Apolipoprotein A-I/C-III/A-IV gene cluster in familial combined hyperlipidemia: effects on LDL-cholesterol and apolipoproteins B and C-III

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Abstract The underlying genetic abnormalities in familial combined hyperlipidemia (FCH) have not been elucidated, although previous association and linkage studies have implicated the apoA-I/C-III/A-IV gene cluster. We now report studies of this cluster in 18 probands, 390 family members (hyperlipidemic relatives, n = 179; normolipidemic relatives, n = 211), and 177 spouses. Three restriction enzyme polymorphisms, XmnI and MspI sites 5′ of the apoA-I gene and the SstI site in the 3′ untranslated region of exon 4 of the apoC-III gene, were examined. In hyperlipidemic relatives and FCH probands, the frequency of each minor allele was significantly higher than in spouses. Associated with the higher frequency of minor alleles were elevated plasma cholesterol, triglycerides, LDL-cholesterol, apoB, and apoC-III levels. Quantitative sib-pair analysis revealed linkage between the MspI minor allele and plasma LDL cholesterol levels ($P < 0.04$). The present data indicate that, while apoA-I/C-III/A-IV gene cluster is not the primary cause of FCH, this cluster has a specific modifying effect on plasma triglyceride and LDL cholesterol levels.—**Dallinga-Thie, G. M., X-D. Bu, M. v L-S. Trip, J. I. Rotter, A. J. Lusis, and T. W. A. de Bruin.** Apolipoprotein A-I/C-III/A-IV gene cluster in familial hyperlipidemia: effects on LDL-cholesterol and apolipoproteins B and C-III. *J. Lipid Res.* 1996. **37:** 136–147.

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Familial combined hyperlipidemia (FCH) was described in 1973 by Goldstein et al. (1) as a genetic lipid disorder with Mendelian dominant inheritance, resulting in dramatically elevated plasma triglyceride levels with a secondary effect on plasma cholesterol levels. Subsequent genetic analysis indicated that the inheritance pattern of the disorder was more consistent with a more complex model (2, 3) with a major gene acting on triglyceride levels (4).

The prevalence of the disease in Western society is about 1–2% of the population, and in survivors of myocardial infarction under 60 years of age, it is approximately 10–20% (1). Thus, FCH results in an approximately 10-fold higher risk for myocardial infarction. The diagnosis is based on the presence of combined hyperlipidemia and expression of multiple type hyperlipidemia in relatives of the proband (5–7). Lipoprotein metabolism in FCH is characterized by an overproduction of apolipoprotein (apo) B-100-containing lipoproteins, which is reflected in elevated plasma concentrations of low density lipoproteins (LDL) and very low density lipoproteins (VLDL) (8–11). Small dense LDL particles are frequently found in FCH (12–16), and the concentration of high density lipoproteins (HDL) tends to be reduced (17, 18).

The genetic defects underlying FCH are as yet unknown. Whereas plasma apoB concentrations are elevated in FCH, several studies have failed to observe linkage between markers near the apoB gene and the FCH phenotype (19, 20). Another candidate gene is the apoA-I/C-III/A-IV gene cluster located on chromosome 11. Linkage between FCH and this gene cluster has been reported by Wojciechowski et al. (21), but this finding has not been confirmed (22). The apolipoproteins encoded by this cluster are involved in the metabolism of HDL and triglyceride-rich lipoprotein particles. ApoA-I and apoA-IV are major constituents of intestinally de-

Abbreviations: HDL, high density lipoprotein; FCH, familial combined hyperlipidemia; apo, apolipoprotein; VLDL, very low density lipoprotein; LDL, low density lipoprotein; PCR, polymerase chain reaction; BMI, body mass index; WHR, waist-hip ratio.

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rived lipoproteins as well as HDL, whereas apoC-III is a major constituent of VLDL, chylomicron remnants, and HDL. Increased expression of these apolipoproteins has been shown to alter lipoprotein metabolism in transgenic mice (23–25). In FCH probands, a delayed elimination of TG-rich remnants was observed that correlated with the increased plasma concentrations of apoC-III (26). Several associations between polymorphisms within this gene cluster and parameters of lipid metabolism have been reported in patient groups and populations (27), but no study of a large number of FCH pedigrees has been published. It was the objective of this study to evaluate polymorphisms in the apoA-I/C-III/A-IV gene cluster in 18 well-characterized FCH kindreds and to characterize associations with lipid and apolipoprotein phenotypes and to use sib-pair analysis as a quantitative trait analysis method that is particularly useful when a mode of inheritance has not been established, as is the case in FCH (6).

SUBJECTS AND MATERIALS

Index subjects

Eighteen unrelated, Dutch Caucasian, index FCH patients were recruited from the Lipid Clinic of the Utrecht University Hospital. These subjects met the criteria described previously (1, 5, 17), 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; *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. In total, 95% of the living relatives over 18 years of age (including 108 first degree, 111 second degree, and 173 third degree relatives) have been included in the present analysis. Hyperlipidemic relatives (n = 179) 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 211 'normolipidemic' relatives. The spouse group (n = 177) represented an environment-matched, nutrition-matched, and age-matched control group for the relatives; 58 spouses (25 men and 33 women) were hyperlipidemic according to the criteria described.

Analytical methods

Venous blood was drawn after subjects had fasted overnight for 12–14 h and abstained from alcohol use for at least 48 h. Plasma was prepared by immediate centrifugation for analytical analysis. Plasma triglyceride and cholesterol were measured in duplicate using a commercial colorimetric assay (GPO-PAP, Boehringer Mannheim no. 701912 and Monotest cholesterol kit, Boehringer Mannheim no. 237574). HDL was prepared from whole plasma by precipitation with phosphotungstate-$MgCl_2$ (28). Low density lipoprotein (LDL) cholesterol was calculated in mmol/l using the Friedewald formula: LDL chol = total plasma chol - (HDLchol + TG/2.18) (29). This formula is known to be less accurate when plasma TG concentrations exceed 4.5 mmol/l. Therefore, LDL cholesterol concentrations were measured in fractions obtained by density gradient ultracentrifugation (d 1.019–1.063 g/ml) (26) in all individuals with plasma TG concentrations > 4.0 mmol/l. Plasma apoB and apoA-I were determined by immunonephelometric assays, using polyclonal rabbit anti-human apoB or apoA-I antiserum, and standards with the assigned values according to the International Federation of Clinical Chemistry (Behringwerke AG, Marburg, Germany) (30). Interassay variability was 7% and 4%, respectively. ApoC-III levels were determined by commercial single radial immunodiffusion assay (Daiichi

TABLE 1. Characteristics of the studied populations

Variable	FCH-Probands	All Relatives	Spouses
Number	18	390	177
Age (yr)	53 ± 11[a]	39 ± 16[b]	48 ± 16
Gender (M/F)	12/6	208/183[c]	70/109
BMI, kg/m²	26.4 ± 3.6[a]	24.8 ± 4.27	25.2 ± 3.8
WHR	0.93 ± 0.08[a]	0.84 ± 0.09	0.84 ± 0.10
Diastolic BP	85 ± 8	84 ± 11	84 ± 11
Systolic BP	131 ± 14[a]	126 ± 18	126 ± 21

Values are expressed as mean ± SD. *P*-values were determined using the *t*-test. FCH, familial combined hyperlipidemia; BMI, body mass index; WHR, waist hip ratio; BP, blood pressure.

[a]Significant difference between FCH-probands versus all relatives and spouses $P < 0.001$.

[b]Significant difference between all relatives and spouses $P < 0.001$.

[c]Significant difference between all relatives versus spouses, $\chi^2 = 9.20$, $P = 0.002$.

Chemicals, Tokyo, Japan). The interassay variability was 8.7%.

DNA amplification

DNA was isolated from 10 ml of EDTA-augmented blood following standard procedures (31) and amplified by the polymerase chain reaction (PCR) technique in a Thermal cycler apparatus (Pharmacia, Uppsala, Sweden). All PCR reactions were carried out in 50 µl reaction volume containing 375 ng genomic DNA, 100 pmol of each primer, all four dNTPs (each at 0.1 mM) (Promega, Madison, WI), 16.6 mM ammonium sulfate, 67 mM Tris-HCl (pH 8.8), 6.7 mM $MgCl_2$, 10 mM β-mercaptoethanol, 10% dimethylsulfoxide, bovine serum albumin (10 µg/ml), and 0.75 units of Taq polymerase (Perkin-Elmer Cetus, Norwalk, CT). Thirty-three cycles were performed under conditions that were specific for each polymorphism, as described below. The PCR products were resolved on 2% agarose gels, using TBE buffer (0.09 M Tris-borate, 0.002 M EDTA, pH 8.3) containing 0.5 µg/ml ethidium bromide. DNA was visualized on an ultraviolet transilluminator. The PCR products were directly used for restriction enzyme typing. Incubations were performed at 37°C for at least 1 h. The products were resolved on 3% agarose gels.

XmnI polymorphism (C-2500T)

Amplification of the region 2.5 kb upstream of the apoA-I gene was achieved using primers 5′-GGAAACAGGGGCCTACACT-3′ (sense) and 5′-GTCTGCAGCCTTTCAGTCT-3′ (antisense) (32) in the protocol described above 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. XmnI restriction enzyme (5 units) and specific restriction buffer (New England Biolabs, Beverly, MA) were added directly to 10 µl PCR reaction mixture to a final volume of 20 µl. Alleles were defined as X1 or X2 based on absence or presence of the XmnI restriction site, respectively.

SstI polymorphism (G3175C)

Amplification of the exon 4 of the apoC-III gene was achieved using primers 3′-ACCTGGAGTCTGTCCAGTGCCCACC-5′ (sense) and 3′-TCGTCCAGTGGGGACATGGGTGTGG-5′ (antisense) (33). PCR conditions were: denaturation for 4 min at 94°C, and 33 cycles of denaturation for 1 min at 94°C, annealing for 2 min at 64°C and extension for 2 min at 72°C. SstI restriction enzyme (5 units) and specific buffer (Boehringer, Mannheim, Germany) were directly added to 10 µl of PCR product to a final volume of 20 µl. Alleles were defined as S1 and S2 based on absence or presence of the SstI restriction site, respectively.

MspI polymorphism (G-78A substitution)

Amplification of the promoter region of the apoA-I gene was achieved with primers 5′-CTGGGGCAAGGCCTGAACCT-3′ (sense) and 5′-CACCCGGGAGACCTGCAAGC-3′ (antisense). Denaturation for 4 min at 94°C, annealing and extension at 55°C, respectively, 72°C for 2 min, followed by 33 cycles of denaturing at 94°C for 1 min, annealing at 55°C for 1 min and extension at 72°C for 2 min (34). MspI restriction enzyme (5 units) and specific restriction buffer (Boehringer, Mannheim, Germany) were added directly to 10 µl of PCR product to a final volume of 20 µl. Alleles were defined as M1 or M2 based on the presence or absence of the MspI restriction site. M1 is equivalent to the G-allele and M2 to the A-allele.

Statistical methods

Results are expressed as mean ± SD. The statistical differences between the plasma parameters of the groups were calculated using the unpaired Student's *t*-test. Data for plasma triglyceride, apoC-III, cholesterol, and apoB were analyzed unadjusted and after log transformation. Frequencies of the three polymorphisms were determined by gene counting and allele counting. Deviations of the Hardy Weinberg equilibrium were tested with a χ^2 goodness-of-fit test. Linkage disequilibrium between the tested markers was tested using the

TABLE 2. Characteristics of the relatives

Variable	Hyperlipidemic Relatives	Normolipidemic Relatives	*P*-Value
Number	179	211	NS
Age (yr)	46 ± 16	32 ± 13	0.001
Gender, M/F	104/76	104/107	NS
BMI, kg/m²	26.2 ± 3.6	24.0 ± 7.0	0.001
WHR	0.88 ± 0.09	0.81 ± 0.08	0.001
Systolic BP	130 ± 19	121 ± 15	0.001
Diastolic BP	90 ± 12	81 ± 11	0.001

Values are expressed as mean ± SD. *P*-values were determined using the *t*-test, except for gender (χ^2). BMI, body mass index; WHR, waist hip ratio; BP, blood pressure.

TABLE 3. Plasma parameters in the studied populations

Variable	FCH-Probands	All Relatives	Spouses
Chol (mmol/l)	11.58 ± 6.06[a]	5.92 ± 1.46	5.65 ± 1.06
HDL-Chol (mmol/l)	1.01 ± 0.23[a]	1.20 ± 0.32	1.27 ± 0.36
LDL-Chol (mmol/l)	5.34 ± 2.12[a]	3.84 ± 1.24	3.66 ± 0.99
TG (mmol/l)	12.84 ± 21.07[a]	1.93 ± 2.00	1.63 ± 1.11
HDL-TG (mmol/l)	0.30 ± 0.07	0.30 ± 0.08	0.30 ± 0.09
ApoA-I (mg/100 ml)	133 ± 45	133 ± 24[b]	138 ± 24
ApoB (mg/100 ml)	150 ± 28[a]	104 ± 24	99 ± 17
ApoC-III (mg/100 ml)	15.0 ± 13.1[a]	9.68 ± 4.36	8.99 ± 3.31

The values are expressed as mean ± SD. The *P*-values were determined using the *t*-test. FCH, familial combined hyperlipidemia.
[a]Significant difference between FCH-probands versus each of all relatives and spouses: $P < 0.001$.
[b]Significant difference between all relatives and spouses: $P < 0.03$.

EHDOS program (35).

Sib-pair linkage analysis

The methodology of robust sib-pair analysis was used to test the hypothesis that there is linkage between a quantitative trait and a polymorphic marker in the apoA-I/C-III/A-IV gene cluster (36). The basis for this approach is to compare the quantitative variation in a trait between siblings as a function of the alleles they share identical by descent (IBD). The sib-pair IBD method does not require prior assumptions about the mode of inheritance, an advantage in the analysis of genetically complex multigenic disease. In this method, the proportion of genes IBD between the members of each pair of sibs is estimated for each of the marker loci. The squared trait differences between members of a sib-pair are regressed on the estimated proportion of genes IBD at the marker loci (zero, one or two shared alleles). The hypothesis of linkage between a trait and a marker locus is tested using the asymptotically normal distribution of regression coefficients. A regression of squared differences was calculated (37). For a trait linked to the locus, the regression coefficient will deviate from zero and is significant when $P < 0.05$ (36, 38, 39).

The actual sib-pair linkage analyses were performed by the SIBPAL program (SAGE version 2.4) (37). The squared trait differences for unadjusted plasma biochemical traits (total cholesterol, triglyceride, HDL-cholesterol, LDL-cholesterol, apoA-I, apoC-III, apoB, BMI, WHR) were calculated for sib-pairs IBD. To avoid false positives in the FCH pedigrees, the analyses were performed before and after exclusion of those sib-pairs with extreme values of a trait (i.e., exceeding 3 SD of the mean), and those sib-pairs with very large squared differences (exceeding 3 SD of the mean). We performed all analyses on the untransformed and logarithmic-transformed data (36, 38, 39).

RESULTS

Subject characteristics

Clinical characteristics of the FCH probands, relatives, and spouse controls are summarized in **Table 1.** FCH probands were characterized by an increased body mass index (BMI) compared to their family members and spouses. Additional differences also included an increased waist-hip ratio (WHR) and an increased sys-

TABLE 4. Plasma characteristics of the relatives

Variable	Hyperlipidemic Relatives	Normolipidemic Relatives	*P*-value
Chol (mmol/l)	7.07 ± 1.22	4.95 ± 0.79	0.001
LDL-Chol (mmol/l)	4.67 ± 1.21	3.14 ± 0.71	0.001
HDL-Chol (mmol/l)	1.15 ± 0.33	1.25 ± 0.31	0.001
TG (mmol/l)	2.76 ± 2.71	1.24 ± 0.39	0.001
HDL-TG (mmol/l)	0.33 ± 0.09	0.28 ± 0.07	0.001
ApoB (mg/100 ml)	129 ± 19	83 ± 11	0.001
ApoA-I (mg/100 ml)	133 ± 27	133 ± 23	NS
ApoC-III (mg/100 ml)	12.2 ± 4.9	7.6 ± 2.4	0.001

The values are expressed as mean ± SD. *P*-values were determined using *t*-test.

tolic blood pressure. The group of FCH probands was composed mostly of male subjects (66% of total) compared to their family members and spouses. For detailed analysis, we divided the relatives into two groups (**Table 2**): hyperlipidemic relatives who expressed the FCH phenotype (cholesterol > 6.5 mmol/l and/or triglyceride > 2.3 mmol/l) versus normolipidemic relatives (chol < 6.5 mmol/l and TG < 2.3 mmol/l). The hyperlipidemic relatives had characteristics similar to those of the probands, including a male preponderance (58%), as well as higher values for BMI, WHR, and diastolic and systolic blood pressures than normolipidemics. Normolipidemic relatives were significantly younger than the hyperlipidemic relatives. It cannot be excluded that in some normolipidemic individuals the FCH phenotype had not yet been fully expressed, as the onset of the FCH phenotype is assumed to be above 20 years of age (1, 7).

Biochemical characteristics of the studied groups are shown in **Table 3** and **Table 4.** Compared to relatives and spouses, FCH probands had significantly higher serum cholesterol, triglycerides, LDL cholesterol, apoB and apoC-III levels. HDL cholesterol levels ($P < 0.001$) were significantly lower in FCH probands compared to spouses (Table 3). The same traits were significantly different between the hyperlipidemic and normolipidemic relatives (Table 4). Plasma apoA-I levels were significantly lower in all relatives compared to spouses.

Polymorphisms of the apoA-I/C-III/A-IV gene cluster

A map of the apoA-I/C-III/A-IV gene cluster is shown in **Fig. 1,** indicating the three restriction sites, XmnI, SstI, and MspI. The observed frequencies for all three polymorphisms behaved within the limits of the Hardy Weinberg law. Because the distribution of men and women was different in probands, relatives, and spouses, we tested whether the gene frequencies were dependent upon gender. No differences between males and females could be observed (data not shown) and this allowed us to combine data from men and women in the analysis. Those subjects who carried the X2 allele invariably had the M2 minor allele, indicating that these sites were in complete linkage disequilibrium ($\chi^2 = 612$, df = 3; $P < 0.00001$). The frequency of the M2-allele was higher than the X2-allele because 21 subjects carried a single M2 allele but no X2 allele. The MspI locus was also in linkage disequilibrium with the SstI locus ($\chi^2 = 10.9$, df = 3; $P < 0.05$), but the XmnI locus was not in significant linkage disequilibrium with the SstI locus ($\chi^2 = 6.1$, df = 3; $P > 0.10$).

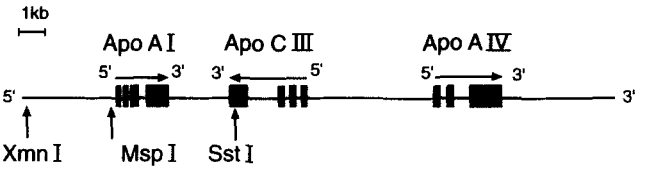

Fig. 1. Restriction enzyme map of the apoA-I/C-III/A-IV gene cluster: XmnI, at -2500 bp from the transcription start site of the apoA-I gene; MspI, at -78 bp from the transcription start site of the apoA-I gene; SstI, at the untranslated region of exon 4 of the apoC-III gene.

Allele frequencies

The allele frequency of the S2 allele was 3-fold increased in FCH probands ($P = 0.006$) compared to relatives and spouses (**Table 5**). At the XmnI and MspI loci, the frequencies of the minor alleles were increased 1.7-(ns) and 2.2-fold ($P = 0.02$) compared to spouses. The X2 and M2 alleles were significantly more frequent (1.5-fold) in relatives than in spouses (X2: $P < 0.0001$ and M2: $P < 0.009$). There was no significant difference in frequencies of the three tested markers between hyperlipidemic and normolipidemic relatives (Table 5).

Genotype frequencies and phenotypic associations

The genotype frequencies of the rare alleles were increased in the FCH probands and hyperlipidemic relatives compared to the spouses, suggesting that the apoA-I/C-III/A-IV locus is involved in the expression of FCH (**Fig. 2**). Genotype frequencies of the minor alleles of XmnI ($P < 0.05$), SstI ($P < 0.025$), and MspI ($P < 0.05$)

TABLE 5. Allelic frequencies of the minor alleles in the populations tested

Population	XmnI	*P*-Value vs Spouses	MspI	*P*-Value vs Spouses	SstI	*P*-Value vs. Spouses	*P*-Value vs. Index
Probands	0.22	NS	0.31	0.02	0.19	0.006	
Relatives, all	0.19	0.0001	0.21	0.009	0.08	NS	0.026
HL	0.19	0.026	0.22	0.011	0.10	NS	NS
NL	0.18	0.035	0.19	0.04	0.06	NS	0.006
Spouses	0.13		0.14		0.06		0.006

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

were higher in FCH probands than in spouses, and XmnI ($P < 0.025$) and MspI ($P < 0.01$) were also higher in hyperlipidemic relatives compared to spouses (Fig. 2). The difference in genotype frequency in the normolipidemic relatives versus the spouse controls only reached significance with the MspI polymorphism ($P < 0.05$). Subsequently, we analyzed the effect of the variations at these loci on plasma lipids and apolipoprotein levels (**Table 6** and **Table 7**). In FCH families, a more severe expression of the phenotype, with significantly higher plasma TG, cholesterol, apoB, and apoC-III concentrations, was observed in those individuals who did have one or more polymorphisms at the tested loci as compared to those individuals without any polymorphisms (Table 6). In spouses, expression of one or more polymorphisms in this gene cluster again resulted in significantly elevated plasma cholesterol, TG, apoB, and apoC-III levels and an additional significant increase in LDL cholesterol levels was also observed (Table 7). This difference was also seen in the relatives, but did not obtain statistical significance (Table 6).

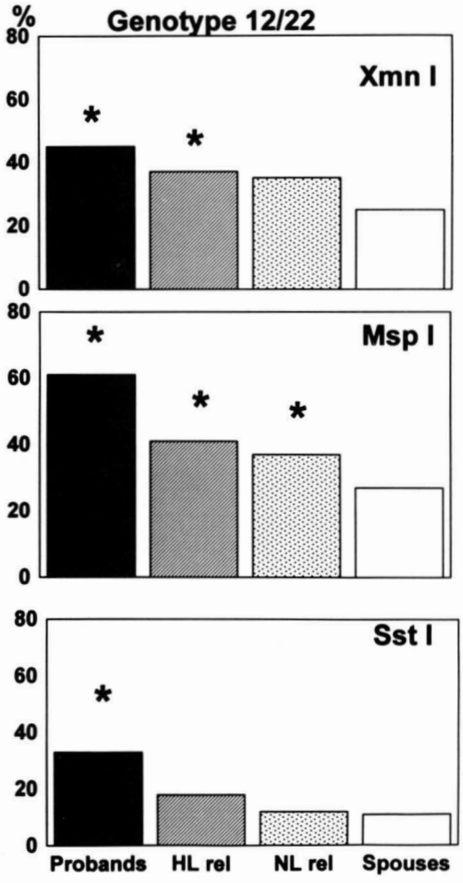

Fig. 2. Relative frequencies of the minor alleles of XmnI, MspI, and SstI restriction enzyme polymorphisms. Probands (n = 18), HL, hyperlipidemic relatives (n = 179), NL, normolipidemic relatives (n = 211), and spouses (n = 177). *P*-values were determined using χ^2-test. *FCH probands versus spouses: XmnI, $P < 0.05$; MspI, $P < 0.05$; SstI, $P < 0.025$. *HL versus spouses: XmnI, $P < 0.025$; MspI, $P < 0.01$. *NL versus spouses: MspI, $P < 0.05$.

To further analyze the contribution of the individual loci to the FCH phenotype, we studied the three polymorphisms separately in FCH probands, hyperlipidemic relatives, normolipidemic relatives, and spouses (**Table 8, Table 9,** and **Table 10**). In probands, the X1X2/X2X2 genotype was significantly associated with elevated plasma cholesterol (2-fold) and TG (10-fold) levels as compared with the X1X1/X1X1 genotype, whereas the M1M2/M2M2 genotype was associated with a 1.5-fold increased total cholesterol (not significantly different) and significantly increased TG levels (6-fold) (Tables 8 and 9). In probands, the S1S2/S2S2 genotype was associated with higher plasma cholesterol and TG plasma concentrations, but these differences were not significant. In hyperlipidemic relatives, the S1S2/S2S2 genotype was associated with significantly higher apoC-III plasma concentrations in combination with elevated plasma TG levels. In both hyperlipidemic and normolipidemic relatives, the X1X2/X2X2 and M1M2/M2M2 genotypes tended to elevate plasma lipid levels, although the differences were not statistically significant. Unique to the SstI polymorphism were the differences in spouses with the S1S2/S2S2 genotype, who showed significantly elevated plasma cholesterol (10%) and TG (14%) concentrations, and also additional effects on plasma levels of LDL cholesterol (14%), apoB (14%), and apoC-III (23%), compared to spouses with the S1S1 genotype (Table 10). Spouses with the X1X2/X2X2 genotype had significantly higher plasma apoB levels (Table 8).

Sib-pair analysis

Sib-pair linkage analysis was performed on a substantial number of pairs for each locus (**Table 11**). The analysis revealed that the MspI locus was significantly linked to quantitative variation in LDL cholesterol plasma concentrations ($P = 0.039$). A similar trend was observed for XmnI ($P = 0.062$); significance was reached after log transformation of LDL cholesterol. In contrast, with the SstI locus there was no evidence for linkage with quantitative variation in plasma LDL cholesterol. No significance was obtained for linkage to quantitative traits such as total plasma cholesterol, HDL cholesterol, triglyceride, apoA-I, apoB, apoC-III or BMI and WHR. The combined haplotype (XmnI, MspI, SstI) exhibited no evidence for linkage, suggesting that a subset of families accounted for the above linkage results.

DISCUSSION

Previous association and linkage studies have suggested that variations of the apoA-I/C-III/A-IV gene

TABLE 6. Relationship between genotype and plasma traits in all relatives including FCH probands

Traits	Relatives without a Minor Allele	Relatives with a Minor Allele	*P*-Value
n	197	211	
Age (yr)	38 ± 15	41 ± 16	NS
Cholesterol (mmol/l)	5.82 ± 1.51	6.50 ± 2.67	0.002
HDL cholesterol (mmol/l)	1.20 ± 0.31	1.19 ± 0.34	NS
LDL cholesterol (mmol/l)	3.79 ± 1.32	4.01 ± 1.31	NS
Triglycerides (mmol/l)	1.82 ± 1.40	3.04 ± 7.24	0.022
ApoB (mg/dl	101 ± 24	110 ± 24	0.006
ApoA-I (mg/dl)	132 ± 23	133 ± 25	NS
ApoC-III (mg/dl)	9.3 ± 4.1	10.6 ± 6.0	0.009

Values are expressed as mean ± SD. All *P*-values were determined using *t*-test.

cluster contribute importantly to FCH (21, 40) but other studies have disputed this conclusion (22). To help resolve the question, we performed an association study using three polymorphisms of the cluster in a large set of FCH families. The results indicate that, whereas the cluster is not a major contributing factor to FCH, it does influence the levels of cholesterol and triglycerides in both normolipidemic and hyperlipidemic individuals.

In the present study, variations in the apoA-I/C-III/A-IV gene cluster had an effect on the expression of apoB-related cholesterol (defined as non-HDL cholesterol) in the spouses. Subjects who were hetero- or homozygous for one of the minor alleles had, even without being hyperlipidemic, significantly elevated plasma LDL cholesterol, total cholesterol, apoB and apoC-III levels. Thus, LDL cholesterol concentrations were 11% higher in spouses with a minor allele. The average difference in LDL cholesterol explained the observed difference in plasma total cholesterol concentration. These results indicated that variations in this gene cluster modify plasma LDL cholesterol concentrations both in hyperlipidemic relatives and in the population at large, and quantitatively to the same extent. As the spouses represent the population, we conclude that the apoA-I/C-III/A-IV gene cluster is identified as one of the genes involved in the expression of multigenic hypercholesterolemia. Sib-pair analysis confirmed that quantitative variation in plasma LDL cholesterol levels showed significant linkage with the MspI locus, and borderline significant linkage with the XmnI locus but not the SstI locus. Linkage disequilibrium was found to exist between the MspI and XmnI loci only. The molecular mechanism that transmits the effect of the gene loci on plasma LDL cholesterol is not known at present. Recently, several studies have reported that the magnitude of the response to cholesterol-lowering diets is dependent on variations in the apoA-I/C-III/A-IV gene cluster, including the MspI locus (41, 42).

The relative frequencies of the three markers, as determined in the group of 177 Caucasian spouses, were similar to published frequencies in Caucasian populations (40, 43–46). In the 18 studied FCH families (n = 408 individuals), increased frequencies of the minor alleles were found compared to spouses, ranging from 1.4- (X1X2/X2X2 genotype), 1.5-(M1M2/M2M2 genotype), to 1.3-(S1S2/S2S2 genotype) fold, respectively. An impressive enrichment of the rare alleles (78%) in probands was observed. Normolipidemic relatives, how-

TABLE 7. Relationship between genotype and plasma traits in spouses

Traits	Spouses without a Minor Allele	Spouses with a Minor Allele	*P*-Value
n	106	71	
Age (yr)	48 ± 15	48 ± 17	NS
Cholesterol (mmol/l)	5.53 ± 1.10	5.99 ± 1.18	0.008
HDL cholesterol (mmol/l)	1.27 ± 0.38	1.28 ± 0.32	NS
LDL cholesterol (mmol/l)	3.54 ± 1.01	3.99 ± 1.09	0.043
Triglycerides (mmol/l)	1.55 ± 0.89	1.76 ± 1.39	NS
ApoB (mg/dl)	97 ± 19	104 ± 17	0.047
ApoA-I (mg/dl)	136 ± 23	141 ± 24	NS
ApoC-III (mg/dl)	8.54 ± 3.17	9.56 ± 3.37	0.043

All values are expressed as mean ± SD. All *P*-values were determined using *t*-test.

TABLE 8. Effect of X1X2/X2X2 genotype on plasma traits

	Probands		Hyperlipidemic Relatives		Normolipidemic Relatives		Spouses	
Traits	X1X1	X1X2/X2X2	X1X1	X1X2/X2X2	X1X1	X1X2/X2X2	X1X1	X1X2/X2X2
n	10	7	113	67	137	74	135	42
Chol	8.70 ± 1.07	16.3 ± 7.73[a]	6.99 ± 1.12	7.20 ± 1.37	4.87 ± 0.82	5.10 ± 0.76	5.56 ± 1.07	5.93 ± 1.02
HDL-C	1.09 ± 0.21	0.95 ± 0.21	1.11 ± 0.33	1.20 ± 0.34	1.23 ± 0.27	1.31 ± 0.37	1.27 ± 0.37	1.29 ± 0.32
LDL-C	5.66 ± 0.95	5.21 ± 3.39	4.65 ± 1.25	4.73 ± 1.16	3.10 ± 0.72	3.23 ± 0.70	3.59 ± 1.00	3.91 ± 0.89
TG	3.19 ± 0.75	27.3 ± 29.3[b]	2.62 ± 1.72	2.93 ± 3.87	1.21 ± 0.39	1.26 ± 0.37	1.56 ± 0.86	1.90 ± 1.69
ApoB	136 ± 21	291 ± 398	129 ± 19	130 ± 20	81 ± 10	86 ± 12	97 ± 19	104 ± 16[a]
ApoA-I	129 ± 31	144 ± 63	131 ± 24	140 ± 29	133 ± 22	133 ± 25	136 ± 23	144 ± 25
ApoC-III	9.9 ± 2.2	25.9 ± 29.8	12.1 ± 4.5	12.3 ± 5.5	7.4 ± 2.4	7.9 ± 2.3	8.8 ± 3.2	9.4 ± 3.7

Values are expressed as mean ± SD. All plasma lipid traits are given as mmol/l, whereas the plasma apolipoprotein traits are given as mg/dl. All *P*-values were determined using the *t*-test.

[a]Significantly different at $P < 0.007$.

[b]Significantly different at $P < 0.02$.

ever, had frequencies similar to those of hyperlipidemic relatives (Table 4). This finding, in combination with the observation that spouses with a minor allele expressed higher LDL cholesterol levels, although still below the 95th percentile for age, and normal triglyceride and HDL cholesterol levels, was consistent with the conclusion that the gene cluster is not the primary cause of FCH, but rather indicated involvement in the expression of multigenic hypercholesterolemia.

In hyperlipidemic relatives and FCH probands, minor alleles were 1.5- to 2.3-times more frequent than in spouses. The presence of minor alleles at each of the three loci was consistently associated with elevated plasma total cholesterol and triglyceride concentrations in addition to already present combined hyperlipidemia (Tables 8–10). Because HDL cholesterol was not affected by the presence of the minor alleles, the expression of hypercholesterolemia was mainly due to higher apoB-related cholesterol. Average LDL cholesterol levels were not significantly different between hyperlipidemic relatives with or without a minor allele, indicating that VLDL and IDL cholesterol levels were elevated in carriers with a minor allele, as reported earlier (17). Therefore, a discrepancy was observed between the effect of the gene cluster on LDL cholesterol in spouses versus hyperlipidemic relatives and FCH probands combined. Elevated LDL cholesterol plasma concentrations are a characteristic metabolic feature of the expression of FCH because VLDL overproduction (10) results in increased conversion to LDL as a result of the lipolytic cascade. We postulate that VLDL overproduction in FCH overrules the specific effect of the apoA-I/C-III/A-IV gene cluster on LDL cholesterol, as observed in spouses. This leads to the conclusion that in hyperlipidemic individuals, genetic variation in the apoA-I/C-III/A-IV gene cluster aggravated the expression of hypercholesterolemia, similar to the definition of a variability gene (47). This was illustrated, in Table 10, by the effect of the S2 rare allele on mean plasma cholesterol levels and observed standard deviations. Mean plasma cholesterol levels increased by 10.4% in spouses, by 3.0% in hyperlipidemic relatives, and 34% in probands, carrying the S2 allele, whereas the standard deviation did not change in spouses but increased by 32% and 48% in hyperlipidemic relatives and probands, respectively.

In 1990 Wojciechowski et al. (21) reported linkage of FCH to the XmnI polymorphism upstream of the apoA-I gene with a low score of 6.7. However, this study had certain ascertainment bias because only 7 FCH probands were selected: those who were characterized by having small dense LDL and heterozygosity for the X2-allele, whereas probands with the X1X1 genotype were excluded. Recently, expression of the small dense LDL phenotype (pattern B) was associated with variations at the apoA-I/C-III/A-IV gene cluster (48). It is possible, therefore, that the original report (21) described the linkage with small dense LDL rather than the FCH phenotype. In the present report, sib-pair analysis revealed statistically significant linkage between the MspI locus and quantitative variation in plasma LDL cholesterol levels. The expression of combined hyperlipidemia in relatives did not depend exclusively upon the presence of polymorphisms at the three loci tested, although an aggravating effect of these polymorphisms on the FCH phenotype was observed. These findings, in conjunction with other data in literature (22), show that the chromosome 11 gene cluster can be excluded as the genetic cause of FCH, but is a contributing gene to the development of a more pronounced FCH phenotype.

It has been reported that the XmnI locus is in linkage disequilibrium with the MspI restriction site in the promoter region of the apoA-I gene (49), which was confirmed in the present study. The MspI locus is located

TABLE 9. Effect of M1M1/M1M2 genotype on plasma traits

	Probands		Hyperlipidemic Relatives		Normolipidemic Relatives		Spouses	
Traits	M1M1	M1M2/M2M2	M1M1	M1M2/M2M2	M1M1	M1M2/M2M2	M1M1	M1M2/M2M2
n	7	11	104	75	131	79	131	46
Chol	8.88 ± 1.18	13.3 ± 7.30	7.01 ± 1.11	7.16 ± 1.36	4.88 ± 0.82	5.04 ± 0.74	5.60 ± 1.07	5.83 ± 0.98
HDL-C	1.12 ± 0.24	0.94 ± 0.21	1.13 ± 0.33	1.15 ± 0.35	1.23 ± 0.27	1.27 ± 0.29	1.27 ± 0.37	1.31 ± 0.32
LDL-C	5.15 ± 2.67	5.15 ± 2.67	4.65 ± 1.25	4.71 ± 1.17	3.10 ± 0.73	3.20 ± 0.68	3.59 ± 1.00	3.82 ± 0.88
TG	3.06 ± 0.76	19.1 ± 25.4[a]	2.62 ± 1.72	2.97 ± 3.66	1.21 ± 0.38	1.26 ± 0.40	1.55 ± 0.87	1.81 ± 1.59
ApoB	121 ± 4	242 ± 314[a]	129 ± 18	129 ± 20	81 ± 10	84 ± 12	99 ± 19	102 ± 15
ApoA-I	137 ± 34	131 ± 53	133 ± 24	135 ± 30	133 ± 22	133 ± 24	136 ± 23	143 ± 25
ApoC-III	10.0 ± 2.3	20.6 ± 24.3	12.1 ± 4.6	12.3 ± 5.2	7.4 ± 2.4	7.8 ± 2.4	8.9 ± 3.2	9.1 ± 3.6

Values are expressed as mean ± SD. All plasma lipid traits are given in mmol/l, all plasma apolipoprotein traits are given in mg/dl. All *P*-values were determined using the *t*-test.

[a]Significantly different at $P < 0.02$.

in the promoter region of the apoA-I gene at position -78 bp of the transcription start site of the apoA-I gene (34, 50) and is the result of a G to A mutation resulting in the loss of a restriction site for MspI. The function of the protein encoded for by the gene is to provide the structural protein, apoA-I, in HDL lipoproteins. In several studies an association has been found between the minor allele of MspI and elevated apoA-I plasma levels (34, 45, 50, 51). Angotti et al. (52) showed that the G to A substitution results in 5- to 7-fold increase of the expression of a reporter gene fused to the apoA-I promoter in human liver and intestinal cells. However, two other studies (53, 54) showed an association with decreased activity of the promoter and decreased apoA-I production rates as a result of this mutation. In the present study, no effect of the M2-allele (G-78A) mutation on plasma apoA-I and plasma HDL cholesterol levels was observed, although the frequency of the M2 allele was significantly increased in FCH subjects. In addition, absence of linkage between the MspI locus and plasma apoA-I levels was observed in the sib-pair analysis. In conclusion, the data reported herein show that the G to A substitution does not substantially affect plasma apoA-I or HDL cholesterol levels in a Dutch population.

The SstI polymorphism is located in the noncoding region of exon 4 of the apoC-III gene. The minor S2 allele has been associated with hypertriglyceridemia (43, 55–57) and elevated apoC-III levels, but this was not observed in all studies (58, 59). Presence of the S2 allele in spouses and hyperlipidemic relatives was associated with increased plasma triglyceride and cholesterol levels. Of the three loci studied, only S2 was associated with significantly increased plasma apoC-III concentrations in spouses and hyperlipidemic relatives (Table 10). In sib-pair analysis, however, no evidence was obtained for an effect of the SstI locus on these plasma traits, including LDL cholesterol. The frequency of the S2-allele was only significantly higher in FCH probands (3-fold) than in relatives and spouses (Fig. 2). Therefore the SstI locus segregated in a different manner than XmnI and MspI loci. Because the SstI locus showed a different contribu-

TABLE 10. Effect of S1S2/S2S2 genotype on plasma traits

	Probands		Hyperlipidemic Relatives		Normolipidemic Relatives		Spouses	
Traits	S1S1	S1S2/S2S2	S1S1	S1S2/S2S2	S1S1	S1S2/S2S2	S1S1	1S2/S2S2
n	12	6	145	33	186	24	157	20
Chol	10.4 ± 5.12	13.9 ± 7.6	7.03 ± 1.15	7.22 ± 1.52	4.93 ± 0.82	5.05 ± 0.58	5.57 ± 1.06	6.15 ± 0.96[a]
HDL-C	1.04 ± 0.28	0.95 ± 0.12	1.15 ± 0.34	1.10 ± 0.32	1.24 ± 0.28	1.27 ± 0.27	1.27 ± 0.37	1.27 ± 0.31
LDL-C	6.01 ± 2.00	4.12 ± 1.91	4.71 ± 1.17	4.51 ± 1.08	3.12 ± 0.74	3.21 ± 0.50	3.60 ± 0.97	4.11 ± 0.96[a]
TG	10.6 ± 21.8	17.3 ± 2.6	2.51 ± 1.50	3.92 ± 5.37	1.23 ± 0.39	1.23 ± 0.36	1.56 ± 0.85	2.15 ± 2.29[a]
ApoB	231 ± 301	124 ± 12	128 ± 20	133 ± 16	82 ± 12	86 ± 6	97 ± 17	111 ± 17[a]
ApoA-I	133 ± 52	136 ± 32	134 ± 26	134 ± 28	132 ± 23	139 ± 23	138 ± 24	139 ± 22
ApoC-III	12.1 ± 5.1	18.7 ± 23.6	11.7 ± 4.1	14.2 ± 7.2[a]	7.5 ± 2.4	8.1 ± 2.1	8.7 ± 3.3	10.7 ± 2.7[a]

Values are expressed as mean ± SD. Plasma lipid traits are given as mmol/l and plasma apolipoprotein traits are given as mg/dl. All *P*-values were determined using *t*-test.

[a]Significantly different at $P < 0.05$.

TABLE 11. *P*-values of sib-pair linkage analysis of plasma LDL cholesterol in relatives from 18 extended families

Gene Locus	Number of Pairs Analyzed	P-Value (untransformed data)	P-Value (log transformed data)
XmnI	513	0.062	0.048[b]
MspI	507	0.039[a]	0.040[a]
SstI	513	0.77	0.73
XMS haplotype	489	0.18	0.17

[a]MspI locus: analysis without outliers, $P = 0.032$; without outliers and with log-transformed data $P = 0.013$.
[b]XmnI locus: analysis without outliers, with log-transformed data $P = 0.016$.

tion towards the expression of hyperlipidemia, a normal genotype frequency in the relatives, and no linkage in the sib-pair analysis, there is the possibility that the rare allele of SstI locus is located on a different haplotype than the rare alleles of XmnI and MspI loci. This is currently under investigation in our laboratories.

The SstI locus is in linkage disequilibrium with two mutations in the apoC-III promotor region at -625 and -482 (57). In this region, specific enhancer elements are present that affect transcriptional activity (60–62). Regulation of transcription of these proteins is likely to be more complex and involves several other regions, as shown by Haase and Stoffel (60). ApoC-III is an apolipoprotein that plays a role in the elimination of remnants of triglyceride-rich particles, potentially inhibiting the lipoprotein lipase activity and receptor-mediated uptake (63). ApoC-III transgenic animals develop lipoproteins enriched in apoC-III which show impaired elimination (23–25), resulting in the accumulation of triglyceride-rich remnant particles and hypertriglyceridemia. Plasma apoC-III concentrations were significantly increased in FCH probands (by 67%) and in FCH relatives (by 36%) compared to spouses (Tables 3 and 4). In a previous report, impaired elimination of triglyceride-rich lipoproteins was associated with increased plasma apoC-III levels (26). It is not presently known whether TG-rich lipoproteins in FCH have an altered composition with excess apoC-III or show an increased number of particles with normal apoC-III content per particle.

The lipoprotein metabolism in FCH is characterized by an overproduction of apolipoprotein (apo) B-100-containing lipoproteins, which is reflected in elevated plasma concentrations of low density lipoproteins (LDL) and very low density lipoproteins (VLDL) (8–11). Plasma apoB concentrations in FCH probands were significantly increased, by 52%, compared to the spouses, and hyperlipidemic relatives had 55% increased apoB levels compared to normolipidemic relatives. In spouses, the presence of a X2 or S2 minor allele had a significant effect on apoB, increasing the levels by 7% and 14%, respectively. Because sib-pair analysis did not reveal linkage between the gene cluster and plasma apoB, it is possible that the effect of the X2 and S2 minor alleles is not causal, but rather the result of interaction with other genes.

In conclusion, the tested loci in the apoA-I/C-III/A-IV gene cluster were shown to aggravate the hypercholesterolemia and hypertriglyceridemia in probands. However, expression of the FCH phenotype per se did not depend upon the presence of minor alleles in the gene cluster. In spouses, representing the normal population, variations in the gene cluster had an effect on plasma LDL cholesterol levels that explained the change in total plasma cholesterol. Analysis of 507 sib-pairs demonstrated evidence for linkage between the MspI locus and quantitative variation in plasma LDL cholesterol. The MspI locus, therefore, contributes to multigenic hypercholesterolemia in the population.

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