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ARTICLE

Genome-wide search for QTLs for apolipoprotein A-I level in elderly Swedish DZ twins: evidence of female-specific locus on 15q11–13

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The effect of genetic variants underlying atherosclerosis is thought to be mediated through intermediate phenotypes such as serum cholesterol levels. Localization of quantitative trait loci influencing levels of serum lipids and (apo)lipoproteins may aid in the search for determinants of susceptibility to atherosclerotic diseases. Since apolipoprotein A-I is the primary protein constituent of high-density lipoprotein, it is considered to be critical for the antiatherogenic effect of high-density lipoproteins. We describe here an effort to map loci influencing apolipoprotein A-I levels. Measurements of apolipoprotein A-I levels and genome scans with more than 1000 microsatellite markers were successfully performed in both members of 501 pairs of fraternal twins from Sweden. Variance component linkage analysis was undertaken to map quantitative trait loci. In the total study sample, two loci showed comparable suggestive evidence of linkage, 6p21–12 (LOD = 2.4) and 12q23 (LOD = 2.4). Sex-limited analyses revealed significant female-specific linkage at marker D15S156 on 15q11–13 (LOD = 4.1). The loci on 12q and 15q in the present study confirm previously reported loci for apolipoprotein A-I, while the peak on chromosome 6p lends further support to a locus influencing several phenotypes related to atherosclerosis. Intriguingly, the presence of genes belonging to the phospholipase A2 superfamily under three out of four observed linkage peaks would lend some support to the view that this group of genes might collectively represent candidates as apolipoprotein A-I level regulators.
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Introduction

Coronary artery/heart disease and other cardiovascular diseases (CVD) are generally attributed to atherosclerosis, a pathological condition arising from interactions between various genetic and environmental risk factors.[1] Disturbances in the metabolism of blood lipids and (apo)lipoproteins are of importance in the etiology of atherosclerosis[2] and several intermediate lipid-related phenotypes, associated with CVD, are well characterized.[3] For instance, high-density lipoproteins (HDLs) are known to be antiatherogenic[4] and low levels of HDL cholesterol strongly increase the risk for development of atherosclerosis.[5] Apolipoprotein A-I (apoA-I) is the major protein

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constituent of HDL[6] and much of the antiatherogenic properties of HDL appear to originate from apoA-I.[7] Interindividual variation in levels of apoA-I is known to be appreciably heritable.[8]

The mapping of genes influencing CVD risk by performing whole-genome linkage analysis of quantitative trait loci (QTLs) for apoA-I may have large impact on the understanding of CVD development. With the intention of localizing QTLs influencing apoA-I levels, we here report on whole autosomal genome linkage scans on 501 fraternal twin pairs from the Swedish Twin Registry.[9,10]

Four genome-wide linkage scans for apoA-I have previously been reported.[11–14] Klos *et al*[14] reported an LOD score of 2.02 on chromosome 12q24 in a randomly ascertained material of 232 families. In families partly ascertained through obese probands, Bosse *et al*[11] reported a number of loci, with the strongest 2-point evidence for linkage obtained on chromosome 15q11 ($P=0.000001$, equivalent to LOD of approximately 4.9). In another sample of female dizygotic (DZ) twin pairs from the United Kingdom, Falchi *et al*[12] found linkage to 9q21.32–33.1 (LOD = 3.28) and further reported two additional loci, 8p21.1–q13.1 (LOD = 3.71) and 10p15.1–p13 (LOD = 5.51), after applying a two-locus ordered subset approach. Because results from such two-locus approach are conditioned on the findings in the first unidimensional scan, they are not directly comparable to standard (unidimensional) genome scans. Finally, Heijmans *et al*[13] performed a meta-analysis on samples from Australia, the Netherlands and Sweden. The strongest signal was seen on chromosome 1q21.3 with an LOD score of 2.1. The combined picture from the previously published studies is scattered with few replicated findings, rather typical of complex traits. Heterogeneity in ethnicity, sex or age as well as differences in techniques used may all be factors explaining the marked variation between studies.

Aside from a smaller sample of 52 sib-pairs, included in the meta-analysis by Heijmans *et al*,[13] the present study is the first report of a genome-wide scan of apoA-I in a Swedish population. The power to map genes by linkage analysis depends, in addition to the QTL effect size, heavily on size of the material (ie, number of families) as well as the available marker information. We utilized a dense marker panel consisting of more than 1000 microsatellite markers (instead of the more frequently used panels of 300–400 markers), assuring complete coverage and substantially improved marker information content throughout the genome.

Materials and methods

Subjects

The participants were recruited from the Swedish Twin Registry.[9] Ascertainment of pairs followed one of two schemes: a smaller number ($N=65$ pairs) of DZ twin pairs were identified because at least one twin had suffered a myocardial infarction or been subjected to revascularization by the time of the study initiation in 2004. Ascertainment of the remaining pairs ($N=436$ pairs) followed age-restricted (>67 years) but otherwise random ascertainment. Median age in the resulting sample was 75 years with a range from 49 to 92 years and 52% of participants were women. The pairwise sex distribution was 144 (28%) female–female, 129 (26%) male–male and 228 (46%) opposite sex pairs. Whole blood was collected at local health-care centers in Sweden and mailed overnight to the Karolinska University Hospital for biochemical measurements including apoA-I and HDL determination. DNA extraction and sample storage was handled by KI Biobank. All subjects have given informed consent to participate. The study was approved by the regional Ethical Review Board at Karolinska Institute (KI D-no. 2005/562-32, dated 18 May 2005).

Pregenotyping information on zygosity status was obtained based on self-reports according to previously described methods.[9] The dizygosity of the twins was confirmed with the results from the genotyping procedure. Five pairs (1%) were found to be monozygous and therefore excluded from analysis.

Phenotyping

Measurement of apoA-I level was performed using a standard immunoturbidimetric method, Syncron LX systems Apo Calibrator (Beckman Coulter Inc.). The subjects were all asked to fast overnight before visiting the health-care center in the morning. Fasting status was confirmed by questionnaire at the time of sample donation. Out of the 1002 subjects, 19 (2%) were found to be non-fasting at blood donation. ApoA-I values of individuals taking statin medications (totally 52 individuals) were included in the analysis without any adjustment since mean apoA-I levels for subjects on statin were found to be practically identical to the grand mean and average effects of statins on apoA-I levels have also been reported to be very modest in magnitude (<5%).[15] The information on statin treatment was obtained from a questionnaire to which all subjects had responded to between 1998 and 2002.[9] Prior to analysis, apoA-I values were Box–Cox transformed and converted into *Z*-scores. For the sex-limited analyses, the transformations were done separately within each sex.

At loci showing significant or suggestive evidence of linkage to apoA-I, we studied possible pleiotropic effects by also performing linkage analyses for HDL levels. Statin treatment has a small but well-established effect on HDL levels (increases HDL by an average of 5–10%[16]), and HDL levels for subjects reported to be on statin were therefore reduced by 7.5%. Aside from this, HDLs were handled the same way as apoA-I levels, with Box–Cox transformation and conversion into *Z*-scores.

Genotyping

All genotyping was performed by deCODE genetics Inc. (Reykjavik, Iceland). After quality control, genotypes from a total of 1064 autosomal genetic markers were available. Genotyping was undertaken in two batches with slightly differing marker sets. The first batch consisting of 235 pairs was genotyped for 1012 autosomal markers, while the second batch of 266 pairs was genotyped for 1061 autosomal markers, providing an average marker density in the total set of approximately 3 cM. In total, there was phenotypic and marker information available for both twins in 501 DZ twin pairs. Cytogenetic positions for markers were taken from an integrated genetic map with interpolated genetic map positions (http://www2.qimr.edu.au/davidD/).[17]

Linkage methods and statistical analysis

Descriptive statistics, transformation of apoA-I and preparation of input data files for linkage analysis were done using the SAS software, version 9.1.3 (SAS Institute Inc., Cary, NC, USA). In the variance component models, age, sex and fasting status were included as covariates. Body mass index (BMI) is correlated with apoA-I levels ($r=-0.24$, $P<0.0001$ in the investigated sample). To increase power to detect loci that influence apoA-I levels independently of BMI, separate analyses were performed when BMI was additionally included as a covariate into the variance component model.

The software package Merlin[18] was used for multipoint variance component linkage analysis. Automation of several parts of the file handling was achieved by use of the AUTOGSCAN program.[19] For variance component linkage analysis, it is crucial that the residual kurtosis (kurtosis of distribution after covariates have been regressed out) is not too high.[20] The kurtosis and skew of the raw apoA-I distribution are 0.69 and 0.62, respectively. After Box–Cox transformation and adjustment for covariates, the residual kurtosis and skew are 0.32 and −0.08, well within the range suggested for assuming multivariate normality in the analysis.[21] Given the normal distribution and the low residual kurtosis, we chose to adopt the Lander and Kruglyak[22] thresholds of LOD scores ≥ 3.60 for significant evidence of linkage and LOD scores ≥ 2.20 for suggestive evidence of linkage, rather than computing empirical *P*-values.

The genome-wide search for sex-specific QTLs was performed in two steps. Firstly, by performing separate genome scans in female–female pairs ($N=144$) and male–male pairs ($N=129$). Secondly, for loci showing suggestive or significant linkage in at least one sex, a formal sex-limited parameterization model was applied as described by Medland.[23] By comparing the likelihood of a full model in which female- and male-specific QTL parameters (QTLf and QTLm, respectively) are estimated and allowed to differ from each other, with the likelihood of a restricted model in which both QTLf and QTLm are set to 0, the evidence for sex-limited linkage is evaluated. Difference in minus twice the log likelihoods of the models is a test statistic that follows a χ^2 distribution with a 25:50:25 mixture of point mass 0, χ^2 with 1 degree of freedom (df) and χ^2 with 2 df. The test statistic is translated into a *P*-value weighted for the mixture of degrees of freedom. The equivalent LOD score is obtained by dividing the 1 df χ^2 value giving the same *P*-value as the test with mixtures of degrees of freedom, by $2\ln(10)$.[23,24]

For loci displaying significant or suggestive evidence of linkage to apoA-I, we investigated if they were also linked to HDL levels in this material. The highest HDL LOD score observed in a ±15 cM window of the initial apoA-I finding is reported.

Results

The mean (SD) apoA-I value from our study subjects was 1.63 g/l (0.32) and the range was 0.71–3.03 g/l among the 1002 subjects with scan data. The distributions of covariates for the total sample as well as for the same sex pairs are reported in Table 1. The intrapair correlation of covariate-adjusted apoA-I levels was twice as high among female–female pairs ($r=0.38$) than that among male–male pairs ($r=0.19$), but the difference was not significant ($P=0.09$). The correlation among opposite sex pairs ($r=0.20$) was not found to be significantly different from male–male pairs ($P=0.46$) or female–female pairs ($P=0.06$).

Marker information content

The entropy-based information content (IC) was estimated at each of the 1064 available marker position using the Merlin program.[18] We investigated the gain in information content associated with using a 1000+ marker set as compared to the most conventional 400 marker set by creating a 40% marker subset consisting of 412 randomly selected markers. Average IC was significantly higher in the more dense 1064 marker map (IC = 0.53) than IC = 0.42 in

Table 1 Characteristics of subjects in phenotyped and genotyped pairs

Variables[a]	*Total set* (n = *501*)[b]	*Set MM* (n = *129*)[b]	*Set FF* (n = *144*)[b]
apoA-I (g/l)	1.63 ± 0.32	1.50 ± 0.26	1.74 ± 0.35
apoA-I range (g/l)	0.71–3.03	0.95–2.47	1.01–3.03
Age (years)	74.6 ± 5.5	75.1 ± 5.3	75.6 ± 5.2
BMI (kg/m^2)	25.2 ± 3.2	25.5 ± 2.7	25.0 ± 3.5
Fasting (%)	98.1	98.8	97.6
Sex (males, %)	48.5	100	0

[a]Continuous variables are presented as mean ± SD, dichotomous variables as relative frequencies.
[b]Number of dizygotic twin pairs.

the 412 marker map ($P<0.0001$), consistent with previously reported results based on simulated data of sib-pairs without parental information.[25]

Linkage analysis

In Figure 1a, the results of the genome-wide linkage analysis are shown graphically for the total set of 501 DZ pairs. At several positions, LOD peaks are over 1, but no evidence of significant or suggestive linkage is observed in these analyses. When further adjustment is made for BMI as covariate, the magnitude of some signals increased, resulting in suggestive evidence of linkage for two loci: 6p21–12 and 12q23 (Table 2).

Sex-stratified results are shown in Figure 1b and c. For female–female pairs, one strong signal was observed on 15q, with an LOD reaching 3.97 at marker D15S156. After

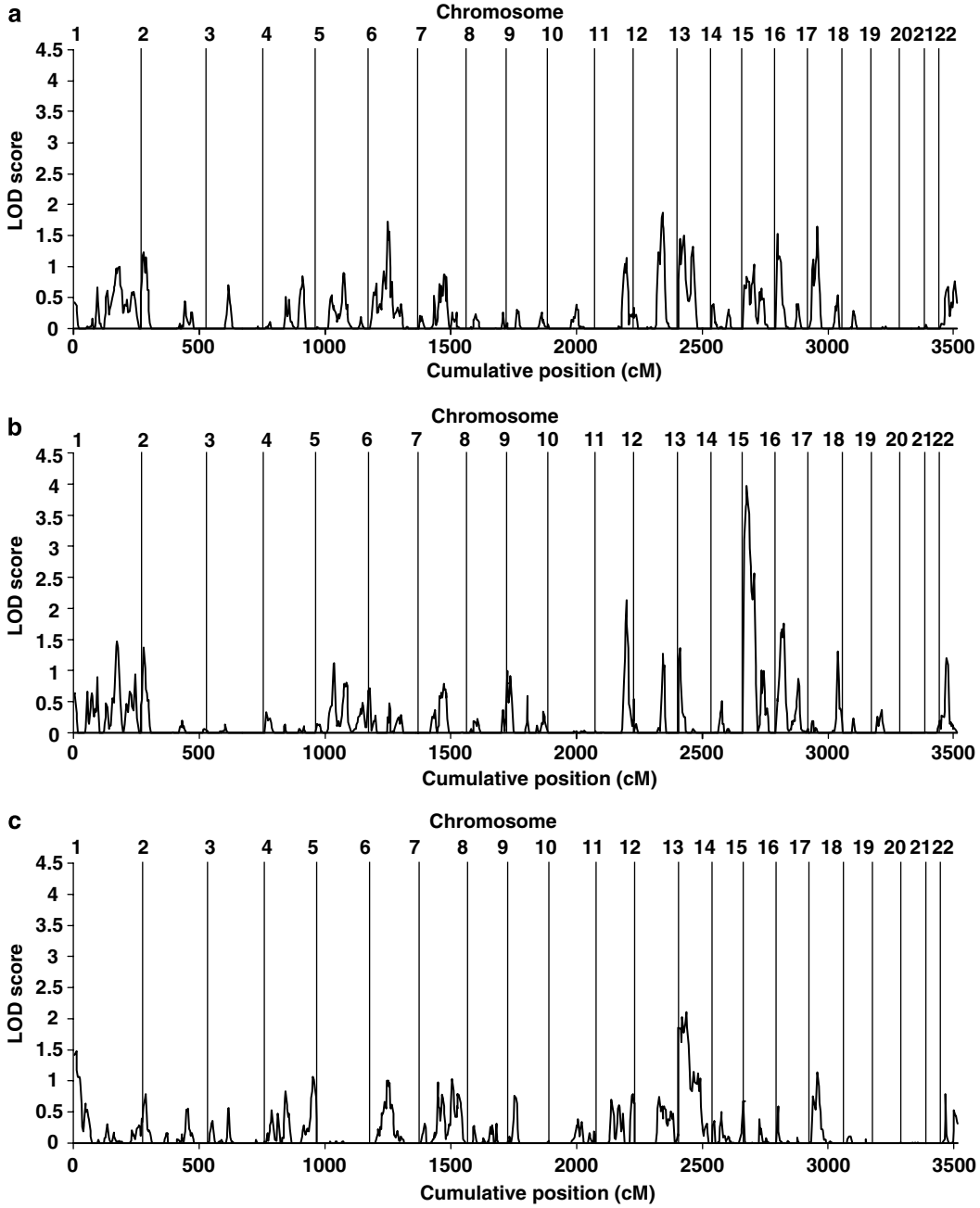

Figure 1 Multipoint variance component linkage of autosomal chromosomes for apoA-I level in (**a**) total set of 501 DZ pairs, (**b**) 144 female–female DZ pairs, (**c**) 129 male–male DZ pairs. The *x*-axis represents genetic distance in cumulative cM (Haldane) and the *y*-axis plots the LOD scores. ApoA-I values adjusted for sex, age and fasting status and Box–Cox transformed before analysis.

Table 2 Suggestive and significant linkage results

Set	Cytogenic region	Marker	Position (cM)	LOD LOD[a]
All pairs	6p21–12	D6S459	70.02	1.72 *2.4*
	12q23	D12S2081	110.27	1.79 *2.38*
		D12S346	112.27	1.87 *2.3*
Female–female pairs	15q11–15q13	D15S128	5.1	*3.11 2.88*
		D15S97	10.18	**3.64** *3.56*
		D15S975	12.31	**3.69** *3.47*
		D15S156	13.84	**3.97** *3.48*
		D15S1019	18.31	**3.66** *2.36*
		D15S165	21.14	*3.52* 2.06
	16p13	D16S404	25.18	1.67 *2.26*
		D16S519	28.61	1.53 *2.29*
		D16S3075	30.88	1.75 *2.62*
		D16S3062	32.8	1.76 *2.5*

LOD, multipoint LOD scores.
Genome-wide significant results are in bold and suggestive linkages in italics.
[a]BMI included as covariate.

additional adjustment for BMI, the 15q peak remained significant but was not increased. Furthermore, suggestive evidence for female-specific linkage was also obtained on 16p13 (Table 2). For male–male pairs (Figure 1c), the strongest signal was seen on 13q12–13 (LOD = 2.10) but no suggestive or significant evidence of linkage could be detected. Including BMI as covariate did not affect these results.

The significance of sex-specific QTLs from the stratified analyses was further tested by adopting a formal sex-limited QTL test, parameterized in the Mx program.[23] The test was applied for the one marker showing the strongest evidence of linkage, marker D15S156 on 15q. The difference in −2 log likelihood value between the null and the sex-limited models was 21.6, corresponding to a *P*-value of 7×10^{-6} (25:50:25 mixture of 0, 1 and 2 df), which in turn corresponds to a χ^2 value (50:50 mixture of 0 and 1 df) of 18.93 and an LOD score of 4.1, somewhat higher than what we observed in the analysis restricted to female–female pairs (Table 2). For the female-specific signal at marker D16S3075 on 16p13, the same procedure was applied. The −2 log likelihood difference was 12.3, corresponding to a *P*-value of 0.0016 (25:50:25 mixture of 0, 1 and 2 df), which is equivalent to a χ^2 value of 10.01 (50:50 mixture of 0 and 1 df) and a sex-limited LOD score of 2.2, similar but not higher than what was obtained in the female-only analysis (Table 2).

At the four loci showing significant or suggestive evidence of linkage to apoA-I, we also investigated linkage to levels of HDL in a window of ± 15 cM (Table 3). At the 6p21–12 locus, the strongest evidence for linkage to HDL was found with the same marker (D6S459) with LOD scores with and without BMI adjustment of 3.13 and 2.17, respectively. The evidence of linkage to HDL at 12q23 locus was weaker with a maximum LOD score of 1.62 (BMI adjusted) at marker D12S1708, 13 cM from the initial apoA-I peak. A female-specific peak on 15q coincided but was less pronounced for HDL with an LOD score of 1.87 (BMI unadjusted) at the marker D15S156. At D16S3062, there was also some weak evidence for female linkage to HDL, LOD = 1.15 (BMI adjusted).

Table 3 Linkage results for HDL at loci showing evidence for linkage to apoA-I

Set	Cytogenic region	Marker	Position (cM)	LOD[a] LOD[a,b]
All pairs	6p21–12	D6S459	70.02	2.17 3.13
	12q21	D12S1708	97.67	1.14 1.62
Female–female pairs	15q11–13	D15S156	13.84	1.87 1.79
	16p13	D16S3062	32.8	0.64 1.15

[a]Peak multipoint LOD score within a ± 15 cM window from the marker with the highest apoA-I LOD score.
[b]BMI included as covariate.

Discussion

It is well acknowledged that the effect of individual QTLs might depend on genetic background and therefore vary considerably between populations. Some QTLs might even be restricted to specific ethnic groups or subgroups. It is thus important to investigate ethnically homogenous samples, ideally harmonized for early life events, as is the case in Swedish twins.

ApoA-I is known to be influenced by sex hormones.[26] Thus, it is not surprising to observe QTLs that differ between the sexes. The most striking signal observed in this study is a female-limited QTL in the 15q11–13 region. This locus has previously been reported to be significantly linked to apoA-I level, with a 2-point *P*-value of <0.00001.[11] It is also intriguing that this region is a heavily imprinted region harboring the Prader-Willi and Angelman syndrome loci and that it has been reported to show marked differences in recombination rates between the sexes.[27,28] The 15q11–13 region is very rich in genes and candidates are plenty, but one gene of special interest might be the phospholipase A2 group 4b (*PLA2G4B*), which hydrolyzes phospholipids and is involved in inflammation by initiating the production of inflammatory mediators (OMIM ref.: #606088). A contradictory observation is that one previous, large genome scan based on female DZ twin pairs found no linkage signal at this locus.[12] However, that sample was on average considerably younger than our sample, while our sample consisted of post-menopausal female subjects. The marked difference in linkage signals between the sexes is intriguing and raises the question whether the genetic variance is equal in males

and females. We found the intrapair correlation to be twice as large in female–female pairs than that in male–male DZ pairs, which could be due to a larger genetic variance in females, but the difference was not significant ($P = 0.09$). Two previous studies investigating sex differences in genetic variance of apoA-I among Swedish twins did not find evidence for such difference.[29,30]

The strongest signals in the full sample were seen on chromosomes 6 and 12 when BMI was included as a covariate in the variance component model. These peaks were of similar height and the only two findings in the total material surpassing suggestive evidence of linkage.

The position 6p21–12 overlaps with QTLs that have previously been reported linked to both level of HDL,[31] which is highly correlated to apoA-I ($r = 0.88$ in the investigated sample), as well as to CVD.[32] Consistent with the previous report,[31] we also found 6p21–12 to be linked to HDL level with an LOD after adjustment for BMI of 3.13 for the same marker (D6S459) that gave the strongest signal for apoA-I. Additional support for linkage to HDL on 6p, albeit approximately 40 cM upstream of our finding, has also been previously published.[33] The 6p21–12 region is rich in genes and contains, besides the large *MHC* region, lymphotoxin-α (formerly *TNFB*), which has been found to be associated with myocardial infarction,[34] and phospholipase A2 group VII (*PLA2G7*), which has been found to be a strong predictor of coronary heart disease.[35]

The QTL we observe on 12q23 is located near two previously implicated regions for apoA-I. Klos *et al*[14] found the highest LOD score peak (LOD = 2.02) for apoA-I at 12q24, about 18 cM downstream from the peak in the present study. By using regression-based linkage, Bosse *et al*[11] replicated this finding with a significant linkage ($P = 0.00003$) of a QTL located at 12q24 about 10 cM from our signal peak. Owing to the low resolution and wide confidence intervals in QTL linkage mapping,[36] all three of these signals can very well be due to the same underlying QTL. Furthermore, the 12q23 region has repeatedly been suggested linked to HDL[13,32,37,38] and bivariately linked to HDL and triglyceride levels.[39] There was some evidence for linkage to HDL at 12q23 in the present material as well (LOD = 1.62), although the highest LOD score was located 13 cM upstream of the apoA-I peak marker. A candidate gene located at 12q24.31, the scavenger receptor class B type 1 (*SRB1*) with a binding affinity for multiple apolipoproteins including apoA-I, has been suggested.[14] Overexpression of SRB1 in mice is associated with a decrease in apoA-I levels.[40] Another gene of potential candidacy may be pancreatic phospholipase A2 group 1b (*PLA2G1B*), located at 12q23–24.1, which has been associated to central fat mass and hypertension.[41]

The suggestive evidence of linkage in the female-only material at 16p13 was not increased in the formal sex-limited test. This locus has, however, also been implicated in previous studies on both apoA-I[11] and atherosclerosis/CVD.[42]

In two previously published genome-wide scans of apoA-I, significant linkage findings have been claimed. First, Falchi *et al* reports a signal on 9q21.32–33.1 with an LOD score of 3.28. It could not be replicated in the present study. After applying a two-locus ordered subset approach, Falchi *et al* reports two additional loci, 8p21.1–q13.1 (LOD = 3.71) and 10p15.1–p13 (LOD = 5.51).[12] Because the two latter results were conditioned on their best unidimensional finding (9q), they are not easily comparable to other genome scans. Second, Bosse *et al*[11] reports several positions to be significantly linked with either two-point (3p25.2, 5q21.3, 9q31.3, 12q24.21 and 15q11.2) or multipoint methods (4q31.21, 13q33.3 and 16q12.2). Our results lend support to the findings on 12q, 15q and 16q but not to the other five loci.

In comparison to using ordinary (non-twin) sib-pairs, there are a number of advantages in using twins for an unprejudiced search for QTLs. Firstly, non-paternity (ie, misspecification of biological father), which is believed to occur in approximately 2–5%[43] of births, is essentially eliminated when the study sample consists of twins. Secondly, because twins are born very close in time, shared environmental experiences will exert effects during the same developmental age. This will lead to a better matching of familial environment within pairs, increasing the likelihood that observed phenotypic differences within pairs are due to genetic causes. Another advantage of the present study is the high density of the marker panel used. Compared to the standard genome-wide panels, it contains more than twice the number of markers. We found this density of markers to increase marker information content by 26%. In linkage studies of complex traits, power is generally limited. High marker information content has been shown to be of particularly importance for power when parental information is missing.[25]

We are aware that the stratification of the study material by sex increases the number of tests performed and thus the risk of finding false-positive results. But given that apoA-I is known to be influenced by sex hormones, we believe that this is balanced by the risk of missing important signals of not performing sex-limited analyses.

In conclusion, this study adds to the understanding of variation in risk for atherosclerotic disease by reporting a locus on 15q showing significant linkage to serum levels of apoA-I among females. It is reassuring to see that among the four tentative loci on which we found evidence for linkage to apoA-I levels in the present material, two (12q and 15q) have previously been significantly linked to apoA-I levels, while all four have been reported linked either to apoA-I/HDL or to atherosclerosis/CVD. There are several interesting candidate genes located in each of these loci that remain for further exploration. The fact that three out of the four loci contain genes coding for members of the phospholipase A2 superfamily might provide an important clue for the involvement of them in atherosclerosis and justifies more detailed study.

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