patient-oriented research

The involvement of upstream stimulatory factor 1 in Dutch patients with familial combined hyperlipidemia[S]

Gerly M. van der Vleuten,* Aaron Isaacs,† Anneke Hijmans,* Cornelia M. van Duijn,† Anton F. H. Stalenhoef,* and Jacqueline de Graaf[1,*]

Department of Medicine,* Division of General Internal Medicine, Radboud University Nijmegen Medical Center, Nijmegen, The Netherlands; and Department of Epidemiology and Biostatistics,† Genetic Epidemiology Unit, Erasmus Medical Center, Rotterdam, The Netherlands

Abstract Recently, the upstream stimulatory factor 1 gene (*USF1*) was proposed as a candidate gene for familial combined hyperlipidemia (FCH). In this study, we examined the previously identified risk haplotype of *USF1* with respect to FCH and its related phenotypes in 36 Dutch FCH families. The diagnosis of FCH was based on both the traditional diagnostic criteria and a nomogram. The two polymorphisms, USF1s1 and USF1s2, were in complete linkage disequilibrium. No association was found for the individual single nucleotide polymorphisms (SNPs) with FCH defined by the nomogram (USF1s1, $P = 0.53$; USF1s2, $P = 0.53$), whereas suggestive associations were found when using the traditional diagnostic criteria for FCH (USF1s1, $P = 0.08$; USF1s2, $P = 0.07$). *USF1* was associated with total cholesterol (USF1s1, $P = 0.05$; USF1s2, $P = 0.04$) and apolipoprotein B (USF1s1, $P = 0.06$; USF1s2, $P = 0.04$). Small dense LDL showed a suggestive association (USF1s1, $P = 0.10$; USF1s2, $P = 0.09$). The results from the haplotype analyses supported the results obtained for the individual SNPs. In conclusion, the previously identified risk haplotype of *USF1* showed a suggestive association with FCH and contributed to the related lipid traits in our Dutch FCH families.— van der Vleuten, G. M., A. Isaacs, A. Hijmans, C. M. van Duijn, A. F. H. Stalenhoef, and J. de Graaf. **The involvement of upstream stimulatory factor 1 in Dutch patients with familial combined hyperlipidemia. *J. Lipid Res.* 2007.** 48: **193–200.**

Supplementary key words single nucleotide polymorphisms • mRNA expression • total cholesterol • apolipoprotein B • small dense low density lipoprotein

Familial combined hyperlipidemia (FCH; Online Mendelian Inheritance in Man 144250) is the most common genetic lipid disorder of unknown cause in humans, affecting up to 5% of the general population (1). Major characteristics of FCH include increased levels of apolipoprotein B (apoB), triglycerides (TGs), and/or plasma total cholesterol (TC). Other FCH phenotypes are decreased levels of high density lipoprotein cholesterol and the presence of small, dense low density lipoprotein (sdLDL). In addition, patients with FCH have a 2-fold increased risk of cardiovascular disease (CVD) and are often obese and insulin-resistant (2).

During the past few years, several groups have performed linkage analyses in an attempt to determine the genetic defects causing FCH (3–8). By doing this, a locus on chromosome 1q21-23 was identified (4, 7–9). The first candidate gene proposed in this region was the thioredoxin-interacting protein gene (*TXNIP*) (10). It was demonstrated, however, that *TXNIP* was not a major contributor to the FCH phenotype (11–13). More recently, a second candidate gene, the upstream stimulatory factor 1 gene (*USF1*), was suggested as the prime candidate gene for FCH in this linkage region in 60 Finnish FCH families (12). Twenty-three single nucleotide polymorphisms (SNPs) were reported in the *USF1* gene. Two of these SNPs, USF1s1 (3′ untranslated region; rs3737787) and USF1s2 (intron 7; rs2073658), individually and combined into a haplotype, showed linkage and association with FCH and multiple phenotypes of FCH, including TG, apoB, TC, and sdLDL, implying that *USF1* contributes to the complex pathophysiology of FCH in these Finnish FCH families (12). The association of *USF1* with FCH, however, was strongest in males with increased levels of TGs (12).

Huertas-Vazquez et al. (5) reported that *USF1* was associated with FCH and increased TG levels in 24 Mexican

Abbreviations: apoB, apolipoprotein B; CHD, coronary heart disease; CVD, cardiovascular disease; FBAT, family-based association test; FCH, familial combined hyperlipidemia; HBAT, haplotype-based association test; HOMA, homeostasis model assessment; HSL, hormone-sensitive lipase; LDL-c, low density lipoprotein cholesterol; lod, log of the odds; PBAT, pedigree-based association test; PBMC, peripheral blood mononuclear cell; sdLDL, small dense low density lipoprotein; SNP, single nucleotide polymorphism; TC, total cholesterol; TG, triglyceride; TXNIP, thioredoxin-interacting protein; USF1, upstream stimulatory factor 1.

[1] To whom correspondence should be addressed.
e-mail: j.degraaf@aig.umcn.nl

[S] The online version of this article (available at http://www.jlr.org) contains two additional tables.

Manuscript received 26 April 2006 and in revised form 30 August 2006 and in re-revised form 17 October 2006.

Published, JLR Papers in Press, October 25, 2006.
DOI 10.1194/jlr.M600184-JLR200

FCH families, whereas other phenotypes were not investigated. No sex-specific effect of *USF1* was found in these families. Coon et al. (14) investigated the association of *USF1* with FCH in Utah pedigrees ascertained for early death attributable to coronary heart disease (CHD), early stroke, or early-onset hypertension. They reported suggestive associations for FCH, TG, and low density lipoprotein cholesterol (LDL-c) levels in the pedigrees ascertained for early stroke and early onset of hypertension. However, in the families ascertained for early death attributable to CHD, in which 60% of the FCH patients were present, no association of *USF1* with FCH was found (14).

The USF1 protein regulates the transcriptional activation of a variety of genes involved in glucose and lipid metabolism and in the development of atherosclerosis (15, 16). USF1 plays a pivotal role in adipose tissue, where it influences de novo lipogenesis by mediating the glucose-regulated expression of hormone-sensitive lipase (HSL), a key enzyme in the regulation of lipid storage in adipose tissue (17). An interaction between SNPs in the *HSL* and *USF1* genes in postprandial TG levels has been reported previously (18). Despite this, HSL activity in adipose tissue was unchanged in Finnish patients with FCH (19). USF1 also influences the transcription of fatty acid synthase (FAS), which is involved in the synthesis of fatty acids (15, 20). FAS might play a role in FCH, as patients with FCH have decreased circulating levels of FAS ligand (21). USF1 also plays a role in the transcription of several apolipoproteins (APOCIII, APOAII, and APOE) (16, 22), glucokinase (23), hepatic lipase (24), angiotensinogen (25), and ABCA1 (26). Genetic variations in *APOCIII*, *APOAII*, and hepatic lipase have been associated with FCH (27–30); however, the role of glucokinase, angiotensinogen, and *ABCA1* in FCH has not been explored yet.

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

METHODS

Study population

The FCH population was ascertained in 1994 and followed up and expanded in 1999. The families were ascertained through probands recruited from the outpatient lipid clinic of the Radboud University Nijmegen Medical Center. In 1994 and 1999, we ascertained families in which the proband exhibited a combined hyperlipidemia, with both plasma TC and TG levels above the age- and gender-related 90th percentile (31), during several periods in which they were not treated with lipid-lowering drugs and despite dietary advice. Additionally, a first-degree relative had increased levels of TC and/or TG above the 90th percentile, and the proband, or a first-degree relative, suffered from premature (before the age of 60 years) CVD. Families were excluded when probands were diagnosed with underlying diseases causing hyperlipidemia (i.e., diabetes mellitus types 1 and 2, hypothyroidism, and hepatic or renal impairment), a first-degree relative had tendon xanthomata, or probands were homozygous for the *APOE2* allele.

In this study, we used the data of all participating subjects in 1999, including the spouses. The total population consists of 36 families from multiple (two to four) generations and comprises 611 subjects with known genealogic and phenotypic data. The diagnosis of FCH was based on *1*) the traditional diagnostic criteria, including TC and/or TG levels above the 90th percentile (32), and *2*) the nomogram, as recently described by our group (33). Plasma TG and TC levels, adjusted for age and gender, and absolute apoB levels were applied to the nomogram to calculate the probability of being affected with FCH. When this probability is >60%, the diagnostic phenotype is present in at least one first-degree relative, and premature CVD (before the age of 60 years) is present in at least one individual in the family, the individual is defined as affected by FCH. In 26 of the 36 FCH families, ascertained through the traditional criteria (32), the proband also fulfilled the criterion of the nomogram for the FCH diagnosis; for the other 10 probands, the nomogram could not be applied because of missing apoB data. The normolipidemic relatives (n = 390), unaffected spouses of both the FCH patients and the normolipidemic relatives (n = 64), and subjects without known phenotypic and/or genotypic data (n = 230) were included in the family-based analyses. After withdrawal of lipid-lowering medication for 4 weeks and an overnight fast, blood was drawn by venipuncture. The maximum waist circumference (cm) at the umbilical level was measured in the late exhalation phase while standing. All included subjects were Caucasian and older than 10 years. The ethical committee of the Radboud University Nijmegen Medical Center approved the study protocol, and the procedures followed were in accordance with institutional guidelines. All subjects gave informed consent.

Biochemical analyses

Plasma TC and TG were determined by commercially available enzymatic reagents (Boehringer-Mannheim, Mannheim, Germany, catalog No. 237574; and Sera Pak, Miles, Belgium, catalog No. 6639, respectively). Total plasma apoB concentrations were measured by immunonephelometry (34). LDL subfractions were separated by single-spin density gradient ultracentrifugation, according to a previously described method (35). A continuous variable, K, represents the LDL subfraction profile of each individual. A negative K value ($K \leq -0.1$) reflects a more dense LDL subfraction profile, and a positive K value ($K > -0.1$) reflects a more buoyant profile. Glucose concentrations were analyzed in duplicate using the oxidation technique (Beckman® Glucose Analyser2; Beckman Instruments, Inc.). Plasma insulin concentrations were ascertained using a double antibody method (36). Insulin resistance was assessed by the homeostasis model assessment (HOMA) method (37).

DNA was obtained from peripheral blood lymphocytes using a standard technique. Microsatellite markers, D1s104 and D1s1677, were amplified by PCR and analyzed on polyacrylamide gels, as described by Hughes (38). D1s104 and D1s1677 were successfully genotyped in 611 and 545 individuals, respectively. The genotyping of D1s1677 failed in 38 FCH patients. For the *USF1* variants, PCRs were performed in a final volume of 50 μl at an annealing temperature of 53.7°C for both polymorphisms. Primer sequences for USF1s1 were 5′-GGTGTGTCCTTGAACTGAG-3′ (forward) and 5′-CAAGCCAGAGCATCACCTG-3′ (reverse), and those for USF1s2 were 5′-CTTTAGTAGAGACAGGGTTTCAC-3′ (forward) and 5′-GATTTAGCAGGTATTAGGAGCA-3′ (reverse). The mismatch (underlined) for USF1s2 was introduced to create a restriction site for BsiHKA I. The PCR products were digested with either 10 units of BstF5 I (USF1s1) or 10 units of BsiHKA I (USF1s2) at 65°C (New England Biolabs); subsequently, the resulting fragments (242, 172, and 70 bp in heterozygotes for

USF1s1 and 154, 136, and 18 bp in heterozygotes for USF1s2) were separated on agarose gels. USF1s1 and USF1s2 were genotyped in all 611 individuals, including 157 FCH patients. The genotyping of USF1s2, however, failed for six individuals, including three FCH patients.

Quantitative real-time PCR analysis of USF1 expression in peripheral blood mononuclear cells

USF1 mRNA expression levels were quantified in peripheral blood mononuclear cells (PBMCs) of 30 FCH patients and 30 sex-matched normolipidemic relatives randomly selected from our study population, as described previously (39). RNA was isolated from PBMCs, including both lymphocytes and monocytes, and reversed-transcribed to cDNA. Quantitative real-time PCR was carried out in a total of 25 μl containing 2 μl of cDNA, 0.25× SYBR® green solution (Invitrogen), 1× fluorescein (Bio-Rad), 2 mM $MgCl_2$, 50 ng of forward (5′-ATGACCCAGGCGGT-GATCCA-3′) and reverse (5′-GACGCTCCACTTCATTATGC-3′) primers, 100 μM deoxynucleoside triphosphates, 1× Ampli*Taq* Gold amplification buffer, and 1.5 unit of Ampli*Taq* Gold DNA polymerase (Applied Biosystems). PCR conditions were as follows: a hot start at 95°C for 10 min, followed by 40 cycles of 95°C for 30 s, 60°C for 30 s, and 72°C for 30 s. Samples were run in duplicate on the Icycler iQ real-time PCR detection system (Bio-Rad) to determine the threshold cycle (40). Expression levels were normalized to beta-2-microglobulin by comparative quantification using the $\Delta\Delta C_t$ method (41).

Statistical analyses

The characteristics of the study population are expressed as means ± SD. Before further statistical analyses, extended Mendelian error-checking was performed for 36 families, including 219 nuclear families (n = 611), with Pedcheck (42). Data were available for only 25 of the 36 probands, because 11 probands were already recruited in 1994 and did not participate in 1999. For families with Mendelian inconsistencies, which can be attributable to paternity problems and/or misgenotyping, problematic genotypes were set to missing for the complete nuclear families or the isolated problematic individuals [n = 10 (1.6%)]. The parental data for each polymorphism were tested for Hardy-Weinberg equilibrium by use of an exact test. Variables with skewed distribution, including TGs and the HOMA index, were logarithmically transformed. Multipoint parametric linkage analysis of the two microsatellite markers on chromosome 1 and the two *USF1* polymorphisms was performed for three traits, the FCH trait, defined by both the traditional diagnostic criteria and the nomogram, and the TG trait, defined by TG levels above the 90th percentile (31), using SIMWALK2 (43). Data were prepared using MEGA2 (44). We assumed a disease allele frequency of 0.01 and 0.10 under the dominant and recessive models of inheritance, respectively. The assumed penetrance in the dominant model was 0.99. In the recessive model, the assumed penetrance values with risk and nonrisk genotypes were 0.99 and 0.50, respectively. A phenocopy rate of 0.01 was assumed. These conditions are comparable to those used by Pajukanta et al. (7). Two-point nonparametric linkage analyses of the two microsatellite markers and the *USF1* polymorphisms were carried out using SOLAR 2.1.4 software (45). The presence of nonparametric linkage was tested for FCH and the related quantitative traits, including apoB, TC, and TG levels, and the presence of sdLDL, represented by the K value. The HAPLOVIEW program (46) was used to calculate allele and haplotype frequencies and to calculate the linkage disequilibrium between the two SNPs. HAPLOVIEW was also used to calculate transmission disequilibrium association with permutation analysis to correct for multiple testing.

Associations between the individual polymorphisms or haplotypes and FCH and its related phenotypes in our extended families were determined using two programs: the family-based association test (FBAT) (47) and the pedigree-based association test (PBAT) (48) software. An additive model of inheritance was used, as the mode of inheritance of FCH is unknown and this model is particularly robust (47). FBAT handles pedigrees by breaking the pedigrees into all possible nuclear families and evaluating their contributions to the test statistics. According to FBAT, our 36 FCH families included 219 nuclear families. The –e option of FBAT, which computes the test statistics using an empirical variance estimator (49), was implemented, because the nuclear families were not independent. The –p option, which performs the Monte Carlo permutation procedure, was used to correct for multiple testing. As Pajukanta et al. (12) reported a sex-specific effect for USF1, adjustments were made for age and gender by calculating the unstandardized residuals. No adjustment for age and gender was made for FCH, as the FCH diagnosis is already corrected for age and gender. For the haplotype analysis, the haplotype-based association test (HBAT) command of the FBAT program was used, using the –e and –p options.

PBAT is capable of handling extended pedigrees without breaking them into nuclear families. Despite this, our extended pedigrees had to be divided into smaller units, because of computational limitations. This resulted in a total of 92 pedigrees, with a maximum of 14 nonfounders per pedigree. Age and gender were used as covariates in the models. All tested variables, except for the dichotomous variables, were transformed to a Z score, as recommended by PBAT (48).

The proportions of trait variations for TC, apoB, and sdLDL explicable by *USF1* polymorphisms were tested by means of generalized estimating equations because of possibly correlated values within families.

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

RESULTS

Subject characteristics

According to both the traditional diagnostic criteria and the nomogram, 135 subjects were affected with FCH. In addition, 38 patients were affected with FCH according to either the traditional diagnostic criteria (n = 16) or the nomogram (n = 22) as the diagnostic criterion. Therefore, in total, 157 subjects were diagnosed with FCH according to the nomogram. Descriptive statistics of anthropometric and metabolic characteristics of the study population, presented below and in **Table 1**, were based on the nomogram as the diagnostic criterion for FCH.

FCH patients were older than normolipidemic relatives but younger than spouses. The higher prevalence of CVD in patients with FCH was evident, compared with normolipidemic relatives and spouses. The mean waist circumference of patients with FCH was significantly higher compared with normolipidemic relatives. By definition, FCH patients were characterized by increased plasma TC, TG, and apoB levels. Furthermore, FCH patients exhibited higher levels of sdLDL, as reflected by a lower K value. Finally, FCH patients were more insulin-resistant, as

TABLE 1. Characteristics of patients with FCH, normolipidemic relatives, and spouses

Characteristics	FCH Patients (n = 157)	Normolipidemic Relatives (n = 390)	Spouses (n = 64)
Allele frequency (USF1s1/USF1s2)	0.75/0.75	0.69/0.69	0.72/0.72
Gender (males)	75 (47.8%)	177 (45.4%)	31 (48.4%)
Age (years)	47.0 (15.6)[a,b]	37.9 (15.7)[c]	56.0 (10.5)
CVD	32 (20.4%)[a,b]	20 (5.1%)	4 (6.3%)
Waist circumference (cm)	87.2 (12.2)[a]	77.7 (10.9)[c]	84.3 (12.5)
TC (mmol/l)	6.5 (1.1)[a,b]	4.9 (0.9)[c]	5.2 (0.8)
TG (mmol/l)	3.1 (1.5)[a,b]	1.2 (0.5)	1.3 (0.6)
TG > 90th percentile	113 (71.6%)[a,b]	11 (2.9%)	2 (3.1%)
ApoB (mg/l)	1,370 (264)[a,b]	960 (220)	996 (175)
LDL-c (mmol/l)	4.1 (1.2)[a,b]	3.2 (0.9)[c]	3.4 (0.7)
K value	−0.26 (0.26)[a,b]	0.05 (0.19)	0.05 (0.25)
HOMA index	3.4 (1.8)[a,b]	2.3 (1.3)[c]	2.7 (1.3)

apoB, apolipoprotein B; CVD, cardiovascular disease; FCH, familial combined hyperlipidemia; HOMA, homeostasis model assessment; LDL-c, low density lipoprotein cholesterol; TC, total cholesterol; TG, triglyceride; USF1, upstream stimulatory factor 1. FCH diagnosis was based on the nomogram. Bivariate variables are presented as number (%); continuous variables are presented as mean (SD). Allele frequencies are given for the wild-type alleles of USF1s1 and USF1s2; for K value, a value <-0.1 represents the presence of small dense LDLs.

[a] $P < 0.05$, compared with normolipidemic relatives.

[b] $P < 0.05$, compared with spouses.

[c] $P < 0.05$, compared with spouses.

reflected by a higher HOMA index. In the total population, 126 subjects showed the TG trait, as defined by TG levels > 90th percentile, including 52 men. In Table 1, the distribution of the TG trait among FCH family members is presented.

For both USF1s1 and USF1s2, the wild-type allele (C and A, respectively) had a frequency of 0.81 in probands. The wild-type allele frequency of 0.72 in spouses was comparable to the frequency found in probands ($P = 0.30$). The distributions of the parental alleles and genotypes for both polymorphisms were in Hardy-Weinberg proportions. Analysis performed with HAPLOVIEW showed that the two polymorphisms were in complete linkage disequilibrium ($D' = 1.0$, $R^2 = 1.0$), resulting in the presence of only two haplotypes in our study population. The C-A haplotype had an overall frequency of 71% (n = 853), and the T-G haplotype frequency was 29% (n = 357).

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

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

Linkage analyses

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

Association and haplotype analyses

In **Table 2**, the associations of the polymorphisms of the *USF1* gene, individually and combined into haplotypes, with FCH and its related phenotypes in the total population are presented. No statistical association of the *USF1* gene with FCH, defined by the nomogram, was found. Applying the traditional diagnostic criteria of FCH revealed a suggestive association of the *USF1* gene with FCH. No association of the *USF1* gene with the TG trait was found. However, after correction for age and gender, suggestive associations for the wild-type haplotype, previously identified as the risk haplotype by Pajukanta et al. (12), were obtained for TC, apoB, and sdLDL when the structures of the pedigrees were taken into account (Table 2). Correcting for multiple testing, with the –p option, resulted in an association of USF1s1 and USF1s2 with TC ($P = 0.05$ and $P = 0.04$, respectively) and apoB ($P = 0.06$ and $P = 0.04$, respectively). sdLDL showed suggestive associations with USF1s1 ($P = 0.10$) and USF1s2 ($P = 0.09$). The *USF1* gene, however, could only explain a small proportion of the variation in plasma TC (1.7%), apoB (1.5%), and sdLDL (0.5%) levels. LDL-c and the obesity- and insulin resistance-related phenotypes of FCH were not associated with *USF1* (Table 2).

TABLE 2. Association of the individual polymorphisms and haplotypes of the *USF1* gene with FCH and its related phenotypes after correction for age and gender in the total population

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

FBAT, family-based association test; HBAT, haplotype-based association test; PBAT, pedigree-based association test. Presented are the obtained *P* values for the individual single nucleotide polymorphisms and haplotypes in the total population. For K value, a value <−0.1 represents the presence of small dense LDLs. –e indicates correction for the nonindependence of the nuclear families; –p indicates correction for multiple testing by permutation tests.

PBAT was also used to analyze the association between the haplotypes of the *USF1* gene and FCH and its associated phenotypes. PBAT does not implement an overall haplotype test but generates a *P* value for each haplotype. As shown in Table 2, the risk haplotype was not associated with FCH when the nomogram was used as the diagnostic criterion, but a suggestive association was found when using the traditional diagnostic criteria for FCH. The analysis of the association between *USF1* with other characteristics of FCH did not reveal any statistically significant association. The associations found for TC and apoB, however, pointed in the same direction as the associations found with the FBAT and HBAT statistics (Table 2).

In view of the fact that Pajukanta et al. (12) demonstrated a gender-specific effect of the *USF1* gene on FCH and TG levels in males, analyses of both the individual SNPs and the haplotypes of *USF1* were also performed in males only (**Table 3**). The male population (n = 283) included 65 FCH patients based on the traditional diagnostic criteria and 75 FCH patients based on the nomogram. In total, 52 men (18.5%) had TG levels > 90th percentile. No significant association of the *USF1* gene with the FCH or the TG trait was found, and only suggestive evidence for an association in males was present for apoB levels and sdLDL, in accordance with the analysis in the complete families. Therefore, no apparent gender-specific effect was present for these parameters (Table 3). For the obesity parameter (waist circumference), we found suggestive evidence for an association with *USF1* in males only (Table 3).

Expression analyses of USF1 in PBMCs

To further explore the role of USF1 in FCH, expression levels of *USF1* in PBMCs were measured in 30 FCH patients (defined by the nomogram) and 30 controls. Anthropometric and metabolic characteristics of these subjects are presented in supplementary Table I. The FCH patients demonstrated a relative expression of 0.9 (0.5–2.2) for the *USF1* gene, compared with the control value of 1.0 (0.3–4.0), which was not significantly different ($P = 0.27$). Of the 30 FCH patients, 14 carried the risk haplotype, 12 were heterozygous for the USF1 mutations, and 4 were homozygous for the rare haplotype. In the control group, 12 subjects carried the risk haplotype, 15 were heterozygous, and 3 were homozygous for the rare haplotype. As the FCH patients carrying the *USF1* risk haplotype had a relative expression of 1.3 (0.5–1.9) and the FCH patients carrying the rare haplotype had a relative expression of 1.0

TABLE 3. Association of the individual polymorphisms and haplotypes of the *USF1* gene with FCH and its related phenotypes after correction for age and gender in males

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

Presented are the obtained *P* values for the individual single nucleotide polymorphisms and haplotypes in males only. For K value, a value <−0.1 represents the presence of small dense LDLs. –e, correction for the nonindependence of the nuclear families; –p, correction for multiple testing by permutation tests.

(0.6–1.6), no differences based on the haplotype were observed within the FCH patients. The results in the controls were comparable.

DISCUSSION

In this study, we investigated the putative role of *USF1* in Dutch FCH families. We show that the wild-type haplotype of *USF1*, previously identified as a risk haplotype (12), was not associated with FCH when using the nomogram to diagnose FCH, which is based on plasma levels of TC, TG, and apoB; however, a suggestive association of *USF1* with FCH in our Dutch families was found when using the traditional lipid criteria to diagnose FCH. Furthermore, we report no association of *USF1* with the TG trait. The risk haplotype of *USF1*, however, was associated with other important phenotypes of FCH, including plasma TC levels, apoB levels, and the presence of sdLDL, accounting for ~1% of the variation in these phenotypes. We conclude that *USF1* shows a suggestive association with FCH and can be considered a modifier gene, contributing to related lipid traits in our Dutch FCH families.

Several groups have tried to replicate the results with regard to *USF1* found in the Finnish FCH families (12) (see supplementary Table II). Huertas-Vazquez et al. (5) reported an association of *USF1* with FCH and TG levels in a Mexican population, although their results were less conclusive and no gender-specific effect was found. Coon et al. (14) also reported an association of *USF1* with FCH in Utah pedigrees ascertained for early death attributable to CHD, early stroke, or early-onset hypertension. When restricting the analyses to the families ascertained for early death attributable to CHD, in which 60% of the FCH patients were present, no evidence for the involvement of *USF1* in FCH, TG, or LDL-c levels was found, although the strongest association would be expected in these families. This lack of association in the families ascertained for early death attributable to CHD could be the result of limited power, as only a few families were ascertained for CHD (n = 17) (14). Another explanation could be the use of different diagnostic criteria for FCH in the different populations. To diagnose FCH, Pajukanta et al. (12) and Huertas-Vazquez et al. (5) used the traditional criteria, based on increased plasma TC and/or TG levels above the 90th percentile, adjusted for age and gender, whereas Coon et al. (14) defined FCH based on LDL-c and/or TG levels above the 90th percentile, adjusted for age and gender. In this study, we applied two diagnostic criteria for FCH: the recently described nomogram (33) and the traditional FCH criteria (32). We show that different diagnostic criteria affect the results of the association analysis. As shown in Table 2, a suggestive *P* value for the association of *USF1* with FCH was found when using the traditional FCH criteria, whereas no association was found when using the nomogram.

In sharp contrast to the results obtained by Pajukanta et al. (12), who reported the strongest association of *USF1* in males with high TG levels, we report here no gender-specific effect and no association of *USF1* with high TG levels. The absence of this association in our population might be explained by the relatively mildly increased levels of TG in our study population compared with the Finnish population (2.8 vs. ~3.3 mmol/l, respectively) (50, 51). FCH remains a multifactorial disease, with extensive genetic heterogeneity, in which the combination of many genes and the environment determine the expression of the phenotype, contributing to the apparently conflicting results in different FCH populations. Our results, however, are supported by a study in Mexican FCH families (5), which also found no gender-specific effect.

Although we did not find an association between *USF1* and TG levels in this study, we were able to replicate some associations for other important phenotypes of FCH, including TC, apoB, and sdLDL. A study performed in 196 healthy obese Scandinavian women (52) and one involving diabetes mellitus type 2 patients (53), however, failed to demonstrate an association between *USF1* and lipid/lipoprotein levels. This suggests that the influence of *USF1*, as a modifier gene, on these lipid levels is specific for patients with FCH.

Recently, a close relationship between *USF1* and catecholamine-stimulated lipolysis in human fat cells was found (52), and based on this, it was suggested that the adipocyte lipolysis-related effects of genetic variation in *USF1* can have clinical consequences against certain backgrounds such as FCH. In healthy young males, it was demonstrated that *USF1* is associated with glucose levels and has a modulating effect on the correlation between body mass index and TC levels (18). In this study, however, we did not find an association between *USF1* and glucose, insulin levels, or obesity parameters. In males, however, a suggestive association was found between *USF1* and the obesity parameter, waist circumference.

In the search for candidate genes underlying complex genetic disorders, haplotype analyses have become a valuable tool. The most convincing results for the association of *USF1* with FCH, from Pajukanta et al. (12) and Huertas-Vazquez et al. (5), were obtained when analyzing the *USF1* SNPs as haplotypes (see supplementary Table II). In our study, however, the two SNPs resulted in only two haplotypes, so no increased power was obtained from haplotype analyses. The complete linkage disequilibrium between these two SNPs has been reported previously in 196 healthy obese women (52). In this study, we used two programs for haplotype analyses, FBAT and PBAT. In FBAT, the presence of linkage is not mandatory for analyzing haplotypes. FBAT, however, splits up the pedigrees into nuclear families; thereby, important information about the family structure is lost. The second program, PBAT, does not require the presence of linkage and is said to be capable of handling extended pedigrees. PBAT, however, could not handle our extended pedigrees, necessitating their division into smaller units. Haplotype analyses in extended pedigrees, therefore, remain a challenge.

For the association analyses of *USF1* with FCH, Pajukanta et al. (12) and Huertas-Vazquez et al. (5) used several programs (see supplementary Table II). In the haplotype-based relative risk and the genotype pedigree

disequilibrium test programs, linkage is assumed to be present in the region under investigation, making these programs unsuitable in our situation of no linkage. The association of *USF1* with FCH in Mexican families was found using the gamete competition test program, which analyzes the presence of both linkage and association, so the significant result can represent the presence of linkage only, as reported for this region in this population (5). Pajukanta et al. (12) resolved this problem in the Finnish FCH families by gene dropping.

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

Although *USF1* was thought to be a good candidate gene for FCH, our results do not support the role of *USF1* as a major gene in our population. This is reinforced by the lack of functionality of the genetic variations in *USF1*, as the two polymorphisms do not result in amino acid changes. The identification of a site for DNA binding proteins in the region of USF1s2 has been reported; however, no evidence was found for the presence of the suggested internal promoter causing the transcription of truncated mRNA (54). Pajukanta and colleagues (54) reported differential expression of three of the USF1-regulated genes in adipose tissue based on the USF1s2 allele. The expression of *USF1* itself, however, was not different in adipose tissue. In this study, we have measured the expression of *USF1* in PBMCs and, consistent with the results of Pajukanta et al. (12), we did not find any haplotype-dependent differences. Our decision to use PBMCs, in the absence of adipose tissue samples from our study population, was justified by a study by Morello et al. (55), showing that the FCH-specific transcription profile was detectable in peripheral blood cells.

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

REFERENCES

1. Hopkins, P. N., G. Heiss, R. C. Ellison, M. A. Province, J. S. Pankow, J. H. Eckfeldt, and S. C. Hunt. 2003. Coronary artery disease risk in familial combined hyperlipidemia and familial hypertriglyceridemia: a case-control comparison from the National Heart, Lung, and Blood Institute Family Heart Study. *Circulation.* **108:** 519–523.
2. de Graaf, J., G. van der Vleuten, and A. F. Stalenhoef. 2004. Diagnostic criteria in relation to the pathogenesis of familial combined hyperlipidemia. *Semin. Vasc. Med.* **4:** 229–240.
3. Aouizerat, B. E., H. Allayee, R. M. Cantor, R. C. Davis, C. D. Lanning, P. Z. Wen, G. M. Linga-Thie, T. W. de Bruin, J. I. Rotter, and A. J. Lusis. 1999. A genome scan for familial combined hyperlipidemia reveals evidence of linkage with a locus on chromosome 11. *Am. J. Hum. Genet.* **65:** 397–412.
4. Coon, H., R. H. Myers, I. B. Borecki, D. K. Arnett, S. C. Hunt, M. A. Province, L. Djousse, and M. F. Leppert. 2000. Replication of linkage of familial combined hyperlipidemia to chromosome 1q with additional heterogeneous effect of apolipoprotein A-I/C-III/A-IV locus. The NHLBI Family Heart Study. *Arterioscler. Thromb. Vasc. Biol.* **20:** 2275–2280.
5. Huertas-Vazquez, A., C. Guilar-Salinas, A. J. Lusis, R. M. Cantor, S. Canizales-Quinteros, J. C. Lee, L. Mariana-Nunez, R. M. L. Riba-Ramirez, A. Jokiaho, T. Tusie-Luna, et al. 2005. Familial combined hyperlipidemia in Mexicans: association with upstream transcription factor 1 and linkage on chromosome 16q24.1. *Arterioscler. Thromb. Vasc. Biol.* **25:** 1985–1991.
6. Naoumova, R. P., S. A. Bonney, S. Eichenbaum-Voline, H. N. Patel, B. Jones, E. L. Jones, J. Amey, S. Colilla, C. K. Neuwirth, R. Allotey, et al. 2003. Confirmed locus on chromosome 11p and candidate loci on 6q and 8p for the triglyceride and cholesterol traits of combined hyperlipidemia. *Arterioscler. Thromb. Vasc. Biol.* **23:** 2070–2077.
7. Pajukanta, P., I. Nuotio, J. D. Terwilliger, K. V. Porkka, K. Ylitalo, J. Pihlajamaki, A. J. Suomalainen, A. C. Syvanen, T. Lehtimaki, J. S. Viikari, et al. 1998. Linkage of familial combined hyperlipidaemia to chromosome 1q21-q23. *Nat. Genet.* **18:** 369–373.
8. Pei, W., H. Baron, B. Muller-Myhsok, H. Knoblauch, S. A. Al Yahyaee, R. Hui, X. Wu, L. Liu, A. Busjahn, F. C. Luft, et al. 2000. Support for linkage of familial combined hyperlipidemia to chromosome 1q21-q23 in Chinese and German families. *Clin. Genet.* **57:** 29–34.
9. Huertas-Vazquez, A., J. P. del Rincon, S. Canizales-Quinteros, L. Riba, G. Vega-Hernandez, S. Ramirez-Jimenez, M. Uron-Gomez, F. J. Gomez-Perez, C. A. Guilar-Salinas, and M. T. Tusie-Luna. 2004. Contribution of chromosome 1q21-q23 to familial combined hyperlipidemia in Mexican families. *Ann. Hum. Genet.* **68:** 419–427.
10. Bodnar, J. S., A. Chatterjee, L. W. Castellani, D. A. Ross, J. Ohmen, J. Cavalcoli, C. Wu, K. M. Dains, J. Catanese, M. Chu, et al. 2002. Positional cloning of the combined hyperlipidemia gene Hyplip1. *Nat. Genet.* **30:** 110–116.
11. Coon, H., N. Singh, D. Dunn, J. H. Eckfeldt, M. A. Province, P. N. Hopkins, R. Weiss, S. C. Hunt, and M. F. Leppert. 2004. TXNIP gene not associated with familial combined hyperlipidemia in the NHLBI Family Heart Study. *Atherosclerosis.* **174:** 357–362.
12. Pajukanta, P., H. E. Lilja, J. S. Sinsheimer, R. M. Cantor, A. J. Lusis, M. Gentile, X. J. Duan, A. Soro-Paavonen, J. Naukkarinen, J. Saarela, et al. 2004. Familial combined hyperlipidemia is associated with upstream transcription factor 1 (USF1). *Nat. Genet.* **36:** 371–376.
13. van der Vleuten, G. M., A. Hijmans, L. A. Kluijtmans, H. J. Blom, A. F. Stalenhoef, and J. de Graaf. 2004. Thioredoxin interacting protein in Dutch families with familial combined hyperlipidemia. *Am. J. Med. Genet. A.* **130:** 73–75.
14. Coon, H., Y. Xin, P. N. Hopkins, R. M. Cawthon, S. J. Hasstedt, and S. C. Hunt. 2005. Upstream stimulatory factor 1 associated with familial combined hyperlipidemia, LDL cholesterol, and triglycerides. *Hum. Genet.* **117:** 444–451.
15. Casado, M., V. S. Vallet, A. Kahn, and S. Vaulont. 1999. Essential role in vivo of upstream stimulatory factors for a normal dietary response of the fatty acid synthase gene in the liver. *J. Biol. Chem.* **274:** 2009–2013.
16. Ribeiro, A., D. Pastier, D. Kardassis, J. Chambaz, and P. Cardot. 1999. Cooperative binding of upstream stimulatory factor and hepatic nuclear factor 4 drives the transcription of the human apolipoprotein A-II gene. *J. Biol. Chem.* **274:** 1216–1225.
17. Smih, F., P. Rouet, S. Lucas, A. Mairal, C. Sengenes, M. Lafontan, S. Vaulont, M. Casado, and D. Langin. 2002. Transcriptional regulation of adipocyte hormone-sensitive lipase by glucose. *Diabetes.* **51:** 293–300.
18. Putt, W., J. Palmen, V. Nicaud, D. A. Tregouet, N. Tahri-Daizadeh, D. M. Flavell, S. E. Humphries, and P. J. Talmud. 2004. Variation in USF1 shows haplotype effects, gene:gene and gene:environment associations with glucose and lipid parameters in the European Atherosclerosis Research Study II. *Hum. Mol. Genet.* **13:** 1587–1597.

19. Ylitalo, K., V. Large, P. Pajukanta, S. Reynisdottir, K. V. Porkka, J. Vakkilainen, I. Nuotio, M. R. Taskinen, and P. Arner. 2000. Reduced hormone-sensitive lipase activity is not a major metabolic defect in Finnish FCHL families. *Atherosclerosis.* **153:** 373–381.
20. Wang, D., and H. S. Sul. 1997. Upstream stimulatory factor binding to the E-box at −65 is required for insulin regulation of the fatty acid synthase promoter. *J. Biol. Chem.* **272:** 26367–26374.
21. Blanco-Colio, L. M., J. L. Martin-Ventura, J. M. Sol, C. Diaz, G. Hernandez, and J. Egido. 2004. Decreased circulating Fas ligand in patients with familial combined hyperlipidemia or carotid atherosclerosis: normalization by atorvastatin. *J. Am. Coll. Cardiol.* **43:** 1188–1194.
22. Pastier, D., J. M. Lacorte, J. Chambaz, P. Cardot, and A. Ribeiro. 2002. Two initiator-like elements are required for the combined activation of the human apolipoprotein C-III promoter by upstream stimulatory factor and hepatic nuclear factor-4. *J. Biol. Chem.* **277:** 15199–15206.
23. Iynedjian, P. B. 1998. Identification of upstream stimulatory factor as transcriptional activator of the liver promoter of the glucokinase gene. *Biochem. J.* **333:** 705–712.
24. Botma, G. J., A. J. Verhoeven, and H. Jansen. 2001. Hepatic lipase promoter activity is reduced by the C-480T and G-216A substitutions present in the common LIPC gene variant, and is increased by upstream stimulatory factor. *Atherosclerosis.* **154:** 625–632.
25. Yanai, K., T. Saito, K. Hirota, H. Kobayashi, K. Murakami, and A. Fukamizu. 1997. Molecular variation of the human angiotensinogen core promoter element located between the TATA box and transcription initiation site affects its transcriptional activity. *J. Biol. Chem.* **272:** 30558–30562.
26. Yang, X. P., L. A. Freeman, C. L. Knapper, M. J. Amar, A. Remaley, H. B. Brewer, Jr., and S. Santamarina-Fojo. 2002. The E-box motif in the proximal ABCA1 promoter mediates transcriptional repression of the ABCA1 gene. *J. Lipid Res.* **43:** 297–306.
27. Martin-Campos, J. M., J. C. Escola-Gil, V. Ribas, and F. Blanco-Vaca. 2004. Apolipoprotein A-II, genetic variation on chromosome 1q21-q24, and disease susceptibility. *Curr. Opin. Lipidol.* **15:** 247–253.
28. Hoffer, M. J., H. Snieder, S. J. Bredie, P. N. Demacker, J. J. Kastelein, R. R. Frants, and A. F. Stalenhoef. 2000. The V73M mutation in the hepatic lipase gene is associated with elevated cholesterol levels in four Dutch pedigrees with familial combined hyperlipidemia. *Atherosclerosis.* **151:** 443–450.
29. McNeely, M. J., K. L. Edwards, S. M. Marcovina, J. D. Brunzell, A. G. Motulsky, and M. A. Austin. 2001. Lipoprotein and apolipoprotein abnormalities in familial combined hyperlipidemia: a 20-year prospective study. *Atherosclerosis.* **159:** 471–481.
30. Groenendijk, M., R. M. Cantor, H. Funke, and G. M. Linga-Thie. 2001. Two newly identified SNPs in the APO AI-CIII intergenic region are strongly associated with familial combined hyperlipidaemia. *Eur. J. Clin. Invest.* **31:** 852–859.
31. Assmann, G., and H. Schulte. 1992. Relation of high-density lipoprotein cholesterol and triglycerides to incidence of atherosclerotic coronary artery disease (the PROCAM experience). Prospective Cardiovascular Munster study. *Am. J. Cardiol.* **70:** 733–737.
32. Goldstein, J. L., H. G. Schrott, W. R. Hazzard, E. L. Bierman, and A. G. Motulsky. 1973. Hyperlipidemia in coronary heart disease. II. Genetic analysis of lipid levels in 176 families and delineation of a new inherited disorder, combined hyperlipidemia. *J. Clin. Invest.* **52:** 1544–1568.
33. Veerkamp, M. J., J. de Graaf, J. C. Hendriks, P. N. Demacker, and A. F. Stalenhoef. 2004. Nomogram to diagnose familial combined hyperlipidemia on the basis of results of a 5-year follow-up study. *Circulation.* **109:** 2980–2985.
34. Demacker, P. N., M. J. Veerkamp, S. J. Bredie, S. M. Marcovina, J. de Graaf, and A. F. Stalenhoef. 2000. 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. *Atherosclerosis.* **153:** 483–490.
35. Swinkels, D. W., H. L. Hak-Lemmers, and P. N. Demacker. 1987. Single spin density gradient ultracentrifugation method for the detection and isolation of light and heavy low density lipoprotein subfractions. *J. Lipid Res.* **28:** 1233–1239.
36. de Galan, B. E., C. J. Tack, J. W. Lenders, J. W. Pasman, L. D. Elving, F. G. Russel, J. A. Lutterman, and P. Smits. 2002. Theophylline improves hypoglycemia unawareness in type 1 diabetes. *Diabetes.* **51:** 790–796.
37. Matthews, D. R., J. P. Hosker, A. S. Rudenski, B. A. Naylor, D. F. Treacher, and R. C. Turner. 1985. Homeostasis model assessment: insulin resistance and beta-cell function from fasting plasma glucose and insulin concentrations in man. *Diabetologia.* **28:** 412–419.
38. Hughes, A. E. 1993. Optimization of microsatellite analysis for genetic mapping. *Genomics.* **15:** 433–434.
39. van der Vleuten, G. M., A. Hijmans, S. Heil, H. J. Blom, A. F. Stalenhoef, and J. de Graaf. 2006. Can we exclude the TXNIP gene as a candidate gene for familial combined hyperlipidemia? *Am. J. Med. Genet. A.* **140:** 1010–1012.
40. Wilhelm, J., and A. Pingoud. 2003. Real-time polymerase chain reaction. *ChemBioChem.* **4:** 1120–1128.
41. Livak, K. J., and T. D. Schmittgen. 2001. Analysis of relative gene expression data using real-time quantitative PCR and the 2(-Delta Delta C(T)) method. *Methods.* **25:** 402–408.
42. O'Connell, J. R., and D. E. Weeks. 1998. PedCheck: a program for identification of genotype incompatibilities in linkage analysis. *Am. J. Hum. Genet.* **63:** 259–266.
43. Sobel, E., and K. Lange. 1996. Descent graphs in pedigree analysis: applications to haplotyping, location scores, and marker-sharing statistics. *Am. J. Hum. Genet.* **58:** 1323–1337.
44. Mukhopadhyay, N., L. Almasy, M. Schroeder, W. P. Mulvihill, and D. E. Weeks. 2005. Mega2: data-handling for facilitating genetic linkage and association analyses. *Bioinformatics.* **21:** 2556–2557.
45. Almasy, L., and J. Blangero. 1998. Multipoint quantitative-trait linkage analysis in general pedigrees. *Am. J. Hum. Genet.* **62:** 1198–1211.
46. Barrett, J. C., B. Fry, J. Maller, and M. J. Daly. 2005. HAPLOVIEW: analysis and visualization of LD and haplotype maps. *Bioinformatics.* **21:** 263–265.
47. Horvath, S., X. Xu, and N. M. Laird. 2001. The family based association test method: strategies for studying general genotype-phenotype associations. *Eur. J. Hum. Genet.* **9:** 301–306.
48. Rabinowitz, D., and N. Laird. 2000. A unified approach to adjusting association tests for population admixture with arbitrary pedigree structure and arbitrary missing marker information. *Hum. Hered.* **50:** 211–223.
49. Lake, S. L., D. Blacker, and N. M. Laird. 2000. Family-based tests of association in the presence of linkage. *Am. J. Hum. Genet.* **67:** 1515–1525.
50. Pajukanta, P., K. V. Porkka, M. Antikainen, M. R. Taskinen, M. Perola, S. Murtomaki-Repo, S. Ehnholm, I. Nuotio, L. Suurinkeroinen, A. T. Lahdenkari, et al. 1997. No evidence of linkage between familial combined hyperlipidemia and genes encoding lipolytic enzymes in Finnish families. *Arterioscler. Thromb. Vasc. Biol.* **17:** 841–850.
51. Vakkilainen, J., P. Pajukanta, R. M. Cantor, I. O. Nuotio, S. Lahdenpera, K. Ylitalo, J. Pihlajamaki, P. T. Kovanen, M. Laakso, J. S. Viikari, et al. 2002. Genetic influences contributing to LDL particle size in familial combined hyperlipidaemia. *Eur. J. Hum. Genet.* **10:** 547–552.
52. Hoffstedt, J., M. Ryden, H. Wahrenberg, V. Van Harmelen, and P. Arner. 2005. Upstream transcription factor-1 gene polymorphism is associated with increased adipocyte lipolysis. *J. Clin. Endocrinol. Metab.* **90:** 5356–5360.
53. Gibson, F., S. Hercberg, and P. Froguel. 2005. Common polymorphisms in the USF1 gene are not associated with type 2 diabetes in French Caucasians. *Diabetes.* **54:** 3040–3042.
54. Naukkarinen, J., M. Gentile, A. Soro-Paavonen, J. Saarela, H. A. Koistinen, P. Pajukanta, M. R. Taskinen, and L. Peltonen. 2005. USF1 and dyslipidemias: converging evidence for a functional intronic variant. *Hum. Mol. Genet.* **14:** 2595–2605.
55. Morello, F., T. W. de Bruin, J. I. Rotter, R. E. Pratt, C. J. van der Kallen, G. A. Hladik, V. J. Dzau, C. C. Liew, and Y. D. Chen. 2004. Differential gene expression of blood-derived cell lines in familial combined hyperlipidemia. *Arterioscler. Thromb. Vasc. Biol.* **24:** 2149–2154.