Familial Combined Hyperlipidemia

A complex multifactorial lipid disorder

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A complex multifactorial lipid disorder

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van de Medische Wetenschappen

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List of abbreviations

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ApM1	Adipose most abundant gene transcript 1
APOA5	Apolipoprotein A5 gene
APOAII	Apolipoprotein AII
APOAV	Apolipoprotein AV
ApoB	Apolipoprotein B
ASP	Acylation stimulatory protein
B2M	Beta-2-microglobulin
BMI	Body mass index
CETP	Cholesteryl ester transfer protein
Ct	Threshold cycle
CVD	Cardiovascular disease
D'	Linkage disequilibrium
DNA	Deoxyribonucleic acid
ELISA	Enzyme-linked immunosorbent assay
FBAT	Family-based association test
FCH	Familial combined hyperlipidemia
FFA	Free fatty acid
GEE	Generalized estimating equations
HBAT	Haplotype-based association test
HDLc	High-density lipoprotein cholesterol
HL	Hepatic lipase
HOMA-index	Homeostasis model assessment-index
HSL	Hormone-sensitive lipase
HyperapoB	Hyperapobetalipoproteinemia
IMT	Intima-media thickness
IR	Insulin resistance
LDLc	Low-density lipoprotein cholesterol
LEPR	Leptin receptor
LPL	Lipoprotein lipase
MnSOD	Manganese superoxide dismutase
mRNA	Messenger ribonucleic acid
NL relatives	Normolipidemic relatives
PBAT	Pedigree-based association test
PBMCs	Peripheral blood mononuclear cells
PCR	Polymerase chain reaction
PCR-RFLP	Polymerase chain reaction restriction fragment length polymorphism
Q-PCR	Real-time quantitative PCR
QTL	Quantitative trait linkage analyses
RLPc	Remnant-like particle cholesterol
sdLDL	Small dense LDL
SNP	Single nucleotide polymorphism

SOLAR	Sequential oligogenic linkage analysis routines
TC	Total cholesterol
TDT	Transmission disequilibrium association tests
TG	Triglycerides
TRL	Triglyceride-rich lipoproteins
TXNIP	Thioredoxin interacting protein
USF1	Upstream stimulatory factor 1
UTR	Untranslated region
VLDL	Very low-density lipoprotein
WHR	Waist-hip ratio

Introduction and outline of thesis

Cardiovascular disease

Since many years, cardiovascular diseases (CVD) are the most frequent cause of death in western civilization. In the Netherlands, 45.445 individuals died of CVD in 2004, representing 33% of the total number of deaths that year [1]. Many risk factors for CVD have been identified, including smoking, diabetes mellitus, high blood pressure, obesity, lack of exercise and a disturbed lipid profile. Obesity (body mass index > 25 kg/m2) is an important risk factor for CVD, because it is present in approximately 48% of the Dutch population. Obesity is responsible for 4% of the newly diagnosed CVD patients [2, 3]. The relation of hyperlipidemia with CVD is stronger, since 20% percent of the total incidence of CVD is attributable to elevated serum total cholesterol levels [3]. Approximately 10% of the Dutch population of 20 years and older, has elevated total cholesterol levels (>6.5 mmol/L) and/or takes cholesterol-lowering drugs [2].

Familial combined hyperlipidemia

Familial Combined Hyperlipidemia (FCH; OMIM144250) is the most common heritable lipid disorder, affecting 1-5% of the general population and up to 20% of the survivors of a premature myocardial infarction [4].

The disturbed lipid profile of FCH is characterized by elevated levels of total cholesterol (TC), triglycerides (TG) and apolipoprotein B (apoB) [5]. Furthermore, increased levels of low-density lipoprotein cholesterol (LDLc), decreased levels of high-density lipoprotein cholesterol (HDLc) and the presence of small dense LDL (sdLDL) are characteristic features of patients with FCH [6, 7]. Besides a disturbed lipid profile, FCH patients are often obese and insulin resistant. So, several cardiovascular risk factors are present in patients with FCH, thereby contributing to the two- to fivefold increased risk of premature CVD (before the age of 60 years) in FCH patients [8-11].

Because of this increased risk of CVD, it is of great importance to correctly identify the patients with FCH and treat them appropriately. The diagnosis of FCH is, however, difficult because of the complex phenotypic heterogeneity and the highly variable expression of FCH, as genetic, metabolic and environmental factors influence its expression [12, 13].

In 1973, FCH was first described by Goldstein et al., based on elevated plasma TC and/or TG levels above the 90th percentile adjusted for age and gender, within a family with multiple types of hyperlipidemia and the presence of premature CVD [14, 15.]

A major problem in the diagnosis of FCH is, however, that the pattern of hyperlipidemia within one individual may vary over time, leading to a non-consistent diagnosis of FCH within a person over time [16]. This makes the traditional diagnostic criteria less suitable for the diagnosis of a genetic lipid disorder like FCH. To establish a more consistent diagnosis of FCH, we have recently evaluated potential new diagnostic criteria for FCH, including sdLDL and apoB, in our 5-year follow-up data of 32 FCH families. A nomogram was developed to calculate the probability of being affected with FCH, based on plasma TC and TG levels, adjusted for age and gender, and absolute apoB levels, as recently published [17]. When this probability is greater than 60% the subject is defined affected by FCH when also at least one first-degree relative exhibits the FCH phenotype and at least one individual in the family has premature CVD.

The genetic origin of familial combined hyperlipidemia

Despite more than 30 years of research the complex genetics of FCH is still not fully understood. Originally, FCH was thought to be a homogeneous single-gene disorder with a major effect on triglyceride levels and a secondary effect on cholesterol levels [14]. Segregation analyses, however, have implicated major genes in FCH that control TG levels, apoB levels, sdLDL and insulin resistance [13, 18-23]. Furthermore, environmental factors, like diet, exercise and smoking, are known to play a role in the expression of FCH [24]. So, at present FCH is known as a multifactorial heterogeneous disorder with numerous genetic, metabolic and environmental factors contributing to its complex phenotype.

Different strategies can be used to identify genetic factors contributing to FCH, including linkage analyses and association studies. For linkage analyses, genome-wide scans are performed within families containing two or more affected individuals by testing many evenly spaced microsatellite markers covering the whole genome. The co-segregation of these markers and FCH is analyzed, resulting in the identification of a chromosomal region that may contain gene(s) involved in the pathophysiology of FCH [25].

The aim in association studies is to detect disease alleles in candidate genes that are more (or less) common in affected subjects than in the general population. Candidate genes include; 1) genes located in a region of interest identified with linkage analyses, 2) genes with a biological action known to be involved in the development or physiology of the disease, and 3) genes involved in similar phenotypes in animal models. The association of the variations in candidate genes with FCH can be tested in a case-control design or family based association studies. When more than one variant is genotyped in a candidate gene, haplotype analyses can be performed. Haplotype analyses are in general more powerful to identify assocation, as the complete genomic region included by the genotyped variants is taken into account.

Linkage analyses

To identify chromosomal regions, containing candidate gene(s) for FCH, linkage analyses have been performed in seven FCH study populations. These linkage analyses have resulted in the identification of multiple loci for FCH, as depicted in figure 1. One locus, on chromosome 1q21-23 has been identified as a major locus for FCH in Finnish FCH families and has subsequently been replicated in four other FCH study populations [26-30]. Another repeatedly identified locus for FCH in Finnish, Dutch and British FCH families, is located on the long arm of chromosome 11, a region including the apolipoprotein *A1/C3/A4/A5* gene cluster [31-33]. Furthermore, loci on seven other chromosomes have been identified for FCH [29, 34-36]. Linkage analyses for the separate traits of FCH, including elevated levels of TC, TG and apoB, decreased levels of HDLc and the presence of sdLDL, resulted in the identification of more than 20 loci spread over the entire genome [27, 29, 31, 33, 36-43].

Candidate genes

Based on mice studies, the thioredoxin interacting protein *(TXNIP)* has been proposed as a candidate gene for FCH underlying the linkage signal on chromosome 1q21-23 [44]. TXNIP is a major regulator of the cellular redox state in mice and humans; by binding and inhibiting thioredoxin, TXNIP inhibits the insulin reducing activity of thioredoxin [45].

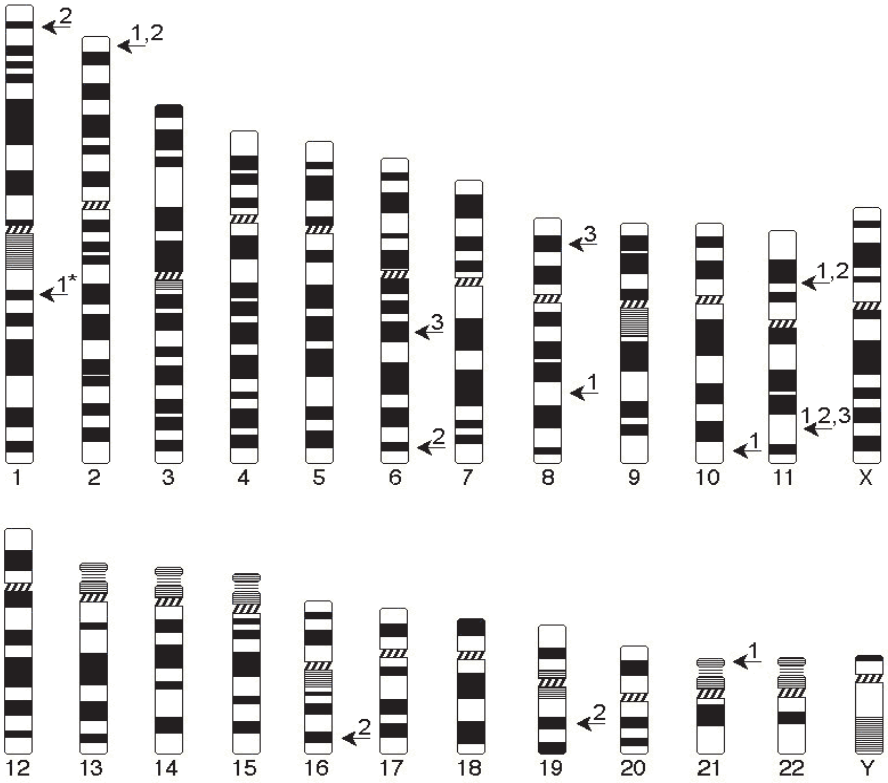

Figure 1
*Presented are all 22 autosomal chromosomes and 2 sex chromosomes present in humans. The arrows indicate previously identified loci for FCH in Finnish (1), Dutch (2) and Brittish (3) FCH study populations. * The locus on chromosome 1q21-23 is replicated in the Mexican, German, Chinese and American FCH populations.*

More recently, a second candidate gene on chromosome 1q21-23, the upstream stimulatory factor 1 *(USF1)* gene, has been suggested as the prime candidate gene for FCH [46]. Two SNPs in the *USF1* gene showed linkage and association with FCH and multiple phenotypes of FCH, including elevated levels of TG, TC, apoB and sdLDL, suggesting that *USF1* contributes to the complex pathophysiology of FCH. Supportive evidence for these associations have been found in two other independent study populations [42, 47]. USF1 regulates the transcriptional activation of more than 40 important enzymes, which are involved in glucose and lipid metabolism, adipose tissue metabolism and blood pressure control, thereby potentially important in the development of atherosclerosis [46, 48].

The repeatedly identified locus on chromosome 11q, includes the apolipoprotein *A1-C3-A4-A5* gene cluster [49]. By combined linkage and association analysis, the apolipoprotein

A5 (*APOA5*) gene, encoding the apolipoprotein AV (APOAV), has been shown to contribute to FCH and its related lipid phenotypes [33, 50, 51]. Based on mice studies, it has been suggested that the APOAV modulates TG levels by influencing VLDL production and secretion [52, 53].

Based on linkage analyses, biological function and animal models several other candidate genes for FCH have been evaluated for their contribution to the FCH phenotype (Table 1). Investigated candidate genes are genes involved in lipid, adipose tissue and glucose metabolism. The most recently investigated candidate gene contributing to FCH is the functional acylation stimulating protein (ASP) receptor C5L2, which is involved in fatty acid uptake in adipose tissue [54].

Table 1 Investigated candidate genes for FCH

Gene	Chromosome	Associated with FCH	References
Lipid metabolism			
Apolipoprotein A1	11q23	+/-	51, 56-62
Apolipoprotein A4	11q23	+	63, 64
Apolipoprotein B	2p24	-	65, 66
Apolipoprotein C3	11q23	+	32, 51, 56, 58, 60-62, 67-69
Apolipoprotein E	19q13	+	65, 70-72
Hepatic lipase	15q21-23	+	73, 74
Lipoprotein lipase	8p22	+/-	59, 75-83
Adipose tissue / FFA metabolism			
ASP receptor C5L2	19q13	+	55
FABP2	4q28-31	-	75, 84
Hormone sensitive lipase	19q13	-	85
PPARα	22q12-13	+	86
PPARγ	3p25	-	87, 88
TNFRSF1B	1p36	+	89
Uncoupling protein 1	4q31	-	69, 90
Glucose metabolism			
Beta3-adrenergic receptor	8p12-11	-	75, 90
Insulin receptor substrate 1	2q36	-	75
Other			
Alpha-adducin	4p16	+	91

ASP receptor C5L2, an acylation stimulating protein receptor; FABP2, Fatty-acid binding protein2; PPAR, Peroxisome proliferator-activated receptors; TNFRSF1B, Tumour necrosis factor receptor 2; +, The candidate gene was associated with FCH; +/-, The association of the candidate gene with FCH is still controversial; -, No association of the candidate gene with FCH was found.

The metabolic origin of familial combined hyperlipidemia

Although several metabolic abnormalities have been suggested to be important for the development of FCH, the exact pathophysiology of FCH is still unknown. Generally, it is thought that FCH is associated with an overproduction of very low-density lipoprotein (VLDL) by the liver, combined with a delayed clearance of atherogenic triglyceride-rich lipoproteins [91, 92]. This is hypothesized to be caused by a disturbed fatty acid (FFA) metabolism. Evidence of a defective postprandial and postabsorptive FFA metabolism in FCH has been demonstrated in vivo [93, 94]. An increased flow of FFA may lead to hepatic overproduction of VLDL [95]. In patients with FCH, this increased flux of FFA to the liver is possibly caused by inadequate FFA trapping in the adipose tissue [96, 97]. So, it is hypothesized that a disturbed adipose tissue metabolism is involved in the pathophysiology of FCH. Presented in figure 2, is a schematic overview of the hypothesized mechanisms by which the adipose tissue contributes to insulin resistance, dyslipidemia and atherosclerosis, all characteristics features of FCH.

Disturbed adipose tissue metabolism

The adipose tissue is the major site for storage and mobilization of free fatty acids (FFAs) [97, 98]. FFAs are transported in the blood bound to albumin to the adipose tissue, where the FFAs are taken up by the adipocytes to be esterified to TG. Furthermore, the lipoprotein lipase (LPL) catalyzed hydrolysis of TG in chylomicrons and VLDL particles, results in the liberation of FFAs, which are also taken up by the adipocyte to be esterified into triglycerides [99]. This process of esterification to TG is mediated by the action of acylation stimulatory protein (ASP) and modulated by insulin [100]. The TG can also be de-esterified again into FFAs. This process of reesterification of FFAs into TG in the adipose tissue is stimulated by hormone-sensitive lipase (HSL) [101]. These FFAs leave the adipose tissue and enter the bloodstream, where they bind to albumin, which transports it to other organs for utilization. FCH patients are suggested to have a defective estification and mobilization of FFA in the adipose tissue, leading to reduced FFA uptake into adipocytes. This results in an increased delivery of FFA to the liver, which in turn leads to enhanced synthesis of VLDL conversion to sdLDL, characteristic for FCH (Figure 2, A) [102].

Adipokines

Adipose tissue was for a long time regarded as a relatively passive site of energy storage. Presently, it is clear that adipose tissue is not only involved in energy storage, but also acts as an endocrinological active tissue which releases numerous proteins involved in inflammation, lipid and glucose metabolism and atherogenesis [103]. Two major adipokines produced by the adipose tissue are leptin and adiponectin.

Leptin is involved in the regulation of the energy expenditure and appetite via hypothalamic receptors, including the leptin receptor (LEPR) [104]. An increase in leptin concentration will lead to more energy expenditure and less appetite in normal persons via the hypothalamus. In obese individuals, however, increased leptin levels appear to fail to influence energy intake or expenditure to restore fat mass to normal. Obesity is therefore believed to be a state of leptin resistance. Since leptin has direct effects on insulin secretion, elevated leptin levels are not only associated with obesity, but also with insulin resistance [105].

Furthermore, leptin levels are associated with CVD (Figure 2, B).

Another major adipokine derived from the adipose tissue is adiponectin, which is abundantly present in human plasma [106]. In contrast to leptin, adiponectin production is inversely correlated with adipose tissue mass [107]. Adiponectin stimulates the oxidation of fatty acids in muscle, enhances insulin sensitivity in muscle and liver and inhibits the inflammatory process and thereby, possibly atherosclerosis [107-110]. In addition, adiponectin had been reported to directly and indirectly modulate plasma lipid levels [111, 112]. Based on these properties, adiponectin is suggested to be the missing link between obesity, insulin resistance and atherosclerosis (Figure 2, B).

Visfatin is a very recently discovered visceral fat-specific protein that may be related to the development of obesity-related diseases such as diabetes mellitus and cardiovascular disease [113]. Other proteins released by the adipose tissue include adipsin, resistin, tumor necrosis factor alpha, interleukine 6 and plasminogen activator inhibitor 1 [114, 115]. These proteins released by the adipose tissue are also potentially involved in dyslipidemia, insulin resistance and atherosclerosis, and may thus contribute to FCH (Figure 2, C).

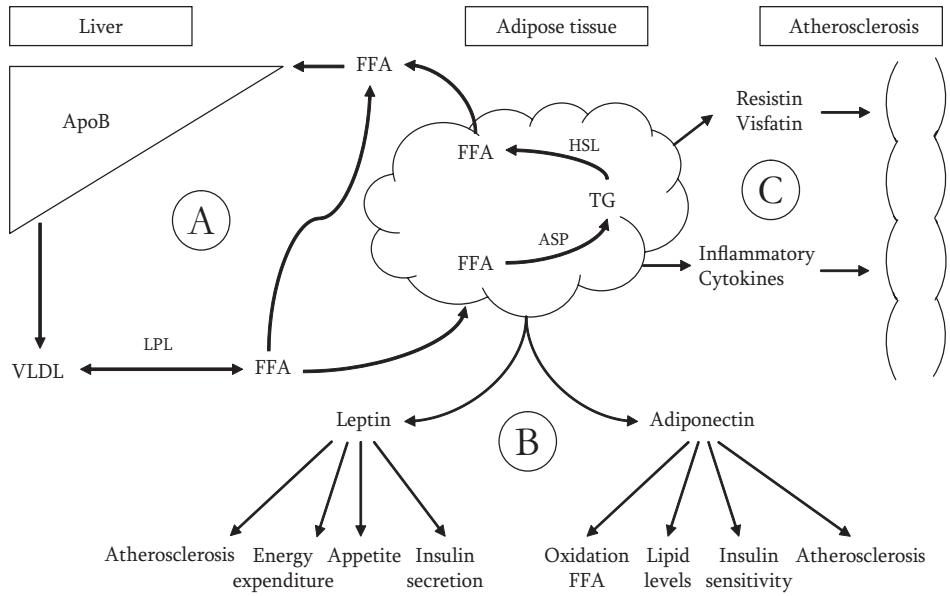

Figure 2
Presented is a schematic overview of the potential central role of adipose tissue in dyslipidemia, insulin resistance and atherosclerosis. Defective FFA trapping in the adipose tissue, leads to an increased delivery of FFA to the liver, which in turn leads to enhanced synthesis of VLDL (A). Furthermore, adipose tissue releases adipocytokines including leptin and adiponectin (B) and numerous other proteins (C), which are involved in insulin resistance, dyslipidemia and atherosclerosis. ApoB, apolipoprotein B; ASP, acylation stimulating protein; FFA, free fatty acids; HSL, hormone-sensitive lipase; LPL, lipoprotein lipase; TG, triglycerides; VLDL, very low-density lipoprotein.

Objectives and outline of this thesis

The central aim of the present thesis was to further explore the genetic origin of FCH by performing both genome-wide linkage analyses in our well-defined FCH families and using the candidate gene approach for *TXNIP*, *USF1* and *APOA5*, three genes reported to be potentially involved in FCH (Part 1).

The second aim was to further evaluate the role of adipose tissue in the etiology of FCH. First we reviewed several metabolic disturbances that may contribute to the pathogenesis of FCH, including the presence of a possible defect in the adipose tissue metabolism in relation to the lipid levels, parameters of obesity and insulin resistance and the presence of atherosclerosis (Figure 3). We have evaluated the plasma levels of two major adipokines, adiponectin and leptin. Finally, variations in two genes, the gene encoding for adiponectin *(ApM1)*, and the gene, encoding for the leptin receptor *(LEPR)*, were determined in relation to FCH (Part 2).

FCH study population

To investigate the genetic and metabolic origin of FCH, we used the Nijmegen FCH study population ascertained in 1994 and followed-up and expanded in 1999. In 2004, all FCH patients and normolipidemic relatives from both 1994 and 1999 were invited for the 10-years follow-up study.

In 1994, 40 FCH families, including both FCH patients, normolipidemic relatives and spouses, were recruited. These multigenerational families varied in size from 12 to 114 members and spanned multiple (between 3 and 5) generations. In total, 201 FCH patients, 357 normolipidemic relatives and 102 spouses were recruited.

Thirty-two of the 40 FCH families, recruited in 1994, participated in the 5-years follow-up study in 1999. These families were further extended by the recruitment of 127 new family members, including 19 FCH patients. Furthermore, 5 new FCH families were recruited, including 121 individuals. So, in 1999 the Nijmegen FCH study population comprised 37 FCH families, including 644 individuals.

In 2004 the 10-years follow-up study was started. In total 31 families from 1994 and 30 families from 1999, including in total 312 subjects, participated in this follow-up study. From 28 families, including 63 patients with FCH and 125 normolipidemic relatives, data were available from 1994, 1999 and 2004. In total 341 individuals of 33 families, including 103 FCH patients, participated in this 10-years follow-up study. Non-invasive measurements of atherosclerosis, including intima-media thickness (IMT) measurements, were performed in all participating individuals.

Part 1: The genetic origin of familial combined hyperlipidemia

A genome-wide linkage scan was performed in the thirteen most informative families of our FCH study population in 1994, consisting of 414 individuals with phenotypic data. In total 377 autosomal markers with an average density of 10 cM and an average heterozygosity of 0.75 were genotyped in the 275 most informative individuals. Linkage analyses were performed for two different diagnostic criteria of FCH, the traditional diagnostic criteria and the nomogram, and for related quantitative traits, including TC, TG, apoB, HDLc and sdLDL levels (**Chapter 1**).

To evaluate the potential role of *TXNIP* in the FCH phenotype in humans, we analyzed

the coding region, 5'UTR and introns of the *TXNIP* gene by direct sequencing in 10 well-defined patients with FCH and 5 healthy controls (**Chapter 2**). The 3'UTR of the *TXNIP* gene, influencing the expression levels of *TXNIP*, has not been sequenced in FCH patients. We have quantified *TXNIP* mRNA expression levels in peripheral blood mononuclear cells (PBMCs) of 30 patients with FCH and 30 healthy controls (**Chapter 3**).

In **chapter 4**, powerfull family-based analyses were used to analyze the role of *USF1* in FCH. Two SNPs of the *USF1* gene were measured and the association of the individual SNPs and their combination into haplotypes with FCH and its associated phenotypes was investigated. Furthermore, *USF1* mRNA expression levels were quantified in PBMCs of 30 FCH patients and 30 healthy controls.

Finally we investigated the potential role of the *APOA5* gene in the etiology of FCH. Two SNPs in this gene, -1131T>C and S19W, were determined and analyzed by family-based association analyses of the individual SNPs and haplotypes (**Chapter 5**).

Part 2: The metabolic origin of familial combined hyperlipidemia

In **chapter 6** we review the characteristic phenotypes of FCH, the central role of FFA in the pathogenesis of FCH and the possibility of a disturbed adipose tissue metabolism in the pathophysiology of FCH. Furthermore, the role of remnant-like particles cholesterol (RLPc), as a characteristic phenotype of FCH, and its contribution to the increased risk of CVD in FCH patients are explored (**Chapter 7**).

To investigate the role of adipose tissue in FCH, we measured plasma leptin levels in 37 FCH families, and analyzed the relation of leptin levels with FCH, obesity and CVD. (**Chapter 8**). In addition, we determined the effect of the Gln223Arg polymorphism in the leptin receptor in our FCH study population (**Chapter 9**).

Plasma adiponectin levels were measured, to evaluate a possible association of adiponectin levels with FCH (**Chapter 10**). To investigate the role of genetic variations in the *ApM1* gene, encoding for adiponectin, in determining the adiponectin levels in patients with FCH, we measured two variations in this gene. Furthermore, we studied the association of these variants in the *ApM1* gene with FCH and its associated atherogenic lipid profile, using a family-based single-nucleotide polymorphism (SNP) and haplotype approach (**Chapter 11**).

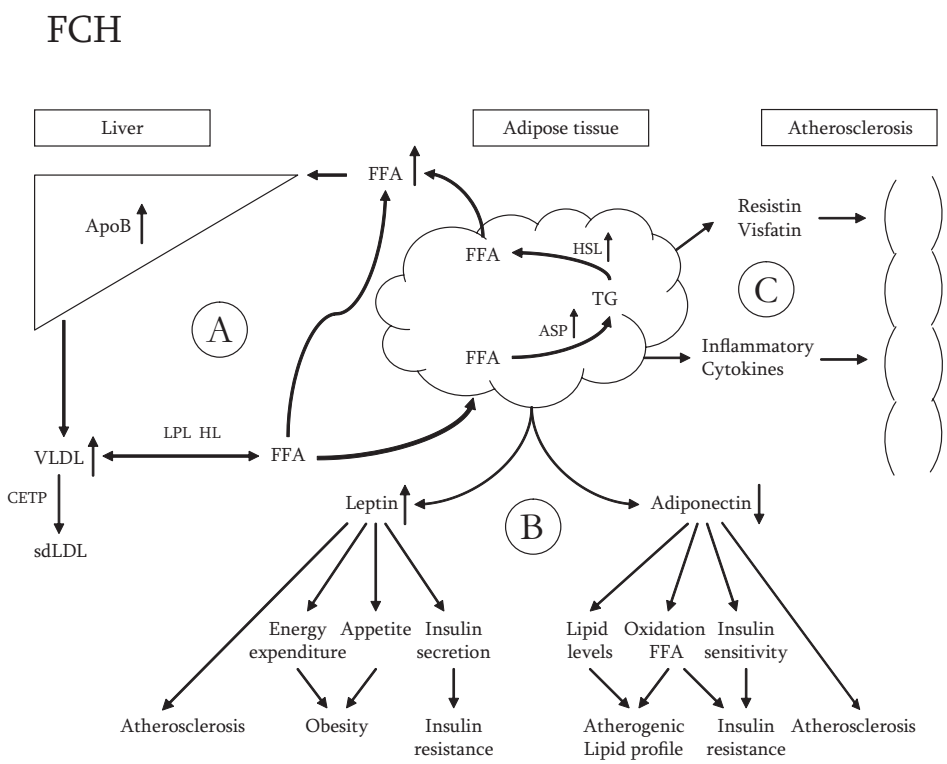

Figure 3
Presented is a schematic overview of the hypothesized role of a disturbed adipose tissue metabolism in the pathophysiology of FCH. A: Defective FFA trapping in the adipose tissue, leads to reduced FFA uptake into adipocytes. Resulting in an increased delivery of FFA to the liver, which in turn leads to enhanced synthesis of VLDL and sdLDL, characteristic for FCH. B: The release of leptin is hypothesized to be increased in patients with FCH, whereas the release of adiponectin is hypothesized to be decreased in patients with FCH. These disturbed adipocytokine levels may contribute to obesity, dyslipidemia, insulin resistance and atherosclerosis in patients with FCH. C: The release of other proteins by the adipose tissue, which are involved in atherogenesis, may also be disturbed and are therefore also potentially involved in FCH.

Chapter 1

Genome-wide linkage analysis in familial combined hyperlipidemia: effect of diagnostic criteria and quantitative traits

Submitted

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Abstract

Background Several linkage analyses for familial combined hyperlipidemia (FCH) have been performed, all using different diagnostic criteria, leading to the identification of multiple loci for FCH. Different diagnostic criteria were, however, used for these linkage analyses. The aim of the present study was to investigate whether different diagnostic criteria for FCH affect the results of linkage analyses. **Methods** For the genome-wide scan we used thirteen informative Dutch FCH families. Multipoint linkage analyses were performed using SOLAR. **Results** The linkage analyses using the traditional diagnostic criteria for FCH did not result in the identification of a region of interest. Linkage analyses using the nomogram did result in the identification of two regions of interest (lod score > 1), on chromosome 6q25-27 (lod score = 1.30) and 12q23-24 (lod score = 1.02). Linkage analysis of two characteristic quantitative traits of FCH, including small, dense LDL (sdLDL) and plasma triglyceride (TG) levels, supported chromosome 6q as potential locus for FCH (sdLDL: lodscore = 1.13; TG: lodscore = 1.92). Linkage analysis of the other quantitative traits of FCH showed four regions with suggestive evidence of linkage, including TG levels (10p14-q21 (lod score = 2.24)), total cholesterol levels (1p32-12 (lod score = 1.98) and 8p22-21 (lod score = 2.11)) and HDL-cholesterol levels (19q13 (lod score = 1.73)). **Conclusion** The use of different diagnostic criteria for FCH affects the results of linkage analyses; only when using the nomogram, a region of interest on chromosome 6q was identified, which was supported by suggestive evidence of linkage for TG levels and a region of interest for sdLDL, two characteristic traits of FCH.

Introduction

Familial combined hyperlipidemia (FCH) is a multifactorial disease, characterized by the presence of multiple lipid and lipoprotein phenotypes, including hypercholesterolemia, hypertriglyceridemia and hyperapobetalipoproteinemia. Decreased levels of high-density lipoprotein cholesterol (HDLc) and the presence of small dense low-density lipoprotein (sdLDL) are also characteristic for FCH [13, 116]. In addition to a disturbed lipid profile, FCH patients are obese and insulin resistant [9].

The diagnosis of FCH, first described in 1973, is traditionally based on elevated plasma total cholesterol (TC) and/or triglyceride (TG) levels above the 90th percentile adjusted for age and gender, within a family with multiple types of hyperlipidemia and the presence of premature CVD [14]. Other groups studying FCH, however, use different cut-off points for elevated TC and/or TG levels, i.e. the 95th percentile or absolute values of TC (> 6.5mmol/l) and/or TG (> 2.3 mmol/l) levels [26-28, 30, 32, 34]. Since 1983, hyperapobetalipoproteinemia has been identified as a characteristic phenotype of FCH [117]. Also for apolipoprotein B (apoB) levels, different cut-off points are used (i.e. the 75th or 90th percentile) as diagnostic criterion for FCH [27, 34]. Recently, we showed that the traditional diagnostic criteria for FCH were not consistent within a person over time, thereby making these criteria less suitable as diagnostic tool [16]. Therefore, we developed a nomogram, based on 5 years follow-up data of 32 FCH families, to establish the diagnosis FCH more consistent over time [17]. This nomogram is based on plasma TG and TC levels, adjusted for age and gender, and absolute apoB levels.

Despite more than 30 years of research, the complex genetics of FCH is still not fully understood. Back in 1973, FCH was reported as a monogenetic disorder with dominant inheritance [14]; however, subsequent studies have proven that the inheritance of FCH is multifactorial. Evidence for a major gene effect on various FCH related traits, including apoB levels [20, 22, 23], TG levels [21], the presence of sdLDL [18, 23], and insulin resistance [19] has been provided by segregation analyses.

Several linkage analyses have been performed in various ethnic groups, leading to the identification of multiple suggestive or significant loci for FCH and its related phenotypes. Using the traditional diagnostic criteria, loci on chromosome 1q, 10q and 21p were identified in Finnish FCH families [28, 29] and additional loci on chromosome 2p and 11p were identified when combined analyses of these Finnish FCH families with a separate Dutch FCH population were performed [35]. Separate linkage analyses in this set of Dutch FCH families, using traditional diagnostic criteria in combination with apoB levels above the 75th percentile, provided evidence of linkage on chromosome 11p, 16q and 19q [34]. Within British FCH families, using more strict criteria with TC or TG plasma levels above the 95th percentile, genome-wide linkage analyses led to the identification of loci on chromosome 6q, 8p and 11p [32, 36]. FCH diagnosis based on TC levels above 250 mg/dL and/or TG levels above 200 mg/dL and/or apoB levels above the 75th percentile, as diagnostic criteria in Dutch FCH families, resulted in the identification of loci on chromosome 6q, 11q and 16q [31]. The linkage analyses in Chinese, German, American and Mexican FCH families, where supportive results for linkage of FCH with chromosome 1q21-23 have been reported, were performed again with other diagnostic criteria [26, 27, 30]. The discrepancy between the linkage analyses in the identified loci may be caused by the use of different diagnostic criteria, in addition to its complex etiology.

The aim of the present study was to evaluate whether the use of different diagnostic criteria affect the results of linkage analyses, thereby contributing to the diversity in identified loci in the different FCH study populations. Therefore we performed linkage analyses for two diagnostic criteria of FCH, using the traditional diagnostic criteria and the nomogram. Here we present the results of never before published genome-wide linkage analyses performed in our group of very well documented Dutch FCH families from Nijmegen with 10-years follow-up data.

Methods

Ascertainment of FCH families

The study population consisted of 40 large FCH families [16, 18, 20]. The families were ascertained through probands, recruited from the outpatient lipid clinic of the Radboud University Nijmegen Medical Centre [18, 20]. Back in 1994, families were included if the proband exhibited a combined hyperlipidemia with both plasma TC and TG levels above the age- and gender-related 90th percentile during several periods in which they were not treated with lipid-lowering drugs and despite dietary advice [18, 20]. In addition, a first-degree relative had to present elevated levels of TC and/or TG above the 90th percentile and the proband or a first-degree relative had to present premature cardiovascular disease (CVD) before the age of 60 years. Families were excluded when probands had underlying diseases causing hyperlipidemia (i.e., diabetes mellitus type 1 and 2, hypothyroidism, and hepatic or renal impairment), a first-degree relative had tendon xanthomata or probands were homozygous for the *APOE2* allele. All included subjects were Caucasian and above the age of 10 years [18, 20].

Diagnostic criteria for FCH

In the present study we have used two diagnostic criteria for FCH, namely the traditional diagnostic criteria and the nomogram, which is based on our 5-years follow-up data and more consistent over time [17]. The traditional diagnostic criteria for FCH are based on TC and/or TG above the age- and sex-related 90th percentile and was used for the ascertainment of the families. The nomogram was used to calculate the probability of being affected with FCH and is based on plasma TC and TG levels, adjusted for age and gender, and absolute apoB levels [17]. In short, in the nomogram for each of the three variables the corresponding number of points was read from a scale and then summed to give a total point score, which was translated into a probability of being affected by FCH. When having a probability of more than 60% the subject was defined as affected by FCH. For both diagnostic criteria for FCH, the diagnostic lipid phenotype had to be present in at least one first-degree relative, and premature CVD, before the age of 60 years, had to be present in at least one individual in the family. The total study population comprised 40 multigenerational families including 663 individuals with phenotypic data [18, 20].

Genome-wide linkage scan

The genome-wide linkage scan was performed in the thirteen most informative families consisting of 414 individuals with phenotypic data. Of these subjects, 275 subjects were genotyped. In 11 of these 13 families, ascertained through the traditional criteria, the proband

also fulfilled the criterion of the nomogram for the FCH diagnosis; for the other 2 probands, the nomogram could not be applied because of missing apoB data. The families varied in size from 12 to 114 members and spanned multiple (between 3 and 5) generations. The selection of the families and individuals, based on their position in the pedigree and informativity, was based on simulation analyses performed with the SLINK software [118]. As in the literature, linkage analyses of a separate independent Dutch FCH population has been published, we will refer to our FCH families as the Nijmegen FCH families.

The ethical committee of the Radboud University Nijmegen Medical Centre approved the study protocol and the procedures were in accordance with institutional guidelines. All subjects gave informed consent.

Biochemical analyses

Plasma TC and TG were determined by enzymatic, commercially available reagents (Boehringer-Mannheim, Germany, catalog No. 237574 and Sera Pak, Miles, Belgium, catalog No. 6639, respectively). TC and TG levels were available for all 414 individuals. Plasma apoB concentrations were successfully determined by immunonephelometry in 395 individuals [119]. HDLc was successfully determined by the polyethylene glycol 6000 method in all individuals. LDL subfractions were separated by single spin density gradient ultracentrifugation [120]. A continuous variable K represents the LDL subfraction profile of each individual. A negative K-value ($K \leq -0.1$) reflects a more dense LDL subfraction profile, and a positive K-value ($K > -0.1$) reflects a more buoyant profile. A K-value was available for 386 individuals.

Genotyping

DNA was obtained from peripheral blood lymphocytes using a standard technique [121]. The genotyping was performed by the Mammalian Genotyping Service at the Marshfield Medical Research Foundation (Marshfield, Wisconsin, USA). The 377 autosomal markers had an average density of 10 cM and an average heterozygosity of 0.75. The markers primarily originated from screening set 9 and in part from screening set 10. Sex-averaged marker maps were obtained from the Marshfield Center of Medical Genetics. To ensure the quality of genotyping data, Mendelian inconsistencies were checked with the PedCheck program and inconsistent marker genotypes were eliminated (0.5%) [122].

Statistical analysis

Descriptive statistics, presented separately for FCH patients, normolipidemic relatives and spouses, are expressed as mean with standard deviation (SD). Heritability estimates and multipoint linkage analysis were performed using the pedigree-based variance components method as implemented in the Sequential Oligogenic Linkage Analysis Routines (SOLAR) package (version 2.1.3 and prerelease version 4.0.0) [123]. Since FCH has a complex pattern of inheritance, nonparametric linkage methods, not requiring assumptions regarding the mode of inheritance, were used. Multipoint linkage analysis increases the power to detect true linkage, decreases the false positive rate and gives a more precise localisation of the linkage regions [123]. Multipoint linkage analyses were performed for FCH using the traditional diagnostic criteria and the nomogram to diagnose FCH, both having a dichotomous distribution. Multipoint linkage analyses were also performed for the traits associated with FCH, including TC, TG, apoB, HDLc

levels and the K-value, as quantitative parameters. These traits were adjusted for age and gender by using the regression modeling option of SOLAR. The variance components method assumes multivariate normality of the trait and is vulnerable to a high kurtosis [124]. In order to obtain normally distributed values, TG was logarithmically transformed and z-scores were calculated for all quantitative parameters. Allele frequencies of the markers were estimated through maximum likelihood techniques and were used to impute missing genotype data and to create identical by descent (IBD) files. The program HOMO, implemented in the SOLAR package, was used to assess genetic heterogeneity. Simulation techniques were applied to calculate empirical pointwise p-values of observed lod scores.

The heritability estimates were calculated for the quantitative traits using SOLAR [123]. We obtained heritability estimates of 0.59 for TC, 0.49 for TG, 0.63 for apoB, 0.40 for HDLc and 0.54 for the K-value. The power to obtain a lod score above 1, representing a region of interest, for a locus that explains at least 20% of the trait heritability was approximately 80%. According to Lander and Kruglyak [125], a lod score above 1.9 provides suggestive evidence of linkage and a lod score above 3.3 provides significant evidence of linkage. As we have only included the autosomal chromosomes of the genome-wide linkage scan in the linkage analyses, the threshold of 1.9 for suggestive and 3.3 for significant evidence of linkage may be to conservative. Therefore, we have applied the statistical methods as described by Lander and Kruglyak [125] for the traits with a lod score above 1.5 to determine if a lower lod score was already suggestive for linkage in our population. Based on these methods, a lod score of 1.7, as discovered in our linkage analyses, provided suggestive evidence of linkage for HDLc, for all other traits a lod score above 1.9 was suggestive for linkage. Regions with suggestive and/or significant evidence of linkage were screened for biologically plausible candidate genes that could explain the observed linkage peaks with the NCBI map viewer, the UCSC genome browser and the ENSEMBL genome browser.

Results

Subject characteristics

According to both the traditional criteria and the nomogram, 106 subjects were affected with FCH. In addition, 24 patients were affected with FCH according to either the traditional diagnostic criteria (n = 12) or the nomogram (n = 12) as diagnostic criterion. So, in total 118 subjects were diagnosed with FCH according to the nomogram. Descriptive statistics of anthropometric and metabolic characteristics of the study population, presented below and in table 1, were based on the nomogram as diagnostic criterion for FCH.

Besides the 118 FCH patients, another 209 normolipidemic relatives and 72 spouses, with available phenotypic data, were included. The percentages of males and females were comparable between the FCH patients, normolipidemic relatives and spouses (Table 1). With a mean age of 46 years, FCH patients were older than the normolipidemic relatives and younger than the spouses. Compared to normolipidemic relatives and spouses, FCH patients had significantly higher levels of plasma TC, TG and apoB and significantly lower levels of HDLc. Furthermore, FCH patients were characterized by the presence of sdLDL, as reflected by a significantly lower K-value. Normolipidemic relatives had lower levels of TC, TG and apoB, compared to the spouses (Table 1).

Table 1 Characteristics of patients with familial combined hyperlipidemia, normolipidemic relatives and spouses

	FCH patients (n = 118)	**NL relatives (n = 209)**	**Spouses (n = 72)**
Gender (males) *	46 (39.0%)	104 (49.8%)	30 (41.7%)
Age (years)	44.6 (16.4) † ‡	35.9 (15.7) §	51.8 (12.0)
Lipids			
- TC (mmol/L)	6.9 (1.2) † ‡	5.2 (1.1) §	6.0 (1.3)
- TG (mmol/L)	2.9 (1.7) † ‡	1.1 (0.5) §	1.4 (0.8)
- ApoB (mg/L)	1413 (253) † ‡	970 (243) §	1133 (277)
- HDL-c (mmol/L)	1.06 (0.29) † ‡	1.30 (0.34)	1.28 (0.38)
- K-value	-0.48 (0.23) † ‡	-0.11 (0.26)	-0.16 (0.28)

*The diagnosis of familial combined hyperlipidemia is based on the nomogram; The results are presented as mean (SD); * Results are presented as number (%); FCH, familial combined hyperlipidemia; NL relatives, normolipidemic relatives; TC, total cholesterol; TG, triglycerides; ApoB, apolipoprotein B; HDL-c, HDL cholesterol; K-value, a value ≤ -0.1 represents the presence of small dense LDL; †, p < 0.05, FCH patients compared to NL relatives; ‡, p <0.05, FCH patients compared to spouses;* §, *p < 0.05, NL relatives compared to spouses.*

Linkage analyses for two diagnostic criteria of FCH

When using the traditional criteria for the diagnosis of FCH, we were unable to identify a region of interest with a lod score > 1 (Figure 1). A region on chromosome 1, where previously a locus for FCH was identified in Finnish FCH families [28], did not show evidence of linkage in our Nijmegen FCH families. A modest peak with a lod score of 0.98 (nominal p-value = 0.021) was observed close to this region, however, with a wide range of approximately 100 cM, overlapping the previously identified locus for FCH in Finnish FCH families. So, no regions of interest with a lod score > 1 were found when using the traditional diagnostic criteria for FCH. However, when using the nomogram as diagnostic criterion for FCH, in our population two regions of interest with lod scores above 1 were observed (Figure 1). A lod score of 1.30 (nominal p-value = 0.008) was located on chromosome 6q25-27 between markers D6s305 and D6s1277. For this locus, a lod score above 1 was present in a moderate interval of 19 cM. On chromosome 12q23-24, a lod score of 1.02 (nominal p-value = 0.017) was observed between the markers D12s2070 and PAH. No genetic heterogeneity was observed for the two identified loci. So, two regions of interest for FCH were observed when using the nomogram as diagnostic criterion for FCH. However, both loci did not reach the level of suggestive (lod score > 1.9) or significant (lod score > 3.3) evidence of linkage.

Linkage analyses for the quantitative traits related to FCH

For the quantitative traits, related to FCH, we identified several loci with suggestive evidence of linkage (Figure 2 and 3). Interestingly, for both TG levels and sdLDL, we identified a locus on chromosome 6q, which co-localized with the peak for FCH, when using the nomogram. For the

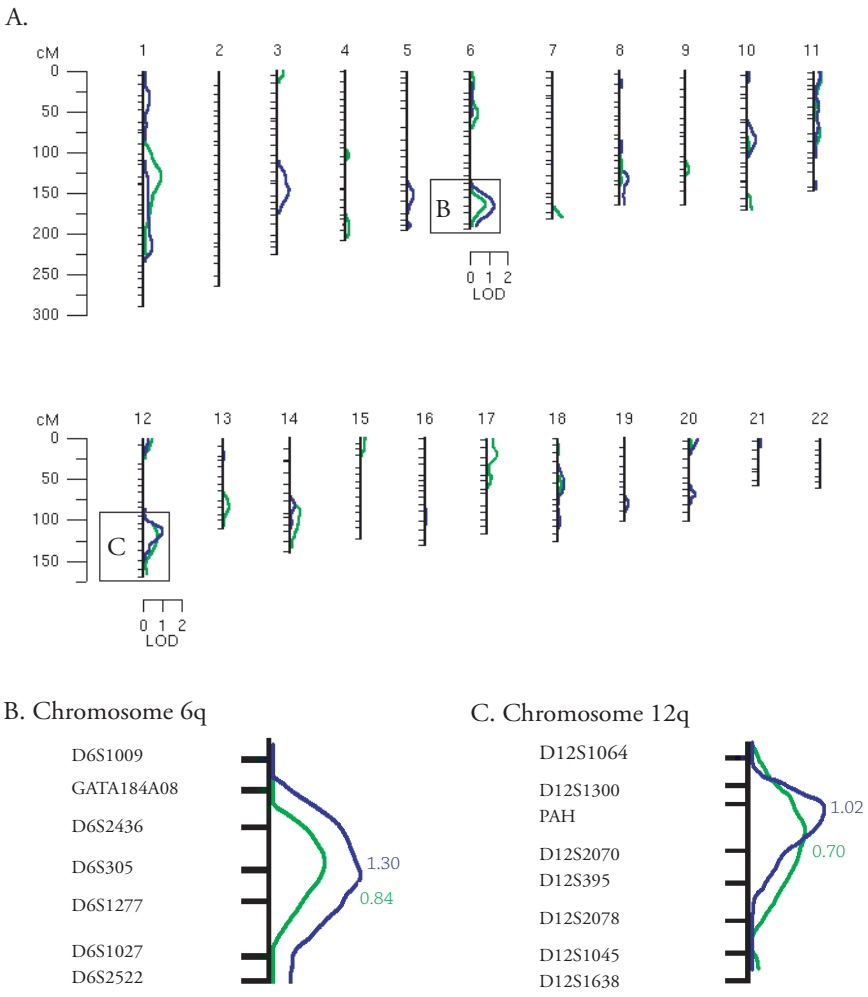

Figure 1
Multipoint lod score for each chromosome in the genome scan for FCH according to the nomogram and the traditional diagnostic criteria. The blue line represents the multipoint lod scores for FCH according to the nomogram; the green line represents the multipoint lod scores for FCh according to the traditional diagnostic criteria based on total cholesterol and/or triglyceride levels above the 90th percentile; ***A*** *shows the lod scores for each chromosome in the genome scan; B shows in detail the region with a lod score above 1 on chromosome 6; C shows in detail the region with a lod score above 1 on chromosome 12; lod scores > 1.0 represent a region of interest; lod scores > 1.9 represent loci with suggestive evidence of linkage; lod score > 3.3 represent loci with significant evidence of linkage.*

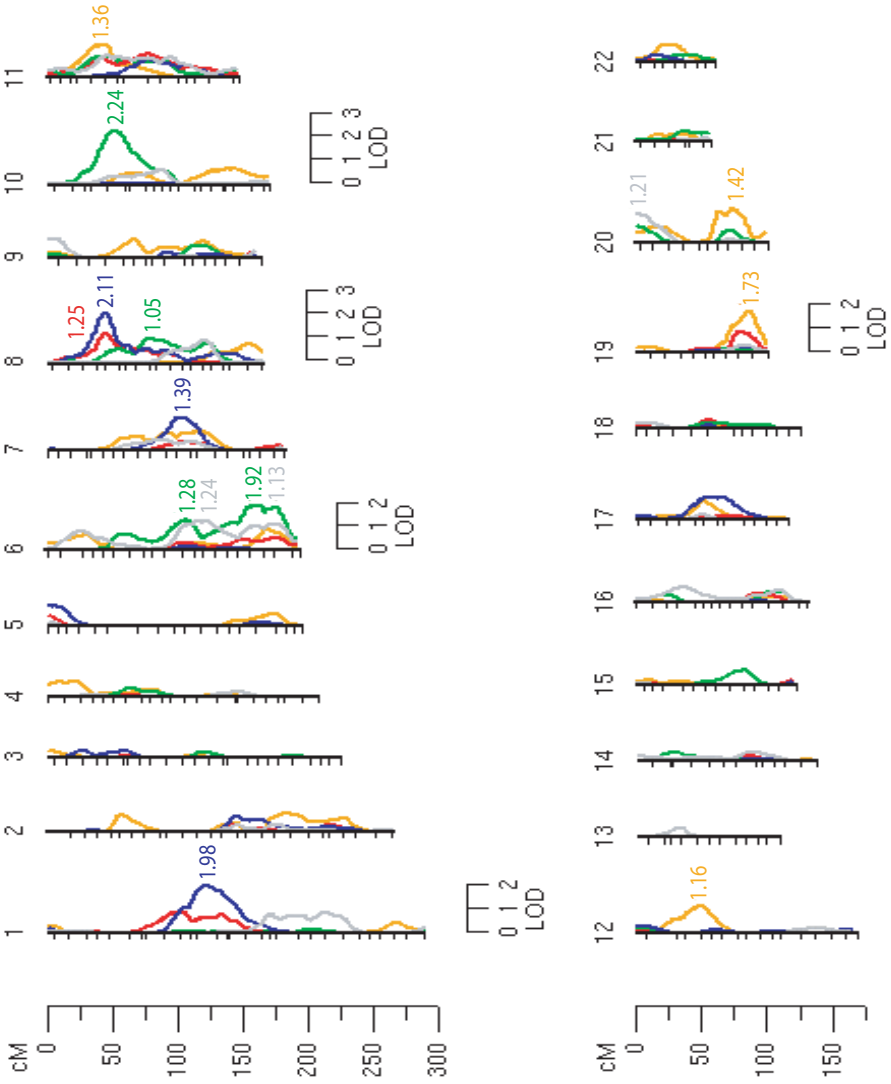

Figure 2
Multipoint lod score for each chromosome in the genome scan for the quantitative lipid and lipoprotein parameters. Represented are the multipoint lod scores for the related phenotypes; the blue line represents the lod score obtained for total cholesterol levels; the green line represents the lod score obtained for triglyceride levels; the red line represents the lod score obtained for apolipoprotein B levels; the orange line represents the lod score obtained for high-density lipoprotein cholesterol; the grey line represents the lod score obtained for the K-value, a marker for the presence of small dense LDL; lod scores > 1 represent a region of interesg; lod scores > 1.9 represent loci with suggestive evidence of linkage; lod scores > 3.3 represent loci with significant evidence of linkage.

quantitative trait TG (green line) suggestive evidence of linkage for chromosome 6q was found with a lod score of 1.92 (nominal p-value = 0.001). This linkage peak on chromosome 6q for TG, had a moderate range of 58 cM, with nearby, another peak with a lod score of 1.28, with a different underlying locus, as their maxima were > 40 cM apart [126]. For the K-value, representing sdLDL (grey line) we report a region of interest on chromosome 6q with a lod score of 1.13.

For TG levels (green line), we identified another locus on chromosome 10p with a lod score of 2.24 (nominal p-value = 0.0002). For the quantitative trait TC (blue line), two loci on chromosome 1p and 8p were found, with lod scores of 1.98 (nominal p-value = 0.002) and 2.11 (nominal p-value = 0.002), respectively (Figure 2). For apoB (red line) no suggestive evidence of linkage was found, although a locus with a lod score of 1.25 was located on chromosome 8p, which co-localized with the peak of TC. Finally, for HDLc (orange line) we identified one locus with suggestive evidence, when applying the statistical methods of Lander and Kruglyak, on chromosome 19q13 with a lod score of 1.73 (nominal p-value = 0.004). No genetic heterogeneity was observed for the identified loci. The exact lod score and position of the peaks with a lod score above 1 are shown for all quantitative traits in figures 2 and 3.

Discussion

In the present study, consisting of 13 Nijmegen FCH families, we performed linkage analyses of two diagnostic criteria for FCH. The linkage analyses using the traditional diagnostic criteria to diagnose FCH did not result in the identification of a region of interest (all lod scores < 1). Linkage analyses using the nomogram did, however, result in two regions of interest with a lodscore above 1, on chromosome 6q25-27 (lod score = 1.30) and 12q23-24 (lod score = 1.02). Most interestingly, this locus on chromosome 6q was supported by suggestive evidence of linkage for plasma TG levels (lod score = 1.92) and a region of interest for sdLDL (LOD score = 1.13), two major characteristics of FCH.

Suggestive evidence of linkage for FCH on chromosome 6q25-27 was previously reported in another Dutch FCH population [31]. In that separate, independent Dutch FCH population, the obtained linkage was supported by suggestive evidence of linkage for LDL particle size, TC, apoB, apoCIII levels and diastolic blood pressure [31, 37, 127]. In another population consisting of families enriched in coronary artery disease, significant evidence of linkage with this region on chromosome 6q was found for LDL particle size [128]. One of the genes located in this region on chromosome 6q is the manganese superoxide dismutase *(MnSOD; SOD2)* gene, the primary defense of cells against free radical mediated damage, suggested as a candidate gene for FCH [31]. The mechanism, however, by which MnSOD may influence the lipid metabolism is yet unclear. Within the supportive region on chromosome 6 in our Nijmegen FCH population, several other candidate genes could also be identified, including the nearby located *Lp(a)* gene (6q26-27). Lp(a) is considered as an independent risk factor for cardiovascular disease [129]. The role of Lp(a) in FCH, however, remains controversial [129, 130].

Previous linkage analyses for FCH have also identified other suggestive loci, located on chromosomes 1q, 2p, 8p, 10q, 11p, 11q, 16q, 19q and 21q [26-32, 34-36]. Only the loci for FCH identified on chromosome 1q and 11q have been identified in more than one FCH study population. A candidate gene, *USF1*, has been proposed to underlie the linkage signal on

chromosome 1q [46]. In our Nijmegen FCH families, *USF1* was identified as a modifier gene [131]. In the present study we show that there is no evidence of linkage for our Nijmegen FCH population in this region. This absence of evidence of linkage for chromosome 1q is supported by linkage analyses performed in the other separate, independent Dutch FCH population, where also no linkage for FCH was found in this region [31, 34, 35]. The apolipoprotein *A1-C3-A4-A5* gene cluster is located on chromosome 11q23-24, a multigene locus repeatedly found to be involved in modulating the expression of FCH [33, 51, 59]. In our FCH families, however, no evidence of linkage for FCH is present in this region.

The discrepancy in identified loci for FCH between different studies, may be caused by the use of different diagnostic criteria. In the present study of Nijmegen FCH families we have used two diagnostic criteria for FCH. Two loci with lod scores above 1, revealing regions of interest, were identified for FCH when using the nomogram as diagnostic criterion, while no loci with a lod score above 1 were identified for FCH based on the traditional criteria. We have already shown that the traditional diagnostic criteria for FCH are not consistent over time and therefore may be less suitable for performing genetic analysis [16]. The nomogram, as previously published, is more consistent over time and therefore clinically a better diagnostic criterion to diagnose FCH [17]. As it is important for genetic studies that the diagnosis of FCH is consistent over time, it was expected that, based on a decrease in misclassification, the nomogram had more power to reveal linkage. Although the lod scores using the nomogram were higher than 1, whereas when using the traditional diagnostic criteria for FCH all lod scores were < 1, linkage analysis of FCH using either diagnostic criteria did not reach the level of suggestive or significant evidence of linkage according to Lander and Kruglyak [125]. Nevertheless, lod scores above 1, revealing regions of interest, are presented in line with previously reported linkage analysis of FCH [26-28, 30, 32, 34].

Also within another separate, independent Dutch FCH population, there was support that the difference in obtained loci by linkage analysis for FCH can be explained by the use of different diagnostic criteria for FCH. Two genome-wide linkage analyses, using different diagnostic criteria for FCH within one population, have been reported [31, 35]. These two studies, performed in the same study population, have identified in total 10 suggestive loci for FCH; however, only 3 comparable loci were obtained in both studies [31, 35]. In the same population, a third linkage analysis of 14 candidate genes was performed, with again other diagnostic criteria for FCH and only 1 of these 3 regions of interest showed supportive evidence of linkage [34]. So, the difference in reported loci obtained within one study population could be explained by the use of different diagnostic criteria for FCH.

Other possible explanations for the diversity of loci reported for FCH by different study populations include ethnic admixture and population stratification. Since FCH is a complex multifactorial lipid disorder and genetic heterogeneity is potentially present in different FCH populations, which may affect the outcome of linkage analyses and contribute to the fact that most studies are underpowered to resolve minor loci of interest.

Our FCH study population for the genome-wide linkage analyses consisted of 13 informative FCH families including 275 individuals with genotypic and phenotypic data. We performed simulation analysis to determine the power of our population to detect linkage. This power calculation revealed that, even when assuming a 50% heterogeneity, our population provided a 61% chance of finding a lod score > 2. So, although our sample size appears relatively small, the power calculations indicated a fair chance of finding a locus for FCH, when present.

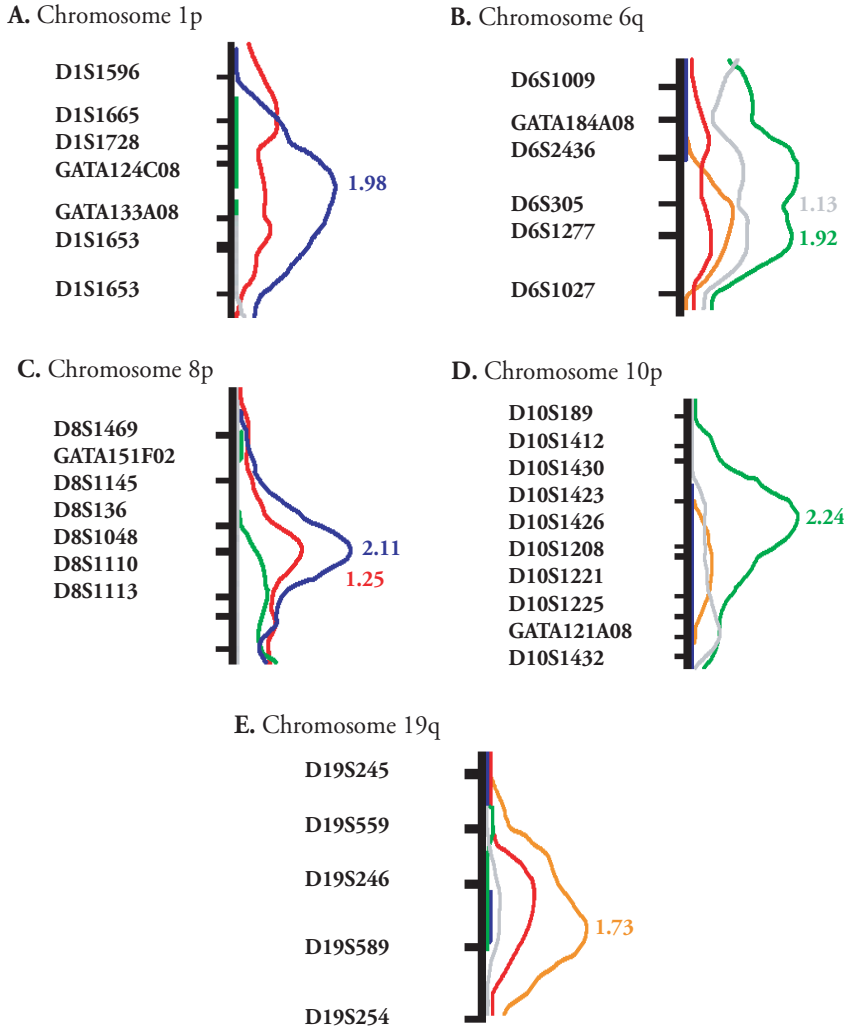

Figure 3
The regions with suggestive evidence of linkage obtained for the quantitative lipid and lipoprotein parameters in detail. Presented are in detail the regions with suggestive evidence of linkage for the related phenotypes; the blue line represents the lod score obtained for total cholesterol levels; the green line represents the lod score obtained for triglyceride levels; the red line represents the lod score obtained for apolipoprotein B levels; the orange line represents the lod score obtained for high-density lipoprotein cholesterol; the grey line represents the lod score obtained for the K-value, a marker for the presentce of small dense LDl; lod scores > 1 represent a region of interest; lod scores > 1.9 represent loci with suggestive evidence of linkage; lod scores > 3.3 represent loci with significant evidence of linkage.

FCH is a multifactorial disease, in which the interplay of multiple genes and environmental factors are involved in the complicated phenotype to be expressed. Therefore, the binary classification of family members into affected and unaffected may not be the best approach for finding disease-predisposing genes. In addition to this, no consensus has been reached on the diagnostic criteria for FCH, making it hard to compare the results of the different linkage analyses. So, to enhance power to detect comparable susceptibility genes for FCH, quantitative trait linkage analyses could be performed for the related lipid profiles. The wide variance of quantitative traits could provide more statistical power to detect linkage than the binary categorization of the FCH diagnosis.

In the present study we performed quantitative trait linkage analyses (QTL) for the following traits; TC, TG, apoB, HDLc and the K-value, resulting in the identification of five loci with suggestive evidence of linkage. On chromosome 1p32-12 we have identified a locus with a lod score of 1.98 for TC, where a lod score above 1 was present within an interval of 50 cM. This same region was also found to be associated with apoB levels in the other Dutch FCH families [38, 40]. The leptin receptor *(LEPR)* gene was previously suggested as a candidate gene for this region. We have shown in a previous study, that the Gln223Arg polymorphism in the *LEPR* gene was associated with an increased risk on FCH and decreased HDLc levels in our Nijmegen FCH families [132]. No increased TC levels were, however, found to be associated with the *LEPR* gene [132]. Also located in this region is the 5'-AMP-activated protein kinase alpha 2 *(AMPK)* gene [133-135]. The AMPK cascade is a sensor of cellular energy status and plays a key role in the regulation of fatty acid and cholesterol metabolism. The AMPK cascade is, furthermore, involved in the regulation of energy expenditure and appetite at the whole body level by mediating, among other things, leptin, adiponectin and ghrelin [135].

In our FCH population, suggestive evidence of linkage was found for TC on chromosome 8p22-21. Within other study populations, this region has previously been identified as a candidate region for FCH, TC, TG, diastolic blood pressure and waist-hip ratio [36, 37]. The lipoprotein lipase *(LPL)* gene is located in this region and it is suggested in the literature that FCH patients have reduced LPL activity [136]. In a previous study in our Nijmegen FCH families, we showed that *LPL* is a susceptibility gene for several types of dyslipidemia, including TC levels [82].

The highest lod score of 2.24 was identified for TG levels on chromosome 10p14-q21. In the Finnish FCH families, suggestive evidence of linkage for FCH and elevated TG levels was found for this region [29]. Suggestive evidence of linkage for TG was also found in the Mexican FCH families; however, in the multipoint analysis no additional support for this region was found [42]. No biologically plausible candidate gene was found in this region.

On chromosome 19q13 a locus for HDLc has been identified. In other FCH study populations, this region has never been reported as an interesting region for FCH or any of the related phenotypes. In the Quebec family study significant evidence of linkage for LDLc has been reported for this region [137]. In the HERITAGE family study, suggestive evidence for a pleiotropic QTL at 19q12-q13 for the covariation of TG levels and adiposity was reported [138] and a meta-analysis of 4 genome scans provided suggestive evidence of linkage for LDLc and apoB [139]. Several candidate genes that influence lipid metabolism are located in this region, including the apolipoprotein E and hormone-sensitive lipase genes [140-142].

In conclusion, we show that the use of different diagnostic criteria for FCH affects the results of the linkage analyses. Only when using the nomogram, we report a region of interest for FCH

on chromosome 6q which is supported by the results of the linkage analysis for TG levels and sdLDL, two characteristic features of FCH. Future research concerning linkage analyses in FCH should focus on unequivocal diagnostic criteria for FCH in addition to underlying quantitative traits that show a high genetic correlation with FCH in multivariate linkage analyses.

Chapter 2

Thioredoxin interacting protein in Dutch families with familial combined hyperlipidemia

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Abstract

Background Familial combined hyperlipidemia (FCH), characterized by multiple lipoprotein phenotypes, is the most common hereditary lipid disorder in humans. A mutant mouse strain, HcB-19, with similar biochemical features as FCH patients, has recently been identified. The mutation causing the FCH phenotype in these mice is located in the thioredoxin interacting protein (*TXNIP*) gene. The *TXNIP* gene in mice is located on chromosome 3F2.2, which is syntenic to chromosome 1q21 in humans, a region where several groups have positioned a locus for FCH. **Methods** To evaluate the potential role of *TXNIP* in the FCH phenotype in humans, we analyzed the coding region, 5' UTR and introns of the *TXNIP* gene by direct sequencing in 10 well-defined patients with FCH and 5 healthy controls. **Results** We did not find any sequence variants in these regions of the *TXNIP* gene in patients with FCH. **Conclusion** Our results suggest that different genes are involved in the FCH phenotype in humans compared to mice. We conclude that in our Dutch FCH patients, the *TXNIP* gene, based on its intronic, exonic and 5' UTR sequences, is not involved as a major contributor to the FCH phenotype.

Introduction

Familial combined hyperlipidemia (FCH) is the most common genetic hyperlipidemia in humans of unknown etiology, affecting 1-2% of the general population. FCH is strongly associated with premature cardiovascular disease and among survivors of a premature myocardial infarction, up to 20% are affected with FCH [4]. FCH is characterized by hypercholesterolemia and/or hypertriglyceridemia. Other phenotypes of FCH include elevated levels of apolipoprotein B (apoB), elevated levels of very low-density lipoprotein (VLDL) remnants, the presence of small dense LDL (sdLDL) [13, 18, 23, 97], obesity and insulin resistance [9, 93].

Recently a mutant mouse strain, HcB-19/Dem (HcB-19), which shows several phenotypic similarities with FCH patients, i.e., increased levels of total cholesterol (TC), triglycerides (TG), apoB and triglyceride-rich lipoproteins, has been identified [143]. Castellani et al. identified a locus (Hyplip1) which strongly linked to the FCH features in these mice [143]. A few years later it was shown that these mice had a decreased expression of the thioredoxin interacting protein *(TXNIP)*. By sequencing, a spontaneous nonsense mutation (T97Z) in the *TXNIP* gene was identified. This mutation changes a tyrosine codon (TAT) into a stop codon (TAA), which leads to a truncated protein of only 97 amino acids [44]. The *TXNIP* gene in mice is located on chromosome 3F2.2, which is syntenic to chromosome 1q21 in humans [144], a region to which a major contributor of the FCH phenotype has been mapped in a Finnish, German and Chinese population [26, 28, 30]. Chen and DeLuca were the first to identify the human homologue of *TXNIP* on 1q21, by differential screening of HL-60 cell lines [145]. The coding region of the human gene spans 2.3 Kb, consists of 8 exons and encodes a polypeptide of 391 amino acids. Exon sizes ranged from 36 bp (exon 8) to 471 bp (exon 1) and intron sizes ranged from 106 bp (intron 5) to 539 bp (intron 1). The 5'UTR region is 212 bp compared with a 1,306 bp long 3'UTR.

TXNIP is a major regulator of the cellular redox state in mice and humans by binding and inhibiting thioredoxin and thereby also inhibiting the insulin reducing activity of thioredoxin [45]. The mutant mice had decreased CO2 production, but increased ketone body synthesis, suggesting that the altered redox status down-regulates the citric acid cycle and so sparing fatty acids for TG and ketone body production. This revealed a new pathway of potentially clinical significance, which quantitatively contributes to plasma lipid metabolism and the FCH phenotype in mice and possibly in humans [143]. In the present study, we evaluated the putative role of the *TXNIP* gene in humans by sequencing the coding region, the 5' UTR and all introns of the *TXNIP* gene in 10 well defined patients with FCH, selected from 32 unrelated FCH families and 5 healthy controls [16].

Methods

In 1994, we recruited 32 large families with FCH (n = 660). In 1999 we completed the first 5 years follow-up study for these families, to investigate the metabolic and genetic aspects of FCH. For sequencing the *TXNIP* gene, we selected 10 well-defined patients with FCH from these 32 unrelated FCH families [16]. The FCH patients were selected based on the presence of FCH, in both 1994 and 1999, according to the standard lipid criteria, i.e., TC and TG levels above

the 90th percentile adjusted for age and gender, according to the PROCAM study [146]. Recently, we showed that elevated apoB levels and the presence of sdLDL are more consistent diagnostic criteria for FCH [16]. So in addition to the traditional lipid diagnostic criteria, all selected patients with FCH required elevated apoB levels (apoB > 1,200 mg/L) and the presence of sdLDL (K-value ≤ -0.1) in both 1994 and 1999 [147]. From the cohort of 1999, five healthy control subjects were randomly selected from the spouses of the families. The ethical committee of the UMC Nijmegen approved the study protocol. Table 1 summarizes the data in patients and controls.

Table 1 Anthropometric and biochemical parameters in patients with familial combined hyperlipidemia and controls both in 1994 and 1999.

				Cohort 1994					**Cohort 1999**				
	Sex	CVD	Age *	BMI	TC	TG	apoB	K	BMI	TC	TG	apoB	K
FCH	M	No	54	27	8.6	1.6	1586	-0.5	27	7.9	3.7	1739	-0.4
	F	No	63	24	7.9	2.0	1695	-0.4	24	6.7	2.8	1473	-0.1
	F	No	61	30	8.0	3.4	1717	-0.7	30	7.2	3.1	1608	-0.3
	M	Yes	48	24	7.4	3.5	1418	-0.6	25	7.7	6.8	1469	-0.6
	F	Yes	71	-	8.9	3.1	2020	-0.7	29	8.6	3.3	2035	-0.6
	F	No	76	22	8.0	3.0	1750	-0.6	22	7.7	2.5	1710	-0.4
	F	No	49	27	8.6	1.6	1808	-	29	8.3	6.7	2000	-0.4
	F	No	51	26	7.6	3.1	1553	-0.6	28	7.9	5.2	1792	-0.7
	M	No	56	33	7.7	2.7	1531	-0.3	36	7.4	4.2	1546	-0.2
	M	No	70	30	7.2	3.6	1604	-0.5	31	7.2	4.4	1531	-0.7
Control	M	No	58						24	3.8	0.5	710	0.1
	F	No	60						37	2.8	0.5	549	0.1
	F	No	36						22	3.7	0.4	638	0.2
	F	No	40						28	4.3	0.8	742	0.3
	M	No	63						18	3.9	0.5	678	0.2

*Sex: F, female; Sex: M, male; CVD, cardiovascular disease; age (years); BMI, body mass index (kg/m²); TC, total cholesterol (mmol/L); TG, triglycerides (mmol/L); apoB, apolipoprotein B (mg/L); K, a K-value ≤ -0.1 represents the presence of small dense LDL (sdLDL); FCH, familial combined hyperlipidemia; *, age in 1999*

Plasma TC and TG were determined enzymatically, using commercially available reagents (Boehringer-Mannheim, Germany and Sera Pak, Miles, Belgium, respectively). LDL subfractions were separated by single-spin density gradient ultracentrifugation [120]. A continuous variable K was defined to characterize the LDL subfraction profile of each individual. A K-value below –0.1 reflects a sdLDL subfraction profile [147]. Total plasma apoB concentrations were determined by immuno-nephelometry as described in detail elsewhere [119].

Genomic DNA was extracted from peripheral blood leukocytes, using the Puregene DNA isolation kit (Biozym, Landgraaf, The Netherlands). The *TXNIP* gene was amplified in six

overlapping fragments (2,573 bp in total) by polymerase chain reaction (PCR) using the following conditions: 20 mM Tris-HCl (pH = 8.4), 50 mM KCl, 1.75 mmol/l MgCl2 (for fragment 5 the MgCl2 was 2.25 mM), 200 µmol of each dNTP, 0.01% gelatin, 100 ng forward and reverse primers, 1.25 U Taq polymerase (Invitrogen, Breda, The Netherlands) and 400 ng DNA in a 50 µl reaction volume. The primer sequences, annealing temperatures, locations, and product sizes of the six fragments are listed in table 2. PCR amplifications were performed, with an initial denaturation (94°C, 3 min) then 40 cycles of 94°C for 1 min, annealing at a temperature specific for each fragment for 1 min (Table 2), extension at 72°C for 30 sec followed by a final extension (72°C, 7 min). The PCR products were column-purified using Roche High Pure PCR purification kits (Roche Applied Science, Mannheim, Germany), and bi-directional sequencing analysis was performed on an ABI Prism 3100 Genetic analyzer using the ABI Prism BigDye Terminator version 3 chemical sequencing kit according to the instructions of the manufacturer (PE Applied Biosystems, Nieuwerkerk a/d Yssel, The Netherlands). The sequences were aligned with the reference sequence to detect sequence variants.

Table 2 Primer sets used for sequencing.

Primer sequence (5' > 3')		Position primer * (bp)	Tm (°C)	Products (bp)
1F	GCTTAGTGTAACCAGCGGCGT	3,004–3,024	53	514
1R	CTCTAATCAGCTTTCACCCTCCAAC	3,518–3,494		
2F	CTTCTGGAAGACCAGCCAACAG	3,554–3,475	53	538
2R	TAGCCATTAGGCTTGCTGTTACAC	3,991–3,969		
3F	CTTCAATACCTTGCATGCCACC	3,915–3,936	62	599
3R	CCATCAGGAATGAACATGCAGG	4,514–4,493		
4F	TCAGACGTCTTGGGCATTAGATTG	4,397–4,420	53	614
4R	GAAACAAGACAGCTGTGGTAACAGATG	5,011–4,985		
5F	GGGTAGATGCAGGGTGGCTTC	4,949–4,969	62	513
5R	CGGTGGTGGCATGAACTTGAAC	5,462–5,441		
6F	GGATGTCATTCCTGAAGATCACCG	5,339–5,362	53	403
6R	CACACTCCATTGCAGAGACTGTTG	5,742–5,719		

[a] *According to GenBank Accession number AB051901*

Results and discussion

The anthropometric and biochemical characteristics of the participants in the present study are presented in table 1. The patients with FCH and controls did not significantly differ in age and gender. Cardiovascular diseases were more prevalent in the FCH patients. FCH patients had a higher BMI compared to controls. By definition the FCH patients had higher plasma TC and TG concentrations compared to the controls. In addition, patients with FCH were characterized

by higher apoB concentrations and a more sdLDL subfraction profile, as reflected by a more negative value of the parameter K.

When sequencing the 5'UTR, the introns and exons of the *TXNIP* gene in 10 unrelated patients with FCH and in 5 healthy controls, we did not detect any sequence variants by direct sequencing.

We therefore conclude that it is unlikely that single nucleotide polymorphisms in the *TXNIP* gene are causally related to the pathophysiology of FCH in our patients. To the best of our knowledge this is the first report to explore the potential role of *TXNIP* in the FCH phenotype in humans. The interest in the *TXNIP* gene as potential contributor to the FCH phenotype in humans is based on the intriguing finding of a mutation in the *TXNIP* gene causing FCH phenotype in HcB-19 mice. Several explanations may be offered for our apparent negative finding that excludes the role of the *TXNIP* gene in the FCH phenotype in humans. First of all, the phenotype of the HcB-19 mice is not completely comparable to FCH in humans. These mutant mice do share common features with patients with FCH, including hypertriglyceridemia, hypercholesterolemia, elevated plasma apoB levels, increased secretion of triglyceride-rich lipoproteins and hyperlipidemia, which is progressive with age. However, in mice TG levels were 10- to 30-fold higher and apoB levels were 2- to 4.5-fold increased compared with the parental strains and other recombinant congenic mice strains derived from the parental strains [143]. Such highly increased TG and apoB levels are not observed in FCH patients and are therefore not representative for FCH in humans. Furthermore, the HcB-19 mice strain is not obese and does not show elevated insulin levels nor decreased levels of HDLc, characteristics of FCH in humans, which suggest another pathophysiology of FCH in these mice. In addition to this, these HcB-19 mice have decreased production of CO2, possibly caused by a decreased oxidation of free fatty acids (FFA) [143]. Therefore more FFA are available for the synthesis of TG and keton bodies. Increased levels of plasma lactate and decreased levels of pyruvate are also present in these mice [143]. These features have not been described in humans with FCH.

Secondly, inconsistencies between research groups in diagnostic criteria for FCH may also be a reason why we did not find a relationship between the *TXNIP* gene and FCH in humans. Linkage of the FCH phenotype to the 1q21-q23 region is based on the standard diagnostic criteria for FCH, i.e., TC and TG levels above the 90th percentile, adjusted for age and gender [26, 28, 30]. We also included apoB levels and the presence of sdLDL as diagnostic criteria for FCH, because our 5-years follow-up study on FCH showed that these parameters are less variable over time and are more consistent in diagnosing FCH than the standard lipid diagnostic criteria [16]. This lack of unequivocal diagnostic criteria of FCH contributes to the heterogeneity between FCH populations, and may result in a lack of consistency between linkage analyses in different FCH populations.

The human *TXNIP* gene has been mapped to 1q21.1, which is approximately 5-10 Mb proximal to the peak marker for linkage of FCH in this region [148]. Therefore, other genes within this linkage region may as well contribute to the FCH phenotype. Other candidate genes for FCH that have been mapped to this region include the apolipoprotein A2 (APOA2) gene and the genes encoding L-, P- and S-selectinm [148].

FCH is a genetic heterogeneous disease, which means that different genes can be involved in different populations. This heterogeneity may account for excluding *TXNIP* as a candidate gene in our patients, but does not necessarily exclude *TXNIP* as a candidate gene in other populations,

in which linkage has been found for this region.

Finally, we cannot completely exclude the *TXNIP* gene as a quantitative contributor of the FCH phenotype, because 3' UTR of the *TXNIP* gene, existing out of 1,307 base pairs, was not completely analyzed for sequence variants.

In conclusion, we did not find sequence variants in the coding regions, nor in the introns and the 5'UTR of the *TXNIP* gene, which argues against an involvement of *TXNIP* in FCH patients. Therefore, we conclude that in our Dutch FCH patients, the *TXNIP* gene is not involved as a major contributor to the FCH phenotype, but further analyses on expression level are warranted to completely exclude the *TXNIP* gene as a candidate gene for FCH.

Chapter 3

Can we exclude the *TXNIP* gene as a candidate gene for familial combined hyperlipidemia?

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To the editor

Familial combined hyperlipidemia (FCH) is the most common genetic hyperlipidemia in humans and is characterized by elevated levels of plasma total cholesterol (TC), triglycerides (TG) and/or apolipoprotein B (apoB) [116]. A mutant mouse strain, HcB-19/Dem (HcB-19), which shows several phenotypic similarities with FCH patients, has been reported [143]. Within these mice, a spontaneous nonsense mutation (T97Z) in the thioredoxin interacting protein (*TXNIP*) gene has been identified, which resulted in a truncated protein of only 97 of the 391 amino acids [44]. In humans the *TXNIP* gene is located on chromosome 1q21 [144], a region where linkage for FCH has been found [26, 28, 30]. Recently, we have published the sequence data of the *TXNIP* gene in humans and reported no sequence variants for this gene in humans [149]. We concluded that, based on the sequence data, the *TXNIP* gene is not involved as a major contributor to FCH. Other groups have also sequenced the *TXNIP* gene and although they did find rare sequence variants, these variants were not associated with FCH nor with the associated phenotypes [46, 150]. However, as none of these studies sequenced the 3'UTR region of the *TXNIP* gene in patients with FCH, the *TXNIP* gene cannot be excluded completely. The 3'UTR is known to influence RNA stability and thereby the expression levels of genes. So, by measuring the expression levels of *TXNIP*, information on a functional mutation in the 3' UTR can be obtained. Apart from the 3'UTR, however, also other factors are known to influence regulation of the *TXNIP* gene expression, such as transcription factors, methylation and cellular stresses, including oxidative stress, ultraviolet irradiation and heat shock [151] and these influences on the expression levels of the *TXNIP* gene are also taken into account when measuring expression levels. Expression levels of *TXNIP* are substantially decreased within the HcB-19 mice [44]. The expression levels of *TXNIP* in human patients with FCH have not been studied before. In the present study, we quantified *TXNIP* mRNA expression levels in peripheral blood mononuclear cells (PBMCs) of 30 FCH patients and 30 healthy controls, selected from our study population [16, 69].

The selection of the FCH patients was based on the presence of FCH in both 1994 and 1999, according to the recently published nomogram, i.e., TC and TG levels above the 90th percentile adjusted for age and gender and elevated apoB levels [17]. The healthy controls were

Table 1 Characteristics of patients with familial combined hyperlipidemia and controls.

	FCH patients (n = 30)	Controls (n = 30)
Total cholesterol (mmol/L)	6.6 (0.9) †	5.3 (0.9)
Triglycerides (mmol/L) *	2.89 (1.50) †	1.13 (1.44)
Apolipoprotein B (mg/L)	1369 (245) †	940 (216)
HDL cholesterol (mmol/L)	1.11 (0.20) †	1.32 (0.29)
BMI (kg/m^2)	29.1 (4.3) †	25.3 (3.4)
HOMA-index [a]	2.5 (1.6) †	1.7 (1.5)

Results are presented as mean (SD); [a] Presented are geometric mean (mean SD); FCH, familial combined hyperlipidemia; HDL cholesterol, high-density lipoprotein cholesterol; BMI, body mass index; HOMA-index, homeostasis model assessment-index; † p-value < 0.05.

randomly selected from the normolipidemic relatives, being unaffected in both 1994 and 1999. After withdrawal of lipid-lowering medication for 4 weeks and an overnight fast, these 60 subjects re-visited the hospital and blood was drawn by venipuncture. The ethical committee of the Radboud University Nijmegen Medical Centre approved the study protocol. RNA was isolated from PBMCs, including both lymphocytes and monocytes, and reversed transcribed to cDNA. Real-time quantitative PCR (Q-PCR) was applied to determine the threshold cycle (C_t). These C_t values were normalized to a housekeeping gene, beta-2-microglobulin (*B2M*) ($\Delta C_t = C_{t,TXNIP} - C_{t,B2M}$) and the difference in expression between patients and controls was calculated ($\Delta\Delta C_t = \Delta C_{t,Patient} - \Delta C_{t,Control}$). Finally, the relative expression was calculated as $2^{-\Delta\Delta Ct}$ [152].

Table 1 summarizes the descriptives of the patients and controls. FCH patients had significantly elevated levels of plasma TC, TG and apoB levels, and decreased high-density lipoprotein cholesterol (HDLc) levels. Furthermore, FCH patients were more obese and more insulin

Table 2 Relative quantification of *TXNIP* mRNA levels in FCH patients and controls.

	ΔC_t	$\Delta\Delta C_t$	$2^{-\Delta\Delta Ct}$	P-value *
FCH (n = 30)	1.98 ± 1.07	0.05 ± 1.07	0.97 (0.5 - 2.2)	0.73
Controls (n = 30)	1.93 ± 2.11	0.00 ± 2.11	1.00 (0.2 - 4.3)	

*$\Delta C_t = C_{t,\,target} - C_{t,\,B2M}$, C_t of TXNIP normalized to the housekeeping gene, B2M; $\Delta\Delta C_t = \Delta C_{t,\,Patient} - \Delta C_{t,\,Control}$, the difference in expression between the FCH patients and controls; $2^{-\Delta\Delta Ct}$, the relative expression; FCH, familial combined hyperlipidemia; *, p-value, significance of difference between FCH patients and controls.*

resistant compared to their normolipidemic relatives (Table 1).

FCH patients demonstrated a relative expression of 0.97 [0.5 – 2.2] for the *TXNIP* gene, this was compared to controls (1.00 [0.2 – 4.3]) not significantly different. From this we concluded that the expression levels of the *TXNIP* gene in PBMCs did not differ between FCH patients and controls (Table 2). In addition, we analyzed the data split up by gender; no differences between males and females were found (data not shown). The correlation between expression levels of the *TXNIP* gene and levels of TC (r = 0.02), TG (r = -0.02), apoB (r = 0.02), HDLc (r = -0.05) or insulin resistance, as represented by the homeostasis model assessment-index (HOMA-index) (r = -0.09), were also investigated. No significant correlation existed between expression levels of the *TXNIP* gene and any of the phenotypes of FCH.

Previously, it has already been shown by others and us that none of the sequence variants found in the *TXNIP* gene in humans were associated either with FCH or with any of the phenotypes of FCH. In the present study, we show that, unlike in mice, there is no differential expression of the *TXNIP* gene in human patients with FCH. Taken together we conclude that the *TXNIP* gene does not contribute to FCH nor to its associated phenotypes, in our population of Dutch patients with FCH.

In the present study we measured the *TXNIP* expression in PBMCs; in the HcB-19 mice the difference in expression of *TXNIP* was found in several tissues including liver, spleen, kidney, brain, lung, skeletal muscle, testis and heart tissue [44]; however, expression levels in PBMCs have

not been reported in mice. Obviously, these tissue are not readily available in FCH patients, in contrast to PBMCs. Morello et al. investigated whether the FCH-specific transcription profile was detectable in immortalized cell lines from peripheral blood [153]. They conclude that it was possible to detect complex disease-specific transcriptional abnormalities of FCH in peripheral blood, which justifies our compelled choice for PBMCs.

The lack of a difference in expression levels of *TXNIP* in human FCH patients compared to mice could be suggestive for another etiology of the FCH phenotype in mice. These mice are not obese nor insulin resistant [143], in contrast to FCH in humans. Furthermore, TG levels were 10- to 30-fold and apoB levels 2- to 4.5-fold increased in mice, whereas such extreme increased levels are not observed in human FCH patients. So, the phenotype of the HcB-19 mice is different from FCH in humans.

Recently, Pajukanta et al. has identified a new candidate gene for FCH, located in the same region as the *TXNIP* gene [46]. This gene, upstream stimulatory factor 1 (*USF1*), is thought to be responsible for the linkage found in this region and not the *TXNIP* gene. So, all data available point to the same direction that the *TXNIP* gene is unlikely to be involved in the pathogenesis of FCH in humans. We conclude that the *TXNIP* gene is not a causal gene for FCH in humans, based on both expression levels and sequence data.

Chapter 4

The involvement of upstream stimulatory factor 1 (*USF1*) in Dutch patients with familial combined hyperlipidemia

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Abstract

Background Familial combined hyperlipidemia (FCH) is the most common hereditary lipid disorder in humans; however, the genetic origin is still unknown. Recently, the upstream stimulatory factor 1 (*USF1*) gene was proposed as a candidate gene. Two polymorphisms in this gene were associated with FCH and its related phenotypes. In the present study, we examined these two SNPs with respect to FCH, and its related phenotypes, in Dutch FCH families. **Methods** The study population consisted of 36 Dutch FCH families, including 157 FCH patients. The diagnosis of FCH was based on both the traditional diagnostic criteria and a nomogram. The polymorphisms, USF1s1 and USF1s2, were genotyped with PCR-RFLP. *USF1* expression levels in PBMCs were measured using a SYBR Green-based quantitative real-time PCR. The family-based association test (FBAT) software was used for statistical analyses. **Results** The two polymorphisms were in complete linkage disequilibrium. No association was found for the individual SNPs with FCH defined by the nomogram (USF1s1: p = 0.53 and USF1s2: p = 0.53) whereas suggestive associations were found when using the traditional diagnostic criteria for FCH (USF1s1: p = 0.08 and USF1s2: p = 0.07). *USF1* was associated with total cholesterol (USF1s1: p = 0.05 and USF1s2: p = 0.04) and apolipoprotein B (USF1s1: p = 0.06 and USF1s2: p = 0.04). Small dense LDL showed suggestive association (USF1s1: p = 0.10 and USF1s2: p = 0.09). The results from the haplotype analyses supported the results obtained for the individual SNPs. Quantitative RT-PCR experiments did not show haplotype-dependent differences in *USF1* expression levels in PBMCs. **Conclusion** The previously identified risk haplotype of *USF1* showed a suggestive association with FCH and contribute to the related lipid traits in our Dutch FCH families.

Introduction

Familial combined hyperlipidemia (FCH, OMIM-144250) is the most common genetic lipid disorder of unknown etiology in humans, affecting up to 5% of the general population [10]. Major characteristics of FCH include elevated levels of apolipoprotein B (apoB), triglycerides (TG) and/or plasma total cholesterol (TC). Other FCH phenotypes are decreased levels of high-density lipoprotein cholesterol (HDLc) and the presence of small, dense low-density lipoprotein (sdLDL). In addition, patients with FCH have a two-fold increased risk of cardiovascular disease (CVD) and are often obese and insulin resistant [97].

Over the past few years, several groups performed linkage analyses in an attempt to determine the genetic defects causing FCH [26, 28, 30, 34, 36, 42]. By doing this, a locus on chromosome 1q21-23 was identified [26-28, 30]. The first candidate gene proposed in this region was the thioredoxin interacting protein (*TXNIP*) [44]. It was demonstrated, however, that the *TXNIP* gene was not a major contributor to the FCH phenotype [46, 149, 150]. More recently, a second candidate gene, the upstream stimulatory factor 1 (*USF1*) gene, was suggested as the prime candidate gene for FCH in this linkage region, in 60 Finnish FCH families [46]. Twenty-three single nucleotide polymorphisms (SNPs) were reported in the *USF1* gene. Two of these SNPs, USF1s1 (3' UTR, rs3737787) and USF1s2 (intron 7, rs2073658), individually, and combined into a haplotype, showed linkage and association with FCH and multiple phenotypes of FCH, including TG, apoB, TC and sdLDL, implying that *USF1* contributes to the complex pathophysiology of FCH in these Finnish FCH families [46]. The association of *USF1* with FCH was, however, strongest in males with elevated levels of triglycerides [46].

Huertas-Vazquez et al. [42] reported that *USF1* was associated with FCH and elevated TG levels in 24 Mexican FCH families, whereas other phenotypes were not investigated. No sex-specific effect of *USF1* was found in these families. The study by Coon et al. [47] investigated the association of *USF1* with FCH in Utah pedigrees ascertained for early death due to coronary heart disease (CHD), early strokes, or early onset hypertension. They reported suggestive associations for FCH, TG and low-density lipoprotein cholesterol (LDLc) levels, in the pedigrees ascertained for early strokes and early onset of hypertension. However, in the families ascertained for early death due to CHD, in which 60% of the FCH patients were present, no assocation of *USF1* with FCH was found [47].

The USF1 protein regulates the transcriptional activation of a variety of genes involved in glucose and lipid metabolism and in the development of atherosclerosis [154, 155]. USF1 plays a pivotal role in adipose tissue where it influences de novo lipogenesis by mediating the glucose-regulated expression of hormone-sensitive lipase (HSL), a key enzyme in the regulation of lipid storage in adipose tissue [156]. An interaction between SNPs in the *HSL* and *USF1* genes on postprandial TG levels has been previously reported [157]. Despite this, HSL activity in adipose tissue was unchanged in Finnish patients with FCH [158]. USF1 also influences the transcription of fatty acid synthase (FAS), which is involved in the synthesis of fatty acids [154, 159]. FAS might play a role in FCH, as patients with FCH have decreased circulating levels of FAS ligand [160]. USF1 also plays a role in the transcription of several apolipoproteins (APOCIII, APOAII and APOE) [155, 161], glucokinase [162], hepatic lipase [163], angiotensinogen [164] and ATP-binding cassette sub-family A member 1 (ABCA1) [165]. Genetic variation in APOCIII, APOAII and hepatic lipase have been associated with FCH [61, 73, 166, 167], however, the role of glucokinase, angiotensinogen and ABCA1

in FCH has not been explored yet.

In the present study, we evaluated the putative role of the *USF1* gene in Dutch patients with FCH. We investigated the effect of two individual SNPs, and the haplotypes formed by these two SNPs, on FCH and its associated phenotypes, including not only lipids and lipoproteins, but also parameters of obesity and insulin resistance.

Methods

Study population

The FCH population was ascertained in 1994 and followed-up and expanded in 1999. The families were ascertained through probands, recruited from the outpatient lipid clinic of the Radboud University Nijmegen Medical Centre. In 1994 and 1999, we ascertained families in which the proband exhibited a combined hyperlipidemia with both plasma TC and TG levels above the age- and gender-related 90th percentile [146], during several periods in which they were not treated with lipid-lowering drugs, and despite dietary advice. Additionally, a first-degree relative had elevated levels of TC and/or TG above the 90th percentile and the proband, or a first-degree relative, suffered from premature (before the age of 60 years) cardiovascular disease. Families were excluded when probands were diagnosed with underlying diseases causing hyperlipidemia (i.e., diabetes mellitus type 1 and 2, hypothyroidism, and hepatic or renal impairment), a first-degree relative had tendon xanthomata or probands were homozygous for the *APOE2* allele.

In the present study we used the data of all participating subjects in 1999, including the spouses. The total population consists of 36 families from multiple (between 2 and 4) generations and comprised of 611 subjects with known genealogic and phenotypic data. The diagnosis of FCH was based on (1) the traditional diagnostic criteria including TC and/or TG levels above the 90th percentile [168] and (2) on the nomogram, as recently published by our group [17]. Plasma TG and TC levels, adjusted for age and gender, and absolute apolipoprotein B levels, were applied to the nomogram to calculate the probability of being affected with FCH. When this probability is greater than 60%, the diagnostic phenotype is present in at least one first-degree relative, and premature CVD (before the age of 60 years) is present in at least one individual in the family, the individual is defined as affected by FCH. In 26 of the 36 FCH families, ascertained through the traditional criteria [169], the proband also fulfilled the criterion of the nomogram for the FCH diagnosis; for the other 10 probands, the nomogram could not be applied because of missing apoB data. The normolipidemic relatives (n = 390), unaffected spouses of both the FCH patients and the normolipidemic relatives (n = 64) and subjects without known phenotypic and/or genotypic data (n = 230) were included in the family-based analyses. After withdrawal of lipid-lowering medication for four weeks and an overnight fast, blood was drawn by venipuncture. The maximum waist circumference (cm) at the umbilical level were measured in the late exhalation phase while standing. All included subjects were Caucasian and above the age of 10 years. The ethical committee of the Radboud University Nijmegen Medical Centre approved the study protocol and the procedures followed were in accordance with institutional guidelines. All subjects gave informed consent.

Biochemical analyses

Plasma TC and TG were determined by enzymatic, commercially available reagents (Boehringer-Mannheim, Germany, catalog No. 237574 and Sera Pak, Miles, Belgium, catalog No. 6639, respectively). Total plasma apoB concentrations were measured by immunonephelometry [119]. LDL subfractions were separated by single spin density gradient ultracentrifugation, according to a previously described method [120]. A continuous variable, K, represents the LDL subfraction profile of each individual. A negative K-value (K ≤ -0.1) reflects a more dense LDL subfraction profile, and a positive K-value (K > -0.1) reflects a more buoyant profile. Glucose concentrations were analyzed in duplicate using the oxidation technique (Beckman®, Glucose Analyser2, Beckman Instruments Inc., USA). Plasma insulin concentrations were ascertained using a double antibody method [170]. Insulin resistance was assessed by the homeostasis model assessment (HOMA) method [171].

DNA was obtained from peripheral blood lymphocytes using a standard technique. Microsatellite markers, D1s104 and D1s1677, were amplified by polymerase chain reaction (PCR) and analyzed on polyacrylamide gels, as described by Hughes [172]. D1s104 and D1s1677 were succesfully genotyped in 611 and 545 individuals, respectively. The genotyping of D1s1677 failed in 38 FCH patients. For the USF1 variants, PCRs were performed in a final volume of 50 µl at an annealing temperature of 53.7°C for both polymorphisms. Primer sequences for USF1s1 were 5'-GGTGTGTCCTTGAACTGAG-3' (forward) and 5'-CAAGCCAGAGCATCACC TG-3' (reverse) and for USF1s2 5'-CTTTAGTAGAGACAGGGTTTCAC-3' (forward) and 5'-GATTTAGCAGGTATTAGGAGCA-3' (reverse). The mismatch (underlined) for USF1s2 was introduced to create a restriction site for BsiHKA I. The PCR products were digested by either 10 U BstF5 I (USF1s1) or 10 U BsiHKA I (USF1s2) at 65°C (New England Biolabs, USA) and, subsequently, the resulting fragments (242, 172 and 70 bp in heterozygotes for USF1s1 or 154, 136 and 18 bp in heterozygotes for USF1s2) were separated on agarose gels. USF1s1 and USF1s2 were genotyped in all 611 individuals, including 157 FCH patients. The genotyping of USF1s2, however, failed for 6 individuals, including 3 FCH patients.

Quantitative real-time PCR analysis of USF1 expression in PBMCs

USF1 mRNA expression levels were quantified in peripheral blood mononuclear cells (PBMCs) of 30 FCH patients and 30 sex-matched normolipidemic relatives randomly selected from our study population, as previously described [173]. RNA was isolated from PBMCs, including both lymphocytes and monocytes, and reversed transcribed to cDNA. A quantitative real-time PCR was carried out in a total 25 µL containing 2 µL cDNA, 0.25xSYBR® green solution (Invitrogen, The Netherlands), 1xfluorescein (Biorad, The Netherlands), 2 mM MgCl2, 50 ng of forward (5'ATGACCCAGGCGGTGATCCA3') and reverse (5'GACGCTCCACTTCATTATGC3') primers, 100 µM dNTPs, 1x Ampli*Taq* Gold amplification buffer and 1.5 unit of AmpliTaq Gold DNA polymerase (Applied Biosystems, The Netherlands). PCR conditions were; a hot-start by 95°C for 10 minutes, followed by 40 cycles of 95°C for 30 s, 60°C for 30 s and 72°C for 30 s. Samples were run in duplicate on the Icycler iQ real-time PCR detection system (Biorad, The Netherlands) to determine the threshold cycle (Ct) [174]. Expression levels were normalized to *B2M* by comparative quantification using the ΔΔCt method [175].

Statistical analyses

The characteristics of the study population are expressed as mean ± standard deviation (SD). Prior to further statistical analyses, extended Mendelian error-checking was performed for 36 families, including 219 nuclear families (n = 611), with Pedcheck [122]. Data was only available for 25 of the 36 probands, as these 11 probands were already recruited in 1994 and did not participate in 1999. For families with Mendelian inconsistencies, which can be due to paternity problems and/or misgenotyping, problematic genotypes were set to missing for the complete nuclear families or the isolated problematic individuals (n = 10 (1.6%)). The parental data for each polymorphism were tested for Hardy-Weinberg equilibrium by use of an exact-test. Variables with a skewed distribution, including triglycerides and the HOMA-index, were logarithmically transformed. Multipoint parametric linkage analysis of the two microsatelite markers on chromosome 1 and the two *USF1* polymorphisms were performed for three traits, the FCH trait, defined by both the traditional diagnostic criteria and the nomogram and the TG trait, defined by TG levels above the 90th percentile [146], using SIMWALK2 [176]. Data was prepared using MEGA2 [177]. We assumed a disease allele frequency of 0.01 and 0.10 under the dominant and recessive mode of inheritance, respectively. The assumed penetrance in the dominant model was 0.99. In the recessive model, the assumed penetrances with risk and non-risk genotypes were 0.99 and 0.50, respectively. A phenocopy rate of 0.01 was assumed. These conditions are comparable to those used by Pajukanta et al. [28]. Two-point nonparametric linkage analysis of the two microsatelite markers and the *USF1* polymorphisms were carried out using the SOLAR 2.1.4 software [123]. The presence of nonparametric linkage was tested for FCH and the related quantitative traits, including apoB, TC and TG levels and the presence of sdLDL, represented by the K-value. The haploview program [178] was used to calculate allele and haplotype frequencies and to calculate the linkage disequilibrium between the two SNPs. Haploview was also used to calculate transmission disequilibrium association tests (TDT) with permutation analysis to correct for multiple testing.

Associations between the individual polymorphisms or haplotypes and FCH and its related phenotypes in our extended families were determined using two programs, namely, the family-based association test (FBAT) [179] and the pedigree-based association test (PBAT) software [180]. An additive model of inheritance was used, as the mode of inheritance of FCH is unknown and this model is particularly robust [179]. FBAT handles pedigrees by breaking the pedigrees into all possible nuclear families, and evaluating their contribution to the test statistics. According to FBAT, our 36 FCH families included 219 nuclear families. The –e option of FBAT, which computes the test statistic using an empirical variance estimator [181], was implemented as the nuclear families were not independent. The –p option, which performs the Monte-Carlo permutation procedure, was used to correct for multiple testing. As Pajukanta et al. [46] reported a sex-specific effect for *USF1*, adjustments were made for age and gender by calculating the unstandardized residuals. No adjustment for age and gender was made for FCH, as the FCH diagnosis is already corrected for age and gender. For the haplotype analysis, the haplotype-based association test (HBAT) command of the FBAT program was used, utilizing the –e and –p options.

PBAT is capable of handling extended pedigrees without breaking them into nuclear families. Despite this, our extended pedigrees had to be divided into smaller units, due to computational limitations. This resulted in a total of 92 pedigrees, with a maximum of 14 non-founders per pedigree. Age and gender were used as covariates in the models. All tested variables, except for

the dichotomous variables, were transformed to a Z-score, as recommended by PBAT [180]. The proportion of trait variation for TC, apoB and sdLDL explicable by *USF1* polymorphisms were tested by means of generalized estimating equations (GEE) because of possibly correlated values within families.

Differences were considered statistically significant at $P \leq 0.05$. These analyses were conducted using SPSS 12.0.1, PedCheck 1.1, MEGA2, SIMWALK2, SOLAR 2.1.4, HAPLOVIEW 3.2, FBAT 3.2 and PBAT 3.0.

Results

Subject characteristics

According to both the traditional diagnostic criteria and the nomogram, 135 subjects were affected with FCH. In addition, 38 patients were affected with FCH according to either the traditional diagnostic criteria (n = 16) or the nomogram (n = 22) as diagnostic criterion. So, in total 157 subjects were diagnosed with FCH according to the nomogram. Descriptive statistics of anthropometric and metabolic characteristics of the study population, presented below and in

Table 1 Characteristics of patients with familial combined hyperlipidemia, normolipidemic relatives and spouses

	FCH patients (n = 157)	NL relatives (n = 390)	Spouses (n = 64)
Allele frequency (Wt) (USF1s1/USF1s2)	0.75 / 0.75	0.69 / 0.69	0.72 / 0.72
Gender (males)	75 (47.8%)	177 (45.4%)	31 (48.4%)
Age (years)	47.0 (15.6) * †	37.9 (15.7) ‡	56.0 (10.5)
CVD	32 (20.4%) * †	20 (5.1%)	4 (6.3%)
Waist (cm)	87.2 (12.2) *	77.7 (10.9) ‡	84.3 (12.5)
TC (mmol/L)	6.5 (1.1) * †	4.9 (0.9) ‡	5.2 (0.8)
TG (mmol/L)	3.1 (1.5) * †	1.2 (0.5)	1.3 (0.6)
TG >90th percentile	113 (71.6%) * †	11 (2.9%)	2 (3.1%)
ApoB (mg/L)	1370 (264) * †	960 (220)	996 (175)
LDLc (mmol/L)	4.1 (1.2) * †	3.2 (0.9) ‡	3.4 (0.7)
K-value	-0.26 (0.26) * †	0.05 (0.19)	0.05 (0.25)
HOMA-index	3.4 (1.8) * †	2.3 (1.3) ‡	2.7 (1.3)

*FCH diagnosis based on the nomogram. Bivariate variables are presented as number (%); Continuous variables are presented as mean (SD); FCH, familial combined hyperlipidemia; NL relatives, normolipidemic relatives; Wt allele (USF1s1/2), allele frequencies for the wildtype alleles of USF1s1 and USF1s2; CVD, cardiovascular disease; waist, waist circumference; TC, total cholesterol; TG, triglycerides; Apo B, apolipoprotein B; K-value, a value < -0.1 represents the presence of small dense LDL; HOMA, homeostasis model assessment-index; *, p-value < 0.05, compared to NL relatives; †, p-value <0.05, compared to spouses; ‡, p-value < 0.05, compared to spouses.*

table 1, were based on the nomogram as diagnostic criterion for FCH.

FCH patients were older than normolipidemic relatives, but younger than spouses. The higher prevalence of CVD in patients with FCH was evident, compared to normolipidemic relatives and spouses. The mean waist circumference of patients with FCH was significantly higher, compared to normolipidemic relatives. By definition, FCH patients were characterized by increased plasma TC, TG and apoB levels. Furthermore, FCH patients exhibited higher levels of sdLDL, as reflected by a lower K-value. Finally, FCH patients were more insulin resistant, as reflected by a higher HOMA-index. In the total population 126 subjects showed the TG trait, as defined by TG levels > 90th percentile, including 52 men. In table 1, the distribution of the TG trait among FCH family members is presented.

For both USF1s1 and USF1s2, the wildtype allele (C or A, respectively) had a frequency of 0.81 in probands. The wildtype allele frequency of 0.72 in spouses was comparable to the frequency found in probands (p = 0.30). The distributions of the parental alleles and genotypes for both polymorphisms were in Hardy-Weinberg proportions. Analysis performed with haploview showed that the two polymorphisms were in complete linkage disequilibrium (D' = 1.0; R2 = 1.0), resulting in the presence of only two haplotypes in our study population. The C–A haplotype had an overall frequency of 71% (n = 853) and the T–G haplotype of 29% (n = 357).

In total 302 subjects (85 FCH patients, 184 normolipidemic relatives and 33 spouses) carried two common alleles for both SNPs. Two hundred and fifty subjects (59 FCH patients, 165 normolipidemic relatives and 26 spouses) were heterozygous fot both SNPs and 53 subjects (9 FCH patients, 39 normolipidemic relatives and 5 spouses) carried two rare alleles for both SNPs.

With a TDT association test, we observed no overtransmission of one of the alleles (USF1s1: p = 0.62 and USF1s2: p = 0.62) or haplotypes (p = 0.32) to the FCH patients. Correction for multiple testing by permutation analysis did not alter these results.

Linkage analyses

Parametric and nonparametric linkage analyses for the 1q21-23 region were performed utilizing the two SNPs in the *USF1* gene and two microsatellite markers on chromosome 1, D1s104 and D1s1677, previously identified as the markers underlying the linkage signal on 1q21-23 in the Finnish population. Two-point nonparametric linkage analyses of these two markers and the two SNPs in the *USF1* gene did not show evidence of linkage with FCH trait (lod score = 0.0 for the nomogram and lod scores = 0.0 – 0.3 for the traditional diagnostic criteria), the TG trait (lod score = 0.0 – 0.2) or any of the related quantitative traits, including apoB, TC, LDL-c levels and the presence of sdLDL (all two-point lod scores < 0.5). As parametric linkage analyses can be more powerful than nonparametric linkage analyses in our extended families, we also performed parametric linkage analyses which also did not show any evidence of linkage with the FCH trait (lod score = 0.0 for the nomogram and lod score = 0.6 for the traditional diagnostic criteria) and the TG trait, defined by TG levels above the 90th percentile (lod score = 0.5).

Association and haplotype analyses

In table 2, the associations of the polymorphisms of the *USF1* gene, individually and combined into haplotypes, with FCH and its related phenotypes in the total population are

presented. No statistical association of the *USF1* gene with FCH, defined by the nomogram, was found. Applying the traditional diagnostic criteria of FCH revealed a suggestive association of the *USF1* gene with FCH. No association of the *USF1* gene with the TG trait was found. However, after correction for age and gender, suggestive associations for the wildtype haplotype, previously identified as the risk haplotype by Pajukanta et al., were obtained for TC, apoB and sdLDL, when the structure of the pedigrees were taken into account (Table 2). Correcting for multiple testing, with the –p option, resulted in an association of USF1s1 and USF1s2 with TC (p = 0.05 and p = 0.04, respectively) and apoB (p = 0.06 and p = 0.04, respectively). sdLDL showed suggestive association with USF1s1 (p = 0.10) and USF1s2 (p = 0.09). The *USF1* gene could, however, only explain a small proportion of the variation in plasma TC (1.7%), apoB (1.5%) and sdLDL (0.5%) levels. LDL-c and the obesity- and insulin resistance-related phenotypes of FCH were not associated with *USF1* (Table 2).

Table 2 Association of the individual polymorphisms and haplotypes of the USF1 gene with familial combined hyperlipidemia and its related phenotypes after correction for age and gender in the total population.

	FBAT -e		FBAT -p		HBAT -e	HBAT -p	PBAT -e
	USF1s1	USF1s2	USF1s1	USF1s2	Overall Haplotype	Overall Haplotype	Risk Haplotype
FCH - NG	0.45	0.52	0.53	0.53	0.52	0.55	0.95
FCH - Trad	0.07	0.06	0.08	0.07	0.06	0.06	0.05
TG *	0.31	0.27	0.39	0.33	0.27	0.38	0.61
TG > 90th	0.66	0.66	0.67	0.69	0.66	0.69	0.96
TC	0.08	0.06	0.05	0.04	0.06	0.05	0.11
ApoB	0.05	0.05	0.06	0.04	0.04	0.05	0.12
LDLc	0.31	0.24	0.24	0.20	0.27	0.22	0.27
K-value	0.09	0.09	0.10	0.09	0.09	0.11	0.46
Waist	0.66	0.71	0.68	0.69	0.65	0.67	0.55
HOMA *	0.43	0.57	0.53	0.69	0.60	0.73	0.43

*Presented are the obtained p-values for the individual SNPs and haplotypes in the total population; *, ln-transformed variable; FCH – NG, FCH diagnosis based on the nomogram; FCH –Trad, FCH diagnosis based on the traditional lipid criteria; TC, total cholesterol; TG, triglycerides; Apo B, apolipoprotein B; LDL-c, LDL-cholesterol; K-value, a value < -0.1 represents the presence of small dense LDL; waist, waist circumference; HOMA, homeostasis model assessment-index; -e, correction for the non-independence of the nuclear families; -p, correction for multiple testing by permutation tests.*

PBAT was also used to analyze the association between the haplotypes of the *USF1* gene and FCH and its associated phenotypes. PBAT does not implement an overall haplotype test, but generates a p-value for each haplotype. As shown in table 2, the risk haplotype was not associated with FCH when the nomogram was used as diagnostic criterion, but a suggestive association was found when using the traditional diagnostic criteria for FCH. The analyses of the association between *USF1* with other characteristics of FCH did not reveal any statistically

significant association. The associations found for TC and apoB, however, pointed in the same direction as the associations found with the FBAT and HBAT statistics (Table 2).

In view of the fact that Pajukanta et al. [46] demonstrated a gender-specific effect of the *USF1* gene on FCH and TG levels in males, the analyses of both the individual SNPs and the haplotypes of *USF1* were also performed in males only (Table 3). The male population (n = 283), included 65 FCH patients based on the traditional diagnostic criteria and 75 FCH patients based on the nomogram. In total, 52 (18.5%) men had TG levels > 90th percentile. No significant association of the *USF1* gene with the FCH or the TG trait was found, only suggestive evidence for an association in males was present for apoB levels and sdLDL, in concordance with the analysis in the complete families. So, no apparent gender-specific effect was present for these parameters (Table 3). For the obesity parameters, waist circumference, we found suggestive evidence for an association with *USF1* in males only (Table 3).

Table 3 Association of the individual polymorphisms and haplotypes of the USF1 gene with familial combined hyperlipidemia and its related phenotypes after correction for age and gender in males.

	FBAT -e		FBAT -p		HBAT -e	HBAT -p	PBAT -e
	USF1s1	USF1s2	USF1s1	USF1s2	Overall Haplotype	Overall Haplotype	Risk Haplotype
FCH - NG	0.45	0.52	0.48	0.56	0.52	0.55	0.70
FCH - Trad	0.41	0.31	0.47	0.29	0.31	0.31	0.83
TG *	0.53	0.44	0.59	0.54	0.44	0.52	0.75
TG > 90th	0.98	0.98	0.94	0.94	0.98	0.94	1.00
TC	0.33	0.30	0.32	0.29	0.30	0.34	0.11
ApoB	0.12	0.10	0.13	0.12	0.10	0.13	0.05
LDLc	0.63	0.64	0.57	0.62	0.64	0.61	0.65
K-value	0.15	0.12	0.23	0.19	0.12	0.22	0.26
Waist	0.11	0.10	0.06	0.06	0.10	0.06	0.15
HOMA *	0.49	0.47	0.64	0.58	0.47	0.59	0.59

*Presented are the obtained p-values for the individual SNPs and haplotypes in males only; *, ln-transformed variable; FCH – NG, FCH diagnosis based on the nomogram; FCH –Trad, FCH diagnosis based on the traditional lipid criteria; TC, total cholesterol; TG, triglycerides; Apo B, apolipoprotein B; LDL-c, LDL-cholesterol; K-value, a value < -0.1 represents the presence of small dense LDL; waist, waist circumference; HOMA, homeostasis model assessment-index; -e, correction for the non-independence of the nuclear families; -p, correction for multiple testing by permutation tests.*

Expression analyses of USF1 in PBMC's

To further explore the role of USF1 in FCH, expression levels of *USF1* in PBMCs were measured in 30 FCH patients (defined by the nomogram) and 30 controls. Anthropometric and metabolic characteristics of these subjects are presented in table 4. The FCH patients demonstrated a relative expression of 0.9 [0.5 – 2.2] for the *USF1* gene, this was compared to

controls (1.0 [0.3 – 4.0]) not significantly different (p-value = 0.27). Of the 30 FCH patients, 14 carried the risk haplotype, 12 were heterozygous for the *USF1* variants and 4 were homozygous for the rare haplotype. In the control group, 12 subjects carried the risk haplotype, 15 were heterozygous and 3 were homozygous for the rare haplotype. As the FCH patients carrying the *USF1* risk haplotype had a relative expression of 1.3 [0.5 – 1.9] and the FCH patients carrying the rare haplotype had a relative expression of 1.0 [0.6 – 1.6], no difference based on the haplotype were observed within the FCH patients. The results in the controls were comparable.

Table 4 Characteristics of the subgroup of subjects with familial combined hyperlipidemia and controls, in whom *USF1* expression levels were determined.

	FCH patients (n = 30)	Controls (n = 30)
Gender (males)	11 (36.7%)	11 (36.7%)
Age (years)	52.0 (14.0)	46.5 (14.5)
Waist (cm)	88.8 (10.2) *	99.2 (10.3)
Total cholesterol (mmol/L)	6.6 (0.9) *	5.3 (0.9)
Triglycerides (mmol/L)	3.17 (1.63) *	1.21 (0.46)
Apolipoprotein B (mg/L)	1369 (245) *	940 (216)
HOMA-index	2.9 (1.8) *	2.3 (1.5)

*Results are presented as mean (SD); FCH patients, familial combined hyperlipidemia; waist, waist circumference; HOMA-index, homeostasis model assessment-index; * p < 0.05.*

Discussion

In the present study, we investigated the putative role of *USF1* in Dutch FCH families. We show that the wildtype haplotype of *USF1*, previously identified by Pajukanta et al. as a risk haplotype [46], is not associated with FCH when using the nomogram to diagnose FCH, which is based on plasma levels of total cholesterol, triglyceride and apolipoprotein B; however, a suggestive association of *USF1* with FCH in our Dutch FCH families was found when using the traditional lipid criteria to diagnose FCH. Furthermore, we report no association of *USF1* with the TG trait. The risk haplotye of *USF1* was, however, associated with other important phenotypes of FCH, including plasma total cholesterol levels, apolipoprotein B levels and the presence of small dense LDL, accounting for approximately 1% of the variation in these phenotypes. We conclude that *USF1* shows suggestive association with FCH and can be considered a modifier gene, contributing to related lipid traits in our Dutch FCH families.

Several groups tried to replicate the results with regard to *USF1* found in the Finnish FCH families [46] (Table 5). Huertas-Vazquez et al. [42] reported an association of *USF1* with FCH and TG levels in a Mexican population, although their results were less conclusive and no gender-specific effect was found (Table 5). Coon et al. [47] also reported an association of *USF1* with FCH in Utah pedigrees ascertained for early death due to CHD, early strokes, or early onset hypertension. When restricting the analyses to the families ascertained for early death due to CHD, in which

Table 5 Summary of the associations of the *USF1* gene previously reported.

			Total population			Males Only		
			HHRR	GAMETE	FBAT	HHRR	GAMETE	FBAT
FCH	USF1s1	Pajukanta et al.	NS	-	-	0.04	0.05	-
		Huertas-Vazquez et al.	0.05	0.01	0.05	-	-	-
		Coon et al. (total group)	-	-	0.02	-	-	NS
		Coon et al. (H/S group)	-	-	NS	-	-	NS
		Coon et al. (CHD group)	-	-	NS	-	-	NS
	USF1s2	Pajukanta et al.	NS	-	-	0.04	NS	-
		Huertas-Vazquez et al.	NS	0.05	NS	-	-	-
		Coon et al. (total group)	-	-	NS	-	-	NS
		Coon et al. (H/S group)	-	-	NS	-	-	NS
		Coon et al. (CHD group)	-	-	NS	-	-	NS
	Haplotype	Pajukanta et al.	NS	0.00004 *	0.02	0.009	0.0004 *	-
		Huertas-Vazquez et al.	-	-	0.03	-	-	-
		Coon et al. (total group)	-	-	NS	-	-	NS
		Coon et al. (H/S group)	-	-	NS	-	-	NS
		Coon et al. (CHD group)	-	-	NS	-	-	NS
TG	USF1s1	Pajukanta et al.	-	-	-	0.0009	0.00001	-
		Huertas-Vazquez et al.	NS	0.005	0.02	-	-	-
		Coon et al. (total group)	-	-	0.02	-	-	0.001
		Coon et al. (H/S group)	-	-	NS	-	-	0.03
		Coon et al. (CHD group)	-	-	NS	-	-	NS

Table 5 Summary of the associations of the *USF1* gene previously reported (Continued).

			Total population			Males Only		
			HHRR	GAMETE	FBAT	HHRR	GAMETE	FBAT
TG	USF1s2	Pajukanta et al.	-	-	-	0.002	0.00006	-
		Huertas-Vazquez et al.	0.04	0.003	NS	-	-	-
		Coon et al. (total group)	-	-	NS	-	-	NS
		Coon et al. (H/S group)	-	-	NS	-	-	NS
		Coon et al. (CHD group)	-	-	NS	-	-	NS
	Haplotype	Pajukanta et al.	0.05	0.00006 *	-	0.00003	0.00001	-
		Huertas-Vazquez et al.	-	-	NS	-	-	-
		Coon et al. (total group)	-	-	NS	-	-	NS
		Coon et al. (H/S group)	-	-	NS	-	-	NS
		Coon et al. (CHD group)	-	-	NS	-	-	NS
TC	Haplotype	Pajukanta et al.	-	0.0001	-	-	0.007	-
apoB	Haplotype	Pajukanta et al.	-	0.00003	-	-	0.0007	-
sdLDL	Haplotype	Pajukanta et al.	-	0.002	-	-	0.01	-
LDL	Haplotype	Coon et al. (total group)	-	-	NS	-	-	0.01
		Coon et al. (H/S group)	-	-	NS	-	-	0.04
		Coon et al. (CHD group)	-	-	NS	-	-	NS

*All results represent p-values, indicating the probability that the SNP or haplotype is transmitted to the affected individuals; the p-values presented for the haplotype analyses are for the transmission of the common haplotype; FCH, familial combined hyperlipidemia; TG, triglycerides; TC, total choleterol; apoB, apolipoprotein B; LDLc, low-density lipoprotein cholesterol; sdLDL, small dense LDL; HHRR, haplotype-based haplotype risk program; GAMETE, gamete competition test program; FBAT, family-based association test program; NS, p-value > 0.05; -, no p-value reported; * gene-dropping is performed; Reference 6 is the stydyby Huertas-Vazquex et al. in Mexican FCH families; Reference 13 is the study by Pajukanta et al. in Finnish FCH families; Reference 15 is the study by Coon et al. in Utah pedigrees for the total group (total group), the group ascertained for early strokes or early onset hypertension (H/S group), and for the subjects ascertained for early death due to CHD (CHD group).*

60% of the FCH patients were present, no evidence for the involvement of *USF1* in FCH, TG or LDLc levels was found, although the strongest association would be expected in these families (Table 5). This lack of association in the families ascertained for early death due to CHD could be due to limited power, as only a few families were ascertained for CHD (n = 17). Another explanation could be the use of different diagnostic criteria for FCH in the different populations. To diagnose FCH, Pajukanta et al. and Huertas-Vazquez et al. used the traditional diagnostic criteria, based on elevated plasma TC and/or TG levels above the 90th percentile, adjusted for age and gender, while Coon et al. defined FCH based on LDLc and/or TG levels above the 90th percentile, adjusted for age and gender. In the present study, we applied two diagnostic criteria for FCH, i.e. the recently published nomogram [17] and the traditional diagnostic criteria [14]. We show that different diagnostic criteria affect the result of the association analyses. As shown in table 2, a suggestive p-value for association of *USF1* with FCH was found when using the traditional FCH criteria, whereas no association was found when using the nomogram.

In sharp contrast to the results obtained by Pajukanta et al., who reported the strongest association of *USF1* in males with high TG levels, we report, in the present study, no gender-specific effect and no association of *USF1* with high TG levels. The absence of this association in our population might be explained by the relatively mildly elevated levels of TG in our population in comparison to the Finnish population (2.8 versus approximately 3.3 mmol/L, respectively) [182, 183]. FCH remains a multifactorial disease, with extensive genetic heterogeneity, in which the combination of many genes and the environment determine the expression of the phenotype, contributing to the apparently conflicting results in different FCH populations. Our results are, however, supported by a study in Mexican FCH families [42], which also found not gender-specific effect.

Although we did not find an association between *USF1* and TG levels in the present study, we were able to replicate some associations for other important phenotypes of FCH, including TC, apoB and sdLDL. A study performed in obese Scandinavian women [184] and diabetes mellitus type 2 [185], however, failed to demonstrate an association between *USF1* and lipid/lipoprotein levels. This suggests that the influence of *USF1*, as a modifier gene, on these lipid levels is specific for patients with FCH.

Recently, a close relationship between *USF1* and catecholamine-stimulated lipolysis in human fat cells was found [184] and, based on this, it was suggested that the adipocyte lipolysis-related effects of genetic variation in *USF1* can have clinical consequences against a certain background such as FCH. In healthy young males it was demonstrated that *USF1* is associated with glucose levels and has a modulating effect on the correlation between BMI and TC levels [157]. In the present study, however, we did not find an association between *USF1* and glucose, insulin levels, or obesity parameters. In males, however, a suggestive association was found between *USF1* and the obesity parameter, waist circumference.

In the search for candidate genes underlying complex genetic disorders, haplotype analyses have become a valuable tool. The most convincing results for the association of *USF1* with FCH from pajukanta et al. [46] and Huertas-Vazquez et al. [42] were obtained when analyzing the *USF1* SNPs as haplotypes (Table 5). In our study, however, the two SNPs resulted in only two haplotypes, so no increased power was obtained from haplotype analyses. The complete linkage disequilibrium between these two SNPs has been reported previously in 196 healthy obese women [184]. In the present study, we used two programs, FBAT and PBAT. In FBAT the presence of linkage is not

mandatory for analyzing haplotypes. FBAT, however, splits up the pedigrees into nuclear families and, thereby, important information about the family structure is lost. The second program, PBAT, does not require the presence of linkage and is said to be capable of handling extended pedigrees. PBAT, however, could not handle our extended pedigrees, necessitating their division into smaller units. Haplotype analyses in extended pedigrees, therefore, remain a challenge.

For the association analyses of *USF1* with FCH, Pajukanta et al. [46] and Huertas-Vazquez et al. [42] used several programs (Table 5). In the HHRR and the genotype pedigree disequilibrium test programs, linkage is assumed to be present in the region under investigation, making these programs unsuitable in our situation of no linkage. The association of *USF1* with FCH in Mexican families was found using the gamete competition test program, which analyzes the presence of both linkage and association, so the significant result can represent the presence of linkage only, as reported for this region in this population [42]. Pajukanta et al. resolved this problem in the Finnish FCH families by gene dropping [46].

In the present study we genotyped two of the SNPs present in the *USF1* gene. In the present study we genotyped two of the SNPs present in the *USF1* gene. By genotyping these two SNPs we did not capture the complete genetic variation in this region. It is possible, therefore, that in our FCH Dutch population, another haplotype is the major risk haplotype. We assume, however, that this is unlikely, as not only in the Finnish families, but also in the Mexican and USA families this particular haplotype was identified as the risk haplotype. Furthermore, associations of this haplotype with underlying phenotypes of FCH were present in these Dutch FCH families. The identification of *USF1* as a modifier gene in our population may, however, be of great importance as genome wide linkage analyses in our population revealed no loci with lod scores reaching the level of suggestive linkage (data not shown), making it unlikely to find major genes for FCH.

Although *USF1* was thought to be a good candidate gene for FCH, our results do not support the role of *USF1* as a major gene in our population. This is reinforced by the lack of functionality of the genetic variations in *USF1*, as the two polymorphisms do not result in amino acid changes. The identification of a site for DNA binding proteins in the region of USF1s2 has been reported; however, no evidence was found for the presence of the suggested internal promoter causing the transcription of truncated mRNA [48]. Pajukanta et al. reported differential expression of three of the *USF1* regulated genes in adipose tissue based on the USF1s2 allele [48]. The expression of *USF1* itself, however, was not different in adipose tissue. In the present study, we have measured the expression of *USF1* in PBMCs and, consistent with the results of Pajukanta et al., we did not find any haplotype-dependent differences. Our decision to use PBMCs, in the absence of adipose tissue samples from our study population, was justified by a study by Morello et al., showing that the FCH-specific transcription profile was detectable in peripheral blood cells [153].

In conclusion, in our Dutch FCH patients, *USF1* shows a suggestive association with FCH and can be considered a modifier gene, contributing to related lipid traits in our Dutch FCH families.

Chapter 5

Haplotype analyses of the *APOA5* gene in patients with familial combined hyperlipidemia

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Abstract

Background Familial combined hyperlipidemia (FCH) is the most common genetic lipid disorder with an undefined genetic etiology. Apolipoprotein A5 gene (*APOA5*) variants were previously shown to contribute to FCH. The aim of the present study was to evaluate the association of *APOA5* variants with FCH and its related phenotypes in Dutch FCH patients. Furthermore, the effects of variants in the *APOA5* gene on carotid intima-media thickness (IMT) and cardiovascular disease (CVD) were examined. **Materials and methods** The study population consisted of 36 Dutch families, including 157 FCH patients. Two polymorphisms in the *APOA5* gene (-1131T>C and S19W) were genotyped. **Results** Haplotype analysis of *APOA5* showed an association with FCH ($p = 0.029$), total cholesterol ($p = 0.031$), triglycerides ($p < 0.001$), apolipoprotein B ($p = 0.011$), HDL-cholesterol ($p = 0.013$), small dense LDL ($p = 0.010$) and remnant-like particle cholesterol ($p = 0.001$). Compared to S19 homozygotes, 19W carriers had an increased risk of FCH (OR = 1.6 [1.0 - 2.6]; $p = 0.026$) and a more atherogenic lipid profile, reflected by higher triglyceride (+22%) and apolipoprotein B levels (+5%), decreased HDL-cholesterol levels (-7%) and an increased prevalence of small dense LDL (16% vs 26%). In carriers of the –1131C allele, small dense LDL was more prevalent than in –1131T homozygotes (29% vs 16%). No association of the *APOA5* gene with IMT and CVD was evident. **Conclusion** In Dutch FCH families, variants in the *APOA5* gene are associated with FCH and an atherogenic lipid profile.

Introduction

Familial combined hyperlipidemia (FCH) is the most common lipid disorder of unknown genetic etiology, affecting 2-5% of the general population [10, 14]. Major characteristics of FCH include elevated plasma levels of apolipoprotein B (apoB), triglycerides (TG) and/or total cholesterol (TC). Other phenotypes include decreased levels of high-density lipoprotein cholesterol (HDLc) and the presence of small, dense low-density lipoproteins (sdLDL). In addition, FCH patients have an increased risk of cardiovascular disease (CVD) and are often obese and insulin resistant [97].

Despite decades of research, the complex genetics of FCH are still not fully understood. Several linkage analyses were performed, leading to the identification of multiple loci for FCH [26, 28, 30, 34, 36, 42]. One repeatedly identified locus is located on chromosome 11q, a site involved in modulating the expression of FCH [31-33]. This region includes the apolipoprotein A1-C3-A4-A5 gene cluster [186]. The apolipoprotein A5 (*APOA5*) gene encodes the apolipoprotein AV protein (APOAV), which is exclusively expressed in the liver. APOAV is found on very low-density lipoprotein (VLDL), HDL and chylomicrons and is, compared to other apolipoproteins, present in very low plasma concentrations [187, 188]. Variations in the *APOA5* gene are related to TG levels, however, the underlying mechanism is not yet fully understood [49]. One hypothesis, based on mouse studies, suggests that APOAV modulates TG levels by guiding VLDL and chylomicrons to proteoglycan-bound lipoprotein lipase for lipolysis and by increasing VLDL clearance [52, 53].

In the human *APOA5* gene, several single-nucleotide polymorphisms (SNPs) have been identified (-1131T>C, -3A>G, S19W (56C>G), IVS3+476G>A and 1259T>C) [189, 190]. The three major haplotypes, representing approximately 98% of all haplotypes in these populations, were defined by the –1131T>C and the S19W SNPs [189, 190]. In Caucasians, the rare alleles of these two SNPs were associated with elevated plasma levels of TC, TG, remnant-like particle cholesterol (RLPc), low-density lipoprotein cholesterol (LDLc) and apoB, decreased HDLc levels and the presence of sdLDL [186, 189, 191]. In patients with FCH, *APOA5* polymorphisms were associated with an increased risk of FCH and related lipid phenotypes, including TG, apoB and HDLc levels and LDL particle size [33, 50, 51]. The relationship between variants in the *APOA5* gene and RLPc levels was not previously studied in FCH patients.

Hypertriglyceridemia is an independent risk factor for the development of CVD [192]. Despite its effect on plasma triglycerides, the role of *APOA5* in the development of CVD remains controversial [189, 193-197]. In 372 Finnish men with CVD, who participated in the LOCAT study, it was demonstrated that only the rare allele of the S19W SNP (19W) was associated with increased progression of atherogenesis [197]. In contrast, in 2,578 subjects from the general population in the Framingham Heart Study, an increased risk of CVD was found only for the rare allele of the –1131T>C SNP (-1131C) [189, 197]. The results for CVD in the Framingham Heart Study, however, conflicted with the results obtained for intima-media thickness (IMT), a surrogate marker of CVD. Differences in IMT were associated with the 19W allele, but an association of the –1131C allele was only present in obese subjects [193]. In FCH patients, who have an increased risk of CVD, the relationship between variants in the *APOA5* gene and CVD was not previously examined.

The aim of the present study was to investigate the association of *APOA5* gene variants (-1131T>C and S19W) with FCH and its associated phenotypes, including RLPc levels, using a family-based SNP and haplotype approach in well-characterized FCH families. Furthermore, the

suggested increased risk of CVD associated with variants in the *APOA5* gene was investigated in our FCH families by taking both intima-media thickness and CVD prevalence into account.

Methods

Study population

Back in 1994, we have recruited FCH families from the outpatient lipid clinic of the Radboud University Nijmegen Medical Centre, ascertained through probands, exhibiting a combined hyperlipidemia with both plasma TC and TG levels above the age- and gender-related 90th percentile [146], during several periods in which they were not treated with lipid-lowering drugs, and despite dietary advice [146]. Additionally, a first-degree relative possesed elevated levels of TC and/or TG above the 90th percentile and the proband, or a first-degree relative, suffered from premature (before the age of 60 years) cardiovascular disease. Families were excluded when probands were diagnosed with underlying diseases causing hyperlipidemia (i.e., diabetes mellitus type 1 and 2, hypothyroidism, and hepatic or renal impairment), a first-degree relative had tendon xanthomata or probands were homozygous for the APOE2 allele. All included subjects were Caucasian and above the age of 10 years. The ascertained families had a mean size of 24 members from multiple (between 2 and 4) generations. The present FCH study population include the 5-year follow-up data of our original FCH families, consisting of 36 Dutch families, comprised of 611 subjects with known genealogic, phenotypic, and genotypic data, of whom 157 individuals were diagnosed as FCH patients [17]. In 1999, the diagnosis of FCH was based on the nomogram [17]. Plasma TG and TC levels, adjusted for age and gender, and absolute apoB levels, were applied to the nomogram to calculate a probability of being affected with FCH. When this probability of being affected with FCH is greater than 60%, the diagnostic phenotype is present in at least one first degree relative, and premature CVD (before the age of 60 years) is present in at least one individual in the family, the individual is defined as affected by FCH. Also included in the family-based analyses were the normolipidemic relatives (n = 390), the unaffected spouses of both FCH patients and normolipidemic relatives (n = 64), and subjects without known phenotypic and/or genotypic data (n = 230) to complete the pedigree structure. After the withdrawal of lipid-lowering medication for four weeks and an overnight fast, blood was drawn by venipuncture. Body mass index (BMI) was calculated as body weight (kilograms) divided by the square of height (meters).

Information concerning CVD was gathered through personal interviews and physical examinations performed by the clinical investigator. When the clinical investigator suspected the presence of CVD, further details and confirmation of the diagnosis were sought from the participant's general practitioner and hospital records. CVD was defined by angina pectoris (AP), myocardial infarction (MI), stroke, peripheral vascular disease or vascular surgery. In our study population (n = 611), 56 subjects were identified with CVD, including 26 subjects with AP, 25 with previous MI, 10 with peripheral vascular disease, seven with stroke and 23 who underwent vascular surgery. In total, 45% (n = 25) of these individuals were diagnosed with CVD based on the presence of two or more manifestations of CVD. The ethical committee of the Radboud University Nijmegen Medical Centre approved the study protocol and all procedures were in accordance with institutional guidelines. All subjects provided written informed consent.

Table 1 Characteristics of patients with familial combined hyperlipidemia, normolipidemic relatives and spouses

	FCH patients (n = 157)	NL relatives (n = 390)	Spouses (n = 64)
Male gender (n (%))	75 (47.8 %)	177 (45.4 %)	31 (48.4 %)
Age (years)	47.0 (15.6) * †	37.9 (15.7) ‡	56.0 (10.5)
CVD (n (%))	32 (20.4 %) * †	20 (5.1 %)	4 (6.3 %)
IMT (mm)	0.80 (0.12) *	0.72 (0.12)	-
BMI (kg/m^2)	27.3 (4.1) *	24.1 (3.8) ‡	26.8 (3.6)
Total cholesterol (mmol/L)	6.5 (1.1) * †	4.9 (0.9) ‡	5.2 (0.8)
Triglycerides (mmol/L)	3.1 (1.5) * †	1.2 (0.5)	1.3 (0.6)
Apolipoprotein B (mg/L)	1370 (264) * †	960 (220)	996 (175)
LDLc (mmol/L)	4.1 (1.2) * †	3.2 (0.9) ‡	3.4 (0.7)
HDLc (mmol/L)	0.98 (0.26) * †	1.22 (0.30)	1.28 (0.40)
K-value	-0.26 (0.26) * †	0.05 (0.19)	0.05 (0.25)
RLPc (mmol/L)	0.57 (0.40) * †	0.26 (0.09) ‡	0.29 (1.3)
HOMA-index	3.4 (1.8) * †	2.3 (1.3) ‡	2.7 (1.3)

*Continuous variables are presented as mean (SD); FCH, familial combined hyperlipidemia; NL relatives, normolipidemic relatives; CVD, cardiovascular disease; IMT, mean common artery intima-media thickness was measured in 61 FCH patients and 155 normolipidemic relatives; BMI, body mass index; LDLc, low-density lipoprotein cholesterol; HDLc, high-density lipoprotein cholesterol; K-value, a value ≤ -0.1 represents the presence of small dense LDL; RLPc, remnant-like particle cholesterol; HOMA-index, homeostasis model assessment-index; *, p-value < 0.05, compared to normolipidemic relatives; †, p-value < 0.05, compared to spouses; ‡, p-value < 0.05, compared to spouses.*

Biochemical analyses

Biochemical analyses were performed as previously described for this population [17]. In short, plasma TC and TG were determined by enzymatic, commercially available reagents (Boehringer-Mannheim, Germany, catalog No. 237574 and Sera Pak, Miles, Belgium, catalog No. 6639, respectively). Total plasma apoB concentrations were measured by immunonephelometry. HDLc was quantified by the polyethylene glycol 6000 method. LDL subfractions were separated by single spin density gradient ultracentrifugation. A continuous variable, K, represented the LDL subfraction profile of each individual. A negative K-value (K ≤ -0.1) reflected the presence of small dense LDL [17]. RLPc levels were measured using an immuno-separation technique as described elsewhere [198]. Glucose concentrations were analyzed using the oxidation technique (Beckman®, Glucose Analyser2, Beckman Instruments Inc., Fullerton, CA 92634, USA). Plasma insulin concentrations were ascertained by a double antibody method. Insulin resistance was assessed by the homeostasis model assessment (HOMA) method.

DNA was extracted from peripheral blood lymphocytes using a standard technique [121]. Genotyping for the S19W and the –1131T>C SNPs was carried out by PCR and restriction enzyme digestion, as previously described [199]. Genotyping of the S19W and –1131T>C SNPs failed in 5% and 3% of the subjects, respectively.

Table 2 Characteristics of the subjects with data on intima media tickness.

	FCH patients (n = 61)	NL relatives (n = 155)
Male gender (n (%))	28 (45.9 %)	70 (45.2 %)
Age (years)	47.4 (14.2) *	37.9(14.7)
CVD (n (%))	9 (14.8 %) *	9 (5.8 %)
IMT (mm)	0.80 (0.12) *	0.72 (0.12)
BMI (kg/m^2)	28.3 (4.05) *	24.1 (3.6)
Total cholesterol (mmol/L)	6.6 (1.3) *	5.4 (1.0)
Triglycerides (mmol/L)	2.9 (1.8) *	1.5 (1.1)
Apolipoprotein B (mg/L)	1327 (297) *	973 (233)
LDLc (mmol/L)	4.2 (1.2) *	3.2 (0.9)
HDLc (mmol/L)	1.14 (0.26) *	1.33 (0.33)
K-value	-0.28 (0.25) *	0.05 (0.21)
RLPc (mmol/L)	0.62 (0.45) *	0.25 (0.09)
HOMA-index	2.4 (1.3)*	1.8 (1.0)

*Continuous variables are presented as mean (SD); FCH, familial combined hyperlipidemia; NL relatives, normolipidemic relatives; CVD, cardiovascular disease; IMT, mean common artery intima-media thickness; BMI, body mass index; LDLc, low-density lipoprotein choleterol; HDLc, high-density lipoprotein cholesterol; K-value, a value ≤ -0.1 represents the presence of small dense LDL; RLPc, remnant-like particle cholesterol; HOMA-index, homeostasis model assessment-index; *, p-value < 0.05.*

Carotid IMT measurements

All our FCH families were invited to participate in an on-going follow-up program. As part of the follow-up screening, non-invasive measurements of atherosclerosis were performed, including carotid intima-media thickness (IMT), in all family members, including both patients and normolipidemic relatives [200]. An AU5 Ultrasound machine (Esaote Biomedica) with a 7.5 MHz linear-array transducer was used to measure the IMT of both common carotid arteries. Longitudinal images of the most distal 10 mm of both the far and the near wall of the common carotid artery were obtained with the optimal projection (anterolateral, lateral or posterolateral). The actual measurement of IMT was performed offline by the sonographer using semi-automatic edge-detection software (M'Ath®Std version 2.0, Metris, Argenteuil, France) [201]. All measurements were carried out in end-diastole using the R-wave of a simultaneously recorded ECG as a reference frame. From each frame the mean IMT was calculated over at least 7.5 mm of the aforementioned 10 mm segment (yielding a minimum quality index of 75%). The outcome variable was defined as the mean IMT of the near and far wall of both common carotid arteries.

Statistical analyses

Descriptive statistics were compared using ANOVA for continuous traits and Pearson's chi-square test for discrete traits. Prior to further statistical analyses, extended Mendelian error-

checking was performed with Pedcheck [122]. For families with Mendelian inconsistencies, problematic genotypes were set to missing for the complete nuclear family or the isolated problematic individual (1%). The parental data for both SNPs were tested for Hardy-Weinberg equilibrium by use of Fisher's exact-test. Variables with a skewed distribution, including TG levels, RLPc levels and the HOMA-index, were logarithmically transformed. The HAPLOVIEW program [178] was used to estimate allele and haplotype frequencies in founders, representing the unrelated individuals of the study population, including probands and spouses. Linkage disequilibrium (LD) and allelic association (R2) between the two SNPs were calculated with HAPLOVIEW using the confidence interval method of Gabriel et al. [202].

To study the relation of *APOA5* in FCH, we used both linkage and association analysis. The SOLAR 2.1.4 software was used to implement two-point linkage analysis [123]. The presence of linkage was tested for FCH and its related phenotypes. As linkage analysis may have limited power to identify common variants with moderate effects for more complex diseases, such as FCH, we also performed association analysis [203].

Associations between the individual polymorphisms (1131T>C and S19W), or haplotypes and FCH, or the related phenotypes, were analyzed using a family based approach, with the family based association test (FBAT) software [179]. FBAT is attractive because it can test for linkage and/or association while avoiding biases due to population admixture or stratification, misspecification of the trait distribution, and/or selection based on the trait. The FBAT test statistics utilizes a general approach to family-based association tests, as proposed by Rabinowitz and Laird (2000), and is based on the distribution of the offspring genotypes conditional on any trait information and on the parental genotypes [179]. An additive model of inheritance was used, as the mode of inheritance of FCH is unknown and this model is particularly robust. FBAT broke down the extended pedigrees into nuclear families (n = 219) and evaluated their contribution to the test statistics. The –e option of FBAT, which computes the test statistic using an empirical variance estimator, was implemented to correct for the non-independence of the nuclear families and for the presence of linkage. The –p option, which performs a Monte-Carlo permutation procedure, was used to estimate empirical p-values. Adjustments for age, gender and BMI were done by calculation of the residuals. For the haplotype analysis, the haplotype-based association test (HBAT) command of FBAT was used, utilizing the –e and –p options. Differences were considered statistically significant at p-value < 0.05.

The explained variance in FCH by the *APOA5* variants was explored using the measured genotype method in the SOLAR Program [123]. The measured genotype analyses uses variance component methodology to estimate the proportion of variance due to environmental and additive genetic effects. By comparing models with and without the *APOA5* genotypes, estimations are calculated of what proportion of the FCH trait variance is attributable to the genotype by examining the changes to the estimates of environmental and additive genetic variance. P-values and the explicable proportion of variance are presented.

To test differences between the different genotypes, generalized estimating equation (GEE) analyses were performed in the STATA 8.0 software. The GEE analyse is especially designed to take into account possible correlated values within families. In the models used for the GEE analyses the link function 'canonical' was used and an equal-correlation population-averaged model was used as the working correlation matrix.

The analyses were conducted using SPSS 12.0.1, PEDCHECK 1.00, HAPLOVIEW 3.2, SOLAR 2.1.4, FBAT 3.2 and STATA 8.0 software.

Results

Characteristics of study population

Anthropometric and metabolic characteristics of FCH patients, normolipidemic relatives and spouses are presented in table 1. FCH patients were older than normolipidemic relatives, but younger than spouses. Compared to normolipidemic relatives and spouses, FCH patients had a higher prevalence of CVD and higher IMT values. When correcting the IMT values for the age difference between FCH patients and normolipidemic relatives, the difference in IMT remained significant (0.78 mm (SD 0.08) in FCH versus 0.73 mm (SD 0.08) in normolipidemic relatives, p = 0.000). FCH patients were characterized by increased lipid levels, including TC, TG, apoB, LDLc and RLPc concentrations, decreased HDLc and an increased prevalence of sdLDL, as reflected by a K-value below -0.1. Finally, FCH patients were more also obese, as reflected by a higher BMI, and insulin resistant, as reflected by a higher HOMA-index (Table 1). The anthropometric and metabolic characteristics of the subgroup of subjects with IMT data are presented in table 2. Characteristics of the subjects with IMT data did not differ significantly from the total group as presented in table 1 (all $p < 0.05$). Anthropometric and

Table 3 Characteristics of subjects with cardiovascular disease

	Subjects with CVD (n = 56)
Male gender (n (%))	35 (62.5 %)
age (years)	62.5 (9.3)
FCH (n (%))	24 (42.9 %)
IMT (mm)	0.88 (0.13)
BMI (kg/m^2)	27.3 (3.4)
Total cholesterol (mmol/L)	6.8 (1.5)
Triglycerides (mmol/L)	2.9 (2.1)
Apolipoprotein B (mg/L)	1339 (302)
LDLc (mmol/L)	4.0 (1.2)
HDLc (mmol/L)	1.19 (0.31)
K-value	-0.18 (0.31)
RLPc (mmol/L)	0.46 (0.27)
HOMA-index	2.3 (1.4)

Continuous variables are presented as mean (SD); CVD, cardiovascular disease; FCH, familial combined hyperlipidemia, IMT, mean common artery intima-media thickness; BMI, body mass index; LDLc, low-density lipoprotein choleterol; HDLc, high-density lipoprotein cholesterol; K-value, a value ≤ -0.1 represents the presence of small dense LDL; RLPc, remnant-like particle cholesterol; HOMA-index, homeostasis model assessment-index.

metabolic characteristics of the subgroup of subjects with CVD are presented in table 3. As expected, patients with CVD were relatively old and predominantly men. Furthermore, patients with CVD had a relatively high IMT value and a more atherogenic lipid profile reflected by high TC and TG levels, low HDLc levels and the presence of sdLDL. Forty three percent of the subjects with CVD were diagnosed FCH.

Analyses of the APOA5 gene with FCH and related lipid phenotypes

In founders, rare allele frequencies for the –1131T>C and S19W SNPs were 0.08 and 0.11, respectively. In the probands, rare allele frequencies for the –1131T>C and S19W SNPs were 0.10 and 0.16, respectively. The genotypic distributions of –1131T>C and S19W were in Hardy-Weinberg proportions. The alleles of the –1131T>C and the S19W SNPs were in complete linkage disequilibrium (D' = 1.00), but with very little correlation (R2 = 0.01). A consequence of this linkage disequilibrium is that, only three of the possible four different haplotypes were observed. Haplotype analyses can, therefore, provide additional information compared to the single SNP analyses, since it accounts for both sources of variation. The wildtype haplotype (APOA5*1) had a frequency of 0.83. The frequencies of the haplotype defined by a rare allele for the –1131T>C (APOA5*2) or by a rare allele for the S19W SNP (APOA5*3) were 0.11 and 0.06, respectively. In total 394 individuals (92 FCH patients, 261 normolipidemic relatives and 41 spouses) carried only common alleles for both SNPs, who are referred to as "wildtypes". 64 subjects (8 FCH patients, 50 normolipidemic relatives and 6 spouses) carried one or two rare for the –1131T>C SNP and wildtype alleles for the S19W SNP and 115 subjects (38 FCH patients, 63 normolipidemic relatives and 14 spouses) carried one or two rare alleles for the S19W and wildtype alleles for the –1131T>C SNP. A rare allele for both the –1131T>C and the S19W SNP were present in 18 subjects (9 FCH patients, 9 normolipidemic relatives and 0 spouses), referred to as "compound heterozygotes".

Linkage analysis of the APOA5 region

Two-point linkage analyses performed utilizing the –1131T>C and S19W SNPs in the *APOA5* gene did not provide evidence of linkage for FCH (-1131T>C; lod score = 0.04 and S19W; lodscore = 0.38). For TG levels a lod score of 1.6 was obtained for the S19W SNP, whereas a lod score of 0.005 was found for the –1131T>C SNP. For all other phenotypes, including apoB, TC, RLPc and HDLc levels, and the presence of sdLDL, no linkage was present (all two-point lod scores < 0.5).

Association analyses of APOA5 variants with FCH and related phenotypes

The associations of the –1131T>C and the S19W SNPs, individually and as haplotypes, with FCH and its related phenotypes are presented in table 4A. These associations are corrected for age, gender and multiple testing. The APOA5*2 haplotype was not associated with FCH, while the APOA5*3 haplotype was overrepresented in patients with FCH (p=0.014). Haplotype analysis showed that the *APOA5* haplotypes were associated with FCH (p=0.029). This resulted in an increased risk of FCH for subjects (n = 119) with the rare allele for the S19W SNP (1.62 [95% C.I.: 1.03 - 2.55]; p = 0.026) and for the compound heterozygotes (n = 19) carrying a rare allele for the –1131T>C and the S19W SNPs (3.28 [95% C.I.: 1.38 - 7.82]; p = 0.008). The APOA5*3 haplotype was also associated with individual lipid and lipoprotein levels,

Table 4 Association of rare alleles of the -1131T>C and the S19W SNPs and haplotypes in the *APOA5* gene with familial combined hyperlipidemia and its related phenotypes.

		SNPs		Haplotypes		
	N	-1131C	19W	APOA5*2	APOA5*3	Overall Haplotype
A						
FCH	611	0.921	0.048	0.931	0.014	0.029
BMI	611	0.519	0.697	0.373	0.799	0.808
TC	611	0.055	0.063	0.272	0.042	0.031
TG *	611	0.041	0.000	0.141	0.000	0.000
ApoB	611	0.097	0.087	0.253	0.024	0.011
LDLc	608	0.403	0.320	0.596	0.630	0.787
HDLc	609	0.445	0.041	0.397	0.019	0.013
K-value	611	0.233	0.045	0.119	0.024	0.010
RLPc *	559	0.031	0.000	0.120	0.001	0.001
HOMA-index *	550	0.534	0.385	0.560	0.273	0.623
B						
CVD	611	0.482	0.379	0.066	0.607	0.202
IMT	216	0.437	0.494	0.797	0.656	0.542

*P-values for the individual SNPs (FBAT) and haplotypes (HBAT) corrected for age, gender and multiple testing are presented; *, ln-transformed variable; N, number of subjects with available data for the relevant phenotype; APOA5*2, haplotype represented by the rare allele of -1131T>C SNP; APOA5*3, haplotype represented by the rare allele of S19W SNP; Overall haplotype, the p-value obtained for the model including all haplotypes; FCH, familial combined hyperlipidemiawas present in 157 subjects; BMI, body mass index; TC, total cholesterol; TG, triglycerides; ApoB, apolipoprotein B; LDLc, low-density lipoprotein cholesterol; HDLc, high-density lipoprotein cholesterol; K-value, a value ≤ -0.1 represents the presence of small dense LDL; RLPc, remnant-like particle cholesterol; HOMA-index, homeostasis model assessment-index; CVD, cardiovascular disease was present in 56 subjects; IMT, mean common carotid artery intima-media thickness data were present in 61 FCH patients and 155 normolipidemic relatives.*

including TC (p = 0.042), TG (p < 0.001), apoB (p = 0.024), HDLc (p = 0.019), K-value (p = 0.024) and RLPc (p = 0.001). No associations were found for the obesity (BMI) and insulin resistance parameters (HOMA-index) (Table 4A). The APOA5*2 haplotype was not significantly associated with any of the characteristics of FCH. Comparable results were found when the non-independence of the families and the possible presence of linkage (-e option) were taken into account (data not shown).

Explained variance in FCH by the APOA5 gene

By measured genotype analyses, it was estimated that 2% of the genetic variance in FCH is attributable to the S19W SNP (p < 0.001). The S19W SNP also explained 3% of the variation in TG levels (p < 0.001), 1% of the variance in HDLc levels (p = 0.005) and 1% of the variation

in K-value (p = 0.005). The -1131T>C SNP did not contribute to the variation in FCH or any of the related phenotypes (TC, TG, ApoB, LDLc, HDLc, K-value, RLPc and HOMA-index); in fact, the point estimates for the combined effects of both genotypes, S19W and –1131T>C, were identical to the point estimate obtained for S19W alone.

Association analyses of the APOA5 gene with IMT and CVD

The associations of the –1131T>C and the S19W SNPs in *APOA5*, and their haplotypes, with IMT values and CVD prevalence, are presented in table 4B. These associations are corrected for age, gender and multiple testing. The APOA5*3 haplotype was not associated with IMT or CVD. The APOA5*2 haplotype did show a trend towards significant association with CVD (p = 0.07), but no association with IMT was present. Overall, no association of the haplotypes with IMT values or CVD prevalence was evident. Further adjustment of IMT values for obesity did not change these results.

Table 5 Differences in phenotypic parameters in the control group including normolipidemic relatives and spouses with a rare allele for the -1131T>C of the S19W SNP in the *APOA5* gene compared to wildtype individuals.

	Wildtypes n = 302 (68.0 %)	-1131C n = 56 (12.6 %)	19W n = 77 (17.4 %)	Compounds n = 9 (2.0 %)
CVD (n (%))	10 (3.3 %)	5 (8.9 %)	6 (7.8 %)	1 (11.1 %)
IMT (mm)	0.74 (0.72 - 0.76)	-1.4 %	-4.0 %	+0.0 %
TC (mmol/L)	4.99 (4.86 - 5.11)	+3.0 %	+2.0 %	-3.4 %
TG (mmol/L) *	1.07 (1.01 - 1.14)	+8.4 %	+22.4 % †	+23.4 %
ApoB (mg/L)	975 (949 - 1001)	+5.3 %	+5.0 % †	+4.6 %
HDLc (mmol/L)	1.24 (1.20 - 1.28)	-2.4 %	-7.3 % †	-17.7 % †
K-value ≤ -0.1 (n (%))	48 (15.9 %)	16 (28.6 %) †	20 (26.0 %) †	4 (44.4 %) †
RLPc (mmol/L) *	0.25 (0.24 - 0.26	+0.0 %	+4.0 %	+8.0 %

*Presented are the data of the combined group of normolipidemic relatives and spouses. For the subjects with wildtype alleles for both SNPs, dichotomous variables are presented as number (%) and the continuous variables, standardized for age and gender, are presented as mean (95% CI); For the other groups, dichotomous variables are presented as number (%) and the continuous variables as percent of difference compared to the wildtype individuals; *, ln-transformed variables; -1131C, subjects carrying either 1 or 2 rare alleles for the 1131T>C SNP, 19W, subjects carrying either 1 or 2 rare alleles for the S19SW SNP; Compounds, subjects carrying 1 rare allele for both SNPs; CVD, vardiovascular disease; IMT, common carotid artery intima-media thickness; TC, total cholesterol; TG, triglycerides; ApoB, apolipoprotein B; HDLc, high-density lipoprotein cholesterol; K-value, a value ≤ -0.1 represents the presence of small dense LDL; RLPc, remnant-like paritcle cholesterol; †, p-value < 0.05 compared to wildtypes.*

Effect of APOA5 variants on lipid and lipoprotein levels, IMT and prevalence of CVD

To determine the effect of the genetic variation in the *APOA5* gene on lipid levels in the general population, we combined the normolipidemic relatives and spouses in one control group, which was allowed because no difference in anthropometric and biochemical parameters, based

on genotypes, were observed between relatives and spouses (data not shown). The results were standardized for age and gender and presented in table 5. Compared to the wildtypes, individuals carrying a rare allele of the S19W SNP had increased TG levels (+22%) apoB levels (+5%) and decreased HDLc levels (-7%). Furthermore, in subjects with a 19W allele, a K-value below –0.1 was present in 26% of the subjects, which was significantly higher than in wildtypes (16%; p = 0.046). Additionally, in carriers of the –1131T>C rare allele, sdLDL was present in more individuals (29%; p = 0.035) compared to wildtypes. The compounds had approximately 15% lower HDLc levels and sdLDL was present in more individuals. Compared to the wildtypes, individuals carrying rare alleles for either or both SNPs had no increased IMT or increased prevalence of CVD (Table 5).

The effect of *APOA5* on lipid levels among FCH patients were comparable (data not shown).

Discussion

The results from this study demonstrate that the S19W variant in the *APOA5* gene are associated with FCH. We show that the APOA5*3 haplotype is over-represented in patients with FCH. Furthermore, we provide evidence for the association of the APOA5*3 haplotype with a more atherogenic lipid profile. However, no association of the –1131T>C and S19W variants in the *APOA5* gene with higher IMT values or an increased prevalence of CVD in patients with FCH was evident.

The 19W allele was previously associated with an increased risk of FCH and elevated levels of TG and apoB [33, 50]. In the present study, we performed family-based haplotype analyses with the FBAT program, corrected either for multiple testing or for the presence of linkage in this region. We confirmed the independent association of the 19W allele with FCH and elevated TG levels. Additionally, we showed that the 19W allele is associated with an atherogenic lipid profile including elevated apoB levels, decreased HDLc levels and the presence of sdLDL. This is in agreement with previously documented associations in general populations [33, 186, 189, 191]. From the literature, it is also suggested that the haplotype containing the 19W is associated with the highest TG levels [190, 199].

In the present study, no independent association for the –1131C allele was found with FCH or any of the related lipid phenotypes whereas the –1131C allele was previously associated with an increased risk of FCH, and elevated levels of TG and sdLDL [33, 50, 51]. In the present study we defined FCH based on the nomogram [17]. To exclude that the lack of association of the –1131T>C SNP with FCH in our population is due to different diagnostic criteria of FCH, we repeated the analyses, now applying the traditional diagnostic criteria of FCH, based on TC and/or TG levels above the 90th percentile. No association of the –1131T>C SNP with FCH, based on the traditional diagnostic criteria was found (Table 6). Most likely, the small number of subjects carrying one or two C alleles of the –1131T>C SNP contribute to the lack of involvement of this SNP to FCH. In contrast, compound heterozygotes did show a significant increased risk on FCH, despite the low number of subjects. However, as the lipid and lipoprotein levels among wubjects who were compound heterozygotes did not differ from wildtype carriers, it may well be possible that the observed increased risk of FCH in compound heterozygotes is a false positive finding and therefore additional studies are required for confirmation of our observed increased

risk on FCH in compound heterozygotes.

Previously, two studies investigated the association of variants in the *APOA5* gene and RLPc levels. In the Framing Heart Study population, both the 19W and –1131C alleles were associated with RLPc levels, while no association was found in the MONICA study population [189, 204]. We report no association of the rare alleles of the –1131T>C and the S19W SNPs in the *APOA5* gene with RLPc levels in our FCH study population, which is in agreement with the findings of the MONICA study [204].

Since the variants in the *APOA5* are suggested to be associated with plasma lipid levels, it is likely that it plays a role in the development of CVD. Several studies investigated possible associations between variants in the *APOA5* gene and the prevalence of CVD in Caucasians, however, these led to contradictory results [189, 193-197, 205]. The LOCAT study showed a trend toward increased progression of atherogenesis in male carriers of the rare allele of the S19W SNP; in contrast, no effect was found for the –1131C allele [197]. In the Framingham Heart Study, an almost 2-fold increased risk of CVD was found in females carrying the rare allele of the –1131T>C SNP, but there was no effect in males. For the 19W allele, no effect was found in either gender [189]. In contrast, in the same Framingham Heart Study, the 19W allele was associated with IMT values in both genders. In obese subjects, this relationship with IMT was also found for the rare allele of the –1131T>C SNP [193].

Table 6 Association of rare allele of the -1131T>C and the S19W SNPs and haplotypes in the APOA5 gene with familial combined hyperlipidemia based on the traditional diagnostic criteria.

		SNPs		Haplotypes		
	N	-1131C	19W	APOA5*2	APOA5*3	Overall Haplotype
FCH	611	0.615	0.124	0.914	0.105	0.429

*P-values for the individual SNPs (FBAT) and haplotypes (HBAT) corrected for age, gender and multiple testing are presented; N, number of subjects with available data for the relevant phenotype; APOA5*2, haplotype represented by the rare allele of -1131T>C SNP; APOA5*3, haplotype represented by the rare allele of S19W SNP; Overall haplotype, the p-value obtained for the model including all haplotypes; FCH, familial combined hyperlipidemiawas present in 151 subjects.*

The association of variants in the *APOA5* gene with CVD and IMT values was not previously studied in FCH patients, which are known to have thicker IMT and an increased risk of CVD [200]. In the present study, we show that, although the rare allele of the S19W variant in the *APOA5* gene was associated with FCH and a disturbed lipid profile, no association with CVD or IMT was evident. This could be a result of the limited number of individuals with CVD in our study population, and the availability of IMT data in only a sub-set of the study population, resulting in limited power for studying these associations. Possible explanations for the contradictory results previously found for CVD could be that the association of variants in the *APOA5* gene with CVD is independent of TG levels, suggesting different mechanisms, or that the influence of *APOA5* gene variants on TG is relatively small and therefore not translated into differences in CVD [193, 195, 197].

In the present study, the measured genotype method and FBAT were used to analyse the assocation of variants in the *APOA5* gene with FCH and its related phenotypes. The measured genotype method is more powerful, uses the whole pedigree and provides an effect estimate for the tested variables, but is not robust in the presence of population stratification. FBAT, however, is robust to stratification, but is less powerful and uses incomplete pedigree information. The results of both statistical analyses were highly concordant, thereby providing strong evidence for the role of the S19W SNP of the *APOA5* gene in FCH patients.

In the general population, the influence of variations in the *APOA5* gene on lipid levels is still controversial. Some studies show that the association of variations in the *APOA5* gene with increased TG levels is limited or not present in the general population [33, 50, 51]. Based on these studies, there is conjecture that *APOA5* gene variants modulate TG levels only when there is an altered genetic and metabolic background. In the present study, we show that the influence of the rare allele of the S19W SNP on lipid levels was not restricted to FCH patients, but also present in normolipidemic relatives and spouses.

In conclusion, in the present study of Dutch FCH families, the rare allele of the S19W SNP in the *APOA5* gene is associated with FCH and a more atherogenic lipid profile.

Chapter 6

Diagnostic criteria in relation to the pathogenesis of familial combined hyperlipidemia

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Abstract

Familial combined hyperlipidemia (FCH) is the most common inherited hyperlipidemia in humans, affecting 1 to 3% of the adult population and up to 20% of patients with premature myocardial infarction. FCH is traditionally diagnosed by total plasma cholesterol and/or triglyceride levels above the 90th percentile adjusted for age and gender; however, the diagnosis of FCH based on these diagnostic criteria is inconsistent in 26% of the subjects over a five-year period, emphesizing the need for re-evaluation of the diagnostic criteria for FCH. Recently, a nomogram was developed based on absolute apolipoprotein B levels in combination with triglyceride and total cholesterol levels adjusted for both age and gender to simply and accurately diagnose FCH. When percentiles of triglyceride and total cholesterol adjusted for age and gender are not available in a population, the definition of FCH can be established based on hypertriglyceridemia (> 1.5 mmol/L) and hyperapoB (> 1200 mg/L).

Standardized and simple diagnostic criteria are necessary to further delineate the pathogenesis of FCH. Several metabolic pathways have been suggested to be important in causing the FCH phenotype including hepatic VLDL overproduction either with or without impaired clearance of triglyceride-rich lipoproteins from plasma. The presence of insulin resistance and obesity in FCH patients further contribute to the expression of the lipid phenotype. A disturbed adipose tissue metabolism that results in an increased plasma free fatty acid pool may be the culprit in the pathogenesis of FCH.

More than three decades ago, familial combined hyperlipidemia (FCH) was identified independently by Goldstein et al., Rose et al., and Nikkila et al. as a new inherited lipid disorder characterized by multiple lipoprotein phenotypes defined by the presence of hypertriglyceridemia, hypercholesterolemia or both in affected individuals and strongly associated with premature cardiovascular disease (CVD) [14, 15, 206]. The lipoprotein phenotype can vary in time, both in affected individuals and in their first-degree relatives, hence the description of this disorder by some as multiple type hyperlipidemia. At present, FCH is the most common inherited hyperlipidemia in humans, affecting 1 to 3% of the adult population and up to 20% of patients with premature myocardial infarction. Despite recent progress, the genetic and metabolic backgrouds of FCH have not been elucidated in detail [9, 13, 116, 207-209]. This review discusses the evolution of the diagnostic criteria of FCH including the complex genetics and different metabolic pathways in the pathogenesis of this disorder.

Characteristic features of FCH

Affected individuals are characterized by variable expression of elevated concentrations of total cholesterol (TC) and/or triglycerides (TG) above the 90th percentile for age and gender. Within a family, multiple type hyperlipidemia in first-degree relatives comprising hypertriglyceridemia, hypercholesterolemia and/or combined hyperlipidemia is obligatory in addition to the presence of atherosclerotic manifestations before the age of 60 years. Usually, FCH becomes manifest only at adult age. Because of the absence of a specific clinical or metabolic marker for the disorder and because of characteristic variability in the presenting lipid phenotype, family studies are necessary to establish the diagnosis of FCH in a single patient [13, 116]. Other major characteristics of FCH include elevated apolipoprotein B (apoB) levels [5, 16, 20, 22, 23], the preponderance of small, dense low-density lipoprotein (sdLDL) [7, 16, 18, 23, 210] and decreased high-density lipoprotein cholesterol (HDLc) levels [6]. Furthermore, FCH is associated with obesity and insulin resistance [9, 11, 211-213].

Thus, FCH patients exhibit several risk factors contributing to an increased risk of CVD. Indeed, several studies have demonstrated that FCH is associated with a two- to fivefold increased risk of premature CVD [8, 10, 11].

Therefore, it is of great importance to identify and treat patients with FCH appropriately. However, the diagnosis of FCH is difficult because of the complex phenotypic heterogeneity and the highly variable nature of FCH as genetic, metabolic and environmental factors influence its expression.

Diagnostic criteria of FCH

There is a lack of consensus on diagnostic criteria for FCH. Traditionally, the diagnosis FCH in a patient is based on the presence of plasma TC and/or TG levels above the 90th percentile adjusted for age and gender in a family with multiple type hyperlipidemia and the presence of premature CVD. Although these internationally accepted diagnostic criteria have been formulated for FCH, not all research groups use the same criteria to define FCH. For example in some studies the 95th percentiles or even absolute values for TC (> 6.5 mmol/L) and

TG (> 2.3 mmol/L) levels, not adjusted for age and gender, are used [214-216].

A major chacteristic of FCH is the variability in lipid phenotype expression in time not only between individuals but also within a patient. This lipoprotein phenotypic heterogeneity in FCH has made the diagnosis of FCH even more difficult.

Total serum cholesterol and triglycerides

FCH phenotype variability at the lipoprotein level has been described in detail [117]. It was shown that a single individual over a 6-year period without drug therapy can present with all three phenotypes at any given time, suggesting that environmental factors strongly influence the variability in the phenotype in a subject with a genetic underlying cause for this disease.

We evaluated the variability in lipid phenotype expression over a 5-year period in 32 FCH families, comprising 299 subjects [16]. We demonstrated that the diagnosis FCH, based on plasma TC and/or TG levels, is consistent in only 74% of the subjects over a 5-year period, suggesting that inaccurate diagnosis of FCH contributes to heterogeneity in FCH populations in the literature [16]. Thus, most important, 26% of the subjects with FCH in 1994 had a sporadic normolipidemic pattern in 1999, defined by TC and TG levels below the 90th percentile, corrected for age and gender.

For the diagnosis FCH the use of only plasma TC and TG levels, age and gender adjusted, is insufficient. When the traditional lipid phenotype is variable and insufficient, accurate clinical diagnosis in individual subjects is not possible. Equally important genetic characterization becomes problematic, and a biologically correct hypothesis may be falsely rejected because of inaccurate diagnosis. Furthermore, the phenotype FCH based on TC and/or TG levels alone incompletely accounts for what is going on physiologically .

ApoB and small, dense LDL

In a previous study we showed that characterization of our FCH cohort was substantially more consistent when using two other major characteristics of FCH, elevated apoB levels and the presence of sdLDL [16]. Subjects affected with FCH in 1994 and/or 1999 had significantly higher apoB levels and an increased amount of sdLDL compared to nonaffected subjects, even when they presented with a normolipidemic phenotype. Together with less variablity in time, measurements of apoB and sdLDL in plasma could potentially lead to more consistent diagnostic criteria for FCH with a better discriminant power to separate affected from non-affected relatives, compared to the use of serum TC and TG levels alone. Ayyobi et al. also reported that sdLDL and elevated apoB are the common characteristics for the three major lipid phenotypes, type IIA, IIB and IV of FCH [5]. Also McNeely et al. showed during a 20-year follow-up of FCH subjects that apoB levels were highest among individuals who were consistently affected with FCH, intermediate among those who switched phenotypes, and lowest among individuals who were consistently normolipidemic [167]. Therefore, it is hypothesized that all lipoprotein phenotypes in FCH, despite great serum TC and TG variability, share fundamental characteristics, that is, sdLDL and elevated apoB levels. These data encourage the identification of unequivocal standardized diagnositic criteria for FCH.

ApoB as diagnostic criterion of FCH

The characteristic metabolic abnormality in FCH is increased production of very-low- density lipoprotein (VLDL) with or without impaired clearance of TG-rich lipoproteins in the majority of patients, resulting in hypertriglyceridemia and elevated plasma apoB (Figure 1, route 1) [91, 217].

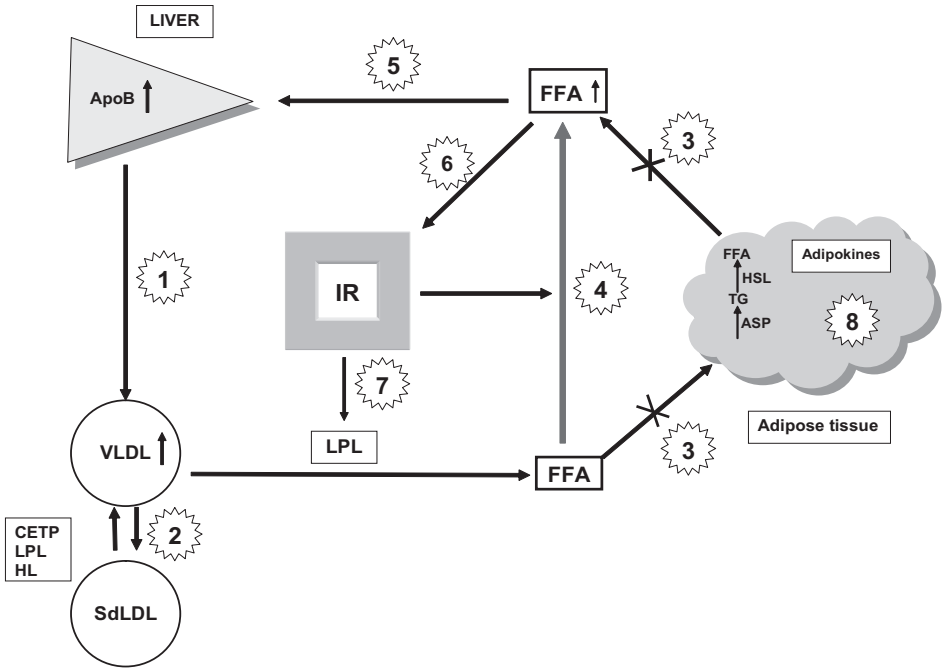

Figure 1
Metabolic pathogenesis of familial combined hyperlipidemia. The characteristic metabolic abnormality in FCH is increased production of apolipoprotein B (apoB) with or without impaired clearance of triglycerides by diminished lipoprotein lipase (LPL) activity, resulting in elevated levels of apoB and hypertriglyceridemia (i.e., elevated VLDL) (route 1). By the action of cholesterol ester transfer protein (CETP) and subsequent action of lipoprotein lipase (LPL) and hepatic lipase (HL), VLDL particles are converted into small, dense LDL (sdLDL) (route 2). As for the metabolic origin of apoB overproduction, emerging evidence points to disturbed trapping of peripheral fatty acids in adipose tissue (route 3), resulting in enhanced flux of free fatty acids (FFAs) to the liver and contributing to increased apoB production (routes 4 and 5). Furthermore, fatty acids activate hepatic gluconeogenesis. Increased production of glucose is a trigger to the pancreas to secrete insulin to maintain nomoglycemia and can thus result in an increased fasting plasma insulin concentration, which is associated with the syndrome of insulin resistance (IR) (route 6). A defect in insulin action at the level of the adipocyte reduces FFA trapping, resulting directly in elevated plasma FFAs (route 4). Finally, increase of FFA in plasma and insulin resistance inhibit LPL and promote the release of LPL from the endothelial cells, resulting in increased uptake of LPL by the liver, which cuold result in slower clearance of triglyceride-rich particles from plasma (route 7). Thus, abnormal metabolism of fatty acids provides a pathophysiological link to the hepatic apoB overproduction, partial LPL deficiency, and insulin resistance syndrome, all characteristics of FCH. The defect in fatty acid metabolism could be in the acylation stimulatory protein (ASP) or hormone-sensitive lipase (HSL) pathway, due to resistance to lipolytic hormones, or due to disturbed adipokine metabolism (e.g. increased leptin concentration, activation of the TNF-TNFR axis (route 8).

Although apoB overproduction is a well-established feature of FCH, the underlying molecular basis is not known. ApoB gene defects have been ruled out by linkage analysis [65, 218]. A locus for elevated apoB levels on chromosome 1p31 in Dutch families with FCH was reported [38]. As for the metabolic origin of apoB overproduction, emerging evidence points to a disturbed trapping of peripheral fatty acids in adipose tissue, resulting in enhanced flux of free fatty acids (FFA) to the liver, contributing to increased apoB production (see later). Several studies have shown that apoB is an important phenotypic measure of FCH. In a previous study we showed that characterization of our FCH cohort was substantially more consistant when using apoB levels [16]. Also, in the follow-up study of McNeely et al., elevated apoB was more persistent than elevated TC or TG levels [167]. Another practical advantage of including apoB levels as new diagnostic criterion is to diagnose FCH at a younger age. Several studies have shown that in teenage children, apoB may be elevated, whereas lipids are not [219-222]. The standardization of the plasma apoB assay has been performed by a committee of the World Health Organisation; apoB can be measured with the same precision and accuracy as the lipoprotein lipids [223]. All these data encourage to involve apoB in the phenotypic definition of FCH and establish a cutoff level for apoB.

We showed in our large FCH cohort with 5-year follow-up that the optimal cutoff point for apoB to predict FCH is an apoB level above 1200 mg/L [17]. Subjects with an apoB level over 1200 mg/L have an increased probability of being affected with FCH (OR = 12.3 [95% CI: 6.99 to 21.69]) compared with subjects with an apoB level below 1200 mg/L [17]. This has also been suggested in other studies. This value corresponds roughly to the 75th percentile in men (results of the Framingham Offspring study) [224] and subjects with apoB concentrations greater than 1200 mg/L were significantly more likely to have CVD than subjects with apoB levels less than 1200 mg/L [225, 226]. In our opinion, measurement of the apoB concentration is imperative for the diagnosis of FCH.

Small dense LDL as diagnostic criterion of FCH

Small, dense LDL is another important feature in FCH [13, 18, 183, 210], The intravascular formation of LDL subfractions involves the conversion of VLDL precursors [227]. Possibly there is also a direct hepatic secretion of different intermediate-density lipoprotein or LDL subspecies into the circulation. In the formation of LDL subfractions, the cholesteryl ester transfer protein (CETP) facilitates the distribution of cholesterol esters among different lipoprotein classes in exchange for TG [228]. The subsequent action of lipoprotein lipase (LPL) or hepatic lipase (HL) on these particles results in the hydrolysis of a significant amount of TG in LDL and thereby in a decrease in LDL particle size (Figure 1, route 2) [229, 230]. In general, this hypothesis can explain the predominance of these sdLDL particles in any form of hypertriglyceridemia. However, in FCH, we and several groups have provided evidence that sdLDL is characteristic of FCH, independent of plasma TG concentration [13, 119, 231]. Genetic influences on the LDL subfractions distribution have been proposed by Bredie et al. and others as well, allthough they are still unclarified [13, 23, 183].

We previously showed that subjects with FCH had significantly more atherogenic sdLDL, compared with nonaffected relatives, even when they had a sporadic normolipidemic phenotype [16]. Similarly, sdLDL has been demonstrated to be the most prominent characteristic shared among

the three FCH phenotypes, independent of the classical plasma lipoprotein levels [5]. However, this was not shown in the follow-up study of McNeely et al., which could be due to different methods used to determine sdLDL [167]. Although sdLDL is an important feature of FCH, it is of interest that in multivariate analysis, LDL heterogeneity did not provide additional information in predicting FCH after including TG; TG levels appeared to be the most important predictor of FCH, most likely because of the strong correlation between LDL heterogeneity and TG levels ($r = -0.68$) [17]. Serum TG concentration has been suggested to be the most important predictor of LDL size in FCH patients [7]. Several studies have shown that plasma TG levels over 1.5 mmol/L distinguish optimally between atherogenic sdLDL and a large buoyant LDL subfraction profile [119, 232].

Hyper-TG hyper-apoB as diagnostic feature of FCH

During an international forum on FCH in Barcelona in 2001, there was a proposal to redefine FCH based on hypertriglyceridemia and elevated plasma apoB (hyperTG hyperapoB) [216]. The cutoff point proposed for apoB was 1200 mg/L and for plasma TG 1.5 mmol/L. The choice of these cutoff points was tentative until recently. We showed for the first time that these cutoff point were justified on the basis of our large FCH cohort with 5-year follow-up [17].

A nomogram to diagnose FCH

Based on the pathophysiology of lipid metabolism in FCH, four major lipid phenotypes of FCH can be distinguished to be used as diagnostic criteria for FCH. Recently, we evaluated which (combination of) diagnostic features most adequately predicts FCH, including serum TC, TG, apoB and sdLDL [17]. The TG level, adjusted for age and gender, was the strongest independent predictor of FCH. The absolute apoB level was the second most important predictor of FCH. Finally, TC levels contributed to the prediction of FCH. Small, dense LDL did not add any significance in the prediction of FCH because of the strong association with TG levels. Thus, absolute apoB levels in combination with TG and TC levels, both age and gender adjusted, are most predictive for diagnosing FCH. To develop a clinical tool, a nomogram was constructed to calculate simply and reliably the probability of FCH in clinical practice in an individual subject on the basis of quantitative serum apoB, TG, and TC values. In case the percentiles of serum TC and TG are not available, we showed that it is acceptable to define FCH by TG levels over 1.5 mmol/L and apoB values over 1200 mg/L [17].

The use of the proposed new diagnostic criteria, included in a nomogram, will make it easier to identify patients and their relatives with FCH. Still, the diagnostic phenotype has to be present in more than one family member and at least one individual in the family must have premature CVD to diagnose FCH. Confirmation of the clinical relevance of this new definition of FCH in other large FCH cohorts is warranted to confirm unequivocal diagnostic criteria for FCH.

Associated nonlipid features of FCH

Patients with familial combined hyperlipidemiat are characterized by the presence of obesity and insulin resistance [9, 11, 211-213]. It is well known that insulin resistance and visceral obesity are both associated with several metabolic abnormalities, including increased TG levels, lower HDLc levels, more sdLDL particles, and increased apoB production rates, all also characteristics of FCH. Insulin resistance or visceral obesity or both may therefore be important factors modulating FCH phenotypes.

Insulin resistance

Impaired insulin action in FCH patients, with respect to the suppression of serum FFA and the stimulation of glucose disposal, has been demonstrated directly by means of the euglycemic hyperinsulinemic clamp technique by several groups [211, 233, 234]. The reported defect in insulin ability to suppress the FFA release results in elevated serum FFA concentration; in peripheral tissues, particular skeletal muscle, high FFA levels block glucose oxidation, thereby causing insulin resistance (Figure 1, route 6) [235]. In the liver, a high flux of FAA most likely drives hepatic overproduction of TG and apoB, contributing to an elevated concentration of VLDL in the plasma (Figure 1, route 5) [95]. Thus, insulin resistance is associated with a combined VLDL + LDL or hypertriglyceridemic lipid phenotype, but it does not explain (isolated) high LDL-cholesterol in FCH. Indeed, most studies on insulin resistance in FCH have been performed in patients who exhibit a combined hyperlipidemia (elevation of both VLDL and LDL) [236, 237].

Therefore, the question arises what the role of insulin resistance is in FCH patients with high LDLc in the absence of hypertriglyceridemia. Several reports have shown that insulin resistance was found in all three FCH lipoprotein phenotypes, being more severe in subjects with hypertriglyceridemia [236, 237]. A major concern in all these studies is that changes in lipid phenotype, which is traditionally considered an important characteristic of FCH, are not taken into account. We reported that 43% of the FCH patients showed a change in lipid phenotype expression after 5 years [16]. The effect of these intra-individual changes in lipid phenotype expression in time on insulin resistance has not been addressed in the literature. It is of great interest to study the interdependence of insulin resistance and change in lipid phenotype expression in time to reveal further the role of insulin resistance in FCH. The data on insulin resistance in FCH imply that FCH defined by the traditional diagnostic criteria, TC and/or TG, includes a heterogenous population in which several metabolic pathways may contribute to the lipid phenotype expression. It will be of great interest to demonstrate an association of the proposed new FCH diagnostic criteria using the nomogram and insulin resistance to reveal the role of insulin resistance in lipid phenotype expression in FCH [17].

Several pathophysiological mechanisms may contribute to insulin resistance in FCH, that is, decreased insulin-induced vasodilation in skeletal muscle or impaired post-prandial fatty acid metabolism, as discussed subsequently, and, last but not least, visceral obesity.

Visceral obesity

A tempting hypothesis that potentially explains the presence of insulin resistance only in patients with hypertriglyceridemia with or without hypercholesterolemia is the frequent presence of visceral obesity in hypertriglyceridemic subjects [238]. The FCH phenotype can be influenced by

various environmental factors such as diet and exercise, which can also alter plasma TG levels. Accordingly, the body mass index (BMI) of hypertriglyceridemic patients was significantly higher than that of the healthy subjects [16]. The FCH subjects affected, on the basis of hypertriglyceridemia or combined hyperlipidemia, had a significantly higher BMI than controls [9, 16]. Furthermore, in our 5-year follow-up cohort, an increase in BMI over 5 years was associated with an increase in the homeostasis model assessment-index (HOMA-index), indicating insulin resistance (correlation between delta BMI and delta HOMA of $r = 0.47$, $p < 0.001$, unpublished data). The interdependence of insulin resistance and visceral obesity in FCH was studied by Purnell et al. [212]. Insulin action was studied by the intravenous glucose tolerance test in 11 male FCH patients with combined hyperlipidemia, and visceral obesity was measured by computed tomography (CT) scan. In the FCH group insulin sensitivity was inversely associated with intra-abdominal fat and BMI but not with subcutaneous fat. Thus, insulin resistance was present in patients with FCH who were viscerally obese. The degree of insulin resistance was proportional with their degree of visceral obesity. In our FCH cohort the HOMA-index, as a measure of insulin sensitivity, was also related to BMI ($r = 0.43$, $p < 0.001$, unpublished data). However, insulin resistance is also present in nonobese FCH patients, as measured by BMI [233]. It is suggested that in nonobese subjects the amount of intra-abdominal fat, as measured by CT scan, can vary up to 10-fold. Which parameter is most sensitive to predict insulin resistance - BMI reflecting degree of adiposity, waist-hip ratio (WHR) reflecting abdominal obesity, or IAF measuring intra-abdominal fat - remains to be determined.

Can insulin resistance and/or visceral obesity explain the FCH lipid phenotype?

The main question is whether insulin resistance or visceral obesity, or both, can explain the FCH lipid phenotype. Purnell et al. examined the relationship between insulin resistance or visceral obesity and increased apoB levels in 11 FCH patients [212]. For any level of insulin sensitivity or intra-abdominal fat, apoB levels remained higher in patients with FCH than in controls. In our FCH cohort, including all subjects with different lipid phenotypes, the majority of the FCH patients had increased apoB levels and more sdLDL at any level of HOMA or BMI (unpublished data). Hence, obesity or insulin resistance or both do not fully account for the elevated levels of apoB or high prevalence of sdLDL in FCH. These results support the physiological concept of separate, but additive, genetic determinants in the etiology of the FCH lipid phenotype. We have reported previously that in our FCH families a major gene influences apoB levels and sdLDL [18, 20, 23], but the gene has not been identified yet.

Central role of free fatty acids in the pathogenesis of FCH

As depicted in figure 1, a disturbed fatty acid metabolism may play a central role in FCH [93, 239, 240]. First, an increased flow of FFA into the portal system has been postulated as a major contributor to dyslipidemia in FCH because it could lead to hepatic overproduction of VLDL particles (Figure 1, route 5) [95]. Second, in vivo evidence has been provided suggesting a pivotal role of increased FFA in insulin resistance (Figure 1, route 6) [95, 211, 235]. Thus, in FCH both overproduction of VLDLapoB and the presence of the syndrome of insulin resistance may be secondary to

impaired postprandial fatty acid metabolism. In vivo evidence of defective postprandial and postabsorptive FFA metabolism in FCH has been demonstrated [94]. Oral fat loading tests during 24 hours were performed in seven FCH patients with combined hyperlipidemia compared with seven nonrelated controls. Changes in FFA and ketone bodies, which represent hepatic products of FFA metabolism, were studied. The postprandial (0 – 8 hour) increase in ketone bodies was almost four times higher in FCH patients than in controls, suggesting an increased flux of FFA to the liver, possibly because of inadequate FFA trapping in the adipocyte (Figure 1, routes 3 and 4). In the postabsorptive period (8 – 24 hours), FFA and ketone bodies significantly decreased in FCH patients in contrast to controls, in whom ketone bodies increased, which may represent diminished release of FFA from adipocytes by hormone-sensitive lipase (HSL) (Figure 1, route 3).

Why is the metabolism of FFA altered in FCH? It is hypothesized that the defect in fatty acid metabolism is likely to be localized in the adipocyte: adipose tissue is the body's major site for storage and mobilization of FFA.

Disturbed adipose tissue metabolism in FCH

In figure 1, a simplified scheme of adipose tissue metabolism is included (routes 3, 4 and 8): The FFA liberated by the continuous hydrolysis of core TG by LPL in VLDL and chylomicrons are stored in adipocytes by intracellular reesterification to TG, a process that is mediated by the action of a basic protein called acylation stimulatory protein (ASP) and modulated by insulin [241]. In the adipocyte, reesterification of FFA results in TG, which can be hydrolyzed again by HSL, releasing FFA from the fat cell. The most important lipolytic hormones are cathecholamines; insulin inhibits the action of HSL [242].

Abnormal adipose tissue FFA metabolism has been identified as a major contributor to the increased plasma FFA in FCH. FCH subjects have insulin-resistant adipose tissue lipid metabolism [213]. Potential defects in adipose metabolism include altered activity of ASP and/or HSL.

Acylation stimulatory protein

The ASP pathway modulates the rate at which fatty acids are trapped by adipocytes and converted to TG. ASP also influences the rate at which fatty acids are released by adipocytes (Figure 1, routes 3 and 4) [241, 242]. Resistance of adipocytes to the effects of ASP and/or impaired ASP activity may contribute to reduced FFA uptake into adipocytes, which may result in an increased flux of FFA to the liver and consequently in an increased hepatic VLDL synthesis, characteristic of FCH (Figure 1, route 5). For example, impaired ASP activity has been reported in hyperapobetalipoproteinemia, a condition apparently related to FCH [243, 244]. However, no difference in plasma ASP levels has been found in FCH patients [245]. It has been suggested that ASP is only locally generated in adipose tissue and cannot be detected in peripheral plasma.

Hormone-sensitive lipase

The release of FFA from visceral adipocytes is mediated by the action of the enzyme HSL (Figure 1, route 3). This lipolytic effect of HSL is inhibited by postprandial hyperinsulinemia, whereas cathecholamines are the most important lipolytic hormones by activating HSL.

In FCH patients from Sweden, a marked resistance to the lipolytic effect of cathecholamines in fat cells has been demonstrated, predominantly related to a defect in HSL [246]. The maximum enzymatic activity of HSL was decreased by 40%. However, in FCH patients from Finland, no difference in adipose tissue HSL activity was found [158]. In addition, in the genetically homogenenous Finnish population the HSL gene was not a major locus responsible for the expression of the FCH phenotype [84, 182]. The discrepancy in HSL activity results between Swedish and Finnish FCH population is most likely explained by the heterogeneity and genetic background of FCH, which may be different in the two populations. In addition, genetic defects may contribute to increased FFA concentrations in FCH.

Leptin

Leptin is a hormone primarily secreted by adipocytes. Serum leptin levels have been associated with obesity, insulin resistance and CVD, all features of FCH. Therefore, it is of great interest to determine whether increased leptin levels, as a marker of disturbed adipose tissue metabolism, contribute to the FCH phenotype and its associated increased risk for CVD. So far, two small studies have studied the relationship between leptin concentrations and FCH, with contrasting results. Jacobson et al. found a significant difference of leptin concentration in a group of young females, and Haluzik et al. found no significant differences in his group of males [247, 248].

Furthermore, adipocytes release a great amount of other hormones/adipokines, including tumor necrosis factor [249] and adiponectin [250], which have been implicated in the pathogenesis of insulin resistance and visceral obesity. The role of these adipokines in the pathogenesis of FCH needs further investigation.

The putative role of adipose tissue in the pathogenesis of FCH has been reviewed [96]. Although more evidence-based arguments are needed, the current hypothesis suggests that a combined defect in lipid storage by reduced ASP and insulin action and in lipolysis by decreased HSL activity results in adipose tissue that is metabolically inactive. As a result, FFA are directly shunted to the liver which drives apoB synthesis and increases the secretion of VLDL by the liver (Figure 1, routes 3, 4 and 5). Several studies have demonstrated differential expressed genes in adipose tissue from patients with FCH, supporting the potential role of adipose tissue in the pathogenesis of FCH [251, 252].

Genetics of FCH

The genetics of FCH and risk of coronary heart disease have been extensively reviewed by Shoulders et al. [209]. At present, the complex genetics in FCH are not fully understood. FCH was orginally described as a monogenic trait, but the inheritance of the FCH-associated phenotypes has been shown to be more complex. Segregation analyses have provided evidence for a major gene effect on apoB levels [20, 22, 23], TG levels [21], the presence of sdLDL [13, 18, 23] and insuline resistance [19].

Genome-wide linkage studies have indicated that several chromosomal regions harbor genes that contribute to the lipid profile of FCH. In Finnish families with FCH, five loci on chromosome 1q21, 2q31, 10p11.2, 10q11.2-10qter, and 21q21 were identified, whereas in Dutch families with FCH four loci, on chromosome 2p, 11p, 16q and 19q were found [28, 29, 31, 34]. Two potential

new loci on 6q and 8p for FCH were identified in two large cohorts of families with FCH from the United Kingdom [36]. The locus on 1q21 has been confirmed in German and Chinese FCH families and in families from the National Heart, Lung, and Blood Institute Family Heart Study [26, 30]. A combined data analysis of Dutch and Finnish genome-wide scans for FCH identified three regions, on chromosomes 16q24.1, 2p25.1 and 9p23 with maximum lod scores of 3.6, 2.2, and 2.1, respectively, which most likely harbor allelic variants for FCH. The 2p25.1 region was detected for the FCH trait wheres the other two regions were detected for the low HDL-cholesterol trait [35]. In addition, regions on 2p, 8q, 16q, and 20q have been detected for low HDL-cholesterol in the Finnish families with FCH and a region on 1p has been linked to apoB in Dutch families with FCH [38]. The current genetic model suggests that FCH might result from the combination of a dominant major gene or genes in combination with several modifier genes influencing plasma lipid levels. The identification of such modifier genes might help in reducing the problem of genetic heterogeneity of FCH. Several candidate genes have been evaluated in their contribution to the FCH phenotype. These include genes involved in lipid metabolism, such as lipoprotein lipase (*LPL*), hepatic lipase (*HL*), *APOA1/C3/A4* and *APOA5* [32, 33, 55, 60, 62, 66, 67, 73, 77, 79, 82, 253]; in adipose tissue metabolism, such as *TNF* and *HSL* [84, 88, 182]; and in insulin resistance and FFA metabolism (fatty acid binding protein (*FABP*), insulin receptor substrate gene and beta3-adrenergic receptor, *PPAR-γ* [83, 86, 87, 89]. To summarize the results of these studies, *LPL* and *APOA1/C3/A4* have been most consistently associated as modifier genes in FCH.

To unravel further the genetics of FCH, animal models have been developed. A locus (Hyplip1) in mutant mouse strain HcB-19 has been identified that was strongly linked to the FCH features in these mice [143]. A few years later it was shown that these mice had decreased expression of the thioredoxin interacting protein (*TXNIP*). By sequencing, a spontaneous nonsense mutation (T97Z) in the *TXNIP* gene was identified [148]. The *TXNIP* gene in mice is located on chromosome 3F2.2, which is syntenic to chromosome 1q21 in humans, a region in which a major contributor of the FCH phenotype has been mapped in the Finnish, German and Chinese populations [28, 30]. However, in humans, we and others did not find any sequence variants in the *TXNIP* gene in patients with FCH, and therefore the *TXNIP* gene is unlikely to be involved as a major contributor to the FCH phenotype [46, 149]. Pajukanta et al. showed that FCH is strongly linked and associated with the gene encoding upstream stimulatory factor 1 (*USF1*)[46]. This is a very intruiging finding as *USF1* is also located at chromosome 1q21 and regulates the expression of several genes involved in glucose and lipid metabolism, making it a potentially good candidate for a major gene involved in the pathogenesis of FCH.

Summary

Studies aimed at resolving the complex metabolic and genetic background of FCH have been going on for over 30 years. Several metabolic abnormalities and genetic backgrounds have been suggested, but the exact cause of these metabolic disturbances is unknown. To delineate further the pathogenesis of FCH, it is essential to simplify and standardize the diagnosis of FCH. In this respect, great progress has been made. The introduction of unequivocal standardized diagnostic criteria of FCH will improve genetic characterization and elucidation of the metabolic pathogenesis of FCH, including both the etiology of the increased pool of apoB100 containing TG-rich lipoproteins and that of the preponderance of small dense LDL particles.

Chapter 7

High plasma level of remnant-like particle cholesterol in familial combined hyperlipidemia

Submitted

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Abstract

Background The traditional lipid and lipoprotein levels in patients with familial combined hyperlipidemia (FCH) are relatively mildly elevated and do not fully explain the increased risk of CVD. In other populations, high remnant-like particle cholesterol (RLPc) levels are an independent risk factor for CVD. In the present study, we investigated whether plasma RLPc concentrations are elevated in patients with FCH, and whether these RLPc levels contribute to the increased prevalence of CVD. **Methods** In this cross-sectional study, we studied RLPc levels in 37 FCH families comprising 582 subjects, of whom 134 subjects were diagnosed as FCH, according to the nomogram. Plasma RLPc concentrations were determined using an immune separation technique. **Results** For both men and women, the mean plasma RLPc level (mmol/l) was extremely elevated in FCH patients (0.59 (0.54 - 0.66) and 0.40 (0.37 - 0.43), respectively) compared to both normolipidemic relatives (0.27 (0.26 - 0.29) in male and 0.22 (0.21 - 0.23) in female, all $p < 0.000$) and spouses (0.27 (0.23 - 0.31) in male and 0.24 (0.21 - 0.27) in female, all $p < 0.000$). Plasma RLPc levels above the 90^{th} percentile were associated with an increased risk for FCH (OR 17.4 [10.8 - 27.8]) and the presence of CVD, independently of nonlipid cardiovascular risk factors (OR 2.18 [1.02 - 4.66]). Most importantly, RLPc levels predicted prevalent CVD independently of triglyceride levels (OR 2.35 [1.15 - 4.83]). however, RLPc did not provide additional information about prevalent CVD over and above non-HDL cholesterol levels. **Conclusion** Patients with FCH have two-fold elevated plasma RLPc levels, which contribute to the increased risk for CVD but not independently of non-HDL cholesterol levels.

Introduction

Familial combined hyperlipidemia (FCH) is the most common genetic hyperlipidemia in humans and affects up to 5% of the general population. It is characterized by multiple lipoprotein phenotypes and is strongly associated with premature cardiovascular disease (CVD). Of the survivors of a premature myocardial infarction, up to 20% are affected with FCH [4, 10, 14]. FCH is characterized by hypercholesterolemia, hypertriglyceridemia and elevated levels of apolipoprotein B (apoB). Other phenotypes of FCH are elevated levels of low-density lipoprotein cholesterol (LDLc), very low-density lipoprotein (VLDL), the presence of small dense LDL (sdLDL) and decreased levels of high-density lipoprotein cholesterol (HDLc) [116]. In addition, FCH is associated with obesity and insulin resistance [9].

The classical concept of the pathogenesis of CVD, based on lipid and lipoproteins, has implicated elevated cholesterol, particularly LDLc, as the central atherogenic lipoprotein class. However, accumulating data suggest that apoB containing lipoproteins other than LDL, particularly in the setting of mild to moderate hypertriglyceridemia, confer additional atherogenic risk beyond that due to LDLc levels alone. FCH is an example of a lipid disorder with only mildly elevated plasma LDLc levels but characteristic high apoB levels due to increased VLDL-cholesterol (VLDLc) and VLDL-triglyceride (VLDLtg) levels, insulin resistance and associated abnormalities. Recently, in a large observational study, the calculated plasma non-HDL cholesterol concentration (LDLc + VLDLc) was a stronger predictor of cardiovascular events than plasma cholesterol alone [254, 255]. Improvement of CVD predictability upon inclusion of VLDLc emphasizes the proatherogenic role of triglyceride rich lipoproteins (TRL). Smaller, partially lipolyzed TRL remnants i.e. remnant-like particles cholesterol (RLPc) are considered to be more atherogenic than larger newly secreted TRL since they can more readily penetrate the endothelial lining of the arterial wall [256]. Several lines of evidence have implicated RLPc as playing an etiologic role in atherogenesis, as recently reviewed [257, 258]. In fact, RLPc have been found to be present in atherosclerotic lesions [259].

Recently, an immuno-affinity separation method was introduced for assaying cholesterol in RLPs as RLPc [198]. Plasma concentration of RLPc have been shown to be higher in patients with CVD [260] and diabetes mellitus [261]. In elderly patients, RLPc rather than LDLc was strongly associated with CVD [262]. Increased RLPc levels are a significant predictor of myocardial infaction in patients with vasospastic angina [263] and CVD [264] and have been shown to be strongly associated with angiographically verified progressions of focal coronary atherosclerosis [262]. Recently, high plasma level of RLPc in patients with the metabolic syndrome was reported [265]. In the metabolic syndrome, elevated levels of RLPc were a risk factor for CVD and endothelial dysfunction, a predictor of coronary events [266].

The aim of the present study was to determine whether increased plasma RLPc levels contribute to the FCH phenotype and its increased risk of CVD.

Methods

Subjects

The study population existed of 37 families, comprising 644 subjects, of whom 158 subjects were diagnosed as FCH patients. The diagnosis of FCH was based on plasma levels of total

cholesterol, triglyceride and apoB using the nomogram, as reported recently [17].

The normolipidemic relatives (n = 390) and spouses (n = 89), also included in this study population, served as two independent reference groups, because of the similar genetic background of the relatives with the FCH patients. Seven subjects had missing data concerning their diagnostic criteria, because of missing data for apoB levels. All subjects filled out a questionnaire about their previous medical history, especially cardiovascular status, medication use and smoking habits, as previously reported [16, 17]. When the presence of CVD was suspected by the clinical investigator, further details and confirmation of the diagnosis were sought from the participants general practitioner, plus, if considered necessary from any relevant hospital records. In our study population 56 subjects were identified with CVD, including 26 subjects with angina pectoris, 25 subjects with previous MI, 10 subjects with peripheral vascular disease, 7 subjects with stroke and 23 subjects experienced vascular surgery. In total 45% (n = 25) of these subjects were diagnosed CVD based on the presence of 2 or more manifestations of CVD.

After withdrawal of lipid-lowering medication for four weeks and an overnight fast, venous blood was drawn by venipuncture. Body mass index (BMI) was calculated as body weight (in kilograms) divided by the square of height (in meters). The maximum hip circumference (H) and waist circumference (W) at the umbilical level were measured in the late exhalation phase while standing. The waist/hip circumference (W/H) ratio was calculated. Systolic and diastolic blood pressure were assessed twice with an automated blood pressure device (Dinamap; Critikon, Tampa, FL, USA) in the right arm in a sitting position after a 5-minute rest period, mean blood pressures were used for analysis. The ethical committee of the Radboud University Nijmegen Medical Centre approved the study protocol and the procedures followed were in accordance with institutional guidelines. All subjects gave informed consent.

Laboratory measurements

VLDL was isolated from whole plasma by ultracentrifugation at density=1.006 g/ml for 16 h at 36,000 rpm in a fixed angle rotor (TFT 45.6 rotor, Kontron), in a Beckman L7-55 ultracentrifuge. HDLc was determined by the polyethylene glycol 6000 method [267]. LDLc was calculated by subtraction of VLDLc and HDLc from plasma total cholesterol. Cholesterol and triglycerides were determined by enzymatic, commercially available reagents (Boehringer-Mannheim, Germany, catalog No. 237574 and Sera Pak, Miles, Belgium, catalog No. 6639, respectively).

Total plasma apoB concentrations were determined by immunonephelometry as recently described in detail elsewhere [119]. LDL subfractions were separated by single spin density gradient ultracentrifugation [120]. A continuous variable K represents the LDL subfraction profile of each individual. A negative K value ($K \leq -0.1$) reflected a more dense LDL subfraction profile, and a positive K value ($K > -0.1$) reflected a more buoyant profile [268].

Glucose concentrations were measured in duplicate using the oxidation method (Beckman®, Glucose Analyser2, Beckman Instruments Inc., Fullerton, CA 92634, USA). Plasma insulin concentrations were determined using a double antibody method with an interassay variability of 10.3% [170]. Insulin resistance was assessed by the Homeostasis model assessment (HOMA-index). The HOMA-index was calculated from the fasting concentrations of insulin and glucose using the following formula: HOMA-index = fasting serum insulin (μU/ml) x fasting plasma glucose (mmol/liter)/22.5 [171].

Table 1 Characteristics of patients with familial combined hyperlipidemia, normolipidemic relatives and spouses.

	FCH patients (n = 134)	NL relatives (n = 387)	Spouses (n = 61)
Gender (males) *	68 (50.8%)	175 (45.2%) ¥	36 (59.0%)
Age (years)	45.5 (16.2) ‡ §	37.7 (17.6) ¥	53.5 (15.8)
Smoking *	34 (25.4%)	89 (23.0%)	10 (16.4%)
Cardiovascular disease *	24 (17.9%) ‡ §	21 (5.4%)	3 (4.9%)
Body mass index (kg/m^2)	27.2 (4.4) §	24.1 (5.3) ¥	26.4 (4.1)
Waist/hip ratio	0.88 (0.08) §	0.83 (0.09) ¥	0.86 (0.08)
Systolic blood pressure (mmHg)	134.4 (17.3) §	128.1 (19.3) ¥	134.8 (16.6)
Diastolic blood pressure (mmHg)	84.7 (10.5) §	80.3 (12.1) ¥	83.1 (9.9)
Total cholesterol (mmol/L)	6.3 (1.0) ‡ §	4.9 (1.1) ¥	5.2 (1.0)
Triglycerides (mmol/L) †	2.7 (1.6) ‡ §	1.1 (1.6)	1.1 (1.6)
VLDL-cholesterol (mmol/L)	1.09 (1.89) ‡ §	0.34 (1.95)	0.35 (1.87)
VLDL-triglycerides (mmol/L)	1.91 (1.91) ‡ §	0.62 (2.01)	0.63 (1.88)
HDL-cholesterol (mmol/L)	0.98 (0.33) ‡ §	1.22 (0.36)	1.30 (0.32)
LDL-cholesterol (mmol/L)	4.07 (0.98) ‡ §	3.22 (1.06)	3.41 (0.95)
Apolipoprotein B (mg/L)	1337 (229) ‡ §	960 (237)	1000 (227)
K-value	-0.23 (0.23) ‡ §	0.04 (0.25)	0.05 (0.22)
non-HDL-cholesterol (mmol/L)	5.34 (1.02) ‡ §	3.63 (1.08)	3.85 (0.99)
Insulin (mU/L) †	12.8 (1.7) ‡ §	9.0 (1.1)	9.6 (1.7)
Glucose (mmol/L) †	5.2 (1.1) §	5.0 (1.1) ¥	5.2 (1.1)
HOMA-index †	2.9 (1.8) ‡ §	2.0 (2.0)	2.3 (1.8)

*Results are presented as means and SD except as indicated; *, presented are number (%); †, presented are geometric mean (geometric SD); FCH, familial combined hyperlipidemia; NL relatives, normolipidemic relatives; VLDL-cholesterol, very low-density lipoprotein cholesterol; VLDL-triglycerides, very low-density lipoprotein triglycerides; K-value, a value ≤ -0.1 prepresents the presence of small dense LDL; HDL-cholesterol, high-density lipoprotein cholesterol; LDL-cholesterol, low-density lipoprotein cholesterol; HOMA-index, homeostasis model assessment-index; ‡, p-value < 0.05, compared to normolipidemic relatives; §, p-value < 0.05, compared to spouses; ¥, p-value < 0.05, compared to spouses.*

RLPc assay

The RLPc fraction was prepared using an immune separation technique described by Nakajima et al. [198]. Plasma RLPc was measured by an enzymatic assay included in the assay kit, using a Cobas Mira S auto-analyzer (ABX Diagnostis, Montpellier, France). The interassay variability was 6%. Due to technical errors, plasma RLPc levels could be determined in 582 subjects, including 134 FCH patients, 387 normolipidemic relatives and 61 spouses.

Statistical analysis

The descriptive statistics expressed as means with standard deviation (SD), are represented separately for FCH patients, normolipidemic relatives and spouses. Variables like plasma RLPc concentration, triglyceride levels, non-HDL cholesterol levels and the HOMA-index, with a skewed distribution were logarithmically transformed for analysis. Differences in characteristics between subjects with FCH, spouses and normolipidemic relatives were tested by generalized estimating equations (GEE) because of possible correlated values within families. Also odds ratios (OR) with corresponding confidence interval as an estimate of risk for FCH or CVD were calculated using GEE. Correlations between plasma RLPc and variables were analyzed using Pearson correlation coefficients. Multiple linear regression test was used to select the variables that contributed independently to plasma RLPc. Probability values < 0.05 were considered statistically significant. All analyses were computed using the STATA 8.0 software.

Results

Subject characteristics

Descriptive statistics for anthropometric measurements and biochemical variables in patients with FCH, normolipidemic relatives and spouses are presented in table 1. The mean age of the group of subjects with FCH was significantly higher compared to the normolipidemic relatives and significantly lower compared to the spouses. As expected, the group of patients with FCH had a higher prevalence of CVD compared to both normolipidemic relatives and spouses. The mean BMI and WHR of FCH patients was significantly higher compared to the normolipidemic relatives. Systolic and diastolic blood pressure were significantly higher in patients with FCH compared to their normolipidemic relatives but did not differ from the spouses. Patients with FCH were more insulin resistant than the normolipidemic relatives and spouses as assessed by the elevated HOMA-index. By definition, FCH patients are characterized by increased plasma total cholesterol, triglyceride and apoB levels compared to normolipidemic relatives and spouses. Furthermore, FCH patients had a more atherogenic lipid and lipoprotein profile as reflected by significantly lower plasma HDLc levels, higher plasma LDLc and non-HDL cholesterol levels and the presence of small, dense LDL (sdLDL), as reflected by a significantly lower K-value. In addition, significantly increased levels of VLDLc and VLDLtg were present in FCH compared to both normolipidemic relatives and spouses. The differences between the spouses and normolipidemic relatives are indicated in table 1.

Remnant-Like Lipoprotein Cholesterol concentration and FCH

The mean plasma RLPc concentration in the FCH patients was significantly higher compared to both the normolipidemic relatives and spouses, indicating a two-fold elevation of RLPc in FCH patients (Table 2). The mean RLPc levels in the normolipidemic relatives did not differ from the spouses. Plasma RLPc levels differed between males and females, i.e. men had higher plasma RLPc levels than women, which did only not reach statistical significance in the group of spouses. After adjustment for gender and age, the mean difference in plasma RLPc concentration between FCH patients versus spouses and normolipidemic relatives remained significant (0.46 (SD 0.38) versus 0.25 (SD 0.45) and 0.23 (SD 0.36) respectively)).

As the plasma RLPc concentration in the normolipidemic relatives and spouses did not differ

significantly, we analyzed the normolipidemic relatives and spouses together defined as the non-FCH control group.

Among the patients with FCH, 91 (68%) had fasting plasma RLPc levels above the 90th percentile as determined in the non-FCH control group without CVD standardized for gender (cut-off point of 0.36 mmol/l) (Table 2).

Table 2 Plasma RLPc concentration in patients with familial combined hyperlipidemia compared to normolipidemic relatives and spouses.

	FCH patients (n = 134)	NL relatives (n = 387)	Spouses (n = 61)
Male	0.59 (0.54 - 0.66) † ‡	0.27 (0.26 - 0.29)	0.27 (0.23 - 0.31)
Female	0.40 (0.37 - 0.43) † ‡	0.22 (0.21 - 0.23)	0.24 (0.21 - 0.27)
RLPc > 90th percentile *	91 (67.9%) † ‡	40 (10.3%)	8 (13.1%)

*Values are means with 95% confidence intervals except as indicated. a presented are number (%); FCH, familial combined hyperlipidemia; NL relatives, normolipidemic relatives; *, 90th percentile of RLPc (0.36 mmol/L) is calculated from controls without CVD and standardized for gender; CVD, cardiovascular disease; †, p-value < 0.05, compared to normolipidemic relatives; †, p-value < 0.05, compared to spouses.*

Characteristics of subjects with plasma RLPc levels above the 90th percentile

In the non-FCH control group, plasma RLPc levels above the 90th percentile were associated with older age, an increased BMI and WHR and elevated blood pressure (Table 3). Furthermore, control subjects with high plasma RLPc levels above the 90th percentile showed a more atherogenic lipid and lipoprotein profile characterized by higher total cholesterol and triglyceride levels, lower HDL cholesterol levels, higher apoB levels and non-HDL cholesterol levels and more small dense LDL i.e. lower K-values (Table 3). Although glucose levels were significantly higher in the control subjects with high RLPc levels above the 90th percentile, no difference in HOMA-index was found compared to the group with RLPc levels below 90th percentile. Similar associations were found in the FCH group (data not shown).

Plasma RLPc levels above the 90th percentile were associated with an increased risk for FCH (OR 19.4 [10.9 - 35.4]). Even after correction for age, gender, CVD, BMI, WHR, blood pressure and HOMA, increased plasma RLPc levels above the 90th percentile remained associated with an increased risk for FCH (OR 17.4 [10.8 - 27.8]).

Plasma RLPc levels in relation to lipid, lipoprotein and insulin resistance parameters

Plasma RLPc level showed significant univariate correlations in the control group with age, BMI, WHR, both systolic and diastolic blood pressure, total cholesterol, triglycerides, VLDLc, VLDLtg, HDLc, non-HDLc, LDLc, apoB, K-value, insulin, glucose and the HOMA-index (Table 4).

Similar correlation coefficients for plasma RLPc with all variables were found among the FCH patients, although not reaching statistical significance for systolic blood pressure, apolipoprotein B and parameters of insulin resistance including HOMA and insulin levels. In FCH patients, an increase in RLPc levels was associated with lower LDLc levels whereas in the control group an

increase in RLPc levels was associated with higher LDLc levels.

Multiple linear regression analyses in the non-FCH control group and in the group of FCH patients showed that the variation in plasma RLPc concentration could be enlightened for approximately 66% and 77%, respectively, by the variables gender and lipid and lipoprotein levels including triglycerides, VLDLc, non-HDLc, LDLc and small, dense LDL.

In both controls and FCH patients, gender, triglyceride levels and non-HDLc levels were the main contributors in the explanation of the variation in RLPc levels (62% in the non-FCH control group and 54% in the FCH-group).

Table 3 Characteristics of non-FCH control subjects stratified by the 90th percentile of plasma RLPc concentration.

	Non-FCH control subjects (n = 448)	
	RLPc < 90th (n = 400)	RLPc ≥ 90th (n = 48)
	Mean (95% CI)	Mean (95% CI)
Age (years)	38.8 (36.8 - 40.9) †	47.4 (42.6 - 52.1)
Cardiovascular disease *	19 (4.8%)	5 (10.4%)
Body mass index (kg/m^2)	24.1 (23.7 - 24.6) †	26.0 (24.9 - 27.1)
Waist/hip ratio	0.82 (0.82 - 0.83) †	0.89 (0.86 - 0.91)
Systolic blood pressure (mmHg)	128.2 (126.2 - 130.2) †	134.1 (132.1 - 136.1)
Diastolic blood pressure (mmHg)	80.0 (78.8 - 81.3) †	84.7 (81.9 - 87.5)
Total cholesterol (mmol/L)	4.84 (4.74 - 4.95) †	5.30 (5.03 - 5.56)
Triglycerides (mmol/L)	0.99 (0.94 - 1.03) †	1.94 (1.73 - 2.18)
VLDL-cholesterol (mmol/L)	0.31 (0.29 - 0.34) †	0.77 (0.65 - 0.91)
VLDL-triglycerides (mmol/L)	0.56 (0.52 - 0.60) †	1.37 (1.16 - 1.63)
HDL-cholesterol (mmol/L)	1.26 (1.23 - 1.30) †	0.97 (0.88 - 1.06)
LDL-cholesterol (mmol/L)	3.22 (3.13 - 3.31)	3.46 (3.21 - 3.70)
Apolipoprotein B (mg/L)	947 (926 - 968) †	1117 (1058 - 1176)
K-value	0.07 (0.05 - 0.10) †	-0.16 (-0.21 - -0.10)
non-HDL-cholesterol (mmol/L)	3.58 (3.48 - 3.68) †	4.32 (4.06 - 4.59)
Insulin (mU/L)	8.98 (8.39 - 9.60)	10.22 (8.62 - 12.12)
Glucose (mmol/L)	4.95 (4.89 - 5.01) †	5.34 (5.18 - 5.50)
HOMA-index	1.97 (1.83 - 2.12)	2.39 (1.99 - 2.87)
RLPc (mmol/L)	0.23 (0.22 - 0.24) †	0.45 (0.42 - 0.48)

*Values are means with 95% confidence intervals except as indicated; *, presented are number (%); FCH, familial combined hyperlipidemia; 90th percentile of RLPc (0.36 mmol/L) is calculated from controls without CVD and standardized for gender; VLDL-cholesterol, very low-density lipoprotein cholesterol; VLDL-triglycerides, very low-density lipoprotein triglycerides; K-value, a value ≤ -0.1 prepresents the presence of small dense LDL; HDL-cholesterol, high-density lipoprotein cholesterol; LDL-cholesterol, low-density lipoprotein cholesterol; HOMA-index, homeostasis model assessment-index; †, p-value < 0.05, compared to subjects with RLPc ≥ 90t^{h} percentile.*

Table 4 Correlation of plasma RLPc level with anthropometric and biochemical parameters in patients with familial combined hyperlipidemia (FCH) and in the non-FCH control group.

	FCH patients (n = 134)	Non-FCH controls (n = 448)
Age (years)	0.19 *	0.33 †
Cardiovascular disease	0.07	0.20 †
Body mass index (kg/m^2)	0.20 *	0.35 †
Waist/hip ratio	0.34 *	0.40 †
Systolic blood pressure (mmHg)	0.13	0.28 †
Diastolic blood pressure (mmHg)	0.27 *	0.30 †
Total cholesterol (mmol/L)	0.20 *	0.45 †
Triglycerides (mmol/L)	0.76 †	0.67 †
VLDL-cholesterol (mmol/L)	0.78 †	0.68 †
VLDL-trglycerides (mmol/L)	0.73 †	0.65 †
HDL-cholesterol (mmol/L)	0.37 †	-0.39 †
LDL-cholesterol (mmol/L)	-0.25 *	0.40 †
Apolipoprotein B (mg/L)	0.12	0.55 †
K-value	-0.46 †	-0.37 †
Non-HDL-cholesterol (mmol/L)	0.29 †	0.57 †
Insulin (mU/L)	0.05	0.19 †
Glucose (mmol/L)	0.33 †	0.33 †
HOMA-index	0.13	0.23 †

*Values are pearson correlation coefficients; VLDL-cholesterol, very low-density lipoprotein cholesterol; VLDL-triglycerides, very low-density lipoprotein triglycerides; K-value, a value ≤ -0.1 prepresents the presence of small dense LDL; HDL-cholesterol, high-density lipoprotein cholesterol; LDL-cholesterol, low-density lipoprotein cholesterol; HOMA-index, homeostasis model assessment-index; *, p-value < 0.05; †, p-value < 0.001.*

Plasma RLPc levels and CVD

In univariate analysis, a plasma RLPc concentration above the standardized 90th percentile was associated with an increased risk for CVD in the non-FCH control group (OR = 2.38 [0.87 – 6.55]) and in FCH subjects (OR = 2.14 [0.75-6.12], reaching statistical significance when the two groups were combined (OR 3.55 [1.97 - 6.39] (Table 5, model I). When putting this in a model together with nonlipid risk factors including age, gender, smoking habits, HOMA, BMI, WHR and blood pressure, plasma RLPc concentration stayed an independent contributor to the prediction of CVD with an OR of 2.18 [1.02 - 4.66] for the total group. Both plasma levels of triglycerides and non-HDLc were also predictors of prevalent CVD in univariate analysis in both FCH and the control group as indicated in table 5 (model II for triglycerides and model III for non-HDLc). The independence of RLPc as a predictor of CVD

when including other lipid variables strongly correlated with RLPc, was explored in model A and B, as presentend in table 5. When putting triglyceride levels and RLPc levels together in one model to predict prevalent CVD (Model A, Table 5), the relative predictive power of RLPc levels for CVD was higher in the FCH group whereas triglycerides contribute more to the prediction of CVD in the control group. For the total group, both plasma RLPc and triglycerides levels contributed independently to the risk of CVD. Adding non-HDLc to the model (model B, table 5) showed that non-HDLc had the best predictive power for CVD in both the FCH and control group.

Table 5 Odds ratios for cardiovascular disease associated with elevated plasma levels of remnant-like particles cholesterol (RLPc), triglycerides and non-HDL cholesterol above the 90th percentile in patients with familial combined hyperlipidemia and non-FCH control subjects.

	FCH patients	Non-FCH controls	Total group
Univariate analyses for			
I RLPc	2.14 [0.75 - 6.12]	2.38 [0.87 - 6.55]	3.55 [1.97 - 6.39]
II TG	1.44 [0.59 - 3.47]	6.27 [1.64 - 24.0]	4.63 [2.64 - 8.12]
III Non-HDLc	3.14 [1.10 - 8.99]	3.59 [1.42 - 9.08]	5.66 [3.18 - 10.1]
Multivariate analyses model A			
RLPc	2.20 [0.74 - 6.50]	1.76 [0.58 - 5.31]	2.35 [1.15 - 4.83]
TG	0.90 [0.33 - 2.44]	4.51 [1.07 - 19.05]	2.14 [1.02 - 4.49]
Multivariate analyses model B			
RLPc	1.61 [0.50 - 5.22]	1.50 [0.49 - 4.62]	1.50 [0.69 - 3.30]
TG	1.20 [0.42 - 3.47]	6.37 [1.45 - 28.04]	1.85 [0.86 - 3.99]
Non-HDLc	2.47 [0.75 - 8.13]	4.14 [1.58 - 10.86]	3.15 [1.61 - 6.19]

Values are presented as odds ratios with 95% confidence intervals (OR [95% CI]); FCH patients, patients with familial combined hyperlipidemia, including 24 patients with cardiovascular disease (CVD) and 110 patients without CVD; Non-FCH controls, including 24 subjects with CVD and 424 subjects without CVD; RLPc, remnant-like particles cholesterol; TG, triglyceride levels; non-HDL-cholesterol, low-density lipoprotein cholesterol and very-low density lipoprotein cholesterol levels.

Discussion

In this study we show that patients with FCH have two-fold elevated plasma RLPc levels compared to their normolipidemic relatives and spouses. In addition, high RLPc levels are associated with an 18-times increased risk for FCH. Therefore, assessment of plasma levels of RLPc completes the atherogenic lipid and lipoprotein profile in FCH and may thus constitute an additional marker for the identification of patients with FCH.

Previous studies have demonstrated that lipoprotein metabolism in FCH patients is disturbed, leading to remnant accumulation in the circulation, using different methodologies to isolate lipoprotein remnants [93, 269]. Only one other study has reported plasma RLPc levels in FCH [269].

In this small study, 12 male FCH patients had a sixfold increased plasma RLPc concentration (1.54 ± 1.84 mmol/l versus 0.24 ± 0.05 (n = 14) in controls) whereas the plasma RLPc concentration was only two-fold higer in female FCH patients (n = 11) as compared to controls (n = 13) (0.44 ± 0.42 versus 0.17 ± 0.04 mmol/l) [269]. This is most likely the result of higher triglyceride levels compared to our population (TG 5.4 ± 5.2 mmol/l versus TG 2.79 ± 1.6 mmol/l).

We show that men have higher RLPc levels compared to women in both FCH patients and controls. In addition, plasma RLPc levels increased with age in both males and females, confirming earlier reports in other populations [261, 270].

In our non-FCH control group, elevated plasma RLPc levels are associated with obesity, insulin resistance and an atherogenic lipid and lipoprotein profile characterized by elevated levels of plasma total cholesterol, triglycerides, apolipoprotein B, decreased levels of plasma HDLc, and more sdLDL. Similar associations have been reported previously [271, 272]. We show that the major determinant of fasting RLPc were gender, plasma triglyceride level and non-HDL-cholesterol accounting for 72% of the variance in fasting RLPc levels.

Strikingly, in the FCH group no significant correlation of RLPc levels with apoB levels and even a negative correlation with LDL-cholesterol was observed. This is most likely explained by the fact that RLPs are highly heterogenous in size and composition; in normolipidemic subjects RLPs are enriched in cholesterol and its plasma concentration was correlated with plasma LDL cholesterol concentration. However, when plasma triglyceride levels increase, as in FCH, then the elution profile of RLP is shifted toward a larger-size particle, similar to that of a larger VLDL subfraction (VLDL1). So, the TG-rich component of RLPc is responsible for the increased RLPc concentration associated with hypertriglyceridemia as plasma triglycerides are then the major determinant of the size and composition of RLPs. Indeed the major determinant of plasma RLPc levels among FCH patients were plasma triglyceride levels.

The importance of plasma TG levels as an independent risk factor for CVD was recognized after the publication of a meta analysis by Austin et al. showing that plasma triglyceride levels predict relative risk for CVD mortality in relatives of FCH patients [8]. Epidemiological data from Framingham study already showed that plasma TG is an important risk indicator of CVD in women [192]. Additional evidence was obtained from by Yarnell et al. in a ten year follow up study [273] and confirmed in several other studies [274]. However, triglycerides are not significantly and independently associated with CVD in most prospective studies [275]. The difficulty with using plasma or serum triglyceride measurements to assess CVD risk may stem from the variability within the subspecies of TRL particles, as well as the inverse association with HDL cholesterol. It is not possible to distinguish TRL that are atherogenic from those that are non-atherogenic simply by measuring plasma triglyceride levels. Therefore, in the present study we use a specific assay to measure atherogenic RLP as plasma RLPc levels have been associated with CVD and its risk factors. First, we show that increased plasma RLPc levels are associated with an increased risk for CVD in non-FCH subjects and in FCH patients, independently of nonlipid cardiovascular risk factors including age, gender, smoking, obesity, insulin resistance and blood pressure. Most importantly, we show that in the total group, RLPc levels conferred additional risk of CVD even when plasma TG levels are taken into account; subjects with RLPc levels above the 90th percentile have a 2.4 times increased risk of prevalent CVD, independent of plasma TG levels. Indeed, several clinical studies demonstrated that plasma RLPc offered independent assessment for CVD

risk in addition to TG [276, 277]. Furthermore we show that in patients with FCH the plasma RLPc level is a better predictor of CVD than plasma triglycerides; these results support the concept of identifying more atherogenic remnant particles by measuring RLPc instead of TG levels. However, in the control group, the plasma triglyceride level is a better predictor for prevalent CVD than the RLPc level. This could be related to the particle heterogeneity in RLP which could affect the ability of RLPc concentration to predict atherosclerotic risk. Therefore, in controls with low plasma TG levels, plasma RLPc may not have the same clinical significance as they do in patients with FCH with hypertriglyceridemia. Indeed, recently, in the Honolulu Heart Study, including normotriglyceridemic healthy men, RLPc levels did not provide additional information about risk of CVD over and above TG levels, whereas the association between RLPc and CVD was significant for the group with elevated TG levels [278]. Further studies should examine whether difference in RLP composition may reflect a different risk on CVD in different populations.

So, RLPc levels predict CVD independent of plasma TG level, however, in our population the addition of non-HDL cholesterol into the model resulted in the loss of RLPc as an independent predictor of CVD. It remains, however, to be studied in large epidemiological studies to what extend the measurement of plasma RLPc provides additional knowledge for the treatment goals of the individual patients.

In summary, patients with FCH have two-fold elevated plasma RLPc levels. RLPc levels predict prevalent CVD independent of nonlipid cardiovascular risk factors. Most importantly, RLPc levels predicted prevalent CVD independently of plasma triglyceride levels, however, RLPc levels did not provide additional information about risk of CVD over and above non-HDL cholesterol levels in FCH.

Chapter 8

Elevated leptin levels in subjects with familial combined hyperlipidemia are associated with the increased risk for CVD

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Abstract

Background Familial Combined Hyperlipidemia (FCH) is characterized by hypercholesterolemia and/or hypertriglyceridemia and is associated with premature cardiovascular disease (CVD). Other features of FCH are obesity and insulin resistance. Serum leptin levels have been associated with obesity, insulin resistance and CVD. The aim of this study was to determine whether increased leptin levels contribute to the FCH phenotype and its increased risk for CVD. **Methods** The study population comprised 644 subjects, including 158 FCH patients. Leptin levels were determined, using a commercially available ELISA. **Results** For both males and females, the mean leptin level (ng/ml) was higher in FCH patients compared to normolipidemic relatives and spouses. However, after standardization for BMI and insulin resistance, these differences disappeared. The 90th percentile of the leptin level, standardized for BMI, insulin resistance and gender, was associated with an increased risk for CVD in FCH patients (odds ratio = 2.9, 95 % CI = 1.1 - 8.0) and in non-FCH subjects (odds ratio = 3.4, 95 % CI = 1.3 - 9.0). The overall increased risk for CVD, associated with a leptin level > 90th percentile, was 3.3 (95 % CI = 1.7 - 6.4). **Conclusion** We conclude that in patients with FCH, leptin levels are increased in proportion to their higher BMI and the presence of insulin resistance. These increased leptin levels are associated with an increased risk for CVD both in FCH patients and non-FCH subjects, independent of BMI, insulin resistance and gender.

Introduction

Familial combined hyperlipidemia (FCH) is the most common genetic hyperlipidemia in humans and affects 1 - 3 % of the general population. It is characterized by multiple lipoprotein phenotypes and is strongly associated with premature cardiovascular disease (CVD). Of the survivors of a premature myocardial infarction, up to 20 % are affected with FCH [4]. FCH is characterized by hypercholesterolemia and/or hypertriglyceridemia. Other phenotypes of FCH are elevated levels of apolipoprotein B (apoB) and low-density lipoprotein cholesterol (LDLc), decreased levels of high-density lipoprotein cholesterol (HDLc) and the presence of small dense LDL (sdLDL). In addition, FCH is associated with obesity and insulin resistance [9].

Obesity results in an increase in number and size of adipocytes. These adipocytes secrete leptin, a hormone, which is increased in obese subjects [279]. Leptin is involved in the regulation of the energy expenditure and appetite via hypothalamic receptors [104]. An increase of leptin level will lead to more energy expenditure and less appetite in normal persons via the hypothalamus. Obese persons have increased levels of leptin, but these high levels appear to fail to influence energy intake or expenditure to restore fat mass to normal. It is, therefore, believed that obesity is a state of leptin resistance.

Leptin has direct effects on insulin secretion by inhibiting insulin gene transcription [280] and insulin secretion [281]. On the other hand, insulin increases leptin production indirectly via its effects to increase glucose utilization and oxidative glucose metabolism in adipocytes at the transcriptional level [281, 282]. Thus, high leptin levels are associated with insulin resistance [105].

Both obesity and insulin resistance are characteristics of FCH, and therefore, it is likely that leptin is elevated in persons with FCH. Furthermore, we hypothesize that leptin could be a marker for a disturbed adipocyte metabolism, one of the mechanisms proposed to play a role in the pathophysiology of FCH [9].

So far, only two small studies have studied the relationship between leptin concentrations and FCH, with conflicting results [247, 248]. Jacobson et al. found a significantly elevated leptin levels in young females with FCH [248], while Haluzik et al. observed no differences in a group of males with and without FCH [247].

The aim of this study was to investigate whether leptin levels in our large cohort of well-defined male and female patients with FCH were elevated, independent of their BMI, indicating that adipose tissue metabolism is disturbed in FCH. The second objective was to investigate whether leptin levels contribute to the increased risk for CVD in FCH.

Methods

Study population

The study population existed of 37 families, comprising 644 subjects, of whom 158 subjects were diagnosed as FCH patients [16, 17]. The normolipidemic relatives (n = 389) and unaffected spouses of both FCH patients and normolipidemic relatives (n = 90), also included in this study population, served as two independent reference groups. We separated the normolipidemic relatives and the spouses into two different reference groups, because of the similar genetic background of the relatives with the FCH patients. The spouses were included in this study

as a separate control group, because they share environment, but are not genetically related to FCH. Seven subjects were not diagnosed because of missing values. For HOMA-index data were available for 143 FCH patients, 346 normolipidemic relatives and 87 spouses, due to technical errors in the insulin measurement. Information about the presence of CVD was gathered by personal interview and physical examination performed by the clinical investigator. CVD was defined by angina pectoris (AP), myocardial infarction (MI), stroke, peripheral vascular disease or vascular surgery. When the presence of CVD was suspected by the clinical investigator, further details and confirmation of the diagnosis were sought from the participants general practitioner, plus, if considered necessary from any relevant hospital records. In our study population 56 subjects were identified with CVD, including 26 subjects with AP, 25 subjects with previous MI, 10 subjects with peripheral vascular disease, 7 subjects with stroke and 23 subjects experienced vascular surgery. In total, 45 % (n = 25) of these subjects were diagnosed CVD based on the presence of two or more manifestations of CVD. Only 21 % of the subjects had only AP, so 79 % of our subjects had well documented CVD. After withdrawal of lipid-lowering medication for 4 weeks and an overnight fast, blood was drawn by venipuncture. The ethical committee of the Radboud University Nijmegen Medical Centre approved the study protocol and the procedures followed were in accordance with institutional guidelines. All subjects gave informed consent.

The diagnosis FCH was based on absolute apoB levels in combination with triglyceride and total cholesterol levels adjusted for age and gender, using a nomogram, as recently described [17].

Body mass index (BMI) was calculated as body weight (in kilograms) divided by the square of height (in meters).

Table 1 Characteristics of patients with familial combined hyperlipidemia, normolipidemic relatives and spouses

	FCH patients (n = 158)	NL relatives (n = 389)	Spouses (n = 90)
Gender (males) *	76 (48.1%)	175 (45.0%)	44 (48.9%)
Age (years)	47.1 (16.3) ‡ §	37.8 (17.9) ¥	52.3 (15.9)
Cardiovascular disease *	32 (20.3%) ‡ §	21 (5.4%)	3 93.3%)
Body mass index (kg/m^2)	27.3 (4.4) ‡	24.2 (5.2) ¥	26.3 (4.2)
Total cholesterol (mmol/L)	6.5 (1.1) ‡ §	4.9 (1.2) ¥	5.2 (1.0)
Triglycerides (mmol/L) †	2.8 (1.0) ‡ §	1.1 (1.0)	1.1 (1.0)
Apolipoprotein B (mg/L)	1371 (236) ‡ §	961 (249)	1001 (233)
HOMA-index †	2.9 (1.1) ‡ §	2.0 (1.0)	2.2 (1.1)

*Values are presented as mean (SD); *, presented are number (%); †, presented are geometric mean (geometric SD); FCH, familial combined hyperlipidemia; NL relatives, normolipidemic relatives; HOMA-index, homeostasis model assessment-index (missing values for HOMA-index in 15 FCH patients, 43 normolipidemic relatives and 3 spouses; ‡, p-value based on GEE < 0.05, compared to normolipidemic relatives; †, p-value based on GEE < 0.05, compared to spouses; ¥, p-value based on GEE < 0.05, compared to spouses.*

Biochemical analyses

Plasma total cholesterol (TC) and total triglycerides (TG) were determined by enzymatic, commercially available reagents (Boehringer-Mannheim, Germany, catalog No. 237574 and Sera Pak, Miles, Belgium, catalog No. 6639, respectively). Total plasma apoB concentrations were determined by immunonephelometry, as recently described in detail [119]. Glucose concentrations were measured in duplicate using the oxidation method (Beckman®, Glucose Analyser2, Beckman Instruments Inc., Fullerton, CA 92634, USA). Plasma insulin was assessed by radioimmunoassay (RIA) using 125I-labeled human insulin and anti-human insulin antiserum raised in guinea pig. Bound and free tracer were separated by sheep anti-guinea pig antiserum; human insulin (Novo Biolabs, Copenhagen, Denmark) was used for standards. The inter-assay coefficient of variation for the insulin measurement was 10.3% at a level of 20.7 mU/l [170]. Insulin resistance was assessed by the Homeostasis model assessment (HOMA). The HOMA-index was calculated from the fasting concentrations of insulin and glucose using the following formula: HOMA-index = fasting serum insulin (μU/ml) x fasting plasma glucose (mmol/L)/22.5 [171].

Serum leptin levels

For measuring leptin levels, serum samples were assayed in duplicate using enzyme-linked immunosorbent assay (R&D Systems, Minneapolis: Elisa Development System; Duoset® Human Leptin, Catalog No. DY398). This assay measured the total amount of leptin present in a sample, independent of the presence of leptin-binding proteins. Inter- and intra-assay coefficients of variance were 6.4 and 3.5%, respectively. The detection limit was 0.1 ng/ml.

Statistical analyses

As it is well documented that, at a given BMI, females have a higher leptin level than males [283, 284], all analyses were stratified or standardized for gender. Variables with a skewed distribution, including leptin levels, triglyceride levels and the HOMA-index, were logarithmically transformed before analysis.

Descriptive statistics, presented separately for FCH patients, normolipidemic relatives and spouses, are expressed as mean with standard deviation (SD), or geomatric mean with geometric SD for logarithmically transformed variables. Parametric Pearson correlation was used for correlation analysis. Differences in characteristics and leptin levels between subjects with FCH, normolipidemic relatives and spouses were tested by means of generalized estimating equations (GEE) because of possible correlated values within families. In the models used for the GEE analyses, with leptin levels and the other descriptive statistics as the dependent and FCH diagnosis as the independent variable, the link function 'canonical' was used and an equal-correlation population-averaged model was used as the working correlation matrix. Also the odds ratios (OR), as an estimator of relative risk (RR), were calculated with logistic GEE. Within the model used for logistic GEE analysis, leptin represented the independent and CVD the dependent variable. To standardize the leptin level for BMI, HOMA-index and gender, for calculation of the 90th percentile, a GEE multiple linear regression model, with leptin as the dependent and BMI, HOMA-index and gender as the independent variable was used to calculate the predicted leptin level for each person. Subsequently, the predicted leptin level was substracted from the measured leptin level, to which the overall mean value of leptin levels was added, resulting in the value of the leptin levels standardized for these variables. The 90th percentile of the leptin levels,

standardized for gender, BMI and HOMA-index, is based on the group of non-FCH subjects without CVD, including spouses. Differences were considered statistically significant at p-values < 0.05. All analyses were computed using the STATA 8.0 software.

Results

Subject characteristics

Descriptive statistics of anthropometric and metabolic characteristics of the study population are presented in table 1. FCH patients are older than normolipidemic relatives, but younger than the spouses. Evident is the higher prevalence of CVD in patients with FCH, compared to normolipidemic relatives and the spouses. The mean BMI of patients with FCH is significantly higher compared to normolipidemic relatives. Compared to normolipidemic relatives and spouses, FCH patients have significant higher levels of total cholesterol, triglycerides and apoB and are more insulin resistant, as reflected by a higher HOMA-index. Normolipidemic relatives are younger, have a lower BMI and have lower total cholesterol levels compared to the spouses (Table 1).

Leptin levels and FCH

Both male and female FCH patients have higher mean leptin level compared to normolipidemic relatives and spouses, only not reaching statistical significance in female FCH patients versus female spouses (Table 2).

Table 2 Mean serum leptin levels in patients with familial combined hyperlipidemia, normolipidemic relatives and spouses.

Leptin levels (ng/ml)		FCH patients (n = 158 (48.1%)) *	NL relatives (n = 389 (45.0%)) *	Spouses (n = 90 44.3%)) *
Crude	Males	8.9 [7.1 - 11.0] † ‡	3.9 [34 - 4.5] §	5.5 [4.2 - 7.3]
	Females	25.8 [21.7 - 30.7] †	18.4 [16.3 - 20.8] §	23.3 [18.6 - 29.2]
Standardized for BMI	Males	6.2 [5.3 - 7.3] † ‡	4.9 [4.4 - 5.4]	4.4 [3.6 - 5.4]
	Females	20.3 [18.0 - 22.9]	20.3 [18.8 - 22.0]	21.7 [18.5 - 25.4]
Standardized for BMI and HOMA-index	Males	5.9 [5.1 - 7.0]	5.0 [4.5 - 5.6]	4.8 [3.9 - 5.8]
	Females	19.1 [16.9 - 21.6]	20.7 [19.1 - 22.5]	21.9 [18.8 - 25.5]

*Values are presented as means [95% confidence interval] (calculated by GEE analyses); FCH, familial combined hyperlipidemia; NL relatives, normolipidemic relatives; BMI, body mass index; HOMA-index, homeostatis model assessment-index; *, percentage males; †, p-value < 0.05 , compared to normolipidemic relatives; ‡, p-value < 0.05, compared to spouses; §, p-value < 0.05, compared to spouses.*

Leptin levels show a strong significant correlation with BMI in both males (r = 0.76) and females (r = 0.73). To determine whether the association of FCH with leptin concentration is dependent on BMI, we standardized the leptin concentration for BMI. After standardization,

no significant differences in leptin levels among females with FCH, normolipidemic relatives and spouses are found. However, male FCH patients still show significant higher leptin levels compared to normolipidemic relatives and spouses (Table 2).

Patients with FCH are not only more obese than their normolipidemic relatives but they are also more insulin resistant. This is not completely contributable to their obesity, because after standardization of the HOMA-index for BMI, the differences in insulin resistance between patients with FCH and normolipidemic relatives and spouses remain significant in our FCH population [9]. Because leptin levels show a strong correlation with the HOMA-index for both males (r = 0.60) and females (r = 0.56), we also standardized leptin levels for the HOMA-index. After standardization of leptin levels for both BMI and HOMA-index, no significant differences in leptin levels are found among FCH patients, normolipidemic relatives and spouses, although among males, a trend can be observed (Table 2).

Leptin concentration in a multiple linear regression model

Multiple linear regression analyses showed that in the total study population, the variation in leptin concentration could be explained for approximately 70% by the variables gender and BMI. When including the HOMA-index in the model, 73 % of the variation in leptin concentration could be explained.

Leptin concentration and CVD

Next, we determined whether elevated serum leptin levels (standardized for gender, BMI and HOMA-index) are associated with an increased risk for CVD. A serum leptin concentration above the standardized 90th percentile of leptin was associated with an increased risk for CVD in FCH patients (OR = 2.9, 95% CI = 1.1 - 8.0) and in non-FCH subjects (OR = 3.4, 95% CI = 1.3 - 9.0). The overall OR for the total group is 3.3 with confidence intervals ranging from 1.7 to 6.4 (Table 3). When performing the analysis with leptin levels as a continuous variable, we also obtained a significant increased risk for CVD to be associated with a 1 SD change in leptin levels (OR = 1.6, 95% CI = 1.2 - 2.2).

Table 3 Odds ratios for cardiovascular disease associated with serum leptin levels above the 90th percentile.

	FCH			Non-FCH [a]			Total group
Leptin	CVD+ (n = 29)	CVD- (n = 110)	OR [95% CI]	CVD+ (n = 29)	CVD- (n = 110)	OR [95% CI]	OR [95% CI]
< 90th	21 (72.4%)	98 (89.1%)	Reference	16 (72.7%)	368 (90.2%)	Reference	Reference
> 90th	8 (27.6%)	12 (10.9%)	2.9 [1.1 - 8.0]	6 (27.3%)	40 (9.8%)	3.4 [1.3 - 9.0]	3.3 [1.7 - 6.4]

Values presented are total number (%) and the odds ratio's (calculated by logistic GEE analyses); serum leptin levels (ng/ml) are standardized for BMI, HOMA-index and gender;FCH, familial combined hyperlipidemia; CVD+, subjects with cardiovascular disease; CVD-, subjects without cardiovascular disease; a, non-FCH subjects include both normolipidemic relatives and spouses; < 90th, leptin levels < 90th percentile; > 90th, leptin levels > 90th percentile;

Discussion

In the present study, we report higher leptin levels in both male and female patients with FCH compared to their normolipidemic relatives and spouses. The extent of increase in leptin levels is in proportion to the degree of overweight and insulin resistance in these FCH patients. In females, the increased leptin levels were completely attributable to BMI, whereas in male FCH patients increased leptin levels were only partially attributable to overweight. However, the remaining difference in leptin levels in male patients with FCH seemed related to insulin resistance, since after correction for HOMA-index, leptin levels were no longer significantly different. So in both male and female FCH patients, leptin is not representing a defect in adipose tissue metabolism. The increased leptin levels in FCH were found to be associated with an increased risk for CVD, independent of gender, BMI and insulin resistance.

So far, only two small studies investigated the relation of leptin concentrations and FCH. Haluzik et al. did not find any difference in the leptin concentration between male subjects with familial combined hyperlipidemia and controls [247]. Another study, performed in a small group of women by Jacobson et al., did show an increased concentration of leptin in females with FCH [248]. However, in this study they used subjects with familial hypercholesterolemia as a reference population and no healthy subjects. Our large study population, with both males and females, confirmed at first the results of Jacobson et al. but after standardization for BMI and insulin resistance, we report no difference in leptin levels of female FCH patients compared to their normolipidemic relatives and spouses, and only a non-significant trend for male FCH patients.

Because we did not find a relation between FCH and leptin levels, independent of BMI and insulin resistance, adipose tissue metabolism with respect to leptin seems not disturbed in FCH. However, we cannot rule out the possibility of a disturbed adipose tissue metabolism in the pathophysiology of FCH because leptin is just one of the many markers of adipose tissue metabolism. Other markers of the adipose tissue, such as adiponectin and resistin [285, 286], need to be investigated before we can judge on involvement of disturbed adipose tissue metabolism in the pathophysiology of FCH indefinitely.

The rate of leptin production is mainly determined by obesity [279, 287-289], but there still is some inter-individual variability in plasma leptin concentration that is independent of body fatness. In our study, 70% of the individual variability in leptin levels could be accounted for by BMI and gender. However, after standardization of leptin levels for BMI only, we still found an association between FCH and leptin levels in men, not in women. Several studies have reported that leptin levels are also influenced by insulin resistance [290-294]. Moreover, from literature, it is known that in men, but not in women, leptin levels correlate negatively with maximum glucose uptake rates and insulin sensitivity is related to serum leptin independently of percent body fat [295]. Indeed, in our population we observed a strong correlation between leptin levels and the HOMA-index, as a measure of insulin resistance. This indicates that leptin levels should not only be standardized for BMI, but also for HOMA-index, as a measure of insulin resistance.

From literature it is known that leptin is an independent risk factor for CVD [289, 292]. So far, the relevance of high leptin concentrations as a risk factor for CVD associated with FCH has not been investigated. In the present study, we show that FCH subjects have elevated leptin levels, explained by the increased BMI and the presence of insulin resistance. Still it is possible that the elevated leptin levels contribute to the increased risk on CVD in FCH. Indeed, we

demonstrate that leptin levels above the 90th percentile are associated with an increased risk for CVD. Similar results were found among non-FCH subjects. This increased risk for CVD was not attributable to differences in gender, BMI and state of insulin resistance because the leptin level was standardized for these variables. Wallace et al. found an increased risk of approximately two-fold on CVD in the highest two quintiles of leptin concentration compared to the lowest quintile [289] in a prospective, nested, case control study of hypercholesterolemic men. Though our groups were not large enough to assess quintiles, the risk found for the 90th percentile in our study was comparable with the risks found for the highest two quintiles (60th and 80th percentile) by Wallace et al. [289]. Moreover, when performing the analyses with leptin as a continuous variable, we also obtained an increased risk for CVD to be associated with higher leptin levels. In another study by Couillard et al., no significant differences in serum leptin concentrations were observed between men with and without ischemic heart disease [287]. This might be explained by the fact that obesity itself was not a risk factor for ischemic heart disease in this study.

Several mechanisms are hypothesized to explain how leptin may increase the risk for cardiovascular events. The fact that leptin and its receptor are expressed in atherosclerotic plaques [296, 297] indicates that leptin may be involved in the development of CVD. Leptin has many potentially atherogenic effects, like stimulation of endothelial production of pro-atherosclerotic endothelin-1 [298], induction of migration and proliferation of vascular smooth muscle cells [299], stimulation of inflammatory cells [300] and induction of calcification of vascular cells [296]. So, leptin is involved in the process of atherosclerosis, which can also be concluded from our results that increased leptin levels are associated with an increased risk for CVD

In summary, serum leptin levels are increased in patients with FCH in proportion to their obesity and state of insulin resistance. Elevated leptin levels are an independent risk factor for CVD in both FCH patients and in healthy controls.

Chapter 9

Gln223Arg polymorphism in the leptin receptor is associated with familial combined hyperlipidemia

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Abstract

Background Familial combined hyperlipidemia (FCH) is characterized by elevated levels of total cholesterol (TC), triglycerides (TG) and apolipoprotein B (apoB) and is associated with premature cardiovascular disease (CVD). Other features of FCH are obesity and insulin resistance. Serum leptin levels have also been associated with obesity, insulin resistance and atherosclerosis. Leptin exerts its effect through the leptin receptor (LEPR). The aim of this study is to determine whether the Gln223Arg polymorphism in the *LEPR* gene contributes to FCH and its associated phenotypes. **Methods** The study population consists of 37 families, comprising 644 subjects, of whom 158 subjects were diagnosed as FCH. The FCH diagnosis was based on plasma TC and TG levels, adjusted for age and gender, and absolute apoB levels, according to our recently published nomogram. The Gln223Arg polymorphism was studied by PCR-restriction fragment length polymorphism. **Results** Carriers of one or two Arg alleles had an increased risk of FCH, compared to subjects homozygous for the Gln allele (OR = 1.6 [95% CI 1.0 – 2.4]). A difference in high-density lipoprotein cholesterol (HDLc) levels was present between carriers and non-carriers of an Arg allele, 1.21 vs. 1.28 mmol/L, respectively (p = 0.04), but no differences in obesity, insulin resistance and other lipid parameters were found. Conclusion The Gln223Arg polymorphism in the *LEPR* gene is associated with FCH, which is supported by a significant association between HDLc levels and the *LEPR* gene.

Introduction

Familial combined hyperlipidemia (FCH) is the most common genetic hyperlipidemia in humans, with a prevalence of 5.7% in the general adult population [10]. FCH is strongly associated with premature cardiovascular disease (CVD), reflected by the fact that up to 20% of the survivors of a premature myocardial infarction are affected with FCH [8]. FCH is characterized by hypercholesterolemia, hypertriglyceridemia and hyperapobetalipoproteinemia (hyperapoB). Other phenotypes of FCH are decreased levels of high-density lipoprotein cholesterol (HDLc) and the presence of small dense low-density lipoprotein (sdLDL) [13, 18, 116]. In addition, patients with FCH are more obese and insulin resistant [9, 93].

At present, the complex genetics of FCH, is still not fully understood [209, 301]. The fact that FCH is a genetic heterogeneous disease complicates the identification of major contributing genes. Based on the pathophysiology of FCH, several candidate genes have been evaluated, showing an association of FCH with lipoprotein lipase (*LPL*) [79], hepatic lipase (*HL*) [73], *APOA1/C3/A4* [50] and *APOE* [69].

Linkage analyses have been performed in several study populations to unravel the genetic defects causing FCH. Several regions of linkage have been identified, including a region on chromosome 1q21-23 [26, 28, 30]. In this region, the upstream stimulatory factor 1 (*USF1*) gene has been suggested to be the gene associated with FCH [46]. Other regions of linkage identified for FCH have been reported on several other chromosomes, including 2p, 6q, 8p, 9p, 10p, 11p, 16q, 19q, 21q, [97] thereby underscoring the genetic heterogeneity of FCH. No other major gene, however, has been identified untill now.

In 2002, a genome scan in 18 Dutch families provided evidence that a locus on the human chromosome 1p31, harboring the leptin receptor (*LEPR*) gene, contributes to apolipoprotein B (apoB) levels in patients with FCH [38]. This region segregated with plasma leptin levels, adiposity and body weight within these FCH families [302]. In the Pima Indians, markers near the *LEPR* gene have also been associated with obesity-related phenotypes [303]. So, the *LEPR* gene is a potential candidate gene of FCH. The leptin receptor is responsible for transmission of the leptin signal in the hypothalamus to several signal transducers and transcription activators and is the major leptin-binding protein in blood [304, 305]. Leptin, mainly produced by the adipose tissue, is involved in the regulation of the energy expenditure and appetite via hypothalamic receptors [104, 306]. Obese individuals have increased levels of leptin, which appear to fail to restore fat mass to normal. It is therefore believed that obesity is a state of leptin resistance [307].The leptin receptor is a class I cytokine receptor and has several isoforms, one of which is the soluble leptin receptor present in blood. In lean subjects the largest part (60 – 98%) of leptin in blood is present in the bound form; however, in obese subjects the majority of leptin is unbound [308]. The leptin receptor is also present in peripheral tissues such as pancreatic beta cells, liver and skeletal muscle [309]. In these tissues, leptin is capable of stimulating lipid oxidation via the LEPR with subsequent increase in insulin sensitivity [309-311].

The human *LEPR* gene is located on chromosome 1p31 [104] and several single nucleotide polymorphisms (SNPs) have been described in this gene [312]. Two polymorphisms have already been described in a population of FCH patients, and were not associated with any feature of FCH38}. A third polymorphism in the *LEPR* gene is Gln223Arg, located within the region encoding the extracellular domain of the leptin receptor and therefore present in all isoforms of

the receptor. The amino-acid change caused by Gln223Arg is associated with a change in leptin-binding activity [313]. The Gln223Arg polymorphism has been associated with body mass index (BMI), fat mass, leptin levels, and systolic and diastolic blood pressure in some studies; [309, 312, 314] however, other studies did not find these associations [315-317].

The aim of this study was to investigate whether there is an association between the presence of the Arg allele defined by the Gln223Arg polymorphism in the LEPR gene and FCH and its associated phenotypes.

Methods

Subjects

The study population consisted of 37 families, comprising 644 subjects, of whom 158 subjects were diagnosed as FCH patients [16, 17]. The diagnosis FCH was based on total cholesterol (TC) and triglycerides (TG) levels adjusted for age and gender in combination with absolute apoB levels, according to our nomogram, as recently published [17]. The normolipidemic relatives (n = 390) and unaffected spouses of both the FCH patients and the normolipidemic relatives (n = 89), served as two independent reference groups, because of the similar genetic background of the relatives with the FCH patients. Seven subjects were not diagnosed because of missing values. Information about the presence of CVD was gathered by personal interview and physical examination performed by the clinical investigator. After withdrawal of lipid-lowering medication for 4 weeks and an overnight fast, blood was drawn by venipuncture. The ethical committee of the Radboud University Nijmegen Medical Centre approved the study protocol and the procedures followed were in accordance with institutional guidelines. All subjects gave informed consent.

BMI was calculated as body weight (in kilograms) divided by the square of height (in meters). Waist circumference was obtained with use of a standard cloth tape measure, measured in mm at the level of the umbilicus in the late exhalation phase while standing. Systolic and diastolic blood pressure were assessed twice with an automated blood pressure device (Dinamap; Critikon, Tampa, Fl, USA) in the right arm in a sitting position after a 5-minute rest period, mean blood pressures were used in our analyses.

Biochemical analyses

Plasma TC and TG were determined by enzymatic, commercially available reagents (Boehringer-Mannheim, Germany, catalog No. 237574 and Sera Pak, Miles, Belgium, catalog No. 6639, respectively). HDLc was determined by the polyethylene glycol 6000 method [267]. Total plasma apoB concentrations were determined by immunonephelometry [119]. LDL subfractions were separated by single spin density gradient ultracentrifugation. A continuous variable K represents the LDL subfraction profile of each individual. A negative K-value ($K \leq -0.1$) reflects a more dense LDL subfraction profile, and a positive K-value ($K > -0.1$) reflects a more buoyant profile [120, 147, 268]. Glucose concentrations were measured in duplicate using the oxidation method (Beckman®, Glucose Analyser 2, Beckman Instruments Inc., Fullerton, CA, USA). Plasma insulin concentrations were determined using a double antibody method [170]. Insulin resistance was assessed by homeostasis model assessment (HOMA), a continuous, quantitative parameter where higher values indicate a more insulin resistant state. The HOMA-index was calculated

from the fasting concentrations of insulin and glucose using the following formula: HOMA-index = fasting serum insulin (μU/ml) x fasting plasma glucose (mmol/L)/22.5 [171]. Leptin levels were measured in duplicate in serum samples using enzyme-linked immunosorbent assay (R&D Systems, Minneapolis: Elisa Development System; Duoset® Human Leptin, Catalog no. DY398) [318].

Table 1 Characteristics of patients with familial combined hyperlipidemia, normolipidemic relatives and spouses.

	FCH patients (n = 158)	NL relatives (n = 390)	Spouses (n = 89)
Gender (males) *	75 (47.5%)	175 (44.9%)	44 (439.4%)
Ager (years)	47.1 (16.3) ‡ §	37.7 (18.0) ¥	52.6 (15.9)
CVD *	32 (20.3%) ‡ §	21 (5.4%)	3 (3.4%)
BMI (kg/m^2)	27.3 (4.4) ‡	24.2 (5.3) ¥	26.4 (4.2)
Waist circumference (cm)	87.4 (14.2) ‡ §	77.6 (12.1) ¥	82.9 (11.3)
Systolic BP (mmHg)	136.4 (17.7) §	128.0 (20.1) ¥	134.3 (17.1)
Diastolic BP (mmHg)	85.2 (10.5) §	80.3 (12.1) ¥	83.6 (10.1)
TC (mmol/L)	6.5 (1.1) ‡ §	4.9 (1.2) ¥	5.2 (1.0)
TG (mmol/L) †	2.8 (1.6) ‡ §	1.1 (1.6)	1.1 (1.6)
ApoB (mg/L)	1372 (236) ‡ §	961 (249)	1001 (233)
HDLc (mmol/L)	0.97 (0.33) ‡ §	1.22 (0.36) ¥	1.29 (0.32)
K-value	-0.25 (0.24) ‡ §	0.04 (0.27)	0.06 (0.23)
HOMA-index †	2.9 (1.8) ‡ §	2.0 (2.0)	2.2 (1.8)
Leptin (ng/mL) male †	8.9 (0.4) ‡ §	3.9 (0.4) ¥	5.5 (0.4)
Leptin (ng/mL) female †	25.8 (0.4) ‡ §	18.4 (0.5) ¥	23.3 (0.4)

*Analyses are performed with GEE and results are presented as mean (sd); *, presented are number (%); †, presented are geometric mean (geometric SD); FCH, familial combined hyperlipidemia; NL relatives, normolipidemic relatives; CVD, cardiovascular disease; BMI, body mass index; systolic BP, systolic blood pressure; diastolic BP, diastolic blood pressure; TC, total cholesterol; TG, triglycerides; apoB, apolipoprotein B; K-value, a value below -0.1 represents the presence of small denxe low-density lipoprotein; HDLc, high-density lipoprotein cholesterol; HOMA-index, homeostasis model assessment-index; ‡, p-value < 0.05, compared to normolipidemic relatives; §, p-value < 0.05, compared to spouses; ¥, p-value < 0.05, compared to spouses.*

DNA extraction and genotyping

DNA was isolated from peripheral blood lymphocytes by a standard method. The Gln223Arg variant was genotyped by PCR-restriction fragment length polymorphism analysis. PCR was carried out in a total volume of 50 μl containing 50 ng of the forward primer 5'-GCCTAATCCAGTAT TTTCATATCTG-3' and 50 ng of the reverse primer 5'-GCCACTCTTAATACCCCCAGTAC-3', 200 μM each dNTP, 10 mM Tris-HCl (pH 8.30), 50 mM KCL, 1.5 mM MgCl2) and 1 unit Taq polymerase (Life Technologies). PCR conditions were: an initial denaturation step of 3 min

at 94°C, followed by 40 cycles of 94°C, 1 min (denaturation), 53.7°C, 1 min (annealing), 72°C, 30 s (extension), and a final extension of 7 min at 72°C. The PCR amplification resulted in a fragment of 416 bp, which was subjected to a MspI restriction enzyme analysis (New England Biolabs, Beverly, MA, USA) to detect the Arg variant; the Arg allele contains an MspI restriction site. Subsequent gel electrophoresis showed a fragment of 416 bp when the Gln allele was present and a fragment of 229 bp and 187 bp when the Arg allele was present.

Statistical analyses

Descriptive statistics, expressed as mean ± standard deviation (SD) or a geometric mean ± geometric SD, were presented for FCH patients, normolipidemic relatives and spouses. Variables with a skewed distribution, including HOMA-index, leptin and triglyceride levels, were logarithmically transformed. The frequencies of the genotypes were tested for Hardy-Weinberg equilibrium by chi-square analysis before the analyses. Differences in anthropometric and metabolic characteristics between the genotypes were tested by means of generalized estimating equations (GEE) because of possible correlated values within families. The odds ratio as an estimate of relative risk of FCH was calculated using logistic GEE. GEE analyses were used to adjust leptin levels for BMI. Differences were considered statistically significant at p-values < 0.05. All analyses described above were computed using the STATA 8.0 software (StataCorp LP, College station, Texas, USA).

Table 2 Risk on FCH associated with Gln223Arg polymorphism in the leptin receptor gene

	FCH patients, n (%)	Controls, n(%)	OR [95% CI]*
Gln223Gln	41 (26.0%)	171 (35.8%)	Reference
Gln223Arg/Arg223Arg	117 (74.0%)	307 (64.2%)	1.6 [1.0 - 2.4]

*Analyses are performed with GEE; FCH, familial combined hyperlipidemia; OR, odds ratio; *, OR represents the risk associated with the presence of the Arg allele in patients with FCH compared to controls, which include both the normolipidemic relatives and spouses.*

Results

Subject characteristics

Descriptive statistics of anthropometric and metabolic characteristics of the study population are presented in Table 1. FCH patients are on average older than normolipidemic relatives, but younger than spouses. Evident is the higher prevalence of CVD in patients with FCH, compared to normolipidemic relatives and spouses. The mean BMI and waist circumference of patients with FCH are significantly higher, compared to normolipidemic relatives and spouses. The systolic and diastolic blood pressures are significantly higher in patients with FCH compared to the normolipidemic relatives, but not compared to the spouses. By definition, FCH patients are characterized by increased plasma TC, TG and apoB levels compared to normolipidemic relatives and spouses. Furthermore, FCH patients have decreased HDLc levels, are more insulin resistant, as reflected by a higher HOMA-index and more sdLDL is present, as reflected by a

lower K-value. Both male and female FCH patients have higher plasma leptin levels compared to normolipidemic relatives and, only within the males, compared to spouses. Normolipidemic relatives are younger, have a lower BMI, waist circumference, systolic and diastolic blood pressure, TC and HDLc levels, compared to the spouses (Table 1).

LEPR polymorphism and FCH

The allele frequencies in the total group are 0.4 for the Arg allele and 0.6 for the Gln allele. The distribution of the alleles in the group of spouses was in Hardy-Weinberg equilibrium (p = 0.75). The Arg223Arg genotype is present within 31 FCH patients (20%), 66 normolipidemic relatives (17%) and 14 spouses (16%). No differences in lipid, lipoprotein and insulin resistance parameters were found between subjects with the Gln223Arg or Arg223Arg genotype, therefore, pooled analyses for the carriers of the Arg allele were performed [319]. As normolipidemic relatives and spouses did not differ in frequencies of the genotypes, they were pooled into one group of controls for further analyses.

Table 3 Mean values of obesity, lipid, lipoprotein and insulin resistance parameters for the carriers and non-carriers of the Arg allele in the control group.

	Gln223Gln	Gln223Arg/Arg223Arg	P-value	P-value *
Age (years)	36.7 (34.1 - 39.3)	42.4 (40.3 - 44.4)	0.000	-
BMI (kg/m^2)	23.9 (23.3 - 24.6)	24.7 (24.2 - 25.2)	0.041	0.501
Waist (cm)	77.1 (75.4 - 78.9)	79.3 (78.0 - 80.7)	0.045	0.565
Syst. BP (mmHg)	127.5 (124.7 - 130.3)	129.7 (127.5 - 132.0)	0.161	0.303
Diast. BP (mmHg)	79.2 (77.6 - 80.9)	81.5 (80.2 - 82.8)	0.013	0.504
TC (mmol/L)	4.82 (4.66 - 4.98)	4.97 (4.85 - 5.10)	0.090	0.955
TG (mmol/L) †	1.01 (0.94 - 1.09)	1.09 (1.03 - 1.15)	0.094	0.422
ApoB (mg/L)	942 (909 - 976)	981 (956 - 1006)	0.061	0.782
HDLc (mmol/L)	1.28 (1.22 - 1.33)	1.21 (1.17 - 1.25)	0.033	0.041
K-value	0.05 (0.02 - 0.09)	0.04 (0.02 - 0.07)	0.589	0.896
HOMA-index †	2.04 (1.84 - 2.26)	2.01 (1.85 - 2.18)	0.795	0.671
Leptin (ng/mL)				
Males †	3.9 (3.1 - 4.9)	4.3 (3.7 - 5.0)	0.517	0.906
Females †	18.7 (15.9 - 21.9)	19.1 (16.8 - 21.7)	0.801	0.875

*Analyses are performed with GEE and results are presented as means (95% confidence interval); BMI, body mass index; K-value, a value < -0.1 represents the presence of small dense low-density lipoprotein; HDLc, high-density lipoprotein cholesterol; HOMA-index, homeostasis model assessment-index; Syst.BP, systolic blood pressure; Diast. BP, diastolic blood pressure; * p-value corrected for age; †, geometric mean with 95% confidence interval is presented.*

In table 2, the genotypic distribution is presented for both the patients with FCH and the controls. In the control group, 64.2% of the subjects were carrier of one or two Arg alleles for the Gln223Arg polymorphism in the *LEPR* gene. In the group of FCH patients, almost 74.0% were carrier of an Arg allele. The presence of an Arg allele was associated with a 1.6 [95% CI 1.0 – 2.4] times increased risk for FCH (Table 2).

LEPR polymorphism and FCH phenotypes

As shown in table 3, controls carrying one or two Arg alleles had a higher BMI, waist circumference and diastolic blood pressure and decreased levels of HDLc compared to control subjects with the Gln223Gln genotype. No differences in other lipid and lipoprotein or insulin resistance parameters were found. The mean leptin level in male and female control carriers of an Arg allele did not differ significantly from non-carriers (Table 3). Also after adjustment of leptin levels for obesity and insulin resistance, as represented by the BMI and HOMA-index, respectively, the leptin levels did not differ between control carriers and non-carriers of an Arg allele (data not shown). Because a difference in age was observed between controls carrying an Arg allele and controls not carrying an Arg allele, analyses were adjusted for age. After adjustment for age, only the HDLc levels were significantly different between the two genotype groups. Within the group of FCH patients there were no significant differences in obesity, blood pressure, lipid levels and insulin resistance parameters between carriers and non-carriers of one or two Arg alleles after adjustment for age (data not shown).

Discussion

In our population of 37 well-defined FCH families, we found that carriers of one or two Arg alleles of the Gln223Arg polymorphism in the leptin receptor gene have a 1.6 times increased risk on FCH and have decreased levels of HDLc.

There are several reasons, which suggest that the *LEPR* gene could be a good candidate gene for FCH. First of all, the *LEPR* gene is located on chromosome 1p31, within a segment to which a QTL for apolipoprotein B has been mapped in another Dutch FCH population [38]. The authors also considered the *LEPR* gene as a candidate gene. However, genotyping two SNPs did not reveal evidence of an association between alleles of the *LEPR* gene and apoB levels in their study population. They did, however, not study the functional Gln223Arg polymorphism in the *LEPR* gene. We show that the Gln223Arg polymorphism is not associated with apoB levels. Most likely, another gene in this chromosomal region contributes to elevated apoB levels in FCH.

Another reason, why the *LEPR* gene has been postulated to be a good candidate gene is the fact that the *LEPR* gene maps to a locus which segregates with adiposity, body weight and plasma leptin levels in pedigrees with FCH [302]. Within these pedigrees with FCH, it was found that leptin levels are associated with two polymorphic markers in the *LEPR* gene [302]. We now show that the Gln223Arg polymorphism is not associated with leptin levels in our population of patients with FCH. Recently, we showed that the increased leptin levels in patients with FCH are attributable to their increased adiposity and insulin resistance, suggesting that the increased leptin levels in FCH patients are not specific for FCH [318]. Reasons for the apparent discrepancy in results between the two different studies could be that van der Kallen et al. did not test a

functional polymorphism like we did, only polymorphic markers within the *LEPR* gene, and they used leptin levels as a surrogate marker of obesity, so the independent role of leptin in FCH was not investigated.

The choice for the functional Gln223Arg polymorphism in the *LEPR* gene was based on previous studies, which suggested that the Gln223Arg polymorphism of the leptin receptor gene is associated with high blood pressure, obesity, lipids and insulin resistance [312, 313, 320, 321], all characteristics of FCH. However, data are conflicting, as there are also many studies reporting no association [317, 322-325]. A recent paper by Heo et al., [322] in which data from nine distinct studies were pooled for analysis, stated that there was no compelling evidence for any of the *LEPR* polymorphisms tested, including the Gln223Arg and the two polymorphisms previously tested in another FCH population, [38] to be associated with BMI and waist circumference in the general population. Our results support the data from Heo et al., [322] as we did not find an association between the Gln223Arg polymorphism in the *LEPR* gene and BMI and waist circumference. In the present study, we also did not find an association with blood pressure, insulin resistance, or any of the lipid and lipoprotein parameters, except for HDLc, which was significantly lower in carriers of the Arg allele.

The mechanistic implication of the association between FCH, decreased HDLc levels and the *LEPR* gene, found in this study, is unclear. It is possible that the association of the Gln223Arg polymorphism in the *LEPR* gene with HDLc originates from chance alone, as it is the only significant association among several tests. However, previous studies in mice and humans support the association between HDLc and the *LEPR* gene. The ob/ob and db/db mice, which have specific mutations in the leptin and the leptin receptor gene, respectively, have elevated levels of HDLc, which can be reversed by treatment with leptin [326, 327]. This suggests that leptin plays a role in the regulation of HDLc. Also a study performed in humans by Mendez-Sanchez et al., [328] found a negative correlation between leptin levels and HDLc. Mendez-Sanchez et al. [328] hypothesized that in obese humans, as they have high leptin levels and are leptin resistant, elevated leptin levels could have direct peripheral actions on the liver, leading to accelerated hepatic degradation of HDLc. Alternatively, leptin is suggested to be capable of influencing several apolipoprotein genes and the hepatic lipase (HL) gene expression. Liang and Tall [326] showed that the ob/ob mice have a down-regulated expression of genes encoding a variety of apolipoprotein and hepatic lipase with reversal upon leptin administration. In humans with FCH, it was recently reported by Soro et al. that HL plays a major role in determining HDLc [6] and also plasma phospholipid transfer protein (PLTP), cholesteryl ester transfer protein (CETP) and lipoprotein lipase (LPL) activity have been suggested to contribute to HDLc in FCH [6, 136]. Leptin or the Arg allele of the *LEPR* gene may thus contribute to low HDLc levels by modifying HL, PLTP, CETP or LPL levels. Recently, a genome-wide linkage analysis in hypertensive sibships indicated that a locus for HDLc levels was located on chromosome 1p, to which also the *LEPR* gene has been mapped [329]. So, it is conceivable that there is an association between the Gln223Arg polymorphism in the *LEPR* gene and HDLc.

The association of the *LEPR* gene with HDLc in our study population is not supported by associations between the polymorphism in the *LEPR* gene and leptin levels or other serum lipid levels, while other studies, which also found an association between the Gln223Arg polymorphism and HDLc levels, do find supportive associations with leptin or other lipid parameters [314, 320].

In conclusion, the Gln223Arg polymorphism in the leptin receptor gene is associated with

Chapter 10

Decreased adiponectin levels in familial combined hyperlipidemia patients contribute to the atherogenic lipid profile

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Abstract

Background Familial combined hyperlipidemia (FCH) is characterized by elevated levels of total cholesterol, triglycerides and/or apolipoprotein B. Other features of FCH are obesity and insulin resistance. Adiponectin is a secretory product of the adipose tissue. Low levels of adiponectin are associated with insulin resistance and accelerated atherosclerosis. The aim of this study was to determine whether decreased adiponectin levels are associated with FCH and its phenotypes. **Methods** The study population comprised 644 subjects, including 158 patients with FCH. Serum adiponectin levels were determined using a commercially available ELISA. **Results** For both males and females, the mean adiponectin level (μg/ml) was significantly lower in FCH patients (2.0 (1.8 - 2.2) and 2.5 (2.3 - 2.8), respectively) compared to normolipidemic relatives (2.3 (2.2 - 2.5) and 3.1 (2.8 - 3.3), respectively), and spouses (2.4 (2.1 - 2.7) and 3.2 (2.8 - 3.6), respectively). These differences remain significant after adjusting for waist circumference and insulin resistance. Low adiponectin level in FCH patients was a superior independent predictor of the atherogenic lipid profile, including high triglyceride levels, low HDL-cholesterol levels and the amount of small, dense LDL present, compared to both obesity and insulin resistance. **Conclusions** Low adiponectin levels may contribute to the atherogenic lipid profile in FCH, independent of insulin resistance and of obesity, as measured by waist circumference. This finding implies a role of adipose tissue metabolism in the pathophysiology of FCH.

Introduction

In the past, the adipose tissue was seen as an energy depot, storing energy in the form of triglycerides and not having a real function of its own. At present, we know that adipose tissue also secretes several signaling proteins, called adipokines [330]. Adiponectin is one of the major adipokines derived from the adipose tissue and is abundantly present in human plasma, at concentrations ranging from 2 to 10 µg/mL in healthy subjects [106]. Adiponectin production is inversely correlated with adipose tissue mass [107].

Low adiponectin levels are found in subjects with obesity [331-333], diabetes mellitus [333, 334] and cardiovascular disease (CVD) [109, 112]. These subjects with low adiponectin levels fail some of the protective actions of adiponectin, including the stimulation of fatty acid oxidation and the improvement of glucose metabolism, by increasing lipid oxidation both in pancreas and muscle, thereby increasing insulin sensitivity [107]. Furthermore, adiponectin has an insulin-sensitizing effect on hepatocytes, resulting in suppression of hepatic glucose output [108]. In addition, adiponectin inhibits the inflammatory process and possibly atherosclerosis by suppressing tumor necrosis factor-alpha-induced adhesion molecule expression [109], the adhesion and migration of monocytes/macrophages, and their transformation into foam cells [110]. Because of these properties, adiponectin is suggested to be the missing link between obesity, insulin resistance and atherosclerosis [106].

Besides the effects of adiponectin on whole body glucose metabolism and insulin sensitivity, adiponectin has also been reported to modulate plasma lipid levels, directly or indirectly [335]. Several studies have reported a negative correlation of adiponectin levels with serum triglycerides (TGs) and small dense low-density lipoprotein (sdLDL) and a positive correlation with high-density lipoprotein cholesterol (HDLc) [111, 336-341]. Furthermore, Chan et al. recently showed that adiponectin regulates triglyceride-rich lipoprotein metabolism [337, 342]. The mechanisms by which adiponectin modulates plasma lipid levels are unknown; however, several possibilities have been proposed. Adiponectin enhances fatty acid oxidation in the circulation and in the skeletal muscle through activation of AMP-kinase [107], so the accumulation of triglycerides occurs with low levels of adiponectin. Recent studies suggest a strong relationship between adiponectin and lipoprotein lipase (LPL) activity [343]. Finally, the effects of adiponectin on lipid levels may be related to the effect of adiponectin on insulin sensitivity.

Familial combined hyperlipidemia (FCH) is the most common genetic hyperlipidemia in humans, affecting 1 - 3% of the general population. It is strongly associated with premature CVD; of the survivors of a premature myocardial infarction, up to 20% are affected with FCH [4]. FCH is characterized by increased levels of plasma total cholesterol, triglycerides, and/or apolipoprotein B (apoB). Other phenotypes of FCH are decreased levels of HDLc and the presence of sdLDL [13, 18, 116]. In addition, FCH is associated with obesity and insulin resistance [9].

The pathophysiology of FCH is still unknown, although several metabolic abnormalities have been suggested, including the hypothesis that FCH is caused by disturbances in adipose tissue [9, 93, 251]. Quantitative and qualitative changes in adipose tissue contribute to fatty acid accumulation in the circulation, which drives apoB secretion in the liver, leading to increased lipid levels and insulin resistance [9, 344]. On the other hand, alterations in adipose tissue can result in dysregulation of the secretion of adipokines. Because adiponectin is one of the major adipokines and, as described above, is associated with obesity, insulin resistance and dyslipidemia, all features of

FCH, adiponectin may play a role in the pathophysiology of FCH or may be a marker for a disturbed adipose tissue metabolism in FCH. Therefore, the inadequate storage and release of fatty acids alone or in combination with dysregulation of secretion of adipokines may contribute to the detrimental metabolic sequelae in FCH.

Here, we address the question of whether in FCH the adiponectin levels are disturbed and whether disturbed adiponectin levels are associated with FCH and its associated phenotypes.

Methods

Study population

Thirty-seven families, comprising 644 subjects (158 subjects diagnosed as FCH patients, 390 normolipidemic relatives, and 89 spouses) were included in the study population, as previously described [16, 17]. Seven subjects were not diagnosed because of missing values. The normolipidemic relatives and spouses are regarded as separate reference groups, because of the similar genetic background of the relatives with the FCH patients. The spouses of both the FCH patients and the normolipidemic relatives were included in this study as a separate control group, because they share environment but are not genetically related to FCH patients. All subjects filled out a questionnaire about their previous medical history. After withdrawal of lipid-lowering medication for 4 weeks and an overnight fast, blood was drawn by venipuncture. The ethical committee of the Radboud University Nijmegen Medical Centre approved the study protocol, and the procedures followed were in accordance with institutional guidelines. All subjects gave informed consent.

The diagnosis FCH was based on the nomogram, as recently published by our group [17]. Plasma triglyceride and total cholesterol levels, adjusted for age and gender, and absolute apoB levels were applied to the nomogram to calculate the probability of being affected by FCH. In short, in the nomogram for each of the three variables, the corresponding number of points is read from a scale and then summed to give a total point score, which is translated into a probability of being affected by FCH. The subjects is defined as affected by FCH when te probability is > 60%, when the diagnostic phenotype is also present in at least one first-degree relative, and when premature CVD, before the age of 60 years, is also present in at least one individual in the family.

Body mass index (BMI) was calculated as body weight (in kilograms) divided by the square of height (in meters). The maximum hip circumference (cm) and waist circumference (cm) at the umbilical level were measured in the late exhalation phase while standing. The waist-hip ratio (WHR) was calculated.

Biochemical analyses

Plasma total cholesterol and total triglycerides were determined by enzymatic, commercially available reagents (catalog number 237574 (Boehringer-Mannheim) and catalog number 6639 (Sera Pak), respectively). HDLc was determined by the polyethylene glycol 6000 method [267]. Low-density lipoprotein cholesterol (LDLc) was calculated by subtraction of very low-density lipoprotein cholesterol (VLDL-c) and HDLc from plasma total cholesterol according to the method of Friedewald, Levy, and Fredrickson [345]. Total plasma apoB concentrations were determined by immunonephelometry [119]. LDL subfractions were separated by single-spin density

gradient ultracentrifugation. A continuous variable, K, represents the LDL subfraction profile of each individual. A negative K-value (K ≤ -0.1) reflects a more dense LDL subfraction profile, and a positive K-value (K > -0.1) reflects a more buoyant profile [120, 147, 268]. Glucose concentrations were measured in duplicate using the oxidation method (Beckman®, Glucose Analyser2, Beckman Instruments Inc., Fullerton, CA 92634, USA). Plasma insulin concentrations were determined using a double-antibody method. Insulin resistance was assessed by the homeostasis model assessment (HOMA). The HOMA-index was calculated from the fasting concentrations of insulin and glucose using the following formula: HOMA-index = fasting plasma insulin (μU/ml) x fasting plasma glucose (mmol/L)/22.5 [171].

Table 1 Characteristics of patients with familial combined hyperlipidemia, normolipidemic relatives, and spouses.

Characteristics	FCH patients (n = 158)	NL relatives (n = 390)	Spouses (n = 89)
Gender (males)	75 947.5%)	175 (44.9%)	44 (49.4%0
Age (years)	47.1 (16.3) * †	37.7 (18.0) ‡	52.6 (15.9)
Cardiovascular disease	32 (20.3%) * †	21 (5.4%)	3 (3.4%)
Body mass index (kg/m^2)	27.3 (4.4) *	24.2 (5.3) ‡	26.4 (4.2)
Waist-to-hip ratio	0.88 (0.08) * †	0.83 (0.08) ‡	0.85 (0.08)
Waist circumference(cm)	87.4 (14.2) * †	77.6 (12.1) ‡	82.9 (11.3)
Total cholesterol (mmol/L)	6.5 (1.1) * †	4.9 (1.2) ‡	5.2 (1.0)
Triglycerides (mmol/L)	2.8 (1.6) * †	1.1 (1.6)	1.1 (1.6)
HDLc (mmol/L)	0.97 (0.33) * †	1.22 (0.36) ‡	1.29 (0.32)
LDLc (mmol/L)	4.2 (1.0) * †	3.2 (1.1) ‡	3.5 (1.0)
Apolipoprotein B (mg/L)	1371 (236) * †	961 (249)	1001 (233)
K-value	-0.25 (0.24) * †	0.04 (0.27)	0.06 (0.23)
Insulin (mU/ml)	12.7 (1.7) * †	9.1 (1.9)	9.5 (1.7)
Glucose (mmol/L)	5.2 (1.1) *	5.0 (1.1) ‡	5.2 (1.1)
HOMA-index	2.9 (1.8) * †	2.0 (2.0)	2.2 (1.8)

*Values presented are means (SD) except as indicated; Gender and CVD data are presented as number (%); TG, insulin, glucose, and HOMA-index data are presented as geometric means (geometric SD); FCH, familial combined hyperlipidemia; K-value, a value ≤ -0.1 represents the presence of small, dense low-density lipoprotein (sdLDL); *, p-value < 0.05, compared to normolipidemic relatives; †, p-value < 0.05, compared to spouses; ‡, p-value < 0.05, compared to spouses.*

Serum adiponectin levels

To measure adiponectin levels, serum samples were assayed in duplicate using a commercially available enzyme-linked immunosorbent assay (catalog number DY1065; R&D Systems, Minneapolis, USA). This assay measures total circulating levels of adiponectin present in the serum. Interassay and intra-assay coefficients of variance were 7.2% and 6.2%, respectively. The detection limit was 0.1 ng/ml.

Statistical analyses

For any given degree of obesity, females have higher adiponectin levels than males. Therefore, all analyses were stratified, standardized, or corrected for gender. Variables with a skewed distribution, including adiponectin levels, triglyceride levels, and the HOMA-index, were logarithmically transformed before analysis.

Descriptive statistics, presented separately for FCH patients, normolipidemic relatives, and spouses, are expressed as a means with SD or geometric means with geometric SD for logarithmically transformed variables. Parametric Pearson correlation was used for correlation analysis. Differences in characteristics and adiponectin levels between patients with FCH, normolipidemic relatives, and spouses were tested by means of generalized estimating equations (GEEs) because of possible correlated values within families. GEE analyses were performed to standardize adiponectin levels for waist circumference and HOMA-index. Tertiles of adiponectin, standardized for gender, were calculated separately for FCH patients and controls. GEE regression models were used to calculate the prediction of the atherogenic lipid profile in FCH patients and controls by adiponectin levels, HOMA-index and waist circumference, adjusted for age and gender. The standardized ß coefficient is calculated by multiplying the ß coefficient with the ratio of the standard deviations for the independent and dependent variables. The predicted change in several variables caused by a 25% model-based decrease in adiponectin or a 5% increase in waist circumference was calculated. The choice of a 25% change in adiponectin level and a 5% change in waist circumference were based on the fact that these changes are associated with a 3 to 4 kg change in body weight [346-348]. Differences were considered statistically significant at p-values < 0.05. All analyses were computed using the STATA 8.0 software.

Results

Subject characteristics

Descriptive statistics of anthropometric and metabolic characteristics of the study population are presented in table 1. FCH patients were older than normolipidemic relatives but younger than the spouses. Evident was the greater incidence of CVD in patients with FCH compared to normolipidemic relatives and spouses. The mean BMI of patients with FCH was significantly

Table 2 Mean adiponectin levels in patients with FCH, normolipidemic relatives, and spouses.

	Males		Females	
Subject	Adiponectin level (ng/mL)	Difference versus Spouses	Adiponectin level (ng/mL)	Difference versus Spouses
FCH patients	2.0 (1.8 - 2.2) * †	-17%	2.5 (2.3 - 2.8) * †	-22%
Normolipidemic relatives	2.3 (2.2 - 2.5)	-4%	3.1 (2.8 - 3.3)	-3%
Spouses	2.4 (2.1 - 2.7)	Reference	3.2 (2.8 - 3.6)	Reference

*Values for adiponectin levels are geometric means with 95% confidence interval (CI); *, p-value < 0.05, compared to normolipidemic relatives; †, p-value < 0.05, compared to spouses.*

higher than that of normolipidemic relatives. Mean WHR and waist circumference was significantly higher in FCH patients compared with normolipidemic relatives and spouses. Compared with normolipidemic relatives and spouses, FCH patients had significantly higher levels of total cholesterol, triglycerides, apoB and LDLc and significantly lower levels of HDLc. Furthermore, FCH patients were characterized by the presence of sdLDL and insulin resistance, as reflected by a significant lower K-value and a significant higher HOMA-index, respectively. Normolipidemic relatives were younger and had lower BMI, WHR, waist circumference, total cholesterol, LDLc, and HDLc compared to the spouses (Table 1).

Adiponectin level and FCH

As presented in table 2, both male and female FCH patients had significantly lower mean serum adiponectin levels compared with both normolipidemic relatives and spouses. Mean adiponectin levels were 17% lower in male and 22% lower in female FCH patients compared with male and female spouses (Table 2). Adiponectin levels in normolipidemic relatives and spouses did not differ significantly.

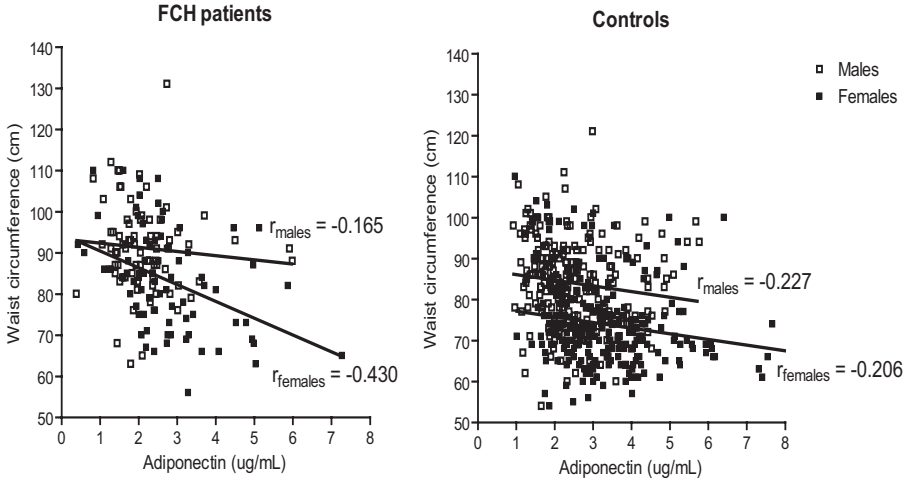

Figure 1
Correlation between waist circumference and serum adiponectin levels in patients with familial combined hyperlipidemia (FCH) and controls. Open squares represent males and closed squares represent females.

Within the total group, adiponectin levels were associated with BMI (males, r = -0.285; females, r = -0.311), WHR (males, r = -0.238; females, r = -0.306), and waist circumference (males, r = -0.246; females, r = -0.339). The correlations of adiponectin levels with markers of obesity were comparable for FCH patients and non-FCH control subjects. The correlations of adiponectin levels with waist circumference for both FCH patients and controls are shown in figure 1.

We adjusted the adiponectin levels for waist circumference because of the good correlation and because it is known from the literature that of these three markers of obesity, waist circumference is the best simple anthropometric predictor of abdominal visceral fat mass [349]. The adiponectin levels remained significantly lower in both male and female patients with FCH (2.1 (1.9 - 2.3)

and 2.8 (2.5 - 3.0), respectively) when comparing with male and female spouses (2.5 (2.2 - 2.8) and 3.2 (2.8 - 3.6), respectively) and borderline significant when comparing with male and female normolipidemic relatives (2.3 (2.1 - 2.4) and 3.0 (2.8 - 3.2), respectively). When we used BMI or WHR as markers for adiposity, the results remained unchanged (data not shown).

It is known that adiponectin is even more related to whole body insulin sensitivity than to adiposity. Indeed, in our population, we observed a correlation of adiponectin with HOMA-index of -0.34 for males and -0.31 for females; therefore, we adjested adiponectin levels not only for waist circumference but also for the HOMA-index. This did not change the point estimates of adiponectin (data not shown). Adiponectin levels in normolipidemic relatives and spouses did not differ significantly, therefore they were combined into one group and referred to as controls.

Table 3 Obesity, insulin resistance, and lipid parameters in patients with FCH stratified by gender-specific tertiles of adiponectin levels.

Characteristics	First tertile (0 - 1.9 ng/mL)	Second tertile (1.9 - 2.6 ng/mL)	Third tertile (2.6 - ∞ ng/mL))
waist (cm)	91.4 (88.0 - 94.9) * †	86.5 (83.0 - 89.9)	84.3 (80.8 - 87.6)
HOMA-index	3.9 (3.3 - 4.5) * †	2.9 (2.5 - 3.1) ‡	2.3 (2.0 - 2.7)
TG (mmol/L)	3.2 (2.9 -3.6) * †	2.7 (2.5 - 3.1)	2.4 (2.2 - 2.7)
HDLc (mmol/L)	0.93 (0.86 - 1.00) †	0.96 (0.89 - 1.03)	1.03 (0.95 - 1.10)
K-value	-0.33 (-0.41 - -0.26) †	-0.24 (-0.31 - -0.16)	-0.19 (-0.26 - -0.12)
TC (mmol/L)	6.5 (6.2 - 6.8)	6.4 (6.1 - 6.8)	6.5 (6.2 - 6.8)
LDLc (mmol/L)	4.07 (3.73 - 4.41)	4.18 (3.82 - 4.52)	4.31 (3.96 - 4.66)
ApoB (mg/L)	1378 (1304 - 1452)	1383 (1307 - 1459)	1358 (1284 - 1432)

*Values shown are means and (95% confidence intervals) except as indicated; HOMA-index and TG data are presented as geometric means (95% confidence intervals); Waist, waist circumference; TG, triglycerides, K-value, a value ≤ -0.1 represents the presence of sdLDL; TC, total cholesterol; LDLc, low-density lipoprotein cholesterol; ApoB, apolipoprotein B; *, p-value < 0.05, compared to second tertile; †, p-value < 0.05, compared to third tertile; ‡, p-value < 0.05, compared to third tertile.*

Adiponectin and the atherogenic lipid profile

FCH patients were stratified by gender-specific tertiles of adiponectin levels, as presented in table 3. FCH patients with low adiponectin levels were more obese, as reflected by a higher waist circumference, and more insulin resistant, as reflected by an increased HOMA-index, compared with FCH patients with intermediate or high adiponectin levels. Furthermore, low adiponectin levels in FCH patients were associated with an atherogenic lipid profile characterized by higher triglyceride levels, low HDLc levels, and the presence of sdLDL. No relationships between adiponectin and total cholesterol, LDLc and apoB levels were found. Similar associations of adiponectin levels with obesity, insulin resistance and the atherogenic lipid profile were found in controls (data not shown).

Multivariate regression analyses showed that, in the total study population, gender, waist circumference, and HOMA-index could explain only 20% of the variation in adiponectin levels.

Expanding the model with age and smoking led to a further 4% of variation that was explained. A maximum of 30% of the variation in adiponectin levels could be explained when HDLc and K-value were also added to the model.

In table 4, the results of regression models are presented, including adiponectin, HOMA-index and waist circumference, as predictors of the atherogenic lipid profile, independent of each other and adjusted for age and gender. Among FCH patients, a reduction in serum adiponectin level was associated with a significant increase in triglyceride levels, a decrease in HDLc level, and a decrease in the K-value (Table 4). A model-based reduction of 25% in serum adiponectin level resulted in a 6.2% increase in triglyceride level, a 3.7% decrease in HDLc level and a 0.06 decrease in absolute value of parameter K, reflecting the presence of more sdLDL. An increase in waist circumference was also associated with a significant increase in triglyceride level, a decrease in HDLc level, and a decreased K-value, indicating the presence of sdLDL. A model-based increase of 5% in waist circumference was associated with an increase in triglyceride level of 3.2%, a decrease of 1.7% in HDLc level and 0.02 decrease in the absolute value of parameter K. A change in HOMA-index was not associated with an independent change in any of these lipid parameters (Table 4). The results found in the control group were comparable to those found in patients with FCH (Table 4). A significant effect for the HOMA-index on triglyceride level and K-value was only found in the controls.

Table 4 Adiponectin levels, HOMA-index, and waist circumference as predictors of the atherogenic lipid profile in patients with FCH and controls.

Variable	Predictor	FCH patients ß	Controls ß
TG (mmol/L)	Adiponectin	-0.219 *	-0.114 *
	Waist circumference	0.222 *	0.185 *
	HOMA-index	-0.066	0.167 *
HDLc (mmol/L)	Adiponectin	0.222 *	0.273 *
	Waist circumference	-0.197 *	-0.260 *
	HOMA-index	0.081	0.050
K-value	Adiponectin	0.329 *	0.129 *
	Waist circumference	-0.173 *	-0.129 *
	HOMA-index	0.124	-0.098 *

*Values shown are standardized ß coefficients of 1 SD change in adiponectin levels (ng/mL) (log-transformed), waist circumference (cm), and HOMA-index (log-transformed) independent of age and gender; TG, triglycerides; HDLc, high-density lipoprotein cholesterol; K-value, a value ≤ -0.1 represents the presence of sdLDL; *, p-value < 0.05.*

Discussion

In this study, we show that patients with FCH have low serum levels of adiponectin, even after adjusting for their body adiposity and degree of insulin resistance. Furthermore, we show that the adiponectin level in patients with FCH is the strongest independent predictor of the atherogenic lipid profile, including high triglyceride level, low HDLc level and the presence of sdLDL.

Several metabolic pathways have been proposed to explain the dyslipidemia in FCH, including an increased production of VLDL, with or without impaired clearance of triglyceride-rich lipoproteins [97]. The increased production of VLDL may be attributable to increased hepatic lipid supply and availability, associated with obesity [350], and to the intrinsic effects of insulin resistance on the hepatic output of VLDL and catabolism of VLDL in peripheral tissue [97]. Therefore, both obesity and insulin resistance are suggested to be involved in the cause of dyslipidemia of FCH. Recently, a defect in adipose tissue metabolism was proposed as the primary cause of this increased production of VLDL in FCH [97]. Our finding that low adiponectin levels contribute to the atherogenic lipid profile in FCH, independent of insulin resistance and obesity, as measured by waist circumference, supports the hypothesis of a role for a disturbed adipose tissue metabolism in the pathophysiology of FCH.

Adiponectin is one of the major adipokines produced by adipose tissue. There is a growing body of evidence that adiponectin is involved in the regulation of both lipid and carbohydrate metabolism. Chan et al. [337] showed that adiponectin exerts an independent role in regulating triglyceride-rich lipoproteins in healthy men. In fact, the mechanism that may underlie the association between serum adiponectin levels and dyslipidemia was recently investigated, and adiponectin was shown to be an independent predictor of VLDLapoB catabolism [342]. We now show for the first time that adiponectin level is decreased in patients with FCH and that low adiponectin levels in FCH, as in non-FCH populations [111, 336-341], are associated with the atherogenic lipid profile, including high levels of triglycerides, low levels of HDLc, and the presence of sdLDL. Obesity and insulin resistance, both characteristic independent features of FCH [344], are also known to be associated with the atherogenic lipid profile. Most importantly, we now demonstrate that in FCH, adiponectin is a superior independent predictor of the atherogenic lipid profile compared with obesity and insulin resistance. Assuming a 3 to 4 kg increase in bodyweight, adiponectin levels will decrease by ~25% [348] and waist circumference will increase by ~5% [346, 347]. This model-based 25% decrease in adiponectin levels resulted in a 2-fold higher increase in triglyceride levels, a 2-fold higher decrease in HDLc levels, and a 3-fold higher decrease in K-value with a 5% increase in waist circumference. In contrast, insulin resistance was not associated with a significant change in these lipid parameters. Therefore, adiponectin has a greater impact on the expression of the atherogenic lipid profile than obesity and insulin resistance. Recently, the increase in adiponectin after weight loss was found to be correlated with serum lipid improvement, independently of insulin sensitivity changes [336]. These results support the role of adiponectin in the lipid phenotype expression of FCH.

Adiponectin levels are linked to triglyceride, HDLc and sdLDL levels, but they do not relate to plasma apoB levels, a characteristic feature of FCH. We propose that the lack of association of adiponectin with apoB supports the concept of a separate, but additive, genetic origin of high apoB levels in patients with FCH [344]. FCH is a multifactorial disease in which the phenotype develops over a lifetime. The most common expression of FCH in children involves high

apoB levels [220, 351, 352]. Therefore, a primary (genetic) defect in FCH results in the hyper-apoB phenotype, supported by segregation and linkage analyses providing evidence of a major gene influencing apoB levels [18]. The fact that the lipid phenotype of FCH is not fully expressed until the third decade of life is possibly associated with the accumulation of central abdominal fat, resulting from changes in adipocyte size and function with aging. Alternatively, as hypothesized by Cnop et al. [111], low adiponectin levels result in hepatic insulin resistance, and this, together with increased plasma nonesterified fatty acid concentrations, will shift the fate of apoB away from degradation towards secretion by the liver, resulting in increased triglyceride levels.

FCH patients are obese, and with this increased body adiposity, large triglyceride-filled visceral adipocytes produce less adiponectin [250], yet the low adiponectin levels in patients with FCH are not completely attributable to the degree of insulin resistance or the degree of obesity, as measured by waist circumference. Even though waist circumference is the best simple anthropometric predictor of abdominal visceral fat mass, we cannot exclude the possibility that the apparent independence of adiponectin levels may be attributable to the fact that intra-abdominal fat was not measured with a more sophisticated technique. However; an intrinsic defect in the adipocytes may also contribute to this hypoadiponectinemia in patients with FCH. To further explore the decreased adiponectin levels in patients with FCH, expression studies of adiponectin in adipose tissue should be performed in patients with FCH. Lihn et al. [353] showed that adiponectin mRNA expression in both visceral and subcutaneous adipose tissue is 6-fold higher in lean individuals compared to obese individuals. Studying expression levels of adiponectin in adipose tissue of patients with FCH can contribute to unravel the cause of the decreased adiponectin levels in these patients that are unexplained by their adiposity and state of insulin resistance.

Furthermore, we demonstrate in our FCH population that body adiposity, insulin sensitivity, HDLc, the presence of sdLDL, smoking and age explained only 30% of the variation in adiponectin levels. Therefore, other factors must contribute to the variation in adiponectin levels. Recent analyses in a predominantly northern European population suggested that variation in serum adiponectin levels had a strong genetic component (heritability estimate = 46%) and adiponectin concentrations were significantly linked (logarithm of the odds = 4.1) to a quantitative trait locus on chromosome 5p [354]. Possibly, a large part of the remaining 70% of the variation in adiponectin levels in FCH could also be explained by a genetic component, which requires further research.

In summary, adiponectin levels are decreased in patients with FCH, independent of their insulin resistance and body adiposity, as measured by waist circumference. Furthermore, low adiponectin level in FCH predicts the presence of the atherogenic lipid profile. Our results support the concept of a disturbed adipose tissue metabolism in the pathophysiology of FCH.

Chapter 11

Variants in the *ApM1* gene in patients with familial combined hyperlipidemia

Submitted

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Abstract

Background Familial combined hyperlipidemia (FCH) is the most common genetic lipid disorder, characterized by elevated lipid and apolipoprotein levels. It is hypothesized that a disturbed adipose tissue metabolism plays an important role in the pathophysiology of FCH. Recently, we have shown that patients with FCH have decreased levels of adiponectin, one of the major adipokines produced by adipose tissue. The aim of the present study was to investigate the association of two variants in the ApM1 gene, encoding for adiponectin, with adiponectin levels and dyslipidemia in FCH patients. **Methods** The study population included 36 Dutch FCH families, including 157 FCH patients. Two polymorphisms in the ApM1 gene (45T>G and 639C>T) were genotyped with PCR-RFLP. The FBAT program was used to analyse the polymorphisms and their haplotypes. **Results** Variants in the ApM1 gene were not associated with FCH. The variants in the ApM1 gene were, however, associated with adiponectin levels, resulting in 19% lower adiponectin levels in FCH patients carrying the 45T allele. Subjects carrying the 639T allele presented a more atherogenic lipid profile. **Conclusions** The association of the ApM1 gene with decreased adiponectin levels and an atherogenic lipid profile was not specific for FCH. We did not find any indication that genetic variations in the ApM1 gene are associated with FCH.

Introduction

Familial combined hyperlipidemia (FCH, OMIM-144250) is the most common genetic lipid disorder, affecting up to 5% of the general population [10]. FCH is strongly associated with premature cardiovascular disease (CVD) and among survivors of a premature myocardial infarction, up to 20% is affected with FCH [4]. Major characteristics of FCH include elevated levels of apolipoprotein B (apoB), triglycerides (TG) and/or plasma total cholesterol (TC). Other FCH phenotypes are decreased levels of high-density lipoprotein cholesterol (HDLc), the presence of small, dense low-density lipoprotein (sdLDL), obesity and insulin resistance [97]. Despite more than 30 years of research, the etiology of FCH is still not fully understood. Several metabolic defects have been proposed to underlie the disturbed lipid profile, including a defect in adipose tissue metabolism [96, 97].

Adiponectin is one of the major adipokines derived from the adipose tissue and is abundantly present in human plasma. The production of adiponectin is inversely correlated with adipose tissue mass [107]. Adiponectin stimulates the oxidation of fatty acids, improves the glucose metabolism and inhibits the inflammatory process and thereby, possibly atherosclerosis [107, 109, 110]. Decreased adiponectin levels were previously found in subjects with obesity, type 2 diabetes mellitus and CVD [109, 112, 331-334]. Based on this, adiponectin is suggested to play an important role in the pathophysiology of FCH [355].

We have recently shown that adiponectin levels are significantly lower in patients with FCH, independent of their obesity and insulin resistance status [355]. These low adiponectin levels were found to be an independent predictor of the atherogenic lipid profile in FCH, including high triglyceride levels, low HDLc levels and the presence of sdLDL.

The gene encoding for adiponectin is the adipose most abundant gene transcript 1 (*ApM1*) gene, located on chromosome 3q27. In total, 87 single nucleotide polymorphisms (SNPs) in this gene can be found the NCBI SNP database. SNPs in the *ApM1* gene are found to be associated with decreased adiponectin levels, although results are conflicting as recently reviewed by Gable et al. [356]. Associations of SNPs in the *ApM1* gene with type 2 diabetes, insulin resistance, hypertension, CVD, obesitas and hyperlipidemia have also been reported [356-358].

The aim of the present study was to investigate the role of genetic variations in the *ApM1* gene in determining adiponectin levels in patients with FCH. For this purpose, we sequenced the *ApM1* gene in 18 FCH patients and determined two common variants. Furthermore, the association of these two variants in the *ApM1* gene with FCH and its associated phenotypes, including obesity, insulin resistance and increased risk of CVD, reflected by intima-media thickness (IMT) values and prevalence of CVD, were studied in our well-characterized Dutch FCH families, using a family-based SNP and haplotype approach.

Methods

Study population

The study population consists of 36 families, comprising 611 subjects with known genealogic, phenotypic, and genotypic data, of whom 157 individuals were diagnosed as FCH patients [17]. These families had a mean size of 24 members from multiple (between 2 and 4) generations. The

diagnosis of FCH was based on the nomogram, as recently published [17]. The normolipidemic relatives (n = 390), unaffected spouses of both the FCH patients and the normolipidemic relatives (n = 64) and subjects without known phenotypic and genotypic data (n = 230) were included for the family-based analyses. After withdrawal of lipid-lowering medication for four weeks and an overnight fast, blood was drawn by venipuncture. The maximum waist circumference (cm) at the umbilical level was measured in the late exhalation phase while standing.

Information concerning CVD was gathered through personal interviews and physical examinations performed by the clinical investigator. When the clinical investigator suspected the presence of CVD, further details and confirmation of the diagnosis were sought from the participant's general practitioner and hospital records. CVD was defined by angina pectoris (AP), myocardial infarction (MI), stroke, peripheral vascular disease or vascular surgery. In our study population 56 subjects were identified with CVD, including 26 subjects with AP, 25 with previous MI, 10 with peripheral vascular disease, seven with stroke and 23 who underwent vascular surgery. In total, 45% (n = 25) of these individuals were diagnosed with CVD based on the presence of two or more manifestations of CVD.

The ethical committee of the Radboud University Nijmegen Medical Centre approved the study protocol and the followed procedures were in accordance with institutional guidelines. All subjects gave written informed consent.

Biochemical analyses

Biochemical analyses were performed as previously described for this population [17]. In short, plasma TC and TG were determined by enzymatic, commercially available reagents (Boehringer-Mannheim, Germany, catalog No. 237574 and Sera Pak, Miles, Belgium, catalog No. 6639, respectively). Total plasma apoB concentrations were measured by immunonephelometry. HDLc was quantified by the polyethylene glycol 6000 method. LDL subfractions were separated by single spin density gradient ultracentrifugation. A continuous variable, K, represents the LDL subfraction profile of each individual. A negative K-value ($K \leq -0.1$) reflects a more dense LDL subfraction profile, and a positive K-value ($K > -0.1$) reflects a more buoyant profile. Glucose concentrations were analyzed, using the oxidation technique (Beckman®, Glucose Analyser2, Beckman Instruments Inc., Fullerton, CA 92634, USA). Plasma insulin concentrations were ascertained by a double antibody method. Insulin resistance was assessed by the homeostasis model assessment (HOMA) method. Adiponectin levels were measured in duplicate using a commercially available enzyme-linked immunosorbent assay (R&D Systems, Minneapolis: Elisa Development System; Human adiponectin/Acrp30 Duoset®, Catalog no. DY1065) [355].

Carotid intima-media thickness measurements

Carotid intima-media thickness (IMT) was measured in 61 FCH patients and 155 normolipidemic relatives who visited our outpatient clinic [200]. An AU5 Ultrasound machine (Esaote Biomedica) with a 7.5 MHz linear-array transducer was used to measure the IMT of both common carotid arteries. Longitudinal images of the most distal 10 mm of both the far wall and the near wall of the common carotid artery were obtained in the optimal projection (anterolateral, lateral or posterolateral). The actual measurement of IMT was performed offline by the sonographer using semi-automatic edge-detection software (M'Ath®Std version 2.0, Metris, Argenteuil, France) [201]. All measurements were carried out in end-diastole using the

R-wave of a simultaneously recorded ECG as a reference frame. From each frame the mean IMT was calculated over at least 7.5 mm of the aforementioned 10 mm segment (yielding a minimum quality index of 75%). The outcome variable was defined as the mean IMT of the near and far wall of both common carotid arteries.

Table 1 Primersets used for sequencing of *ApM1*.

Primer sequence (5' > 3')			Position primer * (#bp)	Product size (bp)
1	F	GAAGTTTCTCCGTCAGATGC	493 - 512	422
1	R	CAGCCTGGAACTTCACAGAA	895 - 914	
2	F	GAAGTAGACTCTGCTGAGATGG	1231 - 1252	369
2	R	CAGTGTAGGAGGTCTGTGATG	1578 - 1601	
3	F	GTGATGGCAGAGATGGCAC	172 - 190	475
3	R	GTAACCACCAACAGAGCCTTG	626 - 646	
4	F	GGCTCAGTCCTGCCTTTGG	574 - 592	474
4	R	CCACTCACAACCTTCTTGAC	1028 - 1047	
5	F	GTGGGAGATATAGAAGGAGG	957 - 976	452
5	R	GTGGTAGGCAAAGTAGTACAG	1388 - 1408	
6	F	CACTATGATGGCTCCACTG	1343 - 1361	501
6	R	CAGGACTGGGAACATAGCAT	1824 - 1843	

** According to GenBank Accession number AB012163S1 (exon 1), AB012163S2 (exon 2) and NT_005962 (intron 2 and exon 3).*

Sequencing and genotyping of the ApM1 gene

DNA was extracted from peripheral blood lymphocytes using a standard technique [121]. All exons and intron 2 of the *ApM1* gene were amplified in 6 fragments by standard polymerase chain reaction (PCR) with an annealing temperature of 53.7 °C [149]. The primer sequences, locations and product sizes of the 6 fragments are listed in table 1. The PCR products were column-purified using Roche High Pure PCR purification kits (Roche Applied Science, Mannheim, Germany), and bi-directional sequencing analysis was performed on an ABI Prism 3100 Genetic analyzer using the ABI Prism BigDye Terminator version 3 chemical sequencing kit according to the instructions of the manufacturer (PE Applied Biosystems, Nieuwerkerk a/d Yssel, The Netherlands). The sequences were aligned with the reference sequence to detect sequence variants. We selected 18 well-defined unrelated FCH patients to sequence the *ApM1* gene. Anthropometric and metabolic characteristics of these 18 FCH patients are presented in table 2.

Genotyping of the 45T>G SNP (rs2241766) and the 639C>T SNP (rs3821799) were carried in a final volume of 50 µl. Primerset 2 was used for the 45T>G SNP and primerset 4 was used for the 639C>T SNP. The PCR products were digested by either 10 U SmaI at 25°C (45T>G) or 10 U BstEII at 60°C (639C>T) (New England Biolabs, Beverly, MA, USA) and, subsequently, the resulting fragments (369, 216 and 153 bp in heterozygotes for 45T>G or 473, 405 and 68

bp in heterozygotes for 639C>T) were separated on agarose gels. The genotyping of the 45T>G and the 639C>T SNPs was successful in all individuals.

Statistical analyses

The characteristics of the study population are expressed as mean ± standard deviation (SD). Prior to further statistical analyses, extended Mendelian error-checking was performed with Pedcheck [122]. For families with Mendelian inconsistencies, problematic genotypes were set to missing for the complete nuclear family or the isolated problematic individual (2%). The parental data for both SNPs were tested for Hardy-Weinberg equilibrium by use of a fisher's exact-test. Variables with a skewed distribution, including triglyceride levels, adiponectin levels, and the HOMA-index, were logarithmically transformed. Nonparametric linkage analysis of the *ApM1* polymorphisms were carried out using the SOLAR 2.1.4 software [123]. The presence of linkage was tested for FCH and the related phenotypes, including apoB, TC and TG levels and the presence of sdLDL, represented by the K-value. The HAPLOVIEW program [178] was used to estimate allele and haplotype frequencies, linkage disequilibrium and allelic association between the two SNPs.

Table 2 Characteristics of patients with familial combined hyperlipidemia, in whom the *ApM1* gene was sequenced

	FCH patients (n = 18)
Gender (males)	10 (55.6%)
Age (years)	58.7 (10.0)
Cardiovascular disease (n (%))	8 (44.4%)
IMT (mm)	0.84 (0.13)
Waist circumference (cm)	87.1 (13.1)
Total cholesterol (mmol/L)	7.2 (0.58)
Triglycerides (mmol/L)	3.6 (1.4)
Apolipoprotein B (mg/L)	1561 (90)
LDLc (mmol/L)	4.4 (0.7)
HDLc (mmol/L)	0.96 (0.20)
K-value	-0.40 (0.19)
HOMA-index	4.6 (2.8)
Adiponectin males (ng/mL)	1.9 (0.6)
Adiponectin females (ng/mL)	2.6 (1.5)

Bivariate variables are presented as number (%); continuous variables are presented as mean (SD); FCH, familial combined hyperlipidemia; IMT, mean common carotid intima-media thickness was measured in 7 FCH patients; LDLc, low-density lipoprotein cholesterol; HDLc, high-density lipoprotein cholesterol; K-value, a value ≤ -0.1 represents the presence of small dense LDL; HOMA-index, homeostasis model assessment-index.

Associations between the individual polymorphisms, 45T>G and 639C>T, or haplotypes with adiponectin levels, FCH and the related phenotypes, in our extended families were determined using the family-based association test (FBAT) software [179]. An additive model of inheritance was used, as the mode of inheritance of FCH is unknown and this model is particularly robust. FBAT broke down the extended pedigrees into nuclear families (n = 219) and evaluated their contribution to the test statistics. The –e option of FBAT, which computes the test statistic using an empirical variance estimator, was implemented as the nuclear families were not independent. The –p option, which performs the Monte-Carlo permutation procedure, was used to correct for multiple testing. Adjustments for age and gender were done by calculation of the residuals. For the haplotype analysis, the haplotype-based association test (HBAT) command of the FBAT program was used, utilizing the –e and –p options. Generalized estimating equations (GEE) analyses were used to test differences between the different genotypes for parameters standardized for age and gender. The subjects carrying wildtype alleles for both SNPs are referred to as 'wildtype individuals' while controls carrying a rare allele for both SNPs, are referred to as 'compound individuals'. Differences were considered statistically significant at p-value < 0.05.

Table 3 Characteristics of patients with familial combined hyperlipidemia, normolipidemic relatives and spouses

	FCH patients (n = 157)	NL relatives (n = 390)	Spouses (n = 64)
Gender (males)	75 (47.8 %)	177 (45.4 %)	31 (48.4 %)
Age (years)	47.0 (15.6) * †	37.9 (15.7) ‡	56.0 (10.5)
CVD	32 (20.4 %) * †	20 (5.1 %)	4 (6.3 %)
IMT (mm)	0.80 (0.12) *	0.72 (0.12)	-
Waist (cm)	87.2 (12.2) *	77.7 (10.9) ‡	84.3 (12.5)
Total cholesterol (mmol/L)	6.5 (1.1) * †	4.9 (0.9) ‡	5.2 (0.8)
Triglycerides (mmol/L)	3.1 (1.5) * †	1.2 (0.5)	1.3 (0.6)
Apolipoprotein B (mg/L)	1370 (264) * †	960 (220)	996 (175)
LDLc (mmol/L)	4.1 (1.2) * †	3.2 (0.9) ‡	3.4 (0.7)
HDLc (mmol/L)	0.98 (0.26) * †	1.22 (0.30)	1.28 (0.40)
K-value	-0.26 (0.26) * †	0.05 (0.19)	0.05 (0.25)
HOMA-index	3.4 (1.8) * †	2.3 (1.3) ‡	2.7 (1.3)
Adiponectin males (ng/mL)	2.1 (0.9) * †	2.5 (0.9) ‡	2.9 (1.3)
Adiponectin females (ng/mL)	2.7 (1.2) * †	3.3 (1.3)	3.4 (1.7)

*Bivariate variables are presented as number (%); continuous variables are presented as mean (SD); FCH, familial combined hyperlipidemia; NL relatives, normolipidemic relatives; CVD, cardiovascular disease; IMT, mean common artery intima-media thickness was measured in 61 FCH patients and 155 normolipidemic relatives; waist, waist circumference; LDLc, low-density lipoprotein cholesterol; HDLc, high-density lipoprotein cholesterol; K-value, a value ≤ -0.1 represents the presence of small dense LDL; HOMA-index, homeostasis model assessment-index; *, p-value < 0.05, compared to normolipidemic relatives; †, p-value < 0.05, compared to spouses; ‡, p-value < 0.05, compared to spouses.*

All above described analyses were computed using the SPSS 12.0.1, PedCheck 1.1, SOLAR 2.1.4, HAPLOVIEW 3.2, FBAT 3.2 software and STATA 8.0.

Results

Characteristics of the study population

Anthropometric and metabolic characteristics of FCH patients, normolipidemic relatives and spouses are presented in table 3. The FCH patients were older than the normolipidemic relatives, but younger than the spouses. FCH patients had a thicker IMT and a higher prevalence of CVD. FCH patients were characterized by a disturbed lipid profile, including increased levels of TC, TG, apoB and LDLc levels and decreased HDLc levels. Furthermore, the presence of sdLDL, as reflected by a K-value ≤ -0.1, is also specific for patients with FCH. FCH patients were more obese, as reflected by a higher waist circumference and more insulin resistant, as reflected by a higher HOMA-index. Serum adiponectin levels, in both males and females, were significantly lower in FCH patients compared to normolipidemic relatives and spouses (Table 3).

Sequencing of variants in the ApM1 gene and linkage analyses

Sequencing all exons and intron 2 of the *ApM1* gene in 18 unrelated FCH patients did not result in the identification of new SNPs. Based on linkage disequilibrium (LD) between SNPs and previously reported associations [357], two SNPs, the 45T>G and the 639C>T, were selected for haplotype analyses of the *ApM1* gene.

In founders, minor allele frequencies for the 45T>G and 639C>T SNPs were 0.14 and 0.40, respectively. For both SNPs, the genotypic distributions were in Hardy-Weinberg proportions. Strong linkage disequilibrium (D' = 0.83) with little correlation between the loci (R^2 = 0.12) was present between the two SNPs. The wildtype haplotype (TC) had a frequency of 0.58. The frequencies of the haplotypes defined by a 45T>G rare allele (GC), a 639C>T rare allele (TT) or a rare allele for both SNPs (GT) were 0.01, 0.31 and 0.10, respectively. The GC haplotype was not taken into account for the statistical analyses, because of the low frequency.

Linkage analyses of the two SNPs in the *ApM1* gene did not show evidence for linkage with adiponectin levels (lod score = 0.0), the diagnosis of FCH (lod score = 0.0) or with any of the related phenotypes, including apoB, TC and TG levels and the presence of sdLDL (all two-point lod scores < 0.5).

Haplotype analyses of the ApM1 gene and adiponectin levels

Family-based association analyses of the individual SNPs in the *ApM1* gene did not result in a significant association with adiponectin levels. When performing haplotype analyses, however, a significant (p = 0.049) association with the wildtype haplotype (TC) was present (Table 4).

The normolipidemic relatives and spouses were combined into one control group, as no differences in anthropometric and biochemical parameters, based on genotypes, were observed between the two groups (data not shown). As shown in table 5 for this combined control group, the lowest adiponectin levels were found in wildtype individuals (2.65 ng/ml) and individuals carrying only a rare allele of the 639C>T SNP (2.64 ng/ml). Compound individuals, carrying the rare allele for both SNPs, presented the highest adiponectin levels (2.90 ng/ml). So, adiponectin levels were approximately 9% lower, when a wildtype allele for the 45T>G SNP was present

(p = 0.042). In patients with FCH carrying a wildtype allele for both SNPs or a rare allele for the 639C>T SNP, mean adiponectin levels (ng/ml) were approximately 19% lower compared to compound individuals (2.08 [95% CI: 1.84 – 2.36] and 2.10 [95% CI: 1.86 – 2.36] versus 2.59 [95% CI: 2.26 – 2.97]; p-values < 0.05). The decrease in adiponectin levels for the individuals with a wildtype allele for the 45T>G allele was not significantly different in FCH patients compared to the controls (p = 0.196).

In our FCH population, 30% of the variation in adiponectin levels is explained by waist circumference, HOMA-index, HDLc, the presence of small dense LDL, smoking and age [355]. In addition to these factors, the 45T>G SNP only explained 1.1% of the variation in adiponectin levels in our complete study population (p = 0.006).

Table 4 Association of the individual SNPs and haplotypes of the ApM1 gene with familial combined hyperlipidemia and its related phenotypes after correction for age and gender.

		SNPs		Haplotypes		
	N	45T>G	639C>T	TC	TT	GT
Adiponectin *	611	0.749	0.126	0.049	0.135	0.384
FCH	611	0.098	0.243	0.170	0.783	0.136
CVD	611	0.459	0.504	0.574	0.679	0.570
IMT	216	0.086	0.526	0.587	0.089	0.212
Waist circumference	611	0.921	0.636	0.885	0.515	0.554
Total cholesterol	611	0.389	0.129	0.058	0.199	0.376
Triglycerides *	611	0.055	0.186	0.193	0.858	0.087
ApolipoproteinB	611	0.138	0.048	0.014	0.130	0.233
LDLc	608	0.483	0.111	0.048	0.163	0.397
HDLc	609	0.155	0.049	0.047	0.251	0.263
K-value	611	0.361	0.115	0.142	0.263	0.353
HOMA-index *	550	0.633	0.598	0.388	0.796	0.501

*P-values for the individual SNPs (FBAT) and haplotypes (HBAT) corrected for age, gender and multiple testing are presented; *, Ln-transformec variable; N, number of subjects with available data for the relevant phenotype; FCH, familial combined hyperlipidemia was present in 157 subjects; CVD, cardiovascular disease was present in 56 subjects; IMT, mean common carotid intima-media thickness; LDLc, low-density lipoprotein cholesterol; HDLc, high-density lipoprotein cholesterol; K-value, a value ≤ -0.1 represents the presence of small dense LDL; HOMA-index, homeostasis model assessment-index.*

The ApM1 gene and related phenotypes of FCH

Single SNP analyses for the related phenotypes of FCH resulted in a significant association of the 639C>T SNP with apoB (p = 0.048) and HDLc levels (p = 0.049) (Table 4). Haplotype analyses showed that these associations were due to undertransmission of the wildtype haplotype (TC) to subjects with high apoB levels (p = 0.014) and to subjects with low HDLc levels (p = 0.047). As presented in table 5, this resulted, in normolipidemic relatives and

spouses, in significant higher apoB levels in subjects carrying only a rare allele for the 639C>T SNP (1008 mg/L; p = 0.003) and a trend towards higher apoB levels in compound individuals (994 mg/L; p = 0.055), compared to the wildtype indivuals (946 mg/L). The lowest HDLc levels were present in subjects carrying only a rare allele for the 639C>T allele (1.19 mmol/L), this was significantly different from levels found in wildtype individuals (1.25 mmol/L; p = 0.043). The HDLc levels in compound individuals (1.24 mml/L) did not differ from the levels found in wildtype individuals (Table 5).

Single SNP analyses also showed a trend towards significance for the 45T>G SNP with TG (p = 0.055), haplotype analyses showed that this was due to undertransmission of the haplotype with a rare allele for both SNPs (GT) (p = 0.087). In normolipidemic relatives and spouses, this resulted in high TG levels in subjects carrying a rare allele for only the 639C>T SNP (1.18 mmol/L) compared to wildtype individuals (1.07; p = 0.028) and compound individuals (1.08 mmol/L; p = 0.072).

Furthermore, haplotype analyses showed an association of the wildtype haplotype with LDLc (p = 0.048) and TC (p = 0.058) levels. The highest LDLc levels were found in compound individuals (3.40 mmol/L [95% CI: 3.24 – 3.56), which were borderline significant different from the levels found in wildtype individuals (3.22 [95% CI: 3.08 – 3.36]; p = 0.077). No significant difference in TC levels was found between the different genotypes (Table 4 and 5).

Table 5 Characteristics of the normolipidemic relatives and spouses according to the *ApM1* gene 45T>G and 639C>T SNPs.

	Wildtypes n = 137 (30.2%)	Rare allele 639C>T n = 207 (45.6%)	Compounds n = 104 (22.9%)
Adiponectin (ng/mL) *	2.65 (2.45 - 2.86)	2.64 (2.47 - 2.82) †	2.90 (2.66 - 3.16) §
CVD (n (%))	6 (4.4%)	13 (6.3%)	4 (3.8%)
IMT (mm)	0.75 (0.72 - 0.77)	0.73 (0.71 - 0.75)	0.73 (0.71 - 0.76)
Total cholesterol (mmol/L)	4.91 (4.76 - 5.07)	5.03 (4.89 - 5.16)	5.07 (4.89 - 5.24)
Triglycerides (mmol/L)	1.07 (0.99 - 1.16)	1.18 (1.11 - 1.27) † ‡	1.08 (1.01 - 1.15) §
Apolipoprotein B (mg/L)	946 (912 - 980)	1008 (979 - 1036)	994 (955 - 1033) §
LDLc (mmol/L)	3.22 (3.08 - 3.36)	3.33 (3.21 - 3.45)	3.40 (3.24 - 3.56)
HDLc (mmol/L)	1.25 (1.20 - 1.31)	1.19 (1.14 - 1.23) †	1.24 (1.18 - 1.30)
K-value ≤ -0.1 (n (%))	53 (28.3%)	96 (35.6%)	56 (38.1%)

*Bivariate variables are presented as number (%) and the continuous variables, standardized for age and gender, are presented as mean (95% confidence interval); *, Ln-transformed variable; Wildtypes, subjects carrying only the common allele for both SNPs; Rare allele 639C>T; subjects carrying either 1 or 2 rare alleles for the 639C>T SNP; Compounds, subjects carrying a rare allele for both SNPs; CVD, cardiovascular disease; IMT, mean common carotid intima-media thickness; LDLc, low-density lipoprotein cholesterol; HDLc, high-density lipoprotein cholesterol; K-value ≤ -0.1 represents the presence of small dense LDL; †, p-value < 0.05, compared to wildtypes; ‡, p-value < 0.10, compared to compounds; §, p-value < 0.10, compared wildtypes.*

No associations were found for any of the other investigated lipid (K-value), obesity (waist circumference) or insulin resistance (HOMA-index) parameters (Table 4 and 5). Taking the non-independence of the families and the possible presence of linkage (-e option) into account, yielded in comparable results (data not shown).

ApM1 gene in relation to FCH and CVD

The 45T>G SNP showed a trend towards an association with FCH (p = 0.098). The 639C>T SNP was not associated with FCH (p = 0.243). The possible association of the 45T>G SNP disappeared when performing haplotype analyses and did not result in a significant increased risk of FCH, when carrying this allele (Table 4).

For the 45T>G SNP we found a non-significant trend towards an association with IMT values (p = 0.086), resulting from an overtransmission of the T allele to subjects with high IMT values. No differences in IMT values or prevalence in CVD were, however, found for carriers of the different genotypes (Table 5).

Discussion

In the present study we show that variants in the *ApM1* gene contribute to low adiponectin levels in patients with FCH. Adiponectin levels were 19% lower in FCH patients carrying the 45T allele. The effect of the *ApM1* gene on adiponectin levels, was, however, not specific for FCH, as in normolipidemic relatives and spouses adiponectin levels were approximately 9% lower in carriers of the 45 T allele.

Initially we sequenced the *ApM1* gene in 18 FCH patients in search for new variants. The *ApM1* gene has previously been sequenced in several different study populations, but not in FCH patients [358-362]. Sequencing the *ApM1* gene in FCH patients did not result in the identification of new SNPs.

The 45T>G SNP is one of the most common and widely studied SNPs of the *ApM1* gene, however, reported associations for this SNP with adiponectin levels are controversial [356]. In Caucasians, both alleles of the 45T>G SNP are reported to be associated with lower adiponectin levels [357, 358, 363, 364]. The two largest studies, both in over 1,000 individuals, report that decreased adiponectin levels are associated with the wildtype T allele, which is in agreement with results of the present study [357, 358].

For the other investigated SNP, the 639C>T, in the *ApM1* gene, no association with adiponectin levels was found in our study of well-defined FCH families. The 639C>T SNP has never been studied before, as it is in strong linkage disequilibrium (LD) with the 276G>T SNP and the 712G>A SNP [357]. The results for these two SNPs are, however, conflicting as both alleles are reported to be associated with lower adiponectin levels [356]. It is suggested that these conflicting results can be caused by the presence of another functional SNP for which these SNPs act as markers [356]. We selected the 639C>T SNP for the haplotype analyses in our FCH study population. No association with adiponectin levels for either allele was found, so the results for the 639C>T SNP could not explain the conflicting results previously found for the 276G>T and the 712G>A SNPs.

Despite the effect of *ApM1* gene on adiponectin levels, the *ApM1* gene was not associated with FCH. This may be explained by the observation that variants in the *ApM1* gene explain only 1.1% of the variation in adiponectin concentration. In the literature the combination of 8 SNPs, including the 45T>G and the 276G>T SNPs, have been found to explain approximately 8% of the variance of adiponectin levels [357]. Patients with FCH have approximately 15 - 20% lower adiponectin concentrations than controls [355]. Considering that this leaves a maximum of 0.2% of the variation in the decreased adiponectin levels in FCH patients that can directly be explained by variants in the *ApM1* gene, it is not surprising that we have missed a possible existing association. From this, and the fact that no association with FCH was found, we conclude that the *ApM1* gene is not a candidate gene for FCH and there are other mechanisms contributing to the decreased adiponectin levels in patients with FCH. An intrinsic defect in the adipocytes, like the presence of more large triglyceride-filled adipocytes, can result in dysregulation of the secretion of adiponectin in patients with FCH [250].

Low adiponectin levels were recently found to be related with the atherogenic lipid profile, including high levels of triglycerides, low levels of HDLc and the presence of small dense LDL, in our population of FCH patients [355]. This association with an atherogenic lipid profile has previously also been reported in other non-FCH study populations [111, 332, 336-339, 341]. In the present study we show that variants in the *ApM1* gene are also associated with a more atherogenic lipid profile. The rare allele of the 639C>T SNP is associated with high apoB levels, high TG levels and decreased HDLc levels. The rare allele of the 45T>G SNP counteracts this effect of the 639C>T SNP on TG and HDLc levels, resulting in normalized levels of TG and HDLc in compound individuals. However, in the present study, variants in the *ApM1* gene explain, independent of age and gender, less than 1% of the variation in the atherogenic lipid profile. So, the clinical relevance of these genetic variants on the atherogenic lipid profile is low. A relation of variants in the *ApM1* gene with a more atherogenic lipid profile has previously been reported in other study populations [357, 365-367].

Despite the association with the atherogenic lipid profile, no effect of the *ApM1* gene on IMT and CVD was found. Previously, decreased adiponectin levels were also not found to be associated with CVD in our population of FCH families [355]. Although a negative association of adiponectin levels with IMT values has recently been reported, it was not supported by other studies [357, 368, 369]. We now report that an association of variants in the *ApM1* gene with IMT values or the prevalence of CVD is not present in our Dutch FCH families.

Linkage analyses performed with the variants in the *ApM1* gene did not result in evidence of linkage for FCH or its related phenotypes. Previously, genome-wide linkage analyses did also not result in the identification of a region on interest on chromosome 3q27, including the *ApM1* gene. From the literature it is, however, known that linkage analyses have limited power to identify genes with moderate effects for complex diseases [203, 370]. Family-based SNP and haplotype association analyses, as performed in this study, have greater power to detect moderate effects [203, 371, 372].

Limitations of the present study are the low number of subjects with CVD and the availability of IMT data in only a sub-set of the study population, resulting in limited power to fully exclude the relevance of the *ApM1* gene in CVD and IMT values.

In summary, variants in the *ApM1* gene were associated with decreased adiponectin levels and an atherogenic lipid profile, in both FCH patients and healthy controls, but the clinical

significance seems limited. Furthermore, no association was found with IMT values and the prevalence of CVD. So, we did not find any indication that genetic variations in the *ApM1* gene are associated with FCH.

Summary, discussion and future perspectives

Despite more than 30 years of research, the genetic and metabolic origin of familial combined hyperlipidemia is still not fully understood. The studies described in the present thesis contribute to the elucidation of the genetic basis and metabolic pathogenesis of FCH. In the first part of the present thesis we have focused on the genetic origin of FCH (**Chapter 1 – 5**), and in the second part we have explored the role of adipose tissue in the metabolic origin of FCH (**Chapter 6 – 11**).

Part 1: The genetic origin of familial combined hyperlipidemia

Linkage analyses

In **chapter 1** we performed linkage analyses for two different diagnostic criteria of FCH in our Nijmegen FCH study population. Genome-wide linkage analyses performed with the traditional diagnostic criteria, based on TC and/or TG above the age- and sex-related 90th percentile, did not result in the identification of a chromosomal region of interest (lod score < 1.0). The nomogram, based on plasma TC and TG levels, adjusted for age and gender, and absolute apoB levels, was also used as a diagnostic criterion for FCH. This resulted in the identification of two regions of interest (lod score > 1), on chromosome 6q25-27 (lod score = 1.30) and 12q23-24 (lod score = 1.02). Linkage analysis of two characteristic quantitative traits of FCH, including TG levels and sdLDL, supported chromosome 6q as potential loci for FCH (lodscore = 1.13 for sdLDL and lodscore = 1.92 for TG levels). Most interestingly, suggestive evidence of linkage for FCH on chromosome 6q has previously been reported in another Dutch FCH population [31]. Linkage analysis of the other quantitative traits of FCH showed four regions with suggestive evidence of linkage, including TG levels (10p14-q21 (lod score = 2.24)), total cholesterol levels (1p32-12 (lod score = 1.98) and 8p22-21 (lod score = 2.11)) and HDL-cholesterol levels (19q13 (lod score = 1.73)).

The results of our linkage analyses are of main importance, as we show that the use of different diagnostic criteria affects the outcome of linkage analyses and that the analyses of the underlying traits are more powerfull. This may be one explanation for the fact that linkage analyses performed by various other research groups, all using different diagnostic criteria for FCH, have resulted in the identification of a diversity of loci spread over the entire genome [26-36, 42]. Other possible explanations for the diversity of loci reported for FCH by different study populations include ethnic admixture and population stratification. Since FCH is a complex multifactorial lipid disorder, genetic heterogeneity is potentially present in different FCH populations, which may affect the outcome of linkage analyses and contribute to the fact that most studies are underpowered to resolve loci of interest.

Candidate genes

In literature two loci, on chromosome 1q and 11q, have repeatedly been reported as regions of interest for FCH [26-33]. Until now, two candidate genes have been proposed to underlie the linkage signal on chromosome 1q, the *TXNIP* and the *USF1* gene. On chromosome 11q, genes of the apolipoprotein *A1/C3/A4/A5* gene cluster have been suggested to underlie the linkage signal for FCH.

The TXNIP gene

Based on a mutant mouse strain with phenotypic similarities with FCH patients, the *TXNIP* gene has been proposed as a candidate gene underlying the linkage signal for FCH on chromosome 1q [44]. In **chapter 2** we investigated the putative role of the *TXNIP* gene in our FCH families, by sequencing the coding region, the 5'UTR and all introns of the *TXNIP* gene in 10 well-defined FCH patients and 5 healthy controls. We did not find any sequence variants in the *TXNIP* gene in patients with FCH or controls. Other research groups have also sequenced the *TXNIP* gene in patients with FCH and although rare sequence variants have been identified in these FCH study populations, none of the variants were associated with FCH [46, 150].

Complete exclusion of the *TXNIP* gene as a candidate gene was, however, not possible as the 3'UTR, which is known to influence the expression levels of genes, has not been sequenced. In **chapter 3** the role of the TXNIP gene in FCH patients was further explored by quantification of TXNIP mRNA expression levels in peripheral blood mononuclear cells (PBMCs) of 30 FCH patients and 30 healthy controls. No differences in expression levels were found between FCH patients and controls. Based on both expression levels and sequence data, we can conclude that the TXNIP gene is not a major contributor to the FCH phenotype.

The USF1 gene

With the *TXNIP* gene excluded, another gene on chromosome 1q, the upstream stimulatory factor 1 (*USF1*) gene, has been suggested as a prime candidate gene for FCH [46]. In a group of Finnish FCH families, two single-nucleotide polymorphisms (SNPs) in the *USF1* gene showed evidence of linkage and association with FCH and multiple phenotypes of FCH [46]. In **chapter 4** we performed family-based SNP and haplotype analyses of these two SNPs in the *USF1* gene, to evaluate the putative role of this gene in Dutch patients with FCH. We show that the previously identified risk haplotype of *USF1* is not associated with FCH defined by the nomogram ($p = 0.55$) whereas suggestive associations were found when using the traditional diagnostic criteria for FCH (USF1s1: $p = 0.08$ and USF1s2: $p = 0.07$). Suggestive associations with important phenotypes of FCH, including total cholesterol levels (USF1s1: $p = 0.05$ and USF1s2: $p = 0.04$), apolipoprotein B levels (USF1s1: $p = 0.06$ and USF1s2: $p = 0.04$) and the presence of small dense LDL (USF1s1: $p = 0.10$ and USF1s2: $p = 0.09$) were found. Several other research groups have also tried to replicate the results found in the Finnish FCH families [42, 47]. The results of these studies, performed in Mexican FCH families and in pedigrees from Utah, were supportive for the role of the *USF1* gene in FCH, although the results were less conclusive than found in the Finnish FCH families [42, 47]. Based on the regulatory function of *USF1* on the transcriptional activation of important enzymes in the glucose and lipid metabolism, *USF1* was thought to be a good candidate gene for FCH. In our study population, however, variants in the *USF1* gene only explain approximately 1% of the variations in total cholesterol, apolipoprotein B and small dense LDL. To further explore the role of *USF1* in FCH, we measured the expression levels of *USF1* mRNA in PBMCs of 30 FCH patients and 30 healthy controls and did not find haplotype-dependent differences in expression of *USF1*. In conclusion, the previously identified risk haplotype of *USF1* showed a suggestive association with FCH and its related lipid traits but explains only 1% of the variation in TC, apoB, sdLDL.

The APOA5 gene

In **chapter 5** of this thesis the association of the *APOA5* gene, located on chromosome 11q, with FCH and its related phenotypes was evaluated. We have genotyped two SNPs in the *APOA5* gene (-1131T>C and S19W) and analyzed the data by family-based SNP and haplotype analyses and the measured genotype method. We demonstrate that the S19W variant in the *APOA5* gene is associated with an increased risk on FCH (OR = 1.6 [95% CI: 1.0, 2.6]) and a more atherogenic lipid profile, including higher TG levels (+22%), higher apoB levels (+5%), decreased HDLc levels (-7%) and the presence of sdLDL (+10%). The S19W explained 1 - 3 % of the genetic variation in FCH and its related phenotypes. So, the *APOA5* gene might be the gene underlying the linkage signal on chromosome 11q. Through both linkage and association analysis, the *APOA5* gene has also been reported to be associated with FCH in other study populations [33, 50, 51]. Despite the association of the variants in the *APOA5* gene with a more atherogenic lipid profile, in literature contradictory results for the possible association of the *APOA5* gene with intima-media thickness (IMT) values and CVD in Caucasian populations have been reported [189, 193-197, 205]. We show that the *APOA5* gene does not contribute to a thicker intima-media thickness of the carotid artery or an increased prevalence of CVD in FCH families. This may be attributable to the low number of subjects with CVD.

Part 2: The metabolic origin of familial combined hyperlipidemia

The pathophysiology of FCH

In **chapter 6** we review the pathophysiology of FCH in relation to the diagnostic criteria and its characteristic phenotypes.

An important diagnostic criterion of FCH is elevated plasma TG levels, which reflect TG-rich lipoproteins. Triglyceride-rich lipoproteins are, however, a heterogeneous group of particles some of which are more atherogenic than others. The remnant-like particles (RLP) are characterized as the most atherogenic triglyceride-rich lipoproteins. In **chapter 7** we explored the role of remnant-like particles cholesterol (RLPc) as a characteristic phenotype of FCH and its contribution to the increased risk of CVD in patients with FCH. Patients with FCH were found to have two-fold higher RLPc levels (males 0.59 mmol/l; females 0.40 mmol/l) compared to normolipidemic relatives (males 0.27 mmol/l; females 0.22 mmol/l) and spouses (males 0.27 mmol/l; females 0.24 mmol/l). Previous studies have also demonstrated that lipoprotein metabolism in FCH patients is disturbed, leading to remnant accumulation in the circulation; however, different methodologies to isolate lipoprotein remnants were used [93, 269, 373]. These increased RLPc levels contribute to the increased prevalence of CVD in FCH patients of our study population, independently of nonlipid cardiovascular risk factors (OR 2.18 [1.02 - 4.66]). RLPc levels did, however, not provide additional information about prevalent CVD over and above non-HDLc levels. So, we conclude that patients with FCH have two-fold elevated plasma RLPc levels, which contribute to the increased risk for CVD but not independent of non-HDL cholesterol levels. It remains, however, to be studied in large epidemiological studies to what extent the measurement of plasma RLPc provides additional knowledge for the treatment goals of the individual patients.

Adipose tissue metabolism

In the pathophysiology of FCH, a disturbed adipose tissue metabolism is hypothesized to play an important role. Generally, it is thought that in patients with FCH, hepatic overproduction of VLDL, combined with a delayed clearance of atherogenic triglyceride-rich lipoproteins, are caused by inadequate FFA trapping in the adipose tissue, as reviewed in **chapter 6** [91, 92, 96]. Besides impaired FFA trapping, adipose tissue may also contribute to the FCH phenotype and its increased risk of CVD by secretion of adipokines, which are involved in glucose and lipid metabolism and atherogenesis.

Adipokines

In the present thesis, the possibility that a disturbed adipose tissue metabolism is the culprit of FCH was approached by the evaluation of two adipokines, leptin and adiponectin.

Leptin

In **chapter 8** we explored the role of leptin in FCH. We showed that leptin levels were increased in patients with FCH; however, these levels are higher, proportional to their increased adiposity and the presence of insulin resistance. Previously, leptin levels were also measured in two small independent FCH populations [247, 248]. The results of these studies were contradictory. Leptin levels were, furthermore, only corrected for the presence of obesity [247, 248]. We conclude that patients with FCH have increased leptin levels, however, this is not attributable to a specific defect in the adipose tissue metabolism in FCH, as leptin levels are higher in proportion to their obesity and insulin resistance state. In our study population, increased leptin levels are related to increased risk for CVD both in FCH patients and non-FCH individuals (overall; OR = 3.3 [1.7 - 6.4]). So, increased leptin levels in FCH do contribute to the increased risk of CVD.

In **chapter 9**, the possible role of genetic variations in the leptin receptor (*LEPR*), which is the transporter of leptin, was explored. For this, we have genotyped the functional Gln223Arg SNP in our FCH study population. The Gln223Arg is located within the region encoding the extracellular domain of the leptin receptor and therefore associated with the binding activity of leptin [38, 313]. The Gln223Arg SNP in the *LEPR* gene is associated with a 1.6 times increased risk of FCH. The association of the *LEPR* gene with FCH was further supported by the presence of decreased HDLc levels in control carriers of one or two Arg alleles (1.21 mmol/L) compared to controls with the Gln223Gln genotype (1.28 mmol/L; p = 0.041). However, as no significant differences were found for other lipid and lipoproteins, it is possible that the association found for HDLc originates from chance alone. Previous studies also show an association of the Gln223Gln with HDLc levels, but always in combination with other lipid and lipoproteins [314, 320]. So, the *LEPR* gene is a modifier gene for FCH.

Adiponectin

In **Chapter 10** we explore the role of adiponectin in FCH. We report significant lower adiponectin levels in both male and female FCH patients (males 2.0 ng/ml (95% CI [1.8 – 2.2]; females 2.5 ng/ml (95% CI [2.3 – 2.8]), compared to normolipidemic relatives (males 2.3 ng/ml (95% CI [2.2 – 2.5]; females 3.1 ng/ml (95% CI [2.8 – 3.3]) and spouses (males 2.4 ng/ml (95% CI [2.1 – 2.7]; females 3.2 ng/ml (95% CI [2.8 – 3.6]). Based on the presence of obesity

and insulin resistance in patients with FCH, these decreased adiponectin levels could be expected [374]. The decreased adiponectin levels in patients with FCH were, however, independent of their body adiposity and degree of insulin resistance. In addition, decreased adiponectin levels were the strongest independent predictor of the atherogenic lipid profile, including high triglyceride levels, low HDLc levels and the presence of small dense LDL. The relation of adiponectin with an atherogenic lipid profile was not specific for FCH patients and has previously been reported in other non-FCH study populations [111, 336-341]. The decreased adiponectin levels found in patients with FCH support the concept of a disturbed adipose tissue metabolism in the pathophysiology of FCH.

In **chapter 11** we describe the screening of the gene encoding for adiponectin, the adipose most abundant gene transcript 1 (*ApM1*) gene. The association of two SNPs, the 45T>G and the 639C>T SNPs, with FCH and its associated atherogenic lipid profile were studied by family based SNP and haplotype analyses. No association of variants in the *ApM1* gene with FCH was found. Variants in the *ApM1* gene were associated with decreased adiponectin levels and an atherogenic lipid profile, however, these associations were not specific for FCH. Previously, the variants in the *ApM1* gene were found to be associated with a more atherogenic lipid profile [111, 332, 336-339, 341]. So, we conclude that the *ApM1* gene is not a candidate gene for FCH and that other mechanisms have to contribute to the decreased adiponectin levels in patients with FCH. For example, an intrinsic defect in the adipocytes, like the presence of more large triglyceride-filled adipocytes, can cause the deregulation of adiponectin in patients with FCH [250].

An association of the *ApM1* gene with intima-media thickness (IMT) values has also previously been reported, but could not be replicated in another study [357, 368, 369]. No association with IMT values and the prevalence of CVD was found in our FCH patients, normolidemic relatives and spouses. Based on these results, we have found no evidence for the ApM1 gene to be a candidate gene for FCH in our Dutch FCH families.

Conclusions

1. The use of different diagnostic criteria for FCH affects the results of linkage analyses.
2. Linkage analyses for FCH and its related phenotypes have resulted in the identification of a locus on chromosome 6q.
3. The investigated candidate genes on chromosome 1q21-23, including *TXNIP* and *USF1*, have not resulted in the identification of a major gene in our Dutch FCH population.
4. The minor allele of the S19W SNP in the *APOA5* gene is associated with FCH and an atherogenic lipid profile, but not with prevalent CVD.
5. Plasma leptin levels are increased in patients with FCH. This is, however, in proportion to the presence of obesity and insulin resistance. The increased plasma leptin levels in patients with FCH contribute to the increased risk of CVD.
6. The Gln223Arg SNP in the *LEPR* gene is associated with an increased risk of FCH and decreased HDLc levels.

7. Plasma adiponectin levels are decreased in patients with FCH, independent of obesity and insulin resistance, supporting a potential role of adipose tissue in the pathophysiology of FCH. Furthermore, adiponectin is the strongest independent predictor of the atherogenic lipid profile, including high triglyceride level, low HDLc level, and the presence of small dense LDL.
8. The two investigated SNPs in the ApM1 gene are not associated with FCH, but do contribute to low adiponectin levels and an atherogenic lipid profile in FCH.

Future perspectives

Part 1: The genetic origin of familial combined hyperlipidemia

To further elucidate the genetic origin of FCH two major issues have to be addressed. First, worldwide consensus about the diagnostic criteria of FCH is obligatory, as the use of different diagnostic criteria influences the outcome of linkage analyses. Secondly, most genome-wide linkage analyses published for FCH are underpowered to detect linkage, resulting in the identification of discrepant loci. These two major issues can be resolved by initiating (inter)national collaborations between the different FCH study groups. Linkage analyses of the combined datasets with the use of an uniform diagnosis of FCH will increase power to detect a major locus for FCH.

Linkage analyses are known to be successful in the discovery of causal genes for monogenetic disorders. From literature it is, however, known that linkage analyses have limited power to identify genes with moderate effects for more complex diseases, like FCH. Association methods have been shown to be more powerful than linkage methods under a number of conditions that typically prevail in complex disease. The power of association methods further improved with the recognition that haplotypes typically provide substantially more power to detect association than individual SNPs. Family-based haplotype analyses in extended pedigrees, however, remain a challenge, as the current programs are not capable of handling extended pedigrees.

As future research probably will focus on the genotyping of many SNPs for every candidate gene, the application of high-throughput sequencing and SNP genotyping technologies are necessary. The recent development of the 500,000 SNP array will allow us to study thousands of SNPs at a time. Although great progress has already been made in genetic epidemiology the challenge remains to analyse these large amounts of data and to deal with the multiple comparison issue.

Part 2: The metabolic origin of familial combined hyperlipidemia

The evidence for an important role of the adipose tissue in the pathophysiology of FCH has become more apparent. In the present thesis we have shown that adiponectin levels in plasma, one of the major adipokines, are significantly lower in patients with FCH, independent of obesity and insulin resistance. Therefore, a primary role of adiponectin in the pathogenesis of FCH is suggested. Because the origin of low plasma adiponectin levels in patients with FCH is still unclear, this should be a major focus for future research.

Recently, different complexes of adiponectin have been identified in the circulation with a different effect on insulin sensitivity and inflammaroty mediators. To further investigate the role of decreased adiponectin levels in patients with FCH, the different complexes of adiponectin

have to be measured. Furthermore, the regulation of the production of different isoforms of adiponectin takes place within the adipocyte, and it is of interest to see if this proces is disturbed in patients with FCH.

The next step to resolve the origin of low adiponectin levels in patients with FCH is to take adipose tissue biopsies to measure RNA and protein expression levels and study the signaling pathway involved in adiponecin production and secretion. In this respect it would be of great interest to explore the role of subcutaneous vs. visceral adipose tissue in adiponectin production and secretion. Furthermore, adipocyte size has been shown to influence cytokine production. These in vitro and ex vivo experiments should be complemented with in vivo studies, using labelled glucose and/or fatty acids in dynamic positron emission tomography (dPET) studies and CT-scan in combination with insulin clamp techniques. These studies will reveal the role of visceral and subcutaneous adipose tissue and the liver in the pathophysiology of FCH.

To further elucidate the role of the adipose tissue in FCH, the expression of other proteins released by the adipose tissue should also be measured in the adipose tissue (e.g. adipsin, resistin, tumor necrosis factor alpha, interleukine 6 and plasminogen activator inhibitor 1). The relation of these proteins with FCH should be investigated not only by measuring protein levels in the circulation or genotyping variants in the encoding genes, but also by measuring expression levels in the adipose tissue. This can be done by expression arrays, which is a powerful tool to investigate many proteins at a time and detect proteins, monitor their expression levels, and investigate protein interactions and functions.

Patients with FCH share many features of patients with the metabolic syndrome (MS) and type 2 diabetus mellitus (DM2), including insulin resistance, obesity, dyslipidemia and increased risk of atherosclerosis. Also in patients with the MS or DM2, low adiponectin levels have

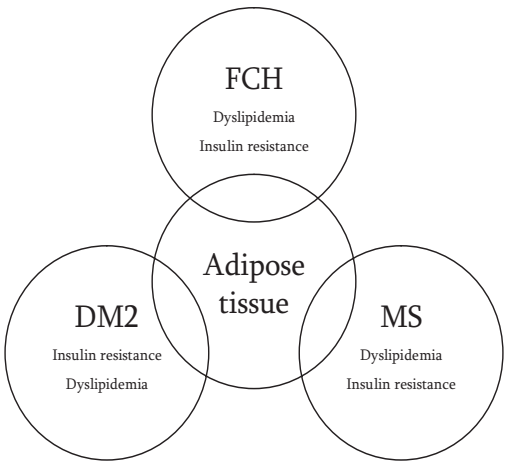

Figure 1
Obesity is a common feature of three metabolic diseases with high prevalence in the general population, including familial combined hyperlipidemia (FCH), type 2 diabetus mellitus (DM2) and the metabolic syndrome (MS). The unique role of a disturbed adipose tissue metabolism in patients with FCH can be explored by evaluating adipose tissue metabolism in patients with FCH, type 2 diabetus mellitus, obesity and the metabolic syndrome.

been reported. To elucidate the unique role of a disturbed adipose tissue metabolism in the pathophysiology of FCH, in vivo and in vitro experiments of the adipose tissue have to be performed not only in patients with FCH and controls, but also in patients with obesity, the metabolic syndrome and type 2 diabetus mellitus (Figure 1).

Nederlandse samenvatting

Hart- en vaatziekten

Al sinds vele jaren zijn hart- en vaatziekten de meest voorkomende doodsoorzaken in westerse landen. In 2004 zijn in Nederland 45.445 mensen ten gevolge hiervan overleden. Dit is gelijk aan 33% van het totale sterftecijfer van dat jaar [1]. Voor hart- en vaatziekten zijn veel risicofactoren bekend, waaronder roken, diabetes mellitus, hoge bloeddruk, overgewicht, gebrek aan beweging en een hoog cholesterolgehalte in het bloed. Omdat tegenwoordig bijna 50% van de volwassen Nederlandse bevolking aan overgewicht lijdt, is overgewicht (BMI > 25 kg/m2) een belangrijke risicofactor voor het krijgen van hart- en vaatziekten. Bij ongeveer 4% van de patiënten met nieuw gediagnosticeerde hart- vaatziekten, was overgewicht te oorzaak [2, 3]. De relatie van een verhoogde cholesterolspiegel met hart- en vaatziekten is echter nog sterker. Bij ongeveer 20% van deze patiënten was een te hoog cholesterol gehalte de oorzaak [3]. Onder personen van de Nederlandse bevolking in de leeftijd van 20 jaar en ouder heeft ongeveer 10% een verhoogd cholesterol (> 6.5 mmol/L) en/of neemt cholesterolverlagende medicijnen [2].

Familiaire gecombineerde hyperlipidemie

In 1973 werd Familiaire geCombineerde Hyperlipidemie (FCH) voor het eerst beschreven als een erfelijke vetstofwisselingsziekte, die gekenmerkt wordt door wisselend verhoogde cholesterol en/of triglyceridegehalte in het bloed. Andere karakteristieke kenmerken van FCH zijn lage spiegels van HDL-cholesterol, hoge apolipoproteïne B-spiegels en de aanwezigheid van veel kleine, dichte, zeer atherogene LDL-cholesteroldeeltjes. Ongeveer 1 tot 5% van de Nederlandse bevolking heeft FCH. FCH wordt niet alleen gekenmerkt door een verstoorde vetstofwisseling, maar de patiënten met FCH zijn ook vaak te dik en hebben een afgenomen gevoeligheid voor de werking van insuline (insuline-resistentie).

Bij mensen met FCH is de kans op hart- en vaatziekten sterk verhoogd. In vergelijking met de hele Nederlandse bevolking hebben mensen met FCH een 2 tot 5 maal verhoogd risico op het krijgen van hart- en vaatziekten voor het 60ste levensjaar.

Omdat de kans op het krijgen van hart- en vaatziekten groot is bij mensen met FCH, is het belangrijk om deze mensen goed te kunnen herkennen en op tijd te starten met de behandeling. Echter, de diagnose van FCH is erg lastig omdat meerdere genetische, metabole en omgevingsfactoren een rol spelen.

Plasma cholesterol- en/of triglyceride-spiegels boven de leeftijd- en geslacht afhankelijke 90ste percentielen worden sinds 1973 als criteria voor de diagnose van FCH gebruikt (traditionele diagnose van FCH) [14, 15]. De variërende cholesterol- en triglyceride-spiegels binnen een persoon zorgen er echter voor dat de diagnose van FCH niet consistent is in de tijd [16]. Hierdoor kan een persoon op een bepaald tijdstip gediagnostiseerd worden als FCH-patiënt, terwijl op een ander tijdstip de persoon niet aan de diagnostische criteria voldoet. Om deze redenen hebben Veerkamp et al. het nomogram ontwikkeld [17]. Dit nomogram is gebaseerd op verhoogde cholesterol-, triglyceride- en/of apolipoproteïne B-spiegels. De diagnose van FCH op basis van het nomogram is consistenter in de tijd.

Ondanks meer dan 30 jaar onderzoek op het gebied van FCH zijn de genetische, metabole en

omgevingsfactoren die een rol spelen bij FCH nog steeds niet in zijn geheel opgehelderd. Met het onderzoek, beschreven in dit proefschrift, hebben wij een bijdrage geleverd aan de verbreding van de kennis over de genetische en metabole factoren die een rol spelen bij de ziekte FCH.

In het eerste gedeelte van dit proefschrift worden genetische factoren die mogelijk een rol spelen, belicht (**hoofdstuk 1 – 5**). In het tweede deel worden de metabole factoren beschreven die mogelijk een relatie hebben met het ontstaan van FCH (**hoofdstuk 6 – 11**).

Deel 1: Genetische factoren en FCH

Voor het ontrafelen van de genetische factoren die een rol spelen bij FCH, kunnen verschillende strategieën gebruikt worden: het zogenaamde koppelingsonderzoek en de associatieanalyses. Voor het koppelingsonderzoek worden genetische variaties (markers) op alle chromosomen getest binnen grote families met twee of meer personen met FCH. Door te onderzoeken of deze markers samen overerven met FCH, kunnen regio's op het chromosoom geïdentificeerd worden waar mogelijk genen liggen die betrokken zijn bij de ziekte FCH.

Het doel van associatieanalyses is het opsporen van ziekte allelen in kandidaat-genen die meer (of minder) vaak voorkomen bij mensen met FCH, dan in mensen zonder FCH.

Kandidaat-genen zijn genen die potentiëel betrokken zijn bij FCH op basis van het feit dat ze bijvoorbeeld liggen in regio's op de chromosomen die gevonden zijn door middel van koppelingsonderzoek. Kandidaat-genen zijn ook genen waarvan we op basis van de bekende functie denken dat ze een rol spelen in de ontwikkeling of verloop van FCH.

Bij associatiestudies wordt het mogelijke verband tussen een variatie in een gen en de ziekte (of de kenmerken van de ziekte) onderzocht. Bij associatiestudies kunnen ook meerdere variaties in een gen tegelijkertijd geanalyseerd worden. Dit noemt men haplotype-onderzoek. Doordat er meerdere variaties tegelijkertijd onderzocht worden, is met deze onderzoeken de kans groter om een bestaande associatie te vinden.

Linkage analyses

In **hoofdstuk 1** beschrijven we de resultaten van het koppelingsonderzoek dat we hebben uitgevoerd voor twee verschillende diagnostische criteria van FCH in onze Nijmeegse FCH populatie. Het koppelingsonderzoek voor FCH waarbij de diagnose is gesteld volgens de traditionele criteria, heeft niet geresulteerd in de identificatie van een relevante regio op een chromosoom.

Het koppelingsonderzoek voor de diagnose van FCH gebaseerd op het nomogram heeft geresulteerd in de identificatie van relevante regio's op chromosoom 6q25-27 en chromosoom 12q23-24. Bij de koppelingsonderzoeken van twee karakteristieke kenmerken van FCH, verhoogde triglyceridespiegels en de aanwezigheid van kleine, dichte LDL-deeltjes, werd suggestief bewijs voor linkage gevonden voor dezelfde regio op chromosoom 6q. Ook in een andere Nederlandse populatie van FCH-families werd suggestief bewijs voor linkage in deze regio op chromosoom 6q gevonden [31]. Het feit dat in deze regio op chromosoom 6q enkele potentiëel relevante kandidaat-genen liggen, ondersteunt onze data dat chromosooom 6q mogelijk betrokken is bij FCH en zeker verder onderzocht dient te worden.

Het koppelingsonderzoek voor de verschillende kenmerken van FCH heeft geresulteerd in

vier regio's met suggestief bewijs voor linkage. Voor triglyceride-spiegels werd op chromosoom 10p14-q21 een interessante regio gevonden, voor cholesterol-spiegels op chromosoom 1p32-12 en 8p22-21, en voor HDL-cholesterol-spiegels op chromosoom 19q13.

Met de door ons uitgevoerde linkage-analyses laten wij zien dat het gebruik van verschillende diagnostische criteria voor FCH van invloed is op de resultaten die gevonden worden met de linkage-analyses. Dit kan dus een verklaring zijn voor het feit dat de reeds eerder uitgevoerde linkage-analyses door andere onderzoeksgroepen hebben geresulteerd in de ontdekking van een grote verscheidenheid van mogelijk relevante gebieden die verspreid liggen over de verschillende chromosomen [26-36, 42]. Mogelijke andere verklaringen voor de diversiteit aan regio's met suggestief of significant bewijs voor linkage voor FCH in de verschillende populaties, zijn etnische verschillen en populatie-stratificatie. Omdat FCH een multifactoriële ziekte is, kan er sprake zijn van genetische heterogeniteit in de verschillende onderzoekspopulaties. Ook dit kan van invloed zijn op de resultaten die gevonden worden met de linkage-analyses en dragen bij aan het feit dat de meeste studies door relatief kleine groepen patiënten met FCH te weinig kans hebben om te leiden tot echt relevante regio's met significant bewijs voor linkage.

Kandidaat-genen

In de literatuur worden twee regio's, op chromosoom 1q en 11q, herhaaldelijk gerapporteerd als regio's met significant bewijs voor linkage met FCH [26-33]. Tot op heden zijn er twee genen, het *TXNIP*-gen en het *USF1*-gen, aangewezen als kandidaat-genen voor de regio op chromosoom 1q. Op chromosoom 11q worden de genen van het apolipoprotein *A1/C3/A4/A5*-gen cluster gesuggereerd als kandidaat-genen.

Het TXNIP-gen

Gebaseerd op resultaten, gevonden in een muizenmodel met kenmerken die overeenkomen met kenmerken van FCH-patiënten, werd het gen coderend voor 'thioredoxin interacting protein' (*TXNIP*) voorgesteld als kandidaat-gen in de regio met linkage voor FCH op chromosoom 1q [44].

In **hoofdstuk 2** hebben we de mogelijke rol van het *TXNIP*-gen in onze FCH families bestudeerd. Dit hebben we gedaan door het coderende gedeelte, het 5'UTR, en alle intronen van het *TXNIP*-gen in tien FCH-patiënten en vijf gezonde personen na te kijken. Er werden geen variaties in de sequentie van het *TXNIP*-gen in de patiënten en gezonde personen gevonden. Ook andere groepen hebben het *TXNIP*-gen bestudeerd bij patiënten met FCH. Ondanks het feit dat deze onderzoeksgroepen wel enkele variaties in de sequentie van het *TXNIP*-gen vonden, was geen van deze variaties geassocieerd met FCH [46, 150].

Volledige uitsluiting van het *TXNIP*-gen als een kandidaat-gen voor FCH was echter nog niet mogelijk omdat het 3'UTR niet bestudeerd is. Van het 3'UTR is bekend dat het invloed kan hebben op het expressieniveau van een gen. In **hoofdstuk 3** hebben we de rol van het *TXNIP*-gen in FCH-patiënten verder onderzocht door het expressieniveau van het *TXNIP*-gen in perifere bloedcellen van 30 FCH-patiënten en 30 gezonde personen te meten. Er werden geen verschillen in expressieniveau van het *TXNIP*-gen gevonden tussen de FCH-patiënten en gezonde personen. Gebaseerd op zowel de gegevens van de sequentie van het *TXNIP*-gen als het expressieniveau van het *TXNIP*-gen, concluderen wij dat het *TXNIP*-gen geen belangrijke bijdrage levert aan de kenmerken van de ziekte FCH.

Het USF1-gen

Op het moment dat het *TXNIP*-gen uitgesloten was als een belangrijk kandidaat-gen, werd een ander gen op chromosoom 1q, het 'upstream stimulatory factor 1' (*USF1*)-gen, als mogelijk kandidaat-gen voor FCH voorgesteld [46]. In een groep van Finse FCH-families is door middel van linkage- en associatie-analyses bewijs geleverd voor de betrokkenheid van twee single-nucleotide polymorfismen (SNPs) in het *USF1*-gen met FCH en enkele karakteristieke kenmerken van FCH [46]. In **hoofdstuk 4** hebben we de mogelijke rol van deze twee SNPs in het *USF1*-gen in Nijmeegse patiënten met FCH onderzocht. Dit hebben we gedaan door analyses op basis van een enkele SNP en op basis van haplotypes. Met de resultaten van deze analyses laten wij zien dat het risico-haplotype van het *USF1*-gen, zoals geïdentificeerd in de Finse FCH-patiënten, niet geassocieerd is met FCH gebaseerd op het nomogram, terwijl een suggestieve associatie met FCH gebaseerd op de traditionele criteria aanwezig was in onze Nijmeegse FCH-families. Het risico-haplotype was tevens geassociëerd met verhoogde cholesterolspiegels, verhoogde apolipoprotein B-spiegels en de aanwezigheid van kleine, dichte LDL-deeltjes. Ook andere onderzoeksgroepen hebben geprobeerd om de resultaten van de Finse onderzoeksgroep te bevestigen [42, 47]. De resultaten van deze studies, uitgevoerd in Mexicaanse FCH-families en families uit Utah, ondersteunen het feit dat variaties in het *USF1*-gen een rol spelen in FCH, hoewel de resultaten van deze studies minder duidelijk waren dan de resultaten van de studie in de Finse FCH-patiënten [42, 47]. *USF1* is gesuggereerd als kandidaat-gen voor FCH op basis van zijn regulerende functie op de transcriptie-activatie van belangrijke enzymen, die betrokken zijn bij de glucose- en vetstofwisseling. In onze studie-populatie konden de twee bestudeerde variaties in het *USF1*-gen echter maar ongeveer 1% van de variatie in cholesterol, apolipoprotein B en kleine dichte LDL-deeltjes spiegels verklaren. Om de rol van *USF1* in FCH-patiënten nog verder te onderzoeken hebben we het expressieniveau van *USF1* in perifere bloedcellen van 30 FCH-patiënten en 30 gezonde personen onderzocht. Wij hebben geen verschillen in expressieniveau van het *USF1*-gen gevonden tussen de verschillende *USF1* haplotypes. Op basis van de gevonden resultaten beschouwen wij het reeds eerder geïndentificeerde risico-haplotype van het *USF1*-gen niet als een belangrijk risico-haplotype voor FCH in onze Nijmeegse populatie van FCH-patiënten.

Het APOA5-gen

In **hoofdstuk 5** hebben we de mogelijke rol van het *APOA5*-gen, dat gelegen is op chromosoom 11q, in FCH onderzocht. Wij hebben het genotype van twee variaties (-1131T>C en S19W) in het *APOA5*-gen bepaald. Deze data hebben we in de FCH-families met behulp van SNP- en haplotype-analyses geanalyseerd. Wij laten zien dat de S19W-variatie in het *APOA5*-gen geassocieerd is met een verhoogd risico op FCH en een meer atherogeen lipidenprofiel. Tot dit atherogeen lipidenprofiel behoren verhoogde triglyceridespiegels, verhoogde apolipoproteïne B-spiegels, verlaagde HDLc-spiegels en de aanwezigheid van kleine, dichte LDL-deeltjes. De S19W variant in het *APOA5*-gen verklaarde 1 tot 3% van de genetische variatie in FCH en de karakteristieke kenmerken van FCH. Het *APOA5*-gen kan dus het gen zijn dat het gevonden bewijs voor linkage op chromosoom 11q kan verklaren. In andere FCH-studiepopulaties is ook gebleken dat het *APOA5*-gen geassocieerd is met FCH [33, 50, 51]. Ondanks de gevonden associaties van variaties in het *APOA5*-gen met een meer atherogeen lipidenprofiel, worden in de literatuur tegenstrijdige resultaten gevonden voor een mogelijke associatie van variaties in *APOA5*-gen met hart- en vaatziekten, dan wel met de dikte van de wand van de halsslagader (intima-media dikte

= IMT) als surrogaat marker voor hart- en vaatziekten [189, 193-197, 205]. In onze studie is geen bewijs gevonden voor een bijdrage van het *APOA5*-gen aan dikkere IMT. Tevens vinden we geen bewijs voor een associatie van de variaties in het *APOA5*-gen met een verhoogd voorkomen van hart- en vaatziekten in onze families met FCH. Een van de verklaringen waarom geen bewijs voor een associatie met IMT of hart- en vaatziekten is gevonden, kan zijn dat maar van een klein aantal personen IMT-data beschikbaar waren en dat maar een klein aantal personen hart- en vaatziekten had.

Deel 2: Metabole factoren en FCH

Verschillende metabole factoren dragen bij aan het tot expressie komen van FCH. Algemeen wordt aangenomen dat er bij patiënten met FCH sprake is van een overproductie van triglyceride-rijke lipoproteïnen (VLDL-deeltjes) door de lever. Dit leidt tot stapeling van triglyceride-rijke lipoproteïnen (o.a. remnant deeltjes genaamd). Echter de oorzaak van de overproduktie van VLDL-deeltjes is niet volledig opgehelderd. Een van de hypotheses is dat het vetweefselmetabolisme in patiënten met FCH is verstoord, hetgeen resulteert in onvoldoende opname van vrije vetzuren door het vetweefsel [91, 92, 96]. De vrije vetzuren blijven in het bloed en bereiken de lever, alwaar de vetzuren de productie van VLDL stimuleren.

Anderzijds kan een gestoord vetweefselmetabolisme leiden tot verandering in de produktie en afgifte van eiwitten (adiponectinen) door het vetweefsel, die betrokken zijn bij het glucose- en lipidenmetabolisme en het ontstaan van hart- en vaatziekten.

De pathofysiologie van FCH

In **hoofdstuk 6** wordt de pathofysiologie van FCH in relatie tot de verschillende diagnostische criteria en de karakteristieke kenmerken van FCH besproken.

Een belangrijk diagnostisch criterium van FCH zijn de verhoogde triglyceridespiegels, welke de aanwezigheid van triglyceride-rijke lipoproteïnen reflecteren. Tot de klasse van triglyceride-rijke lipoproteïnen behoort een grote verscheidenheid aan deeltjes, waarbij het ene deeltje meer atherogeen is dan de andere deeltjes. De remnant-like deeltjes (RLP) worden gekarakteriseerd als de meest atherogene triglyceride-rijke lipoproteïnen.

In **hoofdstuk 7** onderzoeken we de rol van remnant-like particles cholesterol (RLPc) als een karakteristiek kenmerk van FCH en de bijdrage van RLPc aan het verhoogde risico op hart- en vaatziekten van patiënten met FCH. De RLPc spiegels van patiënten met FCH bleken twee maal hoger te zijn in vergelijking tot de familieleden met een normaal lipidenprofiel en de aangetrouwde familieleden. Eerdere studies hebben ook al laten zien dat het lipoproteïne-metabolisme bij patiënten met FCH verstoord is, wat kan leiden tot een opstapeling van remnants in de bloedsomloop. De gebruikte methodes om de lipoproteïne remnants te isoleren verschilden echter van elkaar [93, 269, 373]. Deze verhoogde RLPc-spiegels dragen bij aan het verhoogde risico op hart- en vaatziekten bij patiënten met FCH in onze studiepopulatie. Dit was onafhankelijk van andere, niet-lipiden risicofactoren voor hart- en vaatziekten. RLPc spiegels gaven echter geen extra informatie over het voorkomen van hart- en vaatziekten boven de non-HDLc-spiegels. Daarom concluderen wij dat patiënten met FCH twee maal hogere RLPc-spiegels hebben in het bloed, welke bijdragen aan het verhoogde risico op hart- en vaatziekten, maar niet onafhankelijk

van non-HDL-cholesterolspiegels. In welke mate de meting van RLPc-spiegels extra kennis biedt voor de behandeling van individuele patiënten moet echter nog onderzocht worden in grote epidemiologische studies.

Vetweefselmetabolisme

Het vetweefsel werd lang gezien als een relatief passief orgaan voor opslag van energie. Tegenwoordig is het duidelijk dat het vetweefsel niet alleen betrokken is bij de opslag van energie, maar ook endocrien actief is door het vrijmaken van verscheidene eiwitten. Deze eiwitten zijn betrokken bij ontstekingen, glucose- en lipidenmetabolisme en atherogenese [103].

Adipokines

De mogelijke betrokkenheid van een verstoord vetweefselmetabolisme bij FCH hebben wij onderzocht aan de hand van twee belangrijke eiwitten, de zogenaamde adipokines. Deze twee adipokines zijn leptine en adiponectine.

Leptine

Leptine is betrokken bij de regulatie van het energieverbruik en het hongergevoel door middel van receptoren in de hypothalamus, waaronder de leptine-receptor [104]. Een verhoging van de leptine-spiegels zal leiden tot een hoger energiegebruik en een vermindering van het hongergevoel bij gezonde personen. Echter, bij dikke mensen lijkt de toename in leptine-spiegels te falen bij de beïnvloeding van de energie-inname of om de vetmassa te herstellen naar normale niveau's. Overgewicht wordt daarom gezien als een situatie waarin mensen immuun zijn voor de werking van leptine. Leptine is niet alleen geassociëerd met overgewicht, maar ook met insulineresistentie [105]. Dit komt door de directe werking van leptine op het vrijmaken van insuline. Leptine-spiegels zijn bovendien geassocieerd met hart- en vaatziekten.

In **hoofdstuk 8** onderzoeken we de mogelijke rol van leptine bij patiënten met FCH. Wij tonen aan dat leptine-spiegels verhoogd zijn bij patiënten met FCH. Deze verhoogde spiegels zijn echter evenredig met de mate van vetzucht en de aanwezigheid van insulineresistentie bij patiënten met FCH. Leptine-spiegels werden al eerder in twee andere FCH-populaties onderzocht [247, 248]. De resultaten van deze studies waren echter tegenstrijdig [247, 248]. Wij concluderen dat patiënten met FCH verhoogde leptine-spiegels hebben. Deze verhoogde leptine-spiegels zijn echter niet het gevolg van een specifiek defect in het vetweefselmetabolisme bij patiënten met FCH, omdat de verhoogde leptine-spiegels gerelateerd zijn aan het overgewicht en de mate van insulineresistentie bij deze FCH-patiënten. In onze studiepopulatie laten wij tevens zien dat verhoogde leptine-spiegels bijdragen aan een verhoogd risico op hart- en vaatziekten zowel bij FCH-patiënten als bij mensen zonder FCH. Verhoogde leptine-spiegels dragen dus bij aan het verhoogde risico op hart- en vaatziekten. Dit is echter niet specifiek voor FCH.

In **hoofdstuk 9**, wordt het onderzoek naar de mogelijke rol van genetische variaties in het leptine-receptor (*LEPR*)-gen beschreven. De leptine-receptor zorgt onder andere voor het transport van leptine. Wij hebben de functionele variatie van de *LEPR*-gen, de Gln223Arg-SNP, in onze FCH-studiepopulatie bepaald. De Gln223Arg-variant ligt in de regio die betrokken is bij de binding van deze receptor met leptine, en is daarom mogelijk van invloed op de bindingsactiviteit met leptine [38, 313]. De Gln223Arg variant in het *LEPR*-gen is geassocieerd met een 1,6 keer verhoogd risico op FCH. Deze associatie werd ondersteund door de aanwezigheid

van verlaagde HDLc-spiegels bij dragers van een of twee Arg-allelen door controlepersonen in vergelijking tot controlepersonen zonder een Arg-allel voor het Gln223Arg-variant in het *LEPR*-gen. Het is echter ook mogelijk dat de gevonden associatie voor HDLc-spiegels berust op toeval, omdat er geen significante verschillen werden gevonden voor de andere lipiden en lipoproteïnen. Eerdere studies hebben ook laten zien dat het Gln-allel van de Gln223Arg-variatie in het *LEPR*-gen geassocieerd is met HDLc-spiegels, maar deze associatie werd in deze andere studies altijd in combinatie met andere associaties voor lipiden of lipoproteïnen gevonden [314, 320]. Op basis van deze resultaten concluderen wij dat het *LEPR*-gen een gen is dat betrokken is bij het tot expressie komen van de karakteristieke kenmerken van FCH en het verhoogde risico op hart- en vaatziekten.

Adiponectine

In tegenstelling tot leptine is de productie van adiponectine omgekeerd evenredig gerelateerd aan de hoeveelheid vetweefsel [107]. Adiponectine stimuleert het oxidatie-proces van vrije vetzuren in de spieren, bevordert de insuline-sensitiviteit in de spieren en lever en remt ontstekingsactiviteit [107-110]. Adiponectine heeft bovendien direct en indirect invloed op lipidenbloedspiegels [111, 112]. Op basis van deze eigenschappen van adiponectine is gesuggereerd dat adiponectine de ontbrekende schakel zou kunnen zijn tussen overgewicht, insulinresistentie en atherosclerose.

In **hoofdstuk 10** onderzoeken we de rol van adiponectine in patiënten met FCH. Wij tonen aan dat zowel mannen als vrouwen met FCH significant lagere plasma-adiponectinespiegels hebben in vergelijking met familieleden met een normaal lipidenprofiel en aangetrouwde familieleden. Gebaseerd op het feit dat patiënten met FCH dikker en meer insulineresistent zijn, kunnen deze verlaagde spiegels van adiponectine verwacht worden [374]. De verlaagde adiponectinespiegels bij de patiënten met FCH waren echter onafhankelijk van het overgewicht en de mate van insulinegevoeligheid. Tevens bleek dat de verlaagde adiponectinespiegel de belangrijkste voorspeller was van een atherogeen lipidenprofiel. De relatie van adiponectine met een atherogeen lipidenprofiel is waarschijnlijk niet kenmerkend voor patiënten met FCH en is ook al eerder gevonden in andere niet-FCH-populaties [111, 336-341]. Het feit dat we verlaagde adiponectinespiegels hebben gevonden bij patiënten met FCH, onafhankelijk van overgewicht en de mate van insulinegevoeligheid ondersteunt de hypothese dat een verstoord vetweefselmetabolisme mogelijk een rol speelt in het ontstaan van FCH.

In **hoofdstuk 11** beschrijven we de rol van variatie in het *ApM1*-gen in FCH. Het *ApM1*-gen codeert voor adiponectine. De associatie van twee variaties in het *ApM1*-gen, de 45T>G en de 639C>T-SNPs, met FCH en het geassocieerde atherogeen lipidenprofiel werd bestudeerd in de FCH-families met behulp van SNP- en haplotype-analyses. Er werd geen relatie van de variaties in het *ApM1*-gen met FCH gevonden. Variaties in het *ApM1*-gen bleken wel gerelateerd te zijn aan verlaagde adiponectinespiegels en een atherogeen lipidenprofiel. Deze relaties waren echter niet specifiek voor FCH. In eerdere studies werd de relatie van de variaties in het *ApM1*-gen met verlaagde adiponectinespiegels en een atherogeen lipidenprofiel ook gevonden [111, 332, 336-339, 341]. Wij concluderen dus dat het *ApM1*-gen geen kandidaatgen is voor FCH en dat er andere mechanismen zijn die bijdragen aan de verlaagde adiponectinespiegels die gevonden worden bij patiënten met FCH. Een voorbeeld van zo'n ander mechanisme is de aanwezigheid van een intrinsiek defect in de vetweefselcellen. De aanwezigheid van meer grote met triglyceride-gevulde vetweefselcellen kunnen bijvoorbeeld de adiponectineproductie bij patiënten met FCH

beïnvloeden [250]. De eerder gerapporteerde associatie met IMT werd niet in een andere studie bevestigd [357, 368, 369]. In onze FCH-populatie werd geen bewijs voor een associatie van variatie in het *ApM1*-gen met IMT-waarden of de prevalentie van hart- en vaatziekten gevonden. Gebaseerd op deze resultaten concluderen wij dat er geen bewijs is gevonden voor het *ApM1*-gen als kandidaat gen voor FCH in onze Nijmeegse FCH-populatie.

Conclusies

1. Het gebruik van verschillende criteria voor het stellen van de diagnose FCH is een mogelijke verklaring voor de verschillen in de gevonden resultaten bij koppelingsanalyses.
2. Koppelingsonderzoek voor de diagnose van FCH en de karakteristieke kenmerken van FCH hebben geresulteerd in de identificatie van een regio op chromosoom 6q waar mogelijk gen(en) ligt (liggen) die betrokken is (zijn) bij FCH.
3. De kandidaatgenen, *TXNIP* en *USF1*, op chromosoom 1q21-23 spelen geen belangrijke rol in FCH in de Nijmeegse FCH-populatie
4. Het W-allel van de S19W-variant in het *APOA5*-gen is geassocieerd met FCH en een atherogeen lipidenprofiel.
5. Leptine-spiegels zijn verhoogd bij patiënten met FCH. Dit is echter in verhouding met de mate van overgewicht en insulineresistentie bij deze patiënten. De verhoogde leptine-spiegels in patiënten met FCH dragen bij aan het verhoogde risico op hart- en vaatziekten.
6. De Gln223Arg-variatie in het *LEPR*-gen is geassocieerd met een verhoogd risico op het krijgen van FCH en met verlaagde HDLc-spiegels.
7. Plasma adiponectinespiegels zijn verlaagd bij patiënten met FCH, dit si echter onafhankelijk van de mate van overgewicht en de mate van insulineresistentie. Deze bevinding ondersteunt de potentiële rol van het vetweefsel bij de ontwikkeling en/of verloop van FCH. Bovendien is adiponectine de belangrijkste onafhankelijke voorspeller van het atherogeen lipidenprofiel, waaronder hoge triglyceride-spiegels, lage HDLc-spiegels en de aanwezigheid van kleine, dichte LDL-deeltjes.
8. De twee onderzochte variaties in het *ApM1*-gen zijn niet geassocieerd met FCH, maar zijn wel geassocieerd met lage adiponectinespiegels en een atherogeen lipidenprofiel.

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Dankwoord

Dankwoord

Een promotieonderzoek was iets wat ik erg graag wilde doen na het afronden van mijn studie Biomedische gezondheidswetenschappen. Het is een cliché, maar het voltooien van een promotieonderzoek is een periode van vallen, opstaan en niet opgeven, en zonder al de mensen die mij gedurende deze periode hebben gesteund, was het me niet gelukt. Daarom vind ik het een eer dat ik al diegene in het dankwoord mag noemen.

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Gerly

Curriculum vitae

Curriculum vitae

Gerly Maria van der Vleuten werd geboren op 29 oktober 1978 te Best. In 1997 behaalde ze haar VWO diploma aan het Heerbeeck College te Best. In datzelfde jaar werd aangevangen met de studie Biomedische Gezondheidswetenschappen (thans Biomedische Wetenschappen) aan de Katholieke Universiteit Nijmegen (thans Radboud Universiteit Nijmegen). Tijdens deze studie heeft ze, in het kader van het hoofdvak epidemiologie, stage gelopen bij de afdeling Biostatistiek en Epidemiologie aan de Erasmus Universiteit Rotterdam onder leiding van prof. dr. CM van Duijn. Onder leiding van dr. B. Franke werd, in het kader van het hoofdvak pathobiologie, bij de afdeling antropogenetica stage gelopen. Daarnaast heeft ze, onder leiding van dr. C. Robertson, stage gelopen op de afdeling Biostatistics and Epidemiology bij het European Institute of Oncology te Milaan, Italie. In december 2001 werd het doctoraal examen afgelegd. Van januari 2002 tot en met juni 2006 was zij verbonden als junior onderzoeker aan de afdeling algemeen interne geneeskunde van het Radboud Universiteit Nijmegen Medisch Centrum, alwaar het in dit proefschrift beschreven onderzoek is uitgevoerd. Sinds september 2006 is ze werkzaam als datamanager bij IMRO TRAMARKO.

Curriculum vitae

Gerly Maria van der Vleuten was born on the 29 th of October 1978 in Best. In 1997 she completed her secondary education at the Heerbeeck College in Best. That same year she started the study Biomedische Gezondheidswetenschappen (currently Biomedische Wetenschappen) at the Catholic University Nijmegen (currently Radboud University Nijmegen). During this study she did, in relation to her specialisation epidemiology, an internship at the department of Biostatistics and Epidemiology at the Erasmus University Rotterdam under supervision of prof. dr. CM van Duijn. For the specialisation pathobiology, she did an internship at the department of antropogenetics under supervision of dr. B. Franke. Furthermore, she did an internship in Milan (Italy) at the department of Biostatistics and Epidemiology at the European Institute of Oncology under supervision of dr. C. Robertson. She obtained her MSc degree in December 2001. From January 2002 untill June 2006 she worked as a junior investigator at the department of general internal Medicine at the Radboud University Nijmegen Medical Centre of which the results are described in this thesis. Since September 2006 she is working as a datamanager at IMRO TRAMARKO.

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