QTL Mapping of Lipid Profiles in Mouse

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ABSTRACT The leading cause of death in high-income countries is coronary artery disease (CAD). Plasma triglyceride (TG) and high-density triglyceride (HDL) levels contribute to development of this disease. With the use of a unique mouse cross, we were able to identify two QTL linked to plasma TG and HDL levels. The candidate gene located under the QTL for plasma TG concentration is FK506 binding protein 1A. The candidate gene located under the QTL for plasma HDL concentration is hydroxysteroid (17-beta) dehydrogenase 7. These two genes have not been previously linked to TG and HDL levels and as a result could present possible new drug targets to reduce the risk of CAD in humans. This could reduce the number of lives lost each year to this disease.

KEYWORDS quantitative trait locus; high density lipoprotein; triglyceride; coronary artery disease; atherosclerosis

Introduction

Coronary artery disease (CAD) is the leading cause of death in high-income countries. The most significant risk factor for CAD is atherosclerosis, which is an inflammatory disease characterized by a hardening of the artery walls (Yang *et al.* 2010). The major factors for atherosclerosis development are high levels of plasma triglycerides (TG) and low-density lipoproteins (LDL) as well as low levels of high-density lipoproteins (HDL) (Cohen *et al.* 1997; Stylianou *et al.* 2008). Triglycerides are synthesized in the liver and used for energy. Serum triglyceride concentrations have been linked directly to the risk of coronary heart disease (Sarwar *et al.* 2007). A previous Quantitative Trait Locus (QTL) mapping study for plasma HDL concentration levels found peaks corresponding to a major constituent lipoprotein in HDL, a plasma phospholipid transfer protein, and lipoprotein lipase (Ishimori 2004). The plasma phospholipid transfer protein is bound to HDL and mediates the transfer and exchange of phospholipids among lipoproteins. Lipoprotein lipase plays a major role in lipoprotein metabolism. However, the effect of these QTL only explains 5-10 % of the variation in plasma HDL concentration levels. In an effort to identify more genes that contribute to this variation, this study involves QTL mapping of plasma HDL and TG for a new mouse cross. This cross has been chosen to narrow the QTL region and identify the underlying genes.

Materials and Methods

Animals

C57BL/6J (B6) and C3H/HeJ (C3H) inbred mouse strains and were obtained from The Jackson Laboratory. Mice were housed in a climate-controlled facility with a 12 h light/12 h dark cycle and allowed ad libitum access to water and a chow diet containing 6% fat (LabDiets 5K52; St. Louis, MO). Animal protocols were reviewed and approved by the Animal Care and Use Committee of The Jackson Laboratory. B6 × C3H, a cross with 277 female F2 mice generated from reciprocal mating, fed an atherogenic diet (Ath) as described previously from 8 to 14 weeks of age when they were phenotyped.

Genotyping

DNA was extracted from tail tips using phenol-chloroform. For the cross B6 × C3H, polymorphic MIT microsatellite markers were applied for genotyping using agarose gel electrophoresis (NuSieve 3:1; FMC BioProducts, Rockland, ME). Markers were chosen at evenly spaced intervals where possible. All data from these crosses are available in the Mouse Phenome Database in QTL archive under Su1 (jax.org/phenome; qtlarchive).

Plasma lipid analysis

The mice were fasted for 4 h in the morning and then blood from the retro-orbital sinus was collected in tubes containing EDTA and centrifuged at 9,000 rpm for 5 min. Plasma was frozen at –20°C until assay. Plasma HDL and TG concentrations were measured using enzymatic reagent kits (Beckman Coulter, Fullerton, CA) according to the manufacturer's recommendations on the Synchron CX Delta System (Beckman Coulter).

Statistical Analysis

QTL mapping was performed using QTL Cartographer version 2.5 for windows (Shengchu *et al.* 2012) to identify QTL associated with plasma triglyceride concentration and HDL concentration. Unlike, previous QTL analysis carried on this data (Su *et al.* 2009), the two phenotypic traits were not transformed. Both HDL and TG data exhibited non-normality when submitted to the Shapiro Wilk and qqplot normality test. We expect a mixture of normal to be a more appropriate distribution for the phenotypic traits data, hence the non-normality of the data does not cause much of a concern. We first checked for segregation distortion to determine markers that do not segregate independently. The test statistic for segregation distortion follows a χ^2 distribution with two degrees of freedom (for an F2 population) so that the critical value for rejecting the null hypothesis that the marker is segregating independently using a 0.05 significance level is 5.99 (see Table 1). Since 93 markers were tested for segregation distortion, we also applied a Bonferroni multiple test correction which generated a critical value of 15.06. Under the Bonferroni correction no markers were found to have significant segregation distortion. However, the Bonferroni test is known to be conservative when the number of tests is large. We therefore used the critical value from the non-corrected tests to identify markers with segregation distortion.

The genetic map was estimated using OneMap in R. The package allows to set a threshold for the logarithm (base 10) of odds score (LOD) and a maximum recombination fraction (max.rf). The map was constructed based on a two-point algorithm, Rapid Chain Delineation (RCD). The best threshold of max.rf found was 0.285, which generated 21 linkage groups. The LOD threshold for ordering the markers was set to 3. We attempted to get the same number of linkage groups as chromosomes (19), however, when choosing parameters, we had to choose between breaking apart chromosomes 1 and 10 or combining chromosomes 14 and 15. The former option was chosen since it is generally better not to combine chromosomes (linking unlinked markers) and instead get a few extra linkage groups. Increasing the LOD score to greater than 10 results in the markers at the ends of chromosomes 1 and 12 being lost (i.e. no longer in any linkage group). Increasing the max.rf results in chromosomes 13 and 14 being linked together. For the final map (max.rf = 0.285, LOD = 3), the last marker on chromosome 8 had a recombination frequency that was too large between it and the second to last marker so that it was no longer in a linkage group. Otherwise, all other markers were in a linkage group.The average distance between markers in the linkage group is 17cM with a genome length of 1224.9 cM. Single marker analysis was then performed for plasma triglyceride concentration and HDL concentration where the data were fit to a simple linear regression/ANOVA model and significance levels were used to determine the markers that were linked to a QTL related to the traits under study. Though segregation distortion does exist, we still chose to use all the markers to perform the QTL analysis since only three markers had segregation distortion and we only had 93 molecular markers in this experiment and wanted as much information as possible for a better estimation of the effect and location of QTL.

The significance threshold for interval and composite interval mapping was determined using the permutation test (1,000 times) (Churchill and Doerge 1994; Doerge and Churchill 1995). For a 0.05 significance level, the likelihood ratio statistic (LR) for TG was found to be 16.03, which is equal to a LOD score of 3.5. For a 0.05 significance level, the LR for HDL was found to be 15.44, which is equal to a LOD score of 3.4. The composite interval mapping parameters that were specified in QTL Cartographer were model 6 (which allows for the selection of cofactors which are related to the trait), a stepwise model selection procedure, a walking speed of 2 cM, window size of 10 cM, and a probability for taking a marker in or out of the model of 0.1. When determining the best window size, and in and out probability, a combination of window sizes of 5 cM, 10 cM and 20 cM and probability in and out of 0.05 and 0.1 were tried. For both TG and HDL, these parameter changes did not make a significant difference in our conclusions. For multiple interval mapping for TG, QTL were tested for main effects using the BIC criteria and a stepwise regression method to determine the QTL that were significant. Finally, we searched and tested for possible interaction between the QTL. The same process was repeated for HDL.

Results

Segregation Distortion

There were only three markers with significant segregation distortion, one each on linkage groups 7, 9, and 10 (Table 1). The markers experiencing segregation distortion were left in the analysis and monitored for the presence of QTL near their position. Since, these markers were each at the end of their respective linkage groups we judged it necessary to include them in the analysis to gain more information.

Genetic Map Estimation

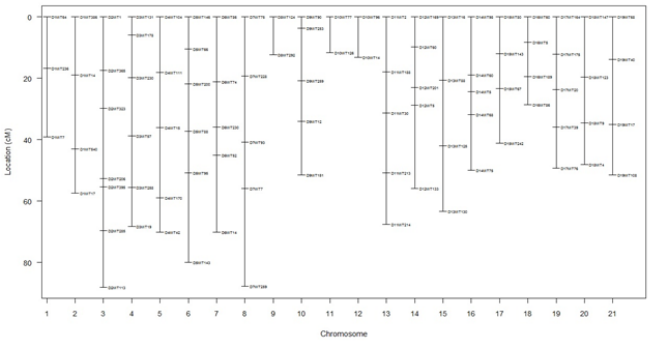

Figure 1 Estimated genetic map for the data using OneMap in R. The genetic map estimation using RCD with max.rf=0.285 and LOD=3 generated 21 linkage groups. Chromosomes 1 and 10 are each split into two linkage groups. The average distance between markers iS 17cM and the entire genome length is 1224.9 cM.

Two subjects were missing a large portion (>50%) of the marker information and as a result, these were excluded from further analysis (Figure 2). No markers were excluded because we wanted to keep as many markers as possible to help determine the location of QTL. After the estimation of the genetic map we checked the recombination fractions and pairwise LOD scores to confirm that we had determined the correct linkage groups. As compared to the published map, in the final estimated genetic map, chromosomes 1 and 10 were both broken into two linkage groups and the last marker on chromosome

Table 1 Markers with significant segregation distortion

Chromosome	Linkage group	Marker	Marker name	χ^2 [a]
9	10	5	D9MIT151	6.14*
8	2	2	D8MIT292	6.25*
6	7	1	D6MIT86	7.92*

[a] χ^2 statistic values are determined significant if they exceed the significance level of $\chi^2_{0.05,2} = 5.99$. The symbol '*' indicates a 0.05 significance level.The Bonferroni multiple test is too conservative given the large number of tests. The critical value under the Bonferroni test is 15.06.

8 was no longer in a linkage group (Figure 1).The order of the markers for this estimated map is nearly the same as the order for the published map.

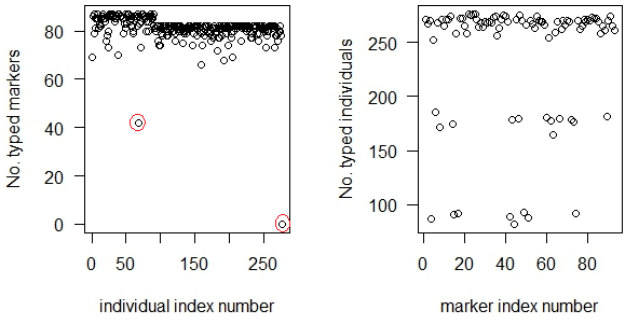

Figure 2 Summary of the missing data including the missing genotypes per individual and the missing genotypes per marker. There are 277 individuals total including two individuals (circled in red) that have a large portion of missing genotypes. These individuals were excluded from the analysis due to their significant amount of missing data. A total of 275 individuals were used in the QTL analysis. There are 93 markers in total. None were excluded from the analysis.

Triglyceride QTL Mapping

The distribution of the plasma TG concentration levels is right skewed and unimodal (Figure 3). Single marker analysis revealed three significant ($p < 0.001$; LOD > 2.5) TG QTL on chromosomes 2, 3 and 18, respectively (Figure 4). The peak on chromosome 2 is the most significant peak with the peaks on chromosomes 3 and 18 being less significant. It is worth noting that the peak on chromosome 2 is located on the end of the chromosome.

Interval mapping found only two of these peaks to be still significant ($p < 0.05$; LOD > 3.5). The QTL on chromosome 3 is no longer found to be significant and the peak on chromosome 2 is now more defined with a slightly higher maximum LOD score and no longer located at the end of the chromosome (Figure 4). Composite interval mapping narrowed these possible QTL further with the QTL on chromosome 18 no longer being significant, but suggestive QTL (window size=10 cM; probability in and out=0.1; $p < 0.05$; LOD > 3.5) (Figure 4). Multiple interval mapping found three significant QTL using the BIC criteria and a stepwise regression model (Figure 6). The final model had a BIC value of 2,880.74. A significant interaction was identified

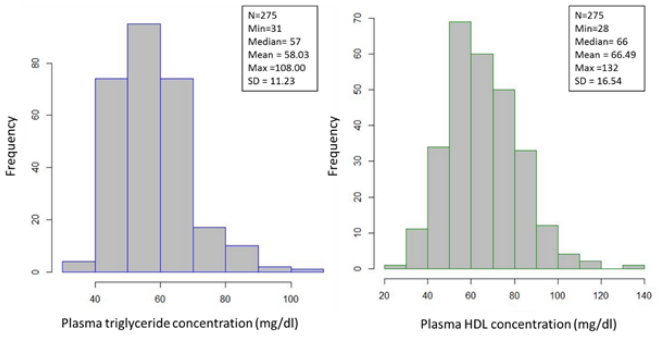

Figure 3 Distribution of the triglyceride and HDL levels for 275 individuals. Measurements were taken at 14 weeks of age. Both HDL and TG are not normally distributed. The distributions of both traits are right skewed and unimodal.The pvalue for the Shapiro-Wilk normality test is 0.0012 for HDL and less than 0.0001 for TG.

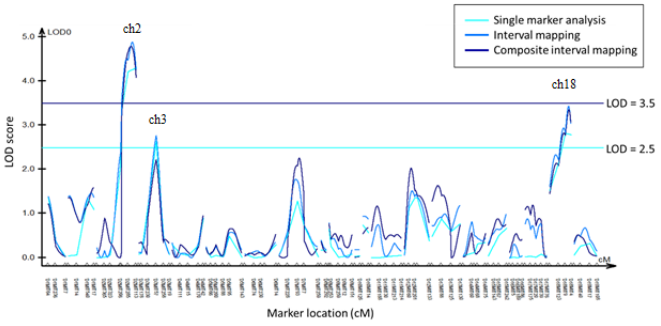

Figure 4 Genome-wide LOD scores for TG. The threshold for single marker analysis is a LOD score of 2.5. The threshold for interval and composite interval mapping is a LOD score of 3.5. There are three significant ($p < 0.05$; LOD > 2.5) TG QTL on chromosomes 2, 3 and 18 for single marker analysis. For interval mapping, there is one significant ($p < 0.05$; LOD > 3.5) TG QTL on chromosome 2. For composite interval mapping, there is one significant ($p < 0.05$; LOD > 3.5) TG QTL on chromosome 2. Model 6, a stepwise model selection, a walking speed of 2 cM, window size of 10 cM, and a probability for taking a marker in or out of the model of 0.1.

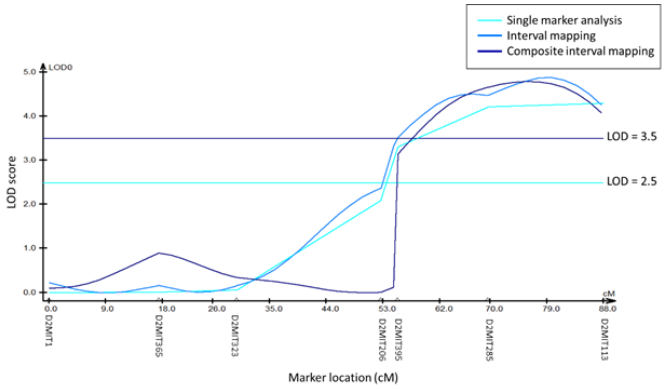

Figure 5 Genome-wide LOD scores for TG. The threshold for single markGenome-wide LOD scores for TG showing chromosome 2 which has the significant peak. The threshold for single marker analysis is a LOD score of 2.5. The threshold for interval and composite interval mapping is a LOD score of 3.5. For composite interval mapping we used model 6, a stepwise model selection, a walking speed of 2 cM, window size of 10 cM, and a probability for taking a marker in or out of the model of 0.1.

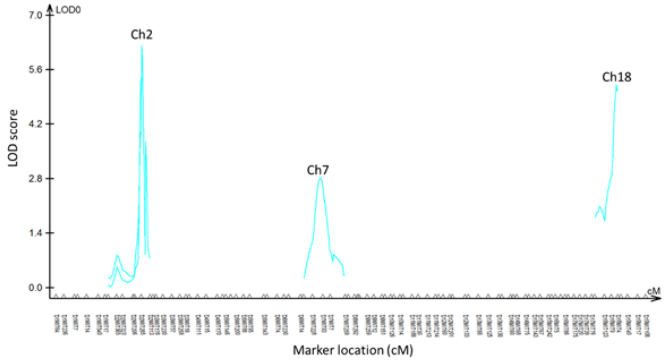

Figure 6 LOD scores for TG under multiple interval mapping showing the three QTL that were tested to be significant using the BIC criteria and a stepwise regression method. The final model had a BIC value of 2,088.74.The QTL on chromosome 2 was found significant in single marker, Interval mapping and composite interval mapping analyses. This QTL is located between markers D2MIT395 and D2MIT113. QTL on chromosomes 2 and 18 interact.

between the QTL on chromosomes 2 and 18. The QTL on chromosome 2 was still significant and is located between markers D2MIT395 and D2MIT113 (Figure 6).

HDL QTL Mapping

The distribution of the plasma HDL concentration levels is slightly right skewed and unimodal (Figure3). Single marker analysis revealed two significant ($p < 0.001$; LOD > 2.5) HDL QTL on chromosomes 1 and 11 (Figure 7). The peak on chromosome 1 is highly significant with the peak on chromosome 11 being less significant. Interval mapping found that the QTL on chromosome 11 was no longer significant ($p < 0.05$; LOD > 3.4). The QTL on chromosome 1, however, is still significant and has a slightly higher maximum LOD score.

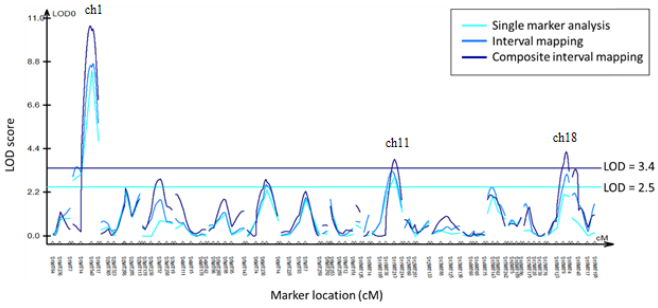

Figure 7 Genome-wide LOD scores for HDL. The threshold for single marker analysis is a LOD score of 2.5. The threshold for interval and composite interval mapping is a LOD score of 3.4. For the single marker analysis, there are two significant ($p < 0.05$; LOD > 2.5) HDL QTL on chromosomes 1 and 11. For the interval mapping, there is one significant ($p < 0.05$; LOD > 3.4) HDL QTL on chromosome 1. For the composite interval mapping, there are three significant ($p < 0.05$; LOD > 3.4) HDL QTL on chromosomes 1, 11 and 18. Composite interval mapping uses model 6, a stepwise model selection, a walking speed of 2 cM, window size of 10 cM, and a probability for taking a marker in or out of the model of 0.1.

Following interval mapping, there is also a small peak on chromosome 1 that is not significant. This peak is very close to the large QTL on chromosome 1 which makes it likely to be a ghost QTL (Figure 7). This is confirmed with the results of the composite interval mapping, where the small peak on chromosome 1 completely disappears. We also found that the peak on chromosome 11 was again significant and a newly significant peak was found on chromosome 18 under the composite interval mapping method (Figure 7). Multiple interval mapping found five significant QTL using the BIC criteria and a stepwise regression model (Figure 9). The final model had a BIC value of 2,287.74. A significant interaction was identified between the QTL on chromosomes 6 and 11. The peak on chromosome 1 is located between markers D1MIT14 and D1MIT17 (Figure 9).

Summary of all significant and suggestive QTL are provided in Table 2.

Joint Trait

The Pearson correlation for the TG and HDL traits was found to be 0.38 (Figure 12). Multiple trait, interval mapping and composite interval mapping revealed five joint QTL ($p < 0.05$;

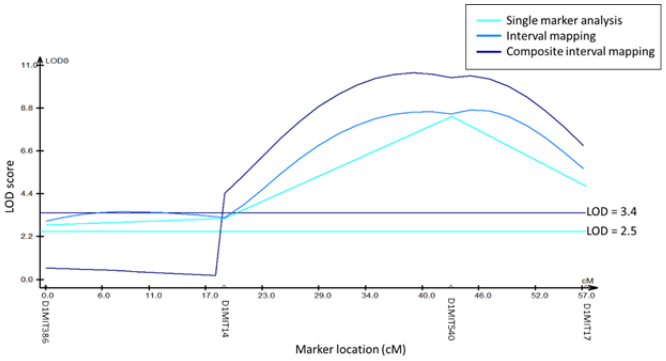

Figure 8 Genome-wide LOD scores for HDL showing the end of chromosome 1 which has the significant peak. The threshold for single marker analysis is a LOD score of 2.5. The threshold for interval and composite interval mapping is a LOD score of 3.4. For composite interval mapping we used model 6, a stepwise model selection, a walking speed of 2 cM, window size of 10 cM, and a probability for taking a marker in or out of the model of 0.1.

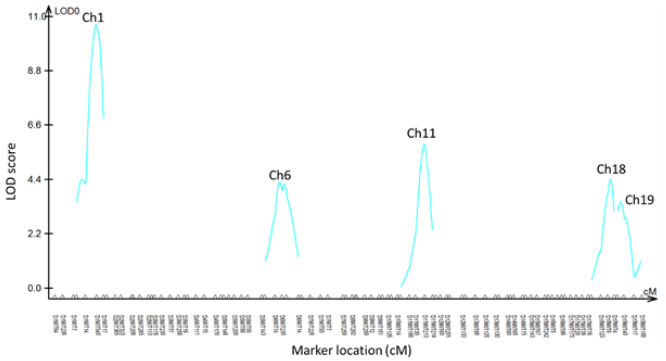

Figure 9 LOD scores for HDL under multiple interval mapping showing the five QTL that were tested to be significant using the BIC criteria and a stepwise regression method. The final model had a BIC value of 2,287.74.

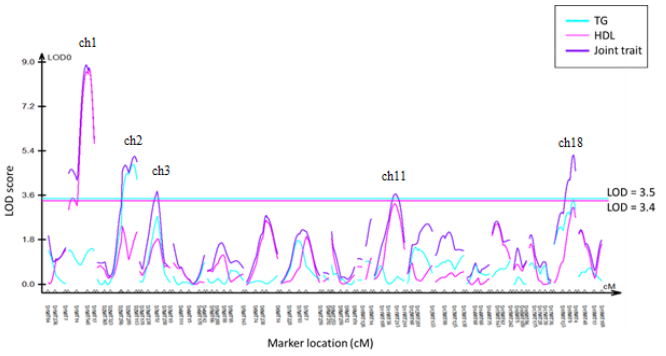

Figure 10 Genome-wide LOD scores for TG, HDL and TG and HDL together (joint trait). Multiple trait, interval mapping. The interval mapping threshold for TG was a LOD score of 3.5. For HDL it was a LOD score of 3.4. There are five joint trait QTL that pass the threshold ($p < 0.05$; LOD > 3.45). There are on chromosomes 1, 2, 3, 11, and 18 and indicate that that region of the genome explains the variation in both traits.

LOD > 3.45) on chromosomes 1, 2, 3, 11, and 18 (Figure 10 and Figure 11) that explain the variation in both traits.

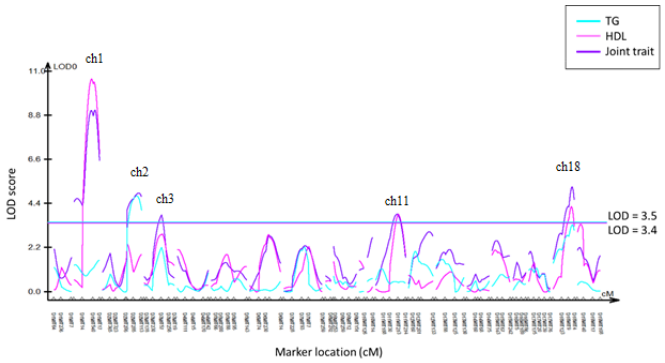

Figure 11 Genome-wide LOD scores for TG, HDL and TG and HDL together (joint trait). Multiple trait, composite interval mapping. The composite interval mapping threshold for TG was a LOD score of 3.5. For HDL it was a LOD score of 3.4. There are five joint trait QTL that pass the threshold ($p < 0.05$; LOD > 3.45). They are on chromosomes 1, 2, 3, 11, and 18 and indicate that that region of the genome explains the variation in both traits.

Discussion

This study utilized a new mouse cross (B6 x C3H) to narrow the QTL regions for plasma TG and HDL levels and be able to identify the underlying genes for these traits. Further understanding of these traits would contribute to studies on reducing coronary artery disease (CAD) in humans, which is the leading cause of death in high-income countries.

For plasma TG concentration, one significant QTL was present in all four analyses (single marker analysis, interval mapping, composite interval mapping and multiple interval mapping) (Figure 4, Figure 5 and Figure 6). This peak is located on chromosome 2 and underneath this peak is the genetic region coding for FK506 binding protein 1A. This QTL accounts for nearly 10% of the variability in TG (see R^2 in Table 2) . Previous studies have linked this gene to cardiovascular health in both mouse and humans. Deletion of this gene in mouse causes a congenital heart disorder known as non-compaction of left ventricular myocardium, which can affect the overall function of the heart and make an individual more likely to get CAD (Chiasson *et al.* 2011). Multiple interval mapping identified a significant QTL on chromosome 18, but these QTL did not pass the threshold for interval mapping or composite interval mapping. It could be considered as a suggestive QTL since its LOD score is very close to the threshold. Further research needs to be done to determine whether this suggestive QTL is a real QTL related with TG concentration levels.

One significant QTL for plasma HDL concentration was present in all four analyses (single marker analysis, interval mapping, composite interval mapping and multiple interval mapping) (Figure 7, Figure 8 and Figure 9). This peak is located on chromosome 1 and explains between 10 to 18% of the variation in HDL (Table 2). Underneath this peak is the genetic region coding for hydroxysteroid (17-beta) dehydrogenase 7. In humans this gene is involved in the biosynthesis of cholesterol (Marijanovic *et al.* 2003). Also in this region on chromosome 1 are genes coding for an apolipoprotein and a phospholipid

Table 2 Significant QTL positions and estimated effects

			TG[a]			
QTL	Chr	Position [b]	A-effect[c]	D-effect[d]	Epistasis [e]	R^2[f]
IM[g]						
1	2	79.7 (58-85.7)	5.25	-0.11		0.11
2	18	42.6 (21.8-44.6)	4.35	-1.30		0.08
CIM[h]						
1	2	75.7 (59.6-85.7)	4.46	1.63		0.08
MIM [i]						0.38
1	2	67.3 (68-71.7)	17.35	-20.01		
2	7	35.2 (23-47.6)	-2.82	-2.35	(2×3)	
3	18	10.0 (0.0-19.7)	3.93	-0.71	(2×3)	
			HDL[j]			
QTL	Chr	Position	A-effect	D-effect	Epistasis	R^2
IM						
1	1	8.0 (0.0-19)	7.31	-0.27		0.10
2	1	45.1 (32.8-51.7)	9.38	2.44		0.16
CIM						
1	1	39.0 (31.4-50.2)	9.72	3.00		0.18
2	11	50.8 (40.3-61.2)	5.13	1.65		0.05
3	18	40.6 (29.6-44.6)	4.71	-6.27		0.07
MIM						0.41
1	1	43.2 (32.6-49.1)	8.95	3.18		
2	6	30.2 (22.2-36)	-3.62	0.06	(2×3)	
3	11	50.9 (40.8-56.2)	5.95	0.99	(2×3)	
4	18	34.7 (29.6-45.6)	4.2	-4.63		
5	18	6.0 (0.0-21.6)	4.2	-4.63		

[a] LOD score of 3.5 used for all QTL analysis for TG.
[b] position of QTL with 1-LOD score interval.
[c] Estimate of additive effect of QTL
[d] Estimate of dominance effect of QTL
[e] Interacting markers are denoted by their name and the markers they interact with. Epistasis results are only available for multiple interval mapping.
[f] For IM and CIM, R^2 denotes the proportion of variation in the trait explained by the given QTL. Under multiple interval mapping R^2 denotes the proportion of total variation explained by all significant markers and their interactions in the model.
[g] The interval mapping analysis was used with a step size of 2 cM.
[h] The composite interval mapping was run with a step size of 2cM a in and out probability of 0.1 and a testing window width of 10cm. Changes in these model specifications resulted in little to no effect on the analysis results.
[i] The BIC model selection criteria and stepwise regression was specified under the multiple interval mapping model. Step size was set to 2cM when searching for main effects and interaction effects.
[j] LOD score of 3.4 used for all QTL analysis for HDL.

binding protein. These genes have been suggested as candidate genes in the original paper where we obtained our data (Su *et al.* 2009). Numerous studies have linked high cholesterol levels to CAD. There are two suggestive QTL that were not significant for interval mapping, but were significant for composite interval and multiple interval mapping and they are located on chromosomes 11 and 18.

In their paper, Su et al, (2009) found a significant QTL on chromosome 18 associated with plasma HDL concentration and on chromosome 2 associated with plasma TG concentration (Su *et al.* 2009). We also identified a QTL on chromosome 2 associated with TG and this appears to be the same QTL that was identified by Su et al, (2009). Moreover, we found a QTL on chromosome 18 as well that was associated with plasma HDL concentration and this appears to be the same QTL that was identified by Su et al, (2009). Underneath this peak is a gene, *Lipg*, that encodes for an endothelial lipase, which hydrolyzes HDL phospholipids in vivo (Maugeais 2003). This gene was identified as the most probable candidate gene by these researchers by using haplotype analysis, sequence analysis and expression analysis to narrow the list of possible genes in this region. Overexpression of *Lipg* in mouse has been found to result in a significant reduction in HDL levels (Ishida *et al.* 2003).

Plasma TG and HDL concentrations were mildly correlated (0.38) in this study. In their paper Yang et al, (2010) also found that these two traits were positively correlated. We know from the literature that there is an inverse relationship between plasma TG and HDL concentrations. A recent study found that lowering TG is inversely correlated with HDL (Miller and Langenberg 2007). Multiple trait, interval mapping and composite interval mapping revealed five joint QTL ($p < 0.05$; LOD > 3.45) on chromosomes 1, 2, 3, 11, and 18 (Figure 10 and 11) that explain the variation in both traits. The peaks on chromosomes 1 and 11 are mostly due to the HDL trait. The peak on chromosome 2 is mostly due to the TG trait and the peaks on chromosomes 3 and 18 appear to be due to both TG and HDL which indicates that these traits are correlated and being controlled by the same genetic regions. The joint peak on chromosome 3 has not been significant in any other analyses.

There are many unannotated genes located underneath the peaks on chromosomes 1 and 2, but based on the phenotype, the most probable candidate genes are the FK506 binding protein 1A for plasma TG concentration and hydroxysteroid (17-beta) dehydrogenase 7 for plasma HDL concentration. These genes are newly identified candidate genes for TG and HDL concentration and could present possible drug targets to reduce the risk of CAD in humans, which could reduce the number of lives lost each year to this disease.

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