Sib-pair linkage analysis of longitudinal changes in lipoprotein risk factors and lipase genes in women twins

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Abstract **Based on longitudinal twin data in women, we have previously demonstrated a genetic influence on changes in lipoprotein risk factors, blood pressure measurements, and body mass index over a decade. The present study examined the linkage between changes in lipoprotein variables and candidate genes encoding the hormone-sensitive lipase (HSL), hepatic lipase (HL), and lipoprotein lipase (LPL). The sample consisted of 126 dizygotic (DZ) pairs of women twins who participated in the two examinations of the Kaiser Permanente Women Twins Study, performed a decade apart. Using quantitative sib-pair linkage analysis, a linkage was demonstrated between the locus for hormone-sensitive lipase and age-adjusted changes in plasma triglyceride ($P = 0.015$), which became more significant after adjustment for environmental factors and the exam-1 level ($P = 0.005$). There was also evidence suggesting linkage between the locus for hepatic lipase and changes in triglyceride ($P = 0.023$), but no linkage was detected for lipoprotein lipase and changes of lipid levels with time. These findings suggest that variation at these candidate gene loci may underlie a portion of the intraindividual variations in these coronary heart disease (CHD) risk factors, and that studies to identify the functional variants could provide new insights into genetic susceptibility to cardiovascular disease.**—Friedlander, Y., P. J. Talmud, K. L. Edwards, S. E. Humphries, and M. A. Austin. **Sib-pair linkage analysis of longitudinal changes in lipoprotein risk factors and lipase genes in women twins.** ***J. Lipid Res.*** **2000.** 41: **1302–1309.**

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A number of studies have demonstrated significant associations between changes in coronary heart disease (CHD) risk factors and subsequent risk of CHD (1–3). It has been proposed that genetic–environmental interactions could reflect the presence of "variability genes." Such variability genes are influenced by environmental factors and may, in turn, cause risk factor variation over time in response to the environmental change (4–7). The variability gene effect can be understood as the idea that some genes are "switched on" in response to specific environmental factors, remain continuously active, and thus regulates the individual's phenotypic response to the ongoing environmental exposure. "Level genes," on the other hand, exhibit an association with absolute risk factor levels. In a longitudinal observation, changes over time probably reflect elements of both processes.

Lipoprotein lipase (LPL) is a major enzyme responsible for the hydrolysis of triglycerides in chylomicrons and very low density lipoproteins (VLDL) (8). It also promotes the exchange of lipids between VLDL and high density lipoproteins (HDL) (9) and is an essential enzyme in the formation of low density lipoprotein (LDL) particles (10). A meta-analysis that combined data of 14 studies, representing 15,000 subjects, has shown that allelic variants in the LPL gene were associated with coronary heart disease risk (11).

More than 70 rare mutations in the LPL gene have been reported in families with inherited LPL deficiency (12). In addition to these, several common variants and genetic polymorphisms have been identified with a more modest effect on LPL catalytic function. The first common mutation described in a coding sequence was Ser447X (exon 9), caused by a C–G transversion that results in the substitution of Ser-447 by a premature stop codon and leads to the truncation of LPL (13). This variant, which does not seem to alter LPL lipolytic activity yet increases the secretion of LPL mass (14), was found to be associated with lower triglyceride levels (15) and with a lower risk of CHD (16). In one study 18.4% of the patients with coronary artery disease carried at least one copy of

Abbreviations: CHD, coronary heart disease; DZ, dizygotic; FCHL, familial combined hyperlipidemia; HDL, high density lipoprotein; HL, hepatic lipase; HSL, hormone-sensitive lipase; IBD, identical by descent; IBS, identity by state; LDL, low density lipoprotein; LPL, lipoprotein lipase; VLDL, very low density lipoprotein.

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the Ser447X allele (17). Carriers of this common variant showed significantly higher LPL activity and HDL cholesterol (HDL-C) levels and lower triglyceride levels than noncarriers. Other investigators, however, did not identify associations of the Ser447X with differences in plasma lipid levels, concluding that it is a neutral polymorphism (18).

Although no convincing epidemiological data are available on the association between hepatic lipase (HL) and CHD it plays a major role in the control of several lipoproteins (19, 20). HL is responsible for the lipolysis of VLDL remnant particles as well as the conversion of larger HDL subclass 2 (HDL_2) to smaller HDL_3 particles. Studies have shown that HL is also involved in lipolysis of large, buoyant LDL and high HL activity is associated with an increase in small, dense LDL particles (21, 22).

Genetic polymorphisms in the promoter region of the HL gene were found to be strongly associated with the observed variation in HL activity (23) and HDL-C (24, 25). In a case-control study of young men with parental history of premature myocardial infarction and age-matched controls, the HL promoter variant C-480T was found to be associated also with concentration of plasma triglyceride and total cholesterol (26). A preliminary study showed that the missense mutation $Val^{73}Met$ of the HL gene was overrepresented in 25 German subjects with familial combined hyperlipidemia (FCHL) compared with controls (27). Yet, in 14 well-documented Finnish pedigrees with premature CHD and FCHL there was no evidence of linkage between FCHL and the HL gene (28).

Hormone-sensitive lipase (HSL) has a key role in the regulation of lipolysis in adipocytes by catalyzing the breakdown of triglyceride to glycerol and free fatty acids (29, 30). In addition, HSL has cholesterol hydrolase activity and generates free cholesterol for steroid synthesis in steroidogenic tissue (31) and macrophages (32).

In a small study of type II diabetics and health controls, variation in the HSL gene was shown to be associated with total cholesterol but not with triglyceride (33). Previous work has suggested that in FCHL there is a lipolytic defect due to impaired function of HSL (34, 35). However, no linkage was found between the well-documented FCHL Finnish pedigrees and the HSL gene (28).

The purpose of this study was to determine whether there was any evidence of genetic linkage between longitudinal changes in lipoprotein risk factors levels and the loci at genes encoding three lipases known to be involved in lipid metabolism. The study makes use of a large sample of dizygotic adult women twins who participated in two examinations of the Kaiser Permanente Women Twins Study a decade apart, using quantitative sib-pair linkage analysis.

MATERIAL AND METHODS

Study subjects

The women twins included in this analysis participated in both the first and second examinations of the Kaiser Permanente Women Twins Study in Oakland, California, conducted during 1978–1979 and 1989–1990, respectively. The sample selection, protocol for data collection, laboratory methodology, and other basic features of this study have been described in detail elsewhere (36, 37). Briefly, the study included 203 monozygotic (MZ) and 145 dizygotic (DZ) twin pairs with mean ages of 41 and 51 years at examinations 1 and 2, respectively.

At both examinations, lipid and lipoprotein determinations were based on blood samples drawn after a 12-h fast. LDL cholesterol was estimated according to the Friedwald formula (38). Subjects and their co-twins were excluded from the lipid analyses, if at either examination, either one of each pair was not fasting, was taking lipid-altering medications, or had missing or extreme values (total cholesterol [TC] >350 mg/dL or triglyceride [TG] >400 mg/dL). Height and weight were measured at both examinations while subjects were dressed in lightweight clothes with shoes removed.

The study was approved by the Kaiser Permanente Institutional Review Board and each woman provided written informed consent for participation in the study.

DNA extraction and genotyping

Genomic DNA was prepared from whole blood after lysis of red blood cells and of the 290 available blood samples from DZ twins, DNA extraction succeeded for 280 (96.6%). The three candidate genes examined in this analysis, and their chromosomal locations, are listed in **Table 1** (39–42). These genes included the hepatic lipase (HL) and hormone-sensitive lipase (HSL). In addition, we identify carriers with the Ser447X variant at the LPL gene. Polymerase chain reaction (PCR) analysis was performed using primers 5′-CATCCATTTTCTTCCACAGGG-3′ (sense) and 5′-GCCCAGAATGCTCACCAGACT-3′ (antisense). After amplification, the PCR product (137 bp) was digested with H*inf*I and the fragments separated by 7.5% microtiter array diagonal gel electrophoresis (MADGE) (43). The $Stop^{447}$ allele after H*inf*I digestion gives two fragments, 114 and 23 bp in length.

Genotypes for the three genes were determined for a total of 126 DZ twin pairs. However, because both co-twins are needed for this analysis, pairs were excluded if a genotype could not obtained for one or both co-twins in a pair. Thus, the sample sizes varied for specific candidate genes and the phenotype examined.

Statistical analysis

Prior to the linkage analyses, the changes in the risk factors between examinations were first adjusted for age, using regres-

TABLE 1. Candidate gene polymorphisms typed for women twins[a]

Candidate Gene	Chromosome	Marker	No. of Alleles	Heterozygosity Index[b]	Reference
LPL	8	H*inf*I	2	0.17	Humphries et al. (40)
HL	15	CA repeat in intron 8	7	0.63	Bhattacharya et al. (41)
HSL	19	GT repeat in intron 7	14	0.67	Levitt et al. (42)

[a] Table reproduced in part from Austin et al. (39).
[b] Based individually on all women in the study.

TABLE 2. Slope and *P* values from quantitative sib-pair linkage analysis on DZ women twins for age, environment, and baseline-adjusted changes in lipid and lipoprotein variables and hormone-sensitive lipase (HSL), hepatic lipase (HL), and lipoprotein lipase (LPL) genes

Lipid Variable	No. of Pairs	Regression Slope (*P* Value)		
		HSL	HL	LPL
Total cholesterol	108	1.307 (0.989)	3.322 (0.981)	0.053 (0.512)
LDL cholesterol	108	−0.177 (0.432)	1.531 (0.884)	2.121 (0.792)
Triglyceride	108	−2.297 (0.005)	−2.108 (0.023)	−2.124 (0.251)
HDL cholesterol	108	−1.077 (0.163)	1.226 (0.823)	2.948 (0.888)

sion analysis. Because co-twins in the same pair tend to share environment as well as genes, in addition to the age adjustment, changes in risk factor levels were adjusted for available environmental and behavioral variables at each of the two examinations (7). Finally, the risk factor value at examination 1 was also included in the regression models to adjust for potential effects of regression to the mean. For details of the adjustment procedure see Friedlander et al. (7)

Linkage between the polymorphisms at various gene loci and the observed variation in changes of risk factors were estimated by using a sib-pair linkage procedure implemented in the SAGE (Statistical Analysis for Genetic Epidemiology) package (44). The underlying basis for this approach is to compare the quantitative variation in the changes of risk factors between co-twins as a function of the number of marker alleles that the twin pair shares identically by descent (IBD) (45). Because parental data are not available in this twin study, IBD was estimated from the observed identity by state (IBS) and based on the frequencies of alleles for each marker (46, 47). As in Austin et al. (39), allele frequencies were estimated from the same sample of DZ twins, because no population-based allele frequencies are currently available for several of the markers used in this study. Allele frequencies for the entire sample were used, because they were similar to frequencies based on one randomly selected co-twin per pair. Because we used a candidate gene approach and in order to reduce the possibility of type I error, an α value of 0.05 has been used as the criterion for statistical significance. The adjusted *P* value ($P_{adjusted}$), correcting for multiple number of genes examined, is also reported for each result (48).

As a supplement to the sib-pair method, when evidence of linkage to a specific marker was found, intraclass correlation of the appropriate phenotype was calculated within twin pairs stratified into those sharing 2, 1, and 0 alleles IBS. When linkage is present, twins sharing 2 alleles IBS are expected to have higher correlation than those sharing 1, which in turn should be higher that those pairs sharing 0 alleles. Because the standard errors of intraclass correlations are difficult to compute, *P* values are reported on the basis of interclass correlations with the same sample size.

RESULTS

Results of the sib-pair linkage analysis for change in lipid and lipoprotein variables, adjusted for age, environ-

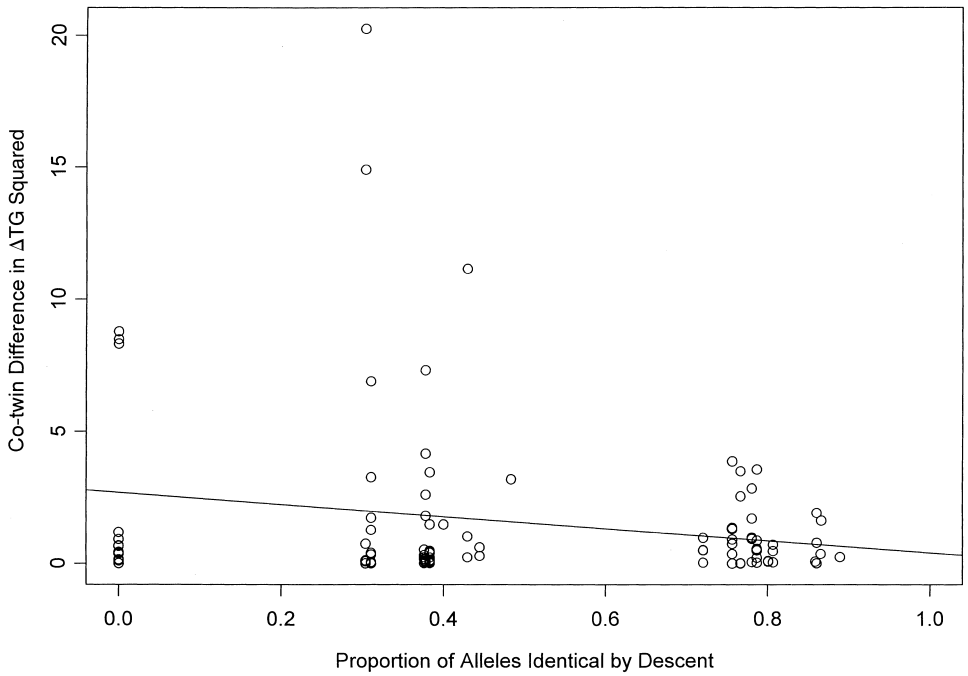

Fig. 1. Results of quantitative sib-pair linkage analysis of the HSL gene in DZ women twins. The x axis indicates the estimated number of alleles shared by co-twins in a pair, at the HSL marker. The y axis indicates the squared co-twin difference in age, environment, and baseline-adjusted ΔTG level (in mg/dl). Note the significant negative slope (b = −2.297; $P = 0.005$) of the regression line, which is consistent with evidence of genetic linkage.

ment, and baseline values, provided evidence of linkage between HSL and HL with ΔTG (**Table 2**). As shown in **Fig. 1**, the slope from the regression of the estimated proportion of alleles identical by descent at the HSL locus versus the co-twin differences in adjusted ΔTG squared has a significantly negative slope (b = −2.297; $P = 0.005$; $P_{adjusted} = 0.149$), demonstrating evidence of linkage between HSL and ΔTG. In addition, the analysis showed evidence of linkage of the HL gene to adjusted ΔTG (b = −2.108; $P = 0.023$; $P_{adjusted} = 0.034$) (**Fig. 2**). No evidence of linkage was obtained for adjusted changes in lipid variables and LPL.

The separate and combined effects of variation in HSL and HL loci were also examined. The distributions of squared twin-pair differences of TG changes among twins sharing different numbers of HSL and HL alleles IBS are presented in **Fig. 3**. The disparity in the distribution of squared twin-pair differences of change in TG among twins sharing no HL alleles IBS was clearly greater than among those twins sharing 1 or 2 HL alleles IBS. Similarly, the distribution of squared twin-pair differences among twins sharing 0 or 1 HSL alleles IBS was considerably greater than among those sharing both HSL alleles IBS. Finally, the combined effects of variation at HL and HSL loci is presented when twin pairs were assigned to sharing 0–2, 3, and 4 alleles at both loci. A clear monotonic decrease in means and standard errors of squared twin-pair differences of ΔTG with an increase in the number of alleles of these genes shared by twins is indicated.

An alternative way to illustrate the linkage of a gene controlling a quantitative trait and a genetic marker is to compare the correlations among those twin pairs sharing 2, 1, or 0 alleles IBS, thus avoiding estimation of IBD status. As expected from the linkage data, we obtained a strong positive correlation for ΔTG, $r = 0.33$ among those twin pairs sharing both HSL alleles IBS and in contrast, a negative correlation, $r = -0.24$, among those twin pairs sharing 0 HSL alleles IBS (**Fig. 4**). Similarly, the intraclass correlations for age-, environment-, and baseline level-adjusted ΔTG were highest among pairs sharing 2 alleles at the HL gene and considerably lower for those sharing 1 allele or 0 alleles (**Fig. 5**).

DISCUSSION

The quantitative sib-pair analysis in this study demonstrated statistically significant evidence of linkage between a chromosomal region within or in the vicinity of the HSL gene and longitudinal change in TG concentrations in women. Furthermore, the finding that there is also significant evidence of linkage between the HSL locus and the longitudinal change in TG, on the adjustment for baseline levels, suggests that this gene may have an effect on "variability" of TG independent of plasma levels of TG. In a linkage analysis, Austin et al. (39) demonstrated no evidence that the HSL gene is linked to plasma TG levels measured at examination 2, indicating that this finding is specific to changes in TG levels over time.

In Japanese subjects with non-insulin-dependent diabe-

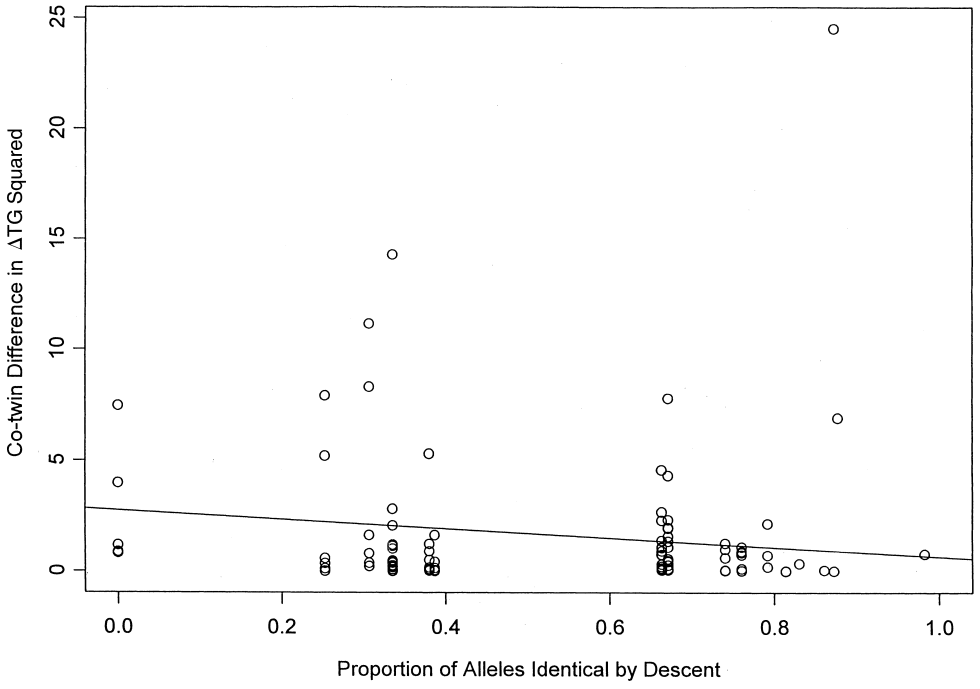

Fig. 2. Results of quantitative sib-pair linkage analysis of the HL gene in DZ women twins. The x axis indicates the estimated number of alleles shared by co-twins in a pair, at the HL marker. The y axis indicates the squared co-twin difference in age, environment, and baseline-adjusted ΔTG level (in mg/dl). Note the significant negative slope (b = −2.108; $P = 0.023$) of the regression line, which is consistent with evidence of genetic linkage.

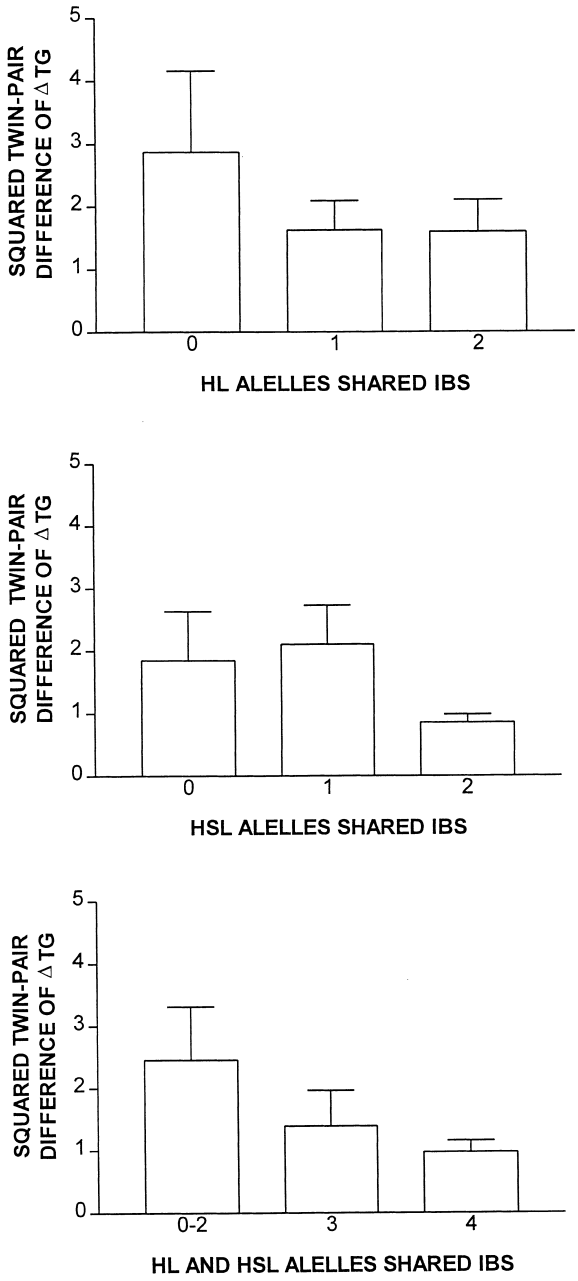

Fig. 3. Squared twin-pair differences of standardized adjusted ΔTG in twins sharing different numbers of alleles IBS. Mean values and standard errors are indicated.

tes mellitus (NIDDM), a mutation in the HSL gene was identified as Arg309Cys (33). A subsequent study by this group has shown that the frequency of this polymorphism was not significantly different between NIDDM patients and nondiabetic subjects. In addition, body mass index, serum triglyceride, and HDL-C were not different in subjects who were carriers or noncarriers of the C309 allele, although total cholesterol was significantly higher in carriers than in noncarriers. Although this evidence supports the view that variation in the HSL gene is involved in determining plasma lipid traits, this polymorphism is itself extremely rare in white individuals (49) and is thus unlikely to be causing the effect seen in this group of twins.

Evidence of linkage was also found between a chromosomal region within or in vicinity of the hepatic lipase gene locus and longitudinal changes in plasma TG after the adjustment for baseline plasma TG levels. This suggests that the relationship is not secondary to HL-mediated variation in plasma TG levels.

At this time, the mechanisms through which HSL and HL are involved in determining TG changes over time are not understood. A study of monozygotic twins suggested that HL activity is strongly influenced by genetic factors (50). In the Heritage Family Study, postheparin plasma lipoprotein lipase and hepatic lipase activities were measured, in addition to plasma lipids and lipoproteins in 171 parents and 266 adult offspring (51). Significant familial resemblance was observed for all the age-adjusted phenotypes. For plasma triglyceride concentrations and HL activity, high genetic heritabilities were estimated ($h^2 = 0.55$ and 0.40, respectively). In another study, the associations between six genetic polymorphisms in the hepatic lipase gene (LIPC) and variation in postheparin HL activity and fasting serum lipoproteins were evaluated in 395 coronary heart disease male patients with low HDL cholesterol concentrations (52). The LIPC promoter polymorphism at position −514 was highly significantly associated with variation in HL activity, with mean activities of 20.4, 17.5, and 13.2 μmol of free fatty acid/ml per hour in subjects having C/C, C/T, and T/T genotypes, respectively. Furthermore, the triglyceride content of low density lipoprotein, intermediate density lipoprotein, and HDL, and the cholesterol content of intermediate density lipoprotein, were found to be associated with variation at LIPC position −514. These data suggest that the LIPC promoter variation is likely to be the basis for variation in HL activity, which may also underlie the variation in change of TG concentrations.

An important question that remains to be answered is the extent of the interaction between HSL and HL on longitudinal change in TG levels. We have examined this question qualitatively by plotting the squared sib-pair differences of TG changes among twins sharing both HSL and HL alleles identical by state (Fig. 3). The disparity in the distributions of the squared twin-pair differences of TG changes among twins sharing different proportions of alleles IBS was clearly greater for HSL and HL considered together than for either locus alone. This observation suggests that the variance in changes of TG levels attributable to the combined effects of these two loci is greater than the fraction contributed by either locus alone.

In our study, the Ser447X variation in the LPL gene was not found to be linked to longitudinal changes of TG or to any other lipid changes. However, in another study of monozygotic twins, the 447X allele was associated with significantly smaller within-pair differences in plasma HDL-C, total cholesterol, and triglyceride levels (53). This suggests that individuals homozygous for the common Ser-447 alleles are more susceptible to fluctuations in their lipid and lipoprotein levels in response to environmental exposure. In addition, the H*ind*III polymorphism, in strong alleleic association with the Ser447X variation, has also been reported to be strongly associated with variability

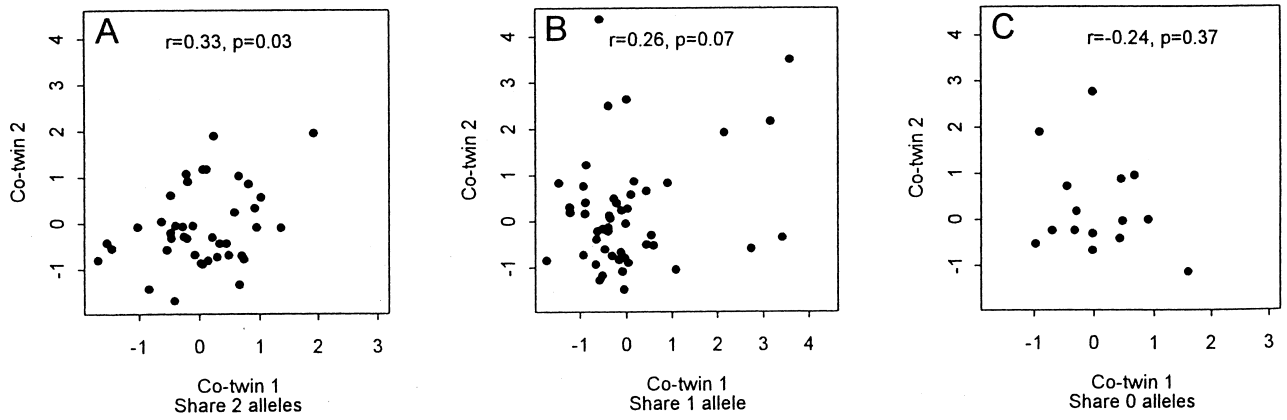

Fig. 4. Similarity of co-twin values for adjusted ΔTG, among pairs sharing (A) 2, (B) 1, or (C) 0 alleles IBS at the HSL marker. Samples sizes are 41, 49, and 15 twin pairs, respectively, and intraclass co-twin correlations are shown. The x axis indicates the value for one randomly selected co-twin/pair, and the y axis indicates the value for the other co-twin of the same pair.

in lipid response to diet manipulations (16, 54). It has been shown that the base change resulting in the Ser447X substitution leads to a truncation of the two carboxyl-terminal amino acids of LPL and mutations affecting the carboxy region have been reported to alter the LPL-specific activity in vitro (55). Yet, these findings are inconsistent (14) and the mechanism through which this mutation exerts level gene and variability gene effects requires further investigation.

The biological interpretation of quantitative genetic data may be strongly influenced by the type of statistical analysis applied (56). In this study we used the sib-pair method of Haseman and Elston (45) because this particular procedure is based on simple genetic and statistical assumptions. When compared with the LOD (log of the odds ratio) score method, this approach has a major advantage of not requiring specification of a genetic model. Furthermore, although the procedure was originally based on a simple biallelic monogenic model (45), it has been shown that it can also be applied to more complex traits influenced by multiple genes with more than two alleles (57). Therefore, the method is appropriate for studying complex traits such as longitudinal changes in lipids and lipoprotein variables. However, it is important to note that the sample size in the current sib-pair analysis may have limited statistical power in detecting true genetic linkages, especially for the LPL Ser447X variant, which is only trialleleic, and has a low heterozygosity index.

In summary, our data provide evidence of genetic linkage between HSL and HL gene loci and 10-year longitudinal changes in TG concentrations. These results emphasize the necessity for identifying additional genetic loci that may be involved in intraindividual variation in these lipid risk factors and their role in genetic susceptibility to coronary heart disease.

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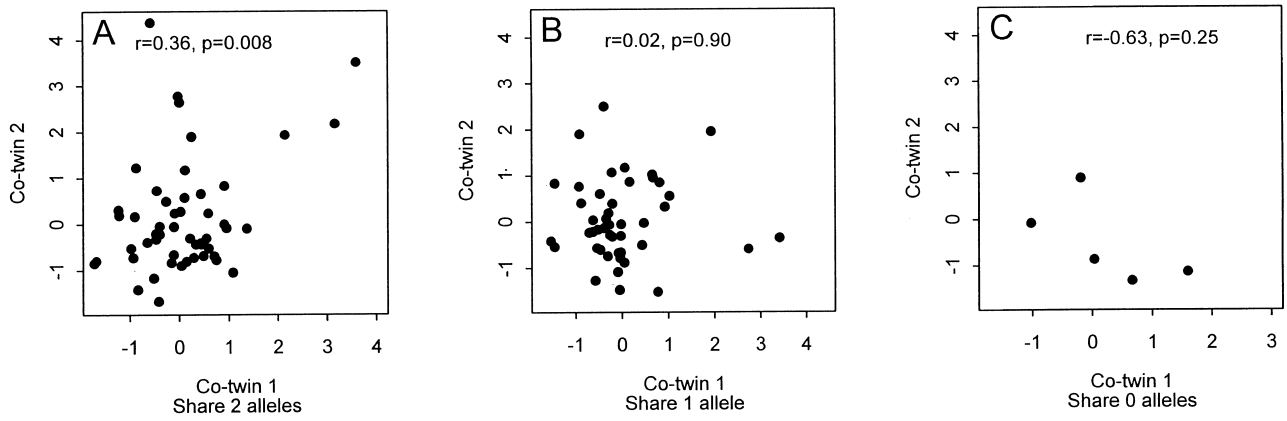

Fig. 5. Similarity of co-twin values for adjusted ΔTG, among pairs sharing (A) 2, (B) 1, or (C) 0 alleles IBS at the HL marker. Samples sizes are 52, 45, and 5 twin pairs, respectively, and intraclass co-twin correlations are shown. The x axis indicates the value for one randomly selected co-twin/pair, and the y axis indicates the value for the other co-twin of the same pair.

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