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Candidate-Gene Studies of the Atherogenic Lipoprotein Phenotype: A Sib-Pair Linkage Analysis of DZ Women Twins

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Summary

There is a growing body of evidence supporting the roles of small, dense LDL and plasma triglyceride (TG), both features of the atherogenic lipoprotein phenotype, as risk factors for coronary heart disease. Although family studies and twin studies have demonstrated genetic influences on these risk factors, the specific genes involved remain to be determined definitively. The purpose of this study was to investigate genetic linkage between LDL size, TG, and related atherogenic lipoproteins and candidate genes known to be involved in lipid metabolism. The linkage analysis was based on a sample of 126 DZ women twin pairs, which avoids the potentially confounding effects of both age and gender, by use of a quantitative sib-pair linkage-analysis approach. Eight candidate genes were examined, including those for microsomal TG-transfer protein (MTP), hepatic lipase, hormone-sensitive lipase, apolipoprotein (apo) B, apo CIII, apo E, insulin receptor, and LDL receptor. The analysis suggested genetic linkage between markers for the apo B gene and LDL size, plasma levels of TG, of HDL cholesterol, and of apo B, all features of the atherogenic lipoprotein phenotype. Furthermore, evidence for linkage was maintained when the analysis was limited to women with a major LDL-subclass diameter >255 Å, indicating that the apo B gene may influence LDL heterogeneity in the intermediate-to-large size range. In addition, linkage was found between the MTP gene and TG, among all the women. These findings add to the growing evidence for genetic influences on the atherogenic lipoprotein phenotype and its role in genetic susceptibility to atherosclerosis.

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Introduction

The importance of elevated plasma LDL cholesterol as a risk factor for coronary heart disease (CHD) is indisputable, especially in light of evidence from intervention trials such as the 4S study (Scandinavian Simvastatin Survival Study Group 1994) and the West of Scotland study (Shepherd et al. 1995). There also is a growing body of evidence to support the roles of LDL heterogeneity and, in particular, small, dense LDL in the risk of CHD. Beginning in the early 1980s, at least nine case/control studies demonstrated that a predominance of small, dense LDL particles is more common in cases than in controls (reviewed in Austin et al. 1994), with relative risks (RRs) ranging from 3.0 (Austin et al. 1988*a*) to 6.9 (Griffin et al. 1994).

Most recently, the results of nested case/control analyses from the Stanford Five City Project, the Physicians' Health Study, and the Quebec Cardiovascular Study have provided the first prospective data evaluating LDL size as a risk factor (Gardner et al. 1996; Stampfer et al. 1996; Lamarche et al. 1997). The Stanford study was based on fatal and nonfatal incident cases of CHD and found a highly significant case/control mean difference in LDL size (−5.1 Å; $P < .001$), demonstrating that smaller LDL size predicted incident CHD in this population (Gardner et al. 1996). In the Physicians' Health Study, baseline LDL-diameter values were smaller for cases, compared with those for matched controls (256 Å vs. 259 Å, respectively; $P < .001$), and the RR was 1.38 (95% confidence interval [CI] of 1.18–1.62) for an 8-Å decrease in LDL size (Stampfer et al. 1996). Finally, the results of the Quebec Cardiovascular Study reported an RR of 3.6 ($P < .01$) for the lowest tertile of LDL size, when incident cases and matched controls were compared. Taken together, these data demonstrate that small, dense LDL is a predictor of CHD, at least in these samples of middle-aged Caucasian individuals.

These studies have led to numerous investigations of the possible biological mechanisms underlying the observed epidemiological associations. Although the met-

abolic mechanisms are still being investigated, at least four explanations of this relationship have been proposed: (1) the association of small, dense LDL with other atherogenic lipoproteins, including increased plasma triglyceride (TG) and decreased HDL cholesterol (Austin et al. 1990*b*); (2) increased oxidative susceptibility of small, dense LDL particles (reviewed in Austin et al. 1994); (3) lower affinity for the LDL receptor (LDL-R), owing to alterations in apolipoprotein (apo) B configuration (Galeano et al. 1994); and/or (4) the presence of insulin-resistance syndrome in subjects with small, dense LDL (Selby et al. 1993). Thus, the small, dense LDL phenotype may confer atherosclerosis susceptibility, through multiple mechanisms that in fact may be synergistic (Austin and Krauss 1995).

Many studies, including several of those noted above, have demonstrated associations between small, dense LDL and plasma concentrations of other lipids and lipoproteins, leading to the designation of small, dense LDL as an "atherogenic lipoprotein phenotype" (Austin et al. 1990*b*). The strongest association is an inverse relationship between LDL size and TG, although small, dense LDL is also simultaneously associated with increased apo B plasma levels and decreased HDL cholesterol and apo AI. The cross-sectional TG association has been confirmed in a sample of Mexican Americans (Haffner et al. 1993) and in a longitudinal study based on the Framingham Offspring Study (McNamara et al. 1992).

Although the role of TG as a risk factor has been controversial for >3 decades (Austin 1991; NIH 1993), accumulating epidemiological evidence is now demonstrating that elevated TG is indeed associated with increased risk of cardiovascular disease (CVD). For example, we recently performed a meta-analysis of results from all population-based prospective studies of TG and CVD that are available from the literature, to determine whether TG is a risk factor for CVD, independent of HDL cholesterol (Hokanson and Austin 1996). The analysis included 17 studies, 16 that studied men and 5 that studied women, including a total of 46,413 men and 10,864 women. Application of meta-analysis approaches resulted in a weighted, summary RR (standardized for a 1 mmol/l or 88 mg/dl increase in plasma TG) of 1.32 (95% CI of 1.26–1.39) for men and 1.76 (95% CI of 1.50–2.07) for women, both of which are statistically highly significant. Of the 6 studies that reported RRs adjusted for HDL cholesterol, the RRs for TG were 1.14 (95% CI of 1.05–1.28) for men and 1.37 (95% CI of 1.13–1.66) for women. Although lower, these values are still statistically significant and confirm that TG is a risk factor for CVD, independent of HDL cholesterol.

Several studies have demonstrated genetic influences on both LDL size and TG levels, possibly reflecting genetic susceptibility to atherosclerosis. On the basis of complex segregation analysis and gradient-gel electrophoresis results, genetic analyses in the United States have found major-gene effects on LDL subclass phenotypes and/or on LDL size, in primarily healthy families and in families with familial combined hyperlipidemia (FCHL) (Austin et al. 1988*b,* 1990*a,* 1993*a*). These single-gene results have been confirmed on the basis of two different samples, of healthy families and families with FCHL, from the Netherlands, by use of a density-gradient ultracentrifugation approach to characterization of LDL heterogeneity (de Graaf et al. 1992; Bredie et al. 1996). Importantly, each of these studies found that the expression of the small, dense LDL trait differed by age and gender and by the hormonal status in women. In combination with data from twin studies of both men and women (Lamon-Fava et al. 1991; Austin et al. 1993*b*), these data provide strong evidence that LDL heterogeneity is genetically influenced.

Another recent complex segregation analysis (Cullen et al. 1994) investigated the inheritance of TG levels in families with FCHL, including families studied in Seattle in the early 1970s (Goldstein et al. 1973) and a sample of families newly recruited through probands ascertained at the Northwick Park Hospital. In both sets of families, a single major-gene effect was seen for TG levels but not for total cholesterol. In the Northwick Park families, a recessive mode of inheritance was found, with a common allele frequency ($q = .25$) for the proposed high-TG allele. Although a recessive model also was found for the Seattle families, the estimated allele frequency ($q = .05$) was lower, and a multifactorial inheritance component also was identified. Thus, these analyses provide evidence for strong major-gene effects on TG levels, at least in FCHL families.

However, the gene(s) controlling LDL size, TG, and related atherogenic lipoproteins remains to be established definitively. In this study, we use a candidate-gene approach to search for linkage to the characteristic lipoproteins of the atherogenic lipoprotein phenotype, building on previous studies that also used this strategy. Two such studies provided evidence against linkage to the apo B gene on chromosome 2 (Austin et al. 1991; LaBelle et al. 1991), whereas two other studies reported linkage with the LDL-receptor (LDL-R) gene, the apo CIII gene, the cholesterol ester–transfer protein (CETP) gene, and the superoxide dismutase (MnSOD) gene (Nishina et al. 1992; Rotter et al. 1996).

The candidate-gene approach has identified a number of genes involved in dyslipidemia and in the risk of coronary artery disease (CAD). The apo B gene is of particular interest, since several polymorphic sites in this gene have been associated with differences in plasma cholesterol and TG levels and demonstrate gene/diet interactions (Humphries et al. 1995). Variation at several other genetic loci, including the apo CIII gene locus, has

been consistently associated with differences in plasma TG levels (Humphries et al. 1996). Furthermore, variation at the apo AI locus (Talmud et al. 1994) is associated with differences in apo AI levels and shows genotype/smoking interactions. Although there are no reports on the association of variation in the microsomal TG-transfer protein (MTP) gene and altered lipid levels, Narcisi et al. (1995) have reported four polymorphic amino acid changes within the amino terminal domain of the MTP gene, suggesting that these polymorphisms may influence plasma lipid levels. Shimada et al. (1996) reported the presence of an R309C variant in the hormone-sensitive lipase (HSL) gene, associated with differences in cholesterol levels, in a Japanese population study. The highly polymorphic dinucleotide repeats in both intron 10 of the MTP gene (Sharp et al. 1994) and intron 7 of the HSL gene (Levitt et al. 1992) are ideal for sib-pair studies.

The purpose of this study was to determine if there is genetic linkage between LDL size, TG, and/or related atherogenic lipoproteins and candidate genes known to be involved in lipid metabolism. The linkage analysis was based on a large, unique sample of DZ women twin pairs, which avoids the potentially confounding effects of both age and gender, by use of quantitative sib-pair linkage analysis.

Subjects and Methods

Subjects

Study subjects were participants in the second examination of the Kaiser Permanente Women Twins Study in Oakland, California. Examination 2 was conducted between 1989 and 1990 and included 704 individuals, representing 81% of the original cohort, examined between 1979 and 1980 (Austin et al. 1987; Selby et al. 1993). This sample included 206 MZ and 146 DZ twin pairs. The study was approved by the Kaiser Permanente Institutional Review Board, and each woman provided written informed consent for participation in examination 2.

At the time of examination 2, each woman completed a health-history questionnaire and a physical examination, including anthropometric and laboratory measurements. The average age at examination 2 was 51 years, and the majority (90%) of women in the sample were white. DZ twin pairs who participated in examination 2 were eligible for the present analysis, with zygosity having been previously determined at examination 1 on the basis of 20 polymorphic loci, such that the probability of misclassification, as MZ, of a pair that was concordant for all these markers was <.001 (Austin et al. 1987).

Laboratory Methods

Lipids, lipoproteins, and apo B.—After the women had completed an overnight fast, 30 ml of whole blood was collected into EDTA-containing tubes. Plasma was separated by centrifugation within 2 h and was stored under refrigeration. Nondenaturing gradient-gel electrophoresis was performed on the plasma by use of 2%–16% polyacrylamide gradient gels (Pharmacia), as described elsewhere (Krauss and Burke 1982; Nichols et al. 1986). The estimated diameter of the major LDL subclass was calculated on the basis of a calibration curve constructed from high-molecular-weight standards run on the same gel (Krauss and Burke 1982). The diameter denoted "LDL peak-particle diameter" (LDL-PPD), or "LDL size," is a continuous variable and is used in this analysis as a measure of LDL heterogeneity.

Total HDL cholesterol (Warnick et al. 1985) and TG (Nagele et al. 1984) were determined, by standardized methods, at the Donner Laboratory (Lawrence Berkeley National Laboratory) (R. M. Krauss, unpublished data), a participating laboratory in the Centers for Disease Control lipid-standardization program. LDL cholesterol was determined by use of the Friedewald formula (Friedewald et al. 1972), when TG values were <400 mg/dl. (Women with TG values $\geqslant$400 mg/dl were excluded from the analysis [see Statistical Genetic Analysis section].)

Apo B plasma levels were initially measured by maximal single-radial immunodiffusion (RID) assay (Oucherony and Nilsson 1978), by use of commercially available plates and reagents (Tago), for 172 of the 291 available samples from the DZ twins. Calibration standards were validated by Northwest Lipid Research Clinic, and in-house control plasma was used to monitor variation of the measurement from plate to plate (coefficient of variation [CV] $\pm$10%). This method was then replaced by a more efficient immunoturbidimetric assay (ITA) method, for the remaining 119 samples (Rifai and King 1986; Smith et al. 1987). Reagents, standards, and reference plasma controls—with and without elevated lipids—are included in the ITA reagent kit (Bacton Assay Systems). Measurements were performed in accordance with the kit instructions, by use of a Corning Express 550 analyzer (Ciba). These measurements are Clinical Laboratory Improvements Amendments-licensed procedures, which are validated twice yearly by College of American Pathologists-test validation. Reference controls supplied with the kit and in-house plasma controls were used to monitor interrun variability (CV $\pm$7.5%). Although these two methods were highly correlated (r = .73, on the basis of 66 twin samples, by use of both methods), the immunoturbidimetric values were adjusted by linear regression to RID values, and these were used in the present analysis.

Table 1

Candidate-Gene Polymorphisms Typed for Women Twins

Candidate Gene	Chromosome	Marker	No. of Alleles	Heterozygosity Index[a]	Reference
Apo E	19	Triallelic: isoforms E2, E3, and E4	3	.43	Bolla et al. (1995)
Apo B	2	VNTR at 3′ end	14	.74	Boerwinkle et al. (1989)
		Tetranucleotide repeat in intron 20	6	.60	Zuliani and Hobbs (1990*a*)
MTP	4	CA repeat in intron 10	13	.74	Sharp et al. (1994)
Apo CIII	11	Tetranucleotide repeat in intron 3	7	.60	Zuliani and Hobbs (1990*b*)
HL	15	CA repeat in intron 8	7	.63	Bhattacharya et al. (1991)
HSL	19	GT repeat in intron 7	14	.67	Levitt et al. (1992)
IR	19	CT repeat in intron 2	12	.51	Xiang et al. (1991)
LDL-R	19	D19S394, 250 kb 5′ to LRL-R gene	21	.91	Day et al. (1997)

[a] Based individually on all women in the study.

DNA extraction.—By use of the same blood samples described above, white and red blood cells were retained after centrifugation and removal of plasma. These samples were frozen at −70°C until 1995. At that time, the samples were transferred from Kaiser Permanente, in Oakland, to the Donner Laboratory, in Berkeley, for isolation of DNA. DNA was extracted from whole blood by use of a NH_4Cl lysis/salt chloroform method (Mullenbach et al. 1989), modified for DNA isolation from buffy coats (A. Recinos and Y. Ma, personal communication). Of the 290 blood samples available from DZ twins, DNA extraction failed for only 10 (3.5%) samples.

DNA samples were then sent to the Rayne Institute, University College London, for genotyping in two batches, one in July 1995 and the other in December 1995. These samples were identified only by anonymous identification numbers and were blinded for zygosity and for co-twin status.

Candidate genes.—The eight candidate genes examined in this analysis and their chromosomal locations are listed in table 1. These genes included those for apo E, apo B, MTP, apo CIII, hepatic lipase (HL), HSL, insulin receptor (IR), and LDL-R. Markers were genotyped for each of these candidate genes, by use of the most highly polymorphic markers available. The number of alleles ranged from 3 (apo E) to 21 (LDL-R), and heterozygosity indices were within the range of .43–.91. Candidate-gene genotypes were determined for a total of 126 DZ twin pairs (252 women). However, since both co-twins are needed for this analysis, pairs were excluded if a genotype could not be obtained for one or both co-twins in a pair. Thus, the sample sizes varied slightly for specific candidate genes.

All PCRs were performed on a Tetrad PTC225 Peltier Thermal Cycler (MJ Research), by use of *Taq* polymerase (Gibco-BRL) and the buffer recommended by the manufacturer, with 8 pmol of each PCR primer. Genotyping of the apo E gene, to identify the three alleles E2, E3, and E4, was determined by use of the method described in the study by Bolla et al. (1995). The apo B VNTR in the 3′ UTR was genotyped in accordance with the method described in the study by Boerwinkle et al. (1989). All other genotyping was performed by use of fluorescent-labeled forward primers (Applied Biosystems [synthesized by Oswell; nonfluorescent reverse primers were synthesized by Gibco-BRL]). Allele sizing was performed on a 373 or a 377 automated sequencer (Applied Biosystems).

The CA repeat in intron 10 of the MTP gene was amplified by use of the following oligonucleotides: forward, TAMRA (N,N,N′N′-tetramethyl-6 carboxyrhodamine)-5′-TCC ACA AGG ATT CAT AAC C-3′, and reverse, 5′-TTC TCC ACT CTT CCC CAT-3′ (R. Gregg, personal communication); and the following PCR-amplification conditions were used: one cycle of 95°C for 4 min and then 30 cycles of 95°C for 1 min, 55°C for 1 min, and 72°C for 1 min, followed by a final extension at 72°C for 5 min. The tetranucleotide repeat in intron 20 of the apo B gene was genotyped with a FAM (6-carboxyflourescein)–labeled forward oligonucleotide, by use of oligonucleotide sequences described in the study

by Zuliani and Hobbs (1990*a*). Apo CIII amplification of intron 3 (Zuliani and Hobbs 1990*b*) was performed with the following oligonucleotides: forward, FAM-5′-GCA GAG ATG ACA GAG TTG AG-3′, and reverse, 5′-GGT TGC AGT GAG CCG AGA TG-3′; and the following PCR conditions were used: one cycle of 95°C for 4 min and then 30 cycles of 95°C for 1 min and 72°C for 3 min. Five pairs of MZ twins were genotyped for this marker, and the co-twins of all five pairs had identical genotypes.

The PCR for dinucleotide-repeat sizing of the HL, HSL, and IR genes was multiplexed in a single reaction tube, by use of the following PCR conditions: one cycle of 95°C for 4 min and then 30 cycles of 95°C for 1 min, 55°C for 30 s, and 72°C for 2.5 min. Oligonucleotide sequences were as reported elsewhere (for HL, Bhattacharya et al. 1991; for HSL, Levitt et al. 1992; and for IR, Xiang et al. 1991). To distinguish the sizing of the three dinucleotide repeats, forward primers were labeled with the following fluorescent ABI labels: for HL, TAMRA; for HSL, FAM; and for IR, TET (4,7,2′,7′-tetracholoro-6-carboxyfluorescein). Analysis of the polymorphic microsatellite D19S394, a marker for the LDL-R—which is located ~250 kb telomeric and 5′ to the LDL-R—was performed as described elsewhere (Day et al. 1997).

Statistical Genetic Analysis

Quantitative sib-pair linkage analysis, using the SIBPAL program from the SAGE (1994) package, was used to test for genetic linkage between the lipoprotein phenotypes and the candidate genes. In this regression approach, quantitative variation in the lipoprotein phenotype, between co-twins, is examined as a function of shared alleles of the polymorphic marker of the candidate gene. A regression line is then calculated, with the squared difference (squaring is used to obtain only positive values) in the lipoprotein phenotypes of co-twins as the dependent variable and the number of shared alleles identical by descent (IBD) as the independent variable (Haseman and Elston 1972). If the slope of this line is negative and is significantly different from zero, with the appropriate df (Blackwelder and Elston 1982), the result is interpreted as evidence for linkage between the lipoprotein phenotype and the candidate gene. For each lipoprotein phenotype, the slope of the line reflects the strength of the relationship; hence, it can be interpreted as a measure of the magnitude of the evidence for linkage and can be compared for different candidate genes (but not for different phenotypes, owing to scale differences). Thus, both the regression slopes and the corresponding *P* values are reported here.

Same-sex twin pairs are ideal for this approach, because they are matched for age and gender, the two most important confounding variables in the evaluation of lipoprotein phenotypes, especially LDL size (Austin et al. 1990*a,* 1990*b*). Thus, no statistical adjustments for these variables were needed. The analysis first focused on LDL size and plasma TG levels. Because sib-pair analysis is sensitive to outliers that may lead to false-positive findings, three pairs in which one co-twin had a TG value >400 mg/dl were excluded from the analysis. In addition, since the TG distribution was skewed, a natural log (ln) transformation was applied to plasma-TG values, to better approximate a Gaussian distribution.

When potential linkage was found for LDL size and/or for TG, by use of an α level of .05, possible linkage to other lipoprotein variables associated with the two phenotypes—including HDL cholesterol, apo B plasma levels, and LDL cholesterol—was then evaluated, to minimize type II error. This approach was used to insure that each potential linkage was detected. However, it is important to note that, owing to the multiple comparisons performed and to the finite nature of the genome, type I error could have led to false-positive results, in this setting.

Since parental data were not available in this twin study, observed allele sharing between co-twins represents identity by state (IBS) rather than IBD. However, IBD can be estimated from IBS, by use of the frequencies of alleles for each marker, resulting in IBD values that differ from 2, 1, and 0. This estimation procedure is implemented in the SIBPAL program, which applies an algorithm described in the study by Amos (1988, pp. 56–58), and was used in this analysis. Ideally, population-based allele frequencies are used for this purpose. However, for several of the markers used in this study, such data are not currently available. Therefore, allele frequencies estimated from the same sample of DZ twins were used. Allele frequencies for the entire sample were used, because they were similar to frequencies based on one randomly selected co-twin/pair. In addition, the lack of parental data precluded construction of definitive haplotypes for the two apo B markers examined.

In order to evaluate IBS data directly, without having to estimate IBD sharing, twin pairs also were stratified into those sharing 2, 1, or 0 alleles IBS, when evidence for linkage was found for a specific marker. Co-twin intraclass correlations of appropriate phenotypes, within these groups, were then calculated by use of the FCORR program, also in the SAGE (1994) package. When linkage is present, twin pairs sharing 2 alleles IBS are expected to have higher correlations than those sharing 1 allele, which, in turn, should be higher than those of pairs sharing 0 alleles. Since the standard errors of intraclass correlations are difficult to compute, *P* values based on interclass correlations of the same sample sizes are reported.

Table 2

Slopes and *P* Values from the Quantitative Sib-Pair Linkage Analysis Based on DZ Women Twins, for LDL-PPD and TG Level

Candidate-Gene Marker	No. of Pairs	Regression Slope (*P* value)	
		LDL-PPD	ln of TG Level
MTP	120	−7.83 (.402)	−.32 (.031)
HL	122	36.28 (.863)	−.14 (.222)
HSL	124	−13.58 (.318)	−.16 (.153)
Apo B	119	−75.71 (.014)	−.34 (.017)
Apo CIII	119	33.24 (.893)	−.11 (.235)
Apo E	117	32.79 (.761)	−.06 (.413)
IR	123	−26.27 (.258)	.19 (.841)
LDL-R	121	−34.52 (.082)	−.20 (.071)

Results

Quantitative Sib-Pair Linkage Analysis of LDL-PPD and TG

The results of the sib-pair linkage analysis for LDL-PPD and TG are summarized in table 2. Evidence for linkage is seen between the MTP gene and TG, at the 0.05 level, but not for MTP and LDL-PPD. As shown in figure 1, the plot of the estimated proportion of alleles IBD at the MTP locus versus the co-twin difference in the squared ln TG level, the regression line has a significantly negative slope (−0.32, $P = .031$), which is consistent with genetic linkage between the MTP gene and variation in plasma TG levels.

No evidence for linkage with either LDL-PPD or TG was obtained for the HL gene, the HSL gene, the apo AI-CIII-AIV gene complex, the apo E gene, or the IR gene (table 2). However, the analysis showed evidence for linkage of the apo B gene to both LDL-PPD and TG ($P = .014$ and $P = .017$, respectively; table 2). Figure 2*A* and *B* shows the regression lines (with slopes of −75.71 and −0.34, respectively) from the sib-pair analyses of the apo B gene, for LDL-PPD and the ln of the squared TG level. The significant negative slopes are clearly shown in both plots, indicating that those pairs who are more alike at the apo B locus have more similar values for LDL size and TG. Finally, borderline statistically significant results for linkage between the LDL-R gene and both of these phenotypes were found ($P = .08$ and $P = .07$, respectively; table 2).

Co-Twin Intraclass Correlations

Each of the significant findings (using $P < .05$) described above was examined further by comparison of intraclass correlations among twin pairs sharing 2, 1, or

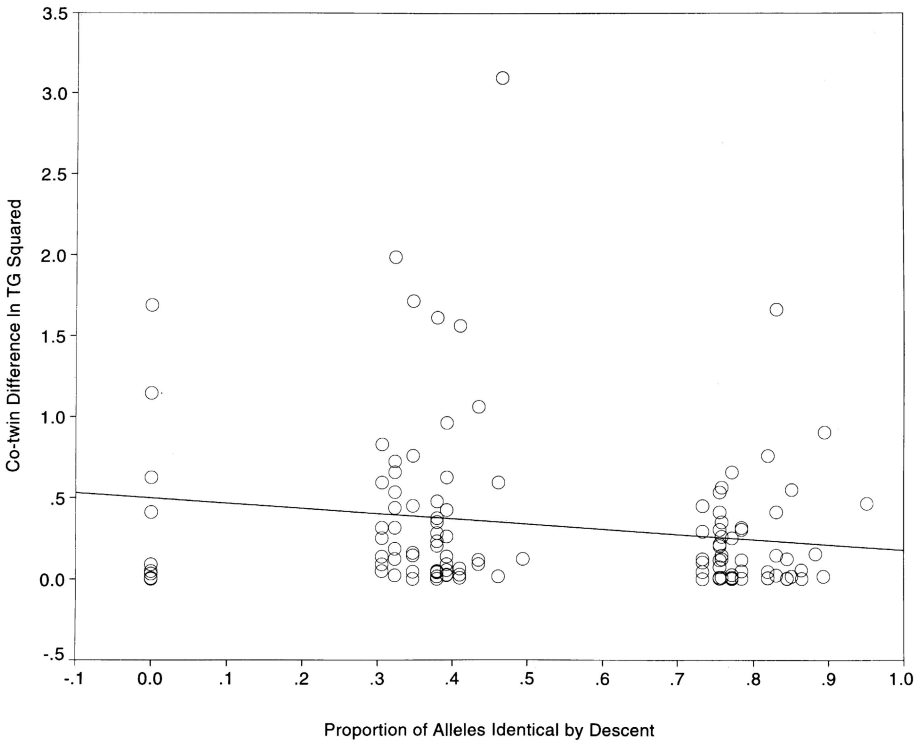

Figure 1 Results of quantitative sib-pair linkage analysis of the MTP gene in DZ women twins. The *X*-axis indicates the estimated number of alleles shared by co-twins in a pair, at the MTP marker (see table 1). The *Y*-axis indicates the squared co-twin difference in TG level (in mg/dl). Note the significant negative slope ($P = .031$) of the regression line, which is consistent with evidence for genetic linkage.

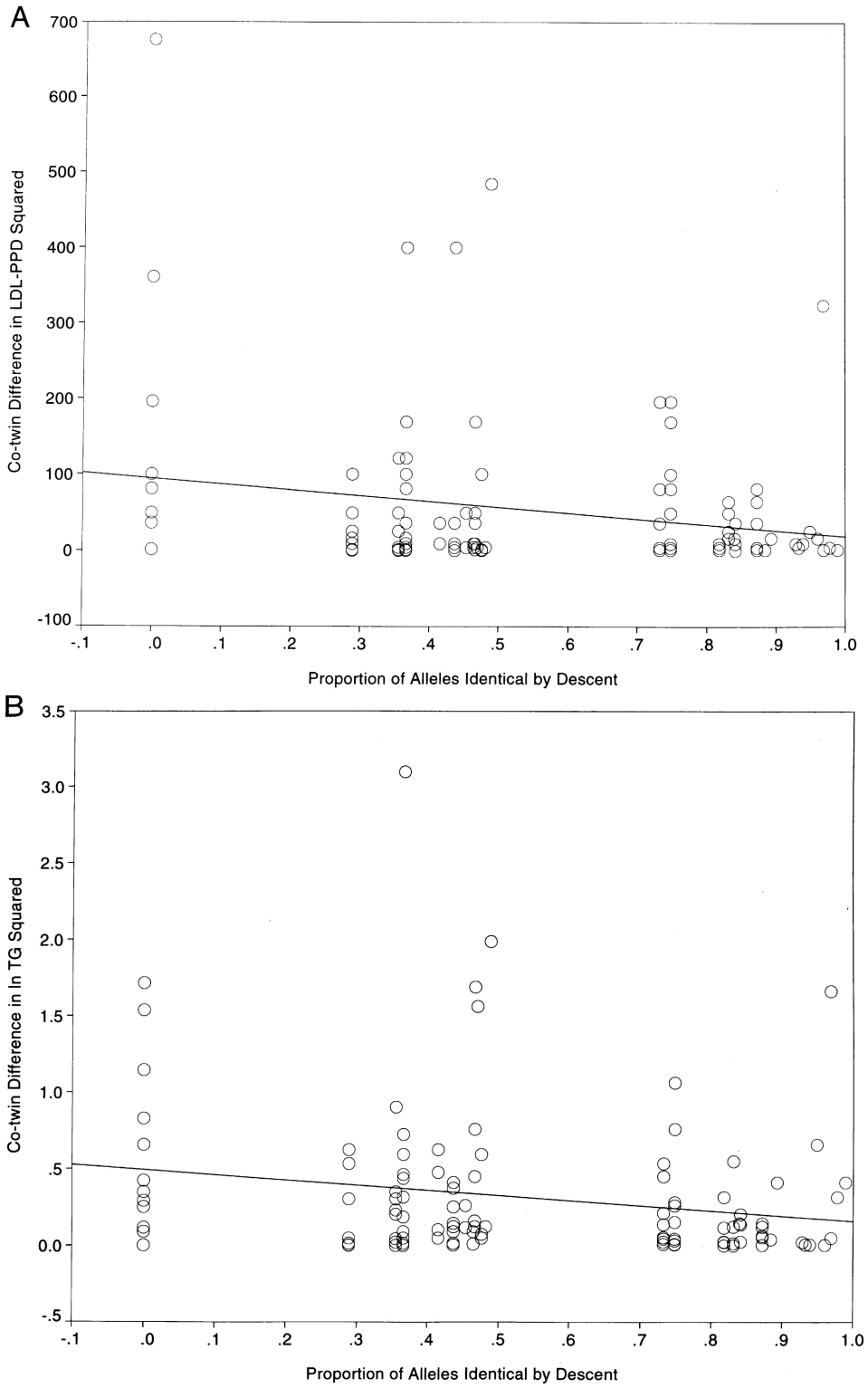

Figure 2 Results of quantitative sib-pair linkage analysis of the apo B gene in DZ women twins. The *X*-axis indicates the estimated number of alleles shared by co-twins in a pair, at the apo B VNTR marker (see table 1). The *Y*-axis indicates the squared co-twin difference in (*A*) LDL-PPD (in Å) and (*B*) TG level (in mg/dl). A significant negative slope (-75.71, $P = .014$, and -0.34, $P = .017$, respectively) is seen for the regression line in each plot, which is consistent with evidence for genetic linkage.

Table 3

Co-Twin Correlations for DZ Women Twins Sharing 2, 1, or 0 Alleles IBS

Marker and No. of Alleles Shared	No. of Pairs	ln of TG Level (mg/dl)	LDL-PPD (Å)
MTP:			
2	52	.624*	…
1	59	.334**	…
0	9	.071	…
Apo B VNTR:			
2	46	.687*	.630*
1	62	.454*	.550*
0	12	−.379	−.255

* $P < .001$.
** $P < .01$.

0 alleles IBS, thus avoiding estimation of IBD status. (Note that the expected .25, .50, and .25 Mendelian frequencies of pairs sharing 2, 1, or 0 alleles IBD were not observed, since IBS was used for stratification [Thomson and Motro 1994]). For MTP and TG, intraclass correlations of .62, .33, and .10 were found among those co-twins sharing 2, 1, or 0 alleles IBS, respectively (table 3). This dose-response relationship of correlations shows that TG values were more similar among co-twins who are genotypically more similar at the MTP locus, possibly reflecting a gene dosage effect.

Twin pairs were also divided into those sharing 2, 1, or 0 alleles IBS at the apo B VNTR-marker locus, and intraclass co-twin correlations were calculated for LDL-PPD and TG. As shown in table 3 and figure 3, significant intraclass correlations of LDL size were seen among pairs sharing 2 alleles or 1 allele at the apo B locus but not among those sharing 0 alleles. The similar magnitude of the correlations among twins sharing 2 alleles and those sharing 1 allele could indicate that an apo B allele is having a dominant or a codominant effect on LDL size. Intraclass correlations for TG were highest among pairs sharing 2 alleles at the apo B gene, decreased among those sharing 1 allele, and were not statistically significant for those sharing 0 alleles.

Quantitative Sib-Pair Linkage Analysis of HDL Cholesterol, Apo B Level, and LDL Cholesterol

For each of the candidate genes showing linkage to LDL-PPD and/or to TG, sib-pair linkage analysis was also performed for other lipoproteins related to the atherogenic lipoprotein phenotype (table 4). For MTP, this analysis provided no evidence for linkage of this gene to HDL cholesterol, apo B plasma levels, or LDL cholesterol. Thus, this result appears to be specific for possible linkage between the MTP gene and TG. In contrast, evidence for linkage of the apo B gene to apo B plasma levels was seen ($P = .007$), but no evidence of linkage was seen for HDL or LDL cholesterol.

Sib-Pair Linkage Analysis among LDL Subclass Phenotype A Twin Pairs

Unlike previous studies of LDL size and the apo B gene (Austin et al. 1991; LaBelle et al. 1991), the frequency of LDL subclass phenotype B was only 11% among the women in this study (Selby et al. 1993), compared with 25%–30% in other studies (Austin et al. 1990*a*). Thus, we hypothesized that the observed linkage may be attributable to LDL size in the intermediate-to-large range. To address this question, we repeated the sib-pair linkage analysis, including only all pairs in which both co-twins had LDL size >255 Å, a group with a high prevalence of LDL subclass phenotype A. As shown in table 5, the *P* value for MTP and TG linkage increased to .099, and the slope for this subsample decreased, indicating that the subjects with LDL particle size <255 Å contributed to the evidence for linkage of this gene to TG.

For the apo B gene VNTR marker, *P* values remained <.05 for linkage with LDL-PPD, TG, and apo B plasma levels, in this subset. To explore this linkage further, genotyping at another apo B marker, located in intron 20 of the apo B gene, was performed (see table 1). Although this marker had fewer alleles, decreasing the statistical power to detect linkage, a similar pattern of results was again seen. In particular, the slopes for LDL-PPD and the two apo B markers were very similar

Table 4

Slopes and *P* Values from the Quantitative Sib-Pair Linkage Analysis Based on DZ Women Twins, for HDL Cholesterol, Apo B Plasma Levels, and LDL Cholesterol

Candidate-Gene Marker	Regression Slope (*P* Value)		
	HDL Cholesterol	Apo B Level	LDL Cholesterol
MTP	390.64 (.926)	−396.69 (.121)	−646.14 (.202)
Apo B VNTR	−322.82 (.113)	−656.95 (.007)	−669.36 (.199)

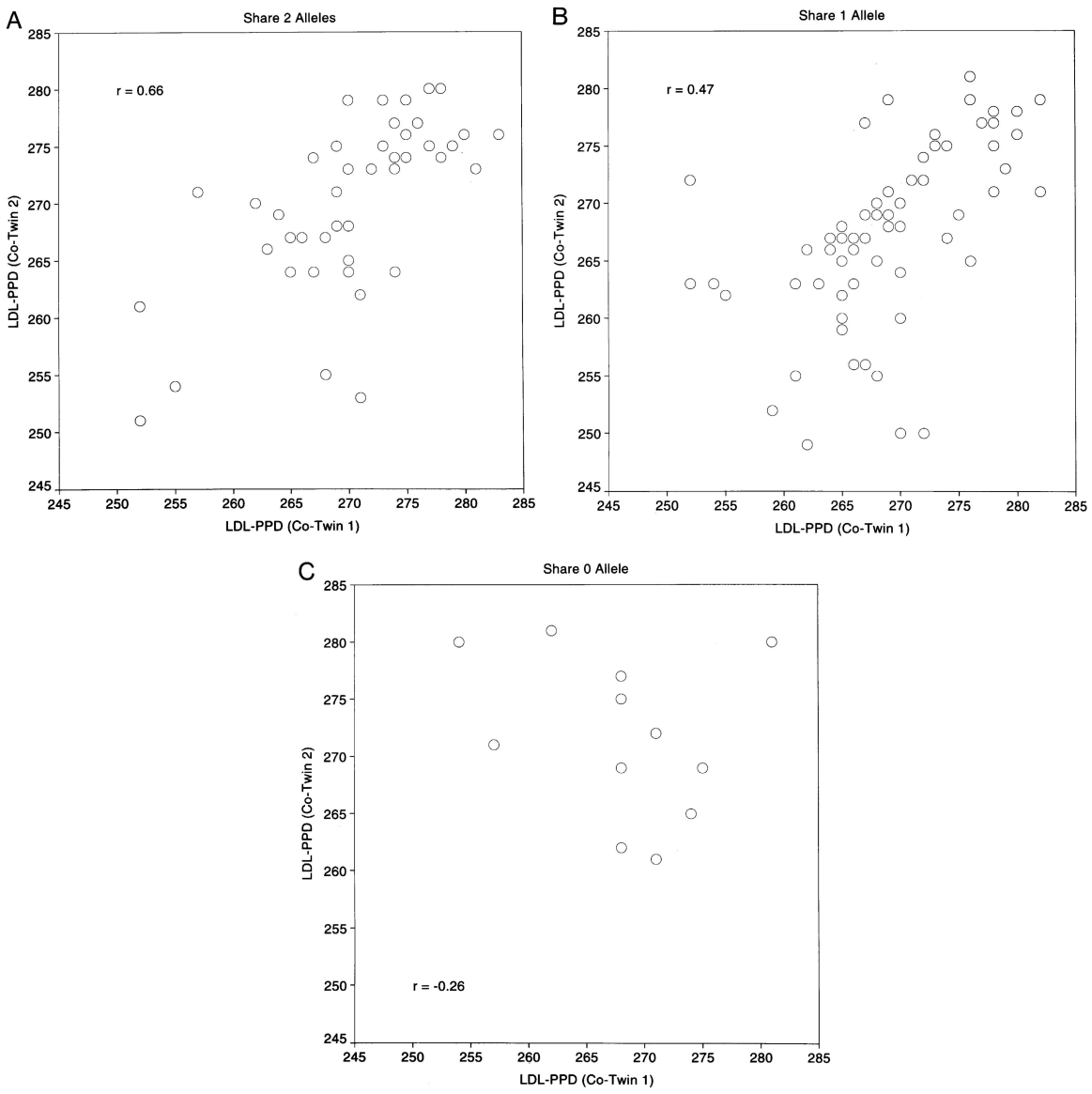

Figure 3 Similarity of co-twin values for phenotypes of LDL-PPD (in Å), among pairs sharing (*A*) 2, (*B*) 1, or (*C*) 0 alleles IBS at the apo B VNTR marker. Samples sizes were 46, 61, and 12 twin pairs, respectively, and intraclass co-twin correlations are shown. The *X*-axis indicates the value for one randomly selected co-twin/pair, and the *Y*-axis indicates the value for the other co-twin of the same pair.

(−43 and −41, respectively), although these values were less than the slope for the VNTR marker in the full sample (−76; table 2). Since the range of LDL-PPD was more limited in the subset of women with LDL-PPD >255 Å, the standard error of the slopes was reduced. Thus, the slopes for the subset remain statistically significant, despite the decrease in absolute value.

Interestingly, a more negative slope and a lower *P* value for linkage to HDL cholesterol were seen for the apo B intron 20 marker than were seen for the apo B VNTR marker (slope of −635 and $P = .032$ vs. slope of −353 and $P = .113$, respectively), suggesting that this phenotype also may be influenced by the apo B locus, among the subset of women with LDL-PPD >255 Å. This notion is further supported by HDL cholesterol co-twin correlations of .39 ($P < .01$), .24 ($P < .05$), and −.19 ($P > .50$), for pairs sharing 2, 1, or 0 apo B intron 20 alleles, respectively.

Discussion

Taken together, these results provide preliminary evidence that the apo B gene is genetically linked to the LDL-PPD, TG, HDL cholesterol, and apo B plasma lev-

Table 5

Slopes and *P* Values from the Quantitative Sib-Pair Linkage Analysis Based on DZ Women Twin Pairs with LDL Subclass Phenotype A (LDL-PPD >255 Å), for Both Co-Twins

Candidate-Gene Marker	No. of Pairs	Regression Slope (*P* Value)				
		LDL-PPD	ln of TG Level	HDL Cholesterol	Apo B Level	LDL Cholesterol
MTP	102	−2.49 (.451)	−.16 (.099)	417.57 (.934)	−312.53 (.055)	−662.71 (.143)
Apo B:						
VNTR	103	−43.25 (.011)	−.28 (.005)	−352.82 (.113)	−325.23 (.045)	−575.57 (.178)
Intron 20	103	−40.83 (.048)	−.16 (.148)	−635.03 (.032)	−320.51 (.094)	−1,070.48 (.085)

els, all components of an atherogenic lipoprotein phenotype, in this sample of adult women twins. Furthermore, these relationships appear to be present among women with LDL in the intermediate-to-large size range, as well as in the entire sample of twins. This implies that the apo B gene contributes to variation of LDL size in the intermediate-to-large size range.

This implication is not inconsistent with two previous studies that had provided definitive evidence against linkage of the apo B gene and LDL subclass phenotype B (by use of the same VNTR marker) in primarily healthy families (LaBelle et al. 1991) and in families with FCHL (Austin et al. 1991) (table 6). That is, both of these previous studies used a dichotomous definition of larger and smaller LDL subclass phenotypes, whereas the current study used LDL-PPD as a continuous variable and found evidence for linkage even when the analysis was limited to the intermediate-to-large size range. Furthermore, these analyses were based on a LOD-score linkage analysis of families that assumes a specific model of single-gene inheritance, rather than on the nonparametric sib-pair approach reported here. However, a recent study of sib pairs from families with CAD also found no evidence for linkage of LDL-PPD to the apo B gene, over the full range of LDL size (Rotter et al. 1996). It is also important to note that, unlike previous studies, the current analysis is based entirely on women.

Because of the strong evidence for major-gene effects on LDL heterogeneity, obtained from complex segregation analysis, several studies have investigated other candidate genes for linkage to LDL size (table 6). The study noted above (Rotter et al. 1996), based on sib pairs from CAD families, showed no linkage to the LPL gene but did find evidence for linkage to the CETP gene and the MnSOD gene, although these findings remain to be confirmed by other studies. In that study, border-

Table 6

Candidate-Gene Linkage Studies of LDL Subclass Phenotypes and LDL Size

Candidate Gene	Chromosome	LOD Score(s) for Families, at $\theta = .001$	*P* Value from Sib-Pair Analysis[a]	
			CAD Families[b]	DZ Women Twins[c]
MTP	4q	…	…	NS
HL	15q	…	…	NS
HSL	19q	…	…	NS
Lipoprotein lipase	8p	…	NS	…
CETP	16q	…	.03	…
MnSOD	6q	…	.001	…
Apo B	2p	−7.5,[d] −6.1[e]	NS	.014
Apo AI-CIII-AIV complex	11q	…	.06	.893
Apo AII	1p	…	NS	…
Apo E	19q	…	NS	NS
IR	19p	1.4[f]	…	NS
LDL-R	19p	4.4[f]	.01	.082

[a] NS = not significant. An ellipsis (…) indicates that the data were not reported.
[b] From the study by Rotter et al. (1996).
[c] From this study.
[d] From the study by LaBelle et al. (1991).
[e] From the study by Austin et al. (1991).
[f] From the study by Nishina et al. (1992).

line statistically significant evidence for linkage to the apo AI-CIII-AIV gene complex also was found, whereas no such evidence emerged from the present study. To date, there is no evidence for linkage to either the apo AII gene or the apo E gene, despite the recent report of an association between LDL size and the apo E isoforms (Haffner et al. 1996). However, because the apo E polymorphism is only triallelic, there was little statistical power to detect linkage, in the present analysis. Of the two studies investigating the IR, neither have found evidence for linkage.

For linkage between the LDL-R gene and both LDL-PPD and TG, the quantitative sib-pair analysis in this study demonstrated borderline statistically significant results, similar to previous findings of linkage between the LDL-R and (*a*) LDL subclass phenotype B in families (Nishina et al. 1992) and (*b*) LDL size in sib pairs from CAD families (Rotter et al. 1996). Furthermore, when twin pairs were divided into groups of those sharing 2, 1, or 0 alleles IBS for the LDL-R–gene marker, the correlations were .66 ($P < .001$), .47 ($P < .001$), and .36 ($P < .05$). However, as in the earlier studies, the LDL-R was not linked to the other lipoprotein measures characteristic of the atherogenic lipoprotein phenotype, including HDL cholesterol, apo B plasma levels, and LDL cholesterol (data not shown). Thus, the role of the LDL-R gene in determination of LDL size remains to be fully elucidated.

In addition to the results for LDL-PPD, evidence for linkage between the MTP gene and plasma TG was found, at the 0.05 level (table 2). Among the entire sample of women, this result was limited to TG and was not found to be the case for other lipoprotein measures. However, when the analysis was specific to women twin pairs with LDL subclass phenotype A, a borderline statistically significant linkage between MTP and apo B plasma levels also was found (slope of -313, $P = .055$). Both of these results are intriguing, since MTP is known to be involved in the assembly of apo B–containing lipoprotein particles in the liver (Jamil et al. 1996) and is a cause of abetalipoproteinemia (Narcisi et al. 1995). If confirmed, these results would imply that the MTP gene influences TG in most individuals, whereas the influence on apo B levels may be limited to those with relatively large LDL size.

In this sample of women twins, heritability analysis demonstrated significant genetic influences on plasma apo B levels. Specifically, the intraclass correlations were .701 ($P < .001$) for MZ twins and .494 ($P < .001$) for DZ twins, resulting in a classic heritability estimate of .413 ($P < .001$). This candidate-gene analysis suggests that both the MTP gene and the apo B gene contribute to this heritability of apo B levels, at least in women.

In contrast to previous association studies of both animal models and human populations (Ito et al. 1990; Dammerman et al. 1993; Zeng et al. 1995), no genetic linkage was observed between the apo CIII gene and TG levels. Because of the relatively limited sample size in this study, it is possible that there was not sufficient statistical power to detect this linkage. Most studies of apo CIII polymorphisms report associations with severe hypertriglyceridemia, whereas all the women included in this study had TG levels <400 mg/dl. Thus, the apo CIII gene may not have a strong influence on TG in this range, despite its role in severe elevations of TG.

It is important to note that the sample size in this sib-pair analysis of 126 pairs is limited, compared with that in many such studies. Although the use of women twins eliminates potentially confounding effects owing to age and sex, the statistical power available may have limited the ability to detect true genetic linkages. To reduce this possibility (type I error), an α value of 0.05 has been used as the criteria for statistical significance, without adjustment for multiple comparisons or consideration of the finite nature of the human genome. However, this approach could have resulted in false-positive results, emphasizing the necessity for replication of these findings, in other populations.

In summary, these candidate-gene sib-pair analyses of adult DZ women twins have suggested genetic linkage between the apo B gene and LDL size, plasma levels of TG, HDL cholesterol, and apo B, all features of the atherogenic lipoprotein phenotype. Furthermore, this result was maintained when the analysis was limited to women with intermediate-to-large LDL, suggesting that the apo B gene may influence LDL heterogeneity in the larger size range. In addition, moderate evidence for linkage between the MTP gene and plasma TG was found among all the women. These findings add to the growing evidence for genetic influences on the features of the atherogenic lipoprotein phenotype and for its role in genetic susceptibility to atherosclerosis.

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