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Cholesterol metabolism in type 2 diabetes

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Academic dissertation

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To my family

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LIST OF ORIGINAL PUBLICATIONS

This thesis is based on the following original articles, which are referred to in the text by their Roman numerals.

I Simonen PP, Gylling HK, Miettinen TA. Diabetes contributes to cholesterol metabolism regardless of obesity. Diabetes Care 2002; 25:1511-1515.

II Simonen PP, Gylling H, Miettinen TA. Body weight modulates cholesterol metabolism in non-insulin dependent type 2 diabetics. Obes Res 2002;10:328-335.

III Simonen P, Gylling H, Miettinen TA. Acute effects of weight reduction on cholesterol metabolism in obese type 2 diabetes. Clin Chim Acta 2002;316:55-61.

IV Simonen P, Gylling H, Howard AN, Miettinen TA. Introducing a new component of the metabolic syndrome: low cholesterol absorption. Am J Clin Nutr 2000;72:82-88.

ABBREVIATIONS

ABC	adenosine triphosphate-binding cassette transporter
ACAT	acyl-CoA cholesterol acyltransferase
ANOVA	analysis of variance
Apo	apolipoprotein
BMI	body mass index
BSEP	bile salt export pump
CETP	cholesteryl ester transfer protein
Cr_2O_3	chromic oxide
CYP7A1	cholesterol 7α-hydroxylase
d	density
Δ^8 – cholestenol	cholestenol
Δ^7 – lathosterol	lathosterol
DM	diabetes group
FCR	fractional catabolic rate
FXR	farnesoid X receptor
HbA1c	glycosylated hemoglobin A1c
GLC	gas-liquid chromatography
HDL	high density lipoprotein
HMG-CoA	3-hydroxy-3-methylglutaryl-coenzyme A
HOMA	homeostasis model assessment
I-BABP	ileal bile-acid binding protein
IDL	intermediate density lipoprotein
LCAT	lecithin:cholesterol acyltransferase
LDL	low density lipoprotein
LED	low energy diet
LRP	LDL receptor-related protein
LXR	liver X receptor
MTP	microsomal triglyceride transfer protein
PLTP	plasma phospholipid transfer protein
RXR	retinoid X receptor
SHBG	sex hormone binding globulin
SR-BI	scavenger receptor BI
SREBP	sterol regulatory element binding protein
TR	transport rate
TRL	triglyceride rich lipoproteins
VLDL	very low density lipoprotein
VLED	very low energy diet

ABSTRACT

Type 2 diabetes is associated with many metabolic disturbances including hyperinsulinemia, insulin resistance, hyperglycemia, dyslipidemia and obesity. The alterations occurring in lipoprotein metabolism in diabetes have been described in detail, but the metabolism of cholesterol and bile acids has been less well characterized, and the results from the few previous studies are controversial. Obesity, in addition to predisposing to the development of diabetes, is associated with abnormal cholesterol metabolism. Accordingly, cholesterol metabolism was studied in obesity with and without type 2 diabetes, and in type 2 diabetes with and without overweight. In addition, the effects of weight reduction were studied on cholesterol and sterol metabolism in a non-stable state during fasting and also in subsequent steady state after a prolonged follow-up.

Cholesterol absorption and cholesterol and bile acid synthesis were studied in 16 obese (BMI > 30 kg/m^2) type 2 diabetic patients and compared to 16 similarly obese controls to reveal the role of diabetes on cholesterol metabolism. The effects of body weight on cholesterol metabolism were investigated with 20 normal-weight (BMI ≤ 26.0 kg/m^2) and 44 overweight (BMI > 26.1 kg/m^2) type 2 diabetic patients. Cholesterol absorption was evaluated with the peroral dual isotope technique and by quantitating serum ratios of phytosterols and cholestanol to cholesterol, cholesterol synthesis with sterol balance as well as serum ratios of squalene and precursor sterols (cholestenol, desmosterol, lathosterol) to cholesterol. In order to clarify the role of weight reduction in modulating cholesterol metabolism in type 2 diabetes, parameters associated with cholesterol and sterol metabolism were determined during weight reduction, and during a 2-year follow-up after weight reduction. Ten obese type 2 diabetic patients consumed a very low energy diet virtually free of cholesterol, cholestanol and plant sterols for 3 months, and serum squalene and non-cholesterol sterol levels were determined before and after the weight reduction program in a non-steady state situation. Sixteen obese type 2 diabetic patients consumed a very low energy or low-energy diet for 3 months, after which they consumed a weight-maintaining diet for up to 2 years. The changes in cholesterol metabolism were determined by assaying cholesterol absorption efficiency, sterol balance, and serum sterols after a 2-year follow-up, at a stable, reduced weight level.

The efficiency of cholesterol absorption and the amounts of absorbed total, dietary and biliary cholesterol were lower in the obese type 2 diabetic patients than obese controls or in normal-weight type 2 diabetic patients. Cholesterol absorption was similar in both diabetics with normal body weight and obese controls. Fecal elimination of cholesterol, mainly as neutral sterols and less as bile acids, was increased, and this enhanced cholesterol synthesis more in obese patients with type 2 diabetes than in obese controls or normal-weight diabetic patients. In addition, fecal bile acids, the total intestinal cholesterol pool, biliary cholesterol secretion and cholesterol turnover were significantly higher in obese diabetic patients than normal-weight diabetic patients, when expressed as mg/d. Moreover, BMI was positively associated with variables of cholesterol synthesis and negatively with cholesterol absorption. Cholesterol absorption and synthesis were inversely related in the diabetic population suggesting that the homeostatic regulation between cholesterol absorption and synthesis was not distrupted by diabetes.

Serum plant sterols and cholestanol ratios correlated with the cholesterol absorption efficiency, and those of cholesterol precursor sterols correlated with variables of cholesterol synthesis and excretion, suggesting that they reflect cholesterol metabolism similarly as in the non-diabetic population. Indeed, lower cholesterol absorption and higher synthesis in obese type 2 diabetes was also seen as lower ratios of serum plant sterols and cholestanol and higher ratios of cholesterol precursor sterols as compared with normal-weight diabetes.

Serum levels of SHBG were lower and serum insulin higher in obese than in normal-weight diabetic patients, suggesting that insulin resistance increased with weight. With high levels of serum insulin and low levels of SHBG, cholesterol absorption was low and cholesterol synthesis was enhanced in obese diabetes. Serum SHBG was positively associated with variables of cholesterol absorption and negatively with cholesterol synthesis. Thus, insulin resistance is related to cholesterol metabolism so that with increasing insulin resistance, cholesterol absorption is lowered and synthesis enhanced.

During effective weight reduction, the ratio of cholestanol increased and those of cholesterol precursor sterols decreased, suggesting that cholesterol absorption was

increased and synthesis decreased in a non-steady state situation. Weight reduction to a steady state caloric balance after the 2-year follow-up increased the efficiency of lowered baseline cholesterol absorption capacity and the ratios of serum plant sterols markedly. In addition, the SHBG level increased and serum insulin level decreased, and SHBG was related to plant sterols and cholestanol after weight reduction. Thus, with weight reduction, variables related to glucose metabolism improved and cholesterol absorption increased, and the improvement of insulin resistance possibly contributed to the enhanced absorption of cholesterol.

In conclusion, type 2 diabetes is associated with low cholesterol absorption and enhanced cholesterol synthesis, and these alterations in cholesterol metabolism are not explained by obesity. In addition, body weight, over its entire range, regulates cholesterol metabolism in type 2 diabetes, so that increasing body weight further lowers cholesterol absorption. Cholesterol and glucose metabolism are closely linked, and the regulation of cholesterol metabolism is related to variables reflecting insulin resistance; the magnitude of the abnormalities in cholesterol absorption and synthesis possibly indicating the severity of the insulin resistance. The abnormalities in cholesterol metabolism are not irreversibile, weight reduction is an efficient way to improve cholesterol metabolism. In addition, the beneficial effects of weight loss on cholesterol metabolism can be seen rather quickly, even in a non-steady state situation.

These studies have increased our knowledge of cholesterol metabolism in type 2 diabetes, and also provided new insights into the beneficial effects of weight reduction as the primary treatment for obese type 2 diabetes.

1. INTRODUCTION

Type 2 diabetes is one of the most common endocrine diseases in all populations throughout the world. Its prevalence has increased in an exponential manner over the last century. The pathophysiology of type 2 diabetes has become clearer during recent years, and both insulin resistance and pancreatic beta-cell dysfunction can affect the development of the disease. Type 2 diabetes is associated with long-term micro- and macrovascular complications, which account for the overall increased morbidity and mortality associated with this disease, with the most common causes of death being cardiovascular diseases (National Diabetes Data Group 1995). Type 2 diabetes is associated with many metabolic disturbances including hyperinsulinemia, insulin resistance, hyperglycemia, dyslipidemia and obesity, all of which contribute to the accelerated atherogenesis in diabetes (National Institutes of Health 1985, 1987). Most patients with type 2 diabetes are obese (American Diabetes Association 1997), and obesity, as an independent risk factor for the diabetes, also complicates the management and exacerbates the metabolic abnormalities in diabetes (Maggio and Pi-Sunyer 1997). Type 2 diabetes, obesity and dyslipidemia are integral parts of the insulin resistance syndrome (DeFronzo and Ferrannini 1991), and insulin resistance with or without compensatory hyperinsulinemia provides a possible cause for the metabolic abnormalities.

Cholesterol is a major component of cell membranes. It is essential for tissue growth and the production of steroid hormones in the body. Cholesterol is acquired either by de novo synthesis or by absorption from the diet, and it is eliminated mainly through biliary secretion into the intestine after conversion to bile acids or secreted into the bile as cholesterol itself. The amount of cholesterol in the body is regulated by absorption and endogenous synthesis of cholesterol. The liver is the major organ responsible for the regulation of total-body cholesterol metabolism. When there is a reduction of dietary cholesterol, and during bile acid or cholesterol malabsorption, there is an increase in the hepatic cholesterol synthesis rate, whereas an increase in dietary cholesterol has an opposite effect, thus compensating for changes that expand or reduce the tissue pools of cholesterol.

The abnormalities occurring in lipoprotein metabolism in type 2 diabetes have been described in detail (e.g. American Diabetes Association 1993, Evans et al. 1999), but the metabolism of cholesterol and bile acids has been less extensively characterized, and the results are controversial. For instance, in some (Bennion and Grundy 1977, Abrams et al. 1982, Naoumova et al. 1996, Gylling and Miettinen 1997), but not all (Briones et al. 1986) studies, cholesterol synthesis and fecal excretion as neutral sterols have been increased compared with non-diabetic subjects. Cholesterol absorption efficiency (Briones et al. 1986, Gylling and Miettinen 1997) and serum plant sterol levels (Sutherland et al. 1992, Gylling and Miettinen 1997), which are indicators of cholesterol absorption, are low in diabetes, even in subjects with high-normal blood glucose levels (Strandberg et al. 1996). However, in these earlier studies, the variable degrees of body mass index, the small and heterogeneous study groups, the different degrees of glucose control, the different types of dyslipidemia and treatments of type 2 diabetes all complicate the interpretation of the results, and the actual abnormalities in cholesterol metabolism in type 2 diabetes remain unclear. In addition, the effects of insulin resistance on cholesterol metabolism are still largely unexplored.

In obesity, cholesterol synthesis (Miettinen 1971a, Nestel et al. 1973) and turnover (Nestel et al. 1969) are markedly enhanced and the cholesterol absorption efficiency is decreased (Miettinen and Gylling 2000). Accordingly, cholesterol metabolism in type 2 diabetes mimics that observed in obesity. Thus, these results raise the question whether being overweight alone, though this is frequently associated with diabetes, is the factor which is responsible for the observed alterations in cholesterol metabolism.

Weight reduction is considered to be the primary treatment for obese patients with type 2 diabetes, because of its beneficial effects on glycemic balance, insulin sensitivity and lipoprotein abnormalities (American Diabetes Association 1999). Following effective weight reduction in obese patients without diabetes, the high levels of cholesterol synthesis and fecal excretion of bile acids and neutral sterols were decreased (Miettinen 1970, Bennion and Grundy 1975, Di Buono et al. 1999), but, despite acute caloric restriction, the cholesterol absorption percentage remained unchanged (Kudchodkar et al. 1977). The effects of weight reduction on cholesterol

metabolism in diabetes have not been documented. Since weight reduction improves cholesterol metabolism in obesity, and ameliorates the metabolic abnormalities associated with diabetes, it could be anticipated also to have beneficial effects on possible abnormal cholesterol metabolism in type 2 diabetes.

Therefore, this study was conducted to compare cholesterol metabolism in obese subjects with and without type 2 diabetes, to compare cholesterol metabolism in type 2 diabetes with and without overweight, and to examine the associations between cholesterol, lipoprotein and glucose metabolism in diabetes. In addition, the effects of weight reduction were studied on cholesterol and sterol metabolism in a non-steady state during fasting and in subsequent steady state after a prolonged follow-up period.

2. REVIEW OF THE LITERATURE

2.1 Type 2 diabetes

Type 2 diabetes is one of the most common chronic diseases in the world. It has been estimated that over 140 million people worldwide currently have diabetes, and by the year 2025, over 300 million people will have this disease (World Health Organization 1999). Type 2 diabetes accounts for around 90% of all diabetes cases. In Finland, there are approximately 150 000 type 2 diabetes patients, and the incidence has been estimated to increase by 70 % by the year 2010 (DEHKO 2000). The main factors contributing to the increasing prevalence of type 2 diabetes are aging of the population, increasing levels of obesity and lack of physical activity.

The most common cause of morbidity and mortality in type 2 diabetes is cardiovascular disease (National Diabetes Data Group 1995). The risk of coronary artery disease is two to four fold, higher in women as compared to men, and following an acute myocardial infarction the risk of death is more than double compared with a non-diabetic population (Stamler et al. 1993, Haffner et al. 1998, Miettinen et al. 1998). A diabetic patient without a previous myocardial infarction has a comparable risk of myocardial infarction as a non-diabetic patient with a previous infarction (Haffner et al. 1998). There are several known cardiovascular risk factors in type 2 diabetes including hyperinsulinemia, hypertension, dyslipidemia, insulin resistance and obesity, all of which are thought to be involved in the accelerated development of atherosclerosis (National Institutes of Health 1985, 1987).

Type 2 diabetes is defined by elevated glucose levels in blood. The principal pathophysiological abnormalities include resistance to insulin action combined with a deficiency in insulin secretion (Taylor et al. 1994, Kahn and Rossetti 1998). These are present to varying degrees in virtually all patients with the common form of type 2 diabetes. Although the molecular basis of type 2 diabetes is not clear, it has been speculated to result from genetic defects that cause both insulin resistance and insulin deficiency. Both of these defects have genetic, environmental and secondary causes, thus no single gene defect or candidate gene causing the common form of type 2 diabetes has been found to contribute to the etiology of the disease (Kahn and

Porte 2001). The general belief is that type 2 diabetes is a polygenic disorder, which probably results from several combined gene defects influenced by enviromental factors, all of which together produce the clinical syndrome.

2.1.1 Insulin resistance

Insulin resistance can be defined as an impaired response to the physiological effects of insulin occurring in peripheral organs and leading to abnormalities in glucose, lipid and protein metabolism (Kahn 1994). Over 66 years ago, Himsworth (1936) observed the phenomenon of insulin sensitivity in some diabetic patients and suggested that diabetes should be sub-divided into two categories according to the insulin sensitivity and insensitivity, the latter condition now being classified as type 2 diabetes (non-insulin dependent diabetes). Insulin resistance is present in the majority of patients with impaired glucose tolerance or type 2 diabetes, and it is also found in up to 25 % of the general, apparently healthy population (Reaven 1988). The genetic background as well as many pathological conditions, such as obesity, contribute to the insulin resistance. Many studies have been performed in order to find candidate genes and possible mutations and abnormalities in the mechanisms of insulin action at the molecular level (Groop and Tuomi 1997, Kahn and Porte 2001). In 1988, Reaven postulated that most individuals with insulin resistance remain non-diabetic because they are able to compensate for their insulin resistance by secreting more insulin, and it is claimed that insulin resistance per se does not cause diabetes as long as the pancreas can secrete more insulin to overcome the insulin resistance (Porte 1991, Leahy et al. 1992).

2.1.2 Insulin secretion

Pancreatic beta-cell dysfunction causes abnormal secretion of insulin, and contributes to the pathogenesis of type 2 diabetes. Normal insulin secretion under basal conditions is phasic, and the hormone is secreted in a pulsatile manner. In non-diabetic subjects after glucose administration, the rapid and immediate increase in insulin secretion, lasting approximately 10 minutes, is defined as the first-phase response, whereas the second-phase insulin secretion is the subsequent sustained increase in insulin secretion which is slower and lasts longer. The first-phase secretion of insulin as a response to glucose is lost in type 2 diabetes (Pratley and Weyer 2001). A secretory defect in the second phase is also characteristic of type 2

diabetes, and the ability of glucose to potentiate the effects of other stimulants of insulin secretion is diminished (Ward et al. 1984, Roder et al. 1998). In addition, the oscillatory insulin release is abnormal in type 2 diabetes (Lang et al. 1981). There are anatomic abnormalities in the pancreatic islet cells in type 2 diabetes, but these cannot account for the beta-cell dysfunctions characteristic of type 2 diabetes (Kahn and Porte 2001). The etiology of the beta-cell dysfunction of type 2 diabetes is incompletely understood, it is thought to result from both genetic and environmental factors (Kahn and Porte 2001, Pratley and Weyer 2001.)

2.1.3 Interaction of insulin resistance and beta-cell dysfunction

Type 2 diabetes is a complex disease, which evolves over many years and progresses through multiple stages, still today, controversy exists about the precise sequence of events and primary causes in the natural history of this disease. It is believed that both insulin resistance and insulin secretion are abnormal before the onset of frank type 2 diabetes, and the hepatic gluconeogenesis is a late phenomenon and determines the degree of hyperglycemia. Thus, the following section is a simplified formula of the progressive evolution of the type 2 diabetes.

Insulin resistance in peripheral organs develops relatively early, leading to an increased need of insulin in the body to be able to maintain glucose uptake and utilisation. In order to maintain the balance, pancreatic beta cells increase their rate of insulin secretion to compensate for the insulin resistance, thus plasma levels of insulin rise and glucose tolerance remains normal. Nevertheless, the compensation might not be complete and/or the ability of beta cells to increase insulin secretion declines, leading to impaired glucose tolerance, and, as the situation evolves, ultimately to diabetes. In addition, increased hepatic glucose production due to hepatic insulin resistance and uninhibited lipolysis in adipose tissue causing overflow of free fatty acids to liver all possibly can accelerate hepatic glucose production leading to severe hyperglycemia.

Several methods have been developed to quantitate insulin action in patients. These include clamps, insulin infusion sensitivity tests, measurement of fasting insulin levels, intravenous glucose tolerance and model assessments (Laakso 1993, Taylor 2001). All of these methods have their limitations, and there is a considerable

variation in the complexity and labour intensity of the various methods. The euglycemic clamp (DeFronzo et al. 1979), “the gold-standard”, is very useful for intense physiological studies on small numbers of subjects. More recently a new method was developed, homeostasis model assessment (HOMA) (Matthews et al. 1985), which utilizes computer aided modeling of fasting glucose and insulin concentrations, and this seems to provide a useful model to assess insulin resistance and beta-cell function in large epidemiological studies (Bonora et al. 1998, Wallace and Matthews 2002)

2.1.4 The metabolic syndrome

The term metabolic syndrome consists of a cluster of metabolic disorders, many of which promote the development of atherosclerosis and increase the risk of cardiovascular disease events. The major components of the metabolic syndrome include abdominal obesity, glucose intolerance/type 2 diabetes, dyslipidemia and hypertension (Hauner 2002). Insulin resistance may lie at the heart of the metabolic syndrome. During the past few years, evidence has accumulated suggesting that there are other abnormalities, secondary to insulin resistance and/or compensatory hyperinsulinemia, that could be added to the cluster of these metabolic events. An impairment of the fibrinolytic system is now mentioned in extended definitions. In 1988, Reaven suggested that this cluster of abnormalities constituted an important clinical syndrome, designated as syndrome X (Reaven 1988). The syndrome has since gained a number of different names including Reaven’s syndrome, insulin resistance syndrome, metabolic syndrome, chronic cardiovascular risk syndrome; with no generally accepted definition.

2.2 Overview of lipoprotein metabolism

The following summary of lipoprotein metabolism is based on several references (e.g. Gotto et al. 1986, Havel and Kane 2001). Fat absorbed from the diet and lipids synthesized by the liver and adipose tissue must be transported between the various tissues for utilisation and storage. Since lipids are insoluble in water, they are transported in plasma as lipoproteins. Lipoprotein particles contain a central core of non-polar lipids, mainly triglycerides and cholesteryl esters, and a surface monolayer of polar lipids, mainly phospholipids, apolipoproteins (apo) and free cholesterol.

Apolipoproteins, excluding apo B, and free cholesterol are readily water soluble and thus have high potential to be easily exchanged between lipoprotein particles. Lipoprotein core lipids and phospholipids need a specific transfer protein in order to be transferred between lipoproteins but free surface cholesterol is freely exchangeable.

Based on density, plasma lipoproteins are separated into five major classes, which have different compositional and functional properties: chylomicrons (d ~ 0.93 g/ml), very low density lipoproteins; (VLDL) (d = 0.93-1.006 g/ml), intermediate density lipoproteins; (IDL) (d = 1.006-1.019 g/ml), low density lipoproteins; (LDL) (d= 1.019-1.063 g/ml), and high density lipoproteins; (HDL) (d = 1.063-1.210 g/ml). The lipoprotein particle size is inversely related to their density, describing the amounts of low-density core lipids and high density apolipoproteins. The core of the two largest classes, chylomicrons and VLDL, contain mainly triglycerides, and are called triglyceride rich lipoproteins (TRL).

Chylomicrons are formed in the enterocytes, and they contain mainly the newly absorbed fatty acids as triglycerides added to smaller amounts of cholesterol esters. The major protein component is apo B-48, and they contain also the A-apolipoproteins. After secretion, chylomicrons acquire apolipoproteins C and E from HDL. These particles are transported via the lymph into blood, where they bind to lipoprotein lipase on the surface of capillary endothelial cells, leading to rapid hydrolysis of most of the triglycerides. Some phospholipids and the apolipoproteins A and C are transferred to HDL resulting in a residual particle called the chylomicron remnant. The remnants are cleared from blood to the liver by several mechamisms. Thus, virtually all cholesterol absorbed from the intestine is delivered to the liver. The cholesterol in hepatocytes can enter metabolic pathways leading to formation of bile acids, be secreted into bile as such, be incorporated into nascent lipoproteins or be stored within the cell.

VLDL is formed mainly in hepatocytes, and provides a pathway for export of excess triglycerides from the liver cells. Triglycerides can be derived from hepatic de novo-production, from plasma free fatty acids taken up by liver or from chylomicron remnants. The VLDL particle consists of a large amount of triglycerides and smaller

amounts of cholesterol and phospholipids. The major protein component of the nascent VLDL is apo B-100, and it contains also some C and E apolipoproteins. In the blood, the triglycerides of VLDL are hydrolyzed in extrahepatic tissues by lipoprotein lipase leading to smaller, remnant particles including particles isolated as IDL. The surface components of the remnant particle, including phospholipids, free cholesterol and soluble apolipoproteins, are transported to HDL facilitated by plasma phospholipid transfer protein (PLTP)(Tall 1995). VLDL remnants can then interact with LDLapo B-receptors on hepatocytes via apo E. The remnant particles, which contain several molecules of apo E, bind effectively to the LDLapo B-receptors and are rapidly taken up from blood to the hepatocytes for catabolism. Particles with smaller amounts of apo E remain longer in the blood. These are transformed to IDL and with further processing by hepatic lipase and the loss of the rest of apo C and E they can form LDL. In most mammals, the majority of VLDL remnants are rapidly taken up by liver, and only a small amount is converted via IDL to LDL. In humans, a much greater fraction of the remnants, perhaps even 50 %, is converted to LDL.

LDL is mainly produced as an end product of the metabolism of VLDL, and it contains predominantly cholesterol esters added to small amounts of triglycerides, phospholipids and free cholesterol. LDL is the main carrier of cholesterol in blood since LDL cholesterol normally accounts for about two-thirds of plasma total cholesterol. The exclusive apolipoprotein of LDL is apo B-100, one LDL particle containing one apo B molecule. LDL can be taken up from the circulation into hepatocytes by LDLapo B- receptors or LDLapo B-receptors on extrahepatic cells. The binding to the receptors is mediated via recognition of apo B-100. Due to the relatively low affinity of LDL for the hepatic LDLapo B-receptors, as compared to the respective affinity of VLDL remnants, LDL circulates in the blood for about three days. Therefore, an appreciable fraction of blood LDL is taken up by many extrahepatic tissues via their LDLapo B-receptors. Thus, LDL is the major particle responsible for transporting cholesterol to peripheral tissues.

Nascent HDL particles are either secreted by the liver or the intestine, or are assembled in the plasma from products of the catabolism of TRL. During the lipolysis of TRL in peripheral tissues, their surface components, phospholipids, cholesterol and apolipoproteins, are transferred to HDL. This is facilitated by PLTP. These

components give rise to new HDL particles, or may be incorporated into pre-existing HDL particles. The major apolipoproteins of HDL are apo A-I and apo A-II. In addition to being transferred from VLDL and chylomicrons, apolipoproteins may be secreted as free apolipoproteins, which then acquire lipids via an interaction with the cellular ATP binding cassette transporter (ABC). In both mechanisms, the discoidal, pre-beta-HDL particles are formed. The plasma cholesterol–esterifying enzyme lecithin: cholesterol acyl-transferase (LCAT) circulates bound to these nascent and discoidal HDLs, and generates cholesteryl esters from free cholesterol. These cholesteryl esters form the core of the spherical, now mature HDL particle. HDL cholesteryl esters may be transferred to apo-B containing lipoproteins by cholesteryl ester transfer protein (CETP) in exchange for triglycerides. The triglycerides of HDL are hydrolyzed by hepatic lipase. The transfer of triglycerides and other surface components from the apo-B containing lipoproteins, and the elevation in the core cholesteryl ester amount due to the function of LCAT both increase the size of the HDL particle. Conversely the transfer of cholesteryl esters out of HDL by CETP and hydrolysis of HDL triglycerides and phospholipids by hepatic lipase will reduce the HDL size. Large HDL particles are often called HDL 2 and the smaller HDL particles are called HDL 3.

HDL is an important mediator of the reverse cholesterol transport, in which cholesterol from peripheral tissues is delivered to the liver: pre-beta HDL particles are specially adapted for mediating free cholesterol efflux from peripheral cells. Cholesterol is then esterified, generating larger cholesteryl ester rich-HDL particles. Next, the cholesteryl esters can be removed from the circulation to the liver with apo-B containing lipoproteins, through selective uptake of special scavenger receptor BI (SR-BI), or as a part of an HDL particle uptake mechanism. The action of the different enzymes affecting and remodelling the HDL composition contributes to the conversion of the mature HDL back to the pre-beta HDL, which is then capable of re-entering the HDL metabolism circle; thus the removal of cholesterol from the extrahepatic cells and the flow of the cholesterol to the liver is maintained.

2.3 Lipoprotein metabolism in diabetes

Type 2 diabetes is associated with abnormal fasting as well as postprandial lipoprotein metabolism. The key features of this dyslipidemia are the elevated levels of triglycerides, the reduced levels of HDL cholesterol, and the increased number of small, dense LDL particles, called LDL subclass pattern B (Howard 1987, American Diabetes Association 1993, Reaven et al. 1993, Evans et al. 1999). In contrast, the levels of total and LDL cholesterol are comparable to those seen in subjects without diabetes. Studies have shown that a dyslipidemic lipoprotein profile characteristic to type 2 diabetes precedes the onset of diabetes (Haffner et al. 1990, Mykkänen et al. 1993) and is present in many conditions where only insulin resistance is observed (American Diabetes Association 1993, Ginsberg 2000).

The mechanism of formation of dyslipidemia in type 2 diabetes remains uncertain, even though many factors are involved including insulin resistance, hyperinsulinemia, disturbed fatty acid metabolism and even hyperglycemia (Evans et al. 1999). The composition and amount of the different lipoproteins are altered. Many studies demonstrate an overproduction of triglyceride-rich VLDL particles and apolipoprotein B-100 (e.g. Ginsberg 1987, Howard 1994). The activity of lipoprotein lipase is diminished leading to a decrease in VLDL catabolism. Despite the expanded VLDL pool, LDL cholesterol levels may be normal due to increased proportion of VLDL particles being metabolized without conversion to LDL (Howard 1987) and to the enhanced fractional catabolic rate of LDL. There is an increased lipid exchange between triglyceride-rich VLDL and both HDL and LDL, possibly due to increased activity of CETP and the excess VLDL pool (Elchebly et al. 1996, Ginsberg 2000). This leads to the decrease of HDL cholesterol and the formation of triglyceride-rich HDL and LDL particles. In addition, the catabolism of HDL is also increased because of the overactivity of hepatic lipase (Howard 1994, De Man et al. 1996). This results in the generation of smaller, more dense lipoprotein particles with abnormal functions. The fractional catabolic rate (FCR) of apo A-I is increased (Golay et al. 1987) leading to a lower HDL cholesterol level (Brinton et al. 1994).

Lipoprotein particles are also modified by glycosylation in the presence of hyperglycemia (American Diabetes Association 1993). The clearance of glycated

LDL particles is prolonged, and they might be more readily oxidized, also leading to their increased uptake by macrophages (Witztum et al. 1982, American Diabetes Association 1993, Bowie et al. 1993).

Insulin resistance is a strong candidate to play a role in evoking these changes: Dyslipidemia appears to be part of the insulin resistance syndrome with or without type 2 diabetes (DeFronzo and Ferrannini 1991, American Diabetes Association 1993, Betteridge 1997). The dyslipidemic lipoprotein profile is more severe in insulin-resistant than in insulin-sensitive type 2 diabetic subjects (Haffner et al. 1999). Prospective studies have shown that hyperinsulinemia predicts the onset of both dyslipidemia and diabetes (Haffner et al. 1992). The antilipolytic effect of insulin is reduced in adipose tissue leading to increased release of fatty acids (Reaven 1988). Especially when there is the presence of high levels of intra-abdominal fat, liver is exposed to a large free fatty acid load, which could induce hepatic insulin resistance (Carey et al. 1996) and provide substrates for increased VLDL production (Björntorp 1991). As a matter of fact, abnormal VLDL production and a deranged activity of lipoprotein lipase have been linked to insulin resistance (Pollare et al. 1991, Malmström et al. 1997). In addition, small dense LDL particles have been shown to be closely related to hypertriglyceridemia in insulin resistance rather than diabetes per se (Austin and Edwards 1996, Lahdenperä et al. 1996, Syvänne and Taskinen 1997).

The combination of hypertriglyceridemia, and increased numbers of small dense LDL particles frequently associated with low levels of HDL cholesterol is nowadays called hypertriglyceridemic hyperapoB. This atherogenic lipoprotein profile is not only seen in type 2 diabetes, but it is also common in subjects prone to develop diabetes, subjects with insulin resistance, and subjects with coronary artery disease.

2.4 Obesity

The prevalence of obesity everywhere in the world is increasing rapidly (Kuczmarski et al. 1994, Kuulasmaa et al. 2000). Obesity is the presence of excessive amount of adipose tissue. It is a physiological response to the environment and behaviour, in which energy intake exceeds energy output, and the interaction between genotypes and the environment all contribute to development of obesity.

The body mass index (BMI kg/m^2) is commonly used for the assessment of obesity. The World Health Organization has proposed that BMI from 18.5 kg/m^2 to 24.9 kg/m^2 is considered as normal (World Health Organization 1998). With respect to the increased incidence of complications, BMI from 25 kg/m^2 to 29.9 kg/m^2 is considered unhealthy, and is defined as overweight. BMI values of 30 kg/m^2 and above are designated as obese. It has been suggested that obesity should be considered as a disease (World Health Organization 1998, National Institutes of Health 1998). Numerous studies have shown that elevated body weight, the consequence of increased body fat, is associated with an increased prevalence of comorbidities, leading to an elevated risk of death (World Health Organization 1998, National Institutes of Health 1998, Leibel et al. 2001). An increased mortality rate is associated with BMI $\geq$ 30 kg/m^2 (National Institutes of Health 1998).There has been debate regarding the impact of overweight on mortality at BMI from 25 kg/m^2 to 30 kg/m^2 (Wooley and Wooley 1984, Ernsberger and Haskew 1987, Kassirer and Angell 1998). However, BMI > 28 kg/m^2 is associated with a three- to fourfold increase in overall risk of morbidity (hypertension, dyslipidemia, diabetes), and a two-fold increase of death (Van Itallie 1985). In cross-sectional studies, there is a progressive positive correlation between BMI and adiposity-related morbidities (World Health Organization 1998), and prospective studies show a significant increase in the incidence of future morbidities when BMI exceeds 27.5 kg/m^2 (Sorkin et al. 1994). Moreover, higher levels of body weight, even within the “normal” range as well as modest weight gains after 18 years of age, increase greatly the risks of coronary heart disease and ischemic stroke in middle-aged women (Willett et al. 1995, Rexrode et al. 1997). Modulating factors such as age, smoking, sex, family history and physical activity can have an impact on the risks of overweight in any given individual, and thus the benefits and risks of overweight must be assessed on an

individual basis (National Institutes of Health 1998). World Health Organization has suggested that individuals with BMI > 25 kg/m^2 should be considered at-risk for adiposity-related morbidity (World Health Organization 1998).

Obesity is associated with many metabolic abnormalities such as insulin resistance with hyperinsulinemia, dyslipidemia, hypertension, cardiovascular diseases and type 2 diabetes (Pi-Sunyer 1993). The risk of development of diabetes increases clearly as the degree of overweight increases (Van Itallie 1985). In fact, several studies reveal an increasing risk at relatively low levels of BMI, as well as with even modest amounts of weight gain after 18 years of age (Chan et al. 1994, Colditz et al. 1995, Sowers 1995). Most patients with type 2 diabetes are obese (Maggio and Pi-Sunyer 1997), and the dramatic increase in obesity during the past decade has been accompanied by a 25 % increase in the prevalence of type 2 diabetes (Harris et al. 1998).

The distribution of body fat plays an important role in the obesity-associated health implications. When body fat is accumulated centrally, e.g., intra-abdominal or visceral obesity, it is associated with a higher risk of concomitant diseases, metabolic abnormalities and mortality than more peripheral distribution of body fat or subcutaneus abdominal fat (Pi-Sunyer 1993, Després 2001). It has been shown that visceral adiposity increases the risk for hyperinsulinemia and glucose intolerance at a given BMI (Kaye et al. 1991, Després 1998), and insulin resistance, hyperinsulinemia and type 2 diabetes are related to increased levels of intra-abdominal fat (Hartz et al.1983, Haffner et al. 1986). Ohlson et al. (1985) in an 8 year prospective longitudinal study showed that central obesity imposed an increased risk of developing diabetes, which was greater than the risk of adiposity per se, a finding that has been confirmed later by others (Lundgren et al. 1989, Haffner et al. 1991).

2.4.1 Lipoprotein metabolism in obesity

Obesity is often associated with abnormal lipoprotein metabolism. The levels of triglycerides are higher and HDL cholesterol lower in obese than in lean subjects (Pi-Sunyer 1993). Total and LDL cholesterol can be elevated, but are also often normal (Barrett-Connor 1985, Grundy and Vega 1990, Pi-Sunyer 1993). Obesity enhances the production of apo B-containing lipoproteins (Kesäniemi and Grundy 1983, Egusa

et al. 1985, Kesäniemi et al. 1985). However, the plasma cholesterol transport by LDL appears to increase relatively modestly, probably due to rapid catabolism of LDL and enhanced removal of VLDL remnants without their conversion to LDL (Kesäniemi and Grundy 1983, Egusa et al. 1985). Increasing BMI is also associated with small, dense triglyceride-enriched LDL particles (Krauss et al. 1998).

It is known that the distribution of body fat has a role in lipoprotein metabolism in obesity. The major accumulation of visceral adipose tissue is characterized by the most severe metabolic disturbances compared to that is seen with subcutaneus accumulation of adipose tissue, including fasting hypertriglyceridemia and reduced HDL cholesterol (Després et al. 1990, Pouliot et al. 1992). Viscerally obese patients have also an increased proportion of small, dense LDL particles compared to obese patients lacking visceral body fat accumulation (Tchernof et al. 1996).

The plasma levels of free fatty acids are elevated in obesity and especially in visceral obesity; this is attributable to their increased elimination from adipose tissue (Jensen et al. 1989). The increased flux of free fatty acids through the hepatic portal circulation provides substrates for triglyceride synthesis, and also promotes hepatic insulin resistance (Björntorp 1991, Grundy 1999, Arner 2001) contributing to the dyslipidemia.

Many investigations have revealed that hypertriglyceridemia is closely linked to insulin resistance (Kissebah et al. 1976, Steiner 1994, Després 1998). Some studies have also shown an association between insulin resistance and small, dense LDL (Haffner et al. 1995, Austin and Edwards 1996) as well as insulin resistance and low HDL cholesterol (Karhapää et al. 1994). Obesity is an insulin resistant state (Reaven 1988, Ferrannini et al. 1997) with compensatory hyperinsulinemia providing one possible cause for the dyslipidemia in obesity.

The detailed mechanisms underlying the dyslipidemia still remain unclear, though many theories exist. However, the major factors causing/influencing these metabolic changes are insulin resistance with compensatory hyperinsulinemia and the degree of intra-abdominal obesity (American Diabetes Association 1993).

2.5 Diabetes and obesity

Insulin resistance is a characteristic feature of both type 2 diabetes and obesity. In the latter, it is acquired due to excessive calorie intake (Sims et al. 1973) with or without predisposing genetic factors, whereas in the former, inheritance of gene(s) that confer insulin resistance are involved (DeFronzo and Ferrannini 1991). In insulin resistance, the normal glucose tolerance is maintained by increased insulin secretion leading to hyperinsulinemia. In obesity, the compensatory response of insulin secretion is nearly perfect and glucose tolerance remains normal. In diabetes, a defect of insulin secretion is present, leading to glucose intolerance and hyperglycemia. As obesity persists or weight is further gained, the excessive secretion of insulin cannot be maintained, thus leading to frank diabetes. Even with inadequate or defective insulin secretion compensatory to the insulin resistance, the plasma insulin levels remain 1.5- to 2-fold elevated compared with age- and weight-matched control subjects (DeFronzo 1988, Golay et al. 1988, Haffner et al. 1988, Reaven et al. 1989, Saad et al. 1989). As the situation evolves, insulin secretion declines, plasma insulin levels normalize or even fall below normal, and severe glucose intolerance develops. A prospective follow-up study, in which obese/diabetic subjects were followed for 6 years, has confirmed the above sequence of events (Jallut et al. 1990), and a prospective study with Pima Indians have shown similar results (Saad et al. 1989).

Many studies have shown that the insulin resistance in normal weight type 2 diabetes patients is of a similar magnitude as in nondiabetic obese patients (Kolterman et al. 1981, Hollenbeck et al. 1984, DeFronzo 1988, Golay et al. 1988). Both diabetes and obesity are characterized by exhibiting hyperinsulinemia, even though there is a difference in the plasma insulin concentrations in these two groups. The plasma insulin response in normal weight type 2 diabetes is higher than in normal weight controls, but it is significantly decreased compared with nondiabetic obese subjects, despite a similar magnitude of insulin resistance (DeFronzo and Ferrannini 1991) .

Lipoatrophy predisposes to diabetes, and it is characterized by insulin resistance probably caused by the absence of fat (Taylor 2001). Gavrilova et al. (2000) have shown that surgical fat transplantation could reverse hyperglycemia, lower insulin

levels and improve insulin sensitivity in lipoatrophic mice, the phenotype of the mice resembled closely that of humans with severe lipoatrophic diabetes.

Therefore, abnormal quantities of adipose tissue, whether too much or too little seems to be important in contributing to insulin resistance and increasing the risk for diabetes mellitus.

2.6 Cholesterol metabolism

Cholesterol is present in every tissue and is transported in plasma lipoproteins either as free cholesterol or combined to long-chain fatty acids as cholesteryl esters. Cholesterol is an essential structural component of cell membranes, and it is a key regulator of membrane fluidity. Cholesterol is also the precursor of all other steroids in the body, such as corticosteroids, sex hormones, bile acids and vitamin D. The transport of mainly water-insoluble cholesterol in the circulation is facilitated by lipoproteins, with LDL being the main carrier.

In humans, cholesterol is acquired from two sources: from the diet and from a cellular de novo cholesterol synthesis from acetyl-CoA. Virtually all cells containing nucleus are able to synthesize cholesterol, and cholesterol in any particular tissue is derived from this de novo synthesis or from the circulating lipoproteins. The liver is the major organ synthetizing cholesterol in the human body. Cholesterol is eliminated from the body mainly as cholesterol and bile acids through biliary secretion into the intestine, from where unabsorbed cholesterol is finally excreted into stools. The main features of cholesterol transport in man are presented in Figure 1.

Homeostatic mechanisms maintain the balance between the input of cholesterol from its two sources, intestinal absorption and de novo synthesis, and its output by two major hepatic mechanisms, irreversible conversion to bile acids and secretion into bile as free cholesterol. The liver is the key organ in the metabolism of cholesterol since it has critical importance in the processes regulating the whole-body cholesterol homeostasis (cholesterol synthesis, plasma clearance of lipoproteins, bile acid synthesis, and biliary cholesterol secretion) (Dietschy et al. 1993). Cholesterol synthesis and dietary cholesterol absorption equals fecal excretion of total steroids in

a steady state at the whole body level, and the amount of cholesterol in hepatocytes (derived from cholesterol synthesis, absorbed from the intestine, produced in other tissues) is equal to the biliary secretion of lipids. According to the homeostatic regulation of cholesterol metabolism, low intestinal absorption of cholesterol upregulates cholesterol synthesis and turnover, whereas an increase in the intestinal cholesterol flux to the liver suppresses cholesterol synthesis. Therefore, any reduction of dietary cholesterol, or bile acid or cholesterol malabsorption, will trigger an increase in hepatic endogenous cholesterol synthesis (Miettinen and Kesäniemi 1986), whereas an increase in dietary cholesterol has an opposite effect.

The sterol balance is defined as the difference between dietary intake and total sterol excretion. The negative balance implies the removal of cholesterol from the body (the fecal excretion of total steroids exceeds the dietary intake of cholesterol), whereas a positive balance indicates that cholesterol is accumulating within the body (dietary intake of cholesterol exceeds the fecal excretion of total steroids). In steady state, this balance is usually negative, and numerically equal to the endogenous synthesis of cholesterol.

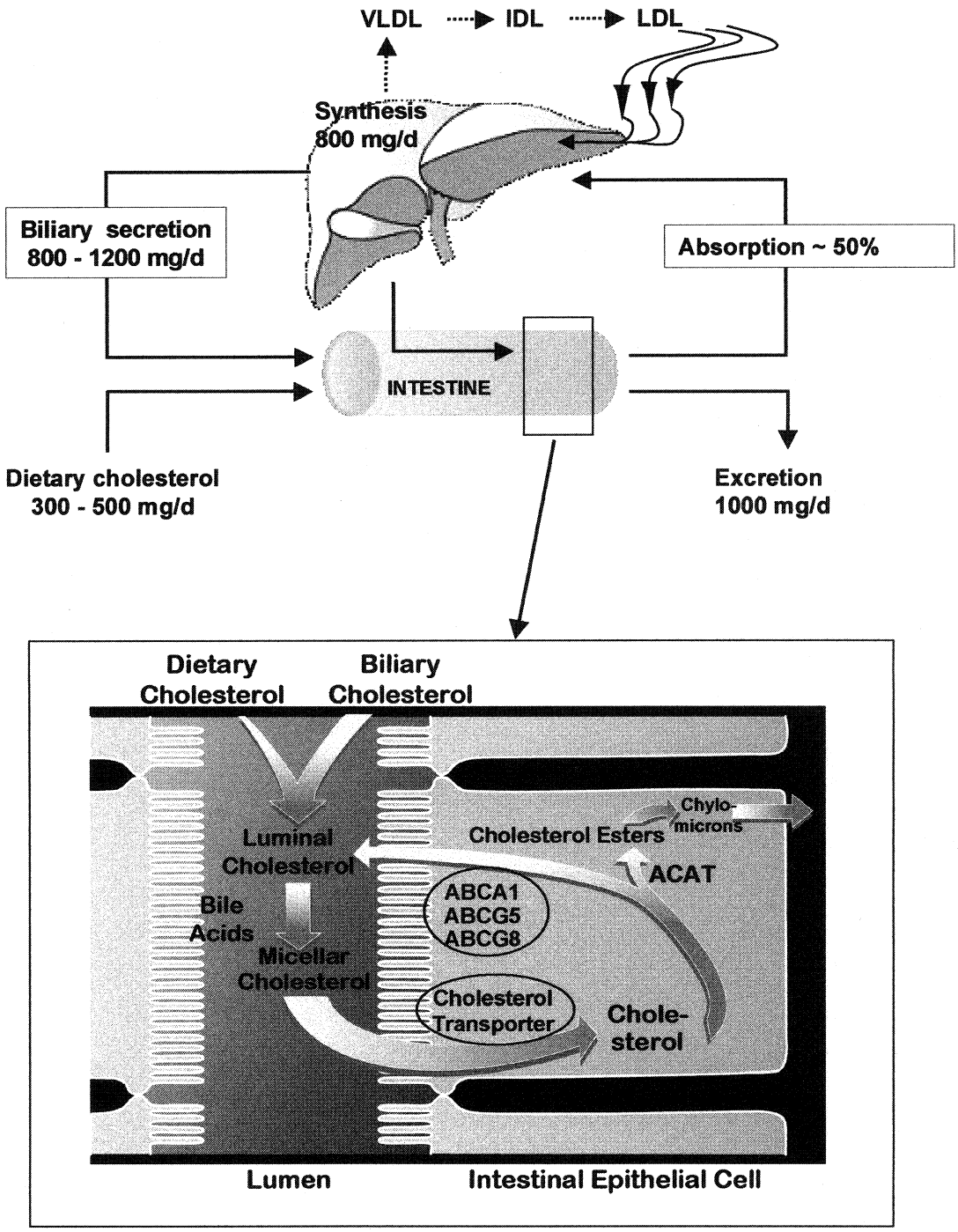


Figure 1. Cholesterol transport in man.

2.6.1 Cholesterol absorption

2.6.1.1 Sources of intraluminal cholesterol

The intraluminal cholesterol available for absorption originates from three sources; the diet, bile and turnover of intestinal mucosal epithelium. The daily Western diet contains 300-500 mg cholesterol, of which a variable proportion is cholesterol esters (8-19%) (Wilson and Rudel 1994). The amount of biliary cholesterol entering the intestine daily varies usually from 800 to 1200 mg (Grundy 1983). All biliary cholesterol is in an unesterified form. The mucosal cell loss and intestinal secretion of cholesterol provide additional sources of cholesterol to the intestinal cholesterol pool, though estimates of this contribution are hard to come by. In three patients with total bile duct obstruction, the intraluminal cholesterol resulting from this source was estimated to be 250-400 mg daily (Cheng and Stanley 1959). The significance of this endogenous cholesterol in humans is still unknown, whereas in rats, it was shown to significantly contribute to the endogenous cholesterol pool (Danielsson 1960). Thus, in humans, over 1 g of cholesterol enters the intestine daily, and approximately one half of this amount is absorbed (Grundy and Ahrens 1969, Wilson and Rudel 1994).

2.6.1.2 Luminal events

Cholesterol absorption is intimately linked to the overall process of lipid absorption. Hydrolysis of dietary fat begins in the stomach, from which the emulsion is delivered to the lumen of the small intestine. The pancreatic secretions contribute hydrolytic enzymes, and bile contributes bile salts, which solubilize the hydrolytic end products of intraluminal fat digestion. Long-chain triglycerides are hydrolysed by pancreatic lipase, with the hydrolysis being accelerated by colipase (Borgström 1975). Phospholipids are hydrolyzed at the 2-position by pancreatic phospholipase A2, to yield lysophospholipid and free fatty acids (Van Deenen et al. 1963). Cholesteryl esters are hydrolyzed by pancreatic cholesterol esterase (Vahouny and Treadwell 1958, Vahouny et al. 1964). This hydrolysis is important, because only free cholesterol is efficiently absorbed (Swell et al. 1955, Swell et al. 1960); this observation has been confirmed later in mice lacking the cholesterol esterase gene (Howles et al. 1996).

Cholesterol is only minimally soluble in aqueous systems, its solubility is dependent on the solubilizing properties of bile salt solutions (Siperstein et al. 1952, Swell et al. 1958). Mixed bile salt micelles and small unilamellar vesicles are thought to be the main natural carriers from which the mucosal uptake of lipids is possible (Carey and Hernell 1992). If there is to be micellar formation and subsequent cholesterol absorption, there must be bile salts present above the critical micellar concentration (Hofmann and Small 1967). In the presence of bile salts, more soluble lipids such as phospholipids, fatty acids and monoglycerides, can increase the solubility of cholesterol and facilitate the micellar solubilization and therefore they can improve the absorption of cholesterol. The known requirement of bile acids for intestinal cholesterol to be absorbed, recently demonstrated with mice lacking an enzyme essential for bile acid formation (Schwarz et al.1998), is met with this model.

2.6.1.3 Mucosal events

2.6.1.3.1 Uptake and re-excretion

Cholesterol absorption occurs through the intestinal mucosal cells, which cover the surface of the intestinal villi, and the uptake occurs mainly in the apical region of the villi (Sylvén and Nordström 1970). The classical model for the molecular mechanism of cholesterol absorption has been that unesterified cholesterol is shuttled in a mixed micelle across the unstirred water layer lining the brush border of enterocytes (Westergaard and Dietschy 1976). Subsequently, the transfer of cholesterol to the cell surface and inside the cell is mediated by passive diffusion according to the concentration gradient. More recently, Thurnhofer and Hauser (1990) suggested that uptake of cholesterol is an active process, and it is protein-mediated, possibly relating to cholesterol-transfer protein in enterocytes (Thurnhofer et al. 1991). Scavenger receptor class B type I (SR-BI), a membrane protein located on the brush border membrane, has been suggested to mediate intestinal cholesterol absorption by facilitating the uptake of dietary cholesterol from either bile salt micelles or phospholipid vesicles (Hauser et al. 1998). This receptor has been suggested to play a role in the absorption of other lipids, such as triglycerides, and even esterified sterols (Compassi et al. 1995). Prior to SR-BI, a direct role for the pancreatic cholesterol esterase (i.e carboxyl ester lipase) in intestinal cholesterol absorption was proposed by Lopez-Candales et al. (1993). However, in knockout mice lacking the cholesterol esterase gene, the efficiency of intestinal cholesterol absorption was

identical to wildtype mice, though intestinal absorption of cholesteryl esters was impaired (Howles et al.1996). This result has been recently confirmed (Weng et al. 1999).

ABC transporters are integral membrane proteins, which transport various molecules across the cellular membrane, and are important in cholesterol metabolism (ABCA1) (Brooks-Wilson et al. 1999, Orso et al. 2000). A study in mice showed that treatment with retinoid X receptor (RXR) ligand could increase the intestinal expression of ABCA1 leading to enhanced efflux of cholesterol from enterocytes back to intestinal lumen (Repa et al. 2000). Further genetic studies have shown that both absorption and secretion of cholesterol are controlled by ABC transporters, ABCG5 and ABCG8, which act in concert to pump cholesterol out of cells (Berge et al. 2000, Lee et al. 2001). In the intestine, they re-excrete cholesterol that has entered enterocytes from the intestinal lumen, thereby limiting cholesterol absorption.

2.6.1.3.2 Mucosal cholesterol

Inside the enterocytes, cholesterol and lipids are esterified, and approximately 75 % of newly absorbed cholesterol appearing in lymph chylomicrons is in an esterified form (Wilson and Rudel 1994). Fatty acids and monoglycerides are re-esterified to form triglycerides and free cholesterol is esterified to form cholesteryl esters. The monoglyceride pathway is used for the synthesis of chylomicron triglycerides (Field and Mathur 1995). Acyl CoA:cholesterol acyltransferase (ACAT) is the enzyme responsible for the esterification of absorbed cholesterol (Purdy and Field 1984, Chang et al. 1997). More recently, two ACAT enzymes were cloned, ACAT 1 and ACAT 2, of which ACAT 2 is most likely mainly responsible for esterification of intestinally absorbed cholesterol (Anderson et al. 1998, Cases et al. 1998, Oelkers et al. 1998). Accordingly, the role of ACAT 2 in cholesterol absorption is the formation of cholesteryl esters for packaging into chylomicrons, which prevents the back diffusion/transport of free cholesterol into intestinal lumen. The effects of ACAT inhibition on cholesterol absorption have been variable, but most studies have shown a decrease in the extent of cholesterol absorption (Krause et al. 1993, Wilson and Rudel 1994). However, with a normal-low cholesterol containing diet, ACAT 2 - deficient mice had plasma cholesterol similar to those of wildtype mice, but when they consumed a high fat, high cholesterol diet, their cholesterol absorption was 85

% lower than in wildtype mice (Buhman et al. 2000). These results emphasize the importance of ACAT, especially when high amounts of cholesterol are available for absorption.

After the esterification, triglycerides and cholesteryl esters are assembled in the endoplasmic reticulum with other lipids and proteins to form the core of nascent chylomicrons. The major protein is apo B-48, which is obligatory for chylomicron formation (Hussain et al. 1996), and which acts in concert with microsomal triglyceride transfer protein (MTP). The assembly of chylomicron particle is completed in the Golgi apparatus (Field and Mathur 1995). MTP has a role in rescuing apo B-48 from intracellular degradation during early lipidation of the protein, this lipidation process possibly being mediated by MTP (van Greevenbroek et al. 1998). When the formation is terminated, chylomicrons enter lacteals in the intestinal villi, and are delivered via the thoracic duct into the bloodstream.

2.6.1.4 Regulation of serum cholesterol level

The cholesterol absorption efficiency has been reported to regulate serum cholesterol levels in a random population of Finnish males (Kesäniemi and Miettinen 1987, Kesäniemi et al. 1987). The cholesterol absorption efficiency was positively associated with serum and LDL cholesterol concentrations, and negatively with cholesterol synthesis (Kesäniemi and Miettinen 1987, Miettinen and Kesäniemi 1989, Gylling and Miettinen 1989, Miettinen et al. 1990). Studies in hyper- and hypo-responding nonhuman primates also indicated that cholesterol absorption and plasma cholesterol level were positively related (Wilson and Rudel 1994). However, in contrast to these previous studies, the efficiency of cholesterol absorption was not related to serum total or LDL cholesterol concentrations in two recent studies (Sehayek et al. 1998a, Bosner et al. 1999). The subjects in the study by Bosner et al. (1999) were from various ethnic groups with a high interindividual variation in their percent cholesterol absorption. In the study by Sehayek et al. (1998a), almost two-thirds of the variation in LDL cholesterol was explained by the dietary cholesterol-induced change in percentage dietary cholesterol absorption, even though the relationship between change in percentage dietary cholesterol absorption versus percent change in LDL cholesterol was non-linear. The use of the plasma isotope ratio method (Sehayek et al. 1998a, Bosner et al. 1999) determines only the

cholesterol absorption percentage, the value is determined from only short period of time, it yields only a single measure of absorption, and the value of cholesterol absorption may be dependent on the composition of the test meal. With the continuous isotope feeding method (Kesäniemi and Miettinen 1987, Kesäniemi et al. 1987, Miettinen and Kesäniemi 1989, Gylling and Miettinen 1989, Miettinen et al. 1990), the cholesterol absorption percentage, amount of cholesterol absorbed and the intestinal influx of endogenous cholesterol can be determined in a balanced, constant state providing more steady and consistent values of cholesterol absorption from day to day during the period of one week. Therefore, the use of different methods for measuring cholesterol absorption in these earlier studies may explain the variation in the obtained results.

The role of cholesterol absorption as a regulator of serum HDL cholesterol seems more evident. Many studies have shown that the cholesterol absorption efficiency as well as plant sterols, indicators of cholesterol absorption, are positively associated with serum HDL cholesterol levels. (Miettinen and Kesäniemi 1989, Miettinen et al. 1990, Miettinen and Gylling 2000)

The fractional and absolute absorption of cholesterol correlates negatively with cholesterol synthesis (Miettinen and Kesäniemi 1989). Accordingly, effective cholesterol absorption will lower cholesterol synthesis and serum levels of cholesterol may increase, whereas with ineffective absorption, the overall cholesterol synthesis is increased (Miettinen et al. 1990). The association between cholesterol synthesis and serum and LDL cholesterol levels are insignificant in many studies (Gylling and Miettinen 1988, Miettinen and Kesäniemi 1989, Miettinen et al. 1989, Gylling et al. 1994), suggesting that serum and LDL cholesterol levels are regulated mainly by cholesterol absorption rather than cholesterol synthesis.

2.6.1.5 Measurement of cholesterol absorption

A variety of different methods have been developed for estimating the absorption of cholesterol in humans. Most of these methods are based on the use of radioactive isotopes. One of the earliest methods used was based on the administration of a single oral or intravenous dose of radioactive ^{3}H- or ^{14}C- , and the absorption of cholesterol was calculated as the difference between the dietary cholesterol and

fecal exogenous neutral steroids (Borgström 1969, Quintao et al. 1971, Sodhi et al. 1974, Samuel et al. 1978). This method has been modified by extending the isotope administration for several days or even as long as several weeks (Quintao et al. 1971), as well as with the use of intestinal intubation (Grundy and Mok 1977). Although these methods provide information about cholesterol absorption, they suffer from some particular disadvantages such as the need for hospitalization in a metabolic ward and inconvenience to the subject. In addition, only one measurement of a single dose of radioactive cholesterol may not accurately estimate the mean absorption over a period of time.

The plasma isotope ratio method, first introduced in rats (Zilversmit 1972), and later validated in humans (Samuel et al. 1978), is based on giving the reference compound ^{3}H-cholesterol intravenously simultaneously with an oral dose of ^{14}C-cholesterol, and the resulting ^{14}C/^{3}H ratio in the plasma gives an estimate of the percentage absorption of cholesterol from the intestine. Despite the simplicity and feasibility for outpatient studies, this method measures only absorption percentage of cholesterol, and the absorption value is determined only from a short period of time, which possibly is not the true reflection of mean overall absorption.

In the continuous isotope feeding method, developed by Crouse and Grundy (1978), the subjects receive peroral low-dose ^{14}C-cholesterol and ^{3}H-sitosterol in capsules three times a day with meals for 7-10 days. The stool is collected on days 3-10. The ratio of isotopes in feces becomes constant after the first 3 days. The percentage absorption of cholesterol is calculated from the difference between the dietary (capsules) and fecal isotope ratios. This measurement gives an accurate value of cholesterol absorption percentage because of the sufficiently long study period which allows one to achieve stable state and constancy in the ratio of the isotopes in feces, and sequential fecal samples diminish the fluctuation in the absorption. In addition, other advantages include the simple administration of isotope, analysis of fecal samples is easy, though laborious, the analysis can be repeated daily, and it is suitable for outpatients. The amount of cholesterol absorbed and the intestinal influx of endogenous cholesterol can be calculated, if the daily intake of cholesterol and fecal neutral steroid excretion are also measured. The only drawback is that children and women of child-bearing age cannot be studied.

More recently, the continuous isotope feeding method was modified by using markers labeled with stable isotopes, deuterated cholesterol, and deuterated sitostanol, quantified by gas-liquid chromatography-selected ion monitoring (Lütjohann et al. 1993). This method is claimed to be safe and reproducible without radioactive exposure. In addition, a nonradioactive modification was developed based on the plasma isotope ratio method (Zilversmit 1972), in which six extra mass units of ^{2}H-cholesterol were given orally and 5 extra mass units of ^{13}C were administered intravenously on day 0: the absorption percentage of cholesterol was calculated on day 3 from the plasma ratio of the two tracers using gas chromatography-mass spectrometry with selected ion monitoring (Bosner et al. 1993). The sensitivity of the analysis was further improved by using negative ion mass spectrometry (Ostlund et al. 1996). Furthermore, the measurement of serum plant sterols and cholestanol ratios to cholesterol, variables reflecting cholesterol absorption (Tilvis and Miettinen 1986, Miettinen et al. 1989, Miettinen et al. 1990) by gas-liquid chromatography provides a less laborious method to evaluate cholesterol absorption compared to methods based on the use of radioactive or stable isotopes.

2.6.1.6 Factors affecting cholesterol absorption

Despite the close relation between intestinal absorption of cholesterol and dietary fats as well as the presence of bile acids as requirements for cholesterol absorption, there are marked differences in the extent of absorption. For instance, the absorption of dietary fat is over 95 % (Carey et al. 1983), that of cholesterol approximately 50 %, and the bile acids are almost quantitatively reclaimed (> 95%)(Wilson and Rudel 1994). The cholesterol absorption efficiency between different individuals shows a high variation, with values ranging widely from 25 % to 80 % (Miettinen and Kesäniemi 1989, Bosner et al. 1999).

Diet

Results from several studies indicate that the cholesterol absorption efficiency remains mainly unaltered even though there are daily variations in the dietary cholesterol content (McMurry et al. 1985, Miettinen and Kesäniemi 1989, Sehayek et al. 1998a). However, with feeding of extra large amounts of cholesterol, the cholesterol absorption efficiency becomes decreased (McNamara et al. 1987, Gylling and Miettinen 1992, Vuoristo and Miettinen 1994). Increasing the amount of dietary

cholesterol resulted in substantial overall reductions in the absorption efficiency of cholesterol, but there was a considerable heterogeneity in the response of different individuals (Ostlund et al. 1999). Studies in Finnish and American populations have shown that ordinary cholesterol intake was not significantly related with cholesterol absorption efficiency (Miettinen and Kesäniemi 1989, Bosner et al. 1999). The association between cholesterol absorption and hypo- and hyper-responsiveness in humans (Beynen et al. 1987, Katan and Beynen 1987) might contribute to the results of dietary cholesterol intake- induced changes in cholesterol absorption. Feeding increasing amounts of cholesterol to mice led to a decrease in the percentage absorption of dietary cholesterol and an increase in the biliary cholesterol concentration, and these two variables were strongly and inversely correlated, suggesting that the biliary cholesterol secretion may have a role in regulating the efficiency of dietary cholesterol absorption (Sehayek et al. 1998b). Biliary cholesterol could saturate the intestinal micelles, preventing the normal absorption of dietary cholesterol. Other studies in mice (Carter et al. 1997) and monkeys (Rudel et al. 1994) have concluded that cholesterol absorption efficiency is significantly lower, when the animals are fed a diet containing extra high amounts of cholesterol compared to feeding a low cholesterol diet.

The results concerning the effects of dietary cholesterol on cholesterol absorption are not clear. The expanded intestinal cholesterol pool due to high amounts of dietary cholesterol may contribute to the dilution of labeled cholesterol resulting in its reduced absorption. The other concern with diets high in cholesterol is the difficulty of ensuring that all the cholesterol is properly micellar-solubilized in the intestine and available for absorption. However, most studies reveal that the amount of cholesterol absorbed is closely related to cholesterol intake, and the increased intake of cholesterol is associated with an increased mass absorption of cholesterol (Miettinen and Kesäniemi 1989).

Intestinal transit time

There are only limited number of studies concerning the effects of intestinal motility and transit time on cholesterol absorption. The reduction of small-bowel transit time by chenodeoxycholic acid (Ponz de Leon et al. 1979) or by metoclopramide (Ponz de Leon et al. 1982) decreases cholesterol absorption. In addition, the cholesterol absorption efficiency has been shown to correlate positively with mouth to anus intestinal transit time (Koivisto and Miettinen 1986). However, the atropine-induced prolongation of the small-bowel transit time did not enhance dietary cholesterol absorption in subjects whose cholesterol absorption percentage was normal (Ponz de Leon et al. 1982). Based on these results, the intestinal transit time seems to be related to steroid absorption under normal physiological conditions.

Obesity

The cholesterol absorption efficiency is decreased in obesity (Miettinen and Gylling 2000). An inverse correlation between BMI and absorption efficiency has been reported in a random population of Finnish men (Miettinen and Kesäniemi 1989). However, in another study, the correlation between BMI and cholesterol absorption percentage remained insignificant (Bosner et al. 1999). The obesity-related increased cholesterol synthesis with increased biliary output could contribute to lowered cholesterol absorption in obesity (Miettinen and Gylling 2000), but the exact mechanisms are not known.

Age

The effect of age on cholesterol absoption is controversial. Cholesterol absorption efficiency was lower in 75 year olds compared to 50 year old subjects (Gylling et al. 1994). On the contrary, the relation between age and the serum plant sterol levels, markers of cholesterol absorption, remained insignificant in another study (Kempen et al. 1991). In a third study, a positive correlation was found between age and the amount of dietary cholesterol absorbed, though the respective correlation for the cholesterol absorption percent was not significant (Bosner et al. 1999)

Apo E

The apo E phenotype is closely associated with the cholesterol absorption efficiency, a finding described first by Kesäniemi et al. (1987). More recent studies have confirmed this relationship (Gylling et al. 1989, Miettinen et al. 1992). The cholesterol absorption efficiency was highest in individuals with the E4/4 and E4/3 phenotypes, lowest with E2/2, and E3/3 was situated in the middle. The serum cholesterol levels were distributed in a similar manner so that highest levels were found in subjects with E4/4 or E4/3 phenotypes (Utermann et al. 1977). These results may confirm the role of cholesterol absorption as the regulator of serum cholesterol levels in a random sample of male population (Kesäniemi and Miettinen 1987, Kesäniemi et al. 1987). In studies with individuals consuming low fat-low cholesterol diets, the relation between apo E and cholesterol absorption efficiency vanished (Miettinen et al. 1992, Bosner et al. 1999).

Plant sterols

Plant sterols have been used for the treatment of hypercholesterolemia since the early 1950s because they lower serum cholesterol by inhibiting cholesterol absorption (Pollak 1953, Lees et al. 1977, Mattson et al. 1982). Further, it was shown that sitostanol, a saturated derivate of sitosterol, could reduce serum cholesterol more efficiently than sitosterol in rats (Sugano et al. 1977, Ikeda et al. 1981) and in humans (Heinemann et al. 1986). In 1993, based on Dr. Miettinen's hypothesis that a fat soluble esterified form of stanol would be more physiological and more palatable, and, accordingly, could inhibit cholesterol absorption and lower serum cholesterol more efficiently than the crystalline form, stanol ester rich mayonnaise (Vanhanen et al. 1993) and later rapeseed oil margarine were developed. Subsequently, many studies have confirmed the effect of stanol ester margarine as a way to safely inhibit cholesterol absorption and decrease serum total and LDL cholesterol levels in normo- and hypercholesterolemic individuals (Miettinen et al. 1995, Gylling et al. 1995), postmenopausal women with prior myocardial infarction (Gylling et al. 1997) as well as in non-insulin dependent diabetes mellitus (Gylling and Miettinen 1994a, 1996a).

Dietary plant sterols interfere with the micellar solubility of cholesterol, which directly impacts on the incorporation of micellar cholesterol to the absorptive enterocytes

(Ikeda and Sugano 1983, Ikeda et al. 1988). Hence, more sterols remain in the intestinal lumen, subsequently cholesterol absorption is depressed and serum cholesterol levels decrease. However, the exact mechanisms by which plant sterols inhibit cholesterol absorption have not been clarified in detail (Björkhem et al. 2001).

2.6.2 Plant sterols and cholestanol

Plant sterols are constituents of plant lipids. The normal human diet contains 200-300 mg/d of plant sterols (Salen et al. 1970), mainly sitosterol (65%), campesterol (32%), and trace amounts of stigmasterol (3%) (Björkhem et al. 2001). These sterols are structurally similar to cholesterol except for a difference in their side chain structure. The side chain of sitosterol contains an ethyl- and campesterol methyl group at the C-24 position. Stigmasterol has the same structure as sitosterol, except for a double-bond at C-22.

The human body is unable to synthesize plant sterols, thus all plant sterols are derived by absorption from the diet. Serum concentrations of plant sterols are regulated by dietary intake, absorption of these sterols from the intestine, and their elimination from the body through secretion into bile. A high cholesterol absorption efficiency is usually associated with high levels in serum of plant sterols, whereas high cholesterol synthesis, usually in conjunction with low absorption, is associated with low serum plant sterol levels as well as increased biliary sterol secretion (Miettinen et al. 1989, Miettinen et al. 1990). The ratios of plant sterols to cholesterol correlate positively with fractional absorption of dietary cholesterol and negatively with cholesterol precursor sterols and cholesterol synthesis, and thus measurement of their ratios to cholesterol can be used as indicators of cholesterol absorption (Tilvis and Miettinen 1986, Miettinen et al. 1989, Miettinen et al. 1990, Gylling and Miettinen 1997, Miettinen and Gylling 2000).

Cholestanol, a 5α-saturated derivate of cholesterol, is enzymatically formed from its precursor. Small amounts of cholestanol accompany cholesterol in most tissues. Dietary intake of cholestanol is normally very small (< 2 mg)(Miettinen et al. 1989), and its absorption is low, about 9 % (Vuoristo and Miettinen 2000). In addition, the serum levels of cholestanol remain constant during consumption of a cholestanol free-diet (Salen and Grundy 1973). Therefore, the body cholestanol is considered to

be mainly derived from endogenous cholesterol, its mean daily synthesis being about 12 mg/d (Salen and Grundy 1973). Cholestanol is eliminated from the body through bile as such or degraded to 5α-bile acids. The accumulation of cholestanol in the tissues and its high serum levels are seen in cerebrotendinous xanthomatosis, a rare inherited disease characterized with a defect in bile acid biosynthesis, and in sitosterolemia (Björkhem et al. 2001), and in cholestasis (Nikkilä et al. 1991). Measuring the serum cholestanol level is used as an indicator of cholesterol absorption (Miettinen et al. 1989).

2.6.2.1 Absorption of plant sterols

The intestinal absorption of plant sterols is normally limited. Only < 5 % of ingested sitosterol is absorbed (Salen et al. 1970), and absorption of campesterol is approximately 10 % (Tilvis and Miettinen 1986). Despite the high inter-individual variation of plasma sitosterol concentrations (Kempen et al. 1991), the plasma plant sterol concentrations seem to be very stable within individuals. The inter-individual variation in the plant sterol concentrations is genetically determined (Berge et al. 2002). Extremely high concentrations of plant sterols occur in a rare hereditary disorder called sitosterolemia (phytosterolemia) (Bhattacharyya and Connor 1974), in which sterol absorption is increased and sterol excretion into bile is decreased (Miettinen 1980, Björkhem et al. 2001). Mutations in the two ABC transporters, ABCG5 and ABCG8, have been described as one cause for this disease (Berge et al. 2000, Lee at al. 2001). Recent studies show that polymorphisms in ABCG8 have a contributing effect to genetic variation in the serum plant sterol concentrations in normal, healthy individuals (Berge et al. 2002).

2.6.2.2 Transport in serum

There are only a few studies, in which the distribution of plant sterols and cholestanol in different serum lipoproteins has been defined. Cholestanol is reported to be transported similarly to cholesterol mainly in LDL (Salen and Grundy 1973). In sitosterolemia, plant sterols are mainly transported in LDL and HDL (Bhattacharyya and Connor 1974), thus following cholesterol distribution. In order to diminish the effect of varying levels of serum cholesterol on the concentrations of plant sterols, they are usually standardized and expressed in relation to cholesterol in serum and lipoproteins as mmol/mol of cholesterol (Vuoristo and Miettinen 2000). In humans

when cholesterol absorption is normal, the ratios of plant sterols to cholesterol seem to accumulate in HDL (Tilvis and Miettinen 1986) this also being seen in rats (Sugano et al. 1978). In familial hypercholesterolemia patients with increased cholesterol synthesis due to ileal bypass, the ratios of serum plant sterols to cholesterol in lipoproteins appear to increase slightly with increasing lipoprotein density (Koivisto and Miettinen 1988b). In rats, plant sterols are transported to bile only in HDL (Robins and Fasulo 1997).

2.6.3 Cholesterol synthesis

Cholesterol is synthesized through several intermediates from acetyl-CoA (Figure 2). The rate-limiting step of cholesterol synthesis is the reduction of 3-hydroxy-3-methylglutaryl-coenzyme A (HMG-CoA) to mevalonate. This process is catalyzed by HMG-CoA reductase, the rate-limiting enzyme in the synthesis of cholesterol. Mevalonate is transformed to squalene, which is converted to lanosterol. Cholesterol is finally formed from lanosterol after several further steps. The synthetic pathway from squalene to cholesterol includes several intermediate methyl- and demethyl sterols released in serum.

Liver is the major organ responsible for cholesterol synthesis. De novo cholesterol synthesis is strictly regulated by the amount of cholesterol in cells (Brown and Goldstein 1983). Hepatocytes receive both exogenous and endogenous cholesterol. Chylomicron remnants transport the absorbed dietary cholesterol from the intestine to the liver, and circulating lipoproteins, mainly LDL, transport the endogenous cholesterol back to the liver for further processing. The key to the uptake process of these particles to the hepatocytes is their binding to the LDLapo B-receptor, thus mediating the endocytosis (Brown and Goldstein 1986). In addition, LDL receptor-related protein (LRP) is another member of the LDL receptor gene family, which binds to chylomicron remnants, probably mediating their uptake (Havel 1998).

Once inside the cell, cholesteryl esters are hydrolyzed in the lysosomes. The level of free cholesterol mediates a feedback control system, which regulates intracellular cholesterol homeostasis. This inhibits the HMG-Coa reductase enzyme leading to the termination of cholesterol synthesis, and it also activates ACAT leading to formation of cholesteryl esters for storage in the cell. It furthermore suppresses the synthesis of

LDL receptors preventing the overaccumulation of cholesterol in the cell. Free cholesterol is also used for membrane synthesis, as a precursor for steroid hormones or is converted to bile acids or secreted to the bile as such.

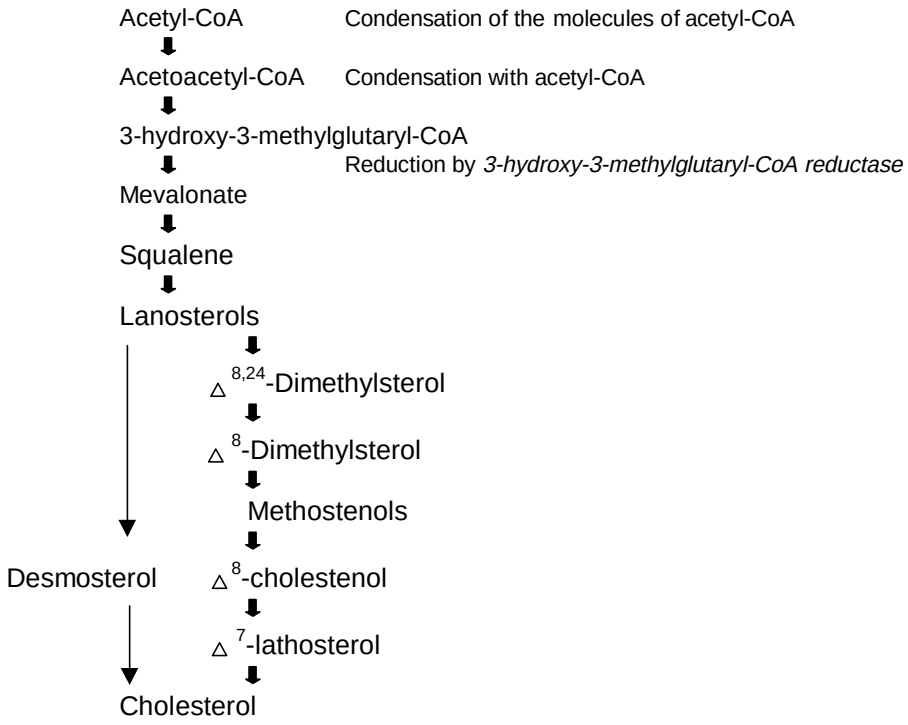


Figure 2. Biosynthesis of cholesterol

The sterol regulatory element-binding proteins (SREBPs) are transcription factors that are important regulators of cholesterol biosynthesis (Horton and Shimomura1999). SREBPs are membrane-bound molecules, whose activity is controlled by sterol-regulated proteolysis (Brown and Goldstein 1997). When the amount of cholesterol in the cell is low, SREBPs are liberated from membranes, and they enter the nucleus. When cellular sterols accumulate, SREBP cleavage is suppressed which leads to decreased levels of nuclear SREBPs.

When they gain access to the nucleus, SREBPs activate the genes involved in the synthesis of cholesterol and fatty acids, and the uptake of cholesterol through transcription of the LDL receptor enzymes. With respect to the cholesterol biosynthetic pathway, SREBPs activate the genes encoding HMGCoA reductase, HMGCoA synthase, farnesyl diphosphate synthase, squalene synthase and lanosterol demethylase (Brown and Goldstein 1997).

To date, three SREBP isoforms have been identified and characterized, SREBP-1a, -1c and SREBP-2 (Brown and Goldstein 1997). There are transgenic mice, which have overexpression of nuclear SREBP-2. This mutation led to marked increases in the mRNAs of cholesterol biosynthetic enzymes and the rate of cholesterol synthesis increased 28 –fold (Horton et al. 1998). SREBP-2 is suggested to be the principal isoform responsible for controlling the cholesterol biosynthetic pathway (Horton and Shimomura 1999).

2.6.3.1 Reverse cholesterol transport

Lipid-rich HDLs arise from lipid poor precursors or from lipid-free apolipoproteins (Fielding and Fielding 1995). Reverse cholesterol transport is initiated by the removal of cholesterol from peripheral tissues, either by passive diffusion to lipid-poor pre-HDL or receptor-mediated efflux to apo A-I.

The active removal is mediated by ABCA1, an ATP- binding cassette transporter, which facilitates the addition of phospholipids and cholesterol from the cells to free apoA-I, initiating the formation of HDL (Schmitz and Langmann 2001). The enzyme LCAT uses phospholipids and free cholesterol as its substrates and forms cholesteryl esters. Cholesteryl esters formed within HDL can be delivered to the liver by three routes.

Cholesteryl esters may be transferred to TRL by CETP (Tall 1993, 1995). These lipoproteins are further metabolized in the circulation to form lipoprotein remnants. The uptake of the remnants to the liver is mediated by binding to the hepatic receptors (LDLapo B-receptors or LRP), proteoglycans and apo E.

HDL cholesteryl esters may also be taken up by the liver via a process of selective lipid uptake. It has been shown recently that this selective uptake is mediated by SR-BI. Hepatocytes and steroid hormone producing cells express SR-BI. This protein binds to HDL and mediates the uptake of cholesteryl esters without internalization and degradation of HDL proteins (Acton et al. 1996, Kozarsky et al. 1997, Rigotti et al. 1997). In addition to cholesteryl ester uptake, SR-BI might mediate free cholesterol uptake of HDL to the liver, providing an important source of biliary cholesterol (Ji et al. 1999, Mardones et al. 2001). Some HDL particles contain apo E, and can be taken up by hepatocytes via the LDLapo B-receptors (Hennessy et al. 1997). The mechanisms of the HDL particle uptake are not clear, and proteoglycans, apoproteins and still unknown receptors might also be involved (Ji et al. 1997).

2.6.4 Squalene and demethylated cholesterol precursors

In addition to cholesterol, some of its precursors including squalene and demethylated sterols, especially Δ^8–cholestenol (cholestenol), desmosterol and Δ^7–lathosterol (lathosterol), are present in detectable amounts in serum. The sterols are transported in serum by lipoproteins similarly to cholesterol, such that the highest amounts are found in LDL. When expressed as ratios to cholesterol, the lathosterol ratio is highest in VLDL in subjects with increased cholesterol synthesis (Koivisto and Miettinen 1988a). Most of the squalene is carried in LDL and HDL in fasting human serum (Miettinen 1982b, Gylling and Miettinen 1994b), but the highest ratio of squalene to cholesterol (Gylling and Miettinen 1994b) and the newly synthesized squalene (Goodman 1964) are found in VLDL. Many studies have shown that serum levels of different precursors to cholesterol are increased under conditions of increased synthesis of cholesterol, and reduced with reduced synthesis of cholesterol (Miettinen 1969, 1970, 1981, Miettinen and Koivisto 1983, Miettinen 1985, Vuoristo and Miettinen 1986). The non-cholesterol precursor sterols correlate positively to HMG-CoA reductase activity and the cholesterol synthesis rate (Björkhem et al. 1987, Kempen et al. 1988, Miettinen et al. 1990) and negatively to cholesterol absorption and to the levels of plant sterols in serum (Miettinen et al. 1990). In addition, treatment with lovastatin reduced the cholesterol synthesis rate and serum lathosterol concentrations as well as the ratio of lathosterol to cholesterol in serum (Kempen et al. 1988, Duane 1995). Thus, non-cholesterol sterols have been demonstrated to reflect the rate of cholesterol synthesis, and thus measurement of

their ratios to cholesterol can be used as indicators of cholesterol synthesis. The serum content of squalene seems to reflect cholesterol synthesis less efficiently (Miettinen et al. 1990).

2.6.5 Elimination of cholesterol

Liver is the major organ responsible for the removal of cholesterol from the body. The removal of cellular cholesterol from peripheral tissues to the liver by HDL mediated extraction occurs by a number of mechanisms including aqueous diffusion, a specific apoprotein-mediated mechanism as well as facilitated efflux mediated by cell surface receptors. In addition, some cells are able eliminate intracellular cholesterol by converting it into more polar metabolites by oxidative mechanisms. These oxysterols are important as transport forms of cholesterol over cell membranes and the blood-brain barrier, because they are easily excreted from the cells and transported to the liver for conversion to bile acids. For instance, cholesterol in the brain is converted into 24S-hydroxycholesterol which can be transported over the blood-brain barrier much more rapidly than unmetabolized cholesterol, and taken up by liver from the circulation for further metabolism (Björkhem and Diczfalusy 2002).

Cholesterol is eliminated from the body primarily by fecal excretion of neutral sterols and bile acids. Cholesterol is secreted either unchanged or as bile acids into bile, and biliary secretion of cholesterol is linked with biliary bile acid output (Schersten et al. 1971). Fecal neutral sterols consist mainly of cholesterol and its bacterial metabolites coprostanol, epicoprostanol and coprostanone, and also variable amounts of cholesterol precursors and plant sterols, and their coprostanol and coprostanone bacterial products (Miettinen et al. 1965, McNamara et al. 1981). The daily excretion rate of cholesterol is 400-1000 mg (Miettinen et al. 1965), mostly being excreted as its metabolite coprostanol.

2.6.5.1 Bile acids

The formation of bile acids from cholesterol accounts for over 30 % of the cholesterol removal from the body. During the past few years it has been shown that the initial step in the formation of the bile acids may start in the extrahepatic tissues (Princen et al. 1997). However, all the subsequent steps in the formation of the bile acids are almost exclusively located in the liver. The hepatic conversion of cholesterol into bile

acids involves many steps and enzymatic processes. The major, “neutral” pathway begins with the rate-limiting step of the conversion of cholesterol into 7 α-hydroxy-cholesterol, catalysed by the cholesterol 7α-hydroxylase (CYP7A1). This enzyme regulates the overall conversion of cholesterol into bile acids in this pathway, and its activity is regulated through negative feedback by reabsorbed bile acids returning to the liver. An alternative pathway, the “acidic pathway”, starts with the 27-hydroxylation of cholesterol followed by a microsomal 7α –hydroxylation by a specific oxysterol 7α -hydroxylase (Björkhem and Eggertsen 2001). This pathway is less responsive to bile acid negative feedback, and is regulated separately from the major pathway (Björkhem et al. 2001). The neutral pathway seems to be responsible for over 50 % of the total synthesis of bile acids, whereas acidic pathway starting in the liver may be responsible for 30-50 % of the total production (Björkhem and Eggertsen 2001). Only a minor amount of the whole bile acid pool is produced by extrahepatic tissues starting with 27-hydroxylation (Duane and Javitt 1999).

The end products of these pathways are the primary bile acids, cholic acid and chenodeoxycholic acid. The primary bile acids are secreted into bile as conjugates with glycine or taurine (3:1). After secretion, these bile acids may be deconjugated and also dehydroxylated by intestinal bacteria. Deconjugation alters both the physical and chemical properties of bile acids. Deoxycholic acid is the dehydroxylated product of cholic acid and lithocholic acid is the corresponding product of chenodeoxycholic acid. Deoxycholic acid is water soluble and it is partly absorbed, whereas lithocholic acid is mainly excreted in the feces. From the intestine, bile acids are reabsorbed and returned to the liver via the portal circulation. Absorbed bile acids are taken up by hepatocytes and secreted again into bile. This enterohepatic circulation is an extremely efficient way to conserve bile acids, of which only 2-5% escape the intestinal absorption to be excreted into feces.

Transformation of cholesterol into bile acids is the major pathway of cholesterol catabolism, and thus bile acids have an important role in cholesterol homeostasis. The activity of CYP7A1 is controlled by negative feedback of the reabsorbed bile acids to guarantee the maintenance of an intact bile acid pool. CYP7A1 enzymatic activity is influenced by a number of dietary and hormonal factors. A close

correlation is found between the activity of HMG-CoA reductase and CYP7A1 (Björkhem and Åkerlund 1988), and their activities usually increase in parallel both in basal conditions and in situations of bile acid malabsorption (Färkkilä and Miettinen 1990, Björkhem et al. 1997). Enhanced fecal elimination of bile acids due to interruption of the enterohepatic circulation, e.g. with cholestyramine or surgical resections, can increase bile acid synthesis, stimulate the expression of hepatic LDL receptors and increase the synthesis of cholesterol (Everson and Kern 1995), which is seen also in an elevation in the levels of the cholesterol precursor sterols (Miettinen 1985, Färkkilä et al. 1988).

Recent studies have shown that the secretion of bile acids and cholesterol from hepatocytes into bile is controlled by ABC transporters. The bile salt export pump (BSEP) is a member of the ABC superfamily of transporters (ABCB11). This pump is located on the canalicular membrane of hepatocytes where it facilitates the transport of bile acids from the hepatocyte into bile (Schmitz et al. 2000). The secretion of cholesterol into bile is mediated by two other ABC transporters, ABCG5 and ABCG8 (Goldstein and Brown 2001).

2.6.6 Nuclear receptors and cholesterol metabolism

Nuclear receptors function as ligand-activated transcription factors that regulate the expression of target genes. Two nuclear hormone receptors involved in the regulation of cholesterol homeostasis are liver X receptor (LXR) and farnesoid X receptor (FXR). The natural ligands for LXR and FXR are oxysterols and bile acids (Accad and Farese 1998, Russell 1999). Ligand-activated LXR or FXR both form heterodimers with the RXR in order to modulate and control transcriptional activity of several important genes regulating cholesterol metabolism.

Three genes coding ABC transporters (ABCA1, ABCG5 and ABCG8) which are involved in the absorption of cholesterol, are the proposed target genes of LXR (Berge et al. 2000, Repa et al. 2000, Lee et al. 2001). In addition, genes coding CYP7A1, (enzyme onvolved in bile acid synthesis), and SREBP-1c (biosynthesis of fatty acids) are activated by the LXR-RXR heterodimer (Repa and Mangelsdorf 1999, Goldstein and Brown 2001). FXR is highly expressed in liver where it appears to function as a bile acid sensor. The genes encoding BSEP and ileal bile-acid binding

protein (I-BABP) (mediates the re-uptake of bile acids from the intestinal lumen) are target genes for FXR (Edwards et al. 2002). In addition, the FXR-RXR heterodimer represses CYP7A1 transcription (Lu et al. 2000).

Therefore, nuclear receptors are important in regulating cholesterol metabolism by controlling the transcription of several important genes involved in cholesterol and bile acid metabolism.

2.7 Cholesterol synthesis and elimination in obesity

Cholesterol synthesis (Miettinen 1971a, Liu et al. 1975, Nestel et al. 1973) and turnover Nestel et al. 1969) are increased in obesity. The serum levels of methyl and demethyl sterols, cholesterol precursor sterols, and squalene are elevated in obesity, reflecting increased synthesis of cholesterol (Miettinen 1970, Liu et al. 1975, Nestel and Kudchodkar 1975, Miettinen and Gylling 2000). Some of the excess cholesterol is produced by the enlarged amount of adipose tissue (Miettinen and Tilvis 1981), though adipose tissue-synthesized cholesterol does not account for all of the enhanced synthesis of cholesterol in obesity (Schreibman and Dell 1975, Miettinen and Tilvis 1981). Increased cholesterol synthesis in liver seems to be responsible for most of the excess cholesterol produced in obesity, associated with increased fatty acid transport to the liver and generally enhanced lipogenesis (Nestel and Goldrick 1976). It has been shown that the activity of hepatic HMG-CoA reductase is increased in obesity (Angelin et al. 1982). More recently, in a study of liver biopsies obtained from obese and non-obese subjects, the activity and mRNA level of HMG-CoA reductase were markedly higher in the liver of obese than non-obese subjects (Ståhlberg et al. 1997).

In addition to enhanced cholesterol synthesis, the elimination of cholesterol to feces is enhanced through its increased secretion into bile and also because of the increased formation of bile acids (Miettinen 1971a, Nestel et al. 1973, Bennion and Grundy 1975, Miettinen and Gylling 2000). In fact, the activity and mRNA level of CYP7A1 are clearly increased in obese vs non-obese subjects (Ståhlberg et al. 1997). The increased secretion of cholesterol into bile increases the risk for gallstone formation in obesity (Miettinen 1987).

2.8 Cholesterol metabolism in diabetes

Type 2 diabetes is associated with many metabolic disturbances, including abnormalities in glucose and lipid metabolism. The alterations in lipoprotein metabolism have been studied and described in detail earlier, and thus are well known (American Diabetes Association 1993, Evans 1999). The study of cholesterol metabolism and bile acids in diabetes has been somewhat neglected, perhaps because of the more common and obvious abnormality in plasma lipids. There are some earlier studies describing cholesterol metabolism in diabetes, though the results are not always consistent.

2.8.1 Cholesterol absorption

A few studies have shown that the cholesterol absorption efficiency might be low in hypercholesterolemic type 2 diabetes (Gylling and Miettinen 1997) or in markedly hypertriglyceridemic diabetes subjects (Briones et al. 1986). However, in the latter study, of 40 diabetic subjects, 22 were receiving insulin treatment at the time of the study, and 6 were insulin –dependent diabetics. Low plant sterol concentrations in type 2 diabetes have been described in two studies (Sutherland et al. 1992, Gylling and Miettinen 1997). The cholesterol absorption efficiency and serum plant sterol levels are higher in type 2 diabetic subjects with coronary artery disease compared to those without this disease (Gylling and Miettinen 1996b). In addition, cholesterol absorption efficiency and serum levels of plant sterols and cholestanol are low in nondiabetic subjects with high-normal blood glucose levels (Strandberg et al. 1996).

2.8.2 Regulation of serum cholesterol level

There are only few studies describing the regulation of serum cholesterol level in type 2 diabetes, but the number of study populations in these reports is are very small. The serum cholesterol level seems to be regulated in type 2 diabetes similarly as in the non-diabetic population by the homeostasis between cholesterol absorption, synthesis and LDL catabolism (Gylling and Miettinen 1997). The serum LDL cholesterol level was inversely related to cholesterol synthesis and FCR of LDL apo B (Gylling and Miettinen 1996b, 1997), and FCR of LDL apo B also correlated positively with the lathosterol ratio (Gylling and Miettinen 1997). However, LDL cholesterol did not correlate significantly with cholesterol absorption. Based on these

results obtained with a few study subjects, the connection between cholesterol absorption and serum LDL levels seen in the normal population, may not necessarily occur in type 2 diabetics.

2.8.3 Cholesterol synthesis and excretion

Bile acid and cholesterol synthesis and cholesterol excretion as neutral sterols are increased in non-obese, mildly hypercholesterolemic (Gylling and Miettinen 1997), mildly hypertriglyceridemic non-obese (Abrams et al. 1982, Naoumova et al. 1996), markedly hypertriglyceridemic (Briones et al. 1986) and markedly overweight (Bennion and Grundy 1977) patients with type 2 diabetes. The ratios of cholesterol precursor sterols to cholesterol in serum were higher in diabetics than in controls (Gylling and Miettinen 1997). The serum level of insulin and lathosterol were correlated positively only in the diabetic but not in control subjects (Sutherland et al. 1992). Scoppola et al. (1995) found that cholesterol synthesis, measured by urinary mevalonic acid excretion, was higher in normolipidemic, non-obese diabetics than controls. In addition, urinary mevalonate excretion was significantly and positively correlated with glycated hemoglobin concentrations (HbA1c) in non-obese normolipidemic type 2 diabetics, suggesting that there is enhanced cholesterol synthesis with a lower degree of blood glucose control (Scoppola et al. 2001). On the contrary, by using sterol balance measurements, Briones et al. (1986) found no difference in cholesterol synthesis between diabetics and controls, when the lipid profile was normal. However, 12 out of the 19 diabetic subjects were receiving insulin treatment, and 4 were type 1 diabetics. Andersen et al. (1986) found that the net steroid balance (total cholesterogenesis) was almost twice as high in diet-treated type 2 diabetics than in controls, and overweight was not a confounding factor. Cholesterol synthesis, indicated by both the cholesterol precursor ratios to cholesterol and by sterol balance data were similar in type 2 diabetes patients with or without coronary artery disease (Gylling and Miettinen 1996b). Cholesterol synthesis and serum lathosterol and desmosterol levels were increased in nondiabetic subjects with high-normal blood glucose level (Strandberg et al. 1996),

and one-third of a randomly selected non-diabetic hypertriglyceridemic population (n=53) had an increased serum lathosterol concentration (Asplund-Carlson et al. 1999).

2.8.4 Insulin treatment

There is no consensus on the effect of insulin treatment on cholesterol metabolism in type 2 diabetes. Enhanced bile acid and cholesterol synthesis was decreased, and gallbladder bile was more saturated after insulin therapy in six obese diabetics, and therefore the net sterol balance was changed (Bennion and Grundy 1977). In five non-obese diabetics, the fecal bile acid excretion decreased and fecal neutral steroid excretion increased with insulin therapy, so that the total sterol balance was not changed (Saudek and Brach 1978). In addition to the controversial results obtained, there were many differences in the protocols of these two studies, such as the duration of the insulin treatment, characteristics of the study populations and their caloric intake. In another study with 14 non-obese diabetics, the increased synthesis rates of cholesterol and bile acids remained elevated after a short treatment with insulin (Abrams et al. 1982). The formation and turnover of bile acids and the fecal excretion of neutral steroids were similar in insulin-treated diabetics and controls, 6 out of 11 diabetics being type 1 diabetics (Andersen et al. 1986). In a study with 6 non-obese type 2 diabetics with higher baseline plasma mevalonic acid concentrations than in 6 controls, the mevalonic acid concentrations decreased with hyperinsulinemic clamp in both groups, but significantly so only in the control group (Naoumova et al. 1996). Intensive insulin therapy decreased the high baseline cholesterol synthesis in ten non-obese normolipidemic type 2 diabetic subjects, when measured by the urinary excretion of mevalonic acid (Scoppola et al. 1995). Moreover, the increased plasma levels of mevalonic acid decreased markedly with a 3-h euglycemic hyperinsulinemic clamp in these same patients.

2.8.5 Summary

Increased synthesis and decreased absorption of cholesterol have been reported in mildly hypercholesterolemic (Gylling and Miettinen 1997), and hypertriglyceridemic (Briones et al. 1986) type 2 diabetes subjects without insulin therapy. However, considering the earlier cholesterol metabolic studies, variable degrees of BMI, small and heterogeneous study groups, different degrees of glucose control, different types

of dyslipidemia and diabetes treatment as well as controversy in results complicate the interpretation of these results, and abnormalities of cholesterol metabolism in type 2 diabetes remain somewhat unclear.

2.9 Treatment with weight reduction

Obesity, especially abdominal obesity, enhances insulin resistance and is a risk factor for the development of type 2 diabetes. Many studies have shown that weight reduction, even a modest degree of sustained weight loss, can reduce the risk for developing type 2 diabetes as well as impaired glucose tolerance progressing to frank diabetes compared to obese diabetics not losing weight (Pi-Sunyer 1996, Leibel et al. 2001). In addition, the reduction in plasma lipids in obese subjects with type 2 diabetes has been suggested to be the cause of the reversibility of insulin resistance after weight loss, rather than the weight loss per se (Mingrone et al. 1997).

2.9.1 Glucose and lipoprotein metabolism

Dattilo and Kris-Etherton (1992) have quantified the effects of weight loss on plasma lipids by meta-analysis of 70 studies; every 1 kg decrease in weight was associated with a reduction of total cholesterol by 0.05 mmol/l, LDL cholesterol by 0.02 mmol/l, triglyceride levels by 0.015 mmol/l, increase in HDL cholesterol by 0.009 mmol/l, as long as the weight reduction was maintained. In addition, HDL cholesterol decreased by 8% or 0.007 mmol/l while subjects were actively losing weight in short term interventions.

Weight reduction is considered as the primary treatment for obese type 2 diabetic subjects, because of its beneficial effects for the improvement of metabolic abnormalities associated with this disorder (American Diabetes Association 1999). Weight loss has been shown to reduce fasting plasma glucose and insulin concentrations (Henry et al. 1985, 1986, Wing et al. 1987), decrease insulin resistance (Henry et al.1986, Henry and Gumbiner 1991) and improve insulin secretion (Henry et al. 1986, Gumbiner et al. 1990). Many studies have shown that weight reduction lowers serum triglyceride levels, total and LDL cholesterol and free fatty acid concentrations, and converts LDL particles to a more normal pattern

(Gumbiner et al. 1998, American Diabetes Association 1999). HDL cholesterol increases with weight reduction (Pi-Sunyer 1996), even though the composition of the diet, the amount of weight loss, and the time after the weight reduction seem to contribute to the changes in the HDL cholesterol level (Henry and Gumbiner 1991). Even energy restriction independent of the weight loss can have marked effects on the glycemic control (Wing et al. 1994), and several studies have shown improved glycemic control within 10 days of imposing energy restriction before any significant weight loss could be achieved (Henry et al. 1985, Bosello et al. 1997). The improvements in the glycemic and lipid abnormalities have been observed even with modest weight reductions, ~ 5 % of the initial weight, and these confer long-term benefits (Wing et al. 1987, Pi-Sunyer 1996). In conclusion, with energy restriction and weight reduction, even with modest weight loss, the glycemic and lipoprotein metabolism is improved, and some of these changes occur quite rapidly. However, obesity is an accentuating factor for abnormal lipoprotein metabolism in type 2 diabetes, but not the only cause, and therefore dyslipidemia is not fully corrected with weight reduction. Triglyceride levels may remain moderately elevated, low HDL levels often persist, and small, dense LDL particles can be retained (Garg et al. 1992).

2.9.2 Cholesterol metabolism

There are only a few studies of weight reduction on cholesterol metabolism in general and their results are inconsistent. In addition, in type 2 diabetes, the effects of calorie restriction and weight reduction on cholesterol metabolism are largely unknown. In non-diabetic subjects, weight reduction reduces the fecal excretion of neutral and acidic steroids (Miettinen 1968a, 1970, 1971a, Bennion and Grundy 1975, Kudchodkar et al. 1977), and lowers effectively cholesterol synthesis (Miettinen 1968a, 1970, 1971a, Bennion and Grundy 1975, Kudchodkar et al. 1977), and serum methyl sterol values (Miettinen 1968b, 1971b). Di Buono et al. (1999) studied cholesterol biosynthesis by measuring the incorporation of deuterium from body water into free cholesterol in the erythrocyte membrane over 24 hours. They found that endogenous cholesterol synthesis was suppressed immediately after the modest weight reduction achieved with energy restriction. Griffin et al. (1998) studied the relationship of postprandial glucose and insulin to cellular cholesterol synthesis in thirteen subjects, characterized with an increased postprandial cholesterol synthesis.

Cellular cholesterol synthesis was measured by quantitating the amount of ^{14}C-acetate incorporated into human peripheral blood mononuclear leucocytes. After an 8% weight reduction to a new-weight stable condition, insulin resistance and insulin secretion fell in seven subjects (3 diabetic and 4 non-diabetic), and their cholesterol synthesis no longer increased postprandially. In six subjects (3 diabetic, 3 non-diabetic), however, despite the occurence of a similar weight loss, insulin resistance, insulin secretion and postprandiallly enhanced cholesterol synthesis did not change.

Acute caloric restriction did reduce cholesterol synthesis and total fecal bile acids, even though cholesterol absorption percentage remained unchanged (Kudchodkar et al. 1977). Bennion and Grundy (1975) also studied the effects of chronic caloric restriction on cholesterol and bile acid metabolism, and found that the outputs of cholesterol, bile acids and phospholipids, and the bile acid pool size were reduced, and in addition, cholesterol synthesis and fecal excretion of cholesterol were also reduced. In another study, cholesterol output and bile acid secretion were decreased in six obese subjects after they consumed a hypocaloric diet for four weeks (Mazzella et al. 1995). In a recent study, the fractional cholesterol synthesis rates were lower after the subjects consumed a diet containing low amounts of fat and/or energy compared with a diet containing an unlimited amount of fat and energy (Raeini-Sarjaz et al. 2001).

The effects of weight reduction on cholesterol metabolism in type 2 diabetes have not been documented in detail. There are only two studies in which the study population included type 2 diabetic patients; in the first study, two out of three subjects were diabetic (Miettinen 1971a), and in the second study, six out of thirteen subjects were diabetic (Griffin et al. 1998).

These earlier studies which were performed mainly in non-diabetic subjects indicate that cholesterol synthesis and fecal excretion of bile acids and neutral sterols are reduced with weight reduction in subjects still actively losing weight or on a stable, reduced weight level regardless of whether some weight loss, even modest, is achieved by total fasting or caloric restriction. However, the effect of weight loss on cholesterol absorption has not been documented.

3. AIMS OF THE STUDY

The risk of coronary artery disease is high in type 2 diabetes, and the frequent metabolic disturbances associated with diabetes including dyslipidemia, hyperinsulinemia, insulin resistance, hyperglycemia and obesity can contribute to the accelerated atherogenesis. Cholesterol metabolism has not been characterized in detail in type 2 diabetes, and very little is known about the interrelation of cholesterol and glucose metabolism in diabetes. The metabolism of cholesterol is disturbed in obesity, and the abnormalities resemble those seen in a few studies with type 2 diabetic subjects. It is not known whether overweight, which is frequently associated with diabetes, is responsible for the alterations observed in the few studies of cholesterol metabolism in type 2 diabetes, or whether diabetes has any independent role in regulating cholesterol metabolism. Accordingly, type 2 diabetes with obesity may be associated with more profound alterations of cholesterol metabolism.

Weight reduction is the primary means to treat overweight diabetic subjects, because of its beneficial effects on glycemic balance, insulin sensitivity and lipoprotein abnormalities. The effects of weight reduction on cholesterol metabolism remain unclear in diabetes. Studies of obesity without diabetes have indicated that cholesterol synthesis decreases with weight loss. Therefore, treatment by weight reduction might change cholesterol metabolism in type 2 diabetes as well. However, the role of effective weight reduction in cholesterol and sterol metabolism acutely after the weight loss is not known, nor the stability of these changes during prolonged follow-up. Thus, the objectives of the present study were

- to investigate the relations of cholesterol, lipoprotein and glucose metabolism in type 2 diabetes.
- to evaluate the independent effect of type 2 diabetes on cholesterol metabolism, when obesity is not a confounding factor, comparing obese patients with and without diabetes with each other.
- to investigate the effect of body weight on cholesterol metabolism in type 2 diabetes, comparing type 2 diabetes patients with and without overweight with each other.

- to investigate cholesterol metabolism, reflected by serum squalene and non-cholesterol sterols, during chronic weight reduction in type 2 diabetes, and

- to investigate the effects of weight reduction on cholesterol, glucose and lipoprotein metabolism in type 2 diabetes after a two year follow-up in a stable, weight maintaining eucaloric phase in type 2 diabetes.

4. MATERIALS AND METHODS

4.1 Subjects and designs

The type 2 diabetic patients for the studies were recruited on the basis of their reply to an advertisement in a newspaper, from health centers of Helsinki, and from the Outpatient Department of Helsinki University Central Hospital. The diagnosis of type 2 diabetes was based on repeated fasting blood glucose level ≥ 7 mmol/l (World Health Organization 1999). The age of the subjects ranged 41 to 74 years. None of the subjects were using insulin therapy or lipid lowering medication. Exclusion criteria were also the presence of diabetic nephropathy and neuropathy, abnormal thyroid, liver or kidney function tests, gastrointestinal disease, unstable angina pectoris, recent myocardial infarction or invasive coronary treatment within one year. All women were postmenopausal and not taking hormone replacement therapy. All subjects volunteered for the studies and gave an informed consent. The study protocols had been accepted by the Ethics Committee of Department of Medicine, University of Helsinki. The studies were conducted according to the principles of the Declaration of Helsinki.

Table 1. Characteristics of the study populations.

Variables	Study I		Study II		Study III	Study IV
	DM	Controls	DM	DM	DM	DM
Number of subjects	16	16	20	44	10	16
Males/females	13/3	10/6	16/4	34/10	8/2	13/3
Age, years	52.2±1.8	50.8±0.5	62.1±2.0	59.0±1.2	51.5±2.8	52.3±1.8
BMI, kg/m^2	32.2±1.0	33.3±0.9	24.1±0.4*	31.0±0.5	31.7±0.9	31.7±0.6
Blood glucose, mmol/l	8.4±0.6*	4.6±0.2	8.5±0.5	8.6±0.4	6.9±0.5	8.4±0.6
Apo E phenotype E2/E3/E4 (n)	1/9/6	2/10/4	3/11/6	8/19/17	1/5/4	1/9/6

Values are mean±SE. * for the difference between groups in the individual studies (Study I, II, III and IV). For apo E phenotype, E2 = phenotypes 2/3 and 2/4; E3 = phenotype 3/3; E4 = phenotypes 4/3 and 4/4.

4.1.1 Study I

To determine the effect of type 2 diabetes on cholesterol metabolism, the study population consisted of obese type 2 diabetic subjects (DM group) and an obese non-diabetic control group (Table 1). The DM group comprised sixteen obese type 2 diabetes patients, thirteen men and three women. Their BMI was > 30 kg/m^2 , and the mean age was 52±2 (SE) years. Diabetes was recently diagnosed (< 2 years). Diabetes was treated with diet in 10 patients, 3 had glibenclamide and 3 had a combination therapy of glibenclamide and biguanide. The control group was recruited

from random population-based age-cohorts of 50 year old men and women. It consisted of sixteen obese, healthy normoglycemic subjects, of which ten were men and six were women. Their BMI and age were similar to the DM group. Their health status was determined with medical examination and laboratory tests.

The DM group consumed a low-fat, low-cholesterol diet, whereas the controls consumed their normal ad libitum home diet. The subjects visited the Outpatient Department twice a week. During the week, the subjects kept a food record for seven days. In order to measure absorption and fecal elimination of cholesterol, they took a marker capsule, the ingredients of which are given in detail in the methods section, three times a day with their regular meals during the 7-day period. A three-day stool collection was performed during the last days of the week. The absorption, synthesis and fecal excretion of cholesterol and bile acids were quantitated from these stool samples.

A blood sample was taken at the beginning and at the end of the study, both after 12-hour fasting, from which serum lipids, lipoproteins and blood glucose were analyzed.

4.1.2 Study II

The modulating effect of body weight on cholesterol metabolism was investigated in type 2 diabetic groups with and without overweight (Table 1). The total study population consisted of 64 DM patients, 51 men and 13 women with a mean age 60±1 years, and BMI of 28.8±0.5 kg/m^2 (range from 20.7 to 40.1 kg/m^2). The diagnosis of diabetes had been made within 5 years. Diabetes was treated with diet in 32 subjects, with sulphonylureas in 14 subjects, with biguanides in 5 subjects, and with a combination of these two drugs in 13 subjects. Twenty-two patients were taking beta-blocking therapy, 25 calcium channel blockers and 6 diuretics.

The subjects were divided into two study groups according to their BMI. Those 20 subjects with BMI $\leq$ 26.0 kg/m^2 formed a normal-weight group, and 44 subjects with BMI > 26.1 kg/m^2 formed the overweight group. The distribution of the different medical treatments were similar between the study groups. The medication and the diet were kept unchanged during the study week.

The subjects consumed their normal home diet during the study. The study protocol was similar as in Study I. The methods and the procedure to measure cholesterol synthesis and absorption, and fecal composition of bile acids, neutral steroids and non-cholesterol sterols were performed similarly as in Study I. Two fasting blood samples were taken one week apart. Serum lipids, lipoproteins, non-cholesterol sterols, squalene, insulin, blood glucose and serum sex hormone binding glodulin (SHBG), an indicator of insulin resistance (Haffner 1996), were analysed from these samples.

4.1.3 Study III

The changes in cholesterol metabolism reflected by serum squalene and non-cholesterol sterols were studied before and during extensive long-term weight reduction, on a non-steady state, in obese patients with type 2 diabetes (Table 1). The study population consisted of 8 men and 2 women with a mean age of 51.5± 2.8 years and BMI > 30 kg/m^2. Diagnosis of diabetes had been made within 2 years. Diabetes had been treated with diet in 8 subjects, one had glibenclamide and one had a combination therapy with glibenclamide and biguanide. Four subjects used a combination therapy of beta- and calcium channel blockers, and three used beta-blockers as a hypotensive medication.

The study consisted of two periods. The first, run-in period, lasted for six weeks. The subjects consumed their normal home diet, and the baseline studies were performed. The second period, the effective weight reduction, lasted for three months. During this period, the subjects consumed daily only 3 servings of a very low energy diet (VLED), which consisted of 97 kJ of energy per day (Cambridge Diet, Howard Foundation, Cambridge, UK). One serving consisted of 14.2 g of protein, 15.0 g of carbohydrates, 2.7 g of fat and essential minerals, trace nutrients and vitamins. The hypoglycemic medication was discontinued during the low calorie period.

For the baseline studies and at the end of the weight reduction period, two fasting blood samples were taken, a week a part. Serum lipids, lipoproteins, squalene and non-cholesterol sterols, blood glucose, serum insulin and SHBG were analysed from serum samples obtained before and at the end of the low calorie period. The amount of sterols of the VLED serving were also quantitated.

4.1.4 Study IV

This study was performed to investigate the effects of weight reduction on cholesterol-, glucose- and lipoprotein metabolism in type 2 diabetes after a two year follow-up in a stable, weight maintaining eucaloric phase (Table 1). Sixteen type 2 diabetic subjects participated in the study. The study population consisted of 13 men and 3 women with a mean age 52.3±1.8 years and BMI > 30 kg/m^2. Diabetes was recently diagnosed (< 2 years).

Ten subjects were randomly selected to intensive weight reduction with a VLED for 3 moths, called the VLED group, and six subjects formed the diet group. In VLED group, diabetes was treated with diet in 8 subjects, one used glibenclamide, and one had a combination therapy of glibenclamide and biguanide. In the diet group, 2 subjects were on diet, 2 had glibenclamide and 2 had combination therapy. Four subjects in the VLED group and two in the diet group used a combination of beta- and calcium channel blockers, and three control subjects had beta-blockers alone, and this medication was kept unchanged during the study.

The study lasted for two years, and it consisted of three periods. During the first, the run-in period, which lasted for six weeks, the subjects were randomized, and baseline metabolic studies were performed. The second, the effective weight reduction period lasted for 3 months. The VLED group ingested daily 3 servings of the same very low energy diet as used in Study III. The diet group was advised to consume a low fat, low cholesterol diet. Biguanides were discontinued, and the glibenclamide dose was adjusted to keep blood glucose < 7 mmol/l. The third period continued after the 3-month weight reduction and lasted up to 2 years. Both groups consumed a weight maintenance diet, in which the daily energy balance was zero, each subject individually advised by a dietician.

Initially, before the low-calorie diet, and at the end of the study, at about two years after active weight reduction, metabolic and kinetic studies of lipoproteins were performed and blood glucose, serum insulin and SHBG were measured from fasting blood samples. The methods and the procedure to measure cholesterol synthesis and absorption, and fecal composition of bile acids, neutral steroids and non-cholesterol sterols were performed similarly as in Studies I and II. LDL and HDL

turnover studies were performed after the stool collection. During these studies, which lasted for two weeks, serum lipids, lipoproteins, apolipoproteins A-I, A-II and B-100, and serum non-cholesterol sterols and squalene were analysed four times from fasting serum samples.

The baseline serum sterols and the fecal samples were frozen and analyzed together with the 2-year samples to avoid the interassay variation. The freezing procedure does not alter the sterol concentrations or the compositions; e.g. the interassay coefficient of variation for serum campesterol was 4.8 %. The lipoprotein assays were performed in fresh serum samples. The interassay coeffiecient of variation was e.g. for LDL cholesterol 6.1 %. Therefore, the laboratory procedures did not confound the obtained results.

4.2 Methods

4.2.1 Inclusion criteria measurements

The diagnosis of diabetes was confirmed by measuring blood glucose levels at least from two fasting blood samples. Serum total, HDL cholesterol, serum triglycerides, variables describing liver (alanine aminotransferase) and kidney functions (creatinine) and thyroid hormones (thyroxine) were also quantitated from these blood samples. LDL cholesterol was calculated by Friedewald formula (Friedewald et al. 1972). Specimens of urine were analysed with commercial dipstics.

The health status of the subjects participating in the studies was determined in a medical examination, which included also weight, height, waist-hip ratio, and blood pressure measurements.

4.2.2 Lipoprotein separation

Lipoproteins were separated from fasting blood samples by ultracentrifugation into density classes: 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, Lipid Research Clinics Program 1974). For this purpose, 2x3.6 ml of plasma was overlayered with a salt solution of density 1.006 g/ml and centrifuged in a Ti 50.4 rotor (Beckman Instruments) at 35 000 rpm for 18 hours. Lipoproteins of less than solvent density were concentrated in a layer at the top of the tubes, thus VLDL was isolated by aspiration of the top supernatant. A

salt solution of density 1.019 g/ml was added to the remaining infranate, centrifuged at same rpm for 18 hours, and IDL was isolated by aspiration. The LDL was isolated with salt solution of density 1.151 g/ml, centrifuged 35 000 rpm for 20 hours, aspirated, and then the remaining infranatant was isolated as HDL.

4.2.3 Lipids and apolipoproteins

Total and free cholesterol, triglycerides and phospholipids from serum and different lipoproteins were analyzed enzymatically with commercial kits (Boehringer Diagnostica, Mannheim, Germany; Wako Chemicals GmbH, Neuss, Germany). Apolipoproteins A-I, A-II and B were quantitated with commercial kits, in which an immunochemical assay is based on immunoprecipitation of antiserum reagent and reference sample (Orion Diagnostica, Espoo, Finland). Apolipoprotein E phenotypes (six different main phenotypes, E2/2, E2/3, E2/4, E3/3, E3/4, and E4/4) were determined electrophoretically by isoelectric focusing from serum (Havekes et al. 1987).

4.2.4 Lipoprotein kinetic studies

LDL and HDL turnover studies were performed in Study IV in order to elucidate the production and clearance of these lipoproteins.

50 ml of fasting plasma was drawn for these kinetic studies. Autologous LDL and HDL were separated by serial density preparative ultracentrifugations. LDL apo B was obtained by method described by Bilheimer et al. (1972), and apolipoprotein A-I was isolated from HDL (Gylling et al. 1992). Briefly; guanidine hydrochloride was added to HDL, solution was incubated, after which it was dialyzed four times against dialysis solution containing 150 mmol/l NaCl, 10mmol/l Tris-chloride, 0.01 % EDTA, pH 8.0. The HDL solution was then density-adjusted to 1.12 g/ml using NaBr, and ultracentrifugated. The protein content of the infranatant contained pure apo A-I, thus the bottom 1.5 ml of the sample was extracted and extensively dialysed aganist 150 mmol/l NaCl, 0,01 % EDTA, pH 7.4.

Apo A-I was iodinated with ^{125}I, and LDL apo B with ^{131}I by a modification of the iodine-monochloride method (McFarlane 1958, Bilheimer et al. 1972). Three days before injection the subjects started to take peroral potassium iodide to maximize the

safety of the thyroid gland. Approximately 1 mg of the labeled autologous LDL apo B and apo A-I were mixed with 5 % human serum albumin, filtered, and injected simultaneously. The total amount of radioactivity did not exceed 60 μCi.

After the injection, blood samples of 10 ml were collected for 14 days and counted. The die-away curves were constructed from plasma for ^{125}I - HDL and ^{131}I - LDL. FCR for LDL apo B and HDL apo A-I were determined using a two-pool model (Matthews 1957). This model assumes the existence of an intravascular pool in dynamic equilibrium with an extravascular pool. Accordingly, both new inputs and exits of apolipoproteins occur from the intravascular pool. Transport rate (TR) was calculated by multiplying FCR by the pool size. The pool size was the apolipoprotein plasma concentration multiplied by plasma volume, which was calculated to be 4.5% of body weight. FCR describes the rate at which lipoprotein is removed from the circulation, and it is expressed in pools/day. TR describes the production rate of the lipoprotein in the circulation, and it is expressed in mg/kg/d.

4.2.5 Analysis of cholesterol metabolism

4.2.5.1 Measurement of cholesterol absorption and elimination

The determination of cholesterol absorption was performed by using the continuous isotope feeding method described by Crouse and Grundy (1978). For this, the study subjects consumed a capsule containing 0.14 Ci of ^{14}C-cholesterol, 0.18 Ci of ^{3}H-sitostanol, and 200 mg of chromic oxide (Cr_2O_3) three times a day, one capsule with each of three major meals, during the 7-day period. The ratio of the isotopes in feces becomes constant after the first 3 days. Feces were collected during the last 3 days for the 7-day period. Cholesterol absorption efficiency was calculated from the difference of the isotope ratios (^{14}C -cholesterol/^{3}H-sitostanol ratio) between the capsule administered and the feces samples collected. Cr_2O_3 was applied to evaluate the fecal flow (Bolin et al. 1952). Fecal cholesterol as fecal neutral sterols (cholesterol, coprostanol and coprostanone) and bile acids, and plant sterols (and their coprostanols and coprostanones), were quantitated by gas-liquid chromatography (GLC) from non-saponifiable material as described earlier (Grundy et al. 1965, Miettinen et al. 1965, Miettinen 1982a), correcting fecal flow by Cr_2O_3 measurement. The measurement of fecal plant sterols indicate the amount of dietary plant sterol intake. Fecal analyses were carried out in studies I, II and IV. In order to

diminish the effect of varying body weight on variables of cholesterol metabolism, the values are standardized and expressed by mg/kg/d. The use of nonstandardized values (mg/d) are separately noted.

4.2.5.2 Determination of squalene and non-cholesterol sterols

Non-cholesterol sterols and squalene were quantitated by GLC from nonsaponifiable serum material on a 50 m long Ultra 1 ^{R} SE-30 capillary column (Hewlett-Packard, Delaware, Little Falls, Wilmington, USA) (Miettinen and Koivisto 1983). This procedure measures total cholesterol, cholesterol precursors: squalene, cholestenol, desmosterol and lathosterol, and plant sterols, campesterol and sitosterol, and cholestanol from serum. In this procedure, 100 µl 5α-cholestane, an internal standard, was added to 200 µl of serum samples. The samples were saponified with 99,5% of ethanol and potassium-hydroxide (10M) (9:1, volume:volume) solution. Non-saponified lipids were extracted by hexane. The lipid extracts were silylated, and sterol fractions were isolated and quantitated with GLC as trimethylsilyl derivates. The sterol composition of the VLED serving was also analysed with GLC.

Serum squalene and non-cholesterol sterols are mainly carried in lipoproteins similarly to cholesterol, and their concentrations are highly dependent on serum and lipoprotein cholesterol levels. To eliminate the effect of variation in serum and lipoprotein cholesterol levels, the squalene and non-cholesterol sterol values are expressed in terms of 10^2 x mmol/mol of cholesterol, i.e ratios to serum total and different lipoprotein cholesterol. When concentrations of compounds are described these are stated separately.

4.2.5.3 Calculations

The dietary intakes of cholesterol, fat and calories were calculated from the 7-day food record (Knuts et al. 1991).

Cholesterol synthesis was obtained as the difference between fecal steroids (neutral and acidic) of cholesterol origin and dietary cholesterol.
Total intestinal cholesterol flux (pool) was the fecal neutral sterols of cholesterol origin divided by (1-fractional cholesterol absorption).

Biliary cholesterol secretion (flux) was measured as total intestinal cholesterol flux minus dietary cholesterol.
The absorbed mass of total, dietary and biliary cholesterol was equal to the respective fluxes multiplied by cholesterol absorption efficiency.
Cholesterol turnover was obtained as the sum of cholesterol synthesis and dietary cholesterol absorbed.
All of the above values represent daily means and are expressed as mg/kg/d of body weight. In special circumstances they are also expressed as mg/d.

The ratios of lathosterol to campesterol and sitosterol were calculated by dividing the concentrations of lathosterol by those of campesterol and sitosterol. Those ratios were considered to indicate cholesterol synthesis (Study II).

4.2.6 Analysis of variables in glucose metabolism

Blood glucose was analyzed enzymatically with the hexokinase method, and HbA1c with high-pressure liquid chromatography. Serum insulin was assayed with radioimmunoassay (Phadeseph ® Insulin RIA, Pharmacia and Upjohn, Uppsala, Sweden) and serum SHBG with fluoro-immunoassay (Delfia ® SHBG, Wallac, Turku, Finland), using commercial kits.

The ratios of SHBG to insulin were calculated by dividing the SHBG concentration by the insulin concentration in order to evaluate any changes in variables describing glucose metabolism after weight reduction.

4.2.7 Statistical analyses

Statistical analysis of data were performed with Microsoft Excel version 6 and the Biomedical Data Processing Program (BMDP Statistical Software, Inc., Los Angeles, California, USA). Differences between the study groups for independent continuous variables were tested with two-tailed t-tests, and for dichotomous variables with Chi-square tests. The differences within the groups of repeated measures were tested with paired t-tests. With three or more independent groups of observations, testing was performed using one way analysis of variance (ANOVA). Correlations between the continuous variables were analysed by calculating Pearson's correlation coefficient or Spearman's rank correlation coefficient. Most variables were normally

distributed. In the case of skewed distributions, transformation to symmetric distribution was obtained by taking logarithms or non-parametric correlation coefficient was calculated.

A stepwise regression analysis was performed to examine the potential role of BMI, the variables of glucose metabolism and cholesterol absorption as independent explanatory variables in the synthesis of cholesterol in Study II. The analysis was carried out using cholesterol synthesis as the dependent variable and BMI, percentage of cholesterol absorption, serum SHBG, blood glucose, dietary cholesterol intake, and LDL cholesterol as the independent variables.

The appropriate sample size for the Study IV was calculated assuming the achieved weight reduction of 5 kg, α=0.05, and β=0.2, i.e., the sample size was expected to give an 80 % power to detect a weight reduction of 5 kg at the level $p < 0.05$.

The value $p < 0.05$ was considered as statistically significant. Values of the continuous variables were expressed as mean±SE.

5. RESULTS

Table 2. Serum and lipoprotein lipids in Studies I and II.

Variables	Study I		Study II	
	DM n=16 BMI>30	Controls n=16 BMI>30	DM n=20 BMI≤26.0	DM n=44 BMI>26.1
Serum cholesterol, Mmol/l	5.90±0.20	6.20±0.30	6.21±0.28	5.85±0.15
VLDL cholesterol, Mmol/l	1.40±0.20**	0.60±0.20	0.61±0.11*	0.96±0.12
LDL cholesterol, Mmol/l	3.20±0.20*	4.00±0.30	3.66±0.18	3.36±0.13
HDL cholesterol, Mmol/l	0.85±0.05***	1.25±0.07	1.26±0.07***	0.95±0.04
Serum triglycerides, Mmol/l	3.80±0.60*	1.90±0.40	1.74±0.20**	2.85±0.29
VLDL triglycerides, Mmol/l	3.10±0.50**	1.10±0.30	1.17±0.17**	2.17±0.25
LDL triglycerides, Mmol/l	0.31±0.02*	0.45±0.05	0.26±0.02	0.30±0.02
HDL triglycerides, Mmol/l	0.18±0.01	0.19±0.01	0.14±0.01*	0.17±0.01

Values are mean ± SE. *p<0.05, **p<0.01, ***p<0.001 for the difference between the groups in the individual studies.

5.1 Diabetes and cholesterol metabolism (Study I)

Since obesity, which frequently accompanies type 2 diabetes, is associated with abnormalities in cholesterol metabolism, it might augment the independent role of diabetes in regulating cholesterol metabolism. In order to evaluate the effects of type 2 diabetes on cholesterol metabolism, cholesterol absorption and sterol balance were determined for 16 obese type 2 DM subjects and 16 similarly obese, non-diabetic controls.

The two study groups were similar according to the demographic variables and apo E phenotype distribution, but the blood glucose level was higher in the DM group (Table 1). In addition serum total cholesterol levels were similar in the two groups (Table 2), and the dietary variables, fat intake, dietary plant sterols, and cholesterol intake did not differ between the study groups (Table 3). The presence or type of hypoglycemic medication had no effect on the different variables evaluated.

Serum and lipoprotein lipids

The lipoprotein metabolism of two study groups differed from each other such that serum total and VLDL triglycerides were higher and LDL triglycerides lower in DM than in controls (Table 2). Despite the similar serum total cholesterol levels, VLDL cholesterol was higher and LDL and HDL cholesterol were lower in DM than controls. The LDL cholesterol level tended to be higher in the non-diabetic controls than in diabetic subjects overall (Study I and II), and their HDL cholesterol and triglyceride levels in serum, VLDL and HDL were similar than in normal-weight diabetics of Study II (ns for all; Table 2).

Table 3. Dietary variables in Studies I and II.

Variables	**Study I**		**Study II**	
	DM n=16 BMI>30	Controls n=16 BMI>30	DM n=20 BMI≤26.0	DM n=44 BMI>26.1
Dietary cholesterol, mg/d	351±47	455±49	340±44	350±22
Dietary fat, g/d	92±9	101±9	86±7	94±5
Dietary calories, kcal/d	2078±141	2187±155	2117±129	2180±86
Plant sterol intake, mg/d *	356±31	352±34	426±26	403±52

Values are mean±SE.

- measured as fecal plant sterols

Cholesterol metabolism

The presence of type 2 diabetes modulated cholesterol metabolism such that cholesterol absorption percentage and the absorbed mass of dietary, total and biliary cholesterol were significantly lower in DM than in controls (Table 4). In the control group, cholesterol absorption efficiency and the absorbed mass of dietary, biliary and total cholesterol were similar to that seen in the normal-weight diabetics in Study II (ns for all; Table 4).

Cholesterol absorption efficiency and the total mass of cholesterol absorbed were significantly related to serum total and HDL cholesterol only in the controls (absorption % vs HDL; r=+0.7098, and mass vs HDL r=+0.7187, $p<0.01$ for both).

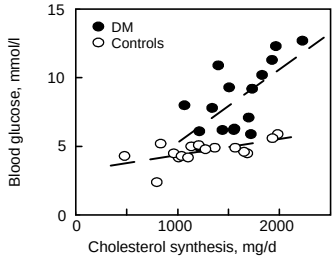


Figure 3. Correlation between blood glucose and cholesterol synthesis. Diabetes group: y=0.005x-0.042, r=0.663, p<0.01; Controls: y=0.001x+3.296, r=0.590, p<0.05

Cholesterol synthesis was higher (p<0.05), and cholesterol excretion as neutral and total steroids, bile acid synthesis and cholesterol turnover tended to be higher in diabetes than in controls (Table 4). In the control group, cholesterol synthesis, excretion as neutral and acidic sterols, cholesterol turnover and total intestinal cholesterol secretion were similar than in diabetics without obesity in Study II (ns for all; Table 4). Blood glucose was significantly related to fecal neutral sterols in both study groups (diabetes; r= +0.501, controls; r=+0.551, p<0.05, for both), and cholesterol synthesis (Figure 3).

Table 4. Cholesterol metabolism in Studies I and II.

Variables	**Study I**		**Study II**	
	DM n=16 BMI>30	Controls n=16 BMI>30	DM n=20 BMI≤26.0	DM n=44 BMI>26.1
Cholesterol absorption, %	29.5 ± 1.3***	41.7 ± 2.3	40.2 ± 2.4**	32.4 ± 1.4
Total cholesterol absorbed, mg/kg/d	5.89 ± 0.48**	8.61 ± 0.65	7.82 ± 0.58*	6.34 ± 0.37
Dietary cholesterol absorbed, mg/kg/d	1.09 ± 0.14**	1.95 ± 0.23	1.90 ± 0.22**	1.24 ± 0.09
Biliary cholesterol absorbed, mg/kg/d	4.80 ± 0.42*	6.66 ± 0.67	5.92 ± 0.47	5.09 ± 0.32
Fecal bile acids, mg/kg/d	7.00±0.64	6.53 ± 0.90	6.26 ± 0.45	7.45 ± 0.54
Fecal neutral sterols †, mg/kg/d	13.96 ± 0.87	11.88 ± 0.63	11.48 ± 0.62*	13.40 ± 0.64
Total fecal steroids †, mg/kg/d	20.96± 1.12	18.40 ± 1.30	17.74 ± 0.75*	20.86 ± 0.98
Biliary cholesterol secretion, mg/kg/d	16.08 ± 1.06	15.81 ± 1.11	14.61 ± 0.71	15.93 ± 0.75
Total intestinal cholesterol pool, mg/kg/d	19.85 ± 1.23	20.49 ± 0.92	19.32 ± 0.82	19.75 ± 0.80
Cholesterol synthesis, mg/kg/d	17.25 ± 0.93*	13.73 ± 1.50	13.04 ± 0.76**	17.02 ± 0.93
Cholesterol turnover, mg/kg/d	18.33 ± 0.96	15.68 ± 1.38	14.96 ± 0.66**	18.28 ± 0.92

Values are mean±SE. *p<0.05, **p<0.01, ***p<0.001 for the difference between the groups in the individual studies.
† of cholesterol origin

Taken together, cholesterol absorption was lower and cholesterol synthesis was higher in obese subjects with diabetes than in those without diabetes, suggesting that diabetes modulates cholesterol metabolism more than would be the case if the subjects were merely obese.

5.2 Body weight and cholesterol metabolism (Study II)

Since obesity is known to exacerbate the abnormalities of lipoprotein metabolism in type 2 diabetes, it might modulate cholesterol metabolism in diabetes as well. To evaluate whether weight interferes with cholesterol metabolism, cholesterol absorption and cholesterol and bile acid synthesis were studied in 64 type 2 diabetic subjects with different weights, ranging from lean to overweight values. The study population was divided by BMI into normal-weight and overweight subgroups. The distributions of age, gender, blood glucose (Table 1), blood pressure and medication did not differ between the two study groups, and the dietary intakes of plant sterols, cholesterol, fat and calories were also similar (Table 3). In addition, age and HbA1c were not related with any of the variables of cholesterol metabolism.

Despite the similar blood glucose levels, the serum level of SHBG was significantly higher, and the serum insulin level lower in the normal-weight than in the overweight group ($p<0.001$, for both) (Table 5). The ratio of serum SHBG to insulin was four times higher in the normal-weight than in the overweight group.

Table 5. Variables of glucose metabolism in Studies II, III and IV. In Study III, variables are at baseline and during weight reduction. In Study IV, variables are at baseline and 2 years after weight reduction (follow-up).

Variables	**Study II**		**Study III**		**Study IV**	
	DM (n=20)	***DM (n=44)***	***DM (n=10)***		***DM (n=16)***	
	BMI≤26.0	BMI>26.1	BMI>30 Baseline	During weight loss	BMI>30 Baseline	After follow-up
Blood glucose, mmol/l	8.5±0.5	8.6±0.4	6.9±0.5	5.3±0.4***	8.4±0.6	7.2±0.5**
SHBG, nmol/l	45.6±5.1	30.9±2.2***	24.7±4.7	45.4±5.8***	24.7±4.7	26.6±2.4
Serum insulin, mU/l	10.2±1.0	20.0±1.4***	18.0±1.7	7.3±0.8***	19.7±1.2	15.1±1.7
SHBG / serum insulin	6.24±1.75	1.44±0.19***	1.26±0.19	8.54±3.10*	1.27±0.18	2.11±0.35*

Values are mean±SE. *p<0.05, **p<0.01, ***p<0.001 for difference between the groups in Study II, baseline and during weight loss in Study III, and baseline and after follow-up in Study IV.

Serum and lipoprotein lipids and sterols

Serum total and LDL cholesterol were similar in the two study groups, but the VLDL cholesterol was higher, and the HDL cholesterol lower in the overweight than in the normal-weight group (Table 2). Serum total, VLDL and HDL triglycerides were

significantly lower in the normal-weight group vs overweight group. HDL and VLDL cholesterol and serum and lipoprotein triglyceride levels were similar in the normal-weight DM group compared to the respective values in the obese subjects without diabetes in Study I (ns for all; Table 2).

The serum campesterol, sitosterol and cholestanol ratios were markedly higher, whereas squalene and the precursor sterol ratios (except lathosterol) were lower in the normal-weight than in the overweight group (Table 6). Lathosterol/campesterol (1.02±0.14 vs 1.47±0.11 p<0.05) and lathosterol/sitosterol ratios were lower in the normal-weight than overweight subjects.

Table 6. Serum squalene and non-cholesterol sterol ratios to cholesterol (10^2 x mmol/mol of cholesterol) in Studies II, III and IV. In Study III, variables are at baseline and during weight reduction. In Study IV, variables are at baseline and 2 years after weight reduction (follow-up).

Variables	**Study II**		**Study III**		**Study IV**	
	DM (n=20)	***DM (n=44)***	***DM (n=9)***		***DM (n=10)***	
	BMI≤26.0	BMI>26.1	BMI>30 Baseline	During weight loss	BMI>30 Baseline	After follow-up
Cholestanol	108.0±4.6	89.1±3.8**	95.6±4.1	125.1±5.6***	85.0±4.0	95.0±5.0
Campesterol	223.5±17.3	182.9±10.7	154.5±15.7	126.3±11.5	162.0±14.0	197.0±14.0*
Sitosterol	121.9±8.6	95.9±4.6**	84.1±9.2	79.2±7.4	87.5±5.0	103.0±8.0*
Squalene	33.4±2.6	42.2±2.9*	47.4±3.2	58.7±13.2	nd	nd
Cholestenol	18.9±1.8	28.7±1.7***	20.4±3.0	15.7±1.2	24.1±1.7	22.3±1.2
Desmosterol	84.7±4.8	116.2±12.2*	82.4±4.7	66.3±4.5***	136.0±29.0	109.0±15.0
Lathosterol	195.5±17.8	233.7±12.0	207.8±14.9	159.2±10.4*	226.0±8.0	218.0±10.0

Values are mean±SE. *p<0.05, **p<0.01, ***p<0.001 for the difference between groups in Study II, baseline and during weight loss in Study III, and baseline and follow-up in Study IV. nd =not determined.

Cholesterol metabolism

Cholesterol absorption efficiency was higher (p<0.01) in the normal-weight subjects compared to the overweight subjects (Table 4, Figure 4). The amounts of absorbed total and dietary cholesterol were higher in normal-weight subjects, but the mass of absorbed biliary cholesterol showed no significant difference between the groups. The variables of cholesterol absorption in the normal-weight diabetes group were similar to the respective values in obese subjects without diabetes in Study I (ns for all; Table 4).

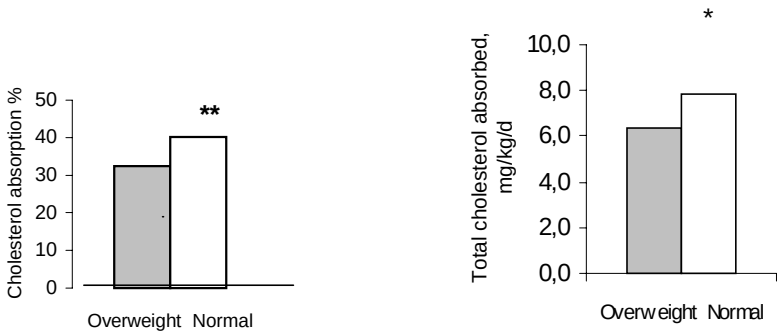


Figure 4. Cholesterol absorption efficiency (%) and the amount of total cholesterol absorbed in overweight and normal-weight type 2 diabetic subjects. *p<0.05, **p<0.01.

Cholesterol synthesis, fecal neutral sterol and total steroid output, and cholesterol turnover were significantly higher in overweight than normal-weight diabetic subjects (Table 4). In addition, when expressed without weight stardardization, also fecal bile acids were significantly higher in the overweight than normal-weight subjects (Figure 5). Biliary cholesterol secretion (overweight; 15.9±0.8 vs. normal 14.6 ±0.7 mg/kg/d; ns) only tended to be higher in the overweight subjects. No difference existed in fecal plant sterols between the groups (normal-weight 5.6±0.7 vs. overweight 4.7±0.3 mg/kg/d; ns) suggesting that the dietary plant sterol intake was similar in the groups. Synthesis and excretion of cholesterol were similar in the normal-weight diabetic and obese non-diabetic subjects in Study I (ns for both; Table 4). In the stepwise multiple regression analysis, the cholesterol absorption percentage explained 24 % of the variability of cholesterol synthesis, and body mass index accounted for 15% of the variability, and these were the only variables explaining the variation in cholesterol synthesis.

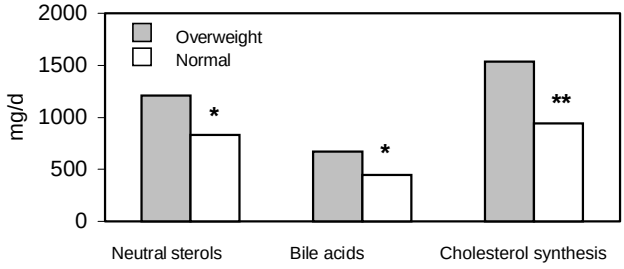


Figure 5. Fecal neutral sterols, bile acids and cholesterol synthesis in overweight and normal-weight type 2 diabetic subjects. *p<0.01, **p<0.001

Correlations

The correlations between BMI and variables of glucose and cholesterol metabolism were determined in the total study population of diabetes (n=64). Cholesterol absorption efficiency was negatively associated with fecal neutral sterol excretion and cholesterol synthesis, and positively with serum cholestanol and sitosterol ratios (Table 7). The serum lathosterol ratio correlated with cholesterol synthesis (r=+0.347, p<0.01), and fecal neutral sterols and bile acids. Cholesterol absorption did not correlate with serum cholesterol level. BMI correlated positively with serum lathosterol ratio, fecal neutral and acidic steroids and cholesterol synthesis (Figure 6), and negatively with serum sitosterol (Figure 6) and cholestanol ratio, but insignificantly with cholesterol absorption percentage (r=-0.137, ns). In addition, BMI was positively related to serum insulin and negatively to SHBG levels (serum insulin; r=+0.470, SHBG; r=-0.469, p< 0.001, for both), but insignificantly to blood glucose.

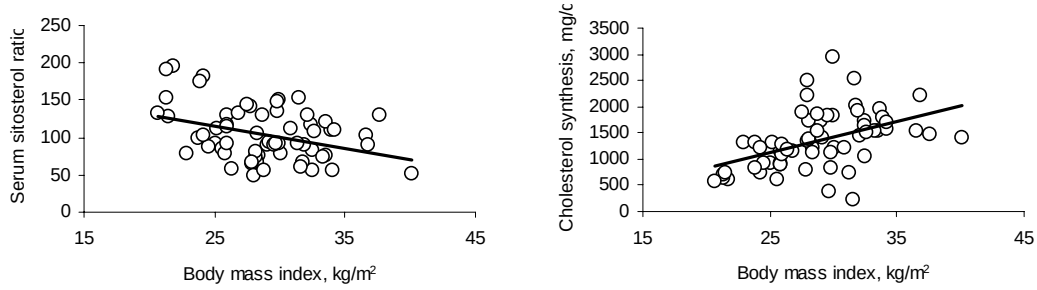
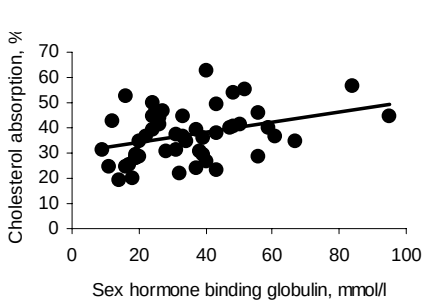


Figure 6. Correlations between body mass index (BMI) and serum sitosterol ratio, and cholesterol synthesis in type 2 diabetes (n=64). Cholesterol synthesis: y= 60.45x-394.09, r=0.476, p<0.001, Serum sitosterol ratio: y= -2.917x+189.33, r= -0.358, p<0.01. Ratio = 10^2 x mmol/mol of cholesterol

Blood glucose was associated with fecal bile acids and cholesterol synthesis, whereas serum insulin was associated with fecal neutral sterols and inversely with serum sitostanol ratio. On the contrary, serum SHBG was inversely related to fecal neutral and acidic steroids and cholesterol synthesis (Figure 7), but positively with cholesterol absorption percentage (Figure 7) and serum sitosterol and cholestanol ratios. Blood glucose, serum insulin, and SHBG were not interrelated. Dietary cholesterol was not associated with any of the variables.



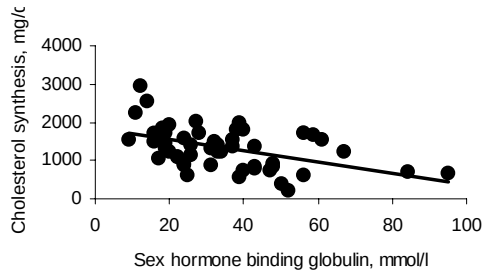


Figure 7. Correlations between sex hormone binding globulin and cholesterol absorption and synthesis in type 2 diabetes (n=64). Cholesterol absorption: y=0.208x+29.32, r=+0.369, p<0.01. Cholesterol synthesis: y=-14.52x+1829.9, r= -0.481, p<0.001.

Table 7. Correlation coefficients between variables of cholesterol and glucose metabolism in type 2 diabetes (Study II) (n=64)

Variables	BMI (kg/m^2)	Cholesterol absorption, %	Cholesterol Synthesis	Blood Glucose	Serum Insulin	Serum SHBG (n=50)
Serum cholesterol, mmol	-0.143	0.007	-0.066	0.136	-0.120	-0.133
LDL cholesterol, mmol/l	-0.165	0.073	-0.091	0.094	-0.024	-0.233
Serum triglycerides, mmol	0.360 **	-0.211	0.199	0.101	0.297 *	-0.346 **
Variables of cholesterol absorption						
Cholesterol absorption (%)	-0.137	1.000	-0.494 ***	-0.069	-0.092	0.369 **
Serum cholestanol [1]	-0.291 *	0.455 ***	-0.450 ***	-0.152	-0.011	0.387 **
Serum sitosterol [1]	-0.358 **	0.565 ***	-0.382 **	-0.032	-0.289 *	0.436 ***
Variables of cholesterol synthesis						
Serum lathosterol[1]	0.265 *	-0.089	0.347 **	0.103	0.297 *	-0.178
Fecal bile acids, mg/d	0.368 **	-0.183	0.811 ***	0.391 **	0.066	-0.431 ***
Fecal neutral sterols, mg/d	0.383 **	-0.550 ***	0.819 ***	0.216	0.282 *	-0.398 **
Cholesterol synthesis, mg/d	0.476 ***	-0.494 ***	1.000	0.303 *	0.222	-0.481 ***

*p<0.05, **p<0.01, ***p<0.001
[1] 10^2x mmol/mol of cholesterol

Taken together, the cholesterol absorption and serum cholestanol and plant sterol ratios were lower, and cholesterol synthesis, excretion and cholesterol precursors were higher in overweight than normal-weight type 2 diabetic subjects. Cholesterol absorption and synthesis were related to variables of glucose metabolism and insulin resistance, but cholesterol absorption and BMI were the only parameters explaining the variability in cholesterol synthesis. Accordingly, cholesterol and glucose metabolism are interrelated, and body weight regulates cholesterol metabolism in type 2 diabetes. In addition, low levels of cholesterol absorption and a high synthesis rate may be part of the insulin resistance syndrome.

5.3 Treatment with weight reduction (Study III and IV)

Weight reduction is the primary treatment for obese type 2 diabetic subjects, because of its beneficial effects for improving the metabolic abnormalities in diabetes. The changes in glycemic control and lipoprotein metabolism occur rather rapidly, even with energy restriction, and these confer long-term benefits to the patients. The effects of weight reduction on cholesterol and sterol metabolism are mainly undocumented in type 2 diabetes. In order to clarify the role of weight reduction in modulating cholesterol metabolism in type 2 diabetes, Study III was performed to determine cholesterol and sterol metabolism during weight reduction in a non-steady state situation, and Study IV investigated the changes in cholesterol metabolism 2 years after weight reduction on a stable eucaloric and reduced weight level.

5.3.1 Serum non-cholesterol sterols and squalene during weight reduction (Study III)

This study was perfomed to determine the effects of chronic caloric restriction and lack of dietary plant sterols on cholesterol and sterol metabolism by measuring serum squalene and non-cholesterol sterol levels before and after a 3-month extensive weight reduction obtained with very low energy diet (VLED) in 10 obese type 2 diabetic patients. One major interest was the elucidation of changes in cholesterol and sterol metabolism during weight reduction in a non-steady state situation.

At the beginning of the study, all subjects were markedly overweight (Table 8). Serum insulin and SHBG levels were within normal limits (reference values, hospital laboratory:2-20mU/l and 10-42 nmol/l). However, the serum insulin level was higher and serum SHBG and its ratio to insulin were markedly lower than in diabetic subjects without obesity ($p<0.001$ for all) (Table 5). Elevated serum total cholesterol and triglyceride levels with low HDL cholesterol provided a typical finding in type 2 diabetes (Table 8). Serum sitosterol and campesterol ratios were significantly lower and squalene ratios higher than in normal-weight diabetic subjects in Study II ($p<0.05$-0.01, for all; Table 6). In addition, body weight and the desmosterol ratio were significantly related ($r=0.680$, $p <0.05$).

Weight reduction

Dietary intake

VLED was well-tolerated. The daily intake contained about 19 % of the prefasting dietary calories. The diet contained only minimal amounts of sterols, because analysis of 15 servings with GLC revealed that the daily intake of cholesterol was 11.4±2.0 mg/d, campesterol 4.1±0.3 mg/d, sitosterol 11.3±0.8 mg/d, and stigmasterol 3.7±0.3 mg/d. The dietary intakes of lathosterol, cholestanol, campestanol, sitostanol, avenasterol and squalene were all < 1 mg/d. Thus, the dietary intake of cholesterol was about 3 % and plant sterols, campesterol and sitosterol, 4% of the prefasting values measured in Study IV.

Body weight and glucose metabolism

Body weight was reduced by 15.5 ± 1.7 kg, (range 8.5-25 kg), the reduction being 16.8±1.5% from the baseline weight (Table 8). Despite the effective weight reduction, several subjects were still overweight, their mean BMI was 26.3±0.7 kg/m^2. Blood glucose was lowered by 23 %, HbA1c by 8%, and serum insulin by 59%. Serum SHBG increased by 108%, and its ratio to blood glucose and serum insulin were increased (Table 5). All of these changes were highly significant (p<0.05-0.001, for all).

Serum lipids and lipoproteins

Serum total cholesterol was reduced by 21 % and triglycerides by 45 % from the baseline values (p< 0.001, for both; Table 8). The HDL cholesterol level remained unchanged, but its ratio to serum total cholesterol was increased by 34 % (p<0.001).

Table 8. Weight, BMI, variables of glucose metabolism and serum and lipoprotein lipids at baseline and during weight reduction in type 2 diabetes (n=10)

Variables	Baseline	During weight reduction
Weight, kg	92.3 ± 4.8	76.7 ± 4.2 *
BMI, kg/m^2	31.7 ± 0.9	26.3 ± 0.7 *
Blood glucose, mmol/l	6.9 ± 0.5	5.3 ± 0.4 *
SHBG, nmol/l	24.7 ± 4.7	45.4 ± 5.8 *
Serum insulin, mU/l	18.0 ± 1.7	7.3 ± 0.8 *
SHBG / serum insulin	1.26 ± 0.19	8.54 ± 3.10 #
Serum cholesterol , mmol/l	5.83 ± 0.24	4.52 ± 0.13 *
Serum triglycerides, mmol/l	2.60 ± 0.26	1.40 ± 0.17 *
HDL cholesterol, mmol/l	0.96 ± 0.08	0.99 ± 0.07

Values are mean±SE. # p < 0.05 , * p < 0.001 by paired t-test.
Reference values from hospital laboratory: serum insulin 2-20 mU/l, SHBG 10-42 nmol/l.

Squalene and non-cholesterol sterols

The desmosterol and lathosterol ratios to cholesterol decreased significantly, whereas the ratio of serum squalene increased by 24 % (ns) (Table 6). The serum cholestanol concentration tended to increase (203.4±14.7 μg/dl vs. 209.4±15.5 μg/dl, ns), whereas the decrease in campesterol and sitosterol concentrations was only significant for campesterol (330.3±39.6 μg/dl vs. 208.2±18.2 μg/dl, $p < 0.01$). As measured as ratios to cholesterol, the increase in cholestanol ratio was highly significant ($p < 0.001$), while the ratios of campesterol and sitosterol only tended to decrease (-12% and –4 %, ns for both) (Table 6).

The cholestanol ratio was markedly higher after the weight reduction than the respective ratio in diabetic subjects with normal weights (Table 6). In addition, cholestenol, desmosterol and lathosterol ratios were lower after weight loss in these still overweight subjects compared to the normal-weight diabetic subjects.

Correlations

The change in body weight tended to be positively associated with that of the desmosterol ratio ($r=0.630$, $p=0.06$) and inversely with final the SHBG level ($r=-0.776$, $p < 0.01$). A significant inverse association existed between the change in serum sitosterol and the baseline ($r=-0.752$) and final ($r=-0.742$, $p< 0.05$, for both) body weight. In addition, the ratios of serum lathosterol and cholestanol were interrelated ($r=-0.823$, $p<0.01$) after the weight reduction, and the change in serum cholestanol correlated inversely with the final serum insulin level ($r=-0.689$, $p<0.05$).

Taken together, the restriction of calories and dietary sterols improved markedly the control of diabetes. It also decreased serum cholesterol precursor sterols and increased serum cholestanol suggesting that cholesterol absorption was increased and synthesis decreased. Serum values of plant sterols only tended to decrease despite their minimal dietary intake. Accordingly, cholesterol metabolism is improved during weight reduction in a non-steady state situation, and the changes in glucose and cholesterol metabolism are interrelated.

5.3.2 Cholesterol, glucose and lipoprotein metabolism after treatment with weight reduction (Study IV)

This study examined the effects of weight reduction on glucose, cholesterol and lipoprotein metabolism after a 2-year follow-up in type 2 diabetes. Cholesterol absorption efficiency was measured with peroral dual isotopes and cholesterol synthesis with sterol balance. The 3-month weight reduction was obtained by very-low-energy diet (VLED; n=10 diabetic subjects) or by low-energy diet (LED; n=6 diabetic subjects). Age, sex, BMI, serum lipids, serum sterols and variables of cholesterol metabolism were similar in these two groups at the baseline. The presence or type of hypoglycemic therapy had no effect on the distribution of BMI, serum glucose, SHBG, serum sterols, and variables of cholesterol metabolism. There was no significant difference in serum lipids and metabolic variables between women and men, and the 16 subjects were analyzed as an aggregate.

At the beginning of the study, the subjects were markedly overweight (Table 9). The subjects had a similar dyslipidemic lipoprotein profile and serum levels of insulin and SHBG as in Study III. Blood glucose was related to bile acid and cholesterol synthesis, and to serum insulin concentration ($r=0.558$, $p<0.05$). The serum insulin level also correlated with fecal neutral sterols and cholesterol synthesis. Serum SHBG was positively associated with body weight and the cholesterol absorption efficiency ($r=+0.575$, $p<0.05$) but not with blood glucose or serum insulin.

Two-year follow-up

All subjects completed the study without side effects, and VLED was well tolerated. After the two year follow up, the mean body weight was 6±1 kg (6.4 %) lower than at baseline (Table 9). The changes in body weight ranged from –18 kg to +1 kg. Most of the subjects were still characterized as obese, because the mean BMI was 30.3±0.8 kg/m^2. The daily intakes of energy, cholesterol, and plant sterols, as expressed by mg/kg/day, were similar, but the fat intake was significantly lower than at the baseline.

Table 9. Weight, BMI and cholesterol metabolism at baseline and 2 years after weight reduction (follow-up) in obese patients with type 2 diabetes (n=16)

Variables	Baseline	After follow-up
Weight, (kg)	93.2 ± 3.7	87.2±3.2 *
BMI, (kg/m^2)	31.7 ± 0.6	30.3 ± 0.8 *
Blood glucose, mmol/l	8.4 ± 0.6	7.2 ± 0.5 **
SHBG, nmol/l	24.7 ± 4.7	26.6 ± 2.4
Serum insulin, mU/l	19.7 ± 1.2	15.1 ± 1.7
SHBG / serum insulin	1.27 ± 0.18	2.11 ± 0.35 *
Cholesterol absorption, %	29.5 ± 1.3	37.6 ±2.1 **
Total cholesterol absorbed, mg/kg/d	5.9 ± 0.5	7.3 ± 0.6 *
Cholesterol synthesis, mg/kg/d	17.3 ± 0.9	17.1 ± 1.4
Fecal bile acids, mg/kg/d	7.0 ± 0.6	7.8 ± 0.9
Fecal neutral sterols, mg/kg/d	14.0 ± 0.9	12.5 ± 1.0 *

Values are mean±SE. *p<0.05, **p<0.01.

The blood glucose level was reduced by 14 %, serum insulin decreased slightly, and SHBG concentrations tended to increase (Table 9). The ratio of serum SHBG to insulin increased by 66 % (Table 9).

Free and esterified cholesterol and phospholipids were practically unchanged in serum and lipoproteins. However, triglyceride levels in serum and VLDL, LDL and HDL decreased significantly (e.g. serum; 3.79±0.56 vs 2.64±0.36, VLDL; 3.08±0.53 vs 2.00±0.32, p<0.05 for both).

Even though the serum and LDL cholesterol levels were unchanged, the FCR for LDL apo B decreased significantly (baseline; 0.331±0.016 vs follow-up; 0.302±0.018 pools/d, p<0.05), whereas the transport rates for LDL apo B remained unchanged. The kinetics for HDL apo A-I did not change significantly.

Cholesterol metabolism

The cholesterol absorption efficiency was increased by 28 %, and the total amount of cholesterol absorbed was increased by 23 % (Table 9). These increased values were similar to those of diabetic subjects without obesity (cholesterol absorption % after follow-up; 37.6±2.1 vs normal-weight diabetes; 40.2±2.4, ns). The serum sitosterol and campesterol ratios increased significantly, whereas the increase in serum cholestanol ratio only tended to be significant (Table 6).

The serum SHBG was positively related to the ratios of sitosterol ($r=+0.779$, $p<0.01$), campesterol ($r=+0.669$) and cholestanol ($r=+0.516$, $p<0.05$ for both). The reduction of body weight was associated with the changes in campesterol ($r=-0.582$) and sitosterol ratios ($r=-0.647$, $p<0.05$ for both).

The synthesis of bile acids and cholesterol remained practically unchanged despite the significant decrease in fecal neutral sterol excretion (Table 9). The ratios of cholesterol precursor sterols, cholestenol, desmosterol and lathosterol, only tended to decrease (Table 6). The significant baseline correlations between serum insulin, blood glucose, and variables of cholesterol synthesis and excretion were not significant after the follow-up.

Therefore, cholesterol absorption (i.e. absolute and relative absorption and the serum ratios of plant sterols and cholestanol) markedly increased after weight reduction suggesting that weight reduction tended to normalize cholesterol metabolism in type 2 diabetes. Hyperinsulinemia and insulin resistance also improved after weight reduction. Variables reflecting insulin resistance were related to variables reflecting cholesterol absorption after weight reduction. Thus, the cholesterol absorption efficiency and insulin resistance are interrelated, and the efficacy of cholesterol absorption seems to be an inverse indicator of insulin resistance.

6. DISCUSSION

6.1 Study population

The study population provided a homogenous and large-enough population to elaborate cholesterol metabolism in type 2 diabetes. The mean age of the subjects was 56 years varying from 41 to 74 years. All women participating in the studies were postmenopausal and did not use any hormone replacement therapy. The variables of cholesterol, glucose and lipoprotein metabolism did not differ between the genders, and therefore male and female subjects in the individual studies could be analyzed together. Gender is known to affect lipoprotein metabolism e.g. women have generally higher HDL cholesterol levels as compared to men. However, HDL cholesterol levels did not differ between women and men in the present study, in which the number of women participants was quite small.

Diabetes had been recently diagnosed, and blood glucose levels were comparable among the diabetic patients. None of the subjects were receiving insulin therapy or using any lipid-lowering medication. The patients with diabetes did not have any detectable diabetic nephro- or neuropathy, and they had normal hepatic, pancreatic, thyroid and intestinal functions. Therefore, the subjects were in good glycemic control and did not suffer any diabetic complications. The presence or type of hypoglycemic medication did not affect the variables of cholesterol and glucose metabolism. In addition, the use of hypotensive treatment was not a confounding factor.

The dietary variables, which could be assumed to affect cholesterol metabolism, i.e., cholesterol and fat intake, type of fat and dietary plant sterols, including stanols, were similar in the different studies. In addition, the study groups were well comparable with regard to serum and LDL cholesterol levels and apo E phenotype distribution suggesting that there were no obvious confounding factors. In the case-control study I, the subjects with and without diabetes were similarly obese and differed from each other only in their blood glucose level.

Cholesterol metabolism was compared between the different studies (e.g Study I and II), even though it was not originally in the protocol. Since the factors possibly

affecting cholesterol metabolism (e.g. demographic and dietary variables and apo E phenotype distribution) did not differ between the two studies, except for age, these comparisons could be performed. In addition, age did not correlate with any variables of diet and cholesterol metabolism, suggesting that it was not a confounding factor.

6.2 Measurement of cholesterol metabolism

The measurement of cholesterol absorption requires differentiation of endogenous from exogenous steroids, which can be achieved by using labeled cholesterol administered both orally and intravenously. The oral dose labels exogenous cholesterol and its steroid products, whereas the intravenous dose labels endogenous steroids. With or without simultaneous measurement of cholesterol excretion, the absorption of dietary cholesterol can be calculated after a leveling period. Several methods have been developed for the determination of dietary cholesterol absorption, and these methods are described in detail in the review of the literature section on pages 35-37.

In the present study, the absorption of cholesterol was measured with the continuous isotope feeding method, which is based on peroral administration of labeled cholesterol and an unabsorbable reference compound. A reduction of the labeled cholesterol/marker ratio in stools reveals the absorption percent of cholesterol. The measurement of cholesterol absorption with this method gives an accurate value of the absorption because the study period is long enough to achieve steady state and constancy of ratio of the isotopes in feces, and sequential fecal samples diminish the fluctuation in the absorption. In addition, other advantages include the simple administration of isotope, analysis of fecal samples is easy, though laborious, the analysis is possible to be repeated daily, and it is suitable for outpatients. Furthermore, the administration of the isotopes in capsules with the regular meals of the study subjects gives the accurate absorption of cholesterol during normal home conditions in everyday life, thus preventing the possible confounding effect of a special test meal. The possible recirculation of absorbed isotopic cholesterol into fecal samples is not a confounding factor, since the fecal isotope ratio remains constant for 10 days after administration of the isotope, and the percent absorption obtained with the present method compared with that of other methods is similar up

to 14 days after isotope administration. Moreover, the amount of cholesterol absorbed and the intestinal influx of endogenous cholesterol can be calculated, if the daily intake of cholesterol and fecal neutral sterol excretion are also measured.

A more simplified way to measure cholesterol absorption is the plasma isotope ratio method, which is based on the simultaneous oral and intravenous administration of labeled cholesterol. Although this method has provided considerable information about cholesterol absorption, it has several drawbacks. It estimates only the cholesterol absorption percentage, the value is determined from only a short period of time, it yields only a single measurement of absorption, and the value of cholesterol absorption may be dependent on the composition of the test meal. In addition, as the method is based on the determination of the ratio of the two isotopes in plasma after days of equilibration in the rapidly miscible pool of body cholesterol, the applicability of this method in subjects with different metabolic conditions or in malabsorption syndromes is uncertain; i.e. in conditions where cholesterol synthesis and elimination is enhanced, the metabolic fate of intravenously administered cholesterol tracers is largely unknown. In these conditions the turnover of the rapidly miscible cholesterol pool is faster and thus invalidates the complete equilibration of the two tracers, resulting bias in the estimation of the cholesterol absorption percentage.

The measurement of serum squalene and non-cholesterol sterol ratios to cholesterol from serum samples by gas-liquid chromatography provides an efficient, easily reproducible and less laborious method to estimate cholesterol absorption and synthesis. The sterol balance technique, in which assaying total-body sterol pool in a steady state by calculating the difference between fecal steroids of cholesterol origin and dietary cholesterol intake, provides a direct and accurate method to determine the endogenous biosynthesis of cholesterol.

In the present study, serum plant sterol and cholestanol ratios correlated with the cholesterol absorption efficiency, and the ratios of serum cholesterol precursor sterols with cholesterol synthesis. Accordingly, serum non-cholesterol sterols reflect cholesterol metabolism in a similar manner to the non-diabetic population (Miettinen 1970, Miettinen and Koivisto 1983, Tilvis and Miettinen 1986, Vuoristo and Miettinen

1986, Kempen et al. 1988, Miettinen et al. 1989, Miettinen et al. 1990, Miettinen and Gylling 2000), and can be used in the evaluation of cholesterol absorption and synthesis. In addition, serum squalene was higher in subjects with elevated cholesterol synthesis compared with those of lower synthesis suggesting that in this diabetic population, serum squalene was also a marker of cholesterol synthesis rate.

6.3 Cholesterol metabolism in diabetes (Study I and II)

6.3.1 Cholesterol absorption

Low cholesterol absorption has been described earlier in a limited number of type 2 diabetes patients with overweight and hypercholesterolemia (Gylling and Miettinen 1997), or those with hypertriglyceridemia (Briones et al. 1986), and in obesity (Miettinen and Gylling 2000). One of the most interesting observations in the present study was the lower cholesterol absorption in obese type 2 diabetes than in normal-weight type 2 diabetes, or when compared with the respective similarly obese, non-diabetic state. In addition, absorption of cholesterol was similar in normal-weight type 2 diabetic patients and obese non-diabetic patients. These results suggest that diabetes with some unknown mechanism inhibits the absorption of cholesterol. In addition, obesity in diabetes still attenuates the efficiency of sterol absorption.

Diet

The effect of dietary cholesterol on cholesterol absorption efficiency is controversial according to earlier results. It may remain mainly unaltered despite high amounts of dietary cholesterol (McMurry et al. 1985, Miettinen and Kesäniemi 1989, Sehayek et al. 1998a) or decreased (McNamara et al. 1987, Gylling and Miettinen 1992, Vuoristo and Miettinen 1994). In some studies, cholesterol intake was not significantly related to cholesterol absorption efficiency (Miettinen and Kesäniemi 1989, Bosner et al. 1999). More recent studies have shown that with a modest increase in dietary cholesterol, there was a decline in the cholesterol absorption efficiency (Ostlund et al. 1999). Along these lines, the absorption percent in the present study might have been even higher in the control group, and the difference in cholesterol absorption efficiency between the groups even larger. However, the dietary cholesterol intake was not associated with the variables of cholesterol, lipoprotein or glucose metabolism. Moreover, the dietary cholesterol intake and cholesterol absorption

efficiency were not interrelated, if anything, the relationship was negative. Therefore, it could be argued that the dietary cholesterol intake was not a confounding factor in the present studies.

Extra amounts of dietary fiber added to the regular diet lower the cholesterol absorption percentage in normal (Salvioli et al. 1985) and hypercholesterolemic subjects (Everson et al. 1992). However, the association between fiber intake and the absorption percentage of cholesterol remained negative in subjects on their normal diet (Miettinen and Kesäniemi 1989, Kesäniemi et al. 1990, Bosner et al. 1999). In the present study, the subjects consumed their normal, regular home diet without extra fiber intake, and did not consume any additional products of gel forming or viscous fibers.

Plant sterols and their stanol esters are known to reduce cholesterol absorption (e.g. Grundy et al. 1969, Mattson et al. 1982, Vanhanen et al. 1993, Vanhanen et al. 1994). Fecal plant sterols were similar in all study groups suggesting they had a similar dietary plant sterol intake. In addition, none of the subjects consumed functional foods consisting of plant stanols and this was confirmed by analysing their levels of fecal stanols.

Apolipoprotein E

Apo E 2/3/4 phenotype is closely associated with intestinal absorption efficiency of cholesterol (Kesäniemi et al. 1987, Miettinen et al. 1992), and the polymorphism of apo E may be one reason for the high interindividual variabily in cholesterol absorption (Miettinen and Kesäniemi 1989). However, the similar distribution of apo E phenotypes between the study groups suggests that the results obtained in cholesterol metabolism were not affected by the apo E phenotype.

Intestinal motility

Gastrointestinal motility disorders are common in patients with diabetes, and their orocaecal transit time is prolonged (Rayner et al. 2001). In addition to the autonomic neuropathy resulting in motility disorders, hyperglycemia has inhibitory effects on gastrointestinal motility to which hyperinsulinemia is contributing (Byrne et al. 1998). In healthy subjects without diabetes, intestinal transit time influences cholesterol

absorption such that a short transit time appears to reduce the cholesterol absorption efficiency (Ponz de Leon et al. 1982, Koivisto and Miettinen 1986). In the present study, diabetes had been recently diagnosed, and subjects did not have any diabetic complications suggesting normal/near normal gastrointestinal motility and transit time. In study II, despite the similar blood glucose levels, the normal-weight type 2 diabetic patients had a greater cholesterol absorption than overweight diabetic patients. Moreover, it could have been anticipated that the possible prolonged intestinal transit time should have enhanced the absorption efficiency of cholesterol in normal-weight diabetes vs obese nondiabetes. However, this was not the case, as their cholesterol absorption efficiencies were similar. Therefore, intestinal motility and intestinal transit time were not confounding factors in the present study.

Clinical conditions and statins

Many diseases and organic dysfunctions, e.g. pancreatic insufficiency, liver, gastric or ileal diseases or dysfunctions, especially celiac disease, alter cholesterol metabolism by inducing cholesterol malabsorption (e.g. Vuoristo et al. 1988, Vuoristo et al. 1992). Hypothyreosis, in which cholesterol synthesis and elimination (Abrams and Grundy 1981), and intestinal motility are impaired, may influence cholesterol absorption. During the initial stage of this study, the possibility of some kind of disease or condition possibly affecting cholesterol metabolism in the subjects was ruled out. Therefore, clinical conditions did not have any effect on the results in the present study.

Statins, HMG-CoA inhibitors, in addition to inhibiting cholesterol biosynthesis, have been found to interfere with cholesterol absorption probably due to the reduction in biliary cholesterol secretion. Statin treatment caused a significant reduction in cholesterol absorption of patients with familial hypercholesterolemia (Miettinen 1991, Vanhanen et al. 1992), but not in nonfamilial mildly hypercholesterolemic patients (Vanhanen and Miettinen 1995). Since the use of statin therapy might interfere with cholesterol absorption in type 2 diabetes as well, the use of statins or any hypolipidemic drug was one of the exclusion criteria in the present studies.

6.3.2 Cholesterol synthesis and excretion

It has been observed earlier in a limited number of diabetic subjects that cholesterol and bile acid synthesis and fecal elimination of cholesterol are increased compared with controls (Bennion and Grundy 1977, Abrams et al. 1982, Briones et al. 1986, Scoppola et al. 1995, Naoumova et al. 1996, Gylling and Miettinen 1997). In all of these previous studies, the subjects were obese and /or hypertriglyceridemic, and some had insulin treatment. However, no difference was found in cholesterol synthesis between diabetic patients and controls, when the lipid profile was normal (Briones et al. 1986). The results from the present study confirm that cholesterol synthesis and excretion are higher in obese than normal-weight type 2 diabetes, and even higher than in obese non-diabetic subjects. Furthermore, the synthesis and excretion of cholesterol is similar in diabetes with normal body weight to non-diabetic state with obesity. Accordingly, diabetes per se seems to upregulate cholesterol synthesis, and obesity further enhances those diabetes-induced alterations.

The constancy of total body cholesterol is maintained by balancing dietary and biliary cholesterol absorption and endogenous cholesterol synthesis with bile acid synthesis and excretion of biliary cholesterol. In addition, there is a homeostatic regulation between cholesterol absorption and synthesis such that a low intestinal absorption of cholesterol will upregulate cholesterol synthesis and turnover. The interrelation of the variables of cholesterol absorption and synthesis in the present study suggests that cholesterol homeostasis is not impaired in type 2 diabetes. Furthermore, the homeostasis is preserved despite the effects of varying body weight.

6.4 Cholesterol and lipoprotein metabolism (Study I and II)

Hypertriglyceridemia with low HDL cholesterol, and moderately elevated serum total and LDL cholesterol is the typical lipoprotein profile in an insulin resistant state (DeFronzo and Ferrannini 1991, American Diabetes Association 1993), this being also a finding in the present study. However, patients with obese type 2 diabetes were characterized with higher triglyceride contents in serum and VLDL and lower HDL cholesterol than in obese non-diabetic patients or in normal-weight diabetes.

Many factors are involved in the development of dyslipidemia, including insulin resistance with compensatory hyperinsulinemia, disturbed fatty acid metabolism, and

hyperglycemia (Evans et al. 1999). Furthermore, the dyslipidemic lipoprotein profile is more severe in insulin-resistant than insulin sensitive type 2 diabetes (Haffner et al. 1999), suggesting that dyslipidemia is aggravated by the increasing degree of insulin resistance. The insulin resistance in type 2 diabetics with normal weight is of similar magnitude as in the nondiabetic obese state (Defronzo 1988, Golay et al. 1988). Therefore, the more aggravated dyslipidemic lipoprotein profile observed in obese type 2 diabetes in the present study suggests that insulin resistance was increased with increasing body weight.

In the non-diabetic population, earlier studies have reported an association between serum total, LDL and HDL cholesterol level, and cholesterol absorption efficiency (Gylling and Miettinen 1989, Miettinen and Kesäniemi 1989, Miettinen et al.1990), suggesting that the higher the cholesterol absorption, the higher the serum cholesterol level. However, not all studies support this observation (Sehayek et al. 1998a, Bosner et al. 1999). The controversy of the results of these studies may be explained by the use of different methods for measuring cholesterol absorption, since the plasma isotope ratio method (Sehayek et al. 1998a, Bosner et al. 1999) determines cholesterol absorption from only a short period of time yielding only a single measure of absorption. Thus, the value of cholesterol absorption may be dependent of the composition of the test meal. On the other hand, the continuous isotope feeding method (Gylling and Miettinen 1989, Miettinen and Kesäniemi 1989, Miettinen et al. 1990) determines cholesterol absorption over a longer period of time in a balanced, constant state resulting in more steady and consistent values from day to day.

The lack of associations between cholesterol synthesis and serum total and LDL cholesterol level is consistent in nondiabetic population (Gylling and Miettinen 1988, Miettinen and Kesäniemi 1989, Miettinen et al. 1989, Gylling et al. 1994). However, earlier studies in a limited number of type 2 diabetic patients, the connection between cholesterol absorption and LDL cholesterol levels is inconsistent (Gylling and Miettinen 1997), whereas LDL cholesterol and cholesterol synthesis have been inversely linked (Gylling and Miettinen 1996b, 1997). In the present study, neither cholesterol absorption nor cholesterol synthesis were associated with serum or lipoprotein cholesterol levels in diabetic patients suggesting that the association

between serum lipids and cholesterol metabolism was more complicated than in non-diabetic subjects.

6.5 Cholesterol and glucose metabolism (Study I and II)

In non-diabetic men with high glucose levels, cholesterol absorption is lower than in low-normal glucose men (Strandberg et al. 1996) suggesting that increasing glucose concentrations occur in parallel with decreasing cholesterol absorption and increasing cholesterol synthesis. However, in the present study, the low cholesterol absorption in obese vs normal-weight diabetic patients with similar blood glucose levels, and the comparable cholesterol absorption between persons with normal-weight diabetes and obese controls do not support the role of hyperglycemia as the only modulator of cholesterol metabolism. In addition, even though blood glucose and cholesterol synthesis were interrelated in an univariate model, the independent effects of blood glucose on cholesterol metabolism attenuated in the regression models.

The serum variables reflecting glucose metabolism and insulin resistance were related to variables reflecting cholesterol metabolism. With high levels of serum insulin and low levels of SHBG, suggesting more aggravated insulin resistance, cholesterol absorption is low and synthesis is enhanced, whereas with low insulin and high SHBG levels, cholesterol absorption is high and cholesterol synthesis low. As a result, the regulation of cholesterol metabolism seems to be closely linked to insulin resistance. In addition, since the cholesterol absorption percentage and BMI were the only variables significantly explaining the variability of cholesterol synthesis, the effects of insulin resistance on cholesterol metabolism may be mediated by BMI in diabetes. The almost 2-fold higher serum insulin levels, and the 32 % lower serum SHBG values in diabetes with obesity compared with normal-weight diabetic patients showed increased insulin resistance as weight increased. Therefore, the abnormalities in cholesterol metabolism reflect the magnitude of insulin resistance in a steady state, the efficacy of cholesterol absorption being an inverse and the rate of cholesterol synthesis being a direct index of insulin resistance.

6.6 Weight reduction (Study III and IV)

6.6.1 Chronic caloric restriction (Study III)

The increase of the ratios of serum cholestanol and the respective decrease of the cholesterol precursor sterols during effective weight reduction in type 2 diabetes suggest that cholesterol absorption was increased and synthesis decreased in this acute non-steady state situation. In addition, the negative correlation between increased cholestanol and decreased lathosterol indicated that decreased synthesis during weight reduction was associated with an increased cholesterol absorption efficiency. This suggests that normal homeostasis of cholesterol metabolism is sustained even in calorie non-steady state.

Actual mechanisms for the increase in serum cholestanol during weight reduction are not known. The serum level of cholestanol can be regulated by its biliary secretion, intestinal absorption or production from cholesterol (Björkhem et al. 2001). Serum cholestanol is very consistently positively related to cholesterol absorption or inversely to cholesterol synthesis, but usually not associated with the synthesis of bile acids (Miettinen et al. 1989). Dietary intake of cholestanol is normally very small (Miettinen et al. 1989), and in the present study virtually zero, its absorption from the intestine low (Vuoristo and Miettinen 2000), and its serum levels remain constant during consumption of a cholestanol free diet (Salen and Grundy 1973). Thus, the mechanism for the relation of serum cholestanol to cholesterol absorption is also unclear. Extensively high serum cholestanol values are found in primary biliary cirrhosis (Nikkilä et al. 1991), and cholestatic conditions increase the serum cholestanol level in a corresponding manner (Hakala et al. 1996). However, in the present series, no signs of cholestasis were found during weight reduction.

A diet with the lowest possible amount of plant sterols is used in treating a patient with sitosterolemia, after which the plasma plant sterol levels usually decrease rapidly (Björkhem et al. 2001). In the present study, serum plant sterol ratios only tended to decrease despite the virtually phytosterol-free diet for 3 months. In fact, the dietary intake of plant sterols was only about 4 % of the prefasting amount. Since plant sterols are not synthesized in human tissues, the serum levels of plant sterols are regulated by dietary intake, absorption efficiency of sterols and biliary sterol

secretion. As for the minimal dietary intake in the present study, the levels of serum plant sterols depended on their absorption efficiency, release from lost adipose tissue and biliary secretion. In normal individuals, the fractional turnover rate of sitosterol is more rapid than cholesterol (Salen et al. 1970) possibly because of restricted intestinal absorption of sitosterol combined with its rapid excretion into bile. Due to the rapid turnover rate, plant sterols do not normally accumulate in the organs, the greatest amounts are normally found in the liver, adrenal glands, ovaries and testes in experimental animals (Subbiah and Kuksis 1973, Sugano et al. 1978). Even though plant sterols are not accumulated in the adipose tissue to any great extent, the high amounts of adipose tissue in obesity might conceal their accumulation. In addition, shrinkage of adipose tissue by calorie restriction results in accumulation of cholesterol and other sterols in adipocytes (Tilvis and Miettinen 1979), release of sterols being slower than that of triglyceride fatty acids. Therefore, the slow mobilization of plant sterols from the shrinken adipocytes during weight reduction may partly prevent the decrease of serum levels. Moreover, reduced synthesis and biliary secretion of cholesterol due to caloric restriction might have decreased the intestinal dilution of plant sterols, increasing their micellar solubilization and thus facilitating the absorption of the minimal amounts of dietary and biliary plant sterols. Therefore, the release of plant sterols from the adipose tissue together with the markedly improved efficiency of cholesterol and sterol absorption prevented the significant decrease in serum plant sterols ratios, despite their minimal dietary intake.

Even though serum desmosterol and lathosterol ratios were reduced, suggesting diminished cholesterol synthesis, serum squalene was not decreased, it even tended to increase. Squalene is both absorbed from the diet and synthesized in adipose tissue, liver and skin. It is not known whether there is an interregulation between different tissue pools and the serum level of squalene. The serum level of squalene is usually low, and adipose tissue is rich in squalene, though most of it is metabolically inactive (80% of total), very slowly released from the adipocytes if at all (Tilvis et al. 1978). After weight reduction due to intestinal bypass, adipose tissue squalene concentrations increased so that the peak values were reached 12 months after the operation, although adipose tissue cholesterol reduced after 6 months (Tilvis and Miettinen 1979). According to the present results, the unchanged serum squalene levels after the 17 % reduction of body weight might suggest that either the elapsed

time was not long enough to reflect changes in squalene metabolism or the metabolism of squalene in adipose tissue was separate from the hepatic pool. Therefore, squalene seems to be a poor marker of cholesterol synthesis in the nonsteady situation.

6.6.2 Steady state after weight loss (Study IV)

Earlier studies have shown that weight reduction can normalize cholesterol metabolism in obesity without diabetes so that cholesterol and bile acid syntheses and fecal excretion of neutral sterols are reduced with weight reduction, even with caloric restriction, measured in a non-steady or in a steady state (Miettinen 1970, 1971a, 1971b, Bennion and Grundy 1975, Kudchodkar et al. 1977, Di Buono et al. 1999). The results from the present study show that in type 2 diabetes during steady state, cholesterol absorption (i.e. absolute or relative absorption and serum plant sterol and cholestanol ratios to cholesterol) were markedly improved after weight reduction so that diminished excretion of cholesterol in feces as neutral sterols was balanced by increased absorption. Accordingly, weight reduction tended to normalize cholesterol metabolism, even though cholesterol synthesis still remained higher and absorption lower than in a random population of similar age (Miettinen et al. 1990). These results point to the reversibility of the abnormalities of cholesterol metabolism in diabetes. In a previous study, enhanced post-prandial cholesterol synthesis, measured by quantitating the amount of ^{14}C-acetate incorporated into human peripheral blood mononuclear leucocytes, did not change with weight reduction, if there was no decrease in insulin resistance (Griffin et al. 1998). Accordingly, despite the modest weight loss achieved in the present study, the subjects were still obese and probably insulin resistant. It seems that even a modest decrease in weight is be enough to diminish insulin resistance, which then could lead to enhancement of cholesterol absorption, whereas a more profound improvement in insulin resistance is needed to see a change in cholesterol synthesis.

Weight reduction in type 2 diabetes improves hyperinsulinemia and insulin resistance (Henry et al. 1986, Henry and Gumbiner 1991), which were also observed in the present study on the basis of serum glucose, insulin, and SHBG concentrations. After weight reduction, the serum SHBG was significantly associated with variables of cholesterol absorption, but not with the variables of cholesterol synthesis. In addition,

weight reduction and the final steady state body weight were inversely related to the changes of the variables reflecting cholesterol absorption, but not to the variables expressing cholesterol synthesis. These associations suggest that weight reduction-induced improvements in insulin resistance and cholesterol absorption are interrelated. Furthermore, the efficacy of cholesterol absorption can also inversely indicate the changes in insulin resistance. Even though the rate of cholesterol synthesis may indicate insulin resistance in a steady state, it does not reflect insulin resistance in a new steady state after weight reduction.

6.7 Mechanisms of abnormal cholesterol metabolism in diabetes

The question then arises, what is initially responsible for the altered cholesterol metabolism in diabetes? The results from the present study suggest that type 2 diabetes modulates cholesterol absorption and synthesis, and it is not only due to obesity alone. However, also body weight can affect cholesterol metabolism in diabetes and thus the possible effects of insulin resistance on cholesterol metabolism are mediated also by BMI. Accordingly, diabetes seems either to up-regulate cholesterol synthesis or down-regulate cholesterol absorption compared with what occurs in the respective non-diabetic state throughout the weight scale. Obesity enhances the diabetes-induced alterations, but the homeostasis between cholesterol absorption and synthesis is still preserved regardless of weight also in type 2 diabetes.

Since the efficient weight loss in diabetes improves cholesterol absorption efficiency and markers of insulin resistance, and these changes are closely interrelated, cholesterol absorption efficiency might be the variable being affected primarily. In addition, based on the multivariate regression analyses, the variability of cholesterol synthesis was explained by cholesterol absorption and BMI. However, the blood glucose level is significantly related to cholesterol synthesis, whereas the respective relation to absorption of cholesterol remains insignificant. Even though the variability of cholesterol synthesis is not independently explained by blood glucose, the role of cholesterol synthesis as a primarily regulated variable can not be ruled out. According to the cholesterol homeostasis theory, when cholesterol absorption is decreased, cholesterol synthesis is increased, whereas with enhanced synthesis of

cholesterol, its absorption decreases. Does the insulin resistance per se affect intestinal sterol transport, or is it the up-regulated cholesterol synthesis that predominates? Both situations are considered separately in the following section.

6.7.1 Cholesterol synthesis

The synthesis of cholesterol will be upregulated if the hepatic cholesterol pool is diminished. This could be due to depressed hepatic cholesterol influx from the tissues, from intestine, or due to increased cholesterol output through bile as bile acids or neutral steroids, or increased VLDL synthesis.

Considering first the hepatic influx of cholesterol from tissues, the HDL cholesterol was within normal limits in the non-obese diabetic patients but lowered in the obese diabetic patients, suggesting that reverse cholesterol transport might be impaired in obesity. However, even if it were, its significance in upregulating cholesterol synthesis remains open. In addition, because of the small amounts of cholesterol carried through this pathway (Tall et al. 2001), this would be unlikely to be sufficient to up-regulate cholesterol synthesis.

Second, insulin resistant fat cells release large amounts of free fatty acids into the circulation, which are taken up by the liver. Lipoprotein synthesis is increased, followed by the assembly and secretion of large amounts of VLDL and this could be observed in the obese type 2 diabetic group. The similar serum total and VLDL triglyceride levels in obese controls and normal-weight diabetic patients suggest that despite the higher amounts of adipose tissue in obesity, the increased flux of free fatty acids to the liver is similar. It can be speculated that the adipose tissue and its fat cells are more prone to insulin resistance in type 2 diabetes. It is not known, whether the enhanced flux of free fatty acids to the liver and increased lipogenesis is able to activate sterol regulatory element binding protein 2 (SREBP), which is needed to be up-regulated to augment cholesterol synthesis (Horton and Shimomura 1999). However, in the present study, the variables of cholesterol synthesis were not associated with serum or VLDL triglyceride levels. Accordingly, the role of enhanced lipogenesis in increasing cholesterol synthesis remains open.

Third, the enhanced production of apo-B containing lipoproteins in obesity is associated with their rapid catabolism and enhanced removal of the remnants (Kesäniemi and Grundy 1983, Egusa et al. 1985). The production rates of LDL apo-B were similar between non-obese type 2 diabetic patients and non-diabetic controls, whereas the LDL apo-B removal was higher in diabetic subjects (Gylling and Miettinen 1997). Since LDL cholesterol levels were higher in the obese controls than obese diabetic patients, it could be speculated that the probably enhanced production of LDL apo B in both groups due to obesity was compensated by their higher removal in the diabetic group. Therefore, the flux of cholesterol to the liver via the LDL receptor pathway compensated by the increased LDL apo B synthesis was not thought to be sufficient to up-regulate cholesterol synthesis.

6.7.2 Cholesterol absorption

The fourth possibility is that cholesterol synthesis is increased in obese type 2 diabetes because of diminished hepatic influx of cholesterol due to decreased cholesterol absorption from the intestine. Even though the relative and absolute absorption (mg/kg/d) of cholesterol was lower in the obese type 2 diabetic subjects, the amount of cholesterol entering the liver was similar to that in non-obese diabetic subjects because of the larger amount of cholesterol entering the intestine from bile. It has been shown in transgenic mice that biliary cholesterol concentration is inversely and almost linearly correlated with the percentage of cholesterol absorption (Sehayek et al. 1998b), suggesting that large amounts of biliary cholesterol are able to saturate the micellar cholesterol pool resulting in poor absorption of cholesterol and other sterols. In subjects without diabetes, similar negative correlations were found for all biliary lipid concentrations, even though the percentage of absorption was only insignificantly negatively related to intestinal cholesterol pool (Miettinen and Gylling 2000), similar to findings in the present diabetic population. In addition, biliary cholesterol secretion did not differ in diabetic or non-diabetic subjects of differing body weight.

The question remains, why was cholesterol absorption lower in the obese type 2 diabetic subjects compared with obese non-diabetes or with normal-weight diabetes? Elevated cholesterol synthesis in obesity increases the biliary secretion of cholesterol, expanding the intestinal cholesterol pool (Miettinen and Gylling 2000).

The large intestinal cholesterol pool may dilute the labeled cholesterol contributing to the reduced cholesterol absorption efficiency. In addition, increased biliary lipids may prevent the entry of labeled dietary cholesterol from the oil phase to the micellar phase, reducing the absorption of labeled cholesterol in obese subjects. The low relative and absolute (mg/kg/d) absorption of cholesterol was probably not due to dilution of the isotope in the larger intestinal cholesterol pool in obese diabetic subjects, because serum plant sterols and cholestanol ratios gave the same result, both being lower in obese type 2 diabetes. Certainly dietary plant sterols can also be diluted in the large intestinal cholesterol mass, slightly lowering their absorption, and effective biliary cholesterol and sterol secretion could have also contributed to their low serum levels. However, despite the increased fecal neutral sterol elimination, the bile acid synthesis, biliary cholesterol secretion and intestinal cholesterol pool tended to be similar in the obese and normal-weight diabetic subjects. In addition, there were increases in cholesterol absorption efficiency, and the serum levels of plant sterols and cholestanol after weight reduction which were not accompanied by any change in intestinal cholesterol pool, suggesting that improved insulin resistance with slightly decreased body weight, enhanced mucosal capacity to absorb cholesterol and sterols.

The question must be asked, whether ABC transporters play a role in regulating sterol absorption in diabetes and obesity, i.e. in the insulin resistance state? A high fat diet increases mRNA expression of the ABCG5 and ABCG8 genes in the liver and intestine of mice (Berge et al. 2000), inhibiting the absorption of sterols. On the contrary, in phytosterolemia, their expression is depressed by mutations (Berge et al. 2000, Lee et al. 2001), resulting in major absorption of sterols and high serum plant sterol levels. Recent studies have shown that polymorphisms in ABCG8 have a contributing effect to the variation in serum plant sterol concentrations in normal, healthy individuals (Berge et al. 2002). Therefore, insulin resistance may play a role in regulating the expression of these ABC transporter genes. It could be argued that type 2 diabetes alone, especially when associated with obesity, could increase the expression of these genes in the liver and intestine similarly to a high fat diet in mice, resulting in low absorption and effective biliary secretion of cholesterol and plant sterols.

7. SUMMARY AND CONCLUSIONS

Type 2 diabetes is associated with abnormalities in glucose and lipoprotein metabolism, which contribute to accelerated atherosclerosis. Cholesterol metabolism in diabetes has been less clearly documented, and the results are controversial. Obesity, in addition to favoring to the development of diabetes, is associated with abnormal cholesterol metabolism. Therefore, the aim of this study was to investigate, first, the metabolism of cholesterol and bile acids in diabetes, and second, whether body weight modulates cholesterol metabolism in diabetes. In addition, the relationships between cholesterol, lipoprotein and glucose metabolism were evaluated. Furthermore, the effects of weight reduction on cholesterol and sterol metabolism in a non-stable state as well as in a steady state after a prolonged follow-up were studied.

Cholesterol metabolism was studied in 16 obese (BMI > 30 kg/m^2) type 2 diabetic patients compared to 16 similarly obese non-diabetic controls to discover the role of diabetes in cholesterol metabolism. Second, the effects of body weight on cholesterol metabolism in diabetes were investigated in patients with normal-weight (n=20) and overweight (n=44) patients with type 2 diabetes.

Cholesterol absorption was evaluated with the peroral dual isotope technique and by quantitating serum ratios of phytosterols and cholestanol to cholesterol, and cholesterol synthesis with sterol balance and by quantitating the serum ratios of squalene and precursor sterols (cholestenol, desmosterol, lathosterol) to cholesterol.

Cholesterol absorption efficiency and the amounts of absorped total, dietary and biliary cholesterol were lower in the obese diabetic patients than obese non-diabetic controls or normal-weight diabetic patients. Cholesterol absorption was similar in diabetes with normal body weight to obese non-diabetes. The differences in cholesterol absorption were not due to hyperglycemia because of lower cholesterol absorption efficiency with similar blood glucose levels in obese diabetic as in normal-weight diabetic subjects, and also because of comparable cholesterol absorption between normal-weight diabetic and obese non-diabetic subjects. Fecal elimination of cholesterol was increased, mainly as neutral sterols rather than bile acids,

enhancing cholesterol synthesis more in patients with obese type 2 diabetes than obese non-diabetic controls or normal-weight diabetes. In addition, fecal bile acids, the total intestinal cholesterol pool, biliary cholesterol secretion and cholesterol turnover were significantly higher in obese diabetics compared to normal-weight diabetics when expressed as mg/d. The variables expressing the synthesis and excretion of cholesterol and bile acids were quite similar in obese non-diabetic and normal-weight diabetic subjects. The ratios of serum plant sterols, sitosterol and campesterol, and cholestanol to cholesterol, indicators of cholesterol absorption, were higher, and those of the cholesterol precursors, markers of cholesterol synthesis, were lower in normal-weight than obese diabetic patients.

In the diabetic population, BMI was positively associated with variables of cholesterol synthesis and negatively with cholesterol absorption. Since the study populations did not differ with respect to the possible confounding factors affecting cholesterol metabolism, e.g. age, gender, apo E phenotype distribution and dietary intakes of cholesterol and plant sterols, the lower cholesterol absorption and higher synthesis in obese type 2 diabetics were probably due to diabetes, which, with some unknown mechanism, can modulate cholesterol metabolism. It can be speculated that diabetes either inhibits the absorption of cholesterol and sterols leading to compensatory increase in cholesterol synthesis, or that it upregulates cholesterol synthesis per se. Furthermore, obesity enhances these diabetes-induced alterations.

There is a homeostatic regulation between cholesterol absorption and synthesis in the normal population such that with a low cholesterol absorption, the synthesis of cholesterol increases. The variables of cholesterol absorption and synthesis (including also squalene and non-cholesterol sterols) were interrelated in diabetic population, suggesting that cholesterol homeostasis was not disturbed in type 2 diabetes. In addition, the negative correlation between cholestanol and lathosterol during weight reduction suggested that normal homeostasis of cholesterol metabolism was sustained also in the calorie non-steady state. Serum plant sterols and cholestanol were correlated with cholesterol absorption efficiency, and cholesterol precursor sterols were correlated with cholesterol synthesis measured with sterol balance, indicating that serum non-cholesterol sterols reflected cholesterol metabolism in a manner similar to that in the non-diabetic population. The higher

serum levels of squalene in those subjects with higher cholesterol synthesis compared to those with lower synthesis could indicate that serum squalene levels reflect cholesterol synthesis rate in diabetes, which is different from the situation in the non-diabetic population.

Serum levels of SHBG, a marker of insulin resistance, and its ratio to serum insulin were higher and serum insulin level lower in normal-weight than obese diabetes, despite the similar blood glucose levels, suggesting that insulin resistance in diabetes increased with weight. Furthermore, BMI was positively associated with serum insulin and negatively with SHBG levels.

Hypertriglyceridemia with low HDL cholesterol, and moderately elevated serum total and LDL cholesterol was characteristic for the diabetic patients and obese non-diabetic subjects. Serum total cholesterol levels did not differ in the subjects with or without diabetes regardless of their body weight despite their differences in cholesterol metabolism. However, obese type 2 diabetic patients had higher triglyceride contents in serum and VLDL, and lower HDL cholesterol than subjects with obese non-diabetes or normal-weight diabetes. Thus, this more aggravated dyslipidemic lipoprotein profile may be related to the higher magnitude of insulin resistance in diabetic patients with obesity. The serum or lipoprotein cholesterol levels were not associated with cholesterol absorption or synthesis in diabetes suggesting that the association between serum lipids and cholesterol metabolism was more complicated than in non-diabetes.

Serum cholestanol levels increased and serum cholesterol precursor levels decreased with effective weight reduction in type 2 diabetes measured in a non-steady state situation. In addition, weight reduction to a steady state caloric balance after a 2-year follow up increased low baseline cholesterol absorption efficiency to the same level as in normal-weight diabetic patients. In addition, ratios of serum plant sterols and cholestanol to cholesterol were higher, and those of cholesterol precursors lower than at baseline at this new, steady state weight level. Thus, the abnormalities of cholesterol metabolism in diabetes are reversibile, and the weight reduction is an efficient way to improve cholesterol metabolism. The increased

cholesterol absorption might be due to the improved insulin resistance with some mechanism leading to enhanced intestinal mucosal capacity to absorb cholesterol.

The serum variables reflecting glucose metabolism and insulin resistance were related to the variables reflecting cholesterol metabolism. When there were high levels of serum insulin and low levels of SHBG, cholesterol absorption was low and its synthesis enhanced, whereas with low insulin and high SHBG levels, cholesterol absorption was high and cholesterol synthesis low. As a result, the regulation of cholesterol metabolism seemed to be closely linked to indicators of insulin resistance. Since the results from multivariate analysis showed that the percentage of cholesterol absorption and BMI were the only variables significantly explaining the variability of cholesterol synthesis, the effects of insulin resistance on cholesterol metabolism may be mediated by BMI. When the subjects under-went weight reduction, the variables of glucose metabolism were improved and cholesterol absorption was increased, with these changes being related to each other. In addition, weight reduction correlated with the changes in variables of cholesterol absorption, but not with cholesterol synthesis, suggesting that improved insulin resistance leads to enhanced absorption of cholesterol, and that the efficacy of cholesterol absorption was an inverse indicator of insulin resistance.

In conclusion, type 2 diabetes is associated with abnormalities of cholesterol metabolism, which are not explained either by hyperglycemia or obesity. However, body weight, through its entire range, can regulate cholesterol metabolism in diabetes so that in conjunction with the increasing insulin resistance evoked by obesity, the cholesterol absorption became lowered and cholesterol synthesis increased. Thus, the regulation of cholesterol metabolism seems to be closely linked to insulin resistance. Despite the disturbances in cholesterol metabolism, the total-body homeostasis between cholesterol absorption and synthesis is not disturbed. The abnormalities in cholesterol metabolism are not irreversible, and weight reduction, even a modest sustained weight loss, is an efficient way to improve cholesterol metabolism by increasing cholesterol absorption. In addition, the beneficial effects of weight loss on cholesterol metabolism can be seen rather rapidly, even in a non-steady calorie state.

Taken together, low cholesterol absorption and high synthesis seem to be part of the insulin resistance syndrome. The exact molecular mechanisms for the modulating effect of insulin resistance on the intracellular trafficking of cholesterol in the liver and intestinal cells are unknown. It could be assumed that insulin resistance in type 2 diabetes, and its higher magnitude especially when associated with obesity, could modulate the expression of genes regulating cholesterol metabolism resulting in low absorption, more effective biliary secretion and elevated synthesis of cholesterol, and low levels of serum plant sterols.

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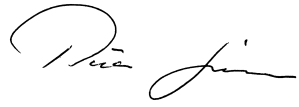

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