ABCB4 mediates diet-induced hypercholesterolemia in laboratory opossums[S]

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Abstract **High-responding opossums are susceptible to developing hypercholesterolemia on a high-cholesterol diet, but low-responding opossums are resistant. The observation of low biliary cholesterol and low biliary phospholipids in high responders suggested that the *ABCB4* gene affects response to dietary cholesterol. Two missense mutations (Arg29Gly and Ile235Leu) were found in the *ABCB4* gene of high responders. High responders (ATHH strain) were bred with low responders (ATHE or ATHL strain) to produce F1 and F2 progeny in two different genetic crosses (KUSH6 and JCX) to determine the effect of *ABCB4* allelic variants on plasma cholesterol concentrations after a dietary challenge. Pedigree-based genetic association analyses consistently implicated a variant in *ABCB4* or a closely linked locus as a major, but not the sole, genetic contributor to variation in the plasma cholesterol response to dietary cholesterol. High responders, but not low responders, developed liver injury as indicated by elevated plasma biomarkers of liver function, probably reflecting damage to the canalicular membrane by bile salts because of impaired phospholipid secretion.[JLR] Our results implicate *ABCB4* as a major determinant of diet-induced hypercholesterolemia in high-responding opossums and suggest that other genes interact with *ABCB4* to regulate lipemic response to dietary cholesterol.**—Chan, J., M. C. Mahaney, R. S. Kushwaha, J. F. VandeBerg, and J. L. VandeBerg. ***ABCB4* mediates diet-induced hypercholesterolemia in laboratory opossums.** ***J. Lipid Res.*** **2010.** 51: **2922–2928.**

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The laboratory opossum (*Monodelphis domestica*) is an omnivorous mammal. Unlike mice, rabbits, and other small mammals typically used in research on genetic control of plasma lipids, this species has a natural diet that is similar to a natural human diet in being balanced between vegetable and animal sources and including moderate levels of cholesterol. The predominant form of hypercholesterolemia in Western human societies is caused by a response to dietary cholesterol and fat. However, identification of the genes that control response has been elusive in research involving human subjects or conventional animal models.

The discovery that some laboratory opossums develop an extreme lipemic response to dietary cholesterol whereas others have a minimal response (1) provided an opportunity to identify one or more genes that affect diet-induced hypercholesterolemia and to determine mechanisms by which those genes influence cholesterol homeostasis. Therefore, we developed a partially inbred strain (ATHH) that develops highly elevated levels (10- to 30-fold) of very low density and low density lipoprotein cholesterol (V+LDLC) when fed a high-cholesterol, high-fat (HCHF) or a high-cholesterol, low-fat (HCLF) diet (2, 3), and two other partially inbred strains (ATHE and ATHL) that only develop slightly elevated levels (<2-fold) of V+LDLC when fed the same cholesterol-enriched diets. The V+LDLC levels of high- and low-responding opossums do not differ on a basal diet.

Using these strains, we demonstrated differences between high and low responders in cholesterol absorption (4), cholesterol metabolism (5), and expression of genes that regulate cholesterol homeostasis (6, 7). Genetic analyses of plasma lipoprotein cholesterol levels from pedigreed families implicated a recessive allele at a single gene locus as largely responsible for diet-induced hypercholesterolemia in high-responding opossums (8), but neither the identity of the locus nor of any candidate gene was identified.

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Abbreviations: ALT, alanine aminotransferase; AST, aspartate aminotransferase; GGT, γ-glutamyl transferase; HCHF, high cholesterol and high fat; HCLF, high cholesterol and low fat; HDLC, high density lipoprotein cholesterol; LD, linkage disequilibrium; SNP, single nucleotide polymorphism; TC, total cholesterol; TG, triglyceride; V+LDLC, very low density and low density lipoprotein cholesterol.

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[S] The online version of this article (available at http://www.jlr.org) contains supplementary data in the form of a table.

In studying the effects of a cholesterol absorption inhibitor on plasma cholesterol, we observed a significant difference in biliary cholesterol concentrations between high and low responders on the HCLF diet, suggesting that biliary cholesterol excretion could be impaired in high responders (9). The association of high concentrations of plasma V+LDLC with low concentrations of biliary cholesterol in high responders led us to hypothesize that impaired secretion of cholesterol into bile affects excretion of cholesterol and thereby elevates plasma V+LDLC concentrations.

In the present study, we considered genes that play a role in biliary cholesterol secretion as candidate genes that control the plasma cholesterol response to the HCHF diet, and we carried out experiments to determine if any of these genes play a major role in diet responsiveness. Genes that are known to play a role in biliary cholesterol secretion are *ABCG5*, *ABCG8*, *ABCB4*, and *NPC1L1*. The *ABCG5* and *ABCG8* genes encode proteins that dimerize to form a functional transporter to efflux cholesterol from the liver into bile (10, 11), whereas the *NPC1L1* gene encodes the Niemann-Pick C1-like 1 protein, which functions in the reverse process to transport cholesterol from canalicular bile back into the liver (12). The protein encoded by the *ABCB4* gene translocates phosphatidylcholine from the inner to the outer leaflet of the canalicular membrane of hepatocytes to form mixed micelles with bile salts in the canalicular lumen (13). Phospholipids are essential for solubilization of cholesterol in bile (13–15).

The *NPC1L1* gene was ruled out as a strong candidate gene due to the observation of lower levels of hepatic *NPC1L1* mRNA in high responders relative to those in low responders on the HCLF diet (9). The downregulation of the *NPC1L1* gene is likely to be a consequence of low levels of biliary cholesterol in high responders (hence, promoting the retention of biliary cholesterol in the gall bladder); therefore, *NPC1L1* is not a logical candidate for causing low biliary cholesterol in high responders. We proceeded to evaluate *ABCG5, ABCG8*, and *ABCB4* as logical candidate genes. Our results implicate *ABCB4* as a major determinant of responsiveness to dietary cholesterol in laboratory opossums.

MATERIALS AND METHODS

Laboratory opossums

The laboratory opossums used in this investigation were produced at the Southwest Foundation for Biomedical Research and were maintained under standard conditions for this species (16). Three partially inbred strains were used. All three strains were derived from nine founders trapped near the town of Exu, Brazil, in 1978 (16). The ATHH strain had been selected for high lipemic response to the HCHF diet, whereas the ATHE and ATHL strains had been selected for low response. F1 and F2 generation animals were produced from matings of ATHH × ATHE (the KUSH6 cross) and ATHH × ATHL (the JCX cross). The inbreeding coefficients of the parentals used in crosses were 0.725–0.768 for ATHH, 0.787–0.872 for ATHE, and 0.881–0.935 for ATHL. As the three strains were derived from the same nine founders, they are closely related. Kinship coefficients of the pairs of strains are 0.69 for ATHH-ATHE, 0.24 for ATHH-ATHL, and 0.24 for ATHE-ATHL.

The research was conducted in conformity with the Public Health Service Policy on Humane Care and Use of Laboratory Animals, and the studies were approved by the Institutional Animal Care and Use Committee.

Diet protocol

The basal diet on which the opossums were maintained was a commercial pelleted fox food (Reproduction Diet, Nutritionally Complete Fox Food Pellets; Milk Specialties Products, New Holstein, WI). The fat content was 10% of dry weight (3% basal fat, 4.7% lard, and 2.3% corn oil), and the cholesterol content was 0.16% of dry weight. The challenge diet, which was fed to the opossums for four weeks, was prepared by adding lard and crystalline cholesterol to the basal diet, as well as water, mixing the ingredients in a Hobart food mixer, and passing the mix through a meat grinder to produce soft pellets which were kept frozen until use. Its fat content was 18.8% (2.7% basal fat, 14.0% lard, and 2.1% corn oil), and the cholesterol content was 0.71% of dry weight (3).

Plasma cholesterol and triglyceride assays

Blood was collected into tubes containing EDTA from animals that were fasted overnight by cardiac puncture under isoflurane anesthesia. Plasma was obtained by centrifugation, and plasma total cholesterol (TC), high density lipoprotein cholesterol (HDLC), and triglycerides (TG) were measured by enzymatic methods with the Ciba-Corning Express Plus Analyzer as described previously (3). V+LDLC concentration was calculated as the difference between TC concentration and HDLC concentration.

Hepatic function markers

Analyses of alanine aminotransferase (ALT), aspartate aminotransferase (AST), γ-glutamyl transferase (GGT), and total bilirubin in plasma samples were performed on a Beckman Unicell DxC600 Chemistry Analyzer (Beckman Coulter, Fullerton, CA).

Biliary phospholipid and biliary cholesterol assays

Bile was collected from the gall bladders of animals 4 h after they were fed. For measurement of biliary phospholipids, a small volume (25–50 μl) of bile was extracted with chloroform and methanol (2:1) by the method of Folch et al. (17). The chloroform extract was dried under air, and the residue was dissolved in a small volume of chloroform and methanol (2:1). The extract was digested with 10 N sulfuric acid at 150–160°C for 3 h. Phosphorus in the digested extract was measured by the method of Bartlett (18). Phospholipid concentration was calculated by multiplying the phosphorus amount by a factor of 25 and expressed as mg/ml of bile.

Biliary cholesterol was measured using the Cholesterol E kit (Wako Diagnostics, Richmond, VA). The manufacturer's protocol was modified to accommodate the small volume of bile samples. The assay was performed by adding 2 μl of a bile sample to 0.2 ml of reagent in a 96-well plate. Color development was read on a Multiskan Spectrum spectrophotometer (Thermo Scientific, Waltham, MA).

Sequencing of opossum *ABCB4* cDNA

Total RNA was isolated from the livers of four high responders from the ATHH stock and four low responders from the ATHE stock using the TRI Reagent (Molecular Research Center, Cincinnati, OH). The *ABCB4* cDNA was amplified from total

RNA by RT-PCR using six sets of overlapping primers (supplementary Table I). The PCR products were sequenced using a BigDye Terminator v3.1 Cycle Sequencing Kit (Applied Biosystems, Foster City, CA) on an ABI Prism 3730 DNA sequencer (Applied Biosystems). Sequence data were analyzed to identify gene mutations using the MacVector software (Cary, NC).

TaqMan genotyping assay

Genomic DNA was isolated from ear pinna and liver samples by the proteinase K-phenol-chloroform extraction method. DNA was quantified using an ND-1000 spectrophotometer (NanoDrop Technologies, Wilmington, DE). Genotyping was carried out with two TaqMan probes for allelic discrimination of the ABCB4 Ile235Leu polymorphism. Forward and reverse primers for the TaqMan assay were 5′-ctatctttccaattttccctaaatatttgggttt-3′ and 5′-tggcataggctgctaattctttgt-3′, respectively. The Vic-labeled TaqMan probe (VIC-attgttattccagttactatc) was complementary to the DNA of high responders, and the FAM-labeled TaqMan probe (FAM-ttgttattccagatactatc) was complementary to the DNA of low responders. PCR amplification was performed on the GeneAmp 9700 thermal cycler (Applied Biosystems), and fluorescence intensity was read on the 7900HT Fast Real-Time PCR System (Applied Biosystems). Fluorescence data were analyzed using the Sequence Detection System software (Applied Biosystems) to assign allele types to the samples.

Statistical analyses of two genetic crosses

For each of the KUSH6 and JCX crosses, we analyzed data on plasma concentrations of TC, HDLC, V+LDLC, and TG measured at two time points in the same group of animals; i.e., at "week 0" prior to a shift from a basal diet low in cholesterol and fat, and then following a four-week exposure to the HCHF challenge diet ("week 4"). Resulting from controlled mating relevant to the current study's aims, the more than 1,300 opossums with data also belong to a large, genetically managed, pedigreed breeding colony with tens of thousands of members in many generations. Approximately 600 KUSH6 animals with data fit into six "trimmed" pedigrees with 675 total members; the largest of these contained F1 and F2 animals with data from four different generations, and the remaining five contained data from only two generations. Five extended pedigrees with 871 total members comprised the JCX cross, with all 700-plus F1 and F2 animals from which we obtained data in two generations. Numbers of relative pairs with data in each cross, an indicator of pedigree complexity and a source of much of the information necessary to estimate genetic effect size, are provided in **Table 1**. Pedigree, phenotype, and genotype data management were accomplished using PEDSYS (19).

We conducted statistical genetic analyses on the data obtained from animals resulting from both crosses to determine if a single nucleotide polymorphism (SNP) within *ABCB4* or another variant with which it is in linkage disequilibrium (LD) exerts a significant mean effect on variation in the phenotypes. All statistical genetic analyses were conducted within a maximum likelihood framework using variance decomposition methods implemented within the computer software package SOLAR (20). The methods implemented in this approach are designed explicitly to be used in the analysis of data from relatives in pedigrees of arbitrary size and complexity, including pedigrees with inbred offspring. We used this approach to estimate the contribution of specific genetic variants and additional genes in the polygenic background on the residual phenotypic variance (i.e., that remaining after accounting for the effects of measured covariates and random environmental factors) in each of the four traits. Tests of significance for parameters of interest were done by means of likelihood ratio tests wherein the $\log_e$ likelihood for the restricted model in which the parameter is constrained to equal 0 is compared with that of a model in which the parameter is estimated.

TABLE 1. Relative pairs with data in two genetic crosses of opossums

Relative-Pair Relationship	KUSH6	JCX
Parent-offspring	1068	1074
Sibling	2858	3102
Other 1st degree	13528	11637
Grandparent-grandchild	356	–
Avuncular	1212	–
Half sibling	1384	1487
Double first cousin	1547	–
Other 2nd degree	47327	21674
3rd degree	53992	28387
4th degree	78	8927

Provided a trait exhibited significant heritability (h^2), we conducted a series of pedigree-based genetic association analyses using the measured genotype approach (21, 22) in which we treated the combination of two alleles at the *ABCB4* marker locus as a fixed effect on the mean of each phenotype. We included (and tested for) both an additive genetic effect and a dominance genetic effect of the locus (or another locus in tight LD with it) in an extension of the same genetic model used to detect and estimate heritability for each of the traits.

RESULTS

Assessment of candidate genes

To decide the most likely candidate among *ABCG5, ABCG8*, and *ABCB4*, we measured cholesterol and phospholipids in bile samples from five high and five low responders fed the HCHF diet for four weeks (**Table 2**). Biliary cholesterol concentrations of high responders were lower (4-fold; $P = 0.0002$) than those of low responders, and biliary phospholipid concentrations of high responders were also lower than those of low responders (4-fold; $P = 0.0001$). The association of low biliary cholesterol with low biliary phospholipids in high responders implicated *ABCB4* (but not *ABCG5* or *ABCG8*) as a strong candidate gene because mutations in the ABCG5 and ABCG8 proteins do not affect phospholipid secretion (15).

ABCB4 facilitates the secretion of phospholipids into the canaliculi, where phospholipids form mixed micelles with bile salts to solubilize cholesterol and protect the canalicular membrane from damage by the detergent effects of bile salts (23, 24). As shown in Table 2, elevated levels of liver function markers AST, ALT, GGT, and bilirubin in the plasma of high responders indicate liver damage, which is consistent with a deficiency in ABCB4.

SNPs in opossum *ABCB4* cDNA

A 3837 bp opossum *ABCB4* cDNA (GenBank accession number GU230140) was amplified by RT-PCR and sequenced. Five SNPs were detected in the *ABCB4* cDNA by comparing the sequences of four low responders from the ATHE stock and four high responders from the ATHH stock (**Table 3**). Two SNPs cause amino acid substitutions in the amino-terminus of ABCB4; Arg at amino acid 29 in

TABLE 2. Biliary lipids and plasma parameters of liver function (mean ± SD) in five high- and five low-responding opossums after four weeks on HCHF diet

Parameters	High Responders	Low Responders
Plasma V+LDLC (mg/dl)	702 ± 445	25 ± 7[a]
Biliary cholesterol (mg/ml)	1.3 ± 0.7	5.7 ± 1.3[a]
Biliary phospholipids (mg/ml)	6.9 ± 5.5	29.7 ± 3.7[a]
ALT (U/l)	456 ± 187	62 ± 15[a]
AST (U/l)	356 ± 152	33 ± 7[a]
GGT (U/l)	44 ± 36	<5[a]
Total bilirubin (mg/dl)	6.3 ± 2.7	0.4 ± 0.1[a]

ALT, alanine aminotransferase; AST, aspartate aminotransferase; GGT, γ-glutamyl transferase; HCHF, high cholesterol and high fat; V+LDLC, very low density and low density lipoprotein cholesterol.

[a]Student *t*-test, significant difference ($P < 0.05$) between high and low responders.

low responders is substituted by Gly in high responders, and Ile at amino acid 235 in low responders is substituted by Leu in high responders. A TaqMan assay was developed to genotype the Ile235Leu polymorphism in opossums from the KUSH6 and JCX crosses. Allele 1 carries the missense mutations whereas allele 2 is the wild-type allele.

Statistical genetic analyses of KUSH6 and JCX crosses

Genotype data. We typed the *ABCB4* SNP Ile235Leu in 424 KUSH6 animals and 640 JCX animals; 404 of the KUSH6 and 536 of the JCX opossums that were genotyped were from the F2 generation. Respectively, the relative frequencies of the two alleles, designated "1" and "2" in this study, were 0.48 and 0.52 (KUSH6) and 0.46 and 0.54 (JCX). The relative genotypic frequencies in animals from the F2 generation of each of these two pedigreed groups (**Table 4**) did not depart from expectations under Hardy-Weinberg equilibrium (KUSH6: $\chi^2_2 = 2.88$, $0.50 > P > 0.10$ and JCX: $\chi^2_2 = 0.121$, $0.975 > P > 0.90$).

Correlation and heritability. As would be expected, the four traits were intercorrelated in both genetic crosses. While the absolute values of the phenotypic correlations (ρ_P) differed by cross and diet, the patterns of trait-pair correlations were similar irrespective of these factors, with the highest correlations between TC and the two major lipoproteins, V+LDLC and HDLC (ρ_P = 0.68–0.84); intermediate correlations between V+LDLC and HDLC (ρ_P = 0.11–0.41); and the lowest correlations between TG and the other traits (ρ_P = 0.06–0.28). Our estimates of heritability for all four traits on the two diets in both crosses were significant and of similar magnitudes, ranging from $h^2 = 0.33$ to $h^2 = 0.58$ in the KUSH6 cross and from $h^2 = 0.19$ to $h^2 = 0.40$ in the JCX cross (**Table 5**).

TABLE 3. SNPs and amino acid substitutions in *ABCB4* cDNA

Codon in Low Responders	Codon in High Responders	Amino Acid Substitution
AGG	GGG	Arg29Gly
ATA	TTA	Ile235Leu
TAC	TAT	Tyr - No change
AAG	AAA	Lys - No change
AAG	AAA	Lys - No change

TABLE 4. Relative genotypic frequencies at the *ABCB4* SNP in the F2 generation of two genetic crosses of opossums

KUSH6			JCX		
Genotype	Frequency (Count)	Relative Frequency	Genotype	Frequency (Count)	Relative Frequency
1/1	85	0.210	1/1	117	0.218
1/2	219	0.542	1/2	262	0.489
2/2	100	0.248	2/2	157	0.293

SNP, single nucleotide polymorphism.

Measured genotype analysis. We detected no evidence for association in the analyses of data from week 0 in either cross. However, in our analyses of week 4 data, we found that variation at the typed SNP in *ABCB4* exerted a significant effect on all four traits in the JCX cross and on the most highly correlated pair of traits, TC and V+LDLC, in the KUSH6 cross, even after employing the overly conservative Bonferroni correction for multiple tests. In all instances, the maximum likelihood estimate of the mean effect (i.e., the "β" in **Table 6**) of the "2" allele(s) at the polymorphic site or the variant in LD with it was negative; i.e., lowering the plasma concentration of the selected lipid/lipoprotein traits (**Figs. 1** and **2**). In the JCX cross, we detected both significant additive and dominance components to the genetic variance for three of the traits measured after four weeks on the challenge diet—TC, V+LDLC, and TG—and additive effects only on HDLC. The level of confidence (i.e., *P* value) for the associations and the magnitudes of the detected mean effects of allelic variation at (or in LD with) this SNP were substantially higher for the highly correlated TC and V+LDLC than for HDLC and TG. Genotypic variation at the *ABCB4* SNP accounted for approximately 16% of the residual phenotypic variance and 65% to 82% of the genetic variance in TC and V+LDLC, respectively; and 4% to 7% of the phenotypic variance and 10% to 37% of the genetic variance in HDLC and TG, respectively. In our analyses of data from the KUSH6 cross, we detected significant additive effects (but not dominance effects) of the *ABCB4* SNP that accounted for roughly 6.5% of the residual phenotypic variance and 16% of the genetic variance in both TC and V+LDLC.

DISCUSSION

The selection of *ABCB4* as a candidate gene for investigation in the present study was prompted entirely by differences in biliary lipids between high- and low-responding opossums. The work presented here was conducted independently of a whole genome scan of lipemic response to the HCHF diet in another genetic cross (neither KUSH6 nor JCX) (Kammerer et al., unpublished observations), although the results obtained from that genome scan implicate a region of opossum chromosome 8 that harbors *ABCB4*.

Detection of significant and substantive genetic effects on plasma lipid and lipoprotein concentrations in the KUSH6 and JCX animals was not unexpected, given our

TABLE 5. Heritability

	KUSH6				JCX			
Week 0	N	h^2	s.e.	$P(h^2 = 0)$	N	h^2	s.e.	$P(h^2 = 0)$
TC	602	0.38	0.064	7.59×10^{-23}	772	0.32	0.075	1.48×10^{-18}
V+LDLC	596	0.34	0.092	5.61×10^{-14}	771	0.25	0.069	9.76×10^{-14}
HDLC	596	0.41	0.087	1.67×10^{-26}	771	0.34	0.073	1.99×10^{-17}
TG	285	0.58	0.211	3.74×10^{-8}	772	0.33	0.064	8.28×10^{-17}
Week 4	N	h^2	s.e.	$P(h^2 = 0)$	N	h^2	s.e.	$P(h^2 = 0)$
TC	584	0.45	0.094	2.58×10^{-19}	734	0.25	0.075	1.48×10^{-18}
V+LDLC	579	0.42	0.098	1.04×10^{-16}	732	0.19	0.069	1.00×10^{-7}
HDLC	579	0.33	0.092	1.18×10^{-12}	732	0.40	0.112	1.43×10^{-14}
TG	293	0.33	0.161	7.11×10^{-5}	733	0.19	0.085	6.00×10^{-6}

Evidence that genes contribute to the phenotypic variance in plasma lipoprotein/lipid concentrations in two genetic crosses of opossums. Abbreviations: h^2, heritability; HDLC, high density lipoprotein cholesterol; s.e., standard error; TC, total cholesterol; TG, triglyceride; V+LDLC, very low density and low density lipoprotein cholesterol.

described breeding protocols for the two crosses. Further, while we consider nonzero heritability estimates to be a logical precondition to testing hypotheses about the effects of *ABCB4* or any other gene on these phenotypes, the magnitude of the total heritability estimate for a trait provides a basis for assessing both the relative genetic importance of the typed gene/variant as well as the likely importance of other genes/variants at other, nontyped (or yet to be discovered) loci.

Given that we observed significant effects of genotypic variation at the typed SNP on the lipoprotein phenotypes in both genetic crosses but only in the analyses of data from the HCHF-challenge diet, we conclude that *ABCB4* is a diet-response gene. This interpretation is consistent with the outcomes of our locus-specific linkage analyses as well (detailed results not presented).

While allelic variation at or in tight LD with the SNP in *ABCB4* accounts for a far greater proportion of the total additive genetic effects on the studied phenotypes in animals from the JCX cross than in those from the KUSH6 cross, it does not account for all the detected genetic effects in either group. Other genes responsible for a significant proportion of the total additive genetic effects on these phenotypes remain to be identified. Based on our estimates of the total heritability and effect size attributable to variation at the SNP, we estimate that these gene(s) could account for at least 10% and up to 40% of the additive genetic effects on lipemic response to diet.

The dominance effect, detected only in the JCX cross (and responsible for the difference in effect sizes between the two crosses), may provide a clue to the nature of the effects of this unidentified gene(s). In studies like the present one in which the effects of both the *ABCB4* locus and the multiple loci making up the polygenic background (i.e., residual heritability) are considered, a significant dominance effect (which, strictly defined, refers to interactions between alleles at one locus) can also implicate epistatic interactions with genes at other loci (25). Although not conclusive evidence for a specific mechanism of gene action or for the existence of additional variants either at the *ABCB4* locus or at other loci, observation of a significant dominance effect in one cross, but not both, is consistent

TABLE 6. Measured genotype analysis

	KUSH6			JCX		
	Additivity			Additivity		
	$P(\beta = 0)$	β	%	$P(\beta = 0)$	β	%
TC	2.00×10^{-6}	−0.31	6.7	1.45×10^{-18}	−0.54	10.3
V+LDLC	5.00×10^{-6}	−0.30	6.3	1.60×10^{-18}	−0.53	9.6
HDLC	1.01×10^{-2}	−0.18	2.1	3.40×10^{-5}	−0.28	4.7
TG	2.82×10^{-1}	—	—	1.60×10^{-5}	−0.28	2.3
	Dominance			Dominance		
	$P(\beta = 0)$	β	%	$P(\beta = 0)$	β	%
TC	4.60×10^{-1}	—	—	4.10×10^{-5}	−0.31	5.9
V+LDLC	4.82×10^{-1}	—	—	1.50×10^{-5}	−0.33	5.9
HDLC	8.38×10^{-1}	—	—	6.95×10^{-2}	−0.15	2.4
TG	6.18×10^{-1}	—	—	4.87×10^{-3}	−0.23	1.9

Evidence that a SNP in *ABCB4* or another variant in LD with it exerts a significant mean effect on plasma lipoprotein/lipid concentrations in two genetic crosses of opossums following a four-week dietary challenge. Maximum likelihood-based, measured-genotype analysis. Levels of significance: $P < 0.00625$ (6.25×10^{-3}) interpreted as evidence for a significant mean effect after Bonferroni correction for multiple testing at nominal $\alpha = 0.05$; $0.0125 > P > 0.00625$ interpreted as evidence for a marginally significant mean effect after Bonferroni correction for multiple testing at nominal $\alpha = 0.10$. Note: Maximum likelihood estimates for β (i.e., the mean effect of the "2" allele(s) in the SNP genotype) not provided if nominal $P > 0.10$ (1.00×10^{-1}). Abbreviations: HDLC, high density lipoprotein cholesterol; TC, total cholesterol; TG, triglyceride; V+LDLC, very low density and low density lipoprotein cholesterol.

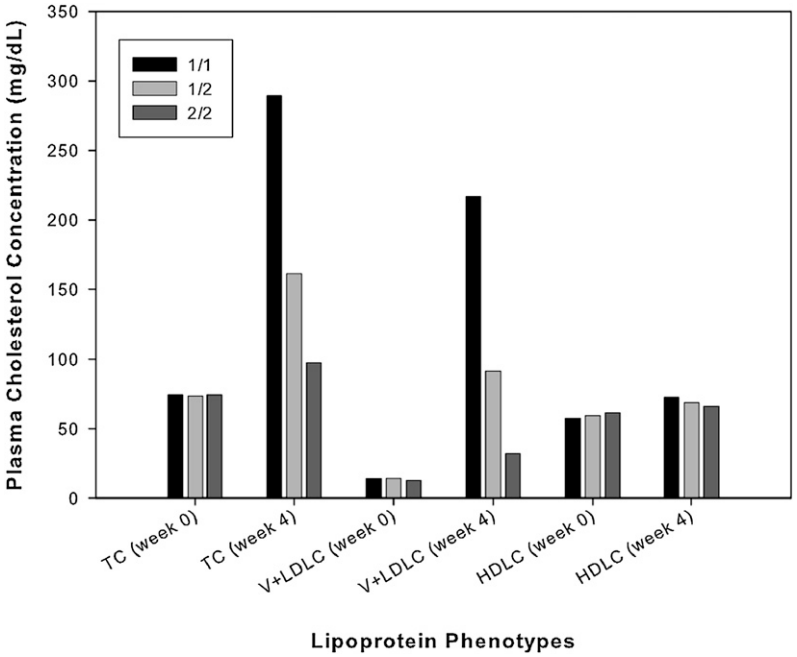

Fig. 1. Plasma lipoprotein cholesterol concentrations by *ABCB4* genotypes in KUSH6 opossums before and after dietary challenge. HDLC, high density lipoprotein cholesterol; TC, total cholesterol; V+LDLC, very low density and low density lipoprotein cholesterol.

with the following simple hypotheses: (1) In the JCX cross, the SNP (or a variant in tight LD with it) interacts with either another variant in *ABCB4* or with a variant in a gene elsewhere in the genome; and (2) This variant is present in a higher frequency in animals from the JCX cross than in those from the KUSH6 cross, in which it could be absent.

Our results do not rule out the possibility that a gene closely linked to *ABCB4*, rather than *ABCB4* itself, is responsible for controlling diet responsiveness in these opossums. However, we again note that *ABCB4* was nominated as a candidate based on biliary lipids data. Moreover, the results of a whole genome scan are consistent with our nomination of *ABCB4* (Kammerer et al., unpublished observations). No other gene is known to affect levels of biliary phospholipids and biliary cholesterol, so the coalescence of the results from the candidate gene strategy and the independently derived results from the genome scan makes it improbable that the results of our statistical genetic analyses are due to the effects of a closely linked gene, rather than to *ABCB4*.

CONCLUSION

We provided evidence suggesting that *ABCB4* is a major determinant of diet-induced hypercholesterolemia in

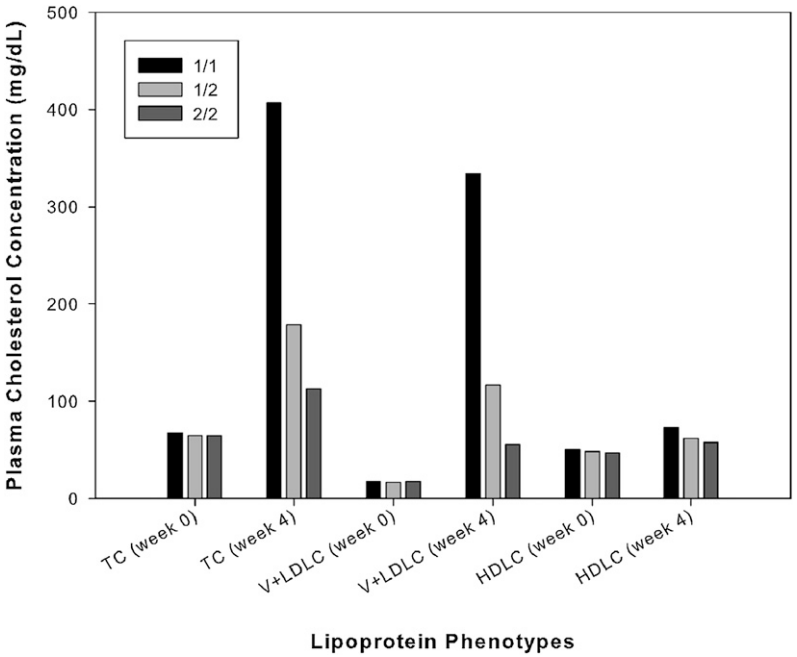

Fig. 2. Plasma lipoprotein cholesterol concentrations by *ABCB4* genotype in JCX opossums before and after dietary challenge. HDLC, high density lipoprotein cholesterol; TC, total cholesterol; V+LDLC, very low density and low density lipoprotein cholesterol.

high-responding opossums. It is known that mutations in the human *ABCB4* gene cause or predispose to hepatobiliary diseases whose clinical symptoms are due to the production of bile with a low phospholipid content and a high cholesterol saturation index (24, 26). Acalovschi et al. (27) examined four human *ABCB4* variants and did not observe an association between high plasma lipid levels and rare *ABCB4* alleles. However, the results of that study do not exclude the possibility that plasma cholesterol in humans can be influenced by other common or rare *ABCB4* variants. This locus should be thoroughly investigated in several human populations in relation to interaction with dietary cholesterol that may affect plasma cholesterol levels.

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