

## ARTICLE

# Genome-wide linkage analysis for identifying quantitative trait loci involved in the regulation of lipoprotein a (Lpa) levels

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Lipoprotein Lp(a) levels are highly heritable and are associated with cardiovascular risk. We performed a genome-wide linkage analysis to delineate the genomic regions that influence the concentration of Lp(a) in families from the Genetic Analysis of Idiopathic Thrombophilia (GAIT) Project. Lp(a) levels were measured in 387 individuals belonging to 21 extended Spanish families. A total of 485 DNA microsatellite markers were genotyped to provide a 7.1 cM genetic map. The variance component linkage method was used to evaluate linkage and to detect quantitative trait loci (QTLs). The main QTL that showed strong evidence of linkage with Lp(a) levels was located at the structural gene for apo(a) on chromosome 6 (LOD score = 13.8). Interestingly, another QTL influencing Lp(a) concentration was located on chromosome 2 with an LOD score of 2.01. This region contains several candidate genes. One of them is the *tissue factor pathway inhibitor (TFPI)*, which has antithrombotic action and also has the ability to bind lipoproteins. However, quantitative trait association analyses performed with 12 SNPs in *TFPI* gene revealed no association with Lp(a) levels. Our study confirms previous results on the genetic basis of Lp(a) levels. In addition, we report a new QTL on chromosome 2 involved in the quantitative variation of Lp(a). These data should serve as the basis for further detection of candidate genes and to elucidate the relationship between the concentration of Lp(a) and cardiovascular risk.

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## Introduction

Lipoprotein Lp(a) is an independent risk factor for atherosclerosis and cardiovascular disease.<sup>1–3</sup> The mechanism by which Lp(a) favours this pathological state may be related to its particular structure. It has many properties in common with low density lipoprotein (LDL), but Lp(a)

contains apolipoprotein apo(a). Apo(a) is an hydrophilic glycoprotein structurally similar to plasminogen. It is linked to apolipoprotein B-100 by a single disulfide bond. The size of the apo(a) gene (*LPA*) is highly variable, resulting in different sized Lp(a) isoforms in the population. The size of the apo(a) isoforms is inversely correlated with Lp(a) concentration. The concentrations vary greatly among individuals and populations. In addition to the apo(a) polymorphism coding for different sized Lp(a) isoforms, an upstream pentanucleotide repeat (TTTTA)<sub>n</sub>;  $n = 5-12$  affects Lp(a) levels. Thus, the variability of the *LPA* gene contributes to the heterogeneity of Lp(a) and influences the metabolic and physiochemical characteristics of this lipoprotein.<sup>4</sup> In fact, the genetic variation in the *LPA* gene has been estimated to be responsible for 42% and even >90% of the variation of Lp(a) concentration.<sup>5,6</sup> According to epidemiological studies, a high concentration of Lp(a) and/or small sizes of apo(a) isoforms increase the risk of cardiovascular disease.<sup>7</sup>

There are several hypotheses that attempt to describe the pathophysiologic mechanism of Lp(a) in atherosclerosis and cardiovascular disease. For example, oxidized phospholipids in plasma bind preferentially to Lp(a) and may contribute significantly to the increased atherogenicity of this lipoprotein.<sup>8</sup> On the other hand, the high homology of Lp(a) to plasminogen suggests that Lp(a) plays a key role as a proatherothrombotic agent in atherosclerosis and cardiovascular disease due to its antifibrinolytic and procoagulant properties.

It is well known that Lp(a) levels are highly heritable. Until recently, most of the studies used genome-wide scan analyses and focused on the genetic determinants that influence Lp(a) levels. Some studies described a strong linkage at the structural gene for apo(a) on chromosome 6q26-q27. So, it is now well-established that Lp(a) levels are genetically determined by a variable number of kringle (K) IV type 2 repeats in *LPA*, which lead to apo(a) isoforms of different sizes. In addition, the wide variability of Lp(a) levels may be affected also by sequence polymorphisms/mutations in coding and noncoding regions of *LPA*. Several functionally significant single-nucleotide polymorphisms (SNPs) have been described in *LPA*. These polymorphisms could be responsible in part for the large quantitative variation of Lp(a) among individuals and populations.<sup>9-12</sup> Apart from the many publications that describe association for Lp(a) located at its structural gene on chromosome 6, several recent studies have described new potential genetic determinants that may control quantitative variation of Lp(a).<sup>13-15</sup>

The major aim of this study was to localize the quantitative trait loci (QTLs) that influence Lp(a) levels in the Spanish population. A genome-wide variance component linkage analysis of data from the GAIT Project was used to accomplish this aim.

## Materials and methods

### Subjects and phenotyping

Our study included 387 individuals belonging to 21 Spanish families from the GAIT project. An extensive description of the GAIT project has been published previously.<sup>16</sup> Each family was composed of 3-5 generations of individuals and the subjects ranged in age from 2 to 87 years, with a mean of 37.8 years. There were an equal number of male subjects (46%) and female subjects (54%) in our study. Twelve families were selected through a proband with idiopathic thrombophilia and the remaining nine families were randomly selected without regard to phenotype. Thrombophilia was defined as recurrent thrombotic episodes (at least one of which was spontaneous), a single spontaneous thrombotic event with a first-degree relative also affected, or early onset thrombosis (before the age of 45 years). The thrombophilia proband was considered idiopathic when all known biological causes of thrombophilia (ie, protein S and protein C deficiencies, antithrombin deficiency, activated protein C resistance and plasminogen deficiency) at the time of recruitment (since 1995-1997) were excluded.

All protocols were approved by the Institutional Review Board of the Hospital de la Santa Creu i Sant Pau (Barcelona). Adult subjects gave informed consent for themselves and for their minor children.

Blood was collected by venipuncture in 1/10 volume of 0.129 mol/l sodium citrate from fasting subjects. Thrombophilic participants were not using oral anticoagulants at the time of sampling. Platelet-poor serum samples were stored at -80°C before assaying for Lp(a) levels. Serum Lp(a) levels were measured by ELISA (Apo-Tek, Organon Teknica, Turnhout, Belgium) as a part of a clinical workup, between 1995 and 1997. Normal levels of Lp(a) in serum were considered to be <300 mg/l.

### Genotyping

DNA was extracted by a standard method.<sup>17</sup> All individuals were genotyped for 485 highly informative DNA microsatellite markers, distributed through the autosomal genome at a density of 7.1 cM. The average heterozygosity of the microsatellite markers was 0.79. Microsatellites consisted primarily of the ABI Prism (Applied Biosystems, Foster City, CA) genotyping set MD-10. Linkage mapping was undertaken with the PE LMS II fluorescent marker set (ABI Prism) with multiplex PCR.<sup>18</sup> The products from the PCR were analyzed on the PE 310, PE 377 and PE 3700 automated sequencers, and genotyped using the Genotyper software. Information on microsatellite markers can be found in the public-accessible genomic database (<http://www.gdb.org>). Marker maps for multipoint analyses were obtained from the Marshfield Medical Research Organization (<http://research.marshfieldclinic.org/genetics/>).

In addition, 12 SNPs (Table 3) in the structural *TFPI* gene were genotyped by using Taqman probes from Applied Biosystems (ABI Prism).

The genotypic data were entered into a database and were analyzed for discrepancies (ie, violations of Mendelian inheritance) using the program INFER (PEDSYS).<sup>19</sup> Mistypings and markers for discrepant individuals were either corrected or excluded from the analyses.

### Linkage analysis

A standard multipoint variance component linkage method was used to assess linkage between autosomal markers and levels of plasma Lp(a). This analysis was performed using the Sequential Oligogenic Linkage Analysis Routines (SOLAR) computer package.<sup>20</sup> Previous studies suggested that such a method might be vulnerable to deviations from multivariate normality and particularly to high levels of kurtosis in the trait's distribution, giving inflated logarithm of odds (LOD) scores.<sup>21</sup> Levels of Lp(a) in the GAIT sample exhibited a kurtosis of 0.8 after a logarithmic transformation, which does not affect the distribution of LOD scores (probability of linkage). Thus, the standard nominal *P*-values for LOD scores are appropriate for the Lp(a) linkage screen.<sup>22</sup>

Quantitative trait association analyses were performed using the measured genotyped method<sup>23</sup> by testing for genotype-specific differences in the means of the trait while allowing for the nonindependence among family members. As 12 of the families were ascertained through thrombophilic probands, all analyses included an ascertainment correction achieved by conditioning the likelihood of these pedigrees on the likelihoods of their respective probands.<sup>24</sup> To control the multiple testing effect, genome-wide *P*-values were calculated using the method of Feingold *et al.*<sup>25</sup>

### Results

The pedigree data extracted from the GAIT database consisted of 21 families with 387 individuals providing data for Lp(a) levels and for the covariates used in the analysis, such as gender, age, total cholesterol, smoking behavior, use of oral contraceptives and the household effect. Table 1 summarizes the phenotypic characteristics of the individuals in our study.

Total cholesterol levels and smoking behavior were the only covariates that showed a significant effect over Lp(a) levels ( $P = 0.0042$  and  $P = 0.019$ , respectively). Neither the gender nor age effects were significant. No significant household effect was found in our samples. The range of Lp(a) levels in the GAIT sample was 0.21–242.30 mg/l, with a mean of 33.38 mg/l.

The overall genetic heritability ( $h^2$ ) of the concentration of Lp(a) was estimated to be  $0.79 \pm 0.07$ , indicating that

**Table 1** Phenotypic characteristics of the 387 individuals included in the study

Characteristics	Frequency (%) or Mean $\pm$ SD
No. of families	21
No. of individuals	387
Sex	
Male	178 (46%)
Female	209 (54%)
Age (years)	37.8 $\pm$ 19.81
Smokers <sup>a</sup>	146 (38%)
Total cholesterol (mmol/l)	5.19 $\pm$ 1.15
Lp(a) (mg/l)	33.38 $\pm$ 38.35

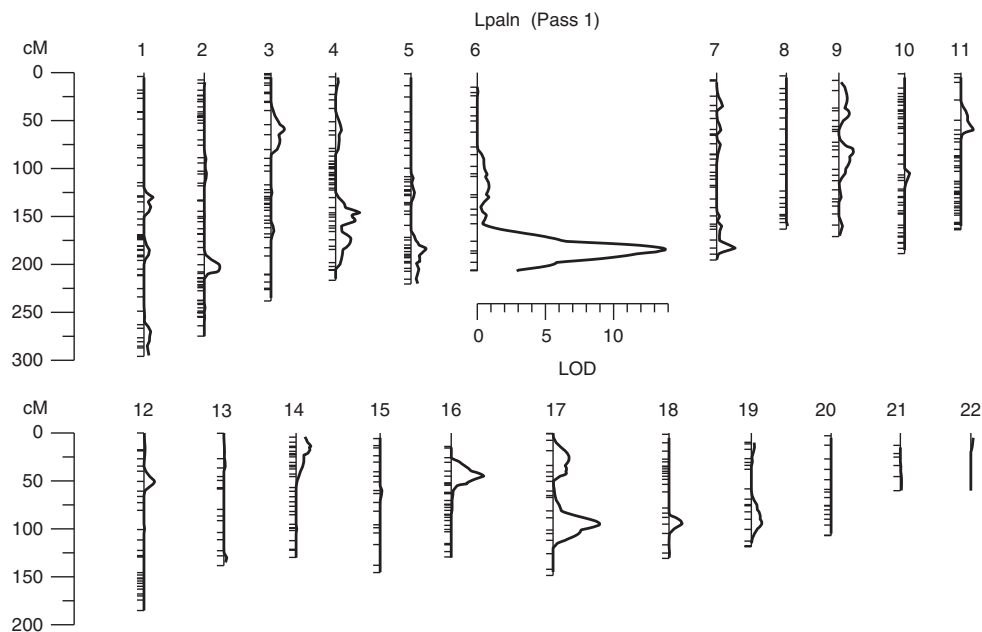
<sup>a</sup>Individuals in the study were defined as smokers when they usually smoke, independently of the number of cigarettes.

79% of the phenotypic variation in this trait is due to the additive effect of genes.

A standard multipoint variance component method was used to assess linkage between autosomal DNA markers, spaced at  $\sim 7.1$  cM, and quantitative values of Lp(a). The proportion of the Lp(a) variance due to the covariates used in the analysis was estimated as 5%. Their effects were estimated simultaneously with the genetic effects.

The results from the linkage analysis revealed strong evidence for a quantitative trait locus (QTL) on the long arm of chromosome 6 (6q), which may influence Lp(a) levels (LOD = 13.8; nominal  $P = 7.77e-16$  and genome-wide  $P = 2.54e-13$ ) (Figure 1). The QTL on chromosome 6 was detected through a peak LOD score located in the interval flanked by markers D6S441 and D6S264 in a region that maps to 6q25.2–6q27. Specific data from the linkage analysis can be found in Table 2. This region contains the structural gene of apo(a). Another linkage signal was detected on the long arm of chromosome 17 (17q) with a genome-wide *P*-value of 0.0179, indicating a potential additional genomic region that may be involved in the control of the concentration of Lp(a).

To look for additional genomic regions controlling Lp(a) levels, we carried out a multilocus linkage analysis, which took into account the effect of the QTL on chromosome 6, by fixing this position in the model and rescanning the genome (Figure 2 and Table 2). In the second linkage pass, which was conditioned on chromosome 6 QTL detected in the initial screen, evidence of linkage on chromosome 17 disappeared (LOD = 0.7) indicating that the initial signal was a false positive. It is important to note that we were able to detect a new genomic region on the long arm of chromosome 2 (2q) with suggestive linkage (LOD = 2.01;  $P = 0.0011$ ). The new QTL on chromosome 2 was located between markers D2S335 and D2S2289, at chromosome position 206cM, in a region that maps to 2q31.1–2q33.2 (Figure 3 and Table 2). A bioinformatic search in this region showed several potential candidate genes. One of them was the *tissue factor pathway inhibitor* (*TFPI*; Ensembl Gene ID:



**Figure 1** Results of the linkage screen for lipoprotein Lp(a). There is a major linkage signal on chromosome 6 (LOD = 13.8). Significant linkage response was detected also on chromosome 17 (LOD = 3.4).

**Table 2** Results of the sequential oligogenic linkage analysis of variation in Lp(a) levels

Chromosome	Flanking markers	Chromosome position (cM)	LOD score	First pass		LOD score	Second pass	
				Nominal P-value	Genome-wide P-value		Nominal P-value	Genome-wide P-value
6	D6S441-D6S264	184	13.8	7.77e-16	2.54e-13	—	—	—
17	D17S787-D17S944	95	3.4	3.61e-05	0.01791	0.7	0.0363	NS
2	D2S335-D2S2289	206	—	—	—	2.01	0.0011	0.5394

NS, not significant.

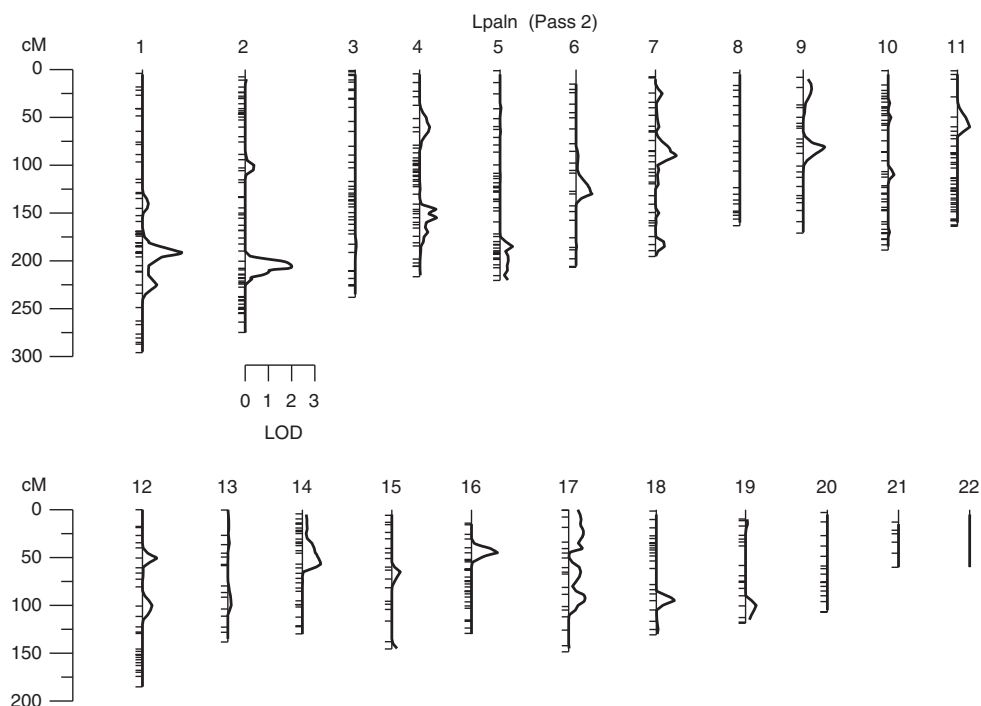
ENSG0000003436). This gene encodes a protein that has an antithrombotic action and also the ability to associate with lipoproteins in plasma. Encouraged by this result, we genotyped 12 SNPs in the *TFPI* gene (Table 3) including three reported as functional (rs5940; rs10153820 and P151L), and looked for the association with Lp(a) levels. However, the measured genotype analysis revealed no significant association of any of these polymorphisms and the Lp(a) levels (Table 3). In addition, 23 haplotypes were analyzed using all these SNPs; however no significant association with Lp(a) levels was found (data not shown).

## Discussion

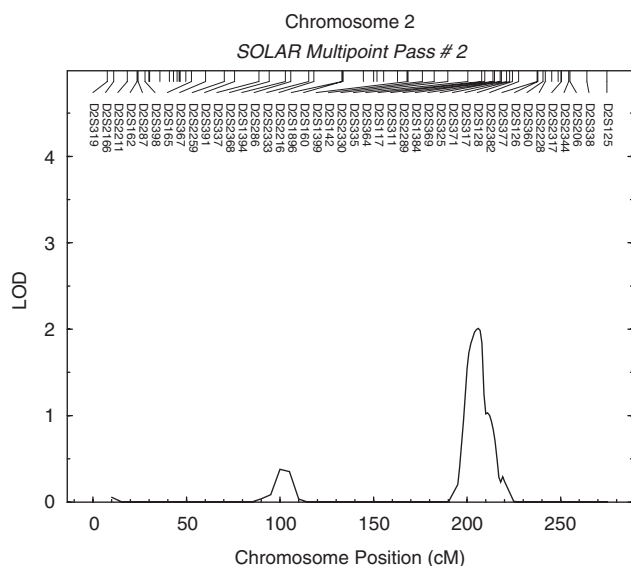
Lipoprotein Lp(a) is a low-density lipoprotein particle linked to apolipoprotein apo(a). It is one of the well-established risk factors for atherosclerosis and cardiovascular disease. The understanding of the physiologic mechanisms and genetic determinants of Lp(a) should

help to elucidate the contribution of this quantitative trait to the risk of cardiovascular disease. Accordingly, the concentration of Lp(a) is highly heritable and a large number of studies have attempted to identify the genomic regions that influence Lp(a) levels. Specifically, heritability ( $h^2$ ) for Lp(a) was estimated in several studies and ranged from 0.69 to 0.94.<sup>13–15,26–28</sup> In agreement with these results, we estimated the  $h^2$  of Lp(a) in the Spanish population in 0.79. This means that 79% of the variance of Lp(a) is attributable to genetic factors. Given the high heritability for Lp(a) obtained in our GAIT sample, we conducted a genome-wide scan analysis to identify the genomic regions that influence the Lp(a) concentration.

We detected a strong linkage signal at the *LPA* gene on Chromosome 6q25.2–q27 with a LOD score of 13.8. This result agrees with the results from previous studies that investigated the Lp(a) trait and found a highly significant linkage at the structural *LPA* gene on chromosome 6.<sup>6,13–15,28,29</sup> On the other hand, it is noteworthy



**Figure 2** Results of the second linkage pass conditioned on chromosome 6 QTL.



**Figure 3** Detailed linkage results for chromosome 2. Significant linkage signal was detected by a peak LOD score of 2.01 (nominal  $P = 0.0011$  and genome-wide  $P = 0.5394$ ) defining a quantitative trait locus for Lp(a) between markers D2S335 and D2S2289 at chromosome position 206cM in a region that maps to 2q31.1–2q33.2.

that when we conditioned the linkage analysis to the strong signal on chromosome 6 (where the structural *LPA* gene maps), a new QTL involved in the control of Lp(a) levels became evident. This QTL is located in a region that

maps to chromosome 2q31.1–2q33.2. According to our results, the genomic regions that influence Lp(a) concentration are not limited to the structural *LPA* gene on chromosome 6. Accordingly, several recent genome-wide scans have described other new potential genetic determinants that may control quantitative variation of Lp(a). However, any of them showed a significant signal on chromosome 2. This may be possible due to the fact that the genetic factors contributing to a complex trait may differ among populations. In agreement with this argument, it is possible that the new QTL on chromosome 2 may play a functional role on Lp(a) levels only in the Spanish population. In addition, given that the major genetic factor that influence Lp(a) levels accounts for >90% of variance, other genes that have a small effect on this trait must be hard to find. Another reason for such differences in reporting QTLs between different studies might be that there is insufficient statistical power because of the study design or the sample size or the small effect of these QTLs. Finally, exists the possibility that these other gene(s) involved in affecting small quantitative variation of Lp(a) in different populations may actually respond to different biological causes that could indeed may be controlling the participation of these genes in the control of Lp(a) levels. A review of these results is summarized in Table 4.

This genomic region influencing Lp(a) on chromosome 2q contains several genes that could be candidate genes involved in the control of Lp(a) levels. One of them is the

**Table 3** List of SNPs genotyped in the *TFPI* gene

SNP	Chromosome 2 position (bp)	P-value <sup>a</sup>	MAF <sup>b</sup>	beta <sup>c</sup>	Ebeta <sup>d</sup>
rs5940	188039949	0.873	0.015	-0.010	-1.010
rs10153820	188127835	0.784	0.124	-0.086	-1.090
P151 L	188057188	0.616	0.004	0.388	1.474
rs2268300	188042980	0.714	0.008	-0.108	-1.114
rs6736363	188067265	0.124	0.031	0.521	1.684
rs2300412	188076120	0.544	0.392	0.044	1.045
rs3755248	188078477	0.506	0.316	0.036	1.037
rs4667168	188101185	0.639	0.107	-0.065	-1.067
rs35935664	188104581	0.768	0.259	-0.051	-1.052
rs13427829	188112528	0.611	0.103	-0.080	-1.084
rs7594359	188117093	0.952	0.448	0.021	1.021
rs6434222	188120489	0.621	0.133	-0.089	-1.093

<sup>a</sup>P-value of association with Lp(a) levels.<sup>b</sup>Minimal allele frequency of the SNP in our sample.<sup>c</sup>Represents the effect of one copy of the rare allele in Lp(a) levels when it is expressed in logarithmic scale.<sup>d</sup>Represents the effect of one copy of the rare allele in Lp(a) levels when it is expressed in original scale.**Table 4** Genomic regions that influence Lp(a) levels reported in recent studies as of this date

Author	Chromosome	LOD score	Flanking markers	Chromosome position (cM)	Population studied
Barlera <i>et al</i> <sup>13</sup>	6	108.3	D6S1581-D6S1599	189	1812 families, including 4012 individuals, from the PROCARDIS coronary artery disease (CAD) study, in which 80% of individuals analyzed were affected by CAD.
	13	7.0	D13S156-D13S265	85	
	11	3.5	D11S902-D11S904	35	
	15	2.9	D15S131-D15S205	76	
	19	2.7	D19S571-D19S418	97	
	1	1.5	D1S498-D1S484	—	
Barkley <i>et al</i> <sup>15</sup>	6	18.62	—	160	1046 Nonhispanic white individuals <sup>b</sup> .
		14.27	—	164	1556 African American individuals <sup>b</sup> .
		12.97	—	163	1061 hispanic white individuals <sup>b</sup> .
	12	1.60	—	38	1046 nonhispanic white individuals <sup>b</sup> .
	14	2.56	—	17	1061 hispanic white individuals <sup>b</sup> .
19	3.8/2.52	—	30/47	1046 nonhispanic white individuals <sup>b</sup> .	
Beekman <i>et al</i> <sup>29</sup>	6	9.8→8.2 <sup>a</sup>	—	163	483 dizygotic and 967 monozygotic twin pairs recruited regardless of their health status.
	1→— <sup>a</sup>	1.6→0 <sup>a</sup>	—	251	
Broeckel <i>et al</i> <sup>14</sup>	6	26.99	D6S305	166	513 western European families, including 1406 individuals, with at least two members affected of early onset myocardial infarction and/or severe premature CAD.
	1	3.81	D1S1679	170	

<sup>a</sup>In the study of Beekman *et al*,<sup>29</sup> evidence for linkage on chromosome 1 completely disappeared (LOD = 0) when further analysis in a two locus model was performed, whereas the strong linkage signal for chromosome 6 remained unaltered.<sup>b</sup>All the individuals were recruited from the Genetic Epidemiology Network of Arteriopathy study of the Family Blood Pressure Program. Further genetic linkage evidence has been found on chromosomes 12 and 19 in nonhispanic whites, as well as on chromosome 14 in hispanic whites. However, there has been a lack of linkage response on these chromosomes in African Americans.

*TFPI*. This gene encodes a protein that inhibits factor X activated (X(a)) directly and, in a Xa-dependent way, inhibits VIIa/tissue-factor activity, presumably by forming a quaternary Xa/LACI/VIIa/TF complex. It has antithrombotic action and also the ability to associate with lipoproteins. Accordingly, a recent study<sup>30</sup> shows that Lp(a) binds and inhibits TFPI *in vitro*. This biologic interaction promotes the prothrombotic action of Lp(a) mediated through the inhibition of the anticoagulant activity of TFPI and suggest a new important link between lipoproteins and thrombosis. With these studies as background, we performed quantitative trait association analyses with an extensive set of SNPs in the *TFPI* gene and Lp(a) concentration. Although, the lack of association of these SNPs and Lp(a) levels does not rule out the possibility that other polymorphisms in the *TFPI* gene could be associated with Lp(a), the most plausible hypothesis is that another gene in this region different from *TFPI* is responsible of the linkage signal.

Interestingly, the genomic region between markers defining the QTL located on chromosome 2 also includes four additional genes that need to be considered. These genes are *OSBPL6* (oxysterol-binding protein-related protein 6); *NDUFB3* (mitochondrial NADH dehydrogenase 1 beta subcomplex subunit 3), *COQ10 B* (mitochondrial precursor protein coenzyme Q10 homolog B) and *AOX1* (aldehyde oxidase).

The *OSBPL6* gene encodes a protein involved in the transport of lipids, in the regulation of cholesterol homeostasis and in signal transduction pathways of lipid metabolism.<sup>31</sup> We believe that the *OSBPL6* protein might be involved in the regulation of Lp(a) levels by an unknown mechanism, probably related with the action of oxysterols as potential signaling molecules, which accumulate in the atherosclerotic lesions in arterial walls.

In addition, both the *NDUFB3* and the *CoQ10 B* genes encode proteins of the mitochondrial respiratory chain. These proteins are part of two important sites of free radicals generated in mitochondria. These free radicals increase the oxidative stress and cellular damage.<sup>32</sup> Furthermore, the enzyme aldehyde oxidase, which belongs to the xanthine dehydrogenase family, also causes an increase in oxidative cell damage by producing hydrogen peroxide and, under certain conditions, catalyzes the formation of superoxide.<sup>33</sup> It is widely known that oxidative stress plays a key role in the development of cardiovascular disease and atherosclerosis. Moreover, oxidized phospholipids in plasma circulate, preferentially, on Lp(a) and may contribute significantly to the increased atherogenicity of this lipoprotein.<sup>8</sup>

In this complex scenario, with several potential candidate genes, which may explain the genetic variability of Lp(a) levels, further analyses are needed that focus on fine mapping. With these new data, the mechanism by which the locus on chromosome 2 modulates Lp(a) levels may

become clear. Our results provide a framework for these additional studies.

Finally, despite the fact that other genes different from *LPA* gene would probably have a small effect on Lp(a) levels, it is prudent to think in terms of networks of genes and systems when considering control of this trait rather than of individual genes.

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