

Heritability of plasma noncholesterol sterols and relationship to DNA sequence polymorphism in *ABCG5* and *ABCG8*

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Abstract The plasma concentrations of cholesterol precursor sterols and plant sterols vary over a 5- to 10-fold range among normolipidemic individuals, and provide indices of the relative rates of cholesterol synthesis and fractional absorption. In the present study, we examined the relative contributions of genetic and environmental factors to variation in the plasma concentrations and sterol-cholesterol ratios of five noncholesterol sterols, including the 5 α -saturated derivative of cholesterol (cholestanol), two precursors in the cholesterol biosynthesis pathway (desmosterol and lathosterol), and two phytosterols (campesterol and sitosterol). Plasma sterol concentrations were highly stable in 30 individuals measured over a 48 week period. Regression of offspring sterol levels on the parental values indicated that plasma levels of all five noncholesterol sterols were highly heritable. Analysis of monozygotic and dizygotic twin pairs also indicated strong heritability of all five sterols. Two common sequence variations (D19H and T400K) in *ABCG8*, an ABC half-transporter defective in sitosterolemia, were associated with lower concentrations of plant sterols in parents, and in their offspring. Taken together, these findings indicate that variation in the plasma concentrations of noncholesterol sterols is highly heritable, and that polymorphism in *ABCG8* contributes to genetic variation in the plasma concentrations of plant sterols.—Berge, K. E., K. von Bergmann, D. Lutjohann, R. Guerra, S. M. Grundy, H. H. Hobbs, and J. C. Cohen. Heritability of plasma noncholesterol sterols and relationship to DNA sequence polymorphism in *ABCG5* and *ABCG8*. *J. Lipid Res.* 2002, 43: 486–494.

Supplementary key words sitosterol • cholesterol absorption • cholesterol synthesis

Adults consuming Western diets ingest ~600 mg of sterols per day, of which one-third is derived from plants, and two-thirds from animal sources (1, 2). In normal individuals, plant sterols are poorly absorbed by the intestine and efficiently secreted in the bile (3). Consequently, the major plant sterols, sitosterol and campesterol, usually com-

prise less than 1% of the total sterol content of human lipoproteins, and large changes in plant sterol consumption result in modest changes in the plasma concentrations of these sterols (4). Although plant sterols appear to play little direct role in sterol metabolism, Miettinen et al. reported that the ratios of plasma sitosterol and campesterol to plasma cholesterol concentrations were positively correlated with the fractional absorption of dietary cholesterol and negatively correlated with cholesterol synthesis (5). Subsequently, coronary patients with low baseline ratios of cholestanol and plant sterols to cholesterol were found to have greater reductions in cholesterol with statin treatment and reduced recurrences of coronary events in the Scandinavian Simvastatin Survival Study (6). Thus the plasma concentrations of plant sterols, and the ratios of these sterols to plasma cholesterol concentrations, may provide useful indices of cholesterol absorption and of responsiveness to statin therapy.

Recently, a critical component of the cellular machinery that limits accumulation of plant sterols was revealed by molecular cloning of the gene defective in sitosterolemia, a rare autosomal recessive disorder of sterol metabolism (7, 8). Patients with sitosterolemia have increased intestinal absorption and markedly impaired biliary excretion of neutral sterols, resulting in ~50-fold elevations in the plasma concentrations of sitosterol and campesterol (9). Sitosterolemia is caused by mutations in two genes that encode the ATP binding cassette (ABC) half-transporters, *ABCG5* and *ABCG8* (7). The two genes are located in a head-to-head orientation on chromosome 2p, and appear to be expressed exclusively in liver and in-

Abbreviations: ABC, ATP binding cassette.

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testine. Cholesterol feeding increases the expression of both genes in mouse liver and (to a lesser extent) intestine (7). The phenotype of sitosterolemic patients, together with the tissue-specific pattern of expression and response to cholesterol feeding of *ABCG5* and *ABCG8*, suggest that the normal function of these genes is to limit the intestinal absorption and promote the biliary excretion of neutral sterols.

Although mutations that cause sitosterolemia are extremely rare, more common sequence variants in *ABCG5* or *ABCG8* may have more subtle effects on sterol metabolism, and may contribute to inter-individual variation in the plasma concentrations of plant sterols. Plasma sitosterol concentrations vary over a 5- to 10-fold range among individuals consuming similar amounts of dietary sitosterol and are poorly correlated with dietary plant sterol intake (10). These findings suggest that inter-individual variation in the plasma concentrations of plant sterols reflect biological differences in sterol metabolism among individuals. The present study was undertaken to test this hypothesis by addressing three questions: *i*) How variable are plasma phytosterol concentrations within an individual?, *ii*) Is variation in plasma phytosterol concentrations heritable?, and *iii*) Do polymorphisms in *ABCG5* and *ABCG8* contribute to variation in plasma phytosterol concentrations? Since the ratio of noncholesterol sterol to cholesterol in the plasma provides an indication of cholesterol biosynthesis and cholesterol absorption, the heritability of these ratios was also determined.

MATERIALS AND METHODS

Subjects

The study protocols were approved by the institutional review boards of the University of Texas Southwestern Medical Center at Dallas and The University of Bonn. Families in which both parents and three or more children were available for sampling were recruited by word of mouth and by advertisements in newspapers, churches, and health centers. Unrelated healthy subjects were also recruited at health promotion events held in the Dallas metropolitan area. Study participants ranged in age from 5 to 79 years. All participants identified themselves as white, not of Hispanic origin, and were ascertained with no a priori knowledge of plasma sterol concentrations. Blood samples were drawn after overnight fast (>10 h) into 10 ml vacuum tubes containing sodium EDTA. Plasma was separated by centrifugation and stored at -20°C , and shipped to Bonn, Germany, for analysis of neutral sterols. Genomic DNA was isolated from the white blood cell pellet using an automated DNA extractor (Applied Biosystems, Foster City, CA). All available family members were sampled, but individuals who were taking lipid-lowering drugs or any form of hormone replacement were excluded from the analyses. All participants completed a detailed questionnaire regarding family history, past and current health status, diet and exercise habits, alcohol and tobacco use, and medications.

Thirty unrelated individuals, 12 pairs of monozygotic (MZ) twins, and 12 pairs of dizygotic (DZ) twins were recruited in Bonn, Germany by newspaper advertisements.

Assay of plasma sterols

Cholesterol precursors (desmosterol and lathosterol), cholesterol, and plant sterols (campesterol and sitosterol) were mea-

sured by gas-liquid chromatography (GLC) after alkaline hydrolysis, extraction, and derivatization as described in detail previously (11, 12). Assay variation was assessed by 12-fold replicate analysis of a single serum sample. Intra-individual variation in sterol levels was estimated from replicate samples drawn at 24- and 48-week intervals from 30 subjects.

Assay of DNA sequence variants in *ABCG5* and *ABCG8*

Five previously identified sequence variants were assayed by PCR amplification and restriction digestion (8, 13). PCR reactions were performed in 20 μl volumes containing 25 ng DNA, 12.5 pmol of each oligonucleotide primer [for primer sequences, see (13)], 4 μmol of each dNTP, and 0.2 U DNA Taq polymerase in the buffer provided by the manufacturer (Roche, Indianapolis, IN). The forward primer for the polymorphism c.55 G>C in *ABCG8* was designed with a single nucleotide substitution to create a restriction enzyme site (*Sbf*I) to distinguish the two alleles. Reactions were subjected to 30 cycles of denaturation at 96°C for 15 s, annealing at 60°C for 30 s, and extension at 72°C for 30 s. The PCR products were digested using the appropriate restriction enzymes (13), size-fractionated by electrophoresis on 3% agarose gels, and visualized by ethidium bromide staining.

Statistical methods

The heritability of plasma noncholesterol sterol concentrations and of the noncholesterol sterol-cholesterol ratios (μg of sterol per mg of cholesterol) was evaluated using family data and twin data. Heritability estimates were obtained from the slope of a regression of mean offspring sterol level on the midparent value in 74 nuclear families. The contribution of shared environment was inferred from spousal correlations. A second test for heritability was performed by comparing estimates of within-twin variation in 12 pairs of MZ and 12 pairs of DZ twins. The statistic for the test is an F-ratio

$$F = \frac{MSW_{DZ}}{MSW_{MZ}}$$

with (n_{DZ} , n_{MZ}) degrees of freedom, where MSW denotes residual mean-square error from separate one-way ANOVAs for each type of twin. For each phenotype MZ and DZ intraclass correlation coefficients were also computed to quantify the degree of similarity within twin pairs.

Linkage disequilibrium was quantified by comparing observed gametic frequencies to those expected under the assumption of linkage equilibrium. The linkage disequilibrium statistic used for analysis is defined by: $D = P_{AB} - P_A P_B$, where the probabilities (P) are estimated by their observed relative frequencies. Letting A and B denote two given alleles at two different loci, the statistic used to test for linkage disequilibrium using $2n$ gametes is

$$X_{AB}^2 = \frac{2nD_{AB}^2}{P_A(1-P_A)P_B(1-P_B)}$$

which is distributed as a chi-square random variable with 1 degree of freedom. Normalized linkage disequilibrium coefficients (D') were calculated by dividing D by its maximal value to give a normalized range of $(-1, 1)$

$$D' = \frac{D_{AB}}{\max(P_A P_B, -P_A P_B)} D_{AB} < 0$$

$$D' = \frac{D_{AB}}{\min(P_A P_B, P_A P_B)} D_{AB} < 0$$

where $P_{\bar{A}} = 1 - P_A$ and similarly $P_{\bar{B}}$. Linkage disequilibrium was evaluated for each of the 10 possible pairs of 5 loci.

Tests for association were performed using five nonsynonymous DNA sequence variants (four in *ABCG8* and one in

TABLE 1. Mean age, plasma sterol concentrations, and plasma sterol-cholesterol ratios in unrelated individuals and twins

	Unrelated Individuals (n = 148)	Monozygotic Twins (n = 24)	Dizygotic Twins (n = 24)
Age (years)	55 ± 11	25 ± 7	25 ± 7
Plasma sterol concentrations			
Cholesterol (mg/dl)	204 ± 38	172 ± 41	182 ± 40
Cholestanol (µg/dl)	420 ± 110	420 ± 140	410 ± 80
Desmosterol (µg/dl)	200 ± 70	130 ± 40	150 ± 50
Lathosterol (µg/dl)	320 ± 150	230 ± 100	250 ± 80
Sitosterol (µg/dl)	250 ± 100	310 ± 80	290 ± 90
Campesterol (µg/dl)	330 ± 140	430 ± 100	420 ± 150
Plasma sterol-cholesterol ratios			
Cholestanol (µg/mg)	2.05 ± 0.46	2.43 ± 0.38	2.32 ± 0.41
Desmosterol (µg/mg)	0.99 ± 0.31	0.80 ± 0.20	0.82 ± 0.17
Lathosterol (µg/mg)	1.53 ± 0.66	1.37 ± 0.50	1.40 ± 0.40
Sitosterol (µg/mg)	1.24 ± 0.47	1.79 ± 0.31	1.62 ± 0.38
Campesterol (µg/mg)	1.66 ± 0.64	2.53 ± 0.41	2.31 ± 0.64

Values are means ± SD. Unrelated individuals are 148 parents from 74 nuclear families.

ABCG5) that were found in more than a single individual. To reduce the risk of false positive association from multiple testing, the analysis of each polymorphism was performed in two stages. First, the parents of the nuclear families were divided into two groups comprising *i*) homozygotes for the common allele, and *ii*) heterozygotes and homozygotes for the less common allele. The mean value of each sterol (and the sterol-cholesterol ratio) was then compared in the two groups using a *t*-test. Second, for those polymorphisms that were associated with the plasma levels of one or more sterols with a nominal $P < 0.05$, the sterol values of siblings who carried the less common allele were compared with those of their siblings who were homozygotes for the common allele using a Wilcoxon test, as described previously (14).

RESULTS

Blood samples were obtained from 502 individuals comprising 74 nuclear families, and from 24 pairs of twins. Ages and plasma sterol concentrations of these individuals are shown in **Table 1**.

Intra-individual variation in plasma noncholesterol sterol concentrations

Plasma noncholesterol sterol concentrations were highly stable within individuals. Within assay variation [(SD/mean) × 100] ranged from 1.5–3.8%. Differences in the noncholesterol sterol levels in the replicate samples drawn 24 weeks apart ranged from 0.2% for desmosterol to 2.6% for campesterol. No appreciable increase in variability was noted at 48 weeks (mean differences for replicate analyses ranged from 0.21–3.1%).

Distribution of plasma noncholesterol sterol concentrations

Plasma concentrations of the cholesterol precursor sterols desmosterol and lathosterol, and the phytosterols campesterol and sitosterol, showed lognormal distributions with significant skewing toward higher levels (**Fig. 1**). The range of inter-individual variation was substantially greater for these sterols (5- to 10-fold) than for plasma

cholesterol levels (~3-fold). The distribution and inter-individual variation of plasma concentrations of cholestanol and cholesterol were similar.

Phenotype correlations

The plasma concentrations of noncholesterol sterols were significantly correlated with sex, age, and plasma cholesterol concentrations. Plasma concentrations of the cholesterol precursor sterols were slightly higher in men than in women (190 ± 70 vs. 160 ± 60 for desmosterol and 270 ± 140 vs. 250 ± 120 for lathosterol), but no sex difference was observed for the other sterols (data not shown). Plasma concentrations of cholesterol, cholestanol, and the cholesterol biosynthetic precursor sterols desmosterol and lathosterol were positively correlated with age (**Table 2**). In contrast, weak negative correlations were observed between age and the plasma concentrations of the two plant sterols campesterol and sitosterol. The plasma concentrations of all five noncholesterol sterols were positively correlated with those of cholesterol (**Table 2**). Plasma concentrations of cholestanol were strongly correlated with those of the two plant sterols sitosterol and campesterol. Plasma concentrations of the cholesterol precursor sterols lathosterol and desmosterol were strongly correlated with each other, as were those of the plant sterols sitosterol and campesterol, but correlations between the cholesterol precursor sterols and the plant sterols were very weak and inconsistent.

Familial correlations and heritability estimation

Regression of mean offspring sterol concentrations on mid-parent values suggested a strong familial component for cholesterol ($H^2 = 0.50$), cholestanol ($H^2 = 0.54$), desmosterol ($H^2 = 0.28$), lathosterol ($H^2 = 0.31$), sitosterol ($H^2 = 0.57$), and campesterol ($H^2 = 0.59$). Heritability estimates were not appreciably affected by preadjustment for age (data not shown), indicating that the familial aggregation of plasma sterol concentrations was not an artifact of confounding factors associated with aging. The

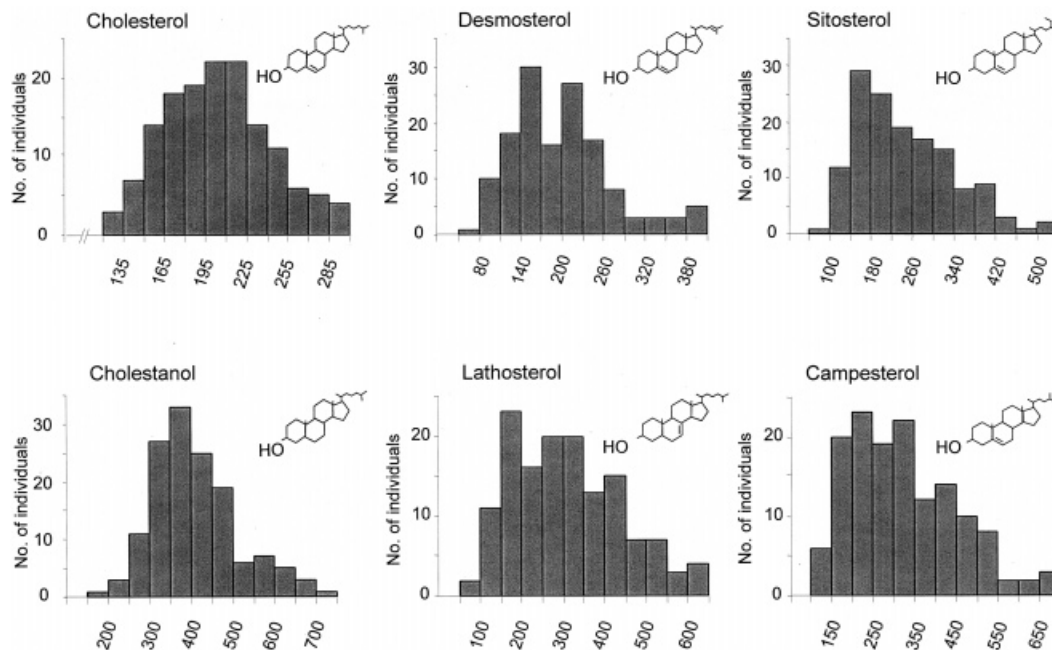


Fig. 1. Distribution of fasting plasma sterol concentrations in 148 unrelated men and women. Values for cholesterol are in mg/dl and for noncholesterol sterols are $\mu\text{g}/\text{dl}$.

heritability estimate for plasma cholesterol level was 0.5, which is similar to that reported for other populations (15–18). Plasma sterol-cholesterol ratios were also highly heritable (**Fig. 2**). Estimates for the ratios of the cholesterol biosynthetic precursors desmosterol ($H^2 = 0.36$) and lathosterol ($H^2 = 0.42$) to cholesterol were lower than the corresponding ratios for cholestanol ($H^2 = 0.81$), sitosterol ($H^2 = 0.81$), and campesterol ($H^2 = 0.84$), and, the three sterols that were previously shown to be correlated with cholesterol absorption (5). To determine if the high heritability estimates for these ratios were due to similar dietary sterol intakes within families, the correlations between the sterol-cholesterol ratios of spouses were determined. A significant spousal correlation was observed for lathosterol-cholesterol ($r = 0.4$), but not for cholestanol-cholesterol ($r = 0.13$), desmosterol-cholesterol ($r = 0.14$), campesterol-cholesterol ($r = -0.04$), or sitosterol-cholesterol ($r = 0.012$). Thus the familial aggregation of plasma sterol-cholesterol ratios cannot be ascribed to similar diets consumed by family members. To further evaluate the heritability of plasma noncholesterol sterols, a second study was performed in adult male MZ and DZ twins. The mean values for plasma lipid levels (Table 1) were similar in the

two sets of twins, but the plasma sterol concentrations (not shown) and the sterol-cholesterol ratios (**Fig. 3**) were significantly more similar among MZ than among DZ twins.

Identification of *ABCG5* and *ABCG8* alleles

Parental haplotypes were determined by analyzing the segregation of the five common polymorphisms in the nuclear families. Nineteen distinct alleles were found among 144 unrelated individuals (**Fig. 4**). The three most common haplotypes accounted for 63.8% of the alleles in this population. The predominance of these alleles is due in part to linkage disequilibrium among the three common sequence variations in *ABCG8* (**Table 3**).

The relationships between the five common, nonsynonymous DNA sequence polymorphisms and plasma concentrations, and sterol cholesterol-ratios of each sterol were determined among unrelated individuals (the parents) (**Table 4**). Analysis of sterol concentrations and sterol-cholesterol ratios yielded similar results, therefore subsequent analyses were performed using plasma sterol-cholesterol ratios only. Two of the polymorphisms were associated with noncholesterol sterol-cholesterol ratios both in the un-

TABLE 2. Correlations among age and plasma sterol concentrations

	Age	Cholesterol	Cholestanol	Desmosterol	Lathosterol	Sitosterol
Cholesterol	0.47					
Cholestanol	0.21	0.71				
Desmosterol	0.33	0.72	0.40			
Lathosterol	0.38	0.61	0.17	0.68		
Sitosterol	-0.1	0.31	0.65	0.06	-0.1	
Campesterol	-0.09	0.39	0.70	0.11	-0.05	0.92

Values are Pearson correlations calculated using 502 individuals from 74 families. $P < 0.05$ for $r > 0.09$.

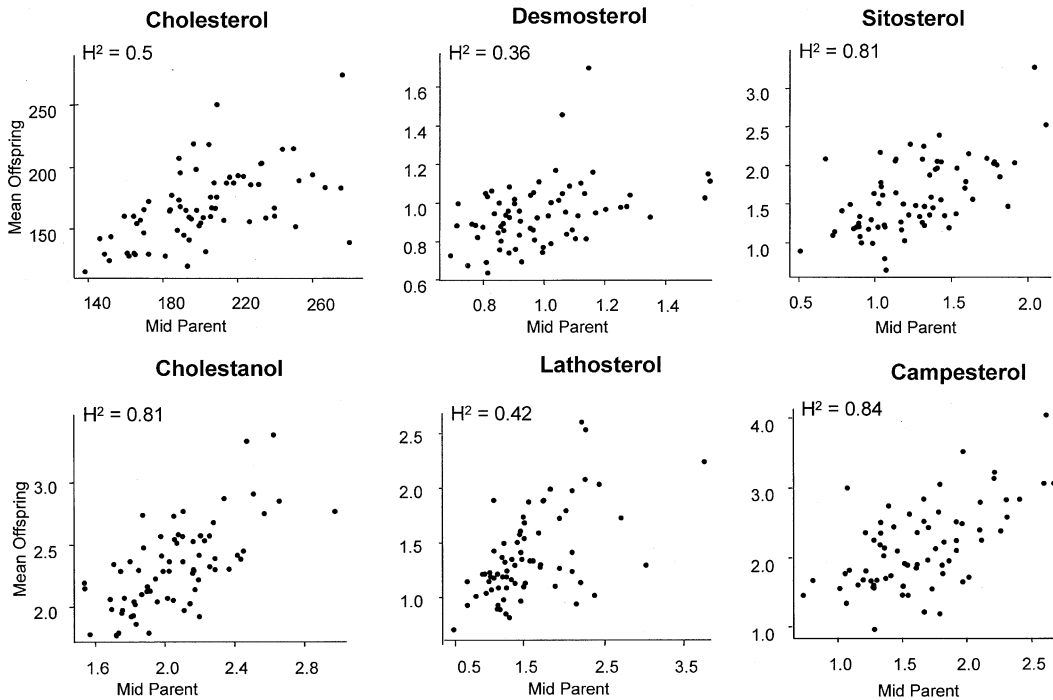


Fig. 2. Plasma sterol-cholesterol ratios in nuclear families. Scatterplots depict mean offspring sterol-cholesterol ratio plotted against the midparental value. Values for cholesterol are in mg/dl and for non-cholesterol sterols in μg per mg of plasma cholesterol.

related individuals (parents) and in the sibling comparison (offspring) (Table 5). The D19H polymorphism in exon 1 of *ABCG8* was associated with the plasma cholestanol-choles-

terol and campesterol-cholesterol ratios, and the T400K polymorphism in *ABCG8* was associated with the plasma sitosterol-cholesterol ratio. Interestingly, the rare allele of

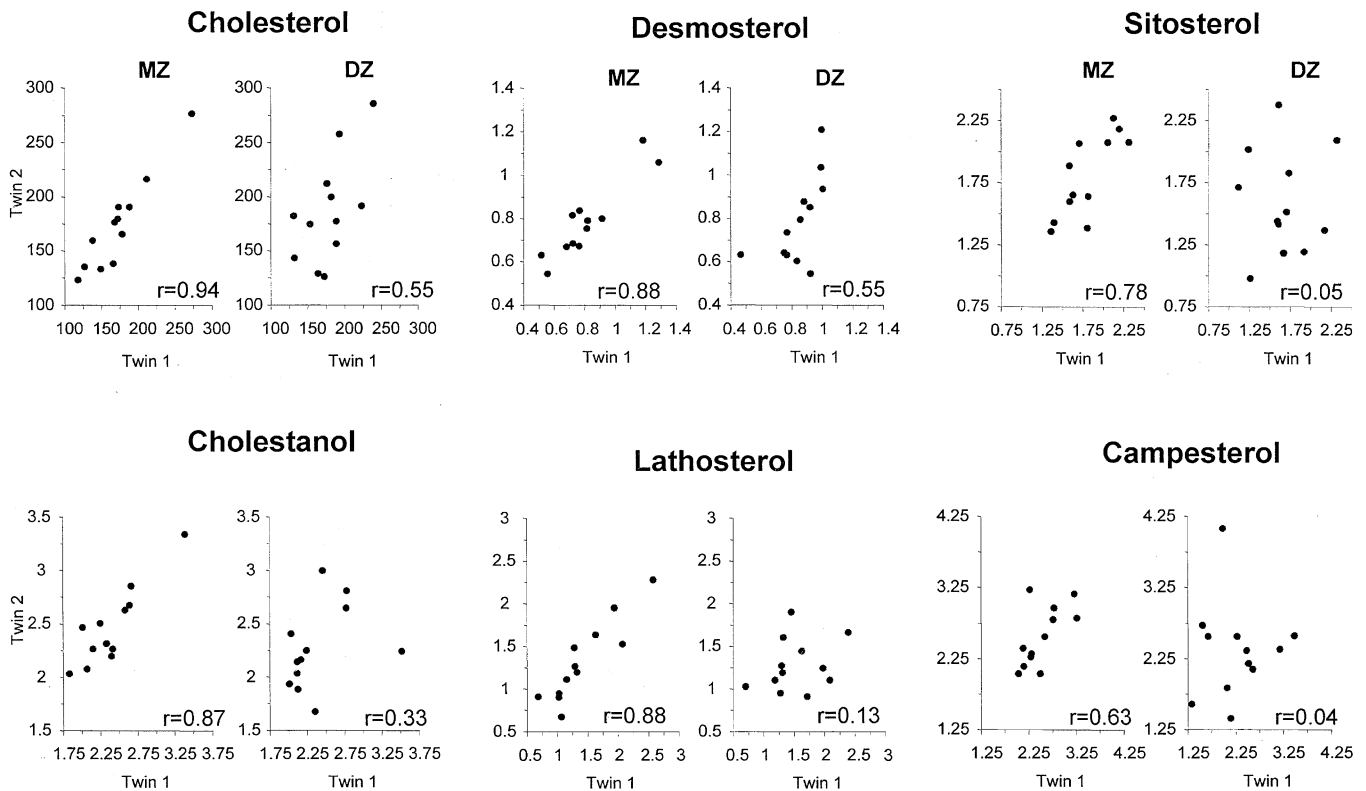


Fig. 3. Plasma sterol-cholesterol ratios in twins. Values for cholesterol are in mg/dl and for non-cholesterol sterols in μg per mg of plasma cholesterol.

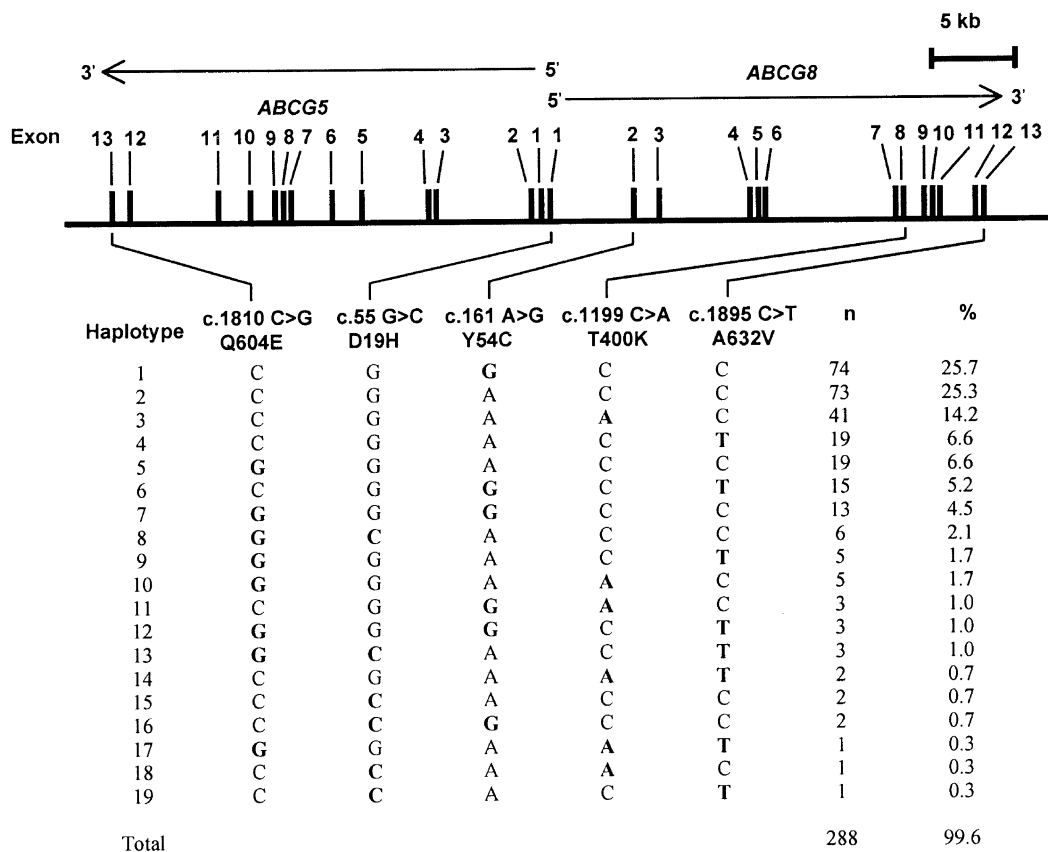


Fig. 4. Haplotypes at the *ABCG5/ABCG8* locus. Linkage phase was determined using 74 nuclear families. Allele counts and frequencies were calculated using the parental alleles only.

both polymorphisms was associated with lower plasma sterol concentrations, and neither polymorphism was associated with plasma cholesterol concentrations. The A632V polymorphism in exon 13 of *ABCG8* was associated with plasma cholesterol concentrations both in the parents and in the offspring, but was not associated with any of the noncholesterol sterols. To further examine the relationship between this polymorphism and plasma cholesterol concentrations, a third analysis was performed in an independent group of white men and women aged 20 years or

older who had plasma LDL cholesterol (LDL-C) concentrations greater than the 90th percentile ($n = 77$), or less than the 10th percentile ($n = 116$). The frequencies of the 632A and 632V alleles were not significantly different between the two groups: The numbers of TT, CT, and CC individuals were 4, 39, and 73 in the low LDL group, and 4, 25, and 48 in the high LDL group ($P > 0.5$).

DISCUSSION

The present study was undertaken to assess the heritability of plasma noncholesterol sterol concentrations, and to determine whether DNA sequence variations in *ABCG5* and *ABCG8* affect the plasma levels of these sterols. Three major observations were made. First, plasma noncholesterol sterol concentrations are very stable within individuals. Intra-individual variation in the plasma concentrations of noncholesterol sterols was minimal during the course of 1 year, and inter-assay variability was negligible. Therefore, most of the variation in plasma noncholesterol sterols reflects true biologic variation among individuals. Second, the plasma concentrations and sterol-cholesterol ratios of each of the noncholesterol sterols was highly heritable. The proportion of phenotypic variation that could be accounted for by shared genes ranged from 36% for plasma lathosterol-cholesterol ratio to 84% for plasma

TABLE 3. Linkage disequilibrium between polymorphisms in *ABCG5* and *ABCG8*

Locus 1	Locus 2	D'	P
<i>ABCG5</i> Q604E	<i>ABCG8</i> D19H	0.47	0.001
	<i>ABCG8</i> Y54C	0.29	0.095
	<i>ABCG8</i> T400K	0.31	0.299
	<i>ABCG8</i> A632V	0.06	0.451
<i>ABCG8</i> D19H	<i>ABCG8</i> Y54C	0.62	0.122
	<i>ABCG8</i> T400K	0.63	0.408
	<i>ABCG8</i> A632V	0.04	0.979
<i>ABCG8</i> Y54C	<i>ABCG8</i> T400K	0.85	0
	<i>ABCG8</i> A632V	0.04	0.959
<i>ABCG8</i> T400K	<i>ABCG8</i> A632V	0.69	0.016

Haplotypes were determined in 74 nuclear families. Offspring were used to determine linkage phase but D' and P values were calculated using the parental alleles only.

TABLE 4. Mean plasma sterol concentrations and sterol-cholesterol ratios in unrelated men and women with different *ABCG5* and *ABCG8* genotypes

Plasma sterol concentrations	<i>ABCG8</i> D19H			<i>ABCG8</i> Y54C			<i>ABCG8</i> T400K			<i>ABCG8</i> A632V			<i>ABCG5</i> Q604E		
	DD n = 128	DH/HH n = 14	YY n = 54	YC/CC n = 85	TT n = 95	TK/KK n = 48	AA n = 94	VA/VV n = 49	QQ n = 91	QE/EE n = 51					
Cholesterol	202 ± 41	194 ± 33	199 ± 39	203 ± 40	197 ± 38	207 ± 42	195 ± 38 ^b	213 ± 40	198 ± 40	204 ± 40					
Cholestanol	420 ± 110 ^b	340 ± 72	400 ± 104	419 ± 114	410 ± 115	418 ± 103	400 ± 97	437 ± 131	411 ± 114	416 ± 105					
Desmosterol	200 ± 70	196 ± 79	195 ± 66	202 ± 74	191 ± 67	221 ± 79	197 ± 75	210 ± 68	197 ± 74	208 ± 70					
Lathosterol	308 ± 135	365 ± 252	308 ± 120	320 ± 168	296 ± 136	354 ± 171	309 ± 165	329 ± 116	314 ± 154	322 ± 145					
Sitosterol	257 ± 105 ^b	177 ± 53	231 ± 93	261 ± 109	256 ± 114	238 ± 83	239 ± 87	274 ± 130	250 ± 107	250 ± 104					
Campesterol	338 ± 147 ^b	233 ± 72	310 ± 133	339 ± 152	332 ± 149	324 ± 144	308 ± 124 ^a	375 ± 177	328 ± 154	332 ± 136					
Plasma sterol-cholesterol ratios (μg/mg)															
Cholestanol	2.09 ± 0.40 ^c	1.76 ± 0.31	2.02 ± 0.37	2.07 ± 0.39	2.09 ± 0.44	2.01 ± 0.31	2.06 ± 0.41	2.05 ± 0.39	2.08 ± 0.40	2.04 ± 0.42					
Desmosterol	0.99 ± 0.26	1.00 ± 0.32	0.97 ± 0.25	0.99 ± 0.28	0.96 ± 0.25 ^a	1.06 ± 0.30	1.00 ± 0.29	0.98 ± 0.23	0.98 ± 0.27	1.02 ± 0.27					
Lathosterol	1.53 ± 0.60	1.84 ± 1.06	1.57 ± 0.60	1.57 ± 0.70	1.48 ± 0.57 ^a	1.73 ± 0.78	1.57 ± 0.70	1.56 ± 0.57	1.57 ± 0.67	1.58 ± 0.63					
Sitosterol	1.28 ± 0.45 ^c	0.94 ± 0.32	1.18 ± 0.45	1.29 ± 0.45	1.30 ± 0.48 ^a	1.15 ± 0.35	1.24 ± 0.42	1.29 ± 0.51	1.27 ± 0.44	1.23 ± 0.48					
Campesterol	1.67 ± 0.62 ^c	1.21 ± 0.36	1.56 ± 0.59	1.66 ± 0.63	1.68 ± 0.62	1.54 ± 0.60	1.58 ± 0.59	1.74 ± 0.65	1.64 ± 0.63	1.61 ± 0.59					

Values are means ± SD. Plasma cholesterol concentrations are in mg/dl. Other sterol concentrations are in μg/dl. Plasma noncholesterol sterol concentrations in μg/dl were divided by the cholesterol concentration in mg/dl. For each polymorphism, mean values among homozygotes for the common allele were compared with the mean values for carriers (heterozygotes and homozygotes) of the rare allele using unpaired *t*-tests. All comparisons resulting in *P* values < 0.05 are shown in bold.

^a Denotes *P* values < 0.05.

^b Denotes *P* values < 0.01.

^c Denotes *P* values < 0.005.

campesterol-cholesterol ratio. Third, common DNA sequence polymorphisms in *ABCG8* contribute to heritable variation in the plasma concentrations of the plant sterols campesterol and sitosterol.

The magnitudes and distributions of the plasma sterol concentrations observed in this study are consistent with those reported by other studies (10, 19), and the plasma concentrations of all five noncholesterol sterols examined were remarkably stable over time. Although the plasma concentrations of any given sterol varied over a 5- to 10-fold range among individuals, analysis of replicate samples drawn over the course of 48 weeks indicated that intra-individual variation was less than 4%. These findings are consistent with the data of Li et al., who reported that the plasma concentrations of sitosterol and campesterol remained stable over a 6 month period in seven premenopausal women (20).

The observation that plasma noncholesterol sterol concentrations were stable in individuals, but highly variable among individuals, suggested that the metabolism of these sterols is strongly influenced by genetic factors. To test this hypothesis, we examined the heritability of plasma noncholesterol sterol concentrations in parents and their offspring, and in MZ and DZ twin pairs. Regression of mean offspring sterol concentrations on the midparent values indicated that the plasma concentrations of all five noncholesterol sterols examined are heritable with estimates of heritability ranging from 28–31% for desmosterol and lathosterol to 59% for campesterol. Heritability estimates for the ratios of plasma sterol-cholesterol were even higher, ranging from 35–40% for desmosterol and lathosterol to greater than 80% for cholestanol, sitosterol, and campesterol. With the exception of lathosterol, spousal values of the noncholesterol sterols were not significantly correlated, indicating that the estimates of heritability for these sterols were not confounded by shared environmental factors. Analysis of MZ and DZ twins provided further evidence that plasma sterol concentrations are highly heritable. Although the number of twins available for study was too small to provide reliable point estimates of heritability, the plasma concentrations and sterol-cholesterol ratios of each sterol were significantly more similar in MZ twins than in DZ twins. Taken together, these findings provide strong evidence that variation in plasma sterol concentrations is strongly influenced by genetic factors.

The ratios of noncholesterol sterols to cholesterol levels reflect the rates of cholesterol biosynthesis and cholesterol absorption. Therefore, the results of the present study indicate that inter-individual variation in both of these processes is heritable. The heritability of cholesterol absorption and biosynthesis has not been directly determined, and only a single previous report has examined the heritability of plasma noncholesterol sterol levels. In a study of 17 MZ and 18 DZ twin pairs, Kesaniemi et al. found that 40–60% of the variation in the plasma concentrations of Δ⁸ methylsterols (which precede lathosterol in the cholesterol biosynthetic pathway) is heritable (21). These data are consistent with our present finding that

TABLE 5. Effect of *ABCG8* polymorphisms on plasma sterol-cholesterol ratios in siblings

	Cholesterol	Cholestanol	Desmosterol	Lathosterol	Sitosterol	Campesterol
<i>ABCG8</i> D19H	0.097	0.08	0.005	0.001	0.13	0.045
<i>ABCG8</i> T400K	0.29	0.02	0.34	0.24	0.05	0.03
<i>ABCG8</i> A632V	0.018	0.29	0.18	0.39	0.26	0.22

The relationships between *ABCG8* polymorphisms and plasma sterol-cholesterol ratios were assessed by comparing the plasma sterol levels of siblings with different genotypes. Sterol-cholesterol ratios were calculated by dividing the sterol concentration ($\mu\text{g}/\text{dl}$) by the cholesterol concentration (mg/dl). Only those polymorphisms that were significantly associated with plasma sterol-cholesterol ratios in unrelated individuals (parents) were examined in the siblings. Sterol levels of homozygotes for the common allele were compared with the corresponding values in their siblings who were carriers (homozygotes or heterozygotes) for the rare allele using Wilcoxon's tests. The table provides *P* values for each comparison. Associations that were significant at the 0.05 threshold in the parents and in the sibling comparison are shown in bold.

plasma concentrations of lathosterol and desmosterol are heritable. Furthermore, the finding that the lathosterol-cholesterol and desmosterol-cholesterol ratios are heritable suggests that rates of cholesterol biosynthesis are determined, at least in part, by genetic variation.

The ratios of plasma cholestanol, the 5α -saturated derivative of cholesterol, and the major plant sterols campesterol and sitosterol to cholesterol are correlated with the fractional absorption of dietary cholesterol (5, 22). The heritability of plasma plant sterol concentrations and of the plant sterol-cholesterol ratios has not been reported previously. Glueck et al. (19) and Kempen et al. (10) measured the plasma levels of these sterols in families, but neither group reported estimates of heritability. Therefore, the results of this study provide the first evidence that individual variation in the plasma sterol-cholesterol ratios of these sterols, and thus fractional absorption of dietary cholesterol, is heritable.

The strong heritable variation in plasma concentrations of campesterol and sitosterol suggested that common DNA sequence polymorphisms influence the metabolism of plant sterols in the general population. To determine whether polymorphisms in *ABCG5* and *ABCG8*, genes that play a key role in sterol absorption and excretion, contribute to heritable variation in plasma sterol concentrations, we tested for association between plasma noncholesterol sterol concentrations and five nonsynonymous polymorphisms in the two genes. The analysis of multiple polymorphisms against multiple traits in a single study population is subject to problems associated with multiple testing, which can increase the likelihood of false positive association. A possible strategy to address this problem is to set a more stringent threshold at which the null hypothesis is rejected, using Bonferroni's correction to calculate the appropriate *P* value. A disadvantage of this approach is that it increases the risk that a true association will be missed, especially if the polymorphism has a modest effect on the trait. To reduce the risk of false positive association while maintaining adequate power to detect modest associations, we analyzed each polymorphism in two steps. First, the mean plasma concentration and sterol-cholesterol ratio associated with each genotype was compared in 148 unrelated individuals (74 pairs of spouses). For those polymorphisms that were associated with the plasma levels of one or more sterols with a nominal $P < 0.05$, a second analysis was performed in which the sterol-cholesterol

ratios were compared among siblings with different genotypes. Both analyses indicated an association between a polymorphism (D19H) in exon 1 of *ABCG8* and the plasma concentrations of campesterol. Similar trends were seen for cholestanol and sitosterol, although these associations did not reach significance at the 0.05 level in the siblings. The finding of a statistically significant association between plasma sitosterol concentrations and this nonconservative substitution suggests that the substitution of histidine for aspartic acid at amino acid 19 alters the function of *ABCG8*. Since the plasma sitosterol concentrations were lower in individuals with histidine at this residue, the predicted change would be expected to increase transporter function. In the absence of an *in vitro* assay, however, the effect of this amino acid substitution on *ABCG8* function cannot be directly determined. Furthermore, we cannot exclude the possibility that the association observed is due to linkage disequilibrium with another polymorphism(s) at this locus, rather than to a direct effect of the D19H substitution.

Two of the other polymorphisms examined were also associated with plasma sterol concentrations. A common nonconservative substitution (T400K) was associated with the plasma concentrations of sitosterol, although the association of this polymorphism with plasma campesterol concentration was marginal. The substitution of valine for alanine at amino acid 632 was associated with plasma cholesterol concentrations in both analyses, but it was not consistently associated with any of the other sterols. It is not clear how a polymorphism in either *ABCG5* or *ABCG8* could affect the plasma concentrations of cholesterol but not those of the plant sterols unless it altered the specificity of the transporter for its substrate. Since no association was observed in a third group selected for plasma LDL-C concentrations greater than the 90th or less than the 10th percentile, we suspect that the association observed between the A632V polymorphism and plasma cholesterol concentrations is spurious. Additional studies will be required to confirm the relationship between these polymorphisms and plasma sterol concentrations.

Since *ABCG5* and *ABCG8* probably function as a heterodimer, multiple polymorphisms in the two genes may interact to affect the function of the complex, as has been observed in the closely related white and brown genes in *Drosophila melanogaster* (23). White and brown are ABC half-transporters required for the uptake of pigment precursors

sors by cells in the developing eye. Some sequence variants in the two genes (G558S in white and N638T in brown) have no phenotypic effect individually but result in impaired eye pigmentation when inherited together (23). In humans, *ABCG5* and *ABCG8* are located within a few hundred base pairs of each other on chromosome 2; therefore we determined the linkage phase of the five polymorphisms examined in the present study by analyzing the segregation of each sequence variant in nuclear families. Nineteen different alleles were identified, of which three accounted for the majority (65%) of the alleles in the population. None of the four most common alleles contained more than one nucleotide substitution corresponding to the less frequent allele at the five loci examined. Because only a very small proportion of individuals carry any given allele that encodes two or more amino acid substitutions, a much larger sample will be required to determine the effect of these alleles on plasma sterol levels.

The demonstration that plasma cholestanol, campesterol, and sitosterol levels are highly heritable may have important clinical implications. Recent data from the Scandinavian Simvastatin Survival Study indicate that statin therapy may reduce the recurrence of coronary events more effectively in individuals with low baseline ratios of serum cholestanol and plant sterols to cholesterol (indicating low cholesterol absorption) than in individuals with high baseline ratios of these sterols. The present study provides strong evidence that these ratios are largely determined by genetic factors. If the efficacy of statin treatment in secondary prevention is constrained by the genetic background of the patient, then more aggressive intervention may be merited in patients with high baseline ratios of cholestanol, campesterol, and sitosterol to cholesterol. ■

The authors thank H. Prange, K. Wilmersdorf, and Yuanlan Liao for excellent technical assistance, and Dick Verstraete and Ned Warner for recruiting the families. This work was supported by grants from the NIH (HL53917 and HL20948), The W. M. Keck Foundation, The Donald W. Reynolds Cardiovascular Clinical Research Center at Dallas, and the Deutsche Forschungsgemeinschaft (BE 1673/1-1). K.E.B. is supported by the Norwegian Research Council and the Thoresen Foundation.

Manuscript received 24 September 2001 and in revised form 2 January 2002.

REFERENCES

- Weihrauch, J. L., and J. M. Gardner. 1978. Sterol content of foods of plant origin. *J. Am. Diet. Assoc.* **73**: 39–47.
- Nair, P. P., N. Turjman, G. Kessie, B. Calkins, G. T. Goodman, H. Davidovitz, and G. Nimmagadda. 1984. Diet, nutrition intake, and metabolism in populations at high and low risk for colon cancer: dietary cholesterol, beta-sitosterol, and stigmasterol. *Am. J. Clin. Nutr.* **40**: 927–930.
- Salen, G., E. H. Ahrens, Jr., and S. M. Grundy. 1970. Metabolism of beta-sitosterol in man. *J. Clin. Invest.* **49**: 952–967.
- Vanhanen, H. T., and T. A. Miettinen. 1992. Effects of unsaturated and saturated dietary plant sterols on their serum contents. *Clin. Chim. Acta.* **205**: 97–107.
- Miettinen, T. A., R. S. Tilvis, and Y. A. Kesaniemi. 1990. Serum plant sterols and cholesterol precursors reflect cholesterol absorption and synthesis in volunteers of a randomly selected male population. *Am. J. Epidemiol.* **131**: 20–31.

- Miettinen, T. A., H. Gylling, T. Strandberg, and S. Sarna. 1998. Baseline serum cholestanol as predictor of recurrent coronary events in subgroup of Scandinavian Simvastatin Survival Study: Finnish 4S investigators. *BMJ.* **316**: 1127–1130.
- Berge, K. E., H. Tian, G. A. Graf, L. Yu, N. V. Grishin, J. Schultz, P. Kwiterovich, B. Shan, R. Barnes, and H. H. Hobbs. 2000. Accumulation of dietary cholesterol in sitosterolemia caused by mutations in adjacent ABC transporters. *Science.* **290**: 1771–1775.
- Lu, K., M. H. Lee, S. Hazard, A. Brooks-Wilson, H. Hidaka, H. Kojima, L. Ose, A. F. Stalenhoef, T. Miettinen, I. Bjorkhem, E. Bruckert, A. Pandya, H. B. Brewer, Jr., G. Salen, M. Dean, A. Srivastava, and S. B. Patel. 2001. Two genes that map to the STSL locus cause sitosterolemia: genomic structure and spectrum of mutations involving sterolin-1 and sterolin-2, encoded by *ABCG5* and *ABCG8*, respectively. *Am. J. Hum. Genet.* **69**: 278–290.
- Bjorkhem, I. J., K. M. Boberg, and E. Leitersdorf. 2001. Inborn errors in bile acid biosynthesis and storage of sterols other than cholesterol. In *The Metabolic and Molecular Basis of Inherited Disease*. C. R. Scriver, A. L. Beaudet, W. S. Sly, and D. Valle, editors. McGraw-Hill, New York. 2961–2988.
- Kempen, H. J., P. de Knijff, D. I. Boomsma, H. A. van der Voort, J. A. Gevers Leuven, and L. Havekes. 1991. Plasma levels of lathosterol and phytosterols in relation to age, sex, anthropometric parameters, plasma lipids, and apolipoprotein E phenotype, in 160 Dutch families. *Metab. Clin. Exper.* **40**: 604–611.
- Lutjohann, D., I. Bjorkhem, U. F. Beil, and K. von Bergmann. 1995. Sterol absorption and sterol balance in phytosterolemia evaluated by deuterium-labeled sterols: effect of sitostanol treatment. *J. Lipid Res.* **36**: 1763–1773.
- Heinemann, T., G. Axtmann, and K. von Bergmann. 1993. Comparison of intestinal absorption of cholesterol with different plant sterols in man. *Eur. J. Clin. Invest.* **23**: 827–831.
- Hubacek, J. A., K. E. Berge, J. C. Cohen, and H. H. Hobbs. 2001. Mutations in ATP-cassette binding proteins G5 (*ABCG5*) and G8 (*ABCG8*) causing sitosterolemia. *Hum. Mutat.* **18**: 359–360.
- Barre, D. E., R. Guerra, R. Verstraete, Z. Wang, S. M. Grundy, and J. C. Cohen. 1994. Genetic analysis of a polymorphism in the human apolipoprotein A-I gene promoter: effect on plasma HDL-cholesterol levels. *J. Lipid Res.* **35**: 1292–1296.
- Perusse, L., J.-P. Despres, A. Tremblay, C. Leblanc, J. Talbot, C. Allard, and C. Bouchard. 1989. Genetic and environmental determinants of serum lipids and lipoproteins in French Canadian families. *Arteriosclerosis.* **9**: 308–318.
- Rice, T., G. P. Vogler, T. S. Perry, P. M. Laskarzewski, and D. C. Rao. 1991. Familial aggregation of lipids and lipoproteins in families ascertained through random and nonrandom probands in the Iowa Lipid Research Clinics Family Study. *Hum. Hered.* **41**: 107–121.
- Austin, M. A., M. C. King, R. D. Bawol, S. B. Hulley, and G. D. Friedman. 1987. Risk factors for coronary heart disease in adult female twins: genetic heritability and shared environmental influences. *Am. J. Epidemiol.* **125**: 308–318.
- Bucher, K. D., Y. Friedlander, E. B. Kaplan, K. K. Nambodiri, J. D. Kark, S. Eisenberg, Y. Stein, and B. M. Rifkind. 1988. Biological and cultural sources of familial resemblance in plasma lipids: a comparison between North America and Israel—the Lipid Research Clinics Program. *Genet. Epidemiol.* **5**: 17–33.
- Glueck, C. J., J. Speirs, T. Tracy, P. Streicher, E. Illig, and J. Vandegrift. 1991. Relationships of serum plant sterols (phytosterols) and cholesterol in 595 hypercholesterolemic subjects, and familial aggregation of phytosterols, cholesterol, and premature coronary heart disease in hyperphytosterolemic probands and their first-degree relatives. *Metabolism.* **40**: 842–848.
- Li, J. H., A. B. Awad, C. S. Fink, Y. W. Wu, M. Trevisan, and P. Muti. 2001. Measurement variability of plasma beta-sitosterol and campesterol, two new biomarkers for cancer prevention. *Eur. J. Cancer Prev.* **10**: 245–249.
- Kesaniemi, Y., M. Koskenvuo, M. Vuoristo, and T. A. Miettinen. 1989. Biliary lipid composition in monozygotic and dizygotic pairs of twins. *Gut.* **30**: 1750–1756.
- Miettinen, T. A., R. S. Tilvis, and Y. A. Kesaniemi. 1989. Serum cholestanol and plant sterol levels in relation to cholesterol metabolism in middle-aged men. *Metabolism.* **38**: 136–140.
- Ewart, G. D., D. Cannell, G. B. Cox, and A. J. Howells. 2001. Mutational analysis of the traffic ATPase (ABC) transporters involved in uptake of eye pigment precursors in drosophila melanogaster. *J. Biol. Chem.* **269**: 10370–10377.