Responders and Non-responders to the therapy of Hypercholesterolemia

Serum ratio of lathosterol to campesterol predicts the outcome of the therapy with Sitostanol ester margarine and Ezetimibe

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Abbreviations

ABCG5	ATP-binding cassette transporters G5
ABCG8	ATP-binding cassette transporters G8
ACAT	Acyl CoA cholesterol acyltransferase
ACE	Angiotensin-converting enzyme
apo	Apolipoprotein
apoA-I	Apolipoprotein A-I
apoB	Apolipoprotein B
BMI	Body mass index
CETP	Cholesteryl ester transfer protein
CHD	Coronary heart disease
CV	Coefficient of variation
CVD	Cardiovascular diseases
DMPP	Di-methylallyl pyrophosphate
DNA	Deoxyribonucleic acid
FFA	Free fatty acid
FPP	Farnesyl pyrophosphate
GPP	Geranyl pyrophosphate
GLC	Gas liquid chromatography
HDL	High-density lipoprotein
HL	Hepatic lipase
HMG-CoA	3-hydroxy-3-methylglutaryl coenzyme-A
IDL	Intermediate-density lipoprotein
IPP	Isopentenyl pyrophosphate
LCAT	Lecithin cholesterol acyltransferase
LDL	Low-density lipoprotein

Abbreviations

Lp(a)	Lipoprotein(a)
LPL	Lipoprotein lipase
LRP	LDL receptor related protein
MI	Myocardial infarction
MTP	Microsomal triglyceride transfer protein
NADPH	Nicotinamide adenine dinucleotide phosphate
NCEP	National Cholesterol Education Program
NPC1L1	Niemann-Pick C1 Like 1 protein
PAI -1	Plasminogen activator inhibitor-1
PLTP	Phospholipid transfer protein
SD	Standard deviation
SNPs	Single nucleotide polymorphisms
SOP	Standard Operating Procedure
TG	Triglycerides
VLDL	Very-low-density lipoprotein
WHO	World Health Organization

1. Introduction

Hypercholesterolemia is a well-known risk factor for coronary heart disease (CHD). The data from the World Health Organization (WHO) indicates that the coronary event rate (per 100,000) in men was highest in Finland (North Karelia, 835) and lowest in China (Beijing, 81). For women the highest event rates were in the United Kingdom (UK) (Glasgow, Scotland, 265) and the lowest in Spain (Catalonia, 35) and China (Beijing, 35) (1). CHD by itself is the single most common cause of death in Europe: accounting for 1.95 million deaths in Europe each year. Over one in five women (23%) and over one in five men (21%) die from the disease (2). The scale of the CHD problem is probably insufficiently appreciated. Although mortality from the disease has fallen in the past two decades, it remains the single leading cause of death for adults, and is expected to remain the leading cause of death and disability in the western world in the 21st century (3). As revealed by the US Framingham Heart Study, the lifetime risk of CHD at the age of 40 years is 1 in 2 for men and 1 in 3 for women. Predictions for the next two decades include tripling of CHD and stroke mortality in Latin America, the Middle East, and sub-Saharan Africa (4). The mortality for all developing countries will increase by 120 percent for women and 137 percent for men.

Hypercholesterolemia is primarily due to elevated LDL (Low-density lipoprotein) concentrations in blood. Elevated LDL cholesterol has several causes. One reason is high dietary intake of cholesterol and/or saturated fat (5) or genetic disorders like familial hypercholesterolemia (6). High LDL cholesterol causes formation of atherosclerotic plaques, which lead to arterial narrowing and heart attacks. The only mechanism of cholesterol removal from the body is its biliary secretion and conversion into bile. However, more than 95% of the bile acids secreted via bile into the duodenum are reabsorbed and returned to the liver (7). In contrast, biliary cholesterol which enters the intestine is reabsorbed between 20 to 80 %.

Major clinical treatment of hypercholesterolemia has focused on reducing the biosynthesis of cholesterol by inhibition of the 3-hydroxy-3-methylglutarate coenzyme A reductase (HMG-CoA reductase). Although statins (HMG-CoA reductase inhibitors) have been shown to be effective in lowering low-density lipoprotein (LDL) cholesterol, many patients do not achieve standard treatment goals as defined by The National Cholesterol Education Program (NCEP) (8).

Although higher doses of statins are more effective in lipid lowering, the risk of serious adverse effects seems to be dose-dependent (9). Thus, the need of additional lipid-lowering compounds not acting as HMG-CoA reductase inhibitors has focused attention on other mechanisms of action such as inhibition of intestinal cholesterol absorption (10) e.g. plant sterol/stanol esters and ezetimibe. Sitostanol, a 5-saturated sitosterol derivative, reduces the intestinal absorption of cholesterol and serum cholesterol (11). It is virtually unabsorbable and a margarine rich in sitostanol ester has been developed and has been shown to reduce cholesterol levels and to be well tolerated which is well documented by short-term and long-term studies when used to replace a part of the daily fat consumption (12-17). Ezetimibe selectively inhibits the intestinal absorption of dietary and biliary cholesterol and related phytosterols (18-22). Ezetimibe localizes and appears to act at the brush border membrane of the small intestine and inhibits cholesterol absorption resulting in the decrease in the delivery of intestinal cholesterol to the liver (23). This translates to a reduction in hepatic cholesterol stores thereby promoting the synthesis of LDL receptors with a subsequent reduction in serum LDL cholesterol concentration (24).

1.1 Aim of the thesis

There is a wide inter-individual variation in response to cholesterol absorption inhibitors in reducing plasma cholesterol concentration. The existence of hypo- and hyper-responders supports the hypothesis that cholesterol absorption is genetically determined. The complex interaction between drugs and genetic factors in lipid metabolism can be studied in a relatively limited study population, assuming that the genetic factor regulates some measurable indicators of the metabolic cascade. It has been clearly demonstrated that the ratio of plant sterols (campesterol and sitosterol) to cholesterol are the indicators of the rate of cholesterol absorption (25). On the other hand, the ratio of lathosterol, a cholesterol precursor, to cholesterol reflects HMG-CoA activity in the liver and total cholesterol synthesis (26). Thus the ratio of lathosterol to campesterol may be an even better marker for response to cholesterol absorption inhibitors. The possible determinants regulating the responsiveness resemble the individual intestinal cholesterol absorption capacity and the individual variation of hepatic cholesterol synthesis.

Therefore, the purpose of the present thesis was:

1. To investigate the responsiveness to sitostanol ester margarine in two selective subgroups with a high and a low ratio of serum lathosterol to campesterol (Responder Study 1).

2. To investigate the responsiveness to ezetimibe in two selective subgroups with a high and a low ratio of serum lathosterol to campesterol and possible genetic variations in the ABCG5/ABCG8 transporters (Responder Study 2).

The studies were conducted to measure the serum cholesterol lowering effect of sitostanol ester margarine and ezetimibe in selected groups with differences in individual intestinal cholesterol absorption capacity and hepatic cholesterol synthesis and also to identify the responders and non-responders to these treatments.

1.2 Review of the literature

1.2.1 CHD risk factors

The concept of CHD risk has changed during the past few decades, and the nature of CHD as a multifactorial disease has become clear. The major risk factors are widely recognized as the primary causes of CHD. It is practical to divide the CHD risk factors into three categories.

1. Personal characteristics
2. Biochemical factors
3. Lifestyle factors.

1.2.1.1 Personal characteristics

Personal characteristics such as age, sex and genes, may play a major role in the development of CHD, but regrettably, these factors cannot be altered by the treatment methods available. A mixture of chronic processes and acute events marks the pathophysiology of atherosclerosis and CHD. The most significant pathogenic processes, which are determined partly genetically and partly environmentally, are dyslipidemia, hypertension, endothelial dysfunction, diabetes, smoking and dietary habits. Thus, the variations of many factors may lead to the disease process, and depending on the combinations of genetic variations in different subjects, atherosclerosis and CHD may be manifested as a wide spectrum of phenotypes (27). Several genes have been analyzed in relation to apolipoproteins, (apo B, apo C-III, apo(a), apo E), lipid transfer proteins (PLTP, CETP), enzymes (LPL, HL, LCAT), receptors (LDL receptor, LDL receptor related protein), thrombogenic factors (fibrinogen, PAI-1, glycoprotein IIIa), and others (ACE, angiotensin II-receptor, paraoxonase, methylene tetrahydrofolate reductase, neuropeptide Y), and their respective regulatory genes are also involved (28).

Genetic factors regulate the age of onset and the intensity of the disease and also the response to treatment, including the response of plasma lipids to dietary behaviour. It has been proposed that common polymorphisms with frequent alleles credibly account for most of the genetic component of atherosclerosis and CHD (29), and rare monogenic disorders affiliated with a high absolute risk for CHD, such as familial hypercholesterolemia, are only observed in a small proportion of the patients with CHD (30-32). The rare genetic disorders consociated with myocardial infarction or atherosclerosis at young age, such as familial hypercholesterolemia and familial defective apo B-100, are relatively well authenticated (33).

1.2.1.2 Biochemical factors

1.2.1.2.1 Plasma lipids and lipoproteins and the risk of atherosclerosis

The most significant biochemical factor is the plasma total and LDL cholesterol concentration. After successful treatment of patients with cholesterol-lowering drugs, it is generally recommended to measure the plasma total, LDL and HDL cholesterol and triglycerides in the case of people at a high risk to develop CHD. A high concentration of plasma total cholesterol is a major risk factor for coronary artery disease (34-36). This risk is arbitrated through the major cholesterol-carrying lipoprotein, LDL, which is regarded as the major atherogenic lipoprotein. The evidence affirming the hypothesis that LDL is atherogenic comes from epidemiologic studies (37-39), clinical trials (40-42), studies in laboratory animals (43), heritable hypercholesterolemias (44), pathologic investigations (24), and studies in model systems (45). Several hypotheses exist concerning the mechanisms by which LDL produces atherosclerosis. The concentration, the size and the chemical modification of LDL are important for atherogenesis. Clinical trials have shown that a reduction of total and LDL cholesterol is followed by a regression of atherosclerotic manifestations (15). Based on these results, the ideal plasma total cholesterol concentration is < 200 mg/dL and the ideal LDL cholesterol concentration < 120 mg/dL (46).

HDL particles are the smallest lipoproteins and therefore enter and also leave the artery wall easily. The plasma concentrations of HDL cholesterol are inversely associated with the risk of CHD (47). The plasma concentrations of apoA-I, the structural protein of HDL, correlate strongly with HDL cholesterol levels and are also inversely associated with the CHD risk. HDL cholesterol and apoA-I are anti-atherogenic, as shown by animal studies and genetic human studies, and the effect has been explained by the reverse cholesterol transport (48, 49). The other potential anti-atherogenic mechanisms of HDL and apoA-I include the protection of LDL from oxidation (50), the protection of endothelial cells from the cytotoxic effect of LDL, and the stimulation and stabilization of the vasodilator prostacyclin (48). The ideal levels for plasma HDL cholesterol concentration are > 50 mg/dL (46).

Although hypertriglyceridemia has been statistically associated with CHD, the independent association of plasma triglycerides with the CHD risk is less certain than that with LDL, and triglycerides may not be causally related to the development of atherosclerosis (51). Hypertriglyceridemia may be a secondary phenomenon that occurs in response to the metabolism of LDL and HDL. ApoB is the major protein component of LDL, IDL, VLDL and chylomicrons, and all apoB-containing lipoproteins are atherogenic. Since there is one apoB molecule per each lipoprotein particle, the apoB concentration is a good indicator of the risk of atherosclerosis and CHD (52).

1.2.1.2.2 Blood pressure

According to epidemiological studies and after adjustment for confounding factors high blood pressure alone is an important risk factor for CHD (53-55). The treatment of hypertension reduces the risk of CHD in clinical drug intervention trials (56), and lifestyle interventions for mildly elevated blood pressure have also been effective in risk reduction. Blood pressure is controlled by both genetic and environmental factors. The mechanism whereby hypertension predisposes to cardiovascular disease is probably related to the fact that high blood pressure accelerates the atherosclerotic process as well as to the pressure effects leading to progressive dilation and rupture of large and small blood vessels. A recommended level of blood pressure is less than 90 mm Hg for diastolic and less than 140 mm Hg for systolic blood pressure (46).

1.2.1.2.3 Obesity

A body mass index (BMI, kg/m^2) over 25 is considered overweight, and BMI over 30 is defined as obesity. Obesity has been associated with excess mortality (57), and prospective studies of cardiovascular morbidity and mortality have shown an association with obesity (58, 59). The risk already begins to increase at a moderate level of obesity. Obesity has an adverse influence on blood pressure, plasma lipids and lipoproteins, and glucose tolerance, and further has adverse hemodynamic effects (38). In obese subjects, the excess lipolysis and release of fatty acids from adipose tissue is followed by increased VLDL production (60) and secretion from the liver (61) and high plasma triglyceride concentrations. The activities of plasma and adipose tissue LPL are high in obese subjects (62). The HDL cholesterol concentration is usually low in obesity (61). The mechanism for this may be partly regulated by elevated CETP activity.

Since obese subjects have supersaturation of cholesterol in bile, increased cholesterol excretion and furthermore, hypomotility of the gallbladder, cholesterol gallstone formation is enhanced, especially in obese women (63). Weight reduction by either caloric restriction or increased energy expenditure is followed by reductions of blood pressure, plasma triglyceride concentration, increase of plasma HDL cholesterol and normalization of blood glucose (64).

1.2.1.3 Lifestyle factors

Lifestyle factors have an important role in the CHD risk both at the population and at the individual level. The lifestyle factors include a diet high in saturated fat, cholesterol and energy, tobacco smoking and physical inactivity. These are factors which, on the one hand, could lower the CHD risk significantly after modification, but, on the other hand, are the most difficult to modify in community-living adult populations.

Smoking is one of the major risk factors for CHD (65, 66). The harmful effect is mediated through altered lipid and lipoprotein metabolism (67) as well as through the induction of vasoactive, thrombogenic and other atherogenic mechanisms (68-70). Also, smokers exhibit several characteristics of insulin resistance syndrome and the impact of smoking on CHD risk is modified by plasma lipid levels (46). Ceasing smoking leads to a considerable risk reduction among CHD patients. The intake of light to moderate amounts of alcohol is associated with reduced morbidity and mortality from several cardiovascular conditions, particularly CHD (71, 72). The beneficial effects of light to moderate alcohol drinking on lipoprotein metabolism (73, 74), coagulation (46, 75) and antioxidative properties (76, 77) have been shown in several studies. Since the adverse effects of excess alcohol use are so well known, alcohol should not be recommended as a general preventive tool against CHD. Sedentary lifestyle is associated with an increased risk of death from cardiovascular disease and an increased CHD risk (78-81), and conversely, a high level of physical activity is followed by a reduction of the CHD risk and favorable effects on overweight and plasma lipids and lipoproteins.

1.2.2 Overview of lipoprotein metabolism

1.2.2.1 Cholesterol synthesis

Although the liver is the major organ for cholesterol synthesis, most of the human organs such as the intestine, skin and muscle are capable of synthesizing cholesterol (82). Cholesterol is synthesized from acetyl-CoA via many biochemical steps in the cytoplasm, endoplasmic reticulum, or peroxisomes (83). In the liver, acetyl -CoA is derived largely from fatty acids. Acetyl-CoA units are converted to mevalonate by a series of reactions that begins with the formation of HMG-CoA (84) (Figure 1). Two moles of acetyl-CoA are condensed to acetoacetyl-CoA. Acetoacetyl-CoA and a third mole of acetyl-CoA are converted to HMG-CoA by the action of HMG-CoA synthase. HMG-CoA is converted to mevalonate by HMG-CoA reductase (82). HMG-CoA reductase 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-CoA reductase is the rate-limiting step of cholesterol biosynthesis. Mevalonate is then activated by three successive phosphorylations, yielding 5-pyrophosphomevalonate (83). In addition to activating mevalonate, the phosphorylations maintain its solubility, since otherwise it is insoluble in water. After phosphorylation, an ATP-dependent decarboxylation yields isopentenyl pyrophosphate (IPP) an activated isoprenoid molecule. Isopentenyl pyrophosphate is in equilibrium with its isomer, dimethylallyl pyrophosphate (DMPP). One molecule of IPP condenses with one molecule of DMPP to generate geranyl pyrophosphate (GPP). GPP further condenses with another IPP molecule to yield farnesyl pyrophosphate (FPP). Finally, the NADPH-requiring enzyme, squalene synthase catalyzes the head-to-tail condensation of two molecules of FPP, yielding squalene. Squalene undergoes a two step cyclization to yield lanosterol (83). The first reaction is catalyzed by squalene monooxygenase. This enzyme uses NADPH as a cofactor to introduce molecular oxygen as an epoxide at the 2, 3 position of squalene. Through a series of 19 additional reactions, lanosterol is converted to cholesterol (84). Theoretically, the features of cholesterol synthesis can be divided into pre-squalene synthesis and post-squalene synthesis. The latter includes cyclization of squalene to lanosterol, and the metabolic reactions leading from lanosterol to cholesterol. These pathways share many enzymes; and deficiency in these enzymes disrupts cholesterol biosynthesis.

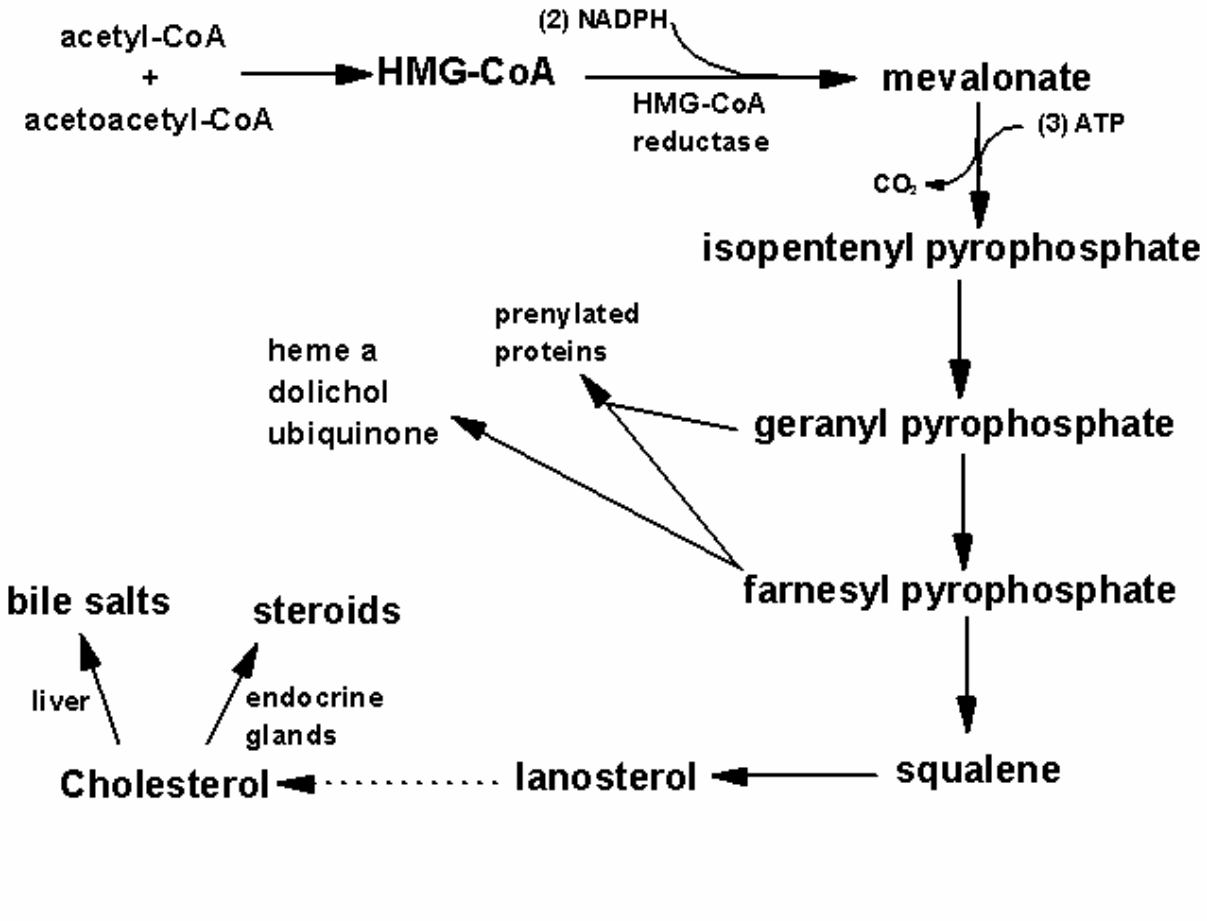


Figure 1: Cholesterol synthesis pathway

1.2.2.2 Plasma lipoproteins

Lipoproteins carry the lipids between the site of their synthesis or absorption i.e., small intestine, the liver, and extra hepatic tissues. Lipoprotein particles contain a hydrophobic core of triglycerides (TG) and cholesterol esters, and a hydrophilic surface of free cholesterol, phospholipids, and apolipoproteins (Figure 2). Since the lipids along with phospholipids and TG are water insoluble, they are transported by lipoproteins in human blood.

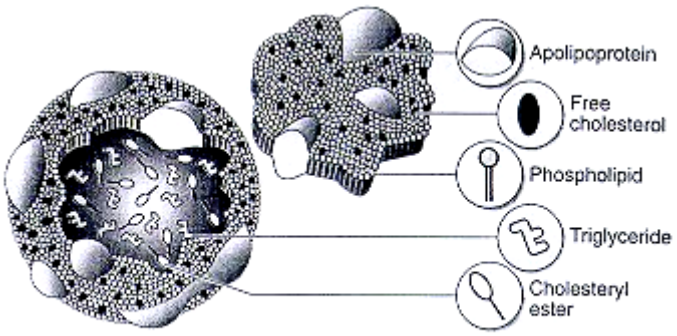


Figure 2: Structure of lipoprotein

Plasma lipoproteins are classified based on their density (d) into five classes:

1. Chylomicrons (CM, d < 0.94 g/ml)
2. Very low density lipoprotein (VLDL, d = 0.94-1.006 g/ml)
3. Intermediate density lipoprotein (IDL, d = 1.006-1.019 g/ml)
4. LDL (d = 1.019-1.063 g/ml)
5. High density lipoprotein (HDL, d = 1.063-1.210 g/ml)

Chylomicrons, which have a particle diameter of 75-1200 nm, are the largest lipoproteins. They are secreted by the enterocyte. ApoB-48, synthesized by the enterocyte, is the primary structural protein of chylomicrons, but chylomicrons contain also small amounts of apoA-I and apoA-II synthesized by the intestine. After absorption, TG, cholesterol, and other sterols are packed in chylomicrons and are transported via lymph to blood where most of the TG of the chylomicrons are hydrolyzed by lipoprotein lipase (LPL) on the surface of capillary endothelial cells to form chylomicrons remnants. The sterols are either stored as esters, excreted into bile as such, or as acidic derivatives, or they can be released into circulation in VLDL (85).

VLDL particles (30-80 nm) are synthesized in the liver and contain apoB-100 as the main structural protein. In addition, VLDL contains also apoC-I-III and apoE. VLDL particles transport not only endogenously synthesized sterols but also exogenously derived TG and sterols that are not excreted in bile. Their release from the liver is followed by hydrolysis of TG by LPL leading to the formation of VLDL remnant particles (86).

As hydrolysis proceeds, phospholipids and most of the apoC are transferred to HDL by PLTP followed by transformation of the remnant particles into IDL particles (25-35 nm) and further by lipolysis by hepatic lipase (HL) into LDL. In humans, about 50% of VLDL is converted to LDL. Part of the remnants and IDL particles are taken up by the liver receptors, mostly by the LDL receptors (85).

LDL, with a particle diameter of 18-25 nm, is a major carrier of cholesterol in plasma. It contains only one molecule of apoB-100 as a structural protein. Most of the LDL particles are taken up into the liver by receptor-mediated pathways by recognition of apoB-100, but peripheral tissues that also contain LDL receptors can take up an appreciable portion of LDL. LDL transports cholesteryl ester to a variety of peripheral tissues, but a significant amount of plasma LDL is eventually removed from the circulation by the liver via the binding of apo B-100 to the hepatic LDL receptor (5). The plasma concentration of LDL depends on the rates of VLDL secretion and the conversion of VLDL to LDL (60) and the fractional clearance rate of LDL, and it is influenced by heritable factors and some environmental factors, the most important being the diet. LDL can undergo an oxidative modification (87), which has a significant role in the process of atherosclerosis (88, 89).

HDL particles (5-12 nm) contain apoA-I, apoA-II, and apoA-IV, which are synthesized by the liver and intestine and are carried to HDL either as free apos or by PLTP from CM. Discoidal nascent pre-ß-HDL contains apoA-I, phospholipids, and cholesterol but not cholesterol esters (90). This nascent HDL carries lecithin cholesterol acyltransferase (LCAT), which is capable of esterifying free cholesterol. The substrate for LCAT is derived from plasma membranes, where the adenosine triphosphate-binding cassette transporter A1 (ABCA1) facilitates the transport of free cholesterol to nascent HDL (91). As the cholesterol is esterified by LCAT, pre-ß-HDL is matured to HDL; cholesterol ester transfer protein (CETP) facilitates the transport of cholesterol esters in exchange for TG to apoB containing lipoproteins, which are taken up by the liver (92). TG derived from apoB containing lipoproteins in HDL are hydrolyzed by HL and smaller discoidal pre-ß-HDL are regenerated to restart the cycle. Thus, HDL accompanied by CETP mediates the reverse cholesterol transport from the peripheral tissues to the liver to be eventually delivered into the bile (93).

1.2.2.3 Cholesterol metabolism

In humans, cholesterol is acquired either by de novo synthesis or by absorption from the diet. In a particular tissue, cholesterol is either synthesized de novo or is derived from circulatory lipoproteins. The human body's cholesterol pool is regulated by the following factors:

- Dietary intake of cholesterol
- Intestinal absorption of cholesterol and formation of postprandial lipoproteins
- Reverse cholesterol transport from peripheral tissues
- Hepatic uptake of lipoproteins by receptors
- Endogenous cholesterol synthesis
- Hepatic secretion of lipoproteins
- Bile acid synthesis
- Uptake of cholesterol by peripheral tissues
- Biliary excretion and fecal elimination of cholesterol and bile acids.

Under normal conditions, endogenous synthesis of cholesterol contributes two-thirds of the total cholesterol input into whole-body pools (94, 95). Cholesterol feeding increases the amount of cholesterol in the body (96, 97). However, accumulation of cholesterol in tissues by increased dietary cholesterol intake is prevented through reduced fractional absorption or synthesis of cholesterol or by enhanced cholesterol excretion (95, 98-100). Cholesterol homeostasis in man is regulated by well-balanced mechanisms of intestinal uptake, endogenous synthesis and metabolism, transport in lipoprotein particles, and biliary excretion.

1.2.2.4 Cholesterol absorption

In addition to 150-300 mg/day of phytosterols, the diet in Western countries contains approximately 50-700 mg/day of cholesterol (101). In addition to dietary sterols, the intestinal sterols consist of biliary sterols (e.g., cholesterol 500-2500 mg/day) and of variable amounts of cholesterol synthesized by the intestinal cells (101, 102). All of the biliary sterols are in a free form, but the proportion of esterified sterols obtained from the diet varies greatly, ranging from 1% up to 73% for cholesterol (103), and from ~10% up to 80% for phytosterols (104). Thus, the bulk of intestinal sterols consist of up to 800-3000 g/day of free and esterified sterols from endogenous and exogenous sources (Figure 3).

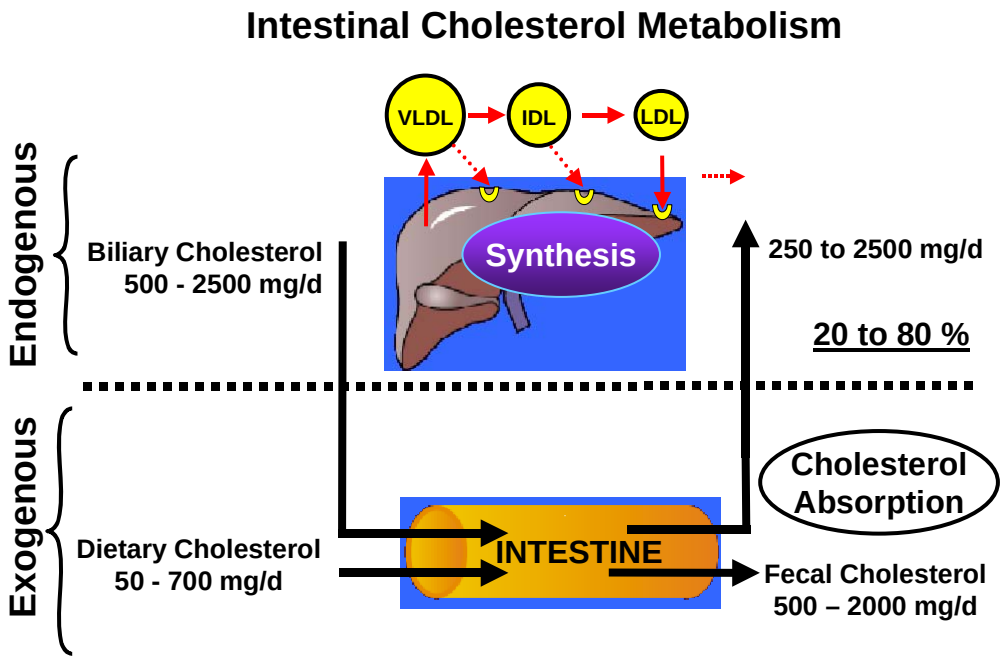


Figure 3: The exogenous and endogenous pathways of plasma lipid transport.

Cholesterol absorption efficiency exhibits a wide interindividual variation, ranging from 20% to 80% (105, 106). The reasons for this variability are not completely understood, but several dietary, physiologic, and genetic factors can influence the absorption of cholesterol. Most studies have evaluated the effect of these factors on cholesterol absorption, whereas much less is known about the regulation of phytosterol absorption. The absorbed mass of dietary cholesterol is increased with the increased intake (98, 106, 107), but the percentual absorption of cholesterol is reported to remain similar (105, 106, 108) or may even be decreased (99, 105). In subjects with a high intake of cholesterol, cholesterol absorption was lower in older subjects (75-year-old) than in the younger ones (50-year-old) (109), while no age-related differences were reported in other studies with lower intakes of cholesterol (105). Some conditions with metabolic disturbances, such as high-normal blood glucose levels in non-diabetic subjects (110), type 2 diabetes (111), and obesity (103) are associated with low cholesterol absorption efficiency.

Phytosterols interfere with cholesterol absorption (112). The intake of phytosterols correlated negatively with the cholesterol absorption efficiency, thus contributing to lower total and LDL cholesterol levels (113). This indicates that phytosterols of normal food explain some variability of cholesterol absorption efficiency.

1.2.2.5 Absorption from intestinal lumen to enterocytes

While the ingested fat is mostly water insoluble, the basic step in fat absorption is conversion of this hydrophobic oil to hydrophilic compounds that can be efficiently absorbed from the intestine. After emulsification and initial hydrolysis by lingual and gastric lipase, the hydrolysis of TG, phospholipids, and esterified sterols takes place in the small intestine principally by pancreatic lipases activated by colipase (114), cholesterol esterase (115), and phospholipase A2 (116). However, in addition to free sterols, some absorption of esterified sterols also occurs (117). While the hydrolyzed sterols still have only limited solubility in an aqueous environment, bile acids are needed to form micelles (118, 119) to assist the sterols to diffuse across the unstirred water layer to reach the mucosal cell membrane (120). The micellar solubility of phytosterols has been shown to be poorer than that of cholesterol (121, 122), thus partly explaining the low absorption rate of the phytosterols.

In the past, cholesterol uptake in the gut was thought to be controlled mainly by 2 enzymes, acyl CoA cholesterol acyltransferase 2 (ACAT2), which enhances intracellular sterol esterification, and the microsomal triglyceride transfer protein (MTP), responsible for intestinal chylomicron assembly. However, two recently discovered cholesterol transporter systems in the human gut ATP-binding cassette (ABC) transporters ABCG5 and ABCG8 and Niemann-Pick C1 Like 1 (NPC1L1) protein brought further insights in the pathways of intestinal cholesterol absorption and also hepatic sterol excretion.

1.2.2.6 ABCG5 and ABCG8 as intestinal cholesterol transporters

Helen Hobbs and coworkers discovered ABCG5/G8 in their studies on a rare disease, sitosterolemia, a recessive disorder characterized by increased absorption of plant sterols and cholesterol and diminished biliary excretion of sterols, hypercholesterolemia, hypersitosterolemia, and early coronary atherosclerosis (123). They found mutations in either of the two ABC monomers (ABCG5, ABCG8) in nine patients with sitosterolemia and concluded that these transporters cooperate to regulate intestinal absorption and promote biliary excretion of sterols. ABCG5 and ABCG8 are present on nearly contiguous genes, and when expressed, the proteins form a heterodimer in the endoplasmic reticulum (124).

Co-expression is required for their movement into the Golgi and onto the apical surface of the cell. Cholesterol feeding increases the expression of both genes in mouse liver and intestine (124). ABCG5/8 are expressed in the liver and intestine but not in other tissues (6). In transgenic animals overexpressing ABCG5/8, biliary cholesterol secretion is markedly increased. The role of ABCG5 and ABCG8 in the regulation of sterol absorption has been confirmed in so far that the overexpression of the two transporter genes reduced the fractional absorption of dietary cholesterol (125). Disruption of ABCG5 and AC5G8 genes in mice greatly inhibited cholesterol secretion into bile, demonstrates that ABC heterodimer is responsible for secretion of biliary cholesterol. In addition, the fact that the polymorphisms in gene coding for ABCG8 have been found to affect the serum plant sterol levels (126), suggests that sterol absorption is heritably controlled.

1.2.2.7 Regulation of intestinal cholesterol absorption by ABCG5 and ABCG8

ABCG5 and ABCG8 genes are located in a head-to-head orientation on chromosome 2p, and appear to be expressed exclusively in liver and intestine. The expression of ABCG5 and ABCG8 transporters seems to be controlled by dietary cholesterol via liver X receptors (LXR) (127). LXR_{α} and LXR_{β} are nuclear receptors which regulate many key genes in cellular sterol metabolism (128). LXRs are expressed in most tissues and are activated by endogenous oxysterols such as 22(R)-hydroxycholesterol, 24(S),25-epoxycholesterol, 24S- and 27-hydroxycholesterol (129). LXRs regulate their target genes in the form of heterodimers with the 9- cis-retinoic acid receptors (RXRs) which bind to LXR response elements in the regulatory regions (130). Besides the regulation of ABCG5 and ABCG8, the target genes also include the ABCA1 and ABCG1 transporters (131). Dietary cholesterol feeding leads to an increase in the expression of the ABCG5 and ABCG8 transporters in mice which is controlled by the activation of LXRs (126, 132). Experiments in LXR_{α} and LXR_{β} knockout mice showed that LXRs are essential for cholesterol-induced up-regulation of ABCG5 and ABCG8 transporters (132). The activation of LXRs is associated with an increase in biliary cholesterol secretion and a decrease in intestinal cholesterol absorption(133). The regulation of intestinal Cholesterol Absorption by ABCG5 and ABCG8 is represented schematically in figure 4.

1.2.2.8 NPC1L1 protein as intestinal cholesterol transporter

Recently, Altmann et al. (2004) and Davis et al. (2004) reported the identification of the NPC1L1 protein as intestinal cholesterol transporter (134, 135). Searching special genomic databases for transcripts containing anticipated features of a sterol transporter they could identify the rat homologue of the human NPC1L1 gene as putative target structure. The NPC1L1 gene has a 50% amino acid homology to the Niemann-Pick C 1 gene which is affected in the cholesterol storage disease Niemann-Pick type C (136, 137). When assessing the NPC1L1 mRNA expression in human tissues, Altmann et al. (2004) found the highest levels in small intestine, gall bladder, and stomach, but also other tissues, such as heart, lung and colon. In rats, they analyzed the distribution of NPC1L1 mRNA in 10-cm segments across the small intestine and found the highest levels in the proximal jejunum with a steady decrease towards the ileocolic valve. Applying in situ hybridization and immunohistochemistry, they were able to identify cell-specific NPC1L1 mRNA and protein expression in the rat jejunum near to the luminal space and suggest that NPC1L1 protein is predominantly associated with the apical membrane of the enterocyte.

Later experiments using a LacZ-Neo cassette and a h-galactosidase histochemical reaction in NPC1L1 knockout mice (NPC1L1–/–) confirmed the predominant expression of NPC1L1 in the proximal jejunum (134). Generating NPC1L1 knockout mice (NPC1L1–/–), the same research team performed numerous experiments on cholesterol and sitosterol absorption in these animals (134). While NPC1L1–/– mice did not show any morphological abnormalities compared with heterozygous and wild-type mice, they showed a significantly lower fractional cholesterol absorption rate of 15.6 % compared to 45.4 % in heterozygous and 51.3 % in wild-type animals (134). Despite the reduced cholesterol absorption rate, the knockout animals had comparable plasma lipids but showed a compensatory 3.3- to 3.8-fold increase of the HMG CoA synthase mRNA in gut and liver without affecting ABCG5 and ABCG8 mRNA expression in the gut. Adding ezetimibe lowers cholesterol absorption rates in the NPC1L1+/– and in the NPC1L1+/+ animals to almost the same level as observed in untreated knockout mice, while the NPC1L1–/– mice did not exert further decrease in cholesterol absorption by ezetimibe. While plasma levels of cholesterol and triglycerides were comparable between wild-type, heterozygous, and knockout animals, significant differences were observed for plant sterols (134).

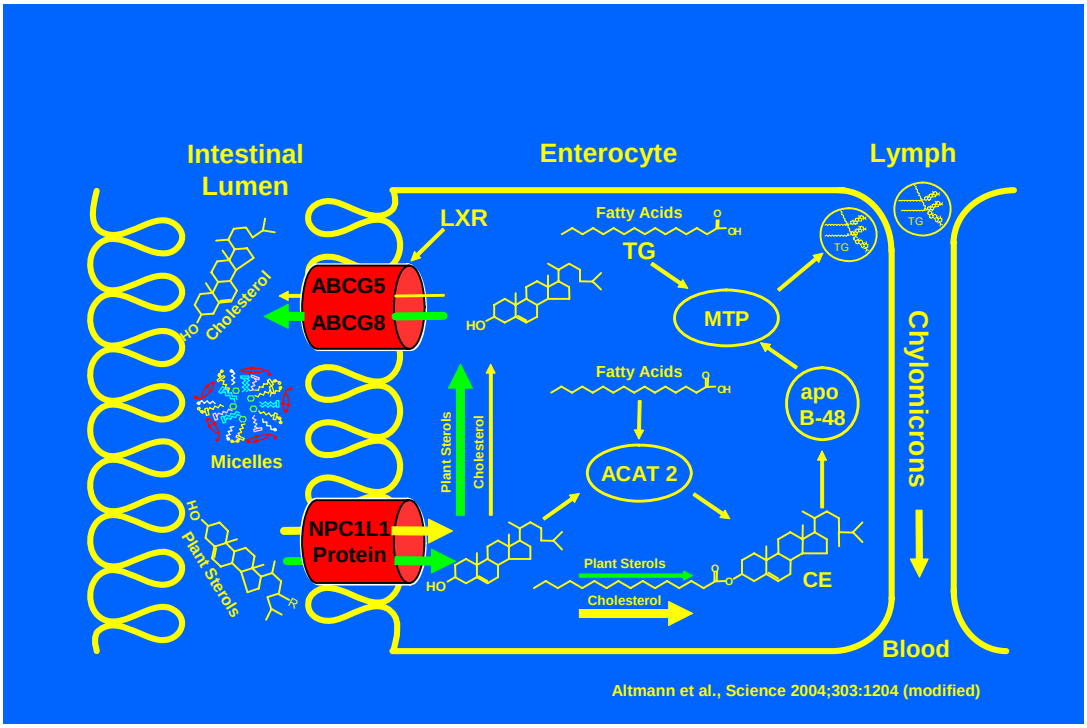


Figure 4: Regulation of intestinal cholesterol absorption by ABCG5, ABCG8 and NPC1L1 transporters

Compared with NPC1L1+/+ animals, heterozygous NPC1L1+/– mice had 37% and 50% lower plasma levels of sitosterol and campesterol, respectively, while NPC1L1–/– mice had even more than 90% lower plasma concentrations of both plant sterols. Using 3H-labeled sitosterol, the authors could also demonstrate a 64% reduced intestinal sitosterol absorption in NPC1L1–/– mice compared to NPC1L1+/+ animals, which was comparable to the effect of a single 10 mg dose of ezetimibe in NPC1L1+/+ mice. Despite the similarity of NPC1L1 knockout and ezetimibe treated wild-type mice, direct binding of ezetimibe to NPC1L1 as well as the reconstitution of a transmembrane cholesterol transporter in nonenterocytes by overexpression of NPC1L1 could not be demonstrated (135). The regulation of intestinal Cholesterol Absorption by NPC1L1 is represented schematically in figure 4.

1.2.2.9 Indicators of the intestinal cholesterol absorption

Measurement of sterol absorption

On average, 20-80% of the dietary cholesterol is absorbed (131, 138), whereas despite the structural similarity, the intestinal absorption of phytosterols is much less. Thus, in humans, when measured with different techniques, the absorption of sitosterol and stigmasterol has been found to be ~5% (139-141) , while the absorption of campesterol is higher (~10-16%) (11, 141). Saturation of the 5-double bond further decreases the absorption leading to absorption portions of ~2% for plant stanols (103, 113), even though up to 13% absorption of campestanol has also been observed (141).

Ostlund et al. (2002) reported extremely low absorption rates for both plant sterols (0.5-1.9%) and stanols (0.04-0.16%) by using a dual stable isotopic tracer technique (142). These results suggest that the reported absorption rates are dependent on the method used, or may be related to the small sample sizes employed (141). Generally, the absorption of different sterols decreases with an increased length of side chain in C-24 (Figure 5) (141, 143, 144).

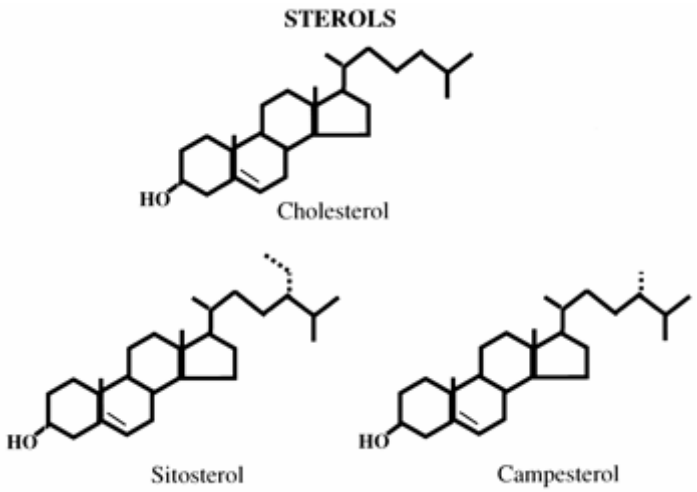


Figure 5: Chemical structures of cholesterol, sitosterol and campesterol

Sterol absorption can be measured by several different methods, mostly based on the use of radioactive compounds (98, 145-147), and thus these are not at all suitable e.g., for children. Even though a recently developed method to study cholesterol absorption by stable isotopes (138, 148) avoids the use of radioactive tracers, the measurement of cholesterol absorption by this method is still laborious. The ratios of serum plant sterols to cholesterol are positively correlated with the cholesterol absorption measured by sterol balance technique (25, 149, 150) and are thus called cholesterol absorption markers. Accordingly, measurement of these non-cholesterol sterols by gas liquid chromatography (GLC) provides a simple method to assess cholesterol absorption and is especially suitable in large-scale studies and for detecting changes in cholesterol absorption during experimental studies.

1.2.2.10 Assessing the rate of whole-body cholesterol synthesis

In addition to cholesterol, serum contains small amounts of squalene and noncholesterol sterols such as the cholesterol precursors cholestenol, desmosterol, and lathosterol, and the plant sterols mainly campesterol and sitosterol. For measurement of cholesterol synthesis in humans, direct and indirect techniques exist (151). These methods are sterol balance (112), assay of HMG CoA reductase activity (152), and tracer incorporation approaches (e.g., incorporation of various [C]-substrates, deuterium, and tritium, and mass 14 isotopomer distribution analysis (94, 153).

The sterol balance technique provides a direct assessment of endogenous biosynthesis rate, which is regarded as the difference between fecal excretion of neutral and acidic steroids and total cholesterol intake. Once internal sterol pools have reached equilibrium, i.e., the steady state, sterol balance provides an accurate method to assay cholesterol synthesis. Alternatively, measuring plasma concentrations of cholesterol precursors such as mevalonate, squalene, methyl sterols, desmosterol, and lathosterol indicates relative changes in cholesterol synthesis rates (151, 154, 155). Cholesterol synthesis rate is regulated by cholesterol feeding as well as by other dietary factors, intestinal cholesterol absorption, and enterohepatic circulation of bile acids. Although this method is well established, it has the disadvantage of being laborious and is not suited to detecting changes in cholesterol synthesis occurring within a few days.

Moreover, the method requires that the subjects be in a steady-state with regard to cholesterol metabolism. For these reasons, serum levels of various pre-cursors along the cholesterol synthesis pathway have been proposed for that purpose, as alternative methods to monitor whole-body cholesterol synthesis. Levels of precursors were shown to correlate significantly with the cholesterol balance in conditions with greatly varying rates of cholesterol synthesis and to fluctuate with a diurnal rhythm (154, 156).

The 1athosterol to total cholesterol ratio in serum has been shown to be an indicator of whole-body cholesterol synthesis in humans (26). Kempen et al. showed directly that lathosterol and the lathosterol to cholesterol ratio are indeed good monitors of whole-body cholesterol synthesis within the range of synthetic rates occurring in healthy people on normal Western diets, in a manner that is apparently independent of the fatty acid composition of the diet. This capacity as an indicator has not been demonstrated for either methylsterols or mevalonate. Bjorkhem et al. (152) reported that serum levels of free methylsterols and of free and total lathosterol were all highly correlated with the hepatic HMG-CoA reductase activity in human patients.

1.2.3 Treatment of hypercholesterolemia

The benefits of lipid-lowering therapy on coronary heart disease (CHD) risk have been clearly established in many large-scale primary and secondary prevention trials (Executive summary of the third report of the national cholesterol education program (NCEP) expert panel on detection, evaluation, and treatment of high blood cholesterol in adults (8).
The major classes of drugs for consideration are:

- HMG-CoA reductase inhibitors (statins) — lovastatin, pravastatin, simvastatin, fluvastatin, atorvastatin, rosuvastatin
- Bile acid sequestrants — cholestyramine, colestipol, colesevelam
- Nicotinic acid — crystalline, timed-release preparations, Niaspan®
- Fibric acid derivatives (fibrates) — gemfibrozil, fenofibrate, clofibrate, bezafibrate
- Cholesterol absorption inhibitors — ezetimibe, sitosterol or sitostanol ester margarine

1.2.3.1 HMG-CoA reductase inhibitors (statins)

Statins inhibit HMG CoA reductase, the rate-limiting step in cholesterol biosynthesis (66). Inhibition of cholesterol synthesis reduces hepatic cholesterol content, resulting in increased expression of LDL receptors, which lowers serum LDL-cholesterol levels (157). Intermediate density lipoprotein (IDL) and VLDL remnants also are removed via the LDL receptor. The latter effect contributes to lowering of triglyceride-rich lipoproteins (TGRLP) by statins (158, 159). Statins also appear to reduce hepatic release of lipoproteins into the circulation (160, 161). Depending upon the specific statin and the dose administered, reductions in LDL cholesterol of 18–55 percent are observed (162, 163). The reductions in LDL cholesterol concentrations are dose-dependent and log-linear. Elevated hepatic transaminases generally occur in 0.5–2.0 percent of cases and are dose-dependent (164, 165). Bradford et al. reported that the 2-year incidence of serum transaminase elevation with lovastatin therapy was 0.1 percent for 20 mg/day and 1.9 percent for 80 mg/day (166). Elevation of creatine kinase levels can also occur and is a good indicator of statin-induced myopathy.

1.2.3.2 Bile acid sequestrants

The sequestrants bind bile acids in the intestine through anion exchange; this binding reduces the enterohepatic recirculation of bile acids, which releases feedback regulation on conversion of cholesterol to bile acids in the liver. The resulting decrease in hepatocyte cholesterol content enhances LDL-receptor expression, which in turn lowers serum LDL-cholesterol concentrations (167). The major action of bile acid sequestrants is to lower LDL cholesterol (168-171). Therapy with cholestyramine reduced the risk of CHD in the Lipid Research Clinics Coronary Primary Prevention Trial (172). Beneficial outcomes also occurred in other clinical trials in which sequestrants were combined with other lipid-modifying drugs. Sequestrants add to the LDL-lowering effects of other drugs, notably statins (173, 174). They remain unabsorbed in their passage through the gastrointestinal tract and lack systemic toxicity. Cholestyramine and colestipol are both administered as powders that must be mixed with water or juice. They usually are given once or twice daily with meals. 8 to 10 g/day cholestyramine or 10–20 g/day colestipol reduce LDL-cholesterol concentrations by 10–20 percent. Sequestrants add to LDL lowering when combined with other cholesterol-lowering drugs.

Their disadvantages are two-fold. Because of their bulk, they lack convenience of administration; they also cause various gastrointestinal symptoms, notably constipation. Since sequestrants tend to raise serum triglycerides, they are contraindicated as monotherapy in persons with high triglycerides (>400 mg/dL) and in familial dysbetalipoproteinemia (175). They generally should be used as monotherapy only in persons with triglyceride levels of <200 mg/dL. Sequestrant therapy can produce a variety of gastrointestinal symptoms, including constipation, abdominal pain, bloating, fullness, nausea, and flatulence (172). Sequestrants are not absorbed from the intestine, but can decrease the absorption of a number of drugs that are administered concomitantly. Colesevelam is a new bile acid binding resin, which is administered in capsules and therefore better tolerated. In addition, it has fewer adverse effects (170).

1.2.3.3 Nicotinic acid

Nicotinic acid or niacin favorably affects all lipids and lipoproteins when given in pharmacological doses. Nicotinic acid lowers serum total and LDL-cholesterol and triglyceride levels and also raises HDL-cholesterol levels. Doses of 2–3 g/day are generally required to produce LDL-cholesterol reductions of 15 percent or greater (176-179).

Nicotinic acid appears to alter lipid levels by inhibiting lipoprotein synthesis and decreasing the production of VLDL particles by the liver. It inhibits the peripheral mobilization of free fatty acids, reducing hepatic secretion of VLDL (180, 181). It decreases the plasma concentrations of triglyceride, VLDL remnants, and IDL (182) and it causes a shift in LDL composition from the small, denser LDL particles to the larger, more buoyant LDL particles (183). Nicotinic acid also is the most effective lipid-lowering drug for raising HDL levels (184). Nicotinic acid therapy can be accompanied by a number of adverse effects (185). Flushing of the skin is common with the crystalline form and is intolerable for some persons (186). However, most persons develop tolerance to the flushing after more prolonged use of the drug (187). Less severe flushing generally occurs when the drug is taken during or after meals, or if aspirin is administered prior to drug ingestion. A variety of gastrointestinal symptoms, including nausea, dyspepsia, flatulence, vomiting, diarrhea, and activation of peptic ulcer may occur. Three other major adverse effects include hepatotoxicity, hyperuricemia and gout, and hyperglycemia (188). The risk of all three is increased with higher doses, especially at doses of 2 g or higher (185, 187).

Although nicotinic acid can be highly efficacious and favorably modify the lipoprotein profile, especially in patients with atherogenic dyslipidemia, its long-term use is limited for many patients by adverse effects (78). Nicotinic acid extended-release tablets (NIASPAN®) show lesser flushing when compared to conventional dosage forms (187).

1.2.3.4 Fibric acid derivatives (fibrates)

The mechanism of action of the fibrates is complex and there may be some variation among the drugs in this class. Research shows fibrates to be agonists for the nuclear transcription factor peroxisome proliferator-activated receptor-alpha (PPAR-alpha) (189). Through this mechanism, fibrates downregulate the apolipoprotein C-III gene and upregulate genes for apolipoprotein A-I, fatty acid transport protein, fatty acid oxidation, and possibly lipoprotein lipase (190). Its effects on lipoprotein lipase and apolipoprotein C-III (an inhibitor of lipoprotein lipase) enhance the catabolism of TGRLP, whereas increased fatty acid oxidation reduces formation of VLDL triglycerides. These effects account for serum triglyceride lowering, which is the major action of fibrates. Serum triglyceride lowering combined with increased synthesis of apolipoprotein A-I and A-II tend to raise HDL-cholesterol levels (191). Triglyceride lowering also transforms small, dense LDL into normal-sized LDL (192). The fibrates are primarily used for lowering triglycerides because the LDL-cholesterol-lowering effects of gemfibrozil and clofibrate are generally in the range of 10 percent or less in persons with primary hypercholesterolemia. Only slight changes in LDL cholesterol are noted in persons with combined hyperlipidemia, and LDL-cholesterol levels generally rise on fibrate therapy in persons with hypertriglyceridemia (193, 194). Fenofibrate frequently reduces LDL-cholesterol levels by 15 to 20 percent when triglycerides are not elevated. Clinical trials indicate a moderate reduction in CHD risk (195). Serious adverse effects seemingly do not occur in the long term, although early studies suggested an increase in non-CHD mortality, dyspepsia, various upper gastrointestinal complaints, cholesterol gallstones, myopathy (196). The fibrates are generally well tolerated in most persons. Gastrointestinal complaints are the most common complaints (197). The fibrates bind strongly to serum albumin and so may displace other drugs that bind with albumin. For example, fibrates displace warfarin from its albumin-binding sites; thereby increasing the latter's anticoagulant effect. Fibrates are excreted primarily by the kidney; consequently, elevated serum levels occur in persons with renal failure and risk for myopathy is greatly increased (198). The combination of a fibrate with a statin also increases the risk for myopathy, which can lead to rhabdomyolysis (199).

1.2.3.5 Cholesterol absorption inhibitors

1.2.3.5.1 Sitosterol/stanol ester margarines

Since the early 1950s, sitosterol was shown to decrease serum cholesterol levels (200). The introduction of sitosterol and sitostanol enriched margarines was an important step towards reduction of total- and LDL cholesterol (201). Earlier studies showed that large amounts of sitosterol (>10 g/d) lowered serum cholesterol levels by 10–20%. The high dosage and the chalky taste of sitosterol limited its use, especially with the advent of the more powerful, well-tolerated, lipid-lowering 3-hydroxy-3-methylglutaryl coenzyme A reductase inhibitors. Grundy and Mok (202) subsequently demonstrated that 3 g/d of sitosterol was sufficient to lower serum cholesterol levels. The differences in the various plant sterols became apparent when saturated derivatives of plant sterols, called plant stanols, were shown to reduce serum cholesterol at low doses. New techniques allowed the incorporation of plant stanols into food forms without affecting the texture and taste. In 1995, the Finnish introduced plant stanol esters in margarine, as dietary adjuncts to lower cholesterol (203). Plant sterols are C-28 or C-29 sterols, differing from cholesterol (C-27) by the presence of an extra methyl or ethyl group on the cholesterol side chain. Cholesterol is an essential component of cell membranes in higher species.

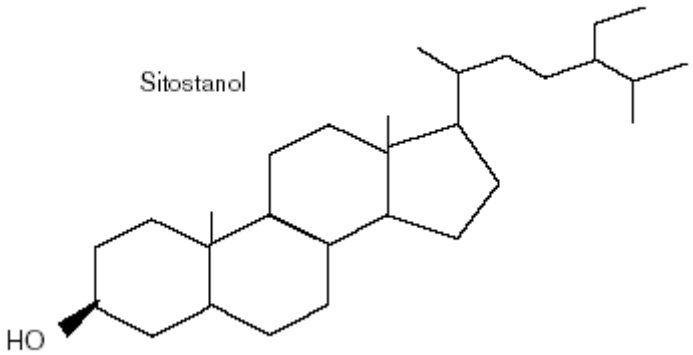


Figure 6: Chemical structure of Sitostanol

Plant sterols play an analogous role in plants; their content is highest in edible oils, seeds and nuts (204). The major dietary sterols are sitosterol (C-29), campesterol (C-28) and stigmasterol (C-29). These represent 50% of the total intake of sterols in the Western diet; the remainder is cholesterol (205). The most common dietary plant stanol, sitostanol, is a saturated derivative of sitosterol (Figure 6). It occurs naturally in wood pulp, tall oil and, in lesser amounts, in soybean oil. The Western daily diet contains 100–300 mg plant sterols and 20–50 mg plant stanols (206).

Absorption and metabolism

The addition of a methyl or ethyl group on the side chain of cholesterol results in poor intestinal absorption of plant sterols in humans (207). Thus, only 1.5–5% of sitosterol is absorbed when typical amounts of sterols are consumed (240–320 mg) (208). Cholesterol absorption is much more efficient, with between 20 and 80% of dietary cholesterol absorbed. Differential absorption rates among plant sterols are related to the length of the side chain. The longer the side chain of the sterol, the less is absorbed because of its increased hydrophobicity (141). Serum levels of sitosterol are 0.3–1.7 mg/dL (140, 209), given a dietary intake of 160–360 mg/d of plant sterols. This wide range in a normal population suggests considerable individual variability in the handling of various plant sterols. Consumption of 3.24 g/d of plant sterols has been shown to increase serum sitosterol and campesterol levels by an average of 40 and 70%, respectively (210). Because dietary plant sterols can initiate the development of atherosclerosis (211) and may increase the risk of premature coronary heart disease (CHD) in hypercholesterolemic patients (209), the lowest serum levels of sterols are desirable. Thus, Lees and Lees (212) suggested that plant sterol preparations that contain more absorbable sterols such as campesterol should not be recommended for therapeutic use.
Hydrogenation of plant sterols to the corresponding stanols renders them virtually unabsorbable (207). Absorption of sitostanol has been estimated to be between 0 and 3%, and serum levels are practically undetectable (210, 213). The absorption of the other major stanol, campestanol, is also very low, in contrast to its unsaturated counterpart, campesterol (214).

Mechanism of action

Plant sterols/stanols interfere with the uptake of both dietary and biliary cholesterol from the intestinal tract in humans (215). The reason for this is not fully understood; however, plant sterols/stanols appear to decrease the solubility of cholesterol in the oil and micellar phases, thus displacing cholesterol from bile salt micelles and interfering with its absorption (216). In humans, intestinal infusion of sitostanol was more efficient in reducing cholesterol absorption than infusion of sitosterol (85% and 50%, respectively) (215). In addition, Becker et al. (1993) showed that 1.5 g/d of sitostanol increased fecal secretion of neutral and acid steroids more effectively (88%) than did 6 g/d of sitosterol (45%) (217). It has been proposed that sitostanol, which is relatively unabsorbable compared with sitosterol, remains in the intestinal lumen where it can interfere continuously and more efficiently with micellar solubility of cholesterol (216). Another important determinant of the effectiveness of these compounds is how well they mix with intestinal contents for proper physical presentation to the gut. When compared with the unesterified stanols, the fatty acid esters of stanols seem to mix more easily with the oil phase of the intestinal contents to interfere with cholesterol absorption and decrease plasma cholesterol concentrations (218). In addition to reducing absorption of cholesterol, plant stanols inhibit absorption of other plant sterols (219).

Hypocholesterolemic effect of plant sterols and stanols

The lower absorbability of sitostanol is thought to be responsible for its greater hypocholesterolemic effect compared with sitosterol (Jones et al. 1997) In addition, Vanhanen et al. (220) showed that the ester form of sitostanol is more efficient than the crystalline form. The majority of the early studies on stanol ester–fortified foods were done in Finnish population studies. Plant stanol esters dosages have ranged in various studies from 0.8 to 3.8 g/d. The data suggest that at least 1 g/d of stanol esters must be consumed to offer a good clinical response. In general, with consumption of 2–3 g/d of plant stanol esters, serum LDL cholesterol (LDL-C) levels were lowered between 10 and 15%. It is difficult to compare these studies in terms of dose response because of differences in background diet, baseline lipid levels and duration of treatment. It has been suggested that consumption of 3 g/d of plant stanol esters may not further decrease the cholesterol-lowering effect (221).

The narrow range of dose responsiveness may be due to the compensatory increase in cholesterol synthesis that can be observed after consumption of higher doses of plant sterols and stanols. Vanhanen et al. (220) calculated that intake of 2 g/d of sitostanol esters increased cholesterol synthesis by 2 mg/d, although there was still a net reduction in serum cholesterol which was not seen with 0.8 g/d of sitostanol esters. Only a few studies have evaluated directly the dose-response relationship of plant stanol esters. Miettinen et al. (222) compared 1.8 and 2.6 g/d of plant stanol esters and showed a significantly greater cholesterol-lowering effect of the higher dose on total cholesterol (TC) (10.2% with 2.6 g/d vs. 9.3% with 1.8 g/d), although the difference was small. Nguyen et al. (223) showed a trend for greater efficacy of 3 g/d of plant stanol esters compared with 2.1 g/d of plant stanol esters. There was a reduction in TC of 9.4 and 5.6%, respectively, after 8 week, compared with the placebo week. Most studies comparing plant stanols with plant sterols have shown the greater potency of plant stanols in lowering serum cholesterol levels.

In contrast to lowering TC and LDL-C, plant stanol esters did not exert a significant effect on HDL-C and TG in most studies (16). In a study of hypercholesterolemic type-2 diabetic patients, 3 g/d of plant stanol esters decreased VLDL cholesterol (VLDL-C) by 12%, intermediate density lipoprotein cholesterol (IDL-C) by 11%, whereas HDL-C increased by 11% ($p < 0.05$) (109). The reason for this is not entirely understood; however, it is thought to result from an increased removal of remnant particles by up-regulation of LDL receptor activity.

1.2.3.5.2 Ezetimibe

Ezetimibe is a novel inhibitor of intestinal cholesterol absorption in humans (224) significantly lowers plasma cholesterol and LDL cholesterol (LDL-C) concentrations in patients with hypercholesterolemia (23, 225, 226). Ezetimibe selectively inhibits the intestinal absorption of cholesterol and related phytosterols. The chemical structure of Ezetimibe is 1-(4-fluorophenyl)-3(R)-[3-(4-fluorophenyl)-3(S)-hydroxypropyl]-4(S)-(4-hydroxyphenyl)-2-azetidinone (Figure 7). Clinical trials have demonstrated that ezetimibe lowers LDL cholesterol and triglycerides, and raises HDL cholesterol slightly in humans (225).

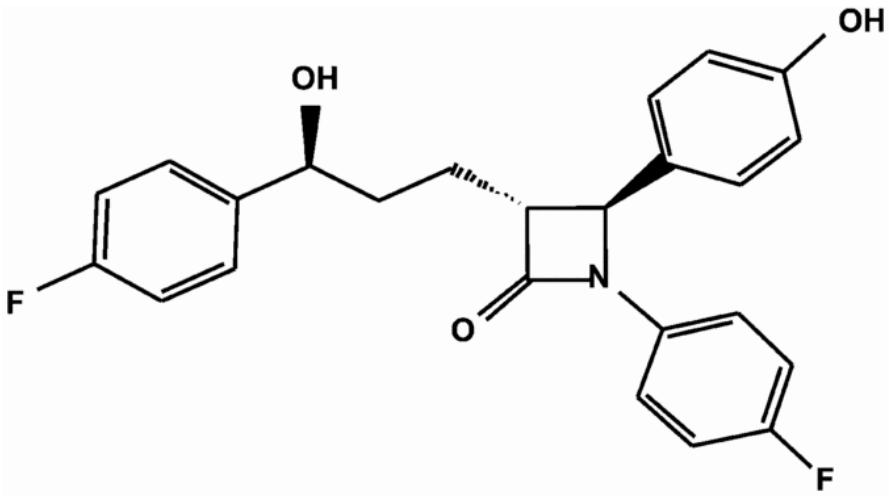


Figure 7: Chemical structure of Ezetimibe

Mode of Action

Ezetimibe reduces plasma cholesterol concentrations by inhibiting the absorption of cholesterol in the small intestine. Ezetimibe localizes and appears to act at the brush border of the small intestine, leading to a decrease in the delivery of intestinal cholesterol to the liver (227, 228). This causes a reduction of hepatic cholesterol stores and an increase in removal of cholesterol from the blood.

Efficacy of ezetimibe in preclinical models of hyperlipidemia

Monotherapy

Ezetimibe has demonstrated efficacy in a variety of preclinical models. Ezetimibe dose-dependently inhibited diet induced hypercholesterolemia in hamsters (227). Ezetimibe attenuated hypercholesterolemia by 60–94% at doses of 0·1–3 mg/kg in rats (229). Ezetimibe also dose dependently reduced intestinal cholesterol absorption in wild type, apolipoprotein E knockout (–/–) and scavenger receptor class B, type I (SR-BI)–/– mice (132, 230). Of all the pre-clinical species studied, ezetimibe has proven to be most potent in monkeys. A single dose of the ezetimibe analogue SCH 48461, when administered to tocynomolgus monkeys fed with a single cholesterol-containing meal caused a significant reduction of cholesterol in chylomicrons and chylomicron remnants during the postprandial phase without affecting triglyceride content (229, 231). In rhesus monkeys, LDL apo B-100 was reduced by nearly 50% after treatment with the ezetimibe analogue. Combined, these data indicate that these cholesterol absorption inhibitors reduce cholesterol content in chylomicrons, which indirectly leads to a decrease in LDL cholesterol and particle number (231).

Combination with statins

Ezetimibe blocks cholesterol absorption and increases neutral sterol excretion, chow-fed animals compensate for the loss of biliary cholesterol by increasing hepatic cholesterol synthesis. The effect of ezetimibe in combination with 3-hydroxy-3-methylglutaryl coenzyme A (HMG-CoA) reductase inhibitors (statins) was determined in chow-fed dogs (232). A synergistic reduction in plasma cholesterol was observed in chow-fed dogs given ezetimibe and the HMG-CoA reductase inhibitor lovastatin. Neither ezetimibe nor lovastatin alone affected plasma cholesterol levels. Their combination for 14 days caused a synergistic 50% reduction in plasma cholesterol levels. Ezetimibe also causes synergistic or additive reductions in plasma cholesterol levels in chow-fed dogs when combined with other HMG-CoA reductase inhibitors for 2 weeks (–41% with pravastatin; –60% with fluvastatin and –30% with simvastatin and atorvastatin) (232).The combination of the cholesterol absorption inhibitor ezetimibe with an HMG-CoA reductase inhibitor may be very effective clinically at reducing plasma cholesterol levels, even with reduced dietary intake of cholesterol.

Effect of ezetimibe on atherosclerosis

The effect of ezetimibe on plasma cholesterol levels and atherogenesis was determined in apo –/– mice, an atherosclerosis model with chylomicron remnant hypercholesterolemia (230). Ezetimibe inhibited cholesterol absorption, reduced plasma cholesterol levels, increased HDL cholesterol levels and inhibited the progression of atherosclerosis under western, low-fat, and cholesterol-free dietary conditions in apo –/– mice. Although apo –/– mice have more severe hypercholesterolemia and more pronounced LDL cholesterol reductions with ezetimibe than humans, these animal data suggest that ezetimibe may inhibit atherogenesis in individuals consuming restricted-fat or western diets.

Pharmacokinetics

After oral administration, ezetimibe is absorbed and extensively conjugated to a pharmacologically active phenolic glucuronide (ezetimibe-glucuronide) (233). After a single 10 mg dose of ezetimibe to fasted adults, mean ezetimibe peak plasma concentrations (C_{max}) of 3.4 to 5.5 ng/mL are attained within 4 to 12 hours (T_{max}). Ezetimibe-glucuronide mean C_{max} values of 45 to 71 ng/mL were achieved between 1 and 2 hours (T_{max}) (233). There is no substantial deviation from dose proportionality between 5 and 20 mg. The absolute bioavailability of ezetimibe cannot be determined, as the compound is virtually insoluble in aqueous media suitable for injection. Ezetimibe has variable bioavailability; the coefficient of variation, based on inter-subject variability, is 35 to 60% for AUC values. Ezetimibe and ezetimibe-glucuronide are highly bound (>90%) to human plasma proteins (234). A number of studies were conducted in animal models to understand the disposition and metabolism of ezetimibe (235). Ezetimibe is rapidly metabolized in the intestine to its phenolic glucuronide; once glucuronidated, it is excreted in the bile, thereby delivering the drug back to the site of action. Cholesterol absorption studies indicated that the glucuronide appeared more potent than ezetimibe itself, and this is likely because glucuronidated ezetimibe localizes more avidly to the intestine. In humans, ezetimibe is rapidly absorbed and primarily metabolized in the small intestine and liver to its glucuronide, with little oxidative cytochrome P450 mediated metabolism (236). Ezetimibe and its glucuronide undergo enterohepatic recycling and have a half-life of approximately 24 hours in humans. Ezetimibe and/or the glucuronide metabolite are excreted in the feces (90%) and urine (10%). Since ezetimibe does not influence the activities of cytochrome P450 enzymes, there are no significant pharmacokinetic interactions with many medications.

Pharmacokinetic interaction studies of ezetimibe in humans have found no significant changes in the plasma levels of other medications including statins (atorvastatin, simvastatin, pravastatin, lovastatin, and fluvastatin), fibrates (gemfibrozil and fenofibrate), digoxin, glipizide, warfarin and oral contraceptives (ethinyl estradiol and levonorgestrel) (237-245).

Effect of ezetimibe on cholesterol absorption in humans

Ezetimibe has been shown to produce a marked inhibition of intestinal cholesterol absorption (up to 96%) in animals (246). In patients with mild to moderate hypercholesterolemia, Ezetimibe reduces plasma concentrations of sitosterol and campesterol (224) mostly by reducing the absorption of the plant sterols. Several clinical trials in humans have revealed LDL cholesterol–lowering effects in the range of 17% to 20% at a dose of 10 mg per day (225, 228, 246, 247). Sudhop et al. showed that ezetimibe inhibited cholesterol absorption on the average by 54% relative to placebo which was associated with a compensatory increase in cholesterol synthesis and led to a 22.3% reduction in plasma LDL cholesterol concentrations (224). Marked reductions in plasma concentrations of the noncholesterol plant sterols sitosterol and campesterol also were observed, suggesting that ezetimibe inhibited the absorption of these compounds as well. When compared to animal studies, ezetimibe had a less extensive effect of on inhibition of cholesterol absorption in humans. Studies in cholesterol fed hamsters and rodents showed an inhibition of cholesterol absorption by 92% to 96% in a dose range of 1 to 10 mg/kg (231, 248). In comparison to other compounds, intestinal cholesterol absorption by ezetimibe was more pronounced than that observed for other known inhibitors of cholesterol absorption including neomycin and plant sterol and stanol esters in humans. Neomycin has been shown to reduce intestinal cholesterol absorption ina dose-dependent manner by 26% to 49% (222, 249-252). Treatment with high-dose plant sterols and stanols has been shown to lower cholesterol absorption by up to 45%, but the maximal effects are observed under circumstances in which the active agents are delivered in fat carriers in conjunction with cholesterol meals (141, 212, 253).

2. Methods

2.1 Materials

Chemicals

Cyclohexane	Merck, Darmstadt
Pyridine	Merck, Darmstadt
1, 1, 1, 3, 3, 3-Hexamethyldisilazan	Merck, Darmstadt
Chlortrimethylsilane	Merck, Darmstadt
Di-ethylether	Merck, Darmstadt
Methanol	Merck, Darmstadt
2,2-Dimethoxypropane	Merck, Darmstadt
Dichlormethane	Merck, Darmstadt
Acetone	Merck, Darmstadt
Sodium hydroxide	Merck, Darmstadt
Ethanol	Merck, Darmstadt
5-alpha-Cholestane	Serva Elektrophoresis GmbH, Heidelberg
Dichlormethane	Merck, Darmstadt
PicoGreen	Molecular Probes, Leiden, The Netherlands

Reagents for Enzymatic methods to measure total - and HDL cholesterol and triglycerides

Calibration serum	Roche Diagnostics GmbH, Mannheim
Quality control serum (Precinorm® L)	Roche Diagnostics GmbH, Mannheim

R1 (Total cholesterol) from Roche Diagnostics GmbH, Mannheim:

Piperazine-1,4-bis(2-ethanesulfonic acid) buffer: 75 mmol/L, pH 6.8; Mg^{2+}: 10 mmol/L; sodium cholate: 0.2 mmol/L; 4-aminophenazone: 0.15 mmol/L; phenol: 4.2 mmol/L; fatty alcohol polyglycol ether: 1%; cholesterol esterase (Pseudomonas spec.): 0.5 U/mL (8.33 µkat/L); cholesterol oxidase (E. coli) : 0.15 U/mL (2.5 µkat/L); peroxidase (horseradish) : 0.25 U/mL (4.17 µkat/L).

R1 (HDL cholesterol) from Roche Diagnostics GmbH, Mannheim:
3-morpholinopropanesulfonic acid buffer: 19.1 mmol/L, pH 7.0; dextran sulfate: 0.5 g/L; magnesium sulfate heptahydrate: 8.11 mmol/L; ascorbate oxidase (Eupenicillium sp., recombinant): 50 µkat/L; peroxidase (horseradish):167 µkat/L.

R2 (HDL cholesterol) from Roche Diagnostics GmbH, Mannheim:
Piperazine-1,4-bis(2-ethanesulfonic acid) buffer: 9.9 mmol/L, pH 7.0; PEG-cholesterol esterase (Pseudomonas spec.) : 3.33 µkat/L; PEG-cholesterol oxidase (Streptomyces sp., recombinant) : 127 µkat/L; peroxidase (horseradish) : 333 µkat/L; 4-amino-antipyrine: 2.46 mmol/L.

R1 (Triglycerides) from Roche Diagnostics GmbH, Mannheim:
Piperazine-1,4-bis(2-ethanesulfonic acid) buffer: 50 mmol/L, pH 6.8; Mg^{2+}: 40 mmol/L; sodium cholate: 0.20 mmol/L; ATP: 1.4 mmol/L; 4-aminophenazone : 0.13 mmol/L; 4-chlorophenol: 4.7 mmol/L; potassium hexacyanoferrate(II): 1 µmol/L; fatty alcohol polyglycol ether: 0.65%; lipoprotein lipase (Pseudomonas spec.) : 5.0 U/mL; glycerokinase (Bacillus stearothermophilus) : 0.19 U/mL; Glycerol phosphate oxidase (E. coli) : 2.5 U/mL; peroxidase (horseradish) : 0.10 U/mL.

Equipment

GC:

Mobile phase:	Hydrogen; 1.1 ml/min
Sample injection:	Splitless injection by an HP 7683 Auto sampler and injector (Hewlett Packard, Waldbronn)
Injection volume:	2µl
Injection temperature:	280°C

Stationary phase:

Column:	DB-XLB; J&W Scientific (Folsom, California, USA)
Length:	30 m
Diameter:	0.25 mm
Film thickness:	0.25 µm

Detection:	6890 Hewlett Packard
Data recording:	Kayak XA Pentium II with the HP GC, ChemStation Software version 2.1.1.0.
Photometer:	Eppendorf Analyzer EPOS 5060 (Eppendorf, Hamburg)

2.2 GC analysis of cholesterol and noncholesterol sterols

2.2.1 Sample collection

Immediately after blood sampling, the collection tube was placed on ice. Then serum was obtained by centrifugation. Butylated hydroxytoluene (BHT) was added to each serum sample (10 µL to 1000 µL serum) and serum was stored at –20°C.

2.2.2 Sample preparation

To 100 µL of serum (at room temperature), 1 µg of epicoprostanol (10 µL from a stock-solution 100 µg/mL in cyclohexane) and 50 µg 5α-cholestane (50 µL from a stock solution mg/mL in cyclohexane), were added as internal and external standards, respectively. Alkaline hydrolysis was performed at 68°C for 1h after addition of 1.0 mL 1M ethanolic (90%) sodium hydroxide solution. After cooling to room temperature and addition of 500 µL of distilled water, the unsaponified lipids were extracted twice into 3 ml of cyclohexane. The combined organic phases were dried under nitrogen at 65°C. The residue was dissolved in 500 µL n-decane and transferred into micro-vials for GC-analysis. The hydroxy-groups of the sterols and stanols are derivatisized to trimethylsilyl (TMSi)-ethers by adding 1.0 mL TMSi-reagent (pyridine-hexamethyldisilazan-trimethylchlorosilane 9:3:1, by volume) to the residue and incubation for 1h at 65°C. All the GC vials are exactly inscribed with study-code, sample-code, and date of work-up.

2.2.3 Chromatographic conditions

The concentration of cholesterol in serum or plasma is about 102-105 fold higher than those of its precursors and metabolites. Therefore, analysis of cholesterol, cholesterol precursors, campesterol and sitosterol is usually performed by GC from the same sample.

The sterols and stanols are separated on a dimethylsilicone capillary column (J&W, Folsom, USA) (30m x 0.25 mm i.d. x0.25 µm film thickness) in an Hewlett Packard (HP) gas chromatograph 5890 after splitless injection by an HP 7683 injector at 280°C**.** Hydrogen is used as carrier gas with an inlet pressure of 9.97 psi, resulting in a total gas-flow of 1.1 mL/min. The oven temperature is kept at 150°C for 3 min. and raises at a rate of 30°C/min to a final temperature of 290°C, keeping for 22.33 min. Cholesterol-TMSi ether is detected with a retention time of 15.88 min by a flame-ionization detector at 280°C with a combined constant column and make-up flow of (hydrogen+nitrogen; 30 mL/min). The whole gas chromatograph is controlled by a computer (Kayak XA) Pentium II with the HP GC ChemStation Software version 2.1.1.0. The samples are injected by an automatic sampler type 7683 Auto sampler and injector (HP). The exact retention times for each run vary with the absolute amounts of the sterols. Figure 8 shows a typical gas chromatogram from a serum sample of a volunteer.

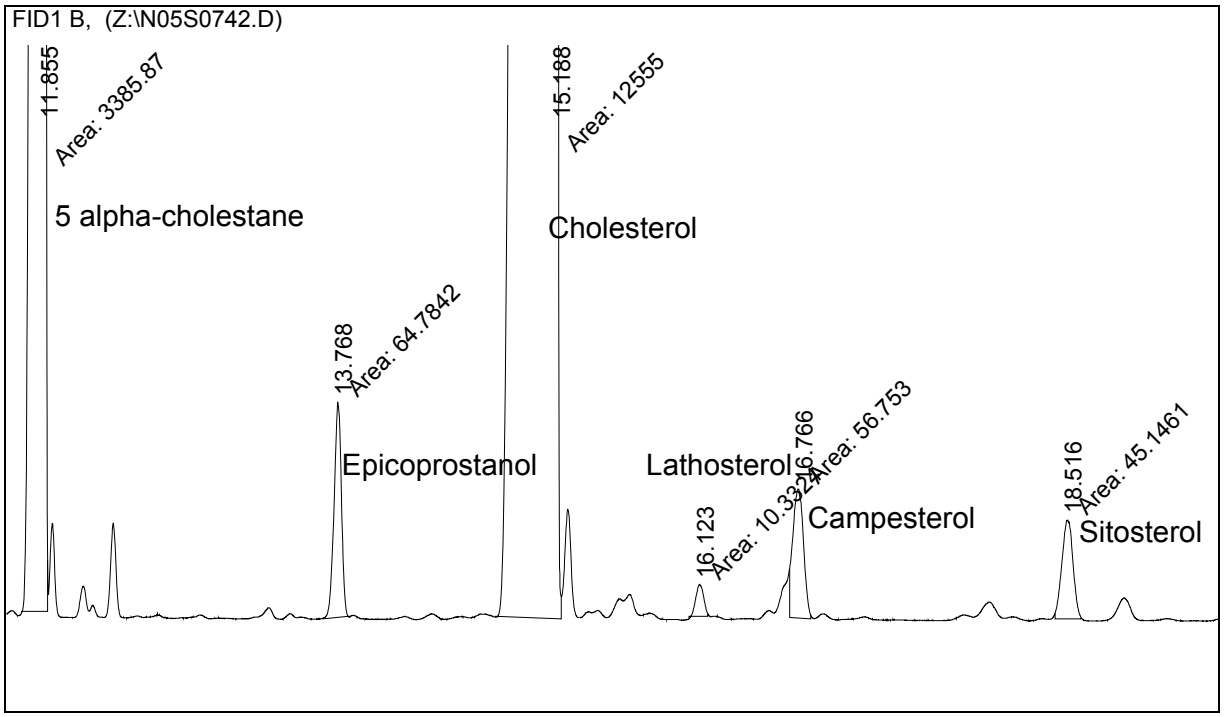


Figure 8: Typical gas chromatogram from a serum sample of a volunteer

The total chromatogram is automatically integrated by the computer's software. The necessary information is taken from a Calibration table, which includes the exact retention times as gained from a serum standard chromatogram.

The calibration table includes information on the sterol retention times, internal standard qualification and amount. The information about the usual integration parameters is saved in an integration event table. After automatically basic integration the integration of each peak is inspected again. If the peak is not integrated correctly, the integration is performed manually. The final integration results including the raw data and a chromatogram of the serum sample is printed out and saved in a file. The raw data and the concentrations for the different sterols are converted from the HP data-file to an EXCEL-data sheet. As 50 µL serum was used, the concentration in µg/sample (50 µL) has to be multiplied by a factor 2 to get the final concentration in µg/100 µL or mg/dL.

2.2.4 Validation including optimization of the derivatization reaction

The analysis of cholesterol and non-cholesterol sterols was performed using a validated GC method which was developed and validated at the Department of Clinical Pharmacology, University of Bonn (VALGCSTER 1.1-Validation of analytical procedures: methodology; Validated by Dr. D. Lütjohann) and its associated SOP (Standard Operating Procedure Serum Sterols GC SOP-KP-3.5, dated 12.08.2002; author: Dr. D. Lütjohann, approved by Prof. Dr. Klaus von Bergmann). There were no changes in the location of equipment, instruments and software platforms, analytical methodology, sample processing procedures, relevant concentration range after the validation or during the course of analysis. This validated method was used to measure the plasma cholesterol and non-cholesterol sterols concentrations within Responder Studies 1 and 2.

2.2.5 Quality assurance during routine analysis

For the routine use of the validated analytical method, its precision should be monitored regularly to ensure that the method continues to perform satisfactorily. To achieve this objective, quality samples were prepared separately and analyzed with processed patient samples at intervals based on the total number of samples. Quality assurance during routine analysis was done by analysis of pooled serum containing known concentrations of cholesterol, lathosterol, campesterol and sitosterol. Thus a pooled serum was analyzed for each visit before the run of patient samples in both studies (Tables 1 and 2).

The precision of an analytical method describes the closeness of individual measures of an analyte when the procedure is applied repeatedly to multiple analysis. Between-day precision or repeatability assesses precision during a single analytical run. The coefficient of variation determined should not exceed 15 % (254).

Table 1: Concentration of cholesterol, lathosterol, campesterol and sitosterol in pooled serum during each run (between-day precision) from Responder study 1.

Run	**Cholesterol concentration** (mg/dL)	**Lathosterol concentration** (mg/dL)	**Campesterol concentration** (mg/dL)	**Sitosterol concentration** (mg/dL)
1	195	0.278	0.443	0.349
2	197	0.263	0.406	0.330
3	199	0.274	0.441	0.344
4	200	0.279	0.459	0.341
5	202	0.291	0.430	0.319
6	204	0.281	0.449	0.344
7	216	0.286	0.452	0.350
8	212	0.261	0.456	0.341
9	205	0.280	0.439	0.345
Mean	204	0.277	0.445	0.340
SD	8.18	0.010	0.018	0.010
CV	4.01 %	3.56 %	4.04 %	2.93 %

SD- Standard deviation; CV- Coefficient of variation

The coefficient of variation of the analysis of quality samples during between-day run was below 5 % (Tables 1 and 2) in both the studies confirming the precision of the method used.

Table 2: Concentration of cholesterol, lathosterol, campesterol and sitosterol in pooled serum during each run (between-day precision) from Responder study 2.

Run	**Cholesterol concentration** (mg/dL)	**Lathosterol concentration** (mg/dL)	**Campesterol concentration** (mg/dL)	**Sitosterol concentration** (mg/dL)
1	197	0.267	0.447	0.337
2	203	0.271	0.440	0.329
3	214	0.261	0.436	0.326
4	211	0.248	0.443	0.328
5	207	0.268	0.427	0.333
6	199	0.250	0.394	0.317
7	201	0.262	0.429	0.332
8	204	0.279	0.417	0.317
9	206	0.269	0.437	0.332
Mean	206	0.263	0.432	0.328
SD	7.48	0.009	0.018	0.007
CV	3.63 %	3.55 %	4.12 %	2.10 %

SD- Standard deviation; CV- Coefficient of variation

2.3 Enzymatic analysis for the measurement of serum lipids

Total cholesterol, HDL cholesterol and triglyceride concentrations were measured by enzymatic methods.

2.3.1 Measurement of total cholesterol concentrations

This method is based on the determination of Δ^4-cholestenone after enzymatic cleavage of the cholesterol ester by cholesterol esterase, conversion of cholesterol by cholesterol oxidase, and subsequent measurement by the Trinder reaction of the hydrogen peroxide formed (255-261). Total cholesterol is determined enzymatically (262), which results in following reaction:

$$\text{Cholesterol esters} + H_2O \xrightarrow{\text{Cholesterol esterase}} \text{cholesterol} + \text{RCOOH}$$

Cholesterol esters are cleaved by the action of cholesterol esterase to yield free cholesterol and fatty acids.

$$\text{Cholesterol} + O_2 \xrightarrow{\text{Cholesterol oxidase}} \text{cholest-4-en-3-one} + H_2O_2$$

Cholesterol is converted by oxygen with the aid of cholesterol oxidase to cholest-4-en-3-one and hydrogen peroxide.

$$2\ H_2O_2 + \text{4-aminophenazone} + \text{phenol} \xrightarrow{\text{Peroxidase}} \text{4-(p-benzoquinone-monoimino)-phenazone} + 4\ H_2O$$

The hydrogen peroxide created forms a red dyestuff by reacting with 4-aminophenazone and phenol under the catalytic action of peroxidase. The color intensity is directly proportional to the concentration of cholesterol and is determined photometrically.

To 100 µL serum sample reagent R1 (Total cholesterol) was added. Along with the serum sample calibration serum (Total cholesterol) and Quality control serum (Precinorm® L) were also analysed. The Eppendorf analyzer EPOS 5060 automatically calculates the analyte concentration of each sample using following formula

$$C = A \times F$$

A = Absorbance of sample

F = Nominal concentration of the standard / Measured absorbance of the sample

The same method was also used for measurement of HDL cholesterol and triglyceride concentrations.

2.3.2 Measurement of HDL cholesterol concentration

The method for direct determination of HDL cholesterol in serum and plasma uses polyethylene glycol (PEG)-modified enzymes and dextran sulfate (256). When cholesterol esterase and cholesterol oxidase enzymes are modified by PEG, they show selective catalytic activities toward lipoprotein fractions (263). In the presence of magnesium sulfate, dextran sulfate selectively forms water-soluble complexes with LDL, VLDL and chylomicrons which are resistant to PEG-modified enzymes. The cholesterol concentration of HDL-cholesterol is determined enzymatically by cholesterol esterase and cholesterol oxidase coupled with PEG to the amino groups (264). The presence of reagents R1 and R2 (HDL cholesterol) results in the following reaction:

$$\text{HDL-cholesterol esters} + H_2O \xrightarrow{\text{PEG-cholesterol esterase}} \text{HDL-cholesterol} + \text{RCOOH}$$

Cholesterol esters are broken down quantitatively into free cholesterol and fatty acids by cholesterol esterase.

$$\text{HDL-cholesterol} + O_2 \xrightarrow{\text{PEG-cholesterol oxidase}} \Delta^4\text{-cholestenone} + H_2O_2$$

In the presence of oxygen, cholesterol is oxidized by cholesterol oxidase to Δ^4-cholestenone and hydrogen peroxide.

$$2\ H_2O_2 + \text{4-amino-antipyrine} + \text{HSDA} + H + H_2O \xrightarrow{\text{Peroxidase}} \text{purple-blue pigment} + 5\ H_2O$$

In the presence of peroxidase, the hydrogen peroxide generated reacts with 4-amino-antipyrine and HSDA (Sodium N-(2-hydroxy-3-sulfopropyl)-3,5-dimethoxyaniline) to form a purple-blue dye. The color intensity of this dye is directly proportional to the HDL cholesterol concentration and is measured photometrically.

2.3.3 Measurement of triglyceride concentrations

The method to measure triglycerides is based on complete hydrolysis of triglycerides to glycerol by lipoprotein lipase followed by oxidation to dihydroxyacetone phosphate and hydrogen peroxide (265). The hydrogen peroxide produced then reacts with 4-aminophenazone and 4-chlorophenol under the catalytic action of peroxidase to form a red dyestuff (266, 267). The addition of reagent R1 (Triglycerides) to the serum sample results in following reaction:

$$\text{Triglycerides} + 3\ H_2O \xrightarrow{\text{LPL}} \text{glycerol} + 3\ \text{RCOOH}$$

$$\text{Glycerol} + \text{ATP} \xrightarrow[Mg^{2+}]{\text{GK}} \text{glycerol-3-phosphate} + \text{ADP}$$

$$\text{Glycerol-3-phosphate} + O_2 \xrightarrow{\text{GPO}} \text{dihydroxyacetone phosphate} + H_2O_2$$

$$H_2O_2 + \text{4-aminophenazone} + \text{4-chlorophenol} \xrightarrow{\text{Peroxidase}} \text{4-(p-benzoquinone-monoimino)-phenazone} + 2\ H_2O + HCl$$

2.3.4 Calculation of LDL cholesterol concentration

LDL cholesterol was calculated by the method of Friedewald et al. (268). It is calculated by subtracting the cholesterol content of the infranatant fraction (the sum of the HDL and Triglyceride) from the total plasma cholesterol. The method requires measurement of the concentrations of plasma total cholesterol, triglycerides, and HDL cholesterol which was done by enzymatic method.

It is based on the observation that the ratio of the mass of triglyceride to that of cholesterol in VLDL is apparently relatively constant and about 5:1 in normal subjects and when chylomicrons are not detectable, most of the triglyceride in plasma is contained in the VLDL (256, 269). Thus, in the vast majority of plasma samples in which chylomicrons are not present, the cholesterol in plasma attributable to VLDL can be approximated by dividing the plasma triglyceride concentration by five (268).

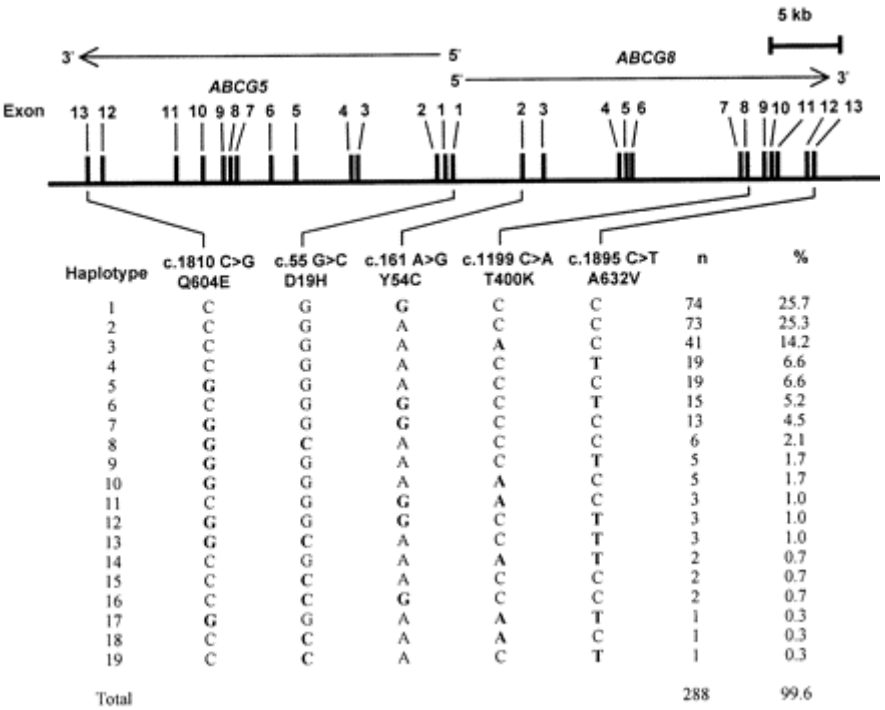
LDL cholesterol = Total cholesterol — [HDL cholesterol + (Triglyceride/ 5)]

2.4 Genotyping of DNA sequence variants in ABCG5 and ABCG8

DNA sequence variants in *ABCG5* and *ABCG8* from all the subjects were genotyped at the laboratory of Dr. Frank Lammert, Department of Internal Medicine I, University of Bonn.

Genomic DNA was isolated from EDTA-anticoagulated whole blood using the QIAamp® protocol (Qiagen, Hilden). DNA concentrations were determined fluorometrically (Biorad), employing the dye PicoGreen (Molecular Probes, Leiden, The Netherlands).

There are five nonsynonymous polymorphisms at the ABCG5/8 loci (Q604E, D19H, Y54C, T400K, and A632V) which were previously identified (126). Q604E indicates Codon 604 glutamic acid subtituted for glutamine, D19H (Codon 19 histidine for aspartic acid), Y54C (Codon 54 cysteine for tyrosine), T400K (Codon 400 lysine for threonine) and A632V (Codon 632 valine for alanine) (Figure 9).



Haplotype	c.1810 C>G Q604E	c.55 G>C D19H	c.161 A>G Y54C	c.1199 C>A T400K	c.1895 C>T A632V	n	%
1	C	G	G	C	C	74	25.7
2	C	G	A	C	C	73	25.3
3	C	G	A	A	C	41	14.2
4	C	G	A	C	T	19	6.6
5	G	G	A	C	C	19	6.6
6	C	G	G	C	T	15	5.2
7	G	G	G	C	C	13	4.5
8	G	C	A	C	C	6	2.1
9	G	G	A	C	T	5	1.7
10	G	G	A	A	C	5	1.7
11	C	G	G	A	C	3	1.0
12	G	G	G	C	T	3	1.0
13	G	C	A	C	T	3	1.0
14	C	G	A	A	T	2	0.7
15	C	C	A	C	C	2	0.7
16	C	C	G	C	C	2	0.7
17	G	G	A	A	T	1	0.3
18	C	C	A	A	C	1	0.3
19	C	C	A	C	T	1	0.3
Total						288	99.6

Figure 9**:** Location of nonsynonymous polymorphisms at the ABCG5/8 loci (126).

These five sequence variants were assayed by PCR amplification and restriction digestion using a method previously described by Hubacek et al. and Lu et al. (123, 270). The patients were genotyped for the *ABCG5* single nucleotide polymorphism (SNP) c.1810C>G (Q604E) and the *ABCG8* SNPs c.55G>C (D19H), c.161A>G (Y54C), c.1199C>A (T400K), and c.1895C>T (A632V) using fluorogenic 5' nuclease (*TaqMan*) assays. Primers and *TaqMan* MGB probes were designed using Primer Express 2.0 software (Applera, Norwalk, CT).

PCR reactions were performed in 25 µl volumes containing 20 ng of DNA, 1x *TaqMan* Universal PCR Master Mix, 900 nM of each primer, and 200 nM of each fluorescent-labelled MGB probe. Reaction mixtures were incubated at 95°C for 10 min and subjected to 40 amplification cycles (92°C, 15 sec; 60°C, 1 min). Fluorescence detection of the amplicons was performed on the ABI PRISM 7000 Sequence Detection System (Applera). The distribution of alleles and genotypes was compared in contingency tables by Armitage's trend test (http://ihg.gsf.de/ihg/snps/).

2.5 Statistical methods

Statistical analysis was performed with the SPSS 12.0 statistical programme (SPSS Inc, Chicago, USA). The data were described by the arithmetic mean and the relative standard deviation (mean ± standard deviation). Probability values were used to describe the changes in lipoprotein profiles, cholesterol and non-cholesterol sterols before and after the treatment periods. They were calculated using Student's paired t-test and p values less than 0.05 were considered to be significant. Probability values were also used to select and categorize the subjects from screening into groups for study period. In this case probability values were used to check that two groups formed did not differ significantly in age, body mass index, lipoprotein profile and noncholesterol sterols.

Pearson's test was used to calculate the correlation coefficients to define the relation between the percentage change of total or LDL cholesterol and the ratio of lathosterol to campesterol. It was also used to define relationship between ratio of lathosterol to campesterol and campesterol to cholesterol during a period of six months from screening to the first week of study.

3 Clinical Design and Study

3.1 Screening and Recruitment

137 male subjects were screened for the following parameters to participate in Responder Study 1 (treatment with sitostanol ester margarine).

Lipoprotein profile:	Total cholesterol, VLDL-, LDL-, HDL-Cholesterol, Triglyceride
Cholesterol precursor:	Lathosterol
Plant Sterols:	Campesterol, Sitosterol

Inclusion criteria:

- Male subjects at the age between 18 and 60 years
- Written informed consent

Exclusion criteria:

- Acute or chronic diseases
- Intake of drugs and any specific diet
- Hypersensitivity to sitostanol ester margarine
- Myopathy with elevated serum concentration of creatinphosphokinase > three times above the upper limit of normal
- Liver disease with elevated serum concentration of transaminases > three times above the upper limit of normal
- Disease of biliary tract
- Chronic intestinal disease
- Condition after cholecystectomy
- Condition after surgery of gastrointestinal tract (excepting appendectomy)
- Cancer

3.2 Study design of Responder Study 1 (treatment with sitostanol ester margarine)

Eight subjects that represented the lowest ratio of lathosterol to campesterol in serum and 8 subjects representing the highest ratio were selected to participate in the study. All subjects gave their written informed consent and the local ethical committee approved the study protocol. Subjects were advised to adhere to their regular diet during the study, but they were prohibited from taking any drugs or other food supplements.

Blood samples were drawn in the morning after an overnight fast, three times at the beginning of the study (week 0), then once at weekly intervals and three times during the last week (week 4) of the study for measurement of serum lipids, serum concentrations of lathosterol, campesterol and sitosterol (Table 3). Serum concentrations of campestanol and sitostanol were measured before and at the end of the study. From week 1 to week 4 subjects received 1 g sitostanol oleat supplemented margarine (Raisio Benecol Ltd., Finland) b.i.d.

Table 3: Study design of Responder Study 1 (treatment with sitostanol ester margarine)

Week	**0**			**1**	**2**	**3**	**4**		
Visit	1	2	3	4	5	6	7	8	9
Sitostanol supplemented margarine consumption				x	x	x	x	x	x
Blood collection	x	x	x	x	x	x	x	x	x

3.3 Study design of Responder Study 2 (treatment with ezetimibe)

121 male normolipemic subjects (age range 18 to 60 years) were screened concerning their serum concentrations of cholesterol, lathosterol, and campesterol to participate in Responder Study 2 (treatment with Ezetimibe). Exclusion criteria were acute or chronic diseases, the intake of drugs, and any specific diets.

Eight subjects that represented the lowest ratio of lathosterol to campesterol in serum and 8 subjects representing the highest ratio were selected to participate in the study. All subjects gave their written informed consent and the local ethical committee approved the study protocol. Subjects were advised to adhere to their regular diet during the study, but they were prohibited from taking any drugs or food supplements. Blood samples were drawn in the morning after an overnight fast at the beginning of the study, and at weekly intervals for measurement of serum lipids. Serum concentrations of lathosterol, campesterol, sitosterol, campestanol, and sitostanol were measured before and at the end of the study. From week 1 to week 4 subjects received ezetimibe (Ezetrol®) 10 mg / day (Table 4).

Table 4: Study design of Responder Study 2 (treatment with Ezetimibe)

Week	**0**			**1**	**2**	**3**	**4**		
Visit	1	2	3	4	5	6	7	8	9
Ezetimibe (Ezetrol®) administered				x	x	x	x	x	x
Blood collection	x	x	x	x	x	x	x	x	x

3.4 Food Records

Subjects were advised to adhere to their regular diet during the study, but they were prohibited from taking any drugs or other food supplements. Food intake was assessed at the beginning the study, using 7-day food records that were evaluated by computerized nutrient analysis.

4. Results

4.1 Responder Study 1 (treatment with sitostanol ester margarine)

4.1.1 Clinical characteristics and compliance

From the 137 subjects screened, 16 were selected to participate in the study according to their ratio of lathosterol to campesterol from fasting serum samples: one group with the lowest ratio (n=8; range 0.19 - 0.71) and the other group with the highest ratio (n=8; range 0.72 - 1.49). The two groups did not differ in age (42 ± 11 vs. 39 ± 11 years) and body mass index (26 ± 1 vs. 25 ± 5 kg/m²) (Table 5).

Table 5: Characteristics of subjects grouped according to the ratio serum lathosterol to campesterol (Lath/Camp).

Parameter	LOW Lath/Camp	HIGH Lath/Camp	p
Lathosterol / Campesterol	0.47 ± 0.22	1.42 ± 0.47	
Range	0.19 - 0.71	0.72 - 1.49	
Age (years)	42 ± 11	39 ± 11	0.241
BMI (kg/m^2)	26 ± 1	25 ± 5	0.686
Total cholesterol (mg/dL)	232 ± 22	238 ± 32	0.663
LDL cholesterol (mg/dL)	160 ± 15	155 ± 47	0.535
HDL cholesterol (mg/dL)	49 ± 13	47 ± 10	0.815
Triglycerides (mg/dL)	155 ± 47	179 ± 60	0.760

4.1.2 Baseline values

The purpose of the study was to investigate the responsiveness to sitostanol ester margarine on serum lipoprotein in two selective subgroups with a high and a low ratio of serum lathosterol to campesterol. Hence, baseline values of serum lipoprotein lipids were measured.

The serum concentration of total cholesterol in the group with the low ratio was 251 ± 27 mg/dL vs 240 ± 34 mg/dL of high ratio group. Serum concentration of LDL cholesterol in the group with the low ratio was 175 ± 24 mg/dL vs 166 ± 31 mg/dL of high ratio group whereas serum concentration of HDL cholesterol in the group with the low ratio was 46 ± 19 mg/dL vs 48 ± 8 mg/dL of high ratio group. The triglycerides in the low ratio group were 134 ± 71 mg/dL vs 145 ± 49 mg/dL (Tables 6 and 7). The two groups did not differ in lipoprotein profiles.

The margarine was well tolerated by all subjects without any adverse event. One subject in the group with the high ratio of lathosterol to cholesterol discontinued the study for personal reasons. There was a good compliance of the sitostanol supplemented margarine intake in all the subjects as indicated by weekly returning of the spread containers and a significant increase of serum campestanol and sitostanol from week 0 to the end of the study (week 4). The serum campestanol concentration increased from 8.1 ± 1.8 to 64.9 ± 37.9 μg/dL in the group with the low ratio and from 6.0 ± 1.8 to 69.8 ± 30.9 μg/dL in the group with the high ratio. Serum sitostanol increased from 6.7 ± 0.8 to 20.0 ± 8.7 μg/dL in the low and from 6.1 ± 0.7 to 20.8 ± 5.9 μg/dL in the high ratio group.

4.1.3 Effect of sitostanol ester margarine on serum lipoproteins

In the group with the low ratio, total cholesterol decreased already significantly after the first week of treatment (Figure 10). After four weeks of treatment serum total cholesterol decreased significantly on the average by -14.2 % ($p<0.01$). In the group with high ratio of lathosterol to campesterol, total cholesterol did not change significantly after four weeks of treatment (Figure 10). After four weeks of treatment serum total cholesterol increased on the average by 2.2 %. There was a highly significant correlation between the percent changes in serum total cholesterol with the ratio of serum lathosterol to campesterol ($r = 0.819$, $p< 0.0002$) in all subjects (Figure 11).

After four weeks of treatment serum LDL cholesterol also decreased by -13.8 % ($p<0.01$) on the average in the group with low lathosterol to campesterol ratio (Figure 12). The average serum LDL cholesterol decreased from 175 ± 24 mg/dL to 157 ± 24 mg/dL during week1, and further decreased to 153 ± 37 mg/dL, and 156 ± 32 mg/dL during week 2 and 3.

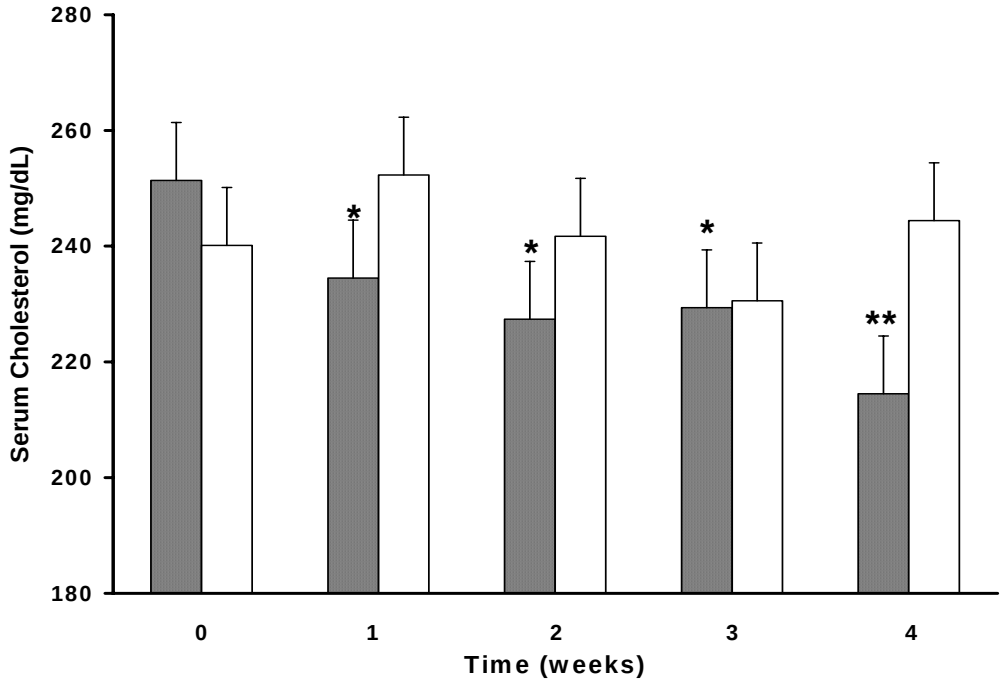


Figure 10: Serum concentrations of total cholesterol during four weeks of treatment with sitostanol ester margarine in subjects grouped according to the ratio lathosterol to campesterol (change from week 0: * $p< 0.02$, ** $p< 0.001$; ■ low and □ high ratio of lathosterol to campesterol)

New steady state concentrations were reached between week 2 and 3. After four weeks of treatment serum LDL cholesterol increased by + 4.3 % on the average in the group with high lathosterol to campesterol ratio (Figure 12). The average serum LDL cholesterol increased from 165 ± 31 mg/dL to 171 ± 24 mg/dL after 4 weeks of sitostanol supplemented margarine consumption. There was a highly significant correlation between the percent changes in serum LDL cholesterol with the ratio of serum lathosterol to campesterol ($r = 0.799$, $p< 0.0001$) in all subjects (Figure 13). Serum HDL cholesterol and triglycerides were not affected significantly in both groups (Table 6 & 7).

Table 6: Serum concentration of lipoproteins before (week 0) and after treatment (week 4) with sitostanol ester margarine in low ratio serum lathosterol to campesterol group.

Low ratio Lath/Camp group Number of subjects = 8	Week 0	Week 4	% Change
Total cholesterol (mg/dL)	251 ± 27	215 ± 26	-14.2 **
LDL cholesterol (mg/dL)	175 ± 24	151 ± 25	-13.8 **
HDL cholesterol (mg/dL)	46 ± 19	43 ± 17	- 5.9
Triglycerides (mg/dL)	134 ± 71	139 ± 52	3.8

Values represent mean ± SD; Level of significance ** $p < 0.01$

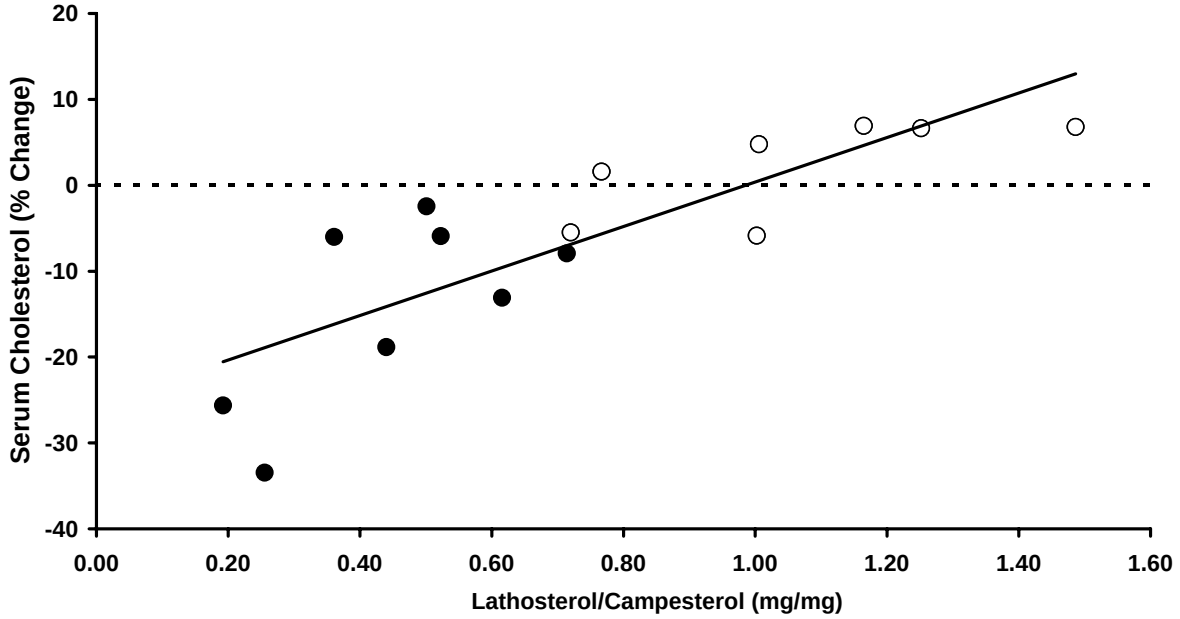


Figure 11: Relationship between percent change of serum total cholesterol between week 0 and week 4 and the ratio lathosterol to campesterol after four weeks of treatment with sitostanol ester margarine ($r = 0.819$, $p < 0.0002$; ● low and ○ high ratio of lathosterol to campesterol)

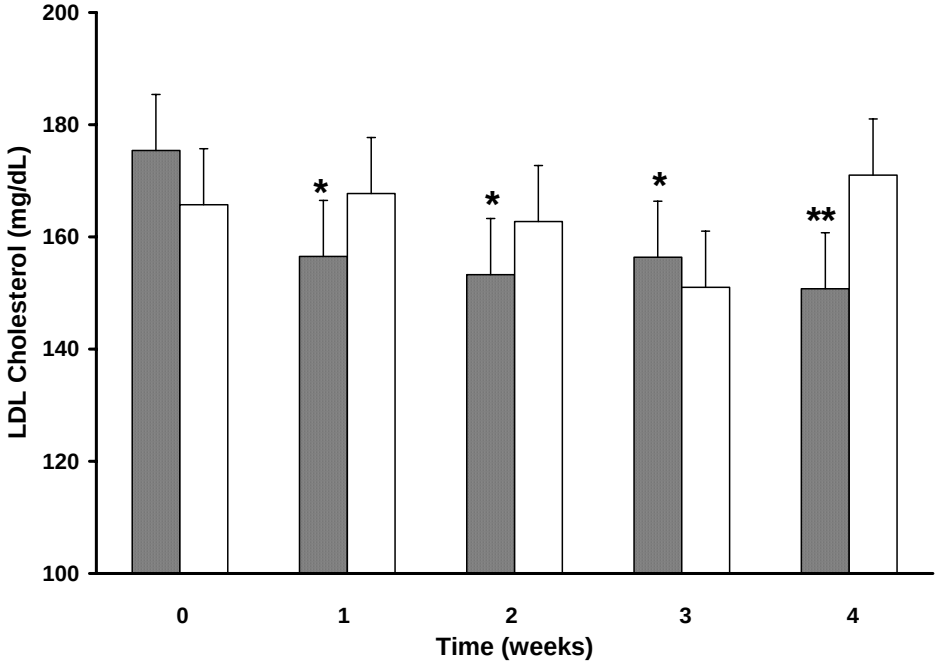


Figure 12: Serum concentrations of LDL cholesterol during four weeks of treatment with sitostanol ester margarine in subjects grouped according to the ratio lathosterol to campesterol (change from week 0: * $p < 0.05$, ** $p < 0.01$; ■ low and □ high ratio of lathosterol to campesterol)

Table 7: Serum concentration of lipoproteins before (week 0) and after treatment (week 4) with sitostanol ester margarine in high ratio serum lathosterol to campesterol group.

High ratio Lath/Camp group Number of subjects = 7	Week 0	Week 4	% Change
Total cholesterol (mg/dL)	240 ± 34	244 ± 29	2.2
LDL cholesterol (mg/dL)	166 ± 31	171 ± 24	4.3
HDL cholesterol (mg/dL)	48 ± 8	48 ± 9	1.6
Triglycerides (mg/dL)	145 ± 49	176 ± 74	20.4

Values represent mean ± SD

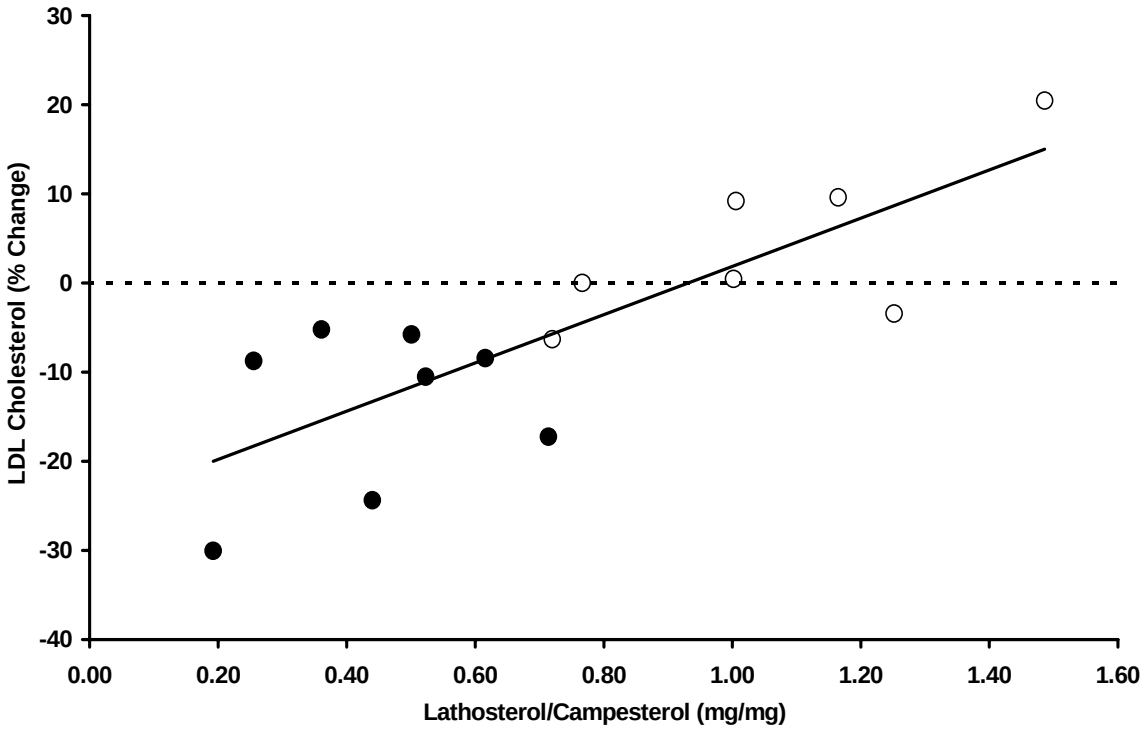


Figure 13: Relationship between percent change of serum LDL cholesterol between week 0 and week 4 and the ratio lathosterol to campesterol after four weeks of treatment with sitostanol ester margarine ($r = 0.799$, $p < 0.0001$; ● low and ○ high ratio of lathosterol to campesterol)

4.1.4 Effect of sitostanol ester margarine on non-cholesterol sterols

The concentration of serum lathosterol increased significantly in the group with low ratio of lathosterol to campesterol from 0.25 ± 0.06 mg/dL at week 0 to 0.31 ± 0.04 mg/dL at the end of study (30.2 %; $p<0.01$) (Table 8). The levels of serum lathosterol increased after the first week of treatment (Figure 14). The ratio of lathosterol to cholesterol also increased significantly by 51.5 % ($p<0.001$) (Table 8 and Figure 15).

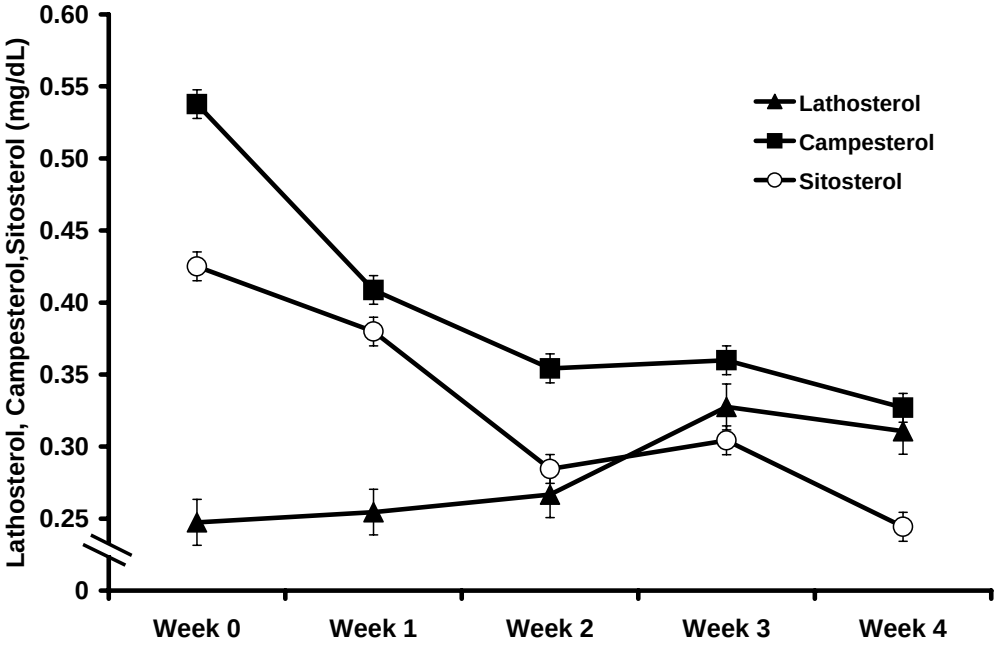


Figure 14: Serum concentrations of lathosterol, campesterol and sitosterol during four weeks of treatment with sitostanol ester margarine in subjects grouped according to low ratio serum lathosterol to campesterol (Lath/Camp).

In the group with low ratio of lathosterol to campesterol, serum campesterol and sitosterol decreased significantly by -37.4 % ($p<0.01$) and -32.4 % ($p<0.05$), respectively (Table 8). The ratio of serum campesterol and sitosterol to cholesterol also decreased significantly by -27.4 % ($p<0.01$) and -23.3 % ($p<0.05$) (Table 8 & Figure 15).

Table 8: Serum concentration of lathosterol, campesterol, sitosterol, and the respective ratios to cholesterol before (week 0) and after treatment (week 4) with sitostanol ester margarine in the low ratio serum lathosterol to campesterol group.

Low ratio Lath/Camp group Number of subjects = 8	Week 0	Week 4	% Change
Lathosterol (mg/dL)	0.25 ± 0.06	0.31 ± 0.04	30.2 **
Campesterol (mg/dL)	0.54 ± 0.08	0.33 ± 0.07	-37.4 **
Sitosterol (mg/dL)	0.43 ± 0.15	0.24 ± 0.05	-32.4 *
Lathosterol/ Cholesterol (µg/mg)	1.02 ± 0.24	1.47 ± 0.16	51.5 ***
Campesterol/Cholesterol (µg/mg)	2.23 ± 0.42	1.57 ± 0.44	-27.4 **
Sitosterol/Cholesterol (µg/mg)	1.77 ± 0.64	1.18 ± 0.35	-23.3 *

Values represent mean ± SD; Level of significance * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$

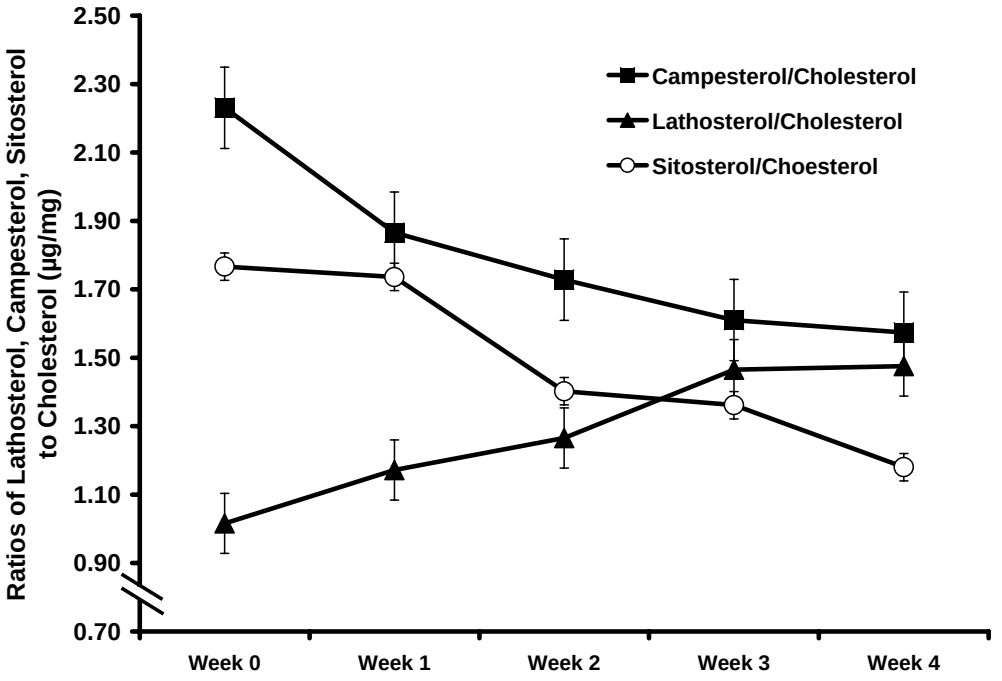


Figure 15: Ratios of serum concentration of lathosterol, campesterol and sitosterol to cholesterol during four weeks of treatment with sitostanol ester margarine in subjects grouped according to low ratio serum lathosterol to campesterol (Lath/Camp).

The changes in the serum concentrations of lathosterol and plant sterols and their ratios to cholesterol were not significantly altered in the high ratio group, although small changes were noted (Table 9, Figure 16 & 17). In the group with high ratio of lathosterol to campesterol, the concentration of serum lathosterol increased by 19.9 % (Figure 16). The ratio of lathosterol to cholesterol increased by 21.1 %. But both the increases did not reach a statistical significance (Figure 17). The serum campesterol and sitosterol decreased by -10.6 % and -3.2 % respectively (Table 9). The ratio of serum campesterol and sitosterol to cholesterol also decreased by -10.2 % and -1.8 % (Table 9 and Figure 17).

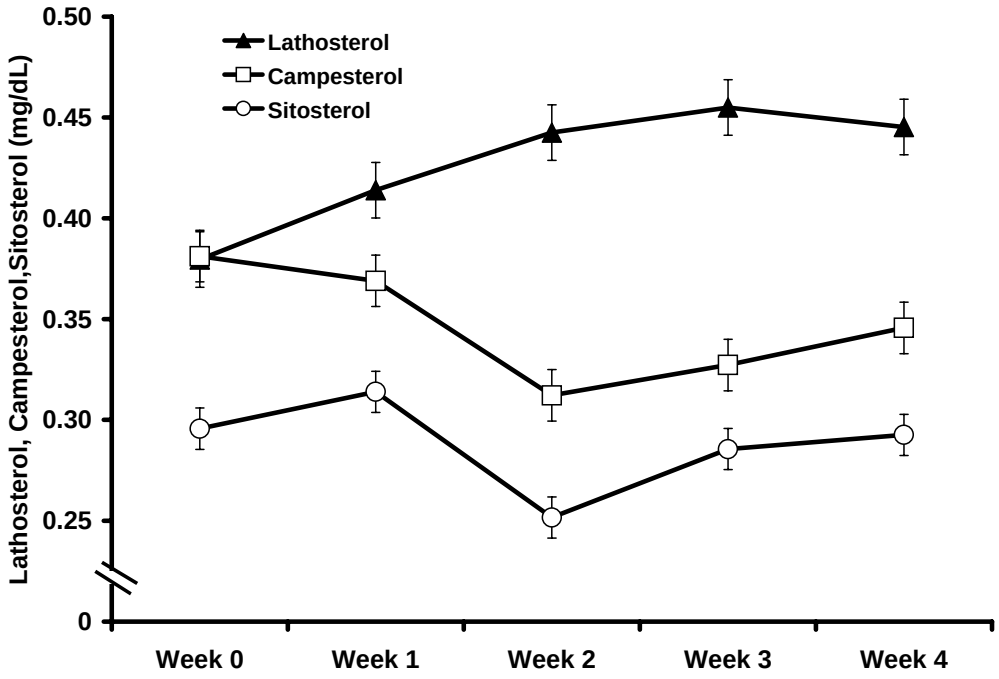


Figure 16: Serum concentrations of lathosterol, campesterol and sitosterol during four weeks of treatment with sitostanol ester margarine in subjects grouped according to high ratio serum lathosterol to campesterol (Lath/Camp).

Table 9: Serum concentration of lathosterol, campesterol, sitosterol, and the respective ratios to cholesterol before (week 0) and after treatment (week 4) with sitostanol ester margarine in high ratio serum lathosterol to campesterol group.

High ratio Lath/Camp group Number of subjects = 7	Week 0	Week 4	% Change
Lathosterol (mg/dL)	0.38 ± 0.05	0.44 ± 0.14	19.9
Campesterol (mg/dL)	0.38 ± 0.06	0.35 ± 0.10	-7.8
Sitosterol (mg/dL)	0.30 ± 0.04	0.29 ± 0.08	-3.2
Lathosterol/ Cholesterol (µg/mg)	1.62 ± 0.28	1.98 ± 0.74	21.1
Campesterol/Cholesterol (µg/mg)	1.68 ± 0.39	1.49 ± 0.47	-10.2
Sitosterol/Cholesterol (µg/mg)	1.33 ± 0.28	1.25 ± 0.30	-1.8

Values represent mean ± SD

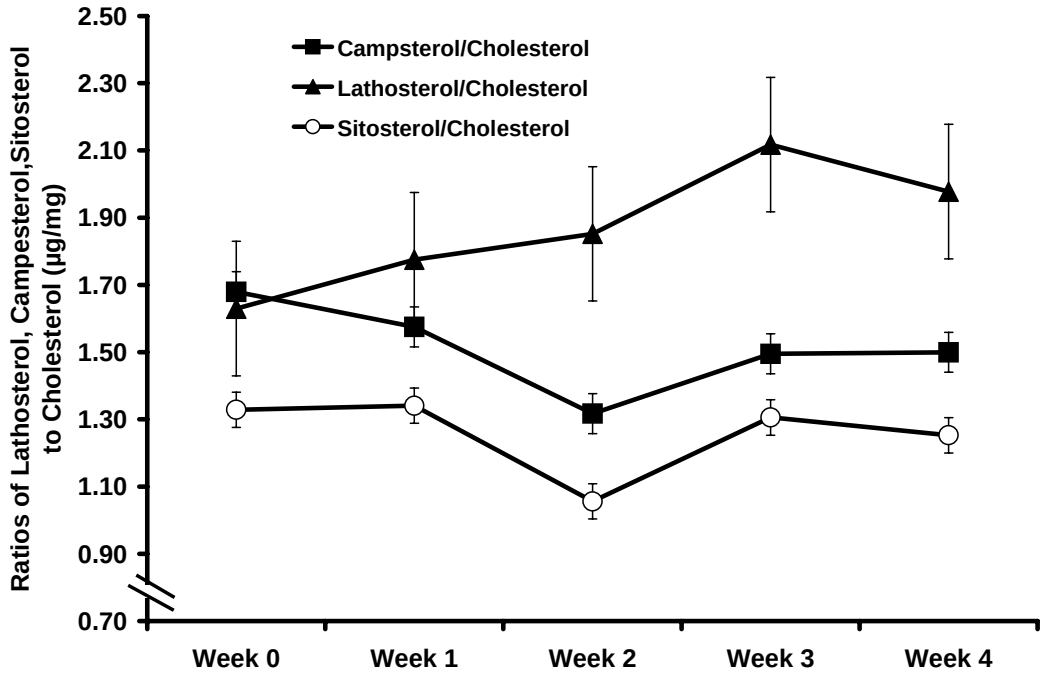


Figure 17: Ratios of serum concentration of lathosterol, campesterol and sitosterol to cholesterol during four weeks of treatment with sitostanol ester margarine in subjects grouped according to high ratio serum lathosterol to campesterol (Lath/Camp).

4.1.5 Post-hoc analysis

In order to evaluate the predictive value of serum parameters other than the ratio lathosterol to campesterol, the data derived were analyzed on the basis of the ratios of lathosterol to sitosterol (Lath/Sit) and serum campesterol to cholesterol (Camp/CH).

Thus, post-hoc analysis was performed with two groups that represented the low values (LOW Lath/Sit, range 0.3 – 1.0) and the high values of this ratio (HIGH Lath/Sit, range 1.2 - 1.88) and that differed significantly at screening (LOW Lath/Sit: 0.62 ± 0.3 µg/mg, HIGH Lath/Sit: 1.41 ± 0.3 µg/mg, $p<0.001$). After 4 weeks of margarine intake total cholesterol decreased significantly by –10.7 ($p<0.05$) % in LOW Lath/Sit, whereas there was an insignificant decrease of - 1.8 % in HIGH Lath/Sit group. There was a significant decrease of –9.8 % ($p<0.05$) in serum LDL of LOW Lath/Sit compared to an insignificant decrease of –0.3 % in HIGH Lath/Sit group. The triglycerides levels in LOW Lath/Sit group increased by 17.2% and by 15.3% HIGH Lath/Sit group and HDL cholesterol decreased by- 4.6 and -0.2 respectively, but the changes were not significant. In the LOW Lath/Sit group serum lathosterol increased significantly by 40.6 % ($p<0.01$) where as the 7.4 % increase in the HIGH Lath/Sit group was statistically insignificant. There was a significant decrease of campesterol and its ratio to cholesterol in the group LOW Lath/Sit group (-30.3 %, $p<0.05$ and -22.7 % $p<0.05$) whereas the decrease in HIGH Lath/Sit was insignificant (-21.5 % and -18.4). Serum concentration of sitosterol and its ratio to cholesterol in the group decreased significantly in the LOW Lath/Sit group (-35.0 %, $p<0.05$ and -28.1 % $p<0.05$), whereas their decreases in the group HIGH Lath/Sit were not significant.

When the post-hoc analysis performed with two groups that represented the low values (LOW Camp/CH, range 1.5 – 2.7) and the high values (HIGH Camp/CH, range 3.1 - 6.4) of the ratio serum campesterol to cholesterol, there was a significant decrease of – 9.0 % ($p<0.05$) in serum LDL of HIGH Camp/CH compared to an insignificant decrease of – 4.0 % in LOW C/CH after 4 weeks of margarine intake. Total cholesterol decreased significantly by – 11.0 % ($p<0.05$) in HIGH Camp/CH, whereas there was an insignificant decrease of – 3.4 % in LOW Camp/CH. There was a significant correlation between changes in serum LDL cholesterol ($r = 0.629$, $p< 0.001$) and serum total cholesterol ($r = 0.570$, $p< 0.01$) from week 0 - week 4 with the ratio of serum campesterol to cholesterol.

The triglycerides levels in LOW Camp/CH group increased significantly by 27.7 % ($p<0.05$) when compared to an insignificant increase of 5.1 % in HIGH Camp/CH group. In the HIGH Camp/CH group serum lathosterol increased significantly by 35.5 % ($p<0.05$) whereas the 13.8 % increase in the LOW Camp/CH was statistically insignificant. There was a significant decrease of campesterol and sitosterol in the group HIGH Camp/CH (-34.7 %; $p<0.05$ and -32 %; $p< 0.05$) whereas the decrease in LOW Camp/CH was not significant.

4.2 Responder Study 2 (treatment with ezetimibe)

4.2.1 Clinical characteristics and compliance

From the 121 patients screened, sixteen were selected to participate in the study according to their ratio of lathosterol to campesterol from fasting serum samples. One patient dropped out due to personal reasons without any relation to the study drug. Two groups were formed: one group with the lowest ratio of lathosterol to campesterol (n = 8; range 0.19 - 0.74 mg/mg) and the other group with the highest ratio (n=7; range 0.99 - 2.39 mg/mg). The two groups did not differ in age, body mass index, or lipoprotein profile before treatment (Table 10).

Table 10: Screening parameters of subjects grouped according to the ratio serum lathosterol to campesterol (Lath/Camp).

Parameter	LOW Lath/Camp	HIGH Lath/Camp	p
Lathosterol / Campesterol (mg/mg)	0.47 ± 0.22	1.42 ± 0.47	
Range	0.19 – 0.74	0.99 – 2.39	
Age (years)	33 ± 8	34 ± 8	0.724
BMI (kg/m2)	23 ± 2	25 ± 3	0.115
Total cholesterol (mg/dL)	198 ± 38	189 ± 24	0.593
LDL cholesterol (mg/dL)	121 ± 28	118 ± 25	0.795
HDL cholesterol (mg/dL)	50 ± 8	53 ± 8	0.530
Triglycerides (mg/dL)	133 ± 78	114 ± 47	0.577

The serum non-cholesterol sterol ratios of subjects were analysed for any difference in their concentration over a period of six months, from screening to the first week of the study. It was observed that plasma noncholesterol sterol concentrations were highly stable within individuals. No appreciable change was noted after six months. The serum ratios of lathosterol to campesterol and campesterol to cholesterol did not change during a period of six months from screening to the first week of the study. There was a highly significant correlation between serum ratios of lathosterol to campesterol ($r = 0.995$; $p<0.0001$) and campesterol to cholesterol ($r = 0.996$; $p<0.0001$) from screening to first week of the study (Figures 18 and 19).

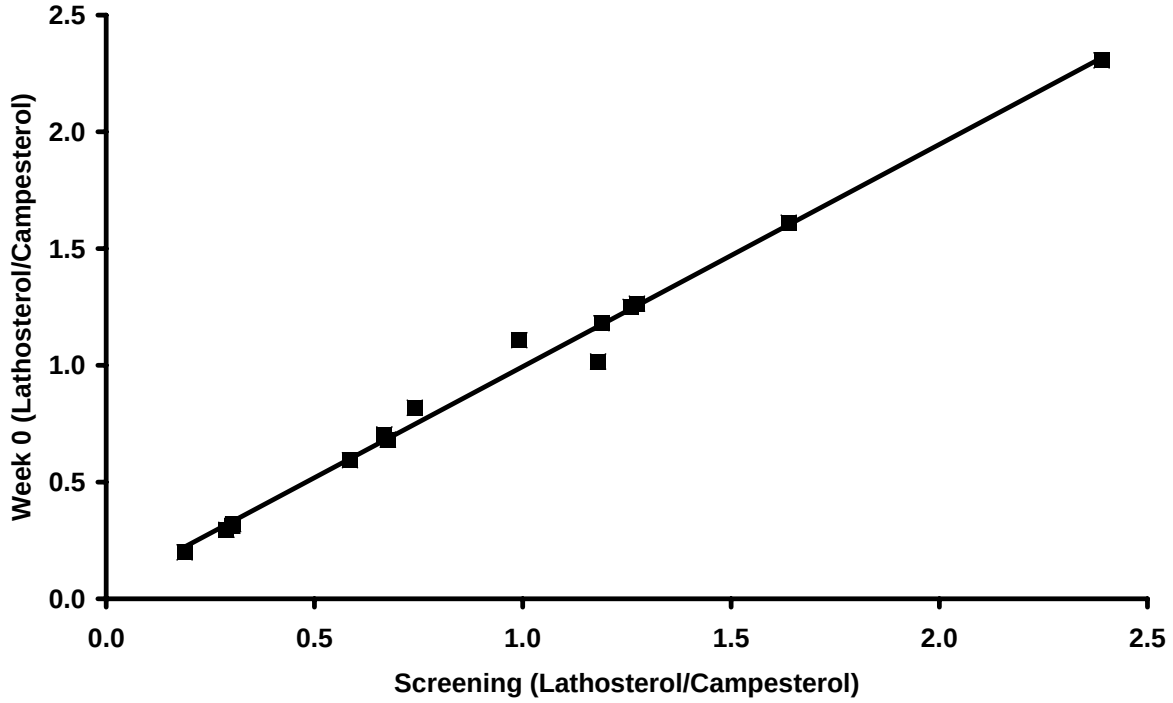


Figure 18: Relationship between lathosterol to campesterol ratio during a period of six months from screening to first week (W0) of the study ($r = 0.995$; $p<0.0001$).

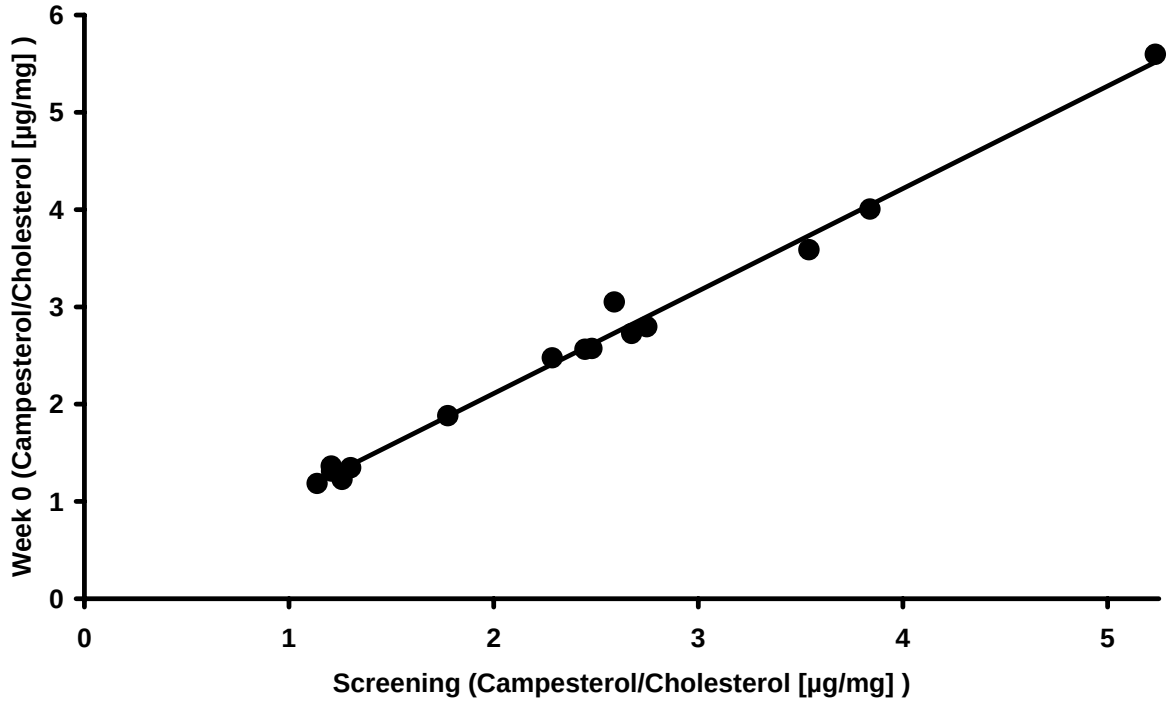


Figure 19: Relationship between campesterol to cholesterol ratio during a period of six months from screening to first week (W0) of the study ($r = 0.996$; $p<0.0001$).

4.2.2 Baseline values

To investigate the responsiveness to ezetimibe on serum lipoprotein in two selective subgroups with a high and a low ratio of serum lathosterol to campesterol, baseline values of serum lipoprotein lipids were measured. The serum concentration of total cholesterol in the group with the low ratio was 193 ± 32 mg/dL vs 184 ± 44 mg/dL of high ratio group. Serum concentration of LDL cholesterol in the group with the low ratio was 116 ± 24 mg/dL vs 102 ± 29 mg/dL of high ratio group whereas serum concentration of HDL cholesterol in the group with the low ratio was 54 ± 9 mg/dL vs 52 ± 15 mg/dL of high ratio group. The triglycerides in low ratio group were 114 ± 58 mg/dL vs 127 ± 62 mg/dL (Tables 11 and 12). The two groups did not differ in lipoprotein profiles.

4.2.3 Effect of Ezetimibe on serum lipoproteins

Ezetimibe was well tolerated by all subjects without any adverse event. After four weeks of treatment serum total and LDL cholesterol concentrations decreased in all the subjects on the average by –17 % ($p<0.0001$) and –22 % ($p<0.0001$), respectively.

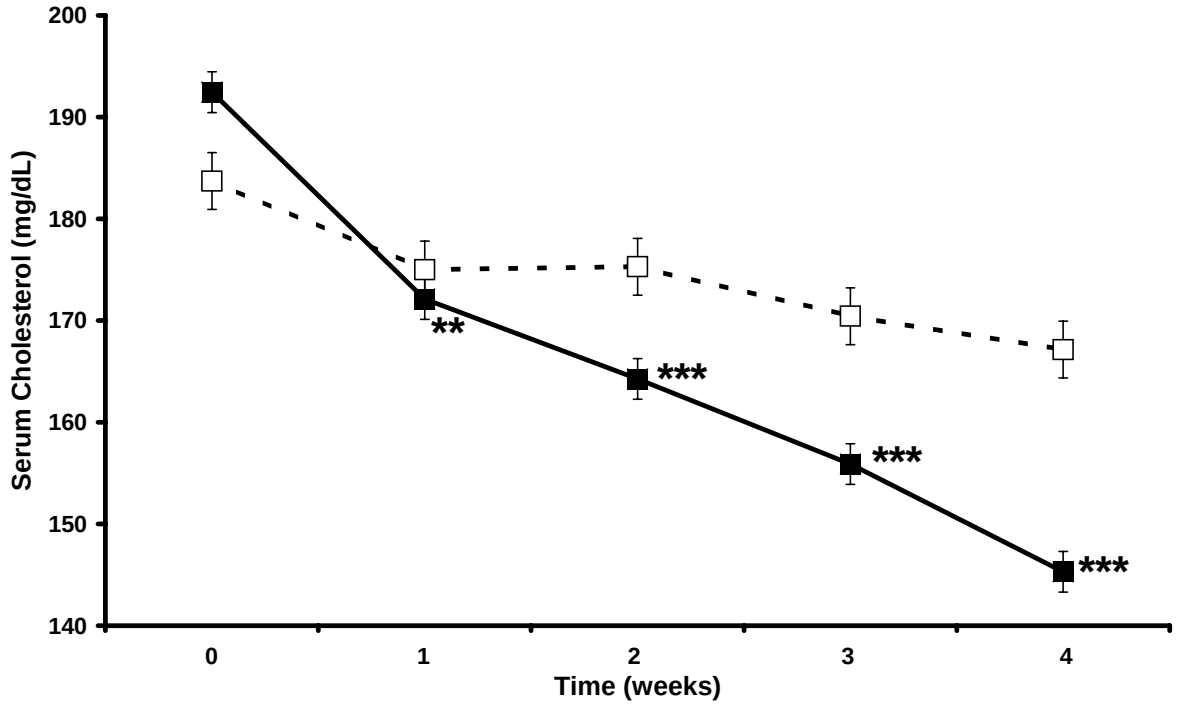


Figure 20: Serum concentrations of total cholesterol during four weeks of treatment with ezetimibe in subjects grouped according to the ratio lathosterol to campesterol (change from week 0: * $p< 0.02$, ** $p< 0.001$; ■ low and □ high ratio of lathosterol to campesterol)

In the group with low lathosterol to campesterol ratio, there was a highly significant decrease of –25 % ($p<0.00001$) in serum total cholesterol. The serum cholesterol decreased from 193 ± 32 mg/dL to 145 ± 27 mg/dL (Figure 20) after four weeks of treatment. In the group with high ratio of lathosterol to campesterol, although total cholesterol decreased by –8 %, it did not change significantly after four weeks of treatment. The serum cholesterol decreased from 184 ± 44 mg/dL to 167 ± 27 mg/dL by the end of week 4 (Table 11). There was a highly significant correlation between the percent changes in serum total cholesterol with the ratio of serum lathosterol to campesterol ($r = 0.773$, $p< 0.001$) in all subjects (Figure 21).

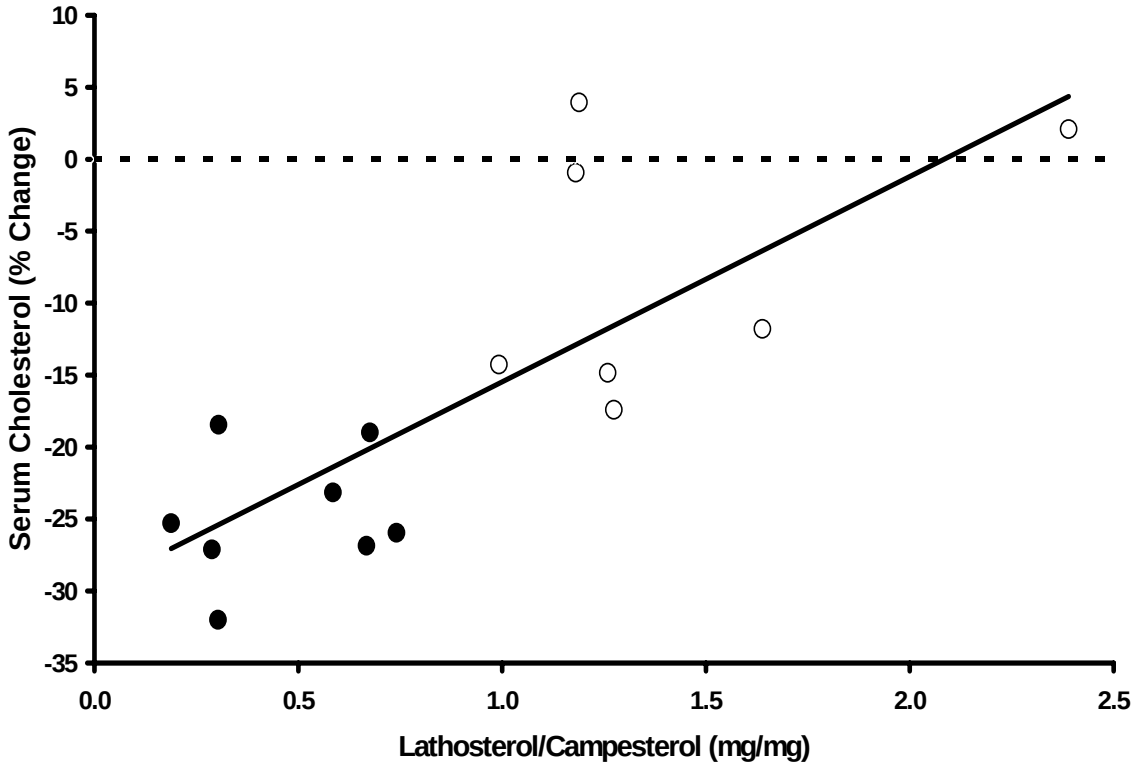


Figure 21: Relationship between percent change of serum total cholesterol between week 0 and week 4 and the ratio lathosterol to campesterol, after four weeks of treatment with ezetimibe ($r = 0.773$, $p < 0.001$; ● low and ○ high ratio of lathosterol to campesterol).

In the group with low lathosterol to campesterol ratio (responder), there was a highly significant decrease of –33 % ($p<0.0001$) in serum LDL cholesterol. The average serum LDL cholesterol decreased from 116 ± 24 mg/dL to 78 ± 16 mg/dL at the end of study (Figure 22). In the group with high lathosterol to campesterol ratio after four weeks of treatment serum LDL cholesterol also decreased by –9 % on the average which was not statistically significant (Table 12). The average serum LDL cholesterol decreased from 102 ± 29 mg/dL to 91 ± 19 mg/dL after 4 weeks of treatment.

There was a highly significant correlation between the percent changes in serum LDL cholesterol with the ratio of serum lathosterol to campesterol ($r = 0.767$, $p < 0.0001$) in all subjects (Figure 23). Serum HDL cholesterol and triglycerides were not affected in both of the groups (Tables 11 and 12).

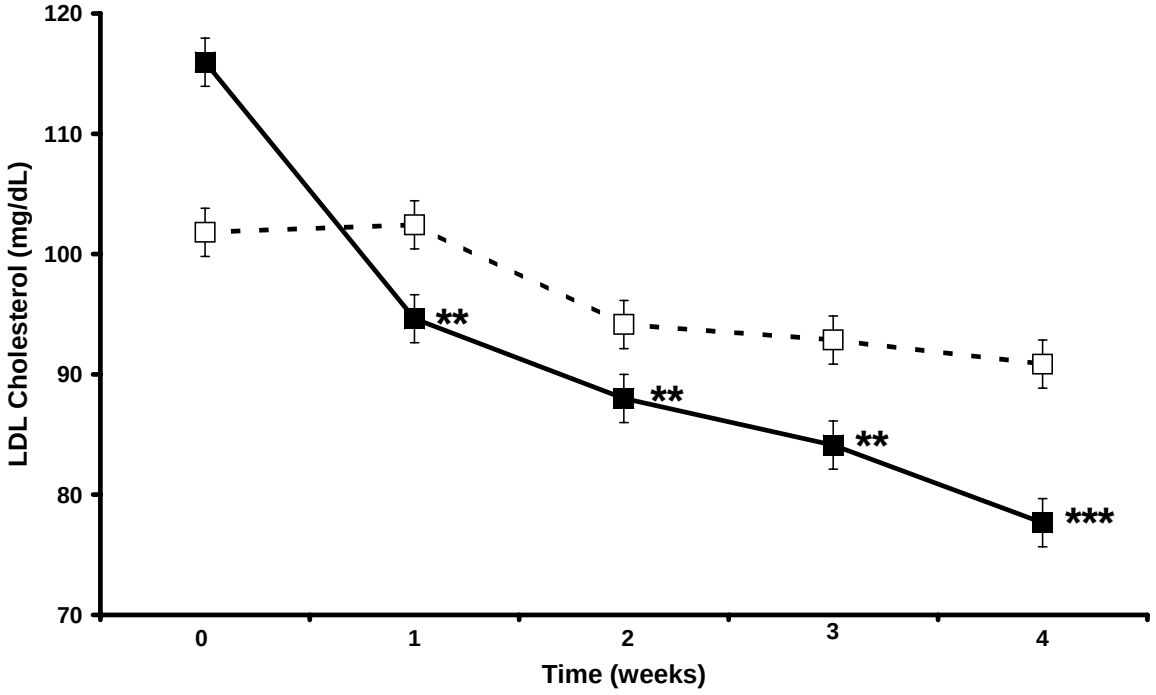


Figure 22: Serum concentrations of LDL cholesterol during four weeks of treatment with ezetimibe in subjects grouped according to the ratio lathosterol to campesterol (change from week 0: * p< 0.02, ** p< 0.001; ■ low and □ high ratio of lathosterol to campesterol)

Table 11: Serum concentration of lipoproteins before (week 0) and after treatment (week 4) with ezetimibe in low ratio serum lathosterol to campesterol group (Lath/Camp).

Low ratio Lath/Camp group Number of subjects = 8	Week 0	Week 4	% Change
Total cholesterol (mg/dL)	193 ± 32	145 ± 27	-25 ***
LDL cholesterol (mg/dL)	116 ± 24	78 ± 16	-33 ***
HDL cholesterol (mg/dL)	54 ± 9	56 ± 12	4
Triglycerides (mg/dL)	114 ± 58	103 ± 34	-10

Values represent mean ± SD; Level of significance *** $p < 0.001$

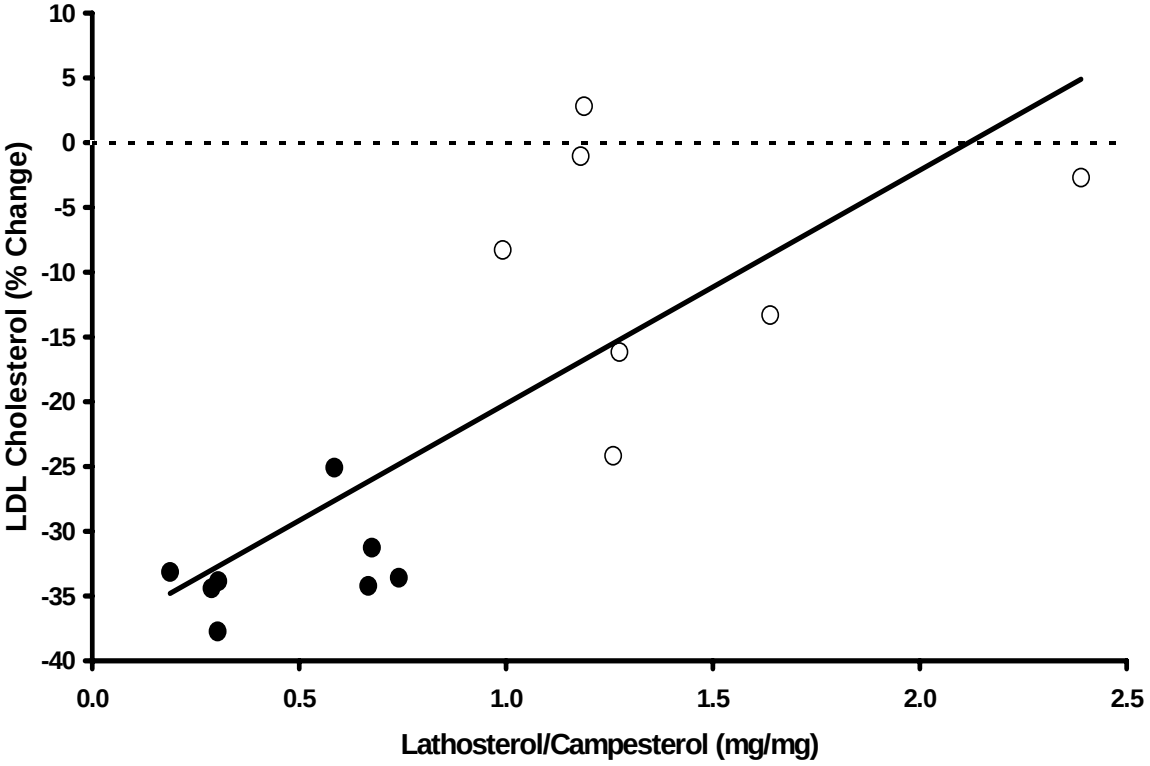


Figure 23: Relationship between percent change of serum LDL cholesterol between week 0 and week 4 and the ratio lathosterol to campesterol, after four weeks of treatment with ezetimibe ($r = 0.767$, $p < 0.0001$; ● low and ○ high ratio of lathosterol to campesterol).

Table 12 Serum concentration of lipoproteins before (week 0) and after treatment (week 4) with ezetimibe in high ratio serum lathosterol to campesterol group (Lath/Camp).

High ratio Lath/Camp group Number of subjects = 7	Week 0	Week 4	% Change
Total cholesterol (mg/dL)	184 ± 44	167 ± 27	-8
LDL cholesterol (mg/dL)	102 ± 29	91 ± 19	-9
HDL cholesterol (mg/dL)	52 ± 15	51 ± 14	-1
Triglycerides (mg/dL)	127 ± 62	130 ± 57	5

Values represent mean ± SD

4.2.4 Effect of ezetimibe on non-cholesterol sterols

The concentration of serum lathosterol increased significantly in the group with low ratio of lathosterol to campesterol by 50 % ($p<0.0001$) the end of study (Table 13). The levels of serum lathosterol started increasing from after first week of treatment (Figure 24). The ratio of lathosterol to cholesterol also increased significantly by 90 % ($p<0.0001$) (Figure 25).

In the group with low ratio of lathosterol to campesterol, serum campesterol decreased significantly by -51 % ($p<0.0001$) (Table 13) and its ratio to cholesterol decreased significantly by -36 % ($p<0.0001$) by the end of study (Table 13). Serum sitosterol and its ratio to cholesterol decreased significantly by -45 % ($p<0.0001$) and -30 % ($p<0.0001$) respectively (Table 13 and figure 25).

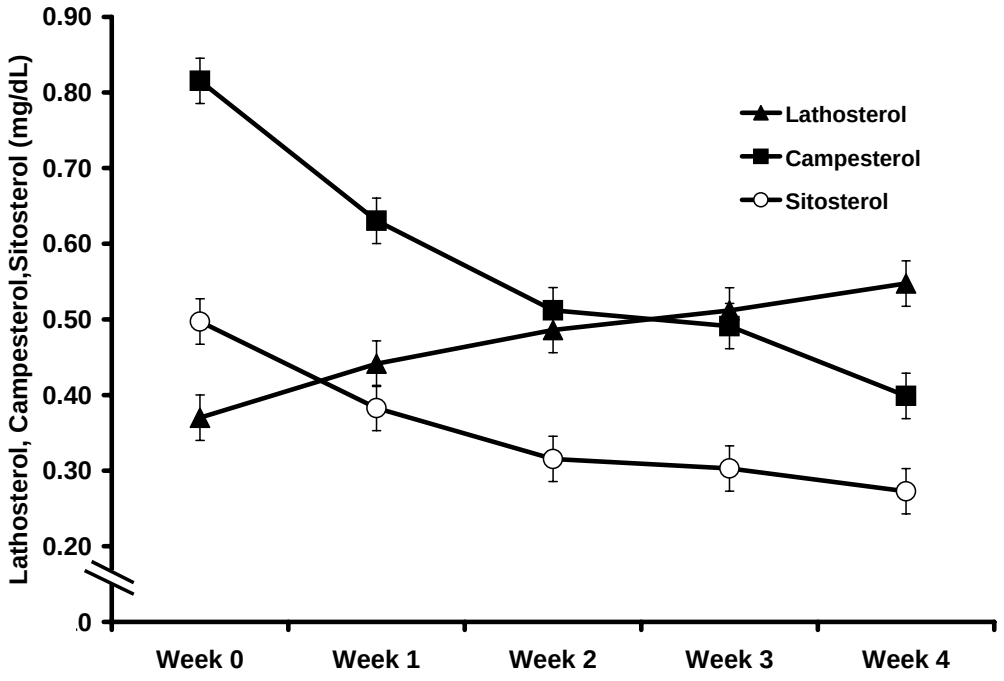


Figure 24: Serum concentrations of lathosterol, campesterol and sitosterol during four weeks of treatment with ezetimibe in subjects grouped according to low ratio serum lathosterol to campesterol (Lath/Camp).

Table 13: Serum concentration of lathosterol, campesterol, sitosterol, and the respective ratios to cholesterol before (week 0) and after treatment (week 4) with ezetimibe in low ratio serum lathosterol to campesterol group (Lath/Camp).

Low ratio Lath/Camp group Number of subjects = 8	Week 0	Week 4	% Change
Lathosterol (mg/dL)	0.37 ± 0.16	0.55 ± 0.22	50 ***
Campesterol (mg/dL)	0.82 ± 0.23	0.40 ± 0.12	-51 ***
Sitosterol (mg/dL)	0.50 ± 0.14	0.27 ± 0.08	-45 ***
Lathosterol/ Cholesterol (µg/mg)	1.51 ± 0.60	2.80 ± 0.95	90 ***
Campesterol/Cholesterol (µg/mg)	3.39 ± 1.06	2.13 ± 0.68	-36 ***
Sitosterol/Cholesterol (µg/mg)	2.05 ± 0.61	1.43 ± 0.47	-30 ***

Values represent mean ± SD; Level of significance *** $p < 0.0001$

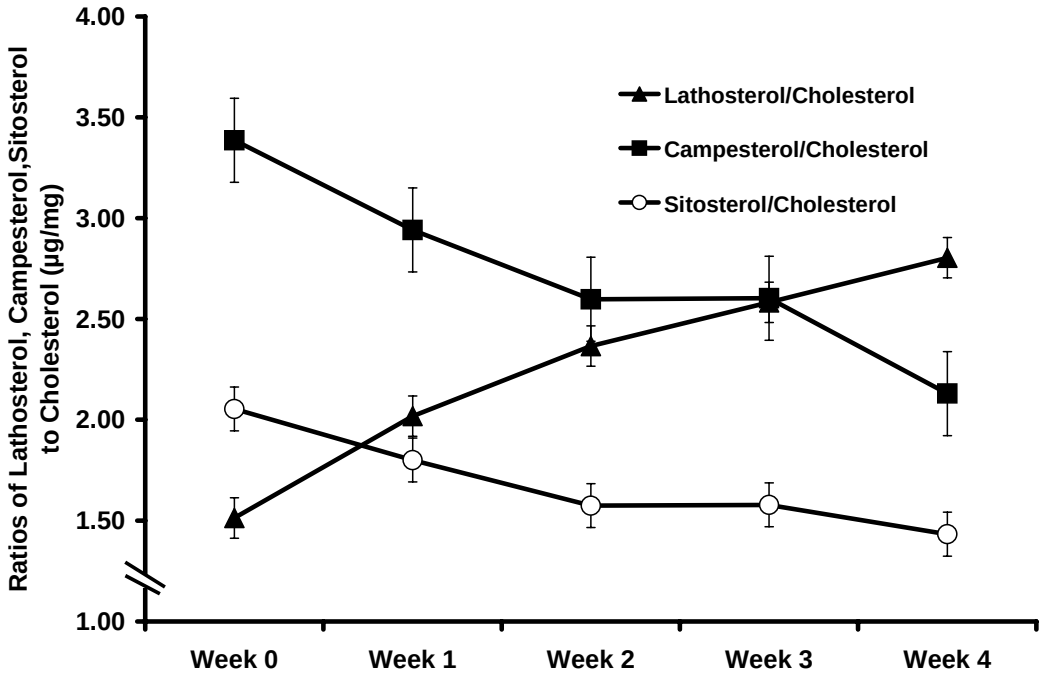


Figure 25: Ratios of serum concentration of lathosterol, campesterol and sitosterol to cholesterol during four weeks of treatment with ezetimibe in subjects grouped according to low ratio serum lathosterol to campesterol (Lath/Camp).

In the high ratio group serum lathosterol increased significantly by 11 % ($p<0.05$) after four weeks of treatment and the ratio to cholesterol increased by 26 % without any statistical significance (Table 14). The serum concentrations of campesterol and sitosterol decreased significantly by –24% ($p<0.01$) and –19% ($p<0.01$) but their ratios to cholesterol were not significantly altered, although decreases were noted (Table 14). (Figures 26 and 27)

Table 14: Serum concentration of lathosterol, campesterol, sitosterol, and the respective ratios to cholesterol before (week 0) and after treatment (week 4) with ezetimibe in high ratio serum lathosterol to campesterol group.

High ratio Lath/Camp group Number of subjects = 7	Week 0	Week 4	% Change
Lathosterol (mg/dL)	0.46 ± 0.11	0.52 ± 0.16	11 *
Campesterol (mg/dL)	0.34 ± 0.12	0.26 ± 0.09	-24 **
Sitosterol (mg/dL)	0.26 ± 0.06	0.21 ± 0.06	-19 **
Lathosterol/ Cholesterol (µg/mg)	2.12 ± 0.56	2.63 ± 0.99	26
Campesterol/Cholesterol (µg/mg)	1.53 ± 0.42	1.33 ± 0.56	-14
Sitosterol/Cholesterol (µg/mg)	1.15 ± 0.23	1.02 ± 0.29	-12

Values represent mean ± SD; Level of significance * $p < 0.05$, ** $p < 0.01$

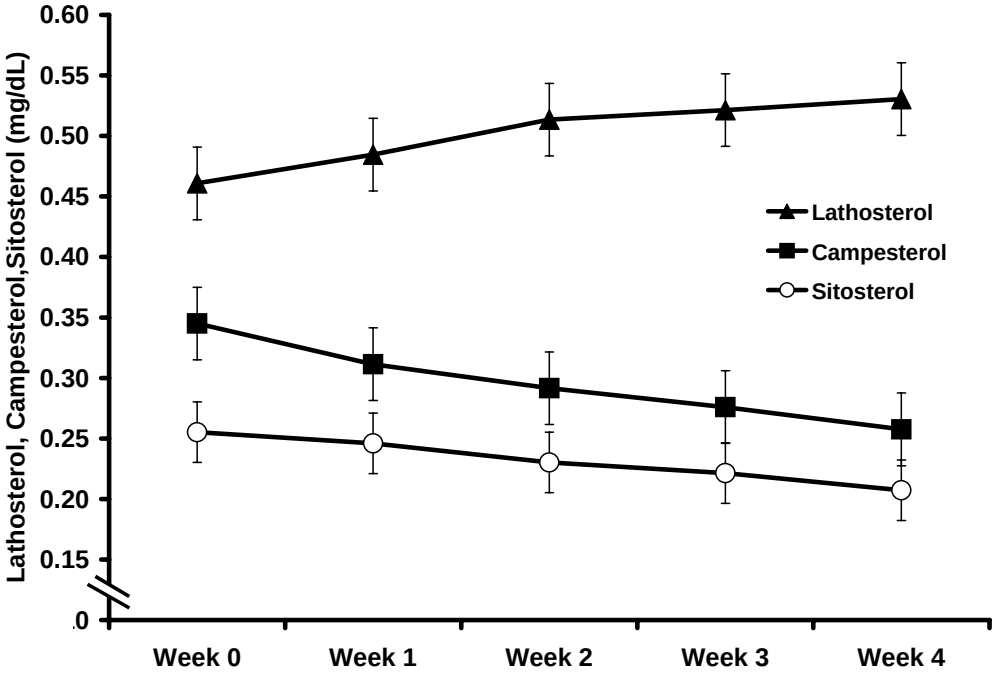


Figure 26: Serum concentrations of lathosterol, campesterol and sitosterol during four weeks of treatment with ezetimibe in subjects grouped according to high ratio serum lathosterol to campesterol (Lath/Camp).

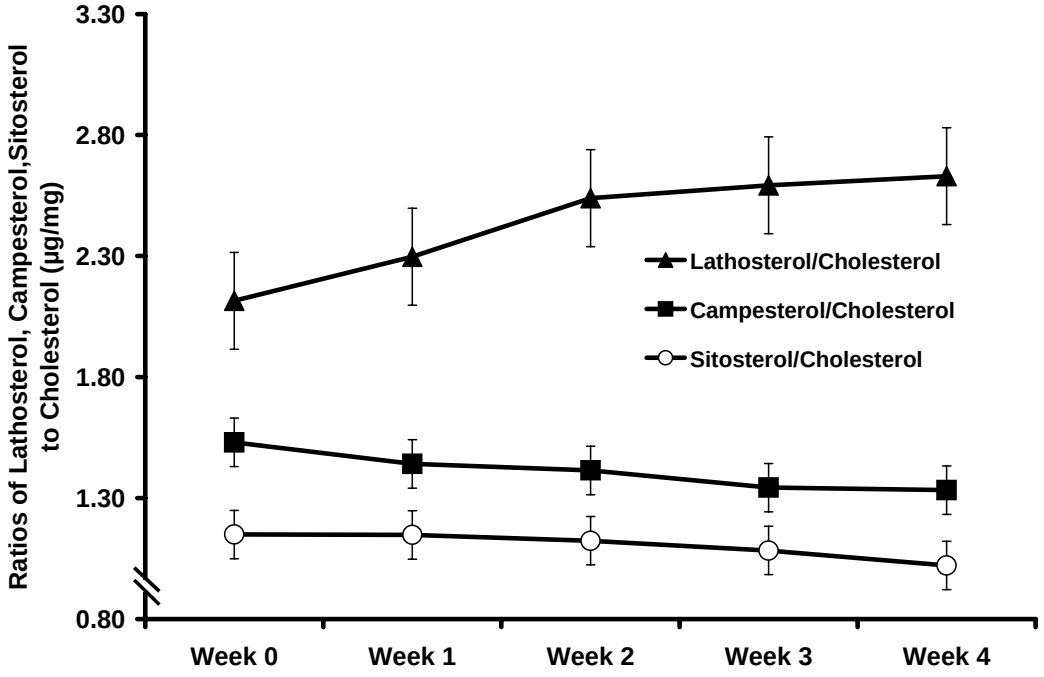


Figure 27: Ratios of serum concentration of lathosterol, campesterol and sitosterol to cholesterol during four weeks of treatment with ezetimibe in subjects grouped according to high ratio serum lathosterol to campesterol (Lath/Camp).

4.2.5 DNA sequence variants in *ABCG5* and *ABCG8*

The genotype frequencies of the different polymorphisms (Table 15) were in Hardy-Weinberg equilibrium in the whole study group, and also in the subgroups of responders and non-responders. The genotype distributions of *ABCG8* SNPs did not differ among responders and non-responders. However, the Q604E polymorphism of the *ABCG5* gene was unevenly distributed in responders and non-responders (Figure 28). Whereas all responders were homozygous or heterozygous carriers of the wild-type Q604 allele, non-responders carried predominantly the minor allele Q604E ($\chi^2 = 3.60$, p=0.05).

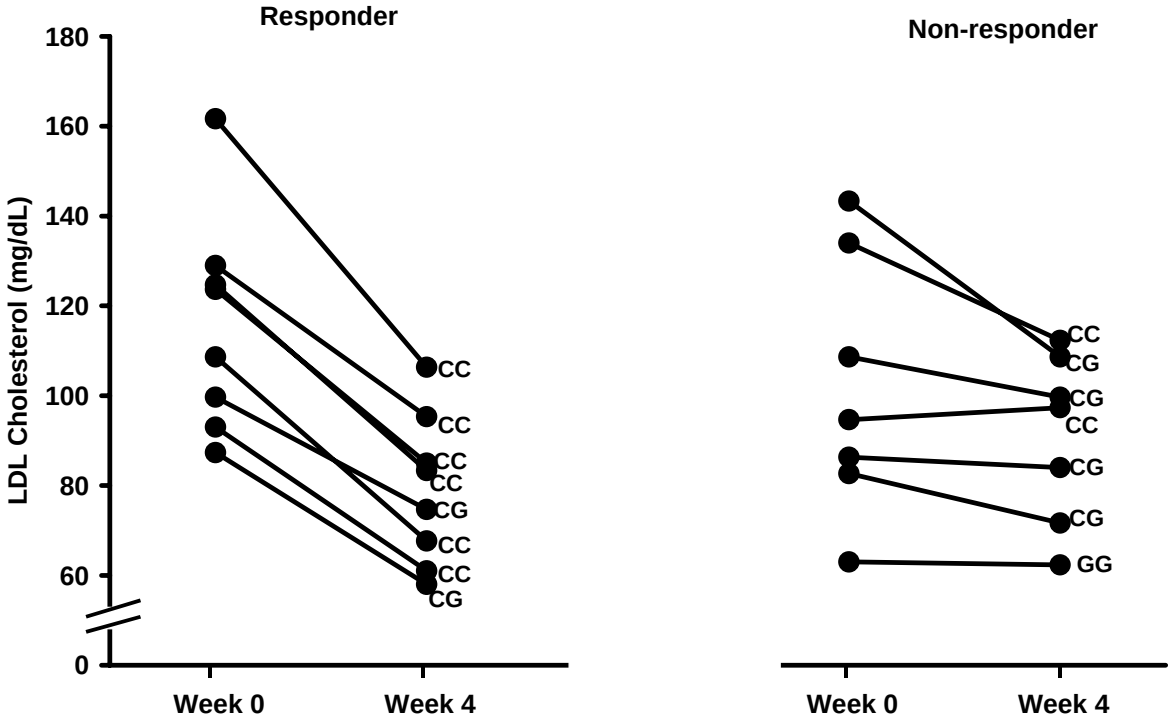


Figure 28: Individual LDL cholesterol concentrations* before and at the end of treatment with ezetimibe in responders and non-responders represented with Q604E polymorphism (CC and CG see Table 15)

*Each point is the mean of three measurements

Table 15: Distribution of polymorphisms at the ABCG5 and ABCG8 (Q604E, D19H, Y54C, T400K, and A632V) in responders and non-responders to ezetimibe

	ABCG5	**ABCG8**			
Subject	**Q604E**	**D19H**	**Y54C**	**T400K**	**A632V**
Responders	**0=CC;1=CG; 2=GG**	**0=GG;1=GC; 2=CC**	**0=AA;1=AG; 2=GG**	**0=CC;1=CA; 2=AA**	**0=CC;1=CT; 2=TT**
1	0	0	0	1	0
2	0	0	0	1	1
3	0	0	0	0	0
4	0	0	1	1	1
5	1	0	1	1	0
6	0	0	2	0	0
7	0	0	2	0	0
8	1	0	0	0	1
No of Allele 1	6	8	4	4	5
No of Heterozygous	2	0	2	4	3
No of Allele 2	0	0	2	0	0
Allele Frequency Allele 1	0.88	1.00	0.63	0.75	0.81
Non-responders					
9	1	0	0	1	0
10	2	0	1	0	0
11	0	0	1	1	0
12	1	0	2	0	1
13	0	0	0	1	1
14	1	0	1	0	0
15	1	1	1	0	0
No of Allele 1	2	6	2	4	5
No of Heterozygous	4	1	4	3	2
No of Allele 2	1	0	1	0	0
Allele Frequency Allele 1	0.57	0.93	0.57	0.79	0.86

Q604E indicates Codon 604 glutamic acid for glutamine; D19H, Codon 19 histidine for aspartic acid; Y54C, Codon 54 cysteine for tyrosine; T400K, Codon 400 lysine for threonine; A632V, Codon 632 valine for alanine.

5 Discussion

Large-scale primary and secondary prevention trials have clearly established the benefits of cholesterol-lowering therapy on coronary heart disease (8). There is a wide inter-individual variation in response to cholesterol absorption inhibitors in cholesterol reduction (99, 106, 271). The inter-individual variations in the plasma concentrations of plant sterols reflect biological differences in sterol metabolism among individuals. Plasma noncholesterol sterol concentrations are very stable within individuals but highly variable among individuals. Most of the variation in plasma noncholesterol sterols reflects true biologic variation among individuals.

5.1 Heritability of non-cholesterol sterols and their ratios to cholesterol

The analysis of serum noncholesterol sterol ratios of subjects for any difference in their concentration over a period of time (six months) from screening to the first week of the ezetimibe study (Responder study 2) revealed that plasma noncholesterol sterol concentrations are very stable within individuals. Intra-individual variation in the plasma concentrations of noncholesterol sterols was minimal during the course of six months. Therefore, most of the variation in plasma noncholesterol sterols reflects true biologic variation among individuals. These findings are consistent with the data of Berge et al., who reported that the plasma concentrations noncholesterol sterols remained stable over a 48 week period (126) and Li et al., where the plasma concentrations noncholesterol sterols remained unchanged over a 6 month period (272).

The observation that plasma noncholesterol sterol concentrations were stable in individuals, but highly variable among individuals, suggested that the metabolism of these sterols is strongly influenced by genetic factors. Kesaniemi et al. reported that 40–60% of the variation in the plasma concentrations of Δ^8 methylsterols which precede lathosterol in the cholesterol biosynthetic pathway is heritable (108). The observations of Berge et al. that plasma concentrations of lathosterol, desmosterol and their ratios to cholesterol and individual variation in the plasma sterol-cholesterol ratios of plant sterols are heritable suggests that rates of cholesterol biosynthesis and fractional absorption of dietary cholesterol are determined by genetic variation (126). The proportion of phenotypic variation that could be accounted for by shared genes ranges from 36% for the plasma lathosterol-cholesterol ratio to 84% for plasma campesterol-cholesterol ratio.

Common DNA sequence polymorphisms in ABCG8 contribute to heritable variation in the plasma concentrations of the plant sterols campesterol and sitosterol. The observations that plasma noncholesterol sterol concentrations and sterol-cholesterol ratios were stable in individuals suggest that the metabolism of the sterols is strongly influenced by genetic factors. The ratios of noncholesterol sterols to cholesterol levels reflect the rates of cholesterol biosynthesis and cholesterol absorption indicating that inter-individual variation in both of these processes is heritable. The strong heritable variation in plasma concentrations of campesterol and sitosterol suggests that common DNA sequence polymorphisms influence the metabolism of plant sterols in the general population.

The existence of hypo- and hyper-responder supports the hypothesis that cholesterol absorption is genetically determined (124) possibly by polymorphisms in ABCG5/G8 co-transporter that selectively pump absorbed phytosterols back into the intestinal lumen (123, 126, 273, 274). Variations in ABCG5 or ABCG8 have effects on sterol metabolism, and contribute to inter-individual variation in the plasma concentrations of plant sterols. Plasma sitosterol concentrations vary over a 5- to 10-fold range among individuals consuming similar amounts of dietary sitosterol and are poorly correlated with dietary plant sterol intake (275). It is not clear how a polymorphism in either ABCG5 or ABCG8 could affect the plasma concentrations of cholesterol but not those of the plant sterols unless it altered the specificity of the transporter for its substrate.

The inhibitory effects of ezetimibe were characterized by considerable inter-individual variation. The basis of these variations could involve intrinsic differences in responsiveness to ezetimibe. It has also been identified that Niemann-Pick C1 Like 1 (NPC1L1) a protein localized in jejunal enterocytes is critical for intestinal cholesterol absorption (134). NPC1L1 is highly expressed in the jejunum and localized on the surface of the absorptive enterocytes. Mice deficient in NPC1L1 exhibit a significant reduction in cholesterol absorption, and the low level of residual cholesterol absorption was insensitive to ezetimibe treatment. Altmann et al also suggested that NPC1L1 resides in an ezetimibe sensitive pathway responsible for cholesterol absorption (135). There is a possibility that non-response to ezetimibe treatment might be a phenotype that is related to genomic variation in NPC1L1 (276).

The complex interaction between drugs and genetic factors in lipid metabolism can be studied in a relatively limited study population, assuming that the genetic factor regulates some measurable indicator of the metabolic cascade. The demonstration that plasma cholestanol, campesterol, sitosterol, lathosterol and desmosterol levels are highly heritable has important clinical implications. The possible determinants regulating the responsiveness resemble the individual intestinal cholesterol absorption capacity and the individual variation of hepatic cholesterol synthesis. Therefore, the thesis investigates the responsiveness to sitostanol ester margarine (Responder Study 1) and ezetimibe (Responder Study 2) in two selective subgroups with a high and a low ratio of serum lathosterol to campesterol.

5.2 Rationale for using ratio of serum lathosterol to campesterol

The ratios of noncholesterol sterols to cholesterol levels reflect the rates of cholesterol biosynthesis and cholesterol absorption. Lathosterol is an intermediate of the cholesterol biosynthetic pathway. The rationale for using serum lathosterol as an indicator of the rate of cholesterol synthesis lies in the assumption that it leaks out of cells and is incorporated into serum lipoproteins at a rate proportional to that of its formation in the cholesterol biosynthetic pathway. In serum, lathosterol concentrations are directly associated with hepatic HMG-CoA reductase activity (152). Miettinen et al. (277) observed that lathosterol changed in a more pronounced manner than free methyl-sterols upon manipulating the enterohepatic circulation. The serum lathosterol is superior to serum free methylsterols as an indicator of cholesterol synthesis. Serum lathosterol or the lathosterol to cholesterol ratio are not only superior as indicators, but quantification of total lathosterol is also considerably simpler than that of free methylsterols, due mainly to the fact that the concentration of lathosterol is of higher magnitude and a laborious sample workup using thin-layer chromatography is not required (155, 278). The ratios of serum plant sterols to cholesterol are proved to be cholesterol absorption markers, as they positively correlated with the cholesterol absorption rate measured by sterol balance technique (25, 150, 279). Measurement of these non-cholesterol sterols by gas liquid chromatography (GLC) provides a simple and convenient method to assess cholesterol absorption and is especially suitable in clinical studies and for detecting changes in cholesterol absorption during experimental studies.

As the data from this study and previous observations indicate that inter-individual, variation in both of these processes is heritable. The ratio of serum lathosterol to campesterol as an indicator to measure response for cholesterol absorption inhibitors is more relevant as it manifests the relative magnitudes of individual intestinal cholesterol absorption and cholesterol synthesis.

5.3 Response to sitostanol ester margarine (Responder study 1)

Dietary therapy remains the cornerstone of strategies to lower serum low-density lipoprotein (LDL) cholesterol levels besides drug treatment (8, 273). Sitosterol was shown to decrease serum cholesterol since the 1950s (200). The differences in the various plant sterols became apparent when saturated derivatives of plant sterols, called plant stanols, were shown to reduce serum cholesterol at low doses. The addition of a methyl or ethyl group on the side chain of cholesterol results in poor intestinal absorption of plant sterols in humans (207). Hydrogenation of plant sterols to the corresponding stanols renders them virtually unabsorbable (207). Sitostanol is relatively unabsorbable compared with sitosterol, remains in the intestinal lumen where it can interfere continuously and more efficiently with micellar solubility of cholesterol (216). Absorption of sitostanol has been estimated to be between 0 and 3%, and serum levels are practically undetectable (210, 213). Another important determinant of the effectiveness of these compounds is how well they mix with intestinal contents for proper physical presentation to the gut. When compared with the unesterified stanols, the fatty acid esters of stanols seem to mix more easily with the oil phase of the intestinal contents to interfere with cholesterol absorption and decrease plasma cholesterol concentrations (218). New techniques allowed the incorporation of plant stanols into food forms without affecting the texture and taste. Vanhanen et al. (220) showed that the ester form of sitostanol is more efficient than the crystalline form. Plant stanol esters dosages have ranged in various studies from 0.8 to 3.8 g/d. The data suggest that at least 1 g/d of stanol esters must be consumed to offer a good clinical response. In general, with consumption of 2–3 g/d of plant stanol esters, serum LDL cholesterol (LDL-C) levels were lowered between 10 and 15%. The introduction of sitosterol and sitostanol enriched margarines was an important step towards reduction of total- and LDL cholesterol (201). Several clinical studies have shown that sterol/stanol ester margarines are effective in lowering total- and LDL cholesterol (12-15).

Plant sterols/stanols lower total- and LDL cholesterol levels by displacing cholesterol from mixed micelles and thereby reducing intestinal cholesterol absorption (280). New steady state concentration of LDL cholesterol is obtained after 1 - 2 weeks and a daily intake of 2 g is sufficient to reach a maximum effect (280, 281).

After four weeks of treatment serum concentration of total- and LDL cholesterol decreased significantly in all subjects on average by 7 % ($p<0.05$) and 6% ($p<0.05$) respectively. Sitostanol ester margarine was tolerated well by all the subjects with no adverse events. The results from the present study indicate that the ratio of serum lathosterol to campesterol predicts the reduction of total- and LDL cholesterol during ingestion of sitostanol supplemented margarine in a dosage of 1 g b.i.d. In subjects with a low lathosterol to campesterol ratio (responder) serum total- and LDL cholesterol decreased significantly compared to subjects with a high ratio where no changes could be observed. The difference was not due to non-compliance of the participants. In all subjects an increase of the serum concentrations of campestanol and sitostanol was noted to the same extent. As a consequence of margarine treatment responders showed a higher decrease in the serum concentrations of the plant sterols campesterol and sitosterol and their respective ratios to serum cholesterol that reflect intestinal cholesterol absorption efficiency (25, 150).

Furthermore, responders showed a higher increase in the serum concentration of lathosterol indicating that the reduction of intestinal cholesterol absorption was more pronounced and was accompanied by a higher compensatory increase in hepatic cholesterol synthesis, since the serum concentration of lathosterol is an indicator of hepatic- and total cholesterol synthesis (26, 150, 152). In addition, responders showed higher pretreatment serum concentrations of plant sterols and lower levels of lathosterol. Thus, the data suggest that responders are characterized by high intestinal sterol absorption as indicated by high pretreatment serum concentrations of plant sterols and low hepatic cholesterol synthesis as indicated by low pretreatment serum lathosterol.

These results are supported by the data published by Gylling and co-workers (282) indicating that mildly hypercholesterolemic patients with low hepatic cholesterol synthesis and high intestinal absorption of cholesterol have the greatest benefit from stanol ester consumption.

These authors also reported that stanol ester feeding induced a reduction of serum cholesterol that is associated with a constant decrease in the ratio of plant sterols to cholesterol and with an increase in lathosterol. Similar results were obtained in the present study. Thus, the ratio of serum lathosterol to campesterol, which reflects the ratio of an individual variation of hepatic cholesterol synthesis to an individual intestinal cholesterol efficiency capacity, is a valuable marker to predict the outcome of therapy with sitostanol supplemented margarine. Similar results were obtained using the ratio of lathosterol to sitosterol.

Recently Plat et al. showed that subjects with the highest plant sterol concentrations had the largest reduction in serum plant sterol concentrations during consumption of plant stanol esters (283) similar to the observation in our study. They examined relationships between changes in serum plant sterol concentrations with ABCG5 and ABCG8 polymorphisms and looked for associations between ABCG5 and ABCG8 polymorphisms with changes in LDL cholesterol after consumption of plant stanol esters. They concluded that, the serum campesterol and sitosterol concentrations, as well as their changes after consumption of plant stanol enriched foods, are related to a variation in the ABCG8 gene, which is present in about 70% of the population. They found no relation with serum lipid and lipoprotein concentrations. This suggests that changes in the functionality of the ABCG5/G8 heterodimer, mainly affects plasma sterol concentrations, but not those of cholesterol. It may also be concluded from the present results that subjects not responding to stanol supplemented margarine have unchanged serum total- and LDL cholesterol levels because there was only a minor effect on plant sterols that indicate intestinal cholesterol absorption and the compensatory upregulation of hepatic cholesterol synthesis as indicated by slight changes of lathosterol or its ratio to cholesterol. The results propose that the most beneficial metabolic profile for dietary stanol ester margarine intake occurs in those individuals whose cholesterol absorption is high resulting in a low cholesterol synthesis. In summary, the ratio of serum lathosterol to campesterol served in this study as a predictive parameter for the response to sitostanol supplemented margarine. Subjects with a high intestinal sterol absorption and low hepatic cholesterol synthesis benefit most concerning reduction of serum total- and LDL cholesterol.

5.4 Response to ezetimibe (Responder study 2)

Ezetimibe is a compound of the 2-azetidinone class that has been shown to produce a marked inhibition of intestinal cholesterol absorption (up to 96%) in animals (246). Ezetimibe undergoes glucuronidation in the intestine and liver, and both the parent compound and its glucuronide localize to the brush border of the small intestine, where they block the absorption of dietary and biliary sources of cholesterol without affecting absorption of triglycerides, bile acids, or fat-soluble vitamins (227, 228). In patients with mild to moderate hypercholesterolemia, ezetimibe reduces plasma concentrations of sitosterol and campesterol (224) mostly by reducing the absorption of the plant sterols. Several clinical trials in humans have revealed LDL cholesterol–lowering effects in the range of 17% to 20% at a dose of 10 mg per day (225, 228, 246, 247). Sudhop et al. showed that ezetimibe inhibited cholesterol absorption by 54% relative to placebo which was was associated with a compensatory increase in cholesterol synthesis and led to a 22.3% reduction in plasma LDL cholesterol concentrations (224). Marked reductions in plasma concentrations of the noncholesterol plant sterols sitosterol and campesterol also were observed, suggesting that ezetimibe inhibited the absorption of these compounds as well.

The results from the Responder study 2 indicate that the ratio of serum lathosterol to campesterol predicts the reduction of total- and LDL cholesterol during treatment with ezetimibe 10 mg/day. Although after four weeks of treatment average serum total and LDL cholesterol decreased in all the subjects, the reduction was only significant in subjects with a low lathosterol to campesterol ratio (Responder).

As a consequence of ezetimibe treatment responders showed a higher decrease in the serum concentrations of the plant sterols campesterol and sitosterol and their respective ratios to serum cholesterol that reflect intestinal cholesterol absorption efficiency (25, 150). In addition, responders showed a higher increase in the serum concentration of lathosterol indicating that the reduction of intestinal cholesterol absorption was more pronounced and was accompanied by a higher compensatory increase in hepatic cholesterol synthesis, since the serum concentration of lathosterol is an indicator of hepatic- and total cholesterol synthesis (26, 150, 152).

Ezetimibe reduced plasma total cholesterol levels rapidly, with essentially the full reduction observed by the second week of treatment. Cholesterol synthesis increased, as evidenced by the 58 % rise in plasma ratio of lathosterol to cholesterol (Table 7). The rise in cholesterol synthesis is due to the decline in cholesterol absorption. Because the human body cannot synthesize plant sterols, progressive decline in these sterols in plasma reflects the reduced cholesterol absorption. In addition, responders showed higher pretreatment serum concentrations of plant sterols and lower levels of lathosterol. Thus, the data suggest that responders are characterized by high intestinal sterol absorption as indicated by high pretreatment serum concentrations of plant sterols and low hepatic cholesterol synthesis as indicated by low pretreatment serum lathosterol. These results indicate that subjects with low hepatic cholesterol synthesis and high intestinal absorption of cholesterol have the greatest benefit from ezetimibe. The results also show that ezetimibe induced a reduction of serum cholesterol that is associated with a constant decrease in the ratio of plant sterols to cholesterol and with an increase in lathosterol.

When five nonsynonymous polymorphisms at the ABCG5/8 loci (Q604E, D19H, Y54C, T400K, and A632V) in 15 subjects were examined, the results suggest that Q604E genotype in ABCG-5 may be associated with a greater total- and LDL cholesterol lowering response to ezetimib. Six of eight subjects in the responder group showed the allele 1 (CC), whereas the others were heterozygous (CG). In the non-responder group only two subjects were homozygous for allele 1 (CC), where as in four subjects the polymorphism was heterozygous (CG). Percent reductions in LDL cholesterol values of subjects having allele 1 (CC) were significantly more than those of CG heterozygous. In addition, significant difference was observed in percent reductions of total cholesterol. None of the remaining polymorphisms were significantly associated with treatment effects.

The results propose that the most beneficial metabolic profile for ezetimibe occurs in those individuals whose cholesterol absorption is high resulting in a low cholesterol synthesis. It appears that the differences in the responses might be due to the common Q604E polymorphism of the *ABCG5* gene, which was unevenly distributed in responders and non-responders. Responders were homozygous or heterozygous carriers of the wild-type Q604 allele; on the other hand non-responders carried predominantly the minor allele Q604E.

It is of note, that these findings are consistent with a previous study demonstrating an association between the wild-type Q604 allele and higher campesterol and sitosterol levels as well as lower cholestanol concentrations in serum (284). Similarly, in our study the carriers of wild-type Q604 allele (responders) had higher serum concentrations of campesterol and sitosterol. There was no contrast in genotype distributions of *ABCG8* SNPs among responders and non-responders. Recently Wang et al. (285) showed that a non-responder to ezetimibe carried two heterozygous non-synonymous polymorphisms in the *NPC1L1* gene. However, these have been observed in a single individual only. Notwithstanding, in addition to the present results, other genetic variations might also help to differentiate between responders and non-responders. Previous studies on transgenic animals suggest that by over expressing of ABCB5/8, biliary cholesterol is markedly increased, and that the absorption of cholesterol and plant sterols is markedly decreased (125). And the findings in patients with sitosterolemia and genetically–engineered animal models suggest that impairment of ABCG5/8 activities significantly reduces cholesterol synthesis in hepatocytes (286). Polymorphism in ABCG5 is associated with total- and LDL cholesterol level reductions is consistent with these observations, due to the fact that lower cholesterol synthesis rate and high absorption capacity results in a greater reduction in total- and LDL cholesterol with ezetimibe therapy.

Thus, the ratio of serum lathosterol to campesterol, which reflects the ratio of an individual variation of hepatic cholesterol synthesis to an individual intestinal cholesterol absorption capacity, is a valuable marker to predict the outcome of therapy with ezetimibe. The results propose that the most beneficial metabolic profile for ezetimibe occurs in those individuals whose cholesterol absorption is high, resulting in a low cholesterol synthesis. Interestingly, the results from ezetimibe study (Responder study 2) were similar with the results of margarine study (Responder study 1). This margarine study (Responder study 1) was performed two years before the beginning of the ezetimibe trial and the participants were completely different (287). Although the proposed mechanism of action of plant sterol and stanol supplemented dietary products is different from the one of ezetimibe, both treatment regimes affect cholesterol absorption and might involve so far unidentified common pathways of intestinal sterol absorption.

However, these conclusions need to be confirmed in other studies with other population groups. In future it would be worthwhile, if further studies are performed in large populations to confirm the present findings. In the Scandinavian Simvastatin Survival study it was observed that in poor responders to statin therapy the baseline ratios of the absorption markers to cholesterol were higher and those of the synthesis markers were lower when compared to good responders (103). It shows that subjects with high cholesterol absorption capacity and lower synthesis rate showed a poor response to simvastatin which is primarily an inhibitor of cholesterol synthesis. The Scandinavian Simvastatin Survival study complements our findings in the meaning that the characteristics of the responders to sitostanol ester margarines or ezetimibe are similar to those of poor responders to statins which act by inhibiting cholesterol synthesis. It would be interesting to check whether the non-responders to sitostanol ester margarines or ezetimibe will show good response to statins or a combination therapy with statins.

In order to rationalize the treatment methods of hypercholesterolemia, the responders and non-responders to treatments can be identified by simple method of measuring serum non-cholesterols (Lathosterol and Campesterol) using GC. After identifying a responder, the therapist is more motivated to continue the selected treatment. After identifying a non-responder, other alternative treatments should be started.

6. Summary

Plant stanol margarines are recommended as a lipid-lowering dietary supplement in the treatment of hypercholesterolemia. Parameters predicting the individual cholesterol-lowering effect have not been elucidated so far. Therefore, the responsiveness to sitostanol-supplemented margarine in a specially selected population was investigated. Eight subjects with the lowest and eight subjects with the highest ratios of lathosterol to campesterol in serum were included in the study from a total number of 137 male subjects with hypercholesterolemia. They received 1 g sitostanol-supplemented margarine b.i.d. for four weeks. Serum lipoproteins, the cholesterol precursor lathosterol, the plant sterols campesterol and sitosterol were measured. Subjects with a low ratio of lathosterol to campesterol had a significant decrease of serum total cholesterol (-14.2%; $p < 0.01$) and LDL cholesterol (-13.8%; $p < 0.01$; responder). In subjects with a high ratio there was no significant change in total cholesterol and LDL cholesterol (2.2 and 4.3%; non-responder). The ratio of serum lathosterol to campesterol predicts the reduction of total cholesterol and LDL cholesterol during administration of sitostanol-supplemented margarine in patients with mild hypercholesterolemia.

Ezetimibe is an cholesterol absorption inhibitor recommended for the treatment of hypercholesterolemia. In order to elucidate the parameters predicting the inter-individual cholesterol lowering effect, the responsiveness of LDL cholesterol lowering of ezetimibe in healthy volunteers was investigated and potential genetic associations between the serum surrogate markers and common polymorphisms of the *ABCG5/ABCG8* genes was assessed. One hundred twenty one male subjects were screened for their ratio of lathosterol to campesterol in serum. Eight subjects with the lowest and 7 subjects with the highest ratio participated in the study. They received ezetimibe 10 mg/day for 4 weeks. Serum lipoproteins, the cholesterol precursor lathosterol as well as the plant sterols campesterol and sitosterol were measured. Subjects with a low ratio of lathosterol to campesterol showed a good response to treatment with a significant decrease of serum total (-25 %; $p< 0.0001$) and LDL cholesterol (-33 %; $p< 0.0001$). In contrast, subjects with a high ratio did not display a significant change in serum total or LDL cholesterol (-9%). The Q604E polymorphism of the *ABCG5* gene was unevenly distributed in responders and non-responders. The responders were homozygous or heterozygous carries of the wild-type Q604 allele, whereas non-responders carried predominantly the minor allele Q604E. The response to ezetimibe in

reduction of total and LDL cholesterol is predicted by the ratio of lathosterol to campesterol in serum and non-response might be due to the predominance of minor allele Q604E polymorphism in *ABCG5* gene.

In both the studies the ratio of serum lathosterol to campesterol served as good predictor for responsiveness to sitostanol ester margarine (Responder Study 1) and ezetimibe (Responder Study 2) in lowering cholesterol.

7. References

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8. Publications originating from this thesis

1. Thuluva SC, Igel M, Giesa U, Lutjohann D, Sudhop T, von Bergmann K. Ratio of lathosterol to campesterol in serum predicts the cholesterol-lowering effect of sitostanol-supplemented margarine. *Int J Clin Pharmacol Ther*. 2005;43(7):305-10.

2. Thuluva SC, Igel M, Lutjohann D, Lammert F, von Bergmann K. Reduction of LDL-Cholesterol by Ezetimibe is predicted by the Ratio of Lathosterol to Campesterol in Serum and is associated with ABCG5 Polymorphism (submitted for publication).

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Jai Hind.

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