A variation in the apolipoprotein C-III gene is associated with an increased number of circulating VLDL and IDL particles in familial combined hyperlipidemia

Josep Ribalta,[1,*] Agnes E. La Ville,* Joan C. Vallvé,* Steve Humphries,[†] Peter R. Turner,* and Lluis Masana*

Unitat de Recerca de Lípids,* Facultat de Medicina, Hospital Universitari de Sant Joan, C/ Sant Llorenç 21, 43201 Reus, Spain, and The Cardiovascular Genetics Division,[†] Department of Medicine, University College London Medical School, London, UK

Abstract Detailed plasma lipoprotein analyses were conducted on 16 familial combined hyperlipidemic (FCHL) probands, all their available family members (n = 106) together with 12 normolipidemic control families (n = 63), and the results were assessed in relation to a C_{1100}-T polymorphism in exon 3 of the apoC-III gene. The frequency of the T_{1100} genotype (CT + TT) was significantly elevated in the probands relative to control subjects (0.64 vs. 0.36; $P < 0.01$) and was associated with elevated concentrations of plasma triglyceride ($P < 0.02$) and apoC-III ($P < 0.03$), VLDL cholesterol ($P < 0.005$), VLDL triglyceride ($P < 0.009$), IDL cholesterol ($P < 0.01$), and IDL triglyceride ($P < 0.007$). The T_{1100} genotype was also associated with elevations in VLDL-apoB ($P < 0.005$) and IDL-apoB ($P < 0.04$) indicating a relationship between this variation and an increased number of triglyceride-rich particles. These findings were confined to the hyperlipidemic members of the FCHL families and showed a strong genotype–status interaction ($P < 0.001$). It is of considerable clinical relevance that the apoC-III gene may be acting as a modifier gene that is only expressed in the presence of other factors (e.g., increased VLDL flux, low LPL activity) and therefore may predispose those members of FCHL families carrying the T_{1100} allele to express the FCHL phenotype.—**Ribalta, J., A. E. La Ville, J. C. Vallvé, S. Humphries, P. R. Turner, and L. Masana.** A variation in the apolipoprotein C-III gene is associated with an increased number of circulating VLDL and IDL particles in familial combined hyperlipidemia. *J. Lipid Res.* 1997. **38:** 1061–1069.

Supplementary key words remnant particles • CAD • genotype–status interaction • metabolic syndrome • A-I–C-III–A-IV gene cluster • genetic marker • predisposition

Familial combined hyperlipidemia (FCHL) was first described in 1973 as a new, autosomal dominant, genetic disorder with an estimated prevalence of 0.5–2% in the general population and was a condition frequently observed among survivors of a premature myocardial infarction (1, 2). Since then it has been shown to be a highly heterogeneous disorder (3, 4) and, although the hyperlipidemia does not normally manifest before the age of 20 years (5), the presence of the FCHL phenotype among children of affected families has been reported (6). Also, FCHL phenotype may vary among family members and, over a temporal sequence, even in the individual patient (7), with phenotypes IIa, IIb, or IV hyperlipidemia being expressed (8).

An increased flux of apolipoprotein (apo) B of very low density lipoprotein (VLDL), together with a decreased catabolism of triglyceride-rich VLDL and IDL has been reported in these patients (9–12). These metabolic data strongly suggest the apoB gene as a candidate in FCHL, but as several studies have demonstrated that apoB does not play a major role in FCHL (13, 14), other modulators of the metabolism of apoB-containing lipoproteins are being studied. One such is apoC-III which is known to inhibit lipoprotein lipase (LPL) activity in vitro (15–17). A decreased LPL activity has been suggested to affect the hepatic re-uptake of nascent VLDL particles (18). Excess of apoC-III delays, by displacement of apoE, the clearance of IDL particles from plasma (19, 20) and causes hypertriglyceridemia in

Abbreviations: FCHL, familial combined hyperlipidemia; VLDL, very low density lipoprotein; IDL, intermediate density lipoprotein; LDL, low density lipoprotein; HDL, high density lipoprotein; apo, apolipoprotein; LPL, lipoprotein lipase; CAD, coronary artery disease; PCR, polymerase chain reaction; Chol, cholesterol; Trig, triglyceride; HL, hyperlipidemis; NL, normolipidemic.

[1]To whom correspondence should be addressed.

transgenic mice (21). Increased concentrations of apoC-III have been observed in FCHL patients (22) and, in case/control studies, is also associated with the risk of coronary artery disease (CAD); elevated apoC-III concentrations were found in patients with carotid artery disease (23) and its concentration in the high density lipoprotein (HDL) fraction was reported as the best predictor of CAD progression (24).

Genetic data suggest that variations at the AI-CIII-AIV locus may be associated with the triglyceride distributions in a normal population as well as with the FCHL phenotype in a subset of hyperlipidemic patients (25–28). The aim of the present study was to investigate the effect of a common variation in the apoC-III gene on lipoprotein distributions in a group of well-characterized FCHL subjects and family members compared to an equivalent group of clinically healthy control individuals.

MATERIALS AND METHODS

Subjects

All subjects recruited into the study gave fully informed written consent and the protocol was approved by the Scientific and Ethical Committee of the Hospital Universitari de Sant Joan.

FCHL families

Sixteen index patients diagnosed as having FCHL were recruited from the Lipid Clinic of the Hospital Universitari de Sant Joan in Reus (Spain). Diagnosis was based on the subject having plasma concentrations of cholesterol (chol) and triglycerides (trig) $\geq$ 6.4 mmol/L and $\geq$ 2.8 mmol/L, respectively, detected at any time in the clinical history and at least one first degree relative with a hyperlipoproteinemic phenotype different from that of the proband.

Biochemical analyses were conducted to rule out secondary causes of hyperlipidemia and apoE genotyping was performed to exclude type III hyperlipidemia. All index subjects were on lipid-lowering diet and had been taken off lipid-lowering medication for a period of at least 2 months when recalled for the study. All available family members of index patients were recruited and totaled 106 individuals. At inclusion in the study, hyperlipidemic relatives (n = 32) were assigned the FCHL phenotype on the basis of the one lipid measurement if they presented plasma cholesterol concentrations $\geq$ 6.4 mmol/L and/or plasma triglycerides $\geq$ 2.8 mmol/L or elevated above the 95th percentile for age and gender in the case of children below the age of 19 years. Relatives who did not meet these criteria (n = 74) were assigned the normolipidemic status.

Normolipidemic control families

Sixty three individuals belonging to 12 normolipidemic families volunteered to participate and were included as controls in this study. The families were from among the clinical and laboratory staff. Subjects undergoing lipid-lowering therapy or with secondary causes of hyperlipidemia were excluded. None of the families met the criteria to be classified as FCHL.

Unrelated normolipidemic controls

Fifty four unrelated healthy normolipidemic controls were included in the study exclusively for comparison of allele and genotypic frequencies.

Analytical methods

A 10-ml venous blood sample was withdrawn after an overnight fast of 12 h. Triglycerides and cholesterol in plasma and lipoprotein fractions were measured using enzymatic kits (Boehringer Mannheim, Germany) adapted for a Cobas Mira centrifugal analyzer (Roche Pharmaceuticals, Switzerland) with Precilip EL® and Precinorm® (Boehringer Mannheim, Germany) as quality controls. Immuno-turbidometry was used for the measurement of the apolipoproteins using specific antiserum purchased from Boehringer Mannheim, Germany (for apoA-I and apoB), Daiichi Chemicals, Japan (for apoC-II and apoC-III), and Incstar Corporation, Stillwater, MN (for Lp[a]).

Sequential preparative ultracentrifugation

Lipoproteins were separated by sequential preparative ultracentrifugation (29), using a Kontron 45.6 fixed-angle rotor in a Centrikon 75 (Kontron Instruments, Italy). The lipoprotein fractions isolated were VLDL (d < 1.006 g/ml), IDL (d 1.006–1.019 g/ml), and LDL (d 1.019–1.063 g/ml). Total HDL and HDL_3 cholesterol were measured subsequent to the precipitation of the apoB-containing lipoproteins with polyethylene glycol (Immuno AG, Austria). HDL_2 cholesterol was calculated from the difference between total HDL and HDL_3 cholesterol.

DNA analyses

DNA was extracted from an aliquot of frozen white cells by the salting out method (30). PCR was carried out to amplify the region containing the C_{1100}-T transition which results in a nonfunctional polymorphism in exon 3 of the apoC-III gene (31). The reaction mixture (50 µl) for amplification contained 250 ng of each of the two primers (Boehringer Mannheim, Germany), 5′ primer, 5′-CAATGGGTGGTCAAGCAGAAGC-3′ and 3′

TABLE 1. Plasma lipid concentrations, in mmol/L, of the 16 FCHL probands

Family	Relatives		Proband	Age	Sex	Chol	Trig	HDLc	ApoE
	All	<19 yr				A / B	A / B		
				yr			*mmol/L*		
1	9	2	1	51	M	7.1/6.5	3.1/2.9	1.2	3/4
2	6	0	2	29	M	8.1/6.8	3.0/2.0	1.1	3/3
3	9	0	3	54	F	8.7/6.0	4.8/2.6	1.7	3/3
4	3	1	4	48	M	8.7/8.2	3.3/3.3	1.4	3/4
5	17	4	5	42	M	6.9/5.5	6.5/2.1	1.0	3/3
6	7	3	6	44	M	6.4/5.8	5.0/2.4	0.9	3/3
7	10	1	7	54	M	7.8/6.5	6.6/2.0	1.1	3/3
8	5	0	8	59	F	7.0/6.1	4.4/2.8	1.5	3/3
9	7	1	9	62	M	8.3/6.7	3.3/3.1	1.1	3/3
10	6	1	10	47	M	7.3/6.3	4.5/3.2	0.7	3/4
11	5	2	11	46	M	6.9/6.7	6.8/2.2	0.7	3/4
12	9	3	12	57	F	9.4/7.6	3.2/2.9	1.3	3/3
13	7	2	13	51	M	7.8/8.2	3.6/3.5	2.1	3/4
14	6	1	14	53	M	6.3/5.2	9.1/1.5	1.7	3/3
15	5	3	15	36	M	6.6/6.3	3.9/1.9	0.9	3/3
16	11	5	16	32	M	9.8/7.6	5.9/2.1	0.9	3/3
All	122	29		48(9)		7.7(1.0)	4.8(1.7)	1.2(0.4)	

Plasma concentrations of cholesterol (Chol), triglyceride (Trig), and HDL cholesterol (HDLc) and apoE (apoE) genotype. Allelic frequencies of apoE are 0.84 for E3 and 0.15 for E4 among probands. Cholesterol and triglyceride mean values correspond to column A and are expressed as mean (SD). Column A: Maximum values for plasma lipid concentrations obtained from patients' files during long-term follow-up (minimum of 1 year in all cases). Column B: Values obtained on entry into the study. Mean values expressed as mean (SD).

primer, 5′ GAGCACCTCCATTCCATTGTTGG-3′, 200 ng of genomic DNA and 1 U of Taq polymerase (Boehringer Mannheim, Germany). Magnesium concentration in the reaction buffer was 1.5 mM. The reactions were performed on a Hybaid Omnigene thermocycler at 95°C for 5 min, 55°C for 1 min, and 72°C for 2 min followed by 35 cycles at 95°C for 1 min, 55°C for 1 min, and 72°C for 2 min. PCR products were run on a 2% agarose gel and double-blotted on hybond-N^+ membrane (Amersham International, U.K.). Membranes were hybridized as described elsewhere (31) with oligonucleotides C: 5′-ATGCAGGGCTACATGAA, T: 5′-TTC ATGTAACCCTGCAT (Boehringer Mannheim, Germany).

Statistical analyses

The χ^2-test was used to compare the frequency of the T_{1100} genotypes among groups. The Z-test for comparison of proportions was used to compare allele frequencies. ANOVA was performed to compare the means of the lipid, lipoprotein and apolipoprotein data adjusted for age, gender, and BMI, and log transformed when the variables were not normally distributed. The Bonferroni corrected-*t*-test was used to adjust the acceptance level when multiple measurements were made. Comparisons using unadjusted data were with the Student's *t*-test. One-way ANOVA was used to assess linearity between genotypes and lipid parameters. Deviation from Hardy Weinberg equilibrium was tested with the χ^2 goodness-of-fit test. Results are expressed as means and standard deviations. Statistical significance was accepted at the 0.05 level.

RESULTS

Probands' lipid profiles on selection together with the family–member composition is presented in **Table 1.** When recalled for the study, five out of 16 FCHL probands showed a clear normolipidemic phenotype in contrast to the hyperlipidemia noted in the extensive clinical history. **Table 2** contains the data on the relatives of FCHL probands who were assigned hyperlipidemic (HL) or normolipidemic (NL) status according to their plasma cholesterol and triglyceride concentra-

TABLE 2. Biometric characteristics of the groups studied

Variable	Probands	HL-rel	NL-rel	Controls
Number	16	32	74	63
Age	48(9)[a,b]	37(24)	33(20)[c]	42(19)
Gender (M/F)	13/3[a,b,c]	13/19	31/43	30/33
BMI (kg/m^2)	29.3(3.7)[a,b,c]	25.4(5.8)	24.5(5.9)	23.9(3.3)
Diastolic BP	81(9)[b]	76(19)	71(15)[c]	76(11)
Systolic BP	128(13)[b]	129(27)[b]	119(23)	125(18)

Values expressed as mean (SD). Gender Male/Female. Hyperlipidemic (HL-rel) and normolipidemic (NL-rel) relatives.
[a]Significantly different from HL-rel.
[b]Significantly different from NL-rel.
[c]Significantly different from controls.

TABLE 3. Lipid, lipoprotein, and apolipoprotein concentrations in the study groups

Variable	Probands n = 16	HL-rel n = 32	NL-rel n = 74	Controls n = 63
Plasma Chol	6.41(1.07)[b,c]	6.12(1.02)[b,c]	4.42(0.82)	5.08(0.93)
Plasma Trig	2.32(0.70)[a,b,c]	1.36(0.82)[b,c]	0.88(0.42)	1.06(0.61)
VLDL				
Chol	0.90(0.31)[a,b,c]	0.41(0.47)[b,c]	0.21(0.22)	0.23(0.20)
Trig	1.56(0.50)[b,c]	0.77(0.73)[b,c]	0.42(0.33)	0.49(0.47)
ApoB	24.39(11.78)[b,c]	14.77(20.92)[b]	5.67(7.36)[c]	10.16(11.00)
IDL				
Chol	0.46(0.19)[b,c]	0.25(0.27)[b,c]	0.14(0.10)	0.17(0.12)
Trig	0.31(0.09)[b,c]	0.17(0.12)[b,c]	0.12(0.06)	0.12(0.06)
ApoB	8.72(5.35)	6.83(6.52)	4.59(1.68)	5.07(3.20)
LDL				
Chol	4.21(0.85)[b,c]	4.02(0.91)[b,c]	2.89(0.70)	3.36(0.76)
Trig	0.43(0.13)[b,c]	0.28(0.10)[b,c]	0.22(0.08)	0.25(0.09)
ApoB	95.63(16.34)[b,c]	90.07(18.76)[b,c]	64.59(17.12)	72.95(16.90)
HDL_2	0.13(0.06)	0.21(0.10)	0.20(0.15)[c]	0.29(0.20)
HDL_3	0.78(0.19)	1.07(0.28)	0.98(0.28)	1.06(0.22)
Total HDL	0.92(0.21)[c]	1.27(0.34)	1.20(0.30)	1.35(0.33)
Plasma apoA-I	113.25(20.52)	128.63(24.16)	114.85(18.57)	123.89(18.79)
Plasma apoB	130.31(19.74)[b,c]	107.44(27.17)[b,c]	71.70(18.72)[c]	87.53(23.24)
Plasma apoC-II	6.85(1.24)[b,c]	6.26(1.40)[b,c]	5.23(0.82)[c]	4.59(0.42)
Plasma apoC-III	15.17(2.97)[b,c]	13.99(5.05)[b,c]	10.77(2.45)	11.04(1.62)
Lp[a]	20.83(19.50)	19.65(16.25)	19.20(17.95)	24.03(14.72)

Hyperlipidemic (HL-rel) and normolipidemic (NL-rel) relatives. Cholesterol and triglycerides in plasma and all fractions are expressed as mmol/L. Apolipoproteins in plasma and all fractions and Lp[a] are expressed in mg/dl. Statistical differences assessed by ANOVA with data adjusted for age, gender, and BMI. All values expressed as means (SD).

[a]Significantly different compared to HL relatives.
[b]Significantly different compared to NL relatives.
[c]Significantly different compared to controls.

tions together with a further group containing the control family members. FCHL probands had a significantly increased BMI and proportion of male subjects compared to HL relatives, NL relatives, and control subjects together with elevated diastolic and systolic blood pressure compared to NL relatives. FCHL probands were significantly older than their relatives but not different from the control subjects. NL relatives were significantly younger and had lower diastolic blood pressure than control subjects. The proportion of premenopausal women in the NL group (n = 35, 47%) and in controls (n = 26, 41%) was not significantly different. Fourteen of the 29 children below the age of 19 years in the FCHL families had concentrations of either plasma cholesterol, plasma triglycerides or LDL-chol ≥ 95th percentile or HDL-chol ≤ 5th percentile cut-off for age and gender for a Spanish population (32). Eight of them were below the age of 15 years.

Plasma lipids, lipoproteins, and apolipoproteins measured in the FCHL probands, HL, NL, and control group are summarized in **Table 3** and the differences were assessed on data adjusted for BMI, gender, and age. Probands and HL relatives had significantly elevated concentrations of cholesterol and triglycerides (plasma, VLDL, IDL, and LDL) as well as increased concentrations of apoB (plasma, VLDL, LDL) and plasma apoC-II and C-III compared to NL relatives and controls. FCHL probands had significantly higher concentrations of plasma triglycerides and VLDL-chol than their HL relatives. Plasma apoB, VLDL-apoB, and HDL_2-chol were significantly increased and apoC-II was significantly decreased in the control group compared to NL relatives. The apoA-I/total HDL-chol ratio was significantly higher ($P < 0.0001$) in FCHL probands (125.7 ± 13.7) and HL relatives (112.3 ± 27.5) compared to NL relatives (97.9 ± 13.8) and control subjects (95.2 ± 14.0).

C_{1100}-T polymorphism in the apoC-III gene

The distributions of genotypic and allelic frequencies in the different groups are displayed in **Fig. 1.** The observed frequencies of the CC, CT, and TT genotypes in FCHL and control families were not different from those predicted by the Hardy-Weinberg distribution.

Comparisons of allelic and genotype frequencies were made with a control group of 54 unrelated subjects (M/F 40/14) of mean age 32 ± 10, BMI 23.8 ± 4.5, plasma cholesterol 5.04 ± 1.06, and triglycerides 0.94 ± 0.62. Frequencies of the T_{1100} genotypes were significantly higher among FCHL probands (0.64; $P < 0.01$) and HL relatives (0.50; $P < 0.001$) compared to con-

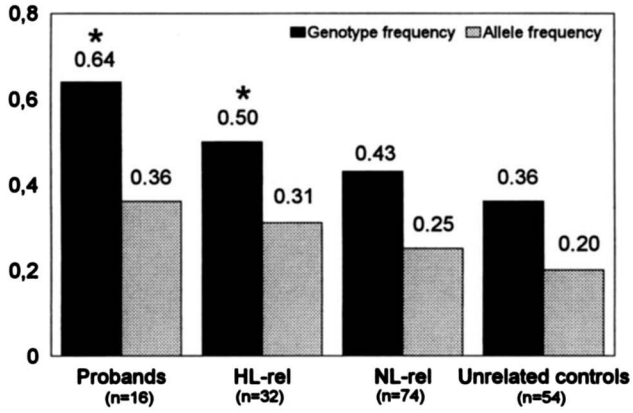

Fig. 1. Genotype (CT/TT) and allelic frequencies of the minor allele (T) in FCHL families: probands, hyperlipidemic relatives (HL-rel), normolipidemic relatives (NL-rel), and unrelated controls. *Statistically different from controls; probands ($P < 0.01$); HL-rel ($P < 0.001$).

trol subjects (0.36). NL relatives did not show higher frequencies of the T_{1100} genotype compared with controls (0.43 and 0.36, respectively). The same, albeit nonsignificant, trend was observed with regard to the allelic frequencies (0.36, 0.31, 0.25, and 0.20; probands, HL relatives, NL relatives and controls, respectively).

The association of the T_{1100} genotype with plasma lipids, lipoproteins and apolipoproteins was studied separately in FCHL and control families and the results are summarized in **Table 4.** Within the FCHL families, the T allele showed a clear association with an increased concentration of plasma triglycerides, apolipoproteins C-III and A-I as well as components (Chol, Trig, and apoB) of VLDL and IDL. Conversely, none of these associations was observed within the control families. Assessed by multiple regression ANOVA after adjustment for age, BMI, and gender, there was a strongly significant interaction ($P < 0.001$) between the effect of the T allele and subject status (FCHL-family member or control-family member) in the above-mentioned parameters. The effect of the T_{1100} genotype on lipids and apolipoproteins, which was observed to be confined to FCHL families, was then assessed in the hyperlipidemic with respect to the normolipidemic family members. The results showed that the T allele was associated with elevated concentrations of all parameters only in HL subjects (probands and HL relatives) while no differences were observed between genotypes in NL relatives. Even when FCHL probands were removed from the statistical analyses, the T allele still showed an association with elevated concentrations of VLDL (Chol, Trig, apoB), IDL-Trig, and apolipoproteins A-I and C-III.

TABLE 4. Lipid, lipoprotein, and apolipoprotein parameters in subjects from FCHL families and control families based on genotype

	FCHL Families		Control Families	
Variable	CC n = 62	CT/TT n = 58	CC n = 39	CT/TT n = 24
Plasma Chol	4.98(1.14)	5.28(1.28)	4.89(0.96)	5.27(0.81)
Plasma Trig	1.06(1.23)	1.45(1.00)[a]	0.95(0.38)	0.97(0.45)
VLDL				
Chol	0.27(0.49)	0.45(0.50)[c]	0.20(0.15)	0.19(0.17)
Trig	0.55(0.97)	0.79(0.81)[b]	0.42(0.31)	0.41(0.32)
ApoB	6.99(10.03)	14.68(17.08)[b]	8.37(8.33)	8.63(5.01)
IDL				
Chol	0.17(0.12)	0.25(0.25)[a]	0.15(0.08)	0.16(0.16)
Trig	0.14(0.11)	0.17(0.13)[b]	0.11(0.05)	0.11(0.07)
ApoB	4.96(2.36)	6.51(5.36)[a]	4.77(3.31)	5.29(3.38)
LDL				
Chol	3.38(0.98)	3.37(1.03)	3.23(0.78)	3.65(0.67)
Trig	0.25(0.12)	0.27(0.11)	0.23(0.08)	0.26(0.11)
ApoB	76.54(21.09)	71.90(22.35)	70.33(17.85)	77.84(13.77)
HDL_2	0.18(0.15)	0.20(0.11)	0.30(0.25)	0.27(0.12)
HDL_3	0.93(0.27)	1.02(0.29)	1.05(0.20)	1.09(0.25)
Total HDL	1.13(0.30)	1.23(0.35)	1.35(0.37)	1.36(0.27)
Plasma apoA-I	113.52(18.14)	122.96(23.20)[b]	122.15(18.56)	121.49(18.96)
Plasma apoB	86.72(31.96)	90.12(33.16)	84.29(20.99)	89.67(19.91)
Plasma apoC-II	5.59(1.63)	5.77(1.30)	4.52(0.40)	4.30(0.36)
Plasma apoC-III	11.51(4.71)	12.75(4.65)[a]	10.78(1.26)	11.34(2.07)

Cholesterol and triglycerides in plasma and all fractions are expressed as mmol/L. Apolipoproteins in plasma and all fractions are expressed in mg/dl. All values expressed as means (SD). Statistical differences assessed by ANOVA with data adjusted for age, gender, and BMI.

[a] $P < 0.05$; [b] $P < 0.01$; [c] $P < 0.001$ compared to the CC genotype in FCHL families.

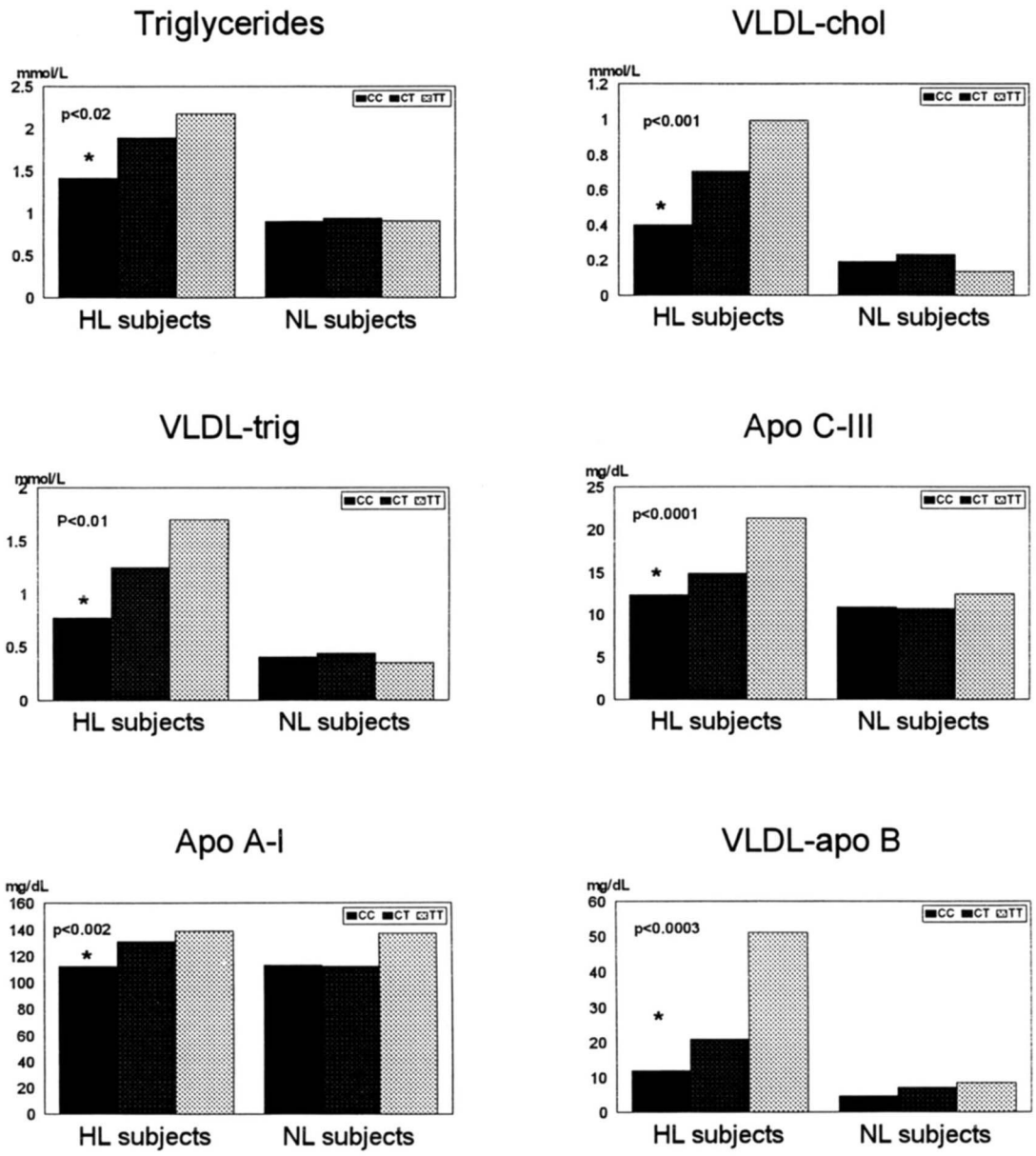

Fig. 2. Genotype-status interaction in relation to concentrations of plasma triglycerides, VLDL-chol, VLDL-trig, VLDL-apoB, apoC-III, and apoA-I. Note: gene dosage observed only among the hyperlipidemic members of FCHL families. *Statistically significant linear correlation.

As a significant association between the T allele and differences in lipid and apolipoprotein parameters was detected only among HL subjects, analysis of gene–dosage effect was performed in this group. The linear relationship between genotypes and those parameters that were affected by the T allele was assessed by one-way ANOVA. Significant gene–dosage effects were observed for plasma triglyceride ($P < 0.02$), VLDL-Chol ($P < 0.01$), VLDL-Trig ($P < 0.01$), VLDL-apoB ($P < 0.0003$), apoA-I ($P < 0.002$), and apoC-III ($P < 0.0005$) (**Fig. 2**).

DISCUSSION

FCHL has traditionally been considered a disorder with a late onset (1, 2) where a hyperlipidemic profile is not observed before the age of 20 years. However, in agreement with the report of Cortner, Coates, and Gallagher (6) we have observed that almost 50% of FCHL children below the age of 19 express hyperlipidemia that was characterized by plasma cholesterol or triglyceride concentrations above the 95th percentile and often accompanied by high LDL-Chol levels (32).

This indicates that individuals affected by FCHL may already express hyperlipidemia at early stages in life and could partially explain the increased risk for CAD seen in adult FCHL patients. Parameters associated with CAD risk, such as male gender and increased age, were over-represented among FCHL probands and might be responsible for additional differences observed in BMI and blood pressure.

The hyperlipidemic phenotype can vary in FCHL patients over time. This was the case in 5 of the 16 well-characterized FCHL probands included in the study who, in contrast to the hyperlipidemia observed during a previous extensive follow-up, presented with a clear normolipidemic profile at the time of sampling for the present study. It confirms the variable expression of hyperlipidemia in these patients and is reflected in the only moderately high mean plasma cholesterol and triglyceride concentrations in the group of FCHL probands who, incidentally, had been on a period of dietary restriction.

As expected for the phenotypic expression of FCHL, increased plasma lipid concentrations, lipid-enriched lipoprotein fractions (VLDL, IDL, LDL), and elevated apoB concentrations were found in subjects expressing the FCHL phenotype. These are well-assessed metabolic features extensively reported in relation to FCHL (9–12).

Several studies have suggested associations between variations at the AI-CIII-AIV gene cluster and FCHL (25, 26, 33) and a more recent work by Dallinga-Thie et al. (28) reports a regulatory role of this gene cluster for the expression of FCHL. These variations are thought to be in linkage disequilibrium with a functional mutation (or mutations) in or around the gene cluster. The mechanism by which this putative locus might modulate lipoprotein metabolism in these patients is not known although it is likely to be via the regulation of triglyceride metabolism (26, 27, 34). To help in clarifying this point we studied the associations between the detailed lipid profile of subjects with FCHL and a C_{1100}-T transition in exon 3 of the apoC-III gene. This mutation is reported to have a significantly elevated frequency among subjects with combined hyperlipidemia (36%) and, with a clear effect in elevating the plasma triglyceride concentrations in these subjects, would appear to be, therefore, a useful marker to assess the potential effect of the apoC-III gene in FCHL. This variation does not result in an altered protein and is, presumably, acting as a marker for a functional mutation (or mutations) elsewhere in the gene cluster, for example, in the up-stream region controlling the synthesis of apoC-III (35), which has been shown to influence triglyceride metabolism. The CT and TT genotypes were more frequent among FCHL probands and HL relatives than in controls and allelic frequencies were in agreement with those reported by Xu et al. (27). Although, due to small numbers, statistical significance was not obtained in all cases, the T allele was consistently more frequent in those groups expressing hyperlipidemia (probands, HL relatives) than in normolipidemic or control subjects, suggesting a contributing role of this gene in the development of hyperlipidemia. The T allele was associated with higher plasma apoC-III and triglyceride concentrations and also to an enrichment in cholesterol and triglycerides in VLDL and IDL. Of particular interest is the observation that the T allele was associated with increased content of apoB in VLDL and IDL which can be interpreted, on the assumption that VLDL and IDL, like LDL, contain one molecule of apoB per particle, as an increased number of these particles. Increased number of VLDL and IDL particles in FCHL may result from hepatic over-production of VLDL-apoB and/or decreased catabolism of these particles as demonstrated by Janus et al. (11). Also, a delayed clearance of postprandial triglyceride-rich lipoproteins has been found to be related with changes in apoC-III concentrations in these patients (22). While the genetic bases of this accumulation of particles are not clear, our data suggest that the apoC-III gene is, indeed, involved in this process. The observed association between the T_{1100} genotype with increased concentrations of apoC-III and the accumulation of VLDL and IDL particles is consistent with the reported role of apoC-III in functionally displacing apoE and leading to an impaired clearance of these lipoprotein particles (19, 20). This, together with the lack of association with total plasma apoB levels, suggests that the apoC-III gene is most probably affecting the catabolism of triglyceride-rich particles rather than the overall hepatic synthesis of the lipoproteins. A further metabolic implication is suggested by the observation that the accumulation of particles results in denser and smaller VLDL particles which, together with IDL, rapidly enter the LDL compartment (5) and therefore may account for an elevation in LDL, also observed in FCHL.

The association between the rarer T allele and increased VLDL, IDL, and apoC-III was observed in FCHL families but was not observed in the control group. More importantly, it was observed only among the hyperlipidemic members of the FCHL families. This is of considerable clinical relevance as this suggests that, in view of the elevated frequency of the T_{1100} genotype among FCHL subjects, the apoC-III gene might be predisposing these individuals to FCHL and that this gene might be displaying a modifying effect in being expressed only in the presence of other factors such as VLDL overproduction or decreased LPL activity. The identification of these factors and the molecular mecha-

nism of this interaction will be of importance in understanding the development and variability of the FCHL phenotype.

We thank Dr. Chun-Fang Xu for invaluable help and advice in the setting-up of the molecular biology techniques; Mercedes Heras, Silvia Olivé, Josefa Girona, and Joaquim Margalef for their excellent technical support; Drs. Núria Plana, Pilar Sardà, Rosa Solà, and Carlos Alonso-Villaverde for their clinical assistance. This study has been supported, in part, by the Fundació Joan Abelló Pascual and the Ministerio de la Salud (DGICYT # PM 92-0209). S. E. Humphries is supported by the British Heart Foundation.

Manuscript received 5 September 1996 and in revised form 3 December 1996.

REFERENCES

1. Rose, H. G., P. Kranz, M. Weinstock, J. Juliano, and J. I. Haft. 1973. Inheritance of combined hyperlipoproteinemia: evidence for a new lipoprotein phenotype. *Am. J. Med.* **54:** 148–160.
2. Goldstein, J. L., H. G. Schrott, W. R. Hazzard, E. L. Bierman, and A. G. Motulsky. 1973. Hyperlipidaemia in coronary heart disease. II. Genetic analysis of lipid levels in 176 families and delineation of a new inherited disorder, combined hyperlipidaemia. *J. Clin. Invest.* **52:** 1544–1568.
3. Kwiterovich, P. O., Jr. 1993. Genetics and molecular biology of familial combined hyperlipidemia. *Curr. Opin. Lipidol.* **4:** 133–143.
4. Castro Cabezas, M., T. W. A. de Bruin, and D. W. Erkelens. 1992. Familial Combined Hyperlipidemia: 1973–1991. *Neth. J. Med.* **40:** 83–95.
5. Grundy, S. M., A. Chait, and J. D. Brunzell. 1987. Familial combined hyperlipidemia workshop. *Arteriosclerosis.* **7:** 203–207.
6. Cortner, J. A., P. M. Coates, and P. R. Gallagher. 1990. Prevalence and expression of familial combined hyperlipidemia in childhood. *J. Pediatr.* **116:** 514–519.
7. Brunzell, J. D., J. J. Albers, A. Chait, S. M. Grundy, E. Groszek, and G. B. Mc Donald. 1983. Plasma lipoproteins in familial combined hyperlipidemia and monogenic familial hypertriglyceridemia. *J. Lipid Res.* **24:** 147–155.
8. Nikkilä, E. A., and A. Aro. 1973. Family study of serum lipids and lipoproteins in coronary heart disease. *Lancet.* **1:** 954–959.
9. Chait, A., J. J. Albers, and J. D. Brunzell. 1980. Very low density lipoprotein overproduction in genetic forms of hypertriglyceridemia. *Eur. J. Clin. Invest.* **10:** 17–22.
10. Cortner, J. A., P. M. Coates, M. J. Bennett, D. R. Cryer, and N. A. Le. 1991. Familial combined hyperlipidemia: use of stable isotopes to demonstrate overproduction of very-low-density lipoprotein apolipoprotein B by the liver. *J. Inherited Dis.* **14:** 915–922.
11. Janus, E. D., A. M. Nicoll, P. R. Turner, P. Magill, and B. Lewis. 1980. Kinetic bases of the primary hyperlipidemias: studies of apolipoprotein B turnover in genetically defined subjects. *Eur. J. Clin. Invest.* **10:** 161–172.
12. Venkatesan, S., P. Cullen, P. Pacy, D. Halliday, and J. Scott. 1993. Stable isotopes show a direct relation between VLDL apoB overproduction and serum triglyceride levels and indicate a metabolically and biochemically coherent basis for familial combined hyperlipidemia. *Arterioscler. Thromb.* **13:** 1110–1118.
13. Raugh, G., H. Schuster, B. Müller, S. Schewe, C. Keller, G. Wolfram, and N. Zöllner. 1990. Genetic evidence from 7 families that the apoB gene is not involved in familial combined hyperlipidemia. *Atherosclerosis.* **83:** 81–87.
14. Austin, M. A., E. Wijsman, S. W. Guo, R. M. Krauss, J. D. Brunzell, and S. Deeb. 1991. Lack of evidence for linkage between low-density lipoprotein subclass phenotypes and the apolipoprotein B locus in familial combined hyperlipidemia. *Genet. Epidemiol.* **8:** 287–297.
15. Ginsberg, H. N., N. A. Le, I. J. Goldberg, J. C. Gibson, A. Rubinstein, P. Wang-Iverson, R. Norum, and V. W. Brown. 1986. Apolipoprotein B metabolism in subjects with deficiency of apolipoprotein C-III and A-I: evidence that apolipoprotein C-III inhibits catabolism of triglyceride-rich lipoproteins by lipoprotein lipase in vivo. *J. Clin. Invest.* **8:** 1287–1295.
16. Brown, W. V., and M. L. Baginsky. 1972. Inhibition of lipoprotein lipase by an apolipoprotein of human very low density lipoprotein. *Biochem. Biophys. Res. Commun.* **46:** 375–382.
17. Auwerx, J., P. Leroy, and K. Schoonjans. 1992. Lipoprotein lipase: recent contributions from molecular biology. *Crit. Rev. Cl. Lab. Sci.* **3,4:** 243–268.
18. Williams, K. J., K. A. Petrie, R. W. Brocia, and T. L. Swenson. 1991. Lipoprotein lipase regulates net secretory output of apolipoprotein B in vitro: a possible pathophysiologic explanation for familial combined hyperlipidemia. *J. Clin. Invest.* **88:** 1300–1306.
19. De Silva, H. V., S. J. Lauer, J. Wang, W. S. Simonet, K. H. Weisgraber, R. W. Mahley, and J. M. Taylor. 1994. Overexpression of human apolipoprotein C-III in transgenic mice results in an accumulation of apolipoprotein B48 remnants that is corrected by excess apolipoprotein E. *J. Biol. Chem.* **269:** 2324–2335.
20. Aalto-Setälä, K., E. A. Fisher, X. Chen, T. Chajek-Shaul, T. Hayek, R. Zechner, A. Walsh, R. Ramakrishnan, H. N. Ginsberg, and J. L. Breslow. 1992. Mechanism of hypertriglyceridemia in human apolipoprotein (apo) C-III transgenic mice. *J. Clin. Invest.* **90:** 1889–1900.
21. Ito, Y., N. Azrolan, A. O'Connell, A. Walsh, and J. L. Breslow. 1990. Hypertriglyceridemia as a result of human apoC-III gene expression in transgenic mice. *Science.* **249:** 790–793.
22. Castro Cabezas, M., T. W. A. de Bruin, H. Jansen, A. W. Kock, W. Kortlandt, and D. W. Erkelens. 1993. Impaired chylomicron remnant clearance in Familial Combined Hyperlipidemia. *Arterioscler. Thromb.* **13:** 804–814.
23. Wiseman, S., J. T. Powell, N. Barber, S. E. Humphries, and R. M. Greenhalgh. 1991. Influence of apolipoproteins on the anatomical distribution of arterial disease. *Atherosclerosis.* **89:** 231–237.
24. Blankenhorn, D. H., P. Alaupovic, E. Wickmaam, H. P. Chin, and S. P. Azen. 1990. Prediction of angiographic change in native human coronary arteries and aortocoronary bypass grafts. *Circulation.* **81:** 470–476.
25. Kessling, A. M., B. Horsthemke, and S. E. Humphries. 1985. A study of DNA polymorphisms around the human apolipoprotein A-I gene in hyperlipidemic and normal individuals. *Clin. Genet.* **28:** 296–306.
26. Wojciechowski, A. P., M. Farrall, P. Cullen, T. M. E. Wilson, J. D. Bayliss, B. Farren, B. A. Griffin, M. J. Caslake, C. J. Packard, J. Shepherd, R. Thakker, and J. Scott. 1991. Familial combined hyperlipidemia linked to the apolipo-

protein AI-CII-AIV gene cluster on chromosome 11q23-q24. *Nature.* **249:** 161–164.
27. Xu, C. F., P. Talmud, H. Schuster, R. Houlston, G. Miller, and S. Humphries. 1994. Association between genetic variation at the apoAI-CIII-AIV gene cluster and familial combined hyperlipidemia. *Clin. Genet.* **46:** 385–397.
28. Dallinga-Thie, G. M., X-D. Bu, M. v L-S. Trip, J. I. Rotter, A. J. Lusis, and T. W. A. de Bruin. 1996. Apolipoprotein A-I/C-III/A-IV gene cluster in familial combined hyperlipidemia: effects on LDL-cholesterol and apolipoproteins B and C-III. *J. Lipid Res.* **37:** 136–147.
29. Schumaker, V. R., and D. L. Puppione. 1986. Sequential flotation ultracentrifugation. *Methods Enzymol.* **128:** 155–170.
30. Miller, S. A., D. D. Dykes, and H. F. Polesky. 1988. A simple salting out method procedure for extracting DNA from human nucleated cells. *Nucleic Acids Res.* **16:** 1215.
31. Xu, C. F., P. Talmud, and S. E. Humphries. 1994. Three new polymorphisms of the apoAI-CIII-AIV gene cluster. *Mol. Cell. Probes.* **8:** 331–332.
32. Lopez, D., I. Plaza, M. T. Muñoz, R. Madero, J. Otero, I. Hidalgo, J. Baeza, M. J. Ceñal, A. Cobaleda, I. Parra, C. Ruiz, and J. Dominguez. 1989. Estudio de Fuenlabrada: Lipidos y lipoproteínas en niños y adolescentes. *An. Esp. Pediatr.* **31:** 342–349.
33. Castro Cabezas, M., T. W. A., de Bruin, H. W. de Valk, C. C. Shoulders, H. Jansen, and D. W. Erkelens. 1993. Impaired fatty acid metabolism in familial combined hyperlipidemia. *J. Clin. Invest.* **92:** 160–168.
34. Hayden, M. R., H. Kirk, C. Clark, J. Frohlich, S. Rabkin, R. McLeod, and J. Hewitt. 1987. DNA polymorphisms in and around the apoAI-CIII-AIV genes and genetic hyperlipidemias. *Am. J. Hum. Genet.* **40:** 421–430.
35. Dammerman, M., L. A. Sandkuijl, J. L. Halaas, and J. L. Breslow. 1993. An apolipoprotein C-III haplotype protective against hypertriglyceridemia is specified by promoter and 3′ untranslated polymorphisms. *Proc. Natl. Acad. Sci. USA.* **90:** 4562–4566.