

GENETIC EPIDEMIOLOGY

Heritabilities, apolipoprotein E, and effects of inbreeding on plasma lipids in a genetically isolated population: The Erasmus Rucphen Family Study

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Abstract. Despite considerable progress in unraveling the genetic basis of dyslipidemias, most findings are based on families with extreme phenotypes. We studied lipid levels in an extended pedigree ascertained irrespective of phenotype from the population of a recent genetic isolate in the Netherlands. Heritabilities of plasma lipid measures were examined; this analysis also included estimates of the proportion of variance attributable to ApoE genotype. The association between inbreeding and lipids was also considered, as a substantial fraction of the population had known inbreeding. A total of 868 individuals from this pedigree, containing more than 60,000 people over 15 generations, were investigated in this study. Laboratory analysis of these subjects included the determination of fasting plasma lipids. ApoE ϵ 2/3/4 status was ascertained using TaqMan assays.

Heritabilities for plasma lipids were estimated with adjustments for multiple covariates using SOLAR. Heritabilities for total cholesterol (TC), high-density lipoprotein cholesterol (HDL), low-density lipoprotein cholesterol (LDL), triglycerides (TG), TC/HDL ratio, and TG/HDL ratio were found to be 0.35, 0.56, 0.30, 0.24, 0.49, and 0.39, respectively. The addition of ApoE genotype in the model significantly decreased these estimates ($\Delta h^2 = -0.030$, -0.004 , -0.054 , and -0.006 for TC, HDL, LDL, and TG). In a further analysis, TC and LDL were positively associated with the extent of inbreeding ($p_{\text{trend}} = 0.02$ and $p_{\text{trend}} = 0.05$, respectively). These data provide estimates of lipid heritability unbiased due to selection and suggest that this population represents a good opportunity to localize novel genes influencing plasma lipid levels.

Key words: ApoE, Cholesterol, Extended pedigree, HDL, Inbreeding, LDL, Triglycerides

Abbreviations: Δh^2 = change in heritability; ApoE = apolipoprotein E; BMI = body mass index; CHD = coronary heart disease; DBP = diastolic blood pressure; ERF = Erasmus Rucphen Family Study; h^2 = narrow-sense heritability; HDL = high-density lipoprotein cholesterol; HRT = hormone replacement therapy; LDL = low-density lipoprotein cholesterol; LLT = lipid lowering therapy; mmol/l = millimoles per liter; SBP = systolic blood pressure; SNP = single nucleotide polymorphism; TC = total cholesterol; TG = triglycerides

Introduction

Cardiovascular disease remains the leading cause of death in the United States, Europe, and portions of Asia [1]. High levels of total cholesterol (TC), triglycerides (TG) and low-density lipoprotein cholesterol (LDL) and low levels of high-density lipoprotein cholesterol (HDL) are well-established coronary heart disease (CHD) risk factors [2]. Plasma lipid levels themselves have emerged as important therapeutic targets, which can reduce the incidence of CHD [3, 4].

Detection of genetic variants leading to altered lipid profiles might prove useful in the diagnosis,

prevention, and treatment of disease. Heritabilities of various plasma lipids in other family-based studies have been previously reported to range from 0.39 to 0.62 for TC, 0.39 to 0.83 for HDL, 0.24 to 0.50 for LDL, 0.20 to 0.55 for TG and 0.34 for TG/HDL ratio [5–14]; even more extreme values were noted in recent twin studies [15, 16]. Several factors may explain the wide variation in these estimates. Until now, genetic studies have been performed in extended families selected on the basis of dyslipidemia, or in affected sib-pairs, usually with comparatively small sizes, which inflate heritability estimates. Furthermore, there is disagreement concerning whether

common traits are caused by numerous common genetic variants (the common disease/common variant theory) or by rare, population specific mutations. If the common disease/rare variant theory holds, specifically ascertained populations might have higher heritabilities than found in less selected populations. By contrast, we studied the heritability of plasma lipids in an extended family, derived from a genetically isolated population. This family was not selected based on the presence of dyslipidemia

The apolipoprotein E (ApoE) $\epsilon 2/3/4$ polymorphism, which results in three ApoE protein isoforms determined by missense mutations in the 112th and 158th amino acids, is one of the most well-described variations affecting plasma lipid values [17]. Differences in binding affinities between the three isoforms are dramatic, and lead to these observed differences in lipid levels [18]. In comparison to the referent ApoE $\epsilon 3/3$ group, the ApoE $\epsilon 4$ allele is strongly associated with increased TC, LDL, and TG, while the ApoE $\epsilon 2$ allele is associated with decreased TC and LDL and increased TG [19, 20]. The effects on HDL are less clear, perhaps context dependent, although $\epsilon 2$ is generally associated with increases, and $\epsilon 4$ with decreases, in HDL [21, 22]. ApoE was further associated with a variety of ailments, such as cardiovascular disease [23] and Alzheimer's Disease [24].

The availability of a genealogical database detailing this population allowed for the construction of a single, large (greater than 60,000 individual) pedigree. This information also enabled us to examine the effects of inbreeding on plasma lipid levels. Despite the anticipation of inbreeding effects on plasma lipids, there was no evidence of this in a prior study, [25] although studies of other cardiovascular disease phenotypes have shown that inbreeding may play a role [26, 27].

The aim of this study was to estimate the heritability of plasma lipids in a recent genetic isolate in the Netherlands and to evaluate the degree to which ApoE contributes to these estimates. A further aim was to evaluate the effect of inbreeding on lipid levels.

Methods

Study population

The present study was conducted in a large family-based cohort study (the Erasmus Rucphen Family [ERF] Study). The cohort was derived from a recent genetic isolate in the southwest Netherlands for which a comprehensive genealogical database exists. This population was founded in the middle of the 18th century by approximately 150 individuals and was isolated until the last few decades. Characterized by rapid growth and minimal immigration, the isolate now includes approximately 20,000 inhabitants. Twenty couples living in the region in the 19th century were chosen. These

couples parented a minimum of 6 children, each of whom was baptized between 1880 and 1900 in the community church. All living descendants of these pairs (as well as their spouses), ascertained on the basis of municipal and baptismal records, were traced and invited to participate ($n \approx 3,000$).

Subjects completed an interview with a physician and a thorough medical examination during a visit to the research center. The interview included questions concerning smoking (number of cigarettes per day) and alcohol consumption (grams per day) habits, lipid lowering and hormone replacement therapy, and diabetic medications. Participants were requested to bring the medications they use to the research center. All details pertaining to their prescriptions were discussed with a research physician. Height and weight data were collected and used to calculate body mass index. Blood pressure was measured twice on the right arm in a sitting position after at least 5 min rest, using an automated device (OMRON 711); the average of the two values was used for analysis.

All participants gave informed consent, and the Medical Ethical Committee of the Erasmus Medical Center approved the study protocol. The present study is based on 868 individuals, from an extended pedigree of almost 60,000 over 15 generations, for whom plasma lipid data and ApoE $\epsilon 2/3/4$ genotype was available at the time of analysis.

Laboratory analysis

Fasting blood samples were collected during the participant's visit to the research center. A Synchron LX20 (Beckman Coulter Inc., Fullerton, CA, U.S.A.) spectrophotometric chemical analyzer was utilized for the determination of plasma lipid values, as well as fasting plasma glucose levels. Diabetes mellitus was defined by one of two common criteria: use of glucose lowering medication or a plasma glucose level greater than 7.0 mmol/l [28].

Genomic DNA was extracted from whole blood samples drawn at the baseline examination, utilizing the salting out method [29]. Subjects were genotyped for the ApoE $\epsilon 2/3/4$ polymorphism with two TaqMan allelic discrimination Assays-By-Design (Applied Biosystems, Foster City, CA), targeting SNPs in the 112th (rs429358) and 158th (rs7412) amino acids of the ApoE gene [30]. Forward and reverse primer sequences for the position 112 polymorphism were 5'-GGGCGCGGACATGGA-3' and 5'-CCTCGCCGCGGTACTG-3', respectively. The minor groove binding probes were 5'-CGGCCGCGCACGT-VIC-3' and 5'-CGGCCGCA-CACGT-FAM-3'. For the SNP at position 158, forward and reverse primer sequences were 5'-TCCGCG-ATGCCGATGAC-3' and 5'-CCCCGGCCTGGTACAC-3', respectively. The minor groove binding probes were 5'-CAGGCGCTTCTGC-VIC-3' and 5'-CAG-GCACTTCTGC-FAM-3'. Both assays were designed using the reverse strand.

The assays utilized 5 ng of genomic DNA and 5 μ l reaction volumes. The amplification and extension protocol was as follows: an initial activation step of 10 min at 95 °C preceded 40 cycles of denaturation at 95 °C for 15 sec and annealing and extension at 50 °C for 60 sec. Allele-specific fluorescence was then analyzed on an ABI Prism 7900HT Sequence Detection System with SDS v 2.1 (Applied Biosystems, Foster City, CA).

Statistical analysis

Multiple linear regression models were fitted in SPSS 11.0 to examine the association of covariates with plasma lipids and to assess the distributional assumption of normality. For heritability estimation, covariates were chosen on the basis of statistical significance ($p < 0.10$). For these analyses, HDL, TG, TG/HDL, and TC/HDL were natural log transformed to yield normally distributed residuals. The normality of residuals was tested using a one-sample Kolmogorov-Smirnov test.

Estimates of heritability, utilizing a variance component approach based on maximum likelihood procedures, were calculated in SOLAR version 2.1.4 [31]. Heritability (h^2) is defined as the ratio of additive genetic variance to total phenotypic variance unexplained by covariates, and does not take into account epistasis or dominance. To determine the proportion of variance attributable to ApoE genotype, heritabilities were computed with and without genotypic data. ApoE $\epsilon 2/3/4$ status was coded using the 3/3 genotype as a baseline, with a separate variable coding for each of the other possible genotypes (2/2, 2/3, 2/4, 3/4, and 4/4). Comparison of the log likelihoods of these models allowed us to assess the significance of the differences. To evaluate the effect of including individuals receiving lipid-lowering therapy, heritabilities were also estimated excluding those individuals.

Inbreeding coefficients were calculated according to Meuwissen's method, as implemented in PEDIG [32]. Spearman's correlation coefficients were calculated for inbreeding versus the lipid outcomes. To predict the impact of these associations on lipid levels, a linear regression model assessing inbreeding coefficient quartile versus plasma lipid levels was realized. The quartiles were used due to the large skew in the distribution resulting from the many individuals who did not possess measurable inbreeding ($n = 200$). These analyses were executed utilizing SPSS 11.0.

Results

Approximately 3,000 individuals were invited to participate in the ERF study. At the time of genotyping and analysis, about 1,000 of these samples

were ready for use; after exclusion of those with missing information and/or genotype, 868 were included in the described study. Table 1 shows descriptive statistics for classical cardiovascular risk factors stratified by gender. Men had higher mean BMI and blood pressure (both systolic and diastolic). In this population, women smoked more often than men, while significantly more men consumed alcohol. HDL and TG levels were substantially different (as, consequently, were the TG/HDL and TC/HDL ratios). There were no significant differences, however, between the sexes with respect to TC or LDL. Men also received lipid-lowering therapy more frequently than women.

The mean levels of TC in this population were relatively high given the age distribution. Considering a clinical cut-off of 6.5 mmol/l to determine hyperlipidemia, the mean TC level was less than one standard deviation lower than this value. Approximately 20% of the population had TC levels greater than, or equal to, 6.5 mmol/l. This pattern was observed in both men and women.

The relationship between lipid levels and classical risk factors revealed that regression coefficients were of anticipated magnitudes and directions (Table 2). Heritability estimates were computed for all plasma lipid outcomes using two adjusted models (Table 3).

Table 1. Summary statistics in the ERF population

	Women (n = 517)	Men (n = 351)
Age (years)	51.00 \pm 14.51	53.80 \pm 13.52*
BMI (kg/m ²)	26.68 \pm 4.82	27.59 \pm 4.29*
Smoking% (n)	47.2 (244)	32.5 (114)*
Alcohol Use% (n)	33.7 (174)	69.8 (245)*
SBP (mm Hg)	138.82 \pm 22.76	144.30 \pm 19.44*
DBP (mm Hg)	78.98 \pm 9.62	82.09 \pm 10.21*
TC (mmol/l)	5.63 \pm 1.17	5.52 \pm 1.11
HDL (mmol/l)	1.37 \pm 0.36	1.13 \pm 0.31*
LDL (mmol/l)	3.72 \pm 1.04	3.73 \pm 0.98
TG (mmol/l)	1.28 \pm 0.63	1.58 \pm 1.00*
TC/HDL Ratio	4.32 \pm 1.19	5.16 \pm 1.41*
TG/HDL Ratio	1.03 \pm 0.67	1.58 \pm 1.27*
Diabetes mellitus% (n)	6.2 (32)	7.1 (25)
LLT% (n)	16.8 (87)	22.2 (78)*
HRT% (n)	3.3 (17)	NA
ApoE $\epsilon 2$ Frequency	0.05	0.05
ApoE $\epsilon 3$ Frequency	0.75	0.74
ApoE $\epsilon 4$ Frequency	0.20	0.20

BMI = Body Mass Index, SBP = Systolic Blood Pressure, DBP = Diastolic Blood Pressure, TC = Total Cholesterol, HDL = High-Density Lipoprotein Cholesterol, LDL = Low-Density Lipoprotein Cholesterol, TG = Triglycerides, LLT = Lipid Lowering Therapy, HRT = Hormone Replacement Therapy.

*Significantly different from women.

Values presented are mean \pm standard deviation for continuous traits and percent (number) for non-continuous traits.

Table 2. Coefficients and *p*-values for covariates by outcome measure

Covariate	TC		HDL		LDL		TG		TG/HDL Ratio		TC/HDL Ratio	
	β	<i>p</i> -value	β	<i>p</i> -value	β	<i>p</i> -value	β	<i>p</i> -value	β	<i>p</i> -value	β	<i>p</i> -value
Age	0.127	<0.001	0.032	0.026	0.106	<0.001	<i>0.049</i>	<i>0.101</i>	<i>0.016</i>	<i>0.665</i>	0.061	<0.001
Sex	-1.140	<0.001	<i>0.338</i>	<i>0.178</i>	-1.108	<0.001	<i>-0.566</i>	<i>0.267</i>	<i>-0.890</i>	<i>0.147</i>	-1.151	<0.001
Smoking	0.007	0.049	-0.015	<0.001	0.010	0.002	0.016	0.021	0.030	<0.001	0.020	<0.001
Alcohol	<i>-0.006</i>	<i>0.165</i>	0.010	0.006	-0.015	<0.001	0.025	0.001	<i>0.015</i>	<i>0.104</i>	-0.014	<0.001
Age*sex	-0.023	<0.001	-0.008	0.079	-0.019	<0.001	<i>-0.005</i>	<i>0.558</i>	<i>0.003</i>	<i>0.798</i>	-0.008	0.081
Age ²	-0.001	<0.001	<i>-0.000</i>	<i>0.206</i>	-0.001	<0.001	<i>-0.000</i>	<i>0.274</i>	<i>-0.000</i>	<i>0.713</i>	-0.000	0.006
DM	-0.248	0.083	-0.270	0.045	-0.338	0.008	0.615	0.022	0.889	0.006	<i>0.109</i>	<i>0.442</i>
Inbreeding	19.113	0.003	11.082	0.079	13.945	0.011	<i>13.978</i>	<i>0.215</i>	<i>4.549</i>	<i>0.751</i>	<i>2.706</i>	<i>0.676</i>
BMI	<i>-0.003</i>	<i>0.741</i>	-0.063	<0.001	<i>0.004</i>	<i>0.594</i>	0.105	<0.001	0.170	<0.001	0.060	<0.001
HRT	<i>-0.276</i>	<i>0.297</i>	<i>0.134</i>	<i>0.593</i>	<i>-0.328</i>	<i>0.162</i>	<i>-0.439</i>	<i>0.381</i>	<i>-0.576</i>	<i>0.341</i>	<i>-0.378</i>	<i>0.152</i>
LLT	-0.908	<0.001	-0.267	0.002	-0.914	<0.001	0.737	<0.001	0.973	<0.001	-0.425	<0.001

DM = Diabetes Mellitus, BMI = Body Mass Index, HRT = Hormone Replacement Therapy, LLT = Lipid Lowering Therapy.

Italicised covariates were excluded from the full model.

Table 3. Plasma Lipid Heritability Estimates in ERF

Outcome	Heritability (SEM)		Proportion of variance due to covariates		ApoE Genotype		
	Age & Sex adjusted	Full Model adjusted	Age & Sex adjusted	Full Model adjusted	Δh^2	Prop. of variance	<i>p</i> -Value
TC	0.19 (0.07)	0.35 (0.09)	0.02	0.16	-0.030	0.031	0.006
HDL	0.51 (0.08)	0.56 (0.08)	0.11	0.21	-0.004	0.011	0.014
LDL	0.17 (0.07)	0.30 (0.09)	0.02	0.19	-0.054	0.053	<0.001
TG	0.28 (0.07)	0.24 (0.07)	0.04	0.14	-0.006	0.008	0.021
TC/HDL Ratio	0.38 (0.07)	0.49 (0.08)	0.10	0.20	-	-	-
TG/HDL Ratio	0.37 (0.07)	0.39 (0.08)	0.08	0.17	-	-	-

TC = Total Cholesterol, HDL = High-Density Lipoprotein Cholesterol, LDL = Low-Density Lipoprotein Cholesterol, TG = Triglycerides, Δh^2 = Change in Heritability, Prop. of variance = Proportion of Variance Attributable to ApoE Genotype.

Full model controlled for age, sex, smoking, alcohol use, age*sex, age2, diabetes mellitus, inbreeding, BMI, hormone replacement therapy and lipid lowering therapy.

The full models included covariates (age, sex, age*sex, age², smoking status, alcohol consumption, lipid-lowering therapy, diabetes status, inbreeding coefficient, body mass index, and hormone replacement therapy) significant at a level of $p < 0.10$. The highest heritability estimates were found for HDL and TC, and, consequently, TC/HDL ratio. The lowest estimates were found for LDL and TG (Table 3). All of these estimates were highly significant; the *p*-values for the full model estimates for each outcome were found to be < 0.0002 .

Excluding individuals on lipid-lowering therapy did not dramatically affect these findings. Heritabilities increased somewhat for each outcome when these individuals were not included; these differences ranged from 0.0005 (for TC/HDL ratio) to 0.039 (for TC and HDL). These increases were of the anticipated magnitude and direction, given the random variance introduced by differing levels of efficacy, compliance, and dosage among treated individuals.

Inbreeding was a significant covariate in the heritabilities estimated for both TC and LDL (Table 2). In light of this result, the relationship between inbreeding and lipids was assessed. Spearman's correlation coefficients for TC, HDL, LDL, and TG (*p*-value) were 0.18 ($p < 0.001$), 0.04 ($p = 0.185$), 0.15 ($p < 0.001$), and 0.09 ($p = 0.006$), respectively. These associations, adjusted for numerous covariates, persisted for both TC and LDL when inbreeding quartiles were considered (Figure 1). TC ($p_{\text{trend}} = 0.02$) and LDL ($p_{\text{trend}} = 0.05$) increased significantly with level of inbreeding. HDL, while not significant, did indicate a tendency for more inbred individuals to have higher levels. In addition, inbred individuals received more frequent prescription of lipid-lowering therapy; 19.9% of the inbred group received such therapy, compared to 13.1% in the non-inbred group ($p = 0.03$).

ApoE genotype was successfully assessed in over 95% of subjects. Mendelian inconsistencies forced the removal of a few individuals/trios ($n = 7$). ApoE

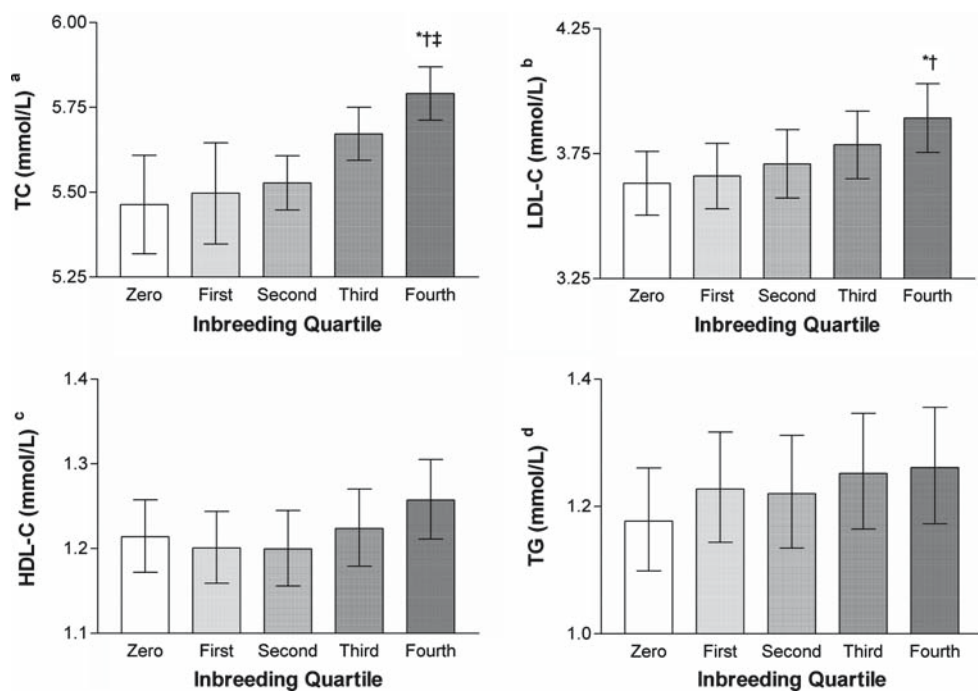


Figure 1. Plasma Lipid Means (95% CI) By Inbreeding Quartiles: All outcomes adjusted for age, sex, smoking, alcohol use, age*sex, age², diabetes mellitus status, hormone replacement therapy, and lipid lowering therapy. * Significantly different from zero inbreeding. † Significantly different from first quartile. ‡ Significantly different from second quartile. ^a p for trend = 0.02, ^b p for trend = 0.05, ^c p for trend = 0.41, ^d p for trend = 0.69.

genotype accounted for significant proportions of the heritabilities of all analysed outcomes (the ratios were not further analysed, as they did not produce informative results) (Table 3). Reductions of 0.030, 0.004, 0.054, and 0.006 were observed for TC, HDL, LDL, and TG, respectively. These values correspond to differences of 3.1%, 1.1%, 5.3%, and 0.8% of the total trait variance.

Discussion

In this large, family-based study in a non-phenotypically selected pedigree, all heritability estimates of fasting plasma lipids (TC, HDL, LDL, TG, TG/HDL ratio, and TC/HDL ratio) were highly significant. These estimates ranged from 0.24 for TG to 0.56 for HDL. The inclusion of ApoE genotype caused significant decreases in these estimates, ranging from 0.70% (HDL) to 17.72% (LDL). A further analysis of the association of inbreeding with plasma lipids revealed significant, if modest, correlations between inbreeding and TC, LDL, and TG. Significant trends of TC and LDL increasing with inbreeding quartile were observed. HDL levels also tended to increase with the extent of inbreeding, although this trend fell well short of the conventional significance level. To our knowledge, this is the first study to document an association between inbreeding and plasma lipids.

These estimates of heritability are in the range of previous estimates, although some publications reported heritabilities higher than those obtained in this study. Several factors may explain this. The simplest revolves around the use of different populations, which may have differing genetic contributions to lipid levels. Another is that prior estimates tended to be obtained from selected pedigrees (i.e., selected on the basis of disease status) [8], whereas this analysis features a “randomly” (i.e., non-phenotypically) selected pedigree. Furthermore, many previous reports have utilized multiple small pedigrees in their analysis [10]. The use of small pedigrees, with closely related individuals, tends to inflate heritability estimates compared to larger pedigrees containing distant relations [33]. This study, which used only one large pedigree, minimizes this problem by virtue of its design.

Although a shared household environment may influence plasma lipids through shared dietary and exercise habits, the homogeneity of the isolated population suggests that this is unlikely to appreciably alter these results. Furthermore, inclusion of BMI as a covariate served as a proxy for these two traits. BMI was significantly associated with all outcomes except TC and LDL; its presence in the model did not alter the significance levels of our estimates of heritability.

Dominance variance, which, in conjunction with additive and environmental variance, comprises broad sense heritability, was also not estimated in this study due to the computational problems involved. Dominance variance has been previously demonstrated to have very little influence on heritability estimates for HDL and TG levels, although it exerted a greater effect on LDL [34]. This would suggest that estimates for HDL and TG are likely accurate, and LDL might be somewhat underestimated in this study.

The ApoE analysis provides a reliable estimate of the proportion of trait variance due to ApoE ϵ 2/3/4 status and demonstrates the power of the ERF study design to detect variations that cause comparatively small changes in a given lipid outcome. The amount of LDL variation attributable to this very well-described variant corresponds well with a previous estimate [19], while the results for the other studied outcomes are also in line with expectations. The large amounts of residual heritability for all traits indicate that other genetic variations also play important roles in the determination of lipid levels in this population. These may include numerous polymorphisms with small effects, or gene-gene interactions, as well as genes with major effects (although these are likely to have been previously detected).

The increase of TC and LDL, and, to a lesser extent HDL, associated with inbreeding suggests that genetic factors, possibly recessive, have a substantial effect on plasma lipids in this population. The lack of clear statistical association between inbreeding and HDL levels may be a result of insufficient statistical power, since the variation in HDL was considerably smaller than for either TC or LDL. Inbreeding has been previously associated with cardiovascular disease, particularly hypertension [26], but not to lipid levels. Estimates that as many as 23% of amino acid mutations are somewhat deleterious, and present mostly in heterozygous individuals [35], suggests that consanguinity should increase homozygosity at these sites and lead to more pronounced effects in inbred populations.

Taken together, the heritability estimates (with and without ApoE) and the association of inbreeding with plasma lipids, offer strong evidence for the presence of genetic variants influencing lipid levels. It is likely that some deleterious recessive alleles occur with increased rates of homozygosity in this population, which ought to feature a smaller number of variants to begin with (due to the founder effect). ApoE does not account for most of the estimated additive genetic variance of these traits. Especially for HDL, with a high heritability estimate, and TC and LDL, with reasonably high heritabilities and an association with inbreeding, this population offers a good opportunity to discover genetic variants associated with these traits.

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