Localization of genes for V+LDL plasma cholesterol levels on two diets in the opossum *Monodelphis domestica*[S]

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Abstract **Plasma cholesterol levels among individuals vary considerably in response to diet. However, the genes that influence this response are largely unknown. Non-HDL (V+LDL) cholesterol levels vary dramatically among gray, short-tailed opossums fed an atherogenic diet, and we previously reported that two quantitative trait loci (QTLs) influenced V+LDL cholesterol on two diets. We used hypothesis-free, genome-wide linkage analyses on data from 325 pedigreed opossums and located one QTL for V+LDL cholesterol on the basal diet on opossum chromosome 1q [logarithm of the odds (LOD) = 3.11, genomic *P* = 0.019] and another QTL for V+LDL on the atherogenic diet (i.e., high levels of cholesterol and fat) on chromosome 8 (LOD = 9.88, genomic $P = 5 \times 10^{-9}$). We then employed a novel strategy involving combined analyses of genomic resources, expression analysis, sequencing, and genotyping to identify candidate genes for the chromosome 8 QTL. A polymorphism in *ABCB4* was strongly associated ($P = 9 \times 10^{-14}$) with the plasma V+LDL cholesterol concentrations on the high-cholesterol, high-fat diet. The results of this study indicate that genetic variation in *ABCB4*, or closely linked genes, is responsible for the dramatic differences among opossums in their V+LDL cholesterol response to an atherogenic diet.**—Kammerer, C. M., D. L. Rainwater, N. Gouin, M. Jasti, K. C. Douglas, A. S. Dressen. P. Ganta, J. L. VandeBerg, and P. B. Samollow. **Localization of genes for V+LDL plasma cholesterol levels on two diets in the opossum *Monodelphis domestica*. *J. Lipid Res.* 2010.** 51: **2929–2939.**

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Plasma lipoprotein phenotypes, especially HDL and LDL cholesterol levels, represent major risk factors for development of atherosclerosis. Several environmental factors, such as diet and exercise, exert important effects on lipoprotein phenotypes, but genetic effects also play a role (1). Using data from human families and animal models of cardiovascular disease, researchers have identified both common, e.g., *APOE* (2), and uncommon, e.g., *PCSK9* (3), variants in candidate genes that influence plasma lipoprotein cholesterol levels. With the development of databases of genetic variation in humans (HapMap, www.hapmap.org), as well as high-throughput genotyping methods, recent genome-wide association analyses of large studies comprising ~20,000 individuals have revealed additional genes that influence plasma lipoprotein levels, especially plasma HDL and LDL cholesterol levels [e.g., (4, 5)]. Taken together, these common genetic variants only account for a small proportion of the known heritable variation in HDL and LDL plasma cholesterol levels, resulting in a phenomenon referred to as the "missing heritability" (6). Some of this missing heritability may result from the effects of genotype-by-environment interactions. Given the known effects of diet on atherogenesis, as well as the wide variation among individuals in response to dietary components, identification of specific genotype-by-diet interaction effects is critical for a deeper understanding of the biological pathways involved, as well as assessment of individual risk (7). Although studies of possible genotype-

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Abbreviations: apoB, apolipoprotein B; HCHF, high-cholesterol, high-fat; IBD, identity by descent; LOD, logarithm of the odds; QTL, quantitative trait locus; RFLP, restriction fragment-length polymorphism; RG-PCR, restriction site-generating PCR; SNP, single-nucleotide polymorphism.

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[S]The online version of this article (available at http://www.jlr.org) contains supplementary data in the form of four figures and nine tables.

by-diet effects on lipid levels have been performed (8), results have been inconsistent, most likely due to relatively small sample sizes or difficulties in measuring dietary components in human populations.

For many of these gaps in our knowledge base, a valid animal model can provide valuable insights. While an animal model may not precisely replicate human metabolism in every detail, effects of specific genes or pathways can be isolated under controlled and consistent conditions, a feature that is required to adequately characterize genotype-by-diet interactions. The gray, short-tailed opossum, *Monodelphis domestica*, is potentially an excellent candidate for this purpose: it is small, reaches sexual maturity in 5–6 months, breeds year-round, has large litters (mean size, eight), is maintained in colonies in at least seven countries, and is used in a variety of research areas, including comparative immunogenetics, neurobiology, carcinogenesis, endocrinology, fetal development, meiotic recombination, and more (9). In particular, lipoprotein metabolism in opossums is similar to human metabolism in many respects. For example, antibodies directed against human apolipoprotein B (apoB) and apoE cross-react with the equivalent opossum proteins (10). Furthermore, metabolic and expression studies indicate that aspects of cholesterol absorption, synthesis, and excretion are similar to humans (11, 12). We also have observed heritability of key aspects of lipoprotein phenotypes that are similar to humans and, in particular, have observed a striking interindividual difference in non-HDL (V+LDL) cholesterol response to a high-cholesterol, high-fat (HCHF) diet (13). The V+LDL response largely involves increases of cholesterol rather than triglyceride levels [~30-fold vs. ~1.6-fold increases, respectively (14)], and the increase in V+LDL cholesterol is elicited by high dietary cholesterol alone but not high fat alone (although the V+LDL response to dietary cholesterol is amplified when dietary fat is also present). In contrast, HDL cholesterol increases in response to dietary fat, and these responses are similar to those seen in other species, including primates (14). We previously reported evidence that plasma levels of HDL cholesterol and V+LDL cholesterol on the basal and HCHF diets are under the control of quantitative trait loci (QTLs) that account for a large proportion of the variation in HDL and V+LDL cholesterol in our population of animals (13). We hypothesized that the metabolic basis for this interaction with diet replicates a similar mechanism in humans and that it may underlie an important component of cardiovascular risk in humans associated with these highly atherogenic lipoproteins.

Recently, the opossum genome was sequenced (15) and we developed a genetic linkage map for this species composed largely of microsatellite markers (16). In the present study, we used these resources to map two of the previously detected QTLs onto the *M. domestica* genome and conduct comparative synteny analyses to derive a group of positional candidate genes, followed by a literature survey to identify high-priority candidates. We then analyzed the expression of these genes in liver (because of its central role in lipid metabolism), sequenced them to detect variants, and genotyped variants in and near the candidate genes in our study population in an effort to identify the QTL influencing V+LDL cholesterol response to the HCHF diet.

MATERIALS AND METHODS

Animals

The opossums used in this study were bred and raised at the Southwest Foundation for Biomedical Research (SFBR), San Antonio, Texas, which is accredited by the Association for Assessment and Accreditation of Laboratory Animal Care International. The origins of the laboratory population of *M. domestica* and the husbandry conditions have been described (17). All experimental protocols were approved by the SFBR Institutional Animal Care and Use Committee.

Plasma and DNA samples were collected from 331 animals (164 males and 167 females) in seven pedigrees (sizes = 106, 89, 84, 36, 10, 3, 3) comprising 28 partially inbred "parent" animals (14 males and 14 females) and their first- and second-generation offspring (see supplementary Fig. I). The 28 parents for these pedigrees were members of the offspring generation from the population studied in an earlier report on the inheritance of lipoprotein cholesterol (13), and their average inbreeding coefficient was 0.50. The first-generation offspring of the 28 parent animals were then mated (one brother with multiple sisters) to produce full-sibship litters within large half-sibships (the second generation). The power of genetic analyses to detect and locate QTLs depends in part on the numbers of different pairs of relatives. For example, in these pedigrees, we have 202 parent-offspring, 308 full-sibling, 168 half-sibling, 158 grandparent-grandchild, and numerous more-distant relationships.

Experimental protocol

All animals were maintained from weaning age on the basal diet, a commercial pelleted fox food diet, which is the standard for this species and which has moderate levels of fat and cholesterol [10.0% and 0.16% of dry weight, respectively (14); see supplementary Table I]. At approximately 5 months of age, animals were fasted overnight and bled via heart puncture while under methoxyflurane-induced anesthesia. Next, they were fed an HCHF diet enriched in fat (from lard) and cholesterol [(18.8% and 0.71% dry weight, respectively (14); see supplementary Table I]. After 8 weeks on the HCHF diet, the animals again were fasted overnight and bled.

Measurement of HDL and V+LDL cholesterol concentrations

From each of the basal and HCHF diet blood samples, EDTA plasma was prepared by low-speed centrifugation and cooled on ice, and plasma cholesterol concentrations were measured enzymatically using a clinical chemistry analyzer and commercial reagents (reagents from Boehringer-Mannheim Diagnostics, Indianapolis, IN; calibrators from Polestar Labs, Escondido, CA). When the measurement exceeded that of the highest calibrator (9.25 mmol/l), the sample was diluted with saline to bring it into range. HDL cholesterol concentrations were measured in the supernatant after precipitation of apoB-containing lipoproteins with heparin-Mg^{2+} (18); V+LDL cholesterol was calculated as the difference between total and HDL cholesterol levels.

Polymorphisms and genotyping

DNA was prepared from liver collected from the 303 first- and second-generation offspring using the Wizard SV 96 Genomic DNA Purification System (Promega). All 303 animals were geno-

typed for 74 anonymous microsatellite markers and a microsatellite within the TDT (terminal deoxynucleotidyltransferase) gene. The map positions of these 75 markers are known from the opossum BBBX linkage map that comprises more than 330 autosomal and X-chromosome-linked loci (16; unpublished observations). Supplemental Table II lists references for the 42 previously published markers and gives descriptions and genotyping methods for the 33 marker systems described here for the first time. Genotyping was then performed on ABI 3100 or ABI 3730XL genetic analysis platforms (19, 20). Quality control procedures were similar to those previously described (21).

Although the total number of base pairs in the opossum genome is similar to that of humans (15), the overall meiotic map length is comparatively small, ~867 cM (21). Therefore, initial genotyping was conducted for 66 marker loci across the eight autosomes: mean intermarker distance on each chromosome ranged from 12 to 17 cM. To further clarify the locations of the putative QTLs, we genotyped an additional nine markers in the QTL regions of interest; the locations of all 75 markers are given in supplementary Table II.

Statistical genetic analyses

All plasma cholesterol distributions were nonnormal. To reduce nonnormality prior to genetic analysis (22), V+LDL cholesterol on the basal diet was transformed by square-root, whereas V+LDL cholesterol on the HCHF diet and HDL cholesterol on both diets were transformed by natural logarithms. In addition, extreme outliers (±4 SD) were removed.

Heritabilities of plasma cholesterol levels were estimated using maximum likelihood variance components methods, in which phenotypic variation was modeled as follows:

$$y_i = \mu + \sum_{i=1}^{n} \beta_j X_{ij} + g_i + e_i,$$

where y_i is the plasma cholesterol level for the ith individual, μ is mean cholesterol level, X_{ij} is the jth covariate for the ith individual, β_j is the corresponding regression coefficient, g_i is the additive genetic effect, and e_i is the residual error effect, which includes unmeasured environmental and nonadditive genetic components. Residual heritability, h^2_r, is the proportion of total trait variance due to the additive genetic component after adjusting for environmental covariates. The likelihood ratio test was used to assess the significance of model parameters by comparing the full model (all covariates and additive genetic effects) with a nested model lacking a specific parameter (23). Because V+LDL on the HCHF diet was bimodal, the effects of sex, age, and weight were assessed within each modal group.

Two-point and multipoint linkage scans were performed using the variance components method, which extends the above model by including the effect of a putative QTL, σ^2_{QTL}, as a component of variance (23). Maximum likelihood methods were used to estimate σ^2_{QTL} based on the expected covariance between relatives due to their identity by descent (IBD) at a given marker (two-point analyses) or at an arbitrary chromosomal location (multipoint analyses) in tight linkage with the presumed QTL. Multipoint IBD probabilities were estimated at 1 cM (Haldane) intervals from map positions 89–200 cM on chromosome 1 (*Mdo* 1) and 0–110 cM on *Mdo* 8 using a Markov Chain Monte Carlo algorithm, as implemented in Loki (24). The likelihood ratio test was used to compare the linkage model to the polygenic (i.e., no linkage, $\sigma^2_{QTL} = 0$) model, and results were reported as the $\log_{10}$ of the likelihood ratio [logarithm of the odds (LOD) score]. Genetic analyses were performed using SOLAR software (23).

After identifying single-nucleotide polymorphisms (SNPs) in high-priority candidate genes (see following section) pedigree-based association analyses were performed to test whether SNP genotypes were associated with V+LDL cholesterol. Specifically, we tested whether the full model (containing parameters for two SNPs) differed from a nested model in which parameters for one or both SNPs were removed. If the nested model does not significantly differ from the full model, then the nested model is accepted as the better (or "best") model because it describes the data as well as the full model with fewer parameters. If the nested model differs significantly from the full model, then the full model is better. We also performed multipoint linkage scans while incorporating the candidate gene marker as a covariate in the model. If no significant evidence for a QTL remained after inclusion of the marker as a covariate, i.e., LOD score <3.0, we interpreted this result to indicate that a variant (or haplotype) near the marker might be causal. Plots and histograms were created in R (R Foundation for Statistical Computing, Vienna, Austria).

Identification of potential candidate genes in QTL regions

We used three unbiased, complementary approaches to identify a list of possible candidate genes in QTL regions of interest, i.e., genomic regions near QTLs that might influence V+LDL cholesterol on the basal and HCHF diets. First, we obtained the current annotated list of genes in the regions of interest from the University of California Santa Cruz (UCSC) Opossum Genome Browser Gateway (http://genome.ucsc.edu/cgi-bin/hgGateway). The opossum genome is extensively annotated; however, it contains many predicted genes of unknown identity ("novel genes") that are likely to have orthologs or paralogs in corresponding regions of the human genome that were not identified by the automated gene prediction algorithms (25). Second, to construct a more comprehensive catalog of genes likely to be present in the QTL regions of interest, we employed a comparative synteny strategy to identify comparable (homologous) regions in the much more thoroughly annotated human genome assembly. Specifically, we utilized the comparative synteny features of the Ensembl Opossum Genome browser (http://www.ensembl.org/Monodelphis_domestica/Info/Index) and UCSC Opossum Genome Browser Gateway to first determine the regions of the human genome that are homologous to the stretch of opossum genome contained within each QTL candidate region. We then used the Biomart feature of Ensembl (http://www.ensembl.org/biomart/martview/bc3dd7b7a5f66472ea30409b20483b62) to determine the annotated gene content and chromosome assembly coordinates for each gene within those homologous regions of the human genome corresponding to the opossum QTL regions. This output was compared with the most recent human genome annotation at the UCSC browser (GRCh37). Wherever the UCSC data set contained a gene prediction not present in Ensembl Biomart output, this gene and its assembly coordinates were merged into the Biomart-based list to produce a comprehensive list of all genes and their genomic locations in the human homologous regions corresponding to the opossum QTL regions. Third, we used two public databases to identify a set of genes that had been tested for association with LDL cholesterol levels in at least one human population. We searched the National Human Genome Research Institute Catalog of Published Genome-Wide Association Studies (http://www.genome.gov/26525384) using the search term "LDL cholesterol" and listed all genes that were significantly associated (at a genome level of significance) with LDL cholesterol. We also searched the HuGE Navigator (http://www.hugenavigator.net/), a database of results from tests of associations between candidate genes and various traits in humans, using the "Phenopedia" option and the search term "Lipid Metabolism Disorders"; this database contains both positive and negative results. From these two databases, we obtained a list of 224 genes potentially associated with LDL metabolism in humans. To obtain the unbiased list of potential

positional candidate genes for the V+LDL QTL on each diet, we merged this list of 224 genes with the comprehensive list of opossum homologs of human genes in the QTL regions of interest. Finally, to identify a subset of high-priority candidate genes from this unbiased list of positional candidate genes, we further assessed the published literature for the specific effects or associations of these genes with LDL-related traits in humans and animal models.

Expression and sequencing of high-priority candidate genes

From the 303 offspring, we identified 12 (6 male and 6 female) opossums with low and 12 (6 male and 6 female) with high V+LDL cholesterol on the HCHF diet, for which we also had stored liver samples. Low and high responders had V+LDL cholesterol <0.3 or >2.2 mmol/l (ln-transformed), respectively (see **Fig. 1**). These animals had liver samples collected immediately after the HCHF diet bleed (6 high and 6 low) or had liver samples collected after subsequently being fed the basal diet for 8–12 weeks (6 high and 6 low). Within each responder-by-diet group, animals were chosen to represent at least three different pedigrees (see supplementary Table III). Total RNA was extracted from the liver tissue of the 24 animals using the TRI-Reagent protocol (Molecular Research Center, Inc.). RNA extracts were DNase treated with the DNA-free Kit (Ambion) and used to synthesize double-stranded cDNA utilizing the SMARTer cDNA Synthesis Kit (Ambion). cDNA concentrations were determined using a Qubit fluorometer (Invitrogen).

Expression assays were conducted for opossum homologs of the high-priority candidate genes (*ABCB1*, *ABCB4*, *INSIG1*) and a ubiquitously expressed housekeeping gene, *GAPDH*, as a control for standardization. The Ensembl-predicted annotations for these genes were used to design forward and reverse PCR primers using PRIMER3.0 software (see supplementary Table IV). Twenty-five nanograms of cDNA were used to conduct real-time PCR (RT-PCR) assays, and the PCR products were detected on a high-throughput gene quantification platform, LightCycler® 480 (Roche). Each reaction was performed in duplicate to confirm results, and the duplicate measures of relative abundance (i.e., threshold cycle or C_T, values) were averaged. For each assay in each animal, we obtained ΔC_T (=C_T gene − C_T *GAPDH*) and tested for mean differences in ΔC_T between the high and low responders on each diet using *t*-tests (26). With this sample size, we had 80% power at $P < 0.05$ to detect 2–5 unit differences in mean ΔC_T, that is, 4-32 fold expression differences, given the standard deviation for the assays ranging from 1–3 units.

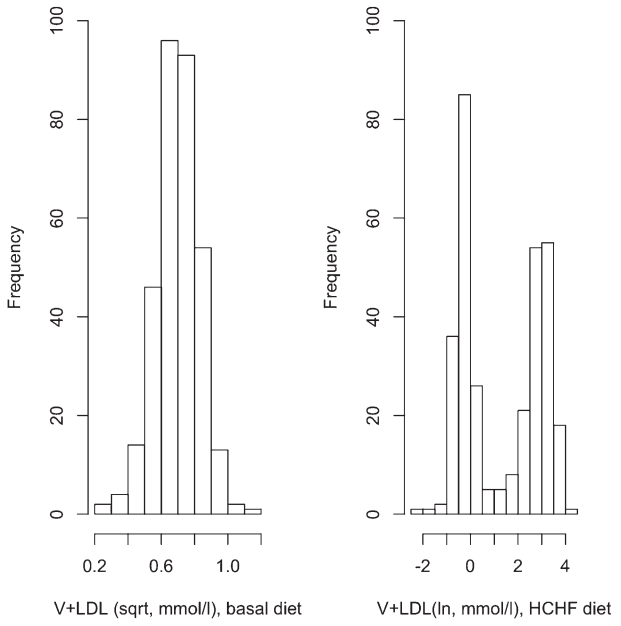

Fig. 1. Distribution of V+LDL cholesterol levels on the basal and high-cholesterol, high-fat (HCHF) diets after transformation by square root (sqrt) and natural logarithms, respectively.

The cDNA sequences of *INSIG1*, *ABCB1*, and *ABCB4* from five high and five low responders (see supplementary Table III) were inspected for SNP discovery. Overlapping primer sets for cDNA sequencing were designed for each gene (see supplementary Table V) and ordered from Bioneer, Inc. Twenty nanograms of cDNA was used as template for PCR with conditions optimized for each primer set. To minimize PCR products resulting from nonspecific priming, all PCR products were separated by 1% agarose gel electrophoresis and gel purified using the QIAquick Gel Extraction Kit (Qiagen). Subsequent PCR was conducted to amplify the gel-purified PCR products, and the resulting second PCR products were again purified using the QIAquick PCR Purification Kit (Qiagen). The forward primer of each primer set was then used to perform DNA sequencing on an ABI 3730 or ABI 3130 DNA analyzer platform using the manufacturer's specifications. All sequence data were viewed, aligned, and assembled using Sequencher4.9© software (Gene Codes Corp.). Regions of *ABCB4* and *INSIG1* cDNAs bearing putative SNPs were resequenced using the reverse primer to verify the allelic variants.

Genotyping polymorphisms in *ABCB4* and *INSIG1*

All offspring were genotyped for polymorphisms in *ABCB4* and near *INSIG1*. For *ABCB4*, the Ile235Leu SNP was genotyped using restriction site-generating PCR (RG-PCR) (27). Specifically, we generated an artificial *Hpy*188I restriction site in the A-bearing amplimer by creating a base mismatch near the 3′ end of the forward primer flanking the SNP site. Primers used for *ABCB4* RG-PCR were F: 5′-TCCCTAAATATTTGGGTTTTATTGTTATTTCAG-3′ and R: 5′-CACCGTCTTAATGGCAGACA-3′; where F is the mismatch primer. This primer design renders the A-bearing amplimer, but not the T-bearing amplimer, susceptible to *Hpy*188I digestion, resulting in an artificial restriction fragment-length polymorphism (RFLP). The RFLP was visualized by electrophoresis of restriction digests on 4% NuSieve 3:1 agarose gels (FMC BioProducts) stained with ethidium bromide. We were unable to design a reliable genotyping strategy for the *INSIG1* Arg24Gly SNP; thus, we developed a microsatellite polymorphism (*8M706*) that was ~8 kb upstream from *INSIG1* as a marker for the *INSIG1* region. Methods for *8M706* follow those described for the other microsatellites used in this study (see supplementary Table II).

RESULTS

Means and heritability of cholesterol levels on two diets

Plasma cholesterol levels were available for 325 animals (163 males and 162 females) with mean age at baseline equal to 161 days (range = 110 – 225 days) and mean weight (±SD) equal to 68 ± 18 g on the basal diet and 77 ± 20 g after 8 weeks on the HCHF diet. On the basal diet, mean (±SD) HDL and V+LDL cholesterol levels were 1.29 ± 0.23 mmol/l and 0.5 ± 0.19 mmol/l, respectively; whereas after 8 weeks on the HCHF diet, they increased to 1.83 ± 0.56 mmol/l and 11.11 ± 12.7 mmol/l, respectively (**Table 1**). Plasma cholesterol levels were transformed prior to genetic analyses, and these transformations revealed the striking bimodality for natural logarithm-transformed

TABLE 1. Means (untransformed) and residual heritabilities (h^2_r) of HDL and V+LDL cholesterol levels on two diets

Trait	N	Mean ± SD	Range	Variance Due to Covariates	h^2_r	*P*
		mmol/l		%		
Basal diet						
HDL	325	1.29 ± 0.23	0.80 – 2.43	0.039	0.53 ± 0.12	10^{-10}
V+LDL	325	0.50 ± 0.19	0.05 – 1.40	0.076	0.38 ± 0.12	10^{-7}
HCHF diet						
HDL	319	1.83 ± 0.56	0.72 – 4.13	0.121	0.13 ± 0.09	0.001
V+LDL	319	11.11 ± 12.7	0.0 – 61.86	0.0	0.67 ± 0.11	10^{-11}

HCHF, high-cholesterol, high-fat.

V+LDL levels (in ln mmol/l) on the HCHF diet (Fig. 1), as has been previously reported (13).

We next estimated the heritability and the effects of age, sex, and weight on the four plasma cholesterol traits. Compared with males, females had higher HDL on the basal diet and the HCHF diet ($P = 0.0009$ and 10^{-9}, respectively) and lower V+LDL cholesterol on the basal diet ($P = 0.001$); however, there was no difference between sexes for V+LDL on the HCHF diet (see supplementary Table VI). V+LDL cholesterol on the basal diet decreased with increasing age and age^2 ($P = 0.003$ for both), but age did not influence any other trait. Weight was not significant for any trait. The significant covariates accounted for 0–12% of the total variation for each trait (Table 1). Residual heritabilities (h^2_r) ranged from 0.13 to 0.67, Table 1) and were highly significant for HDL and V+LDL cholesterol on the basal diet and V+LDL on the HCHF diet. On the other hand, residual heritability was low for HDL cholesterol on the HCHF diet ($h^2_r = 0.13$).

Two-point and multipoint linkage analyses

Two-point variance components linkage analyses (performed using IBD probabilities for each of 66 map markers on the eight autosomes) revealed evidence for linkage with three of the traits on *M. domestica* chromosomes 1 and 8 (*Mdo* 1 and *Mdo* 8) (see supplementary Fig. II). On *Mdo* 8, three markers returned significant LOD scores for V+LDL on the HCHF diet; the highest two-point LOD score was 8.07 at marker *8M253*. On *Mdo* 1, we obtained significant evidence for linkage with V+LDL cholesterol levels on the basal diet, with *1M372* (LOD = 3.81) located near the end of chromosome 1. Although we observed no significant LOD scores for HDL on the basal diet, locus *1M422* had LOD = 1.77 and the adjacent marker (*1M448*) had LOD = 1.52. There were no LOD scores greater than 0.72 for HDL cholesterol levels on the HCHF diet.

On the basis of the two-point results, we genotyped additional markers on *Mdo* 1q and 8q and performed multipoint linkage analyses. On *Mdo* 1, the maximum multipoint LOD score for V+LDL cholesterol on the basal diet was 3.11 at the end of the chromosome near marker *1M372* (**Fig. 2A**); this QTL accounted for ~70% of the additive genetic variation in V+LDL cholesterol on the basal diet. For HDL cholesterol on the basal diet, the maximum multipoint LOD score was 2.41 at 142 cM between markers *1M422* and *1M448* (see supplementary Fig. III). On *Mdo* 8, we obtained very strong evidence for a QTL that accounted for ~65% of the additive genetic variation in V+LDL cholesterol on the HCHF diet. This QTL was located between *8M253* and *8M705*, maximum LOD score = 9.88 at position 85 cM (Fig. 2B). The 2-LOD support interval comprises the QTL region of interest on each chromosome (28). Our region of interest for V+LDL cholesterol on the basal diet on *Mdo* 1 ranged from *1M637* to *1M372* (the q-end of the chromosome); whereas for V+LDL cholesterol on the HCHF diet on *Mdo* 8, the QTL region of interest was from *8M031* to *8M431* (Fig. 2).

Identification of candidate genes

To identify potential candidate genes that might influence V+LDL cholesterol on the basal and HCHF diets, we interrogated the opossum genome database and also identified homologous chromosomal regions in humans (*Homo sapiens*, *Hsa*) that corresponded to our QTL regions of interest on opossum (*Mdo*) chromosomes 1 and 8. The QTL region of interest on *Mdo* 1 exhibited conserved synteny with regions of *Hsa* 2 and 19 that contained a combined total of 17 known genes, whereas the *Mdo* 8 region of interest was homologous to regions of *Hsa* 3, 7, and 10 and contained 215 known genes (see supplementary Table VII). Of the 17 known genes in the QTL region of interest on *Mdo* 1, only two genes, Alstrom syndrome 1 (*ALMS1*) and lipin 1 (*LPIN1*), were also on the list of genes that had been tested for association with lipoprotein-related traits in humans. We did not choose to follow up the *Mdo* 1 QTL in this report (see DISCUSSION).

For the QTL region of interest on *Mdo* 8, we identified eight genes (or their paralogs) that were also on the unbiased list of potential candidates for "LDL cholesterol" or "Lipid Metabolism Disorders" (**Table 2**). These genes included: nitric oxide synthase 3 (eNOS, *NOS3*), insulin-induced gene 1 (*INSIG1*), cubilin (*CUBN*), integrin α8 (*ITGA8*), ATP binding cassette, subfamily B, members 1 and 4 (*ABCB1* and *ABCB4*), calpain 7 (*CAPN7*), and UDP-*N*-acetylgalactosaminyltransferase-like 2 (*GALNTL2*). We next surveyed the literature to determine which specific traits or functions had been associated with these genes in humans or mice (Table 2). Based on their involvement in various aspects of LDL metabolism, *INSIG1*, *ABCB4*, and *ABCB1* were deemed high-priority candidate genes for the QTL on *Mdo* 8 that influences V+LDL cholesterol response to diet.

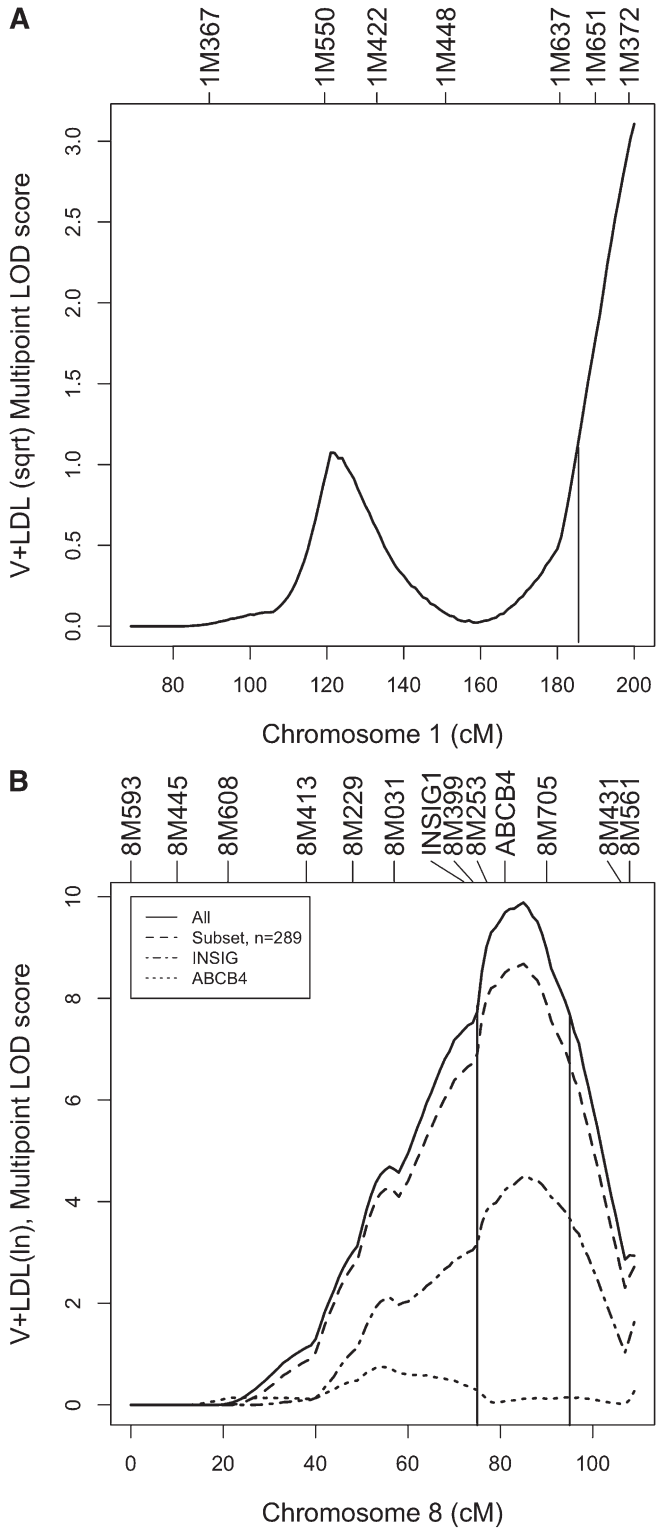

Fig. 2. Multipoint logarithm of the odds (LOD) score plots for (A) V+LDL cholesterol levels on the basal diet on *Mdo* 1 and (B) for V+LDL cholesterol levels on the HCHF diet on *Mdo* 8 using four models: all data; subset, n = 289; *INSIG1* microsatellite included; and *ABCB4* single-nucleotide polymorphism (SNP) included. Solid vertical lines mark the 2-LOD region of interest.

Expression studies of candidate genes for the QTL on *Mdo* 8

Using RT-PCR analysis, we compared expression of *INSIG1*, *ABCB1*, and *ABCB4* in liver samples from both high- and low-V+LDL-responder opossums on both the basal and HCHF diets. Specifically, samples from 6 high- and 6 low-responder animals on the HCHF diet as well as 6 high- and 6 low-responder animals on the basal diet were examined. There was no significant difference between high- and low-V+LDL responders in mean expression for any of the three candidate genes on either diet, although expression of *ABCB1* showed a borderline significant difference on the HCHF diet ($P = 0.052$, **Table 3**).

Sequence analysis of *INSIG1*, *ABCB1*, and *ABCB4*

We next looked for exon variants in the candidate genes. Two SNPs were identified in the *INSIG1* sequence (see supplementary Table VIII), including a nonsynonymous C/G SNP for amino acid 24 in the mRNA sequence predicted to result in an Arg → Gly substitution. Of nine SNPs identified in the *ABCB4* sequence (see supplementary Table VIII), two were predicted to be nonsynonymous; an A/G SNP predicting an Arg → Gly substitution at amino acid 29, and an A/T SNP predicting an Ile → Leu substitution at amino acid 235. SNP detection proved impractical for *ABCB1* due to the apparent coamplification of its sequence with that of an annotated pseudogene, resulting in a very high false SNP rate. Overall protein sequence similarities (human to mouse vs. human to opossum) based on BLAST alignment algorithms were 82% versus 85% for *INSIG1* and 95% versus 94% for *ABCB4*. Complete consensus sequences for *INSIG1* and *ABCB4* and the locations of the nonsynonymous SNPs are shown in supplementary Fig. IV. All three of the nonsynonymous SNPs in *ABCB4* or *INSIG1* were in amino acids that are located in the transmembrane domain and conserved in human, mouse, and opossum (see supplementary Table IX). However, only Ile235 in *ABCB4* was conserved across all tabulated vertebrate species (from human to chicken). Among the 10 sequenced opossums, the genotypes for the nonsynonymous SNPs in *ABCB4* are not strongly correlated with high- versus low-responder group, although the genotypes are correlated with each other (**Table 4**). Because *ABCB1* and *ABCB4* are virtually contiguous in the genome (Table 2), the *ABCB4* SNP marks the region containing both genes. Similarly, the Arg24Gly SNP in *INSIG1* is not strongly correlated with responder group, but is correlated with the microsatellite, *8M706*, which was used to mark the *INSIG1* region (Table 4).

Association and linkage analyses of variants for *INSIG1* and *ABCB4* regions

All animals with DNA were genotyped for the *INSIG1* marker (*8M706*) and the *ABCB4* Ile235Leu SNP. As expected from their genome location (Table 2), the *ABCB4* SNP and *INSIG1* marker showed ~12% recombination (LOD score for linkage = 33.1). We next assessed whether variation at either or both of these polymorphisms was significantly associated with V+LDL cholesterol levels on the

TABLE 2. Location of candidate genes and selected microsatellite markers in the QTL region of interest on *Mdo* 8 (*Hsa* 3, 7, and 10) for V+LDL cholesterol

Gene or Anonymous Map Marker Name	LOD (Max)	Linkage Map Position	Opossum Genome Assembly Starting Coordinate	Opossum Genome Assembly Ending Coordinate	Traits Associated in Humans or Mice	Reference	Gene Name
		cM					
8M031	3.74	57.1	136,982,377	136,982,733			
NOS3	—	—	215,557,058	215,581,589	HDL cholesterol hypertension	29 30	Nitric oxide synthase 3
INSIG1	—	—	221,251,836	221,258,636	Coronary heart disease Cholesterol homeostasis	31 32, 33	Insulin-induced gene 1
8M399	7.76	73.8	234,118,780	234,119,632			
8M253	9.17	77.4	249,523,679	249,523,932			
CUBN	—	—	256,860,498	257,159,613	HDL, apoA-I	34	Cubilin (intrinsic factor-cobalamin receptor)
ITGA8	—	—	258,509,682	258,737,469	Myocardial infarction	35	Integrin, α8 (*ITGA2B* paralog)
ABCB4	—	—	259,416,385	259,502,884	Phospholipid transport cholestasis Plasma cholesterol (LDL)	36 37 38	ATP binding cassette, sub-family B (*MDR/TAP*), member 4
ABCB1	—	—	259,539,949	259,635,355	LDL cholesterol response to drugs	39	ATP binding cassette, sub-family B (*MDR/TAP*), member 1
CAPN7			275,275,816	275,317,137	Intima media thickness Type 2 diabetes	40 41	Calpain 7 (Calpain 10 paralog)
GALNTL2			276,262,481	276,287,420	Hypertriglyceridemia Plasma HDL cholesterol	42 4,5	UDP_ *N*-acetylgalactosaminyl transferase -like 2 (*GALNT2* paralog)
8M705	8.54	90.3	279,550,062	279,550,533			
8M431	2.87	106.3	302,549,232	302,550,062			

apoA-I, apolipoprotein A-I; LOD, logarithm of the odds; QTL, quantitative trait locus.

HCHF diet and whether inclusion of these SNP genotypes as covariates in the linkage analysis models would reduce the QTL signal on *Mdo* 8. For appropriate comparison of results, all association and linkage analyses described below were performed using data on the subset of 289 opossums that had valid V+LDL cholesterol measures on the HCHF diet and valid genotypes at both the *ABCB4* and *INSIG1* markers. Using pedigree-based association methods, the model containing neither SNP was highly significantly different from the model containing both variants ($P = 9 \times 10^{-14}$, **Table 5**), indicating that *ABCB4* or *INSIG1* or both variants were significantly associated with V+LDL cholesterol levels. The model containing *ABCB4* alone was not significantly different ($P = 0.70$) from the full model; thus, *ABCB4* alone described the variation in V+LDL cholesterol as well as a model with both variants, but with fewer parameters. In contrast, the model containing *INSIG1* alone was significantly different from the full model ($P = 7 \times 10^{-9}$), indicating that if *ABCB4* is in the model, *INSIG1* does not provide any additional information. These results indicate that the model containing *ABCB4* genotypes alone best described the variation in V+LDL cholesterol on the HCHF diet and that this model is significantly better than a model with no SNPs ($P = 9 \times 10^{-14}$). Although the *ABCB4* SNP is significantly associated with V+LDL cholesterol on the HCHF diet and accounts for 18% of the total variation in V+LDL cholesterol, the SNP genotype means do not correspond well with the bimodality seen in Fig. 1. Median V+LDL cholesterols for the *AA*, *AT*, and *TT* genotypes were −0.17, 1.58, and 2.87 (ln mmol/l), respectively, and there was considerable variation in V+LDL cholesterol within each genotype (**Fig. 3**), indicating that this variant is not likely to be the sole causal SNP for V+LDL cholesterol variation on the HCHF diet, although it may be in linkage disequilibrium with a causal variant or variants.

TABLE 3. Mean (±SE) of hepatic RT-PCR expression (measured as ΔC_T) on basal and HCHF diets for three candidate genes in high- versus low-V+LDL responders (six animals per group)

Diet	High- or Low-V+LDL Group	*INSIG1*	*ABCB1*	*ABCB4*
Basal	Low	10.14 ± 0.28	24.28 ± 1.36	8.88 ± 0.28
	High	10.00 ± 0.30	24.03 ± 1.41	8.30 ± 0.35
	P	0.713	0.809	0.190
HCHF	Low	9.55 ± 0.33	22.98 ± 1.27	8.20 ± 0.16
	High	8.85 ± 0.18	20.03 ± 0.48	8.13 ± 0.15
	P	0.075	0.052	0.741

We performed two-point QTL analyses between V+LDL cholesterol and these variants; the two-point LOD scores were 7.94 and 1.56 for *ABCB4* and *INSIG1*, respectively. We also reran the multipoint linkage analyses to include *ABCB4* and *INSIG1* marker genotypes as covariates in the linkage analysis model to determine whether significant evidence for linkage remained after including the effects of each polymorphism. Using data on the same subset of 289 animals described above, the maximum LOD score was 8.68 at position 85 cM (Fig. 2B). Inclusion of the *ABCB4* SNP in the multipoint linkage model obliterated the QTL signal on *Mdo* 8; i.e., the maximum LOD score at 85 cM dropped from 8.68 to 0.14. In contrast, inclusion of *INSIG1* in the linkage model did not completely remove evidence for linkage; at position 85, the maximum LOD score was 4.5 ($P = 10^{-5}$), which remains highly significant evidence for a QTL at this position (Fig. 2B). Furthermore,

TABLE 4. Genotypes of five high- and five low-V+LDL responders for three nonsynonymous SNPs and a microsatellite for *INSIG1* and *ABCB4*

		INSIG1		*ABCB4*	
Animal ID	High or Low V+LDL Group	Arg24Gly C/G	*8M706* 170/172	Ile235Leu A/T	Arg29Gly A/G
E4855	High	CC	172/172	TT	GG
E0946	High	CC	172/172	TT	GG
E3023	High	GG	170/170	AT	AG
E3030	High	CG	170/172	AT	AG
E3203	High	GG	170/170	AT	AG
E3026	Low	CG	170/172	AT	AG
E3020	Low	CG	170/172	AT	AG
E3198	Low	CC	172/172	TT	GG
E3205	Low	GG	170/170	AA	AA
E3202	Low	CG	170/172	AT	AG

SNP, single-nucleotide polymorphism.

although the *Mdo* 8 QTL signal was almost abolished, the *ABCB4* SNP only accounted for approximately 1/2 of the additive genetic variation due to the QTL. These association and linkage results indicate that the region marked by the *ABCB4* SNP, but not by the *INSIG1* variant, accounts for much but not all of the variation in V+LDL cholesterol due to the *Mdo* 8 QTL. Thus, additional variants in the *ABCB4* region remain to be detected.

DISCUSSION

In the current study, we performed unbiased, "hypothesis-free" genome-wide linkage analyses of plasma cholesterol levels in opossums fed two different diets. We obtained strong evidence (maximum multipoint LOD = 3.1, genomic $P = 0.019$, Fig. 2A) that a QTL on *Mdo* 1 influenced V+LDL cholesterol levels on the basal diet and that a QTL on *Mdo* 8 influenced V+LDL cholesterol levels on the HCHF diet (maximum multipoint LOD = 9.9, genomic $P = 5 \times 10^{-9}$, Fig. 2B). These two QTLs accounted for ~70% and ~65% of the residual additive genetic variance of V+LDL cholesterol levels on the basal and HCHF diets, respectively, in these pedigrees. The linkage results are completely consistent with our previous analyses, in which we reported that a major gene accounted for ~50% of the genetic variation in V+LDL cholesterol on a basal diet, and a separate major gene accounted for ~80% of the genetic variation in V+LDL cholesterol on the HCHF diet (13). Because we do not have DNA samples from the animals used in our previous studies, we could not perform a combined segregation and linkage analysis to more conclusively demonstrate that the previously detected major genes are, in fact, the *Mdo* 1 and *Mdo* 8 QTLs. However, our current results using data from the first- and second-generation offspring of animals analyzed in the previous study strongly support this conjecture.

TABLE 5. Comparison of models for association of *INSIG1* and *ABCB4* variants (assuming additive allele effect) with V+LDL cholesterol on the HCHF diet

Model	Chi square	df	*P*
ABCB4 + *INSIG1*	—	—	—
ABCB4	0.02	1	0.89
INSIG1	33.58	1	7×10^{-9}
No variants	55.54	2	9×10^{-14}

Locating QTLs for specific traits, either by linkage or association analyses, is the first step toward the goal of identifying candidate genes and, ultimately, causative variants. A potential limitation of the opossum model is that the genome sequence is not as well annotated, nor is the panel of genetic variants as extensive as those of human and some other model species. However, we used a novel approach that incorporated information from a variety of public genomic databases to identify the corresponding conserved syntenic regions in humans and then used the human genome annotation to create a list of known genes in the region. Of the annotated genes that were also orthologs or paralogs of genes on our list of 224 candidate genes (based on two public databases of association studies in humans), we derived a subset of candidate genes (Table 2) residing within the QTL intervals. We then did translated protein BLAST (tBLASTn) analyses of these genes to confirm that the orthologs or paralogs of these genes are indeed present in the opossum chromosomal regions of interest. The power in this multistage analytical approach is that it is unbiased and solely informed by publically available information. After identifying a set of positional candidates, we surveyed the literature to select high-priority candidate genes for follow-up studies.

We identified two potential candidate genes, *ALMS1* and *LPIN1*, located in the QTL region of interest on *Mdo* 1; however, they are relatively distant from the QTL (see supplementary Table VII), and a survey of the literature is not strongly supportive of either as candidates for this QTL. Although one of the characteristics of individuals with Alstrom syndrome (caused by mutations in *ALMS1*) is dyslipidemia, and mice without functional *LPIN1* exhibit hypertriglyceridemia and fatty livers, studies of variants in these two genes in other human populations revealed no strong association with plasma lipid levels (43, 44). These observations suggest that another, potentially novel, gene may influence V+LDL cholesterol on the basal diet. In fact, for future studies, we could expand our list of candidate genes to include all proteins, enzymes, and receptors that are known to be involved in lipoprotein metabolism using, for example, the STRING database of proteins and their

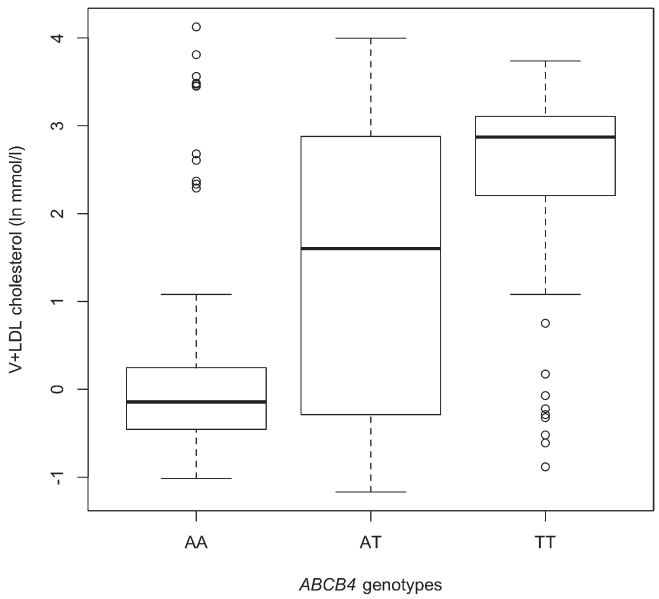

Fig. 3. Box-and-whiskers plots of V+LDL cholesterol (ln mmol/l) showing the significant association ($P = 9 \times 10^{-14}$) with *ABCB4* SNP genotypes. The solid line represents the median, the boxes encompass the interquartile range (IQR) (the middle 50% of data), and the whiskers mark the1.5 × IQR limits of the upper and lower quartiles. Individual circles are points that fall outside the 1.5 × IQR boundaries.

interactions (http://string.embl.de/) and/or the Ingenuity Pathway Analysis program (http://www.ingenuity.com/).

In contrast to the QTL on *Mdo* 1, we identified three high-priority candidate genes (*INSIG1*, *ABCB1*, and *ABCB4*) for the QTL on *Mdo* 8 that influences V+LDL cholesterol on the HCHF diet. *INSIG1* codes for a protein involved in the regulation of the synthesis of cholesterol, fatty acids, triglycerides, and phospholipids (32, 33). *ABCB1* and *ABCB4* are large, membrane-spanning P-glycoprotein hepatobiliary drug transporters that transport amphipathic molecules and phospholipids, respectively (45). *ABCB1* variants have been associated with the LDL cholesterol response to drugs (39); whereas humans and mice with mutations or disruptions in *ABCB4* exhibit an impaired ability to secrete phospholipid into bile and develop liver disease (36, 37). We further investigated these candidate genes using expression studies, sequencing, and association analyses.

First, we assessed whether mean differences in V+LDL cholesterol levels between high versus low responders could be due to differences in mRNA expression of the candidate genes. Although our expression study was limited because we only used liver samples, a strong result could have been informative. There were no significant differences in hepatic mRNA expression of the candidate genes between low- versus high-V+LDL cholesterol responders on the basal diet, or for mean expression of *INSIG1* or *ABCB4* on the HCHF diet, but the difference in *ABCB1* expression was borderline (nominal $P = 0.052$). Mean *ABCB1* expression in the high- versus low-V+LDL cholesterol response groups decreased by ~3 ΔC_T units (or ~8-fold), whereas mean V+LDL cholesterol serum levels increased by ~25-fold. Furthermore, expression of all three genes appears to decline on the HCHF diet. This same pattern of expression in response to the HCHF diet, that is, reduced expression in both responder groups, but lowest in the high-V+LDL responder group, was also observed for genes involved in cholesterol biosynthesis, e.g., HMG-CoA reductase, among opossums fed a high-cholesterol diet (11). Thus, these differences in expression may result as a general response to maintain cholesterol homeostasis. However, this interpretation is limited by our sample size.

To detect SNPs that might affect structural characteristics of the INSIG1, ABCB1, and ABCB4 proteins, and, consequently, differential V+LDL cholesterol response to the HCHF diet, we sequenced all exons of the corresponding mRNAs (isolated from liver) corresponding to our candidate genes from five high- and five low-V+LDL responders. We detected one and two nonsynonymous SNPs in *INSIG1* and *ABCB4*, respectively, but the genotypes at these SNPs were not highly correlated with the high- versus low-V+LDL cholesterol response groups (Table 4). Thus, these structural variants do not, by themselves, account for the differences in V+LDL cholesterol on the HCHF diet. Unfortunately, due to presumed coamplification of sequence from an *ABCB1* pseudogene, we were unable to identify SNPs in this candidate gene.

On the basis of the above expression and sequencing results, we were unable to identify variants in any of our candidate genes that would differentiate between high- and low-V+LDL cholesterol response groups. We next performed association and linkage analyses of polymorphisms in (or near) these candidate genes in an effort to narrow the candidate gene region. Results of association analyses revealed that the *ABCB4* SNP, but not the *INSIG1* marker, was significantly associated with V+LDL cholesterol levels on the HCHF diet ($P = 9 \times 10^{-14}$). Furthermore, inclusion of the *ABCB4* SNP genotypes in the multipoint linkage model removed the QTL signal, whereas inclusion of the *INSIG1* marker genotypes did not (Fig. 2B). However, variation marked by the *ABCB4* Ile235Leu SNP genotype is associated with only ~35% of the additive genetic variation in V+LDL cholesterol (Fig. 3), whereas the *Mdo* 8 QTL accounted for ~65% of the additive genetic variance. Thus, substantial genetic variation at this or other nearby genes remains to be identified, and some of it could well be due to interactions among multiple genetic variants in this region, as well as to epistatic effects of genes on other chromosomes. In support of the latter concept, our previous study (13) indicated that the QTL influencing V+LDL cholesterol on the basal diet also influenced V+LDL cholesterol on the HCHF diet; in the current report, the basal diet QTL is located on *Mdo* 1.

The linkage and association results strongly indicate that the chromosomal region near *ABCB4* contains variants that have large effects on V+LDL cholesterol on the HCHF diet. Mutations in the phospholipid transporter gene *ABCB4* are associated with low levels of biliary phospholipids and a growing list of liver diseases in humans and mice (36, 46). In mice, *ABCG5* and *ABCG8* appear to require intact *ABCB4* (a.k.a. *MDR2*) for secretion of cholesterol into bile and, overall, "to influence the absorption, secretion, and plasma levels of neutral sterols" (47). Although

many patients with impairment of *ABCB4* have cholesterol levels in the normal range (37), a recent study has reported significant associations in humans of plasma cholesterol levels with two tagging SNPs in *ABCB4* (38). These observations are consistent with our hypothesis that genetic variation at or near the *ABCB4* locus is at least partly responsible for the observed differences in V+LDL cholesterol on the HCHF diet. This hypothesis is further supported by previous comparisons of opossums with high- versus low-V+LDL cholesterol: high-responder animals had significantly higher hepatic cholesterol, substantially lower biliary phospholipid and cholesterol levels, and larger livers (12, 48), which are similar to phenotypic differences associated with different *ABCB4* alleles observed in humans and mice. Although *ABCB4* is a plausible candidate gene to influence at least part of the observed V+LDL response to diet, we are approaching the limits of information obtainable from additional statistical genetic analyses of the current population because both our sample size and the opossum meiotic map are relatively small. For example, we are not able to determine whether the *ABCB4* SNP could be marking variants in *ABCB1* or other nearby genes, including microRNAs. Thus, additional biochemical and molecular genetic studies will be needed to identify the specific variants and mechanisms responsible for the differential V+LDL cholesterol response to an HCHF diet.

In conclusion, we observed dramatic interindividual variation in V+LDL cholesterol response to dietary environment in the opossum, and detected evidence of diet-by-genotype interaction, in that the predominant QTLs for V+LDL cholesterol levels on different diets reside on different chromosomes. We adopted a novel, unbiased, "hypothesis-free" approach, combining linkage analyses, comparative synteny/homology methods, and published information on genes associated with lipoprotein physiology in human and mouse models to identify high-priority candidate genes for the V+LDL cholesterol response (*INSIG1*, *ABCB1*, and *ABCB4*). We then performed gene expression and sequencing analyses, followed by association and linkage analyses, in an effort to include or exclude these candidate gene regions. Our results indicate that the chromosomal region encompassing *ABCB1/ABCB4* is likely to harbor variants that affect V+LDL cholesterol response to an HCHF diet. These findings illustrate how combining disparate methodologies can be used to identify candidate genes for quantitative traits, such as V+LDL cholesterol response to diet, in nontraditional animal models such as the opossum.

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