LCAT, HDL Cholesterol and Ischemic Cardiovascular Disease: A Mendelian Randomization Study of HDL Cholesterol in 54,500 Individuals

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Background: Epidemiologically, high-density lipoprotein (HDL) cholesterol levels associate inversely with risk of ischemic cardiovascular disease. Whether this is a causal relation is unclear.

Methods: We studied 10,281 participants in the Copenhagen City Heart Study (CCHS) and 50,523 participants in the Copenhagen General Population Study (CGPS), of which 991 and 1,693 participants, respectively, had developed myocardial infarction (MI) by August 2010. Participants in the CCHS were genotyped for all six variants identified by resequencing lecithin-cholesterol acyltransferase in 380 individuals. One variant, S208T (rs4986970, allele frequency 4%), associated with HDL cholesterol levels in both the CCHS and the CGPS was used to study causality of HDL cholesterol using instrumental variable analysis.

Results: Epidemiologically, in the CCHS, a 13% (0.21 mmol/liter) decrease in plasma HDL cholesterol levels was associated with an 18% increase in risk of MI. S208T associated with a 13% (0.21 mmol/liter) decrease in HDL cholesterol levels but not with increased risk of MI or other ischemic end points. The causal odds ratio for MI for a 50% reduction in plasma HDL cholesterol due to S208T genotype in both studies combined was 0.49 (0.11–2.16), whereas the hazard ratio for MI for a 50% reduction in plasma HDL cholesterol in the CCHS was 2.11 (1.70–2.62) ($P_{comparison} = 0.03$).

Conclusion: Low plasma HDL cholesterol levels robustly associated with increased risk of MI but genetically decreased HDL cholesterol did not. This may suggest that low HDL cholesterol levels *per se* do not cause MI. **(*J Clin Endocrinol Metab* 97: E248–E256, 2012)**

Epidemiologically, high-density lipoprotein (HDL) cholesterol levels associate inversely with risk of ischemic cardiovascular disease (1), an association that has led to the hypothesis that low levels of HDL cholesterol directly cause atherosclerosis. Whether this hypothesis is correct is intensively debated (2–8). Large homogeneous studies of the general population testing whether genetically altered HDL cholesterol levels associate with the expected cardiovascular risk may help to test causality and thus to evaluate HDL cholesterol raising as an antiatherogenic strategy.

A recent genome-wide association study (GWAS) including more than 100,000 individuals identified a single-nucleotide polymorphism (SNP) in the lecithin-cholesterol acyltranferase (*LCAT*) gene as the strongest marker of isolated variation in HDL cholesterol levels (9). This indicates that common variants in *LCAT* associated with levels of HDL cholesterol would be suitable instruments

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Abbreviations: ABCA1, ATP binding cassette transporter A1; APOA1, apolipoprotein A-I; CCHS, Copenhagen City Heart Study; CGPS, Copenhagen General Population Study; CI, confidence interval; GWAS, genome-wide association study; HDL, high-density lipoprotein; ICVD, ischemic cerebrovascular disease; IHD, ischemic heart disease; IS, ischemic stroke; LCAT, lecithin-cholesterol acyltranferase; LDL, low-density lipoprotein; MI, myocardial infarction; SNP, single-nucleotide polymorphism.

for a formal causality test of HDL cholesterol levels for cardiovascular risk using a Mendelian randomization approach. Under the assumptions of Mendelian randomization, the existence of a causal relationship between HDL cholesterol levels and ischemic cardiovascular disease would imply that associations between a gene variant and HDL cholesterol levels will translate into the risk of disease expected from the effect on HDL cholesterol (10). Monogenic HDL cholesterol deficiencies are caused by loss-of-function mutations in genes encoding apolipoprotein A-I (*APOA1*), ATP binding cassette transporter A1 (*ABCA1*), and *LCAT*. In contrast to *ABCA1* and *APOA1* heterozygotes (6, 11), *LCAT* heterozygotes have not been extensively studied in population-based settings (5). To clarify whether heterozygozity for loss-of-function mutations exist at a given frequency in the general population and to obtain an unbiased effect of genetic variation in *LCAT* on lipids and lipoprotein levels and on risk of ischemic cardiovascular disease (2), studies of the general population are needed.

Therefore, we tested the following hypotheses: 1) the genetic variation in *LCAT* affects the levels of lipids, lipoproteins, and apolipoproteins in the general population; and 2) genotypes that are associated with lifelong reductions in HDL cholesterol are associated with an increased risk of ischemic cardiovascular disease and in particular myocardial infarction (MI), suggesting direct causality of HDL cholesterol. These hypotheses were tested in the Copenhagen City Heart Study (CCHS; n = 10,281) and in the Copenhagen General Population Study (CGPS; n = 50,523).

Materials and Methods

Subjects

The studies were approved by the institutional review boards and Danish ethical committees (no. KF-100.2039/91, KF-01-144/01, H-KF-01-144/01) and conducted according to the Declaration of Helsinki. Informed consent was obtained from all participants. All the participants were white and of Danish descent. No participants appeared in more than one of the two studies, permitting independent confirmation of the findings in each group.

The Copenhagen City Heart Study

This is a prospective study of the general population initiated in 1976–1978 with follow-up examinations in 1981–1983, 1991–1994, and 2001–2003. Individuals were selected based on the national Danish Civil Registration System to reflect the adult Danish population aged 20–80+ yr. Data were obtained from a questionnaire, a physical examination, and blood samples. Blood samples for DNA extraction were available on 10,281 participants attending the 1991–1994 and/or 2001–2003 examinations.

The Copenhagen General Population Study

This is a prospective study initiated in 2003 with ongoing enrollment. For the present study, we used CGPS cross-sectionally to include all available events occurring from the establishment of the national Danish Patient Registry and national Danish Causes of Death Registry in 1976 to the end of follow-up in 2010. Participants were recruited from the general population and were examined as in the CCHS. At the time of genotyping, 50,523 had been included.

Gene resequencing

Genomic DNA was isolated from frozen whole blood of the participants in the CCHS with the 2% lowest (n = 190) and 2% highest (n = 190) HDL cholesterol levels for age and gender (QiaAmp4 DNA blood minikit; QIAGEN GmbH, Hilden, Germany). Seven PCR fragments were amplified covering 498 bp upstream of exon 1, all six exons of *LCAT*, and exon-intron boundaries (*LCAT* consensus sequence NC_000016.9). All PCR products were subsequently sequenced on an ABI 3730 DNA analyzer (Applied Biosystems Inc., Foster City, CA).

Genotyping

The ABI PRISM 7900HT sequence detection system (Applied Biosystems) was used to genotype the CCHS for all six *LCAT* variants identified by resequencing [g.-293G>A, g.-249A>G, g.-128G>A, IVS2-10delC, S208T (rs4986970), and L369L (rs5923)], and the CGPS for the common genetic variant S208T with effect on HDL cholesterol levels. TaqMan-based assays were used.

Lipids, lipoproteins, and apolipoproteins

Colorimetric and turbidimetric assays were used to measure HDL cholesterol, total cholesterol, nonfasting plasma levels of triglycerides, apolipoprotein A-I, and apolipoprotein B (Roche Molecular Biochemicals, Mannheim, Germany, and Konelab, Helsinki, Finland). Low-density lipoprotein (LDL) cholesterol was calculated using the Friedewald equation (12) when plasma triglycerides were less than 4.00 mmol/liter (<354.00 mg/dl), and otherwise measured directly (Thermo Fisher Scientific, Waltham, MA).

Events

Information on diagnoses of ischemic heart disease (IHD) (World Health Organization; International Classification of Disease, 8th edition, p. 410–414; 10th edition, p. I20-I25) was collected and verified by reviewing all hospital admissions and diagnoses entered in the national Danish Patient Registry and all causes of death entered in the national Danish Causes of Death Registry. IHD was fatal or nonfatal MI or characteristic symptoms of stable angina pectoris, including revascularization procedures (13). A diagnosis of MI followed the changing definitions over time. After year 2000, the diagnosis was based on either one of the following: 1) typical rise and fall of biochemical markers of myocardial necrosis [troponin or creatine kinase muscle brain fraction (CK-MB)] with at least one of the following: ischemic symptoms, development of pathological Q waves on the electrocardiogram, or electrocardiogram changes indicative of ischemia or coronary artery intervention; or 2) pathological findings of an acute, healed or healing MI (14).

Potential cases with ischemic cerebrovascular disease (ICVD) including ischemic stroke (IS) were gathered from the national

Danish Patient Registry and the national Danish Causes of Death Registry (World Health Organization; International Classification of Diseases, 8th edition, p. 431–438; 10th edition, p. I60-I69, G45). In the CCHS, for each person registered with ICVD, hospital records were requested. Experienced neurologists reviewed and validated all potential cases as described previously (15). The diagnostic criteria for ICVD were IS, transient ischemic attack (focal neurological symptoms lasting less than 24 h), or amaurosis fugax (transient blindness on one eye only).

Follow-up ended in August 2010 and was 100% complete, *i.e.* no individual was lost to follow-up in either study.

Other covariates

Diabetes mellitus, hypertension, and smoking were dichotomized and defined as diabetics (self-reported disease, use of antidiabetic medication, nonfasting plasma glucose >11.00 mmol/liter), hypertension (systolic blood pressure ≥140 mm Hg or diastolic blood pressure ≥90 mm Hg and/or use of antihypertensive drugs), and smokers as current smoking. Body mass index was measured weight (kilograms) divided by measured height squared (square meters). Use of lipid-lowering therapy was self-reported.

Statistical analysis

Data were analyzed using Stata/SE statistical software (version 10.1; StataCorp, College Station, TX). Two-sided $P < 0.05$ was considered significant. A Mann-Whitney *U* test and a Pearson χ^2 test were used for two-group comparisons for continuous and categorical variables, respectively. For trend tests, groups of individuals were classified by *LCAT* genotypes and ranked 0, 1, and 2 with 0 (noncarriers) as the reference group. Power calculations assuming one-sided $P < 0.05$ were performed using NCSS 2001 and PASS 2000 software (NCSS, Kaysville, UT).

To test our first hypothesis that the common genetic variation in *LCAT* (g.-293G>A, S208T, and L369L) affected levels of lipids, lipoproteins, and apolipoproteins in the general population, we used nonparametric Mann-Whitney *U* and trend tests across genotypes with two and three groups, respectively.

To test our second hypothesis that the genotypes associated with lifelong reductions in HDL cholesterol are associated with a risk of ischemic cardiovascular disease in the general population, we used Cox proportional hazards regression models, with age as the time scale and the use of left truncation (delayed entry), to estimate the hazard ratios of IHD, MI, ICVD, and IS in the prospective CCHS; individuals diagnosed with an end point before entry were excluded from the Cox regression analyses, and those dying during follow-up were censored at their death date. In the cross-sectional CGPS and in both studies combined, logistic regression analysis was used to estimate odds ratios. Multifactorial adjustment was for age, gender, hypertension, diabetes mellitus, and smoking. To test causality directly, we first examined whether S208T associated with the risk of MI, the most exact diagnosis of ICVD, to the extent predicted by its effect on HDL cholesterol levels. We used the observed increases in hazard ratios for MI associated with a 1-mmol decrease in HDL cholesterol levels in the CCHS to predict hazard ratios for MI. The predicted hazard ratios as a function of HDL cholesterol levels were corrected for regression dilution bias (16). Second, instrumental variable analysis by two-stage least squares regression was used to assess the potential causal relationship between the decreased HDL cholesterol levels and the increased risk of MI using HDL cholesterol-associated genotypes as instruments for decreased levels of HDL cholesterol in an additive model. Strengths of the instrument (association of genotype with plasma HDL cholesterol) were evaluated by F statistics from the first-stage regression, in which F greater than 10 indicates sufficient strength to ensure the validity of the instrument variable analysis, whereas R^2 in percent is used as a measure of the percent contribution of genotype to the variation in HDL cholesterol levels (17). The risk associated with a 50% reduction in HDL cholesterol levels (observational or genetic) was calculated using the logarithms of HDL cholesterol to base 2 in regression models, multiplying the observational regression coefficient by −1, and exponentiating them to give the hazard/odds ratios. We used Altman's method (18) to compare the causal estimate from the instrumental variable analysis with the observational increased risk of MI from the conventional epidemiology. As we *a priori* tested one hypothesis, we used a one-sided test.

Results

Characteristics of participants and resequencing of *LCAT*

Characteristics of subjects in the CCHS and in the CGPS without any ischemic events, with IHD, or with ICVD are shown in Table 1. Individuals with IHD and ICVD were older and more often men, had lower HDL cholesterol and apolipoprotein A-I levels, and higher triglyceride levels, were more obese, were more often on lipid-lowering therapy, and more often had hypertension or diabetes compared with control subjects. In the CGPS, 80% of IHD and ICVD patients were on lipid-lowering therapy, resulting in lower levels of total cholesterol, LDL cholesterol, and apolipoprotein B compared with all other groups.

Resequencing of the regulatory and coding regions of *LCAT* in individuals from the CCHS with the 2% lowest (n = 180) and 2% highest (n = 180) plasma HDL cholesterol levels identified six genetic variants [Table 2 (19–21)]. All six variants were genotyped in the CCHS, three were rare (g.-249A>G, g.-128G>A, and IVS2-10delC, allele frequency 0.01–0.03%), and three were relatively common (g.-293G>A, S208T, L369L, allele frequency 0.3–5%) (Table 2). None of the common variants were in linkage disequilibrium (R^2 0–0.2%). The common variant associated with HDL cholesterol levels, S208T, was further genotyped in the CGPS. Genotype frequencies did not differ from those predicted by the Hardy-Weinberg equilibrium ($P = 0.24–0.99$).

Genetic variation in *LCAT* and plasma levels of lipids, lipoproteins, and apolipoproteins

Lipids, lipoproteins, and apolipoproteins for the common variants, g.-293G>A, S208T, and L369L, are presented in Fig. 1. Compared with the corresponding non-

TABLE 1. Characteristics of participants by disease status in the Copenhagen City Heart Study and the Copenhagen General Population Study

	Copenhagen City Heart Study			Copenhagen General Population Study		
	No event	**IHD**	**ICVD**	**No event**	**IHD**	**ICVD**
N	7,532	2,090	1,067	44,378	4,105	2,650
Women (%)	58	48[a]	52[a]	57	39[a]	47[a]
Age (yr)	54 (39–66)	68 (60–74)[a]	68 (62–74)[a]	55 (46–65)	67 (60–76)[a]	70 (61–78)[a]
HDL cholesterol (mmol/liter)	1.5 (1.2–1.9)	1.4 (1.1–1.7)[a]	1.5 (1.2–1.9)[a]	1.6 (1.3–2.0)	1.5 (1.2–1.8)[a]	1.6 (1.2–1.9)[b]
Apolipoprotein A-I (mg/dl)	142 (124–162)	135 (118–156)[a]	139 (121–161)	156 (139–177)	151 (133–170)[a]	155 (137–175)[a]
Total cholesterol (mmol/liter)	5.8 (5.0–6.7)	6.4 (5.6–7.3)[a]	6.4 (5.6–7.2)[a]	5.6 (4.9–6.3)	5.3 (4.5–6.1)[a]	5.4 (4.6–6.2)[a]
LDL cholesterol (mmol/liter)	3.5 (2.8–4.3)	4.0 (3.2–4.8)[a]	3.9 (3.2–4.7)[a]	3.2 (2.6–3.9)	2.9 (2.2–3.6)[a]	3.0 (2.3–3.7)[a]
Apolipoprotein B (mg/dl)	83 (69–100)	93 (79–110)[a]	93 (79–109)[a]	107 (88–131)	107 (86–131)[b]	106 (86–129)[a]
Triglycerides (mmol/liter)	1.4 (1.0–2.0)	1.8 (1.3–2.6)[a]	1.8 (1.3–2.5)[a]	1.4 (1.0–2.1)	1.6 (1.1–2.4)[a]	1.5 (1.1–2.2)[a]
Body mass index (kg/m^2)	24 (22–27)	26 (24–29)[a]	26 (24–29)[a]	25 (23–28)	27 (24–30)[a]	26 (24–29)[a]
Lipid-lowering therapy (%)	0	3[a]	2[a]	6	41[a]	33[a]
Hypertension (%)	44	72[a]	76[a]	58	80[a]	80[a]
Smoking (%)	46	49	46	22	23	25[a]
Diabetes mellitus (%)	3	8[a]	9[a]	3	11[a]	9[a]

Values are median (interquartile range) or percentage. A Mann-Whitney *U* test or Pearson χ^2 test was used for continuous and categorical traits, respectively. The risk factors, hypertension, smoking, and diabetes mellitus, were dichotomized and defined as diabetes (self-reported disease, use of antidiabetic medication, and/or nonfasting plasma glucose >11.0 mmol/liter), hypertension (systolic blood pressure ≥140 mm Hg or diastolic blood pressure ≥90 mm Hg and/or use of antihypertensive therapy), and smokers as current smokers.

[a] $P < 0.01$.

[b] $P < 0.05$.

carriers, S208T homozygotes had 0.21 mmol/liter (*P* for trend = 0.004) and 14 mg/dl (*P* for trend = 0.006) reductions in plasma HDL cholesterol and apolipoprotein A-I levels in the CCHS, respectively (Fig. 1, *upper left panels*). Similar effects were seen in the CGPS (*P* for trend <0.001) (Fig. 1, *upper right panels*). No other associations between g.-293G>A, S208T, and L369L and lipids, lipoproteins, and apolipoproteins or between S208T and biochemical

TABLE 2. Genetic variation in regulatory and coding regions of the *LCAT* gene in individuals with the lowest 2% and highest 2% HDL cholesterol levels among 10,281 participants in the Copenhagen City Heart Study

			No. of alleles (allele frequency)					
Gene region	**Nucleotide substitution[a]**	**Amino acid residue[b]**	**Low HDL-C (n = 380)**	**High HDL-C (n = 380)**	**CCHS (n = 20,562)**	***P* value**	**Functional region**	**Previous reports/ rs no.**
LCAT								
Promoter	−293 G>A	—	2	3	65 (0.003)	1.00	−242 bp from TATA-box[c]; −201 bp from element B[d]	New
Promoter	−249 A>G	—	0	1	2	1.00	−198 bp from TATA-box[c]; −157 bp from element B[d]	New
Promoter	−128 G>A	—	1	0	4	1.00	−77 bp from TATA-box[c]; −36 bp from element B[d]	New
Intron 2	IVS2-10 del C	—	0	1	6	1.00	−10 bp from intron-exon boundary	New
Exon 5	694 T>A	S208T	12 (0.03)	7 (0.02)	776 (0.04)	0.35	Near N228[e]	rs4986970
Exon 6	1177 C>T	L369 L	25 (0.07)	21 (0.06)	1045 (0.05)	0.65	Near catalytic residues[f]	rs5923

For *P* values, Fisher's exact test evaluated genotype frequencies in low *vs.* high HDL cholesterol groups. HDL-C, HDL cholesterol.

[a] Nucleotide 1 denotes A in the start codon ATG in exon 1. A *dash* in front of the promoter variants indicates the number of nucleotides 5′ upstream of the start codon ATG.

[b] Residue 1 denotes the first amino acid in the mature LCAT protein, excluding a signal peptide of 24 residues. A *dash* indicates that genetic variants outside the translated region do not affect an amino acid residue.

[c] The closest resemblance to a TATA box is the sequence GATAA 51 bp upstream of the ATG site (19).

[d] Elements A (−46 to −74) and B (−76 to −92) bind transcription factors Sp1 and Sp3, respectively (20).

[e] N228 has been proposed to be one of the residues involved in apolipoprotein A-I activation in addition to residues P10 and T123 (21).

[f] S181, D345, and H377 are proposed to be the catalytic residues (21).

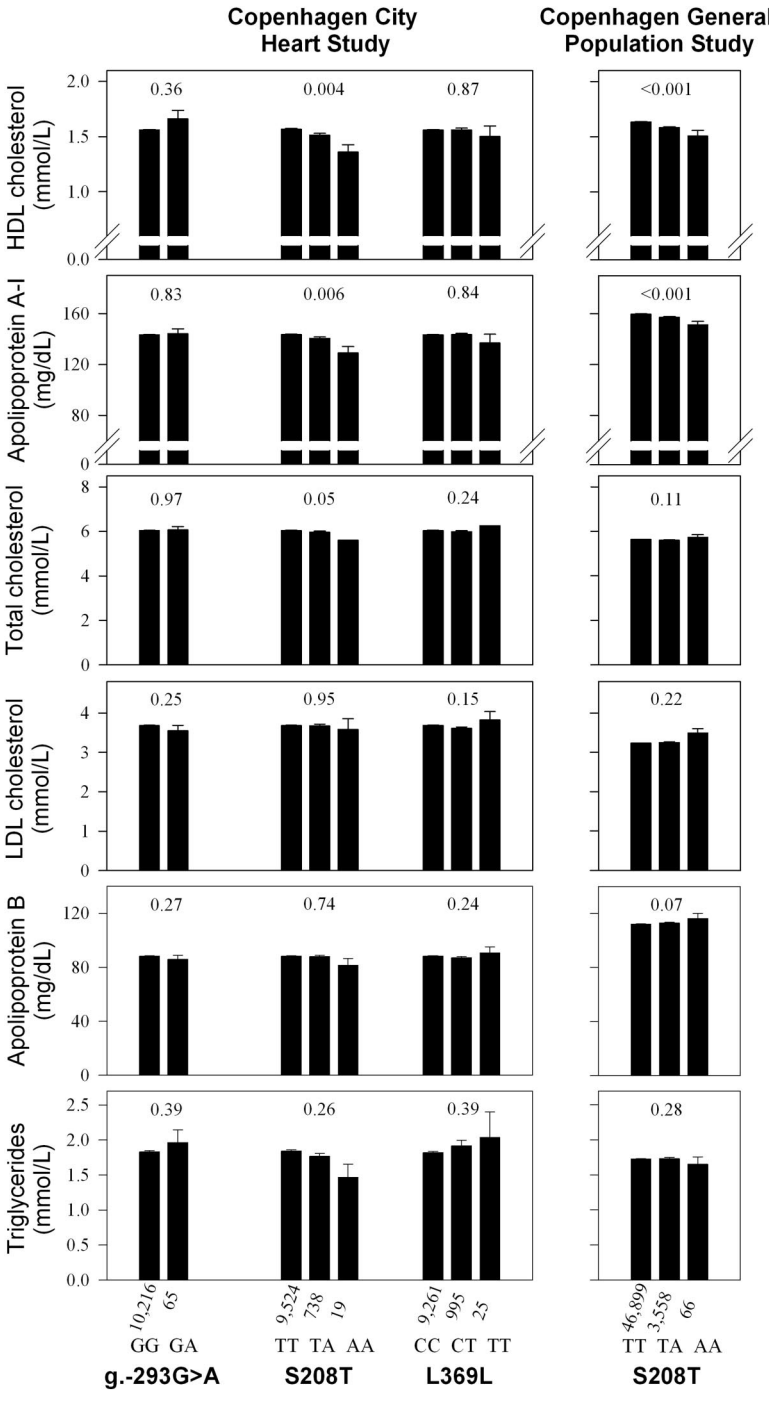


FIG. 1. Mean plasma lipid and lipoprotein levels as a function of the common genetic variants in *LCAT*, g.-293G>A, S208T, and L369L in the CCHS and S208T in the CGPS. Values shown are mean ± SEM. The *P* values are by a nonparametric Mann-Whitney *U* test and by a trend test across genotypes for two- and three-group comparisons, respectively.

markers of inflammation, glucose metabolism, and kidney disease were observed (Fig. 1 and Supplemental Table 1, published on The Endocrine Society's Journals Online web site at http://jcem.endojournals.org).

One of two g.-249A>G heterozygotes and five of six IVS2-10delC heterozygotes had HDL cholesterol and apolipoprotein A-I levels above the 50% percentile, whereas three of four g.-128G>A heterozygotes had HDL cholesterol and apolipoprotein A-I levels below the 50% percentile for age and gender (Supplemental Fig. 1, *red dots*). No other associations between g.-249A>G, g.-128G>A, and IVS-10delC and lipids, lipoproteins, and apolipoproteins were observed (Supplemental Fig. 1).

Common genetic variation in *LCAT* and risk of IHD, MI, ICVD, and IS

We determined the risk of ischemic cardiovascular disease for the S208T variant, which robustly associated with reduced HDL cholesterol levels, in both the CCHS and CGPS. The hazard and odds ratios for IHD, MI, ICVD, or IS (multifactorially adjusted) did not differ significantly from 1.0 for any of the individual genotypes (Fig. 2). All the risk estimates adjusted for age and gender were similar only to multifactorially adjusted hazard and odds ratios. We had 80% statistical power to exclude odds ratios of 1.1 or more for S208T heterozygotes and 1.9 or more for S208T homozygotes for any cardiovascular event in the two studies combined.

Potential causal effect of HDL cholesterol levels on the risk of MI

First, assuming that reduced HDL cholesterol levels have a causal effect on the risk of MI, genetically reduced HDL cholesterol levels should confer a similar increase in the risk of MI as that observed for reduced HDL cholesterol levels in the general population. For example, the reduction of 0.21 mmol/liter (13%) in HDL cholesterol observed in S208T homozygotes (Fig. 3, *left panel*) would theoretically predict an 18% increase in risk of MI [hazard ratio 1.18; 95% confidence interval (CI) 1.12–1.24, multifactorially adjusted] (Fig. 3, *middle panel*). However, the observed risk of MI as a function of the S208T genotype in the combined study of 54,551 individuals did not differ significantly from 1.0 (*P* for trend = 0.52). The risk estimates adjusted for age and gender only were similar to multifactorially adjusted hazard and odds ratios.

Second, we also examined a potential causal effect of decreased HDL cholesterol levels on increased risk of MI using instrumental variable analysis by generalized least squares regression. A 50% reduction in HDL cholesterol

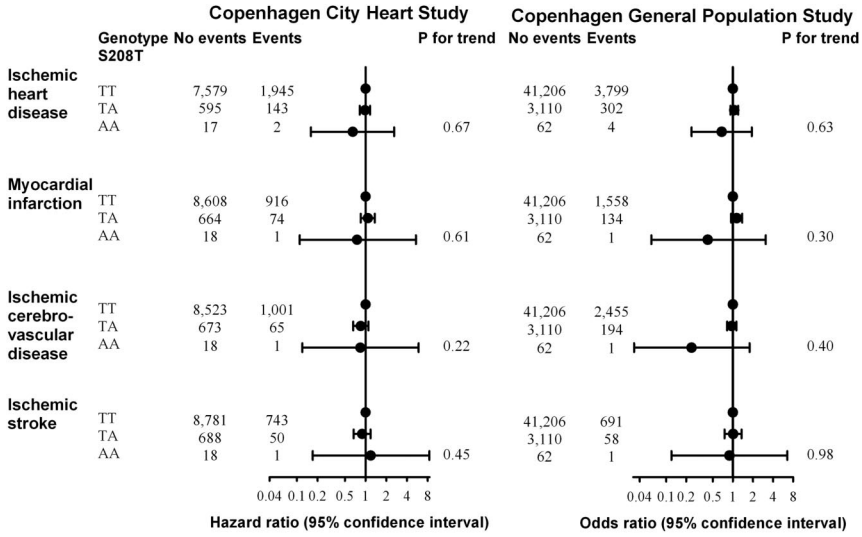


FIG. 2. Risk of IHD, MI, ICVD, and IS as a function of *LCAT* S208T genotype in the CCHS and the CGPS. Hazard ratios and odds ratios were multifactorially adjusted for age, sex, diabetes, smoking, and hypertension. *P* values are from Cox regression trend test.

levels due to the S208T genotype in the combined study associated with a causal odds ratio of 0.49 (95% CI 0.11–2.16, genetic/instrumental variable estimate), whereas the observational hazard ratio associated with a 50% reduction of HDL cholesterol levels of 2.11 (95% CI 1.70–2.62, observational/epidemiological estimate) in the CCHS (observational *vs.* instrumental hazard/odds ratio, $P = 0.03$; Fig. 4). The strength of S208T as an instrumental variable was F = 18 [F statistics >10 indicates sufficient strength of the instrument (17)].

Discussion

The principal findings of the present study are that common genetic variation in *LCAT* associated with decreased HDL cholesterol levels did not associate with increased risk of ICVD. Low HDL cholesterol levels robustly associated with an increased risk of MI; however, genetically decreased HDL cholesterol did not. This may suggest that HDL cholesterol levels are not causally related to risk of MI. Furthermore, loss-of-function mutations in *LCAT* appear to be very rare in the general population, in contrast to HDL deficiency caused by mutations in *ABCA1* and *APOA1*. These findings were observed in two large studies of the general population comprising more than 60,000 individuals. Because we studied Caucasians, the current findings may not be applicable to other ethnic groups.

Candidate gene studies of common variants associated with HDL cholesterol levels but with no other lipids and lipoproteins [*APOA1*, hepatic lipase (*LIPC*), and endothelial lipase (*LIPG*)] do not support that genetic effects on plasma levels of HDL cholesterol *per se* associate with the expected risk of cardiovascular disease (8, 22–25). Additionally, the GWAS of more than 100,000 individuals show that only genetic variants with effects on atherogenic lipids and lipoproteins [LDL cholesterol, apolipoprotein B, triglyceride rich lipoproteins, and lipoprotein(a)] are consistently associated with cardiovascular risk, whereas genetic variants with isolated effects on HDL cholesterol are not (9). The present data on *LCAT* support these findings by contributing with a formal statistical test of causality, suggesting that plasma levels of HDL cholesterol do not cause MI, the most exact diagnosis of ischemic cardiovascular disease. Because the data from epidemiological studies of the inverse relationship between low HDL cholesterol levels and risk of ischemic cardiovascular disease is confounded by high triglycerides, marking the presence of atherogenic remnant lipoproteins (26–30) and because increasing genetic evidence that an isolated increase or decrease in HDL cholesterol does not translate into the expected risk (8, 9), causality for HDL cholesterol levels is at present not sup-

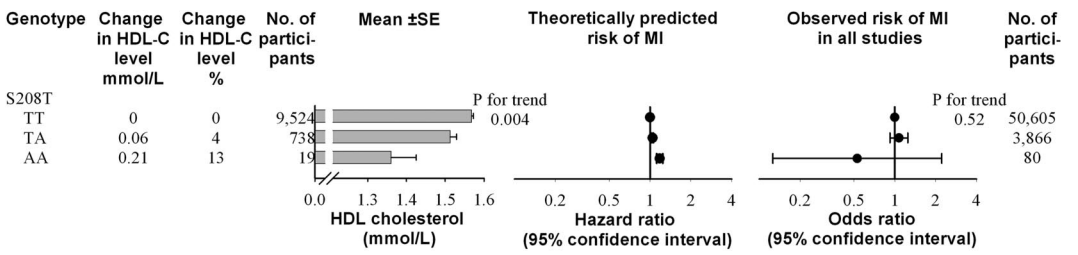
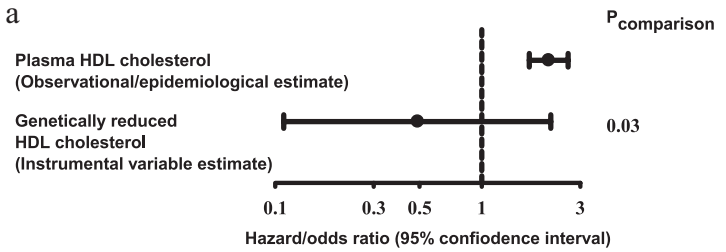


FIG. 3. Effect of *LCAT* S208T genotype on HDL cholesterol levels and theoretically predicted and observed risks of MI. Differences in plasma levels of HDL cholesterol in the CCHS are shown as a function of *LCAT* S208T genotype as change in millimoles per liter and percentage and as mean ± SE, with noncarriers as the reference group (*left panel*). Theoretically predicted risks of MI as a function of change in HDL cholesterol in the CCHS are shown as hazard ratios as a function of *LCAT* genotypes (*middle panel*). Observed risks of MI in the combined study according to *LCAT* genotypes are shown as odds ratios (*right panel*). Hazard ratios and odds ratios were multifactorially adjusted for age, sex, diabetes, smoking, and hypertension. *P* values are from nonparametric and Cox regression trend tests. HDL-C, HDL cholesterol.

a



b

Model	F	R^2	Hazard/odds ratio (95% CI) for MI for a 50% reduction in HDL cholesterol levels	P	$P_{comparison}$ observational versus instrumental
Observational/epidemiological estimate	-	-	2.11 (1.70-2.62)	<0.001	-
Genetic/instrumental variable estimate					
LCAT S208T	18	0.1%	0.49 (0.11-2.16)	0.35	0.03

FIG. 4. Study summary of the causal effect of reduced HDL cholesterol on the increased risk of MI. This was tested in 54,551 individuals from the general population, the CCHS and the CGPS combined. The causal effect of reduced HDL cholesterol on the risk of MI was estimated by the association between the genetically reduced HDL cholesterol and the risk of MI using instrumental variable analysis by two-stage least squares regression and given as an odds ratio with 95% confidence interval (A and B, lower estimate). This risk is compared with the observed epidemiologically increased risk of MI associated with reduced HDL cholesterol levels in the general population, the CCHS, given as a hazard ratio with 95% confidence interval (A and B, upper estimate). F statistics (evaluation of strength of instrument) and R^2 (contribution of genotype to variation in HDL cholesterol levels in percent) are from the first-stage regression analysis. The $P_{comparison}$ value is between the observational estimate from conventional epidemiology and the causal estimate from instrumental variable analysis.

ported. Taken together, these data do not favor isolated HDL cholesterol raising as an attractive antiatherogenic strategy.

Monogenic isolated HDL deficiencies with large reductions in levels of HDL cholesterol are not in general associated with increased risk of ischemic cardiovascular disease (2, 5). This has convincingly been shown for heterozygotes of loss-of-function mutations in *ABCA1* and *APOA1* (6, 31) but remains an unanswered question for *LCAT* heterozygotes (32, 33). In a Dutch study, 47 heterozygotes for *LCAT* mutations presented with a mean 36% decrease in HDL cholesterol levels, a 23% increase in triglyceride levels, a 2.1-fold increase in C-reactive protein levels, and a 5.4% increased mean carotid intima-media thickness compared with family controls (32). In an Italian study, 28 heterozygotes and 12 homozygotes for *LCAT* mutations presented with mean 33 and 84% decreases in HDL cholesterol levels, 29 and 145% increases in triglyceride levels, but unexpectedly with 12 and 20% decreases in mean carotid intima-media thickness (33). A way to obtain an unbiased estimate of the association between heterozygosity for loss-of-function mutations in *LCAT* and risk of ischemic cardiovascular disease is to examine heterozygous carriers identified in the general population prospectively (2). Despite the use of a stringent extreme group resequencing strategy, the present large population study was unfortunately unable to answer this question because no carriers of loss-of-function mutations in *LCAT* were identified, suggesting that *LCAT* mutations associated with low HDL cholesterol are exceedingly rare in the general population, as also observed earlier in a smaller population-based study (34).

Two groups have independently shown that rare alleles of HDL genes are differentially distributed in the extreme tails of the HDL cholesterol distribution in the general population (34, 35). In the CCHS, 6% of Caucasians with the lowest 1% HDL cholesterol levels for age and gender were heterozygous for loss-of-function mutations in *ABCA1* (35), and results were similar in the Dallas Heart Study in different racial groups (34). In the latter study, *APOA1* and *LCAT* were also resequenced, and one heterozygous *APOA1* mutation carrier was identified (0.5% in the low HDL cholesterol group) but no carriers of *LCAT* mutations. Recently Haase *et al.* (23) showed that by using the direct gene product of *APOA1*, apolipoprotein A-I in plasma, 3% of Caucasians with the lowest 1% apolipoprotein A-I levels for age and gender in the general population were heterozygous for loss-of-function mutations in *APOA1*. This suggests that proximity between gene and gene product enhances the chance of detecting loss-of-function mutations in extreme group resequencing. Therefore, we cannot exclude that a few mutation carriers have remained undetected because we used HDL cholesterol levels as the extreme phenotype instead of the direct gene product, levels of LCAT. However, because we know from studies of LCAT-deficient patients that LCAT levels and HDL cholesterol levels correlate well (36–38), this is not likely to have confounded our data. Thus, by using a systematic screening approach of extreme levels of HDL cholesterol in the general population samples, previous data (34) as well as the present data suggest that loss-of-function mutations in *LCAT* are exceedingly rare in the general population.

In a Mendelian randomization approach, naturally occurring genetic variation can be used as instruments to assess causality provided that several requirements are satisfied (10). First, suitable genetic variants for the study of the intermediate phenotype of interest (*in casu* plasma HDL cholesterol levels) need to be identified. The first stage of the instrumental variable analysis ensured that the

S208T SNP had sufficient strength to be used as the genetic instrument [F = 18, F > 10 ensures sufficient strength (17)]. Also, a recent GWAS including more than 100,000 individuals identified a SNP in *LCAT* as the strongest marker of isolated alterations in HDL cholesterol levels (9), supporting that common genetic variants in *LCAT* are suitable instruments to use in Mendelian randomization studies of HDL cholesterol. Second, reliable genotype-to-intermediate phenotype and genotype-to-disease associations need to be established. These associations were obtained in two studies including more than 60,000 individuals. Third, the selected genetic instrument must not display pleiotropic effects. We cannot exclude all potential pleiotropic effects because it is likely that other alternative pathways exist (other than the HDL cholesterol level) for LCAT to exert a potential effect on cardiovascular disease (39, 40). *Ex vivo* studies of normal healthy volunteers (40) and carriers of loss-of-function mutations in *LCAT* (39) suggest that LCAT exerts antioxidant functions independent of the HDL cholesterol level. Whether such *ex vivo* findings have an impact in humans *in vivo* remains to be determined. We have, however, not identified any associations between S208T genotype and other lipids, lipoproteins, and apolipoproteins or any well-known cardiovascular risk factors or any available biochemical quantities involved in inflammation, glucose metabolism, and kidney disease in approximately 60,000 individuals. This supports that S208T in *LCAT* has an isolated HDL phenotype in humans and thus is a suitable instrument to use in a Mendelian randomization design.

In conclusion, low plasma HDL cholesterol levels robustly associated with an increased risk of MI, but genetically decreased HDL cholesterol due to *LCAT* variation did not. This adds to the emerging genetic evidence that the inverse relation between HDL cholesterol levels and ICVD observed in epidemiological studies may not be causal. Finally, loss-of-function mutations in *LCAT* are rare in the general population.

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References

1. **Emerging Risk Factors Collaboration, Di Angelantonio E, Sarwar N, Perry P, Kaptoge S, Ray KK, Thompson A, Wood AM, Lewington S, Sattar N, Packard CJ, Collins R, Thompson SG, Danesh J** 2009 Major lipids, apolipoproteins, and risk of vascular disease. JAMA 302:1993–2000
2. **Vergeer M, Holleboom AG, Kastelein JJ, Kuivenhoven JA** 2010 The HDL hypothesis: does high-density lipoprotein protect from atherosclerosis? J Lipid Res 51:2058–2073
3. **Hausenloy DJ, Yellon DM** 2008 Targeting residual cardiovascular risk: raising high-density lipoprotein cholesterol levels. Heart 94: 706–714
4. **Wild S, Byrne CD** 2008 Time to rethink high-density lipoprotein? Heart 94:692–694
5. **Rader DJ** 2009 Lecithin:cholesterol acyltransferase and atherosclerosis: another high-density lipoprotein story that doesn't quite follow the script. Circulation 120:549–552
6. **Frikke-Schmidt R, Nordestgaard BG, Stene MC Sethi AA, Remaley AT, Schnohr P, Grande P, Tybjaerg-Hansen A** 2008 Association of loss-of-function mutations in the *ABCA1* gene with high-density lipoprotein cholesterol levels and risk of ischemic heart disease. JAMA 299:2524–2532
7. **Frikke-Schmidt R** 2010 Genetic variation in the *ABCA1* gene, HDL cholesterol, and risk of ischemic heart disease in the general population. Atherosclerosis 208:305–316
8. **Nordestgaard BG, Tybjærg-Hansen A** 2011 Genetic determinants of LDL, lipoprotein(a), triglyceride-rich lipoproteins and HDL: concordance and discordance with cardiovascular disease risk. Curr Opin Lipidol 22:113–122
9. **Teslovich TM, Musunuru K, Smith AV, Edmondson AC, Stylianou IM, Koseki M, Pirruccello JP, Ripatti S, Chasman DI, Willer CJ, Johansen CT, Fouchier SW, Isaacs A, Peloso GM, Barbalic M, Ricketts SL, Bis JC, Aulchenko YS, Thorleifsson G, Feitosa MF, Chambers J, Orho-Melander M, Melander O, Johnson T, Li X, *et al.*** 2010 Biological, clinical and population relevance of 95 loci for blood lipids. Nature 466:707–713
10. **Davey Smith G, Ebrahim S** 2003 "Mendelian randomization": can genetic epidemiology contribute to understanding environmental determinants of disease? Int J Epidemiol 32:1–22
11. **Haase CL, Frikke-Schmidt R, Nordestgaard BG, Kateifides AK, Kardassis D, Nielsen LB, Andersen CB, Køber L, Johnsen AH, Grande P, Zannis VI, Tybjaerg-Hansen A** 2011 Mutation in *APOA1* predicts increased risk of ischaemic heart disease and total mortality without low HDL cholesterol levels. J Intern Med 270: 136–146
12. **Friedewald WT, Levy RI, Fredrickson DS** 1972 Estimation of the concentration of low-density lipoprotein cholesterol in plasma,

without use of the preparative ultracentrifuge. Clin Chem 18:499–502

13. **Fox K, Garcia MA, Ardissino D, Buszman P, Camici PG, Crea F, Daly C, De Backer G, Hjemdahl P, Lopez-Sendon J, Marco J, Morais J, Pepper J, Sechtem U, Simoons M, Thygesen K, Priori SG, Blanc JJ, Budaj A, Camm J, Dean V, Deckers J, Dickstein K, Lekakis J, McGregor K, Metra M, Morais J, Osterspey A, Tamargo J, Zamorano JL; Task Force on the Management of Stable Angina Pectoris of the European Society of Cardiology; ESC Committee for Practice Guidelines (CPG)** 2006 Guidelines on the management of stable angina pectoris: executive summary: The Task Force on the Management of Stable Angina Pectoris of the European Society of Cardiology. Eur Heart J 27:1341–1381
14. **Thygesen K, Alpert JS, White HD; Joint ESC/ACCF/AHA/WHF Task Force for the Redefinition of Myocardial Infarction** 2007 Universal definition of myocardial infarction. Eur Heart J 28:2525–2538
15. **Nørskov MS, Frikke-Schmidt R, Loft S, Sillesen H, Grande P, Nordestgaard BG, Tybjaerg-Hansen A** 2011 Copy number variation in glutathione s-transferases M1 and T1 and ischemic vascular disease: four studies and meta-analyses. Circ Cardiovasc Genet 4:418–428
16. **Clarke R, Shipley M, Lewington S, Youngman L, Collins R, Marmot M, Peto R** 1999 Underestimation of risk associations due to regression dilution in long-term follow-up of prospective studies. Am J Epidemiol 150:341–353
17. **Lawlor DA, Harbord RM, Sterne JA, Timpson N, Davey Smith G** 2008 Mendelian randomization: using genes as instruments for making causal inferences in epidemiology. Stat Med 27:1133–1163
18. **Altman DG, Bland JM** 2003 Interaction revisited: the difference between two estimates. BMJ 326:219
19. **McLean J, Fielding C, Drayna D, Dieplinger H, Baer B, Kohr W, Henzel W, Lawn R** 1986 Cloning and expression of human lecithin-cholesterol acyltransferase cDNA. Proc Natl Acad Sci USA 83:2335–2339
20. **Hoppe KL, Francone OL** 1998 Binding and functional effects of transcription factors Sp1 and Sp3 on the proximal human lecithin: cholesterol acyltransferase promoter. J Lipid Res 39:969–977
21. **Santamarina-Fojo S, Hoeg J, Assmann G, Brewer Jr HB** 2001 Lecithin cholesterol acyltransferase deficiency and fish eye disease. In: Scriver CR, Beaudet AL, Sly WS, Valle D, eds. The metabolic and molecular bases of inherited disease. 8th ed. New York: McGraw-Hill; 2817–28133
22. **Johannsen TH, Kamstrup PR, Andersen RV, Jensen GB, Sillesen H, Tybjaerg-Hansen A, Nordestgaard BG** 2009 Hepatic lipase, genetically elevated high-density lipoprotein, and risk of ischemic cardiovascular disease. J Clin Endocrinol Metab 94:1264–1273
23. **Haase CL, Tybjærg-Hansen A, Grande P, Frikke-Schmidt R** 2010 Genetically elevated apolipoprotein A-I, high-density lipoprotein cholesterol levels, and risk of ischemic heart disease. J Clin Endocrinol Metab 95:E500–E510
24. **Jensen MK, Rimm EB, Mukamal KJ, Edmondson AC, Rader DJ, Vogel U, Tjønneland A, Sørensen TI, Schmidt EB, Overvad K** 2009 The T111I variant in the endothelial lipase gene and risk of coronary heart disease in three independent populations. Eur Heart J 30:1584–1589
25. **Hokanson JE, Cheng S, Snell-Bergeon JK, Fijal BA, Grow MA, Hung C, Erlich HA, Ehrlich J, Eckel RH, Rewers M** 2002 A common promoter polymorphism in the hepatic lipase gene (*LIPC*-480C>T) is associated with an increase in coronary calcification in type 1 diabetes. Diabetes 51:1208–1213
26. **Mahley RW, Rall Jr SC** 2001 Type III hyperlipoproteinemia (dysbetalipoproteinemia): the role of apolipoprotein E in normal and abnormal lipoprotein metabolism. In: Scriver CR, Beaudet AL, Sly WS, Valle D, eds. The metabolic and molecular bases of inherited disease. 8th ed. New York: McGraw-Hill; 2835–2862
27. **Austin MA, McKnight B, Edwards KL, Bradley CM, McNeely MJ, Psaty BM, Brunzell JD, Motulsky AG** 2000 Cardiovascular disease mortality in familial forms of hypertriglyceridemia: a 20-year prospective study. Circulation 101:2777–2782
28. **Nestel PJ, Fidge NH, Tan MH** 1982 Increased lipoprotein-remnant formation in chronic renal failure. N Engl J Med 307:329–333
29. **Kasama T, Yoshino G, Iwatani I, Iwai M, Hatanaka H, Kazumi T, Oimomi M, Baba S** 1987 Increased cholesterol concentration in intermediate density lipoprotein fraction of normolipidemic non-insulin-dependent diabetics. Atherosclerosis 63:263–266
30. **Nordestgaard BG, Abildgaard S, Wittrup HH, Steffensen R, Jensen G, Tybjaerg-Hansen A** 1997 Heterozygous lipoprotein lipase deficiency: frequency in the general population, effect on plasma lipid levels, and risk of ischemic heart disease. Circulation 96:1737–1744
31. **Franceschini G, Sirtori CR, Capurso 2nd A, Weisgraber KH, Mahley RW** 1980 A-I Milano apoprotein. Decreased high density lipoprotein cholesterol levels with significant lipoprotein modifications and without clinical atherosclerosis in an Italian family. J Clin Invest 66:892–900
32. **Hovingh GK, Hutten BA, Holleboom AG, Petersen W, Rol P, Stalenhoef A, Zwinderman AH, de Groot E, Kastelein JJ, Kuivenhoven JA** 2005 Compromised LCAT function is associated with increased atherosclerosis. Circulation 112:879–884
33. **Calabresi L, Baldassarre D, Castelnuovo S, Conca P, Bocchi L, Candini C, Frigerio B, Amato M, Sirtori CR, Alessandrini P, Arca M, Boscutti G, Cattin L, Gesualdo L, Sampietro T, Vaudo G, Veglia F, Calandra S, Franceschini G** 2009 Functional lecithin: cholesterol acyltransferase is not required for efficient atheroprotection in humans. Circulation 120:628–635
34. **Cohen JC, Kiss RS, Pertsemlidis A, Marcel YL, McPherson R, Hobbs HH** 2004 Multiple rare alleles contribute to low plasma levels of HDL cholesterol. Science 305:869–872
35. **Frikke-Schmidt R, Nordestgaard BG, Jensen GB, Tybjærg-Hansen A** 2004 Genetic variation in ABC transporter A1 contributes to HDL cholesterol in the general population. J Clin Invest 1141343–1353
36. **Kuivenhoven JA, Weibusch H, Pritchard PH, Funke H, Benne R, Assmann G, Kastelein JJ** 1996 An intronic mutation in a lariat branchpoint sequence is a direct cause of an inherited human disorder (fish-eye disease). J Clin Invest 98:358–364
37. **Kuivenhoven JA, van Voorst tot Voorst EJ, Wiebusch H, Marcovina SM, Funke H, Assmann G, Pritchard PH, Kastelein JJ** 1995 A unique genetic and biochemical presentation of fish-eye disease. J Clin Invest 96:2783–2791
38. **Kuivenhoven JA, Stalenhoef AF, Hill JS, Demacker PN, Errami A, Kastelein JJ, Pritchard PH** 1996 Two novel molecular defects in the *LCAT* gene are associated with fish eye disease. Arterioscler Thromb Vasc Biol 16:294–303
39. **Daniil G, Phedonos AA, Holleboom AG, Motazacker MM, Argyri L, Kuivenhoven JA, Chroni A** 2011 Characterization of antioxidant/anti-inflammatory properties and apoA-I-containing subpopulations of HDL from family subjects with monogenic low HDL disorders. Clin Chim Acta 412:1213–1220
40. **Goyal J, Wang K, Liu M, Subbaiah PV** 1997 Novel function of lecithin-cholesterol acyltransferase. J Biol Chem 272:16231–16239