

Linkage and Association Analyses of Longitudinally Measured Lipid Phenotypes in Adolescence

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The genetic basis of cardiovascular disease (CVD) is complex and still largely elusive. Plasma lipid concentrations are well-established risk factors for cardiovascular disease (CVD), and have adult heritabilities ranging from 0.48 to 0.87. Estimates for adolescents are slightly higher (range 0.71 to 0.82). To identify loci affecting lipid concentrations across adolescence, we analyzed longitudinal lipid data in a sample of 134 monozygotic and 626 dizygotic twin pairs at ages twelve, fourteen and sixteen, and their siblings, from 760 Australian families. Univariate linkage analysis for each phenotype and time point was supplemented by multivariate analysis across the time points. A genome-wide association scan was also performed on a subset of the subjects ($N = 441$). The strongest linkage was seen for triglycerides on chromosome 6p24.3 (multivariate $-\log_{10} p = 6.81$; equivalent LOD = 6.13; $p = 1.55 \times 10^{-7}$). Significant linkage was also found for LDL cholesterol on chromosome 2q35 (multivariate $-\log_{10} p = 5.59$; equivalent LOD = 4.53; $p = 2.57 \times 10^{-6}$). In the association analysis, rs10503840 on 8p21.1 was significantly associated with total cholesterol levels at age fourteen ($p = 8.24 \times 10^{-7}$, estimated significance threshold 2.45×10^{-6}). Association at $p < 2.25 \times 10^{-6}$ was also found between triglycerides at age 12 and rs10507266, in an intron of *THRAP2* (MIM 608771) on 12q24.21; and between HDL-C at age 14 and rs10506325 in an intergenic region of 12q13.13. Suggestive evidence of association at ages twelve and fourteen was found between HDL-C and rs10492859 on 16q23 ($p = 2.42 \times 10^{-5}$ and 2.77×10^{-4} , respectively). Further longitudinal genetic studies of cardiovascular risk factors, focused on critical periods of development or change, are needed.

Keywords: lipids, twin study, adolescents, longitudinal, association, linkage

Elucidation of the genetic basis of cardiovascular disease (CVD) is complicated by the existence of multiple risk factors leading to common clinical endpoints. Plasma lipids such as high-density lipoprotein (usually

measured as high-density lipoprotein cholesterol, HDL-C), low-density lipoprotein (usually estimated by calculation of low-density lipoprotein cholesterol, LDL-C), total cholesterol and triglyceride levels contribute to variation in the risk of cardiovascular disease. The underlying atherosclerotic process has been shown to commence in childhood or adolescence, and has been associated with levels of LDL-C, HDL-C and triglycerides in these age-groups (Berenson et al., 1992; McGill et al., 2000). Identification of genetic causes of variation in these risk factors could lead to targeted intervention, or the discovery of novel targets for drug development.

Cross-sectional twin studies have found substantial heritability for plasma lipid levels. Beekman et al. (2002) found that heritability estimates in a younger twin sample (aged 13–22) were higher (71%–82%) than in an adult sample (aged 34–92, 48%–77%). Similarly high heritabilities (69%–75%) have been reported in a recent study of young European American twins (mean age 17.9 ± 3.2 years) (Iliadou, Snieder et al., 2005). These findings suggest that lipid levels in adolescence are strongly influenced by genetic factors. Numerous genome scans to map quantitative trait loci (QTLs) influencing lipids have found evidence of linkage on multiple chromosomes in various population and ethnic groups. Genome-wide association studies have recently identified further significant regions (Kathiresan et al., 2008). However, most of these studies have focused on adults and were cross-sectional. Longitudinal data are useful to examine whether the same or different genes influence a trait at different ages, and may increase power through better estimation of the long-term mean of the phenotype. It is known that lipid levels change with age at different rates (Friedlander et al., 1997). The present

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study used longitudinal data from 760 Australian twin families to identify QTLs influencing high-density lipoprotein cholesterol (HDL-C), low-density lipoprotein cholesterol (LDL-C), total cholesterol and triglyceride levels. Using a combination of linkage and association analysis, we have investigated whether previous findings found in adults are replicated in adolescents. A further aim was to examine developmental change in the genes influencing lipid levels at 12, 14 and 16 years of age, corresponding roughly to the early, middle and late stages of puberty.

Materials and Methods

Subjects

The data were collected from studies of adolescent twins and their non-twin siblings living in south-east Queensland, Australia. Details of the studies are provided elsewhere (McGregor et al., 1999; Zhu et al., 1999; Wright et al., 2001; Wright & Martin, 2004). In summary, the data were collected from twins and their non-twin siblings, with families comprising up to five siblings (including the twins). A total of 2488 subjects were recruited and most twins participated in more than one study; siblings participated only once. The majority (~98%) of the sample was of mixed European ancestry (mainly British Isles). Blood was collected from twins, siblings, and from 86% of parents for blood grouping and DNA extraction. Zygosity of same-sex twin pairs was diagnosed by typing nine independent highly polymorphic DNA microsatellite markers (AmpFISTR Profiler plus Amplification Kit; Applied Biosystems, Foster City, California) and three blood groups (ABO, MNS and Rh), giving a probability of correct assignment greater than 99.99% (Nyholt, 2006). All participants and their parent/guardian (if younger than 18 years) gave written informed consent to the questionnaire and blood collection, and all studies were approved by the Human Research Ethics Committee of the Queensland Institute of Medical Research.

Serum Lipid Measurements

Total cholesterol, HDL-C and triglycerides were measured using Roche methods on a Hitachi 917 Analyzer. LDL-C was calculated from the total cholesterol, HDL-C, and triglyceride values by the Friedewald formula (Friedewald et al., 1972) if triglycerides were ≤ 4.52 mmol/L. These biochemical analyses were performed on blood samples from the adolescent twins and their siblings, but not on those from the parents.

Genotype Data

Genotypic data were available for 760 families with lipid phenotypes from the adolescent study. Genotype data in this study come from several genome scans performed at two facilities: the Australian Genome Research Facility (AGRF), Melbourne (757 microsatellite and 109,511 SNP markers) and the Centre for Mammalian Genotyping Service, Marshfield, USA (400 microsatellite markers). Details on the cleaning

and error checking of microsatellite genotypic data have been described in detail elsewhere (Zhu et al., 2004). The genome-wide SNP data were obtained using the Affymetrix GeneChip Mapping 100K set, consisting of the GeneChip Human Mapping 50k Array XbaI and the GeneChip Human Mapping 50K Array HindIII. Parents were only genotyped with the Xba array. Data cleaning for SNP genotypes involved checking the expected relationships between individual members and resolving Mendelian errors. First, the expected relationship between individual members was confirmed using identity-by-state across all SNPs using GRR, Graphical Representation of Relationships (Abecasis et al., 2001). Any discordant genotypes between MZ pairs were removed. For the Xba chip, all parent-offspring genotypes were checked for consistency, with genotypes for all family members being removed in the case of a mismatch. Second, genotypes consistent with unlikely recombination events were identified and removed using MERLIN 1.0 (Abecasis & Wigginton, 2005). Families that had genotype errors suggestive of sample swaps or pedigree errors that could not be resolved were dropped from further analysis. In the SNP linkage genotypic data (which is a 15K subset of the complete SNP dataset), any adjacent SNPs with a distance of less than 0.1 cM were excluded to avoid biasing of linkage results by LD between markers.

The final genotypic data available for genome-wide linkage analysis comprised up to 17,249 markers (1186 microsatellite markers and 16,063 SNPs) from 811 families. 760 families had both genotypic and phenotypic data. Details are provided in Table 1.

The final genotypic data available for genome-wide association analysis consisted of a total of 676 individuals from 169 families. Of the 676 individuals, only 441 individuals (163 families) had both genotypic and phenotypic measurements. A total of 109,511 markers were genotyped on these individuals.

Statistical Analysis

Prior to adjustment for covariates and linkage and association analyses, variables were checked for the normality assumption. Only the triglyceride variable required transformation, by logarithm of base 10 (\log_{10}), as the distribution was skewed. Family outliers exceeding four standard deviations from the mean were identified using option %p in *Mx* and *Viewdist* (Beeby et al., 2006) and a total of 32 twin families (LDL-C: 4; HDL-C: 9; total cholesterol:6; triglycerides:13) were excluded from the analysis on this basis.

Genome-Wide Linkage

All linkage analyses were conducted by variance component analysis using full information maximum likelihood methods implemented in *Mx*1.6.1 (Neale, 2005), which uses all raw data. Covariates including age, squared age (age^2), sex, sex x age and sex x age^2 were used. Multipoint estimates of the probabilities of the sib-pairs sharing 0, 1, or 2 alleles identical by

Table 1
General Characteristics of Participants, Genotypic and Phenotypic Data

Characteristics	Age	MZ families (N in SNP set)	DZ families (N in SNP set)
Genotypic			
Number of families		134 (50)	628* (104)
Number of individuals	12	355	1126
	14	236	960
	16	176	824
Number of sibships of size:			
1	12	0	1
	14	1	21
	16	3	19
2	12	21	288
	14	66	340
	16	29	291
3	12	74	130
	14	35	65
	16	33	63
4	12	21	34
	14	0	16
	16	4	6
5	12	2	3
	14	0	0
	16	0	2
Number of markers per person (range)		Microsatellite 23–790, SNP 8009–16028	
Mean number of markers per person (\pm SD)		Microsatellite 543 \pm 193, SNP 15539 \pm 1067	
Mean marker spacing (\pm SD)		Microsatellite 7.75 \pm 3.03, SNP 0.29 \pm 0.18	
Female (%)		50.9	50.7
Phenotypic			
HDL-C (mmol/l)	12	1.46 \pm 0.30	1.45 \pm 0.34
Mean (\pm SD)	14	1.41 \pm 0.28	1.38 \pm 0.30
	16	1.40 \pm 0.31	1.36 \pm 0.30
LDL-C (mmol/l)	12	2.43 \pm 0.61	2.46 \pm 0.69
Mean (\pm SD)	14	2.45 \pm 0.64	2.39 \pm 0.65
	16	2.48 \pm 0.66	2.43 \pm 0.74
Total cholesterol (mmol/l)	12	4.47 \pm 0.70	4.48 \pm 0.76
Mean (\pm SD)	14	4.39 \pm 0.74	4.31 \pm 0.73
	16	4.32 \pm 0.84	4.28 \pm 0.89
Triglycerides (mmol/l, log)	12	0.06 \pm 0.20	0.07 \pm 0.19
Mean (\pm SD)	14	0.03 \pm 0.17	0.04 \pm 0.18
	16	-0.04 \pm 0.17	0.01 \pm 0.18

Note: HDL-C: High-density lipoprotein cholesterol; LDL-C: Low-density lipoprotein cholesterol; SD: standard deviation

* Two families were typed with both the microsatellite and SNP sets.

descent (IBD) were calculated every 5 cM along the genome using the MERLIN program. For each marker locus, the estimated proportion of alleles shared IBD is calculated as $\frac{1}{2} p(\text{IBD}=1) + p(\text{IBD}=2)$ where p is the probability that a given sib-pair shares alleles IBD at a given marker locus. Based on structural equation modeling (SEM) in Mx (Neale, 2005) the proportion of alleles shared IBD was used to estimate the component of variance due to a QTL. The best-fitting model (determined previously in Middelberg et al., [2007]) was extended to incorporate QTL parameters. For the autosomal univariate variance component QTL analysis, for LDL-C, total cholesterol and triglycerides, the total variance was modeled as the sum of the additive (σ_A^2), linked QTL (σ_Q^2) and unique environmental variances (σ_E^2) compared to a null model in which variance

due to the linked QTL was set to zero. For HDL-C, a common environmental component variance (σ_c^2) was also included. To allow familial covariance to be divided into polygenic additive variance and shared environment, 134 MZ twin families were also included in the analysis. Fits of the null and alternate models were compared using the likelihood ratio chi-square test, p values were converted to $-\log_{10}p$ and to LOD scores. The LOD score cut-offs used were 2.19 (equivalent $-\log_{10}p = 3.13$) for suggestive linkage and 3.63 (equivalent $-\log_{10}p = 4.66$) for significant linkage (Lander & Kruglyak, 1995).

For the autosomal multivariate analysis, the use of full information maximum likelihood within Mx analyses allowed the individuals with missing data to be included in the analysis. Similarly, alternate ($H_1; \sigma_p^2$

$= \sigma_A^2 + \sigma_O^2 + \sigma_E^2$) and null ($H_0: \sigma_P^2 = \sigma_A^2 + \sigma_E^2$) models were compared. Two multivariate tests for QTL linkage were performed. First, factor loadings of the QTL (on lipid traits at ages 12, 14, and 16) were unconstrained and were compared to equating the factor loadings of the three measurement occasions set to zero (i.e., difference in minus twice the log-likelihood between the model with unconstrained loadings and model with zero loadings); this was evaluated against a χ^2 distribution with three degrees of freedom ('multivariate test 1'). The second test ('multivariate test 2') involved a model with equated loadings on the QTL, which assumes that the QTL is responsible for the same amount of phenotype variation at each age. The difference in minus twice the log-likelihood between the model with equated loadings and the model with the zero loadings was calculated and evaluated against a χ^2 distribution with one degree of freedom. As linkage results from univariate and multivariate analyses have different distributions, all results are presented as $-\log_{10}p$.

For X-chromosome linkage analyses, a simple extension of the X-linked variance component model (Jardine and Martin, 1984) was implemented, where an extra additive genetic variance component is modeled with the coefficient of relatedness (set to 0.5 in the autosomal case) depending on the sexes of the siblings for each sib-pair combination. Assuming completely random X-inactivation, the coefficient of X-chromosome relationship (Ekstrom, 2004) was set to 0.5 for brother-brother pairs, 0.375 for sister-sister pairs, and 0.25 for opposite-sex pairs. In the multivariate analyses, an additional X-linked additive genetic variance component was patterned as a 3×3 Cholesky decomposition. The QTL model assumed complete random X-inactivation, so that the X-linked QTL variance in females was set to be half that of males.

Genome-Wide Association Analysis

Genome-wide association analysis was carried out using 109,511 markers on 441 individuals (163 families). All association analyses were conducted using the Fulker method (Fulker & Cherny, 1996; Fulker et al., 1999; Abecasis, Cardon et al., 2000; Abecasis, Cookson et al., 2000; Abecasis et al., 2001) to test for evidence of significant association between lipids (HDL-C, LDL-C, total cholesterol or triglycerides) at age 12 and a SNP marker; and similarly to test for association at other ages (14 and 16). The association test was based on a model that decomposed trait variance into additive genetic, unique environmental and quantitative trait locus components (i.e., AEQ model) except for HDL-C where an ACEQ model was fitted (see linkage analysis section). The Fulker test partitioned the effect due to association into two orthogonal components — a between-family component (β_B) and a within-family component (β_w). The between-family component reflects both genuine association and any effects due to population stratification, while the within-family component reflects association free from

the effect of population structure. Thus, the comparison of the likelihood of a model with all parameters free versus a model where $\beta_w = 0$ gives a robust test of association. Twice the difference in log-likelihood between the models is asymptotically distributed as a distribution χ^2 with one degree of freedom. To test for the presence of stratification, a comparison of the likelihood of a model with all parameters free versus a model where $\beta_w = \beta_B$ was fitted (Fulker & Cherny, 1996; Fulker et al., 1999; Abecasis et al., 2000). Again, the test is asymptotically distributed as a χ^2 distribution with one degree of freedom. Analyses were run as described at <http://www.sph.umich.edu/csg/abecasis/QTDT/> using the covariates age, squared age (age^2), sex, sex \times age and sex \times age^2 . Tests for association and population stratification were performed using the QTDT program (version 2.4.3). The genome-wide significant association was obtained using the max(T) permutation procedure in PLINK (v1.03) (<http://pngu.mgh.harvard.edu/purcell/plink/>) (Purcell et al., 2007); 10,000 iterations were used to determine the total number of independent SNPs (or tests) across the genome. Genome-wide p values for association analysis were Bonferroni-corrected using this number of independent SNPs.

Results

Linkage Results

The results from genome-wide univariate and multivariate variance component linkage analyses are shown in Figures 1 to 4. To compare the consistency of results across measurement occasions, each plot displays $-\log_{10}p$ at all three ages and the two multivariate tests. Note that the scale on the y-axis ($-\log_{10}p$) varies to accommodate the data points. Suggestive linkages (those with $-\log_{10}p > 3.13$; equivalent LOD = 2.19) and significant linkages (those with $-\log_{10}p > 4.66$; equivalent LOD = 3.63) based on criteria proposed by Lander and Kruglyak (1995) from univariate and multivariate analyses are summarized in Tables 2 and 3.

The strongest evidence from the univariate variance component tests for linkage was seen in log (triglycerides) at age 12 in region 6p24.3 (20cM, $-\log_{10}p = 4.40$; equivalent LOD = 3.38; $p = .00004$). At age 14, there was also a smaller peak in the same location (20cM, $-\log_{10}p = 2.32$; equivalent LOD = 1.46; $p = .0047$). There was no evidence for linkage to triglycerides at age 16. Multivariate linkage showed stronger evidence of linkage at the same position ($-\log_{10}p = 6.81$; equivalent LOD = 6.13; $p = 1.6 \times 10^{-7}$; Table 3, Figure 5).

Multivariate linkage analysis revealed multiple peaks with LOD greater than 2.2 for LDL-C; most notably on chromosome 2q35 (235cM, $-\log_{10}p = 5.59$; equivalent LOD = 4.53; $p = 2.57 \times 10^{-6}$), but also at 1q32.1, 4p15.1, 5q13.2, 11p14.3 and 18q11.2 (see Table 3). These peaks were not significant in the univariate linkage tests.

For HDL-C at age 14, chromosome 2q31.1 showed suggestive evidence of linkage (180 cM, $-\log_{10}p = 3.82$;

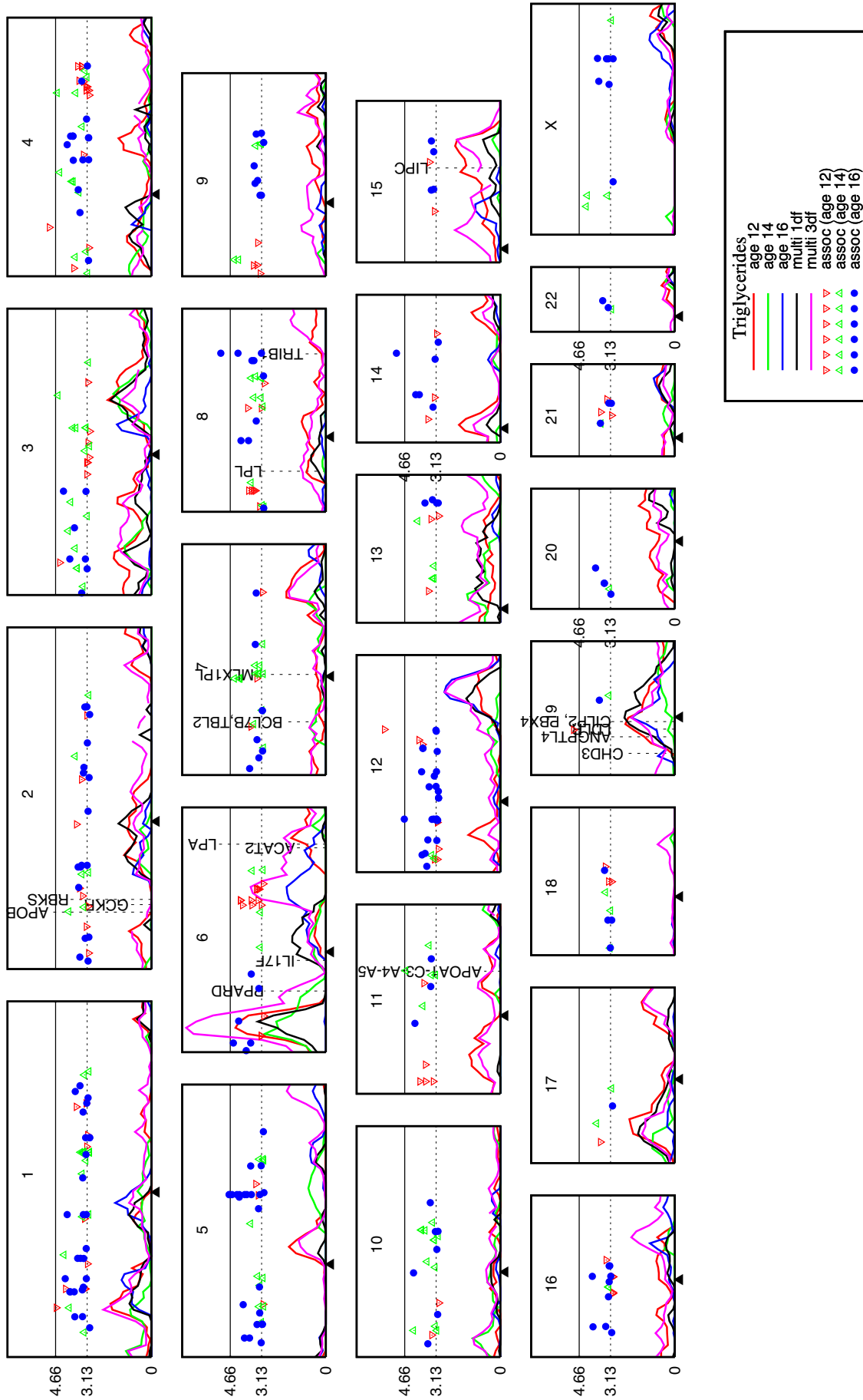


Figure 1

Multipoint linkage analysis of $\log_{10}(\text{triglycerides})$ for autosomal and X chromosomes. X-axis shows the genetic map of each chromosome and y-axis shows the maximized $-\log_{10}(p)$. Lines represent the results from univariate and multivariate linkage analyses. Dots represent results from univariate association analyses Thresholds for significant ($-\log_{10}(p)=3.13$) and suggestive ($-\log_{10}(p)=4.66$) genome wide linkage are shown.

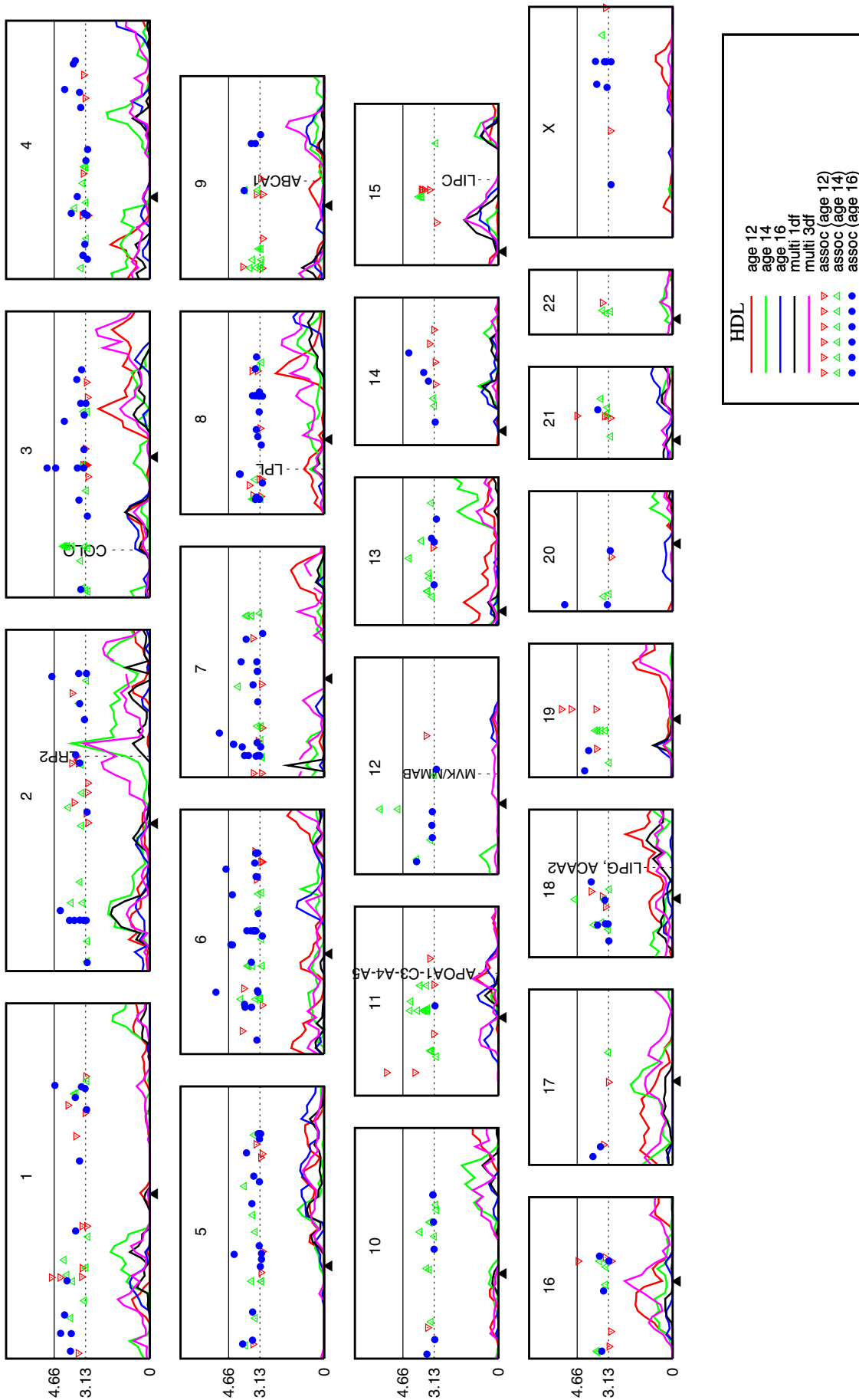


Figure 2

Multipoint linkage analysis of HDL for autosomal and X chromosomes. X-axis shows the genetic map of each chromosome and y-axis shows the maximized $-\log_{10}p$. Lines represent the results from univariate and multivariate linkage analyses. Dots represent results from univariate association analyses thresholds for significant ($-\log_{10}p=3.13$) and suggestive ($-\log_{10}p=4.66$) genome wide linkage are shown.

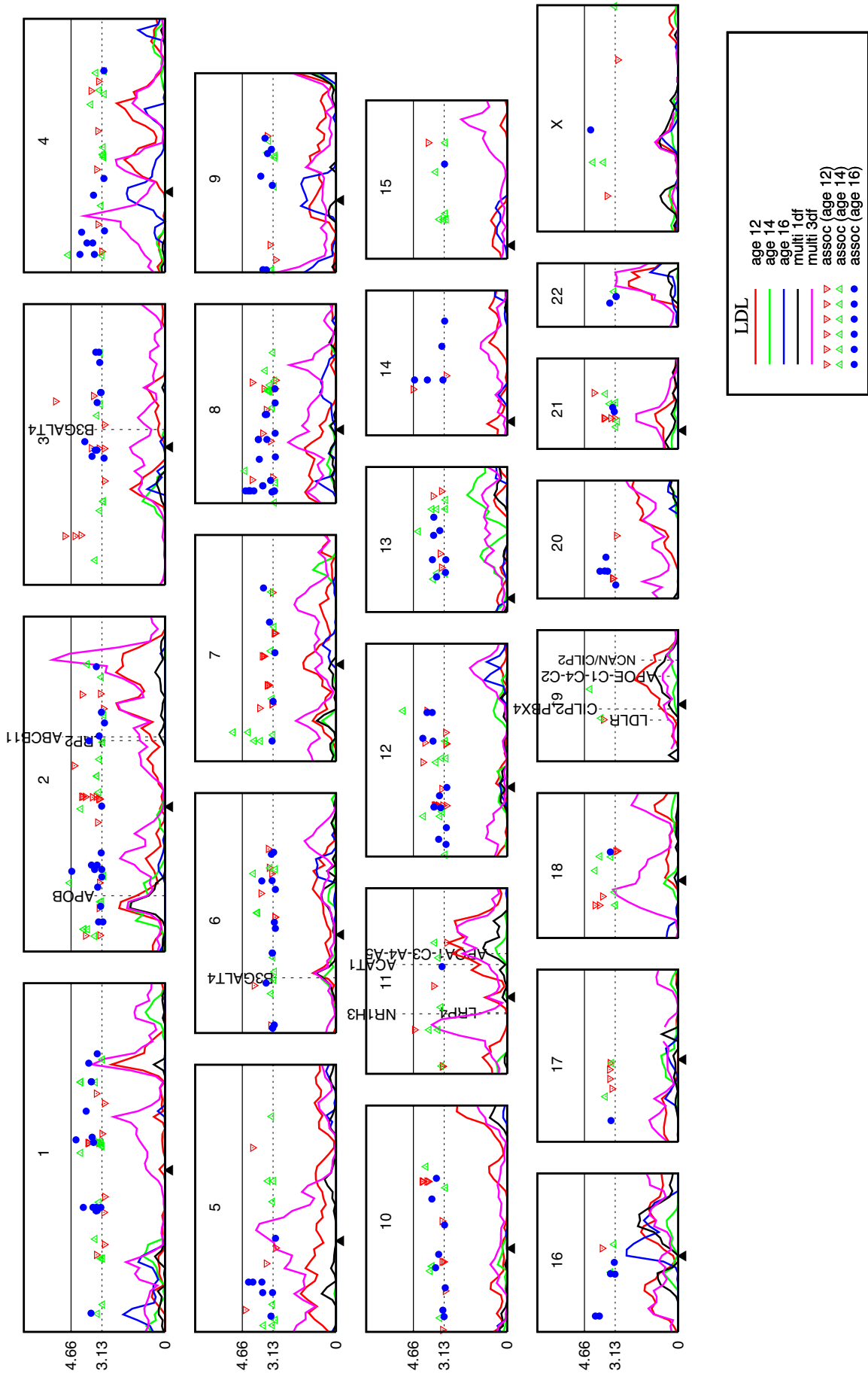


Figure 3

Multipoint linkage analysis of LDL for autosomal and X chromosomes. X-axis shows the genetic map of each chromosome and y-axis shows the maximized $-\log_{10}p$. Lines represent the results from univariate and multivariate linkage analyses. Dots represent results from univariate association analyses for significant ($-\log_{10}p=3.13$) and suggestive ($-\log_{10}p=4.66$) genome wide linkage are shown.

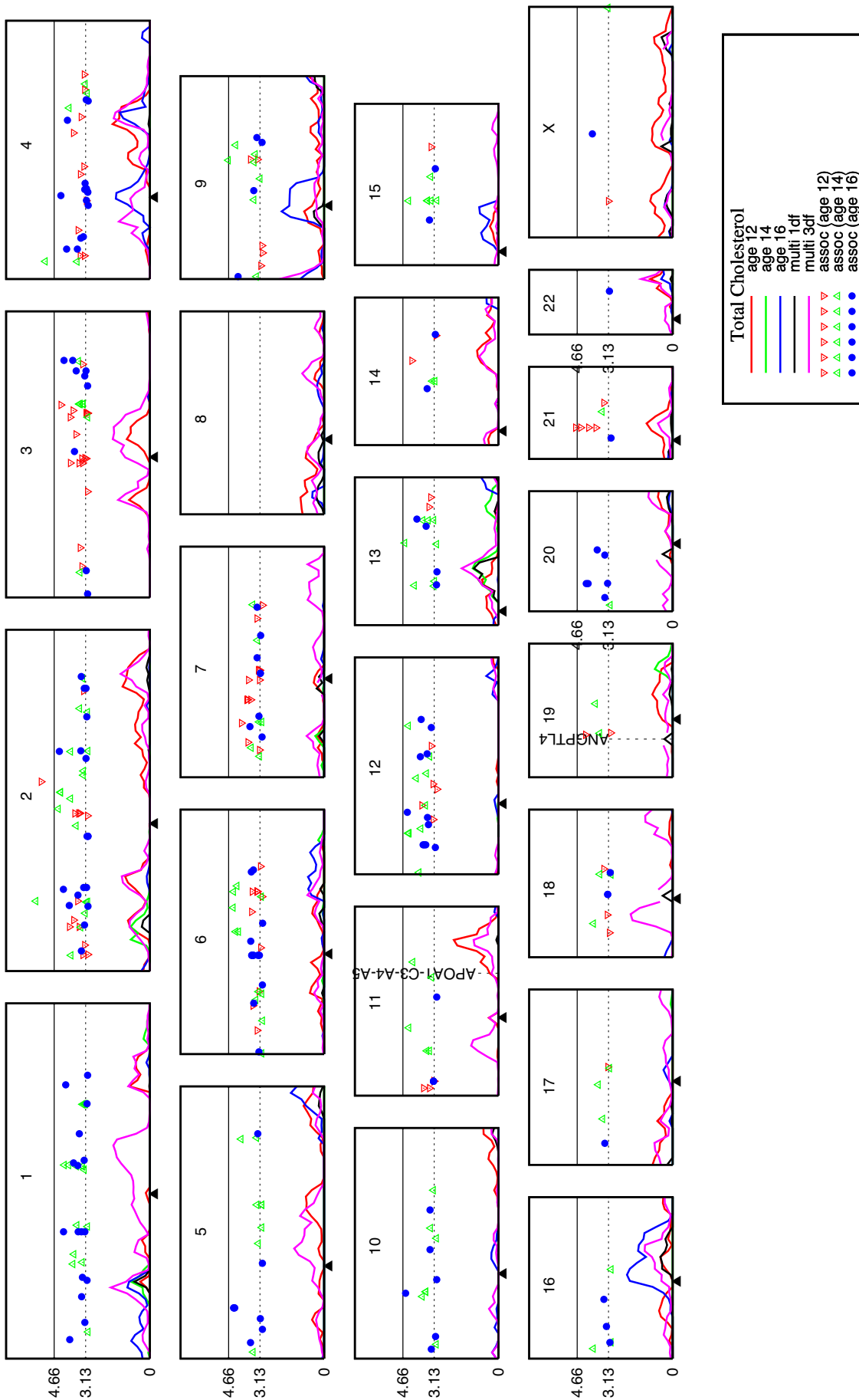


Figure 4

Multipoint linkage analysis of total cholesterol for autosomal and X chromosomes. X-axis shows the genetic map of each chromosome and y-axis shows the maximized $-\log_{10} p$. Lines represent the results from univariate and multivariate linkage analyses. Dots represent results from univariate association analyses thresholds for significant ($-\log_{10} p=3.13$) and suggestive ($-\log_{10} p=4.66$) genome wide linkage are shown.

Table 2Regions of Suggestive Linkage ($-\log_{10}p > 2.2$) in the Univariate Analyses for Lipid Variables

Age	Chr	Position of peak (cM)	Peak Score ($-\log_{10}p$)	Nearest SNP	Physical Position (bp)	Region
HDL-C (mmol/l)						
12	3	155	2.50	rs7639109	144476884	q24
12	8	125	2.36	rs2022962	108262506	q23.1
12	18	100	2.29	rs967631	68505397	q22.3
14	2	175	2.53	rs10497239	164239551	q24.3
14	2	180	3.82	rs10490132	169717207	q31.1
14	13	130	2.20	rs561241	112808035	q34
LDL-C (mmol/l)						
12	1	230	2.65	rs2244783	201454716	q32.1
12	2	35	2.27	rs2345837	18454189	p24.2
12	2	195	2.27	rs1518423	180703408	q31.3
12	2	200	2.42	rs7419276	190027433	q32.2
12	2	225	2.42	rs1510840	216942053	q35
12	4	150	2.40	rs6831717	142498007	q31.21
12	10	180	2.50	rs1491192	133792769	q26.3
12	10	185	2.47	rs2252728	135125348	q26.3
12	11	110	2.70	rs1961370	98257643	q22.1
12	11	115	2.24	rs10488759	102996520	q22.3
12	11	120	2.92	rs1371330	108887902	q22.3
12	11	130	2.46	rs543819	116255283	q23.3
12	11	135	2.51	rs10502233	119398962	q23.3
12	11	140	2.60	rs872414	122170647	q24.1
12	20	100	2.29	rs6089982	61871587	q13.33
12	20	105	2.20	rs2427625	62342703	q13.33
12	22	45	2.46	rs137964	38634527	q13.1
12	22	50	2.46	rs135916	43175021	q13.31
12	22	60	2.28	rs5768312	46835938	q13.31
16	16	65	2.57	rs1420533	51121127	q12.1
16	16	70	2.56	rs2397445	54007250	q12.2
16	16	75	2.21	rs4784833	56206869	q13
Total cholesterol (mmol/l)						
12	11	140	2.17	rs872414	122170647	q24.1
16	16	70	2.23	rs2397445	54007250	q12.2
Triglycerides (log, mmol/l)						
12	6	15	3.84	rs2274393	6127604	p25.1
12	6	20	4.40	rs1537656	8060391	p24.3
12	6	25	4.21	rs10484446	11239151	p24.1
12	6	30	3.77	rs2119483	13514639	p23
12	15	90	2.20	rs1371386	87824056	q26.1
12	17	30	2.21	rs10521217	13219981	p12
12	19	40	2.41	rs876982	18643377	p13.11
12	19	45	2.27	rs2159131	33633026	q12
14	6	15	2.98	rs2274393	6127604	p25.1
14	6	20	2.32	rs1537656	8060391	p24.3
14	6	25	2.42	rs10484446	11239151	p24.1
16	12	140	2.44	rs530454	116604993	q24.22
16	12	145	2.72	rs925531	120637693	q24.31
16	12	150	2.64	rs879940	124319027	q24.31
16	12	155	2.36	rs2111368	126098472	q24.32

Note: HDL-C: High-density lipoprotein cholesterol; LDL-C: Low-density lipoprotein cholesterol; cM, centiMorgans. Results are grouped by variable, then age, then chromosomal location.

equivalent $LOD=2.84$; $p = .00015$). A small peak in the same region was found at age twelve ($-\log_{10}p = 0.12$) but not at age 16. In the multivariate linkage, at the same position the $-\log_{10}p$ score was 3.22 (equivalent $LOD = 2.28$; $p = .00061$).

In the univariate linkage tests, some regions displayed evidence of linkage for more than one variable. For example, peaks were observed at chromosomes 11q24.1 and 16q12.2 for both LDL-C and

total cholesterol at age 12 (Table 2). It must be noted that LDL-C was derived using the total cholesterol measurement, so a linkage peak in the same chromosomal region may simply be due to overlap between these variables.

Association Analysis

Summaries of the results from the genome-wide association scans are displayed in Tables 4 and 5. The study-wide p value with this 100K marker set was

Table 3Linkage Regions with Multivariate $-\log_{10}p \geq 3.13$

Variable	Chr	Region	Location (cM)	Nearest SNP	Univariate $-\log_{10}p$			Multivariate		
					Age 12	Age 14	Age 16	-2LL	$-\log_{10}p$	LOD
HDL-C	2	q31.1	180	rs10490132	0.12	3.82	0.08	17.33	3.22	2.28
LDL-C	1	q32.1	230	rs2244783	2.65	0.03	0.00	19.30	3.62	2.65
	2	q35	235	rs4674417	2.13	0.00	0.00	28.72	5.59	4.53
	4	p15.1	50	rs1373869	0.07	0.00	0.41	21.09	4.00	3.00
	5	q13.2	80	rs6871754	0.98	0.00	0.00	20.99	3.97	2.98
	11	p14.3	45	rs7109666	0.00	0.00	0.00	19.87	3.74	2.76
	18	q11.2	40	rs4800467	0.04	0.26	0.00	17.43	3.24	2.29
	22	q13.31	60	rs5768312	2.28	0.02	1.38	16.90	3.13	2.19
Triglycerides	6	p24.3	20	rs1537656	4.40	2.32	0.00	34.49	6.81	6.13

Note: (Values for univariate analyses at each age are also shown)

HDL-C: High-density lipoprotein cholesterol; LDL-C: Low-density lipoprotein cholesterol; Trig: \log_{10} (triglycerides); cM: centiMorgans; Chr: chromosome.

(0.05/20,439) = 2.45×10^{-6} . In order not to miss potentially significant associations, all loci with a p value less than a liberal threshold of 1×10^{-5} are shown. The most significant finding ($p = 8.24 \times 10^{-7}$) was an association between total cholesterol at age 14 and rs10503840 on chromosome 8p21.1. For triglycerides (at age 12), a significant association ($p = 2.25 \times 10^{-6}$) was found with rs10507266 on 12q24.21. This marker is in an intron of *THRAP2* (MIM 608771), a gene involved in early development of heart and brain (Muncke et al., 2003). For HDL-C (at age 14), the strongest association ($p = 1.74 \times 10^{-6}$) was with rs10506325 on 12q13.13, 70.6 kb from the *HOXC13* gene. For LDL-C, no marker met the significance threshold but the strongest association (at age 12), was $p = 3.67 \times 10^{-6}$ with rs3842879 on 3q25.2, in an intron of the *NELL1* gene.

Table 5 shows the loci associated with lipid traits at more than one time. For HDL-C, at ages 12 and 14 there was some evidence of association at marker rs10492859 ($p = 2.42 \times 10^{-5}$ and $p = 2.77 \times 10^{-4}$ respectively). For total cholesterol, three SNPs (rs4450153, rs4453219 and rs4453220) on 11p15.2 showed some evidence of association across ages 12 and 16 and showed weak evidence of association across all three ages (12: $p < 1 \times 10^{-3}$, 14: $p < 0.05$, 16n: $p < 1 \times 10^{-3}$). For triglycerides, variant rs2363810 on 1p34.2 also showed some evidence of association at ages 12 and 14 and weak evidence of association across all three ages (12: $p = 2.35 \times 10^{-5}$, 14: $p = 1.02 \times 10^{-5}$ and 16: $p = .030$).

Evidence of overlap between the association results and the linkage peaks was also examined. There was weak evidence of association for LDL-C at age 12 and rs7419276 ($p = .010$), corresponding to the linkage peak on chromosome 2 (200cM, $-\log_{10}p = 2.42$); for triglycerides at age 16 and rs879940 ($p = .006$), corresponding to the linkage peak on chromosome 12 (150cM, $-\log_{10}p = 2.64$); for LDL-C at age 16 and rs1420533 ($p = .046$), corresponding to the linkage

peak on chromosome 16 (65 cM, $-\log_{10}p = 2.57$); and for LDL-C at age 12 and marker rs5768312 ($p = .008$), corresponding to the linkage peak found on chromosome 22 (60cM, $-\log_{10}p = 2.28$).

Discussion

This paper reports the use of genome-wide linkage and association scans to find genes whose variation influences lipid levels in adolescence. The most promising result from this study is the linkage peak on chromosome 6p for triglycerides. This was present in both univariate (ages 12 and 14) and multivariate linkage analyses. Several regions of linkage were also identified for LDL-C (chromosomes 1q, 2q, 4p, 5q, 11p and 18q), the most notable being on chromosome 2q35. As expected, multivariate analysis detected linkage at several regions where evidence of linkage in the univariate tests was nonsignificant (Martin et al., 1997). We also found some SNP associations that reached study-wide significance for the 100K marker set used.

Linkage Findings

The largest linkage peak was for triglycerides on chromosome 6p24.3, at 20cM (nearest marker rs1537656), with an observed $-\log_{10}p$ value of 6.81 (equivalent to LOD = 6.13). The multivariate LDL-C peak on chromosome 2q35 (multivariate LOD = 4.53) was also clearly above the significant value of LOD = 3.63. For HDL-C, the linkage peak (LOD = 2.27) observed on chromosome 2q31.1 (180 cM) was suggestive.

Several studies have reported regions on chromosome 6p linked to susceptibility loci influencing cholesterol, triglycerides or the triglycerides/HDL ratio (Coon et al., 2001; Klos et al., 2001; Canizales-Quinteros et al., 2003). There is strong evidence for a QTL on chromosome 6p12.3-q13 (but at 73 to 80cM) that influences HDL-C levels in a multigenerational Mexican kindred (Canizales-Quinteros et al., 2003), triglyceride levels in African-American families from the HyperGEN study (Coon et al., 2001) and triglyc-

Table 4

Summary of Loci Associated with Blood Lipids at Single Time-Points (p Value $< 1 \times 10^{-5}$), Ranked by p Value: The First Three Results are Significant at the Study-Wide Significance Threshold of 2.45×10^{-6}

Trait	SNP	Locus	SNP type	Nearest gene (distance, kb)	Minor allele frequency	P values	Effect of minor allele (s.e.)
Chol (age 14)	rs10503840	8p21.1	intergenic	<i>DUSP4</i> (61.5 kb)	C 0.055	8.24×10^{-7}	-0.943 (0.164)
HDL (age 14)	rs10506325	12q13.13	intergenic	<i>HOXC13</i> (70.6 kb)	A 0.500	1.74×10^{-6}	0.211 (0.039)
Trig (age 12)	rs10507266	12q24.21	intronic	<i>THRAP2</i>	C 0.072	2.25×10^{-6}	-0.167 (0.028)
Chol (age 14)	rs3861571	2p16.1	intronic	<i>KIAA1212</i>	A 0.007	2.84×10^{-6}	-0.092 (0.075)
HDL (age 12)	rs327035	11p15.1	intronic	<i>NELL1</i>	A 0.160	3.43×10^{-6}	-0.169 (0.032)
LDL (age 12)	rs3842879	3q25.2	intronic	<i>MBNL1</i>	C 0.464	3.67×10^{-6}	-0.314 (0.054)
HDL (age 12)	rs2009373	19q13.32	intronic	<i>CARD8</i>	T 0.393	3.69×10^{-6}	-0.151 (0.025)
Chol (age 12)	rs1404683	2q23.2	intronic	<i>LOC130576</i>	T 0.433	5.05×10^{-6}	-0.291 (0.057)
HDL (age 16)	rs2225204	6p12.3	intergenic	<i>CR592675</i> (50.2 kb)	T 0.152	5.32×10^{-6}	0.276 (0.059)
HDL (age 16)	rs6053733	20p12.3	intronic	<i>FLJ2507</i>	C 0.247	5.43×10^{-6}	-0.222 (0.053)
LDL (age 14)	rs674021	12q24.31	intronic	<i>SPPL3</i>	G 0.262	6.94×10^{-6}	-0.383 (0.074)
Trig (age 16)	rs2045856	8q24.3	intergenic	<i>KCNK9</i> (220.4 kb)	C 0.158	7.58×10^{-6}	0.132 (0.030)
LDL (age 14)	rs6972736	7p15.2	intronic	<i>OSBPL3</i>	T 0.359	7.78×10^{-6}	0.429 (0.079)
HDL (age 16)	rs1882075	7p14.2	intronic	<i>ELMO1</i>	C 0.039	7.87×10^{-6}	0.511 (0.111)
Chol (age 14)	rs3796878	4p15.32	intronic	<i>CD38</i>	A 0.141	8.03×10^{-6}	0.635 (0.119)
Trig (age 16)	rs10483873	14q24.3	Intronic	<i>MGC16028</i>	T 0.348	8.78×10^{-6}	-0.119 (0.026)
HDL (age 16)	rs10511235	3q13.11	intergenic	<i>ALCAM</i> (272.1 kb)	G 0.069	9.85×10^{-6}	-0.399 (0.081)

Note: HDL: High-density lipoprotein cholesterol; LDL: Low-density lipoprotein cholesterol; Trig: \log_{10} (triglycerides); Chol: total cholesterol.

erides/HDL ratio in a population from Minnesota (Klos et al., 2001). The only known candidate gene in that region is interleukin 17F (*IL17F*; MIM 606496). The peak found in those studies is quite some distance from the peak identified in our study (see Figure 4). Our peak was closer to the peak previously found by Bielinski et al. (2006) (25 cM, LOD = 2.6) for total cholesterol levels in diabetic populations. There are no obvious known candidate genes in this region of chromosome 6. The nearest candidate gene in this chromosomal region is peroxisome proliferator-activated receptor-delta (*PPARD*; MIM 600409), which is located at a considerable distance (Figure 5). This gene maps to chromosome 6p21.2–p21.1, is a member of the PPARD subfamily of nuclear receptors, and acts as a sensor for polyunsaturated fatty acids and VLDL lipoprotein particles (Barish et al., 2006). It has been shown that PPARD agonists, like those for PPAR-alpha, suppress expression of inflammation genes in macrophages, and thereby suppress the development of atherosclerosis in animal models (Sonoda et al., 2008). PPARD-alpha agonists are under extensive exploration; such agonists are aimed at raising serum HDL in patients with atherogenic dyslipidemia, reducing serum triglycerides and increasing HDL in animal models, as well as humans (Sonoda et al., 2008).

Previous published studies have reported suggestive linkages to lipid-related phenotypes at or near chromosome 2q31. Pajukanta et al. (1999) reported a region on chromosome 2q31 at 186 cM ($Z = 2.25$, $p = .0006$) for triglycerides among Finnish families. Horne et al. (2003) reported suggestive evidence for linkage

on chromosome 2 at 201 cM (LOD = 2.29) for the ratio of triglycerides/HDL. One potential candidate gene related to LDL-C in this chromosomal region is low density lipoprotein receptor-related protein 2 (*LRP2*; MIM 600073), which maps to chromosome 2q24–q31 (160,000–171,600 Kbp). This gene was originally identified as Megalin, a primary antigen in Heyman nephritis (Kerjaschki & Farquhar, 1982; Kerjaschki & Farquhar, 1983). This member of the LDLR-related protein (*LRP*) family is a multiligand receptor that is expressed in a number of different tissues but mainly in glomeruli and proximal tubule cells of the kidney. Members of this family are cell-surface receptors that transport macromolecules. In vitro studies using cultured cells have suggested that *LRP2* participates in LDL catabolism by binding to apoB-100 (Stefansson et al., 1995) and that *LRP2* also cooperates with cubilin to mediate HDL endocytosis (Hammad et al., 2000). Association between *LRP2* and levels of total cholesterol and LDL-C has recently been identified (Mii et al., 2007). Significantly lower total cholesterol in people who carry the C allele +193826T/C polymorphism and lower LDL-C in people who carried G allele IVS55-147A/G polymorphism have been found. The other possible, but more distant, gene on chromosome 2q is ATP-binding cassette, subfamily B, member 11 (*ABCB11*) which is located on chromosome 2q24 (165,900–171,200K bp) (MIM 603201), and has been associated with progressive familial cholestasis.

Several possible candidate genes located in the chromosomal regions that had suggestive (or significant)

Table 5
Summary of Loci Associated With Blood Lipids (Nominal p Values < 0.001) Across Time

SNP	Locus	Type of SNP	Nearest gene (distance, kb)	Minor allele freq At age12 At age14 At age16	Age 12 Effect of minor allele	P values	Age 14 Effect of minor allele	P values	Age 16 Effect of minor allele	P values
LDL-C (mmol/l) rs10492859	16q23.3	intronic	<i>CDH13</i>	G 0.245 G 0.241 G 0.291	0.145 (0.028)	2.42 x 10 ⁻⁵	0.158 (0.035)	2.77 x 10 ⁻⁴	-0.024 (0.057)	0.919
rs5925342	Xq28	intergenic	<i>MAGEA1</i> (53.3 kb)	T 0.227 T 0.243 T 0.160	0.103 (0.024)	5.20 x 10 ⁻⁴	0.087 (0.032)	0.017	0.071 (0.061)	0.36
LDL-C (mmol/l) rs10494412	1q23.3	intergenic	<i>PBX1</i> (316.8 kb)	G 0.060 G 0.062 G 0.116	0.395 (0.111)	1.63x10 ⁻⁴	0.558 (0.135)	7.00x10 ⁻⁴	-0.010 (0.208)	0.878
rs10494413	1q23.3	intergenic	<i>PBX1</i> (316.8kb)	T 0.059 T 0.059 T 0.116	0.395 (0.112)	1.45 x 10 ⁻⁴	0.568 (0.138)	6.56x10 ⁻⁴	-0.010 (0.208)	0.864
rs6697445	1q23.3	intergenic	<i>PS6K43</i> (315 kb)	C 0.060 C 0.063 C 0.116	-0.395 (0.111)	1.63 x 10 ⁻⁴	-0.558 (0.135)	5.02x10 ⁻⁴	0.010 (0.208)	0.868
rs3861571	2p16.1	intergenic	<i>KIAA12</i> (65.6 kb)	A 0.006 A 0.007 A 0.000	-1.17 (0.355)	5.58 x 10 ⁻⁴	-1.841 (0.403)	1.90x10 ⁻⁵	*	-
rs9321341	6q23.2	intronic	<i>MOXD1</i>	G 0.009 G 0.007 G 0.000	-0.957 (0.249)	6.22 x 10 ⁻⁴	-0.967 (0.235)	7.87 x 10 ⁻⁵	*	-
rs10501228	11p12	intergenic	<i>LRR64C</i> (204.5 kb)	G 0.049 G 0.036 G 0.069	0.744 (0.114)	2.47 x 10 ⁻⁵	0.556 (0.195)	3.72 x 10 ⁻⁴	0.409 (0.288)	0.224
rs9308307	12q21.2	intergenic	<i>NAV3</i> (627.2 kb)	G 0.242 G 0.246 G 0.266	-0.197 (0.057)	6.50 x 10 ⁻⁵	-0.382 (0.074)	6.94x10 ⁻⁶	-0.168 (0.168)	0.838
rs674021	12q24.31	Intron (boundary)	<i>SPPL3</i>	G 0.271 G 0.262 G 0.293	0.325 (0.071)	1.02 x 10 ⁻⁴	0.464 (0.092)	8.45 x 10 ⁻⁴	0.196 (0.164)	0.459
rs10520714	15q26.1	intergenic	<i>CHD2</i> (245.2 kb)	A 0.033 A 0.039 A 0.079	-0.584 (0.158)	1.18 x 10 ⁻⁴	-0.512 (0.195)	8.45x10 ⁻⁴	-0.207 (0.287)	0.408

Table 5 (continued)

Summary of Loci Associated With Blood Lipids (Nominal p Values < 0.001) Across Time

SNP	Locus	Type of SNP	Nearest gene (distance, kb)	Minor allele freq At age12 At age14 At age16	Age 12 Effect of minor allele	P values	Age 14 Effect of minor allele	P values	Age 16 Effect of minor allele	P values
rs4966361	17q22	intergenic	<i>YPEL2</i> (94.0 kb)	G 0.139 G 0.141 G 0.167	0.360 (0.077)	4.09 x 10 ⁻⁴	0.392 (0.099)	5.02 x 10 ⁻⁴	-0.277 (0.197)	0.097
Triglycerides (mmol/l)										
rs2363810	1p34.2	intergenic	<i>SCMH1</i> (291.3 kb)	T 0.234 T 0.251 T 0.244	0.090 (0.017)	2.35 x 10 ⁻⁵	0.085 (0.023)	1.02 x 10 ⁻⁵	0.021 (0.029)	0.030
Total cholesterol (mmol/l)										
rs4450153	11p15.2	intergenic	<i>TEAD1</i> (86.5 kb)	T 0.246 T 0.250 T 0.250	0.258 (0.067)	7.11 x 10 ⁻⁴	0.228 (0.092)	0.018	0.413 (0.176)	6.78x10 ⁻⁴
rs4453219	11p15.2	intergenic	<i>TEAD1</i> (86.8 kb)	A 0.246 A 0.250 A 0.250	-0.258 (0.067)	7.11x10 ⁻⁴	-0.228 (0.092)	0.017	-0.413 (0.176)	6.78 x 10 ⁻⁴
rs4453220	11p15.2	intergenic	<i>TEAD1</i> (87.0 kb)	A 0.247 A 0.250 A 0.250	-0.256 (0.067)	6.92 x 10 ⁻⁴	-0.221 (0.092)	0.018	-0.413 (0.176)	6.78x10 ⁻⁴

Note.* These markers were monomorphic among subjects with phenotypic data for age 16.

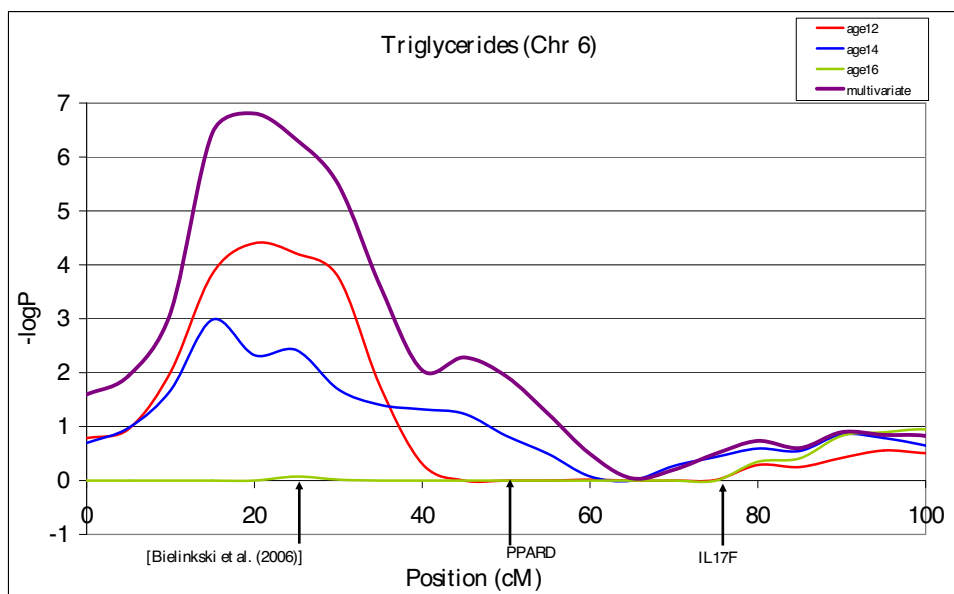


Figure 5

A plot of $-\log_{10}p$ of $\log_{10}(\text{triglycerides})$ adjusted for covariates on chromosome 6 (0 to 100 cM) from genome-wide scan. Nearby candidate genes and previous reported linkage peak are indicated by arrows with gene names and reference respectively.

univariate linkages (highlighted in bold in Table 3) are as follows: *APOB* (18,900–26,100K bp; MIM 107730) on chromosome 2p24, *LRP2* (166,000–171,600K bp; MIM 600073) on chromosome 2q24–q31, *ApoA1/C3/A4* (112,300–117,900K bp) on chromosome 11q23–q24 and *APOEL1* (0–89,000K bp) on chromosome 16q11–q24. The above candidate genes are speculative and will require further investigation.

It is interesting to note that some linkage peaks were present at one age only, particularly given that there is a high correlation between phenotypes across time. One possible explanation is that different QTLs affect the trait at different ages. This is biologically plausible given that puberty is a time of hormone-induced growth and development. Fitting a multivariate model to the data strengthened the evidence of linkage found for triglycerides at ages 12 and 14. However, the sample size at age 12 is larger than at other ages (Table 1), so there is different power at different ages. It is possible that the lack of linkage at age 16 may represent a false negative.

Association Findings

The genome-wide association scan showed significant evidence of association ($p = 8.24 \times 10^{-7}$, compared to genome-wide statistical significance for this set of markers of $p = 2.45 \times 10^{-6}$) between total cholesterol at age 14 and rs10503840 on chromosome 8p21.1 (see Table 4). There was no evidence of significant association for total cholesterol at other ages. Other significant associations occurred for HDL-C on chromosome 12 (at age 14) and for triglyceride elsewhere on chromosome 12 (at age 12).

Potential loci associated with lipid traits at more than one time were found (see Table 5) but the evidence of association was weaker. For example, variants on 11p15.2, located 87kb from the *TEAD1* gene, showed weak evidence of association with total cholesterol across all three ages (12: $p < 1 \times 10^{-4}$; 14: $p < .05$; 16: $p < 1 \times 10^{-4}$); and rs2363810 on 1p34.2, located 291.3 kb from the *SCMH1* gene, also showed weak evidence of association with total cholesterol across all three ages (12: $p = 2.35 \times 10^{-5}$; 14: $p = 1.02 \times 10^{-5}$; 16: $p = .030$). Some evidence of association was also found between rs10492859 in the *CDH13* gene and HDL-C at ages 12 and 14. This variant is located in an intron of the adiponectin receptor cadherin 13 gene (*CDH13*, MIM 601364) on 16q23. *CDH13*, a member of the cadherin superfamily, is expressed in endothelial and smooth muscle cells and acts as a receptor for the hexameric and high molecular weight adiponectin, a hormone secreted by adipocytes that regulates glucose and lipid metabolism (Hug et al., 2004). Another variant in *CDH13*, rs8055236, has been associated with coronary artery disease in the WTCCC study ($p = 9.73 \times 10^{-6}$) (Samani et al., 2007).

Locations of positive association and linkage results were compared to see how well they coincided. There was weak evidence of association for LDL-C at age 12 and rs7419276 ($p = .010$), corresponding to a linkage peak on chromosome 2 (200cM, $-\log_{10}p = 2.42$); for triglycerides at age 16 and rs879940 ($p = .006$) corresponding to the linkage peak found on chromosome 12 (150cM, $-\log_{10}p = 2.64$); for LDL-C at age 16 and rs1420533 ($p = .046$) corresponding to the linkage peak found on chromosome 16 (65 cM, $-\log_{10}p = 2.57$); and for LDL at age 12 and marker rs5768312 (p

= .008), corresponding to the linkage peak found on chromosome 22 (60cM, $-\log_{10}p = 2.28$).

There are several reasons why published results and ours may differ. The subjects in published linkage and association studies were adults and the genes responsible for variation in adult lipid levels may be different from the genes responsible for variation in teenagers. Furthermore, it has been shown that the heritabilities in adolescence are higher (aged 13–22, 71%–82%) compared to adult samples (aged 34–92, 48%–77%) (Beekman et al., 2002), so the power to detect linkage for a QTL of given size in this study may be better (Sham et al., 2000). Another reason is that our association analysis is based on a small number of people, and the 100k marker set gives incomplete coverage of the genome.

Strengths and Limitations

One strength of this study is the availability of longitudinal measurements on the same individuals across time. This allows multivariate QTL linkage analysis, increasing the power to detect QTLs that affect measurements at multiple times (Martin et al. 1997). Here, the multivariate linkage approach has revealed several potential regions where no significant evidence of linkage was found in univariate analyses. Another potential strength is the study design, as the family structure allows combined linkage and association analysis. However, given the small sample size and weak p values, a combined analysis on this dataset was not conducted.

A major limitation of this study is the small sample size available for genome-wide association. Recent GWAS studies (Han et al., 2008; Weedon et al., 2008) have shown that many thousands of subjects are needed to detect most QTLs, so it is hardly surprising that we found little with only approximately 400 subjects. Another limitation of this study is that only 100k SNPs were used, and 370k SNPs (or higher) is now considered the common standard for genome-wide association studies. Also, SNPs in this set do not give even coverage (i.e., they tend to cluster together). Hence, there are large regions along the chromosome where the SNP coverage was not adequate. The third limitation is that the association analyses were performed separately at each time point, instead of a multivariate analysis using all three time points, as the latter analysis would provide a more powerful test. However, at present there are only 83 individuals with data at all three time-points. Multivariate analysis has been deferred until more data become available.

Results from this study showed a poor relationship between significant linkage and significant association. One possible reason is that linkage may be due to multiple rare, or several uncommon, variants in a single gene (Cohen et al., 2004) rather than to a common variant. Even a highly significant association may only account for 1% of variance, which is far too small to be detected by linkage analysis. Nevertheless, results from this study strongly suggest the presence of

a number of potential chromosomal regions that may harbour genes influencing lipid levels. In particular there is evidence for loci on chromosome 6p influencing triglyceride, and chromosome 2q influencing LDL-C and HDL-C, in this adolescent population. These regions have not been previously identified and thus represent new opportunities. Other regions, including chromosomes 1, 4, 5, 11 and 18 for LDL-C, showed evidence of linkage. Genome-wide association analysis suggested a number of potential new loci associated with lipid levels at one time point, as well as loci associated with lipid traits across time. In order to strengthen the above findings, it will be necessary to conduct further studies on larger data sets.

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