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Analysis of Genetic Factors Affecting High Density Lipoprotein Levels and ApoAll Levels in C57 B1/6J and FVB/N Wildtype Mice Using a QTL Mapping Approach

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Analysis of Genetic Factors affecting High Density Lipoprotein Levels and ApoAII levels in C57 Bl/6J and FVB/N wildtype Mice using a QTL Mapping Approach

By
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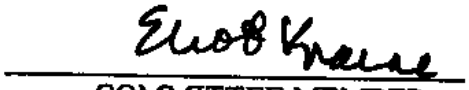

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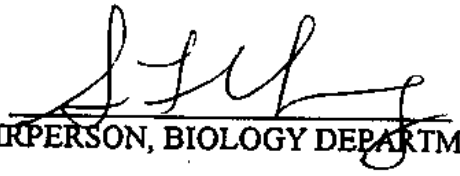

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Abstract

HDL-C levels of C57BL/6J and FVB/N inbred mice strains on chow diets were examined and found to be markedly different with the FVB strain exhibiting elevated levels of total plasma HDL-C (High density lipoprotein cholesterol) while the C57 BL/6J mice showed lower levels of total plasma HDL cholesterol. A cross was generated between C57BL/6J males and FVB females and subsequent crosses to yield 209 F2 progeny. The HDL-C levels of 209 F2 progeny on a chow diet showed HDL-C levels spanning the difference in levels exhibited between the two parental strains suggesting multiple genetic factors may be responsible for the variation in HDL-C. We typed 209 F2 progeny for the genetic marker, D1mit36, which is 3cM away from the apoAII gene suspecting that this locus (apoAII) may influence HDL-C cholesterol in this cross. Statistical analysis (Free regression) revealed that D1mit36 accounts for 55% of the interindividual variation HDL-C in the entire progeny population (n=209). Further investigation of the HDL-C and apoAII levels and an intragenic marker for the apoAII gene showed a gene dose effect relationship between the genotype and phenotype. These results suggest that the apoAII gene is responsible for a major fraction of the genetic variation in HDL-C cholesterol in both males and female F2. Sequencing of the apoAII gene revealed the existence of polymorphisms between C57 and FVB strain resulting in amino acid changes in the apoAII protein. Investigation of the other genetic factors influencing HDL-C cholesterol levels included a genome scan screening of all F2 progeny using SSLP (simple sequence length polymorphisms) at loci located approximately every 10cM throughout the mouse genome. Subsequent linkage analysis has resulted in identification of a locus at 50cM on chromosome 8 (LOD score=4) near the LCAT gene. Thus, a locus on chromosome 1, presumably the apoAII gene, accounts for 55% of the variation seen in HDL-C levels in these inbred mice strains. The chromosome 1 locus accounts for approximately 11% of the variation seen in apoAII protein levels suggesting the involvement of other factors affecting HDL-C and apoAII. Using a QTL mapping approach, one such candidate gene thought to be LCAT (Lecithin Cholinesterase Acetyl Transferase), has been identified to contribute to the variation in HDL-C and apoAII in these mice.

Introduction

Cholesterol is an essential component of cellular structure and necessary for many biological functions involved in maintaining homeostasis. Abnormally high cholesterol levels, however, have long been associated with the development of pathological conditions such as congestive heart disease, coronary artery disease, atherosclerosis and other cardiovascular disorders in which blood circulation is impeded. Extensive research has revealed complex processes involving lipoproteins by which different types of organisms control plasma cholesterol levels. In the blood, cholesterol transport is achieved by lipoproteins including HDL (High Density Lipoproteins), LDL (Low Density lipoproteins) VLDL (Very Low Density Lipoproteins) and variations in blood levels of these lipoproteins have long been associated with the development of cardiovascular diseases. HDL-C has been termed the "good cholesterol" because elevated levels have inverse effects on cardiovascular diseases, mainly atherosclerosis. On the contrary, elevated levels of LDL provide susceptibility for development of atherosclerosis. It is these associations that have fueled research of lipoprotein metabolism, composition and their roles in maintaining cholesterol levels. Vast efforts in the scientific community have also focused on revealing genetic mutations causing variations in lipoprotein structure due to alterations of its components (the apolipoproteins) which play crucial roles in cholesterol homeostasis.

Currently, lipoproteins have been classified according to composition and size. Lipoproteins are composed of lipids-cholesterol, triglycerides and phospholipids, and proteins- apolipoproteins, which serve as an interface between lipids and aqueous environments while aiding in lipid metabolism and transport. Ten apolipoproteins have been classified and studied- apoAI, apoAII, apoCI, apoCII, apoCIII, apoB100, apoB48, apoE, apoAIV and Apo(a). ApoAI, apoAII and apoAIV are found on HDL-C while apoB100 is specific to LDL, VLDL and IDL (intermediate density lipoprotein). The three C-lipoproteins are found on all major lipoproteins but have distinct functions. ApoCII is an activator of lipoprotein lipase while apoCIII is an inhibitor (Ginsberg, 1994). Recent studies have defined the role of apoCI in the uptake of lipoproteins by the liver (Ginsberg,1998). ApoE is synthesized in the liver. It is found on all major lipoproteins and appears to regulate the removal of remnant lipoproteins from the plasma by the liver (Ginsberg, 1994). ApoAI and apoAII are the major components of HDL-C and play crucial roles in cholesterol transport. ApoAI is

synthesized in the liver and small intestines and is secreted on nascent chylomicrons but transfers to HDL-C during lipolysis (Ginsberg,1994).

Cholesterol is the major lipid in both HDL and LDL. In lipoproteins, cholesterol is carried mainly as cholesterol ester at the core of the lipoprotein molecule. Transport of cholesterol in humans can be divided into two transport systems: transport of exogenous (dietary) lipids and transport of endogenous lipids (for review, see Ginsberg,1990). Cholesterol and dietary fat (triglyceride) are absorbed into the cells of the small intestine as fatty acids and cholesterol where reesterification to triglyceride takes place and they are incorporated into chylomicrons. The surface of these chylomicrons are composed of phospholipid, apoB48, apoAI , apoAII and apoAIV (Ginsberg,1994). The chylomicrons also acquire apoC-II, apoC-III and apoE which are transferred from HDL along with free and esterified cholesterol. ApoCII interacts with the enzyme LPL (lipoprotein lipase) to activate it and cause the chylomicron core to be hydrolyzed. This hydrolysis occurs in the capillary beds of adipose tissue, lungs and muscles where LPL is synthesized. After hydrolysis, the remaining chylomicron remnant transfers apoCII, apoCIII, phospholipids and free cholesterol back to HDL maintaining cholesteryl ester rich at its core and apoE on its surface. Remnants are then cleared from circulation by interacting with LDL receptors on hepatocytes, where they are used for bile acid synthesis membrane synthesis and other biological functions. Atherogenesis can result when chylomicron remnants, present in excess, are taken up by artery walls. Transport of endogenous lipids is somewhat distinct from the aforementioned pathway and involves apoB100, LDL, VLDL, and IDL (for review, see Ginsberg, 1994). VLDL are assembled in the ER and Golgi bodies of hepatocytes. Triglycerides and apoB-100 have been packaged together into nascent VLDL. Once in the plasma, VLDL interact with LPL and lose triglyceride to become IDL (intermediate density lipoprotein). VLDL catabolism has two consequences: the transfer of surface cholesterol to HDL occurs and the loss of triglyceride concentrations generates IDL , which after interaction with the liver, becomes LDL. Once LDL are formed, the length of time it circulates in the plasma is determined by the availability of LDL receptors- primarily in the liver. Increased levels of circulating (plasma) LDL results in cardiovascular disorders.

By contrast, elevated levels of circulating HDL-C have been associated with decreased incidence of cardiovascular disorders and protective effects against atherosclerosis. The exact mechanisms through which HDL-C protects against

atherosclerosis and other disorders remains to be discovered. The currently accepted theory of how HDL-C safeguards arteries is that it removes excess cholesterol from the blood and recycles it back to the liver in a process known as "reverse cholesterol transport". Reverse cholesterol transport is mediated by HDL-C in conjunction with the cholesterol esterifying enzyme LCAT (Tall, 1998). Once in the liver, cholesterol ester accumulating in the HDL-C particles may be taken up by resident LDL receptors, transfer to triglyceride rich lipoprotein remnants as a result of CETP (Cholesterol Ester Transfer Protein) or undergo selective uptake mediated by scavenger receptor B1 (SRB1) (Tall, 1998). In humans, the pathway involving CETP is very important because subjects lacking this protein demonstrate a dramatic accumulation of HDL-C (Tall, 1998). By contrast, mice lack CETP and therefore HDL-C mediates reverse cholesterol transport primarily via SRB1 (Tall, 1998).

Recent research has utilized molecular approaches using mouse models to investigate SRB1, HDL-C and apolipoproteins AII and AI as well as other key processes involved in transport of cholesterol from the blood. Breslow (1991) showed that over expression of human apolipoprotein AI in transgenic mice affected HDL-C size and metabolism (Chajek et.al., 1991). Subsequently, it was demonstrated that apoE knockout mice, when over expressing apoAI, lacked severe atherosclerotic lesions (Paszty et.al., 1994). These and other experiments identify the anti-atherogenic effect of AI and HDL-C. Studies have also focused on investigating the associations of HDL-C and SRB1. SRB1 was the first HDL-C receptor to be described and ever since its discovery, has been the focus of intense research. Recent studies focused on the structure of SRB1 and concluded that it is N-Glycosylated , fatty acylated and colocalizes with caveolae (Babbitt et.al., 1997). To test directly the normal role of SRB1 in HDL-C metabolism, *Rigotti et.al (1997).*, generated mice with targeted null mutations in the SRB1 gene. They concluded that there was a significant increase in plasma cholesterol in both homozygous and heterozygous mutants because of the presence of large apoAI containing particles (Rigotti et.al., 1997). These results provide support that the gene encoding SRB1 is impotant in selective uptake of HDL-C by the liver. Although current scientific evidence establishes that SRB1 is an HDL-C receptor, much controversy exists as to the ligands that associate with it. Several proteins such as HBP, apoAII, apoAI, and HB2 (a liver binding protein) are being investigated as possible ligands (Fidge,1999).

Apolipoprotein AII (ApoAII) , which along with apoAI, comprises HDL-C, is studied with regard to its anti-atherogenic effects and its associations with HDL-C metabolism and physiology. *Weng et.al.1996*, used gene knockout technology to create apoAII deficient mice to investigate the role of apoAII in the body. They concluded that apoAII deficient mice had a 70% decrease in HDL-C cholesterol levels and that the HDL-C particles that did exist were diminished in size. These studies also suggest that apoAII functions to influence HDL-C size and metabolism by inhibiting HL- Hepatic Lipase (Weng et.al., 1996). *Guerra et.al. 1997*, associated a hepatic lipase allele with high plasma concentrations of HDL-C. Studies done with apoAI knockout mice demonstrated that low levels of HDL-Cs present in the AI KO mice ,which were enriched with apoAII, apoAIV and apoE, did not lead to diet-induced atherosclerosis (Hughes et. al., 1996). Recent research also presents the hypothesis that PLTP (phospholipid transfer protein) plays a role in determining HDL-C levels and HDL-C distribution (Albers et.al., 1999 and Jiang et.al.,1999). However, the interactions between apoAII and PLTP remain to be elucidated. Recent genetic investigations suggest that mutations in the apoAII gene causing alterations in HDL-C concentrations, affect apoAII concentrations in mice on a chow diet (non-atherogenic) along with differences in HDL-C size (Weng, et.al.,1996). The effect of apoAII on CETP (Cholesterol ester transfer protein) has been investigated by using human CETP transgenic mice cross bred with transgenic mice expressing human apoAII (Zhong et.al., 1994). The results indicate that the coexpression of apoAII with CETP result in increased HDL-C triglyceride and constrain the ability of CETP to reduce HDL-C particle size and protein content (Zhong et. al., 1994). More recent studies investigate the correlation between LPL (lipoprotein lipase) and HDL-C (Clee et. al., 1997).

Thus, research of HDL-C and the reverse cholesterol transport pathway has lead to some understanding of the interactions between HDL-C, SRB1, apoAI, apoAII, LPL, HL, PLTP and CETP and their effects on maintaining increased or normal levels of HDL-C which result in protective effects against atherogenesis. Recent research has focused on using mouse models to study gene interactions affecting cholesterol homeostasis. Mice models are ideal for investigating genes controlling HDL-C because these animals have a small genome that can be easily manipulated to generate knockout mice and large generation sizes. Future scientific investigations may provide the exact mechanisms by which the SRB1 receptor, HDL-C and other

possible ligands such as apoAI and apoAII interact in lipid uptake and the candidate genes that may affect the biological processes involving these proteins.

In this study, we have used QTL (quantitative trait loci) mapping approach to investigate genetic causes of HDL-C levels in two inbred wildtype strains of mice- C57Bl/6J and FVB/N. The study identified several candidate genes associated with HDL-C levels but also revealed a multigene effect controlling HDL-C levels in these mice. In addition, sequencing of the apoAII gene in these two strains of mice was performed in order to determine if polymorphisms in the apoAII gene contribute to the variations in HDL-C (High Density lipoprotein Cholesterol) observed in the FVB and C57 mice. Our study addresses the possibility that HDL-C levels observed in these wildtype inbred mice may be determined by genes other than apoAII and introduces locations for candidate genes that may provide additional knowledge of HDL-C and cholesterol homeostasis in both mice and humans.

Materials and Methods

Animals and Lipoprotein Measurements

The inbred parental strains FVB/N and C57 BL/6J were obtained from The Jackson Laboratory (Bar Harbor, ME) and placed on rodent chow diets supplied by Purina. The FVB females were mated with C57 males to produce the F1 progeny. The F2 animals were produced through sibling matings. After blood samples were obtained, plasma HDL-C levels were determined by separating lipoproteins via ultracentrifugation at 70,000 and then performing a cholesterol assay in which a 1X cholesterol reagent (purchased from Sigma) was added to HDL-C sample aliquots. All samples were observed for color change as an indicator of amount of HDL-C.

Genotypic Analysis

Genomic DNA was isolated from mouse tail tips (from 213 F2 progeny mice) using proteinase K tissue digestion followed by ethanol precipitation. The diluted DNA was stored at 4°C. Genotyping was done by *PCR amplification of SSLPs using PCR primer pairs (Mappairs) purchased from Research Genetics (Huntsville, AL) located at approximately every 10cM distance along the mouse genome. PCR conditions included a hotstart followed by 94°C for 40 sec, 55°C for 50 sec and 72 °C for 30 sec for 35 cycles. PCR samples were run on 8% polyacrylamide gels with 1X TBE running buffer using *GIBCO BRL sequencing system* from *GIBCO Lifetechnologies (Rockville,MD)*. The gels were stained with *Vistra green* purchased from *Amersham Biotech* (Piscataway, NJ) and analyzed using *Fluorescent Scanner Imaging* from *Molecular Dynamics* (Sunnyvale, CA) with *ImageQuant* and *Adobe Photoshop 4.0* software for Microsoft *Windows 98*. Parental DNA was isolated using proK digestion overnight, ethanol precipitation and stored at 4°C. Primer pairs (SSLPs) were tested for polymorphism and for parental genotyping using DNA samples from the FVB/N and C57Bl/6J mice in PCR amplification and manipulated in the same manner as the F2 progeny DNA samples described above.

*PCR reactions done in Perkin Elmer/ Applied Biosystems (Norwalk, CT) Gene Amp 9700

Determination of Plasma Apolipoprotein AII levels

Relative plasma apolipoprotein AII levels were determined using Western Blotting. Diluted plasma samples, from F2 generation mice were run on 16% tricine gels (reducing and denaturing) using a *Novex* (San Diego, CA) western blotting system. Standards used were plasma pooled from FVB/N mice. The gels were transfered to nitrocellulose membranes and exposed to Casein/PBS blocking reagent, 1:1000 Rabbit anti-mouse AII, 1:2000 Goat anti-rabbit IgG HRP and ECL detection reagent from *Amersham.* Relative ApoAII levels determined via autoradiography and computer analysis.

*Rabbit anti-mouse (Neat) purchased from *Biodesign*
**Goat anti-rabbit IgG HRP purchased from *Pierce*, (Rockford, Illinois)

Apolipoprotein AII Gene Sequencing

Apolipoprotein AII gene sequence in the FVB/N and C57Bl/6J mice strains was done by Reverse Transcription PCR reaction using mRNA isolated from liver tissue from FVB and C57Bl/6J mice. The mRNA was isolated using mRNA extraction kit purchased from (Qiagen Inc., Valencia, California). Samples were then submitted to RT PCR using polydt and/or primer 5' AACTCCTTCCGCATTTATTGG 3' primed. The following conditions were used: Heat to 80°C for 5 min, 4°C 10 min., then 42°C for 1 hr and 90°C for 10 min. The cDNA was then subjected to PCR using the following conditions : Hotstart and 30 cycles- 94°C for 50 sec, 56°C for 50 sec and 72°C for 50 sec using 5' GCAGCACAGAATCGCAGCACT and 3' primer AACTCCTTCCGCATTTATTGG pairs. The primer pairs were selected according to data previously published by *Doolittle et. al., 1990.* The PCR product was submitted to the Rockefeller University Protein/DNA Technology center for Automated Fluorescent DNA Sequencing. The facility uses Perkin Elmer/ Applied Biosystems (Norwalk, CT) Model 377 DNA instrument and Taq FS dye terminator cycle sequencing fluorescence-based sequencing method. Data obtained includes unedited sequences and four color electropherograms.

Isolation and Use of ApoAII Intragenic Marker

PCR was performed using genomic DNA from each of the 213 F2 progeny under the following conditions: Hotstart, and 30 cycles -94°C for 50 sec, 59°C for 50 sec and

72°C for 50 sec each cycle. PCR reactions were done using primers 5' GAATTCAACTCCTTCCGCATTTAT 3' and 5'CACTCAATACTTTCAGAGCATGACT 3'. After PCR, reactions were subjected to HaeIII enzyme digest- 37°C 3-4hrs followed by 4°C overnight. HaeIII digest yielded 168 and 164 bp bands in FVB genomic DNA and 160 and 140 bp bands in C57 genomic DNA as a result of cleavage within the apoAII gene previously sequenced. These polymorphisms were visualized by electrophoresis using 4% Nuseive agarose gels prestained with EtBr and/or electrophoresis using 8% polyacrylamide gels as described above.

Computer Data Analysis

Data generated by QTL analysis was analyzed using GraphPad *Prizm* software for Microsoft *Windows 95* which was used to generate graphical associations and corelations between loci and HDL-C. Graph pad software was also used for one way analysis of variance (aANOVA) calculations of the HDL-C data of the parentals, F1 and F2 generation and the apoAII values for the F2 generation. *MapManager QTL version27* for *Apple Computer* was used for Loci / trait analysis. Statistical significance was set at a *p* value of 0.0001. The Mapmanager QTL program generates statistical significance by using Free, Dominant, Additive and Recessive regression models. The free regression model used in this study to analyze the data takes into account variation due to a dominant regression and an additive regression model simultaneously and reports both regression coefficients.

Calculations for Determining Genetic Component of Variation

The following calculation was used to determine the percent variation due to genetic components in the F2 generation mice:

Variance F2- Variance F1/ Variance F2

where *variance F2*= variance of the HDL-C data corrected for gender and D1mit36 of the F2 generation mice

variance F1=variance of the HDL-C data corrected for gender of the F1 generation mice

Note: The variation of the F1 generation is due to environmental factors only

Results

HDL-C Levels In Wildtype and Progeny Mice

Wildtype C57Bl/6J and FVB/N mice, on chow diets, were examined for significant differences in HDL-C (HDL Cholesterol) levels. HDL-C levels in male mice (Figure1) shows an average value of 96 mg/dl ±6 mg/dl for FVB mice as compared to an average of 48±7 for C57 male mice. Analysis of variance shows that the HDL-C levels of these two groups differ significantly with a p value< 0.001. HDL-C levels of F1 generation and F2 generation males were also examined. Differences in HDL-C seen in the F1 and F2 generation mice were significant when compared to levels seen in C57 wildtype mice ($p<0.01$ and $p<0.001$). Data obtained from F2 generation male mice shows HDL-C levels spanning the range represented by the wildtype strains. Data obtained for female wildtype mice yielded similar results (Figure 2). The C57 and FVB female wildtype mice were found to have significant differences in HDL-C levels ($p<0.05$). F1 generation female mice ($n=9$) were found to have significantly higher HDL-C levels than C57 female mice ($p<0.05$). Significant differences were also seen for C57 vs F2 generation mice ($n=96$) with a p value <0.01.

The data obtained for the female mice in this study show that the HDL-C levels of F2 generation female mice spans the range of HDL-C indicated by the parental strains C57 and FVB. Thus, the phenotypic data provides evidence of a striking difference in HDL-C levels between FVB and C57 wildtype mice that is inherited in the F2 progeny mice. The data also shows that the f2 progeny females have a lower average of HDL-C than the males. This difference in sex is also shown in the parentals where the males (both FVB and C57) have higher averages of cholesterol amounts than the females (FVB and C57). The fact that this difference in HDL-C levels is represented among the sexes in the same parental strain indicates that this variation in HDL-C may be due to hormonal causes and not to genetic differences between different sex animals of the same strain. Since the animals obtained for this cross were 99% inbred, it is unlikely that two members of the same strain are genetically different except with regard to the Y chromosome. Thus, HDL-C variation between males and females may be due to sexual variables while variation in HDL-C

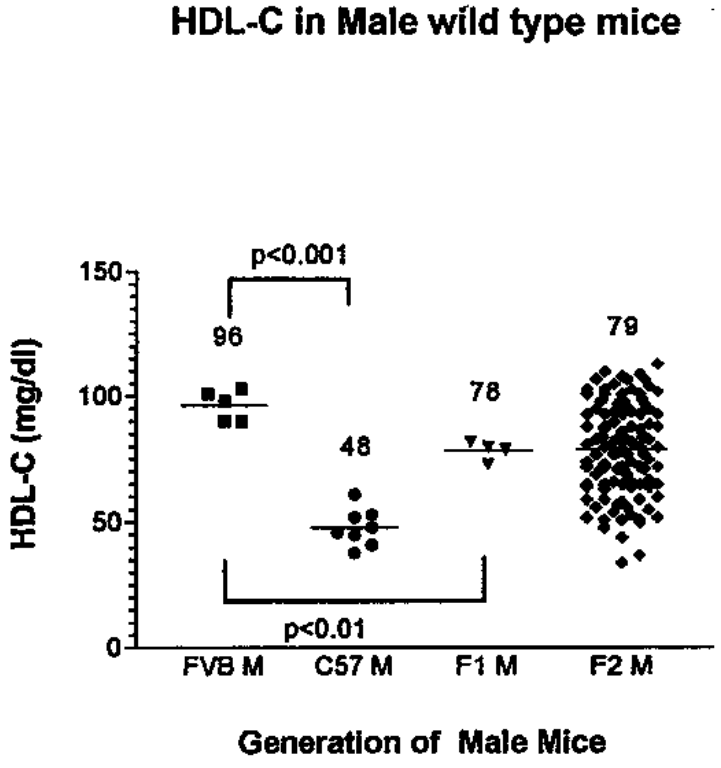


Figure 1 : HDL cholesterol in male wildtype FVB/N, C57BL/6J, F1 generation and F2 generation mice. All values are expressed as milligrams per deciliter. Mean values ± standard error are as follows: FVB Males 96±6.1, C57 males48 ± 7.3, F1 males 78± 3.9 and F2 males 79 ±18.0. Significant differences were found in HDL-C (using one-way *ANOVA* and SNK) between strains C57 vs FVB $p<0.001$, C57 vs F1M $p<0.01$ and, C57 vs F2M $p<0.001$. Sample sizes: FVB n= 5, C57 n= 8, F1M n=4 F2 n= 114.

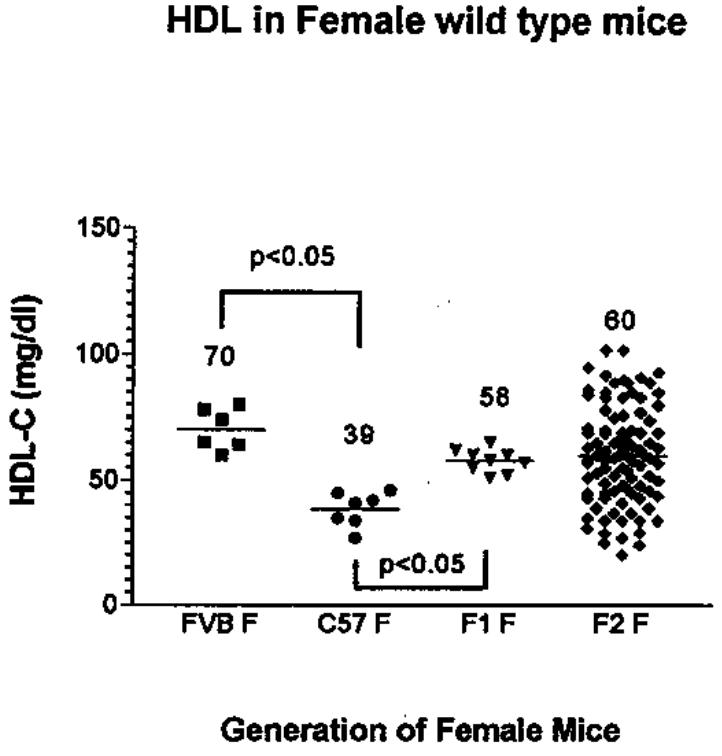


Figure 2 : HDL cholesterol in female FVB/N, C57BL/6J,F1 generation and F2 generation female wildtype mice. All values are expressed as milligrams per deciliter. Mean values±standard deviation are as follows: FVB Females 70±8.8, C57 Females 39±6.8, F1 females 58 ±4.6 and F2 females 60±19.3. Significant differences were found in HDL-C (using one-way *ANOVA* and SNK) between strains C57 vs FVB $p<0.001$, C57 vs F1 $p<0.05$ C57vs F2F$p<0.01$. Sample sizes: FVB n=6 , C57n=7, F1Fn=9, F2F n= 96.

between FVB and C57 parental strains is due to genetic differences between the two strains as indicated by the HDL-C range in the F2 progeny males and females.

Effects of ApoAII Intragenic Marker Genotype on HDL-C Levels in F2 Mice

In order to determine the effect of apoAII gene on HDL-C levels in this cross, the F2 progeny mice were genotyped for an intragenic apoAII marker. Results obtained from genotyping F2 progeny mice (male and female) for ApoAII intragenic marker were compared with the HDL-C levels of animals heterozygous for the locus (FC genotype), homozygous C57 (CC) and homozygous FVB (FF) (Figure 3). In order to compare both males and females together, each female HDL-C level was adjusted by adding 19mg/dl which was the difference in average HDL-C levels observed between malle and female F2 animals (data not shown). Significant differences in HDL-C levels were observed for each apoAII intragenic marker genotype: CC 58±11mg/dl, FC 80±13mg/dl and FF 96±14mg/dl ($p<0.0001$). An overall increase in HDL-C levels was correlated with FVB alleles for the apoAII intragenic marker locus which suggests a gene dosage effect.

Relationship Between ApoAII Intragenic Marker and ApoAII Plasma Levels

Analysis of apoAII levels and apoAII genotype in F2 progeny mice indicates a correlation between plasma apoAII and apoAII marker (Figure 4). Plasma levels of apoAII were obtained using Western Blot analysis as described in Materials and Methods and are relative values based on individual standard curves obtained for each set of samples on a single blot. Comparison of apoAII intragenic marker genotype and relative apoAII plasma levels for F2 generation mice shows that differences in apoAII plasma levels observed for apoAII intragenic marker genotype are significant by ANOVA $p<0.0001$. Furthermore, highest levels of plasma apoAII (19±10) were observed in animals genotyped FF for apoAII intragenic marker, followed by lower levels seen in animals genotyped FC (15±9) and lowest levels in animals genotype CC (11±8). Thus, an increase in apoAII levels in F2 mice was observed in association with apoAII alleles from the FVB parental mice .

HDL-C Levels In Animals Heterozygous for ApoAII Intragenic Marker

HDL-C levels were compared in F2 progeny mice that were genotyped heterozygous for apoAII intragenic marker (Figure 5). N=88 male and female animals are

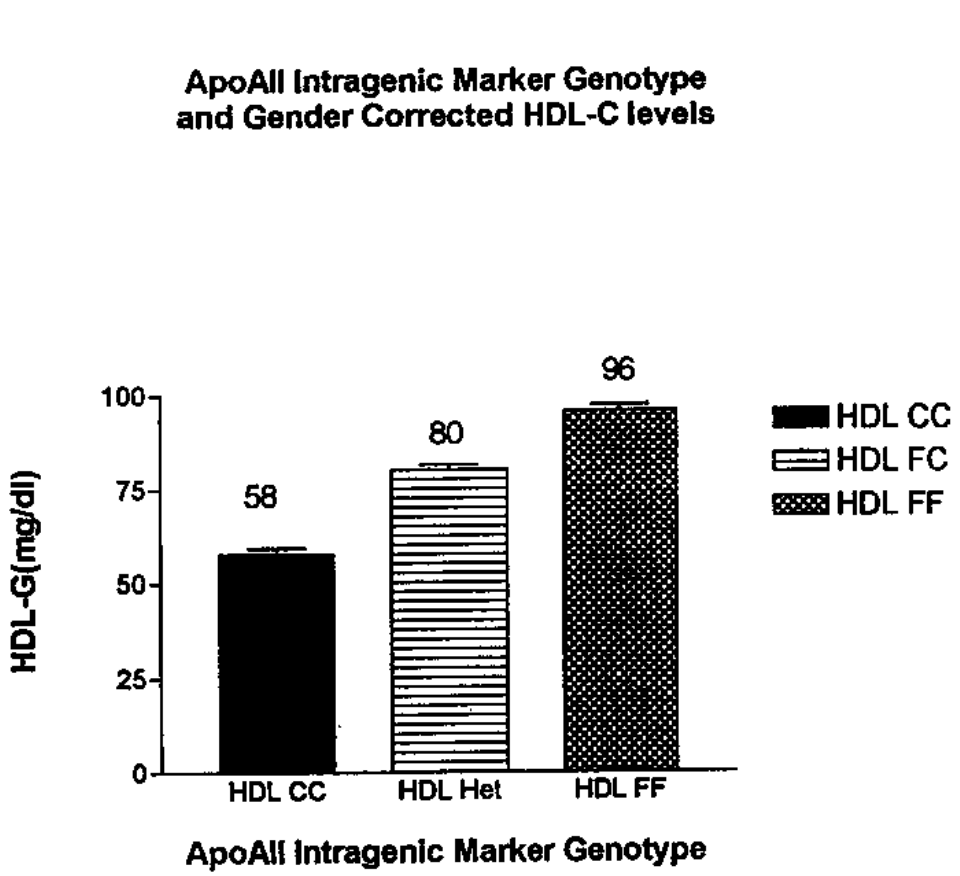


Figure 3: ApoAll intragenic marker genotype and HDL cholesterol in F2 generation mice. HDL levels are expressed as milligrams per deciliter and were corrected for gender effects by adding 19 to all HDL-C values for females. Average HDL-C for each genotype for apo AII are as follows: for homozygous C57 HDL-C =58 ± 11.6, heterozygous(FC) HDL-C =80±12.5, homozygous FVB HDL-C= 96± 13.6. Sample sizes are: CC n=48, Het n=89, FF n=61. Group mean differences found to be significant by ANOVA $p<0.0001$.

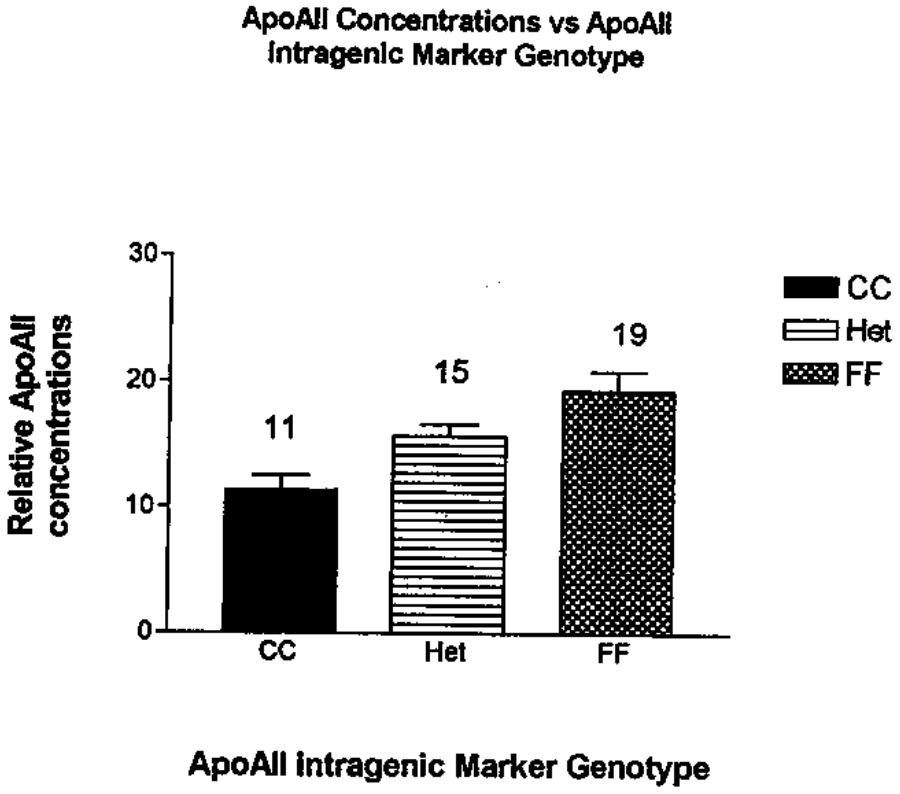


Figure 4: Apo AII concentrations and apoAII intragenic marker genotype. ApoAII concentration values are relative determined by western blotting of apoAII. Average values of relative apoAI concentrationsl ±std dev. are as follows: homozygous C57 [apoAII]=11±7.7 heterozygous FC [apoAII]=15±8.6, homozygous FVB [apoAII]=19±10.3. Sample sizes as follows: CC n=44,Het n=80, FF n=48. Differences in apoAII relative concentrations between groups were determined to be significant by ANOVA $p<0.0001$.

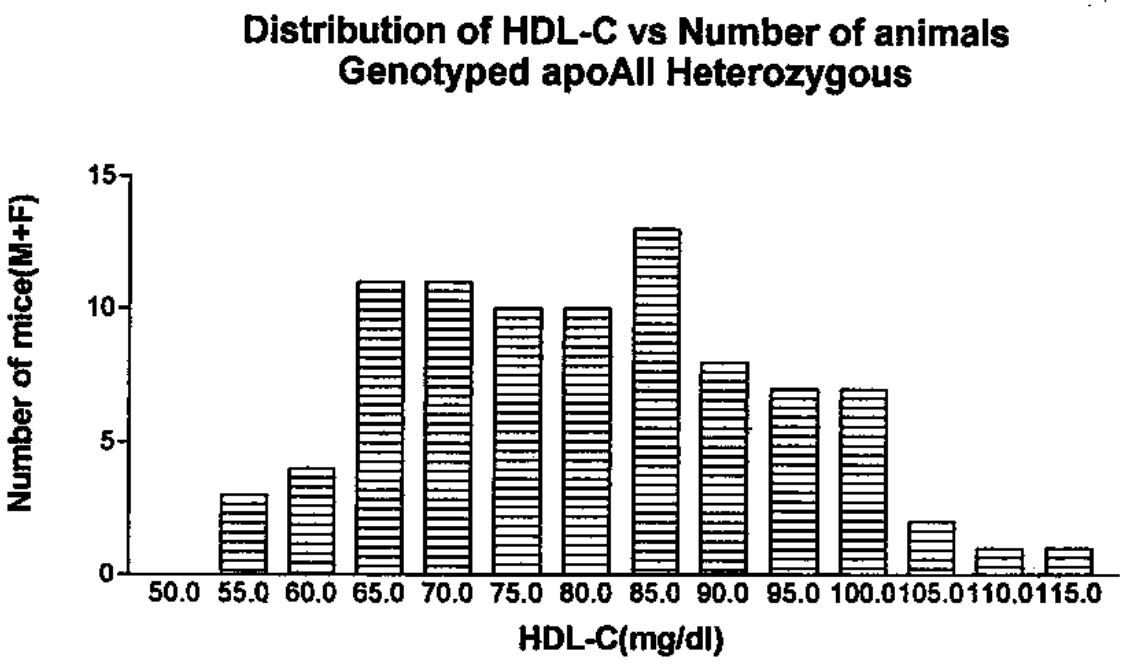


Figure 6 : HDL-C levels corrected for gender and number of F2 progeny animals that are genotyped as heterozygous for apoAII or D1Mit36 locus. N=88 male and female animals represented by graph. HDL-C levels represented in mg/dl and corrected for gender by adding 19 to female HDL-C levels.

represented together after adjusting the HDL-C in males and females by adding 19 (the difference between average HDL-C in F2 males and average HDL-C in F2 females). The results show a gaussian distribution shape with a large number of animals close to the mean HDL-C level and fewer animals with extreme levels of HDL-C. These results deviate from that expected if there were a dominant genetic factor controlling HDL-C levels where 75% of the population would exhibit HDL-C levels similar to one parent and 25% similar to the other. Thus, the wide variation in HDL-C levels seen in the F2 progeny indicate that there may be more than one genetic factor influencing HDL-C levels.

Sequencing of ApoAII gene in Wildtype Mice

Previous research indicates that apoAII structural gene polymorphisms occur between strains (Doolittle et al., 1990). In order to determine if variation in HDL-C levels and/or apoAII levels is due to polymorphisms in the apoAII gene, the apoAII gene was sequenced in FVB and C57 wildtype mice beginning with the end of exon 1 through exon 4 and the translation stop codon. The results show polymorphisms in the nucleotide sequences obtained for C57 as compared to the FVB mice (Figure 6). These polymorphisms resulted in three amino acid changes in the apoAII protein (Figure7). However, the amino acid changes did not alter the charge of the apoAII protein between the FVB and the C57 mice. These results indicate that polymorphisms in the apoAII gene may be responsible for the alteration in HDL-C levels indicated in the parental strains.

Identification of Genetic Loci contributing to plasma HDL-C and ApoAII levels

In order to evaluate the contribution of genetic loci to the phenotypes observed in the F2 mice, we attempted to type genetic makers in the F2 mice at approximately 10 centimorgan distances throughout the mouse genome. Polymorphic simple sequence repeat markers were tested for polymorphisms in parental strains and informative markers were used to type n=48 to n=209 F2 progeny as shown in Figure 8. In some cases, large gaps (>20 centimorgans) resulted from a lack of informative markers in the area whereas other areas of chromosomes were more densely mapped. Altogether, 124 Loci spanning 19 chromosomes were mapped.

Figure 6 : Nucleotide Sequences of ApoAII cDNA from C57 Bl/6J and FVB/N strains

C57Bl/6J

Exon2

←5'*Primer* →

Start

1

GCAGCACAGAATCGCAGCACTGTTCTTAGGCCATAGTCTGCCATCATGAAGCTGCTCGCAATG

FVB/N

GCAGCACAGAATCGCAGCACTGTTCTTAGGCCATAGTCTGCCATCATGAAGCTGCTCGCAATG

↓ Exon3

64

GTCGCACTGCTGGTCACCATCTGTAGCCTGGAAGGAGCTTTGGTTAAGAGACAGGCAGACGA

GTCGCACTGCTGGTCACCATCTGTAGCCTGGAAGGAGCTTTGGTTAAGAGACAGGCGGACGA

126

CCGGATATGCAGAGCCTGTTCACTCAATACTTTCAGAGCATGACTGATTATGGCAAAGATTTG

CCAGATATGCAGAGCCTGTTCANTCAGTACTTTCAGAGCATGACTGAATATGGCAAAGATTTG

↓ Exon4

189

ATGGAGAAGGCCAAGACCTCAGAGATTCAGAGCCAGGCCAAGGCATACTTTGAGAAGACACA

GTGGAGAAGGCCAAGACCTCAGAGATTCAGAGCCAGGTCAAGGCATACTTTGAGAAGACACA

252

CGAGCAGCTGACACCCCTTGTCAGGTCAGCAGGAACTAGTNTGGTGAACTTNTTCAGCAGTTT

CGAGCAGCTGACACCCCTTGTCAGGTCAGCAGGAACTAGTNTGGTGAACTTNTTCAGCAGTTT

Stop

315

355

AATGAACCTTGAGGAGAAACCGGCTCCTGCGGCTAAGTGA

AATGAACCTCGAGGAGAAACCGGCTCCTGCGGCTAAGTGA

Figure 6: Nucleotide sequence of apoAII cDNA obtained from mRNA from C57Bl/6J and FVB/N hepatic tissue via RT PCR as described in *Materials and Methods* .The start and stop codons for translation are highlighted. Differences in nucleotides that change the amino acid sequence between C57 and FVB strains are underlined. The numbers correspond to the number of nucleotide bases sequenced.

Figure 7: Amino Acid Sequences of ApoAII from C57Bl/6J and FVB/N Strain

FVB/N

MKLLAMVALL VTICSLEGAL VKRQADGPDM QSLFXQYFQS MTEYGKDLVE

C57Bl/6J

MKLLAMVALL VTICSLEGAL VKRQADGPDM QSLFTQYFQS MTDYGKDLME

KAKTSEIQSQ VKAYFEKTHE QLTPLVRSAG TSXVNXFSSL MNLEEKPAPA AK.

KAKTSEIQSQ AKAYFEKTHE QLTPLVRSAG TSXVNXFSSL MNLEEKPAPA AK.

Figure 7: Amino acid sequences of apoAII determined for C57Bl/6J and FVB/N wildtype mice predicted from the nucleotide sequence of apoAII gene. Changes in amino acid sequences between the two strains (highlighted) are as follows: aa position 43, position 49, and position 61. These amino acid changes include Gln to Asp, Val to Met and Val to Ala respectively. X represents undetermined amino acid due to unknown base sequence.

Table 1: Free regression analysis using Chi-Square statistics of genotype data in association with quantitative trait HDL (unadjusted). Table shows likelihood ratio statistic (stat), percent variation, additive and dominant regression coefficients and LOD scores(log of odds). Data represented generated by *Mapmanager QTL v 27*

Chi-square Stats. P = 0.01						
locus {chr}	**stat**	**%**	**P**	**add**	**dom**	**LOD**
D1Mit46 {One}	16.8	7	0.00022	-9.93	3.99	3.7
D1Mit102 {One}	48.2	23	3.5e-11	-13.80	2.74	10.5
D1Mit268 {One}	38.7	20	3.9e-9	-12.66	8.53	8.4
D1Mit16 {One}	49.8	25	1.5e-11	-13.94	-3.56	10.8
D1Mit15 {One}	75.3	36	4.5e-17	-18.18	0.06	16.4
D1Mit36 {One}	107.5	40	4.4e-24	-18.63	1.71	23.4
ApoAII {One}	104.7	40	1.9e-23	-18.28	2.36	22.8
D1Mit356 {One}	93.5	37	5.0e-21	-17.88	2.38	20.3
D1Mit359 {One}	71.1	31	3.6e-16	-16.71	-0.58	15.5
D1Mit291 {One}	50.9	24	8.9e-12	-14.23	3.08	11.1
D8Mit121 {Eight }	12.4	5	0.00200	7.06	-4.77	2.7

Table2: Free regression analysis using Chi-Square statistics of Genotype data in association with HDL (mg/dl) values adjusted for gender and D1Mit36 locus. HDL values were adjusted for gender by adding 19 to female values. HDL values were adjusted forD1mit36locus by scaling all values according to the genotype for D1mit36. The table shows a likelihood ratio statistic, percent variation due to locus, Additive and dominant regression coeficients and LOD scores-Log of Odds. Data represented generated by *Mapmanager QTL v 27*. p=0.01

locus {chr}	**stat**	**%**	**P**	**add**	**dom**	**LOD**
D8Mit121 {Eight }	9.6	4	0.00827	5.18	-3.52	2.1

The genetic information obtained was used to statistically determine associations between quantitative traits observed in the F2 mice and genetic loci. These associations were measured using the *MapManager QTL v27* program (Manly et.al., 1999). Free regression analysis was used to determine genetic effects on HDL-C levels in the F2 mice (Table 1). Similar studies suggest that for the density of the markers scored in this cross, a LOD score of >3 indicated the locations of genes responsible for the phenotypes observed (Purcell-Huynh et al., 1995). The results listed in table 1 show that the peak LOD score is located at D1Mit 36 locus with significant loci located at the D1mit46, D1mit102, D1mit268, D1mit16, D1mit15, ApoAII intragenic marker, D1Mit 36, D1Mit 359 and D1Mit 291 markers located at the distal end of chromosome 1. Thus, the peak LOD score implicates a candidate gene affecting HDL-C-C levels in the F2 mice near the apoAII gene mapped at 91cm on chromosome 1. The D8mit121 locus is not as significant in association with the HDL-C trait in the F2 animals. Since the D1Mit36 locus contributes to approximately 40-55% of the variation seen in HDL-C levels in the F2 mice, the HDL-C levels were corrected for the effects of this locus by linear regression. Also, differences in gender were observed in the F2 mice: the average HDL-C for male F2 was 79 mg/dl while the average HDL-C levels in female F2 mice was 60 mg/dl HDL-C (Figures 1 and 2). In order to use all the F2 animals in the genotype analysis, the HDL-C levels were adjusted for these gender effects by adding 19mg/dl to each female HDL-C level. Thus, by adjusting the HDL-C levels for gender, the only variable in HDL-C levels is genetic as opposed to any hormonal interactions causing variation in HDL-C in the females. Calculations for the genetic determinant of variation in HDL-C levels was performed as described in *Materials and Methods* and resulted in 50% of the variation in HDL-C levels was due to genetic affects (data not shown). Moreover, 50% of the variation is not affected by the D1mit36 locus but is caused by genetic components. With this in mind, the genome scan was performed in order to find additional genetic components affecting HDL-C. Table 2 shows free regression analysis of the genotype data in association with HDL-C levels corrected for gender and D1mit36 locus. As observed, the only locus that showed an association with HDL-C levels (corrected) was D8mit 121. However, the resulting LOD score for this locus was not statistically significant (>3) in accordance with the significant LOD scores required for our sample size (n=209).

Table3: Free regression Analysis of genotype data in association with HDL-G (mg/dl) values adjusted for gender by adding 19 to female HDL values. The Table shows a likelihood ratio statistic(stat), percent variation due to a locus, additive and dominant regression coeficients and LOD scores.
Data represented generated by *MapManager QTL v27*. p=0.01

Locus {chr}	stat	%	P	add	dom	LOD
D1Mit46 {One}	20.2	9	4.2e-5	-9.58	3.06	4.4
D1Mit102 {One}	70.0	31	6.2e-16	-14.32	0.53	15.2
D1Mit268 {One}	44.6	23	2.1e-10	-12.73	7.52	9.7
D1Mit16 {One}	39.7	20	2.4e-9	-11.05	0.05	8.6
D1Mit15 {One}	107.7	47	4.2e-24	-17.77	2.69	23.4
D1Mit36 {One}	164.4	55	2.0e-36	-19.22	2.47	35.7
ApoAII {One}	159.0	55	3.0e-35	-18.92	3.49	34.6
D1Mit356 {One}	152.2	53	9.0e-34	-18.93	3.12	33.1
D1Mit359 {One}	112.2	45	4.4e-25	-17.53	0.78	24.4
D1Mit291 {One}	76.2	34	2.9e-17	-15.13	3.68	16.6
D3Mit203 {Three}	9.5	8	0.00844	8.34	1.80	2
D8Mit166 {Eight }	13.4	6	0.00123	4.83	-7.99	3
D8Mit121 {Eight }	19.5	9	5.8e-5	7.36	-6.29	4.2

Figure 8: Chromosomal Map
Male and female F2 progeny mice were genotyped for approximately100-124 markers throughout the 19 chromosomes comprising the mouse genome. Markers were obtained by *Research Genetics* and used in PCR as described in *Materials and methods.* Marker distances are based on information obtained from *The Whitehead Institute for Genome Research, MIT.* Chromosome lengths are based on data collected at *Research Genetics..*
The numbers located next to each marker name represent centimorgan locations of each marker. Numbers below each marker represent number of animals genotyped for that marker. A minimum of *n=47* and maximum *n=209* animals were genotyped for each marker. Markers without an *n* number have not been genotyped. Chromosomes are not drawn to scale
Figure 8A shows chromosomes 1-10, Figure 8B shows chromosomes11-19. The X chromosome has not been genotyped.

Figure 8a: Chromosome Map

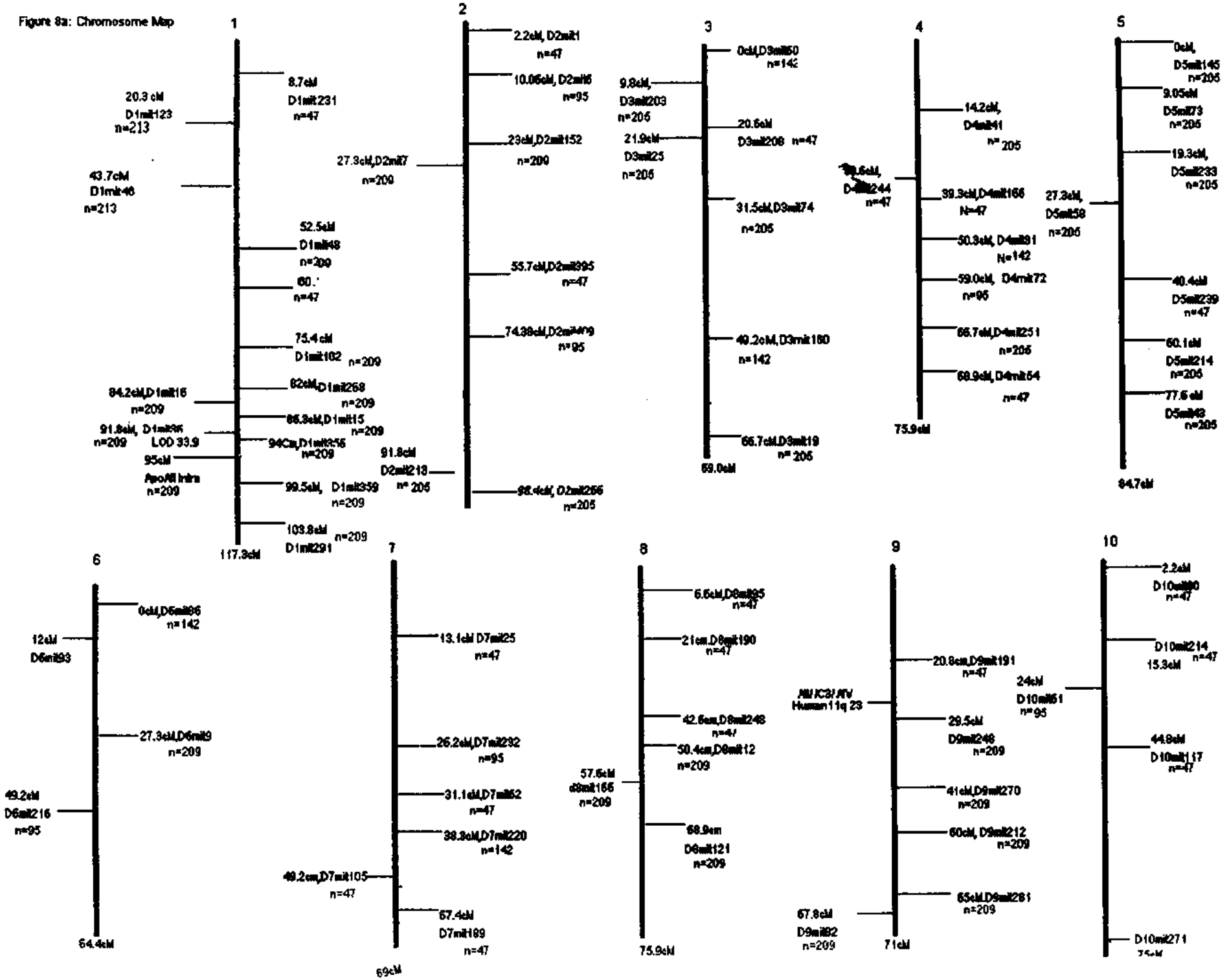
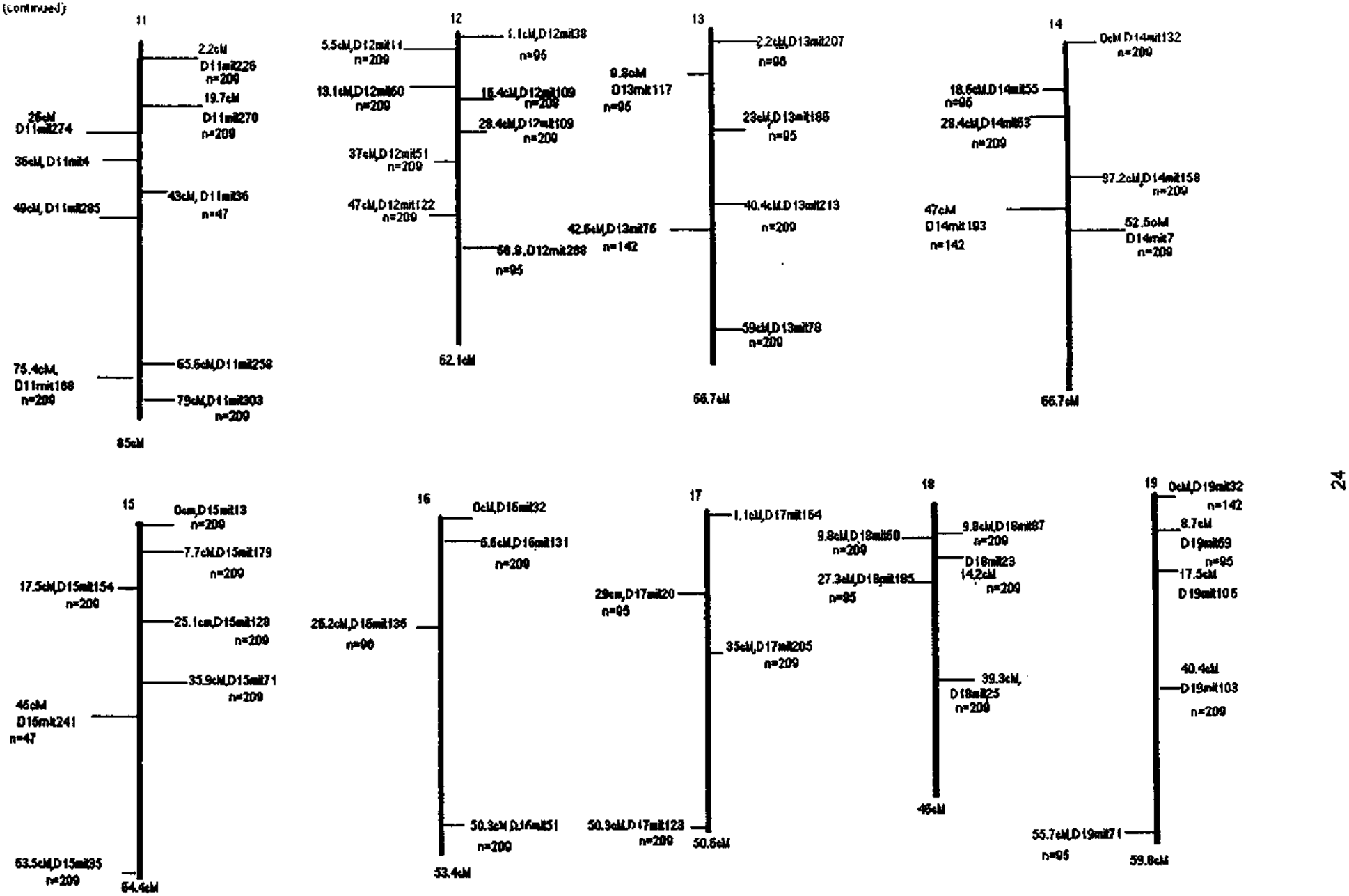


Figure5
(continued)



In order to determine the effect of the loci on chromosome 1 on the entire population, the HDL-C levels were corrected for gender effects (Table3). Free regression analysis of the genotype data in association with HDL-C (gender corrected) results in the same significant loci in chromosome 1 as analysis using uncorrected data. Regression analysis with HDL-C (gender corrected) also resulted in increased significance of D8Mit121 and D8Mit166 loci located at approximately 57-68 cM on chromosome 8. This region of chromosome 8 corresponds to the location of the LCAT gene (lecithin cholinesterase acetyl transferase) at 51 cM which may affect HDL-C physiology and metabolism (Debry et. al., 1996). However, the additive and dominant regression coefficients for these loci indicate that these loci show an opposite effect on HDL-C than that observed for D1Mit36. Moreover, animals typed CC for this locus showed increased levels of HDL-C. Thus, HDL-C levels are positively correlated with C57 alleles for the loci on Chromosome 8 while positive correlation between FVB alleles and HDL-C levels was observed with the significant loci (D1mit36, etc...) on chromosome 1. Free regression analysis of the genotype data was also performed on apoAII levels obtained for the F2 mice (Table 4). As expected, the results indicate a peak LOD score at D1Mit 36 and ApoAII marker locus. Other non-significant loci include D8mit121 and D7mit732. The percent variation of the apoAII levels due to D1mit36 was lower than expected (10% and11%) indicating that there may be other loci affecting apoAII levels in addition to the apoAII gene.

Table4: Free Regression analysis using Chi-Square Statistics of genotype data in association with relative ApoAII values obtained as described in *Materials and Methods*. Table shows a likelihood ratio statistic(stat), percent variation due to locus, additive and dominant regression coefficients and LOD scores. Data represented was generated by *MapManager QTL v27*.p=0.01

locus {chr}	stat	%	P	add	dom	LOD
D1Mit102 {One}	10.0	5	0.00659	-3.14	0.72	2.2
D1Mit268 {One}	14.9	9	0.00059	-4.05	1.62	3.2
D1Mit15 {One}	19.2	12	6.6e-5	-4.85	0.47	4.1
D1Mit36 {One}	20.5	10	3.5e-5	-4.24	0.67	4.5
ApoAII {One}	20.6	11	3.4e-5	-4.31	0.53	4.5
D1Mit356 {One}	20.2	10	4.0e-5	-4.33	0.29	4.4
D1Mit359 {One}	12.4	6	0.00206	-3.44	-0.18	2.7
D1Mit291 {One}	11.7	6	0.00288	-3.43	1.25	2.5
D7Mit232 {Seven }	10.4	5	0.00543	3.25	-0.12	2.3
D8Mit121 {Eight }	11.0	5	0.00414	3.13	-1.83	2.4

Discussion

In this study, we have investigated the genetic causes of elevated HDL-C levels in inbred strains of mice by using quantitative trait loci mapping of the mouse genome represented in the F2 generation from a cross of C57 and FVB wildtype mice. Analysis of the HDL-C levels in male and female wildtype mice and subsequent generations revealed differences in HDL-C levels that were inherited in a Mendelian fashion (see Figures 1 and 2). Also, HDL-C levels represented by the F2 progeny spanned the levels represented by the parental mice indicating that there are a combination of factors affecting the HDL-C in these mice (Figure 1 and 2). Since apoAII comprises HDL-C in the mouse, we genotyped the F2 animals for D1mit36 locus at 91cM on chromosome1. Free regression analysis of HDL-C and the D1mit36 locus revealed that while D1mit36, or presumably the apoAII gene, was responsible for 40-55% of the variation in HDL-C levels observed in the F2 mice, a large amount of the variation was not explained by apoAII (seeTable 1 and 3). These results are in accordance with studies done that link apoAII locus on Chromosome 1 to plasma apoAII and HDL-C levels (Weng et al., 1996 and Welch et. al., 1996). Calculation of the genetic components affecting HDL-C levels in the F2 mice resulted in approximately 88% variation in HDL-C is due to genes affecting HDL-C. The D1mit36 locus, presumably the apoAII gene accounts for 40-55% of this variation. Therefore, there is approximately 40% of the variation not due to the effects of the genes at the D1mit36 locus. Thus, in this study, the genotype data observed together with the phenotype data indicated that there were additional genes affecting HDL-C levels in these mice.

In the current study, we set out to explore the effect of additional genes on ApoAII and HDL-C levels by genotyping the F2 mice for loci at approximately every 10 cM intervals throughout the mouse genome (Figure 8). Similar studies recently performed by Purcell-Huynh et.al. , (1995) provided a model for which to perform a genome scan in the F2 animals from a cross of wildtype mice. Our study reveals a strong correlation between the ApoAII gene (D1mit36) locus and HDL-C levels with a LOD score of 23 resulting from free regression analysis of quantitative trait HDL-C (Table 1). Since male and female F2 mice exhibited gender dependent levels of HDL-

C, the HDL-C data in the F2 mice were corrected for gender specific effects by adding 19mg/dl to the female F2 mice (data not shown). Free regression analysis between the HDL-C data corrected for gender and the genotype data revealed an increase in the LOD score of the D1mit36 locus - LOD=35 (Table 3). Thus, there may be gender specific effects on the apoAII (D1mit36) locus. This is not unexpected since both mice and humans exhibit gender dependent differences in atherosclerosis susceptibility. These results are in accordance with previous studies that identify that the D1mit36 locus explains variation in HDL-C levels on a chow diet (Purcell-Huynh et. al., 1995 and Machleder et. al., 1997). The genome scan results also indicate loci on chromosome 8 (D8mit121and D8Mit166) are linked to variation in gender corrected HDL-C levels and elicit opposite effects from the apoAII locus (see table 3). These loci are only significant (LOD=4.2, 9% variation and LOD=3, 6% variation respectively) when the HDL-C data is corrected for gender because a larger sample size is needed to detect weaker effects. Nevertheless, a small change in HDL-C levels in mice and/or humans can have significant effects on the development of atherosclerosis. Current research has identified one candidate gene –LCAT to be located at approximately 53cM on chromosome 8 (Debry et. al., 1996). Since the D1mit36 locus resulted in contributing to a large percent of the variation (approx. 50%) in HDL-C levels, we analyzed the genotype data by correcting the HDL-C levels for the effects of D1mit 36 using regression (data not shown). Free regression analysis of the HDL-C corrected for gender and D1mit36 locus and the genotype data show that the D8mit121 locus becomes insignificant with a LOD=2.1 (table2). These results introduce the possibility that there may be a gene interaction between gene(s) at the D8mit121 locus and D1mit36 locus such that when the effect of one locus is absent, the other locus alone fails to affect HDL-C levels. The results of the genome scan were also analyzed to determine genetic effects on apoAII levels (Table 4). As expected the most significant locus revealed was the D1mit36 or apoAII gene. These results were in accordance with studies that also resulted in a significant linkage between the apoAII gene and apoAII plasma levels (Warden et. al., 1993). It is of interest to note that while the apoAII gene does significantly influence apoAII levels in this cross ($p<0.0001$), it accounts for only 11% of the variation seen in apoAII levels indicating that other factors may influence apoAII in these mice.

In order to better understand the effects of the apoAII gene on HDL-C levels, closer analysis of the apoAII structural gene in the C57 and FVB wildtype mice was

performed. Polymorphisms in the apoAII gene have been shown to determine HDL-C size and composition which in turn affects HDL-C levels in mice (Doolittle et al., 1990 and Wang et.al., 1997). In this study, we sequenced part of the apoAII gene in order to reveal similar polymorphisms affecting the amino acid sequence for apoAII (Figure 6 and 7). However, analysis of these amino acid changes does not reveal a difference in charge between apoAII proteins in C57 and FVB. Previous studies reveal that polymorphisms in the apoAII gene affect translational efficiency of the apoAII protein (Doolittle, et.al., 1990). Molecular studies suggest that structural differences in the apoAII polypeptide influence clearance of the protein that may affect HDL-C levels (Purcell-Huynh et.al., 1995). Further investigation is necessary to determine if the polymorphisms found between the mice used in this study also influence translational efficiency and/or AII polypeptide structural differences.

Conclusions

In conclusion, the results of this study indicate that variation in the apoAII locus significantly affect plasma HDL-C levels in these mice with the FVB alleles at this locus increasing HDL levels while the C57 alleles lower HDL. While polymorphisms in the apoAII gene were determined between the C57 and FVB mice, the effect of these differences on HDL levels needs to be further investigated. Quantitative trait linkage mapping (QTL) identified another candidate gene, LCAT, on chromosome 8 which may also influence HDL levels in these mice. However, further mapping of the genome in the n=209 F2 progeny mice is necessary to determine if these loci are solely responsible for controlling HDL levels. Future experiments such as compositional analysis of HDL particles and sequencing of the LCAT gene may detail the contributions of these genetic factors on HDL-C in these mice. Thus, this work identifies a possible association between apoAII and LCAT genes in controlling HDL levels but future investigations will reveal their complex roles in lipoprotein metabolism in wildtype mice.

The work documented above was performed at the Breslow Laboratory of Biochemical Genetics and Metabolism, The Rockefeller University, NY, NY., under the guidance and supervision of Dr. Hayes M. Dansky and Dr. Jan L. Breslow.

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