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ARTICLE

Identification of *TNFRSF1B* as a novel modifier gene in familial combined hyperlipidemia

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Familial combined hyperlipidemia (FCHL) is the most commonly inherited hyperlipidemia in man, with a frequency of ±1% in the general population and ~10% in myocardial infarction survivors. A genomic scan in 18 Dutch FCHL families resulted in the identification of several loci with evidence for linkage. One of these regions, 1p36.2, contains *TNFRSF1B* which encodes one of the tumor necrosis factor receptors. An intron 4 polymorphic CA-repeat was used to confirm linkage to FCHL. Linear regression analysis using 79 independent sib pairs showed linkage with a quantitative FCHL discriminant function (*P* = 0.032), and, borderline, with apolipoprotein B levels (*P* = 0.064). Furthermore, in a case–control study, association was demonstrated since the overall CA-repeat genotype distribution was significantly different among 40 unrelated FCHL patients and 48 unrelated healthy spouse controls (*P* = 0.029). This difference was due to a significant increase in allele CA271 homozygotes in the FCHL patients (*P* = 0.019). Mutation analysis of exon 6 in 73 FCHL family members demonstrated the presence of a single nucleotide polymorphism with two alleles, coding for methionine (196M) and arginine (196R). Complete linkage disequilibrium between CA267, CA271 and CA273 and this polymorphism was detected. In 85 hyperlipidemic FCHL subjects, an association was demonstrated between soluble TNFRSF1B plasma concentrations and the CA271-196M haplotype. In conclusion, *TNFRSF1B* was found to be associated with susceptibility to FCHL. Our data suggest that an as yet unknown disease-associated mutation, linked to alleles 196M and CA271, plays a role in the pathophysiology of FCHL.

INTRODUCTION

Familial combined hyperlipidemia [FCHL; MIM 144250: Online Mendelian Inheritance in Man (OMIM), http://www.ncbi.nlm.nih.gov/Omim] is the most commonly inherited hyperlipidemia in man, with a frequency of 1–2% in the general population, and of ~10% in myocardial infarction survivors (1). Affected individuals characteristically show elevated triglycerides and/or cholesterol levels, elevated apolipoprotein B (APOB), insulin resistance, visceral obesity, hypertension and a family history of premature myocardial infarction. In general, this lipid profile is accompanied by an unfavorable decrease in plasma high density lipoprotein cholesterol concentration, and increased prevalence of atherogenic small dense low density lipoprotein (LDL) (2–4). Lipid profiles are usually normal in childhood, followed by manifestation of FCHL in the third decade of life, although some cases of FCHL in childhood and adolescence have been reported (5). Initially, the familial segregation of FCHL was described as consistent with autosomal dominant transmission (1); however, several studies suggest a more complex and heterogeneous mode of inheritance (4,6–9). The genetic pathogenesis of this disease, e.g. the number of genes involved and the magnitude of their effects, is still largely unclear.

Recently, the results from two genome-wide linkage screens were published (10,11). The first group (10) studied linkage of familial combined hyperlipidemia in Finland. To minimize genetic heterogeneity they chose 31 extended FCHL families from the relatively inbred Finnish population, applying strictly defined criteria for phenotype status. They performed linkage analysis with markers from 10 chromosomal regions that contained lipid metabolism candidate genes. One marker, *D1S104*, revealed a LOD score of 3.50, assuming a dominant

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mode of inheritance. Multipoint analysis combining information from *D1S104* and the neighboring marker *D1S1677* resulted in a LOD score of 5.93. Physical positioning of known genes in the area (*APOA2* and three selectin genes) outside the linked region suggested the existence of a novel locus for FCHL on 1q21–q23. The conclusions of this study (10) were bolstered by the work of a second group, who identified a hyperlipidemic locus in an orthologous region of the mouse genome (12).

In a recent report from our laboratories (11), a 10 cM genome-wide scan for novel FCHL-susceptibility genes was described in a panel of 18 Dutch Caucasian FCHL families without type 2 diabetes. The results from this study suggest an oligogenic pathogenesis. Besides a locus on chromosome 11 (LOD score 2.6), three other loci, on chromosomes 2p, 16q and 19q, were identified that showed LOD scores between 1.3 and 2.6 with FCHL. In second-stage analyses, these suggestive loci were further examined in the entire set of pedigrees, comprising of 35 families (782 individuals). Only one locus, near marker *D11S1324*, continued to show evidence for linkage to FCHL. It is of interest to note that the 1q21–23 locus, identified as a hyperlipidemic locus in the Finnish population, showed a LOD score of 1.0 with plasma triglyceride levels in our 18 Dutch FCHL families. In the first stage of our genome scan, an additional locus on chromosome 1p36.2 (near polymorphic marker *D1S1597*) was identified as one of the initial loci with a LOD score of ≥1.0 (11). *TNFRSF1B*, encoding a high-affinity receptor for tumor necrosis factor (TNF) α, is located in this region. This gene was identified as an important candidate gene for FCHL, because of the role of TNF and the TNF receptors (TNFRs) in lipoprotein metabolism and insulin resistance.

The relation between TNF and insulin resistance as well as lipoprotein abnormalities has been described extensively in the literature. Activation of the TNF/TNFR(s) pathway is linked to induction of insulin resistance in a paracrine fashion (13,14). Insulin resistance, or reduced biological activity of insulin, is caused by the ability of TNF to induce serine phosphorylation of insulin receptor substrate 1, resulting in reduced binding of this protein to the juxtamembrane domain of the insulin receptor and reduced insulin sensitivity. Interestingly, FCHL has been associated with insulin resistance (15). An important characteristic of FCHL is the overproduction of hepatic APOB containing very low density lipoproteins (VLDLs). Activation of the TNF–TNFR axis causes increased secretion of VLDLs from the liver. This may result partly from a direct TNF effect on the liver (16,17) and, more importantly, from increased lipolysis in adipocytes, resulting in a greater free fatty acid flux to the liver, thereby promoting lipoprotein secretion secondary to this increased lipid supply (18). Other direct effects on lipoprotein metabolism include induction of *de novo* fatty acid synthesis in adipocytes (18) and hepatocytes (16,17), reduction of lipoprotein lipase expression on the endothelium (19,20), induction of superoxide dismutase 2 activity (21) and reduction of lecithin-cholesterol acyltransferase activity (22). It is of particular interest that these specific genes were shown by our laboratories to be linked to FCHL in the same study population (23) as that in the present study.

TNF signals through at least two known cell surface TNFRs (24,25), tumor necrosis factor receptor superfamily, member 1A [TNFRSF1A (p55)] and tumor necrosis factor receptor superfamily, member 1B [TNFRSF1B (p75)] that are present on virtually all cells of higher mammals. It appears that TNFRSF1A can signal for virtually all known activities of TNF, including apoptosis, differentiation and proliferation, whereas TNFRSF1B is linked to metabolic actions (26). Both receptors for TNF also exist in soluble forms (sTNFRs), apparently derived by proteolytic cleavage from the cell surface forms (27,28). The majority of the functional domains of the TNFRSF1B receptor is encoded by separate exons in the gene (29). Exon 6 encodes a small portion of the transmembrane region and contains the position of the proteolytic cleavage site that produces the soluble form of TNFRSF1B. Receptor shedding provides a mechanism for down-regulating a cell surface receptor and a means of releasing biologically active soluble receptors, which may act as receptor antagonists by capturing free circulating ligand (30).

In this study we report that DNA variability in *TNFRSF1B* contributes to FCHL. An intron 4 polymorphic CA-repeat was used in non-parametric sib pair linkage analyses to verify linkage of the 1p36.2 locus to the FCHL phenotype. In a case–control study, this marker also showed association with susceptibility to FCHL. Finally, to test whether *TNFRSF1B* exon 6 contains a mutation that is associated with susceptibility to FCHL, possibly by affecting receptor shedding, this particular exon was used in mutation detection studies.

RESULTS

Identification of *TNFRSF1B* as an FCHL modifier gene by linkage analysis

To establish the possible role of the *TNFRSF1B* gene in the pathology of FCHL, a polymorphic intron 4 CA-repeat marker was genotyped in 278 Dutch Caucasian FCHL family members (study population 1) and used for additional linkage analyses. We tested several quantitative traits associated with FCHL, including plasma APOB levels, triglycerides, cholesterol, as well as FCHL as a quantitative trait (discriminant function 'FCHL-quant') in non-parametric sib pair linkage analyses using the SIBPAL subprogram of the SAGE package (see Materials and Methods). Table 1 summarizes the results from these analyses. In 79 sib pairs, the intron 4 CA-repeat marker exhibited linkage with FCHL as a quantitative trait (FCHL-quant formula, $P = 0.032$). The FCHL-quant formula includes varia-

Table 1. Two-point non-parametric sib pair linkage results with the *TNFRSF1B* intron 4 CA-repeat marker and FCHL quantitative traits

FCHL quantitative trait	*TNFRSF1B* CA-repeat	
	P-value	Edf
FCHL-quant[a]	0.032[b]	79
APOB	0.064	79
APOC3	NS	79
Cholesterol	NS	79

Edf, effective degrees of freedom; NS, not significant.
[a]$x = -3.7949(\text{APOB}) - 0.1357(\text{APOC3}) - 0.2832$ (cholesterol).
[b]Significant *P*-value.

Table 2. Biochemical and clinical characteristics of the case–control study population (study population 2)

Traits	Probands	Spouses
n (individuals)	40	48
Male/female	27/13	21/27
Age (years)	49.7 ± 10.4	47.6 ± 13.9
BMI	27.2 ± 3.3	24.4 ± 3.1
Cholesterol (mmol/l)	8.7 ± 4.8	5.7 ± 0.9
Triglycerides (mmol/l)	8.3 ± 14.8	1.6 ± 1.1
APOB (g/l)	1.3 ± 0.4	1.0 ± 0.3
APOC3 (mg/100ml)	16.5 ± 16.2	8.8 ± 3.3
LDL-cholesterol (mmol/l)	4.9 ± 1.8	3.7 ± 1.0
LDL-size ($\times 10^{-10}$ m)	261.5 ± 10.8	273.9 ± 7.5

bility in plasma APOB, apolipoprotein CIII (APOC3) and cholesterol levels (see Materials and Methods), representing key abnormalities in the FCHL phenotype. Furthermore, this CA-repeat marker showed borderline linkage to plasma APOB levels (P = 0.064). No significant results were obtained for plasma APOC3 and cholesterol concentrations. These results show that all three traits (APOB, APOC3 and cholesterol) are necessary for linkage, and that APOB is the primary trait. These findings provide further support for linkage of FCHL to this chromosomal region (11).

Case–control study with the intron 4 CA-repeat polymorphic marker in *TNFRSF1B*

Based on the positive linkage results, a case–control study was performed in which distributions of CA-alleles in a group of spouses and unrelated FCHL probands were tested for differences (study population 2). Clinical characteristics of this case–control panel are described in Table 2. The reported frequencies of the alleles in the general population are as follows: CA267 (267 nucleotides), 19.1%; CA269 (269 nucleotides), 2.5%; CA271 (271 nucleotides), 54.2%; CA273 (273 nucleotides), 24.5%; and CA18 (277 nucleotides), 0.8% (31). Two hitherto unreported alleles [CA261 (261 nucleotides), 0.5% and CA275 (275 nucleotides), 1.6%] were identified in this panel. We did not observe the rare CA18 (277 nucleotides), 0.8% allele. The three most common CA-repeat alleles, e.g. CA267, CA271 and CA273 were used in further statistical analysis (Table 3). This case–control study demonstrated that the overall genotype distribution was significantly different (χ^2 = 12.50, df = 5, P = 0.029) (Table 3). Further analyses demonstrated that FCHL probands showed significantly more individuals homozygous for allele CA271 (14 of 40, 35%) than expected on basis of the frequency in the spouses (8 of 48, 16.7%) [adjusted P = 0.019 ($P = 0.006 \times 3$ for testing alleles CA267, CA271 and CA273)] (Table 3). This finding suggested that the presence of both alleles may be needed to confer susceptibility to FCHL. No significant differences in distribution were detected for allele CA267 and CA273 homozygotes. With all three alleles, genotypic distributions were consistent with the Hardy–Weinberg equilibrium. In addition to the positive linkage results, the present data demonstrate that genetic variability within the *TNFRSF1B* gene is associated with the FCHL phenotype.

Table 3. Association of the *TNFRSF1B* polymorphic CA-repeat with susceptibility to FCHL in the Dutch population

Alleles	FCHL, observed (n = 40)		Controls, observed (n = 48)		FCHL, expected (n = 40)	χ^2	df	P-value
	n	%	*n*	%	*n*			
Comparison of genotypes among FCHL patients and controls								
CA267/CA267	1	2.5	4	8.3	3.33			
CA267/CA271	9	22.5	15	31.3	12.5			
CA267/CA273	6	15	5	10.4	4.17			
CA271/CA271	14	35	8	16.7	6.67			
CA271/CA273	9	22.5	15	31.3	12.5			
CA273/CA273	1	2.5	1	2.1	0.83			
Total	40	100	48	100	40	12.5	5	0.029
Comparison of allele CA271 among FCHL patients and controls								
CA271/CA271	14	35	8	16.7	6.68	10.04	2	0.019
CA271/X	18	45	30	62.5	25.0			
X/X	8	20	10	20.8	8.32			
Total	40	100	48	100	40			

[a]*P*-value after Bonferroni correction for multiple comparison. *P*-values were calculated and corrected only for those alleles that were observed >10 times in the total (patient and control) population (e.g. CA267, CA271 and CA273). Based on the observed frequencies in the control group, expected numbers were calculated for FCHL cases (FCHL expected). Differences in distributions were tested between the expected numbers and the numbers actually observed in the FCHL subgroup by χ^2 goodness-of-fit tests.

Mutation analysis of *TNFRSF1B* exon 6

Mutation detection experiments using WAVE denaturing high-performance liquid chromatography (DHPLC) technology (see Materials and Methods) led to the identification of a diallelic polymorphism at amino acid position 196. Direct sequencing revealed a single base substitution at codon 196 (ATG→AGG), which resulted in a non-conservative amino acid substitution [methionine (M)→arginine (R)] within the fourth extracellular domain of TNFRSF1B.

Combining genotype data from the intron 4 CA-repeat with this polymorphism indicated that there is complete linkage disequilibrium between allele 196R and allele CA267. Furthermore, it became apparent that allele 196M is in complete linkage disequilibrium with alleles CA271 and CA273. In the present study population, all individuals homozygous for allele CA267 were also homozygous for allele 196R, whereas allele individuals heterozygous for this allele were heterozygous for position 196 (196M/196R).

Effect of the CA271-196M haplotype on soluble TNFRSF1B plasma concentrations

We recently reported that age-corrected plasma concentrations of soluble tumor necrosis factor receptor superfamily, member 1B (sTNFRSF1B) were significantly lower in hyperlipidemic FCHL relatives than in normolipidemic relatives and spouses (32). To investigate whether this effect could be caused by the CA271-196M haplotype, we studied the effect of this haplotype on sTNFRSF1B plasma concentrations in greater detail using study population 1. These analyses showed that hyperlipidemic individuals homozygous for this haplotype had lower sTNFRSF1B plasma concentrations (1.53 ± 0.31, $n = 29$) than individuals heterozygous for this haplotype (1.64 ± 0.44, $n = 35$) or lacking it (1.74 ± 0.33, $n = 21$). The difference in plasma concentrations between the homozygotes and the individuals lacking this haplotype was significant ($P = 0.023$). No significant effects were detected in the spouse group.

DISCUSSION

This study reports the identification of the *TNFRSF1B* gene as a novel modifier gene in Dutch FCHL families, based on a combination of genetic, biological and statistical evidence. The genetic evidence resulted from a genome-wide scan in 18 FCHL families, which pointed to, amongst other loci, a chromosome 1 region (1p36.2) with a LOD score of ~1.0 (11). It is of interest to note that this chromosomal region was also identified in a genome-wide scan for genes contributing to human systemic lupus erythematosus (SLE), with a similar LOD score of 1.0 (33). In a study by Komata *et al*. (34), the *TNFRSF1B* gene was shown to be associated with susceptibility to SLE in Japanese patients, indicating that scan results with a LOD score of 1 can be useful for the identification of positional candidates. A recent review addressed the fact that linkage methods may have limited power to detect genes with minor impact on the complex disease phenotype, whereas association methods have more power (35). Given the data implicating the TNF–TNFR axis in insulin resistance and lipid abnormalities, we studied the role of *TNFRSF1B* in FCHL. Additional genetic evidence resulted from non-parametric sib pair linkage analyses, in which an intragenic polymorphic CA-repeat marker was analyzed. These analyses demonstrated that quantitative variation in APOB levels showed suggestive linkage with this *TNFRSF1B* CA-repeat, which became significant when a quantitative discriminant function (equation FCHL-quant), in which variation in plasma APOB levels were combined with quantitative variability in APOC3 and cholesterol plasma levels, was applied.

Statistical evidence for association of *TNFRSF1B* with FCHL was provided by a case–control association study. It should be noted that the Dutch population is relatively homogeneous with respect to genetic background, allowing the case–control approach employed in this study. Furthermore, only Dutch Caucasian families were used for all analyses performed. Case–control analysis showed that the distribution of relevant genotypes (CA267, CA271 and CA273) was markedly different among FCHL probands and spouse controls. In the association study, this difference was shown to be caused by a significant increase in the frequency of individuals homozygous for allele CA271 in FCHL patients, compared with the frequency expected on basis of the control group [14 of 40 FCHL patients (35%) were homozygous for CA-allele CA271, which was significantly less frequent in 48 spouses (8 of 48, 16.7%)]. Since impaired TNFRSF1B receptor shedding of the extracellular domain can contribute to the expression of FCHL (32), exon 6 was screened for mutations, as this is the exon containing the position for the proteolytic cleavage site. Mutation analysis demonstrated the presence of a single nucleotide polymorphism at position 196 (M196R). This amino acid substitution does not seem to result in drastic conformational changes of the protein or expression of *TNFRSF1B* (34). The present data showed 100% linkage disequilibrium between alleles 196R and CA267 as well as the 196M allele and alleles CA271 and CA273. Finally, a significant association was shown between sTNFRSF1B plasma concentrations and the CA271-196M haplotype. In view of the fact that the methionine at position 196 in the protein constitutes the wild-type situation and yet this allele, together with allele CA271, was found to be associated with sTNFRSF1B plasma concentrations, it was concluded that this polymorphism by itself is not involved in FCHL pathogenesis, but is merely acting as a marker for another disease-associated mutation. The presence of two alleles CA271 was shown to be obligatory for susceptibility to FCHL.

The role of the TNF–TNFR axis in insulin resistance and lipid abnormalities has been extensively described. A study in which mice were generated with targeted null mutations in the genes encoding both receptors for TNF ($p55^{-/-}$ and $p75^{-/-}$) is of particular interest (36). This study elegantly showed that the lack of either TNFR species did not significantly affect the overall adiposity of these mice. Furthermore, no significant effects were seen on the steady-state lipid profile or β-adrenergic-stimulated lipolysis. On the other hand, the absence of these receptors resulted in significant improvement in insulin sensitivity, which indicates primarily a role for the TNF–TNFRSF1B pathway in the development of insulin resistance, a characteristic feature in FCHL subjects. These data suggest that the presence of cell surface TNFRSF1B receptors modulates insulin sensitivity in human tissues, thereby providing a plausible explanation for the TNFRSF1B effect in FCHL, which is characterized by insulin resistance (15).

It was recently shown by our group that age-corrected plasma concentrations of soluble TNFRSF1B receptor are significantly lower in hyperlipidemic FCHL relatives than in normolipidemic relatives and spouses (32). Moreover, conditional logistic regression analysis demonstrated that low plasma soluble TNFRSF1B levels increased the risk of expressing FCHL. TNFRs exist either as membrane-bound or as soluble forms. Membrane-bound TNFRs become inactive on shedding of their TNF binding domains, either as sTNFRSF1A or sTNFRSF1B. Shedding of TNFRs is an enzymatic process that is regulated by metalloproteases. Pellegrini *et al*. (37) showed that decreased shedding of TNFRSF1B is more related to the development of disease than elevated secretion of TNF, suggesting that the response of cells to TNF can be regulated by the number of remaining functional receptors on the cell surface; this number will be greater if shedding is reduced. Physiological consequences of reduced shedding would be relatively low plasma concentrations of sTNFRSF1B, as seen in our FCHL relatives, with on the other hand relatively high levels of unshed, functionally intact TNFRSF1B remaining on the cell surface. This eventually leads to greater responsiveness to TNF at physiological concentrations, resulting in increased sensitivity of the TNF–TNFRSF1B pathway to TNF, providing FCHL patients with the ability to be hyper-responders to circulating TNF. Hyper-responsiveness to TNF can, at least in part, explain why FCHL patients are extremely sensitive to increases in their fat mass, which contributes to TNF production, and to develop insulin resistance even when mildly obese. Implication of the TNF–TNFRSF1B pathway in the expression of FCHL may therefore have diagnostic as well as therapeutic implications. However, besides decreased receptor shedding, other mechanisms potentially result in low plasma concentration of sTNFRSF1B, including decreased expression of the *TNFRSF1B* gene, increased excretion of sTNFRSF1B and low plasma TNF concentrations. Further analyses will have to be performed in order to elucidate the exact molecular mechanism(s) that causes the low soluble TNFRSF1B plasma concentrations in hyperlipidemic FCHL subjects.

In conclusion, we showed that the *TNFRSF1B* gene is associated with susceptibility to FCHL. Furthermore, our present findings, in combination with earlier obtained results (32), suggest that an as yet unknown disease-associated mutation which is in complete linkage disequilibrium with the 196M allele in exon 6 and with the CA271 allele in intron 4, leads to lower TNFRSF1B plasma concentrations in FCHL. This could be caused by reduced shedding of the TNFRSF1B receptor, rendering FCHL patients hyper-responsive to physiological levels of TNF, although other mechanisms could also be implicated. The data presented in this paper stimulate further research to confirm our results in other insulin-resistant populations such as type 2 diabetes and obesity and to elucidate the underlying molecular and endocrine mechanisms in greater detail.

MATERIALS AND METHODS

Family ascertainment

Dutch Caucasian FCHL families were ascertained as described previously (11,38,39). All probands met three criteria: (i) a primary combined hyperlipidemia with varying phenotypic expression; plasma cholesterol >250 mg/dl (>6.5 mmol/l) and/or triglycerides >200 mg/dl (>2.3 mmol/l), and APOB concentrations >142 mg/dl; (ii) at least one first degree relative with a different hyperlipidemic phenotype; and (iii) a positive family history of premature coronary heart disease defined as myocardial infarction or cardiovascular disease before 60 years of age. Exclusion criteria included diabetes, obesity, familial hypercholesterolemia (tendon xanthomas) and type III hyperlipidemia (*APOE2/E2* genotype). Relatives and spouses of probands were recruited without bias regarding affection status. Over 95% of all living relatives of probands were studied. All subjects gave informed consent to participate in the study protocol that had been approved by the Human Institutional Review Board.

Study populations

TNFRSF1B linkage study population (study population 1). To increase statistical power, an intragenic (intron 4) polymorphic CA-repeat was used for genotyping 278 individuals, including the 240 subjects that were used for the 10 cM genome scan (11) as well as additional probands (n = 22) and extra spouses (n = 16). In total, 125 hyperlipidemic family members (40 probands and 85 hyperlipidemic relatives) were assigned the FCHL phenotype. Family members who did not meet these criteria were classified as normolipidemic (n = 105). A spouse group (n = 48) provided unrelated controls, matched for environment, nutrition and age.

Case–control study population (study population 2). Since case–control association studies have generally more power to detect genes with minor impact on a complex disease phenotype than linkage methods, a case–control association was performed using the *TNFRSF1B* intron 4 polymorphic CA-repeat. In all, 40 unrelated Dutch Caucasian FCHL probands and 48 healthy spouse controls were used.

TNFRSF1B exon 6 mutation detection population (study population 3). To test whether *TNFRSF1B* exon 6 contains a mutation that is associated with susceptibility to FCHL, possibly by affecting receptor shedding, this exon was used for mutation detection analysis. Altogether, 73 individuals [1 proband, 59 relatives (27 hyperlipidemics and 32 normolipidemics) and 13 spouses] were used.

Biochemical analysis

In EDTA-containing (1 mg/ml) tubes, venous blood was collected after an overnight fast (12–14 h). Plasma samples were prepared for analysis by immediate centrifugation. Plasma triglyceride and cholesterol were measured in triploid using a commercial colorimetric assay (GPO-PAP and Monotest cholesterol kit; Boehringer Mannheim, Almere, The Netherlands). Plasma leptin was determined by a commercial radioimmunoassay (Linco Research, St Louis, MO). Plasma concentration of sTNFRSF1B was determined using a sandwich ELISA procedure exactly as published (40). Insulin was measured by a competitive radioimmunoassay, using the polyclonal insulin antibody Caris 46, insulin tracer labeled with 125I (IM.166; Radiochemical Centre, Amersham, UK), and

insulin standards (World Health Organization: first international reference preparation 66/304). Several quantitative variables were used to construct discriminant functions for FCHL.

TNFRSF1B intron 4 CA-repeat genotyping

A *TNFRSF1B* CA-repeat near the 3′ end of intron 4 (31; and GDB:132740, Genome Database, http://www.gdb.org), was used for genotyping the members of study population 1. Genotyping was performed using a fluorescently labeled primer set [upper primer (5′-FAM labeled): 5′-GTG ATC TGC AAG ATG AAC TCA C-3′; lower primer: 5′-ACA CCA CGT CTG ATG TTT CA-3′]. In fluorescent genotyping, all PCR amplifications were carried out in a final volume of 25 µl containing 10 mM Tris–HCl pH 8.4, 2.25 mM $MgCl_2$, 50 mM KCl, 1 mM dNTPs, 10 pmol of each primer, 1 U of *Taq* polymerase (Gibco BRL Life Technologies, Breda, The Netherlands), and 200 ng of template DNA using a GeneAmp PCR System PE 9700 (PE Biosystems, Nieuwerkerk a/d IJssel, The Netherlands). The amplification conditions consisted of initial denaturation at 94°C for 5 min, followed by 32 cycles of denaturation at 94°C for 30 s, annealing at 62°C for 30 s and extension at 72°C for 30 s. The PCR profile ended with a final extension at 72°C for 7 min.

Amplified products were size-resolved by capillary electrophoresis using a commercially available polyacrylamide matrix on an ABI-310 PRISM Genetic Analyzer (PE Biosystems). Alleles were designated according to the size of the PCR fragment (Genescan Analysis 2.0). The following alleles were identified: CA261 (261 nucleotides), CA267 (267 nucleotides), CA269 (269 nucleotides), CA271 (271 nucleotides), CA273 (273 nucleotides) and CA275 (275 nucleotides); two alleles were hitherto unreported, i.e. CA261 and CA275.

Statistical methods

Discriminant function for FCHL. To convert FCHL into a quantitative trait, to be used in sib pair linkage analysis, a discriminant function was constructed. This involved a sample of affecteds and unaffecteds and measurement of FCHL-related quantitative traits. This function is composed of a linear combination of plasma APOB (g/l), APOC3 (mg/100 ml), and cholesterol (mmol/l) concentrations, representing key abnormalities of the FCHL phenotype (11,35,37): FCHL-quant. $x = -3.7949(\text{APOB}) - 0.1357(\text{APOC3}) - 0.2832(\text{cholesterol})$. The percentage of correct classifications of the affecteds and unaffecteds into their correct clinical status was 85% [the means are –1.24 (for affected) and 0.77 (for normal), and the cutpoint is –0.235].

Analysis of sib pairs. Linkage analysis on sib pairs from study population 1 was performed using the Haseman–Elston algorithm by the SIBPAL subprogram of the SAGE package (41). Data from all sib pairs were combined into a linear regression analysis that regresses the squared trait differences (e.g. quantitative disease status) between sib pairs against the estimated proportion of marker alleles that they share identical by descent. A significantly negative regression line slope indicates that those who are phenotypically alike tend to share more alleles and those who are discordant tend to exhibit a lower degree of allele sharing, thus providing evidence for linkage. In the absence of linkage, the slope of the regression line is not expected to be significantly different from zero.

Case–control association study. SPSS 9.0 for Windows (SPSS, Chicago, IL) was used in the statistical analyses. Based on the observed frequencies in the control group, expected numbers were calculated for FCHL cases (FCHL expected). Differences in distributions were tested between these expected numbers and the actually observed numbers in the FCHL subgroup by χ^2 goodness-of-fit tests. Statistical significance was considered at the $P < 0.05$ level. *P*-values were corrected for multiple testing by the Bonferroni correction. Data are expressed as means ± SD. The genotype distributions of the CA-alleles were tested for deviation from the Hardy–Weinberg equilibrium by χ^2 goodness-of-fit tests in the spouse control group.

Effect of TNFRSF1B variability on sTNFRSF1B plasma concentrations

Study population 1 was used to study the influence of variability in the *TNFRSF1B* gene on sTNFRSF1B plasma concentrations by independent sample Student's *t*-tests. Statistical significance was considered at the $P < 0.05$ level. Mann–Whitney tests were performed to correct for small group size ($n < 25$). SPSS 9.0 for Windows (SPSS) was used.

DHPLC analysis

DHPLC-based mutation detection analysis was performed using the WAVE DNA Fragment Analysis System (Transgenomic, Omaha, NB). The DHPLC parameters used for the exon 6 *TNFRSF1B* fragment were calculated using a predictive algorithm (42). All runs were performed at 63°C. Exon 6 was initially selected for mutation detection because of its reported important role in *TNFRSF1B* shedding (30; and GenBank: http://www.ncbi.nlm.nih.gov/Genbank/index.html). Exon 6 was amplified using primer set 5′-CGT GAA TGA GCC CAG CCA CC-3′ (upper primer) and 5′-GAA AGA CAG GCA GAC AGA AGG AGT GA-3′ (lower primer). All PCR amplifications were carried out in a final volume of 25 µl containing 10 mM Tris–HCl pH 8.4, 0.75 mM $MgCl_2$, 50 mM KCl, 1 mM dNTPs, 10 pmol of each primer, 1 U of *Taq* polymerase (Gibco BRL), and 100 ng of template DNA. The amplification conditions consisted of initial denaturation at 94°C for 5 min, followed by 35 cycles of denaturation at 94°C for 30 s, annealing at 60.5°C for 30 s and extension at 72°C for 45 s. The PCR profile ended with a final extension at 72°C for 7 min. PCR reactions were carried out using a GeneAmp PCR system 9700 (PE Biosystems). The nucleotide sequences of the samples showing aberrant WAVE chromatograms were determined by direct sequencing. Fluorescence-based automated sequencing of PCR products was performed on an ABI-310 PRISM Genetic Analyzer (PE Biosystems) using dye-terminator methodology, according to the manufacturer's instructions. It should be noted that individuals homozygous for a DNA variation (mutation/polymorphism) are indistinguishable from individuals with wild-type DNA, both showing normal chromatograms in WAVE analysis. To discriminate between these two populations, DNA from subjects with normal WAVE chromatograms was mixed with wild-type DNA in second-stage analyses.

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