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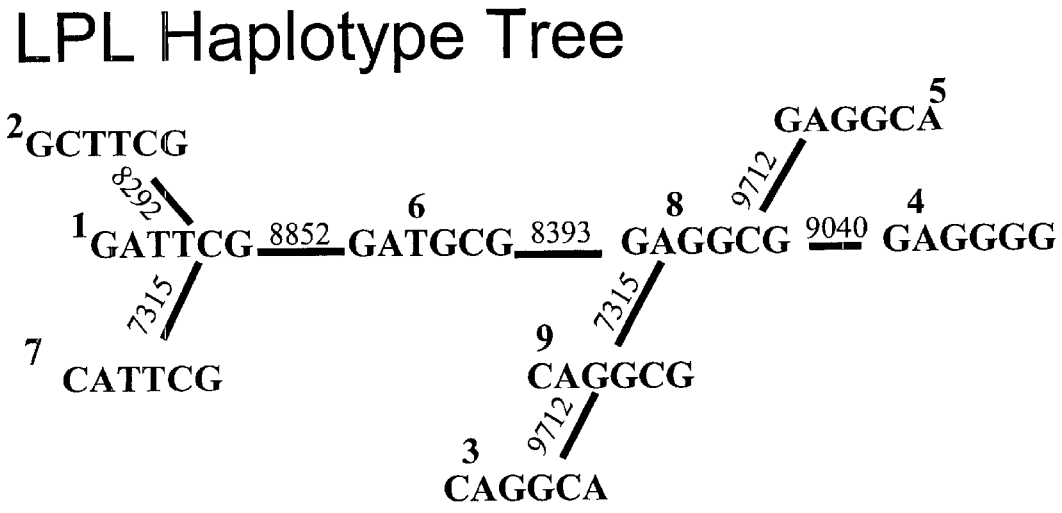

(57) Abstract: Disclosed is a method for determining haplotypes useful for large-scale genetic analysis, within a genomic reference sequence of interest, for a human subpopulation. The method can applied to statistically evaluating the genotypes of subjects for any statistically significant association with a phenotype of interest, such as insulin resistance or coronary artery disease.

WO 03/106652 A2

METHOD OF HAPLOTYPE-BASED GENETIC ANALYSIS FOR DETERMINING RISK FOR DEVELOPING INSULIN RESISTANCE, CORONARY ARTERY DISEASE AND OTHER PHENOTYPES

BACKGROUND OF THE INVENTION

1. Field of the Invention

The present invention relates to the field of medical genetics.

2. Discussion of the Related Art

The insulin resistance syndrome (also called the metabolic syndrome) is a clustering of factors associated with an increased risk of coronary artery disease (CAD; 21). The syndrome affects over 20% of adults in the United States, with the highest age-specific prevalence rates in Mexican-Americans (22). Insulin resistance, whether or not it is accompanied by other features of the metabolic syndrome, has been associated with an increased risk of cardiovascular events and death (23, 24).

There is evidence in the Framingham offspring study that three factors or syndrome clusters, underlie the clustering of basic risk variables that form the insulin resistance syndrome: a diabetic predisposing syndrome characterized by impaired glucose tolerance, a cardiovascular metabolic syndrome, and a hypertension syndrome. Numerous lines of evidence from epidemiological studies support the idea that these factors occur many years prior to the onset of overt coronary artery disease.

The clustering of insulin resistance, hypertension, central obesity, and dyslipidemia in the metabolic syndrome is receiving much attention as a risk factor for cardiovascular disease. The central component of this syndrome, insulin resistance, has been found to increase cardiovascular risk. In the San Antonio Heart Study, insulin resistance, estimated by homeostatic model assessment (HOMA), was an independent predictor of incident cardiovascular events over 8 years of follow-up (24). In the Helsinki Policemen Study, 970 men free of diabetes or CAD at baseline were followed for 22 years; those with the highest levels of insulin resistance as estimated by insulin area under the curve during oral glucose tolerance testing had the highest rates of CAD events and death (23). High fasting insulin concentrations were an independent predictor of ischemic heart disease events among 2103 non-diabetic Canadian men (51). A genetic basis for the components of the insulin resistance syndrome has been demonstrated by familial aggregation (52, 53). For this

reason, investigators have asked the question as to whether genetic determinants of insulin resistance also influence the other components of the metabolic syndrome (54).

As an example, lipoprotein lipase (*LPL*) plays a major role in lipid metabolism. Located on capillary endothelium, *LPL* hydrolyzes triglycerides of chylomicrons and very low density lipoproteins, generating free fatty acids and monoacylglycerol. Complete deficiency of LPL results in the familial chylomicronemia syndrome. Because *LPL* activity affects the concentration of triglycerides, an important cardiovascular risk factor, *LPL* has been studied as a candidate gene for atherosclerosis. Several studies have identified linkage and association of the *LPL* gene with hypertension (25, 1), indirect or surrogate measurements of insulin resistance (2, 3), dyslipidemia (2, 26, 27), obesity (28), and atherosclerosis (4, 29, 30). *LPL* is an excellent candidate connecting insulin resistance to atherosclerosis because it controls the delivery of free fatty acids (FFA) to muscle, adipose tissue, and vascular wall macrophages, wherein lipid uptake influences peripheral insulin sensitivity, central obesity, and foam cell formation (31, 32).

Wu *et al.* demonstrated linkage of the *LPL* locus to systolic blood pressure in non-diabetic relatives of Taiwanese subjects with type 2 diabetes (1). The *Hind*III polymorphism in intron 8 of the *LPL* gene has been associated with measurements of insulin resistance in normoglycemic Caucasian and Hispanic subjects (2) and Chinese subjects (3). The Ser447Stop polymorphism has been found to be associated with decreased atherosclerosis risk (4). Both the *Hind*III and Ser447Stop polymorphisms are in the 3' end of the *LPL* gene, downstream of a recombination hotspot (5).

The *LPL* gene has emerged as a candidate gene for features of metabolic syndrome, including insulin resistance. *LPL* hydrolyzes triglycerides carried in chylomicrons and very low density lipoproteins, the rate-limiting step in delivery of free fatty acids (FFA) to muscle and adipose tissue. By controlling the delivery of FFA to muscle, LPL may affect insulin sensitivity by influencing levels of intramyocellular lipid, which correlate with muscle insulin resistance (55, 56). Also, *LPL* may influence insulin resistance by affecting FFA delivery to visceral adipose tissue, which is increasingly viewed as an endocrine organ, capable of secreting mediators of insulin resistance (57). *LPL* action also regulates the plasma triglyceride concentration, an important atherosclerosis risk factor (58, 59). *LPL* activity indirectly raises HDL-cholesterol levels because *LPL*-mediated hydrolysis of VLDL provides surface components that merge with HDL3 to form HDL2 particles (60). *LPL*-mediated delivery of FFA and lipoprotein remnants to vessel wall macrophages plays

a role in foam cell formation, an early event in the development of atherosclerotic plaque (32). Thus, functional variation in *LPL* may impact both insulin resistance and atherosclerosis.

Most studies that have reported association of the *LPL* gene with insulin resistance used only surrogate measurements of insulin resistance, including fasting glucose (8, 33), fasting insulin (2, 34-36), and insulin area under the curve (AUC) during oral glucose tolerance testing (OGTT; 37). One study evaluated the steady state plasma glucose during the insulin suppression test (3). In addition, all except one (36) of these studies only examined association of the intronic restriction fragment length polymorphisms *Pvu*II and *Hind*III. Thus, current evidence that variation in *LPL* plays a role in insulin sensitivity has been indirect. Assessment of glucose infusion rate (GINF) during the euglycemic hyperinsulinemic clamp study is widely regarded as the most direct physiologic measurement of insulin sensitivity (28, 29). An analysis of indices of insulin sensitivity in the Insulin Resistance Atherosclerosis Study showed that direct physiologic measurements of insulin sensitivity have a higher heritability than measures based on fasting values (such as HOMA; 61). Thus, use of physiologic indices rather than simple fasting indices should provide more power to discover genes that contribute to insulin sensitivity.

While various polymorphisms in the 3' end of *LPL*, such as *Hind*III, have been associated with surrogate measures of insulin resistance and with atherosclerosis (2, 3, 29, 30), published reports of positive linkage or association of variation in *LPL* with indices of insulin sensitivity have typically examined only one or two single nucleotide polymorphisms (e.g., 2, 3, 8, 33-37). However, a haplotype-based analysis recently demonstrated an association of *LPL* 3' end haplotypes with coronary artery disease in Mexican-Americans (30).

Published studies reporting association of the *LPL* gene with insulin resistance used only single variants, usually *Hind*III or *Pvu*II (2, 3, 8, 33-37). In some cases, the results are in conflict; studies have reported the T allele of *Hind*III associated with insulin resistance (2), others report the G allele associated with insulin resistance (3, 37), and others show no association of HindIII with insulin resistance (8). This demonstrates a limitation of the common approach of examining one or two polymorphisms per candidate gene in an association study.

With the sequencing of the human genome it has become apparent that variation in individuals is quite extensive. There is increasing evidence that this variation is best described by groups of associated polymorphisms referred to as haplotypes (13-15).

Recent studies suggest that the extensive variation in human beings is best described by groups of associated polymorphisms referred to as haplotypes (13-15). Haplotypes encompass chromosomal blocks that have remained unbroken by recombination during the population evolutionary history of the gene. Haplotypes are more likely to identify disease associations than single polymorphisms because they reflect global gene structure and encompass the majority of common variation in a gene. Identification of a haplotype associated with increased or decreased disease risk should facilitate identification of the actual functional variant that affects disease risk, because this variant should lie on chromosome regions identified by that haplotype (17).

Thus, haplotypes capture the majority of common variation in a gene; consequently, the use of haplotypes is more likely to identify disease-variation associations than is the use of a random single polymorphism. Identification of a haplotype associated with increased or decreased disease risk should facilitate identification of the actual functional variant that affects disease risk, because this variant should lie on chromosomes identified by that haplotype (16, 17). Genotyping to determine haplotype structure and frequencies is required for this type of analysis. A major challenge is determination and selection of the polymorphisms that will be used to determine haplotypes in a given population.

Currently there is much interest in the use of haplotype data in the genetics of common diseases, such as coronary artery disease and insulin resistance. Investigators are faced with the considerable challenge of how many and which variants or markers to genotype in a given candidate gene for haplotype determination. Gabriel *et al.* (15) sequenced 13 megabases across the genome in subjects from Africa, Europe, and Asia; it was shown that the human genome is organized in haplotype blocks (most of which are longer than 10 kilobases), with three to five commonly occurring (>5%) haplotypes per block. Only six to eight variants were sufficient to define the most common haplotypes in each block. There is a need for a way to select these variants, or markers, efficiently and affordably.

Accordingly, the present invention provides such a method of selecting useful haplotypes, as well particular haplotypes useful for predicting predisposition to insulin resistance in Mexican-Americans. These and other benefits are described hereinbelow.

SUMMARY OF THE INVENTION

The present invention relates to a method for determining haplotypes useful for application to large-scale genetic analysis and screening tests for a human subpopulation, such as Mexican-Americans, within a genomic reference sequence of interest. The method involves detecting the presence of a plurality of genetic markers, or variants, at positions of the genomic reference sequence, in the genotypes of a first number of subjects in the human subpopulation. A frequency hierarchy of the detected markers is identified, and from the frequency hierarchy a set of haplotypes is constructed, each haplotype of the set comprising at least one of the most frequently detected markers. A smaller subset of the set of haplotypes is selected, the smaller subset comprising those haplotypes most frequently occurring in the first number of subjects. The markers needed to define the thus selected smaller subset of the set of haplotypes is identified.

In some embodiments of the present invention, useful in determining genetic associations between specific haplotypes and particular phenotypes, a second number of subjects in the human subpopulation are genotyped for the markers previously identified in accordance with the method; the second number of subjects being larger than the first number of subjects. The genotypes of the second number of subjects are evaluated for any statistically significant association of any members of the thus selected smaller subset of the set of haplotypes with a phenotype of interest, which can be a disease or medical disorder, such as insulin resistance or coronary artery disease.

In accordance with the invention, a method of detecting a genetic predisposition in a Mexican-American human subject for developing insulin resistance is provided. The method involves collecting a biological sample from the subject; genotyping the sample at nucleotide positions 7315, 8292, 8393, 8852, 9040, and 9712, with respect to the Nickerson reference sequence of the human lipoprotein lipase gene (see Table 1 hereinbelow); and assessing whether a haplotype (designated herein "haplotype 4"; see, e.g., Table 5) is present in the sample. The haplotype comprises the following (nucleotide position:variant allele): (i) 7315:G; (ii) 8292:A; (iii) 8393:G; (iv) 8852:G; (v) 9040:G; and (vi) 9712:G. The presence of the haplotype indicates a genetic predisposition for developing insulin resistance in the Mexican-American subject, as demonstrated hereinbelow.

Similarly, in accordance with an inventive method of detecting a lower than normal risk in a Mexican-American human subject for developing insulin resistance, the presence

in the genotyped sample, instead, of a haplotype comprising (nucleotide position:variant allele): (i) 7315:G; (ii) 8292:A; (iii) 8393:T; (iv) 8852:T; (v) 9040:C; and (vi) 9712:G (designated herein "haplotype 1"; see, e.g., Table 5), indicates a lower than normal risk for developing insulin resistance in the subject, as demonstrated hereinbelow.

Alternatively, in accordance with the invention, a method of detecting a lower than normal risk in a Mexican-American human subject for developing coronary artery disease is provided. The method involves collecting a biological sample from the subject; genotyping the sample at nucleotide positions 7315, 8292, 8393, 8852, 9040, and 9712, with respect to the Nickerson reference sequence of the human lipoprotein lipase gene; and assessing whether the sample is homozygous for a haplotype comprising (nucleotide position:variant allele): (i) 7315:G; (ii) 8292:A; (iii) 8393:T; (iv) 8852:T; (v) 9040:C; and (vi) 9712:G (designated herein "haplotype 1"; see, e.g., Table 5). Homozygosity for haplotype 1 indicates a lower than normal risk for developing coronary artery disease in the subject.

If a greater than normal, or lower than normal, risk of developing insulin resistance or coronary artery disease is detected, in accordance with the invention, then suitable treatment or prophylactic modalities can be chosen, as appropriate for the individual with the benefit of this additional clinical information.

The meanings of abbreviations found herein are the following:

LPL, lipoprotein lipase; CAD, coronary artery disease; MACAD, Mexican-American Coronary Artery Disease project; SNP, single nucleotide polymorphism; GINF, glucose infusion rate; S_I, insulin sensitivity.

The present invention is further described by U.S. provisional application 60/388,726, filed June 14, 2002, the disclosures of which are incorporated by reference.

BRIEF DESCRIPTION OF THE DRAWINGS

Figure 1 illustrates the cladistic structure of the *LPL* 3'-end haplotypes. The lines connecting the haplotypes indicate a single nucleotide difference between the connected haplotypes, with the corresponding variant number above the line. The haplotypes are numbered in order of decreasing frequency. Haplotypes 1, 2, 6, and 7 contain *Hind*III allele 1; haplotypes 3, 4, 5, 8, and 9 contain contain *Hind*III allele 2.

Figure 2 shows the effect of LPL 3' end haplotypes on indices of insulin sensitivity. The thick line in the center of each graph represents the mean for the entire haplotyped and clamped population.

Figure 3 shows independent effects of haplotype 1 and haplotype 4 on insulin sensitivity. On the left are haplotype 1 genotypes with haplotype 4 carriers removed. On the right are haplotype 4 genotypes with haplotype 1 carriers removed. The thick line in between represents the mean GINF level for the entire haplotyped and clamped population.

DETAILED DESCRIPTION OF THE PREFERRED EMBODIMENTS

The present invention is directed to a method for determining haplotypes within a genomic reference sequence of interest, which haplotypes are useful for large-scale genetic analysis and genetic screening tests for a human subpopulation. The genomic reference sequence of interest can be any coding or non-coding sequence of interest, for example, the human lipoprotein lipase (*LPL*) gene.

The *LPL* gene is located on the short arm of human chromosome 8, at 8p22. (R.S. Sparkes *et al.*, *Human genes involved lipolysis of plasma lipoproteins: Mapping of loci for lipoprotein lipase to 8p22 and hepatic lipase to 15q21*, Genomics 1:138-44 [1987]). The gene is near microsatellite marker D8S1715 and flanked by microsatellites D8S261 and D8S280. Closer flanking sequences of human *LPL* are defined by GenBank accession numbers M94221 and M94222 (S. Wood et al., *Support for founder effect for two lipoprotein lipase [LPL] gene mutations in French Canadians by analysis of GT microsatellites flanking the LPL gene*, unpublished [1992]). The gene spans about 30 kb and contains 10 exons encoding a 475 amino acid protein including a 27 amino acid secretory signal peptide. (S. Deeb and R. Peng, *Structure of the human lipoprotein lipase gene*, Biochemistry 28(10):4131-35 [1989]; T.G. Kirchgessner et al., *Organization of the human lipoprotein lipase gene and evolution of the lipase gene family*, Proc. Natl. Acad. Sci. USA 86:9647-51 [1989]).

The 3' end of the human lipoprotein lipase gene, for purposes of the present invention, includes nucleotide positions 4801 through 9734 of the Nickerson reference sequence extending from intron 6 into intron 9. (GenBank accession No. AF050163). (D. A. Nickerson et al., *DNA sequence diversity in a 9.7-kb region of the human lipoprotein lipase gene*, Nat. Genet. 19:233-40 [1998]). The complete Nickerson reference sequence is given in Table 1 hereinbelow.

The human subpopulation can be any subpopulation of interest based on ethnicity, gender, age, or other identifiable feature distinguishing the subpopulation from the general population.

In accordance with the method "a first number of subjects" in the human subpopulation is a finite number of subjects with a minimum of 10 or more, and preferably with a minimum number of about 20 to about 40 subjects. The first number can be any number of subjects in the subpopulation up to the total number of individuals in the subpopulation, minus one. The "second number of subjects" can be any number of subjects in the subpopulation up to the total number of individuals in the subpopulation. The minimum of the second number of subjects in the human subpopulation is an appropriate number known to the skilled artisan, depending on several factors, including the frequency of particular haplotypes in the subpopulation, the frequency of particular phenotypes of interest in the subpopulation, the strength of association between a haplotype and the phenotype of interest, the desired level of statistical significance, and other like factors.

Gabriel et al. (15) showed that the human genome is organized in haplotype blocks (most of which are longer than 10 kilobases), with three to five commonly occurring (>5%) haplotypes per block. Only six to eight variants were sufficient to define the most common haplotypes in each block. Genotyping six to eight variants thus allows determination of the most frequently occurring haplotypes in a population for association analysis. The availability of family data assists this approach by facilitating unambiguous determination of haplotypes in a more efficient and less expensive manner, based on genotyping at single variants. Variants of interest can also be selected from available databases, particularly but not exclusively, with respect to a group of non-related individuals.

A benefit of a haplotype-based analysis is that it captures all of the variation across a region, which should improve the ability to detect an association.

The "genome" of an individual member of a species comprises that individual's complete set of genes. Particular locations within the genome of a species are referred to as "loci" or "sites". "Alleles" are varying forms of the genomic DNA located at a given site. In the case of a site where there are two distinct alleles in a species, referred to as "A" and "B", each individual member of the species can have one of four possible combinations: AA; AB; BA; and BB. The first allele of each pair is inherited from one parent, and the second, on a matching chromosome, is inherited from the other parent.

The "genotype"of an individual at a specific site, or in a combination or group of associated polymorphic sites (i.e., haplotype), in the individual's genome refers to the specific combination of alleles that the individual has inherited.

The "phenotype" of an individual refers to one or more of these observable physical characteristics. An individual's phenotype is driven in large part by constituent proteins in the individual's proteome, the collection of all proteins produced by the cells comprising the individual and coded for in the individual's genome, but genetic regulatory elements can also produce a phenotype.

For the purpose of the present invention, a "genetic marker" is a single nucleotide polymorphism (SNP). "Variant", "marker", and "polymorphism" are used interchangeably herein.

For purposes of the present invention, detecting, evaluating, or assessing the presence or absence of a genetic marker (i.e., an allele) or heterozygosity or homozygosity of the subject with respect to the marker, is detected in a biological sample collected from the individual that contains the individual's genomic DNA (such as, but not limited to, a blood, saliva, or tissue biopsy sample, which biological sample can be freshly collected or suitably stored to preserve the DNA) by employing suitable biochemical genotyping analytical assay means. Analytical hybridization or polynucleotide sequencing means are typically employed, optionally after amplification of DNA in the biological sample, for example, by using PCR-based amplification means. High throughput analyses can optionally be achieved by multiplexing techniques known in the art. The genotyping analytical assay means can optionally be performed with commonly available robotic apparati and/or very dense array detection apparati. Probes, primers, and protocols useful in genotyping of a biological sample with respect to markers and haplotypes of the *LPL* gene are described, for example, in Table 2 and the Examples herein, and others are known to the skilled artisan (see, e.g., U.S. Patent No. 6,297,014).

The present invention relates to a method of detecting a genetic predisposition in a Mexican-American human subject for developing insulin resistance. That a genetic "predisposition" is detected means that the subject, who does not currently exhibit insulin resistance, has a greater than normal risk of developing insulin resistance in the future, compared with the Mexican-American subpopulation as a whole.

Similarly, with respect to the inventive methods of detecting a lower than normal risk in a Mexican-American human subject for developing in sulin resistance or coronary artery disease, respectively, "lower than normal" is in comparison with the Mexican-American subpopulation as a whole.

For the purposes of the present invention, a "Mexican-American" is an individual with at least 3 of 4 grandparents native to Mexico. A Mexican-American subpopulation is a human subpopulation (i.e., an ethnic subpopulation of the general human population) consisting of such individuals.

The invention will now be described in greater detail by reference to the following non-limiting examples.

EXAMPLES

Example 1: Lipoprotein Lipase Gene Haplotypes in Mexican-Americans: Structure and Association with Coronary Artery Disease

Briefly, six polymorphisms sufficient to distinguish the most common haplotypes in the 3' end of LPL were identified by genotyping ten polymorphisms in a small pilot population. These were used to haplotype LPL in large family samples of Mexican-Americans and non-Hispanic Caucasians. A case-control association study was performed comparing Mexican-Americans with and without coronary artery disease. The two ethnic groups exhibited significant genetic differences. Among Mexican-Americans, homozygosity for LPL haplotype 1 was protective against coronary artery disease (OR=0.50, 95% CI 0.27-0.91). This study outlines the haplotype structure of the LPL gene, illustrates the utility of haplotype-based analysis in association studies, and demonstrates the importance of defining haplotype frequencies for different ethnic groups.

Materials and Methods

Subjects. The UCLA/Cedars-Sinai Mexican-American Coronary Artery Disease (MACAD) Project enrolls families ascertained through a proband with coronary artery disease, determined by evidence of myocardial infarction on electrocardiogram or hospital record, evidence of atherosclerosis on coronary angiography, or history of coronary artery bypass graft or angioplasty. DNA is obtained from all available family members, and the

adult offspring of the proband and the spouses of those offspring are also asked to undergo a series of tests to characterize their metabolic and cardiovascular phenotype, including indices of insulin resistance determined by euglycemic clamp study, lipid parameters, lipase activities, and carotid intima-media thickness.

In a separate study, non-Hispanic Caucasian families were recruited for a genetic linkage study to determine the influence of specific genes on inter-individual variation in the lipoprotein response to a low-fat, high-carbohydrate diet. Siblings were placed on either a high-fat or a low-fat diet and changes in lipids and lipoproteins were monitored. We examined this population in terms of haplotype frequency for comparison to Mexican-Americans.

Individuals with at least 3 of 4 grandparents native to Mexico were classified as "Mexican American" in our studies.

Genotyping.

An early stage of our haplotyping methodology consists of genotyping a number of single nucleotide polymorphisms (SNPs) spanning a region of a candidate gene in a limited number of subjects. Haplotypes are then constructed using these variants, with subsequent selection of a smaller number of variants that allow discrimination of the most common haplotypes on the majority of chromosomes observed in the population. In the second stage of the haplotyping protocol, the restricted set of SNPs identified in the first stage is genotyped in a large number of individuals using a high-throughput technology and used to determine haplotypes on a population scale.

Twenty-nine subjects from 8 randomly selected families from MACAD were genotyped at 10 single nucleotide polymorphisms (4872, 5168, 5441, 6863, 7315, 8292, 8393, 8852, 9040, 9712) originally delineated in the MDECODE (Molecular Diversity and Epidemiology of Common Disease) project, a study of Finnish, non-Hispanic Caucasian Americans, and African American subjects (9). The numbering of the SNPs corresponds to that reported by Nickerson, et al. (9; see Table 1) and corresponds to Genbank accession number AF050163.

Table 1. Nickerson reference sequence. (GenBank accession No. AF050163). (D. A. Nickerson *et al.*, *DNA sequence diversity in a 9.7-kb region of the human lipoprotein lipase gene*, Nat. Genet. 19:233-40 [1998]). The complete Nickerson reference sequence is the following:

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   1 TGTAACACAA AATTAAAATA AGTAGAATTA GTTTTCAGTA TTTCCTATAT TTGGAAAACA
  61 ATATTTATAT TCATTTTGTT TCTTTTAGTT TTATTTTTGG CAGAACTGTA AGCACCTTCA
 121 TTTTCTTTTT CTTCCAAAGG AGGAGTTTAA CTACCCTCTG GACAATGTCC ATCTCTTGGG
 181 ATACAGCCTT GGAGCCCATG CTGCTGGCAT TGCAGGAAGT CTGACCAATA AGAAAGTCAA
 241 CAGAATTACT GGTAAGAAAG CAATTTCGTT GGTCTTATCA TAAGAGGTGA AAAGACTGTC
 301 ATTCTGAGAG AGAATCAGAA CAAATTTTGT TAAATACCCA CATGTGTGGT GTTCTTCCCG
 361 GAGACATGAC CAGCACTTGA TTATCTCATT GTAGGGCTCT TTATTAGGGA TAAGAAAAAA
 421 CACAGACGCT CTCACTGGCT TACTATCCAC TGGCAATAGC ACAGAAATAA AGCATAATTA
 481 CACACAATGC CTGCAGATTT CTCTGGGAAG CCTGTTTCCT CCCACTCTCA GCTCTGTGTT
 541 TTAGTAGTGT AAATGCACAT CAGTACTAGG AGAAAAGAAG AAGGACCAAT TCCAGAGGCC
 601 ACTTCGAAAG AAGACCGTCA TCTAGGCAAA GGTGTGGCAT ACACACAGAG AGAAAGAACC
 661 CACCACTGTT TATACATCTT CTCGACATAT TCAGAAATAA TCTACAAAAG GAAATCCAGC
 721 CATCCTGAGT GGAAATTGCT GCATAAGGCT AGTTTAAGAG ACTCAAATTC ATTTTAGAAG
 781 GAGCCAAGCC TCCTTTTATG TCTCTCTAAG TAAAGATACC ATGACTGTAG AATAGGAGCT
 841 AATAAGAATC TAAATAGCTG CCAGTGCATT CAAATGATGA GCAGTGACAT GCGAATGTCA
 901 TACGAATGGA AATTTACAAA TCTGTGTTCC TGCTTTTTTC CCTTTTAAGG CCTCGATCCA
 961 GCTGGACCTA ACTTTGAGTA TGCAGAAGCC CCGAGTCGTC TTTCTCCTGA TGATGCAGAT
1021 TTTGTAGACG TCTTACACAC ATTCACCAGA GGGTCCCCTG GTCGAAGCAT TGGAATCCAG
1081 AAACCAGTTG GGCATGTTGA CATTTACCCG AATGGAGGTA CTTTTCAGCC AGGATGTAAC
1141 ATTGGAGAAG CTATCCGCGT GATTGCAGAG AGAGGACTTG GAGGTAAATA TTATTTAGAA
1201 GCGAATTAAA TGTGACTCTT ATCCTTAACC CTTATTGACC CAATGTCCTA CTCAGTAGCT
1261 TCAAAGTATG TAGTTTTCAT ATACACATTT GGCCAAATTA TGTTTCTGAA GAATTCTGCA
1321 ATGTTCAGCA TGACCACCTT AGAGCCAGGC AGACAGCCAT TTTATCTTTT ATTTACTATA
1381 CTGTAGGCTA CACTGAGCAG TGCACTTACA GTAGCAAGAG AAAAAGGTGG GATTTTAGAC
1441 AGGAAGACTC CACTGACCTC AATAATGGCA TCATAAAATG CTATCTGGCC ACATGTTGTC
1501 ATACCTTGAA TGTAGCTGCA AAGCCAATGG AAAGATTTTA GATGTTACTG GAACAGAAGA
1561 TGTTAATTAG CATAAATCTT CCAAAATGTT CAGAACATAA TGTTAGCTTA ATGTTTTACT
1621 TTAATAATGT TAGCTTGTGT TAAATTTATG ATTTTTGTTT GTTTGTTTTT TGAGATAGAG
1681 TCTTATTCTA TTGCCCAAGC TGGGGTGCAG TCACACAATC ACAGGGACTT GCAATGTTGC
1741 CCAGGCTGGT CTCAAACTCC TGGCCTCAAG TGATCCTCCT GCCTCAGCCT CCCAAAGTTC
1801 TGGGATTGCA GCTGTGAGCC ACCACGCCCA GTTTACGATT TATTTTTAAG AGCCCCTTGC
1861 ATACTTTATA GACATTGGGA CCTACCTAGG ATATTCTCGT TATTTTTGTG CACGTAATAG
1921 AACTTAGAGC ATATTGTTAC TATTTTCGAT TGTCCTAAAA ACTTACAAGG AATTCATTCT
1981 TATGGCATTG CTGATTATTT CTATGTTCAT TTGATATAAA AGAGTGTTAG TAGGGGCAGA
2041 ACCCTCAATT GTACATAATA TCAATGATAA AATACAATTC ATTTAACAAT TACCCTCTTA
2101 AGATGTGGTT TCTAGAAATA CAAATTGTCC CTAACTTACA GTTTTCCAAC TTTACAATTG
2161 GGCTGTAACA CCATTTTAAG TTGAGAAGCA CGTGATGGTT TGACTTAAAA CTTTTTGACA
2221 TTATGATGGG TTTTGGGGGT ATTAAGTGCA TTTTGACTTA CAGTATTTTT GACTTATGAA
2281 GAATTTATTG TAAGGCAAGG GGCAGGTATA TGTTTCTAGA AGCACCTAGA AGTGTTAGAC
2341 ACTTTCAATG TAAGAGAAGG ATGAGATAAA CAAGGAAATC ACACCTCCAC CTTGGAGGCT
2401 TATTACAGCT TCATAAACAT ACTCATAAAT ATAAGAAGCA CAAAAGTCAA AAATTCCCTG
2461 TGAACTTGCA ACTTTCACTC TCTTGAAGGT GGGTGGGCCG CTACCACCAA GAATATCTCC
2521 TGAAATAGGG CCTACAATCA TAAATGCACA GGACTATATC CTTGGGTGAT TCTACTCTAA
2581 CACCACATCT CACCTATTTT AGACATGCCA AATGAAACAC TCTTTGTGAA TTTCTGCCGA
2641 GATACAATCT TGGTGTCTCT TTTTTACCCA GATGTGGACC AGCTAGTGAA GTGCTCCCAC
2701 GAGCGCTCCA TTCATCTCTT CATCGACTCT CTGTTGAATG AAGAAAATCC AAGTAAGGCC
2761 TACAGGTGCA GTTCCAAGGA AGCCTTTGAG AAAGGGCTCT GCTTGAGTTG TAGAAAGAAC
2821 CGCTGCAACA ATCTGGGCTA TGAGATCAAT AAAGTCAGAG CCAAAAGAAG CAGCAAAATG
2881 TACCTGAAGA CTCGTTCTCA GATGCCCTAC AAAGGTAGGC TGGAGACTGT TGTAAATAAG
2941 GAAACCAAGG AGTCCTATTT CATCATGCTC ACTGCATCAC ATGTACTGAT TCTGTCCATT
3001 GGAACAGAGA TGATGACTGG TGTTACTAAA CCCTGAGCCC TGGTGTTTCT GTTGATAGGG
3061 GGTTGCATTG ATCCATTTGT CTGAGGCTTC TAATTCCCAT TGTCAGCAAG GTCCCAGTGC
3121 TCAGTGTGGG ATTTGCAGCC TTGCTCGCTG CCCTCCCCTG TAAATGTGGC CATTAGCATG
3181 GGCTAGGCTA TCAGCACAGA GCTCAGAGCT CATTTGGAAC CATCCACCTC GGGTCAACAA
3241 ACTATAACCC TTGTGCCAAA TCCAGCCTAC TTCCTGCTTT TGTAAATAGT TTTTTTAAAA
3301 CTTTTAAGTT CAGGGGTACG TATGTAGGTT TGCTAAAAAG GTAAACTTGT GACATGGGAG
3361 TTTGTTGTCC AGAATATTCC ATCACCCAGG TATTAAGCTT AGTACCCATT AGTTACTTTT
3421 CCTGAAGCTC TCCCTCCTCC CACCCTCTGG GAGGCCCCAG TGTCTGTTGT TCCCCTCTAT
3481 GTGCTCATGC AAAGTTTTAT TAGGACACAG CCACACACAT TCATTACCAT ATTGTCAAAG
3541 GCTGGTTTCA TGCCACCATA ACAGAGTTGA TAGCCCACAG AGCCTAAAAT ATTTACTCCC
3601 TGGCCCTTTA CAGAATGTTC ACAACTTACA TAAAGGCAAG GACCATCTGT CTTATTTATT
3661 TATTTATTTA ATTTGAGATG AAGTCTAGCT TTCTCCTAGG CTGGAGGAGA GGGGCATGAT
3721 CTTGGCTCAC CACAACCTCT GCCTCCCGGG TTCAAATGAT TCCCCTGCCT CAGCCTCCGG
3781 AGTAGCTGGG ATAACAGGCA TGCACCATCA TGCCCAGCTA ATTTTTGTAT TTTTAGTAGA
3841 GAGGGGGTTT CACCGTGTTG ACCAGGCTGG TCTCGAACTG CTGACCTCAG GTGATCTGCC
3901 CTCCTTGGCC TCATCTGTCT TTTTAAATGC AACTATTCCT GGAAGGCAAG AATATCTCAC
3961 ACCTTCTAAG ATACTGCCAT TTTGCCAGGA GTTTGTTTCA CACTTGAATT TCAAGCTTGG
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4021 CCTCTTGTTT AGAGGCAGAC CTAAAGGAAT GGTCGGAAAA TGAGAGAGGA GGTCTTCGGA
4081 TAAATCCGGT GAGAGGGACC AACTTCAGGA AGGGTGGCTT TTGTGGAATC CAGATGGAAA
4141 CCTGAGGGAA GGGATGATAT TAAAGAACAG TGGCCCCAGG TAAAACATAT GGCACCCATG
4201 TGTAAGGTGA TTCTTAGAAT CTGTAGAGGT GTCTTTCGTG GTATAGAGGT TGAGGCACCT
4261 GTGCTTCAAG GAAACCTTAA CTCTTCAAAA TCAGGCAATG CGTATGAGGT AAAGAGAGGA
4321 CTGTGGGACC ATAATCTTGA AGACACAGAC AGGCTTCACT CATCCCTGCC TCCTGCACCA
4381 GTGGGTTCAA GGCTCTGTCA GTGTCCCCTA GGGGCACCTC ACCACTCCCA GCTTCTTCAG
4441 CTCTGGCCTG TCCTGCTGCC TGCAAGGGTT TTGCTTAATT CTCAATTCAA TGTCTCTTCA
4501 TCTTTTAGTA GCTGTGGGGT TTTGTTGTTG TTCTTCTGTT TTTGCTTAGT ATCTGACTAC
4561 TTTTTAATTA TAAAAAGAGA TGTATCTAAA CAAAATAGAG ATTGTTATCA GAAGTTCACA
4621 ACATTTATTA AAAATTTTTT CACCTGGACA AGAGTCTAAA GCAGCATAAA AATATGGTCT
4681 GCTATATTCT AAACCATCAG TCTTAAGAGA TCTGTGTCTC AGCTTAAGAG AAAATACATT
4741 TAATAGACAG TAACACAAAT AAGAAAAAAA TCTGACCAAG GATAGTGGGA TATAGAAGAA
4801 AAAACATTCC AAGAATTATT TTATTTATTT ATTTATTTAT TTATTTATTT ATTTATTTAT
4861 TTTTGAGACA CGGTCTCGCT CAGTTACCCA GGCTGGAGTG CAGCGGCGCA ATCTTAACTC
4921 ACTGCAACCT CTGCTTTCCG GTTCAAGCGA TTCTCCTGCC TCAGCCTCCT GAGTAACTGG
4981 GATTACAGGC ACCCGCCACC ACGCCCAACT AATTTCTGTA TTTTTCTTAG TAGAAACAGG
5041 GTTTCACCAT GTTGGCCAAG CTAGTCTCAA ACTCCTGACC TCAGGTGATT CACCCACCAA
5101 GGCCTCCCAA AGTGCTGGGA TTACAGGCAT GAGCCACCAT GCCTGGCCTC CAAAAACTCT
5161 TTTTTCCTCC ATCATCATGG TTCTATTTTA GTCCTGCTGC CTTTCCTTTT AACCTCTCCC
5221 CAGGCCCATT TGCTCAGGGT TTTTGGTAGA GACCAGAGGA GGGGCAGGGA GGAGATATAG
5281 AAGTTCAACT ACCTGCTTCC AGAGGCTGTC CCTAGTATAG AATACTTTAG GGGCTGGCTT
5341 TACAAGGCAG TCCTTGTGGC CTCACTGATG GCTCAATGAA ATAAGTTCTT TTTTAAAAAA
5401 AATTTTATTT ATTTCCATAG GTTATTGGGG GAACAGGTGG TGTTTGGTTA CATGAGTAAG
5461 TTCTTTAGTA GTGATTTGTG AGATTTTGGT GTGCCCATTA CGGAATGGAA AAATCAACGA
5521 AATAAGTTCT ATGATGCACC TACTAGACAC CTAATCTGCA CTAGATGGTG GGGGAATTAA
5581 GAGCATGGGC ATGATCCTGT GACCGGAAGC CCGCTTACAG TCAGGGTGGA GGACAGACCT
5641 ACTCATGAAA CAAACACAGT GACATATAGT GACACAGAAG CAAATGTCAA ATATGCTTGC
5701 TCCAGATGCT AAGGCACAAG ATGGCCAAGG ATGGCGGAGT TCATGGAGAA AGCATCATGA
5761 GTGTTTTGGC CTTCTGATTT GATCTCCCTA GCACCCCTCA AAGATGGCTA CTTCCTAATG
5821 CTGCTTGGCA ATTCAGACAC ATTTGGGTTT TTCCTATGCA TATAACCACA CTTTTCTGAA
5881 AGGGAGTAGA ATTCAAGGTC TGCATTTTCT AGGTATGAAC ACTGTGCATG ATGAAGTCTT
5941 TCCAAGCCAC ACCAGTGGTT CCATGTGTGT GCACTTCCGG TTTGAGTGCT AGTGAGATAC
6001 TTCTGTGGTT CTGAATTGCC TGACTATTTG GGGTTGTGAT ATTTTCATAA AGATTGATCA
6061 ACATGTTCGA ATTTCCTCCC CAACAGTCTT CCATTACCAA GTAAAGATTC ATTTTTCTGG
6121 GACTGAGAGT GAAACCCATA CCAATCAGGC CTTTGAGATT TCTCTGTATG GCACCGTGGC
6181 CGAGAGTGAG AACATCCCAT TCACTCTGTG AGTAGCACAG GGGGGCGGTC ATCATGGCAC
6241 CAGTCCCTCC CCTGCCATAA CCCTTGGTCT GAGCAGCAGA AGCAGAGAGC GATGCCTAGA
6301 AAACAAGTCT TTAGTTAAAA AAATCAGAAT TTCAAAATTG AGGTCTTTCC TCTATTTGAT
6361 ATTGAGAAAA AAATGCTTCA AATTGGCCAT TTTATTTTCA CTTACTAGTT ATATTTTTTT
6421 ATTTATCATC TTATATCTGT TTATTTCTTT TATAAAGCTG CTGTTAAACA ATATAATTAA
6481 ACTATCTCAA AAGGTTTGAC ATTAAAGAAA ATGAGCAATG GTAACAGGAA ACCACTCTAT
6541 AGATGTACAT ATAATATGTA CAGAAAATAT AAGTAGTAAG AAGTCCATGA CAAAGTGTTA
6601 GCTCTTTTTT TTTTTTTTTT TTTTTTTTTT TTTGAGATGG AGTCTCTCTC CTATTGCCCA
6661 GGCTGGAGTG CAGTGATTCG ATCTCAGCTC ACTGCAACCT CTACCTCCCG AGTTCAAACA
6721 ATTCTTCTGT CTCAGCCTCC CGAGTAGCTG GGGCTGCAGG TGCCCACCAC CATGCCCAGC
6781 TAATTTTTGT ATTTTTAGTA GCGACAGGGT CTCACCATGT TGGCCAAGCT GGTCTTGAAT
6841 TCCTGATCTC AGGTGATCCA CCCGCCTCGG CCTCCCAAAG TGCTGGGATT ACAGGTGTGA
6901 GCCACCATGC CCAGCCTACC CTTTACTACT AATCAAAGAA ATAAAAGTAA GGCAACTTGA
6961 TACTTTTACA ATTACTAGAT GAACAAATCT TTAAAAATAG CCAGTGCAGA CAAGGTGGTG
7021 AAGCAGAACA TGCGAACCTA CCATGCATCA TTCACGGCTA GAACCCTCCA GGTGCGGAAG
7081 GTAGTATTTT AATAACTTTC CATAGCTACA AAATATTATT ACATAGAAGG GAGTGATTTT
7141 TTTCTAATAT TTATCCTAAA GAAATAGTCA ACAAACATTT TTAAAAAACA TCAATTACAG
7201 TCGTACCTAT ACTAGCATAA ATTAGAAACC CAGTATCCAA CATTGAGGCA GTGGGTAAAT
7261 GAATCGTGGT TTATCAAGTC ATTAAAATCA ATCTAGCCTT TAAAAACTAT AATTGTAGGA
7321 AACCCAGGAA AACATAGTAA AAAATGGAAT ATAAAATCTA AAGAGAATAA AGAATAGAGA
7381 ATCGTATGTG TGCTATGATT GTAGCTAAAT AATGTTCAAG TATCAACACA AATTGAAAAG
7441 GAATACATGA AAATGAAAAT TATATTTCTG AATGATTGAC TTCAGGATTT TCTTTTAGAA
7501 TTGTATTAAA TAGTTCATGT CATTAGGATA AATGCTGGAA TGTGGATATA ATTTAAAATA
7561 TACTAAATGC CATCGACCTT CATTTTGAGT TCTTTGTTGG ACATTTTTGT GCATTTTTAA
7621 AATATCCCCT AAATAATAAA GCTATTTATA TTTGGAGAGG AGAAAAAAAA GTGGGGGGCA
7681 GGGAGAGCTG ATCTCTATAA CTAACCAAAT TTATTGCTTT TTTGTTTAGG CCTGAAGTTT
7741 CCACAAATAA GACATACTCC TTCCTAATTT ACACAGAGGT AGATATTGGA GAACTACTCA
7801 TGTTGAAGCT CAAATGGAAG AGTGATTCAT ACTTTAGCTG GTCAGACTGG TGGAGCAGTC
7861 CCGGCTTCGC CATTCAGAAG ATCAGAGTAA AAGCAGGAGA GACTCAGAAA AAGTAATTAA
7921 ATGTATTTTT CTTCCTTCAC TTTAGACCCC CACCTGATGT CAGGACCTAG GGGCTGTATT
7981 TCAGGGGCCT TCACAATTCA GGGAGAGCTT TAGGAAACCT TGTATTTATT ACTGTATGAT
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8041 GTAGATTTTC TTTAGGAGTC TTCTTTTATT TTCTTATTTT TGGGGGGCGG GGGGGGAAGT
8101 GACAGTATTT TTGTATTTCA TGTAAGGAAA ACATAAGCCC TGAATCGCTC ACAGTTATTC
8161 AGTGAGAGCT GGGATTAGAA GTCAGGAATC TCAGCTTCTC ATTTGGCACT GTTTCTTGTA
8221 AGTACAAAAT AGTTAGGGAA CAAACCTCCG AGATGCTACC TGGATAATCA AAGATTCAAA
8281 CCAACCTCTT CAAGAAGGGT GAGATTCCAA GATAATCTCA ACCTGTCTCC GCAGCCCCAC
8341 CCATGTGTAC CCATAAAATG AATTACACAG AGATCGCTAT AGGATTTAAA GCTTTTATAC
8401 TAAATGTGCT GGGATTTTGC AAACTATAGT GTGCTGTTAT TGTTAATTTA AAAAAACTCT
8461 AAGTTAGGAT TGACAAATTA TTTCTCTTTA GTCATTTGCT TGTATCACCA AAGAAGCAAA
8521 CAAACAAACA AAAAAAAAAA GAAAAAGATC TTGGGGATGG AAATGTTATA AAGAATCTTT
8581 TTTACACTAG CAATGTCTAG CTGAAGGCAG ATGCCCTAAT TCCTTAATGC AGATGCTAAG
8641 AGATGGCAGA GTTGATCTTT TATCATCTCT TGGTGAAAGC CCAGTAACAT AAGACTGCTC
8701 TAGGCTGTCT GCATGCCTGT CTATCTAAAT TAACTAGCTT GGTTGCTGAA CACCGGGTTA
8761 GGCTCTCAAA TTACCCTCTG ATTCTGATGT GGCCTGAGTG TGACAGTTAA TTATTGGGAA
8821 TATCAAAACA ATTACCCAGC ATGATCATGT ATTATTTAAA CAGTCCTGAC AGAACTGTAC
8881 CTTTGTGAAC AGTGCTTTTG ATTGTTCTAC ATGGCATATT CACATCCATT TTCTTCCACA
8941 GGGTGATCTT CTGTTCTAGG GAGAAAGTGT CTCATTTGCA GAAAGGAAAG GCACCTGCGG
9001 TATTTGTGAA ATGCCATGAC AAGTCTCTGA ATAAGAAGTC AGGCTGGTGA GCATTCTGGG
9061 CTAAAGCTGA CTGGGCATCC TGAGCTTGCA CCCTAAGGGA GGCAGCTTCA TGCATTCCTC
9121 TTCACCCCAT CACCAGCAGC TTGCCCTGAC TCATGTGATC AAAGCATTCA ATCAGTCTTT
9181 CTTAGTCCTT CTGCATATGT ATCAAATGGG TCTGTTGCTT TATGCAATAC TTCCTCTTTT
9241 TTTCTTTCTC CTCTTGTTTC TCCCAGCCCG GACCTTCAAC CCAGGCACAC ATTTTAGGTT
9301 TTATTTTACT CCTTGAACTA CCCCTGAATC TTCACTTCTC CTTTTTTCTC TACTGCGTCT
9361 CTGCTGACTT TGCAGATGCC ATCTGCAGAG CATGTAACAC AAGTTTAGTA GTTGCCGTTC
9421 TGGCTGTGGG TGCAGCTCTT CCCAGGATGT ATTCAGGGAA GTAAAAAGAT CTCACTGCAT
9481 CACCTGCAGC CACATAGTTC TTGATTCTCC AAGTGCCAGC ATACTCCGGG ACACACAGCC
9541 AACAGGGCTG CCCCAAGCAC CCATCTCAAA ACCCTCAAAG CTGCCAAGCA AACAGAATGA
9601 GAGTTATAGG AAACTGTTCT CTCTTCTATC TCCAAACAAC TCTGTGCCTC TTTCCTACCT
9661 GACCTTTAGG GCTAATCCAT GTGGCAGCTG TTAGCTGCAT CTTTCCAGAG CGTCAGTACT
9721 GAGAGGACAC TAAG//SEQ ID NO:25
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8393 is the *Hind*III variant and 9040 is the Ser447Stop variant. 4872, 5168, and 5441 are in intron 6; 6863 and 7315 are in intron 7; 8292 and 8852 are in intron 8; 9712 is in intron 9; these markers were selected because they spanned a region of the LPL gene downstream of a recombination hotspot and had a minor allele frequency of 15% or greater in MDECODE.12 PCR amplification followed by restriction digest with HindIII was used to genotype the polymorphism at 8393. A single nucleotide primer extension method was used to genotype the remaining nine SNPs (4872, 5168, 5441, 6863, 7315, 8292, 8852, 9040, 9712). Analysis of these initial data showed that a restricted set of six SNPs encompassed all the major 3' end haplotypes.

Large-scale genotyping of these six SNPs in 514 subjects from 85 MACAD families and 629 subjects from 157 non-Hispanic Caucasian families was performed using the 5'-exonuclease (Taqman™ MGB) assay (10). PCR primer and oligonucleotide probe sequences are listed in Table 2 below.

Table 2. Primers and probe sequences used in 5'-exonuclease assay.

Variant	PCR primers	Taqman MGB probes
7315	Forward 5'-TCAAGTCATTAAAATCAATCTAGCCTTT-3'//SEQ ID NO:1; Reverse 5'-TTCTCTTTAGATTTTATATTCCATTTTTTACTATG-3' //SEQ ID NO:2	5'-CCTGGGTTTCCTAcAAT-3'//SEQ ID NO:13; 5'-CCTGGGTTTCCTAgAAT-3'//SEQ ID NO:14
8292	Forward 5'-CCTGGATAATCAAAGATTCAAACCA-3'//SEQ ID NO:3; Reverse 5'-GGAGACAGGTTGAGATTATCTTGGA-3'//SEQ ID NO:4	5'-CTCACCCTTCTtGAAGA-3'//SEQ ID NO:15; 5'-TCACCCTTCTgGAAGA-3'//SEQ ID NO:16
8393	Forward 5'-CATAAAATGAATTACACAGAGATCGCTAT-3'//SEQ ID NO:5; Reverse 5'-TCAATCCTAACTTAGAGTTTTTTTAAATTAACA-3'//SEQ ID NO:6	5'-CACATTTAGTATAAAaGC-3'//SEQ ID NO:17; 5'-CACATTTAGTATAAAcGC-3'//SEQ ID NO:18
8852	Forward 5'-GTGGCCTGAGTGTGACAGTTAATT-3'//SEQ ID NO:7; Reverse 5'-ATCAAAAGCACTGTTCACAAAGGTA-3'//SEQ ID NO:8	5'-AGCATGATCATGTAtTAT-3'//SEQ ID NO:19; 5'-CAGCATGATCATGTAgTAT-3'//SEQ ID NO:20
9040	Forward 5'-TTGTGAAATGCCATGACAAGTCT-3'//SEQ ID NO:9; Reverse 5'-CCAGTCAGCTTTAGCCCAGAA-3'//SEQ ID NO:10	5'-CCAGCCTgACTTC-3'//SEQ ID NO:21; 5'-ACCAGCCTcACTTC-3'//SEQ ID NO:22
9712	Forward 5'-TCCATGTGGCAGCTGTTAGC-3'//SEQ ID NO:11; Reverse 5'-GAGTAGTGAAGGTCACATGCTTAGTGT-3'//SEQ ID NO:12	5'-CCAGAGCgTCAGTAC-3'//SEQ ID NO:23; 5'-CCAGAGCaTCAGTAC-3'//SEQ ID NO:24

In this assay, allele-specific oligonucleotide probes are labeled with different fluorophores (FAM or VIC) at their 5'-ends and with a quencher molecule at the 3'-end. The quencher interacts with the fluorophores by fluorescence resonance energy transfer, quenching their fluorescence. These probes are included in the PCR reaction mixture amplifying a 100-150 base pair segment with the polymorphism at the center. During annealing, the probes hybridize to the PCR products, and during extension, the 5'-3' exonuclease activity of the DNA polymerase degrades perfectly matched annealed probes, separating the fluorophore from the quencher. Imperfectly matched probes are displaced into solution without degradation. Comparison of relative fluorescence from each fluorophore allows determination of genotype.

Data Analysis. Based on pedigree structures and genotype data of all individuals in each pedigree, haplotypes were reconstructed as the most likely set (determined by the maximum likelihood method) of fully-determined parental haplotypes of the marker loci for each individual in the pedigree, using the simulated annealing algorithm implemented in the program Simwalk2. (19) All comparisons between groups of subjects comprised comparisons of unrelated founders, and only founder chromosome data are presented in the tables. Founder haplotypes, i.e. those haplotypes from parents and individuals marrying into the family, were used to calculate haplotype frequencies in 482 chromosomes from 241 Mexican-American founders and in 582 chromosomes from 291 non-Hispanic Caucasian founders. Six Mexican-American and 21 non-Hispanic Caucasian founders were excluded from analysis because their haplotypes could not be unambiguously determined. The χ^2 test was used to compare allele and haplotype frequencies between the Mexican-Americans without coronary artery disease and the non-Hispanic Caucasians.

A case-control association study of coronary artery disease was performed by comparing haplotype frequencies between Mexican-American founders with and those without coronary artery disease. The cases were 77 probands (154 chromosomes) with coronary artery disease; the controls (164 individuals, 328 chromosomes) were their spouses plus the spouses marrying into the offspring generation. Because the cases and controls were genetically unrelated, their allele and haplotype frequencies and gender distribution were compared using the χ^2 test. Student's T test was used to compare the mean age of the cases versus the controls. Odds ratios for coronary artery disease by haplogenotype were calculated, using logistic regression analysis to adjust for any confounding effects of age or sex in the case-control comparison. Analyses were performed using SAS System software (20).

Results

In a pilot study, the haplotypes of 28 unique chromosomes were derived using Mexican-American family data and are shown in Table 3 (below) in order of frequency. These results were used to select the markers genotyped in the large population samples. As seen in Table 3, markers 7315, 8292, 8393, 8852, and 9040 are sufficient to distinguish the haplotypes from each other. In addition to these five SNPs, 9712 was also chosen because it is predicted to distinguish two major ancient clades according to the haplotype tree constructed by Templeton, *et al.* (6) in the Molecular Diversity and Epidemiology of Common Disease (MDECODE) project. The results reported herein are consistent with

their study of the haplotype structure of 9.7 kb of the LPL gene that described four ancient cladistic groups. Markers 7315, 8393, and 9712 are useful to distinguish all four of the ancient 3' LPL clades.

Table 3. Pilot study *LPL* haplotypes.

Haplotype	4872	5168	5441	6863	**7315**	**8292**	**8393H**	**8852**	**9040**	**9712**	Count	Frequency
1	A	T	T	C	**G**	**A**	**T**	**T**	**C**	**G**	13	46.4%
2	G	C	T	C	**G**	**A**	**G**	**G**	**G**	**G**	4	14.3%
3	A	T	T	C	**G**	**A**	**G**	**G**	**C**	**A**	3	10.7%
4	A	T	T	C	**G**	**C**	**T**	**T**	**C**	**G**	3	10.7%
5	G	C	T	T	**C**	**A**	**G**	**G**	**C**	**A**	3	10.7%
6	G	T	T	C	**C**	**A**	**T**	**T**	**C**	**G**	1	3.6%
7	A	T	T	C	**G**	**A**	**T**	**G**	**C**	**G**	1	3.6%

In the second stage, the six selected markers were then genotyped in 514 Mexican-American subjects from 85 families and 629 subjects from 157 non-Hispanic Caucasian families. The allele frequencies are shown in Table 4 (below). The markers from Mexican-Americans without coronary artery disease are presented in Table 4 in order to eliminate any disease-based ascertainment bias in delineating the ethnic comparison.

Table 4. *LPL* SNP allele frequencies in Mexican-Americans and non-Hispanic Caucasians.

Position	Variant	Mexican-American without CAD (328 chromosomes)	Non-Hispanic Caucasian (582 chromosomes)	P value
7315	G → C	0.89	0.85	0.08
8292	A → C	0.85	0.79	0.03
8393	T → G	0.80	0.71	0.003
8852	T → G	0.78	0.70	0.01
9040	C → G	0.93	0.90	0.10
9712	G → A	0.88	0.81	0.02

Of note, while 9040 (Ser447Stop) was extremely rare in the previous MDECODE study subjects (not detected in African Americans or Finns and found with a frequency of 4% in U.S. non-Hispanic Caucasians), in this study it was found with a frequency of 7% in Mexican Americans and 9% in our non-Hispanic Caucasians. Comparing Mexican-Americans to non-Hispanic Caucasians, the allele frequencies were significantly different for four out of the six variants (Table 4).

The founder haplotype frequencies from the Mexican-Americans without coronary artery disease (as determined by EKG or by hospital records of, e.g., angioplasty, coronary artery bypass graft surgery, or angiography) were compared with those of the non-Hispanic Caucasians. The six most common haplotypes, comprising over 99% of the observed haplotypes for each group, are presented in Table 5 (below). Both groups shared haplotype 1 as the most common haplotype. There were several differences between the two groups in regards to the other haplotypes. Haplotypes 2, 3, 4, and 5 were more common in the non-Hispanic Caucasian population; haplotypes 1 and 6 were more common in the Mexican-Americans. These differences reached statistical significance for the three most frequent haplotypes.

In the case-control study, Mexican-American probands with coronary artery disease were compared with their spouses and the spouses of their offspring, none of whom had coronary artery disease. Thus, these case and control individuals were all genetically unrelated. The mean age of the cases was 62.2 years; that of the controls was 42.6 years ($P<0.0001$). This age difference was expected, given that the control group was comprised of individuals from both the parental and offspring generations. The sex distribution was similar between the groups, with males comprising 44% of the cases and 38% of the controls ($\chi^2=0.9$, $P=0.35$).

Table 5. *LPL* haplotype frequencies in Mexican-Americans compared to non-Hispanic Caucasians.

Haplotype	7315	8292	8393	8852	9040	9712	Mexican	Freq	Caucasian	Freq	P value
1.	G	A	T	T	C	G	206	62.8%	284	48.8%	<0.0001
2.	G	C	T	T	C	G	50	15.2%	123	21.1%	0.03
3.	C	A	G	G	C	A	33	10.1%	85	14.6%	0.05
4.	G	A	G	G	G	G	22	6.7%	58	10.0%	0.10
5.	G	A	G	G	C	A	8	2.4%	24	4.1%	0.19
6.	G	A	T	G	C	G	6	1.8%	5	0.9%	0.20
							325	99.1%	579	99.5%	

The genotype frequencies for all six markers were in Hardy-Weinberg equilibrium for both the cases and the controls. Allele frequencies of the six SNPs did not differ significantly among the Mexican-Americans according to coronary artery disease status (Table 6). A comparison of genotype frequencies showed no differences between cases

and controls, except for a modestly significant difference for the 8393 (*Hind*III) variant (P=0.05). However, comparison of the common haplotype frequencies between the Mexican-Americans with and without coronary artery disease revealed a significant decrease in the frequency of the most common haplotype in those with disease (Table 7 below). This implies an increase in frequency of less common haplotypes among cases, the detection of which was hindered by the available sample size. Haplotype 1 was associated with a significantly decreased risk of coronary artery disease (P=0.03). Of the less common haplotypes, haplotype 4 was most prominently associated with the greatest risk of coronary artery disease (P=0.10), though this result did not attain statistical significance with the given sample size. A comparison of subjects homozygous for haplotype 1 with subjects with all other genotypes is presented in Table 8 (below). Homozygosity for haplotype 1 was associated with protection against coronary artery disease with an odds ratio of 0.50 (95% CI 0.27-0.91). Use of the logistic regression model to adjust for age and sex, separately and in combination (Table 7), did not alter the significance of this association (odds ratio estimates from 0.39 to 0.51). None of the haplotypes other than haplotype 1 showed a statistically significant association with coronary artery disease (data not shown).

Table 6. *LPL* SNP allele and genotype frequencies in Mexican-Americans with and without CAD.

SNP		Frequency of major allele	P value[a]	Major allele[b] homozygote	Heterozygote	Minor allele[b] homozygote	P value[c]
7315							
	Cases	0.89	0.46	57	20	0	0.31
	Controls	0.87		131	31	2	
8292							
	Cases	0.85	0.41	52	22	3	0.48
	Controls	0.82		118	42	4	
8393							
	Cases	0.80	0.06	39	33	5	0.05
	Controls	0.72		105	52	7	
8852							
	Cases	0.78	0.08	38	33	6	0.09
	Controls	0.71		100	56	8	
9040							
	Cases	0.93	0.10	61	15	1	0.14
	Controls	0.89		142	22	0	
9712							
	Cases	0.88	0.27	54	21	2	0.37
	Controls	0.84		124	39	1	

[a]For the comparison of allele frequency between cases and controls: χ^2 (1 d.f.)
[b]Major and minor alleles are listed in Table 4.

[c]Major allele homozygotes versus heterozygotes plus minor allele homozygotes, comparing cases and controls: χ^2 (1 d.f.)

Table 7. *LPL* haplotype frequencies in Mexican-Americans with and without coronary artery disease.

Haplotype	7315	8292	8393	8852	9040	9712	CAD	Freq	No CAD	Freq	P value
1.	G	A	T	T	C	G	81	52.6%	206	62.8%	0.03
2.	G	C	T	T	C	G	28	18.2%	50	15.2%	0.42
3.	C	A	G	G	C	A	20	13.0%	33	10.1%	0.34
4.	G	A	G	G	G	G	17	11.0%	22	6.7%	0.10
5.	G	A	G	G	C	A	5	3.3%	8	2.4%	0.61
6.	G	A	T	G	C	G	2	1.3%	6	1.8%	0.67
							153	99.4%	325	99.1%	

Table 8. Logistic regression analysis comparing haplotype 1 homozygotes with all other haplogenotypes.

Adjustment	Odds Ratio	95% CI	P value
None	0.50	0.27-0.91	0.02
Sex	0.51	0.28-0.93	0.03
Age	0.41	0.18-0.93	0.03
Sex and Age	0.39	0.17-0.89	0.03

In comparing two different ethnic groups, we found several differences in the allele and haplotype frequencies observed in the 3' LPL markers. Such differences may affect results of association studies conducted in different populations. In particular, different alleles of *Hind*III occurred at different frequencies, which may account for disparate results of association studies conducted in different populations. For example, a study of postmenopausal Caucasian women found no association of the *Hind*III variant with glucose or insulin levels, while a study in Chinese men with coronary heart disease found an association of *Hind*III with steady state plasma glucose levels, a marker of insulin resistance (3,8).

The haplotypes described here can be very useful in future studies exploring the association of the LPL gene with components of the cardiovascular dysmetabolic

syndrome. This is illustrated here, in that haplotype frequencies were different according to coronary artery disease status. Only one out of six single polymorphic sites was associated with coronary artery disease. This demonstrates that the common approach of examining one or two polymorphisms per candidate gene may fail to detect phenotypic associations. Compared to single-variant analysis, haplotype-based analysis reduces the potential for false negatives in association studies. The benefit of a haplotype-based analysis is that it captures all of the variation across a region, which should, as it did in our study, improve the ability to detect an association. This study thus demonstrates the improved power of haplotyping in eludicating disease gene associations and the importance of ethnic specific haplotype data.

Example 2: Haplotype Analysis of the Association of the Lipoprotein Lipase Gene with Insulin Sensitivity

Lipoprotein lipase (*LPL*) is a candidate gene implicated in features of the cardiovascular dysmetabolic syndrome, atherosclerosis and components of the insulin-resistance syndrome, i.e., hypertension, lipid levels, and fasting insulin.

The aim of this study was to evaluate the relationship between the *LPL* gene and direct measurement of insulin sensitivity in Mexican American families ascertained through patients with CAD, a population and disorder with a high frequency of insulin resistance. Insulin senstivity was evaluated by assessment of the glucose infusion rate (GINF) during a euglycemic hyperinsulinemic clamp study, which is widely regarded as the most direct physiologic measurement of insulin sensitivity (38, 39).

Mexican-American nuclear families were ascertained via a parent with documented CAD in the Los Angeles area. A total of 91 adult offspring underwent euglycemic clamp to determine peripheral glucose disposal. Insulin sensitivity (S_I) was calculated from the glucose infusion rate (GINF) and increment in plasma insulin over basal for each offspring. Both parents and offspring were genotyped for eight polymorphic markers spanning a distance of 6.9 cM at or near the LPL gene on chromosome 8 (D8S261, LPL3, *Hind*III, *Pvu*II, LPL5, D8S258, D8S282, D8S136).

Linkage analysis was conducted using linear regression method as implemented in the SIBPAL program of the SAGE package. Association between HindIII polymorphic markers and S_I was evaluated by comparing the maximum likelihood of the two models

incorporating familial correlation (with or without the marker) as implemented in the ASSOC program.

Results: Multiple markers at or near the LPL gene showed significant evidence of linkage ($0.003p0.05$) to S_I. Furthermore, a significant association between allele 2 of HindIII polymorphism within the LPL gene itself and insulin sensitivity (S_I) was also observed ($p=0.008$).

This shows a linkage of markers near and within LPL to insulin resistance in a family study of Mexican-Americans ascertained by probands with coronary artery disease, and also shows association of the HindIII polymorphism with a direct measurement of insulin sensitivity (S_I, calculated from euglycemic clamp study). HindIII allele 2 is associated with decreased S_I. Thus, in Mexican American families ascertained through CAD probands, we have for the first time shown that the LPL gene is both linked and associated with a direct measure of insulin resistance. This observation provides the most direct evidence as to the importance of the LPL gene in the insulin resistance syndrome and provides a pathophysiologic mechanism for its relation to the development of CAD.

In a further study described hereinbelow our goal was to identify specific haplotypes (groups of alleles on the same chromosome) associated with insulin sensitivity in an expanded family sample undergoing glucose clamps.

Example 3: Evidence of linkage and association between LPL and insulin sensitivity/resistance in Mexican-American hypertension families

We have shown hereinabove that blood pressure (BP) and insulin sensitivity/resistance (IR) cosegregate in Mexican-American families and that there most likely are gene(s) contributing to both BP and IR. Previous studies have shown evidence of linkage and/or association of the HindIII polymorphism in the LPL gene with IR, as well as IR-associated hypertension, dyslipidemia, and atherosclerosis. However, in most cases insulin sensitivity was assessed by indirect methods. To further examine the role of the LPL gene in IR, we genotyped six (7315, 8292, 8393, 8852, 9040, 9712) LPL 3' end single nucleotide polymorphisms (SNPs) in 390 members of 77 Hispanic families ascertained via hypertensive probands. Insulin sensitivity/resistance was directly assessed via hyperinsulinemic euglycemic glucose clamps. Multipoint linkage analyses were performed

using SIBPAL2. Association between the six SNPs, LPL haplotypes and IR-related traits were evaluated using the QTDT program.

Materials and Methods.

Subjects. The UCLA/Cedars-Sinai Mexican-American Coronary Artery Disease (MACAD) Project enrolls families ascertained through a proband with coronary artery disease, determined by evidence of myocardial infarction on electrocardiogram or hospital record, evidence of atherosclerosis on coronary angiography, or history of coronary artery bypass graft or angioplasty (30). DNA was obtained from all available family members, and the adult offspring (age 18 or older) of the proband and the spouses of those offspring were also asked to undergo a series of tests to characterize their metabolic and cardiovascular phenotype.

Genotyping. In a study described hereinabove, we determined a set of six SNPs that are sufficient to identify the most common haplotypes occurring in the 3' end of the *LPL* gene (30). These are 7315, 8292, 8393, 8852, 9040, and 9712. The numbering of the SNPs corresponds to Genbank accession number AF050163, which describes a 9.7 kb segment of the LPL gene originally sequenced in the Molecular Diversity and Epidemiology of Common Disease (MDECODE) project, a study of Finns, non-Hispanic Caucasian Americans, and African-American subjects (9). 8393 is the HindIII variant located in intron 8 and 9040 is the Ser447Stop variant located in exon 9. 7315 is in intron 7; 8292 and 8852 are in intron 8; 9712 is in intron 9.

Large-scale genotyping of the six SNPs in MACAD families was performed using the 5'-exonuclease (Taqman™ MGB) assay (10). PCR primer and oligonucleotide probe sequences are listed in Table 2 (Goodarzi *et al.*; 30). In this assay, allele-specific oligonucleotide probes were labeled with different fluorophores (FAM or VIC) at their 5'-ends and with a quencher molecule at the 3'-end. The quencher interacts with the fluorophores by fluorescence resonance energy transfer, quenching their fluorescence. These probes are included in the PCR reaction mixture amplifying a 100-150 base pair segment with the polymorphism at the center. During annealing, the probes hybridize to the PCR products, and during extension, the 5'-3' exonuclease activity of the DNA polymerase degrades perfectly matched annealed probes, separating the fluorophore from the quencher. Imperfectly matched probes are displaced into solution without degradation.

Comparison of relative fluorescence from each fluorophore allows determination of genotype.

LPL markers were genotyped in 514 individuals from 85 MACAD families. Of these, 29 individual genotypes were discarded because their genotypes were incompatible with their family pedigree, as detected by the program Pedcheck (40). This left 485 individuals genotyped at *LPL*. The genotype frequencies for all six markers were in Hardy-Weinberg equilibrium.

Phenotyping. The adult offspring of the proband and the spouses of the offspring underwent a three-day phenotyping protocol, which includes indices of insulin resistance determined by euglycemic clamp study, lipid parameters, and carotid intima-media thickness. Of the 485 subjects genotyped at *LPL*, 125 were from the parental generation that does not undergo phenotyping, and 69 from the offspring generation were not clamped. Thus, 291 subjects from 74 families were both clamped and genotyped for the *LPL* markers.

Several indices of insulin sensitivity are obtained in the MACAD study. Fasting insulin and glucose, themselves simple surrogate measures of insulin sensitivity, allow calculation of the homeostasis model assessment index (HOMA). Using glucose in mmol/L and insulin in μIU/mL, the HOMA index is (glucose x insulin)/22.5. An ideal, normal-weight person aged < 35 years has a HOMA of 1 (41).

During the hyperinsulinemic euglycemic clamp (38), human insulin (Novolin, Clayton, NC; 60 mU/m^2/min) was infused for 120 minutes at a constant rate to achieve a plasma insulin concentration of 100 μIU/mL or greater. Blood was sampled every 5 minutes, and the rate of 20% dextrose co-infused was adjusted to maintain plasma glucose concentrations at 95 to 100 mg/dL. The glucose infusion rate (GINF, given in mg/min) over the last 30 minutes of steady-state insulin and glucose concentrations reflects glucose uptake by all tissues of the body (primarily insulin-mediated glucose uptake in muscle) and is therefore a direct physiologic measurement of tissue insulin sensitivity. GINF is also often reported divided by body weight, resulting in a trait termed the M value (mg/kg/min; 38).

Data Analysis. Based on the pedigree structures and genotype data of all individuals in each pedigree, haplotypes were reconstructed as the most likely set (determined by the maximum likelihood method) of fully-determined parental haplotypes

of the marker loci for each individual in the pedigree, using the simulated annealing algorithm implemented in the program Simwalk2 (19). Using this method we were able to assign haplotypes to 475 of the 485 genotyped subjects, including 285 of the 291 genotyped and clamped subjects. Founder haplotypes, i.e. those haplotypes from parents and individuals marrying into the families, were used to calculate haplotype frequencies in 482 chromosomes from 241 Mexican-American founders (125 parents, 116 spouses of offspring). The frequencies of the most common haplotypes among 328 chromosomes of the 164 founders (48 parents, 116 spouses) without coronary artery disease are displayed in Table 9 along with the major allele frequencies of the six SNPs. The markers from Mexican-Americans without coronary artery disease are presented in Table 9 in order to eliminate any disease-based ascertainment bias.

Table 9. *LPL* single marker and haplotype frequencies in Mexican-Americans.

SNPs and major allele frequencies:	7315 G→C 0.89	8292 A→C 0.85	8393 T→G 0.80	8852 T→G 0.78	9040 C→G 0.93	9712 G→A 0.88	Subjects	Freq
Haplotype 1	G	A	T	T	C	G	206	62.8%
Haplotype 2	G	C	T	T	C	G	50	15.2%
Haplotype 3	C	A	G	G	C	A	33	10.1%
Haplotype 4	G	A	G	G	G	G	22	6.7%
Haplotype 5	G	A	G	G	C	A	8	2.4%
Haplotype 6	G	A	T	G	C	G	6	1.8%

Log-transformed (anthropometric measurements, fasting glucose, fasting insulin) or square-root-transformed (HOMA, GINF, M) trait values were used to reduce skewness for all statistical analyses. Unpaired, two-sided T tests were used to compare trait values between men and women.

Linkage was assessed using sib pair analysis (42). The basic idea of this approach is that if a locus influences the quantitative trait or phenotype under study, then siblings that share more alleles at that locus will be more similar in phenotype than siblings that share fewer alleles. Conceptually, this procedure first plots the square of the difference in the quantitative trait between each sibpair versus the number of alleles shared, and then uses linear regression to estimate how much of the difference in the trait depends on the number of alleles shared. A significant linkage is shown by a negative regression

coefficient. If there is no linkage, the regression coefficient is expected to be zero. We used the SIBPAL2 program in SAGE 4.2 (43) to implement a sib pair analysis that uses the mean-corrected cross-product instead of the squared difference of the sibs trait values as the dependent variable; this revised method has more power and accommodates multiple sibs in a family (44).

Association was evaluated by quantitative transmission disequilibrium testing for both individual polymorphisms and haplotypes using the QTDT program (45). The transmission disequilibrium test was first developed for dichotomous traits in which alleles transmitted and not transmitted from the parents to affected offspring are compared to determine whether one allele is associated with the disease in question (46). This was later extended to quantitative traits (47). Abecasis developed a general approach for scoring allelic transmission that accommodates families of any size and uses all available genotypic information (45). Family data allows for the construction of an expected genotype for every non-founder, and orthogonal deviates from this expectation are a measure of allelic transmission. The QTDT program implements this general transmission disequilibrium testing using the orthogonal model of Abecasis (48). Age, gender, and body mass index were specified as covariates. Environmental variance, polygenic variance, and additive major locus were specified in the variance model. In all cases of a positive association result, the population stratification model was also executed to confirm the absence of significant population stratification.

Results

The clinical characteristics of the 291 subjects (112 men, 179 women) who had quantitative assessment of insulin resistance are shown in Table 10 below. This is an adult group of Mexican-Americans of mean age 35.3 years. On average, these individuals are overweight. This may account for the degree of insulin resistance observed; however, it is known that Mexican-Americans have a predisposition to visceral adiposity, hyperinsulinemia, and insulin resistance (49, 50). The mean HOMA level suggests that these people are on average almost four times more insulin resistant than normal. The men had statistically significant higher weight ($P<0.0001$) and fasting glucose ($P=0.0023$) levels, while the women had significantly lower GINF ($P=0.0001$) but not M values.

Table 10. Clinical characteristics of 291 genotyped and clamped individuals.

	Mean	SD	Range
Age (yr)			
Men (n=112)	35	9.4	19-60
Women (n=179)	35.5	8.2	18-58
Weight (kg)*			
Men	84.2	15.6	52.5-126.6
Women	72.1	14.0	38.6-128.5
Body mass index (kg/m^2)			
Men	28.9	4.8	17.8-45.4
Women	29.1	5.5	18.1-54.8
Fasting glucose (mg/dL)*			
Men	96.1	9.8	74.0-118.0
Women	92.5	9.4	56.0-117.0
Fasting insulin (μIU/mL)			
Men	15.4	8.9	5.0-62.0
Women	15.5	7.5	2.0-49.0
HOMA (μIU/mL x mmol/L)			
Men	3.7	2.4	1.2-15.9
Women	3.6	1.9	0.5-14.0
GINF (mg/min)*			
Men	428.6	196.8	105.9-1031.5
Women	343.5	147.5	20.7-1010.5
M (mg/kg/min)			
Men	5.4	2.8	1.0-13.9
Women	5.0	2.4	0.2-14.9

*P<0.005 comparing men versus women

Linkage results are shown in Table 11. Of the several indices of insulin sensitivity, linkage was demonstrated only for the direct quantification represented by GINF. The M

value, a clamp-derived index equal to GINF/body weight, was not significantly linked to *LPL* haplotypes.

Table 11. Linkage results for measurements of insulin sensitivity and LPL haplotypes

Phenotype	P value (from SIBPAL)
Fasting glucose	0.57
Fasting insulin	0.44
HOMA	0.34
GINF	0.034
M	0.32

Association was evaluated by quantitative transmission disequilibrium testing. Positive association results for particular haplotypes are shown in Table 12 (below). No haplotype was significantly associated with fasting glucose, fasting insulin, or HOMA, but both haplotypes 1 and 4 were significantly associated with both GINF and the M value. To characterize the nature of the associations of haplotypes 1 and 4 with insulin resistance, we determined the mean levels of insulin sensitivity in carriers of these haplotypes (Table 12 and Figure 2). We observed that haplotype 1 is associated with the most favorable mean insulin sensitivity, while carriers of haplotype 4 had the lowest insulin sensitivity (i.e. the greatest insulin resistance). For fasting insulin, HOMA, GINF, and M, mean insulin sensitivity progressively worsened going from haplotype 1 homozygotes to haplotype 1 heterozygotes to individuals without haplotype 1. Conversely, haplotype 4 heterozygotes were more insulin resistant than those without haplotype 4 (no haplotype 4 homozygotes were observed among the clamped subjects). Figure 3 further explores these associations by examining the effects of haplotypes 1 and 4 on insulin sensitivity independently. Exclusion of subjects with haplotype 4 from haplotype 1 heterozygotes and those without haplotype 1 did not affect the trend of benefit on insulin sensitivity seen with increasing numbers of haplotype 1. Similarly, excluding haplotype 1 carriers from those with and without haplotype 4 did not affect the trend of lower insulin sensitivity in the latter subjects; in fact, the subjects without haplotype 1 who were carriers of haplotype 4 had the lowest insulin sensitivity (most insulin resistance) compared to the other haplogenotype groups. Similar trends were observed with M value.

Table 12. LPL haplotype association results for indices of insulin sensitivity.

Phenotype	Haplotype	P value for association (from QTDT)	Mean trait value for haplotype carriers
GINF	1	0.031	383.0 mg/min
	4	0.007	344.3 mg/min
M	1	0.031	5.3 mg/kg/min
	4	0.005	4.6 mg/kg/min

It is believed that the study described hereinabove is the first that has used insulin sensitivity assessed by the euglycemic clamp as the phenotype in an association study with *LPL*. Two *LPL* haplotypes were associated with variation in GINF. These haplotypes had opposite effects on insulin sensitivity. Haplotype 1, the most common haplotype, was associated with improved insulin sensitivity. As the number of chromosomes in an individual with haplotype 1 decreased (from two, to one, to none), insulin sensitivity by GINF, as well as HOMA and fasting insulin, decreased progressively. Furthermore, haplotype 4 carriers had the lowest insulin sensitivity, i.e. they were the most insulin resistant. The direction of these associations persisted when the haplotypes were considered separately. With the available data we cannot determine whether there is an insulin-sensitizing functional variant on haplotype 1 chromosomes and/or a variant on haplotype 4-bearing chromosomes that promotes insulin resistance. However, in terms of the relation to cardiovascular risk associated with the metabolic syndrome, our previous work has shown that haplotype 1 is associated with protection against coronary artery disease and haplotype 4 may be associated with increased risk of coronary artery disease (see Example 1 hereinabove).

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CLAIMS

1. A method for determining haplotypes, useful for large-scale genetic analysis for a human subpopulation, within a genomic reference sequence of interest, comprising:

detecting the presence of a plurality of genetic markers at positions of the genomic reference sequence, in the genotypes of a first number of subjects in the human subpopulation;

identifying a frequency hierarchy of the detected markers;

constructing from the frequency hierarchy a set of haplotypes, each haplotype of the set comprising at least one of the most frequently detected markers;

selecting a smaller subset of the set of haplotypes, the smaller subset comprising those haplotypes most frequently occurring in the first number of subjects; and

identifying the markers needed to define the thus selected smaller subset of the set of haplotypes.

2. The method of claim 1, further comprising:

genotyping a second number of subjects in the human subpopulation for the identified markers, the second number of subjects being larger than the first number of subjects; and

evaluating the genotypes of the second number of subjects for any statistically significant association of any members of the thus selected smaller subset of the set of haplotypes with a phenotype of interest.

3. The method of claim 2, wherein the phenotype is insulin resistance.

4. The method of claim 2, wherein the phenotype is coronary artery disease.

5. A method of detecting a genetic predisposition in a Mexican-American human subject for developing insulin resistance, comprising:

a) collecting a biological sample from the subject;

b) genotyping the sample at nucleotide positions 7315, 8292, 8393, 8852, 9040, and 9712, with respect to the Nickerson reference sequence of the human lipoprotein lipase gene; and

c) assessing whether a haplotype is present in the sample, the haplotype comprising (nucleotide position:variant allele):

(i) 7315:G;
(ii) 8292:A;
(iii) 8393:G;
(iv) 8852:G;
(v) 9040:G; and
(vi) 9712:G,

wherein the presence of the haplotype indicates a genetic predisposition for developing insulin resistance in the Mexican-American subject.

6. A method of detecting a lower than normal risk in a Mexican-American human subject for developing insulin resistance, comprising:

a) collecting a biological sample from the subject;

b) genotyping the sample at nucleotide positions 7315, 8292, 8393, 8852, 9040, and 9712, with respect to the Nickerson reference sequence of the human lipoprotein lipase gene; and

c) assessing whether a haplotype is present in the sample, the haplotype comprising (nucleotide position:variant allele):

(i) 7315:G;
(ii) 8292:A;
(iii) 8393:T;
(iv) 8852:T;
(v) 9040:C; and
(vi) 9712:G,

wherein the presence of the haplotype indicates a lower than normal risk for developing insulin resistance in the subject.

7. A method of detecting a lower than normal risk in a Mexican-American human subject for developing coronary artery disease, comprising:

a) collecting a biological sample from the subject;

b) genotyping the sample at nucleotide positions 7315, 8292, 8393, 8852, 9040, and 9712, with respect to the Nickerson reference sequence of the human lipoprotein lipase gene; and

c) assessing whether the sample is homozygous for a haplotype comprising (nucleotide position:variant allele):

(i) 7315:G;
(ii) 8292:A;
(iii) 8393:T;
(iv) 8852:T;
(v) 9040:C; and
(vi) 9712:G,

wherein homozygosity for the haplotype indicates a lower than normal risk for developing coronary artery disease in the subject.

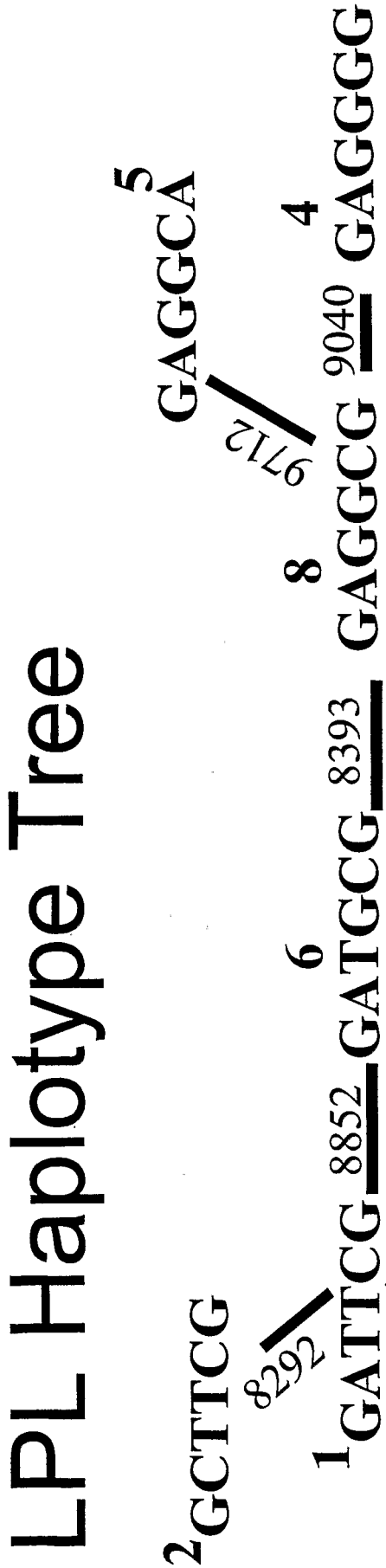
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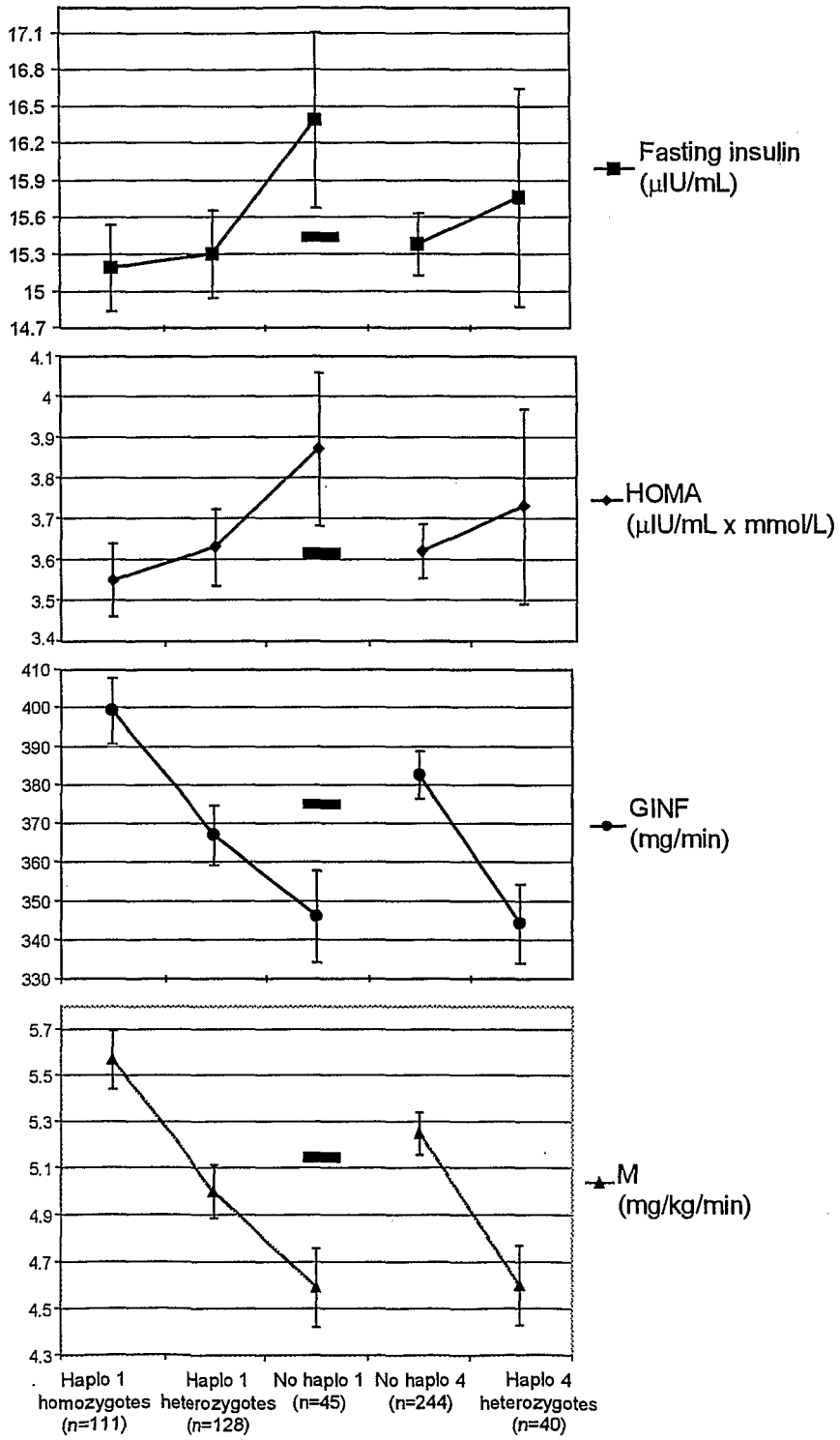
Figure 1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16.8
16.5
16.2
15.9
15.6
15.3
15
14.7
Fasting insulin (μIU/mL)
4.1
4
3.9
3.8
3.7
3.6
3.5
3.4
HOMA (μIU/mL x mmol/L)
410
400
390
380
370
360
350
340
330
GINF (mg/min)
5.7
5.5
5.3
5.1
4.9
4.7
4.5
4.3
M (mg/kg/min)
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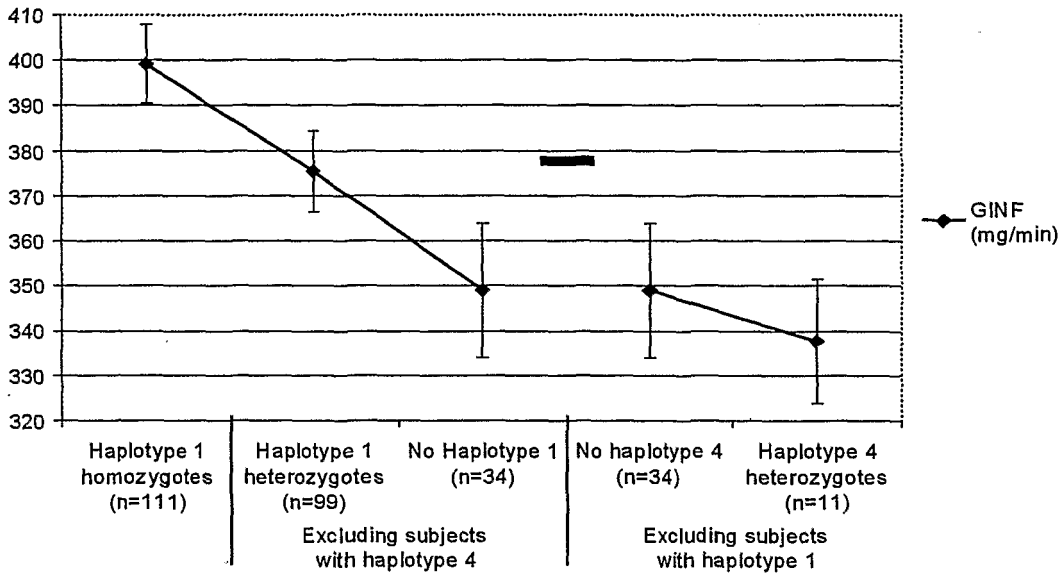
No haplo 4 (n=244)
Haplo 4 heterozygotes (n=40)

FIGURE 2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380
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GINF (mg/min)
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Haplotype 1 heterozygotes (n=99)
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Excluding subjects with haplotype 4
Excluding subjects with haplotype 1

FIGURE 3

SEQUENCE LISTING

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The Regents of The University of California (Assignee)
Kent D. Taylor (Inventor)
Jerome I. Rotter (Inventor)
Huiying Yang (Inventor)
Willa A. Hsueh (Inventor)
Xiuqing Guo (Inventor)
Leslie J. Raffel (Inventor)
Mark O. Goodarzi (Inventor)

<120> Method of Haplotype-Based Genetic Analysis for Determining Risk for Developing Insulin Resistance, Coronary Artery Disease and Other Phenotypes

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