Genetic Variation Related to High-density Lipoprotein Metabolism and Risk of Coronary Heart Disease

PhD thesis

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This thesis is based on the following studies:

Study I: Jensen MK, Pai JK, Mukamal KJ, Overvad K, Rimm EB. Common genetic variation in ABCA1, plasma lipids and risk of coronary heart disease. *Atherosclerosis 2007; 195:e172-80.*

Study II: Jensen MK, Mukamal KJ, Overvad K, Rimm EB. Alcohol consumption, TaqIB polymorphism of cholesteryl ester transfer protein, high-density lipoprotein cholesterol, and risk of coronary heart disease in men and women. *Eur Heart J 2008; 29:104-12*.

Study III: Jensen MK, Rimm EB, Rader DJ, Schmidt EB, Sørensen TIA, Vogel U, Overvad K, Mukamal KJ. The S447X variant of the lipoprotein lipase gene, lipids, and risk of coronary heart disease in three prospective cohort studies. *Submitted.*

Study IV: Jensen MK, Rimm EB, Mukamal KJ, Edmondson AC, Rader DJ, Vogel U, Tjønneland A, Sørensen TIA, Schmidt EB, Overvad K. Variation in the endothelial lipase gene and risk of coronary heart disease in two independent populations. *Manuscript draft.*

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SUMMARIES

English

Much research effort has sought to locate the specific genetic variants that may underlie the heredity in coronary heart disease (CHD) shown in family and twin studies. In candidate gene association studies, variations in genes that are known to be involved in biologic pathways of interest to the etiology of the disease are explored.

Based on the epidemiological observation of an inverse association between high-density lipoprotein (HDL)-cholesterol and risk of CHD, the aim of the present thesis was to investigate if genetic variants that are implicated in the metabolism of HDL were associated with the risk of CHD. Gene-environment interactions, for which there was a strong biological rationale, were also explored.

Four genes that play a major role in the modulation of the HDL particle were chosen. *ABCA1:* mediates the cholesterol efflux from cells to lipid-poor apolipoprotein AI; *CETP*: transfers cholesterol between HDL and apolipoprotein B-containing lipoproteins; *LPL*: hydrolyzes triglycerides and makes apolipoproteins available for HDL; *LIPG*: hydrolyzes phospholipids carried by HDL. One to five polymorphisms in each gene were selected and genotyped in smaller case-control studies nested within prospective cohort studies in US and Denmark. Plasma lipid and lipoprotein concentrations and risk of CHD were investigated according to genotype and in combination with environmental factors.

Among five variants in *ABCA1*, one promoter variant (-565T/C) was associated with risk of CHD, without any pronounced association with HDL-cholesterol concentration. The TaqIB variant in *CETP* was associated with HDL-cholesterol, but not with CHD-risk. This variant appeared to modify the inverse association between alcohol consumption and risk of CHD, such that only moderate alcohol drinkers who were carriers of the variant allele had a lower risk of CHD. The S447X variant in the *LPL* gene was associated with lower plasma triglycerides, higher HDL-cholesterol, and a lower risk of CHD. Three polymorphisms in the *LIPG* gene were not statistically significantly associated with plasma HDL-cholesterol concentration and only weak associations with CHD were observed.

In conclusion, these candidate gene association studies show moderate support for a role of the selected genetic variants in relation to HDL-cholesterol and risk of CHD. The genetic variants that were associated with CHD were generally not associated with HDL-cholesterol concentration. The studied genetic variants may be associated with CHD through a different pathway or measurements of HDL-cholesterol in plasma may not reflect the antiatherogenic properties of the HDL particle well. Improved measures of tissue-specific cholesterol efflux are of great scientific interest for the further exploration of the role HDL metabolism to atherogenesis using the genetic epidemiologic approach.

Danish

Megen forskning har været rettet mod identificeringen af specifikke genetiske varianter der kan være baggrund for den observerede arvelighed i iskæmisk hjertesygdom (IHS). I kandidatgen tilgangen studeres associationen mellem sygdom og variation i gener, der er indblandet i biologiske mekanismer af betydning for opståen af sygdom.

Formålet med denne afhandling var at uddybe forståelsen af den epidemiologisk observerede inverse association mellem high-density lipoprotein (HDL)-kolesterol og risiko for IHS. Vi undersøgte, hvorvidt genetiske varianter af betydning for HDL metabolismen var associeret med risikoen for IHS. Herudover undersøgte vi gen-miljø interaktioner, der havde et stærk biologisk rationale.

Fire gener af stor betydning for moduleringen af HDL partiklen blev valgt. *ABCA1:* medierer efflux af kolesterol fra celler til apolipoprotein AI; *CETP*: overfører kolesterol mellem HDL og apolipoprotein B-holdige lipoproteiner; *LPL*: hydrolyserer triglycerider og gør apolipoproteiner tilgængelige for HDL; *LIPG*: hydrolyserer phospholipider transporteret rundt af HDL. En til fem polymorfier blev genotypebestemt i mindre case-control studier forankret i prospektive kohorte studier i USA og Danmark. Plasma lipider og lipoprotein koncentrationer og risiko for IHS blev undersøgt i henhold til genotype samt i kombination med miljøfaktorer.

Blandt fem varianter i *ABCA1* var en promoter variant (-565T/C) associeret med risikoen for IHS, uden en tydelig association med HDL-kolesterol koncentrationen. TaqIB varianten in *CETP* var associeret med HDL-kolesterol men ikke med risiko for IHS. Denne variant modificerede den tilsyneladende inverse sammenhæng mellem alkohol og IHS, sådan at et moderat alkoholforbrug kun var associeret med en lavere risiko for IHS blandt de, der havde variant allelen. *LPL* varianten, S447X var associeret med lavere plasma triglycerid, højere HDL-kolesterol og en lavere risiko for IHS. Tre polymorfier i *LIPG* genet viste ikke statistisk signifikante associationer med plasma HDL-kolesterol koncentration og kun svage associationer med IHS risiko.

Konkluderende kan siges, at disse kandidatgen studier kun viste moderat støtte for de valgte geners rolle i associationen mellem HDL-kolesterol og risiko for IHS. De genetiske varianter der, var associeret med IHS, var generelt ikke associeret med koncentrationen af HDL-kolesterol. De undersøgte varianter kan være associeret med IHS gennem en anden biologisk sammenhæng, eller de antiatherogene effekter af HDL partiklen kan være dårligt reflekteret i målingen af koncentration af HDL-kolesterol i plasma. Bedre målinger af vævsspecifik kolesterol efflux kan være af stor forskningsmæssig interesse for videre genetisk epidemiologiske undersøgelser af betydning af HDL metabolismen for atherogenese.

CONTENTS

INTRODUCTION

Coronary heart disease (CHD) is among the leading causes of morbidity and mortality in Western nations.[1] Besides age and sex, several modifiable behavioral factors such as smoking, physical activity, alcohol consumption, and diet are recognized as important risk factors for the development and progression of atherosclerosis.[2, 3] However, a hereditary component to CHD has also been shown in epidemiological studies. A family history of early-onset CHD is a strong risk factor for the development of CHD,[4, 5] and a greater concordance in risk of death from CHD has been observed among monozygotic compared to dizygotic twins.[6] Thus, CHD is a common multifactorial disease in which both environmental and genetic risk factors contribute to susceptibility in a complex manner.

The two major cholesterol transporters in circulation: low- and high-density lipoprotein (LDL and HDL) are among some of the important clinical intermediates of the association of both genetic and environmental factors with CHD. That LDL-cholesterol is directly and HDL-cholesterol inversely associated with CHD has been shown in several observational studies.[7-9] Statins are among the most widely used drugs in both primary and secondary prevention of CHD. They efficiently lower LDL-cholesterol levels by inhibiting the de-novo cholesterol production by the liver (HMG-CoA reductase inhibitors).[10] However, considerable residual cardiovascular risk has been documented among patients treated with statins and in the past decades substantial research efforts have been aimed towards the identification of novel drug targets that might lower the risk further by raising the concentration of HDL-cholesterol.[11, 12] Inferences based on epidemiological data suggest that a 2-3% CHD-risk reduction per each 1 mg/dL (0.026 mmol/L) increase in HDL-cholesterol. However, proof of such a causal relationship has, so far, not been fully supported by intervention studies.[13]

Most recently, the HDL-cholesterol raising strategy was questioned by clinical trials of cholesteryl-ester transfer protein (CETP) inhibitors that resulted in surprisingly higher mortality and more cardiovascular events among patients who were treated, despite concurrent great increases in HDL-cholesterol levels.[14, 15] Thus, a deeper insight into the role of the HDL particle in atherosclerosis and improved knowledge about the long-term consequences of HDL-cholesterol modulation is still warranted.

As opposed to clinical interventions where HDL-cholesterol concentration may be therapeutically manipulated over a period of a few years, observational studies of genetically determined variation in HDL-cholesterol concentration allows for insight into the cardiovascular consequences of lifelong exposure to differences in the metabolism of the HDL particle. This may increase our understanding of the metabolic process itself, elucidate potential compensatory mechanisms, and allow for investigations of environmental factors that may render particular

subgroups more susceptible. These scientific aims could hopefully lead to earlier diagnoses and expand current cardiovascular treatment options.

AIMS

The objective of the present thesis was to investigate the role of variation in genes involved in the metabolism of HDL-cholesterol in relation to risk of CHD. Genetic variants were selected based on prior evidence for their impact on HDL-cholesterol concentration or risk of CHD.

Based on the epidemiological observation of an inverse association between HDL-cholesterol and risk of CHD and evidence that HDL-cholesterol level is highly heritable, two aims were formulated as the basis for the four included manuscripts:

- To explore if genetic variants in candidate genes involved in HDL metabolism are associated with HDL-cholesterol levels and risk of CHD
- To investigate whether particularly susceptible subgroups of the population can be identified by examining gene-environment interactions with known HDL-related lifestyle factors (diet, smoking, alcohol, and adiposity)

Genetic variants in four HDL-related genes were selected for the exploration of these aims in prospective studies nested within two US cohorts and the Danish Diet, Cancer and Health study.

BACKGROUND

Candidate gene association studies of coronary heart disease

During the past decade, considerable progress has been achieved in the knowledge of the human genome. The completion of the Human Genome Project in 2003 revealed the sequence of the three billion base pairs in the human DNA and the location of the approximately 20-25000 encoded genes.[16] Although individual genomes are roughly 99.9% identical, this still leaves millions of differences among the base pairs. Patterns of the most common cause of sequence variation, single nucleotide polymorphisms (SNPs; defined as nucleotide variation with >1% frequency), were subsequently identified by the HapMap project. So far, more than three million SNPs have been genotyped in four distinct ethnic groups, providing a unique database for genetic association studies.[17]

In candidate gene association studies, genes are selected for study based on prior molecular knowledge of their role in a biological pathway of major influence to the phenotype under investigation. Traditionally, SNPs within candidate genes were chosen based on characteristics that might render them more likely to be functionally important. For example, the location in the regulatory region or the induction of changes in the encoded amino acid by a SNP located in the protein coding region. Recently, the availability of high-throughput genotyping has allowed gene association studies to include thousands of candidate genetic variants in a single run of a customized gene-chip or hypothesis-free genome-wide screens of millions of variants selected at random or based on HapMap. The present thesis is based on four studies with few SNPs in select candidate genes involved in a biological pathway related to the development of CHD. Genetic variants were primarily selected based on evidence for their implication in the metabolism of HDL. In the perspective, the benefits and limitations of choosing this strategy and study design will be put into context of the recent large-scale genetic epidemiological studies of CHD using high-throughput methods.

HDL metabolism

Observational studies consistently demonstrate that low HDL-cholesterol is a strong risk factor for CHD,[7-9] and some, but not all, controlled clinical trials support a lower cardiovascular risk among high-risk patients treated with HDL-cholesterol raising drugs like niacin and fibrates.[13]

Plausible biological mechanisms for the proposed antiatherogenic effect of HDL-cholesterol have been demonstrated both in vitro and vivo. Besides promoting the transport of cholesterol from peripheral cells back to the liver, HDL has been found to have antioxidant, antiinflammatory, antiapoptotic, nitric oxide-promoting, prostacyclin-stabilizing, and platelet-inhibiting properties.[18]

The pleiotropic effects of HDL underscores that the relationship between plasma levels of HDL-cholesterol and the potentially cardiovascular beneficial functional properties of the HDL particle itself may not be as straightforward as implied by observational epidemiology. This aspect will be further dealt with in the discussion section.

Results from twin and family studies have estimated that genes account for about half of the variation in HDL-cholesterol levels.[19, 20] As opposed to rare Mendelian disorders, where a single infrequent genetic variant influences HDL-cholesterol levels substantially, multiple common genetic variants are likely to contribute with small effects on HDL-cholesterol levels in the general population. Several proteins have been identified that play a key role in the synthesis, processing, and catabolism of HDL (see figure 1). Genes that encode these transporters, apolipoproteins, lipases, receptors, and transfer proteins are all potential candidates for the investigation in relation to risk of CHD.

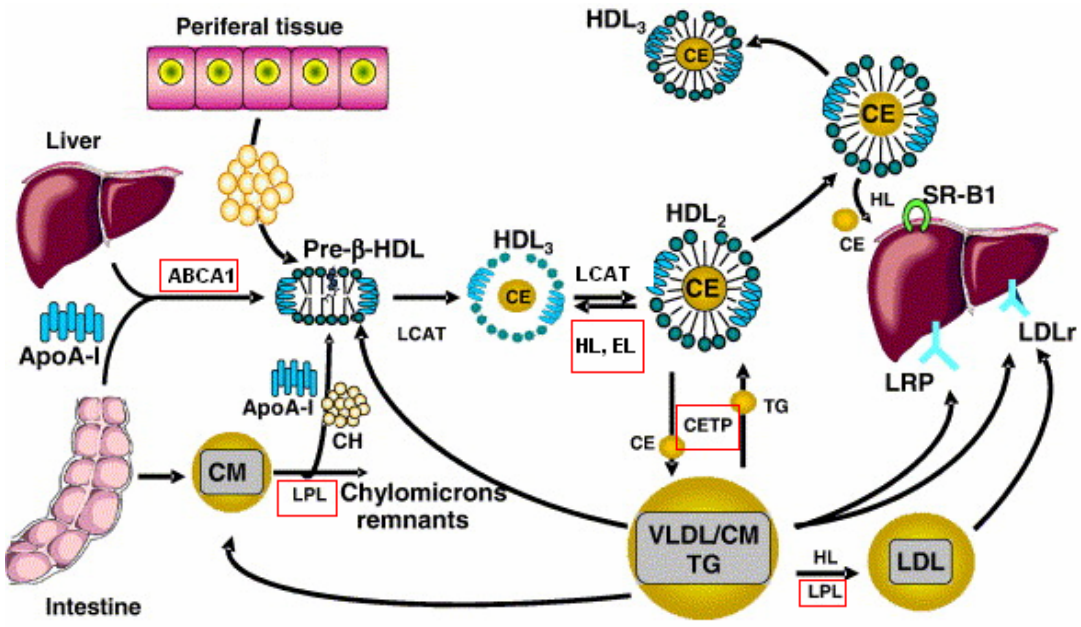

Figure 1. HDL metabolism, modified from[21]
ApoAI: Apolipoprotein AI; CM: Chylomicron; ABCA1: ATP-binding cassette transporter A1; LPL: Lipoprotein lipase; CETP: Cholesteryl ester transfer protein; LCAT: Lecithin cholesterol acyltransferase; HL: Hepatic lipase; EL: Endothelial lipase; CE: Cholesterol ester; TG: Triglyceride, LDLr: LDL-receptor; SR-B1: Scavenger receptor B1

For the present studies, four particular genes were considered: the ATP-binding cassette transporter A1 (*ABCA1*), cholesteryl-ester transfer protein (*CETP*), lipoprotein lipase (*LPL*), and endothelial lipase (EL, gene name: *LIPG*). The encoded proteins are important modulators of HDL at different sites as described in paper I-IV. Briefly, ABCA1 is a large trans-membrane cholesterol transporter that mediates the transferal of cholesterol and phospholipids from the liver and peripheral cells to

lipid-poor apolipoprotein AI (ApoAI). Thus, ABCA1 is thought to be the main mediator of initial HDL lipidation.[22] The circulating HDL particle may receive additional cholesterol and apolipoproteins during LPL-mediated lipolysis of dietary derived triglyceride carried in chylomicrons and very-low density lipoproteins (VLDL).[23] EL is a more recent addition to the list of HDL modulators. EL is mainly a phospholipase, making HDL-cholesterol its preferred substrate.[24] Cholesterol in HDL can be removed by the liver through one of two pathways: In the direct pathway, HDL-cholesterol is either hydrolyzed by hepatic lipase and lipid-poor HDL is released back into circulation, or the entire HDL particle is taken up by endocytosis and degraded. In the indirect pathway, CETP mediates the transferal of cholesterol from HDL to LDL and VLDL, which can then be removed by the liver.[25] Table 1 below summarizes the main functions of the four proteins.

Table 1 The selected candidate genes and their role in HDL-cholesterol metabolism

Protein	Gene	Function
ATP-binding cassette transporter A-1	*ABCA1*	Transmembrane protein. Mediates the transferal of PL and cholesterol to lipid-poor ApoAI
Cholesteryl-ester transfer protein	*CETP*	Transfer protein. Mediates the transfer of CE from HDL to VLDL/LDL in exchange for triglycerides
Lipoprotein lipase	*LPL*	Enzyme. Hydrolyzes triglyceride found in CM and LDL. Makes apolipoproteins and PL available for HDL
Endothelial lipase	*LIPG*	Enzyme. Hydrolyzes mainly PL found in HDL

ApoAI: Apolipoprotein AI; CM: Chylomicron; PL: Phospholipids; CE: Cholesteryl ester

Gene-environment interactions

The etiology of common, complex diseases, such as CHD, involves multiple genetic and environmental factors, and their complex interplay. Stronger associations between genetic variants and risk of CHD may be observed in population subgroups characterized by certain environmental exposures or modification of the behavioral characteristics may be of particular concern among those with certain genetic variants. Failure to account for such potential modification may dilute a genetic association with disease towards the null. Thus, a sub-aim of this project was to examine if the CHD-risk associated with genetic variants differed according to lifestyle factors known to influence HDL-cholesterol levels, or vice versa, to investigate whether the association between lifestyle factors and CHD-risk were modulated by genetic variation.

Several lifestyle factors with influences on HDL-cholesterol concentration have been documented.[26] While the distinction between genetic and environmental factors may not be as straightforward as such a dichotomization implies (because most characteristics will have some underlying genetic causes) common lifestyle factors that were considered in the present thesis were classified as environmental because they were assumed not to be determined by the genetic variants that were investigated in this project (see figure 2).

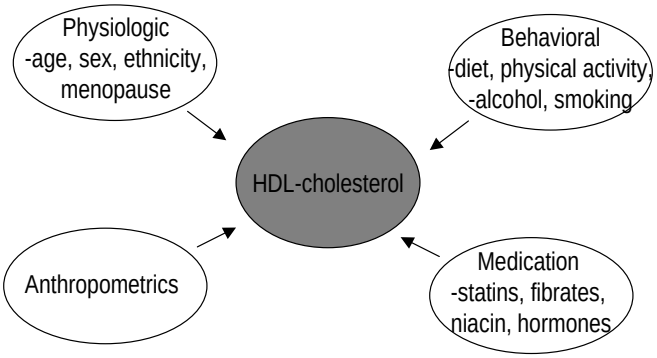

Figure 2. Environmental influences on HDL-cholesterol concentration

Although interactions play a major role for disease development, very little progress has been made in the methodology for their investigation. It is complicated to take interactions into account. Not all studies have collected detailed and validated measures of environmental exposures, and the exploration of several potentially interacting agents quickly becomes overwhelming. The concept of statistical versus biological interaction remains heavily debated. The confusion relates to the difficulty in translating the biological question into a statistical model. While biological interaction is something that is either present or not, evaluation of heterogeneity in the association depends on the chosen statistical model. Whether the observed joint effect deviates from expectation is scale-dependent. Some epidemiologists claim that biological interaction corresponds to interaction on the additive scale,[27] while others claim this to be scenario-specific.[28] The most important point of this debate is probably to consider the implications of the chosen statistical model. In the present thesis, different strategies for the evaluation of gene-environment interactions were used through paper I-IV. Implications of these choices will be covered in the discussion of the results.

STUDY POPULATIONS

The Nurses' Health Studies

In 1976, the first Nurses' Health Study (NHSI) was established in Boston when 121,700 married female registered nurses aged 30 to 55 years answered a detailed lifestyle and medical history questionnaire. The women have since received follow-up questionnaires biennially to update information on exposures and newly diagnosed illnesses. Since 1980, participants have also received a food frequency questionnaire (FFQ) approximately every four years (see figure 3).

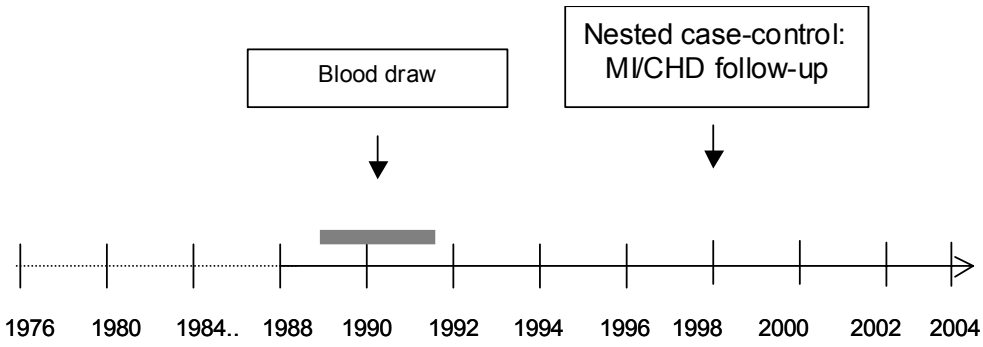

Figure 3. Data collection in the Nurses' Health Study I (NHSI)

Between 1989 and 1990, a blood sample was requested from all active participants in NHSI and collected from 32,826 women. In 1998, a nested case-control of incident CHD was defined by including all incident cases of non-fatal myocardial infarction (MI) and fatal CHD that had occurred among the NHSI participants since blood draw. Participants who had reported an incident CHD on the follow-up questionnaire were contacted for confirmation and permission to review medical records was requested. Medical records for deceased participants were also sought for deaths that were identified by families and postal officials and through the National Death Index. Physicians blinded to the participant's questionnaire reports reviewed all medical records. Cases of MI and fatal CHD were identified primarily through review of medical records, as previously described.[29, 30] Among participants who provided blood samples and who were without cardiovascular disease or cancer at blood draw, 212 women sustained an incident MI and 37 died from fatal CHD between blood draw and June 30, 1998. The NHSI data were used for paper I, II, and III.

The second NHS study (NHSII) was established in 1989 when 116,671 female registered nurses aged 25 to 42 years were enrolled. Health and disease status have been assessed with methods

similar to the NHSI and blood samples were obtained between 1996 and 1998 from more than 29,000 women who were not taking any hormones during the luteal phase of their menstrual cycle. Among women who were without serious medical conditions at blood draw, a subset of 473 women was randomly selected within prespecified alcohol drinking groups (initially, the aim of this sub-study was to address correlations between alcohol drinking patterns and novel biomarkers). The sub-sample of the NHSII cohort was used to explore genetic variants in ABCA1 in relation to lipid markers in Paper I.

The Health Professionals Follow-Up Study

The Health Professionals Follow-Up Study (HPFS) was initiated in 1986 when 51,529 male health professionals between 40 and 75 years of age completed a FFQ and a medical history questionnaire. The participants have been followed with repeated questionnaires on lifestyle and health every 2 years and FFQ's every 4 years (see figure 4). Methods were similar to those described for the Nurses' studies. Blood samples were requested between 1993 and 1996 and obtained from 18,225 participants.[31]

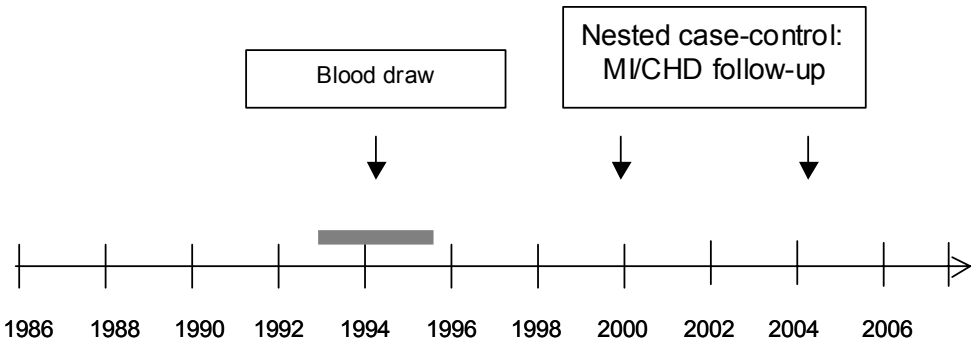

Figure 4. Data collection in the Health Professionals Follow-Up Study (HPFS)

For a nested case-control study, 196 incident nonfatal MI and 70 fatal CHD cases that occurred between blood draw and January 31, 2000 were collected and ascertained with methods similar to those described for NHSI. As a secondary endpoint, 564 men who had coronary artery bypass graft surgery (CABG) or percutaneous transluminal coronary angioplasty (PTCA) were also collected. Confirmation of CABG/PTCA was based on self-report only; hospital records obtained for a sample of 102 men confirmed the procedure for 96% of these.[30] The case-control study with disease follow-up until 2000 was used in paper II and III. Recently, incident CHD cases that occurred between 2000 and 2004 have been included (approximately 250 new cases and newly matched controls 2:1 ratio). These data were only available for paper IV.

The Diet, Cancer and Health study

The Diet, Cancer and Health (DCH) study was initiated in 1993 when a total of 160,725 inhabitants of the greater Copenhagen or Aarhus areas who were born in Denmark and aged 50 to 64 years, were invited to participate. Eligible participants were without a record of cancer in the Danish Cancer Registry at the time of invitation. In total, 27,178 men and 29,875 women participated. Participants received a detailed FFQ by mail prior to the visit to the study clinic, where they also filled in a lifestyle questionnaire, and were asked to provide a blood sample. A detailed description of the cohort has been published previously.[32] The study was conducted in accordance with the Helsinki Declaration II and approved by the Ethical Committees on Human Studies (KF 01-045/93;KF 01-116/96). The sub-studies included in the present thesis have also been approved (KF11-300 421/05).

A case-cohort study was designed using incident acute coronary syndrome (ACS), including unstable angina pectoris, MI, and sudden cardiac death as the outcome.

Information on the disease endpoint was obtained by linkage with central Danish registries via the unique identification number assigned to all Danish citizens.[33] Hospital records of potential cases were retrieved from hospitals for participants who were registered with a first-time discharge diagnosis of ACS (ICD-8 codes 410-410.99, 427.27 and ICD-10 codes I20.0, I21.x, I46.x) in The Danish National Register of Patients, which covers all hospital discharge diagnoses since 1977 and from 1995 all discharge diagnosis from out-patient clinics (until Jan 1, 2004).[34] Cases were classified by three reviewers according to symptoms, signs, coronary biomarkers, ECGs and/or autopsy findings in accordance with the current recommendations of the American Heart Association and the European Society of Cardiology (AHA/ECS).[35] A description of the validation study is in press. In agreement with another Danish validation study, MI was found to be recorded with a high degree of validity in this register,[36] whereas the diagnoses of unstable angina and cardiac arrest were less accurate. Further, linkage to the Cause of Death Register allowed for identification of participants with ACS coded as a primary or secondary cause of death (to Jan 1, 2004). In total, 1150 cases of ACS were identified, however some of these were later excluded because of lacking questionnaire data. For the creation of the cohort sample, 1800 participants were selected from the entire DCH study at random.

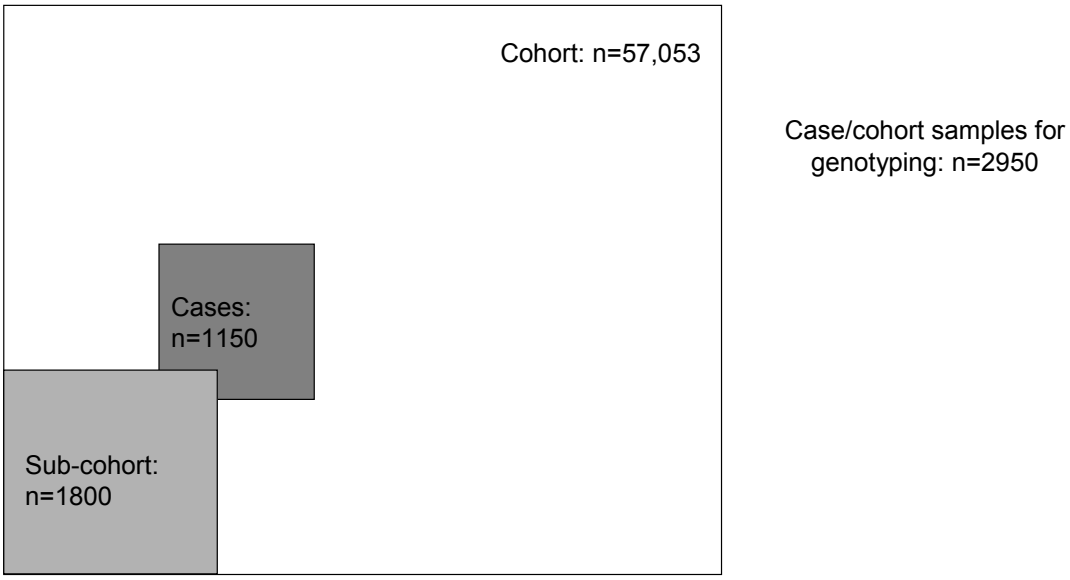

Figure 5. Case-cohort study in the Diet, Cancer and Health study (DCH)

METHODS

Laboratory procedures

In the US cohorts, the collected blood samples were returned to the laboratory associated with the Harvard School of Public Health using overnight courier. Upon arrival, whole blood samples were centrifuged and aliquoted into cryotubes as plasma, buffy coat, and red blood cells, which were stored in the vapor phase of liquid nitrogen freezers.

In the DCH, a total of 30 ml blood was collected from non-fasting participants in citrated (2× 10 ml) and plain (1× 10 ml) Venojects. Plasma, serum, lymphocytes, and erythrocytes were isolated and frozen at −20 ◦C within 2 h. All samples were stored in liquid nitrogen, at −150 ◦C. DNA was isolated from frozen lymphocytes in the laboratory of Ulla Vogel at the Danish Research Centre for the Working Environment.

Blood samples were requested from fasting participants in the NHSI, II and HPFS. The few participants who reported their last meal within 8 hours of blood draw were excluded from analyses of triglyceride levels. In the DCH, participants were not requested to be fasting when blood was obtained.

Genotyping procedures for the three studies are described in the papers. Lipids and lipoprotein fractions were measured by routine laboratory methods in the laboratories of Nader Rifai in Boston and at the Lipid Clinic at the Department of Cardiology, Aalborg Hospital in Denmark. LDL-cholesterol was calculated by the Friedewald formula in DCH (except when triglycerides were greater than 4.5 mmol/L) and directly measured in the US samples.[37] For US samples, the lipid measures were tested under different simulated transport and storage conditions, and were found to be stable and reproducible.[38]

Statistical methods

Details for the statistical analyses are included in paper I-IV.

Because the US case-control studies were designed as matched incidence-density sampled studies, primary analyses were performed using conditional logistic regression. However, because conditional regression is not ideal for stratified analyses in which members of the matched sets do not share levels of covariates, we also used unconditional logistic regression (adjusted for the matching factors) to achieve greater statistical power for the interaction analyses. In the conditional logistic regression, participants in the strata where cases and controls did not report the same level of the modifier would be lost. Results from unconditional logistic regression models were only included in the papers, when they produced essentially the same results as the conditional models.

In the DCH, incidence rate ratios were calculated by means of Cox proportional hazard regression models with age as the time axis. To accommodate the case-cohort design, weights proposed by Kalbfleisch and Lawless were employed for the estimation of the regression coefficient.[39] These weights assign cases the same weight, whether they occurred in the sub-cohort or not, but the censored observations (those that survived until end of follow-up) are weighted with the inverse probability of being in the sub-cohort. Because of the over-sampling of cases, the usual estimates of variance in the Cox model would overestimate the precision of the regression parameter estimate. To take this into account the robust variance estimator was used.

RESULTS

Paper I: Five polymorphism in *ABCA1*, HDL-cholesterol, and risk of CHD

For the investigation of the five SNPs in *ABCA1* and plasma lipids and lipoproteins, data from the cross-sectional NHSII study and the controls from the case-control study in NHSI were combined. Minor allele frequencies were –565C/T: 0.48, -191G/C: 0.47, -17C/G: 0.30, I883M: 0.14, and R1587K: 0.25. The associations were overall weak. In figure 6 below, the results for the association with HDL-cholesterol are presented stratified by age 55.

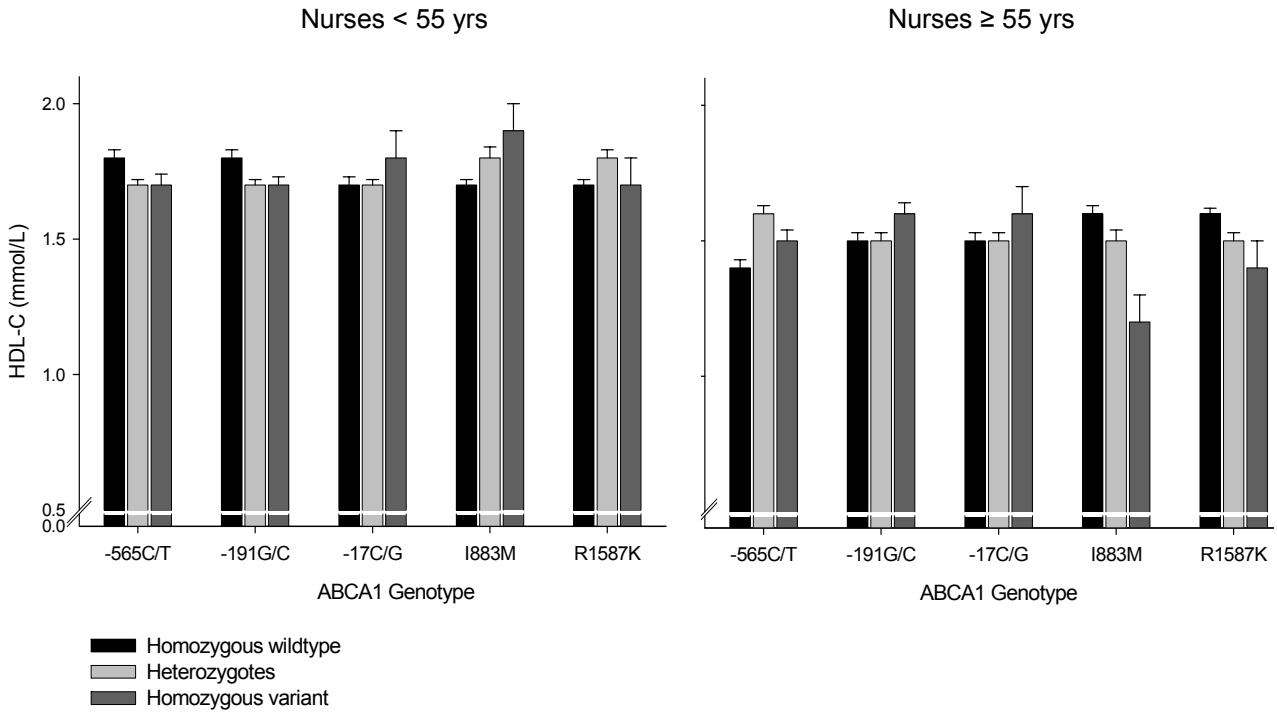

Figure 6. Mean HDL-cholesterol concentrations according to the five *ABCA1* SNPs in the healthy women from NHSI and NHSII, stratified by age 55. Estimates were adjusted for age, smoking, body mass index, alcohol, history of hypertension, parental history of CHD before age 60, diabetes at baseline, and menopausal status

The odds ratios for CHD according to *ABCA1* genotype were assessed in data from the NHSI case-control study. The two tightly linked promoter variants were associated with a lower odds ratio for CHD in an allele dependent manner (odds ratios per minor allele were 0.8 [95% confidence interval; 0.6-1.00] at both loci). The other variants were not statistically significantly associated with the risk of CHD (figure 7). Although the confidence intervals were wide, the –17C/G variant

allele tended to be associated with a higher risk of CHD, and the R1587K variant was associated with a lower risk.

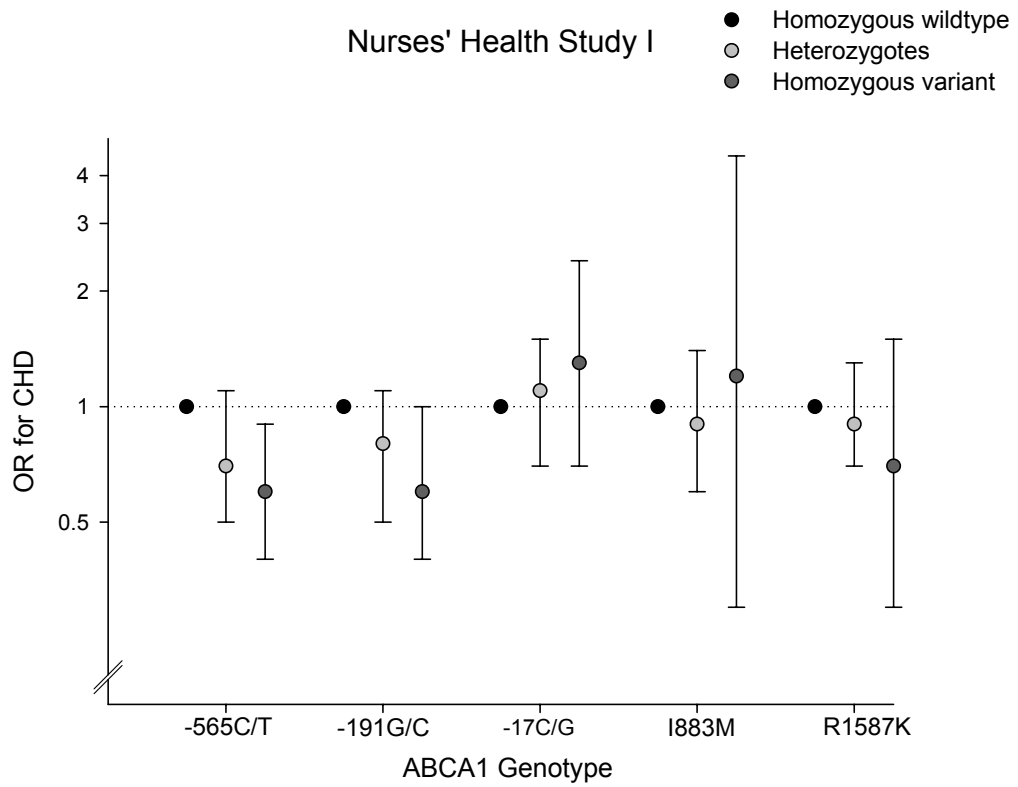

Figure 7. Odds ratios for CHD according to the five *ABCA1* SNPs. Estimates were adjusted for age, smoking, body mass index, alcohol, history of hypertension, parental history of CHD before age 60, diabetes at baseline, and menopausal status

Gene-environment interactions were explored in stratified analyses. There was some evidence that the –191G/C variant was of greater influence among younger women, women who were overweight, or with a low intake of dietary fat (data not shown). However, these analyses did not take into account that the baseline risk of CHD is likely to differ according to the characteristics used for stratification, and p values for the tests of multiplicative interaction were generally weak.

Paper II: TaqIB polymorphism in *CETP*, alcohol, HDL-cholesterol, and risk of CHD

The TaqIB polymorphism in *CETP* was genotyped in the NHSI and HPFS case-control studies. Frequency of the TaqIB variant allele (B2) was 0.4. This did not differ between cases and controls from the NHSI and HPFS. Mean HDL-cholesterol was 0.16 mmol/L higher in B2 homozygotes compared to wildtype homozygotes (data not shown).

We explored the association between alcohol and HDL-cholesterol in strata of TaqIB status because CETP activation has been proposed as one of the explanations for the epidemiological observation of higher HDL-cholesterol and a lower risk of CHD among moderate alcohol drinkers. There was a trend for higher HDL-cholesterol with higher alcohol intake in all three genotype groups (figure 8). However, the association appeared to be strongest among B2B2 homozygotes, especially in the NHSI (p for Wald test of interaction <0.001).

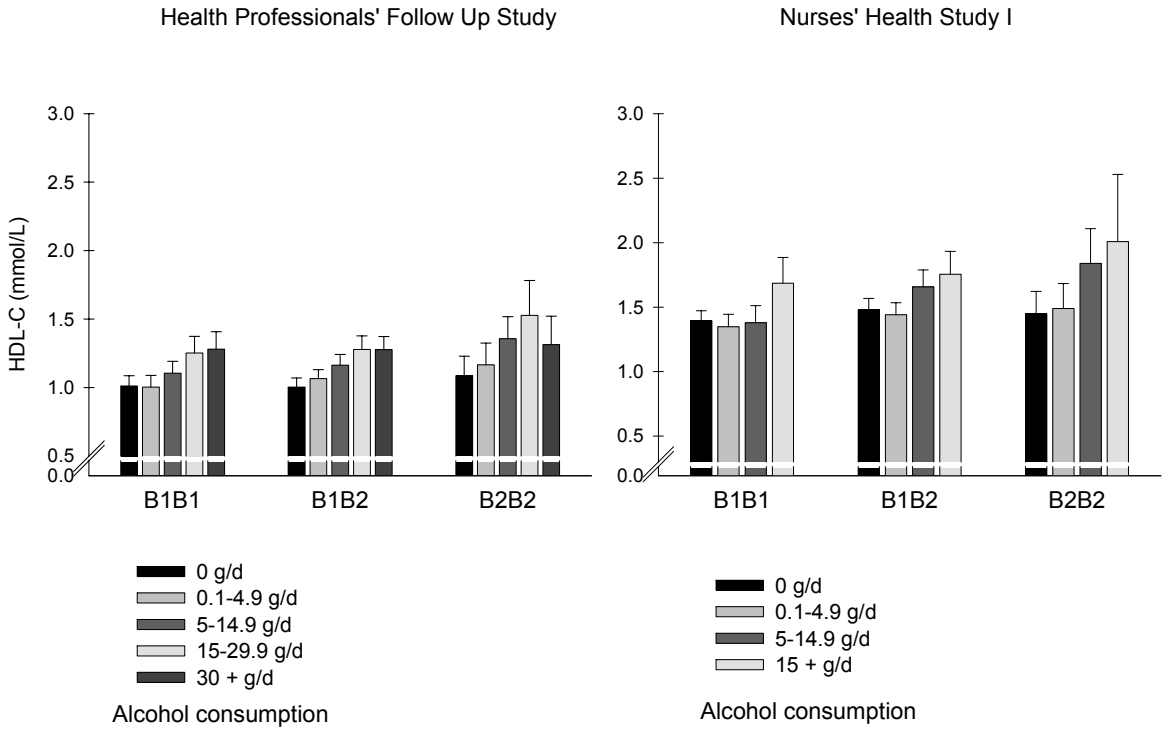

Figure 8. Mean concentration of HDL-cholesterol according to alcohol intake and *CETP* genotype. Estimates were adjusted for age, smoking, time of blood draw, body mass index, family history of MI before age 60, diabetes and hypertension at baseline. Analyses among NHS women also included postmenopausal status

An inverse association between alcohol intake and risk of CHD was only observed in women who were B2-carries, whereas moderate alcohol consumption was not associated with a lower risk of CHD in B1B1 homozygotes. Although tendencies were less strong in the HPFS data, we did not

find statistically significant differences between the two study populations. The result from an analysis where the study specific estimates were pooled using meta-analysis methods is presented in figure 9.

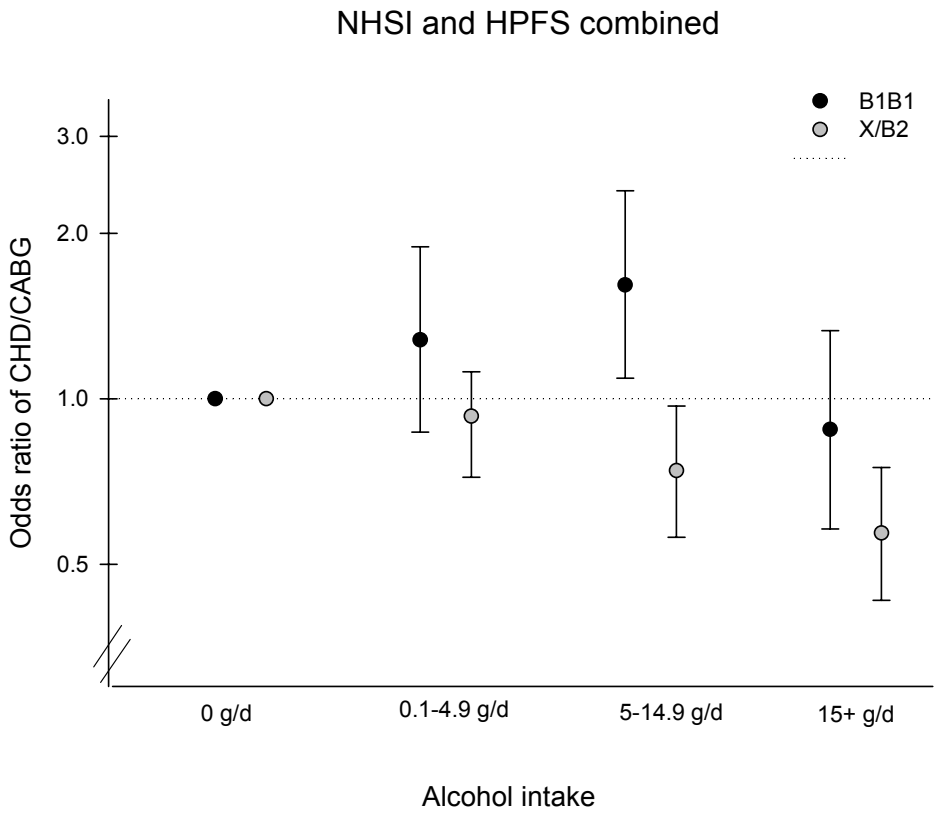

Figure 9. Odds ratio for CHD (including CABG in the HPFS) according to alcohol intake and *CETP* genotype. Estimates were adjusted for age, smoking, time of blood draw, body mass index, family history of MI before age 60, diabetes and hypertension at baseline. Analyses among NHS women also included postmenopausal status. Pooled results were from meta-analysis using a random-effects model

For this analysis, data were stratified by *CETP* genotype. Because the TaqIB variant was not associated with risk of CHD in the overall sample (the baseline risks in the two comparison groups were the same), similar results were obtained when a joint reference group of B1B1 homozygotes, who were non-drinkers, were used. Using likelihood ratios to test nested models with and without all interaction terms between *CETP* (dominant genotype) and the alcohol categories gave a p for interaction of 0.02.

Paper III: The S447X variant in *LPL*, triglycerides, HDL-cholesterol, and risk of CHD

The S447X variant was genotyped in both the US case-control studies and in the DCH study. Minor allele frequencies were 0.12 in the NHSI and HPFS, and 0.10 in DCH controls. Carriers of the S447X variant allele had lower triglyceride and higher HDL-cholesterol levels (data not shown). The variant allele was more frequent among controls than cases in all the studies, and S447X appeared to be associated with an allele-dependent lower risk of CHD (Table 2). This was strongest in the NHSI study, less significant in the HPFS, and not statistically significant in the Danish study. When data were combined, the relative risk was 0.7 (95% confidence interval: 0.6-1.0) per S447X variant allele. The p for heterogeneity between studies was 0.12.

Table 2. Relative risk of CHD according to *LPL* S447X genotype and carrier status

	N (cases/controls)			Relative risk			
	SS	SX	XX	SS	SX	XX	SX/XX
NHSI	211/379	33/100	1/6	1.0 (ref)	0.57 (0.36-0.90)	0.31 (0.04-2.82)	0.56 (0.36-0.87)
HPFS	216/406	40/99	2/10	1.0 (ref)	0.73 (0.49-1.10)	0.37 (0.08-1.69)	0.70 (0.47-1.04)
DCH	840/1386	171/299	4/17	1.0 (ref)	0.93 (0.79-1.19)	0.60 (0.19-1.91)	0.92 (0.72-1.16)
Pooled				1.0 (ref)	0.77 (0.58-1.03)	0.53 (0.29-0.97)	0.74 (0.56-1.00)

Odds ratios for CHD in NHS and HPFS were obtained in conditional logistic regression models. Hazard ratios for ACS in DCH were obtained from Cox proportional hazard regression models. Estimates were adjusted for age, smoking, alcohol, body mass index, and menopausal status (among women). Pooled results were from meta-analysis using a random-effects model

The lower risk of CHD appeared to be modified by body mass index in the NHS sample, to a lesser extent in the HPFS sample, and not in the DCH (figure 10). Results were the same when we used waist circumference as the measure of adiposity. The associations between S447X and HDL-cholesterol or triglycerides were not appreciably modified by adiposity (data not shown).

In the interaction analyses, the jointly unexposed (SS homozygotes who were normal-weight, i.e. body mass index < 25 kg/m^2) were used as the reference group. Thus, all risk estimates were compared to the baseline risk observed in that group.

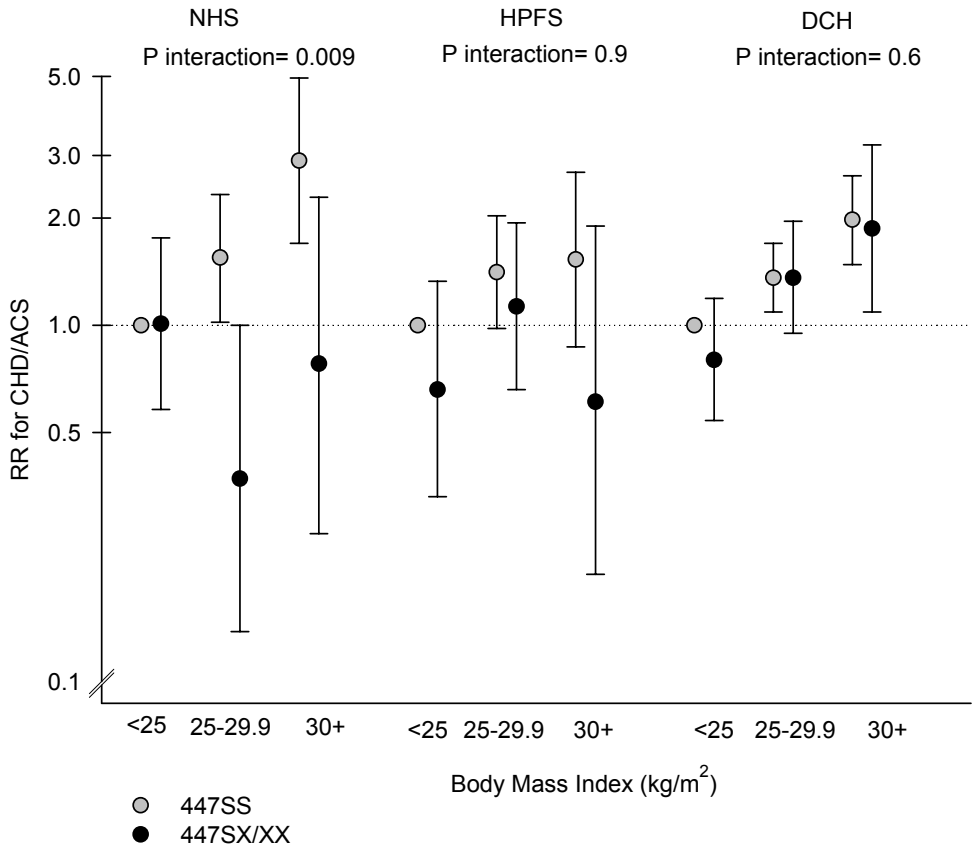

Figure 10. Joint effects of S447X and adiposity on risk of CHD in the three study populations. Odds ratios for CHD in NHS and HPFS were obtained in conditional logistic regression models. Hazard ratios for ACS in DCH were obtained from Cox proportional hazard regression models. Estimates were adjusted for age, smoking, alcohol, body mass index, and menopausal status (among women). Interaction: Wald test of BMI continuously and dominant effect of S447X.

Paper IV: Three variants in *LIPG*, HDL-cholesterol, and risk of CHD

Three *LIPG* variants were genotyped in the DCH and the HPFS. Minor allele frequencies in healthy controls were: -1309A/G=0.23, T111I=0.29, and N396S=0.01. No association between these variants and HDL-cholesterol were detected in the DCH (data not shown).

The SNPs were not statistically significantly associated with the risk of CHD in either study. In figure 11, the relative risk of CHD according to each *LIPG* variant are depicted for the two study populations separately and in a meta-analysis. The association between the –1309A/G and T11I variants and CHD appeared to be allele-dependent, thus, the relative risk per additional variant allele is shown in the figure. In a pooled analysis, the promoter variant was associated with a borderline higher risk per –1309G-allele (RR=1.1 [95% confidence interval, 1.0-1.3]). The risk per T111I variant was 0.9 (95% confidence interval, 0.8-1.1).

The risk among heterozygous carriers of the N396S variant is shown because there were no homozygous variants for this rare mutation.

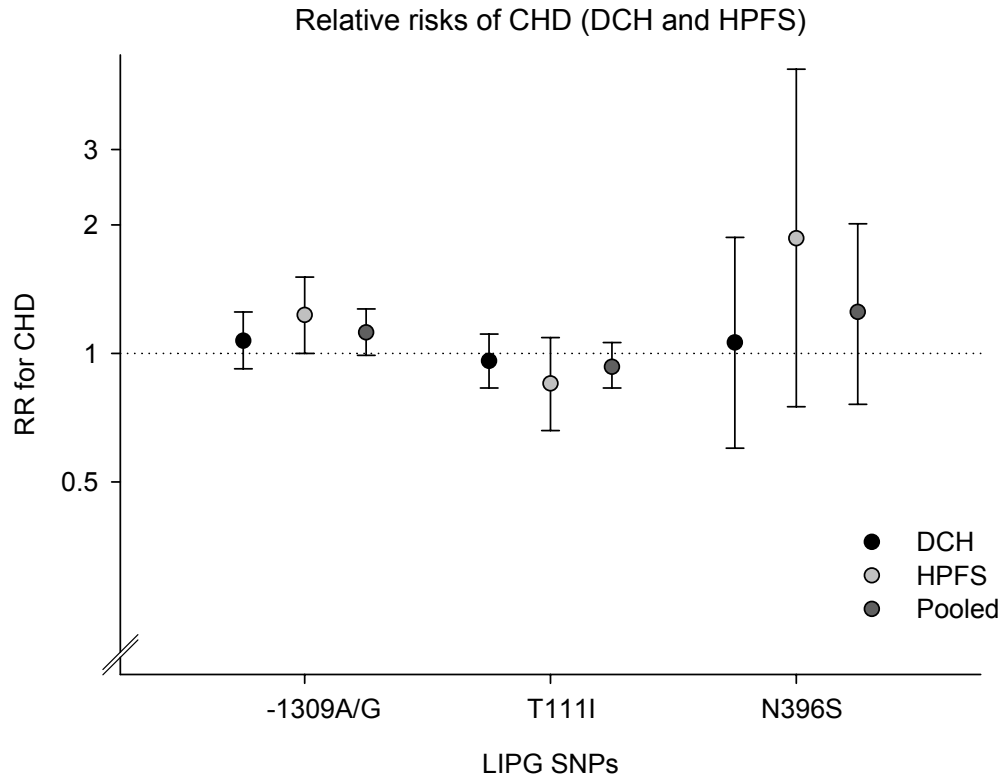

Figure 11.Relative risk of ACS and CHD in data from the DCH and HPFS separately and combined. (Results for –1309A/G and T111I are shown for the co-dominant models and the estimate for the N396S variant is for heterozygotes versus wildtype homozygotes). Estimates were adjusted age, smoking, alcohol, body mass index, and menopausal status (among women). Pooled results were from meta-analysis using a random-effects model

Six groups of diplotypes (combined haplotypes) accounted for >98% of the participants in both studies. The remaining participants included infrequent combinations of the –1309A/G and T111I alleles, but were all characterized by carrying the N396S variant.

Using the homozygous wildtypes for all three variants as the reference group, there was an indication that the risk of CHD was lower among carriers of the T111I variant who were wildtype homozygous at the two other loci (figure 12). When analyses were restricted to participants who were wildtype homozygous at the two other sites, the relative risk per T111I-allele was 0.9 (95% confidence interval, 0.8-1.0) in the meta-analysis.

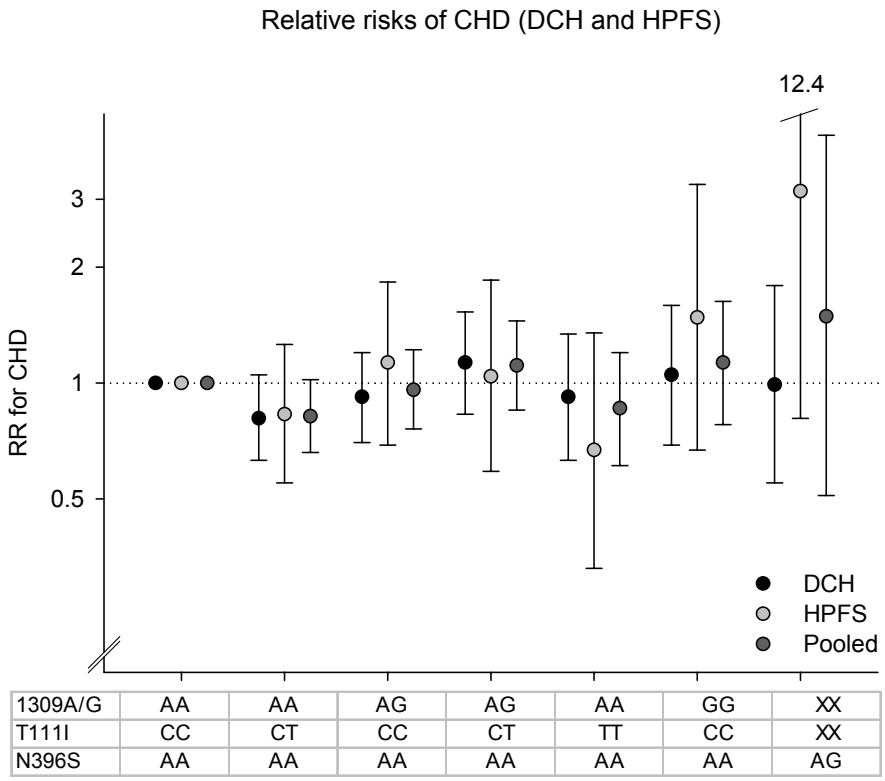

Figure 12. Relative risk of ACS and CHD in data from the DCH and HPFS separately and combined. Estimates shown according to genotype combinations for the *LIPG* variants. Estimates were adjusted age, smoking, alcohol, body mass index, and menopausal status (among women). Pooled results were from meta-analysis using random-effects

Gene-environment interactions between the *LIPG* variants and environmental determinants of HDL-cholesterol concentration were explored by creating a score summarizing the non-genetically determined HDL-cholesterol among the participants. No statistically significant interactions were observed using multiplicative tests of interaction.

DISCUSSION

Does variation in *ABCA1*, *CETP*, *LPL*, and *LIPG* contribute to risk of CHD?

The present thesis presents suggestive evidence for an association with CHD for a common promoter variant in *ABCA1*, the S447X variant in *LPL*, a possible effect-modification of the association with the TaqIB variant in *CETP* by alcohol, and a trend for an association with the T111I variant in *LIPG*.

Whether weak genetic associations reflect true associations or are flawed by methodological biases remains a concern for genetic epidemiologist.[40] Even biologically highly plausible associations should meet standards for assessment and interpretation of genetic association studies. The difficulties encountered in reproducing the results of genetic association studies could be attributable to several issues known to most epidemiologic studies: study design, statistical power, phenotypic heterogeneity, effect modification, and bias. To evaluate the credibility of the proposed relationships of genetic variation in *ABCA1*, *CETP*, *LPL,* and *LIPG* with CHD, a recently developed semi-quantitative index was used to view the results in the context of the existing literature. Ioannidis *et al*. proposed that the evidence for true genetic associations should be evaluated based on three indications of the cumulative literature.[40] The three levels are summarized in the table below:

Table 3. Guide to the assessment of evidence in genetic association studies.[40]

Criteria	Categories
Amount of evidence	A. Large scale evidence (sample size cases >1000)
	B. Moderate evidence (cases 100-1000)
	C. Little evidence
Replication	A. Extensive (includes a well conducted meta-analysis)
	B. Also includes a well-conducted meta-analysis with some methodological limitations or moderate between study heterogeneity
	C. No association, no replications, failed replications, scattered studies
Bias	A. Not likely to be population stratification, genotype and phenotype measurement error, or selective reported
	B. No obvious bias, but missing information
	C. Considerable potential for bias

In the next section, the main findings in the present thesis will be reviewed together with the current literature regarding the particular SNPs in relation to particularly CHD. A summary of the main findings and the assessment of the literature will follow in Table 4.

Amount of evidence and replication

The number of investigations and the number of cases investigated weighs heavily in the assessment of the cumulative evidence for an association. Among the SNPs that were assessed in the present thesis, only the TaqIB variant in *CETP* (included in paper II) and the *LPL* S447X variant (paper III) have been investigated in many candidate gene studies and meta-analyses have also been published for both variants.[41, 42] Nevertheless, reports on the association between the *CETP* TaqIB variant and CHD are rather inconsistent. In the meta-analysis by Boekholdt *et al*., the B2 variant was associated with a lower risk of CHD,[41] whereas the single largest case-control study of more than 4500 cases of MI from UK, which was not included in the meta-analysis, did not find an association between the TaqIB SNP and MI.[43] Because results from meta-analyses may not always be meaningful if there is a high degree of heterogeneity between studies, a single study with a high number of cases may be preferred over the pooled estimates obtained by combining several smaller studies.[40]

Although not all studies have observed a lower risk of CHD among carriers of the *LPL* S447X variant,[44, 45] evidence from several large and well-conducted studies have observed lower plasma triglycerides, higher HDL-cholesterol, and a lower risk of CHD among carriers of this variant.[42, 46, 47] The first meta-analysis supporting this was published in 1999,[48] and later this was confirmed in a second meta-analysis published in 2002.[49] The authors, however, noted that most evidence was based on male participants and that the result was not necessarily generalizeable to women.[49] Subsequent data including men and women, along with the data included in this thesis, have not suggested the association of the S447X variant and CHD to be gender-specific.[47, 50]

The promoter variant in *ABCA1* is not well-investigated and, so far, the literature regarding the role of this variant is inconsistent. In paper I we observed an allele-dependent lower risk of CHD, whereas for instance the Lipoprotein Coronary Atherosclerosis Study found the variant allele associated with a higher number of coronary lesions at baseline and no progression during a short 2.5 years follow-up.[51] The many underlying differences between our analysis of risk of incident CHD in generally healthy populations and the investigation of progression of atherosclerosis in patients with existing cardiovascular disease is just one example of the difficulties encountered when attempting to reconcile evidence from different studies. Whether or not prevalent atherosclerosis in study participants could affect the associations between SNPs and clinical endpoints deserves attention.

Two of the coding SNPs that were included in paper I (I883M and R1587K) have since been found to be associated with a modestly higher risk of CHD in a Danish study that included a prospective analysis with 1107 cases of ischemic heart disease and a case-control study of 900 cases.[52]

Paper IV is the first investigation of *LIPG* variants in relation to risk of CHD among Caucasians, yet the number of cases in the studies categorizes the report as a moderate amount of evidence.

Although our findings were consistent between the HPFS and DCH, they should be replicated in other populations.

Gene-environment interactions

As exemplified in several reviews, relatively few of the initially reported significant findings in candidate gene association studies of CHD have been replicated in later studies.[53, 54] Replication of a genetic association in different population samples implies that the genetic association is independent of environmental factors that may characterize the populations differently. Although this is an unlikely scenario, this is nevertheless the foundation of the assessment of evidence for genetic association when the potential for context dependent effects is ignored or when data are pooled in meta-analyses.

The findings in paper II and III suggest that underlying population characteristics such as alcohol intake and adiposity could play a role for differences between populations regarding the *CETP* TaqIB and *LPL* S447X variants. However, in small studies, there is reason to be concerned about findings of suggestive interaction because the risk of chance findings is high. It has been shown that gene-environment interactions that have been identified in small samples are often not replicated in later studies.[53] Attention to differences in unobserved population characteristics with different prevalence in diverse populations may be of interest to such comparisons.

Gene-environment interactions are evaluated because genes and environmental factors are likely to contribute to pathogenesis through complex interrelationships.[55] The exploration of interactions can be either hypothesis driven, like in the present thesis where interactions for which there was a strong biological rationale were examined, or statistical machinery can be used to detect unsuspected gene-environment combinations that predict disease.[56]

Investigating interactions can be a daunting task as the number of tested models quickly increases beyond the statistical power of even the largest studies. It has been proposed that in order to minimize such efforts, the exploration could be limited to the SNPs that are significantly associated with the outcome.[57] This methodology has been followed in several studies. For instance, Costanza *et al.* found that gene-environment interactions contributed very little to the prediction of variation in HDL-cholesterol when main effects of nine HDL-related polymorphisms and five environmental factors (body mass index, alcohol, smoking status, age, gender) were accounted for.[58] However, leaving out SNPs that are not independently associated with the outcome will ignore the genetic variants that may have pronounced effects under certain environmental conditions. As an example, the *CETP* TaqIB variant was not associated with risk of CHD in the NHSI and the HPFS in paper II. However, when analyses were stratified by alcohol intake, the association appeared stronger among those with a moderate alcohol intake (see also figure 9).

In paper III there was some evidence that the S447X variant in *LPL* may play a bigger role among individuals who were likely to have elevated triglycerides as indicated by the suggested interaction between S447X and obesity. However, this interaction was stronger in the smaller US

samples than in the Danish study. One hypothesis for this finding could be that obesity is a stronger marker of underlying dyslipidemia and other characteristics associated with the metabolic syndrome in the US studies than in the DCH study. Despite similar body mass index across the studies, the prevalence of hypercholesterolemia, type 2 diabetes, and hypertension at baseline was higher in the US sample than in the DCH study, where these characteristics were almost non-existing at baseline. Thus, if S447X plays a bigger role in conditions where increased lipolytic activity would help reduce the proatherogenic effects of such conditions, this might be one explanation for the lack of interaction in the Danish study.

The study populations used in the present thesis did not have enough cases to infer strong conclusions regarding gene-environment interactions. It has been argued that only sample sizes of 500,000 or more will provide a useful study design for the exploration of gene-environment interactions.[59] However, the ability of even such large studies to explore interactions will depend on the number of cases they accumulate. Some of the arguments for initiating the UK BioBank project include that problems associated with pooling data for meta-analyses, such as publication bias and between study heterogeneity, are avoided. It will be interesting to follow this project to see if new and biologically relevant interactions will be elucidated in this large study.

Any investigation of gene-environment interactions ultimately depends on the quality of the information obtained on the exposures.

Bias

Selection bias

Selection bias can be a problem in cohort studies if loss to follow-up differs according to exposure.[60] In the Danish and US studies, loss to follow-up was minimal and unlikely to depend on genotype. However, in the prospective analyses of incident CHD participants who died from other causes were censored. While the causes of these deaths were not considered in these studies there is a possibility that some of the endpoints may be related to CHD. An example could be ischemic stroke, which shares clinical characteristics with ischemic heart disease. If the studied genotypes were associated with the censoring this could lead to selection bias in the association between genotype and CHD. However, as few of the censorings in these studies are likely to be due to death from stroke or other cardiovascular-related diseases, such a bias should be minimal.

In the literature, many of the genetic association studies are based on case-control studies that are not nested within well-defined cohorts. Survival bias is a special case of selection bias that is important in case-control studies where only prevalent and non-fatal cases are included. By only examining those who have survived their MI event, it is difficult to distinguish between the effect of the genotype on disease incidence and disease duration and its progression. Although it may not seem likely that genetic variants affect survival after MI differently than MI incidence, ignoring the cases that are fatal within few days or weeks could lead to erroneous conclusions. For instance, if a genetic variant is observed more frequently among MI survivors than controls it remains a

possibility that genotype plays a role for survival and a better prognosis after the incidence. This may play a role when comparing our prospectively designed studies to the case-control literature.

Information bias

Genotype could be misclassified due to laboratory errors during genotyping. However, quality controls were used and except for very minor deviations, the genotypes conformed to the law of Hardy-Weinberg equilibrium. Furthermore, this would not lead to bias as any misclassification is likely to be non-differential according to the CHD endpoint because the technicians were blinded to case status. Similarly, the prospective nature of the studies included in this thesis excludes the risk of recall bias in the assessment of environmental factors.

The risk of information bias in prospective studies is mostly related to misclassification of endpoints according to exposure. As all cases were assessed independently of information on exposures, this is unlikely to be a problem in the studies used for this thesis.

Confounding

A genetic association may be due to a strong association between the investigated SNP and a nearby causal variant in the same or a different gene close by. Because such linkage disequilibrium decays over distance, the strength of the association for indirect markers may be considerable weaker than investigations based on the true causal variant.[61]

Second, there might be a strong association between the genetic variant and some disease-related characteristic that defines sub-populations within the study population (the most likely scenario being different founder populations).[62] Population stratification is unlikely to explain the observations from the cohorts of US Nurses and Health Professionals where only few of the study participants were of non-Caucasian origin (<2%). In the DCH, participants were only invited to participate if they were born in Denmark. Although, there was no question with regard to ethnicity, we estimate that only very few participants had non-Caucasian parents as they were born in 1943 or earlier.

Genotype is likely to be randomly distributed with regard to behavioral and socioeconomic characteristics that are associated with cardiovascular risk (such as income, education, and lifestyle factors). Thus, confounding is limited in gene association studies.[63] In most of the analyses in papers I-IV important lifestyle factors were included in the multivariate models in order to reduce some of the heterogeneity between the participants. However, with regard to the investigation of gene-environment interactions, there is also a risk that participation could differ among individuals according to certain lifestyle factors that may be important causes of CHD. For instance, moderate alcohol drinkers who chose to participate in the studies may have a different lifestyle and socioeconomic position than moderate alcohol drinkers who chose not to participate. If such related characteristics were the cause of a lower incidence of CHD among the moderate alcohol drinkers who were included in the studies, as opposed to moderate drinkers who did not participate, then it would be important to include such confounders in the multivariate model.[64]

Biologic plausibility

The four candidate genes encode proteins that play major roles in the human HDL metabolism. The genetic variants in *ABCA1*, *CETP*, and *LPL* were selected based on their previously reported significant associations with HDL-cholesterol and CHD-risk. The TaqIB variant in *CETP* is located in an intron and it has been suggested that its association with HDL-cholesterol can be explained by linkage to at least one other promoter variant.[65, 66] Although the other SNPs were located in regions that render them more likely to be functional (promoter and coding regions), these studies were limited in their lack of demonstration of functionality.

The *LIPG* variants were identified by sequencing of the entire gene among individuals in the extreme tails of the HDL-cholesterol distribution in a general population sample. This may increase the likelihood for identifying rare variants with greater phenotypic effects. Much work is still awaited for the further evaluation of the functional implications of these SNPs on gene expression and possibly gene-transfer studies of different *LIPG* variants in animals would be interesting.

Summary of the assessment of evidence for the genetic associations

The above assessment of the findings in this thesis is summarized in the table below. One row is added with a few sentences on the supportive evidence found in the literature. In the last row, the credibility of the associations is summarized by combining the scores for the three categories, as suggested by Ioannidis *et al*.[40]

One major concern when comparing new gene association studies to the existing literature is the risk of publication bias. Such publication or reporting bias may most likely be against studies with null associations. The 'Human Genome Epidemiology Network' (HuGENet) was initiated by a group of researchers in 1998 with the aim to perform systematic meta-analyses and reviews by also including unpublished data from collaborators,[67] in order to avoid the potential for publication bias at least to some extent. Unfortunately, so far, there are no HuGENet meta-analyses of the variants included in this thesis. Thus, the extent of unpublished data is unknown.

Table 4. Summary of main findings in the present thesis and assessment of the cumulative evidence based on the current literature

Gene:SNP / **Criteria**	**Paper I** **ABCA1**: 565T/C (-191G/C)	**Paper II** **CETP**: TaqIB	**Paper III** **LPL**: S447X	**Paper IV** **LIPG**: T111I
Main finding	Allele-dependent lower risk of CHD, no association with HDL-cholesterol	No overall association with CHD, strong association with HDL-cholesterol. More pronounced CHD and HDL-cholesterol findings among moderate alcohol consumers	Variant carriers had higher HDL-cholesterol, and a lower risk of CHD	Suggestive association with CHD
Amount of evidence	B. Moderate	A. Large (for main effects)	A. Large	B. Moderate
Replication	C. Scattered	B. Includes meta-analyses for main effect, but inconsistent findings. C. Scattered for interaction on CHD-risk	A. Includes meta-analyses	C. No replication in a well-designed study
Supporting studies / Functional data	Four studies support a role of this promoter site in CHD-risk. However, not always the same risk allele.[51, 68-70] / Gene expression profile in human plaques suggests opposite allele is the risk allele[69]	Main effect on HDL-cholesterol well-established, inconsistent findings for CHD risk.[41, 43] Three studies support potential modification by alcohol intake.[41, 65, 71] / TaqIB variant consistently associated with lower CETP concentration and activity and higher HDL-cholesterol[72]	Main effect on HDL-cholesterol well-established.[42, 73] Lower risk of CHD among carriers also more or less consistent, but some population heterogeneity.[42, 46, 73] / Variant associated with greater LPL activity[74]	Not consistent[75-77] / No functional studies of variant yet
Bias	A/B. Not likely Possible survival bias in patient studies	A/B. Possible reporting/publication bias	A. Not likely	A/B. Not likely. But the only two published studies were in Asian patient samples
Summary score: 'credibility' of findings	Weak	HDL-cholesterol: Moderate CHD, interaction: Weak	Moderate	Weak

In brief, the existing candidate gene association studies show moderate support for a role of the *CETP* variant in relation to HDL-cholesterol concentration and the *LPL* variant in relation to both levels of HDL-cholesterol and triglycerides and the risk of CHD. Of note, the findings with regard to HDL-cholesterol are more well-investigated. However, it is of interest to note that the SNPs that were associated with CHD were not strongly associated with HDL-cholesterol, and vice versa.

The four studies appear to be generally valid and well-suited for the investigation of SNPs in relation to both plasma lipids and risk of CHD. However, the studies also elucidate some of the limitations of observational epidemiologic studies and the candidate gene - candidate SNP approach. Some considerations that may be of value for future explorations of the role of HDL metabolism in relation to risk of CHD will be discussed in the next section.

Plasma concentration of HDL-cholesterol versus HDL particle function

The use of HDL-cholesterol as an intermediate phenotype for CHD relies on both evidence for its implication in the biological pathway and on epidemiological data showing an inverse association between HDL-cholesterol and CHD. When there is reason to believe that a modifiable phenotype, such as HDL-cholesterol, mediates the association between a gene and CHD it can be useful to combine the evidence from all three associations (gene→HDL, HDL→CHD, and gene→CHD) to assess the causality of the relationship.[78] By exploiting the random segregation of alleles at the time of gamete formation, researchers investigate disease risk according to genetically determined 'exposure' to high or low levels of the intermediate trait without risk of confounding from behavioral lifestyle factors and other biochemical traits. In this Mendelian Randomization approach, it follows that a causal relationship between HDL-cholesterol and CHD could be inferred if the risk of disease conferred by the genetic variant were consistent with the risk expected from its association with higher or lower HDL-cholesterol concentration.[78]

One of the major issues for this approach is the identification of a genetic variant with a strong association with HDL-cholesterol concentration, which ideally would only be associated with risk of CHD through the effect mediated by HDL-cholesterol. In the present thesis, the associations between genetic variation in HDL-related genes and CHD were generally not related to their association with HDL-cholesterol concentration. This finding is consistent with many other candidate gene studies,[79, 80] and the 9p21 loci that was recently identified as the single most strong predictor of CHD in genome-wide association studies (GWAS),[81-83] was also not associated with plasma lipids and lipoproteins.[81]

This cannot be interpreted as a refutation of the potential relationship between HDL and CHD, but clearly there is not evidence to support that any single genetic variation is of major importance for HDL-cholesterol concentration and the genetic variants must be associated with risk of CHD through other mechanistic pathways. The situation where genetic variants have pleiotropic effects, i.e. where their association with disease risk may be explained through several pathways, is not suitable for the use of the Mendelian Randomization approach.[84]

Developmental compensation has also been mentioned as another possibility for lack of relationship between genetic variants and an intermediate phenotype that is important to a clinical endpoint.[63] Individuals who have lived with a genetic variant influencing levels of HDL-cholesterol their entire life could have developed compensatory mechanisms that buffer against a potential health risk associated with very low HDL-cholesterol. The genetic similarity between the different lipases could support some redundancy between them,[85] and alternative pathways for cholesterol efflux may also explain that individuals with Tangiers disease, caused by rare *ABCA1* mutations, do not carry as high a risk of CHD as would be expected from their low HDL-cholesterol.[86] However, individuals who were born with genetic mutations of major influence on intermediate traits, for which no compensatory mechanisms exists, probably would not survive to be included in the observational studies.

In addition, GWAS have identified numerous loci that are associated with HDL-cholesterol concentration that were not identified in the GWAS of CHD.[46, 87] Moreover, sequencing of HDL-related candidate genes in individuals from the general population who are at the extremes of the HDL-cholesterol concentration distribution has identified multiple rare variants.[88, 89] Thus, variation in HDL-cholesterol levels in the general population is likely to be explained by a combination of rare variants with large phenotypic effects and common variants with small effects.

Despite the epidemiologic associations between HDL-cholesterol concentration and CHD, evidence for an antiatherogenic role of HDL-cholesterol is lacking and it has been questioned if concentration of cholesterol in HDL adequately reflects the properties of the HDL particle that may protect against CHD.[90, 91]

Some of the cardiovascular-related functions of the HDL particle were mentioned in the introduction. The genes that were investigated in the present thesis are mainly related to two of these aspects; the reverse cholesterol transport pathway and the modification of the HDL particle. ABCA1 and CETP mainly function in the reverse cholesterol transport pathway where the HDL particle mediates the return of cholesterol to the liver. Because this pathway results in removal of cholesterol from the circulation, it seems reasonable that high levels of HDL-cholesterol would indicate an efficient and beneficial metabolism of cholesterol. However, as most of the cholesterol in HDL is derived from the liver,[92] levels of circulating HDL-cholesterol does not reflect the efflux of cholesterol from peripheral cells such as macrophages well. Macrophage-specific depletion of *ABCA1* has been associated with pronounced atherosclerosis without affecting HDL-cholesterol concentration in mice.[93] Thus, although efflux of cholesterol from macrophages is probably one of the most important roles of the HDL particle, it is not a major determinant of HDL-cholesterol concentration.

The observation that *CETP* deficient individuals had strikingly high HDL-cholesterol levels prompted the development of the CETP inhibitor, Torcetrapib, that raise HDL-cholesterol markedly.[94] However, a recent trial with Torcetrapib was stopped early because there was a higher mortality in the treated group from both cardiovascular and non-cardiovascular events, despite a substantial elevation in HDL-cholesterol and a reduction in triglycerides and LDL-cholesterol.[15] Interestingly, genetic association studies of the TaqIB variant in *CETP* yielded inconsistent results

with regard to the risk of CHD,[41, 43, 80] despite observed higher HDL-cholesterol levels among carriers.[41, 65, 80, 95] Current hypotheses for the mechanisms behind the failure of Torcetrapib include off-target effects of the molecule (not related to inhibition of CETP; for instance the observation of a modest increase in systolic blood pressure in the treated group),[15] reduced rate of return of cholesterol to the liver for excretion,[96] that extremely high levels of HDL-cholesterol, particularly high levels of HDL particles that are large in size, is proatherogenic,[97] and that HDL particles produced in disease states characterized by systemic inflammation may have proinflammatory and proatherogenic effects.[98] Currently, clinical trials of other CETP inhibitors are ongoing which may reveal if the deleterious effect observed in the Torcetrapib trial are attributable to the compound per se or to the principle of CETP inhibition.[99]

These observations all suggest that HDL-cholesterol concentration does not have a straightforward interpretation and other indicators of the efficiency of cholesterol removal from macrophages and smooth muscle cells in atheromas are needed. Cholesterol excretion in feces has been used as a measure of reverse cholesterol efflux in humans.[100] However, this approach does not specifically address the cholesterol efflux from macrophages, which may be particularly important. Assays to assess cholesterol efflux capacity from different cell types in human serum have been developed using for example labeled cholesterol,[101] however, so far they have not been widely used except for smaller clinical studies.[102] Such developments are of scientific interest for deeper insight into the potential metabolic regulators of the cholesterol-efflux promoting capacities of HDL particles.

LPL and EL modulate the composition of the HDL particle by influencing the content of phospholipids and triglyceride and its association with apolipoproteins. The S447X variant has been intensely investigated and beneficial associations with both plasma levels of HDL-cholesterol and triglycerides, and with risk of CHD have been consistent in most of the larger studies. Because there is evidence that this variant encodes a LPL protein with enhanced lipolytic activity, [74] there is some interest in using this variant for gene-therapy in LPL-deficient individuals.[103, 104] In the future, this may also be developed as a novel therapy for dyslipidemia. Suggestive findings from Paper III indicate that future trials may show greater treatment effect if they were performed among overweight individuals.

To further elucidate how genetic variation may affect the composition of the HDL particle, future studies may explore the associations between genetic variants and HDL subclasses.

Finally, whether the strong epidemiologic observation between low HDL-cholesterol and high risk of CHD may reflect the tight, inverse association between HDL-cholesterol and levels of atherogenic remnant lipoproteins remains up for debate.[105] Remnant lipoproteins are the result of lipolysis of chylomicrons and VLDL in the circulation, and these cholesterol-rich low-density lipoproteins are the main source of the initial cholesterol deposition in the arterial wall. Whether or not HDL is able to remove cholesterol from areas of accumulation remains a topic for debate, and more intervention studies are needed to elucidate if HDL infusion can lead to regression in atherosclerosis.[106, 107]

CONCLUSIONS AND PERSPECTIVE

In conclusion, these candidate gene association studies show moderate support for a role of the selected genetic variants in relation to HDL-cholesterol and risk of CHD. Overall, the genetic variants that were associated with CHD were generally not associated with HDL-cholesterol concentration.

The studies highlight some of the complexities in genetic association studies in relation to common disease endpoints. Measurements of HDL-cholesterol in plasma probably does not reflect some of the important roles of the HDL particle, and improved measures of tissue-specific cholesterol efflux are of great scientific interest for the further exploration of the role HDL metabolism to atherogenesis using the genetic epidemiologic approach.

Exploration of context-dependent effects is important for improved understanding of the complex etiology of multifactorial disease such as CHD. However, bigger studies and improved methodology seems necessary before this area can move forward.

Can we use genetic epidemiology to study the role of HDL in relation to CHD?

Can any recommendations for future use of genetic epidemiology for the exploration of biological mechanisms behind the association between HDL and CHD be based on the four studies?

To explore different aspects of the HDL particle, several different measures may be needed for an adequate reflection of the many functionalities of the HDL particle. This could include measures of serum ability to promote cholesterol efflux, antioxidant effects, associations with apolipoproteins, or other.

Another approach would be to explore biomarkers that may reflect some of the key functions of the HDL particle. This could be activity of lipoprotein lipase or levels of apolipoproteins. For novel cardiovascular biomarkers, genetic epidemiologist may find the application of the Mendelian Randomization approach useful in the search of evidence for causal associations. For instance, an ELISA assay has recently been developed to measure concentration of endothelial lipase in circulation.[108] It may be fruitful to apply both the *LIPG* SNPs and endothelial lipase expression to the risk of CHD with the Mendelian Randomization approach. This may be particularly helpful as the investigation of endothelial lipase expression in relation to CHD is difficult because its concentration is highly correlated with other cardiovascular risk factors such as adiposity and markers of inflammation.[109]

For other newly identified proteins that may play a role in the efficiency of cholesterol removal from especially macrophages, it may also be interesting to primarily asses their role in relation to their own gene expression profile instead of HDL-cholesterol.

The selection of appropriate variants remains an area of concern for this approach, however. Using HapMap to select tagSNPs may be one approach to ensure that the entire genetic region is covered, however, this does not give good insight into the potential functional variants. SNP-selecting tools have been developed that make it possible to give high priority for the selection of regulatory or coding region variants.[110]

Although SNPs are the most common cause of variation in the human genome, future work also needs to consider other structural variants, such as copy number variants, rare mutations, and tandem repeats. Further, candidate gene association studies ignore that genes are expressed at different levels and in different tissues, which is not predicted by the DNA sequence. A great challenge awaits in the understanding of heritable modification of DNA such as methylation and histone modification that regulates gene expression.[111] Such epigenetic modifications change the expression of genes without modifying the DNA sequence. Thus, genetic variants would still be successfully genotyped even though the genomic region may not be expressed.

Candidate gene studies in an era of genome-wide studies

Recently, high-throughput technology has made testing of hundreds of thousands of genetic variants for associations with common disease phenotypes feasible. Since the summer 2007, five GWAS of cardiovascular disease outcomes that used 100000 genetic markers or more have been published.[81-83, 112, 113] Although the chips used for GWAS cover less than 10% of the known SNPs in the human genome, the selected SNPs are in strong linkage disequilibrium with up to 96% of all SNPs and therefore they are assumed to provide a high extent of coverage of the human genome. These studies all consistently reported a region located on chromosome 9 (9p21) to be strongly associated with the cardiovascular endpoints.

The most obvious difference between GWAS and candidate gene association studies is that GWAS provides an opportunity to identify new, putative causal, genetic variants of diseases by hypotheses-free testing of numerous loci selected based on HapMap or at random. In comparison candidate gene studies relies heavily on biology. Identification of a strong association with CHD for the 9p21 locus in the recent GWAS does not provide any clues with regard to the potential disease-producing mechanisms. Post hoc hypotheses for the potential role of the 9p21 site were based on speculations about the known molecular role of the close by genes.[81] However, the regulators of expression of a certain gene may be located far away and therefore it may not necessarily be the gene located closest to the site of strong association with the endpoint that explains the observations.

Related to the findings presented in this thesis, it is intriguing that these first GWAS with extensive coverage of the genome did not report any evidence for associations with markers located within the intensely investigated candidate genes. Although the methodology for the assessment of results from GWAS is still developing and new ways that allow researchers to address the huge amounts of data points that sit just below the chosen cut off for statistical significance are urgently

needed, these findings obviously do not replicate the findings using the candidate gene/candidate SNP approach.

At present, increasing use of systems biology approaches have been shown to provide a useful framework for systematic identification of true genetic causes of complex diseases such as CHD by elucidating how genetic variants may affect the atherosclerotic process through their effects on transcription, gene expression, and protein interactions.[114] Network analyses provide the ability to identify genes that affect several different phenotypic characteristics of a disease by including data from all levels of the physiologic process. This may be useful for the identification of potential drug targets, as treatment of e.g. dyslipidemia may initially be focused on the reduction of LDL-cholesterol, however other phenotypes such as body weight, HDL-cholesterol, blood pressure, inflammation, and glucose tolerance may also be affected. Interventions should affect all of these traits favorably. In relation to the genes investigated in the present papers, it is possible that the application of such extensive evaluation of downstream targets would have discouraged the development of CETP inhibitors.

If the GWAS hold promise they are likely to make a significant contribution to the discovery of new pathways involved in disease pathogenesis. The goals of such efforts are the development of novel drugs, personalized medicine, and targeted lifestyle interventions.

However, it is evident that changes in environmental factors explain a great part of the fluctuations in phenotypes, such as blood lipid concentrations and CHD incidence, over the past few decades and that genetic variation contributes little in comparison. As also demonstrated in this thesis, identification of strong gene-environment interactions in relation to common diseases is difficult and so far probably limited by poor quality of data on environmental exposures, as well as lacking methodological developments.

The complexity and challenges that characterize the field of genetic epidemiology in this exciting era does not mean that public health recommendations for cardiovascular disease prevention need to be put on hold. Despite current excitement about the recent release of publicly available genome-wide tests, physician advice to those who are tested for genetically determined elevated risk of CHD remains the same as the primary prevention strategy applied to the general population: abstaining from smoking, being physically active, and consuming a healthy diet. Nevertheless, continued intense research efforts into the secrets of the human genome paves the way for improved understanding of the mechanisms behind disease development and greater insight into underlying differences between individuals that may modify the observed associations behind important environmental risk factors and disease risk. This exciting era gives hope for improved and directed prevention of common diseases.

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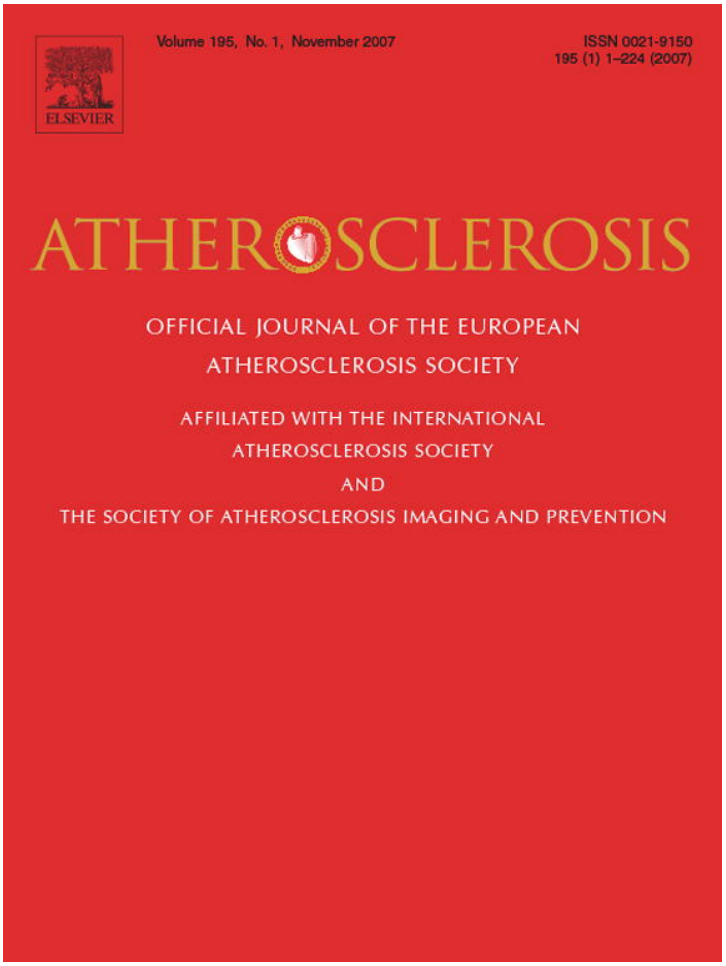

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Common genetic variation in the ATP-binding cassette transporter A1, plasma lipids, and risk of coronary heart disease☆

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Abstract

The ATP-binding cassette transporter A-1 (ABCA1) regulates cholesterol efflux from cells and is involved in high-density lipoprotein (HDL) metabolism and atherogenesis. We investigated whether common *ABCA1* variants, previously reported to have phenotypic effects in humans, were associated with plasma lipids and CHD in a prospective study of coronary heart disease (CHD) in healthy women.

Three polymorphisms in the promoter region (−565C/T, −191G/C, and −17C/G) and two in the coding region (I883M and R1587K) were genotyped in the Nurses' Health Study. During 8 years of follow-up, 249 incident cases of CHD were identified and matched to controls (1:2) on age and smoking.

The I883M variant was associated with higher HDL-cholesterol levels among younger women. Nearly complete linkage disequilibrium was observed between −565C/T and −191G/C and their less common alleles predicted a lower risk of CHD (odds ratio of CHD per −191C allele: 0.8; 95% CI, 0.6–1.0). Neither the −17C/G SNP nor the 2 the coding polymorphisms were associated with risk of CHD. The −565C/T and the −191G/C variants were inversely associated with risk of CHD among healthy women, without pronounced effects on plasma lipids.

Keywords: Reverse cholesterol transport; Genetic epidemiology; ATP-binding cassette transporter A1; Coronary heart disease; Plasma lipids

1. Introduction

Concentration of high-density lipoprotein cholesterol (HDL-C) is inversely associated with risk of coronary heart disease (CHD) [1]. An important mechanism underlying the anti-atherogenic properties of HDL-C is its role in reverse cholesterol transport, the pathway that facilitates the transfer of cholesterol from peripheral tissues back to the liver [2]. The ATP-binding cassette transporter A1 (ABCA1) is a large trans-membrane protein that mediates the cellular efflux of cholesterol and phospholipids to lipid poor HDL apolipoproteins [3]. Homozygosity for mutations in *ABCA1* causes Tangier disease, a rare disorder characterised by HDL-C deficiency and increased susceptibility for atherosclerosis [4]. The gene encoding ABCA1 encompasses 50 exons and more than 100 mutations and single nucleotide polymorphisms (SNPs) have been identified [5].

The relation of more common genetic variants in *ABCA1* with lipid concentrations or risk of CHD remains less clear. Common polymorphisms in *ABCA1* have been inconsistently

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related to plasma lipids or cardiovascular disease [6–16]. In one screening study, 26 SNPs in the promoter and coding region were identified and subsequently genotyped in cases and controls of myocardial infarction [7]. Only the R1587K and the −565C/T promoter variant were found associated with apolipoprotein AI levels, the R219K SNP was associated with risk of myocardial infarction, and none of the 16 identified promoter SNPs were significantly associated with risk [7]. However, this study did not include two common promoter variants (−191G/C and −17C/G) that may influence risk of recurrent cardiovascular events [12]. As the majority of studies that have included a clinical CHD endpoint have been carried out in populations with existing cardiovascular disease [8,9,12–15], the prospective associations of known, common variants in *ABCA1* on plasma lipid concentrations and risk of CHD in generally healthy populations remains unclear.

Furthermore, interactions of environmental factors with *ABCA1* polymorphisms have not been thoroughly explored. For example, an interaction with cigarette smoking has been reported for some *ABCA1* variants [7,13], but most studies have not addressed the potential interaction between *ABCA1* polymorphisms and other factors that are known to influence HDL-levels and risk of CHD, such as age, overweight, alcohol, smoking, postmenopausal hormone use, and dietary fat intake.

The objective of our study was to investigate common SNPs in the coding and promoter regions of *ABCA1* (MAF > 10%) that have been inconsistently related to cardiovascular disease endpoints in previous studies. In total, three polymorphisms in the promoter: −565C/T (also referred to as −477C/T) [13,14], −191G/C (also known as C1176G) [17], and −17C/G (G1355C) [17], and two polymorphisms in the coding region: I883M in exon 18 (A2589G and I823M) [7,9], and R1587K in exon 35 [7] were genotyped in two well-characterized populations of female health professionals; the Nurses Health Studies I and II. We also sought to examine interactions with potentially important environmental exposures.

2. Material and methods

2.1. Study design and population

The Nurses' Health Studies I and II (NHSI and NHSII) are prospective cohort studies among U.S. registered female nurses, respectively, involving 121,700 participants 30–55 years of age at baseline in 1976 and 116,671 participants between 25 and 42 years old at baseline in 1989. The women have received follow-up questionnaires biennially to update information on health and disease and information about diet is obtained through a food frequency questionnaire every 4 years [18]. The validity and reproducibility of the collected data have been reported previously [19]. Between 1989 and 1990, a blood sample was requested from all active participants in NHSI and collected from 32,826 women. Similarly, blood samples were obtained between 1996 and 1998 from 29,614 participants in NHSII. Women who gave blood were similar to those in the overall cohorts.

Among NHSI participants who provided blood samples and who were without cardiovascular disease or cancer in 1990, we performed a nested case–control study of CHD risk. Study physicians blinded to participants' exposure status confirmed nonfatal myocardial infarction if it met World Health Organization criteria (symptoms and either diagnostic electrocardiographic changes or elevated cardiac enzymes). Fatal CHD was confirmed by hospital records or on autopsy, or if CHD was the underlying and most plausible cause, and if evidence of previous CHD was available. We confirmed 212 women with incident nonfatal myocardial infarction (MI) and 37 with fatal CHD between blood draw and June 30, 1998. We randomly selected controls in a 2:1 ratio using risk-set sampling [20], and matching on age, smoking, fasting status, and month of blood return.

In cross-sectional analyses of the NHSII, blood samples were selected from premenopausal women who collected their blood during the luteal phase of their menstrual cycle and who were not using exogenous hormones. We randomly selected 473 participants free of cardiovascular disease, diabetes, gastrointestinal illness, or malignancy within strata of different patterns of self-reported alcohol consumption (the original selection of this subset was to investigate alcohol drinking patterns and novel biomarkers of CHD) [21,22].

2.2. Information on genetic variants, plasma lipids and markers of inflammation

Details on methods for genotyping and measurement of biochemical markers have been submitted as an online supplement (see Supplement I). Plasma lipids available in both study populations included total cholesterol, HDL-C, low-density lipoprotein cholesterol (LDL-C), and triglycerides. Measured markers of inflammation included: interleukin-6, C-reactive protein, soluble tumor necrosis factor-α receptors-1 and -2, and fibrinogen.

Primers and probes used were designed by Applied Biosystems: −565C/T (rs2422493), −191G/C (rs1800976), −17C/G (rs2740483), I883M (rs4149313), and R1587K (rs2230808). Replicate quality control samples were included and genotyped with 100% concordance. A few subjects could not be genotyped with this platform; genotype data were available for 465 women from NHSII and 745 women from NHSI (249 cases and 496 controls).

2.3. Statistical analysis

Allele frequencies were estimated and departure from Hardy-Weinberg equilibrium was tested among NHSI-controls and the NHSII participants separately.

We first evaluated *ABCA1* and plasma lipids. The associations between each *ABCA1* polymorphism and plasma

lipids were initially evaluated separately among NHSI controls and NHSII participants and then pooled when we did not observe discrepancies. We used regression models with robust variance estimation to accommodate deviations from normality (Proc MIXED in SAS) [23]. All models were adjusted for age (5-year intervals), smoking (never, current, former), BMI (<20, 20–24.9, 25–29.9, 30–34.9, ≥35 kg/m^2), alcohol intake (non-drinker, 0–4.9, 5–14.9, 15–29.9, ≥30 g/day), hypertension, parental history of CHD before the age of 60, diabetes, and menopausal status (premenopausal, postmenopausal without hormone replacement therapy [HRT] use, postmenopausal with current HRT use, postmenopausal with past HRT use). Because we identified effect modification by age, we present results stratified by age 55.

We next analyzed the association between each *ABCA1* polymorphism and risk of CHD in the NHSI. We used both conditional and unconditional logistic regression adjusting for the matching factors; because results were similar, only unconditional results are shown. Adjusted models included the covariates noted above. We evaluated recessive, dominant, and codominant (evaluating the risk per each additional variant allele) modes of inheritance. To evaluate the potential interaction between *ABCA1* SNPs and the other risk factors, we examined stratified associations in unconditional logistic regression models and tested significance by including separate interaction terms.

Finally, we evaluated the contribution of haplotypes in *ABCA1*. Extent of linkage disequilibrium (LD) between individual polymorphisms was expressed as Lewontin's D' and r^2. Frequencies of the most common (>1% frequency) haplotypes were estimated using the E–M algorithm (SAS/Genetics package). Participants with missing genotypic data at one or more polymorphic sites were excluded from the haplotype analyses (NHSII: $n = 69$; NHSI: $n = 57$). The association between *ABCA1* gene haplotypes, plasma lipids, and CHD-risk was calculated by imputing subject-specific haplotypes for the regression analysis [24,25]. The likelihood ratio test was used to calculate the global p-value comparing the model with haplotypes to the model without. All analyses were performed using SAS 9 (SAS Institute Inc., Cary, NC).

3. Results

The mean age of the NHSI participants was 60 years, and the mean age of the NHSII participants was 42 years. Expected associations were observed when comparing baseline characteristics of cases and controls in NHSI (Table 1).

The *ABCA1* allele frequencies were identical among NHSI controls and NHSII participants. In these healthy women, the frequency of the −565T allele was 0.48, the −191C allele 0.47, the −17G allele 0.30, the 883M allele 0.14, and the 1587K allele 0.25. There were no significant departures from Hardy–Weinberg equilibrium. Genotype and allele frequencies are shown among cases and controls in NHSI in Table 2.

3.1. Associations between ABCA1 polymorphisms and plasma lipid concentrations

Overall, the associations with plasma lipids appeared stronger in the younger participants (Table 3). Compared to wildtype, the 883M variant was associated with higher HDL-C levels and the 1587K allele with lower triglyceride concentrations. These relationships were not seen among older participants (test for interactions between age and

Table 1
Baseline characteristics of Nurses' Health Study II participants and Nurses' Health Study I cases and matched[a] event free controls

	NHSII	NHSI cases	NHSI controls
N	465	249	496
Age (years[b])	43 (40–45)	62 (56–65)	62 (56–65)
Caucasian, *n* (%)	450 (98)	246 (99)	491 (99)
Fasting at blood draw, *n* (%)	465 (100)	173 (69)	325 (66)
Postmenopausal, *n* (%)	0 (0)	214 (86)	413 (84)
History of diabetes, *n* (%)	0 (0)	49 (20)	33 (7)
History of hypercholesterolemia, *n* (%)	44 (9)	133 (53)	195 (39)
History of hypertension, *n* (%)	22 (5)	143 (57)	144 (29)
Current smoker (%)	33 (7)	80 (32)	156 (32)
Parental history of CHD before age 60, *n* (%)	76 (16)	54 (22)	62 (13)
Overweight (BMI >25 kg/m^2), *n* (%)	150 (32)	137 (55)	224 (45)
Alcohol (g/day)	10 (2–15)	1 (0–4)	2 (0–9)
Plasma levels			
Cholesterol (mmol/L)	5.0 (4.5–5.5)	6.1 (5.5–6.7)	5.8 (5.2–6.6)
Triglycerides (mmol/L[c])	0.8 (0.6–1.1)	1.5 (1.1–2.3)	1.2 (0.8–1.7)
HDL-C (mmol/L)	1.7 (1.5–2.0)	1.3 (1.1–1.6)	1.5 (1.2–1.8)
LDL-C (mmol/L)	2.8 (2.3–3.2)	3.7 (3.1–4.3)	3.4 (2.7–4.0)

[a] Matching criteria in NHSI were age, smoking status, date of blood drawing, and fasting status.
[b] All continuous characteristics are presented as medians with interquartile ranges.
[c] Among fasting participants only (NHSII, $n = 465$; NHSI cases, $n = 164$; NHSI controls, $n = 309$).

Table 2
ABCA1 Genotype and allele distribution among cases and controls in the Nurses' Health Study I

ABCA1 SNP	*n*	Cases ($n=249$)			*n*	Controls ($n=494$)			B-allele frequency		p^*
		AA	AB	BB		AA	AB	BB	Cases	Controls	
−565C/T	243	CC	CT	TT	484	CC	CT	TT	T-allele frequency		
%		35	46	19		29	46	25	0.42	0.48	0.03
−191G/C	247	GG	GC	CC	492	GG	GC	CC	C-allele frequency		
%		35	47	18		30	46	24	0.42	0.47	0.06
−17C/G	235	CC	CG	GG	477	CC	CG	GG	G-allele frequency		
%		45	45	10		49	42	9	0.32	0.30	0.4
I883M	243	AA	AG	GG	482	AA	AG	GG	M-allele frequency		
%		77	21	2		73	25	2	0.13	0.14	0.6
R1587K	246	GG	GA	AA	490	GG	GA	AA	K-allele frequency		
%		58	37	5		56	38	6	0.23	0.25	0.6

Note: ABCA1 genotype frequencies in NHSII were as reported for NHSI controls.
* *p*: chi^2-test of differences in B-allele frequencies between NHSI cases and controls.

I883M: $p=0.004$, and R1587K: $p=0.3$). Other SNPs did not appear to be associated with changes in measured lipid levels.

3.2. Associations between ABCA1 polymorphisms and concentrations of markers of inflammation

We did not observe any strong associations between the ABCA1 polymorphisms and Interleukin-6, CRP, TNFR-1, TNFR-2, or fibrinogen. A tendency for lower concentrations of the inflammatory markers were observed among carriers of the −565T and −191C alleles, however only among women greater than 55 years of age (see Supplementary material).

3.3. Associations between ABCA1 polymorphisms and risk of coronary heart disease

Compared to cases, the −565T and −191C alleles occurred more frequently among the healthy controls, whereas allele frequencies of the other variants did not differ between cases and controls (Table 2).

Table 4 shows the primary results for individual SNPs and adjusted risk of CHD. We did not observe significant associations with CHD for the −17C/G polymorphism or the two polymorphisms in the coding region. The −565C/T and −191G/C polymorphisms were in near complete LD and the −565T allele co-segregated with the −191C allele (Table 5). Both variants were associated with a lower risk of CHD in a codominant fashion. The relative risk of CHD was 0.8 (95% CI, 0.6–1.0) for each additional −565T or −191C allele. The −17C/G polymorphism was in partial LD with the other promoter SNPs. The −17G allele co-occurred with the −565T and −191C alleles less frequently than expected, however, as indicated by the small correlation coefficient, these polymorphisms could not represent each other reliably due to the different allele frequencies ($r^2=0.37$). The polymorphisms in the coding region were not observed to be in LD with each other (Table 5).

From the five *ABCA1* variants, we estimated 11 haplotypes with >1% frequency. Mean lipid concentrations did not differ according to haplotype. Haplotypes including the −565T and the −191C alleles consistently showed lowest risks of CHD, with a global *p*-value of 0.05 (data not shown). Because only the −565C/T and −191G/C SNPs were found to be associated with CHD, we also compared the nested model with the −191G/C polymorphism alone to the model with all haplotypes. The haplotypes did not add to the risk-prediction beyond that of the single SNP (*p*-value, likelihood ratio test: 0.13).

3.4. Potential gene-environment interactions on risk of coronary heart disease

Lastly, we explored whether the association at the −565T/−191C allele was differentially associated with CHD among a variety of subgroups known to have different lipid levels (Table 6). Variation at the −191 site was generally associated with stronger effects on CHD risk among women younger than 60 years of age, with a BMI above 25 kg/m^2, or with a low intake of dietary fat, but these findings did not have strong statistical significance.

4. Discussion

In the present study, two common polymorphisms in the *ABCA1* promoter region were in tight LD and associated with a lower risk of CHD among women. The association could not be explained by differences in circulating plasma lipids or markers of inflammation. No association with CHD was observed for coding region variants.

4.1. ABCA1 polymorphisms and HDL-C levels

We did not find associations between most *ABCA1* variants and plasma lipid levels. The I883M variant was associated with HDL-C concentrations among younger women, but not with CHD risk. The 883M variant has previously been associated with higher HDL-C concentrations in some [10,26], but not all studies [6–9,16]. In other populations, the −565C/T and R1587K polymorphisms have also been

Table 3
Mean lipid concentrations (±S.E.) according to *ABCA1* genotype in the Nurses' Health Studies ($n = 961$)

	N	Cholesterol (mmol/L)	Triglycerides (mmol/L)[a]	HDL (mmol/L)	LDL (mmol/L)	Cholesterol/HDL ratio
Women <55 years of age						
−565C/T						
CC	161	5.3 ± 0.1	1.0 ± 0.1	1.8 ± 0.03	3.0 ± 0.1	3.0 ± 0.1
CT	265	5.1 ± 0.1	1.0 ± 0.04	1.7 ± 0.02	2.8 ± 0.04	3.0 ± 0.1
TT	123	5.2 ± 0.1	1.0 ± 0.1	1.7 ± 0.04	3.0 ± 0.1	3.1 ± 0.1
p trend		0.5	0.5	0.1	0.9	0.4
−191G/C						
GG	161	5.3 ± 0.1	1.0 ± 0.1	1.8 ± 0.03	3. 0 ± 0.1	3.0 ± 0.1
GC	261	5.1 ± 0.1	1.0 ± 0.04	1.7 ± 0.02	2.8 ± 0.04	3.0 ± 0.0
CC	116	5.3 ± 0.1	1.0 ± 0.1	1.7 ± 0.03	3.1 ± 0.1	3.1 ± 0.1
p trend		0.9	0.4	0.2	0.5	0.4
−17C/G						
CC	267	5.2 ± 0.1	1.0 ± 0.04	1.7 ± 0.03	2.9 ± 0.04	3.0 ± 0.0
CG	236	5.1 ± 0.1	1.0 ± 0.04	1.7 ± 0.02	2.9 ± 0.1	3.1 ± 0.1
GG	51	5.3 ± 0.1	1.0 ± 0.1	1.8 ± 0.1	2.9 ± 0.1	2.9 ± 0.1
p trend		0.7	0.6	0.9	0.6	0.8
I883M						
AA	401	5.1 ± 0.04	1.0 ± 0.03	1.7 ± 0.02	2.9 ± 0.04	3.1 ± 0.0
AG	140	5.1 ± 0.1	1.0 ± 0.1	1.8 ± 0.04	2.7 ± 0.1	2.9 ± 0.1
GG	13	5.4 ± 0.2	1.1 ± 0.2	1.9 ± 0.1	2.9 ± 0.1	2.9 ± 0.1
p trend		0.8	0.6	<0.01	<0.01	0.02
R1587K						
GG	310	5.2 ± 0.1	1.0 ± 0.04	1.7 ± 0.02	2.9 ± 0.04	3.1 ± 0.0
GA	211	5.1 ± 0.1	1.0 ± 0.04	1.8 ± 0.03	2.8 ± 0.05	3.0 ± 0.1
AA	35	5.1 ± 0.1	0.8 ± 0.08	1.7 ± 0.1	2.8 ± 0.1	3.0 ± 0.1
p trend		0.2	0.01	0.4	0.1	0.1
Women ≥55 years of age						
−565C/T						
CC	105	5.8 ± 0.3	1.5 ± 0.1	1.5 ± 0.03	3.4 ± 0.1	3.9 ± 0.2
CT	173	7.0 ± 1.1	1.4 ± 0.06	1.6 ± 0.03	3.4 ± 0.1	4.6 ± 0.6
TT	100	6.4 ± 0.4	1.6 ± 0.1	1.5 ± 0.04	3.6 ± 0.1	4.4 ± 0.3
p trend		0.2	0.8	0.9	0.1	0.1
−191G/C						
GG	110	5.8 ± 0.3	1.5 ± 0.1	1.5 ± 0.03	3.5 ± 0.1	3.9 ± 0.1
GC	176	6.9 ± 1.0	1.4 ± 0.1	1.6 ± 0.03	3.4 ± 0.1	4.6 ± 0.6
CC	110	6.4 ± 0.4	1.6 ± 0.1	1.5 ± 0.04	3.7 ± 0.1	4.5 ± 0.3
p trend		0.2	0.6	0.7	0.2	0.03
−17C/G						
CC	187	6.2 ± 0.3	1.5 ± 0.1	1.5 ± 0.03	3.6 ± 0.1	4.3 ± 0.2
CG	153	6.9 ± 1.1	1.4 ± 0.1	1.5 ± 0.03	3.3 ± 0.1	4.6 ± 0.6
GG	34	5.7 ± 0.7	1.6 ± 0.1	1.6 ± 0.1	3.7 ± 0.2	4.3 ± 0.5
p trend		0.7	0.9	0.3	0.3	0.2
I883M						
AA	275	5.9 ± 0.1	1.5 ± 0.1	1.6 ± 0.03	3.4 ± 0.1	4.0 ± 0.1
AG	96	8.1 ± 2.0	1.5 ± 0.1	1.5 ± 0.04	3.6 ± 0.1	5.3 ± 1.1
GG	5	6.3 ± 0.4	3.0 ± 0.6	1.2 ± 0.1	3.6 ± 0.2	4.7 ± 0.4
p trend		0.3	0.3	0.1	0.1	0.3
R1587K						
GG	215	5.7 ± 0.2	1.5 ± 0.1	1.6 ± 0.02	3.4 ± 0.1	3.9 ± 0.1
GA	144	7.8 ± 1.7	1.5 ± 0.1	1.5 ± 0.03	3.5 ± 0.1	5.1 ± 0.9
AA	23	6.1 ± 0.5	1.6 ± 0.1	1.4 ± 0.1	3.6 ± 0.2	4.3 ± 0.3
p trend		0.2	0.9	0.4	0.6	0.2

Linear regression analyses were adjusted for age, smoking, BMI, alcohol intake, history of hypertension, parental history of CHD before age 60, diabetes at baseline, menopausal status and PMH. Nurses Health Study I controls and Nurses' Health Study II.

[a] Fasting participants only ($N = 792$ in total).

Table 4
Odds ratios [OR] and 95% confidence intervals [CI] of coronary heart disease according to *ABCA1* genotype in the Nurses' Health Study I

SNP/inheritance	Indicator			Recessive		Dominant		Codominant
	AA	AB	BB	AA/AB	BB	AA	AB/BB	Per B-allele
−565C/T	CC	CT	TT	CC/CT	TT	CC	CT/TT	
Cases/controls	86/142	112/222	45/120	198/364	45/120	86/142	157/342	
OR (95% CI)[a]	1	0.8 (0.6–1.2)	0.6 (0.4–0.9)	1	0.7 (0.5–1.0)	1	0.7 (0.5–1.0)	0.8 (0.6–1.0)
OR (95% CI)[b]	1	0.7 (0.5–1.1)	0.6 (0.4–0.9)	1	0.7 (0.5–1.1)	1	0.7 (0.5–1.0)	0.8 (0.6–1.0)
−191G/C	GG	GC	CC	GG/GC	CC	GG	GC/CC	
Cases/controls	87/150	115/224	45/118	202/374	45/118	87/150	160/342	
OR (95% CI)[a]	1	0.9 (0.6–1.2)	0.6 (0.4–1.0)	1	0.7 (0.5–1.0)	1	0.8 (0.6–1.1)	0.8 (0.7–1.0)
OR (95% CI)[b]	1	0.8 (0.5–1.1)	0.6 (0.4–1.0)	1	0.7 (0.5–1.1)	1	0.7 (0.5–1.0)	0.8 (0.6–1.0)
−17C/G	CC	CG	GG	CC/CG	GG	CC	CG/GG	
Cases/controls	106/233	106/200	23/44	212/433	23/44	106/233	129/244	
OR (95% CI)[a]	1	1.2 (0.9–1.7)	1.1 (0.6–2.0)	1	1.0 (0.6–1.8)	1	1.2 (0.9–1.6)	1.1 (0.9–1.4)
OR (95% CI)[b]	1	1.1 (0.7–1.5)	1.3 (0.7–2.4)	1	1.3 (0.7–2.3)	1	1.1 (0.8–1.6)	1.1 (0.9–1.5)
I883M	AA	AG	GG	AA/AG	GG	AA	AG/GG	
Cases/controls	184/353	54/121	5/8	238/474	5/8	184/353	59/129	
OR (95% CI)[a]	1	0.8 (0.6–1.2)	1.3 (0.4–4.1)	1	1.4 (0.4–4.2)	1	0.9 (0.6–1.3)	0.9 (0.7–1.3)
OR (95% CI)[b]	1	0.9 (0.6–1.4)	1.2 (0.3–4.5)	1	1.2 (0.3–4.6)	1	0.9 (0.6–1.4)	1.0 (0.7–1.4)
R1587K	GG	GA	AA	GG/GA	AA	GG	GA/AA	
Cases/controls	144/275	90/188	12/27	234/463	12/27	144/275	102/215	
OR (95% CI)[a]	1	0.9 (0.6–1.2)	0.8 (0.4–1.7)	1	0.9 (0.4–1.8)	1	0.9 (0.7–1.2)	0.9 (0.7–1.2)
OR (95% CI)[b]	1	0.9 (0.7–1.3)	0.7 (0.3–1.5)	1	0.7 (0.3–1.5)	1	0.9 (0.6–1.4)	0.9 (0.7–1.2)

[a] Logistic regression models adjusted for age, smoking, time of blood draw.
[b] Logistic regression models additionally adjusted for: BMI, alcohol intake, menopausal status and use of PMH, hypertension, parental history of CHD before age 60, and diabetes at baseline.

associated with plasma apoAI and HDL-C concentrations [6–8,13,14].

4.2. *ABCA1* polymorphisms and risk of coronary heart disease

The role of variation in *ABCA1* in vascular risk remains controversial. To our knowledge, only the ECTIM study has addressed the association between these common *ABCA1* variants and risk of a first CHD event. In that study, none of the 16 identified *ABCA1* promoter SNPs were associated with risk of myocardial infarction [7]. However, the study did not have information available on the −191G/C and −17C/G SNPs because the performed molecular screening failed to detect these common promoter variants. Recently, the MESA study reported a borderline association between the −565T allele and higher prevalence of coronary artery calcification, but not with intima media thickness [27]. The majority of previous studies have investigated angiographic severity or risk of recurrent events among patients with CHD [12–14]. Such studies have found a three-fold higher risk of recurrent CHD events among cardiovascular patients that are −191C homozygotes [12], and a greater number of atherosclerotic lesions among carriers of the −565T allele [13,14]. The latter finding was consistent with a reported lower ABCA1 expression and decreased promoter activity in the atherosclerotic plaques of these patients [13]. Although functional evidence indicates that −565T allele carriers would have a higher risk of CHD-related events, the polymorphism was not associated with progression of atherosclerosis or CHD during a follow-up of 2.5 years [14]. These findings in patients with established atherosclerosis contrast with our findings and another prospective analysis of the −565C/T polymorphism and prognosis after myocardial infarction, where the −565C allele was associated with worse prognosis [15]. Similarly discrepant results have been reported for other *ABCA1* pro-

Table 5
Pairwise linkage disequilibrium (D') and correlation coefficient (r^2) between *ABCA1* polymorphisms

Polymorphism	Linkage disequilibrium (D')				
	−565C/T	−191G/C	−17C/G	I883M	R1587K
Correlation coefficient (r^2)					
−565C/T	–	0.99	0.97	0.15	0.01
−191G/C	0.96	–	1.00	0.13	0.02
−17C/G	0.36	0.37	–	0.02	0.05
I883M	0.0	0.0	0.0	–	0.08
R1587K	0.0	0.0	0.0	0.0	–

Note: Results presented from the Nurses' Health Study II (same estimates as among Nurses' Health Study I controls).

Table 6
The −191G/C *ABCA1* promoter polymorphism and odds ratios [OR] and 95% confidence intervals [CI] of coronary heart disease in groups of HDL-related factors in the Nurses' Health Study I

Characteristic	GG	GC	CC	*p* trend within strata	*p* interaction
Age					
<60 years (*n* = 291)	1 (ref)	0.5 (0.3–0.9)	0.4 (0.2–0.9)	0.01	
≥60 years (*n* = 436)	1 (ref)	1.1 (0.7–1.8)	0.9 (0.5–1.6)	0.7	0.2
BMI					
<25 kg/m^2 (*n* = 376)	1 (ref)	1.1 (0.6–2.0)	1.0 (0.5–1.9)	0.9	
≥25 kg/m^2 (*n* = 351)	1 (ref)	0.7 (0.4–1.1)	0.4 (0.2–0.8)	0.01	0.05
Current smoking					
No (*n* = 497)	1 (ref)	0.6 (0.4–0.9)	0.5 (0.3–0.9)	0.01	
Yes (*n* = 230)	1 (ref)	1.6 (0.8–3.3)	1.2 (0.5–3.1)	0.5	0.1
Alcohol					
<5 g/day (*n* = 517)	1 (ref)	0.9 (0.6–1.4)	0.6 (0.3–1.0)	0.05	
≥5 g/day (*n* = 200)	1 (ref)	0.7 (0.3–1.7)	1.1 (0.4–3.3)	0.8	0.4
Current PMH use[a]					
No (*n* = 388)	1 (ref)	0.9 (0.5–1.6)	0.7 (0.4–1.3)	0.2	
Yes (*n* = 232)	1 (ref)	0.8 (0.4–1.7)	0.5 (0.2–1.2)	0.1	0.6
Dietary fat intake					
≤30% total energy (*n* = 269)	1 (ref)	0.5 (0.2–0.9)	0.2 (0.1–0.5)	<0.01	
>30% total energy (*n* = 448)	1 (ref)	0.9 (0.5–1.5)	1.0 (0.5–1.8)	0.8	0.03

N = 719 (participants with missing genotypic data for the −191G/C SNP were not included). Logistic regression models were adjusted for age, smoking, time of blood draw, BMI, alcohol intake, menopausal status and use of PMH, history of hypertension, parental history of CHD before age 60, diabetes at baseline. P for interaction obtained from models including both genotype and factor of interest and the cross-product.

[a] Among postmenopausal women only.

moter polymorphisms [16,12]. These discrepancies highlight the continuing difficulty in reconciling evidence from patients and healthy populations who are apt to have different pathophysiological processes at work and different distributions of lifestyle characteristics that modify the effects of *ABCA1* variation. We and others report potential effect modification of the effects of *ABCA1* variation on CHD risk by lifestyle characteristics [13]. Thus, differences in these characteristics between populations may explain inconsistencies in the main effects of genetic variation. Moreover, gender may play a role, as associations between *ABCA1* variants and HDL-C concentrations and severity of CAD have generally been found to be stronger among women [10,13,16].

Interestingly, the −191G/C and −565C/T variants were associated with CHD without affecting HDL-C concentration. This finding is consistent with observations of these and other *ABCA1* variants in other studies [7,8,12–14,27]. Recent evidence from in vivo and in vitro studies provides plausible mechanisms for anti-atherogenic properties of ABCA1, independent of its association with plasma HDL-C concentrations. Studies of tissue-specific inactivation of ABCA1 in mice have shown that HDL production is determined primarily by the liver [28]. However, macrophage-specific ABCA1 inactivation markedly increased atherosclerosis in ApoE deficient mice [29], despite little contribution to circulating HDL-C levels. Indeed, macrophages isolated from ABCA1 deficient mice had higher cholesterol content, secreted more chemokines, cytokines, and growth factors, suggesting that the reduced ABCA1 activity increases the proinflammatory properties of the macrophages [30]. We did not find strong associations between *ABCA1* variation and markers of circulating inflammation, but we did observe a tendency for lower concentrations of the inflammatory markers among carriers of the −565T and −191C alleles. However, this was only among women greater than 55 years of age. Other measures of more localized inflammation may be etiologically more important and are of considerable interest in future investigations. Also, population-wide variability in levels of HDL-C is related to both other genetic factors [31] and several lifestyle features [32–35], and HDL-C levels alone may not be sensitive enough to capture the hypothesized effects of ABCA1 on cholesterol efflux.

In the present study, the association between −191G/C and risk of CHD appeared to be modified by specific environmental factors. The observation that the association between the −191G/C polymorphism and CHD was more pronounced among non-smokers is consistent with previous findings [7,13], however the mechanisms behind this observation remain unresolved. To our knowledge, potential interactions between this promoter variant and overweight and dietary fat have not previously been investigated, and further studies are warranted to shed light on this. Preliminary in vitro evidence suggests that elevated fatty acids, which are often associated with overweight and consumption of high fat diets, may promote degradation of ABCA1 [36,37].

4.3. Limitations

Some limitations pertinent to the present study should be noted. We conducted these investigations in two groups of

predominately white health professionals in the U.S., and we cannot necessarily generalize our results to other populations with different distributions of race and ethnicity. The size of the nested case–control study may have limited our power to perform haplotype and interaction analyses. This could affect the reliability of the estimation of haplotypes. Because we examined multiple subgroups, the interaction findings should be interpreted with caution. Additionally, we cannot exclude the possibility that our investigation of five single locus SNPs could be confounded by linkage with other unmeasured polymorphisms. Moreover, we did not have information on the R219K SNP that has consistently been associated with lower CHD risk previously [7–9].

5. Conclusions

In summary, we found that two common *ABCA1* promoter polymorphisms were inversely associated with risk of CHD among women, although the variation was not associated with circulating plasma lipid concentrations. Further research is needed to identify the mechanism for the observed link between *ABCA1* promoter variation and risk of CHD, such as whether these variants confer biologically meaningful tissue-specific effects that could be harnessed to improve CHD prevention.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.atherosclerosis.2007.01.025.

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CLINICAL RESEARCH

Alcohol consumption, TaqIB polymorphism of cholesteryl ester transfer protein, high-density lipoprotein cholesterol, and risk of coronary heart disease in men and women

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Aims To investigate whether a common polymorphism in the cholesteryl ester transfer protein (CETP) gene modifies the relationship of alcohol intake with high-density lipoprotein cholesterol (HDL-C) and risk of coronary heart disease (CHD).

Methods and results Parallel nested case-control studies among women [Nurses' Health Study (NHS)] and men [Health Professionals Follow-up Study (HPFS)] where 246 women and 259 men who developed incident CHD were matched to controls (1:2) on age and smoking. The TaqIB variant and alcohol consumption were associated with higher HDL-C, with the most pronounced effects of alcohol among B2 carriers. In the NHS we did not find an inverse association between alcohol and CHD in B2 non-carriers (*P* trend: 0.5), but did among B2 carriers (*P* trend <0.01). Among non-carriers the odds ratio (OR) for CHD among women with an intake of 5–14 g/day was 1.4 (95% CI: 0.6–3.7) compared with non-drinkers, whereas among B2 carriers the OR was 0.4 (0.2–0.8). Results in men were less suggestive of an interaction; corresponding OR's were 1.9 (0.8–4.5) and 0.9 (0.5–1.6), for B2 non-carriers and carriers, respectively.

Conclusions The association of alcohol with HDL-C levels was modified by CETP TaqIB2 carrier status, and there was also a suggestion of a gene–environment interaction on the risk of CHD.

Keywords Alcohol • Gene–environment interaction • CHD • Cholesterol transport • Lipoproteins

Introduction

In prospective cohort studies, moderate alcohol consumption is associated with a lower risk of coronary heart disease (CHD) than abstention or very light drinking.[1,2] The primary mechanism proposed for this association is the higher levels of high-density lipoprotein cholesterol (HDL-C) found among moderate drinkers.[3] Randomized trials of alcohol administration demonstrate that intake of 30 g of alcohol daily (about 2 drinks) raises HDL-C levels by 0.1 mmol/L (4 mg/dL).[4] In prospective studies with data on alcohol, HDL-C, and incident CHD, about half of the lower risk of CHD among moderate drinkers can be attributed to HDL-C levels.[5–7]

Biological mechanisms underlying the positive association between alcohol and HDL-C are not yet fully understood. One pathway could be through regulation of cholesteryl ester transfer protein (CETP) activity, as CETP mediates transfer of cholesteryl esters from HDL to low-density lipoprotein (LDL) and very-low-density lipoprotein (VLDL) particles. The concentration of CETP is inversely associated with HDL-C levels.[8,9] Observational studies have reported lower CETP activity among both alcohol abusers and young men with moderate alcohol

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intake.[10,11] Genetic variation in the gene encoding CETP is another important determinant of its activity. Although many single nucleotide polymorphisms (SNPs) in the gene have been identified, the most commonly studied occurs at the TaqIB restriction site in intron 1. The TaqIB2 variant allele is associated with lower CETP levels and activity and higher HDL-C levels,[11–14] most likely via its link to one or more functional promoter SNPs.[14–16]

Given that the association of alcohol with HDL-C and possibly cardiovascular risk may be partly mediated by CETP activity, genetic variation in *CETP* may modulate these relationships. Although such an interrelationship has not been extensively studied, two reports suggest that alcohol consumption could interact with the *CETP* TaqIB SNP to alter risk of myocardial infarction (MI).[11,14]

To investigate the importance of alcohol intake and the *CETP* TaqIB SNP in relation to levels of HDL-C and risk of CHD more definitively, we performed two independent nested case-control studies among men and women enrolled in the Health Professionals Follow-up Study (HPFS) and the Nurses' Health Study (NHS).

Methods

Study populations

The NHS cohort was established in 1976 at the Channing Laboratory of the Brigham and Women's Hospital. The study population consists of 121 700 married female registered nurses aged 30–55 years residing in one of 11 larger US states. Women have received follow-up questionnaires biennially to update information on exposures and newly diagnosed illnesses. Since 1980, participants have updated information on diet, alcohol, and vitamin supplements through a food frequency questionnaire approximately every 4 years.

The HPFS was established in 1986 at the Harvard School of Public Health, when 51 529 male health professionals 40–75 years of age from throughout the US completed the initial HPFS questionnaire. The population includes 29 683 dentists, 3745 optometrists, 2218 osteopathic physicians, 4185 pharmacists, 1600 podiatrists, and 10 098 veterinarians. Participants update information biennially, in a manner similar to the NHS.

Nested case-control studies

Between 1989 and 1990, a blood sample was requested from all active participants in NHS and collected from 32 826 women. Similarly, blood samples were obtained from 18 224 men in the HPFS between 1993 and 1995. With the exception of a modestly lower prevalence of smoking, those who returned blood samples did not differ substantially from those who did not in both cohorts, including average alcohol intake of 12.8 vs. 12.2 g/day in the HPFS and 6.5 vs. 6.3 g/day in the NHS. Participants underwent local phlebotomy and returned samples to our laboratory via overnight courier. Upon arrival, whole blood samples were centrifuged and stored in cryotubes as plasma, buffy coat, and red blood cells in the vapour phase of liquid nitrogen freezers.

The outcome for the nested case-control studies was incident CHD, defined as non-fatal MI and fatal CHD. We wrote to participants who reported incident CHD on the follow-up questionnaires to confirm the report and request permission to review medical records. We also sought medical records for deceased participants, whose deaths were identified by families and postal officials and through the National Death Index. Physicians blinded to the participant's questionnaire reports reviewed all medical records. Cases of MI and fatal CHD were identified primarily through review of medical records, as previously described.[17,18] Among participants who provided blood samples and who were without cardiovascular disease or cancer at blood draw, 212 women sustained an incident MI and 37 died from fatal CHD between blood draw and June 30, 1998. The corresponding numbers in HPFS were 196 non-fatal MI and 70 fatal CHD cases prior to January 31, 2000. As a secondary endpoint, we additionally identified 564 men who had coronary artery bypass graft surgery (CABG) or percutaneous transluminal coronary angioplasty (PTCA) during follow-up. Confirmation of CABG/PTCA was based on self-report only; hospital records obtained for a sample of 102 men confirmed the procedure for 96% of these.[18]

Using risk-set sampling,[19] controls were selected randomly and matched in a 2:1 ratio on age, smoking, and month of blood return, among participants who were free of cardiovascular disease at the time CHD was diagnosed in the case patient. In the NHS, we also matched on fasting status.

Information on genetic variants and plasma lipids

DNA was extracted from the buffy coat fraction of centrifuged blood with the QLAmp Blood Kit (Qiagen, Chatsworth, CA, USA). The primary genotyping technique was Taqman SNP allelic discrimination by means of an ABI 7900HT (Applied Biosystems, Foster City, CA, USA), using rs708272.[20] Genotype data were available for 732 women from the NHS (246 cases and 486 controls), 772 men (259 MI cases and 513 controls), and an additional 531 men with CABG/PTCA and their 1075 controls.

Plasma lipids assessed using standard methods with reagents from Roche Diagnostics (Indianapolis, IN, USA) and Genzyme (Cambridge, MA, USA) included triglycerides, total cholesterol, HDL-C, and directly obtained LDL cholesterol (LDL-C). Study samples were sent to the laboratory for analysis in batches where cases were paired with their two controls in random order. The intra-assay coefficient of variation was (CV%) $<2.5\%$ for the lipid parameters. Plasma lipids were measured among for nonfatal MI and fatal CHD cases and their controls (due to limited funding the set of CABG/PTCA cases and controls did not have plasma biomarkers measured).

Assessment of alcohol consumption

We assessed diet with a 131-item semi quantitative food frequency questionnaire that includes separate items for beer, white wine, red wine, and liquor, as described elsewhere.[17] We previously validated estimated alcohol consumption against 3-week dietary records collected approximately 6 months apart from 136 HPFS participants and 173 NHS participants residing in Eastern Massachusetts,[21] with Spearman correlation coefficients between these two measures of 0.90 in women and 0.86 in men.

In these analyses, we used average alcohol consumption assessed in 1990 among women and 1994 among men. Previous assessments were used for 37 women and 25 men with missing information for alcohol intake at the time of the blood draw.

We have previously reported on the association between alcohol consumption and risk of CHD in these nested case-control studies,[7] which was similar to the relationship found in the full NHS and HPFS cohorts.[17,22]

Statistical analysis

Multiple regression analysis was used to address the associations between *CETP* TaqIB genotype and plasma lipids among NHS and HPFS controls. All plasma lipids were log-transformed due to positive

skewness, and geometric means with 95% confidence intervals (CI) are presented. Participants who were homozygous B1 served as the natural reference, and comparisons of means with this group were corrected for mass significance using Dunnett adjustment.[23] Fully adjusted models included age (5-year intervals), smoking (never smoker, current smoker, past smoker), body mass index (BMI) (<20, 20–24.9, 25–29.9, 30–34.9, ≥35 kg/m^2), alcohol intake (0, 0.1–4.9, 5–14.9, 15+ g/day for women, and 15–29.9 and 30+ g/day for men), history of hypertension, diabetes, and parental history of CHD before the age of 60. Analyses among NHS controls were further adjusted for menopause status and postmenopausal hormones (PMH) at blood draw (premenopausal, postmenopausal no PMH, postmenopausal currently taking PMH, postmenopausal past PMH). As expected from the random distribution of alleles, these variables had little impact on the odds ratios (OR), but were kept in the model because of their recognition as risk factors for CHD and because they may account for some of the heterogeneity between study participants. We used covariate information from the time of blood draw, defined as 1994 in the HPFS and 1990 in the NHS. Information from previous questionnaires was used when covariate data from the time of blood draw were missing. In analyses of the combined study populations of NHS and HPFS we further adjusted for study origin and used 15+ g/day as the top category for alcohol consumption among both women and men.

Both conditional and unconditional analyses (adjusted for matching factors) provided essentially the same results for the association between *CETP* and CHD. We present the results from unconditional logistic regression models for all analyses because this parallels the stratified analyses. Due to small numbers among the light-drinkers, we used non-drinkers as the reference group in all analyses. Previous analyses in the entire HPFS cohort have shown that abstainers and very light drinking men have similar CHD risks.[18] We conducted tests of linear trend across increasing categories of alcohol consumption by treating the midpoints of consumption in categories as a continuous variable. Statistical interactions between the *CETP* TaqIB variant and categorical alcohol intake were tested comparing likelihood ratios in models with and without all interaction terms. All statistical tests were two-tailed and *P*-values below 0.05 were considered statistically significant. Analyses were performed using SAS 9 (SAS Institute Inc., Cary, NC, USA).

Results

Table 1 shows baseline characteristics of cases and controls in both studies. As expected, physical activity and HDL-C were lower, and BMI, diabetes, hypertension, LDL-C and triglycerides were higher, among cases than controls (*Table 1*). We found no departures from Hardy–Weinberg equilibrium for CETP in neither the NHS ($P = 0.96$) nor the HPFS ($P = 0.46$). Frequency of the TaqIB2 allele was 0.4. Genotype and allele frequencies did not differ between cases and controls of both studies.

Cholesteryl ester transfer protein TaqIB, alcohol, and plasma lipids

In analyses restricted to the controls, carriers of the B2 allele had higher HDL-C concentrations compared with non-carriers (*Table 2*). There was also a suggestion of higher triglyceride concentrations among B2 homozygotes, however, this was not statistically significant. Alcohol consumption was associated with HDL-C

Table 1 Characteristics of covariates among cases of myocardial infarction and controls in the Nurses' Health Study (NHS) and the Health Professionals Follow-up Study (HPFS)[a]

Variable	NHS		HPFS	
	Cases ($n = 246$)	Controls ($n = 486$)	Cases ($n = 259$)	Controls ($n = 513$)
Alcohol (g/day)	0.9 (0; 24)	1.8 (0; 29)	5.8 (0; 46)	6.8 (0; 77)
Drinking frequency (days/week)	1 (0; 7)	1 (0; 7)	2 (0; 7)	2 (0; 7)
CETP B1B2 genotype	120 (49%)	235 (48%)	126 (49%)	244 (48%)
CETP B2B2 genotype	42 (17%)	85 (17%)	44 (17%)	89 (17%)
Age (years)	62 (47; 69)	62 (48; 68)	66 (50; 78)	66 (51; 78)
Physical activity (METS/week)	11 (0; 47)	12 (1; 53)	24 (1; 125)	27 (1–124)
Body mass index (BMI) (kg/m^2)	24.8 (18.5; 34.2)	23.4 (18.8; 32.4)	25.7 (20.9; 31.9)	25.1 (19.8; 31.8)
Diabetes	49 (20%)	32 (7%)	25 (9%)	24 (5%)
Hypertension	142 (58%)	144 (30%)	111 (42%)	158 (31%)
Hypercholesterolemia	132 (54%)	194 (40%)	128 (49%)	209 (41%)
Postmenopausal hormone (PMH) use	76 (31%)	178 (37%)	N/A	N/A
Plasma lipids				
HDL-C (mmol/L)	1.3 (1.1; 1.6)	1.5 (1.2; 1.8)	1.1 (0.7; 1.7)	1.1 (0.7; 1.8)
LDL-C (mmol/L)	3.7 (3.1; 4.3)	3.4 (2.7; 4.0)	3.5 (2.0; 4.9)	3.2 (2.8; 3.8)
Cholesterol (mmol/L)	6.1 (5.5; 6.7)	5.8 (5.2; 6.6)	5.6 (4.0; 7.1)	5.2 (3.9; 7.0)
Triglycerides (mmol/L)[b]	1.5 (1.1; 2.3)	1.2 (0.8; 1.7)	1.7 (0.6; 5.3)	1.2 (0.5; 3.5)

[a]Medians (5th and 95th percentiles) of continuous covariates. Counts and percentages of categorical covariates.
[b]Fasting participants only (NHS, $n = 302$; HPFS, $n = 298$).

Table 2 Mean [95% confidence intervals (CI)] plasma lipid concentrations according to cholesteryl ester transfer protein (*CETP*) genotype among controls in the Nurses' Health Study (NHS) and the Health Professionals Follow-up Study (HPFS)[a]

	NHS				HPFS			
	B1B1	B1B2	B2B2	P^b	B1B1	B1B2	B2B2	P^b
n	166	235	85		180	244	89	
HDL-C (mmol/L)	1.41 (1.36–1.47)	1.54 (1.49–1.59)	1.57 (1.49–1.66)	<0.01	1.10 (1.07–1.14)	1.14 (1.11–1.18)	1.26 (1.20–1.32)	<0.01
LDL-C (mmol/L)	3.32 (3.18–3.47)	3.24 (3.13–3.36)	3.37 (3.17–3.58)	0.95	3.18 (3.05–3.30)	3.25 (3.14–3.36)	2.97 (2.82–3.16)	0.10
Cholesterol (mmol/L)	5.70 (5.58–5.87)	5.75 (5.61–5.86)	5.98 (5.76–6.20)	0.13	5.15 (5.02–5.29)	5.30 (5.18–5.42)	5.10 (4.93–5.32)	0.91
Triglycerides (mmol/L)[c]	1.20 (1.09–1.32)	1.16 (1.07–1.26)	1.42 (1.22–1.63)	0.13	1.30 (1.16–1.46)	1.32 (1.21–1.45)	1.39 (1.20–1.62)	0.72

[a]Geometric means obtained from regression analyses adjusted for age, smoking, time of blood draw, BMI, alcohol intake, family history of MI before age 60, diabetes and hypertension at baseline. Not all lipid parameters available on all participants.
[b]*P* for test of differences between means of B2 homozygotes and B1 homozygotes (Dunnett adjustment for mass significance applied).
[c]Fasting participants only (NHS, $n = 302$; HPFS, $n = 298$).

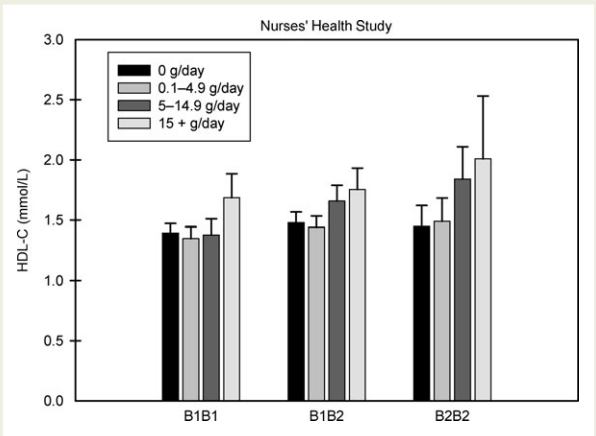

Figure 1 Geometric means of high-density lipoprotein cholesterol (HDL-C) concentrations (error bar represents upper limit of 95% confidence interval) among female controls obtained from regression analyses adjusted for age, smoking, time of blood draw, body mass index (BMI), family history of myocardial infarction (MI) before age 60, diabetes, hypertension at baseline, post-menopausal status and hormone use. All *P* for trend <0.01. *P* for interaction: Wald test for the inclusion of a separate interaction term between cholesteryl ester transfer protein (CETP) (modelled dominantly) and medians of alcohol categories treated linearly, $P < 0.01$

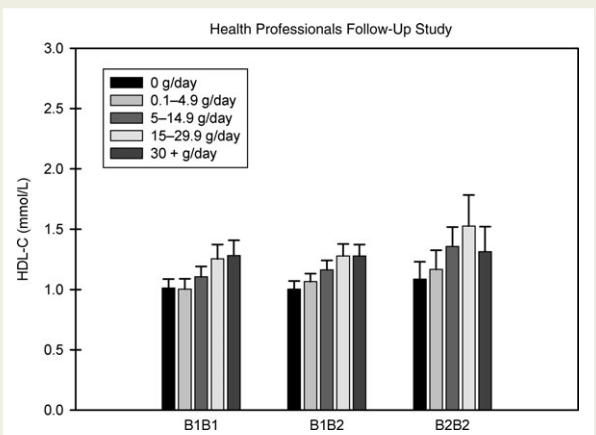

Figure 2 Geometric means of high-density lipoprotein cholesterol (HDL-C) concentrations (error bar represents upper limit of 95% confidence interval) among male controls obtained from regression analyses adjusted for age, smoking, time of blood draw, body mass index (BMI), family history of myocardial infarction (MI) before age 60, diabetes and hypertension at baseline. All *P* for trend: <0.01. *P* for interaction: Wald test for the inclusion of a separate interaction term between cholesteryl ester transfer protein (CETP) (modelled dominantly) and medians of alcohol categories treated linearly, $P < 0.01$

levels in a dose-dependent manner, with the most pronounced effects among both men and women who were homozygous for the B2 allele (*Figures 1* and *2*). B2 homozygous women with an alcohol intake of 15 g/day or more had 0.6 mmol/L (43%) higher HDL-C levels than

Table 3 Odds ratios (OR) and 95% confidence intervals (CI) of coronary heart disease (CHD) [and coronary artery bypass graft surgery (CABG)/CHD] according to cholesteryl ester transfer protein (*CETP*) genotype in the Nurses' Health Study (NHS) and Health Professionals Follow-up Study (HPFS)

	NHS			HPFS		
	B1B1	B1B2	B2B2	B1B1	B1B2	B2B2
CHD cases/controls	84/166	120/235	42/85	89/180	126/244	44/89
OR (95% CI)[a]	1	1.0 (0.7–1.5)	1.0 (0.7–1.6)	1	1.0 (0.7–1.5)	1.0 (0.6–1.6)
OR (95% CI)[b]	1	1.2 (0.8–1.7)	1.2 (0.8–2.1)	1	1.1 (0.8–1.6)	1.1 (0.7–1.7)
CABG and CHD cases/controls	–	–	–	275/530	373/771	142/287
OR (95% CI)[a]				1	0.9 (0.8–1.1)	1.0 (0.7–1.2)
OR (95% CI)[b]				1	0.9 (0.8–1.1)	1.0 (0.7–1.2)

[a]Adjusted for age, smoking, and time of blood draw.
[b]Additional adjustment for BMI, alcohol intake, parental history of CHD before age 60, diabetes, and high blood pressure at baseline. Analyses among women also included postmenopausal status and hormone use.

Table 4 Odds ratios (OR) and 95% confidence intervals (CI) of coronary heart disease (CHD) according to alcohol intake within strata of cholesteryl ester transfer protein (*CETP*) genotype in the Nurses' Health Study (NHS) and the Health Professionals Follow-up Study (HPFS)[a]

		NHS				HPFS			
CETP genotype	Alcohol consumption	Cases (n = 247)	Controls (n = 486)	OR (95% CI) Crude	OR (95% CI) Adjusted	Cases (n = 259)	Controls (n = 513)	OR (95% CI) Crude	OR (95% CI) Adjusted
B1B1	0 g/day	39	65	1	1	17	47	1	1
	0.1–4.9 g/day	22	51	0.8 (0.4–1.6)	1.1 (0.5–2.3)	24	37	1.9 (0.9–4.1)	1.7 (0.7–4.1)
	5–14.9 g/day	14	29	1.0 (0.4–2.2)	1.4 (0.6–3.7)	28	41	1.7 (0.8–3.8)	1.9 (0.8–4.5)
	15–29.9 g/day	8	21	0.9 (0.3–2.2)	1.3 (0.5–3.8)	13	27	1.3 (0.5–3.3)	1.6 (0.6–4.4)
	30+ g/day	–	–	–	–	7	28	0.6 (0.2–1.8)	0.6 (0.2–2.0)
	P trend[b]			0.9	0.5			0.1	0.2
X/B2	0 g/day	79	118	1	1	46	72	1	1
	0.1–4.9 g/day	55	97	0.8 (0.5–1.2)	0.8 (0.5–1.4)	39	78	0.9 (0.5–1.5)	0.9 (0.5–1.6)
	5–14.9 g/day	17	67	0.3 (0.2–0.6)	0.4 (0.2–0.8)	38	75	0.8 (0.5–1.5)	0.9 (0.5–1.6)
	15–29.9 g/day	12	38	0.4 (0.2–0.8)	0.4 (0.2–0.9)	23	53	0.7 (0.4–1.4)	0.8 (0.4–1.5)
	30+ g/day	–	–	–	–	24	55	0.8 (0.4–1.4)	0.8 (0.4–1.6)
	P trend[b]			<0.01	<0.01			0.4	0.5
	P interaction[c]			0.4	0.4			0.2	0.2

[a]Crude model adjusted for age, smoking and time of blood draw. Adjusted models included age, smoking, time of blood draw, BMI, family history of MI before age 60, diabetes and hypertension at baseline. Analyses among women also included postmenopausal status and hormone use.
[b]P trend: median of alcohol categories modelled continuously.
[c]P interaction: Likelihood ratio test of nested models with and without all interaction terms between CETP (dominant effects) and the alcohol categories.

non-drinking, B1 homozygous women. Tests of interaction between *CETP* (modelled with dominant effects of the B2 allele) and alcohol intake on HDL-C were statistically significant ($P < 0.01$).

Cholesteryl ester transfer protein TaqIB, alcohol, and risk of coronary heart disease

The TaqIB polymorphism was not associated with risk of CHD in the NHS and neither with CHD nor the combined endpoint of CHD and CABG in the HPFS (*Table 3*).

An inverse association between average alcohol consumption and risk of CHD was observed among both women and men, as has previously been reported.[7] For power reasons, we modelled the *CETP* genotype according to dominant effects of the B2 allele in the interaction analyses. In the NHS, a strong inverse association between alcohol and CHD was only observed in B2 carriers (P trend <0.01), whereas a light to moderate alcohol intake was not associated with a lower risk of CHD among the homozygous B1 women (P trend = 0.5) (*Table 4*). Among the HPFS men,

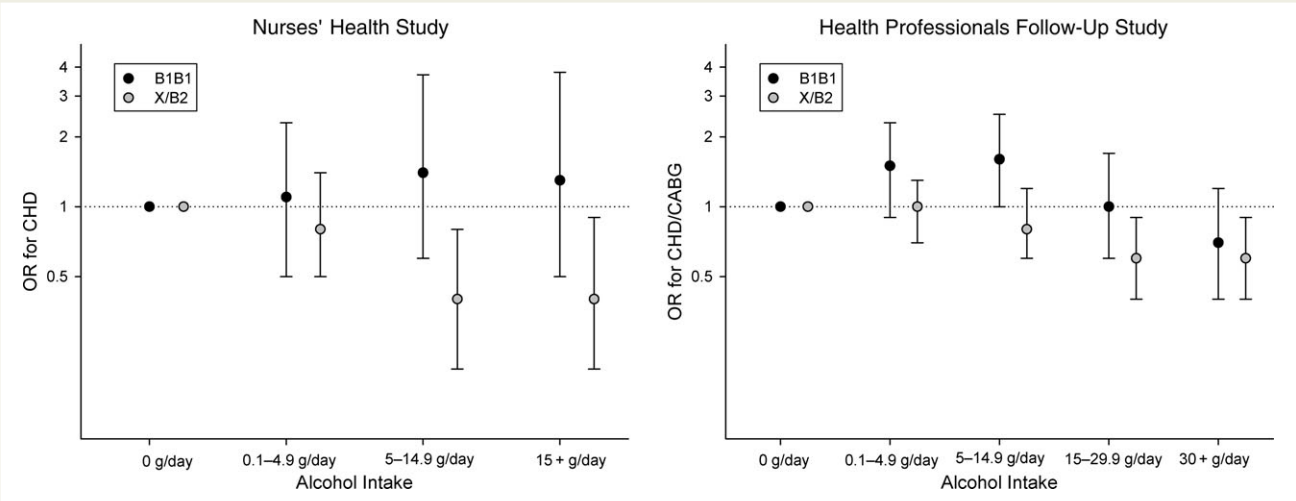

Figure 3 Odds ratio (OR) estimates from analysis in the Nurses' Health Study (NHS) and Health Professionals Follow-Up Study (HPFS), adjusted for age, smoking, time of blood draw, body mass index (BMI), family history of myocardial infarction (MI) before age 60, diabetes and hypertension at baseline. Analyses among women also included postmenopausal status and hormone use. Black dots: B1B1. Grey dots: X/B2. *P* for trend: NHS – B1B1 individuals: 0.5; X/B2 individuals: <0.01, HPFS – B1B1 individuals: 0.03; X/B2 individuals: <0.01. *P* interaction: likelihood ratio test of nested models with and without all interaction terms between cholesteryl ester transfer protein (CETP) (dominant effects) and the alcohol categories. NHS: $P = 0.4$. HPFS: $P = 0.7$

there was a modest difference in the association between alcohol and CHD by genotype, but the CIs were very broad. Moderate alcohol consumption appeared to be associated with a higher risk of CHD among B2 non-carriers while the association between alcohol and CHD was null in B2 carriers. In additional analyses in the HPFS where the CABG/PTCA endpoints were included (531 additional cases and 1075 controls) differences in the association according to B2 carrier status remained modest (*Figure 3*). There was a strong inverse association between alcohol and risk of CHD/CABG among the B2 carriers (*P* trend <0.01), whereas alcohol intake <15 g/day was associated with a higher risk compared with non-drinking in the B1 homozygotes. However, alcohol intake above 30 g/day was associated with the lowest risk of CHD regardless of genotype in the HPFS. Tests of the interaction terms were, however, not significant (NHS, $P = 0.4$; HPFS, CHD only, $P = 0.2$; including CABG, $P = 0.7$).

Given the similar trends in the two study populations, we combined them to achieve greater statistical power. In analyses stratified by genotype, compared with non-drinkers, the OR for CHD among individuals who drank 5–14.9 g/day was 1.6 (95% CI: 1.1–2.3) for B1B1 and 0.7 (95% CI: 0.6–1.0) for B2 carriers (*P* interaction = 0.02) (data not shown).

Discussion

In this prospective study, the association of alcohol with HDL-C levels was modified according to *CETP* TaqIB genotype. Even though the genotype did not have a main effect on CHD risk our results suggest that the inverse association between alcohol and CHD risk is also modified by the CETP TaqIB genotype.

Alcohol, cholesteryl ester transfer protein, and high-density lipoprotein cholesterol

The association between alcohol intake and higher HDL-C levels is well established.[3] However, the metabolic mechanisms for the increase in HDL-C following alcohol consumption are poorly understood. CETP is a key protein in HDL-C metabolism. Congenital CETP deficiency and pharmacological inhibition of CETP both lead to markedly elevated HDL-C levels.[24,25] Limited evidence suggest that alcohol intake is associated with lower CETP activity[11] and lower concentrations among drinkers.[10] Recently it has been suggested that alcohol or a metabolite may inhibit the glycosylation of CETP, which could affect the binding of CETP to lipoproteins in alcohol drinkers.[26] Other metabolic pathways that may also play a role in the association between alcohol and HDL-C concentration include increased transport rate of apolipoproteins, reduced hepatic lipase activity, and greater lecithin cholesterol acyl transferase activity[27,28] and a combination of all may be the most likely scenario.[29–31]

Consistent with our findings, other observational studies have found that genetic variation in *CETP* influences the association between alcohol consumption and HDL-C, such that the highest HDL-C concentrations are found among B2 carriers who drink alcohol.[32,33] Boekholdt *et al.*[11] also found an interaction of borderline statistical significance between alcohol use of any amount and the TaqIB SNP on HDL-C levels, whereas no interaction on HDL-C levels was found in the Northwick Part Heart Study,[34] or in healthy populations from Spain[35] and rural Japan.[36] As higher levels of HDL-C are related to several lifestyle features, including exercise,[37] smoking cessation,[38] PMH,[39] and lower carbohydrate intake,[40] it is possible that other lifestyle characteristics may partly account for these inconsistencies.

Alcohol, cholesteryl ester transfer protein, and risk of coronary heart disease

Although the relationship of CHD risk and the *CETP* TaqIB polymorphism is still debated,[13,41] one meta-analysis found the B2 allele associated with a 22% lower risk of CHD.[11] We did not confirm a statistically significant lower risk associated with the B2 allele among participants in this study, but the CI around these estimates were broad and we cannot exclude a lower risk of the magnitude previously identified. Our results suggest we might have seen a stronger effect of the B2 allele if the prevalence of moderate drinking had been higher. This is consistent with the results of the meta-analysis, which was performed in predominantly European populations where the underlying prevalence of alcohol consumption may be somewhat higher. Another hypothesis that has been put forward is effect modification of the association between CETP activity and CHD risk by plasma triglyceride concentration,[9] suggesting that the B2 allele may be associated with lower CHD risk particularly in hypertriglycedemic populations.[13]

In the present study, a light-to-moderate alcohol intake was associated with a lower risk of CHD among B2 carriers, whereas this was not observed among B1B1 individuals who were not genetically predisposed for higher HDL-C levels. Although our results were not entirely consistent among men and women, this could be related to both lack of statistical power or differences in underlying alcohol consumption pattern between genders. Relatively few studies have addressed the possibility of an interaction between alcohol use and *CETP* genotype on CHD risk (rather than just lipids). In the ECTIM study, Fumeron *et al.*[32] found lower risk of MI associated with the B2 variant allele among heavy drinkers, and in a meta-analysis of more than 13 000 individuals, Boekholdt *et al.*[11] reported a borderline statistically significant interaction ($P = 0.07$) between the SNP and alcohol on cardiovascular events.

Some limitations pertinent to the present study should be noted. We conducted these investigations in two groups of predominately white health professionals in the US, hence we cannot necessarily generalize our results to other populations with different distributions of race and ethnicity. However, the relationships of alcohol intake and the TaqIB SNP with HDL-C levels tend to be consistent in a wide variety of populations.[42,43] Although we documented over 500 incident cases of CHD among over 50 000 individuals in this study, the size of the nested case-control studies limited our power to detect interaction effects.

We measured only a single *CETP* SNP although numerous other, potentially functional, SNPs have been identified in the CETP gene.[14] Other SNPs could interact with alcohol in a manner similar to that of the TaqIB variant, in a different direction, or not at all. Furthermore, our study samples of 250 cases in both men and women were not adequately powered for such exploratory analyses. Thus, in the present study we aimed particularly to investigate the previously hypothesized interrelationship of the widely investigated TaqIB variant and alcohol. Of note, the TaqIB SNP is not itself likely to be functional and may be associated with CETP activity via its link to at least two other SNPs.[14–16] Indeed high linkage disequilibrium between SNPs located in the promoter region of the CETP gene has been demonstrated, suggesting that they all capture the same underlying functional variance.[14] As technological advances make sequencing and genotyping increasingly cost effective, further analyses and in-depth exploration of other CETP variants and alcohol should be performed with appropriate statistical methodology and in larger population samples.

We did not have measures of CETP concentration or activity thus we were not able to directly assess the effect of the TaqIB gene polymorphism on CETP activity. However, we did observe an association between the TaqIB variant and HDL metabolism even using HDL-C as a less sensitive parameter.

The self-reported measures of alcohol consumption used in this study have previously been validated in these populations.[21,44] As in most prospective cohort studies, the NHS and HPFS contain few drinkers who consume alcohol heavily on a regular or even episodic basis. Therefore, we were unable to separately assess heavy drinking or binge drinking, although other studies suggest it may be associated with an increased risk of CHD,[45] and Fumeron *et al.*[32] found that only alcohol consumed in very large quantities modulated the association between the TaqIB SNP and CHD among men.

In summary, we found that the TaqIB SNP in the *CETP* gene modified the association of alcohol intake with HDL-C. Furthermore, our analyses in two independent prospective studies lend support for the suggested interaction between CETP and alcohol on risk of CHD. Further studies with greater information on genetic variation in CETP and wider variation in alcohol consumption among men and women are still warranted to investigate this question further.

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The S447X variant of the lipoprotein lipase gene, lipids, and risk of coronary heart disease in three prospective cohort studies

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Short title: S447X variant in LPL, lipids and CHD

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Aim: To investigate the association between the *LPL* S447X variant with lipids and risk of coronary heart disease (CHD) in three prospective studies.

Methods and Results: The S447X variant was genotyped in nested case-control studies of incident CHD in the Nurses' Health (NHS) and the Health Professionals Follow-up (HPFS) Studies and a case-cohort study of acute coronary syndrome in the Danish Diet, Cancer and Health (DCH) study, totalling 245, 258, and 1015 cases, respectively. Overall, 447X-carriers had lower TG and higher HDL-C concentrations than non-carriers. The 447X variant was associated with a lower risk of CHD in the NHS (Relative Risk [RR]=0.56, 95% CI: 0.36-0.87), weaker in the HPFS (0.70, 0.47-1.04), and statistically insignificant in the DCH (0.92, 0.72-1.16). The pooled RR was 0.74 (0.56-1.00). There was a suggestion that the 447X variant had more pronounced effects in obese individuals in the NHS (p interaction = 0.009), but this finding was not consistent across the studies.

Conclusions: The LPL S447X variant is associated with lower TG and higher HDL-C, and with a lower risk of CHD. LPL is an attractive target for clinical intervention, but studies are needed to clarify whether greater benefit from this variant may be conferred in some subgroups.

Keywords: Genetic epidemiology, CHD, prospective study, lipoprotein lipase, plasma lipids

In prospective observational studies, plasma triglyceride (TG) levels are directly associated and high density lipoprotein (HDL) cholesterol levels inversely associated with risk of coronary heart disease (CHD).[1,2] However, because of the complexity of human lipid metabolism, therapeutic manipulation of TG and HDL-C as new relevant drug targets remain elusive. Studies of DNA sequence variants that are located in genes of major importance to lipid metabolism may provide important knowledge about the role of their encoded proteins in relation to long-term consequences on measures of plasma lipids and risk of clinical endpoints.

The lipoprotein lipase (LPL) enzyme has a prominent role in the metabolism of TG and HDL. LPL hydrolyzes TG carried in very low density lipoprotein (VLDL) and chylomicrons and generates excess phospholipids and apolipoproteins that are transferred to HDL.[3] Thus, high LPL activity is associated with lower TG and higher HDL-C levels.[4] The gene encoding LPL is located on chromosome 8p22, spans close to 30kb and contains 10 exons.[5] The majority of the identified single nucleotide polymorphisms (SNPs) cause loss of enzymatic function and predispose to elevated TG and reduced HDL-C.[6] In contrast, the commonly occurring S447X polymorphism in exon 9 is an intriguing exception: the variant allele encodes a prematurely truncated LPL protein that has increased lipolytic activity in vitro and in vivo in mice.[7] The S447X variant has consistently been associated with lower TG and higher HDL-C levels in candidate gene association studies, [8-14] and it's importance was recently emphasized by its identification as one of 18 loci significantly associated with TG and HLD-C concentration among over 350,000 SNPs examined in a combined analysis of over 8800 individuals.[15, 16] However, the potential interaction with environmental factors have not been explored in detail and data on its association with risk of CHD varies considerably between study populations.[12-14, 17-22]

Some investigations of the S447X variant and the HindIII SNP, which is in high linkage disequilibrium with S447X,[23, 24] suggest that environmental factors, such as smoking,[25, 26] alcohol,[25]

and particularly adiposity,[10, 11, 19, 27, 28] may modulate the association with plasma lipids. Although attempts of combined study of genetic and environmental factors are essential to understand the impact of genetic variation on clinical endpoints at the population level, so far, context-dependent effects of the S447X variant on CHD risk has received little attention. Thus, we aimed to (1) investigate the association between the *LPL* S447X SNP with plasma lipids and risk of CHD in three independent prospective studies of men and women, and (2) examine the role of this functional variant in subgroups of participants based on smoking habits, alcohol, and adiposity.

Methods

Study populations

The Nurses' Health Study (NHS) enrolled 121,701 female nurses aged 35 to 55 who returned a mailed questionnaire in 1976 regarding lifestyle and medical history.[29] The Health Professionals' Follow-up Study (HPFS) enrolled 51,529 males aged 40 to 75 who returned a similar questionnaire in 1986.[30] Participants of both cohorts have received follow-up questionnaires biennially to record newly diagnosed illnesses and to update lifestyle and dietary information. The Diet, Cancer, and Health (DCH) study was initiated in 1993-1997 when 57,053 Danish born residents, aged 50 to 64 years and free of cancer, participated in a clinical examination and detailed lifestyle survey.[31] Details on the assessment of anthropometry have been included in an online supplement.

Endpoint and study designs

To optimize research involving collection and laboratory analyses of human tissue, smaller case-control studies nested within these three cohorts were designed. For the US studies, a blood sample was requested from all active participants in 1989-1990 in NHS and 1993-1995 in the HPFS. A total of 32,826 female participants in NHS returned samples and 18,224 men in the HPFS did the same. Nested case-control studies were designed using incident CHD, with non-fatal myocardial infarction (MI) and fatal CHD as the outcome. Cases of MI and fatal CHD were identified primarily through review of medical records, as previously described.[32, 33] Among participants who provided blood samples and who were without cardiovascular disease or cancer at blood draw, 249 women sustained an incident MI/CHD between blood draw and June 30, 1998, and 266 cases occurred prior to January 31, 2000 in HPFS. Using risk-set sampling,[34] controls were selected randomly and matched in a 2:1 ratio on age, smoking, and month of blood return.

In the DCH, a case-cohort study was designed using incident acute coronary syndrome (ACS), including unstable angina pectoris, MI, and sudden cardiac death as the outcome. Information on the disease endpoint was obtained by linkage with central Danish registries via the unique identification number assigned to all Danish citizens.[35, 36] Details for the assessment of the hospital records have been included in the online supplement. In total, 1150 cases of ACS were identified and validated. For the creation of the case cohort sample, 1800 participants were selected from the entire DCH study at random (for consistency referred to as 'controls', although 35 individuals overlap with the case group).

Laboratory analysis

Details on methods for genotyping and measurement of plasma lipids have been included in the online supplement. Blood was obtained from mostly fasting participants in the US study (for analyses of TG levels, the few non-fasting were excluded) whereas Danes were all non-fasting. The primary genotyping technique was Taqman SNP allelic discrimination by means of an ABI 7900HT (Applied Biosystems, Foster City, CA), using rs328. In addition, two other commonly investigated LPL coding SNPs were genotyped in the US data alone; the D9N variant (rs1801177) and the N291S variant (rs268).

Statistical analysis

Conformity of controls' data with Hardy-Weinberg equilibrium was tested with an exact test and no departures were observed. Multivariable regression analysis was used to address the associations between *LPL* genotype and log-transformed lipids among the controls. Because few participants were homozygous variant carriers, we compared non-carriers (SS447) and carriers (S447X and XX4447). Relative risks (RR) and 95% confidence intervals (CIs) for the association between *LPL*

genotype and CHD were estimated using conditional logistic regression for the US nested case-control data and Cox proportional hazard regression was performed in DCH, using Kalbfleisch and Lawless weights and robust variance suitable for the case-cohort data.[39] Analyses in DCH allowed for sex-specific baseline hazards. Sex-specific analyses and tests of interaction in DCH did not suggest any statistically significant sex-differences.

Despite little impact on the RR's, lifestyle covariates were included in the multivariate models because of their strong association with CHD and because they may account for some of the heterogeneity between study participants. In total, numbers with information available on plasma lipids, genotype, and covariates were: 245 cases, 485 controls in NHS; 258 cases, 515 controls in HPFS; and 2682 (1015 cases) in DCH.

To pool the estimates from the three study populations, we used the weighted average of regression estimates using the DerSimonian and Laird random-effects model.[37]

We explored statistical interactions between the *LPL* genotype and smoking, alcohol, and adiposity, based on prior data suggesting possible interaction on lipids.[10, 11, 19, 25-27] A p-value for interaction was estimated by including the cross-product term between genotype and these factors modelled continuously.

Reported p-values are two-sided and 0.05 was the considered threshold for statistical significance. Analyses were performed using SAS 9 (SAS Institute Inc., Cary, NC) and STATA 9.1 (STATA Corp., College Station, TX).

Results

Table 1 shows baseline characteristics of cases and controls in the three study populations. As expected, controls were generally healthier than cases (**Table 1**). Although the median BMI was very similar across the three study populations, the US participants were more likely to receive a diagnosis of hypertension, hypercholesterolemia, or diabetes than the DCH participants. In contrast, Danes were more often smokers and median alcohol intake was higher.

LPL S447X and lipids

The S447X minor allele frequency was 0.12 in the US studies and 0.10 in DCH. In analyses restricted to controls, carriers of the S447X variant had lower levels of TG, compared with non-carriers. The association was strongest in the NHS where the carriers had 0.21 mmol/L lower TG concentration than non-carriers (p=0.003). The difference in TG concentration was 0.14 mmol/L in HPFS and 0.11 mmol/L in DCH, albeit not statistically significant in HPFS (**Table 2**). The S447X variant was also associated with a slightly higher HDL-C level that was only statistically significant in the larger DCH study. The effect of genotype on TG to HDL-C ratio was similar in magnitude in all three study populations, but not statistically significant in the HPFS. The pooled estimate for the difference in TG/HDL-C ratio between non-carriers and carriers was 0.11 (95% CI: 0.05-0.17, p for test of between study heterogeneity = 0.8).

In the US populations, genotype data were also available on two other LPL coding SNPs; D9N (MAF: 0.01) and N291S (MAF: 0.5). The D9N variant was too rare to pursue any further analyses, and the N291S variant was not associated with triglyceride or HDL-C concentration (data not shown).

LPL genotype and risk of CHD

The S447X variant allele was more frequent among controls than among those who developed CHD in all three populations. The S447X polymorphism was associated with risk of CHD in a co-dominant fashion in the three studies, although only statistically significant in the NHS (**Table 3**). In a pooled analysis, the adjusted RR was 0.77 (95% confidence interval, 0.58-1.03) for heterozygotes and 0.53 (0.29-0.97) for XX447 homozygotes, compared to non-carriers (p for tests of between study heterogeneity were >0.10).

The N291S variant was not statistically significantly associated with CHD in the NHS, but a co-dominant inverse association was observed in the HPFS. OR's were 0.85 (0.59-1.22) in the heterozygous and 0.61 (0.39-0.97) in homozygous carriers (data included in online supplement).

Effect-modification

We examined whether the association between S447X and CHD was modified by sex, smoking, alcohol intake, and adiposity. There were no statistically significant interactions, except suggestive evidence for an interaction between adiposity and risk of CHD in the NHS (p for interaction= 0.01). In analyses stratified by BMI, the S447X variant was not associated with CHD among NHS participants who were normal weight (p=0.5), but the association was stronger in the overweight (p=0.02) and strongest in the obese (p<0.01) (**fig. 1**). When analyses were repeated using WHO cut offs based on waist circumference, we observed very similar results (p for interaction=0.01) (data not shown). Although the S447X variant had a stronger association with CHD among obese HPFS men, the confidence intervals were broad and the interaction was not statistically significant. There was no interaction between BMI or waist circumference on risk of ACS in the Danish population.

We also addressed the potential interaction with adiposity on lipid levels. In all three study populations, the 447X variant appeared to have the strongest association with TG and HDL-C

concentration among the overweight and obese individuals, whereas the association was weaker in the normal weight. Geometric means of the TG to HDL-C ratio are shown according to S447X genotype and BMI in **figure 2**. In the NHS, differences in the TG to HDL-C ratio were largest among the obese. This was also true for HPFS participants, and in the DCH overall differences between genotype groups were smaller in magnitude, but still generally greater in overweight and obese participants. Similar results were observed when waist circumference was used (data not shown). However, tests of interaction between the S447X variant and BMI or waist circumference were not statistically significant in the three studies (all p values >0.05).

We did not observe any modification of the association between S447X genotype and plasma lipids, by smoking, alcohol, or sex (data not shown).

Discussion

In three independent studies, we found that carriers of the *LPL* S447X variant had lower TG and higher HDL-C levels compared to SS447 homozygotes. Concomitantly, the risk of CHD was lower among carriers. There was some evidence that the association of S447X with lipid concentrations and CHD risk might be greatest among the overweight.

The association between the S447X variant and elevated TG and lower HDL-C has been shown in several studies,[8-14] and this association is supported by animal data showing that the S447X variant produces a LPL protein with higher lipolytic function.[7] Although epidemiologic data on the risk of CHD associated with the S447X variant have not been completely consistent,[12-14, 17-22] the lower risk of CHD we observed among S447X carriers was recently supported by a comprehensive candidate-gene association study of 142 loci where only two variants, capturing variation at the S447X site of the *LPL* gene, were consistently associated with coronary artery disease in two case-control studies.[38] The relevance of this genetic variant to cardiovascular risk was also underlined in a candidate gene analysis of the Malmö Diet and Cancer Study, where it was found to independently predict risk of cardiovascular events, although only 238 incident cases of the composite cardiovascular endpoint were documented.[39]

Although low-density lipoprotein cholesterol has been the main therapeutic target for high cardiovascular risk patients, renewed attention has focused on the potential risk reduction that may be achieved by HDL-C manipulation.[40] However, trials of promising HDL-C-increasing drugs such as CETP inhibitors have been consistently negative to date.[41, 42] Given this, other targets have become more attractive, and indeed clinical trials targeting LPL with specific activators (NO-1886) or with gene therapy using the S447X variant are planned.[43, 44] Our results have two important implications for these trials. First, studies of genetic variants appear to provide important insight into the expected effects of pharmacological manipulation of HDL's complex metabolism. For

example, studies of genetic *CETP* variants showed several of the adverse cardiovascular effects – including hypertension - that were ultimately seen in clinical trials, and indeed these genetic studies were rather mixed with respect to risk of CHD despite positive effects on HDL-C levels.[45-47] Thus, we believe it crucial to examine the S447X variant in *LPL* in well-designed prospective studies of healthy population samples prior to use of LPL activators in large-scale trials. In addition, few studies have specifically addressed possible subgroups that may be most appropriate to study in trials of LPL activation on the basis of maximal sensitivity to its potential benefits. Information about specific lifestyle or physiological factors that modify the effects of LPL activation may prove critical for the design and success of clinical trials.

We found some evidence that the association of S447X with lipid concentrations and CHD risk might be greatest among overweight and obese individuals. Differences in TG and HDL-C concentrations between carriers and non-carriers of the S447X variant, and the closely linked HindIII SNP, have previously been reported to be greater in participants characterized by central or general obesity.[10, 11, 27, 28] However, there has been relatively little research on risk of clinical endpoints in potentially susceptible subgroups of the general population. An increased efflux of non-esterified fatty acids from the adipose tissue to the liver among obese individuals and the accompanying increase in the secretion of VLDL may be one of the main forces driving the atherosclerotic effects of obesity.[53] Thus, it is possible that the S447X variant could be of greater importance among individuals where the normal lipid transport system is under stress to maintain normal lipid levels.

One of the strengths of this analysis was the ability to address the role of the same variant in three independent studies of generally healthy populations. However, the populations derived from different countries with some marked differences in lifestyle and levels of cardiovascular

risk factors. Clinical endpoints were also different between the studies because ACS assessed in the Danish cohort includes unstable angina pectoris. However, as expected, exclusion of the 66 cases with this condition did not change the results (data not shown). Furthermore, a slightly higher baseline risk in the Danish cohort could dilute the relative risk of ACS associated with the S447X variant. In further attempts to restrict the cohorts to participants who could be most easily compared across studies, the number of participants in some strata was too limited for definitive exploration of the *LPL* SNP according to these traits. It remains possible that other differences between study populations could explain a greater influence of this *LPL* variant in the US cohorts, but further investigations are needed to fully elucidate whether specific environmental factors modify its significance in the Danish population. Larger studies with greater statistical power to address such gene-environment interactions are needed to evaluate whether chance alone explains the differences across study populations - especially in the modifying effect of obesity – that we observed.

We had information on two other coding *LPL* coding SNPs in the NHS and HPFS populations, but were not able to perform meaningful analyses with the rare D9N variant. A borderline association between the N291S SNP and risk of CHD was observed. Other prospective studies support this finding,[12, 13, 17-22] whereas a recent meta-analysis of 21 highly heterogeneous patient and general population studies (p for between study heterogeneity was 0.003) reported a pooled odds ratio of 1.48 (95% CI: 1.09-2.00).[48]

Limitations of the present study include that we only investigated a few polymorphisms in the *LPL* gene. Although the functional significance of especially the S447X SNP appear well established, other polymorphisms in *LPL* and other candidate genes are needed to fully understand the interplay between genetic variation and environmental factors on lipid metabolism. We had no direct measure of LPL activity in our study, however other studies have found higher LPL activity

in 447X carriers.[6] In addition, LPL also mediates the binding between endothelial cell surface receptors and lipoproteins,[49, 50] and is capable of retaining lipoproteins in the arterial wall, possibly leading to foam cell formation and atherosclerosis. Since the S447X variant is located in the C-terminal region of the protein that is responsible for the non-catalytic effects,[5] it is possible that this variant may affect the bridging function of the protein.[6] This gives rise to some controversy about the pro- and anti-atherosclerotic effects of the LPL protein,[50] and greater knowledge about the atherosclerotic role of tissue-specific LPL expression is needed.

In conclusion, our data support that the S447X SNP in the *LPL* gene is associated with lower TG, a small elevation in HDL-C level, and a modestly reduced risk of CHD. This makes LPL an attractive target for drug developments, although further insights into the molecular mechanism behind the associations are needed.

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Figure Legends:

Figure 1:

Relative risks of CHD in NHS and HPFS and ACS in DCH according to body mass index and S447X genotype. Grey dot: S447 homozygotes. Black dot: 447X carriers (SX and XX).

Figure 2:

Geometric mean triglyceride to HDL-C ratio according to body mass index and S447X genotype in the NHS, HPFS and DCH study populations.

Black bar: S447 homozygotes. Light grey bar: 447X carriers (SX and XX).

Table 1. Characteristics of cases and controls in the Nurses' Health Study (NHS), the Health Professionals Follow-Up Study (HPFS), and the Diet, Cancer and Health study.*

	NHS		HPFS		DCH			
					Women		Men	
Variable	**Cases** (n=245)	**Controls** (n=485)	**Cases** (n=258)	**Controls** (n=515)	**Cases** (n=242)	**Controls**† (n=794)	**Cases** (n=773)	**Controls** † (n=909)
Age (yrs)	62 (47; 69)	62 (48; 68)	66 (50; 78)	66 (51; 78)	60 (52; 64)	56 (51; 63)	58 (52; 64)	56 (51; 63)
BMI (kg/m^2)	24.8 (18.4; 36.2)	23.4 (18.9; 32.0)	25.7 (20.9; 31.9)	25.1 (19.5; 31.9)	26.3 (21.4-32.8)	24.6 (21.0; 30.5)	26.9 (23.3-32.4)	26.4 (22.6; 31.1)
Waist (cm)	81 (66, 107)	76 (64; 97)	99 (86; 118)	97 (84; 117)	85 (71; 101)	80 (69; 96)	97 (87-112)	95 (85; 108)
Diabetes	19.8%	6.7%	9.4%	4.5%	5.3%	1.1%	5.6%	2.8%
Hypercholesterolemia‡	53.6%	39.6%	49.3%	40.6%	17.0%	5.8%	12.3%	9.8%
Hypertension	57.7%	29.1%	42.1%	30.8%	43.9%	17.4%	26.3%	16.0%
Postmenopausal	86.0%	84.1%	N/A	N/A	72.5%	59%	N/A	N/A
Current smoker	31.4	32.0	17.1	17.7	60.6%	36.4%	58.6%	39.0%
Alcohol (g/d)	0.9 (0; 24)	1.8 (0; 29)	5.8 (0; 46)	6.8 (0; 77)	5.7 (0.7; 33)	8.5 (1.1; 34)	17 (2; 61)	20 (3; 62)
Plasma lipids (mmol/L)								
Triglycerides	1.5 (1.1; 2.3)	1.2 (0.8; 1.7)	1.7 (0.6; 5.3)	1.2 (0.5; 3.5)	1.8 (1.0; 3.6)	1.3 (0.8; 2.6)	2.1 (1.1; 4.0)	1.7 (0.9; 3.4)
Cholesterol	6.1 (5.5; 6.7)	5.8 (5.2; 6.6)	5.6 (4.0; 7.1)	5.2 (3.9; 7.0)	6.5 (5.3; 8.1)	6.0 (4.9; 7.4)	6.3 (5.0; 7.7)	6.0 (4.8; 7.3)
HDL-C	1.3 (1.1; 1.6)	1.5 (1.2; 1.8)	1.1 (0.7; 1.7)	1.1 (0.7; 1.8)	1.6 (1.2; 2.1)	1.8 (1.2; 2.1)	1.3 (1.0; 1.8)	1.4 (1.1; 1.9)

*Medians (5th and 95th percentiles) of continuous covariates. Fasting participants in NHS (n=428) and HPFS (n=466). In DCH all lipids were obtained from non-fasting participants (n=2610).

† Random sample of cohort at baseline, includes 33 participants who became cases during follow-up.

‡ Diagnosed with hypercholesterolemia or reporting to use cholesterol lowering medication.

Table 2. Geometric means (95% CI's) blood lipid concentrations according to LPL 447X carrier status in the controls from the Nurses' Health Study (NHS), the Health Professionals Follow-Up Study (HPFS), and the random sub-cohort in Diet, Cancer and Health study (DCH)*.

Blood lipid	Non carrier (SS)	Carrier (SX/XX)	P
Triglycerides (mmol/L)†			
NHS (n=321)	1.26 (1.18-1.35)	1.05 (0.95-1.16)	0.003
HPFS (n=300)	1.38 (1.29-1.48)	1.24 (1.10-1.38)	0.11
DCH (n=1645)	1.70 (1.65-1.75)	1.59 (1.51-1.68)	0.03
HDL-C (mmol/L)			
NHS (n=479)	1.48 (1.45-1.52)	1.56 (1.48-1.64)	0.11
HPFS (n=515)	1.14 (1.11-1.16)	1.16 (1.11-1.20)	0.44
DCH (n=1648)	1.53 (1.51-1.55)	1.58 (1.54-1.62)	0.001
Triglyceride/HDL-C†			
NHS (n=305)	0.87 (0.80-0.95)	0.72 (0.56-0.75)	0.001
HPFS (n=300)	1.19 (1.09-1.30)	1.05 (0.92-1.22)	0.19
DCH (n=1645)	1.11 (1.07-1.15)	1.01 (0.94-1.08)	0.01

*Backtransformed least square means using log-transformed lipid measures in regression models adjusted for smoking, age, alcohol, menopausal status among women, body mass index, and sex in DCH.

† Fasting participants in NHS and HPFS. Non-fasting participants in DCH.

Note: not all lipid parameters available on all subjects.

Table 3. Relative risk [RR] and 95% confidence intervals [CI] of CHD according to Lipoprotein Lipase S447X genotype in the Nurses' Health Study (NHS), the Health Professionals Follow Up Study (HPFS), and the Diet, Cancer and Health (DCH) study.*

	MAF (%)		N (cases/controls)			Relative risk			
	Cases	Control	SS	SX	XX	SS	SX	XX	SX/XX
NHS	7.4	12	211/379	33/100	1/6	1.0 (ref)	0.57 (0.36-0.90)	0.31 (0.04-2.82)	0.56 (0.36-0.87)
HPFS	8.5	12	216/406	40/99	2/10	1.0 (ref)	0.73 (0.49-1.10)	0.37 (0.08-1.69)	0.70 (0.47-1.04)
DCH	8.8	10	840/1386	171/299	4/17	1.0 (ref)	0.93 (0.79-1.19)	0.60 (0.19-1.91)	0.92 (0.72-1.16)
Pooled†						1.0 (ref)	0.77 (0.58-1.03)	0.53 (0.29-0.97)	0.74 (0.56-1.00)

*Conditional logistic regression models were run in NHS and HPFS data (stratified by matching factors). Cox proportional hazard regression models in DCH (stratified by sex). All models adjusted for age, smoking, alcohol intake, menopausal status among women, body mass index.

† Meta-analysis using random effects. P for tests of between study heterogeneity: SX: 0.7; XX: 0.13; dominant (SX/XX): 0.12.

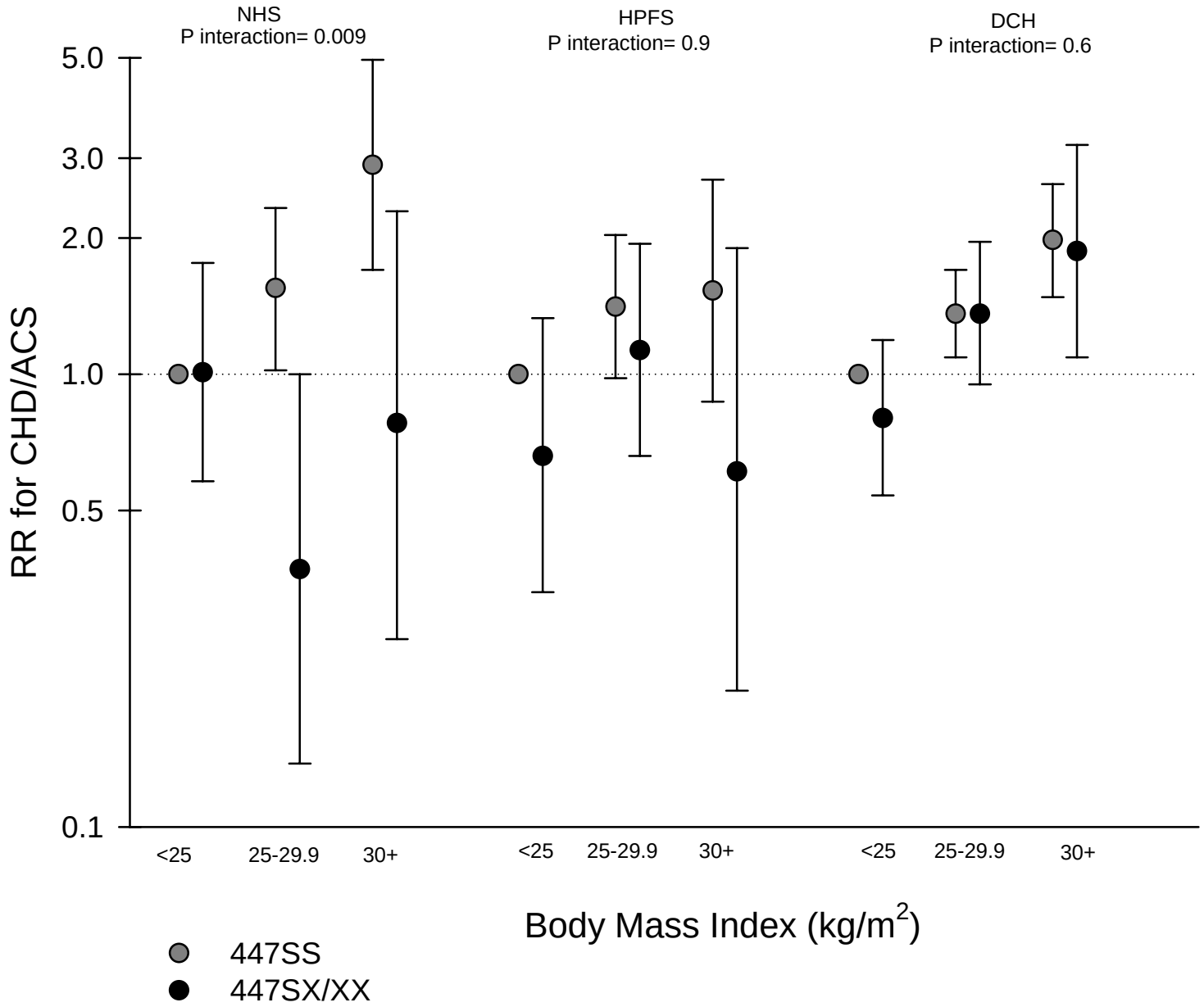
NHS
P interaction= 0.009
HPFS
P interaction= 0.9
DCH
P interaction= 0.6
5.0
3.0
2.0
1.0
0.5
0.1
RR for CHD/ACS
<25
25-29.9
30+
<25
25-29.9
30+
<25
25-29.9
30+
Body Mass Index (kg/m2)
447SS
447SX/XX

Figure 1.

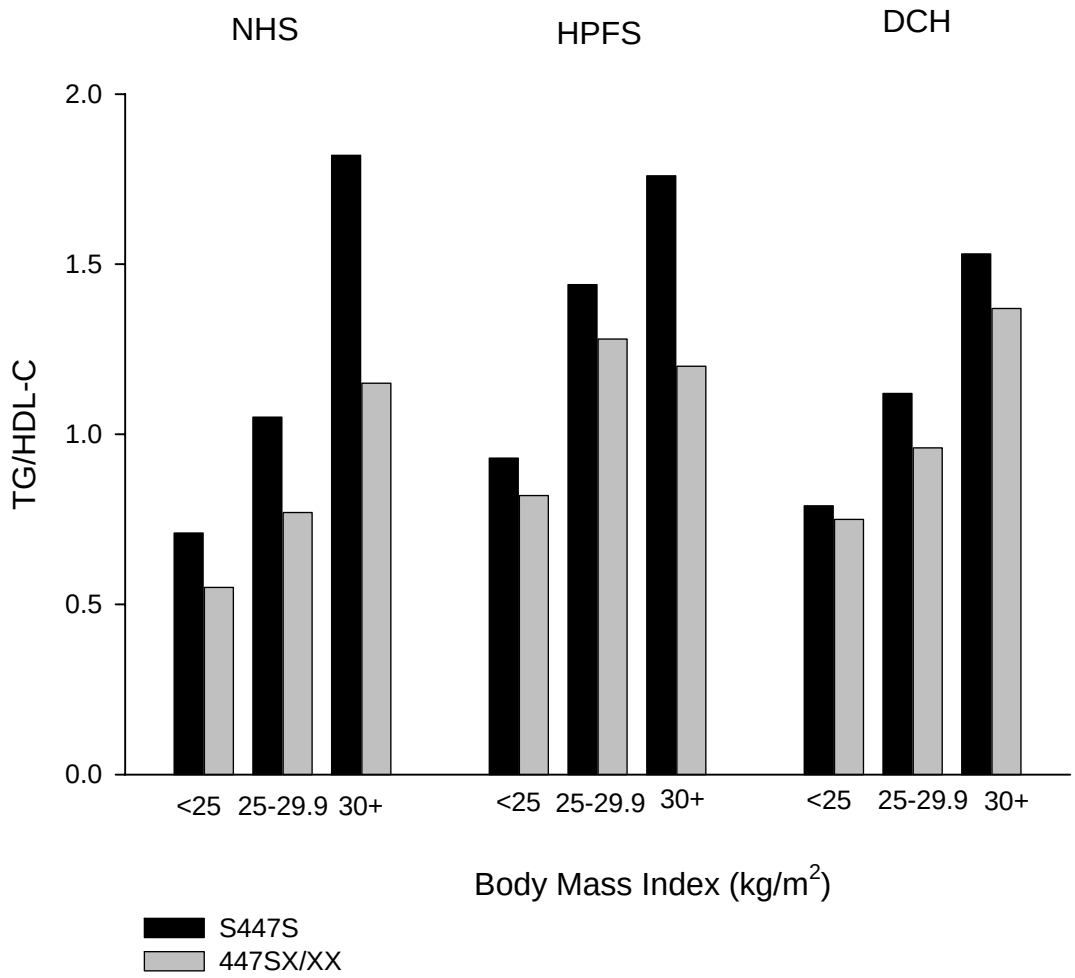
NHS
HPFS
DCH
2.0
1.5
1.0
0.5
0.0
TG/HDL-C
<25
25-29.9
30+
<25
25-29.9
30+
<25
25-29.9
30+
Body Mass Index (kg/m2)
S447S
447SX/XX

Figure 2.

Online Supplement

Supplementary appendix to the manuscript:

The S447X variant of the lipoprotein lipase gene, lipids, and risk of coronary heart disease in three prospective cohort studies

Authors: Majken K. Jensen, Eric B. Rimm, Daniel Rader, Erik B. Schmidt, Thorkild I. A. Sørensen, Ulla Vogel, Kim Overvad, and Kenneth J. Mukamal

Measurements of body weight and waist circumference

In NHS and HPFS, participants were asked to report their height (in inches; 1 in: 2.54 cm) and current body weight (in pounds; 1 lb=0.45 kg); weight was then updated during the biennial follow-up. In 1986, women in the NHS were asked to measure their waist circumference with a paper tape and were given detailed measuring directions. Men were asked to do the same in 1987 and 1996. Self-reports of body weight, height and waist circumference have been shown to be highly correlated with technician-measured weight, height and waist circumference in both HPFS and NHS participants (all $r \geq 0.94$).

In the DCH, a technician took waist, height and weight measurements of all participants at the baseline examination.

Body-mass index (BMI) was calculated as weight (kg) divided by height squared (meters). We used WHO recommended cut-points for BMI; <25 kg/m^2 (normal-weight), 25-29.9 kg/m^2 (overweight), ≥ 30 kg/m^2 (obese) and waist circumference; Men: <94 cm, 94-101.9 cm, and ≥ 102 cm, women: <80 cm, 80-87.9 cm, ≥ 88 cm).

Details for the identification of incident ACS in the Diet, Cancer, and Health study

Information on the disease endpoint was obtained by linkage with central Danish registries via the unique identification number assigned to all Danish citizens.[1] Hospital records of potential cases were retrieved from hospitals for participants who were registered with a first-time discharge diagnosis of ACS (ICD-8 codes 410-410.99, 427.27 and ICD-10 codes I20.0, I21.x, I46.x) in The Danish National Register of Patients, which covers all hospital discharge diagnoses since 1977 and all discharge diagnosis from out-patient clinics since 1995 (until Jan 1, 2004).[2] Cases were classified according to symptoms, signs, coronary biomarkers, ECGs and/or autopsy findings in accordance with the current recommendations of the American Heart Association and the European Society of Cardiology (AHA/ECS).[3] A description of the validation study is submitted. Other validation studies have indicated that MI is recorded with a high degree of validity in this register.[4] Further, linkage to the Cause of Death Register allowed for identification of participants with ACS coded as a primary or secondary cause of death (to Jan 1, 2004).

Laboratory analysis

NHS and HPFS participants underwent local phlebotomy and returned samples to our laboratory via overnight courier. Upon arrival, whole blood samples were centrifuged and stored in cryotubes as plasma, buffy coat, and red blood cells in the vapor phase of liquid nitrogen freezers.

In the DCH study, non-fasting blood from virtually all study participants was sampled at the study clinic and stored at –150◦C.

Triglycerides (TG) and HDL-C were assessed using standard methods with reagents from Roche Diagnostics (Indianapolis, IN) for US data and Bayer Diagnostics (New York, NY) for DCH. In US data, samples were sent to the laboratory for analysis in batches where cases were paired with their two controls in random order. The intra-assay coefficient of variation was (CV) 2% for HDL-C in the three studies. Blood was obtained from mostly fasting participants in the US study (for analyses of TG levels, the few non-fasting were excluded) whereas Danes were all non-fasting. CV's for TG were 2.5% in US samples and 4.5% in the Danish samples.

In US data, DNA was extracted from the buffy coat fraction of centrifuged blood with the QLAmp Blood Kit (Qiagen, Chatsworth, CA). The primary genotyping technique was Taqman SNP allelic discrimination by means of an ABI 7900HT (Applied Biosystems, Foster City, CA), using rs328. In addition, two other commonly investigated LPL coding SNPs were genotyped in the US data alone; the D9N variant (rs1801177) and the N291S variant (rs268).

In the DCH, DNA was isolated from frozen blood samples according to standard procedure as previously described.[5] S447X genotype was determined by Taqman allelic discrimination (ABI 7500, Applied Biosystems, Nærum, Denmark). Technicians were blinded to case status of the samples. Controls were included in each run and repeated genotyping of a random 10% subset yielded 100% identical genotypes.

Supplementary analysis

The RR for CHD among N291S heterozygotes was 1.18 (95% CI: 0.83-1.69) and for SS homozygotes it was 0.76 (0.49-1.19), compared to NN291 homozygous women in NHS. Corresponding results in the HPFS were 0.95 (0.67-1.33) and 0.66 (0.43-1.00).
The rare variant, D9N (MAF: 0.01), did not allow for further analysis.

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Variation in the Endothelial Lipase Gene and Risk of Coronary Heart Disease in Two Independent Populations

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Background*:* The recently identified endothelial lipase (EL) is an important modulator of high-density lipoprotein-cholesterol (HDL-C) metabolism. Over-expression of the gene (encoded by *LIPG*) is associated with decreased HDL-C in mice and genetic variations have also been been associated with HDL-C in humans. So far, no prospective studies have investigated genetic associations with cardiovascular risk in healthy Caucasians.

Objective: To investigate the association between three *LIPG* variants with risk of coronary heart disease (CHD) in two independent studies.

Methods and Results: One promoter variant, –1309A/G, and two non-synonymous variants, N396S and T111I, were genotyped in a case-cohort study of acute coronary syndrome in the Diet, Cancer and Health (DCH) study and validated in a nested case-control study of incident CHD nested within the Health Professionals Follow-up Study (HPFS), totaling 1015 and 418 cases, respectively. In the combined samples, the minor allele frequencies in healthy controls were: -1309A/G=0.23, N396S=0.01 and T111I=0.29. The -1309A/G and the N396S variants were weakly associated with a higher risk in both studies. The pooled estimate for risk of CHD was 1.12 (95% CI: 0.99-1.27) per –1309G-allele. The risk among carriers of the rare N396S variant was 1.25 (0.76-2.01). There was a suggestion that the T111I variant was associated with a lower risk of CHD. The pooled estimate was 0.93 (0.83-1.06) per risk allele.

Conclusions: We did not observe strong associations between three LIPG variants and CHD-risk. Further studies are needed to elucidate the role endothelial lipase in the development of CHD.

High-density lipoprotein cholesterol (HDL-C) is strongly associated with a lower risk of coronary heart disease (CHD), but its complex metabolism has limited efforts to develop pharmacologic therapies.[1] The recently identified endothelial lipase (EL) is considered unique because it is synthesized by endothelial cells and it functions mostly as a phospholipase, making HDL-C its preferred substrate.[2, 3] In animal models, overexpression of EL is associated with substantial reductions in HDL-C concentration and inhibition is associated with relatively higher HDL-C levels.[2, 4, 5]

Evidence that EL plays a role in human lipid metabolism comes from two cross-sectional studies, where an inverse association was observed between plasma EL concentration and HDL-C levels.[6, 7] In addition, a nonsynomous polymorphism (T111I) in exon 3 of the gene encoding EL (gene name: *LIPG*) has been associated with variation in HDL-C concentration in several,[5, 8-10] albeit not all,[11-13] studies.

In humans, data on the association with clinical endpoints are sparse. High EL concentration was positively associated with coronary artery calcification in 858 healthy inviduals.[6] In the small LCAS study of 371 patients with existing coronary artery disease, the T111I variant was not associated with past history of MI or the progression of atherosclerosis during 2.5 years of follow-up.[5] In contrast, recent case-control studies from Japan and China observed significantly lower frequencies of the I111-allele among MI survivors and cardiovascular patients than among healthy controls.[10, 13] To our knowledge, no prospective studies have evaluated the role of genetic variation in *LIPG* and risk of incident CHD in healthy populations.

Several less common *LIPG* variants have been identified in small ethnically mixed studies.[8, 14] (*and Rader/Edmondson, in preparation*). Besides the T111I polymorphism, we selected two specific *LIPG* variants for this study that were identified in population samples that were selected based on high HDL-C levels, and for which preliminary data suggests meaningful changes in HDL-

C levels.[14] (*and Rader/Edmondson, in preparation*) Our aim was to examine their association with risk of CHD in two independent studies of healthy Caucasians. We also aimed to investigate the potential interaction with non-genetically determined variation in HDL-C concentration because substrate availability for EL may influence its potential association with CHD.

Methods

Study populations

The Diet, Cancer, and Health (DCH) study was initiated in 1993-1997 when 57,053 Danish born residents, aged 50 to 64 years and free of cancer, participated in a clinical examination and detailed lifestyle survey. Blood was sampled at baseline in the study clinic and stored as plasma, serum, lymphocytes, and erythrocytes at −150 ◦C.

The Health Professionals Follow-Up Study (HPFS) was established in 1986 when 51,529 male health professionals 40 to 75 years of age from throughout the US completed a mailed questionnaire. Participants have received follow-up questionnaires biennially to update information on lifestyle exposures and newly diagnosed illnesses. A blood sample was requested from all active participants in 1993-1995, and returned by 18,224 men. Participants underwent local phlebotomy and returned samples to the laboratory via overnight courier. Upon arrival, whole blood samples were centrifuged and stored as plasma, buffy coat, and red blood cells in liquid nitrogen freezers. Detailed descriptions of the study cohorts have been published previously.[15, 16]

Endpoint and study designs

In the DCH, a case-cohort study was designed using incident acute coronary syndrome (ACS), including unstable angina pectoris, MI, and sudden cardiac death as the outcome. Information on the disease endpoint was obtained by linking the participants (via the unique identification number assigned to all Danish citizens) with central Danish registries of hospital discharge diagnoses and causes of death (ICD-8 codes 410-410.99, 427.27 and ICD-10 codes I20.0, I21.x, I46.x). We validated the records in the Danish National Register of Patients by retrieving hospital records of all participants who were registered with a first-time discharge diagnosis of ACS. A description of the validation study is in press (AM Joensen *et al*). Our findings were in agreement with other

validation studies that have indicated that MI is recorded with a high degree of validity in this register,[17] whereas unstable angina and sudden death were less accurate. In total, 1150 cases of ACS were identified between baseline and Jan 1st, 2004, the date of the last available update from the hospital discharge register. For the creation of the case cohort sample, 1800 participants were selected from the entire DCH study at random (for consistency, sub-cohort members are designated as 'controls' although 35 individuals overlap with the case group).

In the HPFS, a nested case-control study was designed using incident CHD, with non-fatal MI and fatal CHD as the outcome. Cases of MI and fatal CHD were self-reported followed by review of medical records, as previously described.[18, 19] Among participants who provided blood samples and who were free of diagnosed cardiovascular disease or cancer at blood draw, 418 men sustained an incident MI/CHD between blood draw and January 31, 2004. Using risk-set sampling,[20] controls were selected randomly and matched in a 2:1 ratio on age, smoking, and month of blood return, among participants who were free of cardiovascular disease at the time CHD was diagnosed in the case.

Laboratory analysis methods

Plasma lipids, lipoproteins, and apolipoproteins were assessed using standard methods with reagents from Roche Diagnostics (Indianapolis, IN) and Bayer Diagnostics (New York, NY).[21]

In the DCH, DNA was isolated from frozen lymphocytes according to standard procedure as previously described.[22] Genotypes were determined by Taqman allelic discrimination (ABI 7500, Applied Biosystems, Nærum, Denmark) using rs2000813 (T111I) and rs3829632 (-1309A/G). For N396S primers were: forward GGAGCGGATCGAGCAGAATG; reverse: CAAGTCCTCCTCGGTGTAGAC, probe: CCACCAACACCTTC. The case status of the samples was blinded when analyzing the DNA in the laboratory. Controls were included in each run and

repeated genotyping of a random 10% subset yielded 100% identical genotypes. In HPFS, DNA was extracted from the buffy coat fraction of centrifuged blood with the QLAmp Blood Kit (Qiagen, Chatsworth, CA). The primary genotyping technique was Taqman SNP allelic discrimination by means of an ABI 7900HT (Applied Biosystems, Foster City, CA).

Statistical analysis

Conformity with Hardy-Weinberg equilibrium was tested with an exact test using the controls from both study populations. Multivariable regression analysis was used to address the associations between *LIPG* genotype and log-transformed lipids in the random sub-cohort of the DCH study. The association of the three *LIPG* variants with plasma lipids and lipoproteins has previously been reported for the HPFS (*Rader/Edmondson, in preparation*), and will only be summarized here in the context of a new meta-analyses with the DCH data. All analyses included adjustment for age, sex (DCH only), smoking, alcohol, and BMI, and among women, menopausal status and hormone replacement therapy. Relative risks (RR) and 95% confidence intervals (CIs) for the association between *LIPG* genotype and CHD were estimated using Cox proportional hazard regression with Kalbfleisch and Lawless weights and robust variance suitable for the case-cohort data from DCH,[23] and conditional logistic regression for the HPFS nested case-control data. Analyses in DCH allowed for sex-specific baseline hazards. Sex-specific analyses and tests of interaction in DCH did not suggest any statistically significant sex-differences. We included lifestyle covariates in the model because of their strong association with CHD and because they may partially account for heterogeneity between study participants. In total, the numbers of participants with information available on plasma lipids, genotype, and covariates were: 2621 (985 cases) in DCH and 418 cases, 866 controls in HPFS, except for the T111I variant which was only available on a subset of HPFS cases (n=237).

Three haplotypes could be inferred from the three variants. We defined six groups of diplotypes (combined haplotypes) that accounted for >98% of the participants in both studies. The remaining participants included infrequent combinations of the –1309A/G and T111I variants, but were all characterized by carrying the N396S variant.

To pool the estimates from the two study population, we used the weighted average of regression estimates using the DerSimonian and Laird random-effects model.[24]

Because HDL-C concentration is modulated by several environmental factors, and because EL activity itself may affect risk of CHD indirectly through its influence on HDL-C, we examined whether there was an interaction between *LIPG* variants and characteristics associated with higher HDL-C concentration on the risk of CHD. To avoid running a substantial number of regression models, we created a summary measure of predicted HDL-C based on non-*LIPG* exposures. Using the methodology in Miettinen's original 'multivariate confounder score',[25] we fit the predicted values of HDL-C based on a regression model including the following factors: Age (continuous), sex, education (3 categories), BMI (continuous), physical activity (3 cat), smoking status (5 cat), alcohol (continuous), monounsaturated fat (quintiles), saturated fat (quintiles), diabetes, and hypercholesterolemia. We examined the interaction by comparing the association between the predicted HDL-C and CHD in strata of the *LIPG* variants, and tested their interaction on a multiplicative scale by including the cross-product term.

Reported p-values are two-sided and 0.05 was the considered threshold for statistical significance. Analyses were performed using SAS 9 (SAS Institute Inc., Cary, NC) and STATA 9.1 (STATA Corp., College Station, TX).

Results

Table 1 shows baseline characteristics of cases and controls in the two study populations. As expected, the prevalence of diabetes, hypertension, and hypercholesterolemia was higher among the cases than among the controls, and cases had lower levels of HDL-C than controls.

LIPG genotypes and lipids

Overall, absolute differences in lipid concentrations according to genotypes were small (**Table 2**) Among the randomly selected control group of the DCH cohort, the –1309A/G variant was associated with lower ApoA1 and total cholesterol levels. The T111I variant was not associated with any of the blood lipids. Participants who were heterozygous carriers of the N396S variant had lower LDL-C, ApoB, and total cholesterol levels. Although weaker, the same tendencies for the N396S variant were observed in the HPFS (data not shown). In pooled analyses, N396S heterozygotes had 0.19 mmol/L lower LDL-C (p=0.18), 0.05 mmol/L lower ApoB (p= 0.04), and 0.20 mmol/L lower total cholesterol (p=0.09) concentrations.

LIPG genotype and risk of CHD

Genotypes were in Hardy-Weinberg equilibrium in the control study populations, except for the T111I variant in the HPFS controls (p=0.03). Minor allele frequencies were: -1309A/G=0.23, T111I=0.29 and N396S=0.01 in the DCH sub-cohort. Allele frequencies were very similar in the HPFS controls (**Table 3**).

The –1309A/G variant was associated with risk of CHD in an allele dependent manner in both studies, although only statistically significant in the HPFS (Table 3). In an additive model, the relative risk (RR) was 1.07 (95% confidence interval [CI], 0.92-1.25) in DCH and 1.23 (95% CI, 1.00-1.51) in HPFS. The pooled RR was 1.12 (95% CI, 0.99-1.27). The T111I variant tended to

be associated with a lower risk in both study populations, although not statistically significant (pooled RR = 0.93 [95% CI, 0.83-1.06] per risk allele). The risk of CHD among heterozygous carriers of the rare N396S variant was higher compared to non-carriers, however confidence intervals were broad in both studies separately and in the pooled analysis.

Linkage disequilibrium coefficients are shown in **Table 4**. Because the rare N396S variant seldom co-occurred with the T111I variant, the r^2 was only 0.10 even though the D´ suggested high LD.

Six combinations of the three variants accounted for >98% of the participants in both studies. In **Table 5** we show the associated risk of CHD using the homozygous wildtypes for all three variants as the reference group. These results did not substantially change the interpretation of the results from the main effects models in Table 3. A lower risk of CHD was suggested among carriers of the T111I variant who were wildtype homozygous at the two other loci. The pooled result for heterozygous T111I carriers was 0.82 (95% CI, 0.66-1.02) and for homozygous carriers it was 0.86 (95% CI, 0.61-1.20). In a separate analysis that was restricted to non-carriers of the –1309A/G and N396S variants, the pooled RR for CHD was 0.89 (95% CI, 0.77-1.04) per I111-allele.

We found no statistically significant differences in HDL-C according to *LIPG* diplotype in either study population. The small group that included the S396-carriers had the highest HDL-C in both studies, which was 0.12 mmol/L higher than those who were homozyogous wildtype at all three loci.

Interactions

There was a strong inverse association between estimated HDL-C level based upon environmental determinants and risk of CHD. This association was not significantly modified by any of the three *LIPG* variants (data not shown). In the DCH study, there was a suggestion of a weaker association

between predicted HDL-C and CHD among carriers of the N396S variant, but the statistical power for this analysis was low as only 39 cases occurred among the carriers during follow-up.

Discussion

In the present study we found weak associations between three polymorphisms in the gene encoding EL and plasma lipids and risk of CHD. Both the promoter variant –1309A/G and the N396S variant were associated with lower total cholesterol levels. There was an indication that this was related to lower concentration of ApoA1-containing particles for the –1309A/G variant and lower ApoB-containing particles for the N396S variant. The prospective analysis of two Caucasian cohorts showed suggestive, but consistent, evidence that both these polymorphism were associated with a higher risk of CHD, whereas the T111I polymorphism, which was unrelated to plasma lipid profile, was associated with a lower risk of CHD.

An association between the T111I variant and continuous measures of HDL-C has been reported among US patients with coronary artery disease and among Japanese American and Chinese control samples.[5, 9, 10] In the present study we found no indication that the T111I variant was associated with any of the plasma lipids in two independent samples of generally healthy Caucasian men and women. Our results are similar to those recently reported in a healthy Canadian population and in a small Japanese sample.[11, 13] Minor allele frequencies were close to 30% in all these studies. It is difficult to reconcile such discrepant findings when they originate from patients, healthy individuals, and populations of different ethnicities as different pathophysiological circumstances and different distributions of lifestyle characteristics may modify the observed associations.

In the present study, we genotyped *LIPG* variants that were originally identified by sequencing of the coding and promoter region of the *LIPG* gene in small samples with high HDL-C levels compared to their age and sex.[14](and *Rader/Edmondson, in preparation*). This strategy was chosen to maximize the likelihood of finding variants with a functional role in HDL-C modulation. Rader, Edmondson *et al*. have recently reported that the –1309A/G promoter variant was associated

with lower and the N396S variant with higher HDL-C concentration in a large cohort of more than 5000 participants from the Framingham study.*(Rader/Edmondson in preparation)* While the results from the DCH study are in the same direction, the absolute differences were very small.

The overall main effects of the variant alleles on CHD risk were weak. There was a suggestion that the T111I variant was associated with a lower risk of developing CHD in a co-dominant fashion. The T111I variant was also significantly less common among Japanese MI survivors and Chinese patients with coronary artery disease,[9, 10] whereas no association with progression of atherosclerosis were observed in the LCAS study where CAD patients were followed for a short period of 2.5 years.[2, 4, 5] More well-designed studies, preferably of prospective nature to avoid the potential survival bias in case-control studies are needed to clarify whether this common variant plays a role for the risk of CHD in the general population. Results for both the promoter variant, –1309A/G, and the nonsynomous N396S variant indicated a statistically insignificant higher risk among carriers. To our knowledge these to variants have not previously been examined in relation to risk of cardiovascular endpoints. Unfortunately, we had low statistical power for the analysis of the rare N396S variant, but of interest, this variant was also associated with lower levels of apoB-containing lipoproteins. Recently, it was shown that EL inhibition may raise plasma levels of not only HDL-C but also apoB-containing lipoproteins.[26] This raises questions about the potentially anti- and pro-atherogenic effects of EL. Besides having a high phospholipase activity, EL has, like other lipases, the ability to bind and retain lipoproteins in the arterial wall via a non-catalytic bridging function.[27] While the removal of LDL particles from the circulation may be beneficial, the net effect of the non-enzymatic function of EL may be tissue-specific. EL is found at high levels in macrophages of atherosclerotic lesions, where entrapment of LDL particles might contribute to the atherosclerotic plaque development.[28] This suggests that EL may play a role in the

development of atherosclerosis both directly and indirectly through the modulation of plasma lipids. However, we did not observe strong associations with CHD in the present study.

Overall, our analyses of three *LIPG* SNPs and risk of CHD yielded surprisingly consistent findings between the Danish participants and the American men despite considerable variation in lifestyle characteristics. Although we did not observe any statistically significant modification of the association between any of the *LIPG* variants and risk of CHD, we lacked statistical power to detect such heterogeneity. In depth exploration of gene-environment interactions may be of interest for future investigations in larger studies.

Our study was limited by the lack of functional evidence for the *LIPG* variants. HDL-C concentration may not be a very sensitive marker of the turnover of phospholipids between plasma and lipoproteins. As population-wide variability in levels of HDL-C is related to both genetic factors[29] and several lifestyle features,[30-33] the potential contribution of *LIPG* variants to plasma HDL-C concentration may be small, and thus, very large well defined studies may be needed to detect such differences.

A high degree of linkage disequilibrium has been observed across the *LIPG* gene, and we cannot exclude the possibility that our investigation of three single locus polymorphisms could be confounded by linkage with other unmeasured polymorphisms.

In summary, we found weak evidence that variation in the gene encoding EL may be involved in the development of CHD. Further research is needed to establish the functional role of these polymorphisms. Larger studies to investigate the role of the rare N396S variant in relation to plasma lipids and risk of CHD are of particular interest.

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Table 1. Characteristics of cases and controls in the Diet, Cancer and Health study (DCH) and the Health Professionals Follow-Up Study (HPFS).*

	DCH				HPFS	
	Women		Men			
Variable	**Cases** (n=242)	**Controls**† (n=794)	**Cases** (n=773)	**Controls** † (n=909)	**Cases** (n=418)	**Controls** (n=866)
Age (yrs)	60 (52; 64)	56 (51; 63)	58 (52; 64)	56 (51; 63)	66 (50; 78)	66 (51; 78)
BMI (kg/m^2)	26.3 (21.4-32.8)	24.6 (21.0; 30.5)	26.9 (23.3-32.4)	26.4 (22.6; 31.1)	25.7 (20.9; 31.9)	25.1 (19.5; 31.9)
Waist (cm)	85 (71; 101)	80 (69; 96)	97 (87-112)	95 (85; 108)	99 (86; 118)	97 (84; 117)
Diabetes	5.3%	1.1%	5.6%	2.8%	9.4%	4.5%
Hypercholesterolemia‡	17.0%	5.8%	12.3%	9.8%	49.3%	40.6%
Hypertension	43.9%	17.4%	26.3%	16.0%	42.1%	30.8%
Postmenopausal	72.5%	59%	N/A	N/A	N/A	N/A
Current smoker	60.6%	36.4%	58.6%	39.0%	17.1	17.7
Alcohol (g/d)	5.7 (0.7; 33)	8.5 (1.1; 34)	17 (2; 61)	20 (3; 62)	5.8 (0; 46)	6.8 (0; 77)
Plasma lipids (mmol/L)						
Triglycerides	1.8 (1.0; 3.6)	1.3 (0.8; 2.6)	2.1 (1.1; 4.0)	1.7 (0.9; 3.4)	1.7 (0.6; 5.3)	1.2 (0.5; 3.5)
Total cholesterol	6.5 (5.3; 8.1)	6.0 (4.9; 7.4)	6.3 (5.0; 7.7)	6.0 (4.8; 7.3)	5.6 (4.0; 7.1)	5.2 (3.9; 7.0)
HDL-C	1.6 (1.2; 2.1)	1.8 (1.2; 2.1)	1.3 (1.0; 1.8)	1.4 (1.1; 1.9)	1.1 (0.7; 1.7)	1.1 (0.7; 1.8)

*Medians (10th and 90th percentiles) of continuous covariates. Fasting participants in HPFS (n=298). In DCH all lipid measurements were obtained from non-fasting participants (n=2610).

† Random sample of cohort at baseline, includes 33 participants who became cases during follow-up.

‡ Diagnosed with hypercholesterolemia or reporting to use cholesterol lowering medication.

Table 2. Mean blood lipid, lipoprotein (mmol/L) and apolipoprotein (g/L) concentrations and 95% CI's according to *LIPG* SNPs in 1627 randomly selected participants in the Diet, Cancer, and Health Study.*

EL SNP	Total cholesterol	Triglycerides	HDL-C	LDL-C	ApoA1	ApoB
-1309						
AA‡	5.79 (5.61; 5.97)	1.39 (1.28; 1.51)	1.46 (1.41; 1.51)	3.58 (3.43; 3.74)	1.47 (1.43; 1.50)	1.04 (1.01; 1.08)
AG	5.71 (5.52; 5.90)†	1.37 (1.25; 1.49)	1.47 (1.41; 1.52)	3.51 (3.34; 3.67)	1.47 (1.43; 1.50)	1.03 (0.99; 1.07)
GG	5.73 (5.48; 5.99)†	1.32 (1.16; 1.48)	1.44 (1.36; 1.51)	3.62 (3.39; 3.84)	1.42 (1.36; 1.49)†	1.04 (0.98; 1.10)
Additive	-0.064 (-0.134; 0.006)	-0.029 (-0.108; 0.049)	-0.005 (-0.034; 0.024)	-0.037 (-0.099; 0.026)	-0.014 (-0.033; 0.005)	0.009 (-0.026; 0.009)
T111I						
CC‡	5.68 (5.52; 5.84)	1.36 (1.25; 1.48)	1.47 (1.42; 1.52)	3.55 (3.39; 3.71)	1.46 (1.42; 1.50)	1.05 (1.08; 1.09)
CT	5.60 (5.42; 5.76)	1.39 (1.27; 1.51)	1.46 (1.40; 1.51)	3.57 (3.41; 3.73)	1.46 (1.42; 1.50)	1.04 (1.01; 1.08)
TT	5.63 (5.38; 5.88)	1.46 (1.30; 1.63)	1.48 (1.41; 1.55)	3.58 (3.37; 3.79)	1.49 (1.43; 1.54)	1.05 (1.00; 1.10)
Additive	0.024 (-0.043; 0.091)	0.044 (-0.031; 0.119)	0.001 (-0.027; 0.029)	-0.008 (-0.068; 0.051)	0.006 (-0.013; 0.024)	0.004 (-0.013; 0.02)
N396S						
AA‡	5.77 (5.59; 5.94)	1.36 (1.14; 1.59)	1.46 (1.41; 1.51)	3.57 (3.41-3.72)	1.46 (1.42-1.49)	1.04 (1.00; 1.08)
AG	5.44 (5.08; 5.80)†	1.38 (1.27; 1.49)	1.48 (1.37; 1.59)	3.21 (2.90-3.53) †	1.46 (1.37-1.54)	0.95 (0.88; 1.03) †
GG			-			
Additive	-0.261 (-0.543; 0.020)	0.037 (-0.278; 0.353)	0.021 (-0.097; 0.139)	-0.301 (-0.55; -0.055)	-0.003 (-0.081; 0.074)	-0.070 (-0.140; 0.000)

* Non-fasting lipid concentrations estimated in regression models adjusted for smoking, age, alcohol, BMI, education, diabetes and hypercholesterolemia. Analyses among women also adjusted for postmenopausal status and use of HRT. Geometric means are presented for HDL-C, ApoB, and triglycerides.

† p value <0.05

‡ Letters refers to the nucleotide

Table 3. Minor allele frequencies (MAF), relative risk and 95% confidence intervals of CHD according to *LIPG* genotypes in the Diet, Cancer and Health (DCH) study and the Health Professionals Follow Up Study (HPFS).*

	MAF (s.e)		**N (cases/controls)**			**Relative risk (95% confidence interval)**			
SNP	Cases	Control	Wildtype homozygotes	Heterozygotes	Variant homozygotes	Wildtype homozygotes	Heterozygotes	Variant homozygotes	Per variant allele
-1309A/G			‡ AA	AG	GG	AA	AG	GG	Per G-allele
DCH	0.24 (0.01)	0.23 (0.01)	581/959	344/563	60/99	1.0 (ref)	1.09 (0.89-1.32)	1.13 (0.76-1.66)	1.07 (0.92-1.25)
HPFS	0.21 (0.01)	0.18 (0.01)	262/584	135/246	21/36	1.0 (ref)	1.25 (0.96-1.63)	1.44 (0.80-2.58)	1.23 (1.00-1.51)
Pooled	0.23 (0.01)	0.22 (0.01)	843/1543	479/809	81/135	1.0 (ref)	1.14 (0.98-1.33)	1.21 (0.88-1.67)	1.12 (0.99-1.27)
T111I			CC	CT	TT	CC	CT	TT	Per T-allele
DCH	0.29 (0.01)	0.29 (0.01)	502/826	402/664	81/131	1.0 (ref)	0.97 (0.80-1.17)	0.91 (0.65-1.28)	0.96 (0.83-1.11)
HPFS†	0.28 (0.02)	0.31 (0.02)	135/248	122/249	17/39	1.0 (ref)	0.85 (0.61-1.18)	0.71 (0.38-1.35)	0.85 (0.66-1.09)
Pooled	0.29 (0.01)	0.29 (0.01)	637/1074	524/913	98/170	1.0 (ref)	0.94 (0.80-1.11)	0.87 (0.64-1.17)	0.93 (0.83-1.06)
N396S			AA	AG	GG	AA	AG	GG	
DCH	0.01 (0.003)	0.01 (0.002)	960/1582	25/39	0/0	1.0 (ref)	1.06 (0.60-1.87)	-	-
HPFS	0.01 (0.004)	0.01 (0.002)	407/854	10/12	1/0	1.0 (ref)	1.86 (0.75-4.63)	-	-
Pooled	0.01 (0.002)	0.01 (0.001)	1367/2436	35/51	1/0	1.0 (ref)	1.25 (0.76-2.01)	-	-

*Cox proportional hazard regression models in DCH (stratified by sex). Conditional logistic regression models were run in HPFS data (stratified by matching factors).

All models adjusted for age, smoking, alcohol intake, menopausal status among women, body mass index.

† Restricted to 810 participants because genotypes only available in a subset of cases and controls.

‡ Letters refers to the nucleotide

Table 4 Pairwise linkage disequilibrium (D´) and correlation coefficient (r^2) between *LIPG* polymorphisms.

			Linkage disequilibrium (D´)		
		Polymorphism	-1309A/G	T111I	N396S
Correlation	**coefficient**	**(r^2)**			
		-1309A/G	-	0.97	0.07
		T111I	0.30	-	1.00
		N396S	0.01	0.10	-

Note: results presented from HPFS controls.

Table 5 Combinations of *LIPG* genotypes and relative risk (RR) and 95% confidence intervals (CI) of coronary heart disease in the Diet, Cancer and Health (DCH) study and the Health Professionals Follow Up Study (HPFS).*

SNP	**-1309A/G**	**T111I**	**N396S**	**Frequency**		**RR (95% CI)**		
Nucleotide substitution	A/G	C/T	A/G	DCH	HPFS	DCH	HPFS	Pooled
				Cases/Controls	Cases/Controls			
1	AA	CC	AA	0.25/0.23	0.25/0.25	1 (ref)	1 (ref)	1 (ref)
2	AA	CT	AA	0.25/0.28	0.32/0.35	0.81 (0.63-1.05)	0.83 (0.55-1.26)	0.82 (0.66-1.02)
3	AG	CC	AA	0.20/0.22	0.19/0.18	0.92 (0.70-1.20)	1.13 (0.69-1.83)	0.96 (0.76-1.22)
4	AG	CT	AA	0.14/0.12	0.12/0.10	1.13 (0.83-1.53)	1.04 (0.59-1.85)	1.11 (0.85-1.45)
5	AA	TT	AA	0.08/0.07	0.05/0.07	0.92 (0.63-1.34)	0.67 (0.33-1.35)	0.86 (0.61-1.20)
6	GG	CC	AA	0.06/0.06	0.05/0.04	1.05 (0.69-1.59)	1.48 (0.67-3.28)	1.13 (0.78-1.63)
7	XX	XX	AG	0.03/0.02	0.02/0.01	0.99 (0.55-1.79)	3.15 (0.81-12.4)	1.49 (0.51-4.40)

*Only 3 common haplotypes were observed: ACA (all wildtype alleles), ATA, and GCA.

N=808 in HPFS (participants who were not genotyped for T111I SNP were excluded from the analysis).

Cox proportional hazard regression models in DCH (stratified by sex). Conditional logistic regression models were run in HPFS data (stratified by matching factors). All models adjusted for age, smoking, alcohol intake, menopausal status among women, body mass index.

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