25

6.7 Campesterol/cholesterol ratio

The ratio of campesterol to cholesterol is an indicator for cholesterol and plant sterols absorption. As can be seen in Table 27, it was found that the highest ratio was in case of statins as a monotherapy (80.11) or as a daul therapy (80.45).

Table 27 The ratio of campesterol to cholesterol concentrations in the serum of sitosterolemic patients under different drug treatment (µg/mg).

	Patients without treatment	Patients with statins	Patients with resins	Patients with statins + resins
1	18.66	51.60	48.96	71.94
2	93.30	62.17	43.55	62.87
3	69.98	63.63	82.26	127.48
4	43.89	70.67	107.17	59.52
5	55.75	107.87	34.76	
6	33.48	85.17	171.60	
7	85.91	119.67	43.39	
8	32.32		70.25	
9	67.23		61.23	
10	46.48		37.19	
Mean	54.70	80.11	70.04	80.45
SD	24.24	25.35	42.29	31.78

7 Discussion

The relationship between COPs and atherosclerosis has been widely studied. Most information about their possible atherogenicity is derived from in vitro studies showing that COPs are cytotoxic (91). While no direct evidence of a connection between COPs and atherosclerosis in humans has been established, several findings suggest that such a connection exists. On the other hand, few studies have measured the biological effects of POPs, although some researchers have shown their cytotoxic effects (54, 55). Feeding LDL receptor-deficient and apolipoprotein E-deficient mice a mixture of COPs accelerated fatty streak lesion formation (92); however, exogenous oxyphytosterols were well absorbed and accumulated in the body, but did not promote the development of atherosclerosis in apolipoprotein E-deficient mice (93).

Determination of low concentrations of COPs in biological samples which may contain more than 1,000 times as much cholesterol as COPs is difficult because of the interference with cholesterol. It is important to be aware of this problem when determining concentrations of the common COPs, such as 7α-,7β-hydroxycholesterol and 7-ketocholesterol. In earlier research, very high concentrations of COPs were sometimes reported, probably reflecting significant autoxidation of cholesterol during the sample work-up. As analytical techniques have improved, the reported concentrations of these products have tended to decline.

COPs in living tissues originate from the diet and from non-enzymatic or enzymatic cholesterol oxidation. For example, 7α-hydroxycholesterol is formed from cholesterol by the hepatic enzyme cholesterol 7α-hydroxylase as a normal intermediate in bile acid biosynthesis. In addition, 7α-hydroxycholesterol is formed by autoxidation, if the samples are not protected from oxygen. The presence of dietary COPs in the circulation depends on the rate of absorption from the gut, their

transport to the specific tissue and their elimination, whereas formation of COPs from endogenous cholesterol depends on substrate availability, activity of cholesterol-metabolizing enzymes and the degree of oxidative stress. However, it is difficult to distinguish between oxycholesterols derived from the diet and those endogenously formed.

Little information is available on the presence of POPs in food. Research with rats has shown that some oxyphytosterols were absorbed from the diet and incorporated into mesenteric lymph (57). Extensive information about endogenous formation of oxyphytosterols, whether enzymatically or nonenzymatically, is also lacking. Since it was recently discovered that oxyphytosterols showed similar cytotoxic effects to those caused by oxycholesterols on cultured macrophages (59), we should expand our knowledge concerning the presence of oxyphytosterols in biological materials.

Sitosterolemia is a rare, autosomal recessively inherited sterol-storage disease characterized clinically by premature atherosclerosis and early cardiovascular death and biochemically by increased levels of plant sterols (sitosterol, campesterol, stigmasterol and other phytosterols) and their 5α-stanol derivatives in plasma and other tissues. Sitosterolemia has been shown to be due to mutations in either of the two ATP-binding cassette (ABC) transporters, ABCG5 or ABCG8 (10, 63 and 64). Plat et al. (9) found some oxysitosterols (7-keto, 7β-hydroxy, 5α, 6α-epoxy, and 3β, 5α, 6β-tri-hydroxy sitosterol), in addition to plant sterols, in the plasma of sitosterolemic patients. In this work we were also able to identify 7α-hydroxy, 7β-hydroxy, 7-keto, and 3β, 5α, 6β-tri-hydroxysitosterol/campesterol in sitosterolemic patients, as well as in normal healthy volunteers. The presence of oxidized derivatives of phytosterols in plasma from thirteen normal subjects was also confirmed by Grandgirard et al. (51).

At present, most analytical methods used to study both oxycholesterols and oxyphytosterols are based on GC with flame ionization detection (FID) or GC-MS with selected ion monitoring (SIM). The great advantage of SIM over FID is the specificity of detection, which is necessary for the analysis of oxysterols in complex biological matrices. The choice of GC-MS with multiple deuterium-labelled internal standards, as in the present study, is preferable, because this method is sensitive and specific for the different analytes.

In using this method, the internal standard for each COP was the corresponding multideuterium-labelled COP; thus the behavior of the analytes and the internal standards during the work-up of the samples was similar. This corrected the differences in the recovery of the original compounds. The principles of this method are alkaline hydrolysis (saponification), solid-phase

extraction and GC-MS measurement. A drawback of the method is the relatively laborious sample work-up, which limits the number of samples that can be processed simultaneously. In addition, the synthesis of internal standards is very laborious and expensive.

The principles of analysis for POPs are not different from those for COPs, i.e., saponification, extraction of lipids, enrichment of POPs and subsequent qualitative and quantitative determination by GC-MS. Very few publications on the analysis of POPs are available at this time, and a negligible amount of research has been carried out on their biological effects. This will almost inevitably lead to difficulties in comparing our work with other studies.

To analyze oxidized cholesterol and oxidized plant sterol products in human plasma, we used the GC-MS method first developed by Dezletovic et al. (88). The alkaline hydrolysis parameters chosen were 0.35 M of KOH at 22°C for two hours, where with this protocol 91-94% of the cholesterol esters were hydrolyzed. The solid-phase extraction step was modified by using 10 ml 0.25% isopropanol-cyclohexane (v/v) instead of 8 ml 0.5% isopropanol-hexane (v/v) to elute neutral sterols, and 8 ml 30% isopropanol-cyclohexane instead of 5 ml 30% isopropanol in hexane to elute oxysterols. All possible precautions were taken to reduce undesired autoxidation of cholesterol and plant sterols during the sample work-up. Precautions included keeping the serum samples at -80°C with BHT (25 mg/ml methanol) and saturating them extensively with argon during the work-up. In addition, a carefully controlled alkaline hydrolysis process and rapid separation of neutral sterols from oxycholesterols and oxyphytosterols were carried out. The mass spectrometer was operated in the selected ion-monitoring mode, where the ion used for analysis (*m/z*) and the typical retention time (in minutes) for each deuterated and non-deuterated compound offer the advantage of specificity of detection.

Recovery experiments were performed on spiked serum samples by addition of different volumes of a standard mixture of oxycholesterols and oxyphytosterols. As shown in Table 7c (see page 45), recovery ranged from 82% in case of cholestantriol to 123% in case of 7-ketositosterol, indicating that satisfactory recoveries were obtained for all compounds. Table 8 (see page 46) represents the intra-assay precision, also termed as repeatability. Detection limit in this work was calculated by using the signal-to-noise ratios method. Here the limits of detection were lower than 1 ng/ml, except in case of 7-ketositosterol (5.91 ng/ml) and 7-ketocampesterol (2.22 ng/ml).

The linearity of the GC-MS responses was evaluated by analyzing standard mixtures of oxycholesterols and oxyphytosterols using the same procedure as described for the serum samples, with the regression coefficient of the standard curves ranging from 0.992 for

sitostantriol to 0.9998 for 7α-hydroxycholesterol. The linear range of all compounds was calculated using the correlation coefficient, y-intercept and response factor of the regression line, except in case of 7-ketositosterol and 7-ketocampesterol. For these two compounds, the concentrations were calculated using polynomic curves, which yielded the correlation coefficient better than a linear calibration curve.

In this work we examined the possibility that cholesterol and plant sterol oxidation products were present in two commercially available lipid emulsions that are used as parenteral nutrition. Various oxycholesterols and oxyphytosterols were detected in the emulsions (see Tables 10, 15a and 15b, pages 50 and 55), but the concentrations were lower than those found by Plat et al. (see Table 3, page 35). According to our results, the concentrations of oxyphytosterols in emulsion Lipofundin^{R} 10% were higher than in emulsion Lipovenös^{R} ; in addition, the concentrations of oxysitosterols were always more than those of oxycampesterols, only the concentration of campestantriol which was more than sitostantriol. Whether these oxidized compounds have biological or pathological effects on patients receiving intravenous lipid emulsions, especially infants, requires further investigation.

The presence of oxycholesterols and oxyphytosterols in serum from normal subjects was demonstrated in this study as well as in research by Grandgirard et al. (51); it is important, however, to note that Grandgirard et al. used a different internal standard. These oxidized compounds were also found in the serum of patients receiving lipid emulsions. Although these lipid emulsions contain high amounts of oxidized cholesterol and oxidized plant sterols, we found that the concentrations of oxycholesterols in normal subjects were higher than in patients receiving lipid nutrition; the amounts of oxysitosterols and oxycampesterols for these patients were not different from amounts found in normal subjects. An important note is that we did not investigate the presence of these compounds in those patients before introduction of lipid emulsions. Optimal research design would include measuring the concentration of these oxidized compounds before and after intravenous lipid emulsion introduction.

There is unfortunately very little information available on oxysterols, their absorption and their biological effects in humans. No research has investigated the effects of drug treatment on the level of these compounds in blood serum, whether in normal subjects or in sitosterolemic patients. The intestinal absorption of sterols is still not fully understood, but with the identification of the role of ABCG5 and ABCG8 transporters (10, 63), the introduction of ezetimibe as the first cholesterol absorption inhibitor and the identification of the NPC1L1

protein as a sterol transporter (17), more details about sterol transport processes in the small intestine have become available.

This research aimed to examine the effect of ezetimibe on the concentrations of oxycholesterols and oxyphytosterols in serum from sitosterolemic patients undergoing treatment with bile acid binding resins and/or statins and and in patients who were not receiving any drug treatment. Ezetimibe is a novel inhibitor of intestinal cholesterol absorption in humans, shown to significantly lower plasma cholesterol and LDL-C concentrations in patients with hypercholesterolemia (11). Similarly, in patients with sitosterolemia, ezetimibe also produced a significant and progressive reduction in plasma plant sterol concentrations during short-term treatment (12). In contrast, however, to its effect on neutral sterols, ezetimibe did not decrease the concentrations of oxidized sterols in the blood of sitosterolemic patients.

In looking at the results of this present work (presented in Tables 12, 13, 14, 17a, 17b, 18a, 18b,19a and 19b), we can conclude that ezetimibe probably has no effect on the levels of oxycholesterols, oxysitosterols and oxycampesterols, since the mean values before and after ezetimibe introduction were almost the same except in the case of 7α-hydroxycholesterol and cholestantriol, where it seems that ezetimibe slightly decreased the concentration of these two oxycholesterols in patients being treated with bile acid binding resins. A T-test also showed no effect of ezetimibe on the levels of oxycholesterols, as *P* values were above 0.05, which was taken as significanc level.

It is difficult to explain why ezetimibe has no effect on the level of oxysterols in serum from sitosterolemic patients that is similar to its effect in reducing normal sterols (11, 12). One possible explanation of why ezetimibe has no effect on oxysterols in sitosterolemic patients is the following: the treatment with ezetimibe lasted only for eight weeks, and the half-life of oxysterols is not known. We need to expand our knowledge of the mechanisms of intestinal oxysterol absorption and further investigate the mechanisms underlying ezetimibe’s effects. Future studies should involve larger sample sizes; in this work the number of samples was very limited.

In order to study the effect of different drug treatments on the serum level of cholesterol and plant sterols, serum samples were taken from 31 sitosterolemic patients and analyzed using the GC method, where 5α-cholestane was added as internal standard. Cholesterol, lathosterol, plant sterols and plant stanols were analyzed in those patients, who were divided into four groups, as described above.

Generally, normal to moderately elevated plasma levels of cholesterol were observed in the

patients with sitosterolemia. In this work, the different treatments with bile acids binding resins or with statins as a monotherapy or as a combined therapy did not produce any statistically significant change, although the level of cholesterol under resins treatment was the lowest (136 mg/dl).

Lathosterol, a precursor for cholesterol biosynthesis, was increased in the case of patients who were under bile acids binding resins (0.535 mg/dl) and decreased in those who were on statins as a dual treatment with resins (0.078 mg/dl) or as a monotherapy (0.150 mg/dl). These changes could be confirmed statistically by non-parametric analysis where *P* values showed a significant difference between the four groups. The increasing level of lathosterol in those patients treated with resins is due to interruption of bile acids reabsorption, resulting in increased synthesis of cholesterol in the liver and a decrease in cholesterol plasma level by increased expression of LDL receptors. This could be confirmed by calculating the lathosterol-to-cholesterol ratio, which is an index to cholesterol biosynthesis. Here the higher ratio was in those treated with resins (4.19) and the lower ratios were in the group undergoing combined therapy (0.584) and in those treated with statins (0.838).

Statins, HMG-CoA reductase inhibitors, are the most effective drugs in the treatment of hypercholesterolemia, due to their inhibition of the activity of HMG-CoA reductase, the rate-limiting enzyme in the cholesterol biosynthesis process. This inhibition results in increased expression of LDL receptors, which lowers serum LDL-cholesterol levels.

If we compare the effect of statins on the cholesterol level in hypercholesterolemic patients and their effects on plant sterols in sitosterolemic patients, we find that statins do not reduce plasma plant sterols and stanols. We found in this work that the levels of plant sterols (sitosterol and campesterol) and stanols (sitostanol and campestanol) were increased in the group of patients under statins treatment (see Tables 22, 23, 24 and 25, pages 64-65). This is likely due to decreased biliary cholesterol secretion, which leads to an increased proportion of dietary plant sterols in the intestinal sterol pool, thus favoring their increased absorption. Increased absorption and reduced biliary secretion may increase the serum level of plant sterols during statin treatment. This finding could be confirmed by calculating the campesterol-to-cholesterol concentration ratio, as an indicator of sterol absorption, where the higher ratio was in fact found in the statins group (80,11). This fits in with the conclusions drawn by Miettinen et al. (94).

In comparing dual therapy using resins plus statins with monotherapy using resins alone, there was no significant difference found in the level of sterols and cholesterol. Therefore, this type of

combined therapy is not necessary to decrease the level of sterols in sitosterolemic patients, a finding in agreement with the observations of Cobb et al. (95). The question of whether a combined therapy of ezetimibe plus resins is superior to ezetimibe alone for treatment of sitosterolemia requires further investigation.

In conclusion, ezetimibe did not produce any significant reduction in plasma oxysterols concentrations in patients with sitosterolemia. These oxysterols could be also found in normal subjects and also in two frequently used lipid emulsions. Before ezetimibe introduction, bile acids binding resins were the best drugs used to decrease the plasma level of cholesterol and plant sterols in patients with sitosterolemia as statins were not effective.

8 Summary

Oxycholesterols and oxyphytosterols (oxysterols) are oxidized compounds produced either in vitro or in vivo by oxidation of cholesterol and plant sterols, respectively. They may have implications in the etiology of atherosclerosis.

Sitosterolemia is a very rare inherited disorder, characterized biochemically by elevated plasma levels of the major plant sterols and clinically by the presence of tendon and tuberous xanthomas and by a strong predisposition to premature coronary artery disease, but with normal to moderately elevated plasma cholesterol levels. The genetic defect in sitosterolemic patients is due to mutations in either one of the two ATP-binding cassette (ABC) transporters, ABCG5 and ABCG8 which are expressed in the intestins and liver.

Bile acids binding resins like cholestyramine were firstly used in the treatment of sitosterolemia before ezetimibe was introduced. On the other hand HMG-CoA reductase inhibitors were effective in the treatment of hypercholesterolemia but not in the treatment of sitosterolemia.

Ezetimibe was found to be effective in the treatment of hypercholesterolemia and also in the treatment of sitosterolemia (11,12). This drug is the first member of a new class of lipid lowering drugs known as cholesterol absorption inhibitors. It is suggesting that ezetimibe acts throught the pathway contains NPC1L1 protein (16).

In the first study, oxycholesterols and oxyphytosterols were investigated in 13 patients with sitosterolemia before and after ezetimibe introduction, to see whether ezetimibe has a significant effect on the serum levels of oxysterols. Two frequently used lipid emulsions were also analyzed for the presence of oxycholetserols and oxyphytosterols. In the second study, serum samples from 31 sitosterolemic patients under different drug treatments (bile acids binding resins and statins) were anaylzed to see the effect of these drugs on the serum levels of cholesterol, lathosterol, plant sterols and plant stanols.

For the first study GC-MS method was employed, where deuterium labelled for oxysterols were used as internal standard, whereas in the second study gas chromatography was used with 5α-cholestane s internal standard.

Ezetimibe did not produce any effect on the serum concentrations of oxycholesterols and oxyphytosterols as *P* values of t test were above 0.05 which was taken as a significance level. On the other hand, different oxycholesterols and oxyphytosterls were found in two frequently used lipid emulsions, as well as in the serum of 9 normal subjects.

The lowest cholesterol serum level was found in the patients who treated with resins (136 mg/dl), where lathosterol serum level was increased with resins (0.535 mg/dl) and decreased with statins as a monotherapy (0.150 mg/dl) or a combined therapy with resins (0.078 mg/dl).

Statistically, different drug treatment did not produce any significant effect on the serum levels of plant sterols and sitostanol, as the *P* values of ANOVA test were more than 0.05, but there was a significant difference in the serum level of campestanol between the patients treated with statins and those treated with resins.

Oxidized forms of cholesterol and plant sterols are present in serum from normal subjects, sitosterolemic patients and two frequently used lipid mulsions, where eztimibe had no effect on their serum levels. It is still unknown whether oxyphytosterols affect health, as has been suggested for oxysterols, and that is why expanding our knowledge on circulating oxyphytosterols is of great importance.

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