The very low-density lipoprotein-triacylglycerol pathway and metabolic heterogeneity in familial combined hyperlipidemia

The Very Low-Density Lipoprotein-Triacylglycerol Pathway and Metabolic Heterogeneity in Familial Combined Hyperlipidemia

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На моето семейство, с много обич.

To my family with love.

Contents

List of abbreviations:

Apo	apolipoprotein
ARF-1	ADP-ribosylation factor 1
CAD	coronary artery disease
CE	cholesteryl ester
CETP	cholesteryl ester transfer protein
CM	chylomicrons
DAG	diacylglycerol
FCHL	familial combined hyperlipidemia
FFA	free fatty acids
HDL	high-density lipoprotein
HL	hepatic lipase
HSL	hormone sensitive lipase
IDL	intermediate-density lipoprotein
IMT	intima media thickness
LDL	low-density lipoprotein
LPL	lipoprotein lipase
LRP	LDL receptor related protein
LXR	liver X receptor
PAI-1	plasminogen activator inhibitor 1
PLD	phospholipase D
Sd LDL	small dense LDL
SREBP	sterol response element binding protein
sTF	soluble tissue factor
sTM	soluble Thrombomodulin
TAT	thrombin-antithrombin complex

TG	triglycerides, triacylglycerols
TC	total cholesterol
t-PA	tissue-derived plasminogen activator
TRL	triglyceride-rich lipoproteins
TZD	thiazolidinediones
VLDL	very low-density lipoprotein
vWF	von Willebrand factor
WHR	waist-to-hip ratio
USF1	upstream stimulating factor 1
F XIIa	factor XII activated

General Introduction

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1. Introduction

1.1 Metabolic Profile of FCHL

Familial Combined Hyperlipidemia (FCHL) was delineated three decades ago as a genetic disorder of lipid metabolism occurring in survivors of a myocardial infarction and their relatives[1]. The initial study showed that approximately 10% of the myocardial infarction survivors exhibited familial hyperlipidemia, characterized by variable expression within kindred: i.e. isolated hypercholesterolemia (Fredrickson IIa), combined hyperlipidemia (IIb), or isolated hypertriglyceridemia (IV), whereas other relatives were normolipidemic. A proportion of affected individuals (up to 25%) changes phenotype over time[1, 2]. Subsequent studies have shown that affected FCHL relatives can present with most of the features of the metabolic syndrome: elevated plasma insulin concentrations[3, 4] in the presence of insulin resistance[5-8], visceral obesity[9, 10], hypertension[3, 10], as well as metabolic dyslipidemia, including small dense low-density lipoproteins (sd LDL), decreased plasma high-density lipoprotein (HDL) cholesterol concentrations, and increased plasma Apo B levels[3, 11]. Elevated Apo B levels, which reflect an increased number of lipoprotein particles in plasma, are considered inherent to FCHL[12, 13].

1.2 Metabolic Background

Despite numerous metabolic and genetic studies, the pathophysiology and the genetic background of FCHL remain a matter of debate. Hyperlipidemia, an overt manifestation of FCHL, results from accumulation of Apo B-48 and Apo B-100 containing lipoprotein particles in plasma, especially in the postprandial state[14]. Hence, a significant part of scientific research has focused on the plasma compartment in FCHL. Genetic analysis showed evidence that factors that control plasma lipoprotein metabolism, such as Apo AI[15], Apo AII[16], Apo CIII[15], Apo B[17], Apo AV[15], lipoprotein lipase (LPL)[18], hepatic lipase (HL)[18], and cholesterol ester transfer protein (CETP)[19] are implicated in the etiology of FCHL as susceptibility genes. These genes modify the expression of the FCHL phenotype.

Plasma lipid levels represent the result of acute adaptations (for instance in response to meals), and long-term adaptations to changes in energy homeostasis. Several organs that are implicated in lipid metabolism play an important role in this adaptation, i.e. adipose tissue, liver and skeletal muscle **(Figure 1; Box I)**. These organs direct the flux of energy substrates, glucose and free fatty acid, FFA, in the body in response to insulin. In FCHL, adipose tissue and muscle exhibit an impaired endocrine response to insulin, meaning that these tissues are insulin resistant[5, 6, 8]. There is no evidence in the literature yet to suggest that insulin resistance in liver contributes to FCHL, although this is quite likely. For example, the liver has been implicated in other disorders with insulin resistance, such as type 2 DM[20]. In the following sections, we will review lipid metabolism in FCHL in relation to organ function. We will focus on the role of FFA fluxes and insulin; and discuss hyperlipidemia in FCHL.

Box I Physiology of Fatty Acid Metabolism in Adipose Tissue. Fatty acids constitute an important fuel for many organs in the body. However, at increased concentrations fatty acids can have diabetogenic, or even toxic effects[21]. Intracellular accumulation of fatty acids as triglycerides can impede insulin action (as shown in muscle and liver) [22-24], or in some cases, it can induce apoptosis. This latter mechanism has been proposed as an explanation of apoptosis of the beta-cells of the pancreas[21]. Homeostatic mechanisms prevent increased systemic FFA concentrations. For instance, at times of energy surplus, FFA along with glucose, which is converted to glycerol in the adipocyte, can be stored as triacylglycerols in adipose tissue. The storage potential of adipose tissue can serve as a buffer that protects other organs against the deleterious effect of high plasma FFA[25].
The buffer capacity of subcutaneous adipose tissue is determined by the rate of lipolysis and lipogenesis (esterification) of triacylglycerols **(Figure 1)**[26]. These metabolic pathways are modified by genetic variation and are fine-tuned by a variety of hormonal and nutritional factors. Insulin promotes lipogenesis in adipose tissue and acutely suppresses lipolysis via inhibition of the enzyme hormone sensitive lipase (HSL). The postprandial increase in insulin levels potentiates storage of fatty acids in adipose tissue, and thus prevents extreme plasma concentrations. Several other hormones, e.g. catecholamines, glucagon and growth hormone antagonize insulin's actions, and are known as lipolytic hormones. The resultant of lipolysis and esterification, or in other words the net result of these regulatory processes, determines the nonesterified fatty acid pool in adipose tissue, and contributes to plasma FFA levels[25-27]. Of note, disturbed FFA metabolism in adipose tissue plays a role in various metabolic diseases. Excessive lipolysis or reduced esterification contribute to elevated plasma FFA levels in type 2 DM and obesity[27].

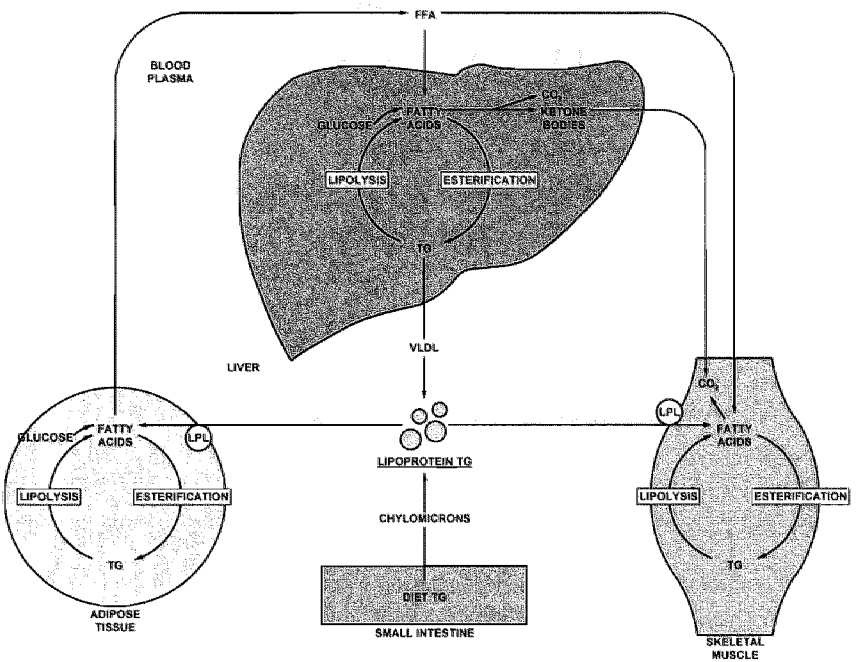

Figure 1. Schematic overview of lipid metabolism, with focus on FFA fluxes.

In the **postprandial state**, dietary fatty acids are transported as Chylomicrons-TG in the blood for storage (adipose tissue), or as fuel supply (muscle). The excess of FFA is accommodated by the liver and oxidized, or stored as TG. In the **fasting state**, liver maintains adequate fuel supply to other organs by secretion of FFA as very low-density lipoprotein-triacylglycerol (VLDL-TG).

Lipid metabolism in FCHL: 1) delayed clearance of TG-rich lipoproteins in the postprandial state because of relative LPL deficiency; 2) impaired FFA trapping for TG synthesis in adipose tissue; 3) increased FFA flux to liver and muscle; 4) excess hepatic production of fatty acids as VLDL-TG; 5) increased fatty acid oxidation in muscle.

2. Organ Insulin Resistance and Lipid Metabolism in FCHL

2.1 Disturbed Adipose Tissue Metabolism Contributes to Elevated Plasma Levels of FFA in FCHL

A number of studies provide evidence that adipose tissue in FCHL shows an impaired response to insulin. FCHL subjects have an **extended postprandial increase of FFA**, that gradually drops to normal in the postabsorptive state[6, 7, 14]. Insulin infusion during an euglycemic hyperinsulinemic clamp, lowers plasma FFA and glycerol in controls, but in FCHL subjects they remain elevated [6-8, 28]. Elevated plasma FFA levels can result from insulin resistant (excessive) lipolysis in adipose tissue, associated with increased activity of HSL[27]. Such a mechanism is operational in type 2 DM, but could not be proven in FCHL[8, 18, 29, 30]. On the contrary, several studies on HSL activity in FCHL demonstrated reduced activity of this enzyme, at least in in vitro studies[8, 29, 30]. The latter seems to be an adaptive mechanism rather than a metabolic defect, because genetic studies have shown no linkage of the HSL gene locus (19q13.1-q13.2) to the FCHL phenotype[8, 18, 31].

Elevation of fasting FFA, however, can be particularly characteristic for FCHL subjects in the highest triglyceride tertile (TG>2.5 mmol/L) [4, 14]. These subjects are characterized by the highest BMI as well[14]. Increased adipose tissue mass, as assessed by BMI, contributes to dyslipidemia, probably through chronically elevated FFA fluxes[32]. In addition, hormones and cytokines, such as leptin, adiponectine, IL-6 and TNF- α, produced by adipose tissue in proportion to adipose tissue mass, can influence whole body insulin resistance and the insulin resistance of adipose tissue in particular. Noteworthy, we have observed a number of differences in gene expression of inflammatory and insulin resistant genes between mature adipocytes of FCHL subjects and controls, including TNF-α (13-fold elevated) and IL-6 (2-fold) [33].

Genetic and metabolic heterogeneity with regard to clearance of triglyceride-rich lipoproteins and FFA metabolism in adipose tissue have been observed within FCHL[34]. These differences can additionally modify FFA levels. For example, delayed clearance of very low density lipoproteins (VLDL), VLDL remnants and chylomicrons, as observed in hypertriglyceridemic FCHL subjects, can contribute to the increase of FFA, both fasting and

postprandial, probably through increased intravascular lipolysis[14, 35]. In addition, genetic variations in PPAR-γ can significantly affect plasma concentrations of fatty acid and glycerol in FCHL[34].

A primary genetic defect, associated with impaired fatty acid metabolism in adipose tissue is plausible in FCHL[6-8]. Studies of our group and others suggest that such a defect is mostly likely associated with impaired fatty acid trapping for triacylglycerol synthesis in FCHL[8]. Others have proposed reduced postprandial fatty acid transport into the adipocyte via acylation-stimulating protein, ASP[36, 37]. However, studies in Finnish families provide evidence against a possible contribution of ASP to the lipid abnormalities in FCHL[38]. Alternatively, we found that the expression of FABP4, a fatty acid translocase necessary for the transport of long-chain fatty acids is upregulated in FCHL adipocytes. In addition, CD36, another fatty acid transporter, was upregulated in FCHL adipocytes as well as in preadipocytes[39]. These latter findings probably represent an adaptive response to defective triacylglycerol storage in FCHL adipocytes, and studies to the underlying gene defects are ongoing.

2.2 Consequences of Increased FFA Flux

2.2.1 Increased FFA Flux and Insulin Resistance in Skeletal Muscle

Skeletal muscle is responsible for 85% of the whole body's glucose uptake. Insulin mediates this process by promoting the availability of glucose transporter GLUT-4, as well as the rate of glucose storage in muscle, i.e. glycogen synthesis. Plenty of clinical and biochemical evidence has been gathered that glucose uptake in response to insulin is impaired in FCHL[6-8, 40]. Skeletal muscle metabolism in FCHL appears to differ from controls: it shows a higher rate of lipid oxidation, and lower rate of glucose oxidation than healthy controls, as measured by indirect calorimetry[6]. This can result from the increased FFA supply to muscle in FCHL, and subsequently greater relative use of FFA, instead of glucose, for oxidation, via the Randle's cycle[41] (**Figure 1**). Alternatively, increased FFA uptake in skeletal muscles can ensue from upregulation of fatty acid transporting proteins, as observed in FCHL adipose

tissue (CD36 and FABP4, as discussed above), and therefore be specific for FCHL. Increased fatty acid uptake can lead to accumulation of fatty acid metabolites in muscle, which can interfere with the intracellular insulin-signaling cascade[22, 42-44]. The extent of muscle insulin resistance in FCHL might be dependent on the rate of FFA supply, or alternatively have a genetic basis. Literature suggests that insulin resistant glucose uptake in muscle is observed predominantly in hypertriglyceridemic FCHL subjects, and is less common in FCHL subjects with hypercholesterolemia only [28]. Failure of insulin's action to stimulate glucose uptake in FCHL muscle can lead to increased plasma glucose concentrations, for which then compensatory hyperinsulinemia is required to maintain euglycemia. Moreover, reduced utilization of glucose by muscle can result in an increased glucose flux to liver, where it can be subsequently stored as glycogen, or converted to triglycerides.

2.2.2 Metabolically Challenged Liver in FCHL

Clinical and biochemical data on liver function in FCHL is scarce. However, literature data indicate that liver can have an important contribution to the expression of the disorder.

An important function of liver is to maintain the homeostasis of body fuels: glucose and FFA. At times of energy surplus, liver temporary accommodates the excess of plasma glucose or FFA, and thus can prevent other tissues from fat (energy) overload[26] (**Figure 1**). In liver, FFA can be oxidized or, under hyperinsulinemic conditions, stored as fat (lipogenesis) [45]. This buffer function of liver can be particularly important in FCHL, where adipose tissue fatty acid storage may be compromised[7, 8]. Additionally, increased flux of glucose to liver is expected due to diminished glucose utilization in muscle, as described above.

Hepatic lipid metabolism has not been addressed in FCHL. Of interest, it was recently shown that FCHL is associated with the gene encoding upstream regulating factor 1, USF-1[46]. This transcription factor can upregulate the transcription of several genes involved in *lipogenesis de novo* in response to high concentrations of intracellular glucose (glucose-responsive genes)[45, 47-49]. USF-1 may represent a switch between glucose and fat metabolism. Lipogenesis can be potentiated by hyperinsulinemia, because insulin upregulates several insulin-responsive genes with a central role in hepatic lipid metabolism, such as Liver X

receptor (LXR)[50], and Sterol Response Element Binding Proteins (SREBPs)[51]. Thus, substrate excess, in combination with hyperinsulinemia can stimulate lipogenesis de novo (i.e. fatty acid synthesis for incorporation in TG) in the liver in FCHL. Enhanced lipogenesis is proposed as one of the mechanisms that leads to fat accumulation in liver[52, 53], and therefore FCHL subjects can be susceptible to develop non-alcoholic fatty liver disease (NAFLD). We report on the high prevalence of fatty liver in ambulatory FCHL patients of the Lipid clinic of the Maastricht University Hospital[54].

2.3 Hepatic Lipoprotein-Apo B 100 Production

Insulin promotes lipid storage and suppresses hepatic glucose and very low density lipoprotein (VLDL) secretion after a meal. Alternatively, at times of energy need, liver can mobilize glucose, or FFA that can be secreted in the form of VLDL-TG. Thus, liver maintains adequate fuel supply to different organs during fasting when plasma insulin levels are low. One important manifestation of hepatic insulin resistance is glucose overproduction, as a result of unsuppressed gluconeogenesis. Another consequence of hepatic insulin resistance can be VLDL overproduction (for plasma lipoproteins see Box II).

Kinetic studies have demonstrated that hepatic overproduction of VLDL particles can explain, at least in part, the frequently observed elevated plasma triglyceride levels in FCHL [13]. The driving mechanism of this overproduction is not known, but literature suggests that hepatic VLDL-Apo B secretion can be a function of hepatic insulin sensitivity[55-57]. The importance of hepatic insulin resistance to triglyceride-rich VLDL production is illustrated by the observation that impaired insulin suppression of VLDL production contributes to increased lipid levels in type 2 DM [20]. Another factor that can mediate VLDL overproduction is accumulation of fat in the liver (fatty liver)[56]. Additional mechanisms can involve enhanced lipogenesis de novo or intrahepatocellular TG breakdown[27, 45, 58, 59].

Box II. Plasma lipoproteins. Plasma lipoproteins and their metabolism can be sorted into 3 major pathways, which interact with each other:

-VLDL pathway[72] VLDL are triglyceride-rich lipoproteins that are secreted by the liver. In plasma, VLDL are depleted from their triglyceride content via the activity of lipases, the main ones being lipoprotein lipase (LPL, localized on extrahepatic capillary endothelium), and hepatic lipase (HL, space of Disse in liver). During a half-life of minutes to a few hours, VLDL is successively converted to smaller particles: VLDL remnants, intermediate-density lipoproteins (IDL) and subsequently to LDL. Approximately, half of VLDL is rapidly cleared at its stage as a VLDL remnant, or IDL via Apo E mediated hepatic uptake. The remainder is progressively delipidated to a LDL particle. The clearance of LDL is mediated by Apo B 100, and is comparatively slow (half-life of LDL is 48-72hours)[72]. Noteworthy, VLDL and LDL consist of distinct, physicochemically heterogeneous subclasses, which differ in respect to production, regulation and catabolism[73]. By convention, VLDL comprises VLDL1 and VLDL2; LDL includes at least four subclasses: buoyant LDL (LDLI and LDLII), and small dense LDL (LDLIII and LDLIV). It appears that there is a metabolic channeling between VLDL and LDL subclasses[73]. VLDL1 can be the precursor of small dense LDL (sd LDL), whereas VLDL2 is rapidly catabolized to LDL of normal density and size (buoyant LDL). Sd LDL is considered to be particularly atherogenic[74, 75]. This is attributed to the ability of sd LDL particles to penetrate relatively easy vascular walls, and their susceptibility to oxidation[76].
Appendix (Table 1) presents enzymes and apolipoproteins implicated in the VLDL-TG pathway.

-HDL pathway. HDL transports cholesterol from the periphery to liver for excretion via the bile, a process known as the reverse cholesterol transport. A part of HDL cholesterol can be delivered to the liver via the VLDL-LDL pathway. This process involves transfer of neutral lipid between HDL and LDL, or VLDL, and is mediated via CETP. Increased plasma VLDL concentrations can result in an enhanced transfer of triglycerides (in exchange of cholesterol esters) to LDL and HDL, and a deteriorated lipoprotein profile[77]. This involves formation of atherogenic sd LDL and decrease in HDL cholesterol. In addition, HDL serves as a reservoir of apolipoproteins (Apo E and Apo C's) that are transferred to VLDLs and chylomicrons in plasma and can influence their catabolism.

-Chylomicron pathway. Chylomicrons characterize postprandial lipemia. These particles are lipolyzed by LPL to chylomicron remnants, and therefore compete with triglyceride-rich VLDL for a common lipolytic pathway[78].

2.3.1 Molecular Mechanisms of Hepatic Lipoprotein Production

Hepatic production of Apo-B 100 containing lipoproteins involves two main steps, which take place in the secretory apparatus of the hepatocyte (**Figure 2**) [60, 61]. **The first step** represents transfer of small amount of lipid (triglycerides and phospholipids) by microsomal triglyceride transfer protein (MTP) to newly synthesized Apo B 100, resulting in the formation of a dense pre-VLDL particle[62, 63]. If proper lipidation does not take place, Apo B 100 is sequestrated for degradation by chaperons, such as heat shock protein 70 (Hsp70) [63, 64]. These processes take place in the rough endoplasmatic reticulum (RER), but some insulin mediated degradation may also occur post-RER [64, 65]. The formation of mature VLDL (**second step**) comprises acquisition of additional lipid in the terminal junctions between the rough and the smooth endoplasmatic reticulum[61, 62, 65]. MTP is implicated in the transfer of neutral lipid to the lumen of the smooth endoplasmatic reticulum, although the exact mechanism is not yet known[63]. The process of fusion between the pre-VLDL particle and Apo B-free lipid droplet involves, at least in part, adenosine diphosphate ribosylation factor-1 (ARF-1)-mediated activation of phospholipase D (PLD) [62, 66]. Apo-B 100 containing lipoproteins produced by the liver are predominantly VLDL, but the density can range from LDL to large VLDL particles, depending on the amount of lipid available for the maturation step. Overproduction of Apo B 100 as VLDL as well as LDL has been reported in FCHL[13, 67].

Insulin regulates lipoprotein synthesis at several steps; it downregulates the MTP gene expression[68], it suppresses ARF-1 activity[62], and it mediates some Apo B degradation[64] among others. Insulin resistance of one or more of these pathways may result in enhanced production of VLDL-Apo B [56, 57, 62, 68-70]. Accumulation of lipid in the secretory apparatus can be another important factor that drives Apo B overproduction[23, 27, 69].

Most of the present knowledge on the molecular mechanism of VLDL production is derived from in vitro and animal studies, and little is known about these processes in humans. Human hepatic tissue is difficult to obtain for studies, which hinders our knowledge on hepatic lipid metabolism in general, and in FCHL in particular. However, genetic studies or markers of hepatic lipid metabolism can advance our understanding. For example, MTP seems less likely as a genetic cause of VLDL-Apo B overproduction, because no evidence of linkage of MTP locus (4q22-q24) to FCHL has been observed so far [71] (see Appendix).

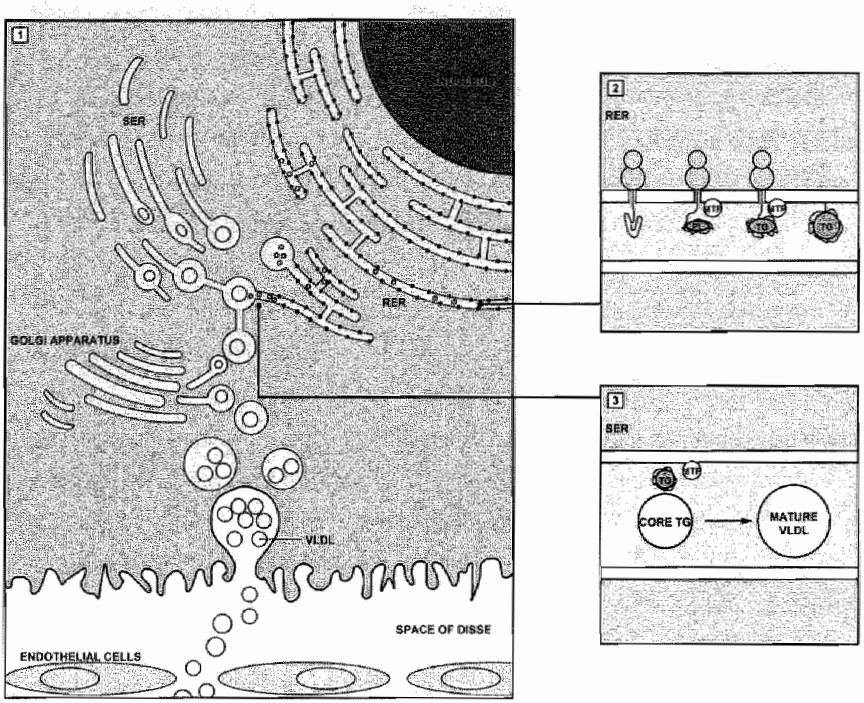

Figure 2. Mechanism of hepatic VLDL-Apo B production

1) Secretory apparatus of the hepatocyte, RER = rough endoplasmatic reticulum; SER = smooth endoplasmatic reticulum; 2) **The first step** of VLDL assembly: Apo B is translated, and simultaneously lipidated by MTP to a pre-VLDL particle in RER, MTP = microsomal triglyceride transfer protein; PL = phospholipids; TG = triglycerides; 3) **The second step** of VLDL assembly: the pre-VLDL particle fuses with an Apo-B free lipid droplet to form a mature VLDL particle.

3. Hyperlipidemia in FCHL

The classical phenomenology of hyperlipidemia describes the lipoprotein abnormalities in FCHL as elevation of plasma VLDL (Fredrickson type IV), or LDL (type IIa), or a combination of both (type IIb). Manifestation of different lipoprotein phenotypes among family members is a hallmark of a FCHL family. This phenomenon has not been fully understood, but genetic and metabolic differences among individuals can be involved.

A more contemporary approach has identified a clustered entity of elevated plasma triglycerides, low HDL cholesterol and predominance of small dense LDL as a phenotype frequently observed in FCHL subjects. In the population, this profile has been named the atherogenic lipoprotein phenotype, because of its association with increased risk for cardiovascular disease[79]. We have studied this adverse phenotype and its metabolic determinants in more detail (chapter 2)[80].

The following factors are known to contribute to the lipoprotein profile in FCHL:

A. Overproduction: discussed above.

B. Disturbed catabolism of lipoprotein particles in FCHL.

- *Impaired clearance by LPL.* Postprandial hyperlipidemia is particularly atherogenic[81]. It involves impaired clearance of VLDL, VLDL remnants and chylomicrons, partly because of impaired conversion of chylomicron and large VLDL particles to remnants[14, 35]. One explanation is relative insufficiency of LPL, because of competition for the common lipolytic pathway[78]. Reduced LPL activity, which may have genetic basis[82], has been reported in a subset of FCHL patients[83]. Although mutations in the LPL do not represent a major gene defect[18], LPL activity may be an important factor in FCHL, taken into consideration its common polymorphisms and impaired function under conditions of insulin resistance[84]. Furthermore, LPL activity reflects PPAR-α activity, because it increases during therapy with fibrates[85], which are PPAR-α activators. The role of PPAR-α as modifier gene in FCHL has been recently described[86].

- *Other enzymes involved in lipoprotein catabolism.* HL and CETP are genetic factors, which contribute to the expression of atherogenic lipoprotein phenotype in FCHL[87, 88].

- *Apolipoprotein composition.* Surface apolipoproteins determine catabolism and distribution of excess VLDL and VLDL remnants to organs in the body including liver,

adipose tissue and vascular wall. For example, Apo CII is a cofactor for LPL, whereas Apo CIII inhibits LPL activity, at least in vitro. Apo E mediates hepatic clearance, as well as peripheral uptake and lipid delivery. Association of the Apo AI-CIII-AIV-AV gene cluster with plasma triglyceride levels has been shown in FCHL[15]. However, Apo CIII content of VLDL particles appears normal in FCHL[89].

- *Hepatic remnant clearance* can be another factor that contributes to hyperlipidemia in FCHL. FCHL carriers of the Apo E2 allele, tend to have higher triglyceride levels than subjects with the Apo E3 or E4 genotype[90]. Hepatic clearance of LDL seems to be relatively insufficient as well, at least in some subjects[91], because treatment with statins, which are known to upregulate hepatic LDL receptors, leads to normalization of plasma LDL-cholesterol levels[92].

All combined, the data clearly indicate that alterations in VLDL pathway can contribute to the expression of FCHL. This pathway carries most of plasma triglycerides in the fasting state, which is the main focus of this thesis. We subsequently refer to it as very low-density lipoprotein-triacylglycerol pathway, **VLDL-TG pathway**.

4 Scope of This Thesis

This thesis focused on the VLDL pathway and hypertriglyceridemia in FCHL. We aimed to identify metabolic factors that can explain the lipoprotein heterogeneity in FCHL. The classical approach to divide FCHL in three major types: Fredrickson Type IIa, IIb and IV on the basis of lipoprotein abnormalities was substituted by simpler one: presence or absence of elevated plasma triglycerides.

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Chapter 2

Subclasses of Low-Density Lipoprotein and Very Low-Density Lipoprotein in Familial Combined Hyperlipidemia. Relationship to Multiple Lipoprotein Phenotype.

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Abstract

The present study addressed the presence of distinct metabolic phenotypes in FCHL, in relation to small dense LDL (sd LDL) and VLDL subclasses. Hyperlipidemic FCHL relatives (n=72) were analyzed for LDL size by gradient gel electrophoresis. Pattern B LDL (sd LDL, particle size < 258 Å), and pattern A LDL (buoyant LDL, particle size ≥ 258 Å) were defined. Analyses showed bimodal distribution of LDL size, associated with distinct phenotypes. Subjects with predominantly large, buoyant LDL showed a hypercholesterolemic phenotype and the highest Apo B levels. Subjects with predominantly sd LDL showed a hypertriglyceridemic, low HDL-cholesterol phenotype, with moderately elevated Apo B, total and LDL cholesterol levels. Subjects with both buoyant LDL and sd LDL (pattern AB, n=7) showed an intermediate phenotype, with high normal plasma triglycerides. VLDL subfraction analysis showed that the sd LDL phenotype was associated with a 10-times higher number of VLDL1 particles of relatively lower Apo AI and Apo E content, as well as smaller VLDL2 particles, in combination with increased plasma insulin concentration in comparison to pattern A. The present observations underscore the importance of the VLDL-TG metabolic pathway in FCHL as an important determinant of the phenotypic heterogeneity of the disorder.

Introduction

FCHL is a metabolic disease, delineated as a genetic disorder of lipid metabolism almost 3 decades ago[1]. It is associated with a two to five-fold increased risk of premature coronary artery disease (CAD)[1, 2]. In spite of recent progress, the genetic and metabolic background of FCHL have not been elucidated in detail. Subjects with FCHL present with a complex phenotype which expression is influenced by genetic, metabolic and environmental factors[3-6]. Affected FCHL relatives are viscerally obese[2, 4, 7], hyperinsulinemic[3], insulin resistant[5, 7] and can show a number of abnormalities in lipid metabolism: hypercholesterolemia and/or hypertriglyceridemia, elevated Apo B levels, small dense LDL (sd LDL), and decreased plasma HDL-cholesterol concentrations.

VLDL and LDL consist of distinct, physicochemically heterogenic subclasses[8]. A practical characterization of the LDL profile divides it into two major phenotypes: pattern A, characterized by a preponderance of large, buoyant particles, with peak particle diameter (PPD) ≥ 258 Å, and pattern B, characterized by predominance of sd LDL particles, with PPD < 258 Å. In the population, sd LDL phenotype and the concurrent metabolic abnormalities (relative hypertriglyceridemia and low HDL-cholesterol) have been designated the Atherogenic Lipoprotein Phenotype[9], consistent with its association with an increased risk of CAD[9, 10]. Furthermore, pattern B LDL has been recognized as a feature of the metabolic syndrome[11] and is characteristic for insulin resistant states, such as type 2 DM[12]. It has been reported that presence of sd LDL is an inherent component of the dyslipidemia in FCHL[6, 13-15] and shares genetic determinants with the expression of FCHL[6, 13, 14].

The aim of the present study was to investigate in detail the sd LDL phenomenon in FCHL. Kinetic studies have shown a metabolic relationship between hepatic VLDL1 production and the appearance of sd LDL in plasma[8, 16, 17]. It has been shown that FCHL subjects exhibit a higher production rate of VLDL-Apo B than controls[18], but no distinction has been made so far between the VLDL1 or VLDL2 subclasses overproduced. We examined whether specific metabolic phenotypes are associated with pattern A or pattern B LDL in hyperlipidemic FCHL relatives, whether a relationship exists with the phenomenon of multiple lipoprotein phenotypes[1], and if specific VLDL subclasses are involved. Therefore, VLDL1 and VLDL2 subclasses have been analyzed with regard to lipid and apolipoprotein

composition in carriers of pattern A, B and AB LDL subspecies. This is the first study, to our knowledge, that addresses this issue in FCHL.

Methods

Subjects

Hyperlipidemic FCHL relatives (n=72, 36 men and 36 women) were recruited at the Lipid Clinic of the Maastricht University Hospital. FCHL families (n=27) were ascertained as previously described[4]. Briefly, FCHL probands had a primary hyperlipidemia with varying phenotypic expression, including fasting plasma cholesterol (TC) > 6.5 mmol/l and/or fasting plasma triglyceride (TG) concentration > 2.3 mmol/l, and a positive family history of premature CAD, i.e. before the age of 60. In addition, FCHL probands had no tendon xanthomas, no Apo E2/E2 genotype, and normal thyroid-stimulating hormone concentrations. Obesity (BMI > 30 kg/m^2) or diabetes was an exclusion criterion for the ascertainment of a FCHL proband. The hyperlipidemic FCHL subjects, who were included in the present study, had been ascertained as an affected relative in a FCHL family, which contained at least one other first-degree relative with a different lipoprotein phenotype[1]. In the present study, 38 subjects exhibited Fredrickson IIa lipoprotein phenotype, 17 type IIb and 17 type IV. The Human Investigation Review Committee of the Academic Hospital Maastricht approved the study protocol and all subjects gave informed consent.

Methods

Subjects were studied after an overnight fast (12-14 h) and at least 3 days without alcohol consumption. Any lipid lowering medication was stopped for two weeks before blood samples were collected. Venous blood was collected in pre-cooled EDTA (1mg/mL) containing tubes; anthropometric measurements, calculation of waist-to-hip ratio (WHR) and BMI, and measurements of fasting plasma concentrations of lipids, lipoproteins, and insulin were performed as described[4]. Analyses of LDL subclass distributions, calculation of LDL peak particle diameter, and assignment of qualitative LDL subclass pattern were done in by means of nondenaturing gradient gel electrophoresis in the Lawrence Berkeley National

Laboratory, University of California, Berkeley, USA, as described elsewhere[8, 14]. VLDL1 and VLDL2 subfractions were separated by density gradient ultracentrifugation, as described by Zhao et al.[19], with minor modifications, which represent ultracentrifugation at 160 000 *g* for 2.5h at 4° C in a SW40 Ti rotor, and collection of fractions starting from the top of the tube, where the upper 1.5 ml represents VLDL1 and the lower 5 ml represent VLDL2. In the VLDL1 and VLDL2 subfractions, concentrations of cholesterol and triglyceride were determined in triplicate by standard laboratory techniques, and apolipoproteins (Apo) AI, AII, B, CII, CIII and E in duplicate by commercial immunoassay Human Apolipoprotein Lincoplex Kit (Cat. APO-62K, Linco Research, Inc., Missouri, USA).

Statistical analyses

A t test was used to analyze differences between the groups. Log transformed values of triglycerides, BMI, insulin were used in the analyses, because these variables did not follow the normal distribution. Kolmogorov-Smirnov statistics was used to test normality of LDL size distribution. Pearson correlation coefficient (r) was used to describe relation between plasma and VLDL subfraction triglycerides in univariate analysis. Mann-Whitney test was used in the VLDL subclass analysis because of the sample size (n=15). In all analyses the statistical package SSPS 11.0 (SSPS Inc) was used.

Results

Distribution of LDL size and associated metabolic phenotypes in hyperlipidemic FCHL subjects

The histogram of LDL particle size in hyperlipidemic FCHL subjects showed a clear, bimodal distribution (p=0.001, Figure 1). The mean diameter of pattern A LDL was 268.1Å, and the mean of pattern B LDL was 250.9 Å. Seven subjects showed an intermediate LDL phenotype[9](pattern AB), with average LDL particle diameter of 261.5 Å, indicating the presence of large, buoyant LDL and sd LDL.

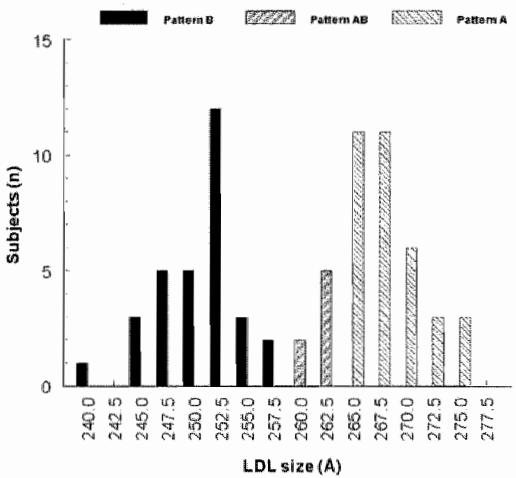

Figure 1.
Frequency distribution of LDL size in hyperlipidemic FCHL relatives.

Potential differences in metabolic phenotypes between carriers of pattern A versus B LDL were evaluated. The hyperlipidemic carriers of pattern B LDL (n=31) showed significantly higher triglycerides (TG=2.8 mmol/l vs. 1.5 mmol/l in carriers of pattern A LDL, p<0.001), but, remarkably, significantly lower total, LDL and HDL cholesterol, Apo B and Apo A1 in comparison with pattern A LDL carriers (Table 1). Of the pattern B LDL carriers, 51.6 % (16 of 31) had total cholesterol < 6.5 mmol/l. Thus, pattern B LDL associated phenotype is consistent with either Fredrickson phenotypes IIb (plasma TG > 2.3 mmol/L and LDL-chol > 4.1 mmol/L), or IV (TG > 2.3 mmol/L and LDL-chol < 4.1 mmol/L).

Furthermore, subjects with pattern B LDL showed statistically significant higher plasma insulin concentrations. By contrast, nearly all (30 of 34, or 88%) hyperlipidemic pattern A LDL subjects showed plasma TC > 6.5 mmol/l in combination with normal TG (<2.3 mmol/l), representing hypercholesterolemia per se. Thus, the pattern A associated phenotype resembles the classical Fredrickson phenotype IIa (LDL cholesterol > 4.1 mmol/L and TG < 2.3 mmol/L). Carriers of pattern AB LDL showed intermediate phenotype, which differed significantly from the pattern B associated metabolic phenotype only in plasma TG. Of the pattern AB subjects, 6 exhibited Fredrickson IIa and 1 subject IIb phenotype.

Table 1. Comparison of metabolic phenotypes between subjects with pattern A, pattern B or pattern AB LDL among hyperlipidemic FCHL subjects (n=72).

	Pattern A LDL	**Pattern B LDL**	**Pattern AB LDL**
No., M/F	34 (17/17)	31 (17/14)	7 (2/5)
Age, yrs	51.9 ± 13.6	51.5 ± 10.0	43.0 ± 17.7
TG, mmol/l	1.5 ± 0.5	2.8 ± 0.6***	1.7 ± 0.4†††
TC, mmol/l	7.4 ± 0.9	6.4 ± 1.4**	7.3 ± 0.8
LDL-chol, mmol/l	5.8 ± 0.9	4.5 ± 1.4***	5.6 ± 0.8
HDL-chol, mmol/l, M/F	0.9 ± 0.2 / 1.1 ± 0.2	0.7 ± 0.2* / 0.8 ± 0.2 **	0.8 ± 0.3 / 0.9 ± 0.2
Apo A1, g/l	1.5 ± 0.2	1.3 ± 0.2**	1.4 ± 0.2
Apo B, g/l	1.5 ± 0.2	1.4 ± 0.2*	1.6 ± 0.3
LDL PPD, Å	268.1± 3.2	251.0± 3.9***	261.5 ± 2.1†††,‡‡
Insulin, μU/ml	7.4 ± 4.2	10.6 ± 6.4*	9.9 ±7.0
BMI	26.8 ± 3.6	28.1 ± 4.6	28.5 ± 5.0
WHR	0.94 ± 0.07	0.95 ± 0.07	0.91 ± 0.11

Values are Mean ± SD.

Statistical difference, adjusted for age and gender: *p< 0.05, **p< 0.01, ***p< 0.001 Pattern A vs. Pattern B; †††p< 0.001 Pattern B vs. AB; ‡‡ p< 0.001 Pattern A vs. AB.

M/F = male/ female, TG = triglycerides, TC = total cholesterol, LDL-chol = LDL cholesterol, HDL-chol = HDL-cholesterol, Apo A1 = apolipoprotein A1, Apo B = apolipoprotein B, LDL PPD = LDL peak particle diameter, BMI = body mass index

Relationship between Plasma and VLDL subfraction triglycerides

Subsequently, the lipid composition of VLDL1 and VLDL2 subclasses were analyzed in typical carriers of pattern A (n = 15), pattern B (n = 15) or AB LDL (n = 6).

In subjects with pattern B LDL, a statistically significant relationship was found between plasma triglycerides and VLDL1-TG (r = .61; p= .015), but not VLDL2. Subjects with pattern AB (n = 6) showed a similar relationship between plasma triglycerides and VLDL1-TG, as observed in subjects with pattern B, although it did not reach statistical significance(r =. 70; p= .12). In contrast, in subjects with pattern A LDL, a statistically significant relationship was found between plasma triglycerides and VLDL2-TG (r =. 52, p =. 047). The relationship with VLDL1-TG approached statistical significance(r =. 47; p= .08). Therefore, the largest contribution to hypertriglyceridemia in pattern B carriers (and probably in pattern AB carriers) comes from VLDL1-TG (Figure 2). In pattern A, VLDL2-TG and, to a lesser extent, VLDL1-TG contribute to plasma TG concentrations. Noteworthy, there was a statistically significant positive relationship between VLDL1-TG and plasma insulin levels in pattern B subjects (r =. 64, p=. 01), but not in pattern A (Figure 3).

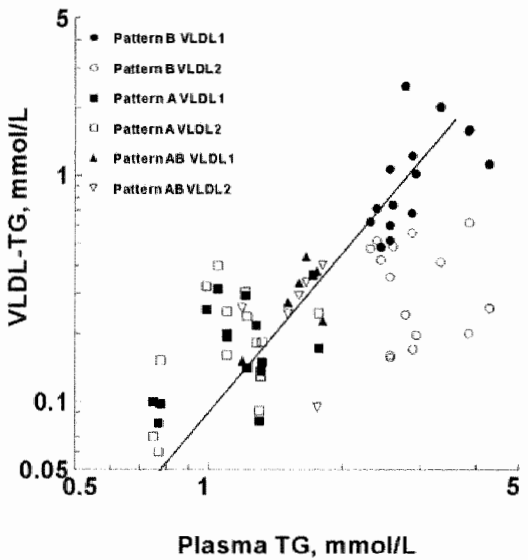

Figure 2.
Plasma TG concentrations in all hyperlipidemic FCHL subjects were correlated with an increase in VLDL1-TG (r=. 91; p < 0.001, regression line shown) and VLDL2-TG (r= 0.47; P<0.01), which reflected increased particle number rather than particle size (as shown in Table 2).

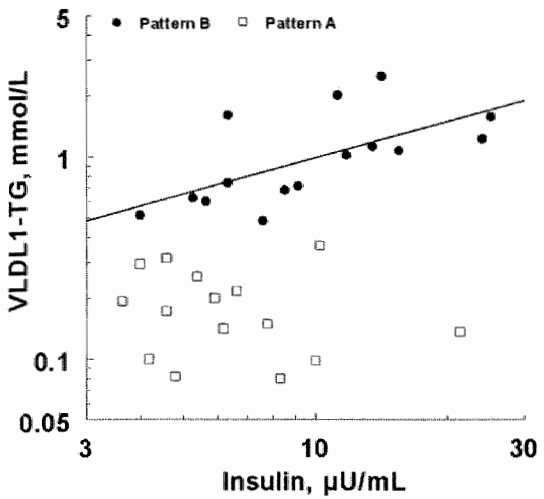

Figure 3.

There was a statistically significant positive relationship between VLDL1-TG and plasma insulin levels in pattern B subjects (r =. 64, p =. 01), but not in pattern A. Data are presented on a log scale.

Lipid profile of VLDL1 and VLDL2 in patterns A, AB or B.

Because there is only one Apo B molecule per VLDL particle, the concentration of Apo B (nmol/L) provides information about the number of VLDL particles in plasma. Pattern B subjects showed a 10-fold higher concentration of Apo B in both VLDL subfractions than pattern A subjects (Table 2), and therefore had around 10 times more VLDL1 and VLDL2 particles in plasma, despite the fact that their mean plasma Apo B concentration was slightly lower (Table 1). Pattern AB subjects showed the most striking increase in their VLDL2 particle number, which was statistically significant when compared to pattern A subjects.

In pattern B subjects, VLDL1 subfraction had a 4-fold higher TG content than the corresponding VLDL2 subfraction (Table 2). By comparison, in carriers of pattern A LDL this ratio was 0.95 ($p<0.001$). Also, VLDL1 in pattern B subjects contained 73 % of all VLDL triglycerides and 65 % of VLDL cholesterol. By contrast, VLDL1 in pattern A subjects contained about half of all VLDL TG, i.e. 48 %, and only 37 % of VLDL cholesterol. With regard to particle lipid composition, pattern A and pattern B subjects showed variations in VLDL1 particle triglyceride and cholesterol content, but on the average there was no statistical difference in lipid composition of theVLDL1 particles between the three groups. (Table 2). Therefore the higher triglyceride content of the VLDL1 subfraction in pattern B

subjects was due to a higher VLDL1 particle number, but not to the presence of triglyceride-richer VLDL1 (larger) particles. Interestingly, pattern A subjects seemed to have triglyceride- and cholesterol-enriched VLDL2, i.e. relatively larger particles, compared to pattern B and pattern AB subjects. Pattern B and pattern AB carriers showed remarkable similarity in VLDL2 lipid content, i.e. reflecting relatively smaller VLDL2 particles.

Table 2. Lipoprotein profiles of VLDL1 and 2 in hyperlipidemic FCHL subjects

	Pattern A LDL	Pattern B LDL	P Value	Pattern AB
No.	15	15		6
VLDL1				
Apo B‡, nmol/L	0.4 ± 0.3	4.6 ± 3.8	0.04	1.0 ± 0.5
TG per Apo B‡, mmol/mg	0.7 ± 0.3	1.2 ± 1.6	NS	0.6 ± 0.3
Total TG (mmol/l)	0.19 ± 0.09	1.10 ± 0.59	p<0.001	0.30 ± 0.10***
% of total VLDL-TG	47.9 ± 6.6	73.1 ± 14.3		52.6 ± 16.1
Chol per Apo B mmol/mg	0.2 ± 0.1	0.56 ± 0.70	NS	0.30 ± 0.50
Total Cholesterol (mmol/l)	0.06 (0.03-0.08)	0.43 (0.22-0.67)	p<0.001	0.15 ± 0.04***· ††
% of total VLDL-Chol	37.0 ± 12.5	65.1 ± 18.0		40.3 ± 16.0
VLDL2				
Apo B‡, nmol/L	1.2 ± 1.4	11.4 ± 1.8	0.004	8.1 ± 2.3††
TG per Apo B‡, mmol/mg	0.7 ± 0.9	0.09 ± 0.01	0.095	0.08 ± 0.01 †
Total TG (mmol/l)	0.21 ± 0.11	0.35 ± 0.16	0.045	0.27 ± 0.10
% of total VLDL-TG	52.1 ± 6.6	26.9 ± 14.3		47.4 ± 16.1
Chol per Apo B mmol/mg	0.3 ± 0.1	0.07 ± 0.02	0.095	0.07 ± 0.01
Total Cholesterol (mmol/l)	0.11 ± 0.10	0.25 ± 0.12	0.003	0.25 ± 0.11†
% of total VLDL-Chol	63.0 ± 12.5	34.9 ± 18.0		59.7 ± 16.0
VLDL1-TG/VLDL2-TG	0.95 ± 0.26	4.06 ± 2.91	p<0.001	1.13 (0.57-1.98)*
VLDL1-Chol/VLDL2-Chol	0.65 ± 0.35	2.20 (0.95-4.67)	p<0.001	0.64 (0.35 – 1.14)*

Values are Mean ± SD, Median (interquartile range), VLDL-TG = triglycerides in VLDL, VLDL-Chol = cholesterol in VLDL, NS = not significant. Pattern B vs. pattern AB: ***$p < 0.001$, **$p < 0.01$, *$p < 0.05$; Pattern A vs. pattern AB: ††$p = 0.01$, †$p < 0.05$. ‡ Pattern A, n = 6; Pattern B, n = 6; Pattern AB, n = 5 in these analyses.

Apolipoprotein profiles of VLDL1 and VLDL2

Concentrations of apolipoproteins (Apo) AI, Apo AII, Apo CII, Apo CIII, Apo E and Apo B were measured in VLDL1 and VLDL2 subfractions in representative subjects of the FCHL patients described in Table 2. Figure 4 represents the apolipoprotein content of the VLDL1 particles in pattern A (n = 6), B (n = 6), or AB (n = 5) subjects, expressed as percent of total apolipoproteins (set at 100 %) calculated per Apo B (i.e. per VLDL particle). Noteworthy, VLDL1 particles of subjects with pattern A exhibited significantly higher Apo AI content in comparison to subjects with pattern B and AB, and significantly higher Apo E than pattern B. A similar tendency was observed for VLDL2 particles, although it did not reach statistical significance. Of note, the Apo E genotype of the subjects studied was analyzed and there was no explanation of the present results by the distribution of alleles.

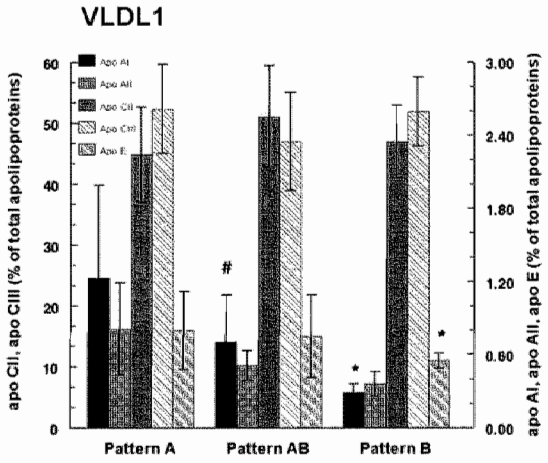

Figure 4.

Apolipoprotein profile of VLDL1 particles in hyperlipidemic FCHL carriers of pattern A, AB or B LDL subspecies. *P<0.05 pattern B vs. pattern A. # P<0.05 Pattern AB vs. A. Data are expressed as percent of total apolipoproteins (mol) per Apo B (mol).

Discussion

The present findings showed that hyperlipidemic relatives from well-defined FCHL families exhibited a bimodal distribution of LDL size, in turn associated with two metabolically distinct phenotypes. Carriers of small dense LDL showed a hypertriglyceridemic, low HDL-cholesterol phenotype, quite similar to the Atherogenic Lipoprotein Phenotype[9], with moderately elevated plasma concentrations of Apo B, total, and LDL cholesterol. Therefore, carriers of sd LDL, also named pattern B LDL in this study, expressed hypertriglyceridemia either as Fredrickson IIb phenotype, considered classical for FCHL, or hypertriglyceridemia per se (type IV). Carriers of large and buoyant LDL particles, named pattern A LDL in this study, were characterized by a hypercholesterolemic phenotype with high Apo B and high LDL cholesterol in combination with near normal plasma TG concentrations. Thus, pattern A LDL is associated with Fredrickson type IIa. The existence of distinct metabolic phenotypes appears to be related to VLDL particle metabolism. This was reflected by a 10-fold higher number of VLDL1 particles, with reduced Apo AI and Apo E content, and in addition, smaller size of VLDL2 in plasma of FCHL pattern B subjects compared to pattern A. The present findings are consistent with the original observation of bimodality of plasma TG in FCHL[1] and offer further insight of the pathophysiology behind the multiple lipoprotein phenotypes in FCHL.

A mechanism that is relevant to VLDL metabolism in insulin resistance[20] and which appears to be important in FCHL as well is hepatic secretion of heterogeneous VLDL subspecies[16]. It has been shown that FCHL subjects exhibit a 2.7-fold overproduction[18] of VLDL-Apo B. However, no distinction has been made thus far between the VLDL subclasses overproduced and their heterogeneous catabolism[8, 21]. A novel finding in the present study is the 10-fold increased number of VLDL1 particles in pattern B subjects compared to pattern A subjects. Moreover, the VLDL1-TG content was the main contributor to the hypertriglyceridemia in pattern B FCHL subjects. In addition, pattern B subjects showed higher plasma insulin concentrations, a surrogate marker of insulin resistance, and a statistically significant positive relationship between the latter and VLDL1-TG, whereas such a relationship was not found in pattern A subjects. Increased VLDL1 secretion in insulin resistance has been formally demonstrated by stable isotope methodology in patients with

type 2 DM[22]. Such a kinetic study has not been performed in FCHL to date, but altogether it is plausible that a similar mechanism is operational in hypertriglyceridemic FCHL subjects as well. VLDL2 secretion, in contrast to VLDL1, is not regulated by insulin[20], but is dependent, at least in part, on cholesterol availability in the liver[8]. VLDL2 are preferentially metabolized to large, buoyant LDL[8]. Accordingly, a relatively increased production rate of VLDL2-Apo B, or direct synthesis of LDL-ApoB[8, 16] can lead to the phenotypic expression of isolated hypercholesterolemia with normal LDL size in pattern A subjects. Therefore, we suggest that differences in liver insulin sensitivity in FCHL cause differences in secretion of VLDL1 and VLDL2 that can explain, at least in part, the present findings. Moreover, a similar mechanism can underlie the change of lipid phenotype observed in FCHL subjects[23, 24].

The present observations underscore the biological importance of the VLDL-TG metabolic pathway in FCHL, especially when put in the perspective of reported linkage and association studies. In FCHL, linkage and association have been described with several genes encoding for apolipoproteins that are part of VLDL lipoproteins: the Apo AI-CIII-AIV-AV gene cluster, Apo AII gene, and plasma concentrations of Apo CIII[6, 25], Apo AII[26] and Apo B[27]. Moreover, linkage between Apo AI-CIII-AIV-AV gene cluster and the presence of sd LDL has been reported[6]. The effect of different apolipoproteins on VLDL catabolism is well known. For instance, Apo E affects the hepatic uptake of VLDL subfractions; Apo E, Apo CII and Apo CIII affect lipolysis, and finally, VLDL apolipoproteins modulate lipid and protein exchange with other lipoproteins. In the present study, Apo AI and Apo E were less abundant on VLDL particles in pattern B FCHL subjects (Figure 4) than in pattern A. Therefore, our data suggest that changes in VLDL apolipoprotein content may be relevant for VLDL metabolism by lipolysis and hepatic clearance, which can contribute to the accumulation of VLDL1 (and VLDL2) lipoproteins observed in plasma in FCHL subjects with sd LDL. Furthermore, it has been shown that accumulation of VLDL1 in plasma, and modifier genes that affect VLDL1 catabolism, such as CETP, hepatic lipase and lipoprotein lipase, affect sd LDL frequency in FCHL[6, 8, 14, 28, 29].

The cross-sectional design of this study prevents a definitive conclusion on the metabolical pathways involved in the phenotypic expression of FCHL. It is worth mentioning, however, that our present findings are consistent with a recent publication by Ayyobi et. al[30], which associates the difference in lipoprotein phenotypes in FCHL with changes in VLDL

and large, buoyant LDL levels. This study[30] and the present observations reflect long-term adaptation changes in FCHL and are therefore difficult to interpret in a simple manner. Further insight in the relative contribution of VLDL subclasses secretion and catabolism to the FCHL phenotype will require stable isotope studies.

In summary, a novel finding in the present study is that hyperlipidemic FCHL subjects showed bimodal distribution of LDL size, and each peak of LDL subclasses corresponded to a distinct phenotype. Subjects with predominance of large buoyant LDL showed a hypercholesterolemic phenotype (Fredrickson type IIa), and the highest Apo B levels. Subjects with predominance of sd LDL presented with a hypertriglyceridemic, low HDL-cholesterol phenotype, moderately elevated Apo B levels, total and LDL cholesterol (type IIb and IV), and in addition, were characterized by 10-times higher number of VLDL particles of lower Apo AI and Apo E content (VLDL1) and smaller size (VLDL2) in plasma, compared to pattern A. The present observations underscore the importance of the VLDL-TG metabolic pathway in FCHL as an important determinant of the phenotypic heterogeneity of the disorder.

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Chapter 3

Radiological Evidence of Nonalcoholic Fatty Liver Disease in Familial Combined Hyperlipidemia.

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Introduction

Familial combined hyperlipidemia is a genetic dyslipidemia (Mendelian Inheritance in Man, MIM 144250) associated with hepatic lipoprotein overproduction, insulin resistance and increased plasma concentrations of free fatty acids[1]. Similar alterations in metabolism are known to contribute to nonalcoholic fatty liver disease[2]. Nonalcoholic fatty liver disease comprises both nonalcoholic fatty liver and nonalcoholic steatohepatitis. The definitive diagnosis of the different stages of nonalcoholic fatty liver disease requires a biopsy, but ultrasonography is regarded as a highly sensitive (89%) and specific (93%) method to assess steatosis[3]. The present study evaluated the prevalence of nonalcoholic fatty liver disease in familial combined hyperlipidemia.

Methods

We evaluated seventeen patients with familial combined hyperlipidemia by upper abdomen ultrasonography, between July 2000 and July 2002. These patients were consecutively referred to the Lipid Clinic of the Maastricht University Hospital, and therefore unselected. Each patient represented a separate familial combined hyperlipidemia family. Diagnostic characteristics of familial combined hyperlipidemia families are premature myocardial infarction (before age 60), and a combination of multiple Fredrickson phenotypes (IIb, IIa, or IV) among first-degree relatives[4]. The patients with familial combined hyperlipidemia, included in this study, had a premature myocardial infarction, or a family history of such a premature myocardial infarction in a parent, uncle or aunt. In addition, these patients showed a primary hyperlipidemia, usually Fredrickson type IIb and IV. Cut-off points are plasma cholesterol > 6.5 mmol/L (250 mg/dL) and TG > 2.3 mmol/L (200 mg/dL). Known causes of nonalcoholic fatty liver disease such as morbid obesity, type 2 diabetes, viral and toxic hepatitis, and ingestion of more than 2 alcoholic beverages per day (20 g of alcohol) were excluded[2]. Sixteen of these seventeen patients were receiving statins; one patient without steatosis was treated with a fibrate. The lipid-lowering medication was not discontinued during the study. The control patients (n=792) represented an unselected sample

of consecutive patients who presented for upper abdominal ultrasonography in the period January 2001 to March 2001 at the University Hospital Maastricht, excluding patients with familial combined hyperlipidemia. No other clinical data was available. The study was performed with the approval of the Human Investigation Review Committee of the Academic Hospital Maastricht.

Ultrasound

Ultrasonography of the liver was done at the Department of Radiology using an ATL HDI 5000 system (Bothell, Washington, USA) with a C 5-2 transducer by independent radiologists. Conventional criteria for nonalcoholic fatty liver disease are increased echogenicity of liver compared to kidney parenchyma, decreased visualization of intrahepatic blood vessels, and posterior beam attenuation[3].

Measurements

Anthropometric measurements and measurements of fasting plasma cholesterol, triglycerides and apolipoprotein B were done as described[4]. Serum alanine and aspartate aminotransferase, and gamma-glutamyl transferase levels were assessed in the Clinical Chemistry Laboratory of the Maastricht University Hospital.

Statistical analyses

Mann-Whitney nonparametric test was used to analyze differences between the groups in Table 1, and Fisher's exact test to compare the prevalence of nonalcoholic fatty liver disease between familial combined hyperlipidemia patients and the control sample. Statistical analyses were performed using the Statistical Package for Social Sciences software (SSPS 11.0 for Windows, SSPS Inc., Chicago, Illinois).

Results

Nonalcoholic fatty liver disease was found in 76% (13 of 17) of patients with familial combined hyperlipidemia (95% CI: 56% to 96%). By contrast, in the control sample, 10% (80

of 792; 95% CI: 8% to 12 %) were diagnosed with nonalcoholic fatty liver disease (P < 0.001). Notably, 92 % (12 of 13) of the patients with familial combined hyperlipidemia and nonalcoholic fatty liver disease exhibited hypertriglyceridemia (Table 1), either as combined hyperlipidemia (Fredrickson type IIb; n=6), or as isolated hypertriglyceridemia (Fredrickson type IV; n=6).

Table 1. Characteristics of FCHL patients with or without radiological evidence of nonalcoholic fatty liver disease.

	Steatosis present (n = 13)	**Steatosis absent (n = 4)**	**P Value**
Male sex	10 (77)	2 (50)	
Age, yrs	51 ± 11	65 ± 10	0.01
FCHL markers:			
Waist-to-hip ratio	0.94 ± 0.06	0.94 ± 0.06	0.95
Body mass index, kg/m^2	27 ± 3	26 ± 4	0.35
Total cholesterol, mmol/L*	6.5 ± 1.5	5.5 ± 0.9	0.20
Triglycerides, mmol/L†	3.3 (2.6 – 7.0)	1.6 ± 0.5	0.002
Apolipoprotein B, g/L	1.3 ± 0.3	1.1 ± 0.2	0.91
Liver biochemistry tests:			
Aspartate aminotransferase, U/L	24 ± 7	29 ± 9	0.10
Alanine aminotransferase, U/L	35 ± 15	23 ± 11	0.32
Gamma glutamyltransferase, U/L	37 ± 25	29 ± 22	0.96

Values are number (%), mean ± SD, or median (range).

*For mg/dL divide by 0.026; † For mg/dL divide by 0.011.

One of these 13 subjects exhibited a hypercholesterolemic phenotype (type IIa) with high normal plasma triglycerides of 1.9 mmol/L. Concentrations of serum aminotransferases and gamma glutamyltransferase were not different between familial combined hyperlipidemia subjects with nonalcoholic fatty liver disease and those without. Four subjects with steatosis presented with slightly elevated alanine aminotransferase (> 40 U/L), probably reflecting the

presence of nonalcoholic steatohepatitis. However, ultrasonography cannot make a distinction between nonalcoholic steatohepatitis and nonalcoholic fatty liver.

Discussion

This report established that nonalcoholic fatty liver disease was present in a considerable fraction (95% CI: 56% to 96%) of unselected subjects with familial combined hyperlipidemia and, specifically, in each of the hypertriglyceridemic subjects. The reported prevalence of nonalcoholic fatty liver disease in the general population varies between 10% and 24%[2], similar to the frequency of 10% (95% CI: 8% to 12 %) in our control patient sample. Given an estimated population frequency of up to 2.0% for familial combined hyperlipidemia[5], it can be concluded that it represents a quantitatively important contribution to the prevalence of nonalcoholic fatty liver disease, potentially representing one in five to ten cases of nonalcoholic fatty liver disease in the population.

Familial combined hyperlipidemia is characterized by excess supply of free fatty acids, lipoproteins and lipoprotein remnants to the liver[6], most likely a consequence of insulin resistance and impaired adipose tissue activity in the postprandial state[1]. Therefore, nonalcoholic fatty liver disease may reflect lipid re-distribution, where fatty acids are deposited over time as triglycerides in liver and visceral fat[4,7]. In addition, genetic mechanisms specific for familial combined hyperlipidemia can contribute to abnormal hepatic lipid metabolism and steatosis. We recently identified Peroxisome-Proliferator-Activated Receptor-alpha and -gamma genes as modifier genes in familial combined hyperlipidemia[8,9]. Peroxisome-Proliferator-Activated Receptor-alpha as well as -gamma activity can affect hepatic lipid content, as shown recently[10,11]. The presence of steatosis in subjects with familial combined hyperlipidemia can contribute to increased production of triglyceride carrying very low-density lipoproteins, which results in hypertriglyceridemia. Elevated plasma triglyceride levels were found in 92 % of the patients with familial combined hyperlipidemia and nonalcoholic fatty liver disease. Thus, the present observation suggests a direct relationship between steatosis and hepatic lipoprotein overproduction in familial combined hyperlipidemia. This finding is in line with previous publications that

hypertriglyceridemia is a common finding in patients with nonalcoholic fatty liver disease and insulin resistance[2, 12].

In summary, there is a high prevalence of nonalcoholic fatty liver disease in familial combined hyperlipidemia, which suggests that familial combined hyperlipidemia potentially underlies one in five to ten population cases with nonalcoholic fatty liver disease. The present data suggest that hepatic steatosis is part of familial combined hyperlipidemia, specifically the hypertriglyceridemic phenotype. Further studies may provide insight in the mechanisms leading to nonalcoholic fatty liver disease in familial combined hyperlipidemia, and contribute to measures to prevent steatosis.

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Prothrombotic Markers in Familial Combined Hyperlipidemia. Evidence of Endothelial Cell Activation and Relation to Metabolic Syndrome.

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Abstract

Familial combined hyperlipidemia is characterized by a varied combination of hypertriglyceridemia and hypercholesterolemia within a family, and a high risk of premature coronary artery disease. The present study evaluated a number of potential prothrombotic markers in familial combined hyperlipidemia, and studied their relationship to the hypercholesterolemic (Fredrickson type IIa) and hypertriglyceridemic (IIb and IV) phenotypes. Selected prothrombotic markers were studied in 68 subjects: 34 hyperlipidemic subjects with familial combined hyperlipidemia and 34 controls. FCHL patients exhibited significantly higher Thrombin-Antithrombin complex (TAT), activated coagulation factor XII (F XIIa), von Willebrand Factor (vWF), Plasminogen Activator Inhibitor-1 (PAI-1) and tissue derived Plasminogen Activator (t-PA) values in comparison to controls. Within the subgroup of familial combined hyperlipidemia subjects, elevated PAI-1 activity and soluble Thrombomodulin levels were particularly associated with features of the metabolic syndrome, including hyperinsulinemia, hypertriglyceridemia, and predominance of small dense LDL.
A general pattern of activated blood coagulation and endothelial activation is present in all hyperlipidemic subjects studied, independent of metabolic phenotype. In those familial combined hyperlipidemia subjects with features of the metabolic syndrome, impaired fibrinolysis can provide an additional cardiovascular risk factor.

Introduction

Familial Combined Hyperlipidemia (FCHL) is a genetic metabolic disorder, associated with a two to five-fold increased risk of premature coronary artery disease[1-3]. Characteristic for FCHL is a primary hyperlipidemia with varied phenotypic expression (Fredrickson type IIa, IIb or IV) among FCHL family members[1]. In addition, FCHL subjects can show features of the metabolic syndrome: elevated plasma insulin concentrations in the presence of insulin resistance, visceral obesity, hypertension, decreased plasma HDL concentrations and predominance of sd LDL[2,4].

Systemic blood coagulation activity contributes importantly to acute coronary events[5,6]. Data from several studies suggest that markers of activated blood coagulation (TAT, factor XIIa, D-dimers), impaired fibrinolysis (PAI-1, t-PA) or endothelial activation (vWF, sTM) have a role as indicators of cardiovascular disease, and predict an increased risk of acute events, especially below 60 years of age[7-11]. Furthermore, heterozygous hemophilia carriers show a mild decrease in coagulation activity, but a reduction by 36% of deaths from CAD[12]. Systemic hypercoagulability[5,6] or endothelial abnormalities[13] may contribute to the higher incidence of coronary artery disease in FCHL as well. The aim of this study was to explore the activity profile of the blood coagulation system, in combination with fibrinolytic markers in patients with FCHL. For this purpose we examined, in a cross-sectional way, typical markers of coagulation and fibrinolysis activity, coupled to markers of endothelial cell activation. Some of these markers (PAI-1, TAT, vWF), have been previously associated with specific lipoprotein phenotypes[14-16]. Here we investigated whether the multiple lipoprotein phenotypes in FCHL (Fredrickson type IIa, IIb and IV) share a common procoagulant profile.

Materials and Methods

In the framework of genetic and metabolic studies 34 hyperlipidemic FCHL subjects and 34 healthy spouses (controls) were recruited at the Lipid Clinic of the Maastricht University Hospital. The FCHL subjects represented 16 FCHL families, ascertained as previously described[17]. Briefly, FCHL subjects had: 1) primary hyperlipidemia with varying

phenotypic expression, including (untreated) fasting plasma cholesterol > 6.5 mmol/l and/or fasting plasma triglyceride concentration > 2.3 mmol/l; 2) they were known with premature CAD (before the age of 60), or had a positive family history of CAD; 3) had at least one other first-degree relative with a different lipoprotein phenotype. Exclusion criteria for diagnosing FCHL in a family were diabetes mellitus type 2, causes of secondary lipidemia (nephrotic syndrome, hypothyroidism, morbid obesity), E2/E2 genotype and tendon xanthomas of the proband. The Human Investigation Review Committee of the Academic Hospital Maastricht approved the study protocol. All subjects gave informed consent.

Subjects were studied in the morning (8.00-11.00h) after an overnight fast (12-14 h) and at least 3 days without alcohol consumption. Any lipid-lowering medication was stopped for two weeks before blood samples were collected. Venous blood was collected in pre-cooled EDTA (1mg/mL) containing tubes for measurement of plasma lipids and insulin, and in 3.8% sodium citrate tubes (dilution 1:10), with limited occlusion of the arm by a tourniquet, for measurements of coagulation proteins TAT, vWF, XIIa, D-dimers, TF, PAI-1 and sTM. Blood collected in Biopool® stabilyte™ tubes was used to measure t-PA. The concentration of the TAT complex was measured by commercial ELISA techniques (Enzygnost® Behring Germany); Free factor XIIa by F XIIa ELISA kit, Shield Diagnostics, Ltd, UK; soluble Tissue Factor by Imubind® TF ELISA kit, American Diagnostica Inc.; PAI-1 activity was measured by Spectrolyse® (pl), and t-PA by TintElize / Biopool, Kordia, NL; D Dimers by VIDAS D-Dimer®, Biomerieux, USA; sTM by Trombomodulin Elisa®, Roche Diagnostics, Switzerland. vWf was measured by a home made ELISA, where Dakopatt® capture and conjugated rabbit anti-human vWf polyclonal antibodies were used (Dakopatt®, Denmark).

Anthropometric measurements, calculation of WHR and BMI, and measurements of blood pressure, plasma concentrations of lipids, lipoproteins and insulin were performed exactly as described[17]. Analyses of LDL subclass distributions, and calculation of LDL peak particle diameter were done in fasting plasma samples by means of nondenaturating gradient gel electrophoresis in the Lawrence Berkeley National Laboratory, University of California, Berkeley, USA, as described elsewhere[18]. IMT was measured by ultrasound at the left and right carotid artery in 17 FCHL subjects as described before[17]. The presence of metabolic

syndrome in FCHL subjects was assessed according to the World Health Organization (WHO) criteria[19].

Statistical analysis

Two-sample *t* test, adjusted for age and gender was used to analyze differences between FCHL subjects and controls. Log transformed values for triglycerides, insulin, TAT, PAI-1, t-PA and vWF were used in the analyses because these variables showed skewed distribution. Differences in frequencies between groups were tested by chi-square test. The age and gender adjusted Mann-Whitney nonparametric test was used to compare subjects with different lipoprotein phenotypes within the FCHL group, because of small sample sizes ($n<30$). Pearson partial correlations controlled for age and gender were used to assess relationship between coagulation factors and metabolic variables in univariate analysis, whereas Spearman rank correlation was used to describe the relationship between vWF and IMT because of smaller sample size ($n = 17$). In all analyses the statistical package SSPS 11.0 (SSPS Inc., Chicago, Ill.) was used. Statistical significance was defined as $P < 0.05$.

Results

Coagulation factors

Anthropometric, metabolic and coagulation system characteristics of FCHL subjects and controls are summarized in Table 1. The hyperlipidemic FCHL relatives differed significantly from controls in the following age and sex adjusted metabolic variables: higher plasma levels of total cholesterol, triglycerides, LDL-cholesterol, Apo B, insulin, lower HDL-cholesterol and predominance of sd LDL particles. FCHL subjects and controls had similar BMI, but differed significantly in WHR. With regard to the activity profile of the coagulation system and fibrinolytic markers, FCHL subjects showed significantly higher plasma levels of TAT, vWF antigen, F XIIa, PAI-1 activity and t-PA. Plasma levels of D-dimers, sTF (with large variations), and sTM did not show significant differences between the groups. There were no group differences in confounding variables (blood pressure, prevalence of CAD, smoking, acetylsalicylate or contraceptive use).

Table 1. Characteristics of the subjects studied

	FCHL Hyperlipidemic Subjects	Controls	P value‡
No. (M/F)	34 (15/19)	34 (18/16)	
Age, yrs	50.1 ± 10.9	48.6 ± 13.0	NS
TC, mmol/l	7.3 ± 1.2	5.1 ± 0.8	p<0.001
TG*, mmol/l	2.0 (1.3 - 2.5)	1.1 (0.8 - 1.4)	p<0.001
HDL, mmol/l	0.9 ± 0.3	1.0 ± 0.2	0.015
LDL, mmol/l	5.3 ±1.2	3.6 ± 0.7	p<0.001
LDL size, Å	259.6 ± 8.2	267.1 ± 5.9	p<0.001
Apo B, g/l	1.5 ± 0.3	1.0 ± 0.2	p<0.001
Insulin*, μU/ml	7.7 (4.3 - 11.2)	3.4 (2 - 6.4)	p<0.001
WHR	0.93 ± 0.07	0.89 ± 0.1	0.002
BMI, kg/m^2	27.3 ± 4.4	26.0± 3.2	NS
SBP, mm Hg	142.9 ± 15.2	136.8 ± 23.4	NS
DBP, mm Hg	89.0 ± 9.3	86.5 ± 12.3	NS
Prevalence of CAD†	8 (23.5)	3 (8.8)	NS
Statins	17 (50)	2 (34)	p<0.001
Smoking†	9 (26.5)	9 (26.5)	NS
Acetylsalicylate†	7 (20.6)	4 (11.8)	NS
Oral contraceptives†	1 (2.9)	4 (11.8)	NS
Coagulation Factors:			
TAT*, µg/L	2.6 (2.1 - 2.9)	2.2 (2.1 - 2.3)	0.004
VWF*, %	109.5 (87.8 - 134.8)	84.0 (60.5 - 123.0)	0.03
F XIIa, ng/mL	1.6 ± 0.7	1.2 ± 0.5	0.008
sTF, pg/mL	53.0 ± 99.7	53.1 ± 118.7	NS
D-dimers (ng/mL)	306.3± 164.4	271.7 ± 180.1	NS
PAI-1*, U/mL	16.0 (11.8 - 25.0)	11.0 (6.5 - 15.0)	0.001
t-PA *, ng/mL	10.7 (8.1 - 13.2)	7.9 (4.7 - 10.9)	p<0.001
sTM, ng/mL	31.0 ± 6.9	30.7 ± 10.4	NS

Values are mean ± SD, *median (interquartile range) and †number (%).

‡ Two-sample *t* test, adjusted for age and gender.

Relation to metabolic syndrome parameters

We have recently shown that elevation of plasma triglycerides in FCHL is associated with insulin resistance and a number of lipid abnormalities (increased number of VLDL particles, low HDL-cholesterol, preponderance of sd LDL)[20]. Therefore, the present study compared coagulation variables between hyperlipidemic FCHL subjects with elevated plasma triglycerides (TG > 2.3 mmol/L) and those with isolated hypercholesterolemia (TG < 2.3 mmol/L, Fredrickson type IIa, n = 18). Hypertriglyceridemic FCHL subjects (n = 16) represented 9 subjects with combined hyperlipidemia (Fredrickson type IIb), and 7 subjects with isolated hypertriglyceridemia (Fredrickson IV). Hypertriglyceridemic FCHL subjects had significantly higher insulin levels and predominance of sd LDL particles in comparison to subjects with hypercholesterolemia (Table 2), compatible with presence of features of insulin resistance. This corresponded to significantly higher prevalence of the metabolic syndrome (62.5%) among FCHL subjects with hypertriglyceridemia. In addition to these metabolic characteristics, hypertriglyceridemic FCHL subjects had significantly higher PAI-1 activity and sTM plasma concentrations. In contrast, no differences were observed in elevated TAT, F XIIa, TF, D-dimers, t-PA plasma concentrations, and vWF antigen between hypertriglyceridemic and hypercholesterolemic FCHL relatives. Furthermore, one-way ANOVA analysis, with Bonferroni correction as a post hoc test, did not show any statistically significant difference in coagulation markers between hypertriglyceridemic FCHL subjects of type IIb or IV (data not shown). These findings suggested that two different patterns of prothrombotic abnormalities were present in FCHL. One pattern with an increased rate of thrombin generation (TAT), coupled to endothelial activation (vWF, t-PA), was present in all FCHL subjects, independent of phenotype. A different pattern, suggesting impaired fibrinolysis and involving PAI-1 and sTM, was predominantly associated to features of the metabolic syndrome, with hypertriglyceridemia as its hallmark in FCHL. To evaluate this possibility further, univariate correlations (controlled for age and gender) between metabolic variables and coagulation factors were evaluated in hyperlipidemic FCHL subjects (Table 3). Notably, PAI-1, t-PA, and sTM were linked to variables of the metabolic syndrome by showing a significantly positive relationship to TG and insulin, and significantly negative correlation with LDL particle size. Furthermore, t-PA correlated positively with WHR. F XIIa was positively correlated with insulin and Apo B. TAT levels and vWF antigen did not show

Table 2. Comparison between FCHL subjects with a hypercholesterolemic phenotype and FCHL subjects with a hypertriglyceridemic phenotype (IIb, IV)

	Hypercholesterolemic Phenotype	Hypertriglyceridemic Phenotype	P Value [#]
No. (M/F)	18 (6/12)	16 (9/7)	
Age, yrs	51.3 ± 11.8	48.7 ± 10.0	NS
TG*,mmol/L	1.4(1.1 - 1.7)	2.5 (2.4 - 2.9)	NA
HDL, mmol/L (M/F)[§]	0.8 ± 0.1/ 1.1 ± 0.2	0.8 ± 0.3/ 0.9 ± 0.2	NS
LDL, mmol/L	5.7 ± 0.8	4.9 ± 1.4	0.039
LDL size[‡], Å	265.7 ± 5.3	254.0 ± 6.7	0.001
Insulin*, μU/ml	5.4 (3.6 - 8.4)	9.3 (5.5 - 14.3)	0.019
WHR	0.92 ± 0.07	0.95 ± 0.05	NS
BMI, kg/m^2	26.0 ± 3.4	27.7 ± 4.9	NS
SBP, mm Hg	142.1 ± 16.7	143.7 ± 13.8	NS
DBP, mm Hg	87.4 ± 8.8	90.9 ± 9.8	NS
Prevalence of CAD†	4 (22.2)	4 (25.0)	NS
Metabolic syndrome†	4 (22.2)	10 (62.5)	0.024
Statins†	12 (66.7)	5 (31.6)	0.039
Smoking†	4 (22.2)	5 (31.3)	NS
Acetylsalicylate†	3 (16.7)	4 (25.0)	NS
Coagulation Factors			
TAT*, μg/L	2.5 (2.1 - 3.2)	2.6 (2.1 - 2.8)	NS
VWF*, %	107.0 (92.0 - 132.5)	109.5 (84.0 - 146.0)	NS
F XIIa, ng/mL	1.7 ± 0.9	1.6 ± 0.6	NS
PAI-1*, U/mL	14.0 (9.0 - 18.0)	24.5 (14.3 - 33.0)	0.002
t-PA, * ng/mL	8.5 (7.7 - 13.6)	12.3 (10.4 - 13.2)	NS
sTM*, ng/mL	28.4 ± 5.6	33.9 ± 7.3	0.046

Values are mean ± SD, *median (interquartile range) and †number (%).

NS= not significant; NA=not applicable, because criteria for selection of cases is based on these parameters.

[‡] LDL size below 256 Å designates presence of sd LDL; [§] HDL cholesterol data are shown for men and women separate

[#] Mann-Whitney nonparametric test, adjusted for age and gender.

a significant correlation with any of the tested metabolic traits, but interestingly there was a positive correlation between vWF antigen levels and IMT (rho =.55, p<0.05), (Figure 1). When coagulation factors were correlated with each other, there was a significant positive correlation between v WF and t-PA (r =. 54, p<0.01), and between vWF and TAT (r =. 47,). The expected correlation between PAI-1 and t-PA was present, although it did not reach statistical significance (r = .39, p = 0.07).

Table 3. Pearson Correlation Coefficients between coagulation factors in plasma and metabolic and anthropometric variables in hyperlipidemic FCHL subjects.

Coagulation Factors	TC	LDL	ApoB	TG	LDL size	Insulin	BMI	WHR
TAT	-	-	-	-	-	-	-	-
VWF	-	-	-	-	-	-	-	-
FXIIa	-	-	.41*	-	-	.42*	.33†	-
PAI-1	-	-	-	.62***	-.58**	.46*	.34‡	-
t-PA	-	-	-	.61**	-.46*	.58**	-	.43*
sTM	-	-	-	.46*	-.36§	.35‡	.32§	-

*p< 0.05, **p< 0.01, ***p< 0.001, age and gender adjusted

† P = 0.09, ‡ P = 0.07, §P = 0.06; "-" = Not statistically significant

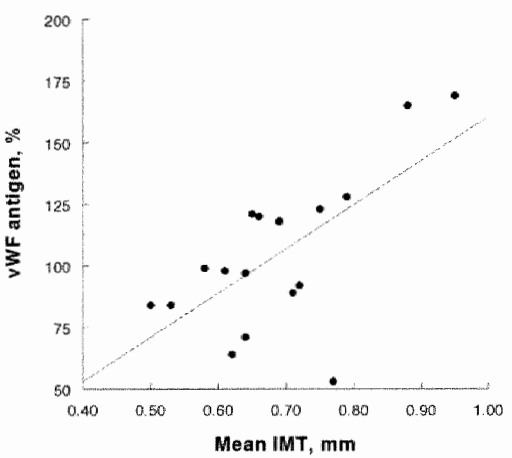

Figure 1.

Relationship between vWF antigen and IMT in FCHL subjects (rho = 0.55, p < 0.05)

Discussion

The present study is the first to evaluate several components of the blood coagulation system in hyperlipidemic FCHL subjects, reported to be at increased risk of premature myocardial infarction[1-3]. Hyperlipidemic FCHL subjects exhibited a prothrombotic phenotype, indicated by significantly higher plasma concentrations of TAT, F XIIa, vWF, and t-PA, in comparison to controls. These results are interpreted as increased coagulation activity coupled to activation of vascular endothelium. Within the subgroup of FCHL subjects with a hypertriglyceridemic phenotype, elevated PAI-1 activity and sTM levels were particularly associated with features of the metabolic syndrome (hyperinsulinemia, hypertriglyceridemia, predominance of sd LDL). Therefore, two different patterns of procoagulant abnormalities in FCHL emerged from the present findings. The limited number of cases with FCHL, however, warrants some caution in interpretation of the results.

The observed increased prothrombotic activity in FCHL subjects can be explained by endothelial activation, or more advanced atherosclerotic lesions. We showed previously that the carotid artery intima-media thickness, a validated surrogate marker of atherosclerosis, is increased in FCHL compared to control subjects[17]. Therefore, it is plausible that the increased prothrombotic activity in hyperlipidemic FCHL subjects reflects a process of active atherothrombosis. According to current insights, the process of atherosclerotic plaque progression is often accompanied by episodes of thrombosis triggered by rupture or erosion, but many of these thrombi remain mural rather than occlusive[6, 21]. Such a mechanism can underlie the increased basal coagulation activity in plasma of hyperlipidemic (both with elevated TG or cholesterol) FCHL subjects, as suggested by significantly elevated TAT plasma levels. TAT complex formation results from thrombin generation and subsequent binding to its inhibitor antithrombin. Enhanced generation of thrombin may have different origins, some of which may be increased exposure of tissue factor on circulating cells or microparticle fragments, or by plaques[21]. However, it is not known at present whether such a mechanism of TAT activation occurs in stable or vulnerable plaques[17], or both. Alternatively, increased TAT plasma concentrations may be explained by increased platelet activation. This alternative explanation is supported by the significantly positive correlation between TAT concentrations and vWF antigen, which is known to play a major role in platelet adhesion,

and perhaps, in the initiation of atherosclerosis. VWF behaves like an acute phase protein that can be released from endothelial cells in response to different types of cell injury. Endothelial dysfunction with reduced NO generating activity has been previously documented in FCHL[13]. Furthermore, vWF antigen showed positive relationship with IMT in this study. Therefore, the present data suggest that activated endothelium[13, 22], plaque development, and possibly platelet activation, underlie increased TAT and vWF plasma levels in FCHL .

The specific significance of F XIIa in FCHL is still unknown. Recent cardiovascular studies suggest it to be a marker of increased risk for coronary complications but the pathophysiological significance of this contact protein for fibrin formation is disputed[8]. The origin of increased factor XIIa activation is generally unknown but the present data suggest that an increased number of lipoprotein particles, measured as elevated plasma Apo B in FCHL, may be involved, probably by providing a surface mediated activation of the zymogen F XII. An unexpected association between F XIIa and the metabolic factors (BMI, insulin levels; Table 3) may suggest an additional role of adipocytes or other cells in this regard.

Hyperlipidemic FCHL subjects showed elevated PAI-1 activity in the present study. Specifically, hypertriglyceridemic FCHL subjects showed a highly significant 2-fold elevated PAI-1 activity, in combination with mild elevation of sTM levels, compared to hypercholesterolemic FCHL subjects. This finding is of interest because recent studies have shown that cardiovascular risk in FCHL is strongly related to the presence of metabolic dyslipidemia[4], and the excess of atherosclerosis and impaired fibrinolysis respectively[28]. Whereas the association of sTM with the metabolic syndrome has not been studied, increased PAI-1 activity is linked to underlying metabolic abnormalities such as hyperinsulinemia, hypertriglyceridemia, and increased plasma FFA [23, 24]. In type 2 DM, another insulin resistant state, adipocytes have been shown to be important factor in the production of PAI-1[25]. However, in FCHL an additional mechanism may be operational because a major difference in PAI-1 activity existed between the hypercholesterolemic and hypertriglyceridemic FCHL subjects, but BMI and WHR were not significantly different (Table 2). Recent studies provided evidence of important contribution of the liver, specifically fatty liver, to plasma PAI-1 levels[26, 27], and the high prevalence of nonalcoholic fatty liver disease in hypertriglyceridemic FCHL subjects[28] is a potential explanation of elevated PAI-1 activity. Furthermore, FCHL is linked and associated with the gene encoding upstream transcription

factor 1 (USF1), especially in subjects with high triglycerides[29]. Interestingly, PAI-1 gene activity can be regulated by USF-1[30], and therefore PAI-1 is a potential marker, that reflects USF-1 activity in FCHL. Thus, elevated activity of PAI-1 in hypertriglyceridemic FCHL subjects can be particularly inherent to the nature of FCHL.

In summary, the present findings are suggestive of two types of prothrombotic abnormalities in hyperlipidemic FCHL subjects. A general pattern of activated blood coagulation and endothelial activation, which may relate to plaque burden, is present in all hyperlipidemic subjects, independent of metabolic phenotype. In those FCHL subjects with features of the metabolic syndrome, specifically the hypertriglyceridemic phenotype, inhibited fibrinolytic mechanisms may provide an additional cardiovascular risk factor. The present findings advance our understanding of the complex relationship between lipoprotein, metabolic phenotypes, and haemostatic characteristics of plasma in FCHL, and may have implications for therapy and prevention of acute coronary events in hyperlipidemic and insulin resistant disorders.

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Chapter 5

Very Low-Density Lipoprotein Subclass Distribution in Familial Combined Hyperlipidemia; Relationship to Hypertriglyceridemia and Metabolic Syndrome.

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Abstract

The objective of the present study was to get more insight on the lipoprotein abnormalities underlying hypertriglyceridemia in FCHL. We examined the relationship between VLDL subclasses and plasma triglycerides in FCHL and controls, and evaluated a potential association between the VLDL heterogeneity and the presence of metabolic syndrome in FCHL. VLDL1 and VLDL2 fractions were isolated from 44 hyperlipidemic FCHL subjects and 18 normolipidemic controls by means of density gradient ultracentrifugation and biochemically characterized. In FCHL subjects a redistribution of plasma triglycerides (TG) towards lipoprotein fractions of lower density, with significantly higher VLDL1-Apo B levels was observed compared to controls ($p < 0.01$). Assessment of TG to Apo B ratios indicated that VLDL1 lipoproteins were of normal size in FCHL. VLDL1-TG explained more than 80 % of the variability of total plasma TG in FCHL study population. FCHL subjects with elevated plasma VLDL1-TG (> 0.5 mmol/L) were more likely to have an extended form of metabolic syndrome, defined as the presence of 3 or more ATPIII criteria in addition to elevated plasma TG ($p < 0.01$). In conclusion, hypertriglyceridemia in FCHL is associated with overproduction and/or delayed elimination of VLDL1 lipoproteins of normal size. Numerical expansion of plasma VLDL1 lipoproteins is a biochemical characteristic of FCHL subjects with full-blown metabolic syndrome.

Introduction

Familial Combined Hyperlipidemia (FCHL) was originally identified as a genetic disorder of lipid metabolism, associated with an increased risk of premature coronary artery disease (CAD)[1]. However, the pathogenesis of FCHL can extend beyond lipid metabolism. Affected FCHL subjects often exhibit a complex insulin resistant phenotype with a high prevalence of metabolic syndrome components, such as abdominal obesity, hypertriglyceridemia and hypertension[2, 3]. A recent study identified the presence of metabolic syndrome, as defined by National Cholesterol Education Program Adult treatment panel III (ATP III), in the majority of FCHL subjects (65 %) compared to 19% in controls [4].

Hypertriglyceridemia in FCHL is known to be associated with the presence of small dense LDL, and often low HDL cholesterol[5]. This phenotype has been previously designated as the Atherogenic Lipoprotein Phenotype (ALP), because of its association with an increased risk for CAD[6]. Such a cluster of lipid abnormalities, which probably results from altered metabolism of VLDL1 particles against a background of insulin resistance[5], is often present in the metabolic syndrome as well [7]. A specific genetic susceptibility for the ALP has been reported in FCHL[6].

Hypersecretion of hepatic Apo B-containing lipoproteins, VLDL-Apo B in particular, can represent a common pathological background of hypertriglyceridemia in FCHL and the metabolic syndrome[8, 9]. Apo-B containing lipoproteins are synthesized in the hepatocyte, where neutral lipid is combined with Apo B protein in a multi-step process, regulated, at least in part, by insulin[10, 11]. The amount of lipid added during lipoprotein assembly determines the size (density range) of VLDL lipoproteins that are secreted by the liver, i.e. VLDL1 (Sf 60 to 400) or VLDL2 (Sf 20 - 60)[12]. Enhanced production rate of VLDL1, but not VLDL2 have been associated with insulin resistance and accounts for the dyslipidemia in type 2 DM and the metabolic syndrome[13, 14]. In order to get more insight on the lipoprotein abnormalities underlying hypertriglyceridemia in FCHL, we characterized VLDL1 and VLDL2 subclasses in FCHL subjects and controls with regard to lipid and apolipoprotein content. The relationship between VLDL subclasses and plasma triglycerides was examined. In addition, association between the presence of the metabolic syndrome and VLDL heterogeneity in FCHL was evaluated.

Subjects and Methods

FCHL probands and controls were recruited through the Lipid Clinic of the Maastricht University Hospital in the framework of genetic and metabolic studies. A total of 44 hyperlipidemic FCHL subjects and 18 normolipidemic controls were included. The subjects represented FCHL families (n=19) ascertained as previously described [3]. Briefly, FCHL probands had: 1) primary hyperlipidemia with varying phenotypic expression, including (untreated) fasting plasma cholesterol > 6.5 mmol/l and/or fasting plasma triglyceride concentration > 2.3 mmol/l; 2) they were known with premature CAD (before the age of 60), or had a positive family history of such; 3) had at least one first-degree relative with a different lipoprotein phenotype. Exclusion criteria for diagnosing FCHL in a family were diabetes mellitus type 2, obesity (BMI >30), causes of secondary hyperlipidemia (nephrotic syndrome, hypothyroidism, morbid obesity), E2/E2 genotype or tendon xanthomas of the proband.

Presence or absence of metabolic syndrome was identified in all subjects according to ATP III definition as three or more of the following criteria [7]: 1) abdominal obesity (waist circumference: >102 cm in men, and >88 cm in women); 2) hypertriglyceridemia ($\geq$ 1.7 mmol/l); 3) low HDL cholesterol (<1.00 mmol/l in men, and <1.29 mmol/l in women); 4) hypertension ($\geq$ 130/$\geq$ 85 mmHg) or medication; 5) impaired whole blood fasting glucose ($\geq$ 5.6 mmol/l).

Subjects were studied after an overnight fast (12-14 h) and 3 days without alcohol consumption. Any lipid lowering medication was stopped for two weeks before blood samples were collected. The Human Investigation Review Committee of the Academic Hospital Maastricht approved the study protocol. All subjects gave informed consent.

Venous blood was collected in pre-cooled EDTA (1mg/mL) containing tubes. Anthropometric measurements (calculation of BMI), and measurements of fasting plasma concentrations of lipids, lipoproteins, and insulin were done as described [3]. Total plasma VLDL (<1.006 g/ml) was isolated according to the method of Redgrave et al. [15] and subsequently fractionated into VLDL1 and VLDL2 by density gradient ultracentrifugation at 160 000g for 2.5 hours at 4°C in SW40 Ti rotor as described [16]. Collection of fractions started from the top of the tube, where the upper 1.5 ml represented VLDL1 and the lower 5 ml

VLDL2. Measurements of triglycerides and cholesterol in the subfractions were done by standard laboratory techniques. Concentration of Apo B in VLDL1 and VLDL2 fractions was measured by gel electrophoresis according to the method of Karpe et al [17]. Apo B concentration in a density fraction reflects the number of lipoproteins, because there is only one Apo B molecule per lipoprotein particle.

Statistical analyses

Log transformation was used for values of triglycerides (TG), insulin, VLDL1-TG, VLDL2-TG, VLDL1-TG/VLDL2-TG ratio, and TG/Cholesterol ratio, because of skewed distribution of these variables. General linear model was used to examine differences between controls and FCHL subjects, and differences in VLDL subclasses, after correction for age, gender and BMI. This correction was done because gender, age and BMI have been shown to affect VLDL metabolism and plasma triglycerides[16, 18]. Mann-Whitney nonparametric test was used for the Apo B (Table 2) and the subgroup analyses (Table 4) because of small sample sizes. Statistical significance was defined at $P < 0.05$. Multiple regression analysis was used to define predictors of plasma TG, and VLDL1/VLDL2-TG ratio. BMI, age, gender, insulin and FCHL status (yes/no) were entered as independent variables in all models. Variables were eliminated by stepwise backwards regression analysis until all remaining variables had a significance level of $P<0.05$. In all analyses, the statistical package SSPS 11.0 (SSPS Inc., Chicago, IL) was used.

Results

Characteristics of the study groups are presented in Table 1. By definition, hyperlipidemic FCHL subjects (n = 44) showed significant differences with normolipidemic controls (n = 18) in plasma lipid profile (corrected for age, gender, and BMI). Of the hyperlipidemic FCHL subjects, 68.2 % had 3 or more metabolic syndrome features, and therefore fulfilled the ATP III definition of the metabolic syndrome, compared to 27.8 % of the normolipidemic controls (χ^2, $p < 0.01$).

Table 1. Characteristics of study groups

	Controls	FCHL	P value*
No. (M/F)	18 (9/9)	44 (20/24)	
Age, yrs	53.5 ± 14.7	52.4 ± 12.2	NS
BMI, kg/m^2	26.2 ± 3.8	27.7 ± 4.5	NS
Waist, cm	92.9 ± 12.0	96.7 ± 11.3	NS
TC, mmol/L	5.1 ± 0.4	6.8 ± 1.2	.0001
Apo B, g/L	1.0 ± 0.2	1.4 ± 0.3	.0001
TG, mmol/L	1.0 ± 0.3	2.1 ± 0.9	.0001
HDL-chol, mmol/L	1.1 ± 0.2	0.9 ± 0.3	.02
Glucose, mmol/L	4.9 ± 0.7	5.1 ± 0.8	NS
Insulin, µU/ml	3.4 (2.0 - 5.4)	7.1 (5.1 - 10.4)	.001
SBP, mmHg	132 ± 15	141 ± 20	NS
DBP, mmHg	84 ± 7	88 ± 11	NS
Prevalence of MetS	5 (27.8)	30 (68.2)	.002

*P values after correction for age, gender, and BMI

Values are mean ± SD, median (interquartile range), and number (%)

Abbreviations: M/F = male/ female, BMI = body mass index, TC= total cholesterol, Apo B=apolipoprotein B, TG= triglycerides, HDL-chol= high density lipoprotein cholesterol, SBP =systolic blood pressure, DBP = diastolic blood pressure, MetS = metabolic syndrome, NS = not significant

Characteristics of VLDL1 and VLDL2 in hyperlipidemic FCHL subjects and normolipidemic controls.

FCHL subjects had significantly higher amount of triglycerides in VLDL1 (3-fold increase, $p < 0.001$) and VLDL2 fractions (1.5-fold, $p < 0.02$) compared to normolipidemic controls (Table 2), as well as more cholesterol in both VLDL1 (5-fold increase, $p < 0.001$) and VLDL2 (2-fold, $p < 0.02$). Moreover, FCHL subjects had a higher number of VLDL1 and VLDL2 lipoproteins than controls, measured as concentration of Apo B, with a major, 5-fold increase in VLDL1-Apo B ($p < 0.01$), and a modest increase in VLDL2 (1.5-fold, $p < 0.05$).

Altogether, the increase in lipid and Apo B was more distinct in VLDL1 in FCHL subjects. This resulted in the significantly 2-fold increased ratio of VLDL1/VLDL2-TG in FCHL compared to controls. These observations combined indicated a shift in distribution of plasma triglyceride towards VLDL lipoproteins of lower density (VLDL1).

Triglyceride content per particle (TG/Apo B ratio) in VLDL subfractions did not differ between FCHL subjects and controls (Table 2). Of note, the size of the VLDL1 particle is largely determined by its triglyceride content, because triglycerides account for more than 65 % of the total lipid content of VLDL1 lipoproteins [19]. Thus, our analysis suggested that VLDL1 lipoproteins in FCHL have similar size (25 ± 9 μmol/mg) as in controls (33 ± 8 μmol/mg, NS). The cholesterol content per particle (Chol/Apo B ratio) however was increased in VLDL1 of FCHL subjects (9 ± 4 μmol/mg) compared to controls (6 ± 1 μmol/mg, NS), and VLDL2 (9 ± 3 μmol/mg vs. 4 ± 2 μmol/mg, $p < 0.01$). Moreover, the TG/Chol molar ratio for both VLDL subfractions was significantly lower in FCHL subjects than in controls. This additional finding supported the observation that VLDL lipoproteins in FCHL were relatively enriched in cholesterol.

Table 2. Characteristics of VLDL1 and VLDL2 in hyperlipidemic FCHL subjects and normolipidemic controls

	Controls	FCHL	P value*
N	18	44	
VLDL 1			
TG, mmol/L	0.22 ± 0.17	0.66 ± 0.58	.0001
Chol, mmol/L	0.06 ± 0.05	0.31 ± 0.39	.0001
Apo B, µg/ml#	9.4 ± 8.4	42.8 ± 25.7	.002
TG/Apo B, µmol/mg#	33 ± 8	25 ± 9	NS
Chol/ Apo B, µmol/mg#	6 ± 1	9 ± 4	NS
TG/ Chol	3.9 (2.6 - 5.4)	2.6 (1.9 - 3.1)	.03
VLDL 2			
TG, mmol/L	0.21 ± 0.09	0.32 ± 0.17	.015
Chol, mmol/L	0.11 ± 0.06	0.22 ± 0.14	.006
Apo B, µg/ml#	24.1 ± 9.6	37.2 ± 7.8	.026
TG/ Apo B, µmol/mg#	12 ± 3	12 ± 3	.NS
Chol/ Apo B, µmol/mg#	4 ± 2	9 ± 3	.005
TG/ Chol	1.9 (1.5 - 3.0)	1.5 (1.1 - 2.0)	.04
VLDL1/VLDL2 -TG	0.7 (0.6 - 1.3)	1.4 (1.0 - 2.4)	.001

*P value, after correction for BMI, age and gender

#For the Apolipoprotein B analysis: controls n=6; FCHL n = 9; differences were evaluated by Mann-Whitney nonparametric test.

Dissection of determinants of plasma TG concentrations

We used several models to evaluate which variables predicted plasma TG (Table 3, Model A). VLDL1-TG was the most important predictor of the variation in plasma TG (Table 3, Figure 1), whereas FCHL status contributed only weakly to the model (R^2 change = .030, $p<0.01$). By contrast, the other predictors in the model: VLDL2-TG, plasma insulin, BMI, age and gender did not contribute significantly to variation in plasma TG. This model suggested that VLDL1-TG explained most of the variation that alternatively would have been carried by "FCHL affected status".

Similarly, when FCHL subjects were analyzed separately, VLDL1-TG predicted 84 % of the variability in plasma TG ($R^2 = .835$, $p < 0.001$). Subsequent analyses showed a highly significant positive relationship between VLDL1-TG concentrations and the amount of VLDL1-Apo B (rho = .946, $p < 0.001$). Combined with the data discussed in the previous paragraph, this analysis confirmed that the increase in plasma triglyceride concentrations in FCHL subjects was associated with an increased number of VLDL1 lipoproteins in plasma, rather than with an increased TG content per VLDL1 particle.

Table 3. Dissection of determinants of plasma TG concentration. Predictors of plasma TG, and VLDL1/VLDL2 TG ratio were evaluated by multiple backwards regression analysis.

Variable	Predictors	N	Stand. β	P	R^2	R^2 Change
A: Plasma TG[1]	VLDL1-TG	62	.809	.000	.820	
	FCHL		.203	.001	.850	.030
B: VLDL1/VLDL2-TG[2]	Plasma TG	62	.620	.000	.556	
	Insulin		.244	.014	.599	.044

[1] Model A included VLDL1-TG, VLDL2-TG, insulin, BMI, age, gender and FCHL status.

[2] Model B included plasma TG, insulin, BMI, age, gender and FCHL status.

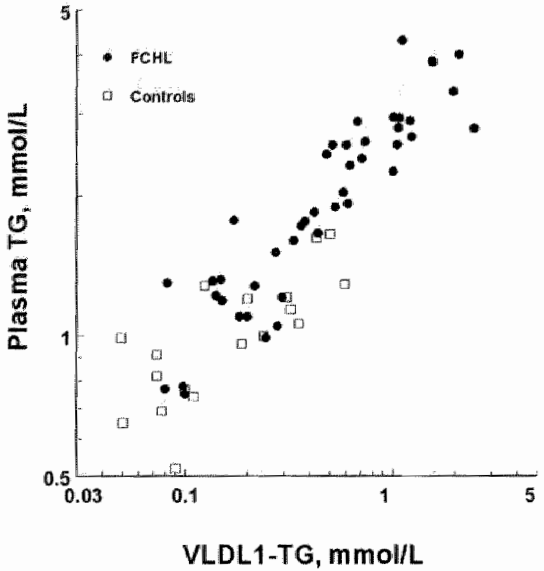

Figure 1.
Relationship between plasma VLDL1-TG and plasma TG in FCHL subjects (R^2=. 835, p<0.001) and controls (R^2 = .575, p <0.001).

Subsequently, we evaluated the factors that determined the distribution of plasma TG between the VLDL subclasses, i.e. VLDL1/VLDL2-TG ratio (Table 3. Model B). Interestingly, plasma insulin predicted significantly the VLDL1/VLDL2-TG ratio, even after correction for plasma triglycerides. This indicated that the shift in triglyceride distribution towards lipoproteins of lower density occurred in the face of elevated plasma insulin levels.

Presence of metabolic syndrome: relationship to VLDL subclasses

We examined the effect of the presence of the metabolic syndrome on the VLDL heterogeneity by subgroup analysis. For the purpose of this analysis only healthy controls, i.e. without metabolic syndrome were included.

Characteristics of VLDL1 and VLDL2 in FCHL subjects with and without metabolic syndrome, and of healthy controls are presented in Table 4. Comparison was made between FCHL subjects with and without metabolic syndrome, as well as between FCHL subjects without metabolic syndrome and healthy controls. FCHL subjects with metabolic syndrome had significantly more triglycerides and cholesterol, particularly in VLDL1 (TG: 0.87 ± 0.61 mmol/l; Chol: 0.41 ± 0.43 mmol/l) than FCHL subjects without the metabolic syndrome (TG: 0.24 ± 0.13 mmol/l, Chol: 0.1 ± 0.07 mmol/l, p< 0.001 for both). Moreover, the

VLDL1/VLDL2 TG ratio was higher in FCHL subjects with metabolic syndrome [1.6 (1.2 – 3.9)] than those without metabolic syndrome [1.1 (0.8 –1.4), p= 0.02]. These observations indicated that expansion of the VLDL1 lipoproteins in plasma in FCHL was typically associated with the presence of metabolic syndrome. The relationship between plasma VLDL1-TG and plasma TG in FCHL is presented in Figure 2 (r^2= .835, p<0.001).

Table 4. VLDL subclasses in relation to metabolic syndrome. VLDL1 and VLDL2 were analyzed in controls and FCHL subjects with and without metabolic syndrome.

	Controls, Met S (-)	FCHL, Met S (-)	FCHL, Met S (+)
N	**13**	**14**	**30**
VLDL 1			
TG, mmol/L	0.22 ± 0.11	0.24 ± 0.13	0.87 ± 0.61 ###
Chol, mmol/L	0.07 ± 0.06	0.1 ± 0.07	0.41 ± 0.43 ###
TG/Chol	3.9 (2.6 – 5.4) #	2.5 (1.8 - 3.2)	2.6 (2.0 – 2.9)
VLDL 2			
TG, mmol/L	0.2 ± 0.1	0.2 ± 0.1	0.4 ± 0.2 #
Chol, mmol/L	0.11 ± 0.06	0.15 ± 0.12	0.25 ± 0.13 #
TG/Chol	1.8 (1.5 – 2.6)	1.7 (1.0 – 2.0)	1.4 (1.3 – 2.0)
VLDL1/VLDL2-TG	0.7 (0.6 –1.3)	1.1 (0.8 – 1.4)	1.6 (1.2 – 3.9) #
Insulin	3.0 ± 1.7	5.3 ± 1.8	10.8 ± 5.8 ##

#P < 0.05, ##P < 0.01 and ### P < 0.001 for difference with FCHL subjects without metabolic syndrome (Met S)

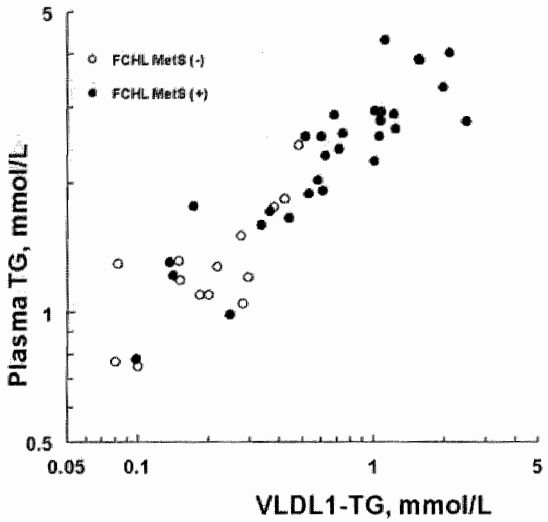

Figure 2.
Relationship between plasma VLDL1-TG and plasma TG in FCHL subjects with or without metabolic syndrome.

Noteworthy, plasma VLDL1-TG concentrations above 0.5 mmol/l were associated with the presence of metabolic syndrome in all FCHL subjects. Because metabolic syndrome is defined in part by high triglycerides, respectively higher VLDL1-TG levels, we tested the relationship between plasma VLDL1-TG and the presence of ATP III criteria of metabolic syndrome, excluding the criterion hypertriglyceridemia. A positive relationship was observed between VLDL1-TG plasma levels and the presence of other ATP III criteria in FCHL, including increased waist circumference, low HDL cholesterol, and hypertension and impaired fasting glucose (rho = .39, $p < 0.01$, data not presented). In addition, we examined the relationship between VLDL-TG > 0.5 mmol/L, and the presence of at least 3 other ATP III criteria in FCHL, excluding hypertriglyceridemia. Figure 3 shows that FCHL subjects with VLDL1-TG > 0.5 mmol/L are more likely to have an extended form of the metabolic syndrome (χ^2, $p < 0.01$).

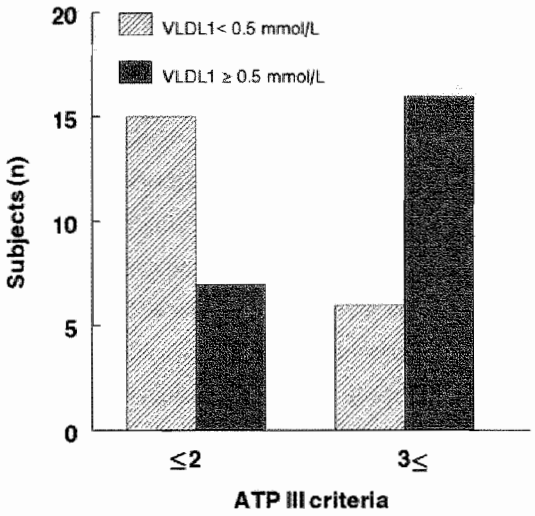

Figure 3.
FCHL subjects with VLDL1-TG > 0.5 mmol/L are more likely to have an extended form of the metabolic syndrome ($p < 0.01$).

Finally, we evaluated if the relative cholesterol enrichment observed in VLDL in FCHL subjects was related to the presence of metabolic syndrome (Table 4). Significantly lower TG/Chol ratio in VLDL1 was observed in all FCHL subjects, both with [2.6 (2.0 – 2.9), $p < 0.01$, p value not shown in Table], and without metabolic syndrome [2.5 (1.8 - 3.2), $p < 0.05$] compared to controls [3.9 (2.6 – 5.4)]. Thus, the presence of low TG/ Chol molar ratio, at least in VLDL1, was not related to the presence of the metabolic syndrome, and may thus present an FCHL specific phenomenon.

Discussion

In the present study, a redistribution of plasma triglycerides towards fractions of lower density (VLDL1) was observed in FCHL subjects compared to controls. This was mainly due to a higher number of VLDL1 lipoproteins (VLDL1-Apo B was more than 5-fold higher in FCHL subjects compared to controls, $p < 0.01$), but not by an increased amount of TG per VLDL1 particle, and can thus be regarded as a numerical expansion of plasma VLDL1 lipoproteins in FCHL. Moreover, VLDL1-TG explained more than 80 % of the variability of plasma triglycerides in the study population, whereas the contribution of "FCHL affected

status" was weak. Remarkably, FCHL subjects with expanded VLDL1 compartment (VLDL1-TG > 0.5 mmol/L) were more likely to have an extended form of metabolic syndrome, defined as the presence of 3 or more ATP III criteria additional to hypertriglyceridemia.

Hypertriglyceridemia in FCHL was associated with increased numbers of VLDL1 lipoproteins of normal size, rather than VLDL2 lipoproteins, similar to findings in normolipidemic subjects [20]. One explanation for accumulation of VLDL1 lipoproteins in plasma in FCHL subjects can be VLDL-Apo B overproduction, as demonstrated in FCHL [8]. Kinetic studies have provided insight on the independent regulation of VLDL1 and VLDL2 production, and their metabolic fate in humans [19]. Hepatic VLDL1 production, but not VLDL2 is inhibited by insulin in healthy subjects [11]. Thus, increased plasma concentration of VLDL1 in the face of elevated plasma insulin levels can indicate hepatic insulin resistance. Preferential hepatic production of VLDL1 over VLDL2 can account for the increased ratio of VLDL1-TG over VLDL2-TG in FCHL reported here. Previous studies suggest that alterations in hepatic metabolism contribute to the expression of FCHL [21]. We have shown that free fatty acid esterification in adipose tissue is insulin resistant in FCHL [22]. This in turn, can lead to an increased flux of fatty acids towards the liver in the postprandial state, contributing to hepatic lipid accumulation. We have reported that fatty liver is present in about 76 % of FCHL subjects [16]. Noteworthy, hepatic lipid accumulation can perturb insulin signaling, i.e. induce hepatic insulin resistance [23]. This may add on to genetic factors that stimulate VLDL1-TG production in FCHL. For instance, propensity to overproduction can relate to a genetic predisposition to lipogenesis de novo in FCHL, mediated by the activity of USF-1, a transcription factor, known to activate fatty acid synthesis [24]. However, the exact biological role of USF-1 remains to be assessed in FCHL.

An alternative explanation for the numerical expansion of plasma VLDL1 lipoproteins may be found in a catabolic defect, independent of overproduction. Decreased activity of the lipoprotein lipase (LPL), which may have a genetic basis [6] or be the result of insulin resistance [25], has been reported in about 30% of FCHL subjects [26]. The VLDL particle itself appears to be a normal substrate for LPL in FCHL, at least in vitro studies [27]. Apparently, overproduction itself can lead to delayed catabolism, because of relative insufficiency of lipolytic pathways [28].

Early studies on FCHL report that VLDL lipoproteins tend to be smaller and cholesterol-enriched compared to controls [27, 29]. However, in those studies VLDL subclasses were not analyzed separately. In the present study we demonstrated that VLDL1 lipoproteins in FCHL subjects showed normal TG to Apo B ratio, but contained relatively more cholesterol. This finding was independent of the presence or absence of the metabolic syndrome, and can indicate prolonged circulation times [30], or alternatively, hepatic production of relatively cholesterol enriched VLDL1 lipoproteins. Of interest, Thompson et al. suggest that hepatic VLDL-Apo B production is regulated, at least in part, by cholesterol availability for incorporation in pre-VLDL lipoproteins [31]. Thus, activated cholesterol synthesis in liver can potentially account for production of cholesterol-enriched atherogenic VLDL lipoproteins in FCHL.

Overproduction of VLDL1-TG is known to contribute to increased plasma triglycerides in type 2 DM, yet another disorder with high prevalence of metabolic syndrome [13, 14]. However, McEneny et al. reported that VLDL1 lipoproteins in subjects with type 2 DM were particularly larger and lipid rich compared to controls [38]. By contrast, our analyses demonstrated that VLDL1 particle size was not different between subjects with FCHL and controls. Therefore, it is likely that genetic and biochemical differences in hepatic lipid metabolism exist between FCHL and other forms of metabolic syndrome.

FCHL is associated with 2 to 5-fold increased risk for premature coronary events[32]. The susceptibility to coronary events can be explained at least in part by the high prevalence of metabolic syndrome in FCHL[4]. The present study identified metabolic syndrome in the majority of hyperlipidemic FCHL subjects (68.2 %) compared to controls (27.8 %), a prevalence quite similar to the reported in literature [4]. Sayle et al. reported that the presence of the metabolic syndrome strongly affected the cardiovascular prognosis among angiographied coronary patients[33]. In subanalysis of the same study, hypertriglyceridemia/low HDL cholesterol significantly predicted the incidence of vascular events in coronary patients with type 2 DM [34]. We showed that particularly FCHL subjects with metabolic syndrome were characterized by high number of plasmaVLDL1 lipoproteins. These observations combined suggest that some of the CAD risk associated with the metabolic syndrome in FCHL can be mediated through VLDL1.

The present findings can have implications for therapy and prevention of CAD in FCHL. Statins successfully lower plasma lipids, specifically LDL and total cholesterol levels, and are currently the treatment of choice for FCHL. However, Verseyden et al. reported that VLDL1-Apo B did not lower in FCHL subjects upon statin treatment[35]. Studies of our laboratory showed that although statins lower plasma TG levels (VLDL1-TG), they remained elevated in most FCHL subjects. Furthermore, statin treatment did not ameliorate insulin resistance, as estimated by surrogate markers (Georgieva et al., unpublished observations). Literature suggests that insulin resistance can drive VLDL1 overproduction[36]. This can be the case in our study as well, because the full-blown metabolic syndrome observed in FCHL subjects with high plasma VLDL1 suggests a severe degree of insulin resistance[33]. Therefore, targeted treatment of the metabolic syndrome and insulin resistance, e.g. by lifestyle changes, can be beneficial for treatment and CAD prevention at least in some FCHL subjects. It has not been investigated whether pharmacological intervention, i.e. thiazolidinediones can have clinical implications for prevention of CAD in FCHL[37].

In conclusion, hypertriglyceridemia in FCHL is associated with overproduction and/or delayed elimination of VLDL1 lipoproteins of normal size. Numerical expansion of plasma VLDL1 lipoproteins is a biochemical characteristic of FCHL subjects with full-blown metabolic syndrome.

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Chapter 6

Lipitor, Inflammation and Gene Expression in Adipose Tissue (LIGEAT) in Familial Combined Hyperlipidemia.
I. Effect on plasma lipid and lipoprotein profile, and insulin resistance.

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Summary of Study Design

Differential gene expression analysis has indicated an inflammatory process in subcutaneous adipose tissue in FCHL subjects compared to controls. This process can represent a response to hyperlipidemia in FCHL. To test this hypothesis, we treated 12 hyperlipidemic FCHL subjects with a statin (atorvastatin). We performed a subcutaneous adipose tissue biopsy in each subject before (t=0) and after 8 weeks of treatment (t=8). Here we present the changes in plasma lipid and lipoprotein profile induced by statin therapy, in combination with plasma inflammatory markers and surrogate markers for insulin resistance. The gene expression analysis can help us identify pathways involved in adipocyte adaptation to hyperlipidemia in FCHL. This latter data however is beyond the scope of this thesis.

Introduction

Familial Combined Hyperlipidemia (FCHL) was delineated as a genetic disorder of lipid metabolism almost 3 decades ago[1]. Affected FCHL subjects show a complex insulin resistant phenotype and show a number of abnormalities in lipid metabolism: hypercholesterolemia (elevated total and LDL cholesterol) and/or hypertriglyceridemia, elevated Apo B, small dense LDL, and decreased plasma HDL cholesterol concentrations[2-5]. FCHL subjects have a higher risk of premature coronary artery disease (CAD) than the population, which can be related at least in part to the higher prevalence of the metabolic syndrome in FCHL[6, 7].

Recently, our laboratory demonstrated abnormalities in gene expression in subcutaneous adipose tissue from FCHL subjects, involving cell cycle genes, insulin resistance genes and inflammatory genes, including TNF-alpha (13-fold elevated vs. controls) and IL-6[8]. Of interest, these adipokines have been associated with systemic insulin resistance and CAD[9]. The observed changes in gene expression can result from a primary genetic defect, or alternatively represent an adaptation to hyperlipidemia, or a combination of both. In the case of adaptation, lipid-lowering with a pharmaceutical compound can result in improved organ function, which in turn can lead to a reduction in systemic inflammation and insulin resistance.

Overproduction of Apo B-containing lipoproteins can explain, at least in part, hyperlipidemia in FCHL[10]. Delayed clearance of VLDL and VLDL remnants contributes to hyperlipidemia as well, as shown in hypertriglyceridemic FCHL subjects[11]. The majority of hypertriglyceridemic FCHL subjects have elevated plasma insulin, a surrogate marker for insulin resistance[2, 12, 13]. Therefore, it is possible that insulin resistance induces alteration in the metabolism of Apo B-containing lipoproteins that results in hypertriglyceridemia. Alternatively, insulin resistance can ensue, at least in part, from elevated triglyceride-rich lipoprotein levels. Again, lipid lowering with a medication can help us evaluate whether elevated plasma insulin is secondary to hypertriglyceridemia in FCHL.

This study was designed to assess if lipid lowering with atorvastatin has a beneficial effect on subcutaneous adipose tissue function of FCHL subjects. In addition, changes in concentration and composition of Apo B-containing lipoproteins, in combination with

surrogate markers for insulin resistance and inflammatory markers have been evaluated. For that purpose, 12 hyperlipidemic FCHL subjects were treated with atorvastatin (Lipitor® 40mg daily, as monotherapy). The following parameters were evaluated before and after 8 weeks of treatment: 1) plasma lipid levels, 2) indirect markers of insulin resistance 3) plasma levels of proinflammatory markers. Here we present in detail the changes in lipoprotein subclasses and metabolic profile, induced by atorvastatin therapy.

Methods

Twelve unrelated FCHL subjects were recruited through the Lipid Clinic of the Maastricht University Hospital. Briefly, FCHL subjects had: 1) primary hyperlipidemia with varying phenotypic expression, including (untreated) fasting plasma cholesterol > 6.5 mmol/l and/or fasting plasma triglyceride concentration > 2.3 mmol/l; 2) they were known with premature CAD (before the age of 60), or had a positive family history of such; 3) had at least one other first-degree relative with a different lipoprotein phenotype. Nine FCHL subjects represented well-defined FCHL families. Three subjects presented with an FCHL phenotype (primary hyperlipidemia, and a positive history of premature CAD), but the diagnosis could not be established in a family, because of nuclear families. The Human Investigation Review Committee of the Academic Hospital Maastricht approved the study protocol. All subjects gave informed consent.

Subjects were asked to stop any lipid-lowering medication 4-week prior to study (5 subjects had been treated with a statin, 3 subjects with a combination therapy of a statin and a fibrate). One subject was treated with Avandia, 4 mg/day and this medication was not discontinued. Before inclusion, after the washout period, 6 subjects presented with combined hyperlipidemia (Fredrickson phenotype IIB, LDL cholesterol > 4.2 mmol/L, triglycerides >2.3 mmol/L), and 6 subjects with isolated hyperlipidemia (Fredrickson type IV, LDL cholesterol < 4.2 mmol/L, triglycerides > 2.3 mmol/l). Two subjects presented with highly elevated plasma triglycerides of 19.0 mmol/ L and 22.7 mmol/L (Fredrickson type IV). Atorvastatin 40 mg at bedtime was prescribed for 8 weeks as monotherapy.

At zero and eight weeks after start of treatment (t=8), venous blood was drawn in pre-cooled EDTA (1 mg/ml) tubes after an overnight fast (12-14 h), for the following analyses: 1) lipid analyses - total cholesterol, HDL cholesterol, plasma triglycerides, Apo B; 2) surrogate markers of insulin resistance – insulin, glucose, FFA; 3) plasma inflammatory markers. For gene expression analysis, abdominal subcutaneous fat biopsies were obtained before (t=0), and after 8 weeks (t=8), of atorvastatin treatment.

VLDL1 and VLDL2 were separated from plasma VLDL (<1.006 g/ml on a 1.006 - 1.250 g/ml KBr density gradient) by density gradient ultracentrifugation at 160 000g for 2.5 hours at 4ºC in SW40 Ti rotor, as described[2]. Measurements of triglycerides and cholesterol in the subfractions were done by standard laboratory techniques. Concentrations of VLDL1 and VLDL2 Apo B was measured by gel electrophoresis according to the method of Karpe et al.[14].

Statistical analyses

Wilcoxon's nonparametric test for two related samples was used to evaluate the effect of treatment. Correlations were assessed by Spearman rank correlation . In all analyses, the statistical package SSPS 11.0 (SSPS Inc.) was used.

Results

Effect of therapy on plasma lipids and markers of insulin resistance

All subjects completed the study. One subject completed the study prematurely at week 6 because of complains of insomnia, constant fatigue and headache. Characteristics of the study population and results are presented in Table 1. Of note, all subjects were viscerally obese and fulfilled the criteria for metabolic syndrome, defined according to Adult treatment panel III of the NCEP (ATP III)[15].

At 8 weeks of treatment, Atorvastatin, 40 mg/d as monotherapy induced significant reduction in fasting total cholesterol (- 41%), triglycerides (- 38%), Apo B (- 26%), as well as significant increase in HDL-cholesterol (19%). The change in triglycerides was significantly correlated with triglycerides at baseline (Rho= - 63, p = 0.03). However, no changes were

seen in plasma insulin, glucose, FFA. Moreover, metabolic syndrome was still present in all subjects at the end of study.

We did not observe significant changes in ALT with atorvastatin treatment. However, nine patients showed a reduction in ALT levels, suggesting an improved liver function.

Table 1. Characteristics of study group. Effect of 8 weeks of Atorvastatin 40 mg per day, monotherapy

	Before treatment	After treatment	P	% Change
Descriptives				
Number (M/F)	12 (7/5)			
Age	52 (34-65)			
BMI kg/m^2	29. 0 (26.9 - 34.1)			
SBP, Hgmm	136 (107 - 161)	132 (107 - 178)	NS	- 3.6 %
DBP, Hgmm	85 (72 - 110)	85 (66 - 107)	NS	- 1.8 %
Lipid profile				
TC, mmol/l	8.1 (5.4 - 12.1)	4.7 (3.4 - 8.4)	.002	- 41 %
Apo B, g/l	1.2 (0.8 - 1.6)	0.8 (0.6 - 1.3)	.003	- 26 %
TG, mmol/l	3.8 (2.3 - 22.7)	2.7 (0.9 - 5.1)	.008	- 38 %
HDL-chol, mmol/l	0.7 (0.4 - 1.3)	0.8 (0.4 - 1.2)	.028	+ 19 %
Markers of Insulin Resistance				
HOMA	3.0 (1.1 - 9.8)	2.8 (0.8 - 9.6)	NS	+ 3 %
Insulin μU/ml	11.1(4.7 - 35.6)	11.7 (3.6 -34.7)	NS	+ 5 %
FFA, mmol/l	0.4 (0.06 - 0.9)	0.3 (0.06 - 0.6)	NS	- 3 %
Liver Function				
ALT U/l	31.4 (11.4 - 118.5)	26.8 (12.5 - 85.4)	NS	- 17.0 %

Data are presented as Median (range)

Abbreviations: M/F = male/female; BMI = body-mass index; SBP = systolic blood pressure; DBP = diastolic blood pressure; TC = total cholesterol; HDL-chol = high density lipoprotein cholesterol; HOMA = homeostatic model assessment index; FFA = free fatty acids; ALT = alanine amino transferase

Effect of therapy on Apo B-containing lipoproteins

Changes in Apo B containing lipoprotein fractions are presented in Table 2.

Table 2. Changes in lipoprotein profile induced by atorvastatin monotherapy (40 mg per day).

	Before treatment	After treatment	P	%% Change
VLDL1				
TG, mmol/l	1.4 (0.6 - 15.0)	0.8 (0.1 - 2.1)	.003	- 52 %
Chol, mmol/l	1.0 (0.3 - 6.4)	0.5 (0.04 - 1.3)	.004	- 77 %
Apo B mg/l	67.7 (16.5 - 285.7)	27.2 (0.8 - 88.6)	.002	- 58 %
VLDL2				
TG, mmol/l	0.8 (0.4 - 3.7)	0.7 (0.3 - 1.6)	.03	- 25 %
Chol, mmol/l	0.5 (0.2 - 2.9)	0.4 (0.05 - 1.0)	.02	- 40 %
Apo B mg/l	68.4 (42.5 - 216.1)	47.7 (13.3 - 130.7)	.003	- 26 %
VLDL1/VLDL2	1.8 (0.9 - 9.6)	1.6 (0.3 - 1.9)	.02	- 38 %
IDL				
TG, mmol/l	0.14 (0.1 - 0.24)	0.12 (0.06 - 0.2)	.003	- 25 %
Chol, mmol/l	0.55 (0.27 - 0.94)	0.33 (0.09 - 0.85)	.004	- 38 %
LDL				
TG, mmol/l	0.25 (0.16 - 0.39)	0.17 (0.1 - 0.33)	.002	-29 %
Chol, mmol/l	3.69 (1.34 - 6.11)	1.90 (1.21 - 5.07)	.003	-37 %
Apo B, g/l	1.2 (0.6 - 1.6)	0.7 (0.6 - 1.3)	.002	- 37 %

Data are presented as Median (range)

Significant decrease of TG and cholesterol were observed in all fractions. The major decrease, was observed for VLDL1 fraction: VLDL1-TG lowered by 52 %, VLDL1-Chol by 77%, and VLDL1 Apo B by 58% ($p<0.01$ for all). For VLDL2, VLDL2-TG lowered by 25 %, VLDL2-Chol by 40 %($p< 0.05$ for both), and VLDL2 Apo B by 26 % ($p<0.01$). This resulted in a significantly lower ratio of VLDL1-TG over VLDL2-TG, which indicated

improvement in fasting plasma TG distribution. IDL-TG lowered by 25 %, IDL Chol by 37 % ($p<0.01$ for both). A similar reduction was observed in the LDL fraction. In addition, we did not see changes in the LDL/Apo B ratio, an estimation of LDL size.

No relationship was observed between changes in lipoprotein levels and surrogate markers for insulin resistance, or lipid variables, or ALT.

Effect of therapy on proinflammatory markers

The most dramatic change after 8 weeks of treatment was the significant reduction of plasma PAI-1 levels (-18%, $p < 0.05$) (Table 3). The reduction in CRP levels by 21 % approached statistical significance. However, the reduction in other proinflammatory markers, such ICAM-1, TNF-α R1 and TNF-α R2 levels was minimal and did not reach statistical significance.

Again, no relationship was observed between changes in PAI-1 or CRP and surrogate markers for insulin resistance.

Table 3. Changes in proinflammatory markers induced by atorvastatin monotherapy.

	Before treatment	After treatment	P	% Change
CRP, ng/ml	4.0 (1.7 - 8.7)	3.0 (1.2 - 7.6)	.099	- 21 %
PAI-1, ng/ml	20.1 (3.4 - 36.9)	15.6 (4.6 - 24.9)	.012	- 18 %
ICAM-1, ng/ml	72.5 (38.5 - 126.4)	70.2 (36.5 - 121.8)	NS	- 5 %
TNF-α R1, ng/ml	1.6 (1.2 - 2.7)	1.6 (1.0 - 2.2)	.087	-7 %
TNF-α R2, ng/ml	1.8 (1.2 - 2.7)	2.0 (0.7 - 2.5)	NS	- 5 %

Data are presented as Median (range)

Abbreviations: CRP = C-reactive protein; PAI-1 = plasminogen activator inhibitor 1; ICAM-1 = intracellular adhesion molecule 1; TNF-α R1 = tumor necrosis factor 1 receptor 1; TNF-α R2 = tumor necrosis factor 1 receptor 2

Discussion

Treatment with atorvastatin 40 mg per day at bedtime for 8 weeks effectively lowered plasma lipids in hypertriglyceridemic FCHL subjects, without concomitant changes in surrogate markers for insulin resistance (plasma insulin, HOMA or plasma FFA concentrations). The main changes in Apo B-containing lipoprotein fractions were observed in VLDL1. However, plasma triglycerides remained elevated (>2 mmol/L) on therapy in most subjects.

Statins effectively lowered plasma Apo B-containing lipoproteins. Several mechanisms are known to mediate this effect in hypertriglyceridemic subjects, the most important of all being increased hepatic uptake via the LDL receptor. Statins inhibit cholesterol synthesis, primarily in liver, and thereby induce upregulation of hepatic LDL receptors. Enhanced hepatic clearance of VLDL-Apo B lipoproteins by 58 % has been observed in subjects with the metabolic syndrome[16,17]. In addition, increased activity of LPL contributes to faster conversion of VLDL1 to VLDL2, and thereby promotes VLDL clearance[17]. Indeed, we saw an improvement in the VLDL1/VLDL2TG ratio, which can at least in part be related to increased LPL activity. Statins decrease the production rate of VLDL-Apo B in healthy subjects, but this mechanism does not operate in subjects with the metabolic syndrome[16]. Insulin resistance in liver seems to mediate overproduction of VLDL-Apo B in the latter subjects[16, 17]. In the present study treatment with atorvastatin did not ameliorate insulin resistance in FCHL subjects, as estimated by surrogate markers (HOMA, or fasting insulin). It is likely that overproduction in these FCHL subjects resulted from relative insulin resistance in liver as well, because treatment with statins improved but did not normalize plasma TG levels[18].

An atherogenic cluster of elevated plasma TG, low HDL and sd LDL is a common finding in FCHL[2]. Atorvastatin treatment resulted in 19 % increase in HDL, but values remained low, at least in men (HDL-cholesterol < 1.00 mmol/l)[15]. Several groups have reported persistence of sd LDL in FCHL upon statin treatment[19, 20]. In agreement, we did not see changes in the LDL/Apo B ratio, which can be used for estimation of LDL size. There are at least two mechanisms that can explain the persistence of the atherogenic profile on statin therapy. First, statins lower VLDL1 levels, but most likely do not resolve overproduction[16].

An expansion of the VLDL1 lipoproteins in plasma mediates low HDL levels and formation of sd LDL[21]. This process involves neutral lipid exchange via the cholesterol ester transfer protein (CETP)[22]. Second, increased activity of hepatic lipase (HL) can contribute to formation of sd LDL and low HDL cholesterol as well. HL activity is increased in insulin resistance[23] and has been implicated as a modifier gene in the expression of FCHL[24]. Moreover, changes in HL activity have not been observed upon atorvastatin therapy in FCHL[25].

The major changes in Apo B containing proteins affected the VLDL1 fraction, where both lipid and apolipoprotein content decreased by more than 50 %. This is in contrast to the findings of Castro Cabezas et al., who did not observe changes in VLDL1- TG and VLDL1-apo B in FCHL subjects upon atorvastatin treatment[26]. The reason for this inconsistence can relate to higher plasma TG on start of atorvastatin therapy in our study. Current insight suggests that the response to statin therapy is phenotype dependent: the higher basal triglycerides (above 1.5 mmol/L), the better the response[17].

Elevated levels of proinflammatory markers and prothrombotic markers, in particular CRP and PAI-1 indicate an increased risk for CAD[27-29]. PAI-1 lowered significantly and there was a tendency for reduction in CRP upon statin treatment. Some but not all studies report changes in PAI-1 levels[30, 31]. The reduction in PAI-1 and CRP was not associated with changes in lipid levels, in agreement with previous reports in literature[30]. Because of these pleiotropic actions, statin therapy can be important for prevention of acute cardiac events in FCHL patients. Moreover, large studies have demonstrated the antiatherogenic effects of statin therapy in the long term[34].

The reduction in PAI-1 observed in the study can be related to improved endothelial function[31, 32], or alternatively reduced inflammatory response in subcutaneous fat tissue, or a combination of both. Additional mechanism that can explain reduction of PAI-1 with statin therapy in FCHL is improvement in liver function. Fatty liver can be an important source of PAI-1 in hypertriglyceridemic FCHL subjects[33]. Noteworthy, we observed reduction in ALT levels after 8 weeks of treatment in the majority of the study subjects, indicating an improved liver function. This observation suggests that statin therapy can be potentially beneficial in the treatment of non-alcoholic fatty liver disease in FCHL. However, elevation in liver transaminases is a known side-effect of statin therapy, and therefore caution is required.

Importantly, the increased risk of CAD in the metabolic syndrome is related to a clustering of metabolic abnormalities, rather than a single factor[15]. Statin treatment did not resolve the insulin resistance, estimated as surrogate markers, and dyslipidemia in FCHL. Therefore, therapies that target insulin resistance are potentially beneficial in FCHL, such as thiazolidinediones (TZD)[35] or life style changes[36]. However, data on the effect of TZD on CAD is still not available.

In conclusion, treatment with atorvastatin effectively lowered plasma lipids, but did not resolve insulin resistance and the atherogenic lipoprotein profile of hypertriglyceridemic FCHL patients. Atorvastatin reduced levels of cardiovascular risk markers, particularly PAI-1 and CRP. Noteworthy, no relationship was observed between changes in PAI-1, or CRP and lipid variables. Because of these pleiotropic effects, statins can be important in treatment of FCHL, especially CAD prevention. Potentially, hypertriglyceridemic FCHL patients might have an additional benefit from therapies that target insulin resistance.

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Chapter 7

General Discussion

Contents

The aim of this thesis was to study the phenotypic heterogeneity in FCHL with regard to lipoprotein subclasses. We assessed the role of VLDL-TG in the expression of the FCHL phenotype, and hypertriglyceridemia in particular. A numerical expansion of plasma VLDL1 lipoproteins can explain, for a large part, major abnormalities in lipid and lipoprotein profile in FCHL subjects, such as hypertriglyceridemia (**chapter 5**), sd LDL, low HDL (**chapter 2**), and potentially relate to perturbations in blood coagulation profile such as elevated PAI-1 levels (**chapter 4**). The cross-sectional design of our studies prevents us from a definite conclusion on the exact pathogenic mechanisms operating in FCHL. It provides us, however, with enough material, which in combination with evidence in literature, allows us to draw a hypothesis. We believe that alterations in liver lipid metabolism mediate the expression of FCHL, especially in the setting of insulin resistance and hepatic lipid accumulation, both of which are very common in FCHL cases.

Here we will discuss our findings on the metabolic disturbances in FCHL in more detail.

1. Dyslipidemia as a Consequence of Ectopic Fat Accumulation

The complex FCHL phenotype results from the interaction of several genes, gene products and the environment[1]. Although their genetic nature is not completely understood, two primary pathogenic mechanisms operate in FCHL: 1) compromised fatty acid handling in response to insulin in adipose tissue, and 2) hepatic overproduction of Apo B 100-containing lipoprotein particles, mostly as VLDL-Apo B.

Current insight suggests a basic defect in adipose tissue lipid metabolism in FCHL subjects. This defect manifests itself as increased fatty acid fluxes mainly in the postprandial state, and may represent an impaired fatty acid storage as triglycerides in response to insulin[2, 3]. Increased FFA fluxes can be redistributed to other organs, and are most likely responsible for the ectopic fat accumulation observed in subjects with FCHL; their abdominal obesity[4], accumulation of fat in liver (**chapter 3**), and potentially in muscle. Clinical observations suggest that expression of FCHL is sensitive to fat mass in the body (obesity)[5], which in turn is responsible for greater fatty acid fluxes[6]. Of note, ectopic accumulation of fatty acids and fatty acid-derived metabolites can result in acquired organ insulin resistance[7].

The adverse effects of ectopic fat accumulation on metabolism, including increased insulin resistance and dyslipidemia have become apparent from clinical practice. Conditions associated with disturbances in TG storage, such as lipodystrophies, are characterized by accumulation of fat in the visceral compartment, liver and skeletal muscles[8]. Adipose tissue dysfunction is a known side effect of highly active antiretroviral therapy of HIV infected patients. Interestingly, these patients show an abnormal metabolic profile, quite similar to that exhibited by hypertriglyceridemic FCHL patients[9].

Ectopic fat accumulation appears to mediate the expression of FCHL[4, 10, 11]. Our laboratory has reported on a strong relationship between abdominal obesity and hypertriglyceridemia in FCHL[4]. However, FCHL subjects show higher plasma Apo B, triglycerides and insulin levels for any degree of abdominal obesity compared to controls[12], suggesting the additional contribution of genetic factors. For instance, the propensity of liver to produce an excess of VLDL particles can relate to genetic predisposition to lipogenesis de novo, mediated by USF-1[13]. USF-1 is a transcription factor known to activate fatty acid synthesis in response to increased plasma glucose[13]. Noteworthy, an increased flux of glucose to the liver in FCHL patients is plausible, because of insulin resistant glucose utilization in the skeletal muscle of these patients[14]. The USF-1 gene has been recently linked to FCHL, but strong biological evidence that implicates it as an aetiogical factor in FCHL is still missing[15]. Yet, enhanced lipogenesis de novo can be an important additional factor in hepatic fat accumulation in FCHL, especially under conditions of increased substrate flux. We were the first to report on the high prevalence of fatty liver in FCHL: it was present in 76% of the FCHL probands that we studied, specifically the hypertriglyceridemic subjects[16] **(chapter 3)**. It is of interest that hepatic lipoprotein production is regulated, among others, by insulin and the intracellular lipid content. These observations combined suggest a potential relationship between steatosis and hepatic lipoprotein overproduction in FCHL, manifested as hypertriglyceridemia.

In summary, increased flux of FFA alone, or in a combination with a genetic propensity to lipogenesis de novo, can challenge the storage capacity of liver, compromise intracellular insulin signalling[7], and result in enhanced VLDL production in FCHL.

2. Possible Mechanism Underlying VLDL-Overproduction in FCHL; Hepatic Substrate Availability

FCHL subjects exhibit overproduction of VLDL-Apo B[17]. There is a discussion in literature as to what drives the VLDL overproduction: altered substrate flux (discussed in the previous paragraph) or excess of cholesterol, phospholipids or triglyceride in the hepatocyte[18]. For example, there is a strong correlation between markers of activated cholesterol synthesis and VLDL-Apo B production rate in man[19]. In vitro, the rate of Apo-B secretion of HepG2 cells is correlated with the intracellular mass of cholesterol esters, and not with the intracellular triglyceride mass[20]. Hyperinsulinemia stimulates cholesterol synthesis in the hepatocyte via enhanced SREBP activity[21]. Thus, an activated cholesterol synthesis pathway in the liver in the setting of hyperinsulinemia, can potentially account for hypersecretion and the presence of cholesterol-enriched atherogenic VLDL particles in FCHL[22-24] (chapter 5).

Other investigators argue that Apo B-lipoprotein secretion is regulated by the rate of the triglyceride biosynthesis and not by the intracellular cholesteryl ester pools, at least in hepatic cell cultures[25]. Availability of phospholipids, particularly phosphatidylcholine, seems to be essential for VLDL assembly as well[26]. Phospholipids comprise the surface of lipoproteins, and can be important in the initiation of VLDL synthesis by formation of Apo B-phospholipids complexes. Besides, phosphatidylcholine can be hydrolyzed through the activity of phospholipase D (PLD) to diacylglycerol, which in turn can be esterified to triacylglycerol for core lipid incorporation in VLDL, or serve as a precursor of phospholipid synthesis[27]. Accordingly, mice with phospatidylcholine deficiency in liver have reduced plasma VLDL-Apo B and VLDL-TG concentration[28].

Taken together, VLDL assembly and secretion is a complex process, subjected to intricate regulation. Elucidating the pathways involved in VLDL production can help identify new target enzymes for effective lipid-lowering drug therapy.

3. VLDL Heterogeneity Can Explain the Phenotypes in FCHL

FCHL subjects exhibit elevated plasma triglycerides (VLDL) and/or cholesterol (LDL), giving rise to three separate lipoprotein phenotypes (Fredrickson IIa, IIb, IV). Family members can show different phenotypes and some subjects can change from phenotype over time[23]. This multiple lipoprotein phenotype is considered intrinsic to FCHL, and still puzzles investigators. To our view, the ability of the liver to produce a population of heterogeneous Apo-B 100 containing lipoproteins provides a plausible biologic explanation for this phenomenon in FCHL (discussed in **chapter 2**). Apo-B 100 containing lipoproteins produced by the liver are predominantly VLDL lipoproteins, but the density can range from LDL to large VLDL particles (VLDL1). Stable isotope studies have shown overproduction of Apo B 100 as VLDL as well as LDL lipoproteins in FCHL[17, 29]. It is possible that the type of Apo-B 100 containing lipoproteins, produced by the liver of a particular FCHL subject is largely determined by the hepatic triglyceride availability for incorporation in lipoprotein particles[29]. **(Figure 1)**. Hepatic triglyceride availability, in turn, is a function of genetic variations that affect hepatic fatty acid handling and storage, as well as fluctuations in FFA flux in response to environment. Of note, we found an increased liver TG content, as measured with ultrasound, in the majority of the FCHL probands we studied, in particular the hypertriglyceridemic ones **(chapter 3).**

Another factor that seems to play an important role in the process of lipoprotein production is hepatic sensitivity to the regulatory role of insulin. Insulin regulates the process of VLDL synthesis and secretion by several mechanisms (see Figure 1 and introduction). Moreover, insulin has a separate regulatory effect on the individual VLDL subclasses. Insulin inhibits hepatic VLDL1 production in healthy subjects, but not VLDL2 production[30-32]. Insufficient suppressive action of insulin on VLDL1 production contributes to increased lipid levels in type 2 DM [33]. Expansion of plasma VLDL1 lipoproteins in FCHL occurs in the face of increased plasma insulin **(chapter 2 and 5)**. Therefore, it is possible that alterations in hepatic lipid metabolism, involving an impaired response to insulin can contribute to excess VLDL1 production at least in some FCHL subjects.

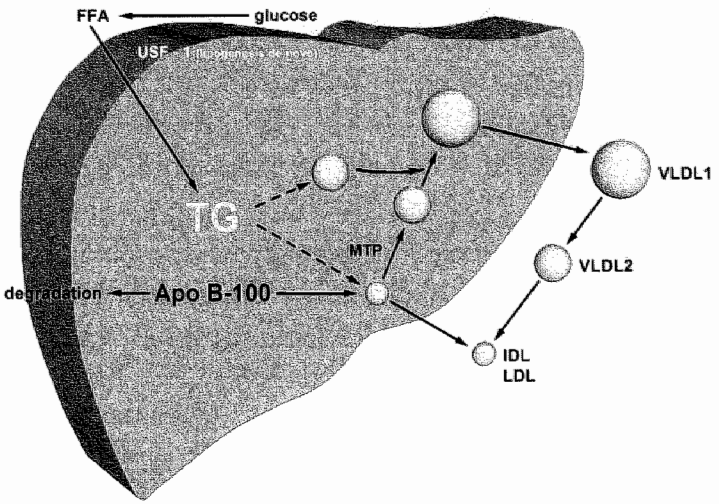

Figure 1. Hepatic production of heterogeneous Apo-B containing lipoproteins can underlie the multiple lipoprotein phenotype in FCHL.

Hepatic synthesis of lipoproteins particles features lipidation of Apo-B 100 protein in several steps: 1) Formation of pre-VLDL particle, catalyzed by microsomal transfer protein, MTP; 2) Addition of Apo B-free lipid mass to the pre-VLDL particle; a VLDL2 or VLDL1 particle can be formed depending on the amount of neutral lipid. For example, VLDL1 contains 2 to 4-fold times more triglyceride molecules per particle than VLDL2 (15-20 000 triglyceride molecules in VLDL1 vs. 5-10 000 in VLDL2). Whether a VLDL1 or a VLDL2, or in some cases IDL or LDL particle is formed, is dependent, among others, on the inhibitory regulation of insulin; the amount of fat in the liver; and the substrate flux (glucose, FFA). These fluxes and the size of TG pool can be increased in FCHL.

Legend: dashed line signifies pathways inhibited by insulin

Importantly, VLDL subclasses appear to have different catabolic fate in plasma: Excess of VLDL1 can induce formation of sd LDL, and are mostly removed as VLDL remnants, whereas VLDL2 are preferentially catabolized to large, buoyant LDL[34, 35] (**Figure 2**). Reciprocal relationship between VLDL and LDL cholesterol was observed in earlier studies on FCHL, suggesting that these lipoprotein classes can be linked through a reciprocal regulation[23]. We extended these observations with VLDL and LDL subclass analysis. The data showed that FCHL subjects who had different VLDL subclasses, differed in plasma triglycerides, LDL size, Apo B concentration and insulin as well, thus giving rise to aggregate metabolic phenotypes (**chapter 2 and 5;** the hypertriglyceridemic phenotype is summarized in Figure 2). Altogether, we suggest that alterations in hepatic Apo-B lipoprotein metabolism in terms of VLDL1 or VLDL2 production offer a coherent metabolic explanation of the multiple lipoprotein phenotype in FCHL.

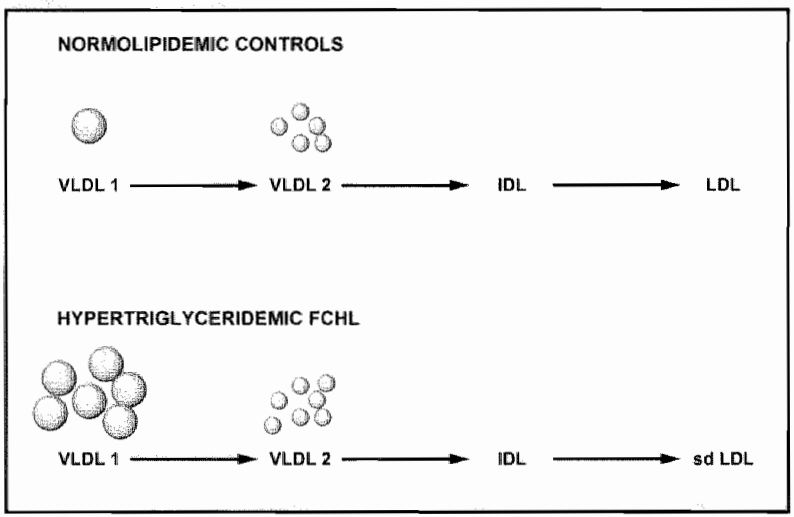

Figure 2. Alteration in the VLDL-TG pathway in hypertriglyceridemic FCHL subjects
Accumulation of triglyceride-rich particles VLDL1 in plasma results in the formation of sd LDL particles in FCHL, because of the existing metabolic channeling between VLDL and LDL subclasses.

4. FCHL and Metabolic Syndrome

FCHL subjects exhibit a complex insulin resistant phenotype with a high prevalence of the metabolic syndrome components such as abdominal obesity, hypertriglyceridemia and hypertension (i.e. dyslipidemic hypertension in FCHL) [4, 11, 36]. A recent study identified the presence of the metabolic syndrome, as defined by National Cholesterol Education Program Adult treatment III panel (ATP III), in the majority of FCHL patients (65%) compared to 19% in controls[37]. Our studies showed similar results (**chapter 5**).

As discussed in the previous paragraph, overproduction of VLDL against a background of insulin resistance can explain hypertriglyceridemia, and the associated changes in HDL and LDL subclasses in FCHL. In addition, VLDL overproduction most likely contributes to the considerable phenotypic overlap between FCHL and the metabolic syndrome (discussed in **chapter 5**). Propensity to overproduction can relate to a genetic polymorphism on chromosome 1q[15]. Pajukanta et al. recently proposed USF-1, located on 1q23-q31 as a candidate gene in hypertriglyceridemic FCHL men[13, 38]. Polymorphisms in USF-1 gene can contribute to metabolic alterations and atherosclerosis, because of its regulatory role in lipogenesis, adipose tissue metabolism, systemic blood pressure, and HDL metabolism[15]. Interestingly, metabolic syndrome was linked to the same genetic locus in an independent study, the Insulin Resistance Atherosclerosis Family Study[39]. Thus, USF-1 activity can mediate, at least in part, expression of the metabolic syndrome in FCHL[13].

It is of interest that patients with type 2 DM, another disorder with high prevalence of the metabolic syndrome, exhibit hepatic production of much larger, lipid enriched VLDL1 particles than controls, at least according to some studies[40]. By contrast, VLDL1 particles in FCHL subjects are of normal size (**chapter 5**). Therefore, it is likely that biochemical and genetic differences in hepatic lipid metabolism exist between FCHL and other forms of the metabolic syndrome or at least subjects with type 2 DM.

FCHL was identified 3 decades ago as genetic disorder of lipid metabolism. Although we have advanced our knowledge on lipid metabolism considerably, the focus on lipid metabolism alone could not help us unravel the genetic nature of FCHL. Numerous studies on FCHL have indicated that the metabolic background of FCHL is much more complex. We believe that the lipid and lipoprotein profiles in FCHL are the consequences of the underlying

genetic and metabolic disturbances such as impaired adipose tissue function, insulin resistance and abdominal obesity. An expansion of plasma VLDL1 lipoproteins characterizes the metabolic syndrome in FCHL, and develops against a background of insulin resistance and visceral obesity. We propose that FCHL can be regarded as a genetic form of the metabolic syndrome, characterized by a propensity to overproduction of VLDL-Apo B particles.

5. Hepatic Lipid Metabolism in FCHL - an Open Field for Research

Hepatic lipid metabolism has not received much attention in FCHL, and one reason is that it is extremely difficult to study. It is difficult to obtain human hepatic tissue for studies, mostly out of ethical reasons. Moreover, a proper animal model for FCHL does not exist, and hepatic culture cells from FCHL subjects are not available. An interesting approach in this regard, are the experiments of van Greevenbroek et al., who cultivated Hep2 cells with hyperlipidemic plasma of FCHL subjects, and observed a different protein response in comparison to cells cultured with control plasma[41]. An alternative to in vitro studies can be to evaluate substrate fluxes and VLDL production with stable isotopes, especially in the framework of an interventional study with a therapy known to improve adipocyte function and insulin resistance, such as thiazolidinediones (TZD) or life-style changes[42]. Moreover, genes involved in VLDL-TG pathway (**Appendix**) can be included as candidate genes in linkage and association studies.

Novel mechanisms of hepatic insulin resistance have been proposed recently. Changes in the redox state in the hepatocyte, related to oxidative stress can induce insulin resistance and affect VLDL production[43]. Endoplasmatic reticulum stress has been implicated in obesity and type 2 DM as a mediator of insulin resistance[44]. The potential role of disturbed endoplasmatic reticulum function in hepatic insulin resistance in FCHL remains to be evaluated.

6. Activated Coagulation Profile Can Be an Important Cardiovascular Risk Factor in FCHL

Hyperlipidemic FCHL subjects exhibited a prothrombotic phenotype **(chapter 4)**. This observation can be interpreted as an activation of the vascular endothelium in response to plaque burden and can relate to the observed susceptibility of premature acute coronary events in FCHL. Moreover, increased PAI-1 activity was specifically observed in hypertriglyceridemic FCHL subjects with features of the metabolic syndrome. An increase in PAI-1 activity indicates impaired fibrinolysis and may provide an additional cardiovascular risk factor in these subjects.

Several mechanisms can explain the increase in PAI-1 activity. Endothelial dysfunction due to the metabolic stress, related to hyperinsulinemia, hypertriglyceridemia or elevated plasma FFA, can lead to increase in PAI-1 levels[45, 46]. Also adipocytes can be an important factor in the production of PAI-1in insulin resistant conditions[47]. A part of our study "Lipitor, Inflammation and Gene Expression in Subcutaneous Adipose Tissue in FCHL (LIGEAT)" has been designed to address this issue **(chapter 6)**. A novel mechanism that can contribute to plasma PAI-1 levels in FCHL subjects is the presence of fatty liver[9, 48]. The high prevalence of nonalcoholic fatty liver disease in hypertriglyceridemic FCHL subjects[16] is a potential explanation for the elevated PAI-1 activity. Interestingly, PAI-1 gene activity can be regulated by USF-1[49], which has been implicated in FCHL subjects with elevated plasma triglycerides[13]. Therefore, PAI-1 is a potential marker of USF-1 activity in FCHL, and elevated activity of PAI-1 in hypertriglyceridemic FCHL subjects can be particularly inherent to the nature of FCHL.

7. Lipoprotein Subclasses as a Diagnostic Approach in FCHL

Preponderance of sd LDL is an inherent feature of FCHL. It has been proposed as a diagnostic marker of the disorder[50], because it captures both pathogenic mechanisms and the increased risk of premature CAD in FCHL. We examined the sd LDL phenomenon in FCHL **(chapter 2)**. We found a close association between sd LDL and the accumulation of

triglyceride-rich VLDL particles, or VLDL1, which characterize the hypertriglyceridemic phenotype in FCHL. Based on this observation and sound evidence from literature, we conclude that in FCHL subjects (who have a background of insulin resistance and propensity to overproduction) hepatic VLDL1 particles are the precursor of sd LDL in plasma.
Sd LDL is a final step in a pathway; therefore all plasma lipid modifier genes contribute to its expression. Thus, presence of sd LDL reflects predominance of VLDL1 particles as well as prolonged circulation times, CETP and HL activities, LDL receptor defects, etc. However, sd LDL is predominantly associated with the hypertriglyceridemic FCHL phenotype (**chapter 2**). Is expansion of plasmaVLDL1 lipoproteins a better marker of FCHL than the presence of sd LDL? More studies are needed to determine how specific this feature is to FCHL. Comparison to other disorders with overproduction with regard to lipid and apolipoprotein composition (e.g. Apo CIII, Apo E) will be required.

The main endpoint in diagnosis and treatment of dyslipidemia is prevention of acute coronary events. Prospective studies have demonstrated that presence of sd LDL is associated with increased cardiovascular risk, independent of plasma lipid levels[51]. Yet, another study in men with cardiovascular disease showed that those with large VLDL particles were 3 to 4 times more likely to have extensive CAD than those without, independent of plasma triglyceride and HDL cholesterol[52]. By contrast, adjustment for these variables greatly reduced the relation of sd LDL to CAD[52]. Taken together, an implication of these and our studies is that detailed analyses of lipoprotein subclasses can advance our knowledge on dyslipidemia, and potentially help identify those patients with increased risk for acute coronary events. However, analytical ultracentrifugation, which we used for VLDL subclasses analysis in this thesis, is laborious and difficult to use on a large scale, or for clinical purposes. Therefore, development of new methods for faster determination of lipoprotein subclasses can offer new insights and advance our capability of diagnosing dyslipidemias and cardiovascular disease[52].

8. Lipid-Lowering Therapy in FCHL

Treatment with statins (atorvastatin, 40 mg daily for 8 weeks) effectively lowered plasma lipid levels in FCHL (**chapter 6**). Increased hepatic uptake of Apo B-containing particles is most likely the mechanism responsible for lowering of plasma Apo-B containing lipoproteins[53]. The observed improvement in lipid profile underscores the contribution of a relative clearance defect of Apo B-containing lipoproteins to hyperlipidemia, especially hypercholesterolemia in FCHL.

FCHL often manifests itself with a combination of elevated plasma triglycerides, low HDL cholesterol, and predominance of sd LDL, a lipoprotein phenotype considered to be particularly atherogenic[54]. We found that statin treatment ameliorated but failed to resolve the atherogenic lipoprotein phenotype in most FCHL subjects, in consistence with previous reports in literature[55]. One explanation can be that statins do not resolve overproduction, because this medication cannot counteract insulin resistance, and insulin resistance is most probably the cause of excessive VLDL1 production[56]. Additional factor that can contribute to the persistence of sd LDL and low HDL cholesterol in FCHL subjects is the activity of HL, which is known to be increased in insulin resistance[32, 54].

Fibrates (PPAR-α agonists) are more efficient than statins in reducing elevated triglyceride levels and increasing HDL cholesterol. A small, but significant increase in LDL size have been observed with fibrate monotherapy in FCHL subjects[55]. However, the same study showed that fibrate monotherapy has little effect on total plasma LDL cholesterol in FCHL[55]. Therefore, a combination of a fibrate with a statin can be the optimal therapy for FCHL patients, especially those with combined hyperlipidemia. In addition, fibrates affect hepatic fatty acid oxidation through activation of PPAR- α, and thus can affect hepatic fat content and potentially improve fatty liver[57]. Fibrate therapy, however, can require extra caution, especially in elderly FCHL patients, because of reported increased risk of rhabdomyolysis. It is of interest that large-scale trials designed to evaluate if fibrate treatment is effective with regard to CAD prevention are being conducted at the moment[58]. The results of these studies are eagerly awaited.

Ectopic fat accumulation and insulin resistance can precede or deteriorate the lipoprotein abnormalities observed in subjects with FCHL (discussed in chapter 5). Therefore,

therapies that aim at ameliorating visceral obesity, fatty liver or insulin resistance are potentially beneficial in FCHL, such as the PPAR-γ agonists, TZD, or life style changes[59]. TZD are a new class of drugs, with an insulin sensitizing action[42]. Treatment with TZD promotes fat mobilization from the liver or the visceral area and its redistribution to the subcutaneous fat depot[42]. However, the effect of TZD on the plasma lipoprotein profile in FCHL has not been addressed yet. It still remains to be established if TZD lower the risk of CAD.

In summary, statin therapy is important for prevention of acute events in FCHL patients, due to its pleiotropic effects, such as lipid-lowering, anti-proinflammatory and anti-prothrombotic action. The atheroprotective effects of statin therapy have been demonstrated in large prospective studies[60]. However, hypertriglyceridemic FCHL patients might have an additional benefit from therapies that target insulin resistance.

9. Conclusions

In conclusion, there is strong evidence that suggests that FCHL should be regarded as a genetic form of the metabolic syndrome, associated with a propensity to overproduction Apo B-100 lipoproteins. Moreover, we argue that alterations in hepatic Apo-B lipoprotein metabolism in terms of VLDL1 or VLDL2 production offer a coherent metabolic explanation of the metabolic phenotypes in FCHL, in combination with altered lipoprotein elimination. The VLDL-TG pathway is central to the plasma lipid and lipoprotein phenotypes observed in FCHL. Recognition of the VLDL-TG pathway in FCHL is important with respect to emerging drug therapies, which target lipid metabolic enzymes, and can potentially manage the increased CAD risk, associated with the disorder.

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Genetic Factors Implicated in the VLDL-TG pathway

Table 1. Enzymes and apolipoproteins of the VLDL-TG pathway (TG and PL synthesis, VLDL particle production and catabolism).

Enzyme/ Apolipoprotein	Chromosomal location	Function	Result[1]	Result[2]
TG synthesis[1]				
GPAT mitochondrial	10q25.3	Acylation of G3P to LPA in mitochondria. Rate-limiting step in esterification	Not studied in FCHL	
GPAT microsomal	-	Acylation of G3P to LPA in ER	Not studied in FCHL	
AGPAT 1-6	6p, 9q, 21q, 6q, 8p, Yp	Acylation of LPA to phosphatide	Not studied in FCHL	
PAP-1	-	Dephosphorylation to DAG	Not studied in FCHL	
DGAT-1	8q24.3	TG synthesis	Not studied in FCHL	
DGAT-2	11q13.3	TG synthesis in liver, inhibited by niacin[2]	Not studied in FCHL	
PL synthesis[3]			Not studied in FCHL	
PCYT1A	3q29	Rate-controlling enzyme in PC biosynthesis in ER		
PCYT1B	-	Cytoplasmic isoform of PCYT1A		
PEMT	-	Alternative pathway for PC biosynthesis,		
Regulation of lipogenesis				
USF-1	1q22-q23	Lipogenesis de novo (FFA)	Linkage and association[4]	
SREBP1-c	17p11.2	FFA and TG biosynthesis	Not studied in FCHL	
SREBP2	22q13	Cholesterol biosynthesis	Not studied in FCHL	

PPAR-α	22q12-q13	Regulation of hepatic FFA oxidation (fasting) and glycerol utilization	No evidence found[5]; Modifier gene[6]	
PPAR-γ	3p25	Adipose tissue differentiation (glycerol), reverse cholesterol transport	Modifier[7]	
LXR	-	Activation of SREBP-1c in response to ↑ intracellular cholesterol and insulin	Not studied in FCHL	
VLDL particle synthesis				
MTP	4q22-24	Transfer of lipid to Apo B for VLDL assembly	No evidence found	5
AADAC[8]	3q21-25	TG hydroxylase (homologous to HSL)	Not studied in FCHL	
ARF-1	1q42	Intracellular vesicular trafficing; activator of PLD	Not studied in FCHL	
PLD	3q26	Intracellular vesicular trafficing. Hydrolysis of PL	Not studied in FCHL	
VLDL catabolism				
LPL	8p22	Hydrolysis of triglyceride-rich lipoproteins	No evidence for linkage[9] Modifier gene[10] Common defect in LPL in approx. 30% of FCHL subjects[11].	5
CETP	16q21	Lipid exchange between VLDL and HDL/ LDL	No evidence [5]	12

Enzyme/ Apolipoprotein	Chromosomal location	Function	Result[1]	Result[2]
HL	15q21-23	Hydrolysis of TG from VLDL2, IDL, LDL, HDL	No evidence for linkage[9]. Modifier[13]	5
PLTP	20q12-13.1	PL transfer between HDL and VLDL	Not studied	
LDL receptor	19p13	Hepatic uptake of ApoE/ApoB100 lipoproteins	No evidence found	5
LRP receptor	12q13	Hepatic uptake of ApoE / Apo B48 lipoproteins	Not studied	
VLDL receptor	9p24	Peripheral uptake of Apo E lipoproteins	No evidence found	5
VLDL apolipoproteins				
Apo B	2p24-p23	Structural protein, ligand for LDL, LRP receptor.	No evidence found[14]	5, 15
Apo CIII	11q23	Inhibits LPL activity	Modifier: TG and sd LDL[16]	5, 17
Apo AI	11q23	Cofactor for LCAT	ApoAI/C3/A4/A5 linkage to TG[17, 18]	17, 18
Apo AII	1q21-23	Apolipoprotein of HDL	Modifier, no evidence for linkage[19]	5, 20
Apo IV	11q23	Reverse cholesterol transport?	Linkage to ApoAI/C3/A4/A5[18]	17
Apo V	11q23	Function unknown	Modifier: effect on plasma TG[18]	17
Apo E	19q13	Clearance of CM and VLDL remnants	No evidence[5]	
Apo CII	19q13	Cofactor for LPL	Not studied	

Abbreviations:

GPAT = Glycerol-3-phosphate acyltransferase; G3P = Glycerol-3 phosphate; LPA = Lysophosphatidic acid; ER = Endoplasmatic Reticulum; AGPAT = AGP acyltransferase/ Lysophosphatidic acid acyltransferase; PAP = Phosphatidic acid phosphatase; DAG = Diacylglycerol; DGAT-1 = Diacylglycerol acyltransferase type 1; DGAT-2 = Diacylglycerol acyltransferase type 2; PL = Phospholipid; PCYT 1A= Phosphate cytidylyltransferase 1A ; PCYT 1B= Phosphate cytidylyltransferase 1B; PC = Phosphatidylcholine; PEMT = Phosphatidylethanolamine *N*-methyltransferase; AADAC = Arylacetamide deacetylase ; ARF-1 = ADP-ribosylation factor 1; PLD = Phospholipase D

Result[2] = study has been confirmed.

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Summary & Samenvatting

Summary

Familial Combined Hyperlipidemia (FCHL) is a genetic disorder of lipid metabolism, defined as a familial hyperlipidemia with a variable expression, i.e. isolated hypercholesterolemia (Fredrickson IIa), combined hyperlipidemia (IIb), or isolated hypertriglyceridemia (IV), in combination with increased familial prevalence of premature myocardial infarction. Affected FCHL subjects exhibit a complex metabolic phenotype, which can feature elevated plasma insulin concentrations in the presence of insulin resistance, abdominal obesity, and hypertension. A clustered entity of elevated plasma triglycerides, low high-density lipoprotein (HDL) cholesterol and predominance of small dense LDL, commonly described as the atherogenic lipoprotein phenotype, is frequently observed in FCHL subjects. We have studied this adverse phenotype and its metabolic determinants in more detail.

There is evidence that the complex FCHL phenotype results, at least in part, from impaired fatty acid metabolism, associated with impaired fatty acid trapping for triacylglycerol synthesis in FCHL adipose tissue and increased fatty acid flux towards other organs. In muscle and liver fatty acid and their metabolites can accumulate and induce insulin resistance. For instance, there is evidence that glucose uptake in FCHL skeletal muscle responds insufficiently to insulin stimulation in relation to the rate of fatty acid supply. There is no literature data, however, to suggest that insulin resistance in liver contributes to FCHL expression. It is of interest to investigate this potential mechanism in FCHL, because hepatic lipoprotein production is a function of hepatic insulin sensitivity. For example, insulin normally suppresses hepatic production of Apo B-100 lipoproteins, especially the production of triglyceride-rich lipoproteins. Another factor that can affect hepatic lipoprotein production can be accumulation of fat in the liver (fatty liver).

The very-low density lipoprotein-triacylglycerol (VLDL-TG) pathway, which reflects the metabolism of Apo B-100 lipoproteins, carries most of plasma triglycerides in the fasting state. In this thesis, we show that alterations in the VLDL-TG pathway contribute to the varied expression of FCHL.

Very low-density lipoprotein (VLDL) and low-density lipoprotein (LDL) each consist of physicochemically and metabolically distinct subclasses. Our FCHL study sample showed a clear bimodal distribution of LDL size. About half of the subjects presented with large buoyant LDL, designated as "pattern A". The other half had small dense LDL, or "pattern B" (*chapter 2*). Subsequently, we assessed whether pattern A or B associate with aggregate metabolic phenotypes. Subjects with predominantly large, buoyant LDL showed a hypercholesterolemic phenotype (Fredrickson IIa) and the highest Apo B levels. Subjects with predominantly small dense LDL showed a hypertriglyceridemic (Fredrickson IIb or IV), low HDL cholesterol phenotype, with moderately elevated Apo B, total cholesterol level, and LDL cholesterol level. The presence of increased numbers of VLDL1 particles accounted for the hypertriglyceridemia, and could explain the formation of sd LDL and the low HDL cholesterol in the latter subjects. Noteworthy, the amount of VLDL1-TG correlated positively with plasma insulin in pattern B FCHL subjects, which indicated that in these subjects hepatic (over)production of VLDL1-TG could result from insulin resistance. Therefore, the existence of distinct metabolic phenotypes in FCHL can relate to VLDL particle metabolism.

Factors that can lead to fat accumulation in liver have been reported in FCHL. These include increased fatty acid fluxes, and enhanced synthesis of fat in liver (lipogenesis de novo) due to activation of transcription factors in response to increased substrate availability and hyperinsulinemia. We examined the prevalence of nonalcoholic fatty liver disease in FCHL patients referred to the outpatient clinics of the Maastricht University Hospital, by ultrasonography (*chapter 3*). We observed a substantial prevalence of nonalcoholic fatty liver disease in FCHL patients, including all of those with elevated plasma triglyceride levels. Fatty liver can contribute to increased production of triglyceride-rich particles through increased availability of substrate for incorporation in VLDL.

We hypothesize that plasma lipoprotein phenotypes in FCHL can be determined by differences in metabolic profile between individuals, which can relate to insulin resistance and the presence or absence of fat accumulation in liver (*chapter 2, 3, 4 and 5*). These differences can result from environmental influences or genetic variants, or a combination of both. Future studies are needed to confirm this hypothesis.

Hyperlipidemic conditions have been associated with a prothrombotic state, but the nature of this association has not been well studied in FCHL. Therefore, we studied the

activity profile of the blood coagulation system, and its relationship to the lipid phenotypes in FCHL. All hyperlipidemic FCHL subjects exhibited a prothrombotic phenotype, including elevated levels of several factors of the coagulation system (*chapter 4*). This observation can be interpreted as an activation of the vascular endothelium in response to plaque burden and can relate to the observed susceptibility of premature acute coronary events in FCHL. Increased plasminogen activator inhibitor 1 (PAI-1) activity was specifically observed in hypertriglyceridemic FCHL subjects with features of the metabolic syndrome. Increase in PAI-1 activity indicates impaired fibrinolysis and may provide an additional cardiovascular risk factor in these subjects.

FCHL subjects exhibit a complex phenotype with a high prevalence of the metabolic syndrome. FCHL subjects with the full-blown metabolic syndrome typically showed numeric expansion of the plasma VLDL1 lipoproteins. VLDL1-TG was the major determinant of the variability of plasma triglycerides. (*chapter 5*). One explanation of VLDL1 plasma expansion in FCHL can be overproduction of VLDL1 that occurs against a background of insulin resistance. It is of interest that patients with type 2 DM, another disorder with high prevalence of the metabolic syndrome, exhibit hepatic production of much larger, lipid enriched VLDL1 particles than controls, as reported elsewhere. By contrast, VLDL1 particles in FCHL subjects were of normal size. Therefore, it is likely that biochemical and genetic differences in hepatic lipid metabolism exist between FCHL and other forms of the metabolic syndrome or at least subjects with type 2 DM. The molecular mechanisms of VLDL overproduction in FCHL remain to be evaluated.

Treatment with atorvastatin 40 mg per day, at bedtime, for 8 weeks effectively lowered plasma lipids in hypertriglyceridemic FCHL, without concomitant changes in surrogate markers of insulin resistance, such as plasma insulin, HOMA or plasma FFA concentrations (*chapter 6*). However, plasma triglycerides remained elevated (>2 mmol/L) upon therapy in most subjects, with no change in LDL size. HDL cholesterol levels improved but remained low on therapy. Atorvastatin reduced levels of cardiovascular risk markers, particularly PAI-1 and C-reactive protein (CRP). Noteworthy, no relationship was observed between changes in PAI-1, or CRP and lipid variables. Because of these pleiotropic effects, statins can be important in treatment of FCHL, especially coronary artery disease prevention.

Potentially, hypertriglyceridemic FCHL patients can have an additional benefit from therapies that target insulin resistance.

Samenvatting

Familiaire Gecombineerde Hyperlipidemie (FGH) is een genetische aandoening die gekenmerkt wordt door een verstoord vetmetabolisme. Aangedane personen afkomstig van FGH families vertonen één van de volgende karakteristieken: een verhoogd cholesterolgehalte in het bloed, bekendstaand als Fredrickson type IIa hyperlipidemie; hypertriglyceridemie, bekend als type IV hyperlipidemie of een combinatie van verhoogd cholesterol- en hypertriglyceridemie, ofwel Fredrickson type IIb. Het frequente optreden van hart- en vaatziekten op jonge leeftijd (vóór 60 jaar) is kenmerkend voor FGH families. Personen met FGH laten dikwijls een verhoging van de insuline plasmaspiegel zien met tevens insulineresistentie, abdominale obesitas en een verhoogde bloeddruk. Een plasma fenotype met verhoogde triglyceriden, laag high-density cholesterol (HDL cholesterol), in combinatie met de aanwezigheid van small dense LDL (sd LDL) komt vaak voor in personen met FGH en wordt "het atherogene lipoprotein fenotype" genoemd. Wij hebben dit fenotype en de metabole determinanten ervan bestudeerd.

Uit onderzoek blijkt dat FGH een gevolg kan zijn van een verstoord vetzuurmetabolisme. Aangedane personen met FGH nemen vetzuren langzamer in hun vetweefsel op en mede daardoor blijven vetzuren na een maaltijd langer in de bloedbaan. Vetzuren die lang blijven circuleren kunnen metabole verstoringen in andere organen veroorzaken. Voorbeelden hiervan zijn de stapeling van vetzuren en hun metabolieten, die in skeletspier en lever tot verminderde gevoeligheid voor de hormonale werking van insuline kunnen leiden. Het is aangetoond dat de door insuline gestimuleerde glucoseopname in de skeletspier in FGH verminderd wordt, afhankelijk van het aanbod van vetzuren. Het is nog niet bewezen dat insulineresistentie in de lever betrokken is bij FGH. Dat is belangrijk om te weten want de lipoproteïnenproductie (Apo B-100) door de lever is insuline gevoelig. Bij gezonde personen onderdrukt insuline de productie van lever lipoproteïnen, vooral de productie van triglyceridenrijke very low-density lipoproteïne (VLDL1). Vetstapeling (vetlever, NASH) in de lever kan ook de lipoproteïnenproductie beïnvloeden. Ook dit werd onderzocht in een FGH patiëntenpopulatie, afkomstig uit de polikliniek van het Academisch Ziekenhuis Maastricht.

De very low-density lipoprotein-triacylglycerol (VLDL-TG) pathway weerspiegelt het metabolisme van plasma Apo-100 lipoproteïne en triglyceriden tijdens de gevaste (nuchtere) toestand. In dit proefschrift hebben we aangetoond dat veranderingen in the VLDL-TG pathway de expressie van FGH beïnvloeden.

Very low-density lipoprotein (VLDL) and low-density lipoprotein (LDL) bestaan uit subklassen, die verschillend zijn in metabool en fysisch-chemisch opzicht. Er was een duidelijk patroon van LDL subklassen te zien in onze steekproef van FGH families: er waren personen met grote LDL deeltjes, ook wel "patroon A" genoemd, en personen met kleine (small dense, sd) LDL deeljes, ofwel "patroon B" (*hoofdstuk 2*). Wij onderzochten wat de verschillen waren tussen "patroon A" en "patroon B" patiënten. Personen met patroon A waren hypercholesterolemisch (Fredrickson IIa) en hadden een sterk verhoogd plasma Apo B gehalte. Personen met patroon B waren hypertriglyceridemisch (Fredrickson IIb or IV) en hadden laag HDL cholesterol, in combinatie met matig verhoogde Apo B, totaal cholesterol en LDL cholesterol waarden. Hypertriglyceridemie in deze personen werd veroorzaakt door toename in plasma VLDL1 deeltjes. VLDL1 wisselt triglyceriden uit met LDL en HDL deeltjes, en een overmatige uitwisseling kan tot gevolg hebben dat er een verlaging van plasma HDL cholesterol en sd LDL stapeling optreedt. Tevens correleerde VLDL1-TG met plasma insuline. Dat kan betekenen dat een (overmatige) VLDL1 productie in personen met patroon B een gevolg kan zijn van insulineresistentie. Bovendien lijkt het VLDL metabolisme de aanwezigheid van aparte fenotypen in FGH te kunnen verklaren.

Een groot aanbod van vrije vetzuren kan bijdragen aan de vetstapeling in de lever. Activatie van transcriptie factoren door hyperinsulinemie speelt ook een belangrijke rol bij de vetstapeling. Wij bestudeerden de prevalentie van vetlever bij FGH patiënten die afkomstig waren van het Academisch Ziekenhuis Maastricht (*hoofdstuk 3*). Het bleek dat de prevalentie van vetlever bij FGH aanzienlijk groter was dan bij de controlepopulatie, eveneens afkomstig van het Academisch Ziekenhuis Maastricht. Bovendien bleken alle FGH patiënten met een vetlever verhoogde plasma triglyceriden te hebben. Dit kan betekenen dat vetstapeling in de lever bijdraagt aan de overmatige productie van grote triglyceridenrijke VLDL1, door de vergrote beschikbaarheid van substraat voor VLDL synthese. Uiteraard is verder onderzoek in een grotere studie populatie nodig om te begrijpen of deze waarneming bevestigd kan worden.

Wij veronderstellen dat plasma lipoproteïne fenotypen in FGH onstaan door verschillen in het metabole profiel tussen individuen. Omgevingseffecten of genetische variatie, alleen of in combinatie, dragen bij aan het ontstaan van de FGH fenotypen door verschillen in insulineresistentie te veroorzaken, en mogelijk mede vetstapeling in de lever te veroorzaken (*hoofdstukken 2, 3 en 5*).

Subklinische activatie van de bloedstolling komt vaak voor in hyperlipidemische omstandigheden, maar deze associatie bleek onvoldoende onderzocht te zijn in FGH. We bestudeerden de activiteit van het bloedstollingssysteem in relatie tot de lipoproteïne fenotypen in FGH. Alle aangedane FGH personen bleken een verhoogd plasma gehalte van een of meerdere stollingsfactoren te vertonen *(hoofdstuk 4)*. Deze subklinische activatie van de stollingssysteem kan een belangrijk rol spelen bij het ontstaan van de symptomen van hart- en vaatziekten in FGH. Een verhoogd plasmagehalte van plasminogen activator inhibitor-1 (PAI-1) was karakteristiek voor hypertriglyceridemische FGH personen met metabool syndroom. Stijging van PAI-1 activiteit duidt op een verstoorde fibrinolyse en dit kan extra risico op hart- en vaatziekten bij deze personen vormen. Op dit moment is er weinig bekend over een specifieke behandeling hiervan.

Aangedane FGH personen tonen een complex fenotype met verhoogde prevalentie van het metabole syndroom. Een verhoogde VLDL1 plasmaspiegel is kenmerkend voor FGH personen met het metabole syndroom en kan leiden tot hypertriglyceridemie *(hoofdstuk 5)*. Insulineresistentie is waarschijnlijk de oorzaak van overmatige VLDL1 productie in personen met FGH en het metabole syndroom. Het is interessant te weten dat personen met type 2 DM, die ook een verhoogd risico op het metabole syndroom hebben, een overmatige leverproductie tonen van VLDL1 deeltjes, die veel groter (triglyceriderijker) zijn dan VLDL1 deeltjes bij gezonde personen. Dit in tegenstelling tot FGH, waar het vetgehalte van de VLDL1 deeltjes bij de FGH patiënten vergelijkbaar was met dat van controles. Die observatie geeft aan dat er verschillen in vetmetabolisme in de lever kunnen bestaan tussen FGH en andere vormen van het metabole syndroom, tenminste met type 2 DM. Deze verschillen moeten nog worden bestudeerd.

Plasmalipiden concentraties, vooral cholesterol en LDL cholesterol, in hypertriglyceridemische FGH personen daalden met atorvastine behandeling (40mg/d), na 8 weken monotherapie, maar er was geen verandering in surrogaat markers voor

insulineresistentie zoals plasma insuline, vrije vetzuren en HOMA *(hoofdstuk 6)*. Ook plasma triglyceriden bleven verhoogd (> 2 mmol/L) in de meeste patiënten en de LDL deeltjes bleven klein. HDL cholesterol steeg met therapie, maar bleef laag. Een verklaring hiervoor kan zijn dat plasma triglyceriden, de grootte van LDL deeltjes en HDL-cholesterol nauw verband houden met het onderliggende ziekteproces in FGH. Het plasmagehalte van PAI-1 en C-reactive protein (CRP) daalde met atorvastatine behandeling. Er was geen verband tussen de veranderingen in lipiden en PAI-1, of CRP. Vanwege gecombineerde effecten blijven statines belangrijk voor de preventie en de behandeling van hart – en vaatziekten bij FGH patiënten. Behandeling van de insulineresistentie kan extra voordeel opleveren bij hypertriglyceridemische FGH patiënten omdat dat het onderliggende ziekteprocess aangrijpt.

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Yours,

Maastricht, 02.09.2000

Anna

List of Publications

Full papers

Georgieva AM, van Greevenbroek M.M.J., Krauss R.M., Brouwers M.C.G.J., Vermeulen V.M.M.-J., Robertus-Teunissen M.G, van der Kallen C.J.H., and de Bruin T.W.A.
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Georgieva A.M., van Greevenbroek M.M.J., Brouwers M.C.G.J., van der Kallen C.J.H., and de Bruin T.W.A.
VLDL subclass distribution in familial combined hyperlipidemia: Relationship to hypertriglyceridemia and metabolic syndrome.
Submitted.

Brouwers M.C.G.J., Georgieva A.M., Bilderbeek-Beckers M.L.A., Robertus-Teunissen M.G., van der Kallen C.J.H., van Greevenbroek M.M.J., and de Bruin T.W.A.
Role of Fatty Liver in Dyslipidemia in Familial Combined Hyperlipidemia: Modulator of Cardiovascular risk?
Submitted.

Brouwers M.C.G.J., Bilderbeek-Beckers M.L.A., Georgieva A.M., van der Kallen C.J.H., van Greevenbroek M.M.J., and de Bruin T.W.A.
Role of Obesity in Nonalcoholic Fatty Liver Disease in Familial Combined Hyperlipidemia
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Abstracts

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VLDL1 rather than VLDL2 number determine plasma triglyceride concentrations in Familial Combined Hyperlipidemia.
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Lipitor, inflammation and gene expression in adipose tissue in familial combined hyperlipidemia.
75th Congress of the European Atherosclerosis Society, 2005, Abstract book, page 20.

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