Association of plasma lipids and apolipoproteins with the insulin response element in the apoC-III promoter region in familial combined hyperlipidemia

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Abstract The apoAI-CIII-AIV gene cluster, located on chromosome 11, contributes to the phenotype of familial combined hyperlipidemia (FCH), but this contribution is genetically complex. Combinations of haplotypes, based on three restriction enzyme polymorphisms: XmnI and MspI sites, 5′ of the start site of the apoA-I gene and SstI polymorphism in the 3′ untranslated region of exon 4 of the apoC-III gene, were analyzed to characterize their effect on the expression of severe hyperlipidemia. An epistatic interaction was demonstrated: the S2 allele on one haplotype was synergistic in its hyperlipidemic effect to the X2M2 allele on the other haplotype (Dallinga-Thie, G. M. et al. ***J. Clin. Invest.* **1997.** 99: **953–961). In the present study two additional polymorphic sites in the insulin response element (IRE) of the apoC-III gene promoter, T-455C: FokI restriction site, C-482T: MspI restriction site, were studied in 34 FCH pedigrees including 34 probands, 220 hyperlipidemic relatives, 300 normolipidemic relatives, and 236 spouses. In contrast to the earlier data for the other polymorphisms in this gene cluster (XmnI, MspI/AI, and SstI), there were no differences in frequency distributions of the T-455C and the C-482T variants between probands, hyperlipidemic and normolipidemic relatives and spouses. No significant associations between plasma lipid traits and DNA variants in the IRE were observed. Analysis of combinations of haplotypes based on the five polymorphisms in the gene cluster provided further evidence for a dominant role of the SstI polymorphism as a major susceptibility locus in FCH. The inclusion of the IRE markers did not improve genetic informativeness, nor our understanding of the observed synergistic relationship associated with the high risk combination of haplotypes in FCH families.**—Groenendijk, M., R. M. Cantor, N. H. H. C. Blom, J. I. Rotter, T. W. A. de Bruin, and G. M. Dallinga-Thie. **Association of plasma lipids and apolipoproteins with the insulin response element in the apoC-III promoter region in familial combined hyperlipidemia.** ***J. Lipid Res.*** **1999.** 40: **1036–1044.**

Supplementary key words familial combined hyperlipidemia • apolipoprotein CIII • insulin response element • gene–gene interaction

Hypertriglyceridemia is correlated with plasma apoC-III levels in the population (1). In FCH subjects, apoC-III levels are specifically related to the impaired postprandial elimination of chylomicron remnants (2), thereby supporting a role for apoC-III in the metabolism of apoB-containing lipoproteins. ApoC-III is a protein of 79 amino acids that is synthesized in the liver and to a lesser degree in the intestine. It is an exchangeable moiety of chylomicron remnants and very low density lipoproteins (VLDL), and to a minor extent of high density lipoproteins (HDL). The in vivo function of apoC-III is poorly understood. A role for apoC-III in the regulation of lipolysis of triglyceride-rich lipoproteins by inhibiting the function of lipoprotein lipase (LPL) has been postulated (3, 4). In vitro studies have shown that apoC-III inhibits lipoprotein lipase (LPL) activity, thereby reducing the capacity to hydrolyze triglyceride-rich particles (5). Furthermore, involvement of apoC-III in the processes of binding of lipoprotein particles to specific receptors in the liver (6–10) has been documented.

The human apoC-III gene has been mapped on chromosome 11 and contains four exons and three introns. It is closely linked to the apoA-IV and apoA-I genes. To further support the concept that apoC-III is involved in the metabolism of triglyceride-rich lipoproteins, studies with transgenic and knockout animals have been performed in several laboratories. Overexpression of the human apoC-III gene in transgenic mice resulted in severe hypertriglyceridemia (11, 12) due to accumulation of large triglyceride-

Abbreviations: bp, base pairs; HDL, high density lipoproteins; VLDL, very low density lipoproteins; LDL, low density lipoproteins; apo, apolipoproteins; PCR, polymerase chain reaction; FCH, familial combined hyperlipidemia; IRE, insulin response element.

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rich particles enriched with apoC-III but poor in apoE. Crossbreeding of apoC-III transgenes with human apoE transgenic mice results in normalization of the hypertriglyceridemia (13), which further supports the observed functional relationship between apoE and apoC-III on the surface of these triglyceride-rich lipoproteins. Further conformation of this hypothesis was obtained from studies using mice with a disrupted apoC-III gene (3). These mice were normotriglyceridemic and their postprandial triglyceride clearance is better than in control mice.

Numerous studies have found an association between the presence of a polymorphic SstI site in the untranslated region of the apoC-III gene with hypertriglyceridemia (1, 14–25). There are exceptions to this relationship, for example, in a cohort of CAD patients from the French Canadian population (26) and in a cohort of CAD patients from Great Britain (27) these associations were not observed. Dammerman et al. (15) showed that the SstI polymorphism is linked to two polymorphic nucleotides changes in the apoC-III gene promoter region at −455 and −482. These two polymorphic sites are mapped within an insulin response element, located at −490 to −449 from the start site of the apoC-III gene (28). In vitro studies (29) showed that insulin was capable of down-regulating apoC-III gene transcription. Presence of the two IRE variants results in a loss of this down-regulatory mechanism (28). The hypothesis was put forward that the −455 and −482 variants may contribute to hypertriglyceridemia in humans, presumably by elevating apoC-III plasma concentrations. However, two recent studies did not support the above-described hypothesis. No contribution of the two DNA variants in the promoter of the apoC-III gene to the variation in plasma apoC-III and triglyceride levels was found in a population of Italian schoolchildren (18) nor in subjects from the ARIC study (17).

Familial combined hyperlipidemia (FCH), described by Goldstein et al. in 1973 (30), is a common genetic lipid disorder in Western society, resulting in elevated plasma triglyceride (TG) and plasma cholesterol concentrations (31). The genetic defects underlying FCH are yet unknown. In a large study using 18 well-characterized FCH kindred, we evaluated three polymorphisms in the apoAI-CIII-AIV gene cluster and their associations with lipid and apolipoprotein phenotypes. It was shown that variations in this gene cluster are not the primary defect in FCH, but confer a specific modifying effect on plasma triglycerides and LDL cholesterol (14). A more detailed analysis revealed a specific high risk combination of haplotypes based on these three polymorphic markers within this cluster, XmnI, MspI, and SstI, indicating that two different susceptibility loci for FCH exist within this gene complex (32). Subjects carrying a combination of haplotypes X2M2S1/X1M1S1 (2-2-1/1-1-1) have elevated plasma concentrations of apoA-I and cholesterol, whereas individuals carrying a combination of haplotypes based on only the minor allele for SstI (S2), have elevated levels of triglycerides and apoC-III. A specific combination of haplotypes with one chromosome carrying the X1M1S2 haplotype and the other chromosome the X2M2S1 haplotype was associated with a more severe expression of the hyperlipidemic phenotype, revealing a synergistic effect (32). Therefore, two susceptibility loci exist in this gene cluster demonstrating the paradigm of complex genetic contribution to FCH.

In the present study we further explored this high risk combination of haplotypes in 34 families including 34 probands, 220 affected relatives, 300 non-affected relatives, and 236 spouses by analyzing two additional markers in the insulin response element in the promoter region of the apo C-III gene: −455 and −482 variants, and asking whether these variants contribute to the FCH lipid phenotype.

SUBJECTS AND MATERIALS

Index subjects

Thirty-four unrelated, Dutch Caucasian index patients were recruited from the Lipid Clinic of the Utrecht University Hospital. These subjects met the criteria described previously (2, 30, 31), including: *a*) a primary hyperlipidemia with varying phenotypic expression, including a fasting plasma cholesterol concentration >6.5 mmol/L or >95th percentile for age, defined according to tables from the Lipid Research Clinics, and/or fasting plasma triglyceride concentration >2.3 mmol/L and elevated plasma apoB concentrations, exceeding the mean ± 2 SD for age adjusted levels; *b*) at least one first degree relative with a different hyperlipidemic phenotype from the proband; *c*) a positive family history of premature coronary artery disease, defined as myocardial infarction or cerebrovascular disease before the age of 60 years in at least one blood-related subject or the index patient; and *d*) absence of xanthomas. Exclusion criteria included diabetes, familial hypercholesterolemia (absence of isolated elevated plasma LDL cholesterol levels and tendon xanthomas), and type III hyperlipidemia (apoE2/E2 genotype). All subjects gave informed consent. The study protocol was approved by the Human Investigation Review Committee of the University Hospital Utrecht. An attempt was made to collect all relatives and spouses of the index patients, without any selection. Hyperlipidemic relatives (n = 220) were assigned the FCH phenotype when they met the following criteria: plasma cholesterol levels >6.5 mmol/L and/or plasma triglycerides >2.3 mmol/L. As a consequence, there were 300 'normolipidemic' relatives. The spouse group (n = 236) represented an environment-matched, nutrition-matched, and age-matched control group for the relatives.

Analytical methods

Venous blood was drawn after an overnight fast of 12–14 h and abstention from alcohol use for at least 48 h. Plasma was prepared by immediate centrifugation for analytical analysis. Lipids and apolipoproteins were quantified by methods as described elsewhere (2, 14, 33). Plasma glucose was determined using the GOD assay as used in routine clinical chemistry and plasma insulin was measured using a radioimmunoassay (RIA).

DNA amplification and genotyping

DNA was isolated from 10 mL of EDTA-augmented blood following standard procedures (34) and amplified by the polymerase chain reaction (PCR) technique in a Thermal cycle apparatus (Pharmacia, Uppsala, Sweden). The XmnI (C-2500T), MspI (G-78A) both at the 5′ site of the apoA-I gene and SstI (G-3175C) in the 3′ untranslated region of the apoC-III gene cluster were typed as described (14). Two polymorphic markers in the insulin response element in the promoter region of the apoC-III gene were amplified using primers 3′-TTCACACTGGAATTTC

AGGCC-5′ (antisense) and 3′-GGATTGAAACCCAGAGATGG AGGTG-5′ (sense) under the following conditions: denaturation for 4 min at 94°C, and 33 cycles of denaturation for 1 min at 94°C, annealing and extension for 1 min at 55°C and 72°C, respectively, and a final extension at 72°C for 5 min. The T-455C variation represents a FokI restriction site whereas the C-482T represents a site for the restriction enzyme MspI. Restriction enzymes were added to 20 μL PCR product (FokI: 4 U and MspI: 10 U (Boehringer Mannheim, Mannheim, Germany)) in a final volume of 30 μL. The incubations were performed at 37°C for at least 1 h. The products were resolved on 3% agarose gels. Alleles were defined as 1 or 2 based on absence or presence of the restriction site.

Statistical methods

Results are expressed as means ± SEM (standard error of the mean). Frequencies of the five polymorphisms were estimated by allele counting. Deviations from the Hardy-Weinberg equilibrium were tested using the chi-square goodness-of-fit test. Linkage disequilibrium coefficients between polymorphic markers in the apoAI-CIII-AIV gene cluster in spouses were estimated using the linkage utility program EH (estimate haplotype frequencies) (35). The chi-square statistics have one degree of freedom because under the hypothesis of no-allelic association there are only two free parameters: the gene frequencies. Proportions of haplotypes and combinations of haplotypes were assigned by examining the cosegregation of individual alleles according to Mendelian inheritance within the 34 families, using the principle of parsimony. The transmission/disequilibrium test (TDT) was used to test for association between alleles and phenotypes. The TDT test requires data from affected individuals and their parents who are heterozygous for a marker allele. It evaluates unequal transmission of alleles from heterozygous parents to the affected offspring (36). Comparisons between plasma lipid levels and apolipoprotein traits for the observed genotypes were tested in unrelated probands and spouses using Student's *t*-test.

RESULTS

Subject characteristics

Clinical and biochemical characteristics of the 34 probands, their hyperlipidemic and normolipidemic relatives and spouse controls are summarized in **Table 1**. The probands and hyperlipidemic relatives were characterized by an increased BMI, WHR, and elevated plasma lipids and apolipoproteins as compared to normolipidemic relatives and spouses. Plasma insulin and glucose levels were elevated in the affected group. Of the affected individuals (probands and hyperlipidemic relatives), 38% and 35%, respectively, showed fasting plasma insulin levels above 11 mU/L, an arbitrary hyperinsulinemic cut-off point used in the clinic to indicate predisposition towards insulin resistance. In the groups of normolipidemic relatives and spouses, 16.6% and 19.2%, respectively, of the individuals had fasting plasma insulin levels above 11 mU/L. From Table 1 it is clear that there is a preponderance of male subjects in both the probands and affected relatives groups, whereas in the spouse group the number of females is increased.

Polymorphisms of the apoAI-CIII-AIV gene cluster

In the promoter region of the apoC-III gene, an insulin response element has been mapped to −449 to −490 (15, 28). Two variations in this element have been described: a T to C substitution at −455 representing a FokI restriction site and at −482 a C to T substitution, representing a MspI restriction site. Downstream from this element three additional variations were mapped: a T deletion at −625, a G to A substitution at −630 and a C to A substitution at −641. These polymorphisms are in strong linkage disequilibrium with each other and with the IRE polymorphisms and are therefore not informative. **Figure 1** shows the genotype frequencies of the two IRE polymorphisms and **Table 2** presents the allele frequencies. The observed frequencies are consistent with the limits of the Hardy-Weinberg law. The frequency distribution of the T-455C polymorphism was similar in the four groups. There was no enrichment of the minor alleles for this marker in either probands or affected relatives as compared to normolipidemic relatives and spouses. The frequencies of the T-455C in the spouse group are very similar to that observed in other populations (15, 17, 18). No difference was observed in the frequency distribution of the C-482T

TABLE 1. Characteristics of the studied population

Variable	Probands	HL Individuals	NL Relatives	Spouses
Number	34	220	300	236
Age (yr)	51 ± 2	46 ± 1	32 ± 1	48 ± 1
Gender (M/F)	25/9	122/97	151/149	95/141
BMI (kg/m^2)	26.8 ± 0.6	25.8 ± 0.3	23.4 ± 0.3	25.1 ± 0.3
Waist Hip Ratio	0.92 ± 0.02	0.89 ± 0.01	0.82 ± 0.01	0.85 ± 0.01
ApoA-I (mg/dL)	127 ± 6	136 ± 2	133 ± 1	140 ± 2
ApoB (mg/dL)	140 ± 6	129 ± 2	85 ± 1	101 ± 2
ApoC-III (mg/dL)	14.6 ± 2.6	11.8 ± 0.3	7.5 ± 0.2	8.7 ± 0.2
Chol (mmol/L)	9.30 ± 0.87	6.96 ± 0.09	4.95 ± 0.05	5.66 ± 0.07
HDL-chol (mmol/L)	1.00 ± 0.05	1.15 ± 0.02	1.21 ± 0.02	1.25 ± 0.02
LDL-chol (mmol/L)	4.89 ± 0.33	4.58 ± 0.09	3.15 ± 0.04	3.70 ± 0.06
TG (mmol/L)	8.20 ± 2.73	2.75 ± 0.17	1.30 ± 0.03	1.63 ± 0.07
NEFA (mmol/l)	0.47 ± 0.04	0.56 ± 0.02	0.51 ± 0.02	0.52 ± 0.02
Insulin (mU/L)	10.8 ± 1.2	10.4 ± 0.8	7.1 ± 0.3	7.8 ± 0.6
Glucose (mmol/L)	5.50 ± 0.54	4.95 ± 0.11	4.55 ± 0.08	4.79 ± 0.06

Values are expressed as means ± SEM; HL, hyperlipidemic; NL, normolipidemic; NEFA, nonesterified free fatty acids.

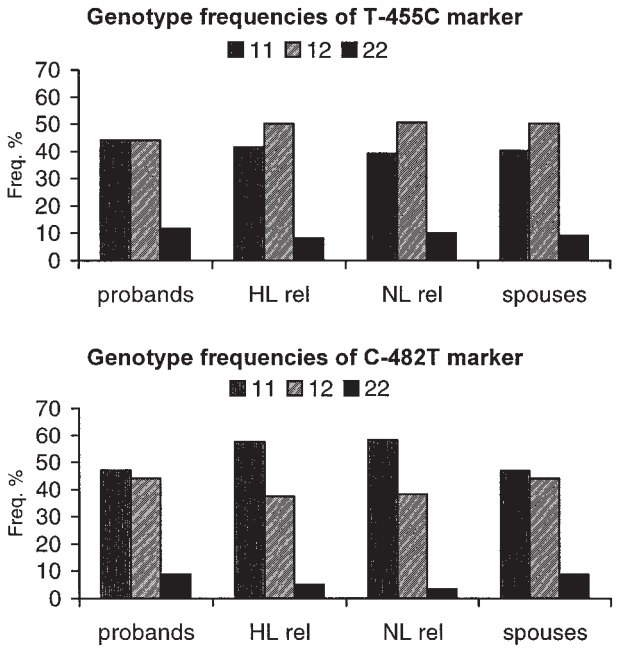

Fig. 1. Genotype frequencies of T-455C (FokI) and C-482T (MspI-CIII) polymorphisms near the insulin response element in the apoC-III gene promoter region; HL rel, hyperlipidemic relatives; NL rel, normolipidemic relatives.

polymorphism in the FCH kindred. The two polymorphisms were in strong linkage disequilibrium with each other and with SstI and XmnI polymorphisms. They were not in linkage disequilibrium with the G-78A (MspI-AI) marker. The SstI marker was not in linkage disequilibrium with the XmnI marker (**Table 3**). TDT analysis showed that the major allele of the C-482T polymorphism was transmitted 42 times to the affected offspring, whereas the minor allele was transmited 32 times ($\chi^2 = 1.35$). Here individuals who were heterozygous for the IRE variants and not for the SstI polymorphism were analyzed to exclude the effect of the SstI polymorphism. TDT analysis of the SstI marker showed that the S2 allele was not transmitted significantly more frequently than the S1 allele ($\chi^2 = 2.13$) (**Table 4**).

Haplotype analysis using five polymorphic markers: XmnI (C-2500T), MspI (G-78A) both at the 5′ end of the apoA-I gene, SstI (G3175C) in the untranslated region of the apoC-III gene, and FokI (T-455C) and MspI (C-482T) in the promoter region of the apoC-III gene was performed. Haplotypes were assigned using parsimony coinheritance in the 34 families, and resulted in 15 observed out of 32 theoretical possible haplotypes (**Table 5**). Because there was no difference in the frequency distribution of the markers between men and women, they were analyzed as one group. The 'wild-type' haplotype 1-1-1-1-1 (in the order: XmnI, MspI, SstI, T-455C, C-482T) had the highest observed frequency in the spouses (53%) and decreased slightly going from spouses to normolipidemic relatives (49%), hyperlipidemic relatives (47%), and probands (44%). This reduced occurrence of the common haplotype in probands was related to an increase in frequency of the 1-1-2-2-2 haplotype ($P < 0.01$, 15% versus 5% in spouses) and the 2-2-1-1-1 haplotype: probands 21%, spouses 11% ($P < 0.01$). The frequencies of the 1-1-1-2-1 haplotype and the 1-1-1-2-2 haplotype were decreased in probands (3% and 12%) versus spouses (12%, $P < 0.03$ and 15%, ns). The 2-2-1-2-2 haplotype was only found in hyperlipidemic relatives (n = 9) and not in probands, normolipidemic relatives or spouses.

Combinations of haplotypes were analyzed, in order to relate the occurring genotype with variations in plasma lipid and apolipoprotein traits. **Table 6** summarizes the most frequently occurring combinations of haplotypes, assigned by inheritance in the FCH families. We only observed 39 combinations of haplotypes, whereas the number of expected combinations based on 15 haplotypes should be 120. One of the reasons for this discrepancy is the fact that some of the markers are in linkage disequilib-

TABLE 2. Allele frequencies of the IRE polymorphisms in the FCH population

	Probands (n = 34)	HL Rel (n = 219)	NL Rel (n = 300)	Spouses (n = 236)
T-455C (FokI)				
1	0.662 (45)	0.667 (292)	0.647 (388)	0.655 (309)
2	0.338 (23)	0.333 (146)	0.353 (212)	0.345 (163)
C-482T (MspI)				
1	0.691 (47)	0.763 (334)	0.775 (465)	0.771 (364)
2	0.309 (21)	0.237 (104)	0.225 (135)	0.229 (108)

P values were tested using chi-square test; HL rel, hyperlipidemic relatives; NL rel, normolipidemic relatives.

TABLE 3. Pairwise linkage disequilibrium coefficients in spouses

	MspI-AI	SstI	FokI	MspI-CIII
XmnI	214[a]	2.7	16.7[a]	7.7[a]
MspI-AI		7.3[a]	3.1	0.06
SstI			15.4[a]	29.3[a]
FokI				122[a]

Chi-squares were calculated using the program EH (35) using 1 degree of freedom.

[a] Significant, $P < 0.0001$.

TABLE 4. Transmission of markers to affected offspring

Transmitted Allele	Nontransmitted Allele		Chi-square
Transmission of the C-482T marker to affected offspring (Sst1 = 11)			
	1	2	1.35, ns
1	129	42	
2	32	3	
Transmission of the T-455 marker to affected offspring (SstI = 11)			
	1	2	0.81, ns
1	104	54	
2	45	3	
Transmission of the SstI marker to affected offspring			
	1	2	2.13, ns
1	197	8	
2	15	3	

P values were tested using the chi-square test; ns, not significant.

TABLE 5. Frequencies of haplotypes in the 34 FCH families

Haplotypes X-M-S-F-m	Probands (n = 68)	HL Rel (n = 436)	NL Rel (n = 600)	Spouses (n = 460)
1-1-1-1-1	44.1 (30)	47.0 (205)	48.5 (291)	52.8 (243)
2-2-1-1-1	20.6 (14)[a]	18.6 (81)	15.7 (94)	10.9 (50)
2-2-1-2-1	—	—	0.2 (1)	0.2 (1)
2-2-1-2-2	—	2.1 (9)	—	—
1-2-1-1-2	—	—	0.2 (1)	0.4 (2)
1-2-1-2-2	4.4 (3)	2.3 (10)	2.3 (14)	2.0 (9)
1-1-2-1-1	—	—	0.5 (3)	0.7 (3)
1-1-2-2-1	—	—	—	0.2 (1)
1-1-2-2-2	14.7 (10)[b]	8.9 (39)	6.2 (37)	5.0 (23)
1-1-1-1-2	—	0.7 (3)	—	0.9 (4)
1-1-1-2-1	2.9 (2)[a]	10.3 (45)	12.5 (75)	12.0 (55)
1-1-1-2-2	11.8 (8)	9.9 (43)	13.8 (83)	14.6 (67)
1-2-1-2-1	—	—	—	0.2 (1)
1-2-1-1-1	1.5 (1)	0.2 (1)	0.2 (1)	—
1-1-2-1-2	—	—	—	0.2 (1)

P values were tested using chi-square test; X, XmnI; M, MspI; S, SstI; F, FokI (T-455C); m, MspI (C-482T).
[a] Significant difference vs. spouses, $P < 0.03$.
[b] Significant difference vs. spouses, $P < 0.01$.

rium with each other, resulting in overrepresentation of certain combinations of alleles.

As discussed earlier, the combinations of haplotypes can be divided into groups based on their modifying effect on lipid metabolism. Based on the observation that in our FCH families the S2 allele always resides on a different haplotype than the M2 allele, we proposed a model wherein the S2 allele was defined as a susceptibility allele

TABLE 6. Frequencies of the most frequently occurring combinations of haplotypes with the IRE polymorphic alleles (−455 and −482)

Haplotype Combinations X-M-S-F-m / X-M-S-F-m	Probands (n = 34)	HL Rel (n = 218)	NL Rel (n = 300)	Spouse (n = 230)
Wild-type				
1-1-1-1-1 / 1-1-1-1-1	**20.6 (7)**	**18.8 (41)**	**21.3 (64)**	**25.7 (59)**
Permissive				
1-1-1-1-1 / 2-2-1-1-1	**17.6 (6)**	**20.2 (44)**	**16.7 (50)**	**13.0 (30)**
2-2-1-1-1 / 1-1-1-2-2	5.9 (2)	5.5 (12)	6.0 (18)	4.8 (11)
2-2-1-1-1 / 1-1-1-2-1	—	4.6 (10)	3.3 (10)	1.7 (4)
2-2-1-1-1 / 1-2-1-1-1	2.9 (1)	0.5 (1)	0.3 (1)	—
1-1-1-1-1 / 1-2-1-2-2	5.9 (2)	3.2 (7)	2.3 (7)	0.9 (2)
1-1-1-1-1 / 2-2-1-2-2	—	2.8 (6)	—	—
Neutral IRE variant				
1-1-1-1-1 / 1-1-1-2-2	**11.8 (4)**	**8.7 (19)**	**14.7 (44)**	**18.3 (42)**
1-1-1-1-1 / 1-1-1-2-1	2.9 (1)	11.5 (25)	12.7 (38)	13.9 (32)
1-1-1-2-2 / 1-1-1-2-2	—	2.3 (5)	0.7 (2)	0.9 (2)
1-1-1-2-2 / 1-1-1-2-1	—	—	3.0 (9)	1.7 (4)
1-1-1-2-1 / 1-1-1-2-1	—	1.4 (3)	2.3 (7)	2.2 (5)
Susceptible				
1-1-1-1-1 / 1-1-2-2-2	**8.8 (3)**	**9.2 (20)**	**7.0 (21)**	**6.1 (14)**
1-1-2-2-2 / 1-1-2-2-2	2.9 (1)	0.9 (2)	—	0.4 (1)
1-1-2-2-2 / 1-1-1-2-2	2.9 (1)	0.5 (1)	1.3 (4)	1.3 (3)
1-1-2-2-2 / 1-1-1-2-1	2.9 (1)	0.9 (2)	1.0 (3)	1.3 (3)
Highly susceptible				
2-2-1-1-1 / 1-1-2-2-2	**8.8 (3)**[a]	**4.6 (10)**	**2.7 (8)**	**0.4 (1)**
2-2-1-2-2 / 1-1-2-2-2	—	0.5 (1)	—	—

P values were tested using chi-square test. Of the 39 observed combinations of haplotypes, only the most frequently occurring combinations are shown; X, XmnI; M, MspI; S, SstI; F, FokI (T-455C); m, MspI (C-482T).
[a] Significant difference versus spouses, $P < 0.001$.

because it behaved like a dominant allele for traits that are part of the FCH phenotype. The effect of the M2 allele remains unclear, however it does not appear to act in a dominant fashion. If the 'wild-type' locus is a resistance locus for hyperlipidemia then the M2 allele renders the locus permissive to the hyperlipidemic effect of other genes, one being the S2 allele when it occurs on the other haplotype. The frequency of the wild-type combination of haplotypes 1-1-1-1-1 / 1-1-1-1-1 (XmnI, MspI, SstI, T-455C, C-482T / XmnI, MspI, SstI, T-455C, C-482T) decreased gradually, with a gradient from 26% in spouses, 21% in normolipidemic relatives, down to 21% and 19% in probands and in hyperlipidemic relatives. If we consider the permissive combinations of haplotypes it will be evident that no difference in frequencies was observed among the groups. The most frequently occurring combination of haplotypes is the 1-1-1-1-1 / 2-2-1-1-1 (13% in spouses, 17% normolipidemic relatives, 20% hyperlipidemic relatives, and 18% in probands). The IRE variants alone account for 32% of the combinations in spouses, 27% in normolipidemic relatives, 20% in hyperlipidemic relatives, and 15% in probands. Because of the higher frequency in spouses as compared to probands ($P = 0.06$) we consider this combination of haplotypes neutral or even protective. The susceptibility combinations of haplotypes are more frequent in the affected groups, but these differences were not statistically significant. If individuals are characterized by the presence of both the S2 allele and the M2 allele in the *trans* configuration, we observe synergism concerning their effect on plasma lipid traits as we described before (14). We were now able to further extend this highly susceptible combination of haplotypes with the two IRE variants. All individuals characterized with this combination of haplotypes were heterozygous for the IRE variants and these variations reside on the same allele as the S2 marker (Table 6). In probands (9%, $P < 0.001$) there was a significant increase in frequency of this combination of haplotypes as compared to spouses (0.4%). Within the kindred, some individuals were characterized by a combination of the X2M2 marker on one allele and the IRE variants on the other allele in *trans* configuration. Only in the hyperlipidemic relative group were we able to identify individuals with a similar combination of haplotypes but then in *cis* configuration. We do not know yet whether this has any functional implications because the number is very small.

Effect of combined haplotypes on quantitative phenotypes

The two IRE markers are in complete linkage disequilibrium (Table 3) which means that we used the observed haplotypes for the association studies. The frequency of the T-455C polymorphism is higher than the C-482T which leads to the inclusion of a group of individuals who were characterized by the combination T-455C (FokI): 12/22 and C-482T (MspI):11. Because the relatives are not independent we cannot use *t*-test statistics to test for differences. In probands (**Table 7**) no significant associations were found between variations in the IRE and plasma lipid traits. In spouses, significant differences were

TABLE 7. Effect of IRE polymorphisms on plasma traits in probands

C-482T	11	11	12	12/22
T-455C	11	12/22	12	22
n	15	1	14	4
ApoA-I (mg/dL)	125 ± 6	93	135 ± 14	122 ± 14
ApoB (mg/dL)	146 ± 11	129	133 ± 11	148 ± 11
ApoC-III (mg/dL)	18.2 ± 5.4	14.2	11.7 ± 1.7	10.5 ± 1.7
Chol (mmol/L)	9.2 ± 1.3	9.0	9.7 ± 1.6	8.2 ± 0.5
LDLC (mmol/L)	5.4 ± 0.6	4.0	4.3 ± 0.5	5.3 ± 0.1
HDLC (mmol/L)	0.98 ± 0.07	1.12	0.99 ± 0.07	1.05 ± 0.19
TG (mmol/L)	9.0 ± 5.1	3.6	9.2 ± 3.9	2.7 ± 0.5
Insulin (mU/L)	12.8 ± 2.0	8.0	10.9 ± 1.8	4.0 ± 2.0
Glucose (mmol/L)	6.1 ± 1.1	5.0	5.0 ± 0.2	4.9 ± 0.2
NEFA (mmol/L)	0.51 ± 0.04	0.21	0.41 ± 0.06	0.58 ± 0.16

Values are expressed as means ± SEM. *P* values were tested using Student's *t*-test.

observed between individuals who were heterozygous only for the T-455C variation and those who were heterozygous for both the T-455C and the C-482T. Plasma apoB and LDL cholesterol were significantly ($P < 0.05$) elevated in this group, while plasma HDL cholesterol was significantly decreased ($P < 0.05$, **Table 8**).

To further analyze the modifying effect of the gene cluster on plasma traits in familial combined hyperlipidemia, association studies using specific combinations of haplotypes including the two IRE polymorphisms were performed. Only those combinations of haplotypes whose occurrence was sufficiently frequent were studied (**Table 9**) and compared to the 'wild-type' combination of haplotypes. Spouses identified with the 1-1-1-1-1/2-2-1-1-1 combination had significantly elevated levels of plasma apoA-I and HDL-cholesterol ($P < 0.01$). These results are identical to those reported before in 18 FCH kindred (14), supporting the evidence for a role of these promoter variants in apoA-I gene expression. The combination of haplotypes with the IRE polymorphisms in *trans* configuration with the XmnI and MspI minor alleles, 2-2-1-1-1/1-1-1-2-2, were associated with significantly higher plasma cholesterol, and plasma LDL-cholesterol concentrations ($P < 0.05$) as compared to spouses with the 'wild-type' combination of haplotypes.

TABLE 8. Effect of IRE polymorphisms on plasma traits in spouses

C-482T	11	11	12	12/22
T-455C	11	12/22	12	22
n	91	48	75	16
ApoA-I (mg/dL)	139 ± 2	144 ± 3	140 ± 3	145 ± 6
ApoB (mg/dL)	101 ± 3	95 ± 4	107 ± 3[a]	93 ± 6
ApoC-III (mg/dL)	8.3 ± 0.3	8.9 ± 0.5	8.8 ± 0.4	9.4 ± 0.6
Chol (mmol/L)	5.6 ± 0.1	5.5 ± 0.2	5.9 ± 0.1	5.5 ± 0.2
LDLC (mmol/L)	3.68 ± 0.09	3.43 ± 0.16	3.97 ± 0.11[a]	3.4 ± 0.2
HDLC (mmol/L)	1.27 ± 0.04	1.29 ± 0.04	1.21 ± 0.04[a]	1.30 ± 0.06
TG (mmol/L)	1.53 ± 0.09	1.64 ± 0.14	1.74 ± 0.17	1.6 ± 0.2
Insulin (mU/L)	7.6 ± 0.9	7.8 ± 0.7	8.0 ± 1.2	7.9 ± 2.2
Glucose (mmol/L)	4.9 ± 0.1	4.8 ± 0.1	4.7 ± 0.1	4.7 ± 0.1
NEFA (mmol/L)	0.50 ± 0.03	0.52 ± 0.03	0.54 ± 0.03	0.50 ± 0.05

Values are expressed as means ± SEM. *P* values were tested using Student's *t*-test.

[a] Significant difference vs. T-455C:12/22 and C-482T:11, $P < 0.05$.

The high susceptibility combination of haplotypes as defined in an earlier study (32) was further extended in the present study. There was only one spouse with this combination (Table 9) and this individual showed increased plasma levels of apoB, apoC-III, cholesterol, LDL-cholesterol, and triglycerides. In probands identified with the high risk combination of haplotypes, the plasma cholesterol and triglyceride levels were significantly elevated ($P < 0.05$, **Table 10**). In the group with hyperlipidemic relatives, 10 individuals were characterized with this high risk combination of haplotypes. These individuals, obtained from five different FCH families, showed increased levels of plasma cholesterol, triglycerides, and apoC-III (Table 10).

DISCUSSION

FCH is the most frequent genetic lipid disorder in the western Caucasian population. The genes involved in the development of the observed clinical phenotype, including the premature atherosclerosis associated with FCH, remain an intriguing question. Several candidate genes have been reported in the literature (32, 37–39). Recently a new locus has been found linked to chromosome 1q21-23 in a Finnish FCH population (40). At the same time, a locus for hyperlipidemia has been mapped in the mouse to a region that is syntenic to the human chromosome region described by the Finnish group (41). We have focused on the role of the apoAI-CIII-AIV gene cluster as a candidate for FCH (14, 32). Although we and others (32, 42) have shown that this cluster does not contain the primary mutation causing FCH, it is evident that it plays a major modifying role in lipid metabolism. Within the gene cluster there are two polymorphisms that are associated with changes in plasma lipid and apolipoprotein traits. The SstI polymorphism in the untranslated region of the apoC-III gene is frequently associated with hypertriglyceridemia (1, 14–25). In our studies the SstI polymorphisms were associated with significantly elevated plasma TG, LDL-cholesterol, and apoC-III levels (14). However, the SstI polymorphism alone was not sufficient to explain the observed role of the apoAI-CIII-AIV gene cluster in FCH. Another interesting polymorphism in the promoter region of the apoA-I gene has been described (43). This G-78A variation, resulting in a loss of the restriction site for MspI, was associated with elevated plasma apoA-I and HDL cholesterol levels (43, 44). Construction of a combination of haplotypes based on these two polymorphisms and the XmnI polymorphism, located 2.5 kb for the apoA-I gene, resulted in the identification of a specific high risk combination of haplotypes: one chromosome containing the M2, X2, and S1 alleles (2-2-1) and the other chromosome containing the X1, M1, and S2 alleles (1-1-2). This relatively rare combination of haplotypes was 12 times more frequent in the group of affected FCH relatives and probands compared to spouses. The high-risk combination of haplotypes was associated with a dramatic increase in plasma cholesterol (52%), triglycerides (485%), and apoC-III (50%) concentrations ($P < 0.05$) (32). Further analysis of the different

TABLE 9. Comparison of most frequent combinations of haplotypes based on the IRE polymorphisms in spouses and their effect on plasma lipid traits

Plasma Traits	1-1-1-1-1-1 / 1-1-1-1-1-1	1-1-1-1-1-1 / 1-1-1-2-2	1-1-1-1-1-1 / 1-1-2-2-2	2-2-1-1-1-1 / 1-1-1-2-2	2-2-1-1-1-1 / 1-1-2-2-2	1-1-1-1-1-1 / 2-2-1-1-1
n	59	42	14	11	1	29
ApoA-I (mg/dL)	134 ± 3	136 ± 5	139 ± 6	148 ± 11	147	148 ± 4[b]
ApoB (mg/dL)	100 ± 4	104 ± 5	110 ± 9	114 ± 7	126	103 ± 4
ApoC-III (mg/dL)	8.5 ± 0.4	8.3 ± 0.5	9.1 ± 0.7	10.7 ± 1.5	14.6	8.0 ± 0.5
Chol (mmol/L)	5.5 ± 0.1	5.7 ± 0.2	6.0 ± 0.2	6.4 ± 0.3[a]	7.2	5.8 ± 0.1
HDL-chol (mmol/L)	1.22 ± 0.05	1.14 ± 0.06	1.28 ± 0.10	1.27 ± 0.11	1.4	1.36 ± 0.06[b]
LDL-chol (mmol/L)	3.6 ± 0.1	3.9 ± 0.2	4.0 ± 0.4	4.3 ± 0.3[a]	5.3	3.8 ± 0.1
TG (mmol/L)	1.6 ± 0.1	1.6 ± 0.2	1.6 ± 0.2	1.8 ± 0.4	11.2	1.5 ± 0.1

Values are expressed as means ± SEM. P values were tested using Student's t-test.
[a] Significant difference vs. 1-1-1-1-1-1 / 1-1-1-1-1-1, $P < 0.05$; [b] $P < 0.01$.

haplotypes showed that the 1-1-2 allele contributes in a more dominant fashion in contrast to its 2-2-1 counterpart. This more dominant action of the 1-1-2 allele on the risk to express FCH can be explained by the fact that the SstI polymorphism might be in strong disequilibrium with a yet unidentified locus in the promoter region of the apoC-III gene or elsewhere in the apoAI-CIII-AIV gene cluster.

Variations in the promoter region could affect the level of transcription of the genes in this cluster. Recently five variations in the apoC-III promoter region were described (15). Two single base pair changes were located in a region which was defined as a possible insulin response element: from −455 till −462 from the start site (28, 29). However, a more detailed sequence comparison between the phosphoenolpyruvate carboxykinase (PEPCK) 'insulin response element' (IRE) and the apoC-III postulated IRE sequence revealed that both DNA basepair changes (−455 and −482) are just outside this specific sequence (45). In the studies of Dammerman et al. (15) the haplotype based on the presence of S2 allele and the two IRE variations was associated with an increased risk for hypertriglyceridemia (relative risk 3.14). In a more recent study using a large sample of individuals from the ARIC study (17), a similar association was found (relative risk: 4.0), but statistical analysis showed that this association was attributable to the effects of the SstI polymorphism and not the two IRE variants. A similar observation was made by Shoulders et al. (18) in a study in 503 Italian school children. In contrast, in a study in 188 aboriginal Canadians, an effect of the T-455C allele was found on plasma TG and HDLC levels (46). In the present study we were unable to demonstrate an effect on the frequency distribution of the two IRE variants between hyperlipidemic relatives, normolipidemic relatives, and spouses. This is already a strong indicator that the IRE variants do not contribute to the development of the atherogenic phenotype in FCH. Detailed studies of association in our families between plasma lipids and apolipoprotein traits and these DNA variations resulted in further evidence to support this. However, in streptozotocine-induced diabetic rats, an up-regulation of the hepatic apoC-III mRNA was measured which was down-regulated upon insulin treatment. In vitro transcription assay studies demonstrated a direct effect of insulin upon apoC-III transcription in cultured HepG2 cells (29), whereas studies using specific apoC-III promoter variants in an in vitro transfection assay in hepatic cells showed that both the −455 and the −482 base pair change resulted in a loss of the insulin responsiveness of the apoC-III promoter (28). The only in vivo evidence for an effect of the apoC-III promoter variants on the transcriptional activity of genes in this gene cluster comes from an elegant study that used human intestinal biopsies from individuals who were heterozygous or homozygous for the two

TABLE 10. Effect of the high susceptibility risk combination of haplotypes in probands and hyperlipidemic relatives

	Probands		HL Relatives	
Plasma Traits	1-1-1-1-1-1 / 1-1-1-1-1-1 Wild-type	2-2-1-1-1-1 / 1-1-2-2-2 High Susceptible	1-1-1-1-1-1 / 1-1-1-1-1-1 Wild-type	2-2-1-1-1-1 / 1-1-2-2-2 High Susceptible
n	7	3	41	10
ApoA-I (mg/dL)	124 ± 10	123 ± 6	132 ± 4	159 ± 13
ApoB (mg/dL)	132 ± 10	140 ± 10	131 ± 4	128 ± 6
ApoC-III (mg/dL)	11.6 ± 2.0	15.3 ± 3.4	11.6 ± 0.8	16.6 ± 3.4
Chol (mmol/L)	7.3 ± 0.7	18.8 ± 4.8[a]	7.0 ± 0.2	8.0 ± 0.7
HDL-chol (mmol/L)	1.03 ± 0.12	0.89 ± 0.07	1.14 ± 0.05	1.25 ± 0.16
LDL-chol (mmol/L)	5.0 ± 0.6	2.8 ± 0.9	4.7 ± 0.2	4.3 ± 0.3
TG (mmol/L)	2.9 ± 0.5	31.5 ± 12.3[a]	2.6 ± 0.3	6.6 ± 2.9

Values are expressed as means ± SEM. P values were tested using Student's t-test.
[a] Significant difference vs. 'wild-type' probands, $P < 0.02$. The data in hyperlipidemic relatives were not statistically tested because some of the individuals were from the same family and are therefore not unrelated.

DNA variants (47). In these subjects, the apoA-I mRNA levels were decreased by 51%. No association was found, however, with plasma apoA-I levels and intestinal mRNA activity, which might be indicative of the fact that other factors are responsible for regulation of plasma apoA-I homeostasis in vivo. In conclusion, the two DNA base pair changes in the insulin response element in the apoC-III promoter region itself do not contribute substantially to the atherogenic phenotype in FCH. Therefore, additional yet unknown processes influence the transcriptional regulation of the apoC-III gene and determine the plasma apoC-III levels.

In the present study the high risk combination of haplotypes was further extended with the two DNA variants in the insulin response element. Interestingly, to create the high risk haplotype, the IRE variants had to be on the same chromosome as the S2 allele. This again supports the importance of the SstI polymorphism which acts in a dominant fashion, whereas the M2 allele acts in a more recessive fashion. The present data indicate that the genetic contribution to the FCH phenotype is complex and that at least two different and separate predisposing genetic susceptibility regions exist within the apoAI-CIII-AIV gene cluster, confirming the paradigm of complex genetic contribution to the expression of FCH. Polymorphisms representing the −455 and −482 IRE variants could not explain the previously described identification of epistasis in the apoAI-CIII-AIV gene cluster resulting in a high susceptibility combination of haplotypes, which results in hyperlipidemia with elevated apoC-III plasma concentrations. There was no evidence of association of specific −455 or −482 IRE variants with any of the haplotypes (resistant or susceptible), although this was predicted by earlier studies (15). The −455 or −482 IRE variants could not explain epistasis between apoAI-CIII-AIV gene cluster haplotypes as observed in the spouse group or in the FCH relatives. The susceptibility associated with the S2 allele to increased expression or increased penetrance of FCH could be explained by the fact that the SstI polymorphism is in strong linkage disequilibrium with a yet unidentified mutation either downstream or upstream, or with an unknown disease gene outside this cluster. We are currently exploring the intergenic regions between the apoA-I gene and the apoC-III gene and the region of the apoA-IV gene in order to explain the observed epistasis and increased susceptibility to FCH associated with this gene cluster (48).

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