Familial Combined Hyperlipidemia

Metabolic features and new diagnostic criteria

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FCH

Familial Combined Hyperlipidemia metabolic features and new diagnostic criteria

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Chapter 1

Introduction and outline of the thesis

Introduction

Cardiovascular disease (CVD) is the principle cause of mortality in all developed countries. The prevalence will increase also in developing countries by their adoption of the "Western" lifestyle with accompanying risk factors like high-fat diet, smoking and lack of exercise. The results of the 'Global burden of disease studies' pointed out that in the coming decennia atherosclerotic disease will be the most common cause of death worldwide (1). In total 49.952 persons died as a consequence of CVD in the Netherlands in 2000, this corresponds with 36% of the total mortality. Hyperlipidemia is like hypertension, obesity, diabetes mellitus and smoking a well-known risk factor of CVD.

Definition of FCH

Familial combined hyperlipidemia (FCH) is the most common heritable lipid disorder, with an estimated prevalence of 1 - 2% in the general population and 10 - 20% in survivors of myocardial infarction (2). Recently, several studies have demonstrated that FCH is associated with a 2- to 5 fold increased risk of premature CVD (3-5). FCH was originally identified in the early 1970s as a new inherited lipid disorder, characterized by multiple lipoprotein phenotypes defined by the presence of elevations of plasma total cholesterol (TC) and/or triglyceride (TG) concentration in affected individuals and strongly associated with premature CVD (6-8). 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. Other major characteristics of FCH include elevated apolipoprotein B (apoB) levels (9-13), the preponderance of atherogenic small dense low density lipoprotein (sdLDL) particles (12-16) and a decreased high density lipoprotein cholesterol (HDL-chol) concentration (17). Furthermore subjects with FCH are frequently obese and insulin resistant (4,18-21). Despite more than 30 years of investigation, the genetic and metabolic backgrounds of this disorder have not been elucidated in detail (21-25). It seems to be a metabolically and genetically heterogeneous disorder (14). Because of the absence of a specific clinical or metabolic marker, and because of the characteristic variability in the presenting phenotype, family studies are necessary to establish the diagnosis of FCH in a single patient.

Metabolic features of FCH

Several disturbances in metabolic pathways have been suggested to be pathophysiologically important for the FCH phenotype. In general, FCH is thought to be caused by hepatic overproduction of very low density lipoprotein (VLDL) particles (26,27) with or without an impaired physiological clearance of TG-rich lipoproteins both of exogenous and of endogenous origin (28,29). Over the past few years, several other metabolic mechanisms that may contribute to the pathogenesis of FCH have been proposed (24). These include an altered metabolism of fatty acids (FA) (18,30), increased plasma free fatty acids (FFA) (30-32), a reduced insulin sensitivity (18,20,33), a prolonged and exaggerated post-prandial lipaemia, an impaired hydrolytic capacity of lipoprotein lipase (LpL) (34), or elevated plasma levels of an inhibitor of LpL, apoCIII (35).

Because no single metabolic defect detected thus far can fully account for the FCH phenotype, it is hypothesized that even with a strict definition, the group of FCH patients might turn out to manifest a mixture of various disease entities, with a number of metabolic defects and possible genetic markers. Therefore, FCH may be considered more like a syndrome showing overlapping characteristics with other entities, such as hyperapobetalipoproteinemia (36), the 'atherogenic lipoprotein phenotype'(37), familial dyslipidemic hypertension (38,39) and the insulin resistance syndrome (40).

Genetic origin of FCH

Genetic analysis so far did also not reveal a genetic marker specific for FCH. FCH was originally assumed to be an autosomal dominant trait (6). However, subsequent reanalysis of the original data of Goldstein et al. provided evidence consistent with a multigenic mode of inheritance. Since then, a number of segregation analyses with multiple sets of families have indicated complex inheritance. These studies have suggested major gene effects on serum TG levels (41), apoB levels (10-12), LDL subfraction profile (12,14,23) and insulin resistance (42). Genome wide linkage studies have indicated that several chromosomal regions harbor genes contributing to the lipid profile of FCH. In Finnish families with FCH, 5 loci on chromosome 1q21, 2q31, 10p11.2, 10q11.2-10qter and 21q21 were identified (43,44), whereas 4 loci on chromosome 2p, 11p, 16q and 19q were found in Dutch families with FCH (45,46). Two potentially new loci on 6q and 8p were identified in 2 large cohorts of families with FCH from England (47). The loci on 1q21 have been confirmed in German and Chinese FCH families and in families from the NHLBI family heart study (48,49). Recently, a combined data analysis of Dutch and Finnish genome wide scans for FCH identified 3 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 whereas the other 2 regions were detected for the low HDL-chol trait (50). In addition, regions on 2p, 8q, 16q and 20q have been detected for low HDL-chol in the Finnish families with FCH and a region on 1p has been detected for apoB in Dutch families with FCH (51). The current genetic model suggests that FCH may result from the combination of a dominant major gene(s) with a number of modifier genes influencing plasma lipid levels. The identification of such modifier genes may help in reducing the problem of genetic heterogeneity of FCH. Several candidate genes have been evaluated in their contribution to the FCH lipid phenotype. These include genes involved in lipid metabolism (LpL (52-54), hepatic lipase (HL) (55,56), apoAI/CIII/AIV (57-62) and apoAV (63)), adipose tissue metabolism (tumor necrosis factor (TNF) (64), hormone sensitive lipase (HSL) (65,66)), insulin resistance and fatty acid (FA) metabolism (intestinal fatty acid binding protein (FABP) (67), insulin receptor substrate gene and beta3-adrenergic receptor (68) and peroxisome proliferator activated receptor (PPAR)-gamma (68,69)). To summarize the results of these studies, only LPL and apoAI/CIII/AIV genes have been most consistently associated as modifier genes in FCH.

To further unravel the genetics of FCH, animal models have been developed. In 1998 a locus (Hyperlip1) in a mutant strain, HcB-19/DEM (HcB-19), has been identified, which strongly links to the FCH features in these mice (70). 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 (71). The TXNIP gene in mice is located on chromosome 3F2.2, which is synthetic to chromosome 1q21 in humans, a region to which a major contributor of the FCH phenotype was mapped in a Finnish, German and Chinese population (43,48). However, we (72) and others (73) did not find any sequence variants in the TXNIP gene in subjects with FCH, so the TXNIP gene is not involved as a major contributor to the FCH phenotype. Recently, Pajukanta et al. (73) showed that FCH is strongly linked and associated with the gene encoding upstream transcription factor 1 (USF1). This is an intriguing finding as USF1 is also located at chromosome 1q21 and regulates the expression of several genes involved in glucose and lipid metabolism. Therefore, it may be a potentially good candidate as a major gene involved in the pathogenesis of FCH.

All these data indicate that FCH may develop from the additive effects of many genes, a high degree of genetic heterogeneity, and gene-gene and gene-environment interactions.

Evaluation of diagnostic criteria

It is clear that FCH is complex and heterogeneous, emphasizing the need for a variety of approaches, both metabolic and genetic, and for studies of multiple populations. Before multiple populations can be compared there has to be agreement about the diagnostic criteria. FCH still lacks a consensus on diagnostic criteria. Traditionally, the diagnosis FCH in a single patient is based on the presence of plasma TC and/or TG levels >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 establish the diagnosis as reviewed (74,75). For example the 95th percentiles or even absolute values for TC and /or TG levels, not adjusted for age and gender, are used in some studies (75-77). But there are more fundamental problems. Amongst the most important is that the lipid phenotype can vary substantially within any individual (78,79). 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 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. It is obvious that there is a need for unequivocal diagnostic criteria to establish the diagnosis of FCH. This may facilitate comparisons of different populations as well as the search for the clue(s) underlying this most common lipid disorder.

Outline of the thesis

The main aim of this thesis was to evaluate the intra-individual variation in lipid phenotype over a period of 5 year (chapter 2) and to elucidate more consistent and unequivocal diagnostic criteria for FCH which can be used in clinical practice (chapter 3). Furthermore, we reviewed about several metabolic disturbances that may contribute to the pathogenesis of FCH (chapter 4) and investigated the contribution of some metabolic factors in relation to the FCH phenotype and its increased risk of CVD (chapter 5-8).

Re-evaluation of the diagnostic criteria of FCH

The intra-individual variation in lipid phenotype is considered to be one important fundamental problem in the research for FCH. In **chapter 2** we evaluate the variability in lipid phenotype expression, apoB level, and LDL subfraction profile

in a large cohort of 299 individuals of 32 well-defined FCH families over a period of 5 year. In addition the factors affecting the lipid phenotype expression are investigated.

A still ongoing problem in the research of FCH is the lack of consistent and worldwide used diagnostic criteria. Until now, different research groups use different definitions of FCH (74,75). In **chapter 3** we evaluate which diagnostic features most adequately predict FCH in our 5-year follow-up cohort. To facilitate the implementation of these new diagnostic criteria in clinical practice we provide a nomogram to simply and accurately diagnose FCH.

Metabolic features of FCH and the increased risk of CVD

The exact pathophysiology of FCH is still unknown. Over the past few years evidence has emerged that a primary defect in adipose tissue metabolism may be the culprit of FCH, resulting in reduced FA trapping in adipose tissue and increased FA flux to the liver resulting in high apoB production and contributing to insulin resistance. In **chapter 4** we present a review about the metabolic pathogenesis of FCH with emphasis on the role of insulin resistance, adipose tissue metabolism and FFA.

Since FCH is accompanied by an increased risk of CVD, the most commonly used approach to estimate coronary heart disease risk is based on the assay of plasma TC, TG and HDL-chol, with calculation of LDL-chol by means of the Friedewald formula. ApoB measurement has been proposed as the first line risk estimator for coronary heart disease instead of the conventional lipid-oriented approach (80). In **chapter 5** we compare in 506 members of families with FCH the validity of the lipid-based and apoB-based approaches in risk evaluation of coronary heart disease.

The plasma lipid and lipoprotein levels are relatively moderately elevated in subjects with FCH and do not fully explain the increased risk of CVD. Hyperhomocysteinemia as a disorder of methionine metabolism is also a well-known independent risk factor for CVD (81,82). In **chapter 6** we investigate whether subjects with FCH have higher plasma homocysteine concentrations than controls, and whether homocysteine contributes to the increased risk of CVD in FCH. Furthermore, we evaluate whether parameters of lipid and lipoprotein metabolism are associated with the homocysteine level.

Insulin resistance coincides with alterations in lipid metabolism, such as hypertriglyceridemia, low HDL- chol, increased apoB levels and a predominance of sdLDL particles. Because all these features are also characteristics of FCH, the existence of insulin resistance may be an important factor modulating FCH phenotype. In **chapter 7** we explore the role of insulin resistance in FCH subjects

with different lipid phenotypes. Moreover, we study the interdependence between insulin resistance and change in lipid phenotype expression over a 5-year period. In addition we investigate whether insulin resistance or obesity can explain the elevated apoB levels and the high concentration of sdLDL in FCH.

Leptin is a hormone increased in obese subjects (83). Elevated levels of leptin are also associated with insulin resistance (84). Both obesity and insulin resistance are characteristics of FCH and therefore leptin may be elevated in FCH subjects. So far, only two small studies have studied the relationship between leptin concentrations and FCH, with conflicting results (85,86). Leptin is an independent risk factor for CVD (87). In **chapter 8** we investigate whether leptin levels in our large cohort of well-defined male and female subjects with FCH are elevated independent of their BMI, indicating that adipose tissue metabolism is disturbed in FCH. The second objective is to consider whether leptin levels contribute to the increased risk for CVD in FCH.

The summary and conclusions are provided in **chapter 9**

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Chapter 2

Diagnosis of Familial Combined Hyperlipidemia based on lipid phenotype expression in 32 families: results of a 5-year follow-up study

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Abstract

Familial Combined Hyperlipidemia (FCH) is characterised by a variable expression of hypercholesterolemia and/or hypertriglyceridemia. We evaluated the variability in lipid phenotype expression over a 5-year period and studied factors affecting the lipid phenotype expression. A total of 32 families (299 subjects) were studied in 1994 and in 1999. Subjects were classified as having FCH when total cholesterol and/or triglyceride levels exceeded the 90th percentile adjusted for age and sex. In 1994, 93 (31%) of the 299 subjects were affected, whereas 206 (69%) of the subjects were unaffected relatives. In 1999, the diagnosis of FCH was consistent in 69 (74%) of the 93 subjects. So, 26% of the FCH subjects in 1994 showed a sporadic normolipidemic pattern (i.e. total cholesterol and/or triglycerides <90th percentile) in 1999. Among the 206 non-affected relatives in 1994, 178 (86%) remained unaffected in 1999, and 28 (14%) developed an FCH lipid phenotype. Multiple regression analysis showed that sex (odds ratio 2.03 (CI 95% 1.09-3.87), p=0.03) and body mass index (odds ratio 1.14 (CI 95% 1.05-1.24), p<0.01) significantly contributed to the variability in lipid phenotype expression. Thus, the diagnosis FCH, based on plasma total cholesterol and/or triglyceride levels, is consistent in only 74% of the subjects over a 5-year period.

Two other major characteristics in our FCH group, compared with the unaffected relatives, included elevated apolipoprotein B (apoB) levels and the presence of small dense low density lipoprotein (LDL), as reflected by a low value of parameter K; (apoB (mg/l) 1461 ± 305 versus 997 ± 249 (p<0.001); K-value –0.22 ± 0.19 versus –0.02 ± 0.19 (p<0.001), respectively). We now report that the apoB concentration and the K-value show less variability in time and are more consistently associated with FCH, inasmuch as affected FCH subjects, compared with the unaffected relatives, persistently show a higher apoB level and a lower value of parameter K, reflecting small dense LDL, even when they present a sporadic normolipidemic pattern.

In conclusion, our results emphasize the need for reevaluation of the diagnostic criteria for FCH. We demonstrate that apoB and small dense LDL are attractive new candidates for defining FCH. Further studies are indicated to evaluate the role of apoB and small dense LDL as diagnostic criteria for FCH.

Introduction

Familial Combined Hyperlipidemia (FCH) was first described as a new autosomal inherited lipid disorder in 1973 by Goldstein et al. (1). FCH is the most common form of heritable lipid disorder with an estimated prevalence of 1.0 to 2.0% in the general population and 10 to 20% in survivors of myocardial infarction (2). The hyperlipidemia is characterised by elevations of plasma total cholesterol (TC) and/or triglyceride (TG) concentration and is therefore also known as 'multiple type hyperlipidemia'. Such a lipid profile is frequently associated with an unfavourable decrease in high-density lipoprotein cholesterol (HDL-C) concentration, an elevated apolipoprotein (apo) B concentration and a preponderance of atherogenic small dense low density lipoprotein (LDL) particles (3-7). In general, FCH is thought to be caused by hepatic very low density lipoprotein (VLDL) overproduction (8,9) with or without impaired clearance of TG-rich lipoproteins (10,11), of exogenous and of endogenous origin. Over the past few years several other different metabolic pathways in the pathogenesis of FCH have been proposed as recently reviewed (12). Other syndromes appear to exhibit overlapping aetiologies with FCH, such as hyperapobetalipoproteinemia, the atherogenic lipoprotein phenotype, familial dyslipidemic hypertension and syndrome X (13-15).

FCH was originally assumed to be an autosomal dominant trait. However, subsequent reanalyses of the original data of Goldstein et al. (1) provided evidence consistent with a multigenic mode of inheritance. Since then, a number of segregation analyses with multiple sets of families have indicated complex inheritance. These studies have suggested major gene effects for serum TG levels (16) and apoB levels (17,18). Bivariate analysis has suggested common genetic mechanisms influencing LDL particle size and apoB levels (19). Despite extensive research in metabolic and genetic fields, there is still no specific metabolic or genetic marker for FCH. Therefore, family studies are still necessary to establish the diagnosis FCH in a single patient.

One of the characteristic features of FCH is its variability in lipid and lipoprotein pattern among family members and even in the individual patient (4). To our knowledge, there are no data published about the variability in lipid phenotype expression over a period of several years. In this 5-year follow-up study, we evaluate the variability in lipid phenotype expression, apoB level and small dense LDL in a large cohort of 32 well-defined FCH families, and study the factors affecting the lipid phenotype expression.

Subjects and Methods

Study population

The study population consisted of kindreds and probands of families with known FCH, who were recruited in 1994 and followed up in 1999. The recruitment of FCH kindreds took place in 1994 via known affected probands who were attending the outpatient clinic as described previously (20). The probands exhibited a combined hyperlipidemia with both plasma TC and TG concentrations >90th percentile using the age- and gender-related 90th percentile upper levels of the prospective cardiovascular Munster (PROCAM) study (21), confirmed by repeated measurement; these subjects were on lipid-lowering diets but were taking no lipid-lowering drugs. Families were included when a multiple type hyperlipidemia with elevated levels of plasma TC and/or TG was present in first-degree relatives. Thus, besides the proband presenting combined hyperlipidemia, the presence of at least one first-degree relative with hypertriglyceridemia (HTG), hypercholesterolemia (HC), or combined hyperlipidemia was obligatory. Furthermore, at least the proband or 1 of the first-degree relatives should have premature cardiovascular disease (CVD) before the age of 60 years. In addition, the 95th percentile for plasma TC and TG was used if the body mass index (BMI) exceeded 30 kg/m^2, or an alcohol consumption of more than 2 units per day was present. Families were excluded when a secondary cause of the hyperlipidemia was diagnosed in the proband (i.e. diabetes mellitus, hypothyroidism and hepatic or renal impairment). None of the probands were homozygous for the apo e2 allele and none of the probands or first-degree relatives had tendon xanthomas. All relatives of probands above the age of 20 years in 1999 and who participated in 1994 and 1999 were included. In this study, all individuals were Caucasian. This resulted in a total of 32 families, providing 299 subjects (excluding spouses). At both points of measurement (1994 and 1999), all subjects filled out a questionnaire about their previous medical histories, especially cardiovascular status, medication, smoking and drinking habits and, hormonal status in women. BMI was determined for all subjects. After withdrawal of lipid-lowering medication for 4 weeks and after an overnight fast, venous blood was drawn by venipuncture. The subjects were classified as having FCH when plasma TC and/or TG levels exceeded the 90th percentile, based on the PROCAM study (21). Note that these percentiles are adjusted for age and sex. Unaffected relatives were defined by TC and TG levels <90th percentile. The study protocol was approved by the ethics committee of the University Medical Center Nijmegen.

Plasma lipid, lipoprotein and apolipoprotein analysis

Plasma TC and TG concentrations were determined by enzymatic, commercially available reagents (Boehringer-Mannheim, Germany, catalog. No. 237574 and Sera

Pak, Miles, Belgium, catalog. No. 6639, respectively). VLDL-cholesterol (VLDL-C) was isolated from whole plasma by ultracentrifugation at density (d)=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. HDL-cholesterol (HDL-C) was determined by the polyethylene glycol 6000 method (22). LDL-cholesterol (LDL-C) was calculated by subtraction of VLDL-C and HDL-C from plasma TC. Total plasma apoB concentrations were determined by immunonephelometry as recently described in detail elsewhere (23,24). To achieve accurate results in relation to the Center for Disease Control Standardization Program, obtained values were recalculated on the basis of an exchange of sera with dr.Marcovina (Northwest Lipid Research Laboratory, Seattle, WA, USA).

Low density lipoprotein subfraction profile analysis

LDL subfractions were separated by single-spin density gradient ultracentrifugation (25). Each individual LDL subfraction profile was defined by a continous variable K, as described in detail previously (26). Briefly, after ultracentrifugation, the LDL subfractions were visible as distinct bands in the middle of the tube. Up to five LDL subfractions could be distinguished i.e. LDL1 (d=1.030-1.033 g/ml), LDL2 (d=1.033-1.040 g/ml), LDL3 (d=1.040-1.045 g/ml), LDL4 (d=1.045-1.049 g/ml) and LDL5 (d=1.049-1.054 g/ml). The subfractions were carefully aspirated by means of a pasteur pipette. The volumes were calculated by weighing after correction for the densities. Subsequently, cholesterol was determined in each fraction, the concentrations were corrected for dilution and incomplete recoveries. The relative cholesterol concentrations (%chol) in the LDL subfractions were used to calculate parameter K as a continuous variable, which best describes each individual LDL subfraction profile (27). A negative value ($K<0$) reflects a more dense LDL subfraction profile, and a positive K value ($K>0$) a more buoyant profile.

Statistical analysis

Differences in baseline characteristics for anthropometric measurements, lifestyle variables including lipid lowering medication use (yes/no), smoking (yes/no), use of >2 alcoholic consumptions a day (yes/no), CVD (yes/no), lipid and lipoprotein parameters, apoB levels and the value of parameter K, between subjects with FCH and their non-affected relatives were tested for statistical significance by using the 2-tailed Fisher exact test for dichotomous variables and the t-test for continuous variables.

Before the final analyses, we were interested in determing whether changes in time in each group were different between men and women. We included sex in the linear mixed model for each dependent variable separately. We were particularly interested in the third-order interaction term among sex, time and group. We found that this item was never significant; therefore the sex variable was removed from the final model.

A linear mixed model with repeated measurements was used to test differences in lipid and lipoprotein concentrations, apoB level, K-value and BMI for statistical significance between the points of measurement (1994 and 1999) and between the groups (I,II,III and IV) for each dependent variable separately. The interaction between time and group and time, group and sex was also included in the model. The dependent variables were TC, TG, LDL-C, HDL-C, apoB, parameter K and BMI. The independent variables were time (1994, 1999), and group (I, II, III and IV) and their interaction. Group I was defined by subjects who were affected (FCH) in 1994 and showed a normolipidemic pattern (NL) in 1999, defined by TC and TG levels <90th percentile, corrected for age and sex. Group II was defined by subjects who were affected both in 1994 and 1999. Group III consisted of normolipidemic FCH relatives in 1994 who developed an FCH lipid phenotype in 1999 and group IV consisted of those subjects who were non-affected i.e. TC and TG <90th percentile in 1994 and in 1999. The mean levels by time and group were estimated using the appropriate least square means. Adjusted p-values according to Tukey-Kramer were presented, and a p-value less than 0.05 was considered to be significant.

Logistic regression was used to test differences in percentages for statistical significance between the points of measurement and between groups, with the appropriate likelihood ratio for each dependent variable assessed separately. The dependent variables were lipid-lowering medication use (yes/no), smoking (yes/no), use of >2 alcoholic consumptions a day (yes/no) and CVD (yes/no). The independent variables were identical to those described in the linear mixed model.

Multiple variable logistic regression with forward selection procedures was used to select the variables that contributed independently to a switch in lipid phenotype. A switch (yes/no) was here defined as change for any reason from FCH in 1994 to NL in 1999, or the reverse. The independent variables were sex, age, BMI, smoking, alcoholic consumption, CVD and the use of lipid-lowering medication.

Statistical analysis was performed by using procedures available in the Statistical Analysis System software package (1996, SAS Institute Inc.).

Results

A total of 32 families (299 subjects) were studied in 1994 and in 1999. In 1994, 93 (31%) of the 299 subjects were affected (FCH), whereas 206 (69%) of the subjects were unaffected, with TC and TG levels <90th percentile. Table 1 shows the anthropometric measurements, life style variables, including smoking habits, use of alcohol and lipid-lowering medication and lipid and lipoprotein variables, apoB levels and parameter K values for FCH and non-FCH (unaffected) relatives in 1994. The ratio of women to men was not statistically significant different between the affected and unaffected FCH family members. The mean age of the

group of affected subjects, compared with the group of unaffected FCH family members, was significantly higher, and they had also a higher BMI. This group also had more subjects with CVD, and the consumption of more than two alcoholic beverages per day was more frequent. In contrast, smoking habits and the use of oral contraceptives , including postmenopausal estrogen replacement therapy, was not significantly different between the affected and unaffected subjects. In 1994, 106 (68%) of the women were premenopausal and 23 (22%) had FCH, whereas 83 (78%) were unaffected. In 1994, among the 50 (32%) postmenopausal women, 27 (54%) had FCH and 23 (46%) were unaffected. Fifteen (14%) premenopausal women in 1994 were postmenopausal in 1999; this finding was associated with a switch from unaffected to an affected FCH lipid phenotype expression in 3 women. Twelve women did not show a change in lipid phenotype expression (7 women were unaffected, and 5 women had FCH in 1994 and also in 1999). Thus, 20% of the women who were premenopausal in 1994 and postmenopausal in 1999 showed a switch in lipid phenotype expression, which was not significantly different from the percentage of lipid phenotype switchers (18%) observed among the postmenopausal women in 1994 and 1999. Therefore, the change to postmenopausal status is not the only reason for the switch in lipid phenotype expression.

All affected FCH subjects with CVD used lipid-lowering medication, whereas 8 subjects used lipid-lowering medication for primary prevention of CVD. In the group of the unaffected subjects, 10 subjects used lipid-lowering medication; 4 of these subjects used it for secondary prevention, whereas the other 6 subjects without a history of CVD and without increased TC and/or TG levels used lipid-lowering medication. In total 5, unaffected subjects with CVD did not use lipid-lowering medication.

In 1994, 3 (1%) of the relatives had diabetes mellitus. Of these 3 relatives, 2 had diabetes mellitus type II and were classified as having FCH, and 1 relative had diabetes mellitus type I and was unaffected. In 1999, 6 subjects had developed diabetes mellitus type II (de novo), which was not associated with a change in lipid phenotype expression; 2 subjects remained unaffected in 1994 and also in 1999, whereas 4 subjects had FCH in 1994 and also in 1999. Note that TC and TG values of these 6 subjects were well within the range of the unaffected and affected FCH group, respectively.

By definition the FCH group had significantly higher TC and TG concentrations. The FCH group exhibited also significantly higher concentrations of VLDL-C, VLDL-triglycerides (VLDL-TG) and LDL-C and lower concentrations of HDL-C. Furthermore, significantly higher levels of apoB and lower values of parameter K, reflecting small dense LDL, were found in the FCH group compared to the group of unaffected relatives (Table 1).

Table 1 Anthropometric measurements, lifestyle variables, lipid and (apo)lipoprotein concentrations, and value of parameter K, reflecting LDL heterogeneity, in 32 FCH families (299 subjects)

	FCH Subjects	unaffected subjects	p*
Subjects, n (%)	93 (31%)	206 (69%)	<0.001
Women/men, n	50 / 43	106 / 100	NS
Age, y	46.6 ± 14.8	38.4 ± 15.2	<0.001
BMI, kg/m^2	26.9 ± 3.5	23.3 ± 3.2	<0.001
CVD, n (%)	14 (15)	9 (4)	<0.001
Lipid-lowering medication, n (%)	23 (25)	10 (5)	<0.001
Smoking (≥1 cigarette/d), n (%)	24 (26)	50 (24)	NS
Alcohol (>2U/d), n (%)	10 (11)	7 (3)	0.013
OAC, n (%)	12 (13)	38 (18)	NS
TC, mmol/L	7.04 ± 1.19	5.30 ± 1.03	<0.001
TG, mmol/L	3.26 ± 2.30	1.16 ± 0.51	<0.001
VLDL-C, mmol/L	1.44 ± 1.01	0.41 ± 0.26	<0.001
VLDL-TG, mmol/L	2.30 ± 1.89	0.66 ± 0.43	<0.001
HDL-C, mmol/L	1.03 ± 0.31	1.25 ± 0.32	<0.001
LDL-C, mmol/L	4.59 ± 1.28	3.65 ± 0.96	<0.001
ApoB, mg/L	1461 ± 305	997 ± 249	<0.001
K-value	-0.22± 0.19	-0.02± 0.19	<0.001

OAC indicates oral contraceptives, including postmenopausal estrogen replacement therapy; K-value <0, small dense LDL; K-value >0,buoyant LDL; NS, not statistically significant (p>0.05). Because of technical errors and/or plasma unavailability, apoB levels and the value of parameter K were determined in 88and 85 FCH subjects, respectively, and 205 and 201 unaffected subjects, respectively. Results are presented as total number (percentages) or mean ± SD.
*Fisher exact test for dichotomous variables (numbers) and t-test for numeric variables (means).

FCH diagnosis in 1994 versus 1999

In 1994, 93 (31%) of the 299 subjects were affected (FCH). These affected subjects presented with hypercholesterolemia (HC), (n=26, 28%), hypertriglyceridemia (HTG), (n=31, 33%) or HC + HTG (n=36, 39%) (Table 2). In 1999 the diagnosis FCH was consistent with the diagnosis in 1994 in only 69 (74%) of the 93 subjects. These subjects were affected in both 1994 and 1999 based on HC (n=9), HTG (n=20) or combined HC+HTG (n=10) levels, and 30 subjects remained affected by FCH but changed in lipid phenotype, as shown in Table 2.

Table 2 Number of subjects in 32 FCH families (299 subjects), stratified by lipid phenotype expression in 1994 and in 1999

	1999				
1994	**HC**	**HTG**	**HC+HTG**	**NL**	**Total**
HC	9	6	3	8	26
HTG	1	20	4	6	31
HC+HTG	2	14	10	10	36
NL	1	26	1	178	206
Total	13	66	18	202	299

HC indicates FCH based on TC >90th percentile, HTG, FCH based on TG >90th percentile and NL, FCH relatives with TC and TG levels ≤90th percentile adjusted for age and sex. Values are absolute number of subjects.

However, most important, a total 24 subjects (26%) with FCH in 1994 according to HC (n=8), HTG (n=6) or HC+HTG (n=10) levels showed a normolipidemic pattern in 1999. Of the 206 non-affected relatives in 1994, 178 (86%) of them remained unaffected in 1999; however, 28 (14%) of the normolipidemic subjects (ie TC and TG levels <90th percentile in 1994 were affected (FCH) in 1999 according to HC (n=1), HTG (n=26) and HC+HTG (n=1) levels. Figure 1 shows the pedigree of one FCH family at both points of measurement (1994 and 1999), indicating the individual change in lipid phenotype expression.

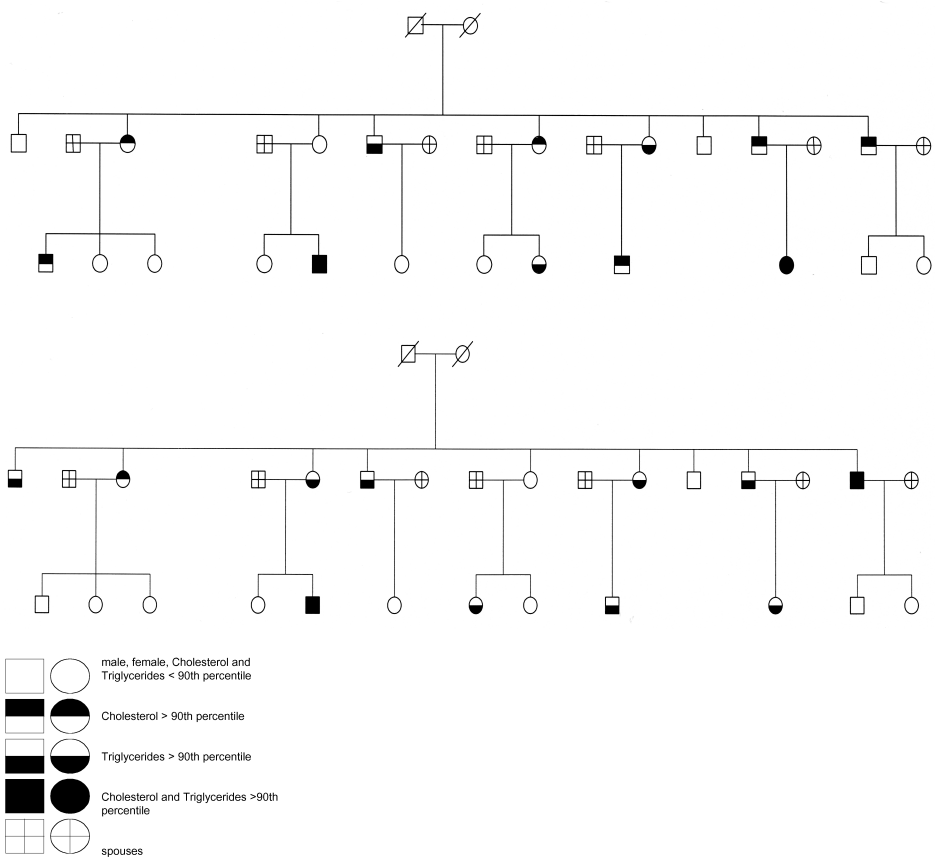

Figure 1 The pedigree of 1 FCH family in 1994 and 1999, indicating the individual change in lipid phenotype expression.

In total, 83 (28%) of the 299 subjects showed a switch in lipid phenotype for any reason (Table 2). The numbers of switches in lipid phenotype that were due to change in only TC concentration (n=27) were not significantly different from the number of switches in lipid phenotype that were due to change in only TG concentration (n=37) (test of Mc Nemar, p>0.05). The cholesterol level of 45 participants in the present study fell above the cut-off point (90th percentile) in 1994 and below this

point in 1999, or vice versa. So, they underwent a switch in phenotype according to the cholesterol level. The mean change in the cholesterol level of these subjects was 1.10 ± 0.66 mmol/L. Fifty-six participants switched in phenotype according to the TG level (mean change: 1.49 ± 1.15 mmol/L). These results show that the switch in lipid phenotype is based on significant TC and/or TG changes that cannot be simply explained by the intra-individual and analytical variation. Therefore, we studied in 4 subgroups (group I, II, III and IV) defined by the switch in lipid phenotype as described in Statistical Analysis.

Anthropometric measurements and lipid and lipoprotein concentrations in 1994 versus 1999

Table 3 shows the anthropometric measurements and mean values of lipid and lipoprotein concentration by the 4 subgroups, each defined by lipid phenotype expression in 1994 versus 1999. Note that a normolipidemic lipid profile is defined by TC and TG levels <90th percentile, corrected for age and sex. The mean age of the unaffected subjects in 1994 and 1999 (group IV) was statistically significantly lower than the mean age of the other groups. The results of the linear mixed model showed that there was a statistical significant difference in each of the lipids and lipoprotein values and of the BMI between 1994 and 1999 and between the groups and that the time differences were different between the groups (ie, interaction). This result indicates that the variability in lipid and lipoprotein concentration observed, especially among FCH subjects (group I, II, and III), is not simply the result of either random biological or analytical variation, or a time effect.

The BMI of the subjects who were affected in 1994 and had a normolipidemic pattern in 1999 (group I) did not change, whereas the BMI of the subjects of the other groups increased significantly from 1994 to 1999 (group II, III, IV).

For TC, a significant decrease was observed in the subjects who were affected in 1994 but had a normolipidemic pattern in 1999 (group I). The decrease in TC concentration of the subjects who remained affected (group II) or unaffected (group IV) and the increase in TC concentration of the subjects who had a normolipidemic pattern in 1994 but were affected in 1999 (group III) did not reach statistical significance. The TG concentration decreased significantly in the subjects who were affected in 1994 but had a normolipidemic pattern in 1999 (group I), whereas the subjects who remained affected (group II) and who had a normolipidemic pattern in 1994 and a FCH phenotype in 1999 (group III) showed a significant increase, most likely reflecting progression of the disease. The unaffected subjects (group IV) showed no significant change in TG concentration. The LDL-C concentration showed a decrease in all 4 groups, reaching statistical significance in the subjects who were affected in 1994 and had a normolipidemic pattern in 1999 (group I) and in the group of subjects who remained affected in both 1994 and 1999 (group II).

Table 3 Anthropometric measurement, lipid and (apo)lipoprotein concentrations and value of parameter K, reflecting LDL heterogeneity, in 299 subjects from 32 FCH families, stratified by switch in lipid phenotype expression in 1994 versus 1999.

	1994 → 1999			
	FCH → NL	**FCH → FCH**	**NL → FCH**	**NL → NL**
	Group I (n=24)	**Group II (n=69)**	**Group III (n=28)**	**Group IV (n=178)**
Male/female,*	16 / 8	27 / 42	17 / 11	83 / 95
Age (1994) † y	47.1 (41.1–53.1)	46.5 (42.9–50.0)	44.0 (38.4–49.6)	37.5|| (35.3–39.8)
BMI,‡ kg/m^2				
1994	26.7 (25.2–28.1)	27.1 (26.3–27.9)	25.4 (24.1–26.7)	23.0 (22.5–23.5)
1999	26.5 (25.0–27.9)	28.6|| (27.7–29.4)	28.0|| (26.7–29.3)	24.2|| (23.7–24.7)
TC,‡ mmol/L				
1994	7.04 (6.61–7.48)	7.05 (6.79–7.30)	5.75 (5.35–6.16)	5.23 (5.07–5.39)
1999	6.03|| (5.60–6.47)	6.93 (6.68–7.19)	5.93 (5.53–6.33)	5.09 (4.93–5.25)
TG,‡ mmol/L				
1994	2.85 (2.19–3.50)	3.40 (3.02–3.79)	1.55 (0.93–2.16)	1.10 (0.85–1.34)
1999	1.86|| (1.20–2.52)	4.27|| (3.88–4.66)	2.89|| (2.28–3.51)	1.20 (0.96–1.45)
LDL-C,‡ mmol/L				
1994	4.70 (4.28–5.12)	4.55 (4.30–4.80)	4.11 (3.72–4.50)	3.58 (3.43–3.74)
1999	4.21|| (3.78–4.63)	4.13|| (3.87–4.38)	3.73 (3.34–4.06)	3.44 (3.29–3.60)
HDL-C,‡ mmol/L				
1994	0.97 (0.85–1.10)	1.05 (0.98–1.12)	1.03 (0.92–1.15)	1.29 (1.24–1.33)
1999	1.0 (0.88–1.12)	0.92|| (0.85–0.99)	0.93 (0.81–1.04)	1.23|| (1.18–1.27)
ApoB,§ mg/L				
1994	1434 (1331–1536)	1477 (1415–1539)	1174 (1078–1269)	973 (935–1011)
1999	1271|| (1169–1374)	1452 (1390–1514)	1285|| (1190–1380)	1006 (969–1044)
K-value§				
1994	-0.25 (-0.34– -0.17)	-0.21 (-0.26– -0.15)	-0.14 (-0.22– -0.06)	0.0 (-0.04–0.03)
1999	-0.13 (-0.21– -0.04)	-0.31|| (-0.36– -0.26)	-0.26 (-0.34– -0.19)	0.05 (0.02–0.08)

Values are estimated mean (95% CI) and absolute numbers for sex distribution.
* = χ^2-test ($p<0.05$).
† = ANOVA ($p<0.05$), indicating that only the mean age is significanlyt lower in group IV compared with all other groups,
‡ Statistically significant effect for each of the following, (1) time, (2) group and (3) time-group interaction with use of a linear mixed model ($p<0.05$).
§ In 1994 and 1999, the mean value in groups I, II, III is significantly different compared with that in group IV ($p<0.05$ by adjusted Tukey-Kramer test).
|| p-value <0.05 by adjusted Tukey-Kramer for differences between 1994 and 1999.

The HDL-C concentration in the subjects who were affected FCH in 1994 and had a normolipidemic pattern in 1999 (group I) did not change. In all the other groups (II, III and IV) the HDL-C concentration decreased, reaching statistical significance in the subjects consistently had FCH and in those who were consistently unaffected (groups II and IV, respectively).

These results show that, on average, the subjects who switch from affected to a normolipidemic pattern (group I) improve on all parameters (although less for

HDL-C). Most likely, the healthier lifestyle in 1999, as indicated by the stable BMI, contributed to the improved lipid and lipoprotein profile. However, the lipid phenotype remained more atherogenic: TC, TG and LDL-C were significantly higher and HDL-C was significant lower than in the unaffected relatives, suggesting that these subjects are genetically predisposed to FCH. Also, on average, the subjects with a normolipidemic pattern in 1994 but with FCH in 1999 (group III) deteriorated. Although the deterioration was not more than that in the unaffected subjects (group IV) for all parameters, the assumption that these subjects already had a predisposition to FCH is more likely because these subjects already had higher baseline levels in 1994.

Because the change in lipid and lipoprotein parameters, as described above, is not simply the result of a random effect (including biological and analytical variation), further statistical analysis was performed to identify whether sex, age, use of lipid-lowering medication and BMI contribute to the change in lipid phenotype expression (ie, from FCH to a normolipidemic pattern or from a normolipidemic pattern to FCH). The final model, in which we used selection procedures with multivariate logistic regression analyses, showed that only sex (adjusted odds ratio 2.03 (95% CI 1.09-3.87), p=0.03) and BMI (adjusted odds ratio 1.14 (95% CI 1.05-1.24), p<0.01) independently contributed to the change in lipid phenotype expression between 1994 and 1999. Thus, the estimated adjusted relative risk for switch in lipid phenotype is 1.13 times higher per unit (kg/m^2) gain in BMI and 2.03 times higher in men compared to women, (ie adjusted for the other variable in this model). This is in line with the general observation that men with a high BMI are most likely to vary in lipid phenotype.

ApoB and LDL heterogeneity in 1994 versus 1999

ApoB levels and values of parameter K, reflecting LDL heterogeneity, are shown in Table 3, stratified by 4 groups, defined by switch in lipid phenotype expression in 1994 versus 1999. In contrast to the lipid and lipoprotein levels, the difference between time points (1994-1999) in the values of apoB and parameter K among FCH subjects (group I, II and III) was not significantly different from that observed among the group of non-affected relatives (group IV). This result indicates that apoB and parameter K are less variable in time than are TC and TG levels. Although variability in apoB levels and the value of parameter K is present, it is most intriguing to observe that, on average, subjects with an affected FCH phenotype in 1994 and/or 1999 (group I, II and III) have significantly higher apoB concentrations and lower values of parameter K, reflecting small, dense LDL, than do the unaffected relatives (group IV), even when they have a sporadic normolipidemic phenotype (group I in 1999 and group III in 1994, Table 3).

Presence of CVD, use of lipid-lowering medication and smoking/drinking habits in 1994 versus 1999

Table 4 shows the use of lipid-lowering medication smoking and drinking habits and the presence of CVD by group (I, II, III and IV) in 1994 and 1999.

The percentage those consuming >2 alcoholic beverages per day and smokers was not statistical significantly different between points of measurement (1994 and 1999). The percentage users of those consuming >2 alcoholic beverages per day was significantly higher in the group of subjects who remained affected (group II) compared with the subjects who remained normolipidemic between 1994 and 1999 (group IV). The percentage of smokers in the group of subjects who were affected in 1994 but had a normolipidemic pattern in 1999 (group I) was significantly higher than the percentage of smokers in the group of subjects who remained affected (group II) and in the group of subjects who remained normolipidemic (group IV). The percentage of subjects using lipid-lowering medication significantly increased (odds ratio 3.28, CI 95% 1.96-5.5, p<0.001) in 1999 compared to 1994, and this was similar for all groups. The use of lipid-lowering medication of the subjects who remained affected in 1994 and also in 1999 (group II) was significantly higher than in all other groups (group I, III, IV). This percentage increased from 29 to 61% between 1994 and 1999. In group IV (the unaffected subjects), the use of lipid-lowering medication was significantly lower than in the other groups (group I, II, III), as expected.

Table 4 Smoking and drinking habits, the use of lipid-lowering medication and the presence of CVD in 299 subjects from 32 FCH families stratified by switch in lipid phenotype expression in 1994 versus 1999.

	1994 → 199			
	FCH → NL	**FCH → FCH**	**NL → FCH**	**NL → NL**
	Group I (n=24)	**Group II (n=69)**	**Group III (n=28)**	**Group IV (n=178)**
Alcohol >2U/d, †				
1994	3 (13)	7 (10)	2 (7)	5 (3)
1999	1 (4)	5 (7)	0 (0)	9 (5)
Smoking >1/d ‡				
1994	8 (33)	16 (23)	8 (29)	42 (24)
1999	11 (46)	14 (20)	7 (25)	44 (25)
LL med *				
1994	3 (13)	20 (29)	6 (21)	4 (2)
1999	11 (46)	42 (61)	10 (36)	8 (4)
CVD *				
1994	4 (17)	10 (14)	4 (14)	5 (3)
1999	6 (25)	17 (25)	8 (29)	11 (6)

LL med indicates lipid-lowering medication, Values are absolute number (percentages),
† the use of alcohol is significantly higher in group II compared with group IV, by multivariate logistic regression. ‡ smoking is significanlyt higher in group I compared with groups II and IV, by multivariate logistic regression. *Statistically significant (p<0.05) increase in 1999 compared with 1994 for all groups, by multivariate logistic regression.

The percentage of subjects with CVD increased significantly between 1994 and 1999 (odds ratio 2.05, CI 95% 1.18-3.56, p=0.011), but the increase was not significantly different between the 4 groups. However, the total percentage of subjects with CVD was significantly lower in the subjects who remained normolipidemic (group IV) than in each of the other groups (group I, II, III), as expected, and there were no significant differences between group I, II and III.

Discussion

This is the first long-term follow-up study of a large cohort of FCH families. This study shows that the diagnosis FCH, based on internationally accepted criteria (including plasma TC and/or TG level above the 90th percentile adjusted for age and sex), is consistent in only 74% of the subjects over a 5-year period. So 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 sex. The literature shows that variability in lipid phenotype expression is a characteristic of FCH (1,2,4). However, this variability is usually determined as a change from HTG to HC or vice versa. Indeed, in our large cohort of FCH families, 32% of the affected subjects showed such a change in lipid phenotype over a period of 5 years, but the change was still consistent with the diagnosis of FCH. Our present results show that this variability in lipid phenotype may also lead to a sporadic "normolipidemic" pattern, because 26% of the affected subjects in 1994 showed a normolipidemic pattern in 1999, 5 years after the initial diagnosis FCH (Table 2).

The switch from affected FCH to a normolipidemic phenotype was due to a significant decrease in plasma TC, LDL-C and TG level (Table 3), which could not be explained by a random effect, including biological and analytical variation. Further statistical analysis revealed that BMI contributes to a change in lipid phenotype expression. Indeed, these subjects who switched from FCH to a sporadic normolipidemic pattern did not change in body weight, whereas the subjects who remained affected increased in body weight. Knowledge of the presence of FCH in 1994 might have resulted in the patients leading a healthier lifestyle, contributing to a stable body weight and a reduction in lipid and lipoprotein levels. However, this knowledge of having FCH did not affect BMI and lipid and lipoprotein levels in subjects who remained affected in 1994 and also in 1999. From the present study, we cannot deduce which other factors contribute to the confounding effect of knowledge of the presence of FCH.

The subjects who developed an FCH phenotype between 1994 and 1999 showed a significant increase in BMI, which was associated with a significant increase in TG level, whereas TC, LDL-C and HDL-C levels did not change significantly. As

discussed above, BMI influences the FCH lipid phenotype expression. Thus, our data suggest that body weight control in FCH subjects should be an important issue in lifestyle intervention programs. However, the significant increase in BMI among the unaffected subjects in 1994 and 1999 is not associated with expression of FCH phenotype, suggesting that these subjects are most likely not genetically predisposed to develop FCH. The expression of FCH has been suggested to be age-dependent (1) with increased prevalence in adolescence. However, in the present study, the mean age of the subjects who developed an FCH phenotype between 1994 and 1999 was 42.9 years. In additional statistical analysis, age was not an independent variable contributing to FCH expression. Also Porkka et al. (28) did not find clear signs of age dependence with TC and/or TG criteria.

With aging, the plasma TC and TG levels usually increase, so it may be surprising that in our cohort after 5 years, the TC concentration did not change and even decreased in some groups (Table 3). The change in TC concentration in our FCH cohort reflects the total Dutch population in which the plasma TC concentration had decreased with 0.5 mmol/L between 1987 and 1997 (29).

Thus, we demonstrate that the lipid profiles in FCH families are highly variable in time, leading to misclassification in diagnosis. Our results help to explain why, despite extensive research, until now no single diagnostic marker has been identified for FCH. For example, until now, 3 genome screens have been reported in an attempt to locate the genetic region for FCH (30-32) but no unique locus has been found in the different family sets. To be able to delineate the metabolic and genetic factors contributing to the aetiology of FCH, it is essential to have consistent diagnostic tools to establish the diagnosis of FCH. Although internationally accepted diagnostic criteria are formulated for FCH, not all research groups use the same criteria to establish the diagnosis as recently reviewed (28,33). For example, in some studies the 95th percentiles or even absolute values for TC and /or TG levels, not adjusted for age and sex, are used, whereas in other studies apoB also served as a diagnostic criterion (11). However, a change in threshold or cut-off points for TC and TG levels will not improve the diagnosis of FCH because of the considerable intraindividual variability in lipid phenotype expression in time, as demonstrated by our results: with use of the 90th percentile cut point for TC and/or TG level adjusted for age and sex, 26% of the FCH subjects in our cohort had a normolipidemic pattern after a 5-year period, leading to a false-negative disease status at that point of time. So, for the diagnosis FCH, the inclusion of only plasma TC and TG levels is insufficient due to this considerable variability in time.

Literature has shown that FCH is associated with other traits that may contribute to the increased risk of CVD, including unfavourable increases in plasma apoB and an increased prevalence of atherogenic small dense LDL. Previously, we reported that in FCH, apoB is as effective as lipid levels in classifying subjects at increased risk for CVD (24). In the present study, a significant higher apoB level and a lower

value of parameter K, reflecting small dense LDL, was found in the affected FCH group. Most important, we show that the variabilities in the plasma levels of apoB and in the value of parameter K in time is considerably less than those for TC and TG levels and not different from those for unaffected relatives. Most intriguingly, we demonstrate that compared with unaffected subjects, subjects with FCH have significantly higher apoB levels and increased prevalence of small dense LDL (ie, lower value of parameter K), even when they present a normolipidemic phenotype. So, even the affected FCH subjects in 1994 who showed a normolipidemic lipid profile in 1999 had significantly higher levels of apoB and more small dense LDL in 1994 and 1999. Similarly, the subjects who were unaffected in 1994 but who developed the FCH phenotype in 1999 showed a significantly higher apoB concentration and a lower value of K in 1994 and 1999. These data suggest that subjects genetically predisposed to FCH have increased levels of apoB and increased prevalence of small dense LDL, independent of the lipid profile. Together with less variability in time, apoB and small dense LDL, compared with TC and TGs, could potentially contribute to more consistent diagnostic criteria for FCH with a better discriminant power to separate affected from unaffected relatives.

FCH is accompanied with an increased risk of CVD (1). The total percentage of subjects with CVD increased significantly between 1994 and 1999, but the increase was not significantly different between the 4 groups. However, the total percentage of subjects with CVD in the group of subjects who had an FCH phenotype in 1994 and also in 1999 was significantly higher than in each of the other groups; in the group of subjects who were normolipidemic in 1994 and also in 1999, the percentage subjects with CVD was significantly lower than in each of the other groups. It might appear somewhat surprising that the percentage of subjects who develop CVD was similar among FCH subjects despite the switch to a normolipidemic phenotype in 20% of the subjects versus a switch to an FCH phenotype in 23% of the subjects. The explanation to this apparent contradiction is most likely that once a subject shows an FCH lipid phenotype, he or she is genetically predisposed to FCH, associated with an increased risk of CVD. In FCH, several potential risk factors have been suggested in literature to contribute to its increased atherogenicity, including small dense LDL, an increase in apoB, low HDL levels, and insulin resistance (12). We show that affected FCH subjects (group I, II and III) have an increased apoB level and more small dense LDL (low value of parameter K), independent of the lipid phenotype expression. So, even when a relative with FCH shows a sporadic normolipidemic pattern, increased levels of apoB and lower values of parameter K are found, most likely contributing to an increased risk of CVD and, thus, contributing to the explanation for the similar increase in the percentage of subjects who develop CVD within 5 years.

In 1994, only 23 of the 93 affected subjects used lipid-lowering medication. Thus, <25% of the subjects were treated properly. In 1999, 53% of the affected subjects

used lipid-lowering medication, showing that FCH, (most likely due to relatively mild hyperlipidemia) is still not treated properly. The recommendations for treating subjects with mild hyperlipidemia in a family with FCH must be stricter than the recommendations for treating subjects with mild hyperlipidemia in a family without FCH, because a positive family history is an independent risk factor for CVD (34). Besides lipid- lowering medication, lifestyle intervention programs should reinforce a cessation of smoking, because, unfortunately >20% of all the subjects were still smoking in 1999, despite their known FCH status in 1994.

A major problem in clinical medicine, contributing to the undertreatment of FCH patients, is the difficulty in diagnosing FCH, because with the present diagnostic criteria (TC and TG levels), the diagnosis cannot be made in a single patient without family screening. Therefore, a major effort should be undertaken to redefine the diagnostic criteria of FCH by 1 standardized parameter, which, measured at 1 time point, discriminates between affected and unaffected relatives. We now demonstrate that apoB and small dense LDL are potentially attractive parameters for improving the diagnostic criteria for FCH.

This unique study of a large cohort of FCH families with 5-year follow-up indicates that the diagnosis FCH, based on plasma TC and/or TG levels above the 90th percentile adjusted for age and sex, is consistent in only 74% of the subjects. Twenty-six percent of the affected subjects showed a normolipidemic pattern after 5 years. Therefore, our results emphasize the need for reevaluation of the diagnostic criteria. Furthermore, we demonstrate that plasma apoB and small dense LDL are potentially valuable diagnostic criteria for FCH. Further studies are indicated to determine the role of apoB and the presence of small dense LDL and, thus, to improve the diagnostic criteria for FCH.

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Chapter 3

Nomogram to diagnose Familial Combined Hyperlipidemia on the basis of results of a 5-year follow-up study

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Abstract

Background: Familial combined hyperlipidemia (FCH) is traditionally diagnosed by total plasma cholesterol and/or triglyceride levels above the 90th percentile adjusted for age and gender. In a recent study we showed that the diagnosis FCH based on these diagnostic criteria was inconsistent in 26% of the subjects over a 5-year period. This results emphasizes the need for re-evaluation of the diagnostic criteria for FCH.

Methods and Results: A total of 32 families (299 subjects) were studied in 1994 and in 1999. A subject was defined 'truly'-FCH, when diagnosed FCH in 1994 and/or 1999 based on the traditional plasma lipid criteria. Additional lipid and lipoprotein parameters, including apolipoprotein B (apoB) and small dense LDL, were measured at both time points. In total, 121 subjects (40%) were defined as truly FCH. Multivariate analysis revealed that absolute apoB values combined with triglyceride and total cholesterol levels adjusted for age and gender best predicted truly FCH. A nomogram including these parameters is provided to simply and accurately calculate the probability to be affected by FCH. Furthermore, it is shown that 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).

Conclusions: The diagnosis of FCH is best predicted by absolute apoB levels combined with triglyceride and total cholesterol levels adjusted for age and gender and can accurately be calculated by a nomogram. This definition is also a good predictor of cardiovascular risk in FCH.

Introduction

Familial Combined Hyperlipidemia (FCH) was first described in 1973 as a common familial disorder characterized by multiple lipoprotein phenotypes and increased risk of premature cardiovascular disease (CVD) (1) . In the 30 years ago since it was described, the genetic basis for FCH has remained elusive; indeed even the mode of inheritance remains controversial. Obviously, no genetic hypothesis can be reliably tested when there is no consistent phenotype to establish the diagnosis FCH.

FCH is characterized by several phenotypes, including increased total cholesterol (TC), increased triglycerides (TG), decreased high-density lipoprotein cholesterol (HDL-C), increased apolipoprotein B (apoB) and the presence of small dense low-density lipoprotein (LDL). Not all research groups use the same criteria to establish the diagnosis of FCH (2,3). There are even more fundamental problems. Amongst the most important is that the lipid phenotype can vary substantially within any individual. Recently, we showed in a 5-year follow-up study (1994 to 1999) of 32 families that the diagnosis of FCH, based on plasma TC and/or TG level above the 90th percentile (pTC or pTG >90th) adjusted for age and gender is consistent in only 74% of the subjects (4). Most importantly, 26% of the subjects with FCH in 1994 had a sporadic normolipidemic pattern in 1999 defined by pTC and pTG levels <90th corrected for age and gender. Thus, the classical lipid phenotypes are not only variable amongst family members, but also within individuals. This has previously been suspected, but has now been convincingly demonstrated by our data, and by recent data of Mc Neely et al (5).

Although the genetic origin of FCH has remained obscure, much has been learned about its pathophysiology. The characteristic abnormality is an increased production of very low density lipoprotein (VLDL) with or without impaired clearance of TG-rich lipoproteins in the majority of patients (6) that results in the generation of increased numbers of small dense LDL particles. This suggests that just as a variable lipid phenotype is the hallmark of FCH within a family, an elevated plasma apoB might be the common hallmark of FCH within an individual. Indeed, this was a principal finding of our previous study (4), which demonstrated that subjects affected FCH in 1994 and/or 1999 had significantly higher apoB levels and an increased amount of small dense LDL compared with nonaffected subjects, even when they presented a normolipidemic phenotype. Together with less variability in time, measurements of apoB and small dense LDL in plasma could potentially lead to more consistent diagnostic criteria for FCH with a better discriminant power to separate affected from nonaffected relatives compared to TC and TG levels alone.

The objective of the present study is to evaluate several diagnostic features of FCH and to provide a nomogram that can simply and accurately predict FCH in clinical practice.

Subjects and Methods

Study population

The study population consisted of kindreds and probands of families with known FCH, who were recruited in 1994 and followed up in 1999 as recently described elsewhere (4). At both time points of investigation all subjects filled out a questionnaire about their medical history, especially cardiovascular status. Body mass index (BMI) was determined in all subjects. After withdrawal of lipid-lowering medication for four weeks and after an overnight fast, venous blood was drawn by venipuncture. The subjects were classified affected FCH when plasma pTC and/or pTG >90th on the basis of the PROCAM study (7). These percentiles are adjusted for age and gender. Nonaffected relatives were defined by TC and TG levels <90th percentile (pTC and pTG <90th). The ethics committee of the University Medical Center Nijmegen approved the study protocol.

In total, 32 families, including 299 subjects, were studied in both 1994 and 1999. In 1994, 93 of the 299 subjects (31%) were affected by FCH. The diagnosis of FCH in 1999 was consistent with the diagnosis in 1994 in only 69 of the 93 subjects (74%). Most importantly, however, 24 subjects (26%) with FCH in 1994 showed a normolipidemic pattern in 1999 and 28 (14%) of the normolipidemic subjects i.e. pTC and pTG levels <90th, in 1994 were affected in 1999. Of the 206 nonaffected relatives in 1994, 178 (86%) remained nonaffected in 1999 (4). In the present study we define all subjects who were diagnosed FCH in 1994 and/or 1999, on the basis of the traditional lipid criteria (pTC and/or pTG >90th), as affected by FCH. Thus, this definition also includes the subjects with FCH in 1994 who show a normolipidemic pattern in 1999 (Table 1). We refer to these affected FCH subjects further as 'truly'-FCH.

Table 1 Subjects in 32 families defined affected by familial combined hyperlipidemia (FCH) or normolipidemic relatives (NL) in 1994 and 1999.

	1999, n (%)		
1994	**FCH**	**NL**	**Total**
FCH	69 (74) *	24 (26) *	93 (31)
NL	28 (14) *	178 (86)	206 (69)
Total	97 (32)	202 (68)	299 (100)

NL indicates normolipidemic relatives defined by pTC and pTG levels <90th. FCH was based on pTC and/or pTG levels >90th.

*Subjects classified as truly FCH defined by affected FCH based on pTC and/or pTG>90th in 1994 and/or 1999. In total, 121 subjects (40%) of the study population were defined as truly FCH.

Plasma lipid, lipoprotein and apolipoprotein analysis

Plasma TC and TG concentrations were determined by enzymatic, commercially available reagents (Boehringer-Mannheim, Germany, catalog. No. 237574 and Sera Pak, Miles, Belgium, catalog. No.6639, respectively). HDL-cholesterol (HDL-C) was determined by the polyethylene glycol 6000 method (8). LDL-cholesterol (LDL-C) was calculated by subtraction of VLDL-C and HDL-C from plasma TC. Total plasma apoB concentrations were determined by immunonephelometry (9). Coefficient of variation estimates of the analytical and biological variation of apoB in our laboratory were <7%.

LDL subfractions were separated by single spin density gradient ultracentrifugation (10). Each individual LDL subfraction profile was defined by a continuous variable K, as described in detail previously (11). A negative value (K<0) reflects a more dense LDL subfraction profile, and a positive K value (K>0) a more buoyant profile.

Statistical analyses

The Mann-Whitney-test was used to test differences at baseline between truly FCH subjects and their nonaffected relatives for statistical significance in the case of quantitative variables and the 2-tailed Fisher exact test in the case of qualitative variables.

Univariate logistic regression was used to evaluate the prognostic ability of the variables, separately, to discriminate between truly FCH subjects and their nonaffected relatives. Because the apoB level, K-value and TC and TG levels in particular were expected to be non-linearly related in the logistic model, reasonable cut-off values for these variables to discriminate truly FCH subjects from their unaffected relatives were constructed. At this point the condition of equal 'costs' of misclassification of cases and noncases was used. In other words, the optimal cut-off value for a particular variable was chosen so that the sum of the sensitivity and the specificity to discriminate truly FCH subjects from their unaffected relatives was maximal. Crude ORs with 95% CIs are presented.

Multivariate logistic regression analysis with selection procedures was used to determine the variables, that were sufficient and complete to contribute independently to the prediction of truly FCH. Because forward selection procedures do not identify other important variables, probability values for entry into the model were considered to find close alternatives to the variables selected. Adjusted ORs with 95% CIs are presented. With the multivariable prognostic model, a boundary value of the probability to be truly FCH, given the values of the prognostic variables only, was constructed again under the condition of equal 'costs' of misclassification of cases and noncases. In case of a two variable prognostic model a straight line dissociates cases (i.e. truly FCH with high probability) and noncases. In case of a three (or more) variable prognostic model a nomogram is constructed.

Finally, univariate logistic regression was used to evaluate the prognostic ability of variables, separately, to identify patients with CVD compared to normal. Statistical analysis was performed by using procedures available in SAS (software package 2000, SAS Institute Inc).

Results

In total, 32 families, comprising 299 subjects, were studied in both 1994 and 1999. One hundred and twenty one subjects were defined truly FCH, based on plasma pTC and/or pTG >90th adjusted for age and gender in 1994 and/or 1999 (Table 1). Thus, 40% of the study population was 'truly'-FCH, whereas 31% in 1994 and 32% in 1999 were affected by FCH. Table 2 shows the anthropometric measurements, lipid and (apo)lipoprotein concentrations and value of parameter K, reflecting LDL heterogeneity, in truly FCH subjects and nonaffected relatives in 1994.

Table 2 Anthropometric measurements, lipid and (apo)lipoprotein concentrations and value of parameter K, in truly FCH subjects and normolipidemic relatives in 1994.

	Truly FCH (n=121)		**Normolipidemic relatives (n=178)**	
Age, y	48	(19-73) *	34	(13-71)
BMI, kg/m^2	26	(18-37) *	23	(15-35)
CVD, n (%)	18	(15%) #	5	(3%)
TC, mmol/L	6.9	(3.7-9.0) *	5.2	(2.4-7.5)
TG, mmol/L	2.4	(0.2-15.0) *	1.0	(0.3-2.9)
LDL-C, mmol/L	4.48	(1.67-6.63) *	3.47	(1.25-5.71)
HDL-C, mmol/L	0.98	(0.53-2.56) *	1.25	(0.55-2.47)
Apo B, mg/L	1374	(604-2640) *	914	(467-1640)
K-value	-0.23	(-0.58-0.59) *	0.03	(-0.48-0.41)

BMI indicates body mass index. Values are median (minimum-maximum). Values of K<0 reflect small, dense LDL.

* p<0.001 Mann-Whitney test, #: p<0.001 Fisher's exact test.

Truly FCH subjects were significantly older and had a higher BMI than normolipidemic relatives. As expected, among subjects with FCH, CVD was more prevalent. By definition, the truly FCH group had higher TC and TG concentrations. The truly FCH group also exhibited significantly higher concentrations of LDL-C and lower concentrations of HDL-C. Furthermore, significantly higher levels of apo B and lower values of parameter K, reflecting small dense LDL, were found in the truly FCH group compared with the unaffected relatives.

Crude ORs to predict truly FCH are indicated in Table 3; all variables except gender, contributed independently to the diagnosis truly FCH.

Table 3 Crude ORs to predict truly FCH with univariate logistic regression

Independent variables	OR	95% CI	R2 ,(%)	AUC,(%)
Gender (m vs f)	1.13	0.71–1.79	1	52
Age, y	1.20	1.11–1.30	7	65
BMI, kg/m^2	1.41	1.29–1.55	31	79
TC absolute, mmol/L	3.08	2.36– 4.02	40	82
pTC	2.32	1.88–2.85	37	79
TC>6.0 mmol/L	7.19	4.29–12.07	25	73
TG absolute, mmol/L	11.51	6.43–20.61	58	89
pTG	3.24	2.48–4.24	54	87
TG>1.5 mmol/L	15.19	8.53–27.03	41	80
ApoB absolute (per 100 mg/L)	1.76	1.54–2.01	49	86
ApoB>1200 mg/L	12.31	6.99–21.69	37	78
K-value absolute (per 0.1)	0.60	0.51–0.69	27	78
K-value <-0.1	9.62	5.50–16.82	31	76

AUC indicates area under the curve as a measure of predictive discrimination (ie 50% is equivalent to random guessing and 100% is the perfect prediction); CI: confidence interval; R2: rescaled R-square, indicating the percentage explained variance; BMI: body mass index.

ROC analysis shows that the K-value, reflecting LDL heterogeneity, and apoB levels are nearly as good predictive for truly FCH as the traditional lipid criteria including TC and TG levels. Next the variables were dichotomized by determining the optimal cut-off values of apoB, TC, TG and K-value for prediction of truly FCH (Table 4). In our cohort cut-off values of apoB >1200 mg/l, parameter K <-0.10, TG levels >1.5 mmol/l (pTG ≥6) and TC levels >6.0 mmol/l (pTC ≥7) were found.

Table 4 Otimal cutoff values with receiver-operating curve parameters for apoB, parameter K, absolute TG and TC, pTG and pTC for prediction of truly FCH with univariate logistic regression under the condition of equal cost of misclassification.

	Cut-off point	Probability	Maximum J	Sensitivity	Specificity
ApoB, mg/L	>1200	0.44	0.55	0.77	0.79
K-value	<-0.10	0.40	0.51	0.77	0.74
TG, mmol/L	>1.5	0.35	0.59	0.81	0.78
TC, mmol/L	>6.0	0.42	0.46	0.70	0.75
PTG	≥6	0.58	0.61	0.77	0.84
PTC	≥7	0.74	0.51	0.51	1.00

To evaluate which (combination of) phenotype(s) best predicted truly FCH, we included all the variables presented in Table 3 in a multivariate logistic regression model. This analysis revealed that the absolute apoB value in combination with pTG and pTC best predicted truly FCH with an R^2 = 69%. pTG was the strongest independent predictor of truly FCH (R^2=58%); when percentiles are not available, TG concentrations >1.5 mmol/l can be used to predict truly FCH. When TG levels in percentiles were included in the model, the K-value did not provide additional information. In the next step of the regression analysis, apoB was the second most important predictor of FCH. Including apoB levels besides pTG in the model improved R^2 to 68%. Finally pTC contributed significantly to truly FCH, although the R^2 did not increase substantially. The R-square (i.e. the amount of variation) with the combination of these three variables was 69%.

By logistic regression the probability of classification affected FCH based on absolute apoB values and pTG and pTC was assessed at 60%. In Figure 1, the two most informative variables in predicting truly FCH, including apoB levels and the pTG levels, are plotted for all individuals.

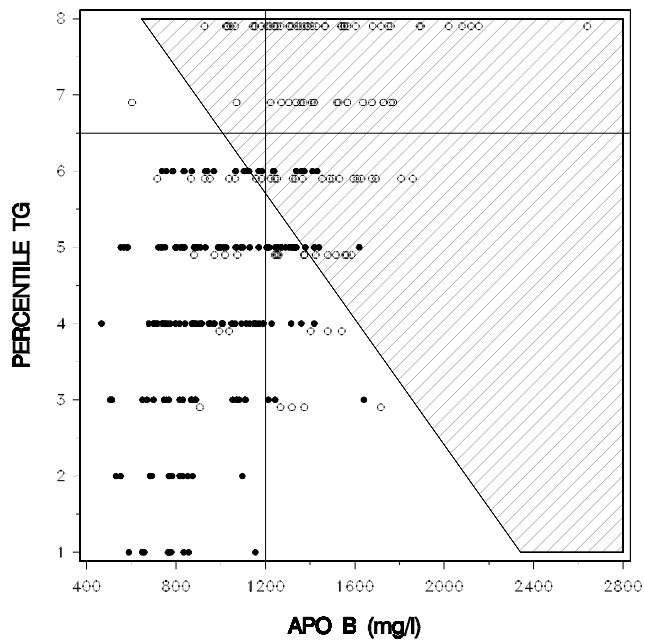

Figure 1 Probability of classification of affected by truly FCH on the basis of absolute apoB concentration and TG levels adjusted for age and gender.
Shaded area indicates probability of truly FCH of >0.52 on the basis of absolute apoB concentration and TG levels adjusted for age and gender. This area was optimal to discriminate those truly FCH from unaffected subjects under condition of equal costs of misclassification. For ease of presentation, unaffected subjects are shifted upward, truly FCH subjects are shifted downward, and cutoff point of pTG (90th percentile) is presented at 6.5. For pTG and pTC; 1= 0-5%; 2= 6-10%; 3= 11-25%; 4= 26-50%; 5= 51-75%; 6= 76-90%; 7= 91-95%; 8= 96-100%. ° indicates truly FCH subjects; •, unaffected subjects.

The optimal cut-off value to predict truly FCH based on the combination of absolute apoB with pTG is 0.52. The shaded area indicates high probability of predicting truly FCH, using the observed apoB and pTG values. This figure shows that the prediction area makes a fairly good distinction between truly FCH subjects and nonaffected subjects.

Finally, a nomogram was constructed to calculate the probability of affected by FCH (Figure 2). For each of the three variables (pTG, pTC and absolute apoB levels), the corresponding number of points is read from the scale below. These are then summed to give a total points score, which can be translated into a probability of being affected by FCH by using the 2 scales at the bottom. The optimal cutoff point for the probability of affected FCH was determined under the condition of equal costs of misclassification according to sensitivity and specificity. When having a probability >60% the subject is defined as affected by FCH when also at least 1 other family member also exhibits the FCH phenotype and at least 1 individual in the family has premature CVD. For example, a subject with a pTG of 7 (between 91 and 95%, 8 points), a pTC of 6 (between 76 and 90%, 2.8 points) and an apoB level of 1600 mg/l (4.3 points) will receive a total amount of 15.1 points. The probability of being affected by FCH is then 0.92. Thus, according to this test, this individual subject (if a member of a FCH family) is being affected by FCH.

Diagnosis FCH as predictor for CVD

In 1999, 41 subjects (14%) had a history of CVD. To define the ability of the different FCH definitions to predict CVD, sensitivity and specificity were determined. FCH diagnosis based on traditional lipid criteria, including pTC and/or pTG >90th adjusted for age and gender, showed a sensitivity and specificity to predict CVD of 45% and 27% respectively. The proposed new FCH diagnosis based on pTG, pTC and absolute apoB had a sensitivity and specificity for CVD risk prediction of 59% and 72%, respectively. In literature, the diagnosis FCH has also recently been defined as hyper-TG (TG >1.5 mmol/l) and hyper-apoB (apoB >1200 mg/l). In our cohort, the diagnosis of FCH on the basis of these criteria (hyperTG plus hyperapoB) had corresponding values of sensitivity and specificity to predict FCH (64% and 72%, respectively). Thus, both new definitions of FCH improved CVD risk prediction compared with the FCH diagnosis based on traditional lipid criteria.

The predictive value of the nomogram for CVD analyzed as a quantitative trait shows that the median probability of being affected by FCH is >3.5 times higher in patients with CVD (median (95% CI): 0.71 (0.54 to 0.80)) compared with patients without CVD (median (95% CI): 0.20 (0.28 to 0.38)) ($p<0.001$; t-test using angular transformed data).

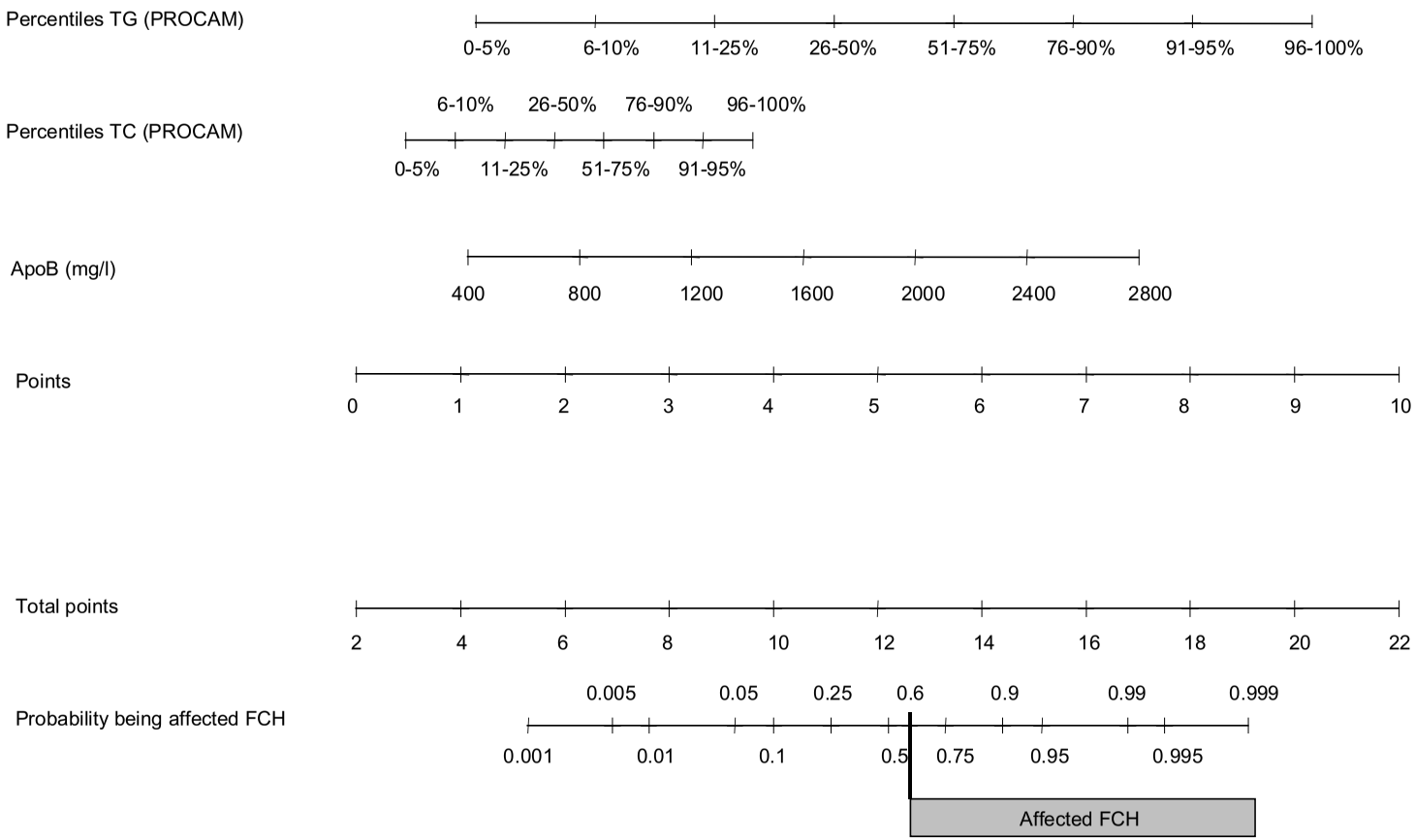

Figure 2 Nomogram to calculate probability of being affected by FCH using absolute apoB and TG-TC values adjusted for age and gender. In each of 3 variables, points are calculated by reading from point scale. Total point score is then translated into probability of affected FCH using 2 bottom scales.

Discussion

We propose a new definition of FCH based on absolute apoB levels and TG and TC levels, both adjusted for age and gender. A nomogram is provided to simply and reliably read the probability to be affected FCH in clinical practice (Figure 2). This nomogram has been constructed using data from our large FCH cohort including 299 subjects with 5-year follow-up. This new definition of FCH also reflects increased CVD risk in contrast to the traditional criteria of FCH.

Until now, different research groups used different definitions of FCH (1-3). In a recent follow-up study we showed that for the diagnosis FCH, only plasma TC and TG levels adjusted for age and gender, is insufficient (4). Furthermore, the phenotype FCH based on TC and/or TG levels alone is physiologically incoherent (12). 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 biologically correct hypotheses may be falsely rejected because of inaccurate diagnosis. For all these reasons, it is necessary to come to unequivocal standardized diagnostic criteria of FCH. The present study shows that absolute apoB levels combined with pTG and pTC are most informative to predict FCH.

ApoB as a diagnostic feature of FCH

In our previous study, subjects with FCH had significantly higher apoB concentrations compared to nonaffected relatives, even when they had a sporadic normolipidemic phenotype (4). Additionally, in the follow-up study of Mc Neely et al. (5) apoB levels were highest among individuals who were consistently affected by FCH, intermediate among those who switched phenotypes, and lowest among individuals who were consistently normolipidemic. In addition, several other studies have shown that apoB is an important phenotypic measure in FCH (4,13-16). ApoB fits with the most important pathophysiological feature of FCH, increased VLDL secretion and impaired clearance of postprandial lipoproteins, resulting in hypertriglyceridemia, elevated plasma apoB and small dense LDL. Another practical advantage of including apoB level as a new diagnostic criterion of FCH is diagnosing FCH at a younger age. A number of studies have shown that in teenaged children, apoB may be elevated, whereas lipids are not (2,17). A limitation in using apoB as a diagnostic criteria for FCH is the need for an apo B measurement standardized according to the WHO-IFCC standardization program. ApoB can be measured with the same precision and accuracy as the lipoprotein lipids (18). All these data encourage the inclusion of apoB in the phenotypic definition of FCH.

The present study shows in a large FCH cohort with 5-year follow-up that the optimal cutoff point for apoB to predict FCH is >1200 mg/L. Subjects with an apoB level >1200 mg/L have an increased probability to be affected by FCH (OR, 12.3 (95%

CI: 6.99 to 21.69)) compared with subjects with an apoB level <1200 mg/L. The level of apoB >1200 mg/L has been suggested in previous studies. It corresponds to roughly the 75th percentile in men (results of the Framingham Offspring study) (19), and subjects with apoB concentrations >1200 mg/L were significantly more likely to have CVD than subjects with apoB levels <1200 mg/L (20). Thus, our data now show convincingly that the measurement of apoB concentration is imperative for the diagnosis of FCH.

Small dense LDL and TG levels as diagnostic feature of FCH

Small dense LDL, reflected by the parameter K, is also an important feature in FCH (16,21-23). We previously showed that subjects with FCH had significantly lower levels of parameter K, reflecting atherogenic small dense LDL, compared with nonaffected relatives, even when they had a sporadic normolipidemic phenotype (4). However, this was not shown in the follow-up study of Mc. Neely et al. (5), maybe because different methods were used to determine small dense LDL. The present study shows that the optimal cutoff point for K-value to predict FCH is <-0.10, reflecting small dense LDL.

However, although small dense LDL is an important feature of FCH, it is of interest that in multivariate analysis, parameter K, reflecting LDL heterogeneity, did not provide additional information in predicting FCH after inclusion of TG, which is the most important predictor of truly FCH, most likely because of the strong correlation between LDL heterogeneity and TG levels (r=-0.68). Serum TG concentration has been suggested to be the most important predictor of LDL size in FCH patients (23). In both in earlier studies (9,24) and this study, we have confirmed that plasma TG levels >1.5 mmol/L distinguish optimally between atherogenic small dense LDL and a large buoyant LDL subfraction profile.

Hyper-TG and hyper-apoB as diagnostic feature of FCH

In an international forum on FCH in 2001, a proposal to redefine FCH was made that was based on hypertriglyceridemia and elevated plasma apoB (hyperTG hyperapoB) (12). The cutoff points proposed were >1200 mg/L for apoB and >1.5 mmol/L for plasma TG. The choice of these cutoff points was tentative and based on the reports mentioned earlier. We show here for the first time that cutoff points of >1200 mg/L for apoB and >1.5 mmol/L for TG are justified on the basis of our large FCH cohort with 5-year follow-up.

Comparing the absolute apoB value combined with pTG and pTC as diagnostic criteria for FCH with hyperTG and hyperapoB reveals that the diagnosis based on these combined values better predicted truly FCH (R^2 =69% versus 50% respectively).

The disadvantage of using percentiles of lipid levels in the definition of FCH is that these levels vary substantially across the world and therefore will affect decisions on cutoff levels. Lipid levels are changing, and up-to-date, accurate information

on lipid levels and threshold values is not available in many populations. An advantage of using percentiles is the correction for age and gender, but assignment of percentiles still depends on the population.

To take into account the great interindividual variability in apoB and TG levels, we recommend using apoB and TG levels as quantitative traits instead of a dichotomy i.e. apoB>1200 mg/L and TG>1.5 mmol/L. Much information is lost because we do not know whether an individual is close to or far from the threshold. Moreover, where family data are used, quantitative traits of relatives are often more informative than their affected status. As a clinical tool, a nomogram was constructed to simply and reliably calculate the probability of FCH in an individual subject on the basis of quantitative apoB, pTG and pTC values. In case pTC and pTG are not available, we show that it is acceptable to define FCH by TG levels >1.5 mmol/L and apoB values >1200 mg/L.

Finally, we found that our new definition of FCH is of value in predicting CVD risk. Subjects fulfilling the new FCH definition have an increased risk of CVD (OR=3.8 (95%CI: 7.0 to 21.7)). Subjects fulfilling the definition hyperTG and hyperapoB, have a comparable OR (4.6, 95% CI: 2.3 to 9.2) in contrast to the diagnosis of FCH based on traditional lipid criteria (pTC and/or pTG >90th), which had an OR of only 0.3 (95% CI: 0.2 to 0.6). Similar results have been reported for intima-media thickness, a surrogate end point of CVD (25).

Using the proposed new diagnostic criteria, included in a nomogram will make it easier to identify patients and test relatives to diagnose FCH. Still, the diagnostic phenotype has to be present in >1 family member, and ≥1 individual in the family must have premature CVD to diagnose FCH. Confirmation of the relevance of this new definition of FCH in other large FCH cohorts is warranted to confirm the unequivocal diagnostic criteria for FCH.

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Chapter 4

Metabolic pathogenesis of Familial Combined Hyperlipidemia with emphasis on insulin resistance, adipose tissue metabolism and free fatty acids

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Introduction

Familial combined hyperlipidemia (FCH) was originally identified by Goldstein et al, Rose et al, and Nikkila et al in the early 1970s as a new inherited lipid disorder, characterized by multiple lipoprotein phenotypes and strongly associated with premature cardiovascular disease (1-3). At present, FCH is the most common inherited hyperlipidemia in humans, affecting 1% to 3% of the population and up to 20% of patients with premature myocardial infarction. The genetic and metabolic basis of the disorder has not yet been defined (4-6). This review discusses the different metabolic pathways with emphasis on the role of insulin resistance, adipose tissue metabolism and free fatty acids (FFA).

Definition of FCH

Individuals with FCH are characterized by variable expression of elevated cholesterol and/or triglyceride (TG) levels, defined by concentrations 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 atherosclerosis before the age of 60 years. 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 (4,5). Recently, we evaluated the variability in lipid phenotype expression over a 5-year period in 32 FCH families, comprising 299 subjects (7). We demonstrated that the diagnosis of FCH, based on plasma total cholesterol 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 (4,5). Characterization of our FCH cohort was substantially more consistent when using two other major characteristics of FCH: elevated apolipoprotein B (apoB) levels and the presence of small dense low-density lipoprotein (sdLDL). Further studies are necessary to evaluate the role of apoB and sdLDL as potential new diagnostic criteria for FCH (7,8).

Cardiovascular risk factors in FCH

In FCH, total cholesterol and TG levels are frequently elevated only mildly. In our large cohort of 42 FCH families, comprising 161 FCH patients and 401 normolipidemic relatives, mean total cholesterol and TG levels among FCH patients and non-affected relatives were 6.4 ± 1.4 mmol/L and 3.6 ± 2.9 mmol/L

versus 4.9 ± 1.0 mmol/L and 1.2 ± 0.5 mmmol/L, respectively. This FCH lipid profile is often associated with an unfavourable decrease in high-density lipoprotein (HDL) cholesterol concentration and elevated apoB concentration (4,5,7). In addition, other traits in FCH have been proposed to contribute to accelerated atherosclerosis in vivo, including the presence of increased numbers of sdLDL, increased oxidative stress, low-grade inflammation, genetic heterogeneity, insulin resistance and impaired metabolism of fatty acids (4-6).

Metabolic pathogenesis of FCH

Abnormalities in several metabolic pathways have been suggested to be important in causing the FCH phenotype (Figure 1).

FCH and metabolism of apo-b100 containing lipoproteins

In general, FCH is thought to be caused by [1] hepatic very low-density lipoprotein (VLDL) overproduction either with or without [2] impaired clearance of TG-rich lipoproteins from plasma. These TG-rich lipoproteins, of exogenous and endogenous origin, result in an increase in the pool of apoB100 containing TG-rich lipoprotein (9). Although apoB overproduction is a well-established feature of FCH, the underlying mechanism is not known. ApoB gene defects have been ruled out by linkage analysis in FCH kindreds (10). The most likely contribution to increased VLDL apoB production is increased concentrations of plasma FFA; their role is discussed in more detail below.

FCH and lipoprotein lipase

The enzyme lipoprotein lipase (LPL), activated by the cofactor apoCII, liberates FFA from TG-rich lipoproteins of both exogenous (chylomicrons) and endogenous (VLDL) origin. FA then pass into adipose tissue or skeletal muscle cells to be oxidized or stored (11). During this process, LPL converts VLDL into smaller apoB100-containing VLDL remnants, intermediate-density lipoprotein (IDL) and LDL (12).

Of patients with FCH, 36% have diminished postheparin LPL activity and mass values (13). Reduced LPL activity due to LPL gene mutations has been reported repeatedly in subsets of FCH populations. Our data confirm that previous studies have shown that LPL mutations have significant additional effects on lipid and lipoprotein phenotype expression in FCH (14). However, although linkage analysis ruled out LPL as a major gene in FCH, it may act as a modifier gene (15).

Another possible explanation for the reduced LPL activity and mass found in some FCH families may be an adaptation to changes in the transport of FFA in plasma, as increased concentrations of FFA decrease LPL activity (16). Furthermore,

insulin is the principal hormone that regulates LPL, i.e. stimulating expression and activity of adipocyte LPL (17). FCH is associated with insulin resistance, which also contributes to decreased LPL activity. Finally, increased apoCIII levels are associated with impaired clearance of TG-rich lipoprotein due to direct inhibition of LPL by apoCIII (18).

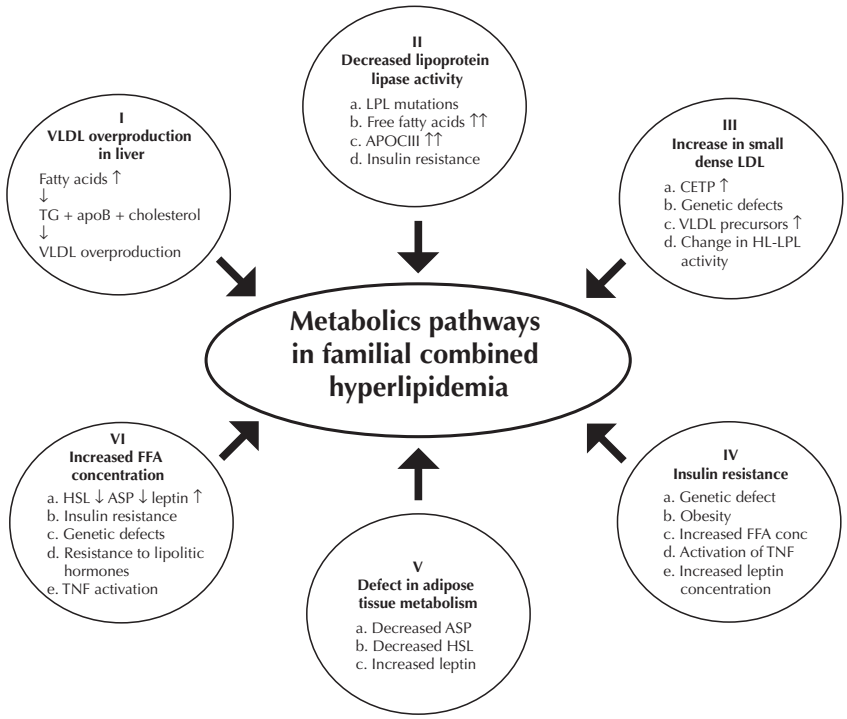

Figure 1 Different metabolic pathways in Familial Combined Hyperlipidemia
(I) Hepatic very low-density lipoprotein (VLDL) overproduction may result from an increased supply of fatty acids (FFA) to the liver. (II) Impaired clearance of triglyceride-rich lipoproteins may be caused by diminished lipoprotein lipase (LPL) activity resulting from a. LPL mutations; b. increased FFA concentrations; c. increased apoCIII concentrations; d. insulin resistance. (III) Small dense LDL may be present due to a. increased cholesteryl ester transfer protein (CETP) activity; b. major gene effect - genetic defect c. increased concentrations of precursor molecule VLDL; d. change in hepatic lipase (HL) and/or LPL activity. (IV) Insulin resistance may be caused by a. genetic defect; b. obesity; c. defects in fatty acid metabolism; d. activation of TNF system; e. increased leptin concentration. Insulin resistance itself results in impaired suppression of HSL (V), increased FFA concentrations (VI) and diminished LPL activity (II). (V) Defects in adipose tissue metabolism may result from a. decreased activity of acylation stimulatory protein (ASP); b. decreased activity of hormone sensitive lipase (HSL); c. increased leptin concentration. These defects contribute to insulin resistance (IV) and increased plasma FFA concentration (VI) which results in increased supply of FFA to the liver (I). (VI) Increased FFA concentrations may be induced by a. (genetic) defects in HSL, ASP or leptin (V); b. insulin resistance (IV); c. genetic defects; d. resistance to lipolytic hormones; e. TNF activation. Increased concentration of plasma FFA results in increased supply of FFA to the liver (I), insulin resistance (IV) and decreased LPL activity (II).

FCH and insulin resistance

Resistance to the normal action of insulin is related to alterations in lipid metabolism, such as hypertriglyceridemia, low HDL cholesterol and an excessive postprandial release of FFA. An increased supply of FFA to liver cells is associated with VLDL overproduction. Furthermore, the normal insulin-mediated activation of LPL is diminished resulting in a reduced clearance of TG-rich lipoproteins (19). In addition, insulin resistance may coincide with a predominance of sdLDL particles (20). Since all these features are also characteristics of FCH, the existence of insulin resistance may be an important factor modulating FCH phenotypes.

Impaired insulin action in FCH patients, both on the suppression of serum FFA and the stimulation of glucose disposal, has been demonstrated directly by several groups using the euglycemic hyperinsulinemic clamp technique (21-24).

In 1997, we demonstrated that in male FCH patients, who presented with combined hyperlipidemia, mean whole-body glucose uptake was lower compared with the non-affected relatives (21). Most importantly, these observations were not confounded by gender/age and were found in the absence of obesity, as measured by body mass index (BMI), hypertension or impaired glucose tolerance. A similar study was reported by Aitman et al (22), who also used the clamp technique to study insulin action on both carbohydrate and fatty acid metabolism in 8 FCH patients with combined hyperlipidemia compared with 6 normolipidemic relatives and 2 non-related controls. FCH patients showed impaired insulin- mediated glucose uptake in peripheral tissues, which was dependent on increased upper- body fat, as measured by dual-energy X-ray absorptiometry scan. Furthermore, impaired insulin mediated suppression of serum nonesterified fatty acids (NEFA) was found, although this was independent of BMI or fat distribution. This is consistent with elevated fasting and postprandial NEFA in hypertriglyceridemic FCH patients (25). These findings suggest that a defect in insulin-mediated suppression of NEFA in patients with FCH may play a primary role in the development of FCH phenotype. These results were confirmed and extended by a study of Karjalainen et al (23) who studied 28 FCH patients with combined hyperlipidemia, 30 non-affected relatives and 72 non-related controls. Their data also showed that defects in both glucose oxidation and FFA suppression were also observed in relatives without dyslipidemia.

In conclusion, the suppressive effect of insulin on FFA levels is impaired in both patients with FCH and their first-degree relatives. This impairment may precede dyslipidaemia in FCH.

Several pathophysiological mechanisms may contribute to insulin resistance in FCH i.e., increased upper body-fat, decreased insulin-induced vasodilatation in skeletal muscle (21) or impaired post-prandial FA metabolism, as discussed below. Another potential mechanism contributing to insulin resistance is the activation of the tumor necrosis factor (TNF) / TNFreceptor axis. TNF has the ability to modulate the binding of insulin receptor substrate 1 (IRS-1) to the insulin receptor, thus

contributing to reduced insulin sensitivity (26). Activation of the TNF-TNFR axis also causes increased secretion of VLDL from the liver (27), an important characteristic of FCH. Other effects of TNF include induction of de novo FA synthesis in adipocytes and hepatocytes and reduction of LPL expression on the endothelium (28). Finally, the increased leptin concentration reported in FCH (29) may also result in insulin resistance, due to the effect of leptin on pancreatic insulin secretion and on peripheral insulin action and sensitivity.

FCH lipid phenotype and insulin resistance

All studies on insulin resistance in FCH have been performed in patients who exhibit a combined hyperlipidemia. The reported defect in the ability of insulin to suppress FFA release results in elevated serum FFA concentration, and in peripheral tissues, particularly skeletal muscle, high FFA levels block glucose oxidation, causing insulin resistance (30). In the liver, high flux of FAA is the most likely driver of hepatic overproduction of TG and apoB, thereby contributing to an elevation in the concentration of VLDL (31). Insulin resistance is associated with a combined or hypertriglyceridemic lipid phenotype. However, this does not explain (isolated) high LDL-cholesterol in FCH, and the question arises as to the role of insulin resistance is in FCH patients with high LDL choleserol in the absence of hypertriglyceridemia.

This question was addressed by Pihlajamaki et al (24), who studied insulin action in 55 FCH patients, including 19 subjects with hypercholesterolemia only, 22 subjects with combined hyperlipidemia, 14 subjects with isolated hypertriglyceridemia, and 50 non-affected relatives compared with 110 non-related controls. All FCH family members, irrespective of their lipid profile, had higher FFA levels than controls. In contrast, insulin-stimulated glucose uptake was impaired only in patients with hypertriglyceridemia or combined hyperlipidemia. This is the first study to report that insulin action is selectively impaired only in hypertriglyceridemic/combined hyperlipidemic subjects. In 1998, Vakkilainen et al (32) also investigated whether glucose tolerance/insulin resistance is an inherant feature of FCH, independent of lipid phenotype or secondary to existing hypertriglyceridemia. In 253 FCH patients, an oral glucose tolerance test was performed. Among controls 94% had a normal glucose tolerance versus 80% of the hypercholesterolemic FCH patients (type IIA). In FCH patients with hypertriglyceridemia or combined hyperlipidemia, glucose tolerance was impaired, with a prevalence of 58.3% and 54.3%, respectively. In our large FCH cohort, including 23 patients with only hypercholesterolemia, 96 patients with hypertriglyceridemia and 30 patients with combined hyperlipidemia, we evaluated insulin sensitivity using the homeostasis model assessment (HOMA, fasting insulin x fasting glucose / 22.5), which correlates closely with the glucose clamp technique in the assessment of insulin sensitivity. The HOMA index in patients who presented with hypertriglyceridemia or combined hyperlipidemia was significantly higher compared to patients with isolated hypercholesterolemia and

normolipidemic relatives (3.89 ± 3.04 and 4.42 ± 2.67 versus 2.67 ± 1.28 and 2.37 ± 1.41, unpublished data).

However, a major concern in all these studies is that a change in lipid phenotype, which is considered an important characteristic of FCH, is not taken into account. We reported that 43% of the FCH patients showed a change in lipid phenotype expression after 5 years (7). The effect of these intra-individual changes in lipid phenotype expression with time on insulin resistance has not been addressed in literature. It would be of great interest to study the interdependence of insulin resistance on change in lipid phenotype expression with time to reveal further the role of insulin resistance in FCH.

FCH and insulin resistance and visceral obesity

A tempting hypothesis that potentially explains the presence of insulin resistance in hypertriglyceridemia alone, with or without hypercholesterolemia, is the frequent presence of visceral obesity in hypertriglyceridemic subjects (33). The interdependence of insulin resistance and visceral obesity was studied by Purnell et al (34). Insulin action was studied using 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 associated inversely with intra-abdominal fat and BMI but not with subcutaneous fat. Therefore, insulin resistance was present in patients with FCH who were viscerally obese. The degree of insulin resistance was related to their degree of visceral obesity. In our FCH cohort, the HOMA index, used as a measure of insulin sensitivity, was also related to BMI ($r=0.43$, $p<0.001$, unpublished data). Insulin resistance was also present in non-obese FCH patients (obesity measured by BMI) (21). However, it has been suggested that intra-abdominal fat levels, as measured by CT-scan, can vary up to 10-fold in non-obese subjects. The most sensitive parameter for predicting insulin resistance (e.g. BMI- reflecting degree of adiposity, waist-hip-ratio (WHR)- reflecting abdominal obesity, or CT-scan measuring intra-abdominal fat) remains to be determined.

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

It is well-known that insulin resistance and visceral obesity are both associated with a number of metabolic abnormalities, including increased TG levels, lower HDL cholesterol, more sdLDL particles, and increased apoB production rates. These abnormalities are all characteristics of FCH (19).

The main question of whether insulin resistance / visceral obesity can explain the FCH lipid phenotype remains unanswered. Purnell et al (34) examined the relationship between insulin resistance / visceral obesity and increased apoB levels in 11 FCH patients. For any level of insulin sensitivity or intra-abdominal fat, apoB

levels remained higher in FCH patients than 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 (unpublished data). Therefore, visceral obesity and/or insulin resistance, do not account fully 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 aetiology of the FCH lipid phenotype. We have reported previously that in our FCH families, a major gene influences apoB levels and sdLDL (35), although, this gene has not yet been found.

FCH and adipose tissue metabolism

So, FCH is associated with reduced insulin-mediated NEFA suppression, which is consistent with elevated fasting and postprandial FFA in hypertriglyceridemic FCH patients (25). Why are FFA suppressed poorly by insulin in FCH patients? It is hypothesized that a defect in FA metabolism is a primary defect in FCH, which is likely to be localized in the adipocyte: adipose tissue is the major site for storage and mobilization of FFA within the body.

A simplified scheme of adipose tissue metabolism is shown in figure 2. The FFA liberated by the continuous hydrolysis of core TG by LPL in VLDL and chylomicrons are stored in adipocytes by intracellular re-esterification to TG, a process that is mediated by the action of a basic protein called acylation stimulatory protein (ASP) and modulated by insulin (36). In the adipocyte re-esterification of FFA results in TG that can be hydrolysed again by hormone sensitive lipase (HSL), releasing FFA from the fat cell. While the most important lipolytic hormones are cathecholamines, insulin is important in the inhibition of HSL (37).

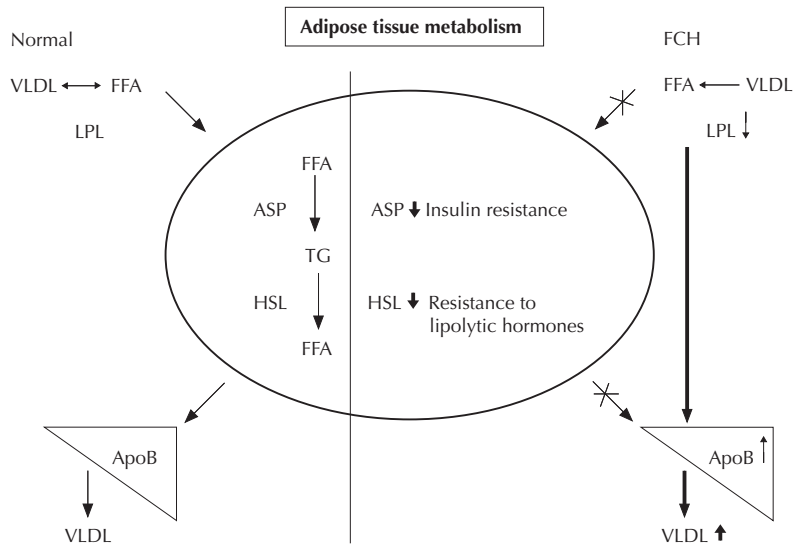

Figure 2 Potential defects in adipose tissue metabolism in Familial Combined Hyperlipidemia, see figure 1 for key to abbreviations

FCH and the role of ASP in adipose tissue metabolism

ASP was first characterized by Cianflone et al in 1989 (38). Adipocytes are able to secrete complement factors, including C3, from which ASP is generated through activation of the alternative complement pathway. The ASP pathway modulates the rate at which FA are trapped by adipocytes and converted to TG. ASP also influences the rate at which FA are released by adipocytes. In vivo evidence on the role of the ASP pathway in determining the effectiveness of FA trapping and storage by adipocytes has recently been reviewed extensively (39).

Resistance of adipocytes to the effects of ASP and/or impaired ASP activity may contribute to reduced FFA uptake into adipocytes, potentially resulting in an increased flux of FFA to the liver and consequently in an increased hepatic VLDL synthesis, characteristic of FCH. For example, impaired ASP activity has been reported in hyperapobetalipoproteinemia, a condition apparently related to FCH (40,41). However, no elevation of apoB lipids were found in ASP-knock-out- mice (42). It is possible that species variations may account for this difference. In contrast, Cianflone et al (43) reported increased ASP levels in patients with coronary heart disease (CHD) and speculated that elevated ASP levels would counteract the reduced number (or impaired function) of the putative ASP receptor. Recently, Ylitalo et al (44) studied plasma ASP levels in 66 hypertriglyceridemic FCH patients compared with 84 normotriglyceridemic relatives. No differences in plasma ASP levels was found. A key step in the ASP concept is that chylomicrons will promote ASP production. However, no response in plasma ASP after a fatty meal was found in FCH subjects (44) or in healthy controls (45). However, Cianflone et al (46) did report increased ASP levels after oral fat load. It has been suggested that ASP is generated only locally in adipose tissue and cannot be detected in peripheral plasma. Indeed, the work of Saleh et al (47) demonstrated that ASP generation in adipose tissue was accentuated postprandially in vivo. Although no significant difference in plasma ASP levels were found in FCH patients compared with their normolipidemic relatives, FCH patients were found to have significantly elevated serum levels of C3 (44). It remains to be determined whether C3 is an independent risk factor or a surrogate marker of vascular inflammation and atherosclerosis.

FCH and the role of HSL in adipose tissue metabolism

The release of FFA from visceral adipocytes is mediated by the action of the enzyme HSL. This lipolytic effect of HSL is inhibited by postprandial hyperinsulinemia. As discussed above, FCH is associated with increased insulin resistance, which accounts for the increased VLDL production via increasing supply of FFA to liver cells.

Cathecholamines are the most important lipolytic hormones, and they activate HSL. In FCH patients from Sweden a marked resistance to the lipolytic effect of cathecholamines in fat cells has been demonstrated, predominantly due to a defect

in HSL (48). The maximum enzymatic activity of HSL was decreased by 40%. This lipolytic defect correlated with the serum lipid abnormalities typical for FCH. However, in FCH patients from Finland, no difference in adipose tissue HSL activity was found (49). In addition, in the genetically homogeneous Finnish population the HSL gene was not a major locus responsible for the expression of the FCH phenotype (50). The most likely explanation for the discrepancy in HSL activity results between the Swedish and Finnish FCH populations is the heterogeneity and background of FCH between the two populations. For example, a substantial number of the Finnish FCH patients had only hypercholesterolemia and a number of the patients with hypertriglyceridemia had normal apoB levels. Therefore, many of the Finnish patients would not have been expected to have impaired FFA trapping.

Recently, the putative role of adipose tissue in the pathogenesis of FCH has been reviewed (51). Although more evidence-based arguments are needed, the current hypothesis suggests that a combined defect in lipid storage (as a result of reduced ASP and insulin action) and in lipolysis (following decreased HSL activity) results in adipose tissue that is metabolically inactive. As a result, FFA are shunted directly to the liver which drives apoB synthesis and increases VLDL levels (Figure 2).

FCH and increased free fatty acid concentration

In vivo evidence has been provided suggesting a pivotal role for increased FFA in insulin resistance (22,31,52): FFA activate gluconeogenesis by supplying acetyl CoA which stimulates one of the enzymes involved in hepatic gluconeogenesis, namely pyruvate carboxylase. Increased glucose production acts as a trigger to stimulate insulin secretion from the pancreas in order to maintain normoglycemia. Therefore, this can result in an increased fasting plasma insulin concentration. In FCH, increased fasting plasma insulin concentrations associated with the insulin resistance syndrome may be secondary to impaired postprandial FA metabolism. High FFA levels may lead to both a decrease in insulin-stimulated glucose uptake in adipose tissue and skeletal muscle, according to the scheme proposed by Randle et al (30), and to an increase in the synthesis of lipoproteins in the liver (31).

Recently, in vivo evidence of defective postprandial and postabsorptive FFA metabolism in FCH was demonstrated (53). 24 hr oral fat loading tests were performed with 7 FCH patients with combined hyperlipidemia and 7 non-related controls. Changes in FFA and ketone bodies, which represent hepatic products of FFA metabolism, were studied. The post-prandial (0-8hr) increase in ketone bodies was almost 4 times higher in FCH patients compared with controls, suggesting an increased flux of FFA to the liver, possibly due to inadequate FFA trapping in the adipocyte. In the post-absorptive period (8-24hr), FFA and ketone bodies were decreased significantly in FCH patients compared with controls, in whom ketone bodies increased. This may represent a diminished release of FFA from adipocytes by HSL.

Why is the metabolism of FFA altered in FCH? Potential mechanisms include altered activity of ASP and/or HSL. Furthermore, insulin resistance has been associated with impaired inhibition of HSL, resulting in increased FFA release from adipocytes. In addition, genetic defects, resistance to lipolytic hormones, increased leptin levels and TNF activation may contribute to increased FFA concentrations in FCH (26-29).

Conclusions

Studies aiming to solve the complex metabolic background of FCH have been going on for nearly 25 years. Several metabolic abnormalities have been suggested, but the exact cause of these metabolic disturbances is unknown. The abnormal metabolism of FA may be a primary defect in FCH which provides a pathophysiological link to the hepatic apoB overproduction, partial LPL deficiency and the insulin resistance sydrome, all of which are characteristic features of FCH (Figure 3).

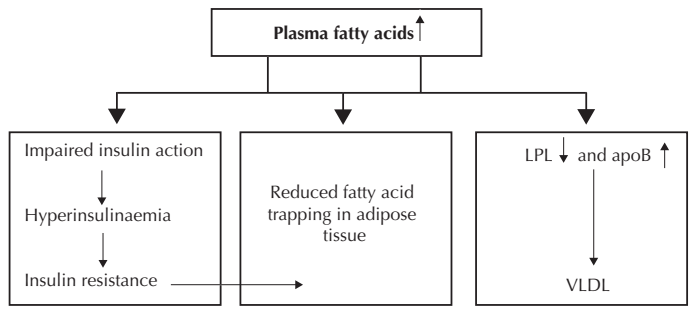

Figure 3 Possible central role of fatty acids in the pathogenesis of FCH
Free fatty acids (FFA) activate hepatic gluconeogenesis. Increased production of glucose is a trigger to the pancreas to secrete insulin in order to maintain normoglycemia and can thus result in increased fasting plasma insulin concentration, which is associated with the syndrome of insulin resistance. A defect in insulin action at the level of the adipocyte reduces FFA trapping, resulting directly in elevated plasma FFAs. Increased hepatic availability of FFA stimulates VLDL-apoB and triglyceride production. Furthermore, increase of FFA in plasma inhibits lipoprotein lipase (LPL) and promotes the release of LPL from the endothelial cells resulting in increased uptake of LPL by the liver which could result in slower clearance of TG-rich particles from plasma. So, an abnormal metabolism of fatty acids provides a pathophysiological link to the hepatic apoB overproduction, partial LPL deficiency and insulin resistance syndrome, all characteristic features of FCH. The defect in fatty acid metabolism could be in the acylation stimulatory protein (ASP) or hormone sensitive lipase (HSL) pathway, or due to resistance to lipolytic hormones, increased leptin concentration or activation of the TNF/TNFR axis.

To further delineate the pathogenesis of FCH, it is essential to simplify and standardize the diagnosis of FCH (7,8). In this respect, both the aetiology of the increased pool of apo B100 containing TG-rich lipoproteins and that of the preponderance of sdLDL particles in FCH patients have to be elucidated.

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Chapter 5

Comparison of the measurement of lipids and lipoproteins versus assay of apolipoprotein B for estimation of coronary heart disease risk: a study in Familial Combined Hyperlipidemia

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Abstract

We compared in 506 members of families with familial combined hyperlipidaemia (FCH), two approaches to select subjects with an apparent increased risk for coronary heart disease: assay of only apolipoprotein (apo) B versus measurement of plasma lipids and lipoproteins. When comparing both criteria, there was an overlap of 81.2% at apo B levels ≤1250 mg/l and of 86.9% at apo B levels >1250 mg/l. At apo B ≤1250 mg/l all subjects were normolipidemic. However, 18.8% of these subjects had sub-normal HDL-cholesterol concentrations (<0.9 mmol/l) who were not considered to have an increased risk because of very low LDL-cholesterol levels (<2.5 mmol/l). At apo B concentrations >1250 mg/l we observed a group with normal plasma lipid levels (13.1%). This group was defined as normolipidemic hyperapobetalipoproteinemia who are considered to have an increased risk for coronary heart disease. In this group, apo B determination was thus most informative. The selection of the subgroup with "normolipidemic hyperapobetalipoproteinemia" on the basis of the conventional approach could be refined using a cut off limit for plasma triglycerides <1.5 mmol/l. This limit distinguished optimally between an atherogenic very dense LDL pattern versus a dense and buoyant pattern.

Thus, based on the results of our study, the determination of apo B appeared to be, if not superior, at least as effective as the conventional lipid and lipoprotein parameters in classifying subjects at increased risk for coronary heart disease.

Introduction

At present, the most commonly used approach to estimate coronary heart disease risk is based on the measurement of plasma cholesterol, triglycerides and high-density lipoprotein cholesterol (HDL-chol) with calculation of low-density lipoprotein cholesterol (LDL-chol) by means of the Friedewald formula (1-4). The variable fasting conditions of the examined patients constitute the major limitation of this approach. In addition, the information that dense LDL is more atherogenic than buoyant LDL is not included (5). There is consensus that apoprotein (apo) B of LDL is a more accurate clinical measure of atherogenic risk than is total cholesterol or LDL-chol (6-10), but it is doubtful whether this is also true when risk estimation includes plasma lipids and HDL-chol. Sniderman and Cianflone (11) recently proposed apo B measurement as the first line risk estimator for coronary heart disease in stead of the conventional lipoprotein-oriented approach.

This proposal prompted us to review our results on measurement of lipids, lipoproteins, LDL-subfractions and apo B measurement in 40 families with familial combined hyperlipidemia (FCH). FCH is the most common form of heritable lipid disorder accompanied by an increased incidence of cardiovascular disease (12). In these families, the occurrence of dense LDL is typical (13) and normolipidemic hyperapobetalipoproteinemia is more frequent (14). Thus, this sample group provides interesting study material to compare the validity of the lipoprotein-based and apoprotein B-based approaches in risk evaluation of coronary heart disease.

Subjects and Methods

Design

The recruitment of FCH families took place through probands exhibiting a combined hyperlipidemia with both plasma cholesterol and triglycerides above the 90th percentile, adjusted for age and gender, as derived from the Prospective Cardiovascular Munster (PROCAM) study (15). These values were consistent over several measurements in which the probands had not been given any lipid-lowering drug. Families were included when a multiple type hyperlipidemia with levels of total plasma cholesterol and/or triglycerides above the 90th percentile was present. Besides a proband presenting a combined hyperlipidemia, the presence of at least one first-degree relative with hypertriglyceridemia or hypercholesterolemia was obligatory. Furthermore, at least one of the first-degree relatives should have cardiovascular disease before the age of 60 years. All probands were tested for an underlying cause of hyperlipidemia (i.e., diabetes mellitus, hypothyroidism and hepatic or renal impairment). The presence of one of these causes excluded them and their families from further analyses. None of the probands in these families was

homozygous for the apo E2 allele and none of the first-degree relatives had tendon xanthoma. In addition, to refine the selection procedure, the 95th percentile for plasma cholesterol and triglycerides was used if the body mass index exceeded 30 kg/m^2, or an alcohol consumption of more than two units per day was present. In total 506 members including the probands were studied from multigenerational families. All individuals were Caucasian above the age of 12 years. Everyone filled out a questionnaire in order to collect information on medical status, medication use, alcohol intake and smoking habits. The ethical committee of the University Hospital of Nijmegen approved the study protocol. Details about the study population have been described elsewhere (13,14).

Blood sampling procedure

Patients did not taken lipid-lowering medication for at least four weeks. After an overnight fast blood was drawn by venipuncture into EDTA-containing vacutainer tubes. Non-local participants were visited at their homes, and blood was transported immediately to the laboratory. Plasma was isolated within 3 hours for determination of the lipid, lipoprotein, apolipoprotein B levels and the LDL subfraction profile.

Analytical methods

Plasma cholesterol and triglycerides were measured on the Hitachi 747 analyser with enzymatic, commercially available reagents (Boehringer-Mannheim, Germany). HDL-chol was determined by the PEG-6000 method (16), LDL-chol was calculated by the Friedewald formula (4). For other purposes, not reported here, VLDL-chol and LDL-chol were determined by ultracentrifugation (17). Both series of LDL values obtained with different methods agreed well (Pearson correlation coefficient r = 0.97, n = 506) and were used in the risk estimation study. For risk evaluation, the upper limits for total cholesterol, LDL-chol, and triglycerides were 6.5, 4.5 and 2.0 mmol/l, respectively; as normal limits for HDLchol we used ≥0.90 mmol/l (18). Subjects were defined to have abnormal plasma lipids or lipoproteins when at least one of the values was abnormal.

Total plasma apo B concentrations were determined by immunonephelometry following modifications of a previously reported approach (19). As antigen we used LDL (d 1.025 – 1.038 g/ml). The antiserum appeared monospecific and did not react with purified apo A-I, apo A-II, apo C-II, apo C-III, apo E or human albumin as tested by immunodiffusion. The analyses were performed on the Behring Nephelometer II (Behringwerke AG, Marburg). Before use, the antiserum was treated with 7.5% (w/v) PEG-4000 1:2 (v/v). After incubating overnight at room temperature, the mixture was centrifuged and filtered through a 0.20 um filter. Sample (30 uL), diluted 20 or 100 times with saline, was incubated with 40 uL of antibody solution for 20 min followed by immunonephelometry. Results of both dilutions had to be within 5% and, after acceptance, were averaged. The assay was calibrated against freshly

isolated LDL. The LDL protein content was determined by the method of Lowry et al. (20) with chloroform extraction of the coloured solution before reading the absorbance. To evaluate the standardisation of the measurements, two sets of fresh-frozen plasma samples were shipped in dry ice to the Northwest Lipid Research Laboratories at the University of Washington, Seattle. One set was composed by 14 untreated plasma samples while the other set was composed by 14 samples to which we added 600 mg/L of saccharose prior to being frozen at -80º C. Multiple apo B analyses were performed on these samples at the Northwest Lipid Research Laboratories using a standardised nephelometric approach calibrated by the use of the World Health Organisation International Federation of Clinical Chemistry (WHO-IFCC) First International Reference preparation for apo B (21).

LDL subfractions were detected by single spin density gradient ultracentrifugation, according to a method described in detail elsewhere (22). Up to five LDL subfractions could be distinguished concentrated in the following density ranges: LDL1 (1.030-1.033 g/ml), LDL2 (1.033-1.040 g/ml), LDL3 (1.040-1.045 g/ml), LDL4 (1.045-1.049 g/ml) and LDL5 (1.049-1.054 g/ml). The ultacentrifugation tubes, containing the LDL subfractions stained with Coomassie Briljant Blue R, were placed in a specially designed rack and photographed (23). Accurate documentation of the different LDL subfraction patterns was obtained by scanning the obtained slides in triplicate on a LKB 2202 ultrascan laser densitometer gel scan program (Pharmacia-LKB, Upsala, Sweden), The mean peak heights (h1-h5) of the LDL subfractions (LDL1-LDL5) on the three scans were used to calculate the parameter K as a continuous parameter, that best describes each individual LDL subfraction pattern (24). The relative contribution of each LDL subfraction, expressed by its peak height (%hl -%h5), relative to the total LDL subfraction profile (total LDL [100%] = %hl+%h2+%h3+%h4+%h5) was calculated. The relative peak heights of LDL3 and the less frequently occurring LDL4 and/or LDL5 were added to give %h3' = (%h3+%h4+%h5), where LDL (100%) = LDL 1 [%h 1] + LDL2 [%h2] +LDL3 [%h3'], When a subfraction profile was characterised by a predominance of buoyant LDL particles (h1-h3 >0), parameter K was calculated by K=(%h1-%h3')/(%h2-%h3' + 1). In the case of a predominance of dense LDL subfraction (h1-h3 <0), parameter K was calculated by: K=(%h1-%h3)/(%h2-%h1 + 1). A buoyant LDL subfraction profile was defined by values of K≥0, whereas a dense LDL subfraction profile was reflected by parameter K<0 (24). In those subjects with predominantly dense LDL subfractions LDL3, LDL4 and LDL5, parameter K was <-0.25. So, the dense LDL subfraction profile (K<0) was subdivided into 2 groups: dense (-0.25≤ K≤ 0) and very dense (K<-0.25) LDL subfraction profile.

Statistics

A one-way analysis of variance (ANOVA) was used to analyse the differences in plasma lipid and lipoprotein levels between subjects stratified by three different LDL subfraction profiles. The student t-test was used to analyse the differences in mean values between the distinctive groups. Pearson correlation coefficients were computed to determine the correlation between LDL values calculated by Friedewald formula and determined by ultracentrifugation. The correlation between apo B values obtained in our laboratory and these obtained at the Northwest Lipid Research Laboratories (NWLRL) in Seattle was also determined by computing Pearson correlation coefficients. All values are presented as mean ± S.D.. Statistical analysis were performed with procedures available in SPSS/PC software (Statistical Package for the Social Sciences) Software package version 4.0 (SPSS Inc., Chicago, IL, USA).

Results

Standardisation of apo B measurements

To evaluate the accuracy of the apo B measurements, we prepared two sets of fresh-frozen samples, encompassing a large range of apo B values. Saccharose was added as a cryopreservative to one set of samples while the other set did not contain any additive. Multiple apo B analyses were performed on each sample, in our laboratory and at the Northwest Lipid Research Laboratories (NWLRL) in Seattle. The NWLRL has been the leading laboratory of the IFCC International Standardisation of apo A-1 and apo B and consequently these parameters are measured by a highly standardised nephelometric approach calibrated against the WHO-IFCC International Reference Materials with accuracy-based target values assigned as previously described in details (21,25). An excellent agreement was found between the apo B values obtained in our laboratory and those obtained at the NWLRL with a correlation coefficient of 0.995 for the untreated samples and a correlation coefficient of 0.998 for the samples containing saccharose. However, there was a systematic bias between the apo B values obtained in our laboratory and those obtained at the NWLRL and this bias was practically identical in the two sets of data. The following equation, $y=0.73x + 49$, where x represents the Nijmegen data, provides the magnitude of the bias and this equation was used to harmonise our data to those traceable to the WHO-IFCC International Reference Material. When corrected, the average bias and the absolute bias for apo B measurements were 0.5% and 2.7%, respectively. These biases were independent of the plasma triglyceride concentration up to a value of 8.0 mmol/l. Considering the excellent correlation between the two methods, the inaccuracy of our data, prior to correction, was most likely related to the assay calibration due to differences in the method used for LDL purification and/or in the Lowry method used to assign a target value to our primary calibrator.

The diagnostic power of apolipoprotein B versus lipid and lipoprotein profile

We compared the validity of apo B assay as a selection criterion for coronary heart disease versus conventional risk estimation on the basis of measurement of lipids and lipoproteins. Subjects were grouped according to the cut off limits for total cholesterol, LDL-chol, and triglycerides (6.5, 4.5, and 2.0 mmol/l, respectively) (18), and stratified as having an increased risk of coronary heart disease when a value was outside the normal range. In total 246 subjects had an elevated total cholesterol and/or triglycerides and/or LDL-chol level and were defined as subjects at risk. In all these subjects the apo B level was >1250 mg/l. So, values of apo B above 1250 mg/l were considered to identify subjects at risk for coronary heart disease.
In table 1 we show the comparison of both diagnostic criteria for selecting subjects with an apparent increased risk for coronary heart disease.

Table 1 Comparison of both diagnostic criteria (apolipoprotein B values versus lipid and lipoprotein profile) for selecting subjects with an apparent increased risk for coronary heart disease.

	Apo B ≤ 1250 mg/l	**Apo B > 1250 mg/l**
Total group (n=506)	223	283
Normal lipids	181	37
Elevated TC and/or TG and/or LDL-chol *	0	246
Decreased HDL-chol only **	42	0

TC = total cholesterol, TG = triglycerides
* defined as TC >6.5 mmol/l and/or TG >2.0 mmol/l and/or LDL-chol >4.5 mmol/l
** defined as HDL-chol<0.9 mmol/l without other abnormal lipid concentrations

Within the group of subjects with apo B concentration ≤1250 mg/l no subject had elevated total cholesterol or triglyceride concentrations. A subgroup of 42 subjects (18.8%) was found with HDL-chol <0.9 mmol/l without other abnormal lipid levels, apparently not being at an increased risk because of the concomitant sub-normal total cholesterol and low LDL-chol concentrations (<2.5 mmol/l). Thus, at an apo B concentration ≤1250 mg/L, there was an overlap between both diagnostic criteria in 81.2% of the subjects. With inclusion of a second risk estimator of the lipid/lipoprotein approach, there was complete identity between both selection criteria. On the other hand, the group with apo B >1250 mg/L included 37 subjects (13.1%) with normal plasma lipid and lipoprotein parameters (Table 1). This subgroup is referred to normolipidemic hyperapobetalipoproteinemia in whom the risk estimation on the basis of apo B appears thus more accurate. At an apo B concentration >1250 mg/L, there was an overlap between both diagnostic criteria in 86.9% of the subjects.

The phenomenon of normolipidemic hyperapobetalipoproteinemie subjects (n=37) was studied further with regard to their triglyceride concentrations. All

these 37 subjects had a plasma triglyceride concentration between 1.5 and 2.0 mmol/l. When the cut off limit for normal triglycerides was lowered to 1.5 mmol/l, the overlap between both selection criteria in the subjects with apo B>1250 mg/l increased from 86.9 to 97.8%. Decreasing the cut off limit for plasma triglycerides from 2 mmol/l to 1.5 mmol/l could thus enhance the selection power. To underscore this, we re-analysed, subjects with a plasma triglyceride concentration between 1.5 and 2 mmol/l (n=76). Of the 76 subjects, only 9 had a normal apo B concentration which stresses the additional power of lowering the cut-off limit for normal triglycerides from 2.0 to 1.5 mmol/l for inclusion of the patients with normal versus hyperlipidemic hyperapobetalipoproteinemia.

Dense LDL as another risk estimator

The total group of 506 subjects was subdivided according to their LDL-subfraction profile defined by parameter K. In 132 subjects a buoyant LDL subfraction profile (K>0, group 1) was found. A dense LDL subfraction profile, defined by parameter K≥-0.25 and K≤0, was found in 151 subjects (group 2), whereas 223 subjects showed a very dense LDL subfraction profile (K<-0.25, group 3). As expected, in FCH a very dense LDL subfraction profile was frequently observed (Table 2).

Table 2 Plasma lipids, lipoproteins and apolipoprotein B concentrations in subjects stratified according to their LDL-subfraction profile.

	Buoyant LDL	**Dense LDL**	**Very dense LDL**
Parameter K[a]	**K>0**	**-0.25≤K≤0**	**K<-0.25**
	Group 1	**Group 2**	**Group 3**
Total group (n)	132	151	223
Age (years)	36.5 ± 16.3	37.7 ± 16.6	46.4 ± 15.9[c]
Plasma cholesterol	5.23 ± 1.13	5.48 ± 1.35	6.40 ± 1.30[c]
Plasma triglycerides[d]	0.88 ± 0.51	1.42 ± 1.42[b]	2.69 ± 1.84[c]
HDL-chol[d]	1.45 ± 0.33	1.24 ± 0.33[b]	0. 99 ± 0.25[c]
LDL-chol[d]	3.40 ± 1.04	3.68 ± 1.26[b]	4.27 ± 1.09[c]
Plasma apo B[d]	1050 ± 272	1218 ± 338[b]	1616 ± 378[c]
LDL-chol/plasma apoB[d]	3.31 ± 0.40	3.06 ± 0.50[b]	2.65 ± 0.57[c]

Plasma concentrations of lipids and lipoproteins are given in mmol/L and of apo B in mg/L.

a) parameter K is defined in Materials and Methods section
b) dense LDL versus buoyant LDL $p<0.05$, student's t-test
c) very dense LDL versus dense LDL $p<0.05$, student's t-test
d) significant difference for the presented variable ANOVA $p<0.005$

Table 2 shows the concentrations of plasma lipids, lipoproteins and apolipoprotein B stratified by LDL-subfraction profile. A significant difference for all parameters was found (ANOVA, $p<0.05$). A very dense LDL subfraction profile was associated with significantly increased levels of total cholesterol, triglycerides, LDL-chol, apo B and decreased concentration of HDL-chol and ratio LDL-chol/plasma apo B compared to a dense and buoyant LDL subfraction profile (Table 2).

In Figure 1 we show that a triglyceride concentration of 1.5 mmol/l is a sharp cut off limit for segregating subjects with a very dense LDL versus dense and buoyant LDL confirming the rationale to lower the threshold for normal triglyceride value from 2 to 1.5 mmol/l. In addition figure 2 shows that an apo B value of 1370 mg/l, near to the cut off limit of 1250 mg/l used in the present evaluation, can optimally distinguish subjects with very dense versus dense and buoyant LDL.

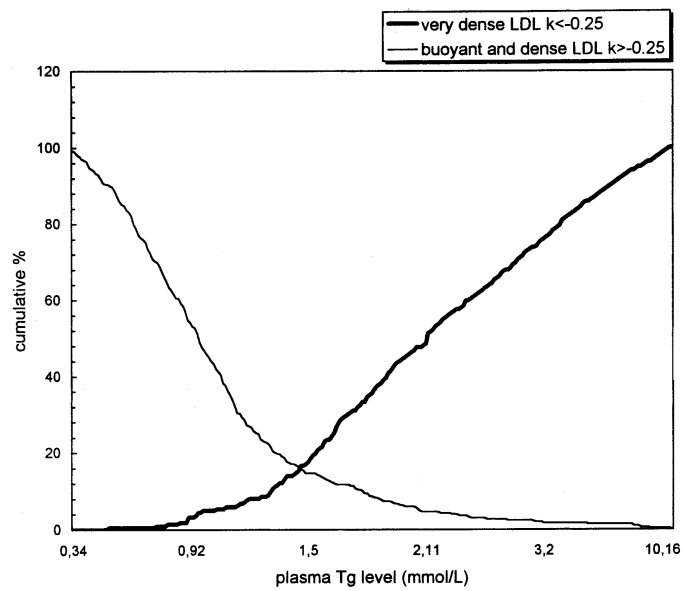

Figure 1 Cumulative frequency distribution of plasma triglycerides in the groups with very dense versus dense and buoyant LDL. Note: the frequency distribution for the dense and buoyant group is combined and reversed for better establishing the cut off limit that optimally distinguishes both groups. The intercept of both curves is at a triglyceride concentration of 1.5 mmol/l.

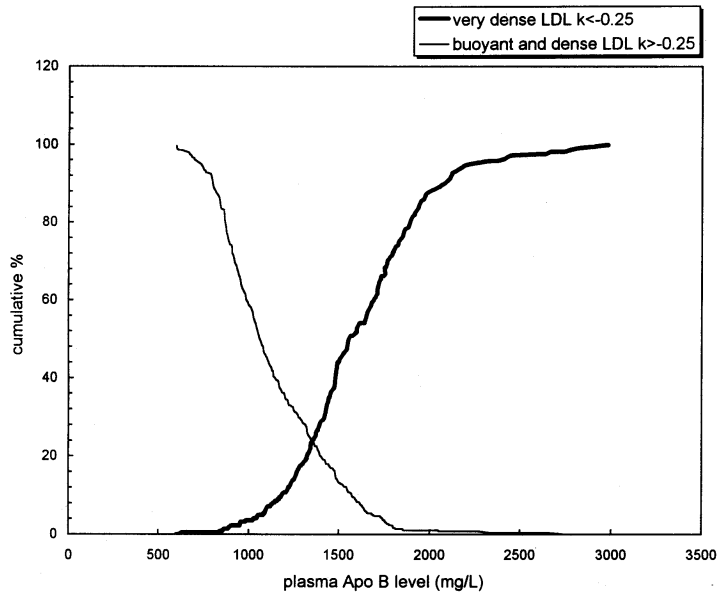

Figure 2 Cumulative frequency distribution of plasma apo B in the groups with very dense versus dense and buoyant LDL. For explanation see figure 1. The intercept of both curves lays at an apoB concentration of 1370 mg/l.

Discussion

In this study, using subjects with familial combined hyperlipidemia and their relatives, we show that assay of apo B results in almost the same selection of subjects with an increased risk for coronary heart disease as measurement of lipids and lipoproteins. Thus, on the basis of one single deviating parameter (apolipoprotein B) we obtained 81.2% of agreement between both criteria in the subjects with normal apo B concentrations (<1250 mg/l) and 86.9% agreement in the subjects with increased apo B concentrations (>1250 mg/l). With inclusion of a second parameter, we obtained 100% agreement in the first group, whereas, in the group with increased apo B concentration, an additional 10.9% of subjects with normal lipid concentrations could be included if the upper limit for triglycerides was decreased to 1.5 mmol/l. Thus, irrespective of knowing the coronary heart disease conditions assessed by invasive techniques we were able to show that the claim of Sniderman and Cianflone (11) about the predictive value of total apo B measurement in plasma is indeed justified. The predictive value of apo B is slightly superior to that obtained with the conventional risk estimation for which a fasting condition is needed and three different parameters should be measured according to strict quality control criteria in combination with calculation of LDL-cholesterol. Evaluation of treatment by diet or drugs would be less problematic if only the

change in one parameter has to be taken into account instead of positive and/or negative changes in three to four different parameters. The additional value of apo B measurement is especially relevant in subjects with the so called normolipidemic hyperapobetalipoproteinemia (6). Patients with this phenotype are characterised by a typical LDL-subfraction pattern with a predominance of very dense LDL; the protein to cholesterol content of this LDL is increased (13,14,26) This explains their preferential selection on the basis of apo B versus LDL-chol. A dense LDL subfraction is usually associated with an increased plasma triglyceride concentration (22-24). It is surprising that we were able to detect a group of normolipidemic subjects with a plasma triglyceride concentration in the upper part of the normal range (between 1.5 and 2.0 mmol/l). lt is likely that the subjects participating in our family study strictly adhered to the prescriptions for fasting.

Regarding the large biological variation of the fasting plasma triglyceride concentration (27), it cannot be excluded that these patients have temporary episodes of hypertriglyceridemia throughout the year. The half-life of VLDL, the main triglyceride carrier is several hours (28,29); while that of LDL is several days (29,30). In addition, the parameter LDL-apo B is independent of typical lipid exchange reactions, in contrast to LDL-chol. Apo B is thus a more stable metabolic estimate of the lipoprotein status similarly as HbA1c which is a long term metabolic control of glucose homeostasis. Consequently, strict fasting conditions on the day preceding the blood sampling procedure or an increased physical activity in the evening or morning before blood sampling can normalise plasma triglycerides in contrast to LDL-chol and especially to LDL-apo B. The fact that fasting conditions are mandatory for the lipid-oriented approach makes it more attractive to concentrate on apo B measurements. On the other hand, increased apo B concentrations are the result of quantitative changes in LDL rather than qualitative changes. Indeed, only 10.9% of our subjects with increased apo B concentrations belong to the last group with mainly qualitative changes in LDL. The apo B approach is thus primarily directed towards the atherogenic LDL particle. Consequently, this approach diminishes the significance of an altered triglyceride mechanism as a risk factor for coronary heart disease. On the basis of epidemiological and case control studies, hypertriglyceridemia in combination with slightly decreased HDL-chol level is considered atherogenic (15). However, the group with normolipidemic hyperapobetalipoproteinemia had, in our study, apo B values that were lower than those in the group with hypertriglyceridemia and decreased HDL-chol levels. That decreasing the cut off limit for normal triglycerides to 1.5 mmol/l results in the inclusion of this group, could mean that normolipidemic hyperapobetalipoprotein emia is a marginal problem within two valid diagnostic approaches. Nevertheless, these patients have a very dense LDL pattern. On the basis of this it is tempting to decrease, in the classical lipid approach, the normal limit for plasma triglycerides to

1.5 mmol/l. A similar approach was followed by Austin et al (27). This cut off value could have relevance for the metabolic control of VLDL secretion.

In our study, the apo B value appeared to be, if not superior, at least as effective as lipid and lipoprotein values in identifying subjects at increased risk for coronary artery disease. However, to reach uniformity across the studies, it is imperative that apo B is measured by standardized methods that are documented to be traceable to the WHO-IFCC International Reference Material. Recently, population-based reference values for apo B obtained by WHO-IFCC standardised methods showed an excellent agreement between the apo B cut-off values obtained in the different populations (31-33). Another potential problem in the accuracy of apo B measurements may be related to improper storage of the samples, which may result in denaturation of apo B (34). In this study, we show that the storage at -80º C does not affect the apo B assay and that addition of saccharose to fresh-frozen quality control samples is not needed. However, in lyophilized samples used as assay calibrator or quality control, saccharose addition may be important to prevent LDL denaturation (35).

In conclusion, we have shown that apo B values obtained on accurate assay are if not superior at least as effective as the conventional lipid and lipoprotein parameters in classifying subjects at increased risk for coronary artery disease.

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Chapter 6

Plasma homocysteine in subjects with Familial Combined Hyperlipidemia

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Abstract

Familial Combined Hyperlipidemia (FCH) is characterised by hypercholesterolemia and/or hypertriglyceridemia and associated with an increased risk of cardiovascular disease (CVD). The plasma lipid and lipoprotein levels in subjects with FCH are relatively moderately elevated and do not fully explain the increased risk of CVD. Hyperhomocysteinemia is a disorder of methionine metabolism and also a well-known independent risk factor for CVD. We investigated whether subjects with FCH have higher plasma homocysteine concentrations than controls, and whether homocysteine contributes to the increased risk of CVD in FCH. Furthermore we evaluated whether parameters of lipid and lipoprotein metabolism and/or insulin resistance are associated with the homocysteine level.

In total 667 subjects, including 161 subjects with FCH, 109 spouses who referenced as control group and 397 normolipidemic relatives were studied. FCH was defined by the presence of plasma total cholesterol and/or triglyceride levels above the 90th percentile adjusted for age and gender. The mean homocysteine concentration of the FCH group did not significantly differ from the control group. The risk for CVD due to hyperhomocysteinemia in subjects with FCH was not higher than in subjects without FCH. No associations were observed between plasma homocysteine concentration and plasma lipid and lipoprotein levels, including small dense LDL, nor between homocysteine concentration and insulin resistance.

Introduction

Familial combined hyperlipidemia (FCH) is the most common form of heritable lipid disorder with an estimated prevalence of 1.0 to 2.0% in the general population and 10 to 20% in survivors of premature myocardial infarction (1). The hyperlipidemia is characterised by elevations of plasma total cholesterol (TC) and/or triglyceride (TG) concentration and therefore known as 'multiple type hyperlipidemia'. Features of FCH include an increased production and decreased clearance of very low density lipoproteins (VLDL), an elevated apolipoprotein B (apo B) concentration and a preponderance of atherogenic small dense low density lipoprotein (LDL) subfractions (2,3). In addition, FCH is associated with insulin resistance. The lipids and lipoprotein levels in FCH are relatively moderately elevated and do not fully explain the total increased risk of cardiovascular disease (CVD).

Hyperhomocysteinemia is a disorder of methionine metabolism. Several studies reported that even moderate hyperhomocysteinemia is an independent risk factor for CVD (4-6). An increase of 5 µmol/L homocysteine may increase the risk of CVD by as much as does an increase in cholesterol level of 0.5 mmol/L (7). It is estimated that approximately 10% of the CVD in the general population could be attributed to hyperhomocysteinemia (7). However the results of some population-based prospective cohort studies have reported no association between homocysteine level and risk of CVD (8-10) or only in subpopulations (11,12).

The aim of this study was to investigate whether subjects with FCH have higher plasma homocysteine levels than controls and whether this metabolic factor contributes to the increased risk of CVD in FCH. Furthermore, we studied whether parameters of lipid and lipoprotein metabolism, including apo B and small dense LDL, and/or insulin resistance are related to the homocysteine level.

Subjects and Methods

Study population

Recently, we performed a large family study (n=667 individuals) to investigate the metabolic and genetic aspects of FCH. For the current study plasma homocysteine levels were measured in all subjects with FCH (probands and relatives) and compared with homocysteine levels of spouses, who referenced as control group. Furthermore, the normolipidemic relatives were included to evaluate which factors are related to the homocysteine level. The relatives and spouses in this study were ascertained through probands exhibiting a combined hyperlipidemia, with both plasma TC and TG concentrations above the 90th percentile, adjusted for age and gender, as obtained from the Prospective Cardiovascular Munster (PROCAM) study (13). These values were confirmed by repeated measurement, on a lipid

lowering diet and without lipid-lowering drugs. At least one first-degree relative of the proband had a multiple type hyperlipidemia with elevated levels of plasma TC and/or TG. Furthermore, at least the proband or one of the first-degree relatives should have premature CVD before the age of 60 years. All probands were tested for an underlying cause of hyperlipidemia (i.e., diabetes mellitus, hypothyroidism, and hepatic or renal impairment). The presence of one of these causes excluded the proband and his/her family from further analysis. None of the probands in these families were homozygous for the apo E2 allele and none of them and their first-degree relatives had tendon xanthoma. Relatives were included in this study in the FCH group when affected by FCH (having a plasma TC and/or TG level above the 90th percentile adjusted for age and gender (PROCAM)). The spouses of these probands and relatives referenced as control group. All individuals were Caucasian above the age of 12 years. All subjects filled out a questionnaire about their previous medical history, especially cardiovascular status, medication, and smoking habits. Body mass index (BMI), waist/hip ratio (WHR) and blood pressure were determined in all subjects. After a withdrawal of four weeks of lipid-lowering medication and an overnight fast, venous blood was drawn by venipuncture. Samples were put on ice immediately and transported to the laboratory. The ethical committee of the University Medical Center Nijmegen approved the study protocol.

Laboratory measurements

Serum levels of plasma TC, TG, and high density lipoprotein cholesterol (HDL-C) were determined enzymatically on a Hitachi 747 analyser. LDL-C levels were calculated according to the Friedewald method (14). ApoB concentrations were determined by immunonephelometry (15,16). LDL subfraction analysis was performed by density gradient ultracentrifugation (17). Each individual LDL subfraction profile was defined by a continuous parameter K, as described in detail previously (18), recently modified by using the relative cholesterol concentrations in the LDL subfractions to calculate parameter K. $K>0$ reflects a light LDL subfraction profile and $K\leq 0$ a small dense LDL subfraction profile (19).

The total homocysteine concentration in plasma was measured by an automated high-performance liquid chromatography method with reverse phase separation and fluorescence detection (Gilson 232-401 sample processor, Spectra Physics 8800 solvent delivery system and Spectra Physics LC 304 fluormeter), essentially according to the method by Fiskerstrand et al.(20). Glucose concentrations were measured 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 6%. Insulin resistance was assessed by the Homeostasis Model Assessment index (HOMA). The HOMA index was calculated by following formula as described by Matthews et al.: fasting insulin x fasting glucose / 22.5 (21). The methylenetetra-

hydrofolate reductase (MTHFR) genotype was tested by polymerase-chain reaction (PCR) essentially according to Frosst et al. (22).

Statistical analysis

Descriptive values were expressed as mean ± SD or absolute numbers with percentages. Differences in characteristics between subjects with FCH, controls and normolipidemic relatives were tested by means of Generalized Estimating Equations (GEE) because of possible correlated values within families. Also odds ratios as an estimate of risk of cardiovascular disease were calculated using GEE. Correlations between plasma homocysteine level (log transformed) and variables were analysed using Pearson's correlation coefficients. Multiple linear regression test was used to select the variables that contributed independently to homocysteine level. P-values <0.05 were considered statistically significant.

Results

In total 667 subjects, including 161 subjects with FCH, 109 spouses, who referenced as control group and 397 normolipidemic relatives from 37 families, were included in the study. Table 1 shows the anthropometric measurements, life style variables, including smoking and biochemical variables by subjects with FCH, controls and normolipidemic relatives. The ratio women/men was not significantly different between the groups. The mean age of the group of subjects with FCH was significantly younger compared to controls and significantly older compared to the normolipidemic relatives. As expected, the group of subjects with FCH had a higher prevalence of CVD compared to controls and normolipidemic relatives. Smoking habits did not differ between the studied groups. The BMI and WHR were significantly higher in the FCH group compared to the control group and normolipidemic relatives. Systolic blood pressure (SBP) and diastolic blood pressure (DBP) were not significantly different between the FCH group and controls, but were significantly higher in the FCH group compared to the normolipidemic relatives. By definition the FCH group had significantly higher plasma TC and TG concentrations compared to controls and normolipidemic relatives. In addition, the subjects with FCH had a more atherogenic lipid and lipoprotein profile as reflected by decreased HDL-C-, increased LDL-C- and increased apo B-concentrations. The subjects with FCH also had a more dense LDL subfraction profile as reflected by a more negative value of parameter K compared to the control group and normolipidemic relatives.

Subjects with FCH appeared to be more insulin resistant as assessed by the HOMA index, which was significantly higher, compared to controls and normolipidemic relatives. The glucose concentration was not significantly different between the FCH

group and control group whereas the subjects with FCH had significantly higher insulin levels comparing to both other groups. The prevalence of 677 TT genotype was not significantly different between the groups. The differences between the control group and normolipidemic relatives are indicated in Table 1.

Table 1 Anthropometric and biochemical parameters in subjects with familial combined hyperlipidemia (FCH) compared to controls and normolipidemic relatives (NL)

	FCH n=161	**Controls n=109**	**NL N=397**
M/F	71/90	50/59	188/209
Age (years)	47 ± 16 # *	53 ± 13 ‡	39 ± 16
CVD (y/n)	34 (21%) # *	4 (4%)	24 (6%)
Smoking (y/n)	35 (22%)	19 (17%)	95 (24%)
BMI (kg/m^2)	27.6 ± 4.1 # *	26.3 ± 3.8 ‡	24.1 ± 3.7
WHR	0.88 ± 0.09 # *	0.86 ± 0.09 ‡	0.83 ± 0.08
SBP (mmHg)	138 ± 17 *	135 ± 16 ‡	128 ± 16
DBP (mmHg)	86 ± 8 *	84 ± 9 ‡	80 ± 10
TC (mmol/l)	6.4 ± 1.4 # *	5.4 ± 1.0 ‡	4.9 ± 1.0
TG (mmol/l)	3.6 ± 2.9 # *	1.4 ± 0.9 ‡	1.2 ± 0.5
HDL-C (mmol/l)	0.95 ± 0.28 # *	1.27 ± 0.36	1.22 ± 0.32
LDL-C (mmol/l)	3.92 ± 1.33 # *	3.62 ± 0.96 ‡	3.30 ± 0.91
Apo B (mg/l)	1341 ± 292 # *	1057 ± 232 ‡	983 ± 240
K-value	-0.26 ± 0.28 # *	0.03 ± 0.24	0.04 ± 0.20
Glucose (mmol/l)	5.5 ± 1.5 *	5.3 ± 0.8 ‡	5.0 ± 0.6
Insulin (mU/ml)	15.1 ± 8.4 # *	11.5 ± 5.3	10.6 ± 5.6
HOMA	3.8 ± 2.8 # *	2.8 ± 1.4 ‡	2.4 ± 1.4
MTHFR TT677	19 (12%)	9 (8%)	32 (8%)

M/F: male/female, CVD: cardiovascular disease, BMI: body mass index, WHR: waist/hip ratio, SBP: systolic blood pressure, DBP: diastolic blood pressure, TC: total cholesterol, TG: triglycerides, HDL-C: high density lipoprotein cholesterol, LDL-C: low density lipoprotein cholesterol, apo B: apolipoprotein B, K-value <0 reflects small dense LDL, HOMA: Homeostasis model assessment for insulin resistance defined by fasting plasma insulin x fasting plasma glucose / 22.5, MTHFR: methylenetetrahydrofolate reductase. Results are presented as mean values ± SD, #: statistical significant FCH compared to controls, *: statistical significant FCH compared to normolipidemic relatives, , ‡: statistical significant controls versus normolipidemic relatives, P<0.05= statistical significant

FCH and plasma homocysteine

The first aim of this study was to investigate whether subjects with FCH have higher plasma homocysteine levels than controls. The mean plasma homocysteine concentration (Table 2) in the FCH group was not significantly different from the control group with a mean difference of 0.9 µmol/l (CI 95% -0.5 to 2.3). After adjustment for age and sex, the mean difference in homocysteine levels between FCH and control subjects was 0.9 µmol/l (CI 95% -0.4 to 2.5). Figure 1 shows the individual fasting plasma homocysteine values for the subjects with FCH and control subjects. The cut-off point was defined as the 90th percentile in control

subjects (=18.9 µmol/l). Among the subjects with FCH, 16 (10 %) had fasting homocysteine concentrations above the cut-off point of 18.9 µmol/l, versus 10 (9 %) in the control group (Table 2).

Table 2 Plasma homocysteine concentration in subjects with familial combined hyperlipidemia (FCH) compared to controls

	FCH n=161	**Controls n=109**	
Homocysteine (µmol/l)	14.1 ± 6.5	13.2 ± 4.8	NS
>90th perc. (>18.9 µmol/l)	16 (10)	10 (9)	NS

Values are mean ± SD and absolute numbers (percentages), NS: not significant, perc.: percentiles

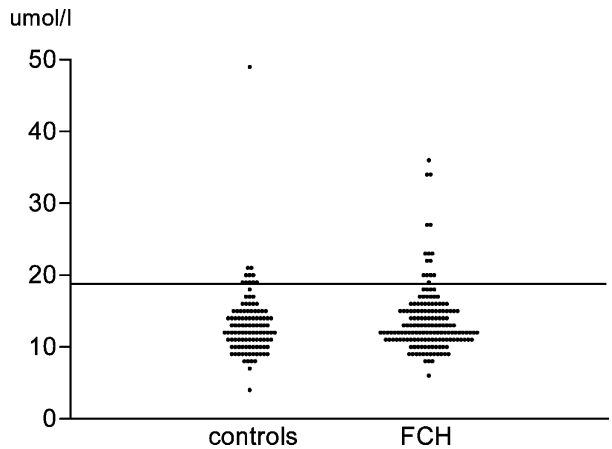

Figure 1 Fasting homocysteine concentrations (µmol/l) in subjects with Familial Combined Hyperlipidemia (FCH) (n=161) and controls (n=109). The straight line indicates the 90th percentile of the homocysteine distribution in the control subjects.

Homocysteine and risk of cardiovascular disease

Hyperhomocysteinemia is associated with CVD. Therefore, we stratified the subjects by the presence or absence of CVD (Table 3). Thirty-four (21%) of the 161 subjects with FCH had CVD. The group of control subjects with CVD consisted of only 4 subjects. As FCH is not associated with hyperhomocysteinemia, we analysed normolipidemic relatives and non-related control subjects together in 1 group, defined as non-FCH. In this non-FCH group 28 (6%) of the 506 subjects had CVD.

In the non-FCH group increased levels of homocysteine, defined by levels above the 90th percentile (18.9 µmol/l), were associated with a 2.8 times increased risk of CVD compared to subjects with lower homocysteine levels (Table 3). In the FCH group increased levels of homocysteine were also associated with increased risk of CVD although the risk estimate was lower.

Table 3 Odds ratios for cardiovascular disease associated with hyperhomocysteinemia in subjects with familial combined hyperlipidemia (FCH) and non-FCH subjects

	FCH			Non-FCH		
	CVD + n=34	CVD – n=127	OR	CVD + n=28	CVD – N=478	OR
Homocysteine >90th perc.	5 (15)	11 (9)	1.8 (0.7 to 4.6)	5 (18)	35 (7)	2.8 (1.1 to 7.1)

Values are absolute numbers (percentages), OR: Odds ratios (95% CI), CVD: cardiovascular disease, perc.: percentiles

Effect of parameters of lipid and lipoprotein metabolism and insulin resistance on plasma homocysteine levels

Log transformed plasma homocysteine level showed significant correlations in the total group (n=667) with sex (r=0.246), age (r=0.08), CVD (r=0.123), WHR (r=0.131), SBP (r=0.158), TC (r=0.091), HDL-C (r=-0.099), LDL-C (r=0.116), apo B (r=0.079), but no significant correlations with the other measurements were found (Table 4). With the multiple regression model using log homocysteine as the dependent variable and sex, age, CVD, WHR, SBP, TC, HDL-C, LDL-C, apo B as the independent variables, only sex, age, WHR, SBP proved to correlate independently with plasma homocysteine level (Table 5).

Table 4 Pearson correlation coefficient between plasma homocysteine level (log transformed) and anthropometric and biochemical parameters in the total group (n=667).

	R	p-value
Sex	0.246	<0.001
Age (yr)	0.08	0.039
Smoking (y/n)	-0.03	0.41
CVD (y/n)	0.123	0.001
BMI (kg/m2)	0.05	0.18
WHR	0.131	0.001
SBP (mm Hg)	0.158	<0.001
DBP (mm Hg)	0.105	0.07
Biochemical parameters		
TC (mmol/l)	0.091	0.019
TG (mmol/l)	0.008	0.832
HDL-C (mmol/l)	-0.099	0.011
LDL-C (mmol/l)	0.116	0.003
Apo B (mg/l)	0.079	0.043
K-value	-0.040	0.296
Insulin (mU/ml)	-0.015	0.713
Glucose (mmol/l)	0.029	0.455
HOMA	0.004	0.918

R: Pearson's correlation-coefficient, CVD: cardiovascular disease, BMI: body mass index, WHR: waist/hip ratio, SBP: systolic blood pressure, DBP: diastolic blood pressure, TC: total cholesterol, TG: triglycerides, HDL-C: high density lipoprotein cholesterol, LDL-C: low density lipoprotein cholesterol, apo B: apolipoprotein B, K-value <0 reflects small dense LDL, HOMA: Homeostasis model assessment for insulin resistance defined by fasting plasma insulin x fasting plasma glucose / 22.5.

Table 5 Multiple regression model for variables associated with homocysteine level (log transformed) in the total group (n=667)

Variable	Unstandard. Coefficients B	S.E.	p-value
Sex	0.06	0.012	<0.001
Age	-0.001	0.00	0.003
CVD	0.03	0.018	0.079
WHR	-0.152	0.077	0.048
SBP	0.001	0.00	0.004
TC	0.09	0.014	0.485
HDL-C	0.003	0.021	0.160
LDL-C	0.016	0.010	0.114
ApoB	0.00008	0.00	0.142

CVD: cardiovascular disease, WHR: waist/hip ratio, SBP: systolic blood pressure, TC: total cholesterol, HDL-C: high density lipoprotein cholesterol, LDL-C: low density lipoprotein cholesterol, apo B: apolipoprotein B.

Discussion

This study shows that subjects with FCH have no increased levels of plasma homocysteine compared to controls. In FCH, homocysteine does not contribute significantly to the increased risk of CVD. Furthermore plasma homocysteine level is not associated with parameters of lipid and lipoprotein metabolism, including small dense LDL, nor by an estimate of insulin resistance.

FCH is a disorder in which multiple risk factors interact to increase the risk of CVD. Our interest in associations between plasma homocysteine concentration and FCH has been spurred by the search for novel metabolic factors accounting for the increased risk of CVD in FCH. Sofar, the relevance of plasma homocysteine concentration as risk factor in FCH has not been investigated.

As described in the literature, oxidative modification of LDL is believed to constitute the link between increased cholesterol concentrations and atherosclerosis (23). Subjects with FCH have more small dense LDL, which are more susceptible to oxidative modification (24). According to Welch et al. high plasma homocysteine concentrations may exert an atherothrombotic effect through increasing oxidative stress (6), although not confirmed in other studies (25). So, it is unclear, if high plasma homocysteine concentrations could be even more harmful in this particular group.

In our study we report that mean plasma homocysteine levels are not elevated in subjects with FCH compared to control subjects. The frequency of the 677C→T mutation in the MTHFR gene, which is a determinant of elevated homocysteine levels (22), was quite similar between FCH patients, controls and normolipidemic relatives. Similar to the control group, 10% of the subjects with FCH had plasma homocysteine levels above the 90th percentile. The 90th percentile cut off point among the control subjects found in our population (Table 2) is comparable to

the cut-off point of 18.6 μmol/l found in another large cohort in the Netherlands (n=220) (26).

Both the FCH and non-FCH subjects with a plasma homocysteine concentration >90th percentile showed an increased risk of CVD, although the risk estimates were lower in the FCH group (Table 3). So, there seems to be no synergism between hyperhomocysteinemia and FCH as risk factors for CVD. This is in line with data of the European Concerted Action Project demonstrating no more than an additional effect between elevated cholesterol and hyperhomocysteinemia (27). The odds ratios found in our cohort correspond to odds ratios found in other studies (28).

In several studies the plasma homocysteine concentration is associated with different anthropometric and biochemical parameters. The correlations found in our study are in agreement with those found in literature. The modest correlations suggest that these parameters potentially account for some of the variation in homocysteine levels, although, most likely not resulting in clinically important elevations in homocysteine concentrations. A significant correlation was found between homocysteine concentration and sex. Sex proved to affect homocysteine level independently. It is well known that homocysteine levels of men are higher compared to women (29). Also age correlated with plasma homocysteine concentration but did not influence this parameter independently. In the Hordoland study, the plasma homocysteine level was positively associated with increased SBP and DBP (28). Like in our study, Sutton-Tyrell et al. (30) and Malinow et al. (31) found homocysteine to be strongly and independently associated with SBP.

We found a positive correlation between plasma homocysteine concentration and TC, HDL-C, LDL-C and apo B, but none of the plasma lipid and lipoprotein levels (TC, TG, HDL-C, LDL-C but also apolipoprotein B and small dense LDL) was associated with the homocysteine level independently. There are no data earlier published whether small dense LDL, which is more susceptible to oxidative modification, is associated with homocysteine levels. A positive correlation between homocysteine concentration and total or LDL-C level has been demonstrated in a few previous reports (32-35). The Hordaland homocysteine study by Nygard et al. found a positive relation between homocysteine level and TC level, which was particularly strong in the younger age group (28). Other studies showed no significant correlations between homocysteine concentration and serum plasma lipids (36,37). Our data support the hypothesis that plasma homocysteine concentration and lipids and lipoproteins are independent parameters.

Insulin resistance and hyperhomocysteinemia may both be associated with endothelial dysfunction and CVD. Plasma insulin levels could influence homocysteine metabolism, possibly through effects on glomerular filtration or by influencing activity of key enzymes in homocysteine metabolism (38). In agreement with previous reports (39), we show that subjects with FCH are more insulin resistant as reflected by a higher HOMA index. However, plasma homocysteine

concentration was not related with insulin resistance. This is in accordance with studies, which also found no relation between insulin resistance and homocysteine level (40,41). In some other previous studies hyperhomocysteinemia is associated with insulin resistance (29,34,42). In the Framingham Offspring study (n=2011) significant but low correlations between fasting glucose, insulin and homocysteine concentration were found (r=0.11 and r=0.07, respectively) (43).

In conclusion, subjects with FCH have no increased levels of plasma homocysteine compared to controls and hyperhomocysteinemia does not disproportionally increase the risk of CVD in FCH subjects. No associations were observed between plasma homocysteine concentration and plasma lipid and lipoprotein levels, including small dense LDL, nor between homocysteine concentration and insulin resistance.

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Chapter 7

The role of insulin resistance in Familial Combined Hyperlipidemia

Submitted

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Abstract

Objective: Insulin resistance is associated with increased triglyceride levels, low HDL cholesterol, small dense LDL and increased apoB levels, all characteristics of Familial Combined Hyperlipidemia (FCH). Therefore, we explored the role of insulin resistance in FCH lipid phenotype expression.

Methods and results: FCH was defined by traditional diagnostic criteria including plasma total cholesterol and/or triglyceride levels >90th percentile. Insulin resistance was assessed by the Homeostasis Model Assessment index (HOMA). In total 132 subjects with FCH, 350 normolipidemic relatives and 81 spouses, who referenced as controls were studied. FCH subjects were significantly more insulin resistant compared to controls and normolipidemic relatives (HOMA index 2.9 (95% CI 2.6-3.2), 2.2 (95% CI 2.0-2.5) and 2.0 (95% CI 1.9-2.2), respectively), even after correction for sex, age and BMI. The degree of insulin resistance was dependent on lipid phenotype expression and a change in lipid phenotype expression over 5 year was associated with a change in insulin resistant-state. For any level of insulin resistance and degree of obesity, FCH subjects had increased levels of apoB and more small dense LDL compared to controls.

Conclusion: Insulin resistance is a characteristic feature of FCH, which is not fully explained by their increased BMI and is associated with (change in) lipid phenotype expression. Furthermore, our results support the concept of genetic origin of high apoB and small dense LDL in FCH, which is modulated by insulin resistance and obesity.

Introduction

Familial Combined Hyperlipidemia (FCH) is the most common familial form of hyperlipidemia with an estimated prevalence of 1 to 3% in the general population and up to 20% of patients with premature myocardial infarction (1). FCH was originally identified in the early 1970s as a new inherited lipid disorder, characterized by multiple phenotypes (2). The genetic and metabolic basis of the disorder has not yet been identified. In general FCH is thought to be caused by hepatic very low density lipoprotein (VLDL) overproduction with or without impaired clearance of triglyceride-rich lipoproteins (3). So, FCH is characterized by elevated apoB levels and high occurrence of small dense LDL, which are both attractive new candidates to redefine FCH, as recently described (4). Furthermore FCH has been associated with the presence of insulin resistance and obesity (5).

Resistance to normal action of insulin is related to an excessive postprandial release of free fatty acids (FFA). An increased supply of FFA to liver cells is associated with VLDL overproduction. Furthermore, the normal insulin mediated activation of lipoprotein lipase (LPL) is diminished, resulting in a reduced clearance of triglyceride-rich lipoproteins (6). So, insulin resistance may coincide with alterations in lipid metabolism, such as hypertriglyceridemia, low HDL cholesterol, increased apoB levels and a predominance of small dense LDL particles (7). Because all these features are also characteristics of FCH, the presence of insulin resistance may be an important factor modulating FCH phenotypes.

The aim of this study was to explore the role of insulin resistance in FCH lipid phenotype expression. Therefore, we studied in our large FCH cohort, FCH subjects with different lipid phenotypes and the effect of intra-individual changes in lipid phenotype on insulin resistance over a 5- year period. Furthermore, we investigated whether the elevated apoB levels and the presence of small dense LDL in FCH could be explained by the degree of insulin resistance and/or obesity.

Subjects and Methods

Study population

Recently, we performed a large study in FCH families (n=667 individuals), including a 5-year follow-up period (1994-1999), to investigate the metabolic and genetic aspects of FCH (4). For the current study we included all subjects with FCH, normolipidemic relatives and spouses of whom glucose and insulin levels were available in 1999, resulting in a study population of 132 FCH subjects, 350 normolipidemic relatives and 81 spouses, who referenced as controls. Among the 132 FCH subjects, data of lipids and glucose/insulin levels were also available in 1994 in 76 subjects (58%). Fifty-five of these patients were affected FCH in both

1994 and 1999 (72%). These subjects were included in the follow-up to study the interdependence of change in lipid phenotype expression in time on insulin resistance. Among the 350 normolipidemic relatives data of lipids and glucose/insulin levels were also available in 1994 in 180 subjects (51%). One hundred and fifty-six of the 180 subjects were normolipidemic relatives in both 1994 and 1999 (87%).

The relatives and spouses as described previously (4) were ascertained through probands exhibiting a combined hyperlipidemia, with both plasma TC and TG concentrations above the 90th percentile, adjusted for age and gender, as obtained from the Prospective Cardiovascular Munster (PROCAM) study (8). These values were confirmed by repeated measurement, on a lipid lowering diet and without lipid-lowering drugs. At least one first-degree relative of the proband had a multiple type hyperlipidemia with elevated levels of plasma TC and/or TG. Furthermore, at least the proband or one of the first-degree relatives should have premature CVD before the age of 60 years. All probands were tested for an underlying cause of hyperlipidemia (i.e., diabetes mellitus, hypothyroidism, and hepatic or renal impairment). The presence of one of these causes excluded the proband and his/her family from further analysis. None of the probands in these families were homozygous for the apo E2 allele and none of them and their first-degree relatives had tendon xanthomas.

The subjects were classified affected FCH when plasma TC and/or TG levels exceeded the 90th percentile, based on the PROCAM study (8). These percentiles are age and gender adjusted. Normolipidemic relatives were defined by TC and TG levels <90th percentile. All individuals were Caucasian above the age of 12 years. All subjects filled out a questionnaire about their previous medical history, especially cardiovascular status. Body mass index (BMI), waist/hip ratio (WHR) and blood pressure were determined in all subjects. After a withdrawal of four weeks of lipid-lowering medication and an overnight fast, venous blood was drawn by venipuncture. The study protocol was approved by the ethical committee of the University Medical Center Nijmegen.

Plasma lipid, lipoprotein and apolipoprotein analysis

Plasma TC and TG concentrations were determined by enzymatic, commercially available reagents (Boehringer-Mannheim, Germany, catalog. No. 237574 and Sera Pak, Miles, Belgium, catalog. No. 6639, respectively). Total plasma apo B concentrations were determined by immunonephelometry as recently described in detail elsewhere (4,9).

Low density lipoprotein subfraction profile analysis

LDL subfractions were separated by single spin density gradient ultracentrifugation(10). Each individual LDL subfraction profile was defined by a continuous variable K, as

described in detail previously (11,12). A negative value (K<0) reflected a more dense LDL subfraction profile, and a positive K value (K>0) a more buoyant profile.

Glucose, Insulin and Insulin resistance analysis

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 6%. Insulin resistance was assessed by the Homeostasis Model Assessment Index (HOMA) and calculated using the formula: HOMA = fasting serum insulin (mU/l) x fasting plasma glucose (mmol/l) / 22.5 (13).

Statistical analysis

Descriptive values were expressed as mean (95% CI) or absolute numbers with percentages. TG, HOMA, insulin and glucose were logarithmically transformed to obtain normal distributions before statistical analysis. Differences in characteristics between subjects with FCH, controls and normolipidemic relatives were tested by Generalized Estimating Equations (GEE) because of possible correlated values within families. Correlations between HOMA and variables were analyzed using Spearman correlation coefficients. The 90th percentile of HOMA, apoB concentration and K-value were based on the group of controls. Multiple linear regression test was used to select the variables that contributed independently to HOMA. Probability values <0.05 were considered statistically significant. All analysis were computed using STATA 8.0 software.

Results

In total 563 subjects, including 132 subjects with FCH, 350 normolipidemic relatives and 81 controls, were included in the study. Table 1 shows the anthropometric and biochemical variables. The ratio women/men was not significantly different between the groups. The mean age of the group of subjects with FCH was significantly lower compared to controls and significantly higher compared to the normolipidemic relatives. The BMI was significantly higher in the FCH group compared to the control group and normolipidemic relatives. By definition the FCH group had significantly higher plasma TC and TG concentrations compared to controls and normolipidemic relatives. In addition, the subjects with FCH had a more atherogenic lipid and lipoprotein profile as reflected by increased LDL-C-, decreased HDL-C, increased apoB concentrations and a more small dense LDL subfraction profile as reflected by a more negative value of parameter K compared to the control group and normolipidemic relatives. The differences between the control group and normolipidemic relatives are indicated in Table 1.

Table 1 Anthropometric and biochemical parameters in subjects with familial combined hyperlipidemia (FCH) compared to controls and normolipidemic relatives (NL)

	FCH n=132	Controls n=81	NL N=350
Sex (%male)	58 (44%)	39 (48%)	168 (48%)
Age (years)	46.4 (43.6 – 49.2) # *	52.4 (48.9 – 56.0) +	38.4 (36.5 – 40.3)
BMI (kg/m2)	27.7 (27.0 – 28.4) # *	26.5 (25.6 – 27.3) +	24.2 (23.7 – 24.7)
WHR	0.88 (0.86 – 0.89) *	0.86 (0.84 – 0.87) +	0.83 (0.82 – 0.84)
TC (mmol/l)	6.3 (6.1 – 6.5) # *	5.2 (4.9 – 5.4)	5.0 (4.8 – 5.1)
TG (mmol/l)	2.8 (2.6 – 3.0) # *	1.1 (1.0 – 1.2)	1.1 (1.0 – 1.1)
LDL-C (mmol/l)	4.0 (3.8 – 4.2) # *	3.5 (3.2 – 3.7)	3.3 (3.2 – 3.4)
HDL-C (mmol/l)	0.95 (0.90 – 1.00) # *	1.29 (1.22 – 1.35)	1.22 (1.18 – 1.26)
Apo B (mg/l)	1328 (1285 – 1370) # *	993 (939 – 1047)	981 (954 – 1008)
K-value	-0.26 (-0.31 - -0.22) # *	0.07 (0.02 – 0.12)	0.04 (0.01 – 0.07)

Values are estimated mean (95% CI) and absolute numbers for sex distribution, FCH: familial combined hyperlipidemia, NL: normolipidemic relatives, BMI: body mass index, WHR: waist hip ratio, TC: total cholesterol, TG: triglyceride, LDL-C: low density lipoprotein cholesterol, HDL-C: high density lipoprotein cholesterol, apoB: apolipoprotein B, K-value <0: reflecting small dense LDL, #: p<0.05 FCH compared to controls, *:P<0.05 FCH compared to normolipidemic relatives, +: P<0.05 controls compared to normolipidemic relatives, p<0.05= statistical significant

FCH and insulin resistance

Subjects with FCH were more insulin resistant as assessed by the HOMA index, which was significantly higher in FCH subjects compared to the normolipidemic relatives and group of controls (Table 2) with a mean difference of 0.9 (CI 95%: 0.79 to 0.92) between FCH subjects and controls. Even after adjustment for age, sex and BMI, subjects with FCH were still significantly more insulin resistant with a mean difference in HOMA of 0.9 (CI 95%: 0.83 to 0.95). Controls and normolipidemic relatives did not differ in HOMA. A significantly higher number of FCH subjects (18%) had a HOMA index above the 90th percentile compared to 6% in the group of normolipidemic relatives (Table 2).

Table 2 Degree of insulin resistance (HOMA) in subjects with familial combined hyperlipidemia (FCH) compared to controls and normolipidemic relatives (NL)

	FCH n=132	Controls n=81	NL n=350
HOMA	2.9 (2.6 – 3.2) # *	2.2 (2.0 – 2.5)	2.0 (1.9 – 2.2)
HOMA >90th perc.	24 (18%) # *	8 (10%)	22 (6%)
Insulin (mU/l)	12.5 (11.4 – 13.7) # *	9.7 (8.6 – 10.8)	9.2 (8.6 – 9.8)
Glucose (mmol/l)	5.2 (5.1 – 5.3) *	5.2 (5.0 – 5.3) +	4.9 (4.9 – 5.0)

Values are estimated mean (95% CI) and absolute numbers (%) for homa >90th percentile (4.3), FCH: familial combined hyperlipidemia, NL: normolipidemic relatives, HOMA: homeostasis model assessment for insulin resistance defined by fasting plasma insulin x fasting plasma glucose / 22.5, #: p<0.05 FCH compared to controls, *: p<0.05 FCH compared to normolipidemic relatives, +: p<0.05 controls compared to normolipidemic relatives, p<0.05= statistical significant

The glucose concentration was not significantly different between the FCH group and control group, whereas the subjects with FCH had significantly higher insulin levels compared to both other groups. HOMA was not statistically different between males and females in the three different groups (data not shown). We evaluated the effect of different variables on insulin resistance. The degree of insulin resistance (HOMA) had significant correlations with BMI (R=0.51, p<0.001), WHR (R=0.35, p<0.001), TG (R=0.32, p<0.001), HDL-C (R=-0.27, p<0.01), K-value (R=-0.19, p=0.02), systolic blood pressure (0.21, p=0.01) and diastolic blood pressure (0.30, p<0.001) evaluated in the FCH group (n= 132). With the linear regression model using log transformed HOMA as the dependent variable and sex, BMI, WHR, TG, HDL-C, K-value and blood pressure as the independent variables, only BMI and TG proved to contribute independently to the degree of insulin resistance. BMI alone explained 22% and the combination of BMI and TG explained 28% of the variance in HOMA. Similar results were found for the control group and the group of normolipidemic relatives (data not shown).

Lipid phenotype and insulin resistance in FCH

Of the 132 FCH subjects, 86 subjects were affected based on the presence of isolated hypertriglyceridemia (hyperTG: TG >90th percentile according to PROCAM(8)) , 24 subjects based on the presence of combined hyperlipidemia (combined HLP: TC and TG >90th percentile) and 22 subjects based on isolated hypercholesterolemia (hyperTC: TC >90th percentile). Lipid and lipoprotein profiles are given in Table 3.

Table 3 Anthropometric and biochemical parameters of FCH subjects with different lipid phenotypes compared to controls

	Affected FCH based on							
	HyperTG N=86		**Combined HLP N=24**		**HyperTC N=22**		**Controls N=81**	
Age (years)	46.7	(43.5-49.8) #	48.7	(42.9-54.6)	42.0	(35.9- 48.1) ⊥	52.4	(49.2-55.7)
BMI (kg/m²)	28.1	(27.2-29.0) #	27.8	(26.3-29.4)	26.7	(25.1-28.3)	26.3	(25.5-27.2)
TC (mmol/l)	5.7	(5.5-5.9) *‡#	7.8	(7.4-8.1) †¶	7.2	(6.9-7.6) ⊥	5.2	(5.0-5.4)
TG (mmol/l)	2.9	(2.8-3.1) *‡#	3.8	(3.3-4.4) †¶	1.5	(1.3-1.8) ⊥	1.1	(1.0-1.1)
ApoB (mg/l)	1205	(1158-1252) *‡#	1629	(1543-1714) †¶	1505	(1416-1594) ⊥	999	(950-1048)
K-value	-0.30	(-0.36- -0.24) *#	-0.39	(-0.49- -0.29) †¶	-0.03	(-0.13-0.07)	0.06	(0.003-0.12)
HOMA	3.0	(2.7 – 3.4) *#	3.3	(2.7 – 4.1) †¶	2.3	(1.9– 2.9)	2.2	(2.0-2.5)

Values are estimated mean (95% CI), FCH: familial combined hyperlipidemia, BMI: body mass index, TC: total cholesterol, TG: triglycerides, apoB: apolipoprotein B, , K-value <0: reflecting small dense LDL , HOMA: homeostasis model assessment for insulin resistance defined by fasting plasma insulin x fasting plasma glucose / 22.5, hyperTC: FCH subjects based on TC >90th percentile, hyperTG: FCH subjects based on TG>90th percentile, combined HLP: FCH subjects based on TC and TG >90th percentile, * :p<0.05 hyperTG vs hyper TC, :‡: hyperTG vs combined HLP, #: p<0.05 hyperTG vs controls, †: p<0.05 combined vs hyperTC, ¶: p<0.05 combined HLP vs controls, ⊥: p<0.05 hyperTC vs controls, p<0.05= statistic significant

The FCH subjects affected based on hyperTG or combined HLP have a significantly higher BMI, TC, TG, apoB level and more small dense LDL compared to controls. The HOMA index of FCH subjects who presented with hyperTG or combined HLP was significantly higher compared to controls even after correction for BMI. FCH subjects with combined HLP have higher TC, TG and apoB levels and more small dense LDL compared to hyperTG subjects but they do not differ in BMI or HOMA index. The FCH subjects affected based on hyperTC have higher TC, TG and apoB levels compared to controls. Strikingly, although all affected FCH subjects had higher apoB, TC and TG levels compared to controls, subjects with FCH based on hyperTC did not show a small dense LDL subfraction profile and were not insulin resistant.

Lipid phenotype and insulin resistance over a period of 5 year

To evaluate the interdependence of change in lipid phenotype expression on insulin resistance we studied 55 subjects who were affected FCH in both 1994 and 1999, including 156 subjects who were normolipidemic in both 1994 and 1999 (Table 4).

Table 4 Insulin resistance (HOMA) and obesity (BMI) in FCH subjects stratified by different lipid phenotype expression compared to normolipidemic relatives in 1994 and 1999.

		Affected FCH 1994 -- 1999 based on lipid phenotype expression			
		hyperTC – hyperTC (n=7)	**hyperTC – hyperTG or combined HLP (n=8)**	**hyperTG or combined HLP – hyperTG or combined HLP (n=39)**	**normolipidemic – normolipidemic (n=156)**
HOMA	1994	2.6 (1.7 – 4.1) #	1.9 (1.0 – 3.6)	2.6 (2.1 – 3.3) §	1.5 (1.4 – 1.7)
	1999	2.6 (2.0 – 3.4) #	3.8 (2.0 – 7.3) *¶	3.0 (2.6 – 3.6) * §	1.9 (1.7 – 2.1) *
BMI	1994	27.6 (25.3 – 29.9) #	27.0 (23.6 – 30.4) ¶	26.7 (25.5 – 28.0) §	22.8 (22.3 – 23.6)
	1999	28.5 (26.1 – 30.9) #	29.3 (24.8 – 33.7) *¶	28.3 (26.5 – 30.1) §	24.2 (23.6 – 24.8) *

Values are mean (95% CI), TC: FCH based on hypercholesterolemia, TG: FCH based on hypertriglyceridemia, * : $p<0.05$ 1994 versus 1999, # : $p<0.05$ hyperTC – hyperTC vs NL - NL, ¶ : $p<0.05$ hyperTC – hyperTG or combined HLP vs NL – NL, § : $p<0.05$ hyperTG or combined HLP – hyperTG or combined HLP vs NL – NL, $p<0.05$ = statistical significant

Among the normolipidemic subjects the HOMA index and BMI increased significantly over 5 year, with a correlation between ΔBMI and ΔHOMA of $r=0.24$, $p=0.001$. Also among all FCH subjects an increase in BMI over 5 year was associated with an increase in HOMA index ($r=0.47$, $p<0.001$). Seven subjects were affected FCH based on hyperTC in both 1994 and 1999. BMI increased in 5 year but this increase did not reach statistical significance. HOMA index did not change over this period. Both in 1994 and 1999, HOMA and BMI were significantly higher compared to NL subjects. Eight subjects were affected FCH in 1994 based on hyperTC and in 1999 based on combined HLP or hyperTG. Their BMI and HOMA index increased significantly over the 5- year period. Both in 1994 and 1999, BMI

and HOMA index were higher compared to the NL subjects, however, the HOMA in 1994 did not reach statistical significance. Among the 39 subjects who remained affected FCH based on hyperTG or combined HLP, BMI showed a trend of increase and the HOMA index increased significantly. Again, both BMI and HOMA, among these FCH subjects are significantly higher compared to the NL group. There was only one subject who changed from hyperTG to hyperTC; the HOMA index of this individual decreased from 1.96 in 1994 to 0.43 in 1999, whereas, the BMI increased slightly from 24.0 to 24.1 kg/m^2.

Can insulin resistance and/or obesity explain the elevated apo B levels and high occurrence of small dense LDL in FCH?

Scatter plots were generated to explore the relationship between insulin resistance / obesity and apoB levels / presence of small dense LDL in FCH and control subjects (Figure 1). Sixty-four percent of the subjects with FCH had an apoB level above the 90th percentile, also 58% of the subjects with FCH had a K-value <-0.24. This figure shows that for any level of insulin resistance and/or degree of obesity most FCH subjects have higher apoB levels and more small dense LDL compared to controls.

So, insulin resistance and/or obesity do not fully account for the increased plasma levels of apoB or high occurrence of small dense LDL in FCH.

Insulin resistance and risk of cardiovascular disease

Insulin resistance is related to cardiovascular risk. An HOMA index above the 90th percentile (>4.3) was associated with an increased risk for CVD in FCH subjects (OR=1.4 (95% CI: 0.49 to 3.93) and in non-FCH subjects (normolipidemic subjects and controls together) (OR=3.1 (95% CI: 0.97 to 9.83). The overall OR for the total group is 2.7 (95% CI:1.24 to 5.72) (Table 5). Thus, insulin resistance is associated with an increased risk of CVD. In the FCH group, the HOMA did not proportionally increase the OR. So insulin resistance does not appear to be an additional risk factor for CVD particularly in FCH subjects.

Table 5 Odds ratios for CVD associated with HOMA index above the 90th percentile

	FCH			Non-FCH			Total group
	CVD + n=27	CVD – n=105	OR [95%CI]	CVD + N=23	CVD - n=408	OR [95%CI]	OR [95%CI]
HOMA >90th percentile	6 (22)	18 (17)	1.4 [0.49-3.93]	4 (17)	26 (6)	3.1 [0.97-9.83]	2.7 [1.24-5.72]

Values are total numbers (percentages), FCH: familial combined hyperlipidemia, CVD: cardiovascular disease, CVD +: subjects with CVD, CVD -: subjects without CVD, OR: odds ratio CI: confidence interval, HOMA: homeostasis model assessment , Non-FCH includes both normolipidemic relatives and controls. The 90th percentile for HOMA as defined in controls was found at a level of >4.3

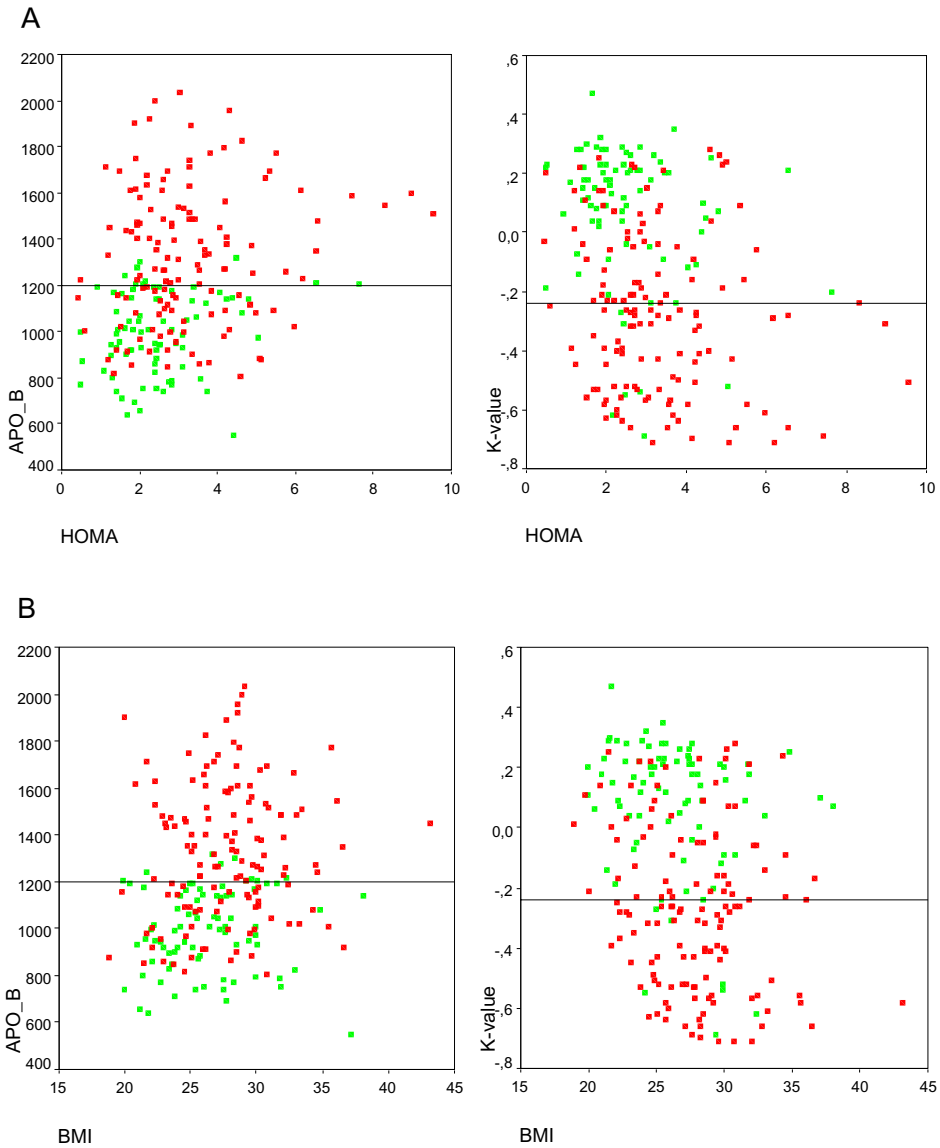

Figure 1 Scatterplots to explore the relationship between insulin resistance (A) and degree of obesity (B) with apoB levels and the presence of small dense LDL.

HOMA: homeostasis model assessment for insulin resistance defined by fasting plasma insulin x fasting plasma glucose / 22.5, BMI:body mass index (kg/m2) , apoB: apolipoprotein B (mg/l), K-value <0: reflecting small dense LDL , blacksquares: FCH subjects, lightsquares : control subjects. The line indicates the 90th percentile for apoB levels ((>1200 mg/l) and the value of parameter K (<-0.24) as evaluated in the control group.

Discussion

This study shows that subjects with FCH are more insulin resistant compared to controls, even after correction for sex, age and BMI. The insulin resistant state in FCH is dependent on lipid phenotype, as subjects with FCH based on hyperTG or combined HLP were more insulin resistant compared to FCH subjects based on hyperTC. For the first time we show in our 5 -year follow-up study that a change in lipid phenotype expression is associated with a change in insulin resistance. Moreover, insulin resistance does not fully account for the increased plasma levels of apoB or high occurrence of small dense LDL in FCH, both major characteristics of FCH. Thus, our results support the concept of genetic origin of high apoB and small dense LDL in FCH, which is modulated by BMI and insulin resistance.

Several groups including ourselves have demonstrated directly by hyperinsulinemic clamp technique the impaired insulin action in FCH patients (14-17). These studies were performed in a small number of subjects. In the present study we included a large population of 563 subjects, including 132 FCH subjects, 81 controls and 350 normolipidemic relatives. Therefore the level of insulin resistance was determined by HOMA index. The accuracy and precision of the HOMA index as a measure of insulin resistance has been determined in literature by comparison with euglycemic and hyperglycemic clamps and the intravenous glucose tolerance test (13). In this large population we confirm that FCH subjects are more insulin resistant compared to controls. Pathofysiologically this could be related to the higher BMI in FCH subjects. However, we show that even after correction for BMI, FCH subjects are more insulin resistant compared to normolipidemic relatives and controls. So, our results suggest that a genetic component influences the degree of insulin resistance in FCH beside the traditional environmental influences such as BMI. Indeed, recently a major gene effect on insulin resistance in FCH has been suggested (18).

FCH is characterized by multiple lipoprotein phenotype expression; in one family affected subjects may present with hyperTC, hyperTG or combined HLP. We demonstrate that insulin resistance depends on lipid phenotype as FCH subjects based on hyperTG or combined HLP, were more insulin resistant compared to FCH subjects based on hyperTC. This was previously also shown by Pihlajamaki et al. (16) and Vakkilainen et al. (19). This could be related to their increased BMI frequently reported in hyperTG subjects, however, even after correction for BMI, subjects with hyperTG or combined HLP were more insulin resistant compared to FCH subjects based on hyperTC. Thus, the degree of insulin resistance appears to depend on lipid phenotype expression.

The main feature of FCH is the variability in lipid phenotype expression within an individual in time. Recently, we showed that 43% of the FCH patients had a change in lipid phenotype expression after 5 years (4). The effect of these intra-individual changes in lipid phenotype expression in time on insulin resistance has not been

addressed in literature. Twenty-four of the 55 FCH subjects (44%) in this study show an intra-individual change in lipid phenotype expression over the period of 5 year. Independent of lipid phenotype expression is an increase in BMI in time associated with an increase in insulin resistance ($r=0.47$, $p<0.001$). A switch in lipid phenotype from hyperTC to hyperTG or combined HLP is associated with an increase in insulin resistance.

Thus, most strikingly, our data show that FCH defined by the traditional criteria (TC and/or TG levels >90th percentile) potentially comprise a heterogeneous group; subjects defined FCH by the presence of hyperTG or combined HLP are characterized by the presence of small dense LDL and insulin resistance whereas the FCH subjects based on hyperTC do not show these major characteristics of FCH. It appears that subjects with hyperTC comprise a distinct group within FCH. These results suggest that several metabolic pathways may contribute to the lipid phenotype expression in FCH.

The diagnostic criteria for FCH have been debated in literature (4,20). Recently, we and others have shown that apoB and small dense LDL are important potential new diagnostic characteristics (4,21,22). Insulin resistance and obesity are also associated with increased levels of apoB and the presence of small dense LDL (6). Therefore, we explored whether insulin resistance and/or obesity can explain the increased apoB levels or high occurrence of small dense LDL in FCH.

Purnell et al. examined this question for only apo B in a population of 11 FCH subjects (23). In our large cohort of 132 FCH subjects we confirmed that most FCH subjects had increased apo B levels for any level of insulin resistance (HOMA) or BMI compared to controls. In addition we show that FCH subjects have also more small dense LDL for any level of insulin resistance (HOMA) or BMI compared to controls. So, obesity and/or insulin resistance, do not fully account for the elevated levels of apoB but also not for the high prevalence of small dense LDL in FCH. These results support the physiological concept of separate, but additive, genetic determinants in the etiology of the FCH lipid phenotype with modulation by BMI and insulin resistance. We have reported previously that in our FCH families a major gene influences apoB levels and small, dense LDL (24), however, the gene has not been found yet. Recently, Pajukanta et al. published interesting data about association between FCH and upstream transcription factor 1 (USF1) (25). USF1 encodes a transcription factor known to regulate several genes of glucose and lipid metabolism.

FCH subjects have increased risk of CVD, which can only to a certain extent be explained by the disturbed lipid profile. Insulin resistance is also associated with an increased risk of CVD (26). The question arise if the presence of insulin resistance in FCH subjects could be responsible for a part of the increased risk of CVD. We show that an HOMA index above the 90th percentile gives an increased risk of CVD in the total group but there was no proportionally increased risk in the FCH group

separately, however the number of subjects is small. So, insulin resistance does not appear to be an additional risk factor for CVD particularly in FCH subjects.

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Chapter 8

Elevated leptin levels in subjects with Familial Combined Hyperlipidemia contribute to the increased risk of CVD

Submitted

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Abstract

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. The study population comprised 651 subjects, including 158 FCH patients. Leptin levels were determined, using a commercially available ELISA. 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 (OR = 3.4 [1.2-9.6]) and in non-FCH subjects (OR = 3.4 [1.3-9.3]). The overall OR on CVD was 3.5 [1.7-6.9].

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 to 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 (1) FCH is characterized by hypercholesterolemia and/or hypertriglyceridemia. Other phenotypes of FCH are elevated levels of apolipoprotein B (apo B) 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 (2).

Obesity results in an increase in number and size of adipocytes. These adipocytes secrete leptin, a hormone which is increased in obese subjects (3). Leptin is involved in the regulation of the energy expenditure and appetite via hypothalamic receptors (4). 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 (5) and insulin secretion (6). 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 (6,7). Thus high leptin levels are associated with insulin resistance (8).

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 (2).

So far, only two small studies have studied the relationship between leptin concentrations and FCH, with conflicting results (9,10). Jacobson et al. found a significantly elevated leptin levels in young females with FCH (10), while Haluzik et al observed no differences in a group of males with and without FCH (9).

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.

Subjects and methods

Study population

The study population existed of 37 families, comprising 651 subjects, of whom 158 subjects were diagnosed as FCH patients (11,12). The normolipidemic relatives (n=389) and spouses (n=97), also included in this study population, served as two independent reference groups. Eight subjects were not diagnosed because of missing values. 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. All subjects filled out a questionnaire about their previous medical history, especially cardiovascular status, medication, smoking and drinking habits and hormonal status in women. CVD was defined by stroke, myocardial infarction, peripheral vascular disease and angina pectoris. After withdrawal of lipid-lowering medication for four weeks and an overnight fast, blood was drawn by venipuncture. The ethical committee of the University Medical Center Nijmegen 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 apo B levels in combination with triglyceride and total cholesterol levels adjusted for age and gender, using a nomogram, as recently described (12).

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

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 apo B concentrations were determined by immunonephelometry as recently described in detail (13). 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 serum insulin (μU/ml) x fasting plasma glucose (mmol/L)/ 22.5 (14).

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 both approximately 3%, 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 levels than males (15,16), 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, expressed as means with 95% confidence intervals (95% CI), are presented separately for FCH patients, normolipidemic relatives and spouses. 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. Also the odds ratios (OR) as an estimate of risk for CVD were calculated using GEE. Multiple linear regression analyses were performed to standardize leptin levels for BMI, HOMA-index and gender. 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 incidence 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 apo B and are more insulin resistant, as reflected by a higher HOMA-index. Normolipidemic relatives are younger, have a lower BMI, are less insulin resistant and have lower total cholesterol and apo B levels compared to the spouses (Table 1).

Table 1 Characteristics of patients with Familial Combined Hyperlipidemia, normolipidemic relatives and spouses

	FCH patients (n = 158)	NL relatives (n = 389)	Spouses (n = 97)
	Mean (95% CI)	**Mean (95% CI)**	**Mean (95% CI)**
Gender (males) *	76 (48.1%)	175 (45.0%)	43 (44.3%)
Age (years)	47.1 (44.5-49.6) †‡	37.8 (36.0-39.6) §	52.2 (49.0-55.4)
CVD *	32 (20.3%) †‡	21 (5.4%)	3 (3.1%)
BMI (Kg/m^2)	27.3 (26.6-28.0) †	24.2 (23.7-24.7) §	26.4 (25.6-27.2)
Total Cholesterol (mmol/L)	6.5 (6.3-6.6) †‡	4.9 (4.7-5.0) §	5.3 (5.0-5.5)
Triglycerides (mmol/L)	2.7 (2.6-3.0) †‡	1.1 (1.0-1.1)	1.2 (1.1-1.3)
Apo B (mg/L)	1370 (1333-1407) †‡	961 (936-985) §	1018 (971-1065)
HOMA-index	2.9 (2.6-3.2) †‡	2.0 (1.9-2.1) §	2.3 (2.0-2.6)

* Presented are number (%), FCH; familial combined hyperlipidemia, NL relatives; normolipidemic relatives, CVD; cardiovascular disease, BMI; body mass index, Apo B; apolipoprotein B, HOMA-index; homeostasis model assessment- index

† p <0.05; FCH patients compared to normolipidemic relatives

‡ p <0.05; FCH patients compared to spouses

§ p <0.05; Normolipidemic relatives compared to spouses

Leptin concentration 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

		FCH patients n = 158 (48.1%) *	NL relatives n = 389 (45.0%) *	Spouses n = 97 (44.3%) *
Leptin levels (ng/ml)	Males	8.9 (7.1-11.0) §†	3.9 (3.4-4.5) ‡	5.4 (4.1-7.2)
	Females	26.4 (22.3-31.4) §	18.4 (16.4-20.7) ‡	23.9 (19.4-29.4)
Leptin levels (ng/ml) standardized for BMI	Males	6.2 (5.3-7.2) §†	4.9 (4.4-5.4)	4.4 (3.6-5.4)
	Females	20.8 (18.5-23.5)	20.5 (19.0-22.2)	21.3 (18.5-24.7)
Leptin levels (ng/ml) standardized for BMI and HOMA-index	Males	5.9 (5.0-6.9)	5.0 (4.5-5.6)	4.7 (3.9-5.8)
	Females	19.7 (17.5-22.2)	20.9 (19.3-22.7)	21.4 (18.6-24.6)

Values are means with 95% CI, FCH; Familial combined hyperlipidemia, NL relatives; Normolipidemic relatives, BMI; Body mass index, HOMA-index; homeostasis model assessment insulin resistance index, § P <0.05 between FCH patients and Normolipidemic (NL) relatives; † P <0.05 between FCH patients and spouses; ‡ P <0.05 between Normolipidemic (NL) relatives and spouses; * Percentage males

Leptin levels show a strong significant correlation with BMI in both males (r = 0.75) 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 (2). Because leptin levels show a strong correlation with the HOMA-index for both males (r = 0.59) and females (r = 0.46), 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 71% by the variables gender and BMI. When including the HOMA-index in the model, 74 % 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 = 3.4 [1.3-9.4]) and in non-FCH subjects (OR = 3.4 [1.3-9.2]. The overall OR for the total group is 3.5 with confidence intervals ranging from 1.8 to 6.9 (Table 3). When performing the analysis with leptin levels as a continuous variable, we also obtained a significant increased risk on CVD associated with higher leptin levels (data not shown).

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

	FCH			Non-FCH*			Total Group
	CVD+ (n = 20)	CVD- (n = 84)	OR [95% CI]	CVD+ (n = 35)	CVD- (n = 497)	OR [95% CI]	OR [95% CI]
Leptin level >90th percentile	8 (27.6%)	12 (10.0%)	3.4 [1.2-9.6]	6 (27.3%)	40 (9.9%)	3.4 [1.3-9.3]	3.5 [1.7 – 6.9]

* Non-FCH includes both normolipidemic relatives and spouses

Presented are total number (%) and the Odds Ratio's. 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

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, suggesting a defect in adipose tissue metabolism in male FCH patients. 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. The increased leptin levels in FCH were found to contribute to the 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 (9). Another study, performed in a small group of women by Jacobson et al, did show an increased concentration of leptin in females with FCH (10). 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 (17,18), 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 (3,19-21), but there still is some inter-individual variability in plasma leptin concentration that is independent of body fatness. In our study 71 % 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 (22-26). 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 (27). 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 (20,24). 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 of CVD in FCH. Indeed, we demonstrate that leptin levels above the 90th percentile give 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 2-fold on CVD in the highest two quintiles of leptin concentration compared to the lowest quintile (20) 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 (20). 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 (21). 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 (28,29) 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 (30), induction of migration and proliferation of vascular smooth muscle cells (31), stimulation of inflammatory cells (32) and induction of calcification of vascular cells (29). 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

So 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.

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Chapter 9

Summary and conclusions

Summary

Familial Combined Hyperlipidemia (FCH) was originally identified in the early 1970's as a new inherited lipid disorder, characterized by multiple lipoprotein phenotypes and strongly associated with premature cardiovascular disease (CVD). At present, FCH is the most common inherited hyperlipidemia in humans, affecting 1-3% of the population and up to 20% of patients with premature myocardial infarction. Subjects with FCH have a 2- to 5 fold increased risk of premature CVD. Therefore, it is of great importance to identify and treat subjects with FCH appropriately. For more than 30 years studies have been going on aiming to solve the metabolic background of FCH. Several metabolic abnormalities have been suggested, but the exact pathophysiology of FCH is still unknown. Also, the genetic basis for FCH has remained obscure. Because there is still no specific marker for FCH in clinical practice it demands time-consuming family investigation before the diagnosis can be established in individuals.

The search for metabolic and genetic origin of this lipid disorder has some fundamental problems. Not all research groups use the same criteria to establish the diagnosis FCH, thereby the lipid phenotype can vary substantially within any individual.

The main aim of this thesis was to evaluate this intra-individual variation in lipid phenotype over a period of 5 year and to come to more consistent and unequivocal diagnostic criteria for FCH which can be used in clinical practice. Furthermore, we investigated the contribution of other metabolic components and factors in relation to FCH phenotype and the increased risk of CVD.

Re-evaluation of diagnostic criteria of FCH

The most fundamental problem in the research of FCH is that the lipid phenotype can vary substantially within an individual. This has been suspected but was not convincingly demonstrated. In 1994, blood samples and required data of 687 family members from 40 well-defined FCH families were collected. In 1999 we collected again blood samples and required data of 299 of these 687 family members from 32 families.

In **chapter 2** we evaluated the variability in lipid phenotype expression over this 5-year period (1994-1999). A total of 32 families including 299 subjects were studied in 1994 and 1999. Subjects were classified affected FCH when total cholesterol (TC) and/or triglyceride (TG) levels exceeded the 90th percentile adjusted for age and gender. In 1994, 93 of the 299 subjects (31%) were affected, whereas 206 subjects (69%) were non-affected relatives. In 1999 the diagnosis FCH was consistent in 69 of the 93 subjects (74%). So, 26% of the affected FCH subjects in 1994 showed a sporadic normolipidemic pattern (i.e. TC and/or TG < 90th percentile) in 1999. Among the 206 non-affected relatives in 1994, 178 subjects (86%) remained non-affected in 1999 and 28 (14%) developed a FCH lipid phenotype. So, the diagnosis FCH, based on plasma TC and/or TG levels, is consistent in only 74% of the subjects over a 5-year period. Thereafter, we studied which characteristics of FCH are more consistent and show less intra-individual variability in time.

Two important characteristics of FCH are an elevated apolipoprotein B (apoB) concentration and the increased prevalence of small dense low-density lipoprotein (sdLDL). We reported that both the apoB concentration and the LDL subfraction profile show less variability in time and are more consistently associated with FCH. In conclusion, the results emphasize the need for re-evaluation of the diagnostic criteria for FCH. We demonstrated in chapter 2 that apoB and sdLDL are attractive new candidates to define FCH. Further studies are indicated to evaluate the role of apoB and sdLDL as diagnostic criteria for FCH (chapter 3).

In **chapter 3** we intended to define unequivocal diagnostic criteria for FCH based on our large FCH cohort with 5-year follow-up. We defined a subject 'truly'-FCH when diagnosed FCH in 1994 and/or 1999 based on the traditional lipid criteria (TC and/or TG level above the 90th percentile). One hundred and twenty one of the 299 subjects (40%) were affected 'truly'-FCH. It appeared that the combination of TG level adjusted for age and gender in combination with absolute apoB and TC level adjusted for age and gender most adequately predict FCH. The TG concentration is well associated with the concentration of sdLDL. To facilitate the implementation of these new diagnostic criteria in clinical practice we provided a nomogram to simply and accurately diagnose FCH. In the literature a proposal to redefine FCH was undertaken based on hypertriglyceridemia (TG level>1.5 mmol/l) and hyperapoB (apoB concentration > 1200 mg/l). The choice of these cut-off points was tentative.

Now we showed for the first time that a cut-off point for apoB >1200 mg/l and for TG >1.5 mmol/l is justified, based on our large FCH cohort with 5 year follow-up. So, our data provided evidence that the definition of FCH based on hyperTG and hyperapoB, as suggested in the literature, is an alternative when percentiles of TG and TC are not available. However, the diagnosis based on the absolute apoB value in combination with TG and TC concentrations, both adjusted for age and gender, better predicts 'truly'-FCH compared to hyperTG and hyperapoB (R2 =69% versus 50%, respectively). Using the proposed new diagnostic criteria included in a nomogram will make it easier to identify patients and test relatives to diagnose 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 relevance of this new definition of FCH in other large FCH cohorts is warranted to confirm the unequivocal diagnostic criteria for FCH.

Metabolic features of FCH and its increased risk of CVD

Several disturbances in metabolic pathways have been suggested to be pathophysiologically important for the FCH phenotype. The exact pathophysiology of FCH is unknown. FCH is, in general thought to be caused by hepatic overproduction of very low density lipoprotein (VLDL) particles with or without an impaired clearance of TG-rich lipoproteins. There is still more evidence that a primary defect in adipose tissue may be the culprit of FCH, resulting in reduced fatty acid (FA) trapping in adipose tissue and increased FA flux to the liver resulting in high apoB production and contributing to insulin resistance.

In **chapter 4** we presented a review about the known pathogenesis of FCH with emphasis on the role of insulin resistance, adipose tissue metabolism and FA. Abnormalities in several metabolic pathways have been suggested to be important in causing the FCH phenotype. Figure 1 shows different possible metabolic disturbances in FCH.

The overproduction of VLDL in the liver (I) may result from an increased supply of FA to the liver. Thereby the impaired clearance of TG-rich lipoproteins is thought to be caused by the diminished lipoprotein lipase (LPL) activity (II) which is caused by LPL mutations, increased FFA concentrations, increased apoCIII concentration and/or insulin resistance. A characteristic of FCH is the increased prevalence of sdLDL (III). This may be present due to an increased cholesteryl ester transfer protein (CETP) activity, a major gene effect or genetic defect, increased concentrations of precursor molecule VLDL, a change in hepatic lipase (HL) and/or LPL activity. The existence of insulin resistance (IV), which is a characteristic of FCH, and an important factor modulating the FCH phenotype may be caused by a genetic defect, obesity, defects in FA metabolism, activation of TNF-α system and an increased leptin concentration.

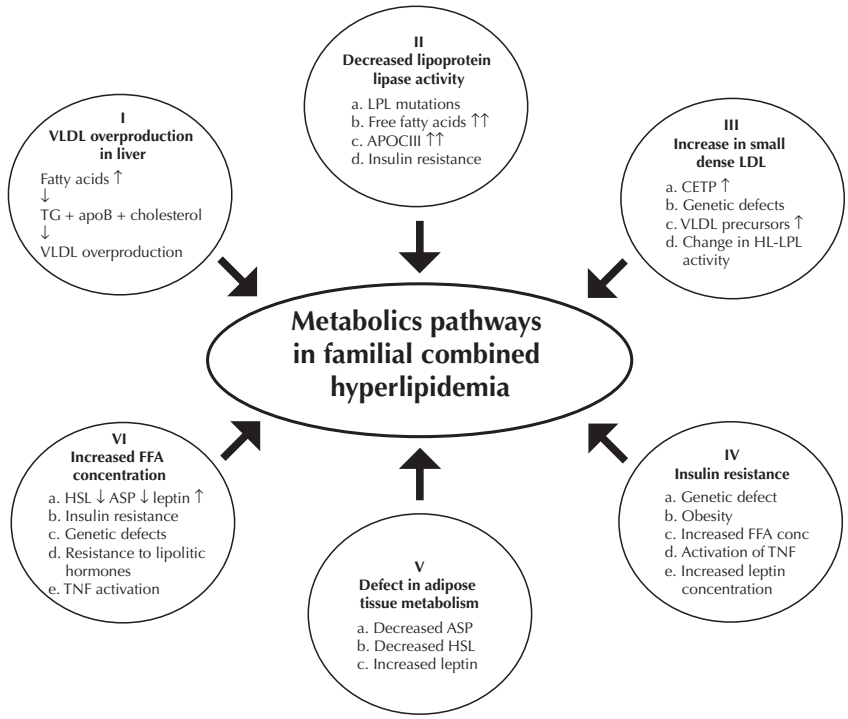


Figure 1 Different possible metabolic disturbances in Familial Combined Hyperlipidemia

Insulin resistance itself results in impaired suppression of hormone sensitive lipase (HSL) (V), increased FFA concentrations (VI) and diminished LPL activity (II).

Over the past few years evidence has emerged to support the hypothesis that a primary defect in adipose tissue metabolism may be the culprit of FCH. The defects in adipose tissue metabolism (V) may result from a decreased activity of acylation stimulatory protein (ASP), a decreased activity of HSL, and/or an increased leptin concentration. These defects contribute to insulin resistance (IV) and an increased plasma FFA concentration (VI). Other recently proposed defects in adipose tissue metabolism are an increased adiponectin concentration or an altered adipocyte fatty acid-binding protein (A-FABP). A disturbed FA metabolism may play a central role in FCH (VI). Increased FFA may be caused by (genetic) defects in HSL, ASP or leptin (V), insulin resistance (IV), genetic defects, resistance to lipogenic hormones and TNF-α activation. The increased concentration of plasma FFA results in increased supply of FFA to the liver (I), insulin resistance (IV) and decreased LPL activity (II).

ApoB as risk predictor in FCH

Hyperlipidemia is strongly correlated with increased risk of CVD. The most commonly used approach to estimate CVD risk is based on the measurement of plasma TC, TG and HDL-chol with calculation of LDL-chol by means of the Friedewald formula. Recently apoB has been suggested to be a more accurate clinical measure of atherogenic risk than TC or LDL-chol.

Therefore, we compared in **chapter 5** the measurement of lipids and lipoproteins versus assay of apoB for estimation of CVD in our FCH cohort. We compared in 506 members of families with FCH these two approaches to select subjects with an apparent increased risk of CVD: assay of apoB versus measurement of plasma lipids and lipoproteins. When comparing both criteria, there was an overlap of 81.2% at apoB levels ≤ 1250 mg/l and of 86.9% at apoB levels > 1250 mg/l. At apoB ≤ 1250 mg/l all subjects were normolipidemic. At apoB concentrations > 1250 mg/l we observed a group with normal plasma lipid levels (13.1%). In this group, defined as normolipidemic hyperapobetalipoproteinemia, and considered to have an increased risk for CVD, apoB determination was thus most informative. The selection of the subgroup with "normolipidemic hyperapobetalipoproteinemia" on the basis of the conventional approach could be refined using a cut off limit for plasma TG < 1. 5 mmol/l. This limit distinguished optimally between an atherogenic very dense LDL pattern versus a dense and buoyant LDL pattern.

Thus, based on the results of this study, the determination of apo B appeared to be, if not superior, at least as effective as the conventional lipid and lipoprotein parameters in classifying subjects at increased risk for CVD.

Homocysteine and FCH

FCH is associated with an increased risk of CVD. The plasma lipid and lipoprotein levels in subjects with FCH are relatively moderately elevated and do not fully explain the increased risk of CVD. Hyperhomocysteinemia, a disorder of methionine metabolism is also a well-known independent risk factor for CVD.

In **chapter 6** we investigated whether subjects with FCH have a higher plasma homocysteine concentration than controls and whether homocysteine contributes to the increased risk of CVD in FCH. In total 667 subjects, including 161 subjects with FCH, 109 spouses who referenced as control group and 397 normolipidemic relatives were studied. FCH was defined by the traditional accepted criteria: plasma TC and/or TG levels above the 90th percentile adjusted for age and gender. The mean plasma homocysteine concentration in the FCH group was not significantly different from the control group with a mean difference of 0.9 µmol/l (CI 95% -0.5 to 2.3). Also after adjustment for age and gender the mean difference in homocysteine levels between FCH subjects and controls was 0.9 µmol/l (CI 95% -0.4 to 2.5). So, the mean plasma homocysteine levels are not elevated in subjects with FCH compared to control subjects.

Thirty-four (21%) of the 161 subjects with FCH had CVD. As FCH is not associated with hyperhomocysteinemia, we analyzed normolipidemic relatives and non-related control subjects together in one group, defined as non-FCH. In this non-FCH group 28 (6%) of the 506 subjects had CVD and increased levels of homocysteine, defined by levels above the 90th percentile (18.9 µmol/l). They were associated with a 2.8 times increased risk of CVD compared to subjects with

lower homocysteine levels. In the FCH group increased levels of homocysteine were also associated with an increased risk of CVD although the risk estimator was lower (1.8 times). So, hyperhomocysteinemia does not disproportionally increase the risk of CVD in FCH subjects. We found a positive correlation between plasma homocysteine concentration and TC, HDL-chol, LDL-chol and apoB, but none of the plasma lipid and lipoprotein levels (TC, TG, HDL-chol, LDL-chol but also apoB and sdLDL) were independently associated with the homocysteine level. Our data support the hypothesis that plasma concentrations of homocysteine and lipids and lipoproteins are independent parameters. Also, insulin resistance was not related with plasma homocysteine concentration.

Insulin resistance and FCH

Insulin resistance is associated with a number of metabolic abnormalities, including increased TG levels, low HDL-chol, increased sdLDL and increased apoB levels, all characteristics of FCH. The presence of insulin resistance may be an important factor modulating FCH lipid phenotype expression.

In **chapter 7** we explored the role of insulin resistance in FCH lipid phenotype expression and the effect of intra-individual changes in lipid phenotype on insulin resistance over a 5-year period. FCH was defined by the traditional diagnostic criteria including plasma TC and/or TG levels >90th percentile. Insulin resistance was assessed by the Homeostasis Model Assessment index (HOMA). For the current study we included all subjects with FCH, normolipidemic relatives and spouses of whom glucose and insulin levels were available in 1999, resulting in a study population of 132 FCH subjects, 350 normolipidemic relatives and 81 spouses, who referenced as controls. Among the 132 subjects with FCH, data of lipid and glucose/insulin levels were also available in 1994 in 76 subjects. These subjects were included in the follow-up to study the interdependence of change in lipid phenotype expression in time on insulin resistance. We showed that insulin resistance is a characteristic feature of FCH. FCH subjects were more insulin resistant compared to controls and normolipidemic relatives (HOMA index 2.9 (95% CI 2.7-3.2), 2.2 (95% CI 2.0-2.5) and 2.0 (95% CI 1.9-2.2), respectively), even after correction for sex, age and BMI. The degree of insulin resistance is dependent on lipid phenotype expression. FCH subjects affected based on isolated hypertriglyceridemia or combined hyperlipidemia were more insulin resistant, whereas, FCH subjects based on isolated hypercholesterolemia were not more insulin resistant compared to controls. It appears that subjects with isolated hypercholesterolemia may comprise a distinct group within FCH. The mean feature of FCH is the variability in lipid phenotype expression within an individual in time. In our 5-year follow-up we studied the effect of these intra-individual changes in lipid phenotype expression in time on insulin resistance. A change in lipid phenotype expression over 5 year was associated with a change in insulin resistance, which is strongly related to the increase in BMI.

Increase of apoB and sdLDL are important characteristics of FCH. Insulin resistance and obesity are also associated with increased levels of apoB and increased prevalence of sdLDL. Therefore we explored whether insulin resistance and/or obesity could explain the increased apoB levels and high amount of sdLDL in FCH. In chapter 7 we showed that obesity and/or insulin resistance do not fully account for the elevated levels of apoB and the 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 with modulation by BMI and insulin resistance.

Leptin and FCH

The hormone leptin is involved in the regulation of the energy expenditure and appetite via hypothalamic receptors. Obese persons have increased levels of leptin and 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. Elevated levels of leptin are also associated with insulin resistance. Both obesity and insulin resistance are characteristics of FCH and therefore leptin may be elevated in FCH subjects. So far, only two small studies have studied the relationship between leptin concentrations and FCH, with conflicting results. Leptin concentration is also associated with an increased risk of CVD.

In **chapter 8** we investigated whether leptin levels in subjects with FCH were elevated, independent of their BMI. The second objective was to investigate whether leptin levels contribute to the increased risk for CVD in FCH. The study population existed of 37 families, comprising 651 subjects, of whom 158 subjects were diagnosed as affected FCH, 389 as normolipidemic relatives and 97 subjects were spouses. The diagnosis FCH was in this study based on absolute apoB levels in combination with TG and TC levels adjusted for age and gender, using a nomogram, as described in chapter 3. We observed higher leptin levels in both male and female subjects with FCH compared to their normolipidemic relatives and controls, which is in proportion to the presence of overweight and insulin resistance in FCH subjects. In females, the increased leptin levels were completely attributable to BMI, whereas in male FCH subjects increased leptin levels were found, independent of overweight, suggesting a defect in adipose tissue metabolism in male subjects with FCH. After standardization of leptin levels for both BMI and HOMA, no significant difference in leptin levels were found among, subjects with FCH, normolipidemic relatives and controls, although the trend of male subjects with FCH having higher leptin levels was still present. Multiple linear regression analyses showed that the variation in leptin concentration could be explained for approximately 71% simply by the variables gender and BMI. When including the degree of insulin resistance (HOMA index) in the model, even 74% of the variation in leptin concentration could be explained. A serum leptin concentration

(standardized for gender and BMI) above the 90th percentile was associated with an increased risk of CVD in FCH subjects (OR=3.4 (95% CI: 1.3-9.4)) and in non-FCH subjects (normolipidemic relatives and spouses together) (OR=3.4 (95% CI: 1.3-9.2)). The overall OR for the total group was 3.5 (95% CI: 1.8-6.9). So, there is an increased risk for CVD associated with leptin levels (standardized for gender and BMI) above the 90th percentile in both subjects with FCH and in healthy controls.

Conclusions

1. The diagnosis FCH, based on plasma TC and/or TG levels above the 90th percentile adjusted for age and gender, is consistent in only 74% of 307 subjects over a 5-year period. This emphasizes the need for more consistent diagnostic criteria for FCH.
2. Absolute apoB levels in combination with TG and TC levels, both adjusted for age and gender, are most predictive for diagnosing FCH. A nomogram can be used to simply and accurately calculate the probability to be affected FCH in clinical practice.
3. The definition of FCH based on hyperTG (TG levels > 1.5 mmol/l) hyperapoB (apoB>1200 mg/l) is an alternative when percentiles of TG and TC are not available.
4. The culprit of the pathophysiology of FCH may be a defect in adipose tissue metabolism and/or adipose tissue hormonal factors, resulting in reduced fatty acid (FA) trapping in adipose tissue and increased FA flux to the liver resulting in high apoB production and contributing to insulin resistance.
5. ApoB values are, if not superior, at least as effective as the conventional lipid and lipoprotein parameters in classifying subjects with FCH at increased risk for CVD.
6. Subjects with FCH have no increased levels of plasma homocysteine compared to controls. Plasma homocysteine concentration and lipids and lipoproteins are independent parameters. In FCH, plasma homocysteine does not disproportional contribute to the increased risk of CVD.
7. Insulin resistance is a characteristic feature of FCH, independent of BMI and dependent on lipid phenotype expression.
8. The increased levels of apoB and the prevalence of small dense LDL in FCH are determined by genetic factors and are modulated by BMI and insulin resistance.
9. Serum leptin levels are increased in subjects with FCH in proportion to their obesity and state of insulin resistance. Increased leptin levels in FCH contribute to the increased risk for CVD, independent of gender, BMI and insulin resistance.

Future perspectives

Confirmation of the new diagnostic criteria of FCH

FCH is a heterogeneous disorder with absence of a specific clinical, metabolic or genetic marker. A major step forward in the process of unravelling the metabolic and genetic background of this disease is the introduction of unequivocal diagnostic criteria, as we proposed using the nomogram. Confirmation of the relevance of the new definition of FCH in other large FCH cohorts is therefore warranted.

Genetic studies

After confirmation of the diagnostic criteria, re-evaluation of the genome-scans in all FCH cohorts should be performed, preferably by combining data sets to increase power. Recently, a genome scan was performed in our FCH cohort in collaboration with the NIH (USA). Currently, linkage analysis and quantitative trait analyses are in process.

Depending on the results of the linkage analysis, potential candidate genes in the regions of linkage can be further explored. Recently, the thioredoxin interacting protein (TXNIP) gene and the upstream transcription factor 1 (USF1) gene were found to be associated with FCH as a result of linkage analysis in the Finnish FCH cohort. However, in our FCH cohort we could exclude TXNIP to be involved in the pathogenesis of FCH. Again, differences in diagnostic criteria could be the culprit in the opposing results.

Furthermore, the candidate gene approach can be used based on the pathophysiology of FCH. For example, evidence for a disturbed adipose tissue metabolism as the culprit of FCH accumulates. Therefore several genes of adipocytokines potentially involved in a disturbed adipose tissue metabolism are of interest, including leptin, adiponectin and resistin. Not only genetic variations in the promotor regions of the genes of adipocytokines but also mRNA expression studies in biopsies of adipose tissue should be performed to further evaluate the role of adipose tissue in the pathophysiology of FCH.

Just recently, an exciting new type of genomic variation emerged, the so-called large-scale copy number variation (LCV), involving gain or loss of relatively large stretches of genomic DNA. It is anticipated that at least some of the LCVs may serve as predisposing factors for the development of human multifactorial diseases. So, systematic LCV studies will be of large interest in FCH populations.

Metabolic studies

To further explore the role of adipose tissue in the pathophysiology of FCH it is of interest to measure several adipocytokines in plasma of FCH patients, including leptin, adiponectin and resistin in combination with in vivo turnover studies in adipose tissue.

FCH is characterised by a number of abnormalities which correspond with symptoms of the insulin resistance syndrome. Interventions purely aimed at improving insulin resistance should be performed to further evaluate the role of insulin resistance in the pathophysiology of FCH. Therefore, it is of interest to study the effect of thiazolidinediones, a class of drug which improves insulin sensitivity, on the FCH phenotype.

FCH is the most common inherited lipid disorder with increased risk of CVD, which cannot only be explained by the relatively mildly elevated lipid levels. Additional risk factors contributing to the increased risk of CVD should be explored, including both pro-inflammatory and oxidative stress parameters. Interventions aimed at reducing these risk factors should be developed.

Imaging of atherosclerosis

To estimate the risk of CVD without the need to screen or know all potential risk factors it is necessary to be able to measure the burden of atherosclerosis. Non-invasive measurements of atherosclerosis including both functional and structural changes in the arterial wall of FCH patients will be informative to calculate the risk of CVD and evaluate the effect of interventions aimed at eliminating risk factors.

Chapter 10

Samenvatting en conclusies

Samenvatting

Familiaire Gecombineerde Hyperlipidemie (FCH) werd voor het eerst beschreven in 1973 als een erfelijke vetstofwisselingsziekte. Deze ziekte wordt gekenmerkt door verhoogde plasmalipiden, dit wil zeggen een verhoogd totaal cholesterol-(TC) gehalte met of zonder een verhoogd triglyceride- (TG) gehalte en geeft een verhoogde kans op hart- en vaatziekten al op jonge leeftijd; patiënten met FCH hebben een 2 - 5 maal verhoogde kans op hart- en vaatziekten voor het 60e levensjaar. Momenteel is FCH de meest voorkomende erfelijke vetstofwisselingsstoornis, 1-3% van de bevolking en 20% van de patiënten met een hartinfarct voor het 60e levensjaar zijn aangedaan. Daarom is het van groot belang patiënten met FCH zo vroeg mogelijk op te sporen en te behandelen. Al meer dan 30 jaar wordt er onderzoek verricht naar de oorzaak van FCH. Vele metabole stoornissen zijn gesuggereerd, maar de exacte oorzaak van FCH is nog steeds onbekend. Ook de genetische oorzaak van FCH is nog niet opgehelderd. Omdat er nog steeds geen specifieke marker voor FCH is in de klinische praktijk, is tijdrovend familieonderzoek nodig voor het stellen van de diagnose FCH in een individuele patiënt; om de diagnose FCH te stellen moet zowel bij de patiënt als bij tenminste 1 familielid een verhoogd TC- en/of TG-gehalte gemeten worden in combinatie met het voorkomen van hart- en vaatziekten voor het 60e levensjaar in de familie.

Het onderzoek naar de metabole en genetische achtergrond van deze ziekte kent enkele belangrijke problemen. Ten eerste gebruiken de verschillende onderzoeksgroepen over de wereld die zich bezig houden met FCH niet altijd dezelfde diagnostische criteria. Daarnaast kunnen de TC- en TG-waarden binnen een individu variëren in de tijd (wisselend lipidenfenotype).

Het doel van dit onderzoek was om de variatie in lipidenfenotype binnen een individu te evalueren over een periode van 5 jaar en daarmee te komen tot meer consistente en duidelijke diagnostische criteria voor FCH die gebruikt kunnen worden in de klinische praktijk. Daarnaast hebben we de bijdrage van diverse metabole variabelen onderzocht in relatie tot FCH en het verhoogde risico op hart- en vaatziekten.

Her-evaluatie van de diagnostische criteria van FCH

Het belangrijkste probleem bij het onderzoek naar FCH is het wisselende lipidenfenotype dat kan optreden binnen een individu. Dit wisselende fenotype werd vermoed maar was nog niet overtuigend bewezen. In 1994 waren klinische gegevens en bloedmonsters van 687 familieleden van 40 FCH families verzameld. In 1999 hebben we opnieuw bloedmonsters en klinische gegevens verzameld bij 299 van deze 687 personen uit 32 families.

In **hoofdstuk 2** werd de variabiliteit in lipidenfenotype over een periode van 5 jaar (1994-1999) geëvalueerd. In totaal 299 personen van 32 families werden bestudeerd. Personen werden geclassificeerd als aangedaan met FCH wanneer zij een TC- en/of TG-gehalte boven de 90^{e} percentiel hadden, gecorrigeerd voor leeftijd en geslacht. In 1994, waren 93 (31%) van de 299 personen aangedaan, 206 (69%) waren niet aangedane familieleden. In 1999 bleken 69 (74%) van de 93 personen weer aangedaan te zijn. Dus, 26% van de met FCH aangedane personen in 1994 waren niet aangedaan in 1999 (TC and/of TG <90^{e} percentiel). Van de 206 niet aangedane familieleden in 1994 bleken 178 (86%) personen ook niet aangedaan te zijn in 1999 en 28 (14%) waren in 1999 wel aangedaan. Dus, de diagnose FCH, gebaseerd op plasma TC- and/of TG-concentratie was slechts consistent in 74 % van de personen over een periode van 5 jaar. Hierna bestudeerden we welke andere karakteristieken van FCH potentieel meer betrouwbaar zijn en minder intra-individuele variatie vertonen in de tijd. Twee belangrijke karakteristieken van FCH zijn een verhoogde apolipoproteine B- (apoB) concentratie en de aanwezigheid van meer small dense low-density lipoprotein (sdLDL). We toonden aan dat deze beide karakteristieken minder variabel zijn in de tijd en consistenter geassocieerd met FCH. Als conclusie tonen de resultaten van deze studie de noodzaak aan tot her-evaluatie van de bestaande diagnostische criteria voor FCH.

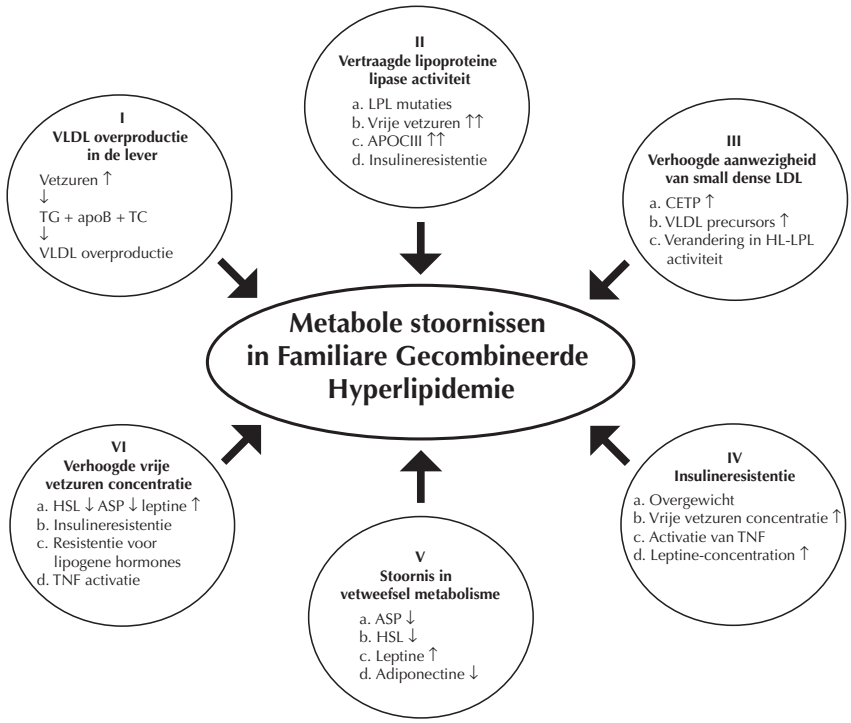
In **hoofdstuk 3** stelden wij ons tot doel om te komen tot nieuwe, meer consistente diagnostische criteria voor FCH gebaseerd op ons grote FCH cohort met 5 jaar follow-up. We definieerden een persoon als hebbende FCH wanneer in 1994 **dan wel** in 1999 de diagnose FCH gesteld was gebaseerd op de traditionele criteria (TC en/of TG >90^{e} percentiel (gecorrigeerd voor leeftijd en geslacht)). Op basis van deze criteria hadden 121 (40%) van de 299 personen FCH. Vervolgens werd geëvalueerd welke (combinatie van) karakteristieken van FCH het meest betrouwbaar FCH voorspellen. De combinatie van TG-waarde (gecorrigeerd voor leeftijd en geslacht) met absoluut apoB- en TC-gehalte (gecorrigeerd voor leeftijd en geslacht) bleek het meest adequaat voor de diagnosestelling FCH. Om deze nieuwe definitie van FCH makkelijk toe te passen in de klinische praktijk werd een nomogram ontworpen waarmee op simpele en accurate wijze de diagnose FCH gesteld kan worden.

In de literatuur was een voorstel verschenen om FCH te definiëren op basis van hypertriglyceridemie (TG >1.5 mmol/l) en hyperapoB (apoB concentratie >1200 mg/l). De keuze van deze afkappunten was tentatief. In onze studie tonen we aan dat de afkappunten voor apoB >1200 mg/l en TG >1.5 mmol/l gerechtvaardigd zijn. De definitie FCH gebaseerd op hyperTG en hyperapoB is dus een alternatief wanneer de percentielen voor TC en TG niet voorhanden zijn. Echter de diagnose FCH gebaseerd op de absolute apoB-concentratie in combinatie met TC- en TG-concentraties (beide gecorrigeerd voor leeftijd en geslacht) heeft een betere voorspelling voor de diagnose FCH vergeleken met hyperTG en hyperapoB (R^2=69% versus 50%, respectievelijk). Het gebruik maken van de voorgestelde nieuwe criteria met behulp van het nomogram maakt het makkelijker FCH patiënten te definiëren en familieleden te testen. Wel blijft de voorwaarde bestaan dat meer dan één familielid moet zijn aangedaan en er hart- en vaatziekte moet voorkomen in de familie onder het 60^e levensjaar voor men de diagnose FCH mag stellen.

Metabole factoren bij FCH en het verhoogde risico op hart- en vaatziekten

Verschillende stoornissen in metabole processen dragen bij aan het ontstaan van FCH. De exacte pathofysiologie van FCH is echter nog steeds onbekend. Overproductie van TG-rijke very-low density lipoprotein (VLDL) deeltjes door de lever met of zonder een afgenomen afbraak van TG-rijke deeltjes zijn twee belangrijke stoornissen die leiden tot FCH. Er komt steeds meer bewijs dat een primaire stoornis in het vetweefsel mogelijk de belangrijkste oorzaak is voor FCH. Een gestoord vetweefselmetabolisme resulteert in een verlaagde opname van vetzuren in het vetweefsel en dus een verhoogde stroom van vetzuren naar de lever. Een verhoogd aanbod van vetzuren aan de lever leidt tot een verhoogde apoB-productie en draagt bij tot insulineresistentie, een ander belangrijk kenmerk van FCH.

In **hoofdstuk 4** wordt een overzicht gegeven over de tot op heden bekende oorzaken van FCH met de nadruk op de rol van insulineresistentie, vetweefsel en vetzuren. Stoornissen in meerdere metabole processen dragen bij aan het ontstaan van FCH (Figuur 1).



Figuur 1 Verschillende metabole stoornissen die mogelijk bijdragen aan het ontstaan van FCH.

(I) VLDL overproductie door de lever wordt veroorzaakt door verhoogde aanvoer van vrije vetzuren aan de lever. (II) Verminderde afbraak van TG-rijke deeltjes kan veroorzaakt worden door verminderde lipoproteine-lipase (LPL) activiteit hetgeen een gevolg kan zijn van (a) LPL mutaties, (b) verhoogde vrije-vetzuren-concentratie, (c) verhoogde apolipoprotein CIII-concentratie of (d) insulineresistentie. (III) Meer small dense LDL kan het gevolg zijn van (a) verhoogde cholesteryl ester transfer protein (CETP) activiteit, (b) verhoogde concentratie van precursor VLDL of (c) verandering in leverlipase (HL) en/of LPL-activiteit. (IV) Insulineresistentie kan het resultaat zijn van (a) overgewicht, (b) stoornissen in vetweefselmetabolisme, (c) activatie van tumor necrosis factor (TNF) of (d) verhoogde leptine-concentratie (V) stoornissen in het vetweefselmetabolisme kunnen het resultaat zijn van (a) verlaagde acylation-stimulatory-protein (ASP)-activiteit, (b) verlaagde hormone-sensitive lipase-(HSL) activiteit , (c) verhoogde leptine-concentratie of (d) verlaagde adiponectine-concentratie (VI) verhoogde concentratie van vrije vetzuren is potentieel het gevolg van verlaagde HSL, ASP activiteit, verhoogde leptine-concentratie, (b) insulineresistentie, (c) resistentie voor lipogene hormonen, (d) TNF activatie. Al deze metabole stoornissen (I – VI) kunnen ook berusten op genetische afwijkingen.

ApoB als risicovoorspeller voor hart- en vaatziekten in FCH

Hyperlipidemie is sterk geassocieerd met een verhoogd risico op hart- en vaatziekten. De meest gebruikte methode om het risico op hart- en vaatziekten te voorspellen is gebaseerd op de bepaling van plasma TC, TG en high-density lipoprotein cholesterol (HDL-c) met berekening van LDL-c met de Friedewald formule. Recente studies laten zien dat apoB mogelijk de beste parameter is om het risico op hart- en vaatziekten te voorspellen.

In **hoofdstuk 5** is de bepaling van lipiden versus die van apoB in het classificeren van personen met een verhoogd risico op hart- en vaatziekten in onze FCH-populatie bestudeerd. Bij het vergelijken van beide criteria bleken alle personen met een apoB-concentratie ≤1250 mg/l ook normale plasmalipidenwaarden te hebben. Echter bij een apoB concentratie >1250 mg/l bleek er een groep personen (13,1%) te zijn met normale plasmalipidenwaarden. Een verhoogde apoB-concentratie >1250 mg/l is geassocieerd in de literatuur met een verhoogd risico op hart- en vaatziekten. Bij deze personen, die we definieerden als normolipidemisch hyperapoB, is de bepaling van apoB dus meer informatief dan de lipidenwaarden. Dus, gebaseerd op de resultaten van deze studie, blijkt de bepaling van apoB in ieder geval even effectief en mogelijk superieur te zijn boven de conventionele bepaling van lipiden bij het classificeren van personen met een verhoogd risico op hart- en vaatziekten.

Homocysteine en FCH

FCH is geassocieerd met een verhoogd risico op hart- en vaatziekten. De plasmalipiden en lipoproteinenconcentraties in patiënten met FCH zijn relatief slechts matig verhoogd en kunnen niet volledig het verhoogde risico op hart- en vaatziekten verklaren. Een te hoog homocysteinegehalte in het bloed (hyperhomocysteinemie) is ook een bekende onafhankelijke risicofactor voor hart- en vaatziekten. Hyperhomocysteinemie is het gevolg van een stoornis in het methionine metabolisme.

In **hoofdstuk 6** werd onderzocht of patiënten met FCH een hogere plasma-homocysteineconcentratie hebben in vergelijking met controles, en of de homocysteineconcentratie bijdraagt tot een verhoogd risico op hart- en vaatziekten in FCH. In totaal werden 667 personen bestudeerd waaronder 161 patiënten met FCH, 109 partners (controle groep) en 397 normolipidemische familieleden.

FCH werd gedefinieerd op basis van de traditionele criteria (TC- en/of TG-concentratie boven het 90e percentiel gecorrigeerd voor leeftijd en geslacht). De gemiddelde plasmahomocysteineconcentratie in de FCH groep was niet significant verschillend van de controle groep met een gemiddeld verschil van 0.9 µmol/l (CI 95% -0.5 tot 2.3). Ook na correctie voor leeftijd en geslacht bleef het gemiddelde verschil in homocysteineconcentratie 0.9 µmol/l (CI 95% -0.4 tot 2.5). De gemiddelde plasmahomocysteineconcentratie is dus niet verhoogd in FCH patiënten vergeleken met controles.

Vier en dertig (21%) van de 161 patiënten met FCH hadden hart- en vaatziekten. Daar FCH niet is geassocieerd met hyperhomocysteinemie analyseerden we de normolipidemische familieleden en controles in één groep gedefinieerd als niet-FCH. In deze niet-FCH groep hadden 28 (6%) van de 506 personen hart- en vaatziekten en een verhoogde homocysteineconcentratie, gedefinieerd als een concentratie boven het 90e percentiel (18.9 µmol/l). Dit was geassocieerd met

een 2.8 maal verhoogd risico op hart- en vaatziekten in vergelijking met personen van de niet-FCH groep met een homocysteineconcentratie <18.9 µmol/l. In de FCH groep waren verhoogde homocysteineconcentraties ook geassocieerd met verhoogd risico op hart- en vaatziekten ofschoon de risico schatting lager was (1.8 maal). Hyperhomocysteinemie verhoogt dus niet disproportioneel het risico op hart- en vaatziekten in FCH patiënten. Daarnaast vonden we een positieve correlatie tussen plasmahomocysteineconcentratie en TC, HDL-c, LDL-c en apoB, maar geen enkele van de plasmalipiden of lipoproteinen (TC, TG, HDL-c, LDL-c, apoB of sdLDL) was onafhankelijk geassocieerd met de homocysteineconcentratie. Onze gegevens ondersteunen dan ook de hypothese dat de plasmaconcentraties van homocysteine en lipiden en lipoproteinen onafhankelijke parameters zijn. Ook insulineresistentie was niet gerelateerd aan de plasmahomocysteineconcentratie.

Insulineresistentie en FCH

Insulineresistentie wordt geassocieerd met een aantal metabole afwijkingen, zoals een verhoogde concentratie van TG, verlaagde HDL-c-concentratie, meer sdLDL en een verhoogde apoB-concentratie, welke ook allen karakteristieken zijn van FCH. Insulineresistentie is dus een potentieel belangrijke factor die van invloed zou kunnen zijn op de expressie van het lipidenfenotype FCH.

In **hoofdstuk 7** werd de rol van insulineresistentie op de expressie van het FCH lipidenfenotype en het effect van intra-individuele veranderingen in lipidenfenotype op insulineresistentie over een periode van 5 jaar bestudeerd. FCH werd gedefinieerd volgens de traditionele criteria (plasma TC en/of TG >90^{e} percentiel, gecorrigeerd voor leeftijd en geslacht). Insulineresistentie werd bepaald met behulp van de Homeostasis Model Assessment Index (HOMA). De studie populatie bestond uit 132 FCH patiënten, 350 normolipidemische familieleden en 81 controles. Van 76 van de 132 FCH patiënten waren ook gegevens van lipiden-, glucose- en insuline-waarden aanwezig uit 1994. Deze 76 personen werden opgenomen in de follow-up studie. Insulineresistentie bleek een karakteristiek van FCH te zijn. FCH patiënten waren meer insulineresistent vergeleken met controles en normolipidemische familieleden (HOMA index 2.9 (CI 95% 2.6 –3.2), 2.2 (CI 95% 2.0-2.5) en 2.0 (CI95% 1.9-2.2), respectievelijk), ook na correctie voor geslacht, leeftijd en body mass index (BMI). De ernst van insulineresistentie was geassocieerd met het lipidenfenotype; FCH patiënten aangedaan gebaseerd op een geïsoleerd verhoogd TG-gehalte of een combinatie van verhoogd TC- en TG-gehalte waren meer insulineresistent dan de controle personen, terwijl patiënten met FCH gebaseerd op een geïsoleerd verhoogd TC-gehalte niet meer insulineresistent waren dan controles. Deze data suggereren dat personen met een geïsoleerd verhoogd TC-gehalte mogelijk een aparte groep vormen binnen FCH.

Een belangrijk kenmerk van FCH is de intra-individuele variabiliteit in lipidenfenotype in de tijd. In onze 5 jaar follow-up bestudeerden we de effecten van

deze intra-individuele veranderingen in lipidenfenotype op insulineresistentie. Een verandering in lipidenfenotype over 5 jaar was geassocieerd met een verandering in insulineresistentie, welke sterk gecorreleerd was aan BMI.

Toename van apoB en sdLDL zijn belangrijke karakteristieken van FCH. Insulineresistentie en overgewicht zijn ook geassocieerd met een verhoogde apoB-concentratie en een verhoogde prevalentie van sdLDL. We onderzochten of insulineresistentie en/of overgewicht de verhoogde apoB-concentratie en toegenomen prevalentie van sdLDL in FCH konden verklaren. In **hoofdstuk 7** toonden we aan dat overgewicht en/of insulineresistentie de verhoogde apoB-concentratie en de prevalentie van sdLDL niet konden verklaren. Deze resultaten ondersteunen het fysiologische concept dat afzonderlijke, genetische determinanten bijdragen aan het FCH lipidenfenotype, met modulatie door overgewicht en insulineresistentie.

Leptine en FCH

Het hormoon leptine is betrokken bij de regulatie van het energieverbruik en eetlust via hypothalame receptoren. Mensen met overgewicht hebben een verhoogde concentratie van leptine, echter deze verhoogde leptinewaarden leiden niet tot een normalisering van energieverbruik/opname dan wel herstel van de vetmassa. Overgewicht lijkt dus een toestand van leptineresistentie te zijn. Een verhoogde leptineconcentratie is ook geassocieerd met insulineresistentie en hart- en vaatziekten. Overgewicht en insulineresistentie zijn beide karakteristieken van FCH. Een verhoogd leptinegehalte zou dus potentieel aanwezig kunnen zijn in FCH. Tot dusver hadden slechts twee studies de relatie tussen leptineconcentratie en FCH onderzocht met tegengestelde resultaten.

In **hoofdstuk 8** onderzochten we of de leptineconcentratie verhoogd is bij patiënten met FCH, onafhankelijk van BMI en insulineresistentie. Ten tweede bestudeerden we of de leptineconcentratie bijdraagt tot het verhoogde risico op hart- en vaatziekten in patiënten met FCH. De studiepopulatie bestond uit 37 families, met in totaal 651 personen, 158 patiënten met FCH, 389 normolipidemische familieleden en 97 controles. De diagnose FCH werd in deze studie gebaseerd op absolute apoB- concentratie in combinatie met TC- en TG-concentraties, beide gecorrigeerd voor leeftijd en geslacht, gebruik makend van het nomogram, zoals beschreven in **hoofdstuk 3**. We vonden hogere concentraties van leptine in zowel mannen als vrouwen in de FCH groep vergeleken met de normolipidemische familieleden en controles, hetgeen was toe te schrijven aan het feit dat patiënten met FCH meer insulineresistent zijn en er sprake is van overgewicht. Bij de vrouwen was de verhoogde leptineconcentratie volledig toe te schrijven aan overgewicht, echter bij de mannen met FCH was de verhoogde concentratie leptine onafhankelijk van het overgewicht. Dit suggereert een defect in het vetweefselmetabolisme in mannelijke personen met FCH. Na correctie voor

beide, zowel overgewicht als insulineresistentie, werd er geen significant verschil meer in leptineconcentratie gevonden tussen de FCH groep, normolipidemische groep en controles, ofschoon de trend dat mannelijke patiënten met FCH een hogere concentratie leptine hebben nog aanwezig was. Multiple lineaire regressie toonde aan dat de variatie in leptineconcentratie voor bijna 71% verklaard kon worden door de variabelen, geslacht en overgewicht en 74% door de variabelen, geslacht, overgewicht en insulineresistentie. Een serumleptineconcentratie (gecorrigeerd voor geslacht en BMI) boven het 90^{e} percentiel was geassocieerd met een verhoogd risico op hart- en vaatziekten in FCH patiënten (OR=3.4 (95%CI: 1.3-9.4)) en in niet-FCH personen (normolipidemische familieleden en controles) (OR=3.4 (95%CI: 1.3-9.2)). De OR voor de totale groep was 3.5 (95%CI: 1.8-6.9). Een verhoogde leptineconcentratie is dus geassocieerd met een verhoogd risico op hart- en vaatziekten in patiënten met FCH en in gezonde controles.

Conclusies

1. De diagnose FCH, gebaseerd op plasma TC- en/of TG-concentratie >90e percentiel (gecorrigeerd voor leeftijd en geslacht), is consistent in slechts 74% van de 307 onderzochte personen over een periode van 5 jaar. Dit ondersteunt de noodzaak om te komen tot meer consistente diagnostische criteria voor FCH.
2. De absolute apoB-concentratie in combinatie met TC- en TG-concentraties, (beide gecorrigeerd voor leeftijd en geslacht) voorspellen het best de diagnose FCH. Het ontworpen nomogram kan gebruikt worden in de klinische praktijk om makkelijk en adequaat de kans te berekenen of iemand FCH heeft.
3. De diagnose FCH gebaseerd op hyperTG (TG concentratie >1.5 mmol/l) en hyperapoB (apoB >1200 mg/l) is een alternatief wanneer de percentielen van TC- en TG-waarden niet voorhanden zijn.
4. Een stoornis in het vetweefselmetabolisme is mogelijk een van de belangrijkste factoren in de pathofysiologie van FCH. Een gestoord vetweefselmetabolisme leidt tot een verlaagde opname van vetzuren in het vetweefsel en een verhoogde stroom van vetzuren naar de lever. Dit veroorzaakt een verhoogde apoB-aanmaak en draagt bij tot insulineresistentie, beiden belangrijke kenmerken van FCH.
5. ApoB-waarden zijn, indien niet superieur, tenminste even effectief in vergelijking met de conventionele lipiden- en lipoproteinen-waarden in het classificeren van FCH patiënten met een verhoogd risico op hart- en vaatziekten.
6. Patiënten met FCH hebben geen verhoogde plasmahomocysteinespiegels vergeleken met controles. De plasmahomocysteineconcentratie en lipiden- en lipoproteinen-waarden zijn onafhankelijke parameters. In patiënten met FCH, draagt plasmahomocysteine niet disproportioneel bij aan het verhoogde risico op hart- en vaatziekten.
7. Insulineresistentie is een karakteristiek van FCH, onafhankelijk van overgewicht en afhankelijk van lipidenfenotype expressie.
8. De verhoogde concentratie van apoB en de prevalentie van sdLDL in FCH worden bepaald door genetische factoren en gemoduleerd door overgewicht en insulineresistentie.
9. De serumleptineconcentratie is verhoogd in patiënten met FCH evenredig met de mate van overgewicht en insulineresistentie. Verhoogde leptineconcentraties in FCH dragen bij tot het verhoogde risico op hart- en vaatziekten, onafhankelijk van geslacht, BMI en insulineresistentie.

Dankwoord

Dankwoord

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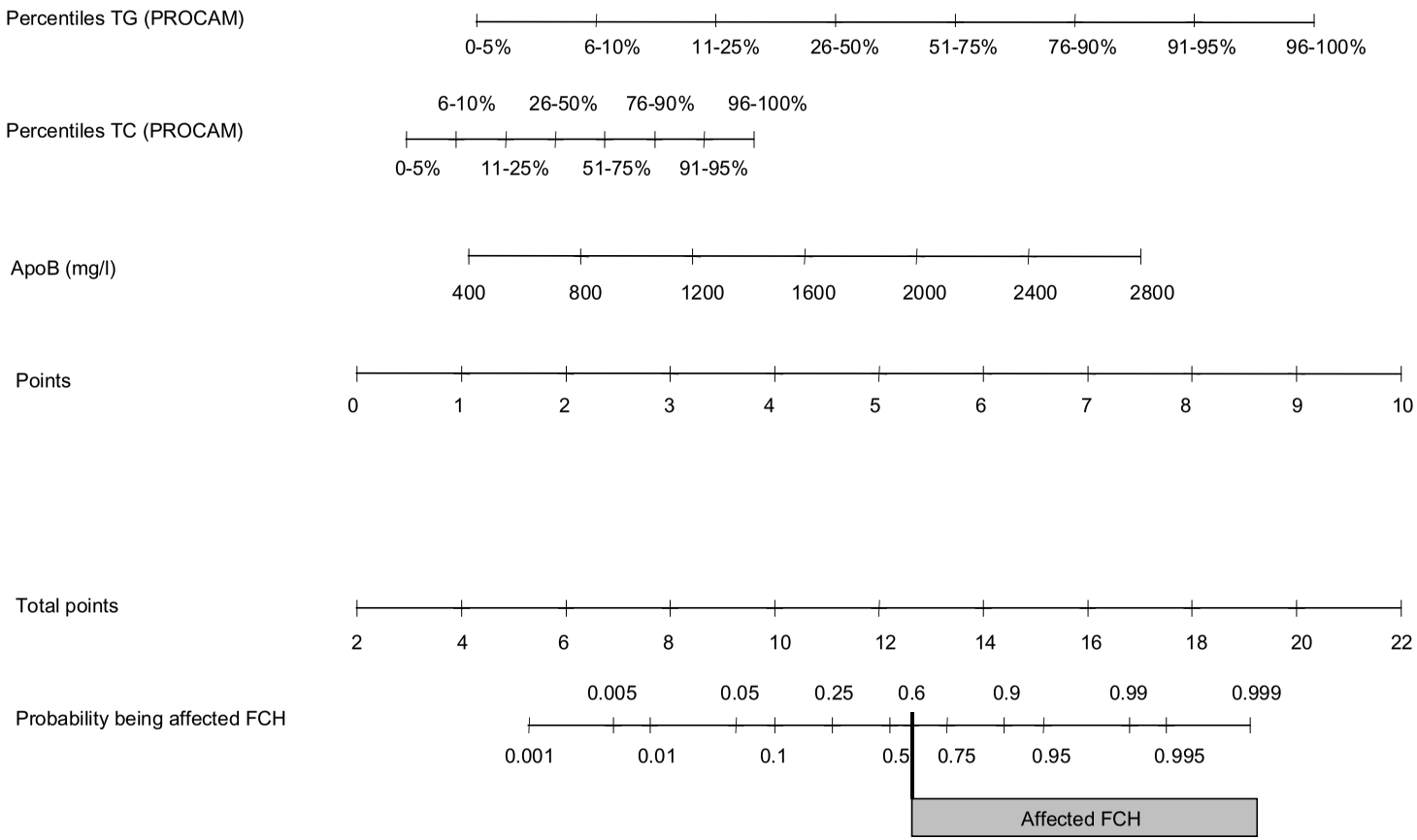
Curriculum vitae

Curriculum Vitae

Mario Veerkamp werd geboren op 8 november 1967 te Nijmegen. In 1986 behaalde zij het VWO diploma aan het Canisius College te Nijmegen. Het jaar daarop volgend heeft zij de propedeuse fysiotherapie behaald. Zij startte met de studie geneeskunde in 1987 aan de Katholieke Universiteit Nijmegen. In 1991 ontving zij haar doctoraal diploma geneeskunde en in 1994 haar arts diploma.
Van augustus 1994 tot juli 1995 is zij werkzaam geweest als arts-assistent geneeskunde niet in opleiding op de afdeling interne geneeskunde van het Universitair Medisch Centrum St. Radboud, waarna zij startte met de opleiding tot internist in het St. Maartensgasthuis te Venlo (opleider Dr. J.J.J. Mattousch). Vanaf april 1997 vervolgde zij haar opleiding in het Universitair Medisch Centrum St. Radboud (opleider prof. dr. J.W.M. van der Meer). Van januari 1999 tot november 2002 werkte zij deels fulltime, deels parttime aan het onderzoek waarvan de resultaten verwerkt zijn in dit proefschrift. In juli 2003 volgde de registratie tot internist. Vanaf januari 2004 is zij in opleiding tot reumatoloog in het Universitair Medisch Centrum St. Radboud (opleider prof. dr. P.L.C.M. van Riel). Zij is getrouwd met Guus Franssen en samen hebben zij twee kinderen, Lieke (2001), Bart (2003) en is er één op komst.



Nomogram to calculate probability of being affected by FCH using absolute apoB and TG-TC values adjusted for age and gender. In each of 3 variables, points are calculated by reading from point scale. Total point score is then translated into probability of affected FCH using 2 bottom scales.