METABOLISM OF SQUALENE IN TRIGLYCERIDE-RICH LIPOPROTEINS IN HUMANS

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Academic dissertation

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To my family

CONTENTS

ABBREVIATIONS

ACAT	Acylcoenzyme A:cholesterol acyltransferase
ANOVA	Analysis of variance
Apo	Apolipoprotein
AUC	Area under curve
AUIC	Area under incremental curve
BMI	Body mass index
CETP	Cholesteryl ester transfer protein
d	Density
GLC	Gas-liquid chromatography
HDL	High-density lipoprotein
HMG-CoA	3-hydroxy-3-methylglutaryl coenzyme A
HPLC	High-pressure liquid chromatography
HSPG	Heparan sulphate proteoglycan
IDL	Intermediate-density lipoprotein
LCAT	Lecithin:cholesterol acyltransferase
LDL	Low-density lipoprotein
LPL	Lipoprotein lipase
LRP	LDL receptor-related protein
PLTP	Phospholipid transfer protein
SR-BI	Scavenger receptor class B type I
VLDL	Very low-density lipoprotein

LIST OF ORIGINAL PUBLICATIONS

This thesis is based on the following original articles, which are referred to in the text by their Roman numerals. In addition, some unpublished data are presented.

I Relas H, Gylling H, Rajaratnam RA, Miettinen TA. Postprandial retinyl palmitate and squalene metabolism is age dependent. J Gerontol Biol Sci 2000;55:B515-B521.

II Relas H, Gylling H, Miettinen TA. Dietary squalene increases cholesterol synthesis measured with serum non-cholesterol sterols after a single oral dose in humans. Atherosclerosis 2000;152:377-383.

III Relas H, Gylling H, Miettinen TA. Fate of intravenously administered squalene and plant sterols in human subjects. J Lipid Res 2001;42. In press.

IV Relas H, Gylling H, Miettinen TA. Effect of stanol ester on postabsorptive squalene and retinyl palmitate. Metabolism 2000;49:473-478.

V Gylling H, Relas H, Miettinen TA. Postprandial vitamin A and squalene clearances and cholesterol synthesis off and on lovastatin treatment in type III hyperlipoproteinemia. Atherosclerosis 1995;115:17-26.

ABSTRACT

Squalene, a non-steroid obligate precursor of cholesterol, is derived from both de novo synthesis from acetyl coenzyme A and from diet. Little is known of the postprandial metabolism of squalene. However, recent evidence exists that squalene may play a role in the development of atherosclerosis such that the removal of postprandial squalene was retarded in women with coronary artery disease. Accordingly, the kinetics and metabolism of squalene in the postprandial state and after intravenous administration in humans were studied.

An oral fat load test with 0.5 g of squalene was performed in six young (22-25 years) and eight old (78-79 years) males to learn whether postprandial squalene metabolism is affected by age. The acute effects of a single dose of squalene on cholesterol synthesis as measured by cholesterol precursor sterols in postprandial lipoproteins were studied in 16 subjects who underwent two oral fat challenges in random order, one with 0.5 g of squalene supplement and the other without. Removal of squalene from chylomicrons was investigated in six subjects by giving an intravenous bolus injection of artificial chylomicron-like lipid emulsion (Intralipid^{R}) enriched with squalene. To learn whether inhibition of cholesterol absorption with a subsequent increase in cholesterol synthesis reduces postprandial squalene concentrations, three oral fat challenges with 0.5 g of squalene in each were performed in 11 mildly hypercholesterolemic subjects before and during stanol ester (3g/day) consumption. The latter two tests were performed during stanol ester consumption with and without a stanol ester (1 g) supplement in the test meal. To explore whether inhibition of cholesterol synthesis influences postprandial squalene metabolism, oral fat loads with 1 g of squalene were performed before and during lovastatin (40 mg/d) treatment in five type-III hyperlipoproteinemic subjects.

A prolonged increase in squalene concentrations up to 12 hours was seen in the aged in contrast to the decrease after 9 hours which occurred in the young, reflecting reduced removal of postprandial remnant lipoproteins in the aged. A single squalene dose raised postprandial squalene concentrations in chylomicrons, very low-density lipoproteins (VLDL), and intermediate-density lipoproteins but not in low-density lipoproteins and high-density lipoproteins. After 9 to 12 hours, the lathosterol-to-campesterol ratio increased significantly in the chylomicrons, VLDL, and VLDL infranatant, compared to ratios for fat loads without squalene. This suggests that cholesterol synthesis was increased. Obviously, some squalene was already converted to cholesterol in the intestinal mucosa, probably inhibiting endogenous synthesis of squalene temporarily. After intravenous injection, the clearance of squalene, plant sterols, and triglycerides was monoexponential. The half-life of chylomicron squalene was 74±8 min, which was longer than those of plant sterols (15-36 min) and triglycerides (17±2 min). The half-life of squalene correlated positively with fasting chylomicron concentrations of squalene, suggesting that its

removal is retarded by high fasting concentrations. The injection did not increase squalene concentrations in lipoproteins other than chylomicrons and VLDL. Inhibition of cholesterol absorption efficiency by stanol ester consumption did not affect fasting serum squalene concentrations, but squalene peak times in chylomicrons and VLDL, as well as squalene peak concentrations and areas under incremental curves in VLDL were reduced, suggesting that VLDL removal was enhanced. Inhibition of cholesterol synthesis by lovastatin in type III hyperlipoproteinemic subjects reduced fasting concentrations of squalene, but despite normalized fasting serum cholesterol and triglyceride concentrations, did not reduce postprandial squalene concentrations.

In conclusion, that orally ingested and intravenously administered squalene labeled triglyceride-rich lipoproteins, suggests that postprandial squalene concentrations reflect postprandial chylomicron and remnant lipoprotein metabolism. A single oral dose of squalene increased cholesterol synthesis at 9 to 12 hours after the ingestion. Inhibition of cholesterol absorption efficiency inhibited the accumulation of postprandial lipoproteins, whereas inhibition of cholesterol synthesis did not influence postprandial lipoprotein clearance, at least in type III hyperlipidemia. These studies have increased knowledge of squalene metabolism in triglyceride-rich lipoproteins, and also given new insights into postprandial lipoprotein metabolism during either inhibition of cholesterol absorption or its synthesis.

1. INTRODUCTION

Squalene is a naturally occurring non-steroid intermediate of cholesterol synthesis. It is widely distributed in nature and is found in plant materials and various human tissues (Liu et al. 1976, Tilvis and Miettinen 1980). Because squalene is an intermediate of cholesterol synthesis that is carried in serum by lipoproteins (Goodman 1964), information on cholesterol metabolism can be obtained by measuring its serum levels (Miettinen 1969, Nestel and Kudhodkar 1975). Serum levels of cholesterol precursor sterols, however, reflect cholesterol synthesis more consistently than do serum levels of squalene (Miettinen 1970, Björkhem et al. 1987), but evidence shows that squalene reflects rapid changes in cholesterol synthesis (Gylling et al. 1998) including those that occur in the circadian cycle (Miettinen 1982a). Newly absorbed squalene is carried in chylomicrons, and its postprandial fate resembles that of retinyl palmitate (Gylling and Miettinen 1994a), an indirect marker of postprandial lipoproteins (Hazzard and Bierman 1976).

Squalene content varies widely among different foodstuffs, the richest in squalene being olive oil (Liu et al. 1976). Serum levels of squalene reflect its dietary intake (Liu et al. 1976), and daily squalene consumption increases cholesterol synthesis as measured by cholesterol precursor sterols (Miettinen and Vanhanen 1994a). Results are contradictory as to the effects of dietary squalene on the serum and low-density lipoprotein (LDL) cholesterol level (Strandberg et al. 1990, Miettinen and Vanhanen 1994a, Chan et al. 1996), a high level of which is a risk factor for coronary artery disease. However, an increased squalene level has been found in atheromatous plaques (Lewis 1975a), and more lately in sera of women with coronary artery disease (Rajaratnam et al. 1999a, Rajaratnam et al. 2000). Accumulation of postprandial lipoproteins has been suggested to be a risk factor for coronary artery disease, as well (Moreton 1947, Zilversmit 1979). Few studies have measured postprandial squalene concentrations, but reveal an association between increased postprandial accumulation of squalene-containing lipoproteins following a squalene-supplemented oral fat load and coronary artery disease (Rajaratnam et al. 1999b).

An elevated level of serum cholesterol can be treated in several ways, when diet is insufficient to keep it at the recommended level. Statins, inhibitors of cholesterol synthesis, have become the drugs of choice during the past two decades (Knopp 1999). It is also possible to improve postprandial lipemia by statin treatment (Weintraub et al. 1989, Cianflone et al. 1990), but effects of statins on postprandial squalene metabolism remain unknown. The use of plant sterols and stanols, inhibitors of cholesterol absorption (Becker et al. 1993), have, since the mid-nineties, attracted renewed interest as lipid-lowering agents (Miettinen et al. 1995, Nguyen 1999). It is as yet unknown whether plant sterol treatment affects postprandial lipid metabolism.

Accordingly, the present work concentrated on the postprandial aspects of squalene metabolism. A single dose of squalene comparable to daily intake from a squalene-rich diet was administered with an oral fat challenge, after which metabolism of postprandial squalene was investigated in subjects of different ages, and the acute effects of the squalene dose on cholesterol synthesis were studied. Metabolism of postprandial squalene was studied by two interventions, in which cholesterol metabolism had been influenced by either inhibited cholesterol absorption or by inhibited synthesis. In addition, the kinetics of squalene was investigated after intravenous administration of squalene in a lipid emulsion which mimics endogenous chylomicrons.

2. REVIEW OF THE LITERATURE

2.1. Squalene - structure and occurrence in nature

Squalene ($C_{30}H_{50}$, mol wt 411) is a polyunsaturated non-steroid hydrocarbon intermediate (Fig 1) of cholesterol synthesis preceding the formation of the steroid nucleus (Langdon and Bloch 1953a, Langdon and Bloch 1953b). It is a non-polar compound, and at room temperature, a colorless free-flowing oil. Named by Tsujimoto (1916), squalene was first isolated from shark liver oil; it is widely distributed in nature, identified in leaves (Alam et al. 1962), fungi (Wirth et al. 1961), and in plant oils (Liu et al. 1976). Olive oil is especially rich in squalene compared to other common human food fats and oils, with a concentration of 2 to 7 mg/g of oil (Liu et al. 1976).

Liu et al. (1976) have examined the squalene concentrations of various human tissues, and the highest levels were detected in skin (475 µg/g dry weight) and adipose tissue (275 µg/g), while moderate concentrations were found in organs with high cholesterol synthesis capacity such as liver (75 µg/g) and small intestine (42 µg/g). Human skin surface lipids contain 6 to 12% squalene (Boughton et al. 1957, Greene et al. 1970). Squalene has also been detected in human and rat blood plasma, and is carried in lipoproteins (Goodman 1964).

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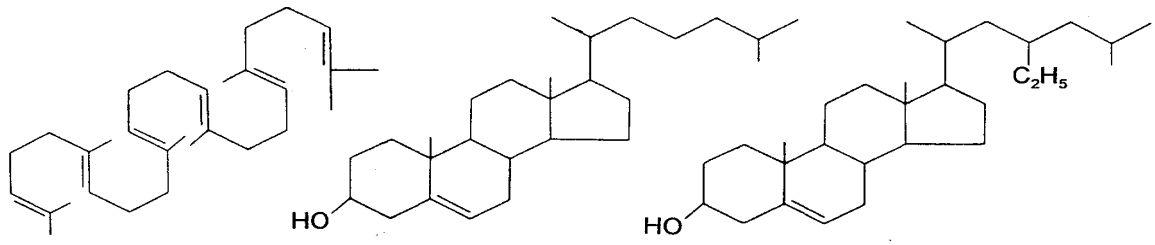

Fig 1. Structures of squalene (A), cholesterol (B), and sitosterol (C).

2.2. Classification of lipoproteins

Since lipids including cholesterol, squalene, cholesterol precursor sterols, triglycerides, and phospholipids are water-insoluble, they are carried by lipoproteins in plasma. A lipoprotein is a complex of proteins and lipids with a characteristic density, size, and chemical composition. Lipoproteins transport lipids in blood

between the sites of their absorption, the liver and various tissues that utilize lipids. The most common classification of lipoproteins is based on the criterion of density. The major lipoprotein classes are chylomicrons, very low-density lipoproteins (VLDL), intermediate-density lipoproteins (IDL), LDL, and high-density lipoproteins (HDL) (Table 1). Chylomicrons and VLDL are, due to their high triglyceride content, called triglyceride-rich lipoproteins.

Table 1. Characteristics of lipoproteins.

	CM	VLDL	IDL	LDL	HDL
Density (g/ml)	<0.95	0.95-1.006	1.006-1.019	1.019-1.063	1.063-1.210
Particle diameter (nm)	75-1200	30-80	25-35	18-25	5-12
Total protein (%)	1.5-2.5	5-10	15-20	20-25	40-55
Total lipid (%)	97-99	90-95	80-85	75-80	50-55
Phospholipids (%[a])	7-9	15-20	22	15-20	20-35
Esterified cholesterol (%[a])	3-5	10-15	22	35-40	12
Free cholesterol (%[a])	1-3	5-10	8	7-10	3-4
Triglycerides (%[a])	84-89	50-65	30	7-10	3-5
Apolipoproteins	B-48, AI, AII, C-I, C-II, C-III, E	B-100, C-I, C-II, C-III, E	B-100, C-II, C-III, E	B-100, (C-I, C-III)	A-I, A-II, C-I, C-II, C-III, D, E

[a] Percent composition of lipid fraction. CM, chylomicrons.
Modified from: Gotto et al. 1986, Shultz 1986.

Chylomicrons are secreted by the small intestine and are the largest of the lipoproteins. Apolipoprotein (apo) B-48, a low molecular-weight form of apo B produced by the intestine, is their major structural protein (Kane et al. 1980). In addition, apolipoproteins A are synthesized by the intestine and are associated with chylomicrons (Gotto et al. 1986). Chylomicrons also contain apolipoproteins C and E (Table 1). VLDL particles are synthesized by the liver and contain apo B-100 as their major structural protein (Gotto et al. 1986). VLDL contain also apolipoproteins C and E (Table 1) and are generally called pre-β lipoproteins based on their electrophoretic mobility. Lipids synthesized by the liver are carried by VLDL to tissues (Fig 2). IDL (slow pre-β lipoproteins) and LDL (β-lipoproteins) are products of VLDL catabolism (Sigurdsson et al. 1975), and become depleted of apolipoproteins C and E. HDL, also called α-lipoproteins, play an important role in reverse cholesterol transport, delivering extrahepatic cholesterol to the liver (Fielding and Fielding 1995). The major HDL proteins, apo AI and apo AII, are elaborated in precursor form in the liver and intestine (Wu and Windmueller 1979).

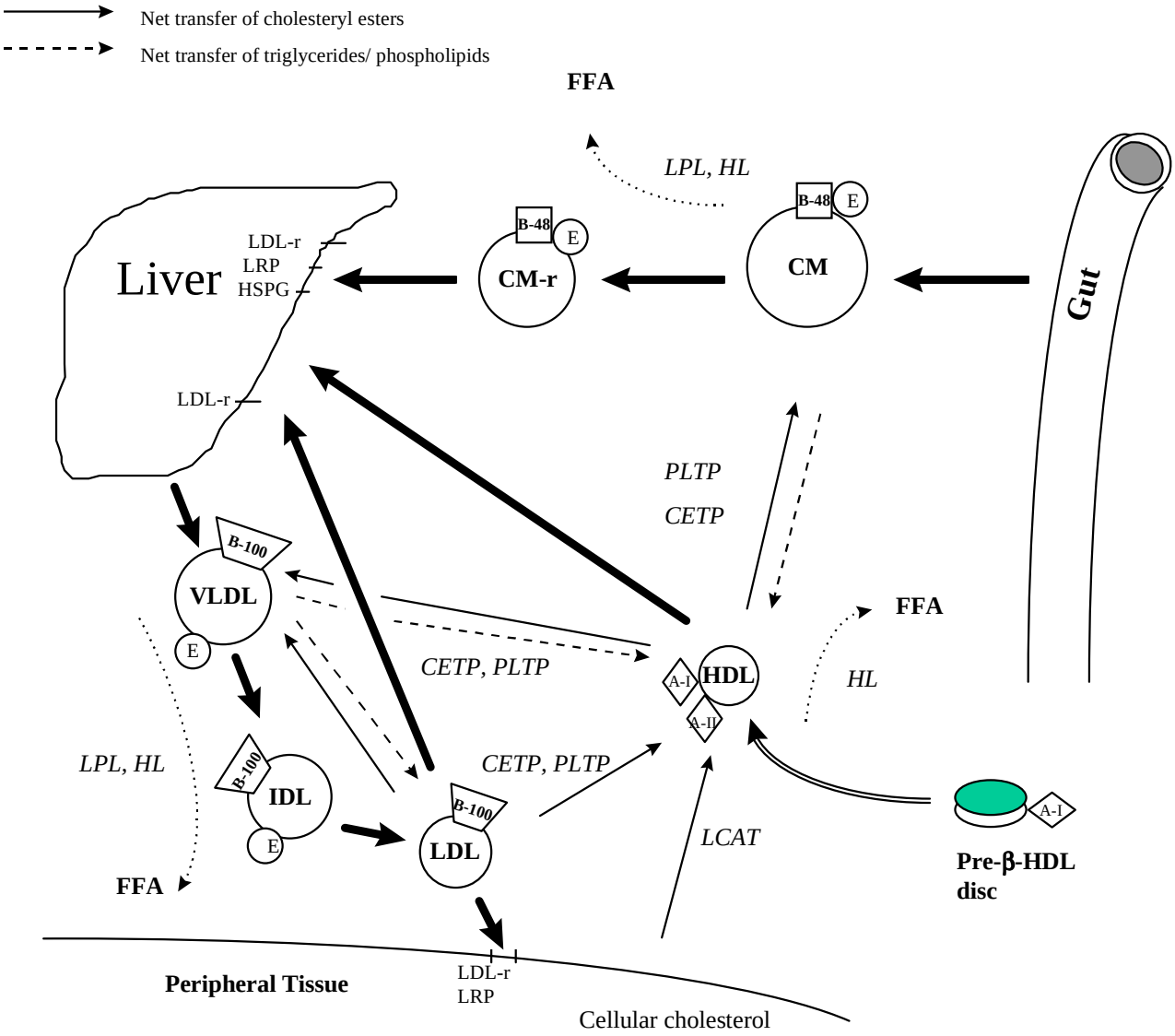

Fig 2. Schematic illustration of lipoprotein metabolism. CETP, cholesteryl ester transfer protein; CM, chylomicrons; CM-r, chylomicron remnants; FFA, free fatty acids; HL, hepatic lipase; HSPG, heparan sulphate proteoglycans; LDL-r, LDL receptor; LPL, lipoprotein lipase; LRP, LDL receptor-related protein; PLTP, phospholipid transfer protein. A-I, A-II, B-48, B-100, and E refer to the respective apolipoproteins.

These discoidal lipid-poor particles (nascent HDL) contain mainly proteins and phospholipids, and are transformed by enzymatic and transfer activities of plasma into mature spherical HDL (Hamilton et al. 1976).

Lipid transfer proteins in plasma mediate the transfer and exchange of neutral lipids and phospholipids between the plasma lipoproteins (Fig 2). Lecithin:cholesterol acyltransferace (LCAT) catalyzes the formation of cholesteryl esters in human plasma and increases HDL cholesteryl ester content (Glomset 1968), and is also capable of esterifying sitosterol (Nordby and Norum 1975). Cholesteryl ester

transfer protein (CETP) facilitates the exchange of neutral lipids between HDL and triglyceride-rich lipoproteins (Tall 1995). Phospholipid transfer protein (PLTP), which facilitates the transfer and exchange of phospholipids but not the exchange of neutral lipids (Tollefson et al. 1988), has been suggested to enhance the transfer of unesterified cholesterol from phosphatidylcholine and cholesterol-containing bilayer vesicles to HDL (Nishida and Nishida 1997), suggesting that transfer of unesterified sterols from triglyceride-rich lipoproteins to HDL is possible. However, it is unknown whether lipoprotein squalene is influenced by lipid transfer proteins.

2.3. Metabolism of squalene, cholesterol, and non-cholesterol sterols

2.3.1. Lipid absorption

2.3.1.1. Lipid absorption from intestinal lumen to enterocyte

More than 95% of dietary fat is long-chain triglycerides, and the remaining fat consists of phospholipids (intake 2-4 g/day), free fatty acids, cholesterol (intake 200-600 mg/day), and fat-soluble vitamins (Alfin-Slater and Aftergood 1980). Intake of dietary squalene is approximately 24 mg/day in the United States (Liu et al. 1976), but a diet rich in fish, vegetables, and especially olive oil could comprise as much as 500 to 1000 mg squalene daily. The main events in intestinal fat digestion and absorption are emulsification, hydrolysis of fatty acid ester bonds, dispersion of lipolytic products in bile acid micelles, and the absorption taking place in the jejunum. Hydrolysis of dietary fat begins by the action of lingual and gastric lipases, and is continued in the small intestine by the pancreatic lipases. Cholesterol esterase, a lipolytic enzyme capable of hydrolyzing triglycerides, phospholipids, and cholesteryl esters, mediates absorption of cholesteryl esters but does not play a primary role in free cholesterol absorption (Howles et al. 1996). Bile acids are needed in the absorption of cholesterol (Siperstein et al. 1952) to form micelles (Carey and Small 1978), but cholesterol in bile may also occur in phospholipid vesicles independently of bile salts (Somjen and Gilat 1983).

The belief has been that only unesterified cholesterol is absorbed (Vahouny et al. 1964), but more recent evidence indicates that long-chain cholesteryl esters may also be taken up as such by the brush border membrane (Compassi et al. 1995). It was also suggested earlier that the uptake of cholesterol to enterocytes is a passive diffusion process. There is, however, evidence that mucosal uptake of cholesterol is an active process (Thurnhofer and Hauser 1990) related to cholesterol transfer protein in enterocytes (Thurnhofer et al. 1991). Scavenger receptor class B type I (SR-BI), located on the brush border membrane, facilitates the mucosal uptake of cholesterol (Hauser et al. 1998) and probably also other sterols including the esterified ones (Compassi et al. 1995), but data on the role of SR-BI in the mucosal

lipid uptake is still limited. Cholesterol is re-esterified by acylcoenzyme A:cholesterol acyltransferase (ACAT) in the enterocyte (Norum et al. 1979, Helgerud et al. 1981), and is subsequently secreted into the blood circulation in chylomicrons (Goodman 1962). ACAT also has the capacity to esterify sitosterol, but only a limited one (Field and Mathur 1983), which may be a factor contributing to the poor absorption of sitosterol.

Marked differences occur in the intestinal absorption of cholesterol and other lipids. The absorption percentage for dietary triglycerides is more than 95% (Carey et al. 1983), that for cholesterol is up to 50% (Hellman et al. 1955, Grundy and Ahrens 1969, Bosner et al. 1999), and for the plant sterols campesterol, 10% (Heinemann et al. 1993) and sitosterol, 5% (Gould et al. 1969, Salen et al. 1970). Interindividual differences in cholesterol absorption are wide, ranging from 20% to 80% (Sehayek et al. 1998, Bosner et al. 1999).

Like other lipids, squalene is probably solubilized in micelles before being taken up by the enterocytes. Since squalene does not have any hydroxyl groups, and therefore is not esterified to fatty acids, it is not influenced by the specific hydrolytic actions of esterases. It is possible that the mucosal uptake of squalene is a passive diffusion process. However, present knowledge of the mechanisms of squalene absorption is limited. In an experimental rat study, the absorption of dietary ^{3}H-squalene was 42%, and that of ^{14}C-cholesterol 48% (Tilvis and Miettinen 1983b). In human subjects, fecal analyses revealed that 60% of squalene was absorbed during squalene feeding (0.9 g/day for 7 to 30 days) (Strandberg et al. 1990), and that 85% was absorbed during squalene feeding of 1 g/day for 9 weeks (Miettinen and Vanhanen 1994a). In general, squalene absorption seems to be efficient in humans but this has not been widely studied.

2.3.1.2. Methods to study sterol absorption

The absorption of cholesterol has been extensively studied in humans. One possibility to study sterol absorption is the use of radioactive isotopes. Accordingly, in the plasma isotope ratio method (Samuel et al. 1978), oral and intravenous ^{3}H- and ^{14}C-cholesterol are used, whereas the continuous isotope feeding method (Crouse and Grundy 1978) is based on oral administration of ^{14}C-cholesterol and ^{3}H-β-sitosterol. Methods to study cholesterol absorption by using stable isotopes have been introduced more recently (Bosner et al. 1993, Lütjohann et al. 1993). Stable isotopes give the benefit of avoiding radioactive isotopes, but the method is laborious. Serum levels of campesterol and sitosterol, and their ratios to cholesterol, are positively correlated with absorption of cholesterol (Tilvis and Miettinen 1986, Miettinen et al. 1990). In addition, serum concentration of cholestanol, a metabolite of cholesterol, is also correlated with the absorption efficiency of cholesterol (Miettinen et al. 1989), suggesting that these serum sterols can be used as indicators

of cholesterol absorption. Accordingly, measurement of serum plant sterols or cholestanol by gas-liquid chromatography (GLC) provides a less laborious means to study cholesterol absorption than methods based on the use of radioactive or stable isotopes.

2.3.1.3. Factors affecting sterol and squalene absorption

Cholesterol absorption is more widely studied than is absorption of squalene or non-cholesterol sterols. Several genetic, physiological, and dietary factors affect cholesterol absorption. The apo E phenotype is associated with the intestinal absorption efficiency of cholesterol. The highest absorption efficiency is related to apo E4/4 and E4/3 phenotypes (Kesäniemi et al. 1987). However, the apo E phenotype was not related to cholesterol absorption efficiency during a diet of low fat and cholesterol content (Miettinen et al. 1992a). Cholesterol absorption efficiency, on the other hand, is positively correlated with serum total and LDL cholesterol levels (Kesäniemi and Miettinen 1987, Miettinen and Kesäniemi 1989), thus being one determinant of serum cholesterol levels.

Whether cholesterol absorption is affected by age is not completely clear. No age-related differences existed in subjects on a diet relatively low in cholesterol (Kempen et al. 1991, Bosner et al. 1999), whereas lower absorption efficiency was observed in 75-year-old subjects as compared to 50-year-old subjects (Gylling et al. 1994). One explanation for this discrepancy could be a higher cholesterol intake for the subjects in the latter study. However, while the mass of dietary cholesterol absorbed increases with the intake (Quintao et al. 1971, Miettinen and Kesäniemi 1989, Sehayhek et al. 1998, Ostlund et al. 1999), a high cholesterol intake compared to low has no effect on the absorption efficiency of dietary cholesterol (McNamara et al. 1987, Miettinen and Kesäniemi 1989, Sehayhek et al. 1998), or it reduces it (Ostlund et al. 1999). High cholesterol intake is thought to reduce absorption efficiency because of the limited capacity of micellar solubilization in the presence of excessive amounts of sterol molecules.

Reduced cholesterol absorption efficiency has been documented in bile acid malabsorption (Hofmann 1967), in obesity as a result of increased cholesterol synthesis and neutral sterol excretion through bile (Miettinen and Kesäniemi 1989, Miettinen and Gylling 2000), and in diabetes mellitus (Gylling and Miettinen 1997, Simonen et al. 1999). In addition, shortened small bowel transit time reduces cholesterol absorption (Ponz de Leon et al. 1982) by reducing the exposure time for absorption.

Several dietary supplements and pharmacological agents reduce cholesterol absorption. Since the early 1950s, plant sterols have been known to lower serum cholesterol levels (Pollak 1953, Lees et al. 1977), probably by interfering the

micellar solubility of cholesterol (Ikeda and Sugano 1983). Sitostanol, a saturated derivative of sitosterol, is virtually unabsorbable and reduces cholesterol absorption more effectively than does sitosterol, with a 50% reduction in cholesterol absorption at low doses and up to a 85% decrease at high doses (Heinemann et al. 1991). Sucrose polyester (Olestra^{R}) is a mixture of transesterified sugars that is unabsorbable (Mattson and Volpenhein 1972). Substitution of a proportion of dietary fat by sucrose polyester reduced cholesterol absorption by up to 40% (Crouse and Grundy 1979) and serum level of LDL cholesterol by 26% (Grundy et al. 1986). Resins such as cholestyramine and cholestipol bind to bile acids in the intestinal lumen irreversibly, and thereby enhance their fecal elimination. As a consequence, a reduction of 38% in cholesterol absorption can be achieved by cholestyramine (McNamara et al. 1980). Fibrates (McNamara et al. 1980) and 3-hydroxy-3-methylglutaryl coenzyme A (HMG-CoA) reductase inhibitors (Miettinen 1991) have been reported to have minor inhibitory effects on cholesterol absorption. It has been speculated that low cholesterol content in the enterocytes and subsequently reduced ACAT activity are the mechanisms of action of HMG-CoA reductase inhibitors in reducing cholesterol absorption (Miettinen 1991). In addition, it has been shown that neomycin (Sedaghat et al. 1975), ketoconazole (Miettinen 1988), ursodeoxycholic acid (Fromm 1984), and tetrahydrolipstatin (Borgström 1988) reduce cholesterol absorption. Squalene feeding, on the other hand, has no consistent effect on cholesterol absorption efficiency (Strandberg et al. 1990).

Little is known about the regulation of squalene absorption, and the same is true for plant sterols. In sitosterolemia, an inherited condition, serum plant sterol levels far exceed normal levels, and the absorption of sitosterol is increased 5 to 10 times; decreased biliary secretion of plant sterols further enhances the development of high serum plant sterol levels (Bhattacharya and Connor 1974, Miettinen 1980). Both squalene (Salvioli et al. 1984, Nosaka et al. 1985) and plant sterols (Hay and Carey 1990) are secreted in bile, and their serum levels are thus partly regulated by biliary excretion. Accordingly, treatment with cholestyramine, which binds bile acids, has succeeded in reducing plant sterol levels by 45% in phytosterolemia (Salen et al. 1985). Serum plant sterol levels are reduced in celiac disease, a condition characterized by reduced cholesterol absorption, and their levels are normalized by adequate treatment with a gluten-free diet (Vuoristo et al. 1988). It is evident that dietary sitosterol raises serum sitosterol concentrations, whereas the saturated derivative sitostanol reduces both serum sitosterol and campesterol concentrations (Vanhanen and Miettinen 1992, Hallikainen et al. 2000). This suggests that serum levels of plant sterols reflect their dietary intakes, as observed, for instance, in vegetarians (Vuoristo and Miettinen 1994), but dietary plant stanols interfere with the absorption of plant sterols.

2.3.2. Postprandial lipids

2.3.2.1. Chylomicrons and chylomicron remnants

Chylomicrons, which contain newly absorbed lipids including cholesterol, triglycerides, squalene, and fat-soluble vitamins, appear in plasma after a fatty meal (Fig 2). In the circulation, the endothelial lipoprotein lipase (LPL) hydrolyzes chylomicron triglycerides, and chylomicrons thus become cholesterol-enriched remants (Redgrave 1970). One molecule of apo B-48 is present in one chylomicron particle (Phillips et al. 1997), and apo B-48 molecules are not exchanged between the different lipoproteins (Stalenhoef et al. 1984). The small intestine, however, secretes lipoproteins directly into the VLDL density class, especially in the fasting condition (Ockner et al. 1969), and some evidence exists that the intestine is capable of producing apo B-100, as well (Hoeg et al. 1990), an apolipoprotein characteristic of VLDL of hepatic origin (Gotto et al. 1986). The impact of intestinal apo B-100 in contributing to the magnitude of postprandial lipemia remains unknown.

Hydrolyzed remnants of chylomicrons contain ingested cholesterol, and are taken up by the liver (Sherrill and Dietchy 1978). This uptake involves LDL receptor, LDL receptor-related protein (LRP) and heparan sulfate proteoglycans (HSPG) via apo E, a process, which has been reviewed recently by Mahley and Ji (1999). Chylomicrons and chylomicron remnants of intestinal origin and VLDL synthesized by the liver share the same lipolytic pathways, which, after a fatty meal become saturated (Brunzell et al. 1973). This explains why up to 80% of the increase in postprandial particle number is due to apo B-100-containing VLDL particles (Cohn et al. 1993, Karpe et al. 1993b, Schneeman et al. 1993).

A hypothesis of postprandial lipemia as a risk factor in the development of atherosclerosis was formulated over 20 years ago (Zilversmit 1979). Abnormal postprandial accumulation of intestinally derived apo B-48-containing lipoproteins has been associated with coronary artery disease (Karpe et al. 1993a, Karpe et al. 1994). Since the apo B-48 and apo B-100-containing lipoprotein particles overlap in size and compete for the same lipolytic pathway (Cohn et al. 1988b, Cohn et al. 1993, Karpe et al. 1993b, Schneeman et al. 1993), it is possible that the accumulation of the postprandial apo B-100 triglyceride-rich particles of hepatic origin may also be of significance in developing atherosclerosis (Gianturco and Bradley 1988). In fact, evidence exists that postprandial dyslipidemia in coronary artery disease may be related even more closely to postprandial accumulation of the endogenous apo B-100-containing lipoproteins of hepatic origin (Sharrett et al. 1995, Karpe 1999, Karpe et al. 1999).

2.3.2.2. Squalene and postprandial lipoproteins

Lewis (1975b) reported that after a meal containing 1.5 mg of squalene the newly absorbed squalene was found in human subjects' LDL, with maximum serum squalene concentration reached at 3 hours in three of five. However, this small dose of squalene caused only minor increases in serum squalene, and unfortunately, squalene was not studied in any triglyceride-rich lipoproteins. Thus, the fate of postabsorptive squalene remained poorly studied in humans. It was later found that squalene peak concentrations were reached at 8.2±0.3 hours after a fat meal containing 1.0 g of squalene; the postprandial squalene was transported in chylomicrons and in VLDL (Gylling and Miettinen 1994a) producing curves resembling the postprandial vitamin A concentration curves widely studied (Goodman et al. 1966, Blomhoff et al. 1991). After a test meal containing squalene and retinyl palmitate, however, as measured in non-triglyceride-rich lipoproteins, postprandial squalene peaked almost 3 hours earlier than did retinyl palmitate; and in contrast to the palmitate, the 24 hour squalene concentrations returned to their baseline level (Rajaratnam et al. 1999b). This suggests that differences exist in the fates of postprandial squalene and retinyl palmitate.

2.3.2.3. Measurement of chylomicron metabolism by oral fat loads

Retinyl esters have been widely used as markers of chylomicrons and chylomicron remnants in human studies (Hazzard and Bierman 1976, Weintraub et al. 1987a, Weintraub et al. 1987b, Groot et al. 1991, Cohen and Grundy 1992, Hadjadj et al. 1999, Weintraub et al. 1999). Dietary vitamin A (retinol) is taken up by enterocytes and incorporated into the core of chylomicrons as retinyl esters (Goodman et al. 1965, Goodman et al. 1966) and is carried to the liver in chylomicron remnants. There is evidence that in humans 6% or less of dietary retinyl esters appear in LDL after 6 to 12 hours following ingestion (Berr and Kern 1984, Wilson et al. 1983), and the in vitro transfer of retinyl palmitate between chylomicrons and other lipoproteins has been insignificant (Berr and Kern 1984). On the other hand, up to 25% of postprandial retinyl palmitate has been detected in the apo B-100 triglyceride-rich lipoproteins (Cohn et al. 1993), suggesting that plasma retinyl palmitate is not tightly associated with lipoproteins of intestinal origin. Determination of apo B-48 has become a popular means to study intestinally derived postprandial lipoproteins (Cohn et al. 1988b, Karpe et al. 1993b). Determination of apo B-48, however, is laborious. Postprandial concentration curves of dietary squalene resemble closely those of vitamin A (Gylling and Miettinen 1994a), which suggests that squalene could be used as a marker for postprandial lipoproteins. In fact, squalene labeled postprandial lipoproteins more specifically than did vitamin A when compared to postprandial apo B-48 levels (Rajaratnam et al. 1999b). Thus, squalene may be a better marker than vitamin A to detect intestinally derived lipoproteins. However,

whether there is a substantial exchange of squalene between the lipoprotein fractions has not been studied.

2.3.2.4. Measurement of chylomicron metabolism by chylomicron-like emulsions

Since the oral fat tolerance tests have certain disadvantages due to variation in intestinal absorptive capacities, intravenously administered labeled lipoprotein particles may be able to give more accurate results as to the metabolism of triglyceride-rich lipoproteins. Thus, models to study lipoprotein clearance by intravenous chylomicron-like emulsions have been under study since the sixties (Hallberg 1965, Carlson and Hallberg 1963, Carlson and Rössner 1972, Nilsson and Zilversmit 1972). A commercially available lipid emulsion, Intralipid^{R}, has been used in several studies as a model of chylomicrons (Carlson and Hallberg 1963, Carlson and Rössner 1972, Hultin et al. 1992, Björkegren et al. 1996), and such emulsions are generally called chylomicron-like emulsions. Intralipid^{R}, which has soybean oil as a constituent, is rich in plant sterols. Campesterol and sitosterol, the major plant sterols in serum, are transported in lipoproteins similarly to cholesterol (Björkhem and Boberg 1995).

Chylomicron-like emulsion particles, like chylomicrons, are cleared in two steps (Bradley and Gianturco 1986, Hultin et al. 1995). Triglycerides are first removed by the lipolytic action of LPL, and the remaining remnant particles rich in cholesterol and other sterols are then removed from circulation by the liver. Like dietary fat intake, infusion of chylomicron-like triglyceride emulsions causes accumulation of VLDL of hepatic origin, since they are degraded by the same pathways (Björkegren et al. 1996). Intravenously administered chylomicron-like emulsions acquire apolipoproteins C and E from circulating lipoproteins (Carlson 1980, Hultin et al. 1995) including apo C-II, an essential activator of LPL (Jong et al. 1999). These emulsions subsequently activate LPL (Peterson et al. 1985, Nordenström et al. 1991, Hultin et al. 1992), PLTP, CETP, and LCAT (Riemens et al. 1999). Apo E is necessary to mediate binding to the LDL receptor of at least large triglyceride-rich lipoprotein particles (Bradley and Gianturco 1986, Rensen et al. 1997).

Chylomicron-like emulsions are cleared similarly to chylomicrons in vivo (Carlson and Rössner 1972, Redgrave and Maranhao 1985, Redgrave et al. 1987), but somewhat slower clearance rates have also been documented (Hultin et al. 1995), suggesting that chylomicron-like emulsions do not completely mimic chylomicron metabolism. The presence of unesterified cholesterol is necessary for the effective clearance of chylomicron-like emulsions (Mortimer et al. 1995, Redgrave et al. 1987). In addition, the elimination of triglycerides follows first-order kinetics after intravenous doses of less than 0.2 g/kg body weight (Hallberg 1965, Carlson and Rössner 1972). Recently, chylomicron-like emulsions have been traced by radioactive labels in order to study lipoprotein clearance in patients with coronary

artery disease (Maranhão et al. 1996) and to investigate effects of lipid-lowering therapy on human lipoprotein (Santos et al. 2000, Sposito et al. 2001).

2.3.2.5. Factors affecting postprandial lipoproteins

Apo E plays an important role in the clearance of postprandial lipoproteins. Slower clearance of postprandial lipoproteins has been associated with apo E phenotype E2/3 as compared to apo E3/3 carriers, and those with E4/3 and E4/4 may have even increased clearance of postprandial lipoproteins (Weintraub et al. 1987b). Type III hyperlipidemia (dysbetalipoproteinemia) is a rare monogenic disorder characterized by elevated serum cholesterol and triglyceride concentrations, and by accumulation of chylomicron remnants of intestinal origin and of VLDL of hepatic origin (Mahley and Rall 1989). The primary molecular cause of type III hyperlipoproteinemia is the apo E2/2 phenotype's (Utermann et al. 1977) having a defective affinity to the LDL receptor (Havel et al. 1980). Other genetic determinants of postprandial lipemia include defects in LPL (Brunzell 1995), in hepatic lipase (Hegele et al. 1993), and in LCAT (Gylling and Miettinen 1993). However, postprandial lipids are not excessively accumulated in familial hypercholesterolemia with absent LDL receptors (Eriksson et al. 1991a) suggesting that apo E-dependent pathways other than LDL receptors (Mahley and Ji 1999), i.e., LRP and HSPG on liver cells, may play an important role in chylomicron clearance.

Fasting serum triglyceride concentration is a well-known predictor of the magnitude of postprandial lipemia (Cohn et al. 1988b, Cohen et al. 1992, Ooi et al. 1992, Karpe et al. 1993b). Retarded postprandial lipemia is also an inherent feature of diabetes mellitus (Chen et al. 1993, Lewis et al. 1991). Obesity is associated with impaired clearance of triglyceride-rich lipoproteins (Lewis et al. 1990). Whether age has a consistent effect on postprandial lipemia is not completely clear. Increases in postprandial triglyceridemia (Cohn et al. 1988a) and of retinyl ester response (Krasinski et al. 1990) with advancing age have been reported, but the study of Eriksson et al. (1991b) showed no difference in postprandial triglyceridemia between young and old subjects. Moreover, two studies have shown decreased postprandial retinyl palmitate concentrations even in old subjects (Borel et al. 1998, Weintraub et al. 1992). Postprandial triglyceride responses in males appear to be higher than in females (Redard et al. 1990, Ryu et al. 1992). In addition, physical exercise diminishes the accumulation of postprandial lipids (Hartung et al. 1993), whereas smoking (Mero et al. 1997), alcohol (Hartung et al. 1993), and mental stress (Le Fur et al. 1999) increase their magnitude.

Fibrates resemble short-chain fatty acids and increase the oxidation of fatty acids in liver and muscles resulting in decreased secretion of triglyceride-rich lipoproteins. Fibric acid derivatives increase LPL activity, resulting in enhanced lipolysis and clearance of VLDL and their remnants (Saku et al. 1985). Fibrates both reduce

hepatic VLDL production and enhance VLDL catabolism (Grundy and Vega 1987, Todd and Ward 1988). The underlying fibrate mechanism is to activate peroxisome proliferator-activated receptor α, up-regulating expression of apo A-I genes and down-regulating apo C-II genes (Knopp 1999). Gemfibrozil reduced postprandial lipids in hypertriglyceridemia patients (Weintraub et al. 1987a, Bhatnagar et al. 1992), in type 2 diabetic patients (Syvänne et al. 1993), and in hypoalphalipoproteinemia and hypertriglyceridemia syndrome (Simo et al. 1993), as did fenofibrate in both normo- and hypercholesterolemic subjects (Simpson et al. 1990). Common to all these studies was that fasting triglycerides were also reduced. Thus, the underlying mechanism of improved postprandial lipemia may be related to the normalization of fasting triglyceride levels.

Statins are structurally similar to HMG-CoA and inhibit HMG-CoA reductase activity competitively. They reduce serum cholesterol by inhibiting HMG-CoA reductase activity, which in turn increases the hepatic expression of LDL receptors and so enhances catabolism of both IDL and LDL (Kovanen et al. 1981, Brown et al. 1981, Grundy 1988). In general, statins reduce serum triglyceride levels by 10 to 29% (Knopp 1999, Vaughan et al. 2000). Lovastatin failed to reduce postprandial lipemia in hypoalphalipoproteinemia and hypertriglyceridemia syndrome (Simo et al. 1993) and in normotriglyceridemic subjects (Weintraub et al. 1989). On the other hand, lovastatin reduced postprandial lipoprotein levels in mildly hypertriglyceridemic subjects (Weintraub et al. 1989) and in patients with elevated plasma LDL apo B levels (Cianflone et al. 1990). Common to these latter studies was that fasting triglycerides were also significantly lowered. However, simvastatin improved chylomicron remnant clearance and increased postheparin LPL activity in patients with familial combined hyperlipidemia with no concomitant reduction of serum triglycerides (Castro Cabezas et al. 1993). In type 2 diabetic patients, cerivastatin, on the other hand, reduced fasting triglyceride concentrations and postprandial accumulation of chylomicron apo B-100, triglycerides, and cholesterol, but not the levels of postprandial apo B-48 (Battula et al. 2000). Accordingly, statins have variable effects on postprandial lipids, and the underlying mechanisms probably involve decreased VLDL production and increased expression of hepatic LDL receptors.

Tetrahydrolipstatin, a pancreatic lipase inhibitor, reduces cholesterol absorption by inhibiting both gastric and pancreatic lipases (Borgström 1988). Reduction of postprandial lipemia by tetrahydrolipstatin without reduction of fasting triglycerides (Reitsma et al. 1994) suggests that the underlying mechanism was either a direct reduction in fat absorption or an indirect upregulation of hepatic LDL receptors. Metformin reduces postprandial retinyl palmitate areas under curves (AUC) in glucose-intolerant subjects (Weintraub et al. 1998) without any significant change in fasting triglycerides. Since insulin has an inhibitory effect on the hepatic secretion of large VLDL (Malmström et al. 1997), the underlying mechanism of the metformin

effect can be metformin-induced improvement in hepatic insulin sensitivity. Furthermore, estrogens may improve the clearance of postprandial lipoproteins (Westerveld et al. 1995, Weintraub et al. 1999). It is also possible to reduce the magnitude of postprandial lipemia by altering long-term diet, for instance by using diets rich in fiber (Redard et al. 1990) or in fish oils (Harris et al. 1988). Possible effects on postprandial lipids of the plant sterols and stanols which induce cholesterol malabsorption have not been studied.

2.3.3. Cholesterol synthesis

Cholesterol is derived from de novo biosynthesis and from diet. De novo synthesis occurs in virtually all cells. The capacity of cholesterol synthesis is greatest in the liver, intestine, adrenal cortex, and the gonads. Plasma plant sterols are totally of dietary origin and cannot be synthesized by the human body (Björkhem and Boberg 1995). The rate of cholesterol synthesis can be investigated by a sterol balance method (Grundy and Ahrens 1969), or by studies of cholesterol kinetics using compartmental analysis (Goodman et al. 1973), or by input-output analysis after administration of radioactive cholesterol (Samuel and Lieberman 1973). Measurement of cholesterol precursor sterols, e.g., lathosterol, provides indirect means to study cholesterol synthesis (Miettinen 1970). In addition, methodologies based on mass isotopomer distribution analysis (Neese et al. 1993) and deuterium (Taylor et al. 1966) have been developed.

Hepatic cholesterol synthesis is regulated by the amount of cholesterol in hepatocytes (Brown and Goldstein 1983). When the amount of dietary cholesterol is reduced, cholesterol synthesis in the liver and intestine is compensatorily increased. Such increased dietary intake of cholesterol can suppress de novo synthesis of cholesterol in the liver (Quintao et al. 1971, Brown and Goldstein 1986). The estimated daily whole-body cholesterol synthesis rate in humans is 0.4 to 3 g (Nestel et al. 1969, McNamara et al. 1977). Total cholesterol synthesis in hypercholesterolemia is low (Miettinen 1970, Miettinen 1971a) but in hypertriglyceridemia (Briones et al. 1986), diabetes mellitus (Bennion and Grundy 1977), and obesity (Miettinen 1971b) is high. Statins reduce cholesterol synthesis by inhibiting HMG-CoA reductase (Grundy 1988). Cholesterol synthesis is increased in response to cholesterol malabsorption induced by neomycin (Miettinen 1982b), resins (Strandberg et al. 1990), high-fiber diet (Kesäniemi et al. 1990), and plant stanols (Vanhanen et al. 1993, Vanhanen et al. 1994, Gylling et al. 1999a), and by bile acid malabsorption caused by ileal exclusion (Koivisto and Miettinen 1988).

Circadian changes in cholesterol synthesis occur in experimental animals (Kandutsch and Saucier 1969, Edwards et al. 1972). The circadian rhythm is caused by diurnal changes in the activity of HMG-CoA reductase, and cholesterol synthesis

is highest at night (Hamprecht et al. 1969). This phenomenon has also been observed in the rat in a study of incorporation of ^{14}C-acetate into precursor sterols in the dark phase (Strandberg et al. 1984). Plasma squalene and methyl sterol contents have been observed in human subjects to peak between midnight and 4 a.m., indicating increased nocturnal cholesterol synthesis (Miettinen 1982a) and revealing diurnal variation in cholesterol synthesis also in humans. This has been confirmed by a finding that in human plasma, mevalonic acid concentrations and cholesterol synthesis increase between the midnight and 3 a.m. (Parker et al. 1982).

2.3.3.1. Squalene in cholesterol synthesis

Mevalonic acid is the first compound in the process which is unique to the cholesterol biosynthesis pathway (Fig 3). It is formed from HMG-CoA by a reaction catalyzed by the microsomal enzyme HMG-CoA reductase, which is the rate-limiting reaction of cholesterol biosynthesis (Rodwell et al. 1976). Evidence that squalene is an intermediate precursor in the biosynthesis of cholesterol was obtained in rat studies when ^{14}C-acetate was converted to labeled squalene and post-squalene sterols located further along the cholesterol synthesis chain (Langdon and Bloch 1953a, Langdon and Bloch 1953b). Mevalonic acid is converted via farnesyl pyrophosphate to squalene by squalene synthetase (Goodman and Popjak 1960, Popják and Agnew 1979, Goldstein and Brown 1990), and squalene is further cyclized by squalene epoxidase to lanosterol (Bloch 1960). Squalene is bound to sterol carrier protein$_1$ in liver microsomes during its enzymatic conversion to lanosterol (Srikantaiah et al. 1976, Gavey and Scallen 1978). Continuous feeding of squalene results in depression of the synthesis of ^{14}C-cholesterol from ^{14}C-acetate in plasma (McGuire and Lipsky 1955), suggesting a negative feedback by exogenous squalene for acetate conversion. In humans, intravenously administered ^{14}C-mevalonic acid is converted to ^{14}C-squalene and further to ^{14}C-cholesterol (Goodman 1964, Liu et al. 1975). Enzymatic conversion of squalene into cholesterol occurs via many steps; the most abundant precursors are lanosterol and other methylated sterols, and the demethylated C27 sterols including Δ8-cholestenol, desmosterol, and lathosterol (Fig 3).

It has been proposed that plasma methyl sterols (Miettinen 1968, Miettinen 1969) and squalene (Miettinen 1969) provide a qualitative estimate of cholesterol synthesis, a finding confirmed by Nestel and Kudhodkar (1975). Later it was shown that the serum contents of methyl sterols, Δ8-cholestenol, desmosterol, and lathosterol correlate positively with changes in overall cholesterol synthesis, whereas serum squalene reflects cholesterol synthesis less consistently (Miettinen 1970, Miettinen 1985, Liu et al. 1976). A similar discrepancy was observed in patients with end-stage liver disease, in whom serum and liver noncholesterol sterol levels were equal, whereas serum squalene levels were markedly lower than hepatic levels (Nikkilä et al. 1992), suggesting that serum squalene does not consistently reflect

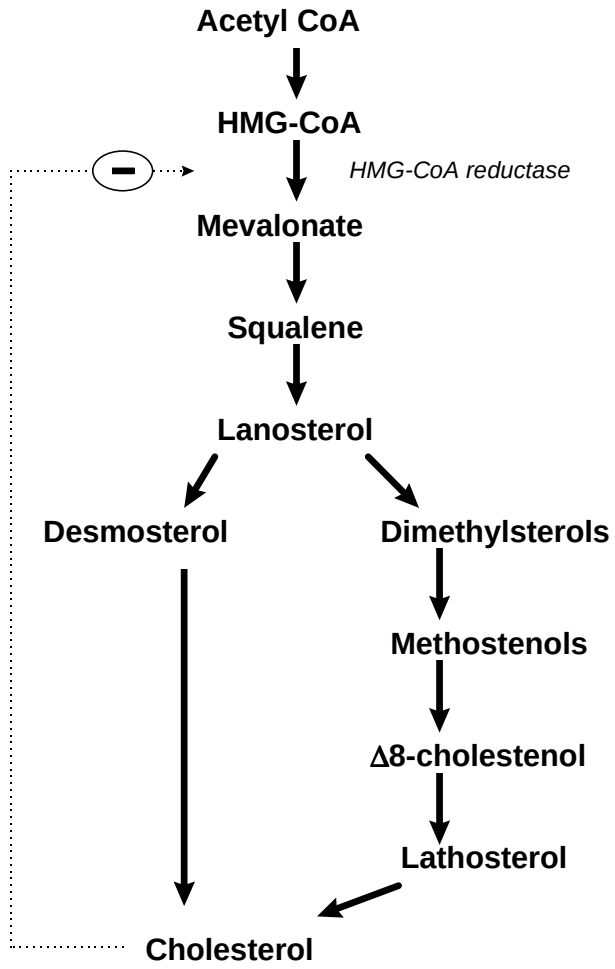

Fig 3. Simplified illustration of cholesterol synthesis and the negative feedback loop for cholesterol synthesis exerted by cholesterol on HMG-CoA reductase, the rate-regulating enzyme of the pathway.

cholesterol synthesis in chronic liver disease, either. In addition, in patients with bile acid malabsorption, serum unesterified methyl sterols showed a constant diurnal rhythm (Miettinen 1985), indicating a diurnal rhythm for cholesterol synthesis also at a high rate of cholesterol production, whereas serum squalene exhibited no such constant rhythm. These findings suggest that the metabolism of squalene compared to that of precursor sterols is complicated, and its use in estimating cholesterol synthesis can be questioned.

In human subjects, plasma mevalonic acid (Parker et al. 1982, Parker et al. 1984), serum total lathosterol (Björkhem et al. 1987), and proportions of desmosterol or lathosterol to cholesterol (Kempen et al. 1988, Miettinen et al. 1990) correlate positively with cholesterol synthesis. In addition, high levels of serum cholestanol reflect low cholesterol synthesis (Miettinen et al. 1989). On the other hand, serum cholesterol precursor sterols are negatively correlated with cholesterol absorption (Miettinen et al. 1990). Ratios of lathosterol and desmosterol, but also those of

squalene and lanosterol to cholesterol, correlate highly with results obtained by the deuterium-uptake method (Matthan et al. 2000). It can thus be concluded that determination of lathosterol provides a useful means to estimate cholesterol synthesis indirectly.

2.3.3.2. Hepatic squalene and sterol metabolism

In rat liver, squalene is divided into a small metabolically active pool which functions in cholesterol synthesis, and into a large metabolically inactive pool, in which squalene is stored (Loud and Bucher 1957). After intravenous administration of labeled mevalonic acid, most of the newly synthesized squalene was carried in VLDL (Goodman 1964), suggesting that it originated from the liver. This is in agreement with the later observation that intravenously administered ^{3}H-squalene was eliminated rapidly and cyclized to sterols by the liver, in the rat did not accumulate in other tissues (Tilvis and Miettinen 1982). Squalene feeding increases the concentrations of squalene and methyl sterols in serum and liver (Tilvis and Miettinen 1983a, Strandberg et al. 1989a), suggesting that dietary squalene enhances cholesterol synthesis. In accordance with this, squalene feeding increases cholesterol synthesis in humans, as measured by the sterol balance method (Strandberg et al. 1990). Absorbed dietary ^{3}H-squalene is already partly cyclized to plasma sterols during its transit through the rat intestinal wall (Tilvis and Miettinen 1983b), which in turn indicates that squalene also stimulates intestinal cholesterol synthesis. The activity of ACAT is stimulated, and the activity of HMG-CoA reductase is strongly inhibited by squalene feeding (Strandberg et al. 1989a). Cyclization of squalene, on the other hand, is inhibited by dietary cholesterol (Rao and Olson 1967, Johnson and Shah 1974) or by lipoproteins in vitro (Srikantaiah et al. 1980), suggesting that alimentation is a major factor regulating squalene metabolism. Interestingly, the activation of hepatic cholesterol synthesis and increased ACAT activity by squalene feeding leads to depletion of hepatic plant sterols (Strandberg et al. 1989b), which can be explained by the poor capacity of the liver to esterify plant sterols, leading to their better accessibility to biliary secretion.

2.3.3.3. Adipose tissue squalene

Adipose tissue contains cholesterol in dynamic equilibrium with plasma cholesterol (Chobanian and Hollander 1962). It has been estimated that the rate of cholesterol synthesis in the adipose tissue of the rat is 4% of that in the liver (Kovanen et al. 1975). Human adipose tissue, which is exceptionally rich in squalene (Liu et al. 1976, Tilvis and Miettinen 1980), is also capable of synthesizing squalene de novo (Tilvis et al. 1978). Squalene in adipose tissue is mainly located in fat cells, and the metabolically active fraction of squalene (20% of total) is located in microsomal membranes. The metabolically inactive, slowly exchanging fraction of squalene (80% of total) is located in the central lipid droplet (Tilvis et al. 1982b). The rate of

adipocyte cholesterol synthesis increases with fat cell size, but fasting suppresses it (Kovanen et al. 1975). After weight reduction by gastric bypass, fat cells decrease in size and the sensitivity to inhibitors of lipolysis is normalized (Ohisalo et al. 1992). Squalene concentration increased rapidly and remained high in adipose tissue after ileo-jejunal bypass for up to 12 months, although adipose tissue cholesterol was reduced after 6 months, indicating that after weight reduction mobilization of squalene from the shrunken fat cells is slower than that of cholesterol (Tilvis and Miettinen 1979).

2.3.4. Serum and lipoprotein squalene

Fasting serum concentration of squalene varies from about 34 µg/dl in plasma of healthy normolipidemic subjects (Liu et al. 1976) to about 65 µg/dl in hypercholesterolemic subjects (Gylling and Miettinen 1994a) and postmenopausal mildly hypercholesterolemic women (Rajaratnam et al. 1999a). However, a diet rich in squalene can result in several-fold increases in serum squalene (Liu et al. 1976), suggesting that serum squalene levels are regulated by dietary intake. In fasting human serum, most of the squalene is carried in LDL and HDL (Miettinen 1982a, Gylling and Miettinen 1994a), but the highest ratio of squalene to cholesterol is in VLDL (Strandberg et al. 1990, Gylling and Miettinen 1994a). In postabsorptive plasma without preceding squalene ingestion, more than 50% of squalene occurred in triglyceride-rich lipoproteins (Saudek et al. 1978), and plasma squalene correlates positively with plasma triglycerides (Liu et al. 1976, Saudek et al. 1978).

Serum squalene levels vary also among different clinical conditions. Gallin et al. (1970) reported increased concentrations of serum squalene up to 190 µg/dl in patients with acute influenza. This increased concentration may represent either an increased rate of squalene production or a decreased rate of squalene conversion to cholesterol precursor sterols as a result of infection, or both. Liver function was normal, so that the effect of influenza on squalene levels was probably due to some specific interference with the metabolism of squalene rather than to liver malfunction. In addition, increased squalene concentrations (125±15 µg/dl) that correlated with serum cholesterol levels appeared in hepatocellular carcinoma (Hirayama et al. 1979), whereas decreased serum squalene concentrations have been detected under cholestatic conditions in which cholesterol synthesis was reduced (Yamanishi et al. 1978, Hirayama et al. 1979, Nikkilä and Miettinen 1988).

More recently, increased serum concentrations of squalene have been shown to be associated with coronary artery disease in postmenopausal women (Rajaratnam et al. 1999a, Rajaratnam et al. 2000), suggesting that squalene may be involved in the development of atherosclerosis. Squalene has been shown to accumulate in human atheromatous plaques (Lewis 1975a). In animal studies, it exhibits antioxidant

activities at least in vitro (Govind Rao and Achaya 1968), and possibly has radioprotective (Storm et al. 1993) and antitumor properties (Rao et al. 1998, Smith et al. 1998). Thus, squalene itself could be considered a beneficial rather than a harmful compound. It appears, however, that even the accumulated squalene cannot prevent the development of atherosclerotic lesions. Despite the potentially beneficial effects of squalene, it cannot be ruled out that squalene exerts some harmful effects in atherosclerotic plaques. Therefore, it must be emphasized that the role of squalene in the development of coronary artery disease and other diseases is unknown and needs further investigation.

2.3.5. Effect of dietary squalene on human serum cholesterol levels

The effect of squalene feeding on human serum cholesterol levels has received only limited attention, and the results have been variable. At a dose of 0.9 g/day, squalene had no consistent effect on serum cholesterol levels in long-term hospital patients suffering from cerebrovascular disorders (Strandberg et al. 1990), while a similar daily dose significantly reduced serum total and LDL cholesterol levels in elderly patients with hypercholesterolemia (Chan et al. 1996). In the former study, the feeding period was only 7 to 30 days, which may explain the lack of effect. In a third study, addition of 1 g of squalene in rapeseed oil caused significant increases in serum total and LDL cholesterol in 4 to 9 weeks, but a subsequent period on 0.5 g squalene/day normalized serum cholesterol levels (Miettinen and Vanhanen 1994a). Summing up, studies on the effects of dietary squalene on serum cholesterol levels are quite contradictory and call for further investigation.

2.3.6. Elimination of squalene and cholesterol

The most important pathway for the elimination of cholesterol in mammals is the formation and excretion of bile acids. Cholesterol 7α-hydroxylase and 27-hydroxylase are the enzymes responsible for regulating the overall conversion of cholesterol into bile acids (Shefer et al. 1970, Björkhem 1992), of which the primary ones are cholic acid and chenodeoxycholic acid, both synthesized in the liver. Cholesterol is also secreted by the hepatocytes into bile and partly eliminated unchanged in the feces. In human subjects in Western countries, some 400 to 1000 mg of cholesterol and 100 to 500 mg of bile acids pass daily through the colon (Macdonald et al. 1983). More than 97% of bile acids are reabsorbed in distal ileum and returned to the liver. The remaining bile acids enter the large intestine, where they undergo extensive bacterial degradation (Macdonald et al. 1983). Outside the liver, cholesterol is removed by steroid hormone production and desquamation of epithelial cells.

Biliary squalene levels exceed plasma levels (Liu et al. 1976). The origin of squalene in bile is unknown, but it can be assumed that it is partly derived from de novo synthesis in the liver and partly from circulating lipoproteins similarly to cholesterol (Schwartz et al. 1978). Squalene is detected in small amounts (0.5-3 mg/day) in the feces of subjects on squalene-free diet (Liu et al. 1976), showing that it is partly derived from endogenous synthesis. On the other hand, it is obvious that squalene secreted in bile is effectively reabsorbed, since in humans only small amounts of squalene are excreted in the feces or urine (Liu et al. 1975, Liu et al. 1976). Squalene levels in bile decrease in parenchymal liver diseases, especially liver cirrhosis, in which HMG-CoA reductase activity is reduced (Ponz de Leon et al. 1978), but are unchanged in patients with cholelithiasis (Nosaka et al. 1985) and acalculous cholesterolosis (Tilvis et al. 1982a). This suggests that biliary squalene is partly synthesized by the liver. Treatment with ursodeoxycholic acid reduced the squalene-to-cholesterol ratio in the bile but not in the serum of patients with gallstones (Salvioli et al. 1984) which, in turn, suggests that a decreased biliary squalene-to-cholesterol ratio may indicate reduced cholesterol synthesis. The content of squalene in gallstones, however, is unknown. Squalene is also secreted with skin surface lipids. The secretion of squalene by the skin is substantial, ranging from 125 to 475 mg/day (Nikkari et al. 1974), but a major proportion of this secreted squalene is made de novo in the skin (Nikkari et al. 1975). Thus, it is likely that skin excretion of squalene does not affect serum squalene levels.

2.4. Hypolipidemic interventions and serum squalene

Since statins inhibit cholesterol synthesis some steps before squalene (Fig 3), they might be expected to reduce serum squalene concentrations. Despite decreased cholesterol synthesis as indicated by reduced lathosterol ratios, squalene remained unchanged in most (Miettinen et al. 1992b, Uusitupa et al. 1992, Miettinen et al. 2000a), but not all (Vanhanen and Miettinen 1995) studies performed on statins (Table 2). This is in agreement with the earlier assumptions that serum squalene levels do not consistently reflect cholesterol synthesis.

Fibrates are the most effective triglyceride-lowering drugs. Since there is a linear correlation between serum concentrations of triglycerides and squalene, one might also expect fibrates to reduce serum squalene levels. In the few studies available, however, it seems that serum squalene is not consistently changed, whereas levels of lathosterol are elevated by gemfibrozil treatment (Table 2), which has been shown to raise cholesterol synthesis also by the sterol balance method (Vanhanen and Miettinen 1995).

Dietary plant sterols and stanols are effective in reducing serum total and LDL cholesterol levels. Sitostanol incorporated into margarine inhibits cholesterol

absorption and reduces LDL cholesterol by up to 14% (Miettinen et al. 1995). Several studies (Gylling and Miettinen 1994b, Gylling and Miettinen 1996, Miettinen et al. 2000b) observe that cholesterol synthesis is upregulated during stanol ester treatment as indicated by increased serum precursor sterol-to-cholesterol ratios, whereas the respective serum squalene ratios have been unaffected by such treatment. However, in a recent report by Hallikainen et al. (2000), the squalene-to-cholesterol ratio was increased both by stanol ester and by sterol ester treatment (Table 2).

Table 2. Changes in serum cholesterol, triglycerides, squalene, lathosterol, and sitosterol in clinical studies. All subjects were hypercholesterolemic, unless otherwise noted.

			Change				
Reference	n	Treatment	CH	TG	SQ	LA	SI
Vanhanen and Miettinen 1995	38	Pravastatin 40 mg/d	↓	=	↓	↓	↑
Uusitupa et al. 1992	62	Lovastatin 80 mg/d	↓	NR	=	↓	↑
Miettinen et al. 1992b	10	Simvastatin 40 mg/d	↓	=	=	↓	=
Miettinen et al. 2000a	434	Simvastatin 20-40 mg/d	↓	NR	=	↓	↑
Vanhanen et al. 1993	67	Stanol ester 3.4 g/d	↓	=	=	↑	↓
Hallikainen et al. 2000	34	Stanol ester 2 g/d	↓	=	↑	↑	↓
		Sterol ester 2 g/d	↓	=	↑	↑	↑
Gylling et al. 1995[+]	14	Stanol ester 3 g/d	↓	=	=	↑	↓
Gylling and Miettinen 1994b[++]	11	Stanol ester 3 g/d	↓	=	=	↑	↓
Miettinen et al. 1992b	9	Gemfibrozil 1200 mg/d	↓	↓	=	↑	↓
Vanhanen and Miettinen 1995	38	Gemfibrozil 1200 mg/d	↓	↓	=	↑	↓

CH, cholesterol; TG, triglycerides; SQ, squalene; LA, lathosterol; SI, sitosterol; NR, not reported. ↑ increase, ↓ decrease, = no change.
[+] Familial hypercholesterolemia, [++] Type 2 diabetes.

Resins such as cholestyramine bind bile acids, and by interrupting the enterohepatic circulation, lower LDL cholesterol. Increased cholesterol synthesis during resin treatment is indicated by increased lathosterol ratios with no concomitant increase in squalene levels (Strandberg et al. 1990), although cholestyramine feeding increases squalene synthetase activity (Cohen et al. 1986). Few reports concerning other cholesterol-lowering treatments measure levels of serum squalene. A transient increase in serum squalene but not in precursor sterol ratios occurred during an oat bran diet with no reduction in serum cholesterol levels (Uusitupa et al. 1997). Charcoal reduced serum levels of cholesterol and increased serum lathosterol but not serum squalene ratios (Neuvonen et al. 1989). Ursodeoxycholic acid feeding increases whole-body cholesterol synthesis (Miettinen TE et al. 1998), but when ursodeoxycholic acid was administered to gallstone patients, serum squalene remained unchanged (Salvioli et al. 1984).

Plasma mevalonic acid concentration and the lathosterol-to-cholesterol ratio were unchanged immediately after LDL apheresis, but increased on the day after the procedure (Pfohl et al. 1994, Pfohl et al. 1997), indicating an increase in cholesterol synthesis after 24 hours. In agreement with this, serum lathosterol-to-cholesterol ratio did not change, but the serum squalene-to-cholesterol ratio increased immediately after LDL apheresis in patients with familial hypercholesterolemia (Gylling et al. 1998), suggesting that squalene, known to have a rapid turnover in serum (Liu et al. 1975, Saudek et al. 1978), may be a sensitive indicator of cholesterol synthesis in unsteady states like those immediately after apheresis.

In conclusion, dietary squalene is efficiently absorbed and is carried by chylomicrons in the postprandial state until taken up into the liver by the hepatocytes. In the liver, squalene is either cyclized to sterols, secreted into the blood circulation in lipoproteins, or is eliminated in bile.

3. AIMS OF THE STUDY

Squalene is an obligatory endogenous precursor of cholesterol, and it is present also in the diet. Dietary squalene is carried in postprandial lipoproteins, and an oral fat load enriched with squalene can thus be used to study the fate of postprandial squalene in lipoproteins. Recent evidence suggests that squalene may play some role in the development of atherosclerosis, and that the removal of squalene-labeled postprandial lipoproteins is known to be retarded at least in women who have coronary artery disease. Accordingly, the metabolism of postprandial squalene may be of marked clinical relevance. Relatively little is known, however, about the human postprandial squalene metabolism. In general, because the results of the few studies concerning human squalene metabolism have been variable, the postprandial metabolism of squalene was considered to be worth investigating in detail in humans. The effects of the absorption process, which may considerably interfere with the evaluation of postprandial lipoprotein clearance, can be avoided by use of chylomicron-like intravenous lipid emulsions instead of oral fat loads. Thus, Intralipid^{R} with added squalene was considered an additional possibility for studying squalene clearance in triglyceride-rich lipoproteins after their intravenous administration.

The aims of the present study were to investigate

- postprandial metabolism of squalene after its administration as a single oral dose in a test meal

- relation of postprandial squalene metabolism to age

- acute effects of dietary squalene on cholesterol synthesis as measured by cholesterol precursor sterols in postprandial lipoproteins

- removal of squalene from circulation and its relation to that of plant sterols after their intravenous administration in chylomicron-like emulsion particles (Intralipid^{R} enriched with squalene)

- whether inhibition of cholesterol absorption and compensatory increase in cholesterol synthesis by plant stanol ester consumption interfere with postprandial levels of squalene after an oral squalene dose

- whether inhibition of cholesterol synthesis by lovastatin interferes with postprandial levels of squalene after an oral squalene dose

4. SUBJECTS AND METHODS

4.1. Subjects and study design

Study I
The study group comprised of 6 healthy young male students aged 22 to 25, and 8 home-living old men aged 78 to 79 (Table 3), volunteers from a random age-cohort of the inhabitants of Helsinki. All were non-smokers. Two young and four old men had the apo E4/3 phenotype, and all the others had the apo E3/3 phenotype. Subjects with the ε2 allele were excluded. Each subject received an oral fat load containing 0.5 g of squalene and 345 000 IU of retinyl palmitate as postprandial markers. A detailed description of the oral fat load test is given in 4.2. Blood samples were collected before and 3, 4, 6, 9, 12, and 24 hours after the test meal.

Study II
The study group comprised of 16 males aged 22 to 79 (Table 3). One subject had the apo E2/3, 9 had the apo E3/3, and 6 subjects had the apo E4/3 phenotype. Each subject underwent two oral fat load tests in random order: one was supplemented with 0.5 g of squalene and the other was without squalene addition. Both fat loads contained 345 000 IU of retinyl palmitate to label postprandial lipoproteins. The blood samples were collected as in Study I.

Study III
Six healthy men participated in an intravenous chylomicron-like emulsion clearance test (Table 3). All subjects were non-smokers and took no medications. All had the apo E3/3 phenotype. An intravenous bolus of Intralipid^{R} enriched with squalene was given to each subject in the fasting state, and blood samples were drawn before and 2½, 5, 10, 20, 30, 45, 60, 120, and 180 min after the injection. A detailed description of the intravenous study design is given in 4.3.

Study IV
The study group consisted of 11 males (Table 3). One subject had the apo E2/3 phenotype, 6 had the apo E3/3 and 4 the apo E4/3. The subjects visited the outpatient clinic three times at an least one-week interval, and a 24-hour postprandial fat clearance test was performed during each visit. At baseline, an oral fat load containing 0.5 g of squalene and 345 000 IU of retinyl palmitate as postprandial markers was administered. After the last blood sample on the following morning, the subjects were instructed to replace 24 g of fat from their normal diet with stanol ester margarine, but otherwise to keep their diets unchanged. The margarine was provided in containers of 8 g of rapeseed oil margarine each, in which 1 g of stanol was dissolved as rapeseed oil fatty acid esters (Raisio Corporation, Raisio, Finland). The subjects used one container three times a day, so

that the intake of stanol was expected to be 3 g/day. The postprandial fat clearance test was repeated after one and two weeks on stanol ester margarine in the diet. The two test meals contained, in random order, margarine without or with 1 g of stanol esters. The blood samples were collected as in Study I.

Study V

Five subjects with apo E2/2 phenotype and type III hyperlipidemia (called type III group hereafter) were enrolled in the study (Table 3). The diagnosis was based on elevated serum cholesterol (>6.5 mmol/l) and triglyceride (>2.5 mmol/l) values, elevated VLDL cholesterol-to-total serum triglycerides, and elevated ratios of VLDL cholesterol to VLDL triglycerides, and apo E2 homozygosity. Sixteen mildly hyperlipidemic subjects with apo E3/3 phenotype volunteered as controls. Each subject underwent an oral fat load test containing 1.0 g of squalene and 345 000 IU of retinyl palmitate. After the baseline fat load test, the type III subjects started lovastatin treatment (40 mg/day) for 8 to 12 weeks, after which the fat load test was repeated. Blood samples were collected before and 3, 4, 6, 9, and 24 hours after the test meal. Cholesterol synthesis was measured with the sterol balance technique in all type III patients before and during lovastatin treatment and in 13 volunteer controls.

Table 3. Clinical characteristics of the study populations.

Variables	Study I		Study II	Study III	Study IV	Study V	
	Young	Old				Type III	Controls
Number	6	8	16	6	11	5	16
Age, years	24±1	79±0	56±7	33±4	58±7	55±4	55±2
BMI, kg/m^2	22.4±0.8	26.6±1.0*	24.5±0.5	22.1±0.5	25.8±0.9	25.4±1.4	25.9±1.1

Means±SE. BMI, body mass index. * P=0.01 from the young.

All subjects volunteered for the studies. The study protocols were accepted by the Ethics Committee of the Department of Medicine, University of Helsinki. The subjects had no renal, liver or gastrointestinal diseases. They took no medications except for one elderly patient, whose well-controlled type 2 diabetes mellitus was treated with glibenclamide (Studies I and II), and one subject whose stable coronary artery disease was treated with beta-blocking agent, nitrate, and acetylsalicylic acid without any change in the dosage (Study IV).

4.2. Standard oral fat load test

A standard oral fat load test contained 90 g of milk fat and 432 mg of cholesterol. In addition, 0.5 g (1.0 g in Study V) of squalene and 345 000 IU of aqueous vitamin A were added to the standard fatty meal to label postprandial lipoproteins. The meal was given as a cream-eggshake containing 1 200 kcal. The test was started at 8 a.m. after a 12-hour fast. After the meal, the subjects fasted for 9 hours, after which they consumed a standard low-cholesterol, low-fat hospital meal. Blood samples were drawn before the meal and after 3, 4, 6, 9, 12, and 24 hours. The blood samples were taken into dark heparin-containing tubes.

4.3. Intravenous study

A chylomicron-like lipid emulsion containing squalene, hereafter called squalene emulsion, was prepared by dissolving oily squalene in the commercially available Intralipid^{R} 200 mg/ml fat emulsion (Pharmacia, Stockholm, Sweden) as follows: liquid 98-100% squalene (Sigma Chemicals Co, St. Louis, MO, USA) was first filtered by a sterile Millex-GV 0.22 µm filter unit (Millipore S.A., Molsheim, France) to ensure its sterility. To make squalene soluble, 80 mg of squalene was dissolved in 0.9 ml of 99.5% ethanol (Apoteksbolaget, Umeå, Sweden). The resulting squalene-ethanol solution (1:9, vol:vol) was subsequently dissolved in 100 ml of Intralipid^{R}.

The subjects were admitted to the laboratory in the morning after an overnight fast. After the baseline blood sample, 30 ml of squalene emulsion was injected as an intravenous bolus into a forearm vein in 2 to 3 minutes. The samples were collected via an intravenous cannula (Venflon2^{R} 1.2/45mm cannula, BOC Ohmeda AB, Helsingborg, Sweden) 2½, 5, 10, 20, 30, 45, 60, 120, and 180 minutes after the injection into 10 ml tubes (Venoject Autosep^{R}, Terumo Europe, Leuven, Belgium) from the opposite side. No side-effects occurred. Control infusions with saline were performed on two subjects.

4.4. Lipoprotein ultracentrifugation

Chylomicrons were separated after a 30-minute ultracentrifugation with carefully overlayered 1.006 g/ml NaCl salt solution in a fixed-angle Type 50 Ti rotor (Beckman Instruments Inc., Fullerton, CA, USA), and the other lipoproteins were separated according to densities (d) as follows: VLDL, <1.006 g/ml; IDL, 1.006-1.019 g/ml; LDL, 1.019-1.063 g/ml and HDL, 1.063-1.210 g/ml (Havel et al. 1955, Warnick and Alberts 1982). Postprandial plasma samples were separated into chylomicrons, VLDL, and VLDL infranatant (d 1.006-1.210 g/ml).

4.5. Gas-liquid chromatography

Serum cholesterol, squalene, and noncholesterol sterols (cholestanol, Δ8-cholestenol, desmosterol, lathosterol, lanosterol, campesterol, stigmasterol, and sitosterol) were quantitated by gas liquid chromatography (GLC) on a 50 m long Ultra 1^R SE-30 column (Hewlett-Packard Co, Palo Alto, CA, USA) from saponified serum or lipoprotein fractions as their trimethylsilyl derivatives using 5α-cholestane as internal standard (Miettinen and Koivisto 1983, Miettinen 1988). The procedure was as follows: 100 µl of 5α-cholestane was added to serum, plasma, or lipoprotein samples, usually in 200 to 1000 µl depending on the lipoprotein fraction. Subsequently, the samples were saponified with 99% ethanol and kalium-hydroxide (10M) (9:1, vol:vol). Nonsaponified lipids were extracted in hexane. Trimethylsilylation was performed to bind free O^--groups of sterols before the GLC run.

Free and esterified cholesterol and plant sterols from chylomicron samples (Study III) were quantitated by GLC after the free and esterified sterols had been separated by thin-layer chromatography as described by Miettinen (1988). Briefly, a chloroform-methanol (2:1, vol:vol) extract of chylomicrons was applied to the thin-layer chromatography plate. After a one-hour run with ethyl ether-heptane, with cholesterol and esterified cholesterol serving as standards, free and esterified sterols were separated based on their mobility. Separated fractions containing free and esterified sterols were added, with 5α-cholestane as the internal standard, and extracted with ethyl-ether. The esterified fraction was saponified with ethanol-KOH. Subsequently, a GLC run was performed on both fractions as described above.

4.6. Other analytical procedures

Retinyl palmitate was quantitated by high-pressure liquid chromatography (HPLC) using a 250 mm long normal-phase Supelcosil^R LC-Si column (Supelco Inc, Bellefonte, PA, USA) at 326 nm wavelength (Ruotolo et al. 1992). Mobile phase buffer consisted of hexane (82% by vol), n-butyl chloride (14% by vol), and acetonitrile (4% by vol). In addition, 50 µl acetic acid was added. Plasma or lipoprotein samples, usually 100 to 500 µl, were added with 500 µl of methanol and 600 µl of buffer for lipid extraction. Retinyl acetate was added as an internal standard, and the final volume was adjusted to 1.8 ml with saline. The supernatant, after mixing and centrifugation, was aspirated and dried under nitrogen. The lipid fraction was then dissolved in buffer and injected into the HPLC system to determine retinyl palmitate.

Commercial enzymatic kits were used to analyze serum and lipoprotein cholesterol, triglycerides (Roche Diagnostics, Hoffman-La Roche Ltd, Basel, Swizerland), and phospholipids (Waco Chemicals GmbH, Neuss, Germany). Apo E phenotyping was performed by isoelectric focusing of serum (Havekes et al. 1987).

Elimination of cholesterol from the body and cholesterol absorption efficiency were measured from 3-day stool collections. Each subject consumed one capsule containing 0.07 µCi of ^{14}C-cholesterol, 0.11 µCi of ^{3}H-sitosterol, and chromic oxide (Cr_2O_3, 200 mg) three times daily with major meals during a 7-day dietary recording. Stools were collected during the last 3 days of the 7-day period and pooled. Fecal cholesterol as neutral sterols (cholestenol, coprostanol, coprostanone) and bile acids were quantitated by GLC (Miettinen 1982c). Dietary records were analyzed by a computerized program (Knuts et al. 1991).

4.7. Calculations

Postprandial triglyceride, retinyl palmitate, and squalene were expressed as concentrations or were given as incremental concentrations calculated by subtracting the respective basal fasting value from each postprandial value. To determine postprandial responses, the area under the 9-hour concentration curves (AUC) for cholesterol and triglycerides and the 24-hour AUC for squalene and retinyl palmitate were calculated for each subject. Areas under incremental curves (AUIC) were also calculated. Retinyl palmitate and squalene to triglyceride ratios were calculated by dividing retinyl palmitate and squalene concentrations by the respective triglyceride concentration (Study I) in order to evaluate any changes in the composition of postprandial lipoproteins. Serum cholesterol precursor sterols are given as ratios to serum plant sterols in order to elucidate de novo cholesterol synthesis (Study II). Ratios of squalene and noncholesterols to cholesterol were also used to minimize the effect of variation in lipoprotein cholesterol levels.

Cholesterol absorption efficiency was calculated by the ^{14}C/^{3}H ratio in stools as compared to the ratio fed (Crouse and Grundy 1978), and the Cr_2O_3 measurement (Bolin et al. 1952) was applied to measure fecal flow. Cholesterol synthesis was obtained as the difference between the fecal steroids (neutral and acidic) of cholesterol origin and dietary cholesterol. Intestinal cholesterol flux was calculated by dividing fecal neutral sterols by 1 minus fractional cholesterol absorption. Biliary cholesterol secretion was the difference between intestinal cholesterol flux and dietary cholesterol.

4.8. Statistical analysis

Data analyses were performed by the Biomedical Data Program (BMDP Statistical Software, Inc. Los Angeles, CA, USA). Continuous values are given as mean±SE. Differences between continuous variables were tested by two-tailed t-test or by Mann-Whitney rank sum test. Chi-square or Fisher's exact test were used for discrete variables. The paired t-test was used to evaluate treatment effects (Studies II, IV, and V). In case of more than two groups, one-way analysis of variance (ANOVA) was used. Statistical significance of within-group changes and between-group differences in postprandial and post-injection studies were tested with ANOVA for repeated measures. The follow-up comparisons were performed by paired t-test, two-tailed t-test, or one-way ANOVA. Possible interactions of potential confounding variables (fasting lipids; body mass index, BMI) with postprandial values were studied with two-way ANOVA. Logarithmic transformations were used when the distributions were skewed. Correlations were analyzed by calculating Pearson's correlation test, or by Spearman rank correlation test in case of skewed distributions. A P value of <0.05 was considered statistically significant, except for Study I, in which only P values <0.01 were considered significant in order to avoid type I error. To evaluate the differences in clearance rates for chylomicron lipids in Study III, die-away curves expressing the percentage of peak concentration were constructed. Half-lives of injected lipids were determined with nonlinear least squares analysis.

5. RESULTS

5.1. Baseline values

The study subjects were almost normolipidemic, except for some of the old subjects in Study I and part of the control subjects in Study V, who were mildly or moderately hypercholesterolemic, and the type III hyperlipoproteinemic subjects in Study V (Table 4). Baseline serum squalene concentration, shown in Table 5, was highest in type III subjects, with levels up to twice as high as in the other groups. In addition, baseline serum Δ8-cholestenol and lathosterol concentrations in type III subjects exceeded the respective levels in the other groups. Fasting serum squalene was distributed in lipoproteins as follows: 7.5±1.4% in chylomicrons, 12.1±2.7% in VLDL, 6.8±1.1% in IDL, 40.6±3.5% in LDL, and 33.0±3.9% in HDL. No correlation existed between fasting serum triglyceride and squalene concentrations (data not shown).

Table 4. Baseline lipid values for the study population.

Variables	Study I		Study II	Study III	Study IV	Study V	
	Young, n=6	Old, n=8	n=16	n=6	n=11	Type III, n=5	Controls, n=16
Serum CH, mmol/l	4.4±0.2	5.6±0.3[a]	5.2±0.2	5.1±0.4	5.5±0.4	8.2±0.7[c]	6.8±0.3
LDL CH, mmol/l	2.41±0.25	3.48±0.23[b]	3.05±0.19	2.81±0.30	3.26±0.31	1.5±0.3[c]	4.4±0.4
HDL CH, mmol/l	1.48±0.07	1.15±0.11	1.26±0.07	1.38±0.07	1.24±0.08	1.0±0.2	1.3±0.1
Serum TG, mmol/l	0.91±0.09	1.38±0.20	1.18±0.09	0.87±0.04	1.42±0.17	6.2±1.8[c]	1.6±0.3

Mean±SE. CH, cholesterol; TG, triglycerides.
[a] P <0.05, [b] P<0.01 for difference from the young, Study I. [c] P<0.05 from controls, Study V.

5.2. Postprandial metabolism of squalene, retinyl palmitate, and triglycerides

Postprandial squalene metabolism was studied in relation to age and in different lipoproteins by means of oral fat load tests supplemented with squalene and retinyl palmitate. In addition, postprandial squalene concentrations were compared to postprandial retinyl palmitate and triglyceride concentrations.

Table 5. Baseline serum sterol and squalene concentrations in the study population.

Variables	n=42 [a]	Type III, n=5
Cholesterol, mg/dl	203±9	264±23*
Squalene, µg/dl	106±7	217±50*
Δ8-cholestenol, µg/dl	38±4	97±32*
Desmosterol, µg/dl	133±7	173±21
Lathosterol, µg/dl	293±18	541±91*
Campesterol, µg/dl	534±46	811±134
Sitosterol, µg/dl	299±22	401±36
Cholestanol, µg/dl	262±18	281±35

Mean±SE. * P<0.05. [a] The first column includes the subjects from Studies I, II, III, IV, and V. Eight subjects participating in more than one study (Studies I, II, and IV) appear only once.

5.2.1. Postprandial responses of squalene, retinyl palmitate, and triglycerides in young and old subjects (Study I)

The postprandial squalene peak concentration occurred at 7 to 8 hours in the young and 9 to 10 hours in the old (NS) in plasma, chylomicrons, and VLDL (Table 6). Squalene peak concentrations and AUCs in plasma and lipoproteins were similar in both groups. However, VLDL squalene concentrations began to diverge after 9 hours, such that in the young, VLDL squalene was decreasing, when in the old it continued to increase (Fig 3 in Study I). Accordingly, 12-hour VLDL squalene concentration tended to be higher in the old (P=0.03). Due to the different response after 9 hours, the 0 to 24 hour curves of VLDL squalene differed significantly between the age-groups (P<0.01, ANOVA for repeated measures).

The peak times for retinyl palmitate were similar to those for squalene in plasma, chylomicrons, and VLDL, and peak times did not differ between age-groups. However, plasma retinyl palmitate peak concentrations tended to be higher (P=0.01) and AUICs were higher in the old subjects (P<0.01) largely due to their high plasma 24-hour retinyl palmitate concentrations. Chylomicron and VLDL retinyl palmitate peak concentrations and AUICs showed only a tendency toward higher values in the old.

Table 6. Plasma and lipoprotein squalene peak concentrations, peak times, and AUCs.

Variables	Study I		Study IV	
	Young	Old	Baseline	Stanol esters
Plasma peak c, µg/dl	674±119	677±93	806±147	743±116
CM peak c, µg/dl	310±83	282±55	312±59	375±72
VLDL peak c, µg/dl	161±37	212±42	337±89	242±48*
Plasma peak time, h	7.2±0.9	9.8±0.9	10.4±0.6	8.3±0.4*
CM peak time, h	7.2±0.9	9.4±1.1	9.8±0.8	7.6±0.5*
VLDL peak time, h	7.7±0.9	9.8±0.9	10.9±0.4	8.6±0.5*
Plasma AUC, mg/dl · h	7.5±0.9	8.1±0.9	9.6±1.4	7.9±1.1*
CM AUC, mg/dl · h	2.4±0.6	2.7±0.5	3.0±0.6	2.8±0.5
VLDL AUC, mg/dl · h	1.5±0.3	2.5±0.4	3.6±0.8	2.5±0.6*

Mean±SE. c, concentration; CM, chylomicron.
* $p<0.05$ for difference between groups within Study IV.

Postprandial triglyceride peak times occurred significantly earlier than those of squalene and retinyl palmitate in plasma, chylomicrons, and VLDL. Triglyceride peak times were age-dependent, such that they occurred later in chylomicrons ($P<0.01$) and less consistently in VLDL ($P=0.04$) in the old. However, the triglyceride peak concentrations and AUICs in plasma, chylomicrons and VLDL were similar in the two age-groups.

Since the postprandial concentration curves of triglycerides differed from those of squalene and retinyl palmitate, the ratios of squalene and retinyl palmitate to triglycerides were calculated to evaluate changes in the composition of postprandial lipoproteins. Squalene-to-triglyceride ratios differed significantly in both groups between chylomicrons and VLDL (Fig 4), with the ratio being higher in chylomicrons at 9 and 24 hours ($P<0.01$ for both). Retinyl palmitate-to-triglyceride ratios were higher in chylomicrons than in VLDL at 6 and 9 hours ($P<0.01$ for both). Squalene and retinyl palmitate-to-triglyceride ratios in VLDL increased relatively little, suggesting that the VLDL-sized particles were rich in triglycerides at all postprandial time points.

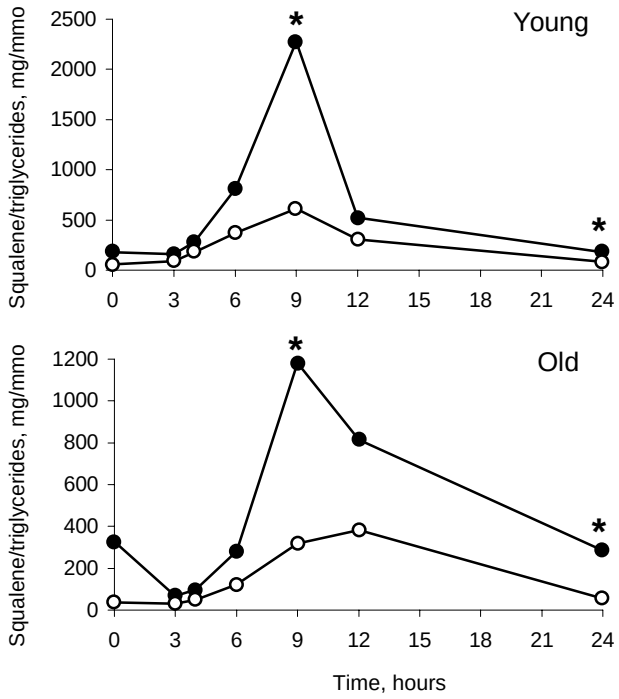

Fig 4. Squalene-to-triglyceride ratios in chylomicrons (—•—) and in VLDL (—o—) of young (upper panel) and old subjects (lower). P<0.01 between the chylomicron and VLDL curves, ANOVA for repeated measures. * P<0.01 from VLDL, one-way ANOVA.

Neither postprandial squalene and retinyl palmitate concentrations nor AUICs correlated with BMI, fasting triglycerides or cholesterol (data not shown). The plasma triglyceride peak hour was correlated with fasting triglycerides (r=0.707, P<0.01), and VLDL triglyceride peak hour with fasting serum cholesterol (r=0.886, P<0.001). Although fasting triglycerides, a determinant of postprandial lipemia, differed between the groups, albeit not statistically significantly, the possible effects of fasting triglycerides on postprandial results were studied by two-way ANOVA, which showed that postprandial squalene and retinyl palmitate concentrations or AUICs had not been affected by fasting triglycerides.

These results suggest that the response of postprandial VLDL squalene was delayed in the old subjects.

5.2.2. Postprandial plasma and lipoprotein lipids after squalene-supplemented test meals (Study II)

Without supplementation, postprandial squalene levels were practically unchanged from the baseline. Addition of a single dose of 0.5 g of squalene to the test meal had no detectable effect on postprandial cholesterol, triglyceride or retinyl palmitate levels or on AUCs for either plasma or lipoproteins. However, after supplementation, postprandial squalene concentrations increased significantly in

chylomicrons, VLDL, and in six random subjects, also in IDL; whereas LDL and HDL squalene only tended to increase (Fig 2 in Study II). These results suggest that orally ingested squalene labels triglyceride-rich lipoproteins but not LDL and HDL.

Squalene AUCs were significantly increased most in chylomicrons (868±113%), somewhat less in VLDL (530±103%), and least in VLDL infranatant (92±17%) compared with the test meals without squalene supplementation, so that roughly two-thirds of the postprandial squalene was carried in chylomicrons and VLDL. Squalene peak times in chylomicrons occurred at 8.5±0.7, in VLDL at 9.3±0.6 hours, and in VLDL infranatant at 9.8±0.5 hours after squalene supplementation. The respective times for chylomicron and VLDL retinyl palmitate were 8.1±0.8 and 8.8±0.7 hours, and for triglycerides 4.3±0.3 and 4.8±0.6 hours, and these were similar before and after squalene addition. The peak for VLDL infranatant retinyl palmitate occurred significantly later than that for squalene, at 14.9±1.3 hours ($P<0.05$). Thus, the fates of postprandial squalene and retinyl palmitate resembled each other in chylomicrons and VLDL, but not in VLDL infranatant.

5.2.3. Relationship between postprandial squalene and retinyl palmitate (Studies II and IV)

Correlation coefficients calculated at each time-point for the concentrations of squalene and retinyl palmitate after the test meal were significant from 3 to 12 hours in chylomicrons and from 3 to 24 hours in VLDL (Fig 5). Inhibition of cholesterol absorption by stanol esters did not interfere with the postprandial relationship between squalene and retinyl palmitate. No significant correlations were found with VLDL infranatant, in which squalene and retinyl palmitate peak times differed significantly. These results suggest that postprandial squalene and retinyl palmitate configurations closely resemble each other in chylomicrons and VLDL, but not in the more dense lipoproteins.

5.3. Metabolism of intravenous squalene and plant sterols (Study III)

After intravenous administration, the metabolism of commercially available lipid emulsions, such as Intralipid^{R}, resembles that of chylomicrons of endogenous origin. The advantage of using intravenous chylomicron-like emulsions is to avoid the intestinal absorption process, a possible confounder in postprandial studies. Thus, we studied removal of squalene and plant sterols of Intralipid^{R} enriched with squalene after an intravenous bolus injection.

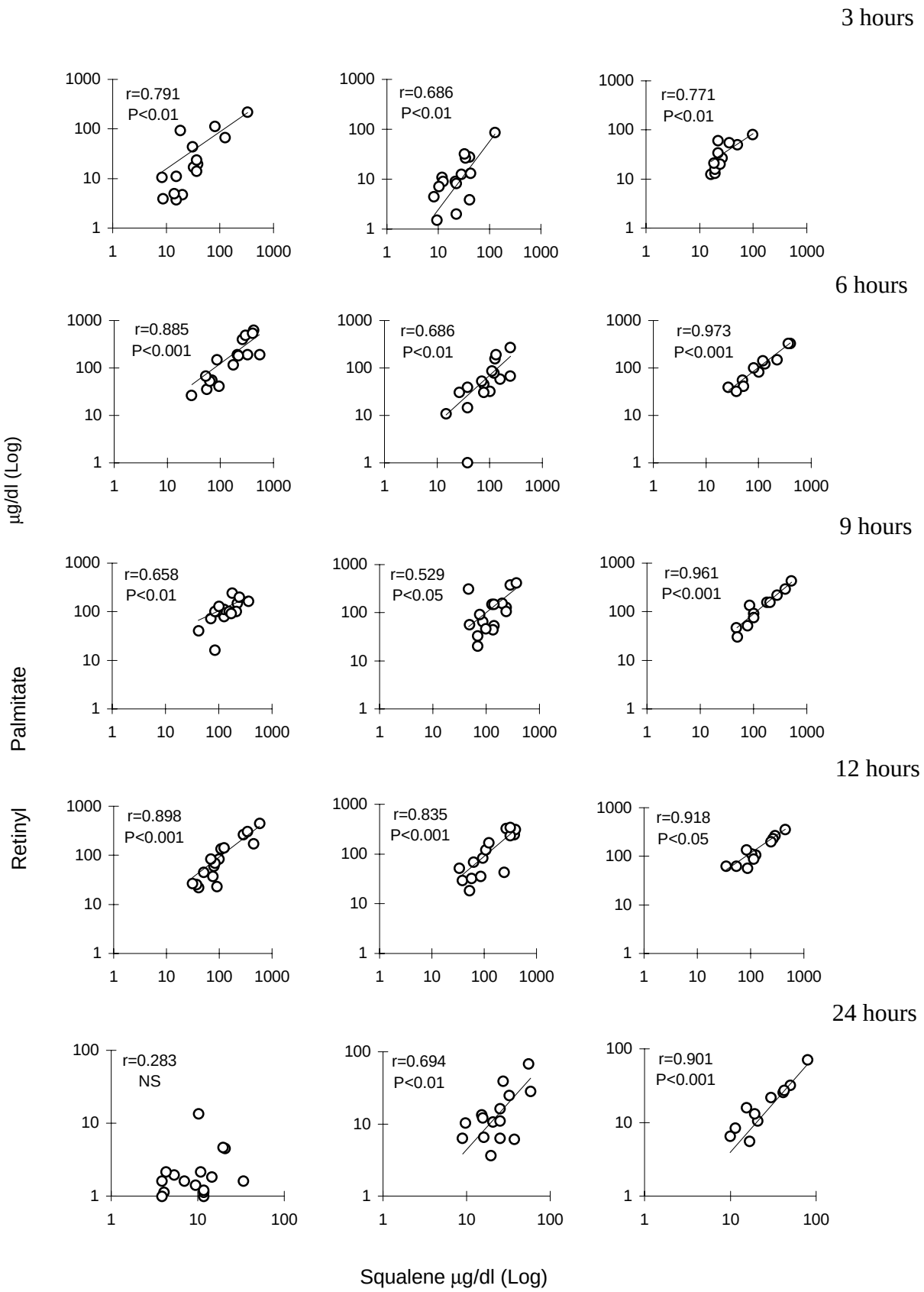

Fig 5. Correlation of postprandial squalene and retinyl palmitate concentrations in chylomicrons (left panel), VLDL (middle), and VLDL during stanol esters (right).

5.3.1. Properties of squalene emulsion

Originally, the concentration of squalene in Intralipid^{R} was 0.80±0.12 mg/dl. After dissolution of squalene, its concentration in the emulsion was 60.3±1.4 mg/dl. The emulsion used in the experiments contained 21.0 mg/dl of cholesterol, 6.4 mg/dl campesterol, 19.0 mg/dl sitosterol, and 5.3 mg/dl stigmasterol. The dose given by an injection of 30 ml thus contained on average 18.1 mg of squalene, 6.0 mg of cholesterol, 5.7 mg of sitosterol, 1.9 mg of campesterol, and 1.6 mg of stigmasterol. The dose of injected triglycerides was 6 g. Only 1% of Intralipid^{R} cholesterol was esterified, whereas the respective values for the plant sterols ranged from 6 to 25% (Table 2 in Study III). In addition, ultracentrifugation of the squalene emulsion revealed that the majority of sterols and of squalene were detected in lipoproteins of densities (d) <0.95 g/ml, but small amounts were found also in d 0.95 to 1.063 g/ml.

5.3.2. Clearance of squalene, plant sterols, and triglycerides in chylomicrons

After the injection, peak squalene concentrations were detected in chylomicrons at 2½ min in four, and at 5 min in two subjects (Fig 1 in Study III). The disappearance of chylomicron squalene, plant sterols, and triglycerides was monoexponential, and no improvement could be achieved by introducing more exponents to the data fitting. Chylomicron squalene with a half-life of 74±8 min was cleared more slowly than plant sterols and triglycerides, and squalene concentration in chylomicrons at 180 min was still higher than at the baseline (P=0.02). The half-life of squalene was correlated with fasting chylomicron squalene concentration (r=0.899, P<0.05).

Peak sitosterol and triglyceride concentrations as well as those of campesterol and stigmasterol were observed in each subject at 2½ min, except in one subject with a peak time at 5 min. The injection did not significantly affect chylomicron cholesterol concentrations (data not shown), since the amount of injected cholesterol was small in relation to the fasting concentrations of cholesterol. The half-lives of injected campesterol (37±5 min), sitosterol (17±2 min), stigmasterol (15±1 min), and triglycerides (17±2 min), were all significantly shorter than that of squalene (Fig 2 in Study III). In addition, the half-life of campesterol exceeded the half-lives of the other plant sterols and triglycerides. Thus, sitosterol, stigmasterol, and triglycerides exhibited a similar pattern of chylomicron clearance, and their baseline levels were reached between 60 and 120 min. Chylomicron campesterol half-life correlated with fasting chylomicron campesterol concentration (r=0.841, P<0.05), whereas the respective correlation coefficient for sitosterol was less consistent (r=0.600, NS). Although 75% of injected sitosterol remained unesterified, concentrations of unesterified and esterified sitosterol increased in chylomicrons almost similarly. After the injection, unesterified and esterified sitosterol (Fig 3 in Study III) and other plant sterols were removed similarly from chylomicrons.

5.3.3. Squalene, plant sterols, and triglycerides in non-chylomicron lipoproteins

After the injection, incremental VLDL squalene, plant sterol, cholesterol, and triglyceride concentrations increased rapidly (Fig 4 in Study III). VLDL squalene, sitosterol, and stigmasterol increments were significant from 2½ min up to 120 min, and campesterol and triglyceride increments from 2½ min up to 60 min. VLDL cholesterol increments, on the other hand, were significant at 30 to 60 min. IDL squalene, campesterol, cholesterol, and triglyceride concentrations were unchanged, whereas those of sitosterol and stigmasterol rose significantly (data not shown). LDL and HDL concentrations of squalene, campesterol, and triglycerides were not significantly altered, whereas again those of sitosterol and stigmasterol rose. In addition, cholesterol precursor sterols were unchanged after the injection (data not shown).

These results suggest that squalene removal from chylomicrons differs from that of plant sterols and triglycerides. Squalene, a hydrophobic compound with no hydroxyl groups, is assumed to remain in the core of the emulsion particles until taken up by the liver, thus labeling the emulsion particles more specifically than do plant sterols.

5.4. Dietary squalene and intestinal cholesterol synthesis (Study II)

Dietary squalene is absorbed quite effectively. It may be assumed that part of the squalene absorbed by mucosal cells may be converted to cholesterol, so that precursor sterol concentrations would be increased in postprandial lipoproteins. Thus, we measured those sterols in chylomicrons, VLDL, and VLDL infranatant postprandially after a squalene test meal. In fact, cholesterol synthesis evaluated by lathosterol-to-campesterol ratios in chylomicrons, VLDL, and VLDL infranatant was diminished after the fat load without squalene up to 12 hours, after which the ratio returned to baseline at 24 hours or remained significantly above it in VLDL infranatant (Fig 5 in Study II). Postprandial plant sterol levels after the test meal remained practically unchanged (data not shown).

However, after the fat load with squalene supplementation, the lathosterol-to-campesterol ratios in the three lipoprotein fractions differed from their respective non-supplemented ratios (Fig 5 in Study II). Squalene addition caused an increase in the chylomicron and VLDL lathosterol-to-campesterol ratio from 6 hours on, and reached significantly higher values at 12 hours from the non-squalene results. In VLDL infranatant, squalene-supplemented values diverged after 6 hours from the non-squalene values and were significantly higher at 9 and 12 hours. The lathosterol-to-sitosterol ratio gave results equal to the respective campesterol ratios for VLDL (Fig 6) and for VLDL infranatant. The postprandial Δ^8-cholestenol-to-campesterol ratio was not influenced by squalene supplementation, but the

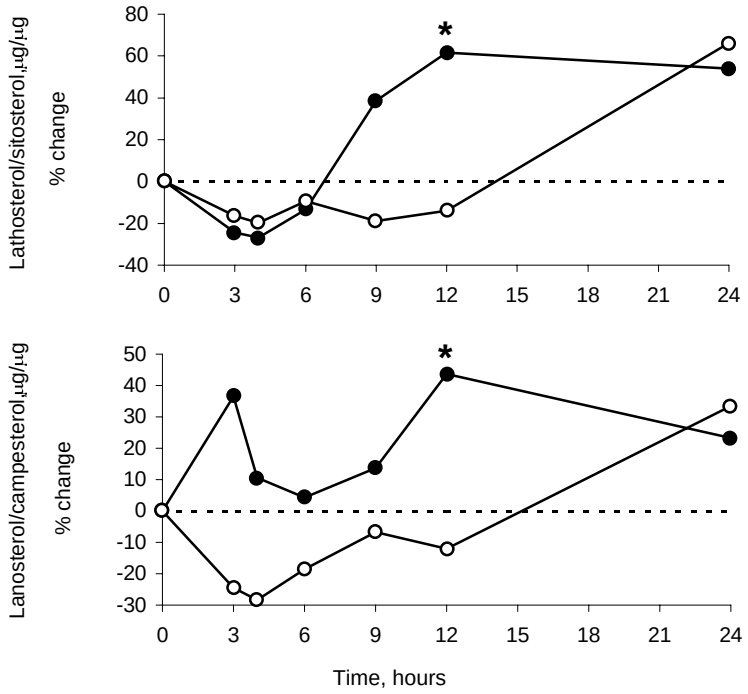

Fig 6. Percentage changes in lathosterol-to-sitosterol (upper panel) and lanosterol-to-campesterol (lower) ratios after squalene-supplemented (—•—) and and non-squalene-supplemented (—o—) test meals. P<0.05 between the studies, ANOVA for repeated measures. * P<0.05 between the studies, one-way ANOVA.

desmosterol-to-campesterol ratio in VLDL differed significantly from the respective non-squalene values. Similarly, the VLDL lanosterol-to-campesterol ratio was significantly increased by squalene-supplemented meals (Fig 6). Thus, even though the changes in other methyl sterols remained inconsistent (data not shown), the findings for precursor sterols suggest that some of the absorbed squalene was converted to cholesterol in intestinal mucosal cells.

5.5. Effect of inhibited cholesterol absorption with increased synthesis of cholesterol on squalene metabolism (Study IV)

Inhibition of cholesterol absorption by stanol esters leads to a compensatory increase in cholesterol synthesis and upregulation of hepatic receptors. Thus, the postprandial metabolism of squalene and retinyl palmitate was studied, before and during stanol ester consumption in oral fat loads enriched with squalene and retinyl palmitate, to learn whether postprandial lipoprotein metabolism is altered during consumption of stanol esters. In addition, the test meals supplemented with additional stanol esters were used to study whether they have any acute effect on postprandial lipoprotein metabolism.

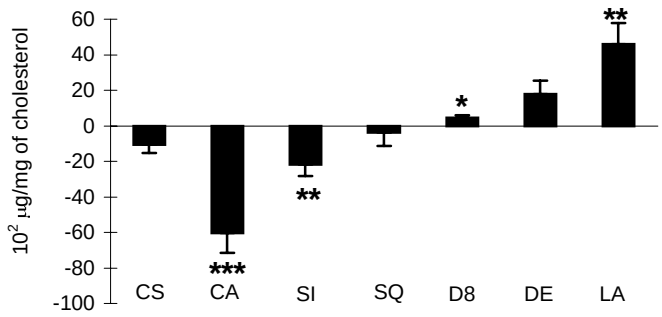

Fig 7. Change in serum squalene and various sterols caused by stanol ester consumption. The value for squalene is the difference obtained by subtracting the squalene-to-cholesterol ratio (10^2 µg/mg) before stanol ester consumption from that during consumption. Values for the various sterols were obtained analogously. CS, cholestanol; CA, campesterol; SI, sitosterol; SQ, squalene; D8, Δ8-cholestenol; DE, desmosterol; LA, lathosterol. * P<0.05, ** P<0.01, *** P<0.001. Mean±SE.

Because postprandial squalene and retinyl palmitate responses did not differ significantly between the fat loads either with or without stanol ester supplementation in the test meal, (Fig 2 in Study IV), the two fat-loading experiments during stanol ester consumption were thus combined.

During the 2-week stanol ester margarine consumption, serum cholesterol and triglycerides were almost unchanged, whereas the ratios of serum campesterol and sitosterol to cholesterol fell significantly (Fig 7), reflecting inhibited cholesterol absorption efficiency. Simultaneously, the serum Δ8-cholestenol and lathosterol-to-cholesterol ratios rose, reflecting compensatory stimulation of cholesterol synthesis. Squalene, desmosterol, and cholestanol levels, however, remained unchanged. Squalene in the test meal had a similar impact on cholesterol synthesis, as assayed by the lathosterol-to-campesterol ratio in postprandial chylomicrons and VLDL, both before and during stanol ester intake (Fig 8). The baseline ratios were, however, higher during stanol esters.

Postprandial VLDL squalene peak concentrations occurred earlier and were 23±8% lower, and AUICs were also significantly reduced during stanol esters by 32±7% (Table 6). Accordingly, the pattern of postprandial squalene curves between the before- and during stanol-ester periods were different (P=0.002, ANOVA for repeated measures). However, only the peak concentration times of postprandial VLDL retinyl palmitate were reduced by stanol esters; peak concentrations and AUICs were not reduced as were the respective squalene values. The postprandial responses of triglycerides and cholesterol in VLDL were not consistently changed during stanol ester consumption.

Postprandial chylomicron squalene and retinyl palmitate concentrations increased and fell earlier during inhibited cholesterol absorption, so that their peak hours

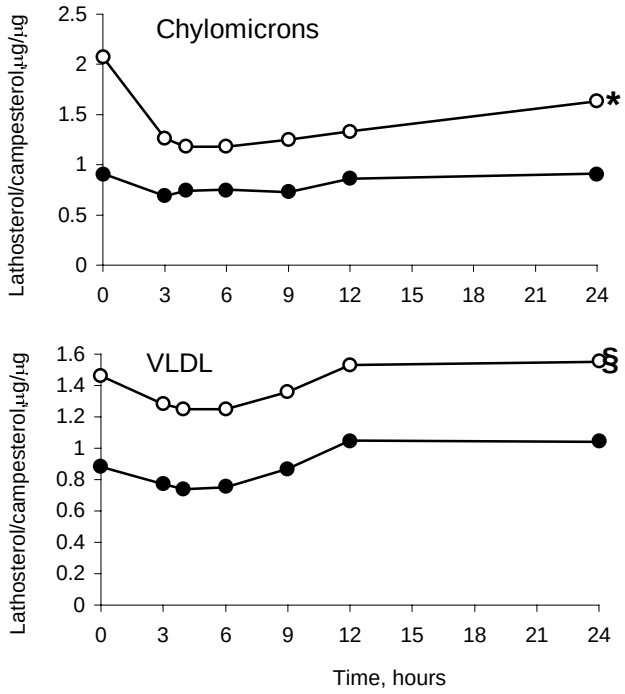

Fig 8. Postprandial lathosterol-to-campesterol ratio before (—•—) and during (—o—) stanol ester consumption in chylomicrons (upper panel) and VLDL (lower). * P=0.02 for stanol ester main effect, P=0.28 for time x stanol ester interaction. § P=0.03 for stanol ester main effect, P=0.91 for time x stanol ester interaction, ANOVA for repeated measures.

occurred significantly earlier than before the consumption. Chylomicron AUCs and AUICs, however, were similar for both markers. Thus, squalene and retinyl palmitate responses were shifted to earlier points in time. The chylomicron triglyceride response was similar before and during stanol esters, but chylomicron cholesterol AUIC was actually even increased by stanol esters.

These results suggest that chylomicron formation was not diminished by inhibited cholesterol absorption efficiency, but the removal of squalene-containing VLDL particles was enhanced during stanol ester consumption.

5.6. Effect of inhibition of cholesterol synthesis on serum and lipoprotein squalene and sterols (Study V)

This study was performed to discover whether inhibition of cholesterol synthesis has any effect on postprandial squalene and vitamin A metabolism. The rationale in recruiting type III hyperlipoproteinemic subjects was to investigate the effect of inhibited cholesterol synthesis in subjects with extreme postprandial lipemia and increased fasting serum squalene levels.

Fasting serum squalene concentration (Table 5) and the squalene-to-cholesterol ratio (Table 2 in Study V) were more than two-fold higher than in the controls, and lathosterol as well as the campesterol-to-cholesterol ratio were also elevated in type III patients. The respective precursor sterol to plant sterol ratios were not increased. However, cholesterol synthesis as measured by the cholesterol balance method and cholesterol absorption efficiency in type III patients were normal.

Before treatment with lovastatin, type III patients had higher postprandial plasma, chylomicron, and VLDL squalene peak concentrations than did controls (Table 4 in Study V). In addition, squalene peak concentration times occurred 2.5 to 6 hours later than in the control group in plasma, chylomicrons, and VLDL ($P<0.05$). This delay in peak time was most prominent in VLDL. As a result, squalene plasma and lipoprotein AUCs were significantly higher in type III patients than in controls.

Cholesterol synthesis measured by the sterol balance method was reduced below the levels of control subjects after treatment with lovastatin (Table 3 in Study V). Similarly, after the treatment, lathosterol-to-plant sterol ratios fell, whereas the lathosterol-to-cholesterol ratio did not differ from that of controls (Table 2 in Study V). In addition to cholesterol levels, lovastatin reduced fasting serum squalene and triglyceride concentrations to close to the levels of control subjects. However, the postprandial squalene response was almost unchanged (Table 4 in Study V). The only effect of synthesis inhibition on postprandial squalene metabolism was a reduction in the time of IDL squalene peak concentration in the type III group. Similar results were obtained with vitamin A serving as a marker, except that the VLDL peak time was also reduced.

Accordingly, although serum lipids as well as cholesterol synthesis evaluated by the sterol balance method and the lathosterol-to-plant sterol ratio were all reduced, postprandial squalene metabolism and that of postprandial lipoproteins in general remained unchanged.

Table 7. Study population.

Subject	Study	Age, years	BMI, kg/m^2	Apo E	Serum C	HDL-C	Serum TG	Diagnosis	Medication
1	I, II	22	21	4 /3	5.1	1.4	1.1		
2	I, II	23	20	3 /3	4.9	1.2	0.6		
3	I, II	23	23	3 /3	4.0	1.6	0.9		
4	II, IV	24	23	2 /3	4.0	1.4	0.8		
5	I, II, IV	25	22	3 /3	4.4	1.7	1.2		
6	I, II, IV	25	25	4 /3	4.4	1.4	0.7		
7	I	25	25	3 /3	3.9	1.6	1.0		
8	IV	58	28	4 /3	6.9	0.9	2.0	CAD	ASA, β-blocker, nitrate
9	II	63	21	3 /3	5.2	1.1	1.0		
10	II, IV	64	27	3 /3	5.8	1.2	1.6		
11	IV	66	27	3 /3	6.3	1.2	2.2		
12	IV	67	23	3 /3	7.4	1.3	1.2		
13	I, II	78	25	4 /3	5.2	0.8	1.5		
14	I, II	79	27	3 /3	5.3	0.9	1.9		
15	I, II, IV	79	25	3 /3	6.5	1.2	1.0		
16	I, II, IV	79	30	4 /3	4.8	1.1	1.2		
17	I, II	79	26	4 /3	5.7	1.3	0.9	DM	ASA, glibenclamide
18	I, II, IV	79	27	3 /3	6.1	0.8	2.3		
19	I, II, IV	79	24	4 /3	5.0	1.6	0.9		
20	I, II	79	26	4 /3	5.2	1.6	0.8		
21	III	21	23	3 /3	4.4	1.4	1.0		
22	III	27	22	3 /3	4.0	1.5	0.9		
23	III	30	23	3 /3	4.7	1.4	0.7		
24	III	31	25	3 /3	6.1	1.2	1.0		
25	III	35	21	3 /3	5.4	1.2	0.9		
26	III	51	22	3 /3	6.0	1.7	0.8		
27		37	25	3 /3	5.5	1.4	1.2		
28		44	26	3 /3	6.3	1.0	1.3		
29		45	22	3 /3	4.3	-	1.0		
30		46	27	3 /3	7.2	1.1	2.1		
31		47	33	3 /3	8.6	1.6	1.0		
32		50	30	3 /3	6.3	1.0	1.7		
33		51	23	3 /3	7.6	2.8	0.5		
34		52	21	3 /3	7.4	1.0	2.0		
35		53	28	3 /3	6.4	1.1	1.4		
36		59	27	3 /3	5.1	1.3	0.8		
37	V	60	24	3 /3	6.8	1.6	1.0		
38		61	-	3 /3	7.5	1.1	2.5		
39		62	23	3 /3	7.5	1.5	1.1		
40		67	26	3 /3	6.4	0.9	1.8		
41		67	23	3 /3	4.0	-	1.3		
42		73	-	3 /3	5.4	0.6	3.2		
43		44	29	2 /2	7.9	1.5	4.4	Type III	
44		47	27	2 /2	6.7	0.8	6.6	Type III	
45		56	30	2 /2	12.0	1.0	7.8	Type III, CAD	ASA, β-blocker, nitrate
46*		63	23	2 /2	10.2	0.6	11.0	Type III	
47		64	24	2 /2	7.2	1.6	2.5	Type III	

Apo, apolipoprotein; ASA, acetylsalicylic acid; BMI, body mass index; C, concentration; CAD, coronary artery disease; DM, Type 2 diabetes mellitus; TG, triglycerides; Type III, Type III hyperlipidemia. * female.

6 DISCUSSION

6.1. Study population

The study population, altogether 47 subjects, consisted of males with normolipidemia or mild to moderate hypercholesterolemia (Studies I-V), and in Study V, one female and 4 males with type III dyslipidemia (Table 7). All subjects in Study I participated also in Study II, and 8 subjects participated both in Studies II and IV. The age of the subjects ranged from 21 to 79 years, serum cholesterol from 3.9 to 12 mmol/l, and serum triglycerides from 0.6 to 11 mmol/l. The wide variation in subjects' ages and serum cholesterol and triglyceride levels was intended to give a more reliable profile of squalene metabolism; had only subjects with a narrow range of ages or normolipidemic subjects been studied, the number of subjects should have had to be extremely large. Since the subjects were their own controls, except for those in Studies I and V, the variation in baseline lipids or body composition did not confound the results. Type III hyperlipidemic subjects were recruited to study the effects of inhibited cholesterol synthesis on postprandial squalene metabolism in order to evaluate this question in extreme conditions, i.e., in subjects with excessively delayed chylomicron clearance. However, Study I, a case-control study had some variation in baseline lipids. Thus, fasting serum triglyceride level, a predictor of postprandial response (Cohn et al. 1988b, Cohen et al. 1992), even though not statistically differing between the young and old subjects, was taken into account in the comparisons of postprandial squalene and retinyl palmitate values. In addition, to avoid type I error, only values of $P<0.01$ were considered statistically significant, since the study population was small, and several statistical comparisons were made. The subject with type 2 diabetes mellitus was not excluded although diabetes is known to influence postprandial lipid metabolism (Chen et al. 1993), since neither his baseline lipids nor postprandial results differed from the other old subjects.

6.2. Squalene in fasting and postprandial serum and lipoproteins

Overall, mean fasting serum squalene concentration ranged from 66 to 163 µg/dl. No population reference values for squalene are available in the literature. In addition, variation in the dietary intake of squalene affects serum levels (Liu et al. 1976, Strandberg et al. 1990, Miettinen and Vanhanen 1994a), which is one possible explanation for the present variation. Other factors affecting serum squalene levels include serum triglyceride concentration (Saudek et al. 1978) and the activity of cholesterol synthesis. However, serum triglycerides did not correlate with serum squalene levels in the present study. In addition, the present study revealed that fasting serum squalene was not correlated with age. It remains unknown whether

gender differences exist in squalene metabolism. In the fasting state, squalene was mainly present in LDL and HDL, although the squalene-to-cholesterol ratio was highest in VLDL and lowest in LDL, as observed previously (Strandberg et al. 1990).

The present squalene-enriched fat challenges were performed with an oral squalene dose of 0.5 g. In chylomicrons and VLDL, postprandial squalene peak times occurred 8 to 10 hours after the ingestion and then declined to baseline almost as did the respective postprandial retinyl palmitate configurations. We also observed that squalene concentrations were not consistently increased in LDL and HDL after oral ingestion, and that postprandial increases in VLDL infranatant squalene mainly comprised increases in IDL squalene. In sum, postprandial squalene was carried in triglyceride-rich lipoproteins.

Postprandial retinyl palmitate peaked later than squalene in VLDL infranatant, as in the study by Rajaratnam et al. (1999b), in which squalene labeled postprandial lipoproteins with higher specificity than did vitamin A as compared to postprandial apo B-48 levels. Thus, it is possible that squalene is a more reliable marker of postprandial remnant lipoproteins than is retinyl palmitate. In fact, use of retinyl palmitate as a postprandial marker has been criticized because of a marked exchange of retinyl palmitate between lipoproteins of intestinal and hepatic origin (Cohn et al. 1993). Apo B-48 and retinyl palmitate do not label the same components of postprandial intestinal lipoproteins (Lemieux et al. 1998), more specifically apo B-48 reflects the number of intestinal particles, and retinyl palmitate the lipid of intestinal origin. It must be emphasized that when squalene is used as a marker of postprandial lipoproteins and lipids in general, it does not specifically reflect the number of apo B-48-containing lipoproteins derived from the intestine. Lipoproteins containing apo B-48 and B-100 particles may, however, be of the same size and share common lipolytic pathways (Cohn et al. 1988b, Cohn et al. 1993, Karpe et al. 1993b, Schneeman et al. 1993). Thus, both may be of pathophysiological importance in the development of coronary artery disease (Karpe 1999, Karpe et al. 1999).

6.3. Fate of postprandial squalene in young vs. old subjects (Study I)

LDL cholesterol levels increase with advancing age (Miller 1984, Eriksson et al. 1991b), and results concerning age from earlier postprandial studies have been variable. Thus, we investigated whether age affects postprandial squalene metabolism. In chylomicrons, there were no consistent age-related differences in postprandial squalene, retinyl palmitate, or triglyceride metabolism. However, the postprandial pattern of VLDL squalene concentrations differed between the young and old subjects in our study. Squalene concentrations increased similarly in both groups up to 9 hours, but after this point in time, concentrations in the young group

started to decline, whereas a marked increase up to 12 hours was observed in the aged. Then, at 24 hours, squalene returned to baseline levels also in the aged. This difference in pattern may be caused by delayed absorption of squalene or defective removal of postprandial lipoproteins.

In concordance with Borel et al. (1998), in the old subjects chylomicron triglyceride peaks were reached later. One possible explanation for this may be related to the reduced gastric emptying rate in the aged (Evans et al. 1981, Wegener et al. 1988). However, despite delayed gastric emptying, the mouth-to-cecum or whole-gut transit time in the aged is not necessarily decreased (Wegener et al. 1988, Husebye and Engedal 1992). Squalene absorption or chylomicron formation were not expected to be delayed, since the ascending parts of the chylomicron triglyceride, squalene, and retinyl palmitate curves in both age groups were similar.

Postprandial plasma triglycerides reached their maximum earlier and disappeared faster than did squalene and retinyl palmitate in both age-groups, a common observation in postprandial studies. Thus, both chylomicrons and VLDL had low squalene and retinyl palmitate-to-triglyceride ratios in the early stage of absorption. The values increased markedly in the later samples at 6 to 9 hours only in chylomicrons. However, the resulting ratio should be higher also for the remnant VLDL than for chylomicrons if VLDL is derived from chylomicrons as a result of lipolysis. Thus, it could be assumed that, in addition to chylomicrons, VLDL particles containing squalene and retinyl palmitate were also released directly from the intestine, which is known to secrete lipoproteins also directly into the VLDL density class (Ockner et al. 1969). In addition, the triglyceride content of VLDL of hepatic origin is considered to remain unchanged in postprandial lipemia (Björkegren et al. 1997), but the meal consumed at 5 p.m. (at 9 hours) may affect the squalene-to-triglyceride ratio at 12 hours due to the increased triglyceride levels. Recent evidence suggests, however, that under postprandial conditions, retinyl esters are secreted with chylomicrons but not with VLDL (Nayak et al. 2001).

Increase in VLDL apo B-100 production (Millar et al. 1995) and decrease in LPL activity but not that of hepatic lipase (Huttunen et al. 1976) have been associated with increasing age. The removal of LDL apo B, on the other hand, was similar in 75-year-old and 50-year-old men (Gylling et al. 1994), suggesting that LDL receptor activity was normal. On the other hand, Eriksson et al. (1991a) hypothesized that the age-associated increase in LDL cholesterol was mediated via reduced hepatic LDL receptor expression. Although the LDL receptor is a critical participant in the uptake of remnants by hepatocytes, other molecules, including the LRP and cell-surface HSPG, are also involved in the process (Mahley and Ji 1999). We suggest that increased 12-hour VLDL squalene concentrations in the aged more likely represent defective removal of remnant lipoproteins than delayed absorption of squalene. This

could be caused in the aged, in addition to the LDL receptor, by defects in LRP or HSPG pathways.

6.4. Clearance of intravenously administered squalene

Our purpose was to study chylomicron clearance of squalene and plant sterols after an intravenous bolus injection in human subjects. We also examined nonchylomicron lipoprotein fractions since little is known about the metabolism of intravenously administered squalene and plant sterols in human lipoproteins. Preliminary studies were performed to study squalene clearance in endogenous squalene-labeled lipoproteins and to compare this to the clearance of squalene emulsion, but due to low squalene concentrations in endogenous lipoproteins no reliably detectable squalene levels were reached after their intravenous administration.

After the injection of squalene emulsion, squalene, campesterol, and triglyceride concentrations increased only in chylomicrons and VLDL, whereas the concentrations of sitosterol and stigmasterol increased also in LDL, and those of sitosterol even in HDL. Chylomicron squalene was cleared more slowly than triglycerides, which suggests that it remains in the remnant particles after LPL-mediated lipolysis until finally taken up by the liver. We assumed that plant sterols also would be removed more slowly than triglycerides, since, in humans, labeled cholesteryl esters are cleared at a slower rate than labeled triglycerides injected with chylomicron-like emulsions (Redgrave et al. 1993, Maranhão et al. 1996, Santos et al. 2000). However, sitosterol and stigmasterol were removed from chylomicrons more rapidly than expected up to a rate comparable to that of triglycerides.

After simultaneous pulse labeling with ^{3}H-sitosterol and ^{14}C-cholesterol by the intravenous route, the half-life of sitosterol was shorter than that of cholesterol (Salen et al. 1970), suggesting that in humans, the removal rates for cholesterol and plant sterols are not similar. Different side chains of sitosterol and campesterol, however, do not affect their serum clearances in the rat (Mortimer et al. 1995). On the other hand, campesterol is absorbed more efficiently than is sitosterol (Heinemann et al. 1993), and serum levels of campesterol are higher than sitosterol's (Miettinen et al. 1990). Thus, one explanation for the slower clearance of campesterol than of sitosterol may be the higher basal concentration of campesterol.

Increased concentrations of VLDL squalene, plant sterols, and triglycerides may originate directly from the emulsion particles immediately after the injection. However, the increase in these lipids in VLDL after the initial decreases at 5 to 10 min may derive from emulsion remnants, although evidence exists that a large fraction of the Intralipid^{R} emulsion particles are removed with little or no lipolysis

occurring during the circulation (Hultin et al. 1995). Unlike sitosterol, changes in squalene concentrations were observed only in chylomicrons and VLDL, indicating that squalene probably may not be transferred between the lipoprotein fractions. It is possible that the rapid clearance of chylomicron sitosterol and stigmasterol, and the appearance of sitosterol in LDL and HDL are caused by an nonspecific transfer of surface-associated unesterified sterols according to concentration gradients, or the phenomenon is related to the activity of lipid transfer proteins including PLTP. Nishida and Nishida (1997) suggested that PLTP could facilitate transfer of unesterified cholesterol to HDL. On the other hand, if plant sterols are rapidly esterified after an intravenous injection like cholesterol (Viikari et al. 1977), esterification may be one mechanism contributing to the more extensive initial disappearance of unesterified sitosterol observed in the present study (Fig 3 in Study III).

The finding in the rat that squalene disappears faster than cholesterol from serum after an intravenous injection, and is partly cyclized to sterols, and partly re-enters the circulation (Tilvis and Miettinen 1982) could not be observed in the present human studies. It is possible that the follow-up time of 3 hours was not long enough to observe any changes in cholesterol synthesis, or the squalene dose (1/25 of the oral dose in Study II) was too small to increase cholesterol synthesis detectably.

These results suggest that squalene labels chylomicron-like triglyceride-rich particles and can be used to measure the clearance of chylomicron-like emulsions without the use of radioactive isotopes. However, whether squalene clearance from endogenous chylomicrons is comparable to squalene emulsions remains to be studied.

6.5. Acute effect of dietary squalene on cholesterol synthesis

Squalene feeding increases cholesterol synthesis, as indicated by lathosterol or other precursor sterols both in the rat (Tilvis and Miettinen 1983a) and in humans (Miettinen and Vanhanen 1994a). Reports concerning effects of daily squalene consumption on serum cholesterol levels present variable results for humans. Intake of squalene (1 g/day) mimicking a diet very rich in squalene led to elevated cholesterol precursor sterol and cholesterol concentrations, but during a smaller dose (0.5 g/day), serum precursor sterols and cholesterol were normalized (Miettinen and Vanhanen 1994a). In addition, LDL cholesterol levels were significantly higher during an olive oil diet with its daily squalene intake of 0.2 g than during rapeseed or sunflower oil diets with a low squalene content (Pedersen et al. 2000), suggesting that intake of squalene may have unfavorable effects on serum lipids. On the contrary, squalene consumption of 0.86 g/day has been found even to reduce serum cholesterol in hypercholesterolemic subjects (Chan et al. 1996). Accordingly, we

examined whether a single dietary dose of squalene would increase cholesterol synthesis, as measured by precursor sterols within 24-hour period of time.

The lathosterol-to-cholesterol ratio has earlier been used to measure acute changes in cholesterol synthesis induced by simvastatin (Pfohl et al. 1998). Since a substantial amount of cholesterol is ingested with a fat meal, we decided to use the precursor sterol-to-plant sterol ratios as an indicator of cholesterol synthesis. Intestinal absorption of squalene has been estimated to be as high as 85% in human subjects (Strandberg et al. 1990, Miettinen and Vanhanen 1994a) and 0.5 g of oral squalene produces 10-fold increases in plasma squalene concentrations (Rajaratnam et al. 1999b). We assumed that a single dose of 0.5 g of squalene might detectably affect cholesterol precursor sterols in postprandial lipoproteins. In fact, cholesterol synthesis was increased from 9 to 12 hours after the test meal as indicated by an increased lathosterol-to-campesterol ratio in chylomicrons, VLDL, and VLDL infranatant. The present data also suggest that 0.5 g of squalene given with a cream-eggshake meal does not interfere with postprandial cholesterol, triglyceride or retinyl palmitate concentrations.

Without a squalene supplement, cholesterol synthesis was reduced as indicated by a lowered precursor sterol-to-plant sterol ratio during the daytime, and not until the next morning did it reach baseline level. This situation reflects the diurnal variation in cholesterol synthesis observed in experimental animals (Kandutsch and Saucier 1969, Edwards et al. 1972, Strandberg et al. 1984) and in human subjects (Miettinen 1982a). Decrease in cholesterol synthesis is indicated by lowered precursor sterols in serum at noon and afternoon hours, followed by their rise towards the evening and the maximum at 2 to 4 a.m. (Miettinen 1982a).

Since it is known from the earlier studies (Gylling and Miettinen 1994a, Rajaratnam et al. 1999b) that postprandial squalene peak concentrations in triglyceride-rich lipoproteins occur most often between 6 and 9 hours after oral ingestion, the effect of squalene on cholesterol synthesis was expected to be observable no earlier than 6 hours after oral administration. The increases in precursor sterol-to-plant sterol ratios in chylomicrons suggest that squalene is already partly converted to cholesterol precursors in the intestinal mucosa, which has a significant capacity to synthesize cholesterol (Lindsey and Wilson 1965). In fact, it has been shown that in the rat, dietary squalene is converted to sterols during its transit through the intestinal wall (Tilvis and Miettinen 1983b). It is impossible to distinguish between intestinal and hepatic cholesterol synthesis using precursor sterol-to-plant sterol ratios, but it is probable that the newly absorbed squalene was converted to precursor sterols and further to cholesterol also in the liver as suggested by the elevated precursor sterol ratios also in VLDL and VLDL infranatant. Overall cholesterol synthesis, however, need not be increased if endogenous cholesterol synthesis is temporarily reduced by squalene.

If cholesterol synthesis is continuously stimulated by dietary squalene (Strandberg et al. 1990), it is possible that newly absorbed squalene is converted into bile acids to a significantly higher extent than is cholesterol as shown earlier in the rat (Tilvis et al. 1983b). The activity of HMG-CoA reductase, on the other hand, can be suppressed by a negative feedback mechanism in response to an increased precursor flow (Brown and Goldstein 1986, Strandberg et al. 1989a). These factors may counteract squalene-induced increases in serum cholesterol levels.

6.6. Effect of inhibited cholesterol absorption on postprandial squalene metabolism

Assuming that de novo cholesterol synthesis is not upregulated in the cell when cholesterol absorption is inhibited, less cholesterol is available in the enterocyte to be assembled into the chylomicron particle. It is not known, how cholesterol malabsorption affects the postprandial lipoprotein metabolism. In fact, daily consumption of stanol ester margarine diminished postprandial chylomicron and VLDL squalene and retinyl palmitate peak times and postprandial VLDL squalene peak concentrations and AUICs. However, postprandial triglyceride concentrations were not consistently affected.

Consumption of stanol ester margarine for two weeks only tended to decrease serum cholesterol levels. The earlier stanol ester studies of serum total and LDL cholesterol-lowering, the treatment periods have lasted markedly longer, 6 weeks as a minimum (Heinemann et al. 1986, Miettinen and Vanhanen 1994b, Vanhanen et al. 1994, Miettinen et al. 1995, Vuorio et al. 2000), but in cholectomized subjects a reduction in lipids was detectable within a few days (Miettinen et al. 2000b). However, in the present study, that serum plant sterol-to-cholesterol ratios decreased and those of precursor sterols increased, suggests that compliance was good, and that cholesterol absorption was inhibited and cholesterol synthesis was compensatorily increased. Serum squalene, on the other hand, was unaffected, which is actually a common finding in stanol ester studies (Table 2). Accordingly, these results suggest that the 1 to 2-week stanol ester consumption had altered the whole-body cholesterol metabolism, but the reduction in serum cholesterol level was only modest, possibly due to the compensatory increase in cholesterol synthesis.

The underlying mechanism of malabsorption caused by plant sterols and stanols is most probably related to the reduced micellar solubilization of cholesterol (Hassan and Rampone 1980). Dietary stanols unexpectedly even increased chylomicron cholesterol AUC, suggesting that the chylomicron formation was not interfered with. On the other hand, the similar or increased postprandial cholesterol concentrations before and during administration of stanol esters suggest that increased cholesterol

synthesis, most likely also in the intestinal mucosa, compensates for the stanol-induced inhibition of cholesterol absorption. However, the acute stimulation of cholesterol synthesis by squalene was similar before and during stanol esters.

Similar postprandial chylomicron lipid configurations together with the observation that no additional effect was obtained by stanol esters added to the test meal suggest that it is not the acute stanol ester-induced impairment of cholesterol absorption but altered metabolic phenomena caused by daily treatment responsible for altered postprandial lipid metabolism. On the other hand, the finding that the postprandial concentrations of squalene and retinyl palmitate in chylomicrons were not reduced by margarine (8 g) with or without stanol esters suggests that neither squalene nor retinyl palmitate absorption was decreased.

Consumption of stanol esters more frequently caused significant effects on postprandial squalene than on retinyl palmitate values. As a hydrocarbon, squalene is more fat-soluble than is retinyl palmitate, which could lead to a more effective replacement of squalene from intestinal micelles by stanols. In addition, it has been shown that stanol esters do not affect serum levels of retinol, whereas serum levels of ß-carotene, which is less polar than retinol, are reduced (Gylling et al. 1999b, Hallikainen and Uusitupa 1999).

Despite the suggested increase also in mucosal cholesterol synthesis, inhibited cholesterol absorption is expected to lead to overall diminished cholesterol flux to the liver, because plant sterols also inhibit absorption of cholesterol secreted by the liver into bile (Mattson et al. 1982, Heinemann et al. 1991). Reduced cholesterol absorption may lower the hepatic cholesterol pool despite compensatorily enhanced cholesterol synthesis (Gylling et al. 1999a). In general, an increase in number of LDL receptors results in the lowering of LDL cholesterol levels during such treatments as with resins and statins (Kovanen 1987). Stanol esters reduce the LDL apo B transport rate and also VLDL and IDL cholesterol levels (Gylling and Miettinen 1996), and possibly may increase the hepatic uptake of these precursor lipoproteins by upregulated LDL receptor activity. Upregulation of LDL receptors is suggested to improve the removal of postprandial lipids during tetrahydrolipstatin treatment, as well (Reitsma et al. 1994).

Thus, reduction of postprandial VLDL squalene peak concentration and AUICs with no respective changes in chylomicrons by dietary stanol ester consumption, and that addition of stanol esters to the test meal caused no further effect, together suggest that the hepatic removal of squalene-containing remnant lipoproteins was enhanced.

6.7. Effect of inhibited cholesterol synthesis on postprandial squalene metabolism in type III lipoproteinemia

A favorable lipid-lowering effect in type III hyperlipidemia has been attained by lovastatin (Illingworth and O'Malley 1990), but whether statins lessen the delayed removal of postprandial lipids in type III hyperlipidemia is unknown. To this end, postprandial squalene metabolism was studied before and during lovastatin treatment in type III hyperlipoproteinemic subjects known to have increased fasting and postprandial levels of squalene (Gylling and Miettinen 1994a).

Lovastatin treatment normalized the abnormally high fasting serum and lipoprotein lipid levels. In addition, increased squalene and lathosterol-to-plant sterol ratios were lowered to levels comparable to those of controls. Since cholesterol synthesis measured by the sterol balance method was normal in type III patients before statin treatment, it can be suggested that increased precursor-to-cholesterol ratios do not reflect cholesterol synthesis in this extreme remnant dyslipidemia. However, the lathosterol-to-plant sterol ratio was a reliable indicator of cholesterol synthesis confirmed by sterol balance data. This suggests that the precursor sterol-to-plant sterol rather than the respective cholesterol ratio may be a more reliable indicator of cholesterol synthesis in certain clinical conditions with an altered lipid pattern such as type III hyperlipidemia. Increased serum squalene levels, known to correlate with serum triglycerides (Saudek et al. 1978), were probably related to hyper-triglyceridemia in the type III group. It has been observed that statin treatment may reduce fasting serum squalene levels both with (Vanhanen et al. 1992) and without (Vanhanen and Miettinen 1995) concomitantly reduced serum triglycerides. On the other hand, reduction of serum triglycerides during pravastatin treatment does not necessarily lead to reduced squalene levels (Gylling and Miettinen 1996). Thus, despite reduced cholesterol synthesis during statin treatment (Table 2), serum squalene levels can be highly variable.

As fasting triglycerides and squalene were reduced to normal levels by lovastatin in type III subjects, a reduction in postprandial squalene response could be expected. However, only insignificant reductions in squalene AUCs occurred, and the AUCs in the type III group still significantly exceeded those of controls. Type III hyperlipoproteinemia is caused by forms of apo E that are defective in binding to lipoprotein receptors (Mahley and Rall 1989) or by apo E deficiency (Schaefer et al. 1986). Upregulation of hepatic LDL receptors occurs in type III hyperlipoproteinemia, since cholesterol delivery to the liver is reduced due to the defective binding of apo E2-containing lipoproteins to the LDL receptors (Davignon et al. 1988, Mahley and Rall 1989). Thus, in type III hyperlipoproteinemia, inhibition of cholesterol synthesis by statin treatment, which in turn increases hepatic LDL receptor activity (Brown et al. 1981), probably does not cause sufficient induction of LDL receptors to reduce the levels of postprandial lipids. This might

explain why, after the fat load, despite the normalized fasting triglycerides, postprandial concentrations of triglyceride-rich lipoprotein squalene were unaltered by lovastatin treatment.

These results suggest that in type III hyperlipoproteinemia neither serum squalene nor precursor sterol-to-cholesterol ratios reflect cholesterol synthesis, and that normalization of serum lipids by lovastatin treatment does not lead to enhanced postprandial lipoprotein removal, as measured by squalene. Whether inhibition of cholesterol synthesis by statins has any effect on postprandial squalene metabolism in other populations remains to be studied.

7. SUMMARY AND CONCLUSIONS

Postprandial metabolism of squalene in humans has been infrequently studied. Some of the absorbed squalene is incorporated into de novo synthesis of cholesterol, but its serum levels reflect cholesterol synthesis less consistently than do the cholesterol precursors possessing the sterol ring. Elevated fasting serum and postprandial lipoprotein squalene levels have recently been associated with coronary artery disease, at least in women. Therefore, the postprandial metabolism of dietary squalene was studied in order to discover whether it is age-related, and whether inhibition of cholesterol synthesis or absorption with subsequently increased cholesterol synthesis interferes with it, and to investigate whether exogenous squalene can rapidly increase cholesterol synthesis as measured by cholesterol precursor sterols in postprandial lipoproteins. In addition, the clearance of intravenously administered squalene in lipid-emulsion was measured and related to clearances of plant sterols and triglycerides to learn whether squalene in artificial chylomicron-like emulsions reflects postprandial lipoprotein metabolism.

Postprandial squalene metabolism was examined in young compared to old subjects after an oral fat challenge test with 0.5 g of squalene. Fasting serum concentrations of squalene in young and old subjects did not differ. The pattern of postprandial squalene concentration in chylomicrons after oral administration of squalene did not differ between the age-groups, suggesting that chylomicron formation was not age-dependent. Postprandial squalene concentrations in VLDL were similar for up to 9 hours in both groups, but thereafter squalene concentrations showed a prolonged increase up to 12 hours in the old men, whereas in the young the concentrations had already declined after 9 hours. Plasma squalene levels returned to baseline at 24 hours in both groups. This suggests that the clearance of postprandial VLDL squalene was age-dependent and delayed in old subjects.

Acute effects of oral squalene on cholesterol synthesis were studied by measuring lipoprotein precursor sterols after a test meal with and without 0.5 g of squalene supplementation. The squalene dose increased squalene areas under curves 9-fold in chylomicrons, 5-fold in VLDL, and 1.7-fold in VLDL infranatant. Significant increases in squalene concentrations were observed also in IDL but not in LDL and HDL. Cholesterol synthesis measured by cholesterol precursor sterol-to-plant sterol ratios in chylomicrons, VLDL, and VLDL infranatant was increased 9 to 12 hours after the ingestion of squalene. These results suggest that orally ingested squalene labels only the postprandial triglyceride-rich lipoproteins and leads to increased cholesterol synthesis obviously even in the intestinal mucosa, but owing to possible inhibition of HMG-CoA reductase activity, overall synthesis may not be changed.

The removal of intravenously administered squalene and plant sterols in lipid emulsion (Intralipid^{R}) enriched with squalene was examined in healthy volunteers.

Clearance of the squalene in chylomicrons occurred more slowly than that of triglycerides and of plant sterols. Campesterol removal, on the other hand, was slower than that of sitosterol or stigmasterol, but was more rapid than that of squalene. In contrast to sitosterol, the concentrations of squalene and campesterol were not increased in IDL, LDL, and HDL. Thus, squalene obviously remained tightly associated with the remnants of triglyceride-rich particles until taken up by the liver. In addition, the clearance rates of squalene and campesterol were correlated negatively with their fasting concentrations, suggesting that their removal may depend on fasting concentrations. This indicates that squalene clearance of intravenous of lipid emulsion may be a suitable means for measurement of postprandial lipoproteins.

We considered that chronic cholesterol malabsorption and the subsequent increase in cholesterol synthesis may enhance removal of postprandial lipoprotein squalene. Postprandial metabolism of dietary squalene was thus studied before and during inhibition of cholesterol absorption by stanol ester consumption. Fasting levels of serum squalene were not altered by inhibition of cholesterol absorption associated with increased cholesterol synthesis during such consumption. The peak chylomicron squalene concentration occurred earlier during stanol ester consumption, but neither chylomicron peak concentrations nor areas under incremental curves were affected by stanol esters, suggesting that chylomicron formation was not interfered with during cholesterol malabsorption. VLDL squalene peak concentrations and areas under incremental curves as well as peak times fell during stanol ester consumption, suggesting that chylomicron remnant formation was reduced or the uptake to liver enhanced. Addition of stanol esters to the test meal did not alter postprandial squalene metabolism further. These results suggest that inhibition of cholesterol absorption enhances postprandial VLDL squalene clearance and, in general, postprandial lipoprotein clearance. Since postprandial lipemia is considered atherogenic, inhibition of cholesterol absorption would seem to diminish the postprandial atherogenic burden.

Postprandial metabolism of dietary squalene was also studied during inhibition of cholesterol synthesis by lovastatin in type III hyperlipoproteinemic patients with retarded postprandial lipoprotein removal. The fasting serum squalene level was higher in type III hyperlipidemic subjects than in controls, but their cholesterol synthesis was normal as measured by the sterol balance technique. Postprandial plasma, chylomicron, and VLDL squalene areas under curves and peak concentrations were significantly higher, and the time of peak concentrations in plasma, chylomicrons, and VLDL occurred significantly later in type III patients than in controls. Inhibition of cholesterol synthesis normalized fasting serum lipids including fasting serum squalene levels, but that postprandial plasma and lipoprotein squalene levels remained unchanged suggests that, in type III patients, postprandial squalene metabolism is unaffected by cholesterol synthesis inhibition.

The present studies have expanded our knowledge of postprandial squalene metabolism in human beings. Since most of the exogenous, i.e., oral or intravenous squalene was carried in triglyceride-rich lipoproteins, postprandial lipoprotein squalene measurement offers an additional means to evaluate lipoprotein clearance. Postprandial squalene and lipoprotein removal is age-dependent and retarded in elderly men. A single dose of squalene even partially increases cholesterol synthesis in the intestinal mucosa, revealing why large amounts of squalene in the diet, for example in olive oil, do not necessarily reduce serum total and LDL cholesterol levels. Inhibition of cholesterol synthesis does not normalize postprandial squalene and lipoprotein metabolism, at least in type III hyperlipidemic subjects with retarded uptake of postprandial lipoproteins, but, however, the postprandial clearance of squalene and, in general, that of lipids, can indeed be enhanced by inhibition of cholesterol absorption efficiency.

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