Research Article

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Low-density lipoprotein cholesterol and risk of gallstone disease: A Mendelian randomization study and meta-analyses

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Background & Aims: Drugs which reduce plasma low-density lipoprotein cholesterol (LDL-C) may protect against gallstone disease. Whether plasma levels of LDL-C *per se* predict risk of gallstone disease remains unclear. We tested the hypothesis that elevated LDL-C is a causal risk factor for symptomatic gallstone disease.

Methods: We used a Mendelian randomization approach and genotyped 63,051 individuals from a prospective cohort study of the general Danish population, including 3323 subjects with symptomatic gallstones. We selected eight genetic variants in *APOE*, *APOB*, *LDLR*, and *PCSK9* affecting LDL-C. Furthermore, studies of *APOE* rs429358/rs7412 (defining ε2/ε3/ε4 alleles; 12 studies) and *APOB* rs693 (eight studies) were included in meta-analyses.

Results: The observational hazard ratio (HR) for symptomatic gallstone disease for the fifth *versus* first quintile of LDL-C was 0.94 (95% confidence interval: 0.76–1.17), despite a corresponding 134% increase in LDL-C. Furthermore, although individual genetic variants in *APOE*, *APOB*, *LDLR*, and *PCSK9* associated with stepwise increases/decreases in LDL-C of up to +59% compared with non-carriers (p <0.001), none predicted the risk of symptomatic gallstone disease. Combining all variants into 10 genotypes, carriers of 9 *versus* ⩽3 LDL-C increasing alleles associated with 41% increased LDL-C (p <0.001), but predicted a HR for symptomatic gallstone disease of 1.09 (0.70–1.69). Finally, in meta-analyses, random effects odds ratios for gallstone disease were 0.91 (0.78–1.06) for carriers of *APOE* ε4 *versus* non-carriers, and 1.25 (0.95–1.63) for *APOB* rs693 CT + TT *versus* CC.

Conclusions: Results from the observational study, genetic studies, and meta-analyses suggest that elevated plasma levels of LDL-C are not causally associated with increased risk of symptomatic gallstone disease.

Keywords: Cholelithiasis; Genetics; Lipids; Lipoproteins; Epidemiology; Mendelian randomization.

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Abbreviations: APOB, apolipoprotein B gene; APOE, apolipoprotein E gene; BMI, body mass index; CCHS, Copenhagen City Heart Study; CGPS, Copenhagen General Population Study; CI, confidence interval; HR, hazard ratio; LDL-C, low-density lipoprotein cholesterol; LDLR, low-density lipoprotein receptor gene; OR, odds ratio; PCSK9, proprotein convertase subtilisin/kexin type 9 gene.

Introduction

Cholesterol plays a pivotal role in gallstone disease, one of the most common and costly diseases in the Western world [1]. Circulating cholesterol carried by lipoproteins is taken up by the liver, metabolized, and eventually secreted into bile [2]. Biliary supersaturation with cholesterol is an important predisposing factor for the precipitation of cholesterol gallstones [1,2]. Hypothetically, elevated plasma cholesterol might lead to an elevated transhepatic cholesterol flux and consequently to an increase in risk of gallstone disease [2]. The clinical relevance of this hypothesis has recently been underscored by studies suggesting that use of plasma low-density lipoprotein cholesterol (LDL-C) lowering therapeutics (statins and ezetimibe) associates with a decreased risk of gallstones in humans [3–6]. However, the combined results from decades of functional animal and human studies on the role of different plasma lipoproteins in transhepatic cholesterol flux are far from being clear [2]. Although LDL-C lowering appears to associate with a decreased risk of gallstones, the association between LDL-C *per se* and gallstone disease is unclear, with reports of both null [7], positive [8,9], and inverse [10] associations. Whether plasma levels of LDL-C are causally associated with the risk of gallstone disease in humans is a clinically important question that might open new avenues towards the medical treatment or prevention of gallstone disease based on existing LDL-C lowering therapeutics [11].

To test the hypothesis that LDL-C is causally associated with the risk of symptomatic gallstone disease, we used Mendelian randomization, an epidemiological approach that utilizes the random inheritance of genetic variants from parents to offspring [12]. Genetic variants with effect on plasma LDL-C represent an ideal system for circumventing confounding and reverse

causation, limitations inherent to observational epidemiological studies [12]. We asked the following three questions: (1) Do plasma levels of LDL-C measured at baseline predict risk of symptomatic gallstones? (2) Are genetic variants in *APOE*, *APOB*, *PCSK9*, and *LDLR* associated with plasma levels of LDL-C? (3) Do genetic variants with effect on LDL-C predict risk of symptomatic gallstone disease? To answer these questions, we measured LDL-C in plasma, and genotyped eight genetic variants in *APOE*, *APOB*, *LDLR*, and *PCSK9* with documented effects on LDL-C in a large study of the Danish general population, comprising 63,051 individuals, including 3323 subjects with symptomatic gallstone disease. To study mainly the clinically relevant *symptomatic* gallstones, we defined symptomatic gallstone disease as ICD codes for cholelithiasis or cholecystitis diagnosed in hospital. Furthermore, we performed meta-analyses of studies on genetic variants with effect on plasma LDL-C in, respectively, *APOE* (12 studies; 5115 cases and 73,895 controls), and *APOB* (eight studies; 4459 cases and 61,155 controls).

Materials and methods

Studies were approved by institutional review boards and Danish ethical committees, and were conducted according to the Declaration of Helsinki. Written informed consent was obtained from participants. There was no overlap of individuals between the studies. All participants were white and of Danish descent: The National Danish Central Person Register registers date and place of birth and death, gender and descent, and all immigration/emigration to/from Denmark of all inhabitants in Denmark. Danish descent is defined as an individual born in Denmark with Danish citizenship with both parents also born in Denmark with Danish citizenship. Furthermore, at the time of examination, the examiner registered the ethnicity of the participants.

Participants

We included participants in two similar studies of the Danish general population, The Copenhagen General Population Study (CGPS) and The Copenhagen City Heart Study (CCHS). Combining these two studies yielded a total of 63,051 participants, of whom 3323 developed symptomatic gallstone disease.

The Copenhagen General Population Study (CGPS)

The CGPS is a prospective study of the Danish general population initiated in 2003 with ongoing enrollment [13,14]. Individuals were selected based on the National Danish Civil Registration System to reflect the adult Danish population aged 20–80+ years. Data were obtained from a questionnaire, a physical examination, and from blood samples drawn at study entry, including DNA extraction. We included the first 54,024 participants from this study in the present analysis. Of these, 2730 developed symptomatic gallstone disease.

The Copenhagen City Heart Study (CCHS)

The CCHS is a prospective study of the Danish general population initiated in 1976–78 with follow-up examinations in 1981–83, 1991–94, and 2001–03 [13,14]. Participants were recruited and examined exactly as in the CGPS. Blood samples for laboratory analyses and DNA extraction were drawn at the 1991–94 and 2001–2003 examinations. We included 9027 participants in the present analysis. Of these, 593 developed symptomatic gallstone disease.

Follow-up time for symptomatic gallstone disease for each participant in either study began at the establishment of the National Danish Patient Registry (January 1, 1977) or on the participant's birthday, whichever came last. For all end points, follow-up ended at the date of death, occurrence of event, emigration, or on August 8th, 2010 (last update of the registry), whichever came first. Follow-up was 100% complete (no individual was lost to follow-up).

Symptomatic gallstone disease

We defined 'symptomatic gallstone disease' as ICD-codes for cholelithiasis or cholecystitis (ICD8: 574 and 575; ICD10: K80 and K81) diagnosed in hospital. Information on diagnoses of symptomatic gallstone disease was collected from the National Danish Patient Registry and the National Danish Causes of Death Registry. The National Danish Patient Registry has information on all patient contacts with all clinical hospital departments and outpatient clinics in Denmark, including emergency wards since 1994. The National Danish Causes of Death Registry contains data on the causes of all deaths in Denmark, as reported by hospitals and general practitioners. Information on cholecystectomies (from 1977 to 2009) was also collected from the National Danish Patient Registry.

Laboratory analyses

Non-fasting plasma levels of total cholesterol, high-density lipoprotein cholesterol, and triglycerides were measured daily on fresh samples using standard hospital assays (Boehringer Mannheim) at the time of study entry (CGPS: 2003 and ongoing; CCHS 1991–94 or 2001–03). LDL-C was calculated with the use of the Friedewald equation if triglycerides were $\leqslant$4 mmol/L (<352 mg/dl), and were measured directly for higher triglyceride levels (Thermo Fisher Scientific and Konelab). Participants were asked the time of their last meal at the time of blood sampling. The times of last food intake were categorized as 0–1, 1–2, 2–3, 3–4, 4–5, 5–6, 6–7, 7–8, or >8 h ago.

Genotypes

Genotyping of *APOB* rs693 (= *Xba*I) and rs5742904 (R3500Q), *APOE* rs429358 (R112C) and rs7412 (R158C) (which together define the ε2/ε3/ε4 alleles), *LDLR* W23X, W66G, and W556S, *PCSK9* rs11591147 (R46L), and *ABCG8* rs11887534 (D19H), a well-known risk factor for gallstone disease, as previously shown in this [13] and other cohorts, only used in the sensitivity analysis, was performed by Taqman based assays (Applied Biosystems), or by restriction enzyme analysis. See Supplementary Material for references to our previous studies on these genotypes [Supplementary references 7–11].

Other covariates

For details regarding definitions of covariates: body mass index (BMI), physical activity, hormone replacement therapy, alcohol consumption, and lipid-lowering therapy, see Supplementary Material.

Meta-analysis

All studies investigating the association between *APOB* rs693 (T2488T; *Xba*I+/− in older studies) or *APOE* rs429358 (R112C) and rs7412 (R158C) and risk of gallstone disease published until May 2012 were considered in the meta-analysis. Studies in English were identified in PubMed, Embase, and Web of Science databases using the search criteria (APOB or APOE) and (gallstones or cholelithiasis), and references were reviewed. We included studies with available genotype frequencies for *APOB* rs693 and/or *APOE* ε2/ε3/ε4 in both cases and controls. We excluded studies if the controls were stated as not healthy. When a study reported on different ethnic groups as subpopulations, they were treated as separate studies.

Statistical analysis

For an expanded description of the statistical analyses, see Supplementary Material. Data were analyzed by SS, RF-S, and AT-H using STATA/SE 12. Two-sided probability values p <0.05 were considered significant. Chi-square tests evaluated the Hardy–Weinberg equilibrium (HWE). Mann–Whitney U test or Pearson's χ^2-test were used to compare characteristics in individuals by disease status. For statistical analyses, genotypes were coded 0, 1, and 2 for wild type homozygotes, heterozygotes, and rare homozygotes, respectively, and 0–6 for combined genotypes.

First, to test whether plasma levels of LDL-C at baseline predicted an increased risk of symptomatic gallstones, Cox regression models with age as the time scale and left truncation (delayed entry) were used to prospectively estimate hazard ratios (HRs) for symptomatic gallstone disease. Analyses were conducted from the time of blood sampling (baseline: CGPS: 2003 and onwards; CCHS: 1991–94 or 2001–03) through 2010. Individuals with symptomatic