

Association of the *APOLIPOPROTEIN A1/C3/A4/A5* Gene Cluster With Triglyceride Levels and LDL Particle Size in Familial Combined Hyperlipidemia

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Abstract—The *APOLIPOPROTEIN (APO)A1/C3/A4/A5* gene cluster on chromosome 11 has been hypothesized to be a modifier of plasma triglycerides in FCH. In the present study, we extended previous association analyses of the gene cluster to include *APOA5*, a newly discovered member of the cluster. Eight SNPs across the *APOA1/C3/A4/A5* gene region were analyzed in 78 FCH probands and their normolipidemic spouses as well as in 27 Dutch FCH families. Of the individual SNPs tested in the case-control panel, the strongest evidence of association was obtained with SNPs in *APOA1* ($P=0.001$) and *APOA5* ($P=0.001$). A single haplotype defined by a missense mutation in *APOA5* was enriched 3-fold in FCH probands when compared with the normolipidemic spouses ($P=0.001$) and a second haplotype was significantly enriched in the spouses ($P=0.001$). Family-based tests also indicated significant association of triglyceride levels and LDL particle size with the investigated SNPs of *APOC3* and *APOA5*. These findings suggest that genetic variation in the *APOA1/C3/A4/A5* gene cluster acts as a modifier of plasma triglyceride levels and LDL particle size within FCH families and furthermore indicate that a number of haplotypes may contribute to FCH. (*Circ Res.* 2004;94:993-999.)

Key Words: genetics ■ coronary artery disease ■ apolipoproteins

Familial combined hyperlipidemia (FCH; see Mendelian Inheritance in Man [MIM-144250], which can be accessed online [OMIM] at <http://www.ncbi.nlm.nih.gov/Omim/>) is a complex lipid disorder characterized by elevated plasma triglyceride and cholesterol levels.¹ The prevalence of FCH is about 1% to 2% in Western societies, whereas in survivors of myocardial infarction under 60 years of age, it is enriched about 10-fold, indicating an important role in coronary artery disease.¹ FCH is often accompanied by an overproduction of apolipoprotein (apo) B-100-containing lipoproteins, accumulation of small, dense low-density lipoprotein (LDL) particles, and reduced concentrations of high-density lipoproteins (HDL).²⁻⁵ Although the disorder was first recognized about 30 years ago, the underlying genetic abnormalities in FCH remain unknown.

In a landmark study, Pennacchio and colleagues⁶ identified *APOA5*, a new member of the *APOA1/C3/A4* gene cluster that modulates plasma triglycerides in mice as well as in humans. Two distinct alleles of *APOA5* were shown to be independently associated with ~30% increase in plasma

triglyceride levels in two independent populations.⁷ Several additional studies have confirmed the association of *APOA5* SNPs with increased plasma triglyceride levels in the general population and in dyslipidemic populations.⁸⁻¹²

Strong linkage between the *APOA1/C3/A4* gene cluster and FCH was first reported in 1991 by Wojciechowski and colleagues.¹³ We have previously reported modest evidence for linkage of the region to FCH,¹⁴ although these results have not been confirmed by other studies.^{15,16} Furthermore, association of the locus with FCH has been observed in cohorts where there was no evidence for linkage.¹⁷⁻¹⁹ The differences in the results among these studies may be explained by the complex nature of FCH in which multiple genes and environmental factors are thought to play a role. Population-based differences as well as differences in ascertainment and diagnosis of FCH between studies could also explain the conflicting results.

To test whether variation in the *APOA1/C3/A4/A5* gene cluster is associated with FCH and its associated lipid parameters, we used both a case-control and family-based

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TABLE 1. Clinical Characteristics of Probands and Spouses

Trait	FCH		TG Probands
	Probands	Spouses	
Male/female, n	51/31	50/74	24/27
Age, years	52±11	51±11	49±12
Total cholesterol, mmol/L	7.10±2.66	5.54±1.02	7.25±3.04
Triglycerides, mmol/L	4.63±8.99	1.33±0.61	6.10±10.94
HDL-C, mmol/L	0.92±0.26	1.22±0.39	0.92±0.28
LDL-C, mmol/L	4.39±1.62	3.72±0.92	3.98±1.47
apoB, g/L	1.39±0.34	1.00±0.25	1.36±0.35
Body mass index, kg/m ²	27.62±3.57	25.37±3.83	28.00±4.10
Waist-to-hip ratio	0.94±0.08	0.86±0.09	0.94±0.09

Values are expressed as mean±SD.

approach. In the present study, we provide evidence confirming the association of the *APOA1/C3/A4/A5* gene cluster with FCH and report the novel finding of an association of the cluster with LDL particle size. The results demonstrate that the *APOA1/C3/A4/A5* gene cluster is an important genetic determinant of plasma triglyceride levels and LDL particle size in FCH.

Materials and Methods

Subjects

The 82 FCH probands and 124 spouses were recruited through the Lipid Clinics of the Utrecht and Maastricht University Hospitals, the Netherlands. The clinical characteristics of the study populations are listed in Tables 1 and 2. Twenty-seven FCH families from Utrecht were ascertained through probands as previously described²⁰ and in the expanded Materials and Methods section in the online data supplement available at <http://circres.ahajournals.org>. All subjects gave informed consent, and the study protocol was approved by the Human Investigation Review Committees of the University Hospitals of Utrecht and Maastricht, the Netherlands, and the University of California, Los Angeles.

Biochemical Analyses

Venous blood was drawn after an overnight fast, and plasma was prepared by immediate centrifugation. Lipids and apolipoproteins were quantified by methods as described.²⁰ LDL particle diameters were measured as described.²¹ Probands and hyperlipidemic rela-

tives who used lipid-lowering drugs were studied after their lipid-lowering treatment was withheld for 3 weeks.

Dichotomization of Triglyceride Status

Triglyceride levels are age- and sex-dependent. Therefore, the triglyceride trait of the FCH probands and family members was dichotomized based on the 90th age- and sex-specific population percentiles for triglyceride levels. Because no triglyceride percentiles for the Dutch population are publicly available, we used Finnish population percentiles for this qualitative approach. This method of reclassification of Dutch FCH individuals is identical to the criteria reported elsewhere.²² Because the Finnish triglyceride levels are, on average, somewhat higher than the Dutch, the Finnish population percentiles can be considered to be a more stringent criteria than if population averages from the Netherlands were used. Using these criteria, there were 152 affected individuals and 671 unaffected individuals in the family study sample.

Genotyping

The *XmnI*, *MspI*, *SstI*, and the *HinfI* polymorphisms were genotyped as described.²⁰ *APOA5* SNPs 1, 2, and 4 were genotyped by pyrosequencing using the PSQ96 instrument and the SNP Reagent kit (Pyrosequencing AB).²³ *APOA5* SNP3 was genotyped as described.⁷ Primer design information and accession numbers for the SNPs are given in the online data supplement.

Statistical Analyses

Pairwise linkage disequilibrium (LD) was estimated using the Genetic Equilibrium option of the MENDEL 5.0 program.²⁴ Statistical analyses of association in probands and spouses were performed using the SAS computer program.²⁵ One-sided Fisher's exact probability values are reported because association of the investigated SNPs has been observed in other studies (see reviews^{26,27}). A Bonferroni correction was used to account for multiple testing. SNPs in strong LD with each other were not considered as independent tests, thus the observed probability value was multiplied by the number of groups of SNPs in complete LD. ANOVA was used to initially assess genotypic differences in mean triglyceride levels across the three genotypic groups. However, due to a low number of homozygotes, individuals who were either heterozygous or homozygous for the minor allele were grouped together. Unpaired *t* tests were then used to assess genotypic differences in the spouse and proband groups separately. Triglyceride values of the spouses and probands were not adjusted for age and sex because these factors accounted for only a small part of the variation in triglyceride levels ($R^2 < 0.05$).

For spouses and probands from the 27 FCH families, SNP haplotypes were constructed using the GENEHUNTER program.²⁸

TABLE 2. Clinical Characteristics of the FCH Family Members

Trait	Hyperlipidemic Individuals	Normolipidemic Individuals	Spouse Controls
Male/female, n	173/125	144/151	87/121
Age, years	48±16	34±16	48±15
Total cholesterol, mmol/L	7.37±2.37	4.95±0.81	5.70±1.06
Triglycerides, mmol/L	3.57±6.78	1.27±0.41	1.64±1.04
HDL-C, mmol/L	1.14±0.32	1.21±0.32	1.25±0.34
LDL-C, mmol/L	4.66±1.36	3.13±0.74	3.73±0.99
apoB, g/L	1.32±0.30	0.83±0.21	1.02±0.28
PPD* (Å)	265.21±10.91	274.42±6.91	273.44±7.19
Body mass index, kg/m ²	26.14±3.57	23.09±3.16	25.33±3.76
Waist-to-hip ratio	0.89±0.09	0.82±0.08	0.85±0.10

*Peak LDL particle size.

Values are expressed as mean±SD.

When using pedigree data, Mendelian errors were identified with the PedCheck program.²⁹ For haplotype construction, APOA5 SNP2 was chosen as the representative marker for APOA5 SNPs 1, 2, and 4 due to extensive LD; however, for clarity, all three SNPs are shown in Figure 2B. The haplotypes identified in the 27 families were then used to assign the haplotypes in the spouses and probands. Haplotypes present in fewer than 10 individuals were binned. Fisher's exact test was used to compare the haplotype distributions of FCH probands and spouse controls and to test for enrichment of each haplotype versus all other haplotypes.

The SNPs were tested for linkage or association in the FCH families through use of the gamete competition test³⁰ and the haplotype-based haplotype relative risk test (HHRR).³¹ The gamete competition test was performed using OPTION 8 of MENDEL 5.0²⁴ and the HHRR analysis was performed using the HRRLAMB program.³² The gamete competition provides a parametric extension of the transmission/disequilibrium test (TDT) and views transmission of marker alleles from heterozygous parents to affected children as a contest between the alleles, making effective use of full pedigree data.³⁰ The gamete competition method is not purely a test of association, because the null hypothesis is no association and no linkage, and thus, linkage could result in a significant probability value. Using the gamete competition test, the SNPs were tested for association or linkage with FCH and a binary triglyceride trait as well as with the following quantitative traits: triglyceride levels, total cholesterol, BMI, WHR, LDL levels, HDL levels, LDL particle size, apoA-I levels, apoB levels, and apoC-III levels. Due to nonnormality, triglycerides were log transformed before all analyses. The HHRR is an extension of the TDT, which includes one affected individual per pedigree when there is no linkage to the region, yet it is also able to incorporate data from triads in which the parents are either homozygous or have missing genotypes.³² All genotyped SNPs were tested for association with FCH and a binary triglyceride trait using this method.

Results

Pairwise Linkage Disequilibrium

Setting a criterion of $P < 0.001$, the observed pattern of LD was the same in spouses and probands (online Tables I and II, available at <http://circres.ahajournals.org>, in the online data supplement). APOA5 SNPs 1, 2, and 4 were found to be in strong LD with each other and with the SstI polymorphism of APOC3. Both of the APOA1 polymorphisms, XmnI and MspI, were in LD with each other and with APOA5 SNP3. HinfI was not in significant LD with any of the polymorphisms studied (online Tables I and II).

Case-Control Association Studies With Individual SNPs

The allele frequencies of the eight polymorphisms in APOA1/C3/A4/A5 were compared between FCH probands and their spouses (Figure 1). The XmnI, MspI, SstI, and HinfI polymorphisms were chosen on the basis of previously reported associations with FCH.^{14,20,33} The studied APOA5 SNPs were chosen on the basis of reported associations with triglyceride levels (SNPs 1 and 4)⁶ and the likelihood of altering the level of expression or function of apoAV (SNPs 2 and 3). APOA5 SNP2 is located in the putative Kozak sequence of APOA5 and thus, may alter the level of APOA5 expression. APOA5 SNP3 results in a nonsynonymous substitution of tryptophan for serine and therefore may alter apoAV function. With the exception of HinfI, the minor alleles were about 2-fold increased in the probands over controls, although only XmnI, MspI, SstI, and APOA5 SNP3 reached statistical significance

(corrected $P=0.001$, $P=0.001$, $P=0.01$, and $P=0.001$, respectively). The minor alleles of XmnI, MspI, and APOA5 SNP3 were associated with higher triglyceride levels in FCH probands, although only XmnI reached statistical significance (4.47 ± 3.5 versus 3.25 ± 2.3 mmol/L; $P=0.02$ for carriers versus noncarriers; data not shown). The four FCH probands that were homozygous for the minor allele of APOA5 SNP3 exhibited a marked increase in plasma triglycerides over probands homozygous for the major allele (7.46 ± 5.7 versus 4.45 ± 10.3 mmol/L, respectively), although due to a small number of homozygotes, statistical significance was not reached. In contrast to the other polymorphisms studied, the frequency of the minor allele of HinfI was significantly increased in spouse controls ($P=0.004$). Within FCH probands, carriers of the minor allele of HinfI exhibited lower plasma triglycerides (3.15 ± 1.40 versus 5.12 ± 10.7 mmol/L, for carriers versus noncarriers), although this decrease was not significant ($P=0.39$).

In a subset of 40 hypertriglyceridemic probands (see Materials and Methods), the frequency of the rare alleles of XmnI, MspI, and APOA5 SNP3 were three times more frequent in probands than in spouse controls ($P=0.000003$, $P=0.0001$, and $P=0.0001$, respectively); however, no associations with SstI, HinfI, or APOA5 SNPs 1, 2, or 4 were observed.

Haplotype Distributions in FCH Cases and Controls

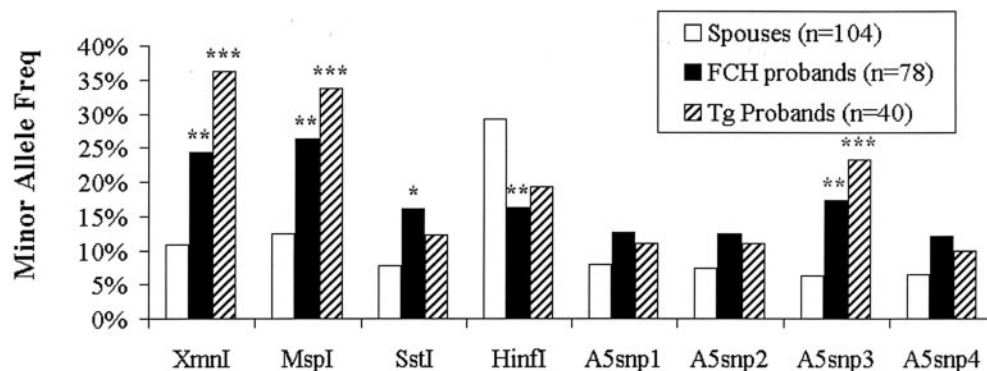
The locations of the SNPs used to define each haplotype are shown in Figure 2A. The six haplotypes shown in Figure 2B represent 95% of those observed in spouses and probands. The haplotype distribution of the probands was significantly different from that of the spouse controls ($P=0.0001$). Of the six haplotypes, there was significant association of haplotypes 3 and 6 with FCH (Figure 2B). When compared with the spouse controls, the frequency of haplotype 3, which carries the minor alleles of XmnI, MspI, and APOA5 SNP3, was 3-fold enriched in the probands ($P=0.001$). Interestingly, no difference in the frequency of haplotype 4, which carries the minor alleles of XmnI and MspI but not of APOA5 SNP3, was observed between the spouses and the probands ($P=0.4$). Consistent with the increased frequency of the minor allele of HinfI in normolipidemic spouses, the frequency of the haplotype that carries this allele was also significantly increased in spouses ($P=0.001$).

Haplotype-Based Haplotype Relative Risk Test

Next, we investigated the associations of the eight SNPs and their haplotypes on the 27 Dutch FCH families, which avoids some potential problems with population association. Suggestive evidence of association of the XmnI polymorphism with FCH was observed ($P=0.03$). For the binary triglyceride trait, evidence of association was observed with HinfI ($P=0.04$) and APOA5 SNP2 ($P=0.05$) (Table 3).

Gamete Competition Test

The gamete competition test was also used to investigate the contribution of this locus to FCH. Significant evidence of preferential transmission to those with FCH was observed



	XmnI ^a	MspI ^b	SstI ^c	HinfI ^d	A5SNP1 ^e	A5SNP2 ^f	A5SNP3 ^g	A5 SNP4 ^h
FCH ^f vs.	0.001	0.001	0.01	0.004	NS	NS	0.001	0.04
Spouses	(0.003)	(0.002)	(0.03)	(0.01)			(0.002)	(0.1)
TG ^g vs.	0.000003	0.0001	NS	NS	NS	NS	0.0001	NS
Spouses	(0.00001)	(0.0002)					(0.0003)	

NS = not significant

^fFCH probands

^gHypertriglyceridemic FCH probands (see Methods)

^a*APOA1* -2500C>T

^b*APOA1* -75G>A

^c*APOC3* 3238G>C

^d*APOA4* 347A>T

^e*APOA5* -1131T>C

^f*APOA5* -3A>G

^g*APOA5* S19W

^h*APOA5* exon3 + 476G>A

Figure 1. Allele frequencies of the 8 polymorphisms within the *APOA1/C3/A4/A5* gene cluster. Results are represented as the frequency of the minor allele in normolipidemic spouses (white bars), FCH probands (black bars), and hypertriglyceridemic FCH probands (striped bars). Probability values were tested by Fisher's exact test. Bonferroni-corrected probability values are shown in parentheses. * $P \leq 0.05$, ** $P \leq 0.005$, and *** $P \leq 0.0005$.

with the minor allele of the *SstI* polymorphism ($P=0.01$). In hypertriglyceridemic individuals, evidence of preferential transmission of the minor alleles of *SstI* ($P=0.001$), *APOA5* SNPs 2 and 4 ($P=0.002$ and $P=0.004$, respectively), and the major allele of *HinfI* ($P=0.003$) was observed. Similar results were obtained with quantitative triglyceride levels (Table 3, right). *SstI* and *APOA5* SNPs 1, 2, and 4 were also strongly associated with LDL particle size ($P=0.004$, $P=0.005$, $P=0.0001$, and $P=0.0004$, respectively). Because a number of studies have demonstrated that waist-to-hip ratio (WHR) is strongly correlated with LDL particle size, we tested for association of the SNPs with WHR.^{34–38} Only the minor allele of *SstI* showed evidence of preferential transmission to individuals with a high WHR ($P=0.01$, data not shown). There was also evidence of association of *APOA5* SNP2 and SNP4 with HDL levels ($P=0.008$ and $P=0.02$), but no evidence of association was observed with total cholesterol, LDL, apoA-I, apoB, or apoC-III levels (data not shown).

Discussion

Our results indicate that variation in the *APOA1/C3/A4/A5* gene cluster is associated with a difference in both plasma

triglyceride levels and LDL particle size in FCH. This study is of particular importance because linkage and association results of the *APOA1/C3/A4* gene cluster in FCH have not always been consistent. Although we and others have previously reported both linkage and association of this locus with FCH, several other studies have not observed the same results.^{13,16} The discrepancies among studies regarding the involvement of this gene cluster in FCH may be explained both by the complexity of its contribution to FCH as well as the potential genetic heterogeneity underlying the disease.

Although the association of *APOA5* SNPs with triglyceride levels has been firmly established, no direct effect on LDL particle size has previously been reported, although it has been associated with WHR, a strong predictor of LDL particle size.⁹ By using a quantitative gamete competition test, we were able to investigate the effects of *APOA1/C3/A4/A5* variation on several lipid parameters within FCH families. The presence of sdLDL particles, which often accompanies the FCH phenotype, has been postulated to be a consequence of VLDL overproduction, and has been associated with CAD.^{39,40} Indeed, it has been shown that the most consistent lipid abnormality among FCH patients is the

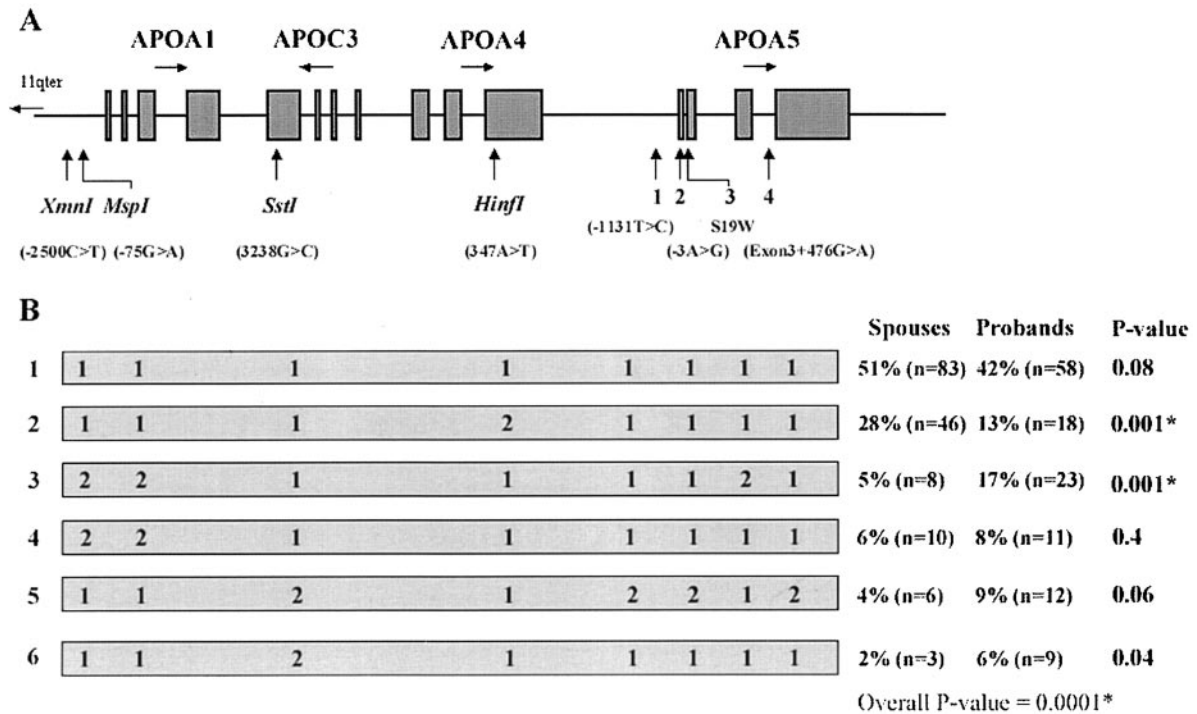


Figure 2. A, APOA1/C3/A4/A5 genomic structure and location of polymorphisms. Exons are depicted as gray boxes. Arrows below the name of each gene indicate the transcriptional orientation of each gene. Location of each SNP is given in parentheses. B, Comparison of APOA1/C3/A4/A5 haplotype distributions in spouses and FCH probands (Fisher's exact value of $P=0.0001$). Major alleles of each SNP are represented as 1 and minor alleles are represented as 2. Asterisks indicate significant differences in frequencies between spouses and FCH probands.

persistence of sdLDL.⁴¹ Therefore, genes influencing LDL particle size might be key factors contributing to the development of FCH, and by extension, the development of coronary artery disease (CAD). It has been shown that the minor allele of the *SstI* polymorphism may help to predict coronary artery disease within FCH families.⁴² Additionally, we have previously reported evidence that the APOA1/C3/A4 gene cluster is a shared genetic determinant of LDL particle size in FCH families and families enriched for CAD.⁴³

TABLE 3. Family-Based Association of Polymorphisms in the APOA1/C3/A4/A5 Gene Cluster With FCH, Triglycerides, and LDL Particle Size

	Haplotype-Based Haplotype Relative Risk		Gamete Competition			
	FCH	TG†	FCH	TG†	TG-Q‡	PPD§
<i>XmnI</i>	0.03*	0.9	0.2	0.04*	0.4	0.8
<i>MspI</i>	0.3	0.5	0.2	0.04*	0.3	0.6
<i>SstI</i>	0.9	0.1	0.01*	0.001**	0.01*	0.004**
<i>HinfI</i>	0.6	0.04*	0.4	0.003**	0.002**	0.1
A5snp1	0.7	0.1	0.5	0.07	0.03*	0.005**
A5snp2	0.10	0.05*	0.08	0.002**	0.01*	0.0001**
A5snp3	0.6	0.2	0.6	0.05*	0.2	0.4
A5snp4	0.3	0.07	0.2	0.004**	0.003**	0.0004**

* $P \leq 0.05$, ** $P \leq 0.005$.

†Age- and sex-dependent binary triglyceride trait; ‡Quantitative triglyceride levels; §Peak LDL particle diameter.

Consistent with this finding, polymorphisms within *APOC3* as well as *APOA5* were significantly associated with LDL particle size ($P=0.004$ and $P=0.0001$, respectively) (Table 3), providing evidence that these genes could contribute to the production and/or development of sdLDL, and thereby may be significant risk factors for the development of CAD.

The gamete competition test provided only modest results with the combined hyperlipidemia trait ($P=0.01$ for *SstI*) and with a binary triglyceride trait ($P=0.001$). This result is comparable to that obtained in a similar study of 115 FCH families in which the minor allele of *SstI* (alternatively named *APOC3*^{c.386G}) was associated with combined hyperlipidemia ($P=0.014$) and with a binary triglyceride trait ($P=0.049$).¹² However, in that study, the minor allele of *APOA5* SNP3 (alternatively named *APOA5*^{v.56G}) was significantly associated with FCH ($P=0.003$), whereas in the present study, this SNP was not significantly associated with FCH in our family study sample ($P=0.6$). The differences in these results are most likely due to differences in FCH diagnostic criteria, because when an age- and sex-adjusted criterion is applied to the triglyceride trait, a significant association is observed for this SNP ($P=0.05$).

In the case-control study, the investigated SNPs were associated with FCH, and with binary and quantitative triglyceride traits within FCH probands. *APOA5* SNP1 has been associated with increased VLDL-cholesterol in a population of 627 dyslipidemic individuals and with increased VLDL-apo B in 16 FCH families.^{8,11} Although these traits were not available for analysis in the present study, no significant differences in total cholesterol, LDL-cholesterol,

HDL-cholesterol, or plasma apoB were observed between carriers and noncarriers in the case-control sample. However, these negative results could be due to the limited size of our case-control data set.

It has been postulated that additional genetic factors are necessary for expression of the triglyceride-raising effect of *APOA5* variants.^{10,11} Consistent with this hypothesis, an increase in plasma triglyceride levels was observed only in FCH probands who carried the minor allele of *APOA5* SNP3, whereas spouse controls who carried the minor allele did not exhibit increased triglyceride levels, suggesting that the dyslipidemic background present in FCH individuals augments the triglyceride-raising effect of this allele. A similar result was observed in a study by Ribalta et al¹¹ in which proband carriers of the *APOA5* SNP1 (alternatively named -1131^{T>C}) allele exhibited higher plasma triglyceride levels than that of noncarrier probands (2.52 ± 1.34 versus 1.76 ± 0.90 mmol/L), whereas this effect on triglyceride levels was not observed in normolipidemic relatives (0.82 ± 0.47 versus 0.94 ± 0.44 mmol/L, for carriers versus noncarriers, respectively).¹¹ Therefore, analysis of multilocus associations with other FCH candidate genes may help to elucidate possible gene-gene interactions that affect plasma triglyceride levels within FCH individuals. One such possible candidate is the gene encoding the peroxisome proliferator-activated receptor α (*PPARA*). *PPARA* gene expression has been shown to affect the expression of *APOA1*, *APOC3*, and *APOA5*.^{44,45} Indeed, variation near the *PPARA* locus has been shown to have a modifying effect on plasma concentrations of apoC-III in FCH subjects, raising the possibility of an additional effect on plasma apoAV, which could subsequently influence triglyceride levels.⁴⁶

Lastly, we were also able to place *APOA5* within the context of a previously defined FCH susceptibility haplotype at this locus. Previously, we reported the identification of two possible susceptibility haplotypes, one which carries the minor alleles of *XmnI* and *MspI* (X2M2S1) and the other which carries the minor allele of *SstI* (X1M1S2).¹⁴ With the addition of *APOA5* SNP3, the X2M2S1 haplotype has now been dichotomized into two haplotypes, indicated by haplotypes 3 and 4 in Figure 2B. In the present study, only the haplotype carrying the minor allele of *APOA5* SNP3 (haplotype 3) was enriched in FCH probands, suggesting that the previous association with the X2M2S1 haplotype was due to LD with *APOA5* SNP3. This finding indicates that it may be the presence of *APOA5* SNP3 (S19W) that is important in the development of FCH rather than the *XmnI* and *MspI* polymorphisms. This hypothesis is further supported by the findings of Eichenbaum-Voline et al¹² who reported a significant preferential transmission of the *APOA5* SNP3 minor allele to FCH individuals. With the inclusion of *APOA5* SNPs, the second susceptibility haplotype, the X1M1S2 haplotype, is also now dichotomized into two haplotypes: haplotype 5, which carries the *SstI* minor allele as well as the minor alleles of *APOA5* SNPs 1, 2, and 4, and haplotype 6, which carries the *SstI* minor allele and common alleles at all other loci (Figure 2B). Both haplotypes 5 and 6 are enriched in FCH probands, although the low frequency of these haplotypes in our data sample limits our ability to detect a statistically significant association.

Although the primary purpose of this study was to investigate the role of *APOA5* in FCH, the contribution of *APOC3* cannot be overlooked. The *SstI* polymorphism has been shown to be significantly associated with a decrease in LDL particle size in men and has been consistently associated with increased plasma triglyceride levels in several populations.^{19,47-51} The *SstI* polymorphism of *APOC3* was associated with FCH in our case-control and family study samples, and with LDL particle size in FCH families. The *SstI* minor allele also exhibited preferential transmission to affected individuals in British FCH families.¹² Taken together, these data strongly suggest that *APOC3* contributes significantly to FCH, and in particular, to LDL particle size.

In conclusion, although it is likely that variation within the *APOA1/C3/A4/A5* gene region primarily affects triglyceride levels, the novel association with LDL particle size provides additional evidence of the contribution of this locus to FCH. The data also demonstrate the existence of a specific haplotype that is associated with FCH and further support the notion that *APOA5* acts as a modifier of FCH. Thus, incorporation of these data into a multilocus genome scan approach may enhance the signals of existing FCH loci and may also lead to the identification of other novel loci that contribute to FCH.

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Association of the *APOLIPOPROTEIN A1/C3/A4/A5* Gene Cluster With Triglyceride Levels and LDL Particle Size in Familial Combined Hyperlipidemia

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Expanded Material and Methods

Detailed Ascertainment Criteria

The probands met the following criteria: (1) a primary combined hyperlipidemia with varying phenotypic expression, including a fasting plasma cholesterol > 6.5 mmol/l, or $> 90^{\text{th}}$ percentile for age, defined according to tables from the Lipid Research Clinics, and fasting plasma triglycerides > 2.3 mmol/l; (2) at least one first degree relative with a different hyperlipidemic phenotype; (3) a positive family history of premature CAD defined as a myocardial infarction or cardiovascular disease before 60 years of age. Additional singleton FCH probands in the case-control study sample were single individuals diagnosed as FCH when the following criteria were met: (1) evidence of documented premature coronary artery disease in the family or in the patient and (2) documented evidence of multiple lipoprotein phenotype present among first-degree relatives. Exclusion criteria for the probands included diabetes, obesity (BMI > 30), tendon xanthomas indicative of familial hypercholesterolemia, or type III hyperlipidemia (apoE2/E2). Relatives were assigned the FCH phenotype when they met the following criteria: fasting plasma cholesterol > 6.5 mmol/l, and/or fasting plasma triglycerides > 2.3 mmol/l. Using these criteria, there were 275 affected individuals and 548 unaffected relatives in the study sample. The spouses (n=208) represent a population-, environment-, nutrition-, and age-matched control group for the probands and their hyperlipidemic relatives. Additional normolipidemic spouses of FCH family members were included in the control study sample.

SNP Primer Information and Accession Numbers

Primers for PCR were designed using the Primer3 program, available at the Whitehead Institute for Biomedical Research Web site. Detection primers for pyrosequencing were

designed through the use of the SNP primer Design Software, version 1.01 (Pyrosequencing AB). Oligo Analyzer 2.5, available at the Integrated DNA Technologies Web site, was used to calculate the melting temperature of the primers and to check all the primers for primer dimers and hairpins to prevent possible background signals in the SNP genotyping³. *APOA5* SNPS 1, 2, 3 and 4 were previously identified¹ and are available at the dbSNP home page under accession numbers ss3199915, ss4383596, ss4383597, and ss3199914, respectively.

Supplementary Tables

Table I. Pairwise Linkage Disequilibrium in Normolipidemic Spouses.

	XmnI ^a	MspI ^b	SstI ^c	HinfI ^d	A5snp1 ^e	A5snp2 ^f	A5snp3 ^g	A5snp4 ^h
XmnI ^a		0.000001	NS	NS	NS	NS	0.0003	NS
MspI ^b			NS	NS	NS	NS	0.000001	NS
SstI ^c				NS	0.000001	0.000001	NS	0.00003
HinfI ^d					NS	NS	0.04	NS
A5snp1 ^e						0.000001	NS	0.000001
A5snp2 ^f							NS	0.000001
A5snp3 ^g								NS
A5snp4 ^h								

Table II. Pairwise Linkage Disequilibrium in FCH probands.

	XmnI ^a	MspI ^b	SstI ^c	HinfI ^d	A5snp1 ^e	A5snp2 ^f	A5snp3 ^g	A5snp4 ^h
XmnI ^a		0.000001	NS	0.02	NS	NS	0.000001	NS
MspI ^b			NS	NS	NS	NS	0.000001	NS
SstI ^c				NS	0.000001	0.000001	NS	0.000001
HinfI ^d					NS	NS	NS	NS
A5snp1 ^e						0.000001	NS	0.000001
A5snp2 ^f							NS	0.000001
A5snp3 ^g								NS
A5snp4 ^h								

Pairwise linkage disequilibrium between SNPs was estimated using the Genetic Equilibrium option of MENDEL 5.0. Fisher's exact P-values are shown above (spouses) and below (probands) the gray boxes. NS = not significant

^aAPOA1 -2500C>T

^bAPOA1 -75G>A

^cAPOC3 3238G>C

^dAPOA4 347A>T

^eAPOA5 -1131T>C

^fAPOA5 -3A>G

^gAPOA5 S19W

^hAPOA5 exon3+476G>A

References for Online Data Supplements

1. Pennacchio LA, Olivier M, Hubacek JA, Krauss RM, Rubin EM, Cohen JC. Two independent apolipoprotein A5 haplotypes influence human plasma triglyceride levels. *Hum Mol Genet.* 2002;11:3031-8.

Electronic Database Information

URLs for data presented herein are as follows:

Integrated DNA Technologies, <http://www.idtdna.com/> (for Oligo Analyzer 2.5)

Whitehead Institute for Biomedical Research, <http://www->

genome.wi.mit.edu/genome_software/other/primer3.html (for the Primer3 program)

dbSNP Home Page, <http://www.ncbi.nlm.nih.gov/SNP/>