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Hypertriglyceridemia and the apolipoprotein CIII gene locus: lack of association with the variant insulin response element in Italian school children

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Abstract Hypertriglyceridemia is a common metabolic disorder with a major inherited component. In some individuals the condition is suspected to occur as a result of overproduction of apolipoprotein (apo)CIII, a major constituent of triglyceride-rich lipoproteins. Population studies have established an association with the apoCIII gene but the identity of the causal mutation remains unknown. In the present study we have examined a series of six 5′ polymorphic nucleotides (G^{-935} to A, C^{-641} to A, G^{-630} to A, deletion of T^{-625}, C^{-482} to T, and T^{-455} to C) that lie within the promoter region of the apoCIII gene for evidence of possible involvement in disease susceptibility. The polymorphic nucleotides at positions –455 and –482 reside within a negative insulin-response element. We show, in a community-based sample of 503 school children, that a DNA polymorphism (S2 allele) within the 3′-noncoding region of the apoCIII gene was associated with elevated apoCIII and triglyceride levels, but that the polymorphic nucleotides of the promoter were not. In addition, no obvious effect of any extended apoCIII promoter haplotype on plasma apoCIII or triglyceride levels, over and above that conferred by the presence of the S2 polymorphic nucleotide, was detected. These results demonstrate that none of the 5′ apoCIII polymorphisms can account for the association of the apoCIII gene locus with hypertriglyceridemia and, moreover, owing to linkage disequilibrium, raise the possibility that the region conferring susceptibility maps downstream, rather than upstream, of the apoCIII gene promoter sequences.

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Introduction

Hypertriglyceridemia is a common, heterogeneous, metabolic disorder that may constitute a risk factor for the premature development of coronary heart disease (Austin 1991; Criqui et al. 1993). In some individuals it is caused by mutations of the lipoprotein lipase (LPL) gene, while in others, defects of the apolipoprotein (apo)CII gene are responsible (Santamarina-Fojo 1992). Due to their rarity, however, these single-gene defects contribute little to the wide variation observed in plasma triglyceride levels both within and between populations (Goldstein and Brown 1984). Instead, most of the genetic variation is suspected to occur as a result of subtle alterations in one or more of the many other genes that regulate the production and/or clearance of triglyceride-rich lipoproteins (Berg 1983; Deeb et al. 1986). One such group of genes is the apoAI/CIII/AIV complex (Karathanasis et al. 1983, 1986).

Numerous reports have now shown, in both healthy and hyperlipidemic populations, an association between specific variant alleles of the apoAI/CIII/AIV gene cluster and hypertriglyceridemia (Rees et al. 1983; Ordovas et al. 1991; Hegele et al. 1995). For example, the frequency of one allele of the apoCIII gene, designated S2, has been shown to be increased about fourfold in hypertriglyceridemic individuals attending specialist lipid clinics in London, Finland, Arabia, South Africa and America compared with control normolipidemic populations (Rees et al. 1983; Aalto-Setälä et al. 1987; Henderson et al. 1987; Sinan 1989; Dammerman et al. 1993). Likewise, a variant allele of the apoAI gene, designated X2, has been implicated in the pathogenesis of familial combined hyperlipidemia (Hayden et al. 1987; Monsalve et al. 1989; Wojciechowski et al. 1991; Tybjaerg-Hansen et al. 1993). However, since the association of the S2 and X2 alleles with hyperlipidemia has not been found in all studies (Rees et al. 1986; Paul et al. 1987; Price et al. 1989; Xu

et al. 1994), it has been speculated that the linkage disequilibrium between these polymorphic sites and the causative mutation(s) is weakened or absent in some populations.

Although the biochemical basis for the association of the S2 and/or the X2 allele with hyperlipidemia has yet to be established, a defect in the regulation of apoCIII gene expression has been implicated. ApoCIII is a major component of chylomicrons and very low density lipoprotein (VLDL) and in vitro it inhibits the hydrolysis of triglyceride by LPL (Wang et al. 1985; McConathy et al. 1992). In vivo it modulates the postprandial management of triglycerides (Maeda et al. 1994) and inhibits the hepatic uptake of VLDL remnants (Windler and Havel 1985). Plasma apoCIII levels are highly correlated with plasma triglyceride levels (Kashyap et al. 1981; Shoulders et al. 1991) and, in two studies, were significantly higher in individuals with an S1S2 genotype compared with those with an S1S1 genotype (Anderson et al. 1989; Shoulders et al. 1991). In transgenic mice, overexpression of the human apoCIII gene causes hypertriglyceridemia (Ito et al. 1990; Aalto-Setälä et al. 1992), while the disruption of the apoCIII gene in "knockout" mice reduces plasma triglyceride levels to 70% of normal (Maeda et al. 1994). Moreover, fibrates [which efficiently treat diet-resistant hypertriglyceridemia (Larsen and Illingworth 1993)] lower the apoCIII mRNA levels of rat liver by up to 90% in a dose- and time-dependent manner (Staels et al. 1995).

An analysis of the sequences regulating apoCIII gene expression has revealed that the S2 polymorphic nucleotide of the apoAI/CIII/AIV complex is in strong linkage disequilibrium with five polymorphic nucleotides lying within the promoter sequences of the apoCIII gene (Dammerman et al. 1993). Two of these polymorphic nucleotides, at positions –455 and –482 relative to the transcriptional start site of the gene, reside within a negative insulin-response element (IRE) (Li and Todd 1994; Li et al. 1994), prompting speculation that the association of the S2 allele with hypertriglyceridemia might be due to a defective IRE that results in constitutive overexpression of apoCIII (Li et al. 1994). In support of this suggestion, transcriptional activity of a reporter construct containing wild-type apoCIII sequences at positions –455 and –482 was down-regulated in response to insulin in transiently transfected HepG2 cells, whereas a construct containing the variant sequence at these positions was not (Li et al. 1994). Further evidence implicating a role for insulin in regulating apoCIII gene expression derives from several clinical observations. Hyperinsulinemia is associated with Familial Combined Hyperlipidemia (Cabezas et al. 1993) and Syndrome X (Haffner et al. 1992), and an increased prevalence of hypertriglyceridemia exists in diabetes mellitus (Dunn 1990). Furthermore, in an animal model of insulin-dependent diabetes, treatment with insulin suppressed apoCIII gene expression and normalized plasma triglyceride levels (Chen et al. 1994). There is still no direct evidence, however, specifically to connect the apoCIII promoter polymorphisms with abnormal levels of plasma apoCIII and/or triglyceride.

In this study we have examined the contribution of the apoCIII promoter polymorphisms to plasma lipid levels in a community-based sample of 503 Italian school children. We show that the S2 polymorphic nucleotide, situated in the 3′-noncoding region of the apoCIII gene, contributes to the variance observed in plasma apoCIII and triglyceride levels even within this young and healthy group, but that the polymorphic nucleotides at positions –935, –641, –630, –625, –482 and –455 of the apoCIII gene and the X2 polymorphic nucleotide do not.

Materials and methods

Subjects and biochemical analysis

Five hundred and three unrelated, healthy Italian children between the ages of 11 and 13 years were recruited from five schools in the 10th district of Rome, as described (Crea et al. 1994). Permission was obtained from the local hospital ethics committees. Following written parental permission, fasting blood samples were taken. Total cholesterol, high-density lipoprotein (HDL)-cholesterol, triglycerides, apoB and apoAI were measured by standard methods (Bucolo and David 1973; Allain et al. 1974; Grove 1979; Rosseneu et al. 1981a, b). ApoCIII was measured by radial immunodiffusion using antisera-containing plates with a range of apoCIII standards (Daichi Pure Chemicals, Tokyo) and interassay controls. The diameter of the precipitation ring was measured by an investigator unaware of the specimen's identity. The correlation coefficient of the assay, determined on 46 duplicate measurements, was 0.98.

Sequence analysis of the 5′-flanking region of the apoCIII gene

The –1010 to +123 region of the apoCIII gene (Ogami et al. 1990) was amplified by the polymerase chain reaction (PCR) (Saiki et al. 1988) using oligonucleotides 5′ATCTGCAGTCCCTGCTGCGGCT3′ and 5′GTGCTGCAGCAGGCTTGCTGGCT3′ as the forward and reverse primers, respectively. PCRs were carried out in a volume of 50 μl containing 2–8 μl of DNA prepared as described (Shoulders et al. 1993), 50–100 ng of each primer, and 1 U of *Taq* DNA polymerase. The buffer contained 30 mM TRIS, pH 8.4, 2 mM $MgCl_2$, 400 μM each of dATP, dCTP, dGTP, dTTP and 0.1 mM dithiothreitol (DTT). Following an initial 5-min denaturation at 95°C, 30 reaction cycles were performed. Denaturing and annealing were for 1 min each at 94°C and 63°C, respectively. Extensions were for 6 min at 72°C. Gel-purified PCR products were cloned into pGem-T vector (Promega) and sequenced as described (Sanger et al. 1977), using appropriate oligonucleotide primers. Three to six clones were analyzed. Differences contained in two or more clones were assumed to be polymorphic and analyzed further.

Allelic notation and genotype analysis

The S2 polymorphic nucleotide, identified by an *Sst*I restriction fragment length polymorphism (RFLP), arises from a C to G transversion at position 40 of the 3′-noncoding region of the apoCIII gene (Karathanasis et al. 1985; Sharpe 1985). The X2 polymorphic nucleotide resides approximately 2.6 kb upstream of the apoAI gene and is identified by an *Xmn*I RFLP (Kessling et al. 1985; Shoulders et al. 1993). Common and rare alleles at each locus are designated n:1 and n:2, respectively, where n identifies, with the exception of the S and X alleles, the site of the apoCIII gene under consideration. Four regions of the apoAI/CIII/AIV gene cluster were amplified by PCR. Oligonucleotide primers, some of which contained a genetically engineered restriction enzyme site, were: 5′ATCTGCAGTCCCTGCTGCGGCT3′ and 5′GGCTGAGCTC-

TCACAGCC3′ [region 1; nucleotides –1010 to –511 of the apoC-III gene (Ogami et al. 1990)]; 5′AACCCAGAGATGGAGGT-GCT3′ and 5′CCCTGCAGCCCAGATGAG3′ (region 2; nucleotides –554 to –193 of the apoCIII gene); 5′TCCGTCGACTT-GCCTACAGAGGAGTTCTCA3′ and 5′CCTGACGACTGCCC-ACCCACAGAACA3′ [region 3; spanning exon 4 and 3′-noncoding region of apoCIII, positions 2960 to 3393 (Protter et al. 1984)] and 5′GGAAACAGGGGCCTACACT3′ and 5′GTCTGCAGCC-TTTGCAGTCT3′ [region 4; a 390-bp fragment, spanning the *Xmn*I RFLP (Shoulders et al. 1993)]. PCRs were typically carried out in a volume of 25 µl containing 2–8 µl of DNA prepared as described (Shoulders et al. 1993), 10–50 ng of each primer, and 1–2 U of *Taq* DNA polymerase. The PCR buffer consisted of 30 mM TRIS, pH 8.4, 2 mM $MgCl_2$, 400 µM dNTPs, and 0.1 mM DTT (region 1); 10 mM TRIS, pH 8.4, 50 mM KCl, 2.5 mM $MgCl_2$, 200 µM dNTPs (region 2); and 10 mM TRIS, pH 8.4, 50 mM KCl, 2.5 mM $MgCl_2$, 200 µM dNTPs (region 3). The PCR buffer and amplification conditions for region 4 were as described (Shoulders et al. 1993). Thirty reaction cycles were performed for all regions, with the initial denaturing step at 95°C for 5 min and the final extension at 72°C for 9 min. Annealing conditions for regions 1, 2 and 3 were at 57°C for 2 min, 56°C for 3 min and 60°C for 2 min, respectively. Denaturing and extensions were at 95°C for 1 min and 72°C for 2 min, respectively.

Determination of S1/S2 and X1/X2 genotypic status was undertaken by digestion of the appropriate PCR products with *Sst*I and *Xmn*I, respectively. In addition, blots of PCR products spanning region 3 were hybridized to radiolabeled S1 and S2 allele-specific oligonucleotides (ASOs) (Shoulders et al. 1991). Blots of PCR products spanning regions 1 and 2 were hybridized with four and two sets of ASOs, respectively. Prior to rehybridization, blots were immersed in 0.5% SDS at 100°C. Once the solution had cooled to 80°C they were removed and rinsed in 0.1xSSC. The sequences of the ASOs used to determine the –641 and –630 genotype status were as described (Dammerman et al. 1993). ASOs for positions –935, –625, –482 and –455 were: –935:1, 5′GGGGAG-TCGGTGGTCCA3′ and –935:2, 5′TGGACCACC*A*ACTCCCC3′; –625:1, 5′GCGG*T*GGGGCAC3′ and –625:2, 5′GCGGGGGGCAC3′; –482:1, 5′CACTGATGCC*C*GGTCTTCT3′ and –482:2, 5′AGAA-GACC*A*GGCATCAGTG3′; –455:1, 5′TGGGGATGTTTGGAG-TA3′ and –455:2, 5′TACTCCAAACA*C*CCCCA3′. Blots were washed in 6xSSC, 0.1% SDS at 54°C (–935:1), 52°C (–935:2); 45°C (–641:1), 43°C (–641:2); 43°C (–630:1), 41°C (–630:2); 40°C (–625:1), 38°C (–625:2); 60°C (–482:1), 58°C (–482:2); 50°C (–455:1) and 52°C (–455:2) for 3 min. To validate methodology and to assess quality control, 40–80 samples spanning each of the polymorphic loci were reamplified and reanalyzed. No discrepancies were found.

Statistical analysis

Raw values of triglyceride and apoCIII were $\log_e$ transformed to normalize the distribution. Plasma triglyceride levels were adjusted for the effect of body mass index (BMI) and sex. BMIs were unavailable for 53 children; therefore all analyses involving plasma triglyceride levels were undertaken on a sample of 450, rather than 503. ApoCIII levels did not vary with BMI, sex or menarche. Associations between specific polymorphisms and plasma apoCIII and triglyceride levels were tested using Student's *t*-test. Quartiles were constructed by sorting the data and examining the cutoff points for exact quartile deviations. When multiple individuals with the same value spanned the cutoff point, it was adjusted to keep all individuals with the same values together while maintaining it as close as possible to the quartile cut. Evidence for differences in the distribution of allelic frequencies between the upper quartile versus the rest was assessed by means of a likelihood-ratio χ^2 *te*st with one degree of freedom. Values of *P* for individual loci were not adjusted for the number of comparisons made since the pattern and direction of the (uncorrected) significant results were in agreement with previous findings. The percentage of variation in plasma apoCIII and triglyceride levels that was accounted for by the S2 allele was determined by regression analysis. The average effect of the S2 allele on apoCIII and triglyceride levels was estimated as described (Sing and Davignon 1985). Correlation coefficients (*R*) are equal to the square root of the coefficients of determination (i.e., R^2).

Maximum-likelihood estimates of linkage disequilibrium between two diallelic loci were determined as described (Hill and Robertson 1968). The standardized disequilibrium statistic (D′) provides information on linkage disequilibrium relative to the maximum possible, given the allelic frequencies (Lewontin 1964). Values of D′ are shown as negative if the rare allele at one locus was associated with the common allele at the second, and positive when the rare alleles at each locus were associated. Haplotype frequencies were estimated by means of the myriad haplotypes algorithm (Maclean and Morton 1985). Evidence for differences between the groups in the distribution of estimated haplotype frequencies was assessed by means of a likelihood-ratio χ^2 test.

Results

Genotype analysis of the apoAI/CIII/AIV complex

Genomic DNA from five children, two with an S1S1 genotype and three with an S2S2 genotype, was screened for polymorphic sequences within the –1010 to +123 region of the apoCIII gene (Ogami et al. 1990). Six were identified: G^{-935} to A, C^{-641} to A, G^{-630} to A, deletion of T^{-625}, C^{-482} to T and T^{-455} to C (Fig. 1). All six polymorphic sites were in strong linkage disequilibrium with each other and with the *Sst*I and *Xmn*I polymorphic loci (Table 1). For instance, the genotype at site –625 of the apoCIII gene predicted the genotype at sites –630 and –641 in every child and furthermore predicted the genotype at site –455 in all but 17 of the 503 children.

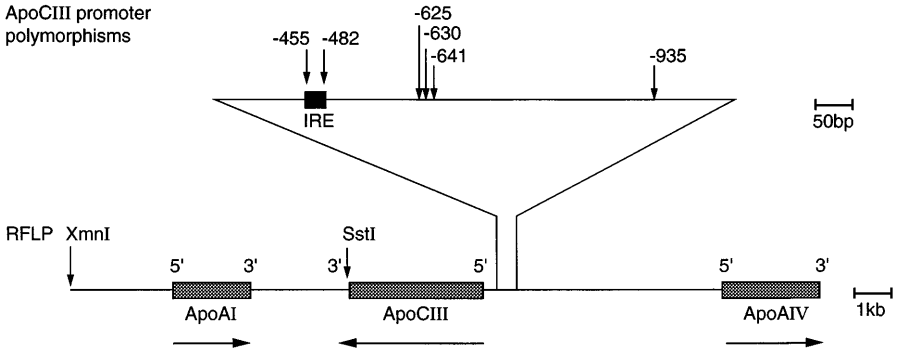

Fig. 1 Location of eight polymorphic nucleotides within the apolipoprotein (apo)AI/CIII/AIV gene cluster. Numbering of the apoCIII promoter sequences is according to Dammerman et al. (1993). *IRE* represents the negative insulin response element of the apoC-III gene (Li and Todd 1994; Li et al. 1994)

Table 1 Pairwise linkage disequilibria between six polymorphic markers of the apo AI/CIII/AIV complex in 503 children*

	Locus	*D′*
*Xmn*I	*Sst*I	–0.99
	–455	–0.80
	–482	–0.83
	–625	–0.83
	–935	–0.84
*Sst*I	–455	0.83
	–482	0.80
	–625	0.91
	–935	–0.85
–455	–482	0.91
	–625	0.98
	–935	1.00
–482	–625	0.99
	–935	0.45
–625	–935	1.00

* All *P* values are highly significant and < 0.00001, even after correcting for multiple testing. χ^2 values ranged from 24–826, each with 1 *df*

Table 2 Mean ± SD plasma apoCIII and triglyceride levels in 503 children stratified by aopAI/CIII/AIV genotypes

Polymorphic site	Genotype	Number (frequency)	ApoCIII (mg/dl)	Triglyceride[a] (mg/dl)
*Xmn*I	X1/X1	349 (0.69)	5.71 ± 1.40	58.60 ± 1.51
	X1/X2	138 (0.27)	5.71 ± 1.41	58.87 ± 1.55
	X2/X2	16 (0.03)	5.54 ± 1.40	61.87 ± 1.43
*Sst*I	S1S1	382 (0.76)	5.54 ± 1.41	56.74 ± 1.47
	S1S2	112 (0.22)	6.23 ± 1.37*	63.94 ± 1.52**
	S2S2	9 (0.02)	6.64 ± 1.46	96.12 ± 1.74
–455	1/1	173 (0.34)	5.59 ± 1.41	55.62 ± 1.55
	1/2	254 (0.50)	5.76 ± 1.41	61.23 ± 1.56
	2/2	76 (0.15)	5.77 ± 1.42	57.85 ± 1.48
–482	1/1	245 (0.49)	5.67 ± 1.41	56.10 ± 1.50
	1/2	215 (0.43)	5.81 ± 1.39	62.37 ± 1.56***
	2/2	43 (0.09)	5.37 ± 1.47	56.26 ± 1.61
–625	1/1	169 (0.34)	5.57 ± 1.41	55.25 ± 1.49
	1/2	254 (0.50)	5.77 ± 1.40	60.94 ± 1.55
	2/2	80 (0.16)	5.77 ± 1.44	59.38 ± 1.58
–935	1/1	313 (0.62)	5.88 ± 1.40****	59.96 ± 1.53
	1/2	172 (0.34)	5.45 ± 1.41	57.18 ± 1.55
	2/2	18 (0.04)	5.29 + 1.50	54.14 ± 1.43

* *P* = 0.0015 for the difference between the means for apoCIII in the S1S1 group versus the S1S2 group. ** *P* = 0.012 for the difference between the means for triglyceride in the S1S1 group versus the S1S2 group. *** *P* = 0.019 between the means for the –482:1/1 group versus the –482:1/2 group. **** *P* = 0.0197 for the difference between the means of the –935:1/1 versus the –935:1/2 group

[a] Triglyceride levels were corrected for body mass index and gender

The minor –625:2, –482:2 and –455:2 alleles were in strong linkage disequilibrium with the X1, S2 and –935:2 alleles. Thus, 16 out of the 18 S2-bearing chromosomes in children with an S2S2 genotype were of the same haplotype, bearing the less frequent sequence variant at sites –625, –482 and –455 and the more common sequence variant at the –935 and the *Xmn*I polymorphic sites. However, since the frequencies of the minor –625, –482 and –455 alleles were 0.411, 0.299, and 0.403, respectively, compared with a frequency of 0.129 for the S2 allele, many S1 chromosomes also bore the less common –641, –630, –625, –482 and –455 apoCIII promoter sequences.

Table 3 Plasma apoCIII and triglyceride levels (mean ± SD) in S1S1 children stratified by an additional apoAI/CIII/AIV genotype

Polymorphic site	Genotype	Number (frequency)	ApoCIII (mg/dl)	Triglyceride (mg/dl)
*Xmn*I	X1/X1	254	5.51 ± 1.41	56.09 ± 1.41
	X1/X2	112	5.61 ± 1.42	57.72 ± 1.41
	X2/X2	16	5.54 ± 1.40	61.87 ± 1.43
–455	1/1	165	5.52 ± 1.40	55.17 ± 1.49
	1/2	177	5.56 ± 1.42	58.93 ± 1.58
	2/2	40	5.54 ± 1.45	53.75 ± 1.40
–482	1/1	233	5.62 ± 1.41	55.72 ± 1.50
	1/2	127	5.46 ± 1.39	59.06 ± 1.58
	2/2	22	5.20 ± 1.50	54.38 ± 1.50
–625	1/1	165	5.55 ± 1.41	55.17 ± 1.49
	1/2	173	5.53 ± 1.41	58.92 ± 1.58
	2/2	44	5.55 ± 1.41	54.30 ± 1.50
–935	1/1	222	5.62 ± 1.40	56.56 ± 1.53
	1/2	142	5.45 ± 1.43	57.37 ± 1.54
	2/2	18	5.29 + 1.50	54.14 ± 1.43

Single-site associations

Genetic variation within the apoAI/CIII/AIV complex contributed to the variance observed in plasma levels of both apoCIII and triglyceride (Table 2). The S2 and –935:1 alleles of the complex were found to be associated with elevated plasma levels of apoCIII (*P* = 0.0015 and 0.0197, respectively), while the S2 and –482:2 alleles were associated with raised plasma triglyceride levels (*P* = 0.012 and 0.019, respectively). However, as predicted by the strong linkage disequilibrium between the three polymorphic loci, when all 121 individuals with an S2 allele were excluded from the statistical analysis the association between the –935:1 allele with elevated plasma levels of apoCIII and the –482:2 allele with elevated triglyceride levels no longer remained (Table 3).

An association between each of the apoCIII alleles, plasma levels of apoCIII and triglyceride was also examined by comparing their prevalence in children comprising the top quartile of the apoCIII and triglyceride distribution with the remaining children. The only significant association found was with the S2 allele, this being present at a significantly increased frequency in children comprising the top quartile relative to the rest (Table 4). Likewise, in a complementary analysis the S2 allele was present at a

Table 4 Frequency of apoAI/CIII/AIV alleles in the top quartiles of the apoCIII and triglyceride distributions compared with quartiles 1–3

Site	Allele	Parameter	Quartiles	
			1–3	4
*Xmn*I	2	ApoCIII	0.17	0.16
	2	Triglyceride	0.16	0.15
*Sst*I	2	ApoCIII	0.11	0.18*
	2	Triglyceride	0.11	0.18*
–455	2	ApoCIII	0.40	0.42
	2	Triglyceride	0.40	0.43
–482	2	ApoCIII	0.31	0.28
	2	Triglyceride	0.29	0.31
–625	2	ApoCIII	0.41	0.43
	2	Triglyceride	0.40	0.45
–935	2	ApoCIII	0.22	0.17
	2	Triglyceride	0.22	0.18

* $P = 0.001$ for difference in allele frequency of quartile 4 versus quartiles 1–3 of the apoCIII and triglyceride distributions

decreased frequency in children comprising the bottom half of the apoCIII and triglyceride distribution compared with the top half (0.086 and 0.052 versus 0.174 and 0.156 for apoCIII and triglyceride, respectively). The frequency of the S2 allele in the lowest 10th percentile of the apoC-III and triglyceride distributions was 0.049 and 0.067, respectively, compared with a peak frequency of 0.233 and 0.22 in the 70–80th and 90–100th percentile, respectively.

Regression analysis revealed that the S2 allele accounted for 2.4% and 2.7% of the total variance in plasma apoCIII and triglyceride levels, respectively ($R^2 = 0.024$ and 0.027, $P = 0.0009$ and 0.0005). In comparison, variation in BMI accounted for 1.9% of the total variance in plasma triglyceride levels ($R^2 = 0.019$, $P = 0.015$). The average effect of the S2 allele was to increase apoCIII and triglyceride levels by 0.59 mg/dl and 10.13 mg/dl, respectively, compared with the S1 allele. In marked contrast to plasma apoCIII and triglyceride levels, none of the genetic variants of the apoAI/CIII/AIV cluster investigated in this study was associated with variability in plasma cholesterol, HDL-cholesterol, apoAI or apoB levels (data not shown).

Plasma apoCIII and triglyceride levels were highly correlated (Fig. 2). In children with an S1S2 genotype mean plasma levels of apoCIII and triglyceride were similarly elevated, being 12.1% and 12.7%, respectively. Linear regression showed that a 2.7-fold increase in plasma apoCIII levels was associated with a 1.9- and 2.4-fold rise in plasma triglyceride levels in S1S1 and S1S2 children, respectively. Thus, children with an S1S2 genotype do not appear to have apoCIII-enriched triglyceride particles.

Nine children had an S2S2 genotype. These had higher mean plasma levels of apoCIII and triglyceride than their S1S2 peers but this did not reach statistical significance (Table 5). Plasma levels of apoCIII ranged from 3.9 to

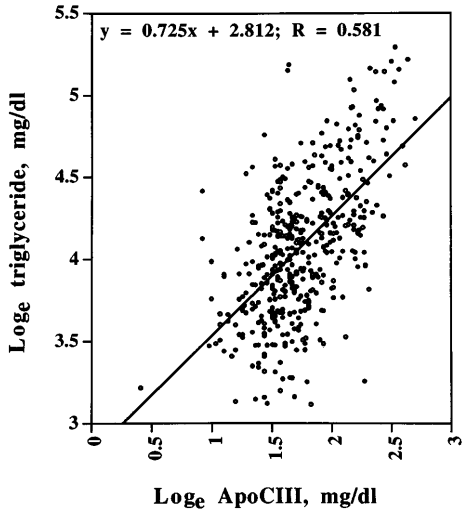

Fig. 2 Linear regression of plasma triglyceride levels on plasma apoCIII levels in 450 children. $y = \log_e$ triglyceride, $x = \log_e$ apoC-III, $P < 0.01 \times 10^{-8}$

Table 5 Plasma ApoCIII and triglyceride Levels in children with an S2S2 genotype. (*ND* not available)

Subject	ApoCIII (mg/dl)	Triglyceride (mg/dl)
1	4.7	ND
2	9.3	131.3
3	12.6	198.7
4	7.6	124.3[a]
5	6.1	ND
6	7.3	94.7[a]
7	4.5	54.9
8	7.7	ND
9	3.9	46.8
S1S1	5.5 ± 1.4	56.7 ± 1.5
S1S2	6.2 ± 1.4	63.9 ± 1.5

[a] Children 4 and 6 were heterozygous for the –935:1 and –482:1 alleles, respectively

12.6 mg/dl while levels of triglyceride ranged from 46.8 to 198.7 mg/dl. Plasma apoCIII and triglyceride levels in three S2S2 children were below the mean levels found in children with an S1S1 genotype. These results suggest that either the deleterious effect of an S2S2 genotype on plasma triglyceride levels is dependent upon the presence of additional genetic, environmental or physiological factors, or that only a proportion of S2-bearing chromosomes bear a mutation predisposing to the development of elevated levels of apoCIII and/or triglyceride.

Haplotype analysis

Genotypes at all eight loci were in Hardy-Weinberg equilibrium and alleles at all loci were in strong linkage disequilibrium (Table 1). Thus, most individuals with the polymorphic S2 nucleotide were predicted to have one of three S2 haplotypes (Table 6). They were: (i) X1, S2, –455:2, –482:2, –625:2, –935:1; (ii) X1, S2, –455:2, –482:1, –625:2, –935:1; and (iii) X1, S2, –455:1, –482:1,

Table 6 Estimated frequencies of apoAI/CIII/AIV haplotypes in children with apoCIII and triglyceride levels < and > 75th percentile of the sample distribution

Haplotype	*Xmn*I	*Sst*I	–455	–482	–625	–935	ApoCIII		Triglyceride	
							< 75%	> 75%	< 75%	> 75%
1	1	1	1	1	1	1	0.422	0.416	0.434	0.413
2	1	1	1	2	1	1	0.002	0.000	0.003	0.000
3	1	1	1	2	2	1	0.008	0.008	0.008	0.014
4	1	1	2	1	1	1	0.001	0.008	0.003	0.005
5	1	1	2	1	2	1	0.017	0.046	0.026	0.027
6	1	1	2	1	2	2	0.082	0.080	0.082	0.093
7	1	1	2	2	2	1	0.058	0.033	0.053	0.049
8	1	1	2	2	2	2	0.129	0.072	0.129	0.062
9	1	2	1	1	1	1	0.008	0.000	0.012	0.000
10	1	2	1	1	2	1	0.000	0.004	0.001	0.000
11	1	2	1	2	2	1	0.002	0.008	0.004	0.005
12	1	2	2	1	1	1	0.001	0.000	0.002	0.000
13	1	2	2	1	2	1	0.004	0.017	0.005	0.020
14	1	2	2	2	2	1	0.088	0.142	0.079	0.156
15	1	2	2	2	2	2	0.004	0.004	0.003	0.005
16	2	1	1	1	1	1	0.157	0.144	0.143	0.136
17	2	1	1	2	1	1	0.001	0.000	0.001	0.000
18	2	1	2	1	1	1	0.001	0.000	0.001	0.000
19	2	1	2	2	2	1	0.004	0.001	0.002	0.000
20	2	1	2	2	2	2	0.004	0.011	0.005	0.017
21	2	2	1	1	1	1	0.000	0.004	0.000	0.000
22	2	2	2	2	2	1	0.004	0.000	0.006	0.000

–625:1, –935:1. Likewise, five haplotypic arrangements accounted for nearly 90% of the predicted S1 haplotypes. The predominant S1 haplotype was of the form X1, S1, –455:1, –482:1, –625:1, –935:1. A comparison of the structure of the S1 and S2 haplotypes indicates that a single recombination event of the predominant S2 haplotype (i.e., X1, S2, –455:2, –482:2, –625:2, –935:1) with a common S1 haplotype could account for the existence of all of the predicted S2 haplotypes.

Evidence for a significant association between specific haplotypes of the apoAI/CIII/AIV locus and plasma levels of apoCIII and triglyceride was examined by comparing the distribution of the estimated apoAI/CIII/AIV haplotype frequencies in the top quartile of the apoCIII and triglyceride distribution with the rest (Table 6). The predominant S2 haplotype (X1, S2, –455:2, –482:2, –625:2, –935:1) was estimated to be 1.6-fold more common in children with plasma apoCIII levels comprising the top quartile of the distribution compared with the rest, a magnitude remarkably similar to the 1.64-fold increase observed for the S2 polymorphic nucleotide alone (Table 4). Likewise, the estimated frequency of the second most common S2 haplotype (X1, S2, –455:2, –482:1, –625:2, –935:1) was also increased, around 4-fold, in children comprising the top quartile of both the apoCIII and triglyceride distributions relative to the rest. In contrast, the frequency of the third most common S2 haplotype (X1, S2, –455:1, –482:1, –625:1, –935:1) was estimated to be lower in children comprising the top quartile of the apoCIII and triglyceride distributions relative to the rest. Of the less common S2 haplotypes, three (X1, S2, –455:1, –482:1, –625:2, –935:1; X1, S2, –455:1, –482:2, –625:2, –935:1; and X2, S2, –455:1, –482:1, –625:1, –935:1) were estimated to be higher in children comprising the top quartile of the apoCIII distribution compared with the rest. Thus, the association of the polymorphic S2 nucleotide with elevated plasma levels of apoCIII and triglyceride was not confined to a specific S2 haplotype.

The distribution of the estimated S1 and S2 haplotype frequencies did not differ significantly between children with plasma levels of apoCIII and triglyceride comprising the top quartile of the distribution compared with the rest (Table 6). Likewise, when the estimated haplotype frequencies in the bottom quartile were compared with the rest, no significant associations emerged (data not shown). All of the information distinguishing between the two groups of children (i.e., top quartile versus the rest) was provided by the S2 polymorphic nucleotide itself; there was no obvious effect of any extended S2 haplotype on plasma apoCIII or triglyceride levels.

Discussion

In the present study we have applied multiple DNA variant association analysis (Julier et al. 1994) to the problem of identifying a locus within the apoAI/CIII/AIV complex that confers susceptibility to hypertriglyceridemia. Previously, this approach was used successfully to refine the map of the sequences conferring susceptibility to diabetes mellitus to a 4.1-kb segment of DNA spanning the insulin gene (Lucassen et al. 1993). Using a series of 19 poly-

morphisms, Lucassen et al. (1993) showed that 10 spanning the 4.1-kb region were highly associated with this condition whereas 9 flanking the region were not. In our study of 503 children, we found that a DNA polymorphism within the 3′-noncoding region of the apoCIII gene (S2 allele) was associated with elevated plasma levels of apoCIII and triglyceride whereas a series of six polymorphisms 5′ of this gene were not. This result indicates that none of the 5′ apoCIII polymorphisms can account for the association of the apoCIII gene region with hypertriglyceridemia and moreover raises the possibility that the locus conferring susceptibility to hypertriglyceridemia maps downstream, rather than upstream, of the apoCIII gene promoter sequences.

Our results complement and extend the findings of two smaller studies, which also showed that the S2 allele is associated with elevated plasma levels of apoCIII. Anderson et al. (1989) found this association in American female, but not male, students of west-European origin aged between 11 and 14 years of age. Likewise, Shoulders et al. (1991) showed that the S2 allele was associated with elevated plasma apoCIII levels in both men and women in a healthy "English" population with a mean age of 35 years. Here we show that mean plasma apoCIII levels were 12% higher in children of both sexes with an S1S2 genotype as compared with children with an S1S1 genotype (Table 2), a magnitude that, compared with the population distribution, is similar to the 22.5% and 25% elevation found by Anderson et al. (1989) and Shoulders et al. (1991), respectively. Moreover, while this manuscript was under review, Dallinga-Thie et al. (1996) reported that the S2 allele was associated with elevated plasma apoCIII levels in the hyperlipidemic relatives and spouses of Dutch probands with familial combined hyperlipidemia. Hyperlipidemic relatives with an S2 allele had 21% higher apoCIII levels compared with hyperlipidemic relatives without the S2 allele (14.2 ± 7.2 versus 11.7 ± 4.1 mg/dl), while S2-bearing spouses had 23% higher levels than S1S1 spouses (10.7 ± 3.3 versus 8.7 ± 3.3 mg/dl).

Plasma levels of apoCIII and triglyceride showed a high positive correlation ($R = 0.58$, $P < 0.01 \times 10^{-8}$) in our population sample. In consequence, children with an S1S2 genotype had 12.7% higher plasma triglyceride levels than their S1S1 peers. This figure is comparable to findings in other healthy populations (Anderson et al. 1989; Mendis et al. 1990; Ordovas et al. 1991; Shoulders et al. 1991; Hegele et al. 1995). In these populations, participants with an S1S2 genotype had on average 23% (range 7.7–38.1%) higher plasma triglyceride levels than those without an S2 allele. In contrast, plasma levels of apoAI and HDL-cholesterol did not significantly differ between the two groups of children, despite a strong inverse correlation between plasma triglyceride and HDL-cholesterol levels ($R = 0.244$, $P < 0.01 \times 10^{-5}$). These results indicate that the effect of the S2 allele on plasma triglyceride levels is not secondary to variation in plasma HDL-cholesterol levels.

In view of the central role of apoCIII in regulating the clearance of triglyceride-rich lipoproteins, a prime candidate for the site of a sequence variant conferring susceptibility to hypertriglyceridemia in individuals with the S2 allele would be within the apoCIII gene itself. However, two studies of the exon sequences of the gene have failed to identify any such mutation (Karathanasis et al. 1985; Sharpe 1985). Moreover, in the present study, screening of the known 5′ transcription-regulating sequences of the gene in three children with an S2S2 genotype also revealed no sequence change that individually associated with raised levels of apoCIII and/or triglycerides (Table 3). Instead, six polymorphic nucleotides located at positions –455, –482, –625, –630, –641 and –935 were identified. The minor alleles at five of these polymorphic sites were in strong linkage disequilibrium with each other and with the S2 polymorphic nucleotide (Table 1). Thus, the predominant S2 haplotype in our sample was S2, –455:2, –482:2, –625:2, –630:2, –641:2, –935:1 (Table 6). Likewise, in the smaller, more restricted haplotype analysis of Dammermann et al. (1993), 12 of 13 S2 chromosomes in a control group of 78 normolipidemic adults were estimated to be of the form S2, –482:2, –625:2. The 13th was predicted to be S2, –482:1, –625:1.

The S2, –482:2, –625:2 haplotype was also estimated by Dammerman et al. (1993) to be 3.8-fold more common in a group of patients with severe hypertriglyceridemia compared with a group of normolipidemic individuals: 36 of the 41 S2-bearing chromosomes were predicted to be of this form, compared with just 5 with the S2, –482:1, –625:2 haplotype. In the present study we were unable to detect an association of any specific S2 haplotype with elevated levels of plasma apoCIII and/or triglyceride (Table 6), over and above that conferred by the presence of the S2 polymorphic nucleotide alone. In other words, all of the genetic information distinguishing between the top quartile of the apoCIII and triglyceride distribution and the rest was provided by the S2 polymorphic nucleotide itself (Table 4). This result is remarkably similar to the findings of Dammerman et al. (1993). In their study, the relative risk of hypertriglyceridemia conferred by the S2, –482:2, –625:2 haplotype was 3.14 compared with a relative risk of 3.85 for the S2 allele alone.

The combination of a significant association of the S2 polymorphism with elevated plasma levels of apoCIII and triglyceride, but no association with one or more of the 5′ apoCIII promoter polymorphisms and/or a specific S2 haplotype, invites several possible interpretations. One is that the increased susceptibility to hypertriglyceridemia may result from the interaction of the S2 polymorphic nucleotide with several different combinations of sequences at the 5′ end of the apoCIII gene. The second is that two or more of the observed S2 haplotypes bear the causal mutation, and that the present study had insufficient power to demonstrate this. The third is that the S2 polymorphic nucleotide is itself the causal mutation, but that the genetic and environmental backgrounds of certain individuals prevent the phenotype being expressed. At present the only argument against this possibility is that its location, the 40th nucleotide of the apoCIII 3′-noncoding sequence, is without precedent for a single base pair

change to affect gene expression. The fourth possibility is that the region of increased susceptibility to hypertriglyceridemia lies downstream from the –455 nucleotide of the apoCIII gene, in a region of the complex that is still relatively uncharted territory. Such a region would encompass the apoCIII and AI genes but not the apoAIV gene. In this regard, it is of note that Haase and Stoffel (1990) have shown in HepG2 cells that the intergenic region of the apoAI and CIII genes contains a number of *cis*-acting elements and that these appear to regulate gene expression in cooperation with 5′-flanking elements of the apoAI and CIII genes.

A significant difference between the present study and that of Dammerman et al. (1993) is that we have examined an unbiased community-based sample rather than a preselected group of patients drawn from a lipid clinic. We found that the average effect of the S2 allele on plasma triglyceride levels was small, as have other community-based studies (Anderson et al. 1989; Mendis et al. 1990; Ordovas et al. 1991; Shoulders et al. 1991; Hegele et al. 1995). In contrast, each patient with the S2 allele in the study of Dammerman et al. (1993) had, on at least one occasion, a fasting triglyceride level in excess of 1000 mg/dl. Other studies based in lipid clinics have likewise found an increased frequency of the S2 allele in groups of patients with mean triglyceride levels raised to well above 200 mg/dl, relative to control groups of normolipidemic individuals (Rees et al. 1983; Aalto-Setälä et al. 1987; Henderson et al. 1987; Shoulders et al. 1989; Sinan 1989). While it is true that the lack of association of the S2 allele with more severe forms of hypertriglyceridemia in community-based samples may, in part, be due to weak linkage disequilibrium between the S2 polymorphic site and the causative mutation(s), alternative explanations are possible. First, the development of hypertriglyceridemia in individuals with the S2 allele could arise from gene-environment interactions that require sufficient time and/or dose to exert their full effect. Second, the outcome of the S2-associated defect could be determined by its interaction with one or more of the many genes regulating the production and/or catabolism of triglyceride-rich lipoproteins. For example polymorphic alleles of LPL (Chamberlain et al. 1989; Hegele et al. 1995) and apoB (Law et al. 1986) influence plasma triglyceride levels. Third, the development of severe hypertriglyceridemia in persons with a genetically determined susceptibility at the apoCIII locus may require interaction with another metabolic disorder such as diabetes mellitus and/or insulin resistance. In the study of Dammerman et al. (1993), for example, many of the hypertriglyceridemic individuals had Syndrome X, a metabolic condition characterized by decreased HDL-cholesterol levels, hypertension, glucose intolerance and insulin resistance, in which the latter is believed to be the primary defect (Gwynne 1992).

In conclusion, our data indicate that the genetically determined susceptibility to hypertriglyceridemia at the apoCIII locus is not caused by either of the polymorphic nucleotides contained within its IRE. Instead, sequences downstream of position –455 are implicated.

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