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Identification of the PPARA locus on chromosome 22q13.3 as a modifier gene in familial combined hyperlipidemia

Petra M.H. Eurlings,[a,*] Carla J.H. van der Kallen,[a] Jan M.W. Geurts,[a] David M. Flavell,[b] and Tjerk W.A. de Bruin[a]

[a] *Department of Internal Medicine, Laboratory of Molecular Metabolism and Endocrinology, Cardiovascular Research Institute Maastricht, University of Maastricht, PO Box 616, Maastricht, NL-6200 MD, The Netherlands*
[b] *Department of Medicine, Centre for Cardiovascular Genetics, Royal Free and University College London Medical School, The Rayne Institute, London, UK*

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Abstract

Familial combined hyperlipidemia (FCHL) is a common genetic lipid disorder that is present in 10% of patients with premature coronary artery disease (CAD). It was the objective of the present study to evaluate the possible involvement of the *PPARA* locus in the pathophysiology of FCHL. Mutation detection analyses of the six coding *PPARA* exons resulted in the identification of four novel variants, [C/T] intron 3, S234G, [G/A] intron 5, and [C/A] 3′ UTR in three FCHL probands, whereas no novel variants were identified in spouses. In a case-control study, markers D22S275 and D22S928 were shown not to be associated with FCHL. However, D22S928, mapped within 1 Mb of the *PPARA* gene, was shown to have a modifying effect on plasma apoCIII concentrations ($P = 0.011$) and the combined hyperlipidemic FCHL phenotype ($P = 0.038$). In addition two PPARA polymorphisms in intron 2 and 7 were studied, but these were not associated with FCHL. The frequency of the L162V variant was less in FCHL probands (1.98%) compared to that in spouses (4.84%). These results clearly demonstrate the genetically complex nature of FCHL and identify the *PPARA* gene as a modifier of the FCHL phenotype.

Keywords: Hyperlipidemia; Peroxisome proliferator-activated receptor α; FCHL; ApoCIII; Polymorphism

Introduction

Familial combined hyperlipidemia (FCHL) is a common genetic lipid disorder that is present in 10% of patients with premature coronary artery disease (CAD) [1]. FCHL is defined by elevated plasma cholesterol (>6.5 mmol/L) and/or triglyceride levels (>2.3 mmol/L). In addition, hepatic apolipoprotein (apo) B overproduction, elevated plasma apoB concentrations, small dense low density lipoprotein (LDL), low levels of high density lipoprotein (HDL), elevated postprandial free fatty acids (FFA) and insulin resistance have been associated with FCHL [2–4]. FCHL was presumed to have a Mendelian dominant pattern of inheritance [1], but recent analyses indicated that this disorder is genetically more complex [5,6]. At present, a major FCHL locus, *Hyplip 1*, has been identified on chromosome 1 in a Finnish population [7]. In our Dutch FCHL families, chromosomal loci have been identified that contribute to hyperlipidemia, plasma leptin levels (a marker of adiposity), and systolic blood pressure, indicating a genetic propensity to the insulin resistance syndrome [8–10].

In search of novel genes for FCHL, the peroxisome proliferator-activated receptor α (*PPARA*) gene is an interesting candidate. PPARα acts as a nuclear transcription factor regulating genes involved in intra- and extracellular lipid metabolism, such as lipolysis and mitochondrial β-oxidation [11]. PPARα is activated by natural ligands derived from fatty acids such as 8(S)hydroxyeicosatetraenoic acid, 8(S)hydroxyeicosa-

* Corresponding author. Fax: +31-43-367-0916.
E-mail address: p.eurlings@intmed.unimaas.nl (P.M.H. Eurlings).

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pentaenoic acid and leucotreine B_4 [12]. Moreover, hypolipidemic drugs, such as fibrates, are ligands for PPARα [13]. Fibrate treatment has been shown to be effective in the management of FCHL, with a predominant effect on the correction of hypertriglyceridemia and low HDL-cholesterol levels [14]. In addition, PPARα has also been implicated in changes in plasma apoB concentrations [15].

Recently, we reported a linkage study with 14 candidate gene loci in Dutch FCHL families [5]. In this report, the *PPARA* gene (marker D22S275) showed no linkage with FCHL (adjusted P value for multiple testing: $P = 0.06$), but in the report it was not mentioned that the unadjusted P value was significant ($P = 0.004$). Although of considerable interest, this study may have lacked sufficient power to detect significant linkage, since multiple candidate genes and a limited number of genetically independent FCHL individuals were tested. Therefore, the present objective was to assess the involvement of the *PPARA* locus on chromosome 22q13.3 in FCHL by mutation detection experiments and a formal association study.

Methods

Subjects

The case-control study population consisted of 102 genetically unrelated FCHL probands (of which 18 were used in the candidate gene study), each representing one FCHL family, and 124 unrelated spouses, recruited through the Lipid Clinics of both the Maastricht and Utrecht University Hospitals. FCHL families were ascertained as previously described [5]. Probands had a primary hyperlipidemia with a varying phenotypic expression including a fasting plasma cholesterol >6.5 mmol/L and/or a fasting plasma triglyceride >2.3 mmol/L sometimes combined with the presence of CAD. Spouses represent a common environment-, nutrition-, and age-matched control group without hyperlipidemia. The excess of spouse individuals in this study is explained by the inclusion of (i) spouses whose partner had deceased due to complications of FCHL, and (ii) spouses of unaffected FCHL relatives. The study protocol was approved by both of the Human Investigation Review Committees of the University Hospitals of Maastricht and Utrecht, and all subjects gave informed consent.

Biochemical analyses

Venous blood was drawn after an overnight fast (12–14 h) in pre-cooled EDTA (1 mg/ml) tubes and prepared by immediate centrifugation for analytical analyses. All subjects on hyperlipidemic drugs were withheld from therapy for two weeks in order to obtain untreated plasma samples from all subjects. It is known that subjects who were originally hyperlipidemic then return to a hyperlipidemic phenotype (unpublished observations). Plasma concentrations of total cholesterol (TC), triglycerides (TG), HDL-cholesterol (HDL-C), HDL-triglycerides (HDL-TG), FFA, apoAI, and apoB were measured as described before [16]. LDL particle size was also measured as previously reported [16]. Plasma apoCIII concentrations were measured using the Hydragel LP CIII particle kit (SEBIA, Issy-les-Moulineaux, France) according to the manufacturers instructions.

PPARA mutation analysis

Denaturing high-performance liquid chromatography (DHPLC)-based mutation detection analysis was performed using the WAVE DNA Fragment Analysis System (Transgenomic, Omaha, NB) [16]. All six coding *PPARA* exons (exons 3–8) were screened in 52 FCHL probands and 78 spouses from the case-control population.

The following primer sets were used for polymerase chain reaction (PCR) amplification

Exon 3	forward 5′-CAA GTG AAC GTT GTT ATA ACG-3′ reverse 5′-GTT TCT TCC TCC AAG AAA AG-3′
Exon 4	forward 5′-GAA GCC TCG TAT GCG AAA TC-3′ reverse 5′-TGT TAT CCG GGA CTT TCT GC-3′
Exon 5	forward 5′-AGT AAA GCA AGT GCG CTG GT-3′ reverse 5′-GAT CTG TTC CCT TGG AGC AA-3′
Exon 6	forward 5′-TCC ATA GTG GAA AGC CGA AT-3′ reverse 5′-GGT TCC ATG TTG CCA AGA GA-3′
Exon 7	forward 5′-ATA GCG CAT CCC ACA TCA C-3′ reverse 5′-ACA GCT CAG GCT GGT ACT GG-3′
Exon 8	forward 5′-TGA TAA GCA GTT CTT GGG TGA-3′ reverse 5′-GCA TTG ATT AAC ATC GGG TGA-3′

Runs were performed at temperatures varying from 57 to 61 °C. The nucleotide sequences of the samples showing aberrant WAVE chromatograms were determined by fluorescence-based automated sequencing on an ABI-310 PRISM Genetic analyzer (Applied Biosystems, Nieuwerkerk a/d/ IJssel, The Netherlands).

PPARA intragenic genotyping

Two intragenic *PPARA* single nucleotide polymorphisms (SNP) in intron 2 and intron 7 were studied. The intron 2 SNP (G to A substitution) was determined as described before [17]. A restriction enzyme digestion assay was designed for the intron 7 SNP (G to C substitution, nucleotide 82158 of GenBank sequence AL032818). PCR amplification was performed using forward primer 5′-ACA ATC ACT CCT TAA ATA TGG TGG-3′ and reverse primer 5′-AAG TAG GGA CAG ACA GGA CCA GTA-3′. The polymorphism was subsequently digested with TaqI (New England Biolabs, Beverly, MA). In addition the L162V variant was determined by WAVE analysis as described above and a forced-site assay as described previously [17].

PPARA locus genotyping

The CA-repeat markers D22S275, and D22S928, were used for genotyping. PCR amplification was performed using the following primer sets:

D22S275	forward	(FAM) 5′-CTC CAG CCT GTG CAA CAGA G-3′
	reverse	5′-GGC TCA ACC CAT CCT CCT-3′
D22S928	forward	(FAM) 5′-TGC AAA GTG CTG GAG G-3′
	reverse	5′-TGA AGA TGG CTA GTA CGG G-3′

The heterozygosity index of the markers D22S275 and D22S928 is 0.82 and 0.79, respectively [18]. Amplified products were size-resolved by capillary electrophoresis on an ABI-310 PRISM Genetic Analyzer (Applied Biosystems) using performance optimized polymer 4 (Applied Biosystems). Alleles were designated according to the size of the PCR product (Genescan analysis 2.0), using the Genescan-400 HD ROX or the Genescan-500 TAMRA size standard (Applied Biosystems). Alleles with a frequency of more than 5% in both the FCHL and spouse groups were used for analyses. These alleles were for each marker:

D22S275:	CA160 (160 nucleotides), CA162, CA164, CA166, CA168,
D22S928:	CA149, CA157, CA173, CA175.

Statistical analyses

Differences in allelic distribution between cases and controls were determined by χ^2 test. P values were corrected for multiple testing using a Bonferroni correction when individual allele frequencies were tested. Deviations of the Hardy–Weinberg equilibrium were tested with a χ^2 test. The relationship between each of the PPARA genotypes and plasma parameters was analyzed by Student's t test, and by logistic regression analysis adjusting for age and body mass index (BMI). Plasma TG was not normally distributed and therefore log transformed. Statistical significance was considered at the $P < 0.05$ level (two-sided).

Results

Novel variations in the PPARA gene

PPARA gene mutation detection experiments were done by WAVE DHPLC technology [16] in search for mutations or variants associated with FCHL. Each of the coding *PPARA* exons were screened in 52 FCHL probands and 78 spouses. We identified four novel variants in three separate FCHL subjects, but none in spouses (Fig. 1). In one proband two variants were identified, a C to T substitution −71 bases of exon 4 ([C/T] intron 3, affecting nucleotide 101787 of GenBank sequence AL032818), and an A to G substitution

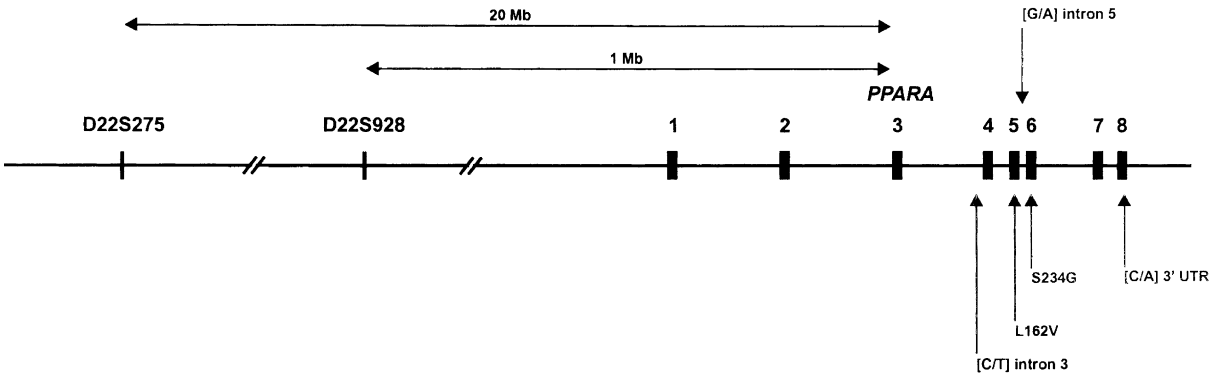

Fig. 1. Schematic overview marker positions in relation to the *PPARA* locus. Positions of markers D22S275, D22S928, and the genomic organization of the *PPARA* gene are based on data available in the Ensemble, Whitehead, and Sanger public databases (last consultation 24 January 2002). Ensemble: http://www.ensemble.org/, Whitehead: http://www-genome.wi.mit-edu/, and Sanger: http://www.sanger.ac.uk/HGP/Chr22/.

resulting in a Serine to Glycine substitution at codon 234 (S234G, affecting nucleotide 96886 of GenBank sequence AL032818). Moreover, a G to A substitution -84 bp from exon 6 ([G/A] intron 5, affecting nucleotide 97161 of GenBank sequence AL032818), and a C to A substitution in the 3′-UTR ([C/A] 3′ UTR exon 8, affecting nucleotide 81336 of GenBank sequence AL032818) were found in two different FCHL probands. The 3 FCHL carriers of the variant alleles [C/T] intron 3, S234G and [C/A] 3′ UTR, or [G/A] intron 5 did not present with exceptional phenotypes except for remarkably low plasma HDL-C concentrations being 0.79, 0.71, and 0.68 mmol/L, respectively. Analyses of the genotypes of first-degree relatives of the FCHL probands carrying the [C/T] intron 3, S234G, [G/A] intron 5, and [C/A] 5′ UTR variant did not result in the identification of additional carriers.

PPARA intragenic genotyping

The frequency and phenotypic associations of *PPARA* genotypes were analyzed in 102 FCHL probands and 124 spouses. Table 1 presents the clinical characteristics of the case-control study population.

The WAVE DHPLC technology resulted in the identification of the recently reported L162V variant as well [17]. The V162 allele was present in 1.98% (95% Cl 0.042–3.88) of the probands and in 4.84% (95% Cl 2.15–7.55%) of the spouses ($P = 0.10$; Table 2). Interestingly, the L162V frequency in FCHL probands (1.98%) is notably lower than that reported in other Caucasian populations (varying between 2.8 and 12.8%, on average being 7%), suggesting that the 162V allele protects against expression of FCHL. Based on the limited number of individuals in the case-control panel carrying the L162V variant no additional statistical analyses were warranted. Therefore, a case-control association study was performed using two more frequent *PPARA* polymorphisms, an intron 2 [G/A] [17] and an intron 7 [G/C] polymorphism, which had become available after completion of the mutation detection analyses [17,19]. These polymorphisms were studied in one hundred and twenty-four spouses and one hundred and two unrelated FCHL probands. The genotype distribution of both polymorphisms was in Hardy–Weinberg equilibrium in the case-control panel. The intron 2 A allele was present in 9.41% (95% Cl 5.35–13%) of the FCHL probands and 9.68% (95% Cl 5.97–13%) spouses ($P = 0.92$), whereas the intron 7 C allele was present in 17% (95% Cl 11–22%) of the FCHL probands and 17% (95% Cl 12–22%) of the spouses ($P = 0.98$). No homozygotes for either variant allele were detected in the case-control panel.

Subsequently, the potential effects of the intron 2 A allele and intron 7 C allele on plasma TC, TG, HDL-C, FFA, apoAI, apoB, and apoCIII concentrations, and LDL-size were evaluated. In FCHL patients and spouses

Table 1
Clinical characteristics of the case-control study population

Trait	FCHL ($n = 102$)	Spouses ($n = 124$)	P
Male/female	41/61	73/51	0.003
Age (years)	51.4 ± 10.8	51.1 ± 11.1	NS
BMI (kg/m^2)	27.4 ± 3.25	25.3 ± 3.88	$\leqslant 0.001$
TC (mmol/L)	6.90 ± 2.4	5.5 ± 1.0	$\leqslant 0.001$
HDL-C (mmol/L)	0.92 ± 0.26	1.22 ± 0.39	$\leqslant 0.001$
TG (mmol/L)	4.4 ± 8.3	1.3 ± 0.6	$\leqslant 0.001$
ApoAI (g/L)	1.33 ± 0.27	1.44 ± 0.24	0.004
ApoB (g/L)	1.4 ± 0.34	1.0 ± 0.25	$\leqslant 0.001$
ApoCIII (mg/100 ml)	5.87 ± 1.85	4.37 ± 1.17	$\leqslant 0.001$
LDL-size (1×10^{-10} m)	259.7 ± 1.73	271.2 ± 7.88	$\leqslant 0.001$

Data represent means ± SD.

Table 2
Clinical characteristics of *PPARA* L162L and L162V allele carriers

Trait	FCHL probands ($n = 102$)		Spouses ($n = 124$)	
	L/L ($n = 98$)	L/V ($n = 4$)	L/L ($n = 112$)	L/V ($n = 12$)
Gender (F/M)	48/60	3/1	68/44	6/6
Age	51.4 ± 10.8	52.3 ± 13.5	51.4 ± 11.3	48.3 ± 9.37
BMI (kg/m^2)	27.3 ± 3.2	30.1 ± 3.5	25.2 ± 3.9	26.6 ± 3.4
TC (mmol/L)	6.93 ± 2.46	6.29 ± 1.49	5.52 ± 1.02	5.69 ± 1.02
HDL-C (mmol/L)	0.91 ± 0.25	1.14 ± 0.30	1.23 ± 0.38	1.12 ± 0.42
TG (mmol/L)	4.35 ± 8.27	1.74 ± 0.27	1.31 ± 0.59	1.47 ± 0.73
ApoAI (g/L)	1.34 ± 0.28	1.25 ± 0.25	1.45 ± 0.23	1.34 ± 0.31
ApoB (g/L)	1.40 ± 0.32	1.23 ± 0.61	0.99 ± 0.25	1.01 ± 0.30

Data represent means ± SD.

both the intron 2 A allele and intron 7 C allele carriers showed no significant associations with any of the traits tested compared to wildtype individuals (data not shown). In summary, the intragenic *PPARA* genotyping yielded no evidence of association with FCHL.

PPARA locus genotyping

In order to confirm or reject the suggestive linkage between the *PPARA* locus and FCHL in Dutch FCHL families [5], we examined the association between linkage marker D22S275 and FCHL in the case-control panel. The allele distribution of D22S275 in the case-control panel was fully consistent with the distribution predicted by Hardy–Weinberg equilibrium. The overall allele frequency distribution of D22S275 did not differ between FCHL probands and spouses ($P = 0.74$).

The complete sequencing of chromosome 22 has resulted in the availability of more reliable marker and gene position data in public databases. These novel data showed that D22S275 is situated 20 Mb from the *PPARA* gene, whereas D22S928, located within 1 Mb, is the nearest informative marker (Fig. 1). Therefore, association of this marker with FCHL was examined in the case-control panel. The allele distribution of D22S928 in the case-control panel was fully consistent with the distribution predicted by Hardy–Weinberg equilibrium. The overall allele frequency of D22S928 in FCHL probands did not differ significantly ($P = 0.34$) from that in spouses.

To study whether the *PPARA* locus is a modifier locus in FCHL, instead of a causal locus, we evaluated whether genetic variation at the *PPARA* locus associated with certain sub-phenotypes from the overall complex FCHL phenotype. Notably, the allele frequency distribution of D22S928 differed significantly ($P = 0.038$) in FCHL probands with a combined hyperlipidemic phenotype compared to isolated hyperlipidemic probands, i.e., hypercholesterolemia or hypertriglyceridemia alone (Table 3A). Analyses of individual alleles revealed that allele CA157 was significantly enriched in combined hyperlipidemic FCHL probands (56.5 vs 37.9% in isolated hyperlipidemic probands: $P = 0.010$).

PPARA gene expression has been shown to affect *APOCIII* and *APOAI* gene transcription [20] and, moreover, the *APOAI-CIII-AIV* gene cluster has been shown to affect the FCHL phenotype [21]. Therefore, associations between D22S928 alleles and plasma apoCIII and apoAI concentrations were tested. In FCHL probands with a plasma apoCIII concentration greater than the 75th percentile (apoCIII $\geqslant$ 5.9 mg/100 ml), a significantly different distribution of D22S928 alleles ($P = 0.011$) was found compared to probands with a plasma apoCIII concentration under the 75th percentile (apoCIII < 5.9 mg/100 ml: Table 3B). It was observed that D22S928 allele CA149 was significantly less frequent in FCHL probands with a high plasma apoCIII concentration (10.2 vs 33.3% in probands with plasma apoCIII < 5.9 mg/100 ml: $P < 0.0001$). Notably, the distribution of allele CA157 was similar to that found with the combined hyperlipidemia phenotype (Table 3A), but this was not statistically significant. In spouses no associations with plasma apoCIII concentrations were found, and no associations were found with plasma apoAI concentrations in both FCHL probands and spouses.

Table 3
D22S275 and D22S928 allele frequency differences

D22S928	Combined hyperlipidemic FCHL probands *N* (%)	Isolated hyperlipidemic probands *N* (%)	*P**
(A) D22S928 allele frequencies in combined hyperlipidemic FCHL probands vs isolated hyperlipidemic FCHL probands			
CA149	11 (17.7)	23 (24.2)	NS
CA157	35 (56.5)	36 (37.9)	<0.010
CA173	0 (0.0)	7 (7.4)	NS
CA175	16 (25.8)	29 (30.5)	NS
Overall frequency difference			0.038
Number of chromosomes (%)	62 (100%)	95 (100%)	
D22S928	FCHL probands with ApoCIII >75th percentile *N* (%)	FCHL probands with apoCIII <75th percentile *N* (%)	*P**
(B) D22S928 allele frequencies in FCHL probands with apoCIII >75th percentile vs FCHL probands with apoCIII <75th percentile			
CA149	5 (10.2)	18 (33.3)	<0.0001
CA157	32 (65.3)	20 (37)	NS
CA173	2 (4.1)	1 (1.9)	NS
CA175	10 (20.4)	15 (27.8)	NS
Overall frequency difference			0.011
Number of chromosomes (%)	49 (100%)	54 (100%)	

*P** represents Bonferroni corrected values.

Table 4
Effects of D22S928 CA149 and CA157 alleles on FCHL quantitative traits in FCHL probands and spouses

Traits (mmol/L)	FCHL probands ($N = 102$)				Spouses ($N = 124$)			
	x/x ($N = 69$)	x/149 and 149/149 ($N = 33$)	P^*	$P^\#$	x/x ($N = 61$)	x/149 and 149/149 ($N = 57$)	P^*	$P^\#$
(A) Effects of CA149 allele on quantitative traits in FCHL probands and spouses								
BMI (kg/m^2)	27.4 ± 2.90	27.3 ± 3.96	NS	NS	25.3 ± 4.0	25.5 ± 3.5	NS	NS
TC	6.8 ± 1.6	7.2 ± 3.6	NS	NS	5.5 ± 1.1	5.6 ± 0.9	NS	NS
HDL-C	0.93 ± 0.29	0.92 ± 0.19	NS	NS	1.21 ± 0.38	1.23 ± 0.43	NS	NS
TG	2.8 ± 1.7	3.2 ± 2.4	NS	NS	1.2 ± 1.6	1.2 ± 1.4	NS	NS
FFA	0.42 ± 0.18	0.57 ± 0.24	0.007	0.014	0.49 ± 0.21	0.47 ± 0.19	NS	NS
ApoAI (g/L)	1.33 ± 0.30	1.33 ± 0.23	NS	NS	1.44 ± 0.24	1.43 ± 0.25	NS	NS
ApoB (g/L)	1.39 ± 0.34	1.39 ± 0.34	NS	NS	1.00 ± 0.26	0.97 ± 0.22	NS	NS
ApoCIII (mg/100 ml)	6.23 ± 1.73	5.23 ± 1.91	0.035	0.082	4.22 ± 1.04	4.79 ± 1.43	NS	NS
LDL-size (1×10^{10} m)	260.4 ± 10.5	258.3 ± 9.96	NS	NS	270.6 ± 8.04	270.6 ± 7.38	NS	NS
Traits (mmol/L)	FCHL probands ($N = 102$)				Spouses ($N = 124$)			
	x/x ($N = 45$)	x/157 and 157/157 ($N = 57$)	P^*	$P^\#$	x/x ($N = 51$)	x/157 and 157/157 ($N = 73$)	P^*	$P^\#$
(B) Effects of CA157 allele on FCHL quantitative traits in FCHL probands and spouses								
BMI (kg/m^2)	27.8 ± 3.47	26.9 ± 3.02	NS	NS	25.4 ± 3.3	25.3 ± 4.3	NS	NS
TC	6.7 ± 1.7	7.1 ± 2.9	NS	NS	5.6 ± 1.1	5.5 ± 1.0	NS	NS
HDL-C	0.92 ± 0.22	0.93 ± 0.28	NS	NS	1.11 ± 0.26	1.30 ± 0.44	0.004	0.011
TG	2.7 ± 1.7	3.2 ± 2.1	NS	NS	1.2 ± 1.5	1.21 ± 1.6	NS	NS
FFA	0.49 ± 0.25	0.46 ± 0.18	NS	NS	0.46 ± 0.19	0.50 ± 0.22	NS	NS
ApoAI (g/L)	1.31 ± 0.23	1.35 ± 0.31	NS	NS	1.41 ± 0.22	1.46 ± 0.25	NS	NS
ApoB (g/L)	1.37 ± 0.29	1.41 ± 0.38	NS	NS	1.02 ± 0.22	0.98 ± 0.27	NS	NS
ApoCIII (mg/100 ml)	5.47 ± 1.83	6.11 ± 1.84	NS	NS	4.22 ± 1.31	4.45 ± 1.07	NS	NS
LDL-size (1×10^{10} m)	257.1 ± 9.15	261.7 ± 10.8	NS	NS	269.8 ± 7.66	272.2 ± 7.96	NS	NS

Data represent means ± SD.
P values are provided for two separate analyses. *Unadjusted using Student's t test. #Adjusted for age and BMI using logistic regression.

Genotype–phenotype relations

Based on the observed association in FCHL probands of D22S928 allele CA149 with plasma apoCIII concentrations and allele CA157 with combined hyperlipidemia, we studied the effect of these alleles on quantitative lipid traits (Table 4). FCHL probands carrying allele CA149 had significantly decreased plasma apoCIII concentrations (unadjusted $P = 0.035$, adjusted $P = 0.082$: Table 4A), and elevated plasma FFA concentrations (unadjusted $P = 0.007$, adjusted $P = 0.014$). In spouses, allele CA149 carriers showed no significant associations with plasma lipid traits compared to non-carriers (Table 4A). When the effect of the CA157 allele was studied in FCHL probands no significant associations were found (Table 4B). In contrast, spouses carrying allele CA157 had significantly higher plasma HDL-C levels compared to non-carriers (unadjusted $P = 0.004$, adjusted $P = 0.011$; Table 4B).

Discussion

The objective of the present study was to establish the involvement of the *PPARA* locus (chromosome 22q13.3) in FCHL. A previous linkage study detected at best suggestive linkage between the *PPARA* locus and FCHL when multiple genes were tested, and a limited number of FCHL families were used [5]. The present data show that the *PPARA* locus influences plasma apoCIII concentrations and the combined hyperlipidemic phenotype in FCHL subjects, but not in spouses, indicating that this gene modifies the expression instead of contributing to the FCHL phenotype in a direct manner.

Mutation detection experiments of the coding region of the *PPARA* gene, led to the identification of four novel mutations and the previously reported L162V variant in FCHL probands. No exceptional lipid phenotypes were observed except that the three FCHL probands each carrying one of the novel variants had remarkably low plasma HDL-C levels. The prevalence of the L162V variant in the spouse group was similar to previously reported general population frequencies [15,17], but unfortunately, in FCHL probands there were not enough carriers to enable an association study. Therefore, two recently identified non-coding *PPARA* polymorphisms in intron 2 and 7 were studied, but these did not associate with FCHL. Our results do not exclude *PPARA* as a modifier gene in FCHL since the biological role of these polymorphisms remains to be established. Ideally, a polymorphism should represent a functional change in physiology, but at present no coding polymorphisms

with a frequency greater than 10% are available for the *PPARA* gene [19]. The lack of association with these noncoding SNPs could, in theory, also be due to the limited number of subjects tested. However, in populations of similar size significant associations with the intron 2 polymorphism have been reported [17].

The absence of association between markers D22S275 and D22S928 and FCHL, indicates that DNA variation in the *PPARA* locus does not primarily contribute to FCHL, but is more likely a modifier locus. The strategy of sub-phenotyping has previously been shown to be very effective in other disorders [22], and therefore this strategy was applied using D22S928, the nearest informative marker to the *PPARA* locus. D22S928 showed significant association with plasma apoCIII levels and, remarkably, also with the combined hyperlipidemic phenotype in FCHL probands, showing that the *PPARA* locus modifies the combined lipoprotein phenotype.

Marker D22S928 is situated within 1 Mb upstream of the *PPARA* promoter, and given its modifying effect on characteristic FCHL traits that are under transcriptional control of the *PPARA* gene, *PPARA* is most likely responsible for this effect. Specifically, CA157 was shown to increase the likelihood of FCHL subjects to present with combined hyperlipidemia, while all other D22S928 alleles were underrepresented, suggesting that they protect against the expression of the combined hyperlipidemic phenotype of FCHL. The data also suggest that the CA157 allele is a marker for decreased PPARα activity. The frequency of allele CA149 was significantly higher in FCHL probands with low apoCIII (<75th percentile) which suggests, given the role of PPARα in the inhibition of apoCIII expression [23,24], that CA149 is in allelic association with variants which are associated with increased PPARα activity. Intragenic screening of *PPARA* could not confirm nor exclude this. In our view, the fact that D22S928 is situated 1 Mb from *PPARA* implies that one or more functional polymorphism(s) that are in linkage disequilibrium with D22S928 alleles CA149 and CA157 may be present in the *PPARA* promoter or other regulatory sequences. Promoter polymorphisms can affect transcription of the *PPARA* gene and result in either increased or decreased expression.

ApoC-III is an apolipoprotein that, when present in high concentrations, limits the clearance of triglycerides from the circulation. Under normal circumstances, PPARα activation increases lipolysis through increased lipoprotein lipase gene expression [25] and promotes hepatic clearance of triglycerides through a decrease in apoCIII expression. PPARα also enhances transcription of the HDL apolipoproteins AI and AII, thereby increasing HDL-C production. The observed modifying effect of the *PPARA* locus on plasma apoCIII concentrations in combination with the positive effects of fibrate treatment on lipid traits in FCHL patients, such as HDL-C, apoCIII, and TG [14], imply a role of *PPARA* in the pathophysiology of the FCHL phenotype [20]. Elevated plasma apoCIII concentrations, and low plasma HDL-C concentrations, both well known FCHL characteristics [26] which have been shown to be associated with *PPARA* alleles in this study, imply decreased PPARα function. Decreased PPARα function can result in decreased hepatic FFA uptake secondary to lowered fatty acid binding protein (FABP) and fatty acid transport protein (FATP) expression [13,22]. Moreover, those FFAs taken up will be preferably used for TG synthesis because of decreased FFA β-oxidation. Increased hepatic triglyceride synthesis will contribute to increased VLDL production, more apoC-III containing remnant lipoproteins, and a decrease in HDL-cholesterol through decreased apoA-I and apoA-II gene expression [11]. Thus, reduced PPARα activity can contribute to the FCHL atherogenic lipoprotein phenotype in multiple ways. In addition, other genes have been identified recently that are directly or indirectly under transcriptional control of PPARα, such as sterol regulatory element-binding protein (SERBP)-regulated genes, involved in cholesterol synthesis [28], phospholipid transfer protein (PLTP), involved in HDL particle size and lipid composition modulation [29,30], and cholesterol 7α-hydroxylase (CYP7A1), involved in the conversion of cholesterol in bile-acids [31]. It is possible that one PPARα metabolic pathway will contribute to the complex phenotype of FCHL. To unequivocally include *PPARA* as a modifier gene in FCHL, its biological function in human FCHL hepatic tissue should be studied, for instance by gene-expression studies, but at this time human FCHL liver samples are very hard to obtain. Until such data are available, another gene responsible for the observed effects cannot be excluded.

In conclusion, the present data showed that *PPARA* has a modifying effect on plasma apoCIII concentrations and the combined hyperlipidemic phenotype in FCHL subjects but not in spouses. In spouses, a modifying effect on HDL-C concentrations was found. These results clearly demonstrate the genetically complex nature of FCHL and identify the *PPARA* locus as a modifier locus of the FCHL lipid-phenotype.

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