Genetic variation in the cholesterol transporter NPC1L1, ischaemic vascular disease, and gallstone disease

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Aims

Ezetimibe reduces plasma levels of low-density lipoprotein (LDL) cholesterol by inhibiting Niemann-Pick C1-Like protein 1 (NPC1L1), the transporter responsible for cholesterol uptake from the intestine into enterocytes and from the bile into hepatocytes. We tested the hypothesis that genetic variation in NPC1L1, mimicking the effect of ezetimibe, was associated with reduced risk of ischaemic vascular disease (IVD) and with increased risk of symptomatic gallstone disease.

Methods and results

We included 67,385 individuals from the general population. Of these, 5255 and 3886 individuals developed IVD or symptomatic gallstone disease, respectively, during follow-up from 1977 to 2013. We genotyped four common NPC1L1 variants, previously associated with reduced LDL cholesterol levels, thus mimicking the effect of ezetimibe, and calculated a weighted genotype score. With increasing genotype score, LDL cholesterol decreased stepwise up to 3.5% (0.12 mmol/L) and total cholesterol up to 1.9% (0.11 mmol/L) (P-trend: 2 × 10−12 and 2 × 10−9). The cumulative incidence by age of IVD decreased, while that of symptomatic gallstone disease increased as a function of increasing genotype score (P-trend: 0.005 and 0.01). Hazard ratios for genotype scores ≥ 5.0 vs. < 2.0 were 0.82 (95% confidence interval: 0.70–0.95) for IVD and 1.22 (0.99–1.49) for gallstone disease (P-trend across genotype scores: 0.004 and 0.01).

Conclusion

Genetic variation in NPC1L1 is associated with a reduction in risk of IVD, with a corresponding reduction in LDL cholesterol, but with a concomitant increased risk of gallstone disease. These data support the hypothesis that treatment with ezetimibe protects against IVD but raise the question whether long-term treatment increases the risk of gallstone disease.

Keywords

Genetics • Cholesterol • Cardiovascular diseases

Introduction

Treatment with statins reduces plasma levels of low-density lipoprotein (LDL) cholesterol and consequently protects against ischaemic vascular disease (IVD).1 Moreover, a growing body of evidence indicates that reductions in LDL cholesterol to levels lower than what is typically achieved by statins might further reduce the risk of IVD.2 To achieve such additional LDL cholesterol reduction, ezetimibe has been the focus of intense clinical and scientific interest as a potential add-on drug to statins.3–8 Ezetimibe inhibits Niemann-Pick C1-Like protein 1 (NPC1L1), a transporter responsible for intestinal sterol uptake into enterocytes, thereby reducing plasma levels of total and LDL cholesterol.9 Until the recent presentation of the data from the IMPROved Reduction of Outcomes: Vytorin Efficacy International Trial (IMPROVE-IT), (http://www.eas-society.org/fileArchive/IMPROVE%20IT%20-%20PRESENTATION%202014-11-17.pdf, accessed 27 March 2015) it has been unclear, however, whether the addition of ezetimibe to conventional statin treatment confers additional protection against IVD.10,11

Ezetimibe has been safe and well-tolerated in randomized controlled trials, with no major adverse effects reported (http://www.eas-society.org/fileArchive/IMPROVE%20IT%20-%20PRESENTATION%202014-11-17.pdf)
However, these trials have been of relatively short duration (up to 7 years) (http://www.eas-society.org/fileArchive/IMPROVE%20IT%20-%20PRESENTATION%202014-11-17.pdf, accessed 27 March 2015), and thus, the safety profile of long-term treatment with ezetimibe remains unknown. It was recently shown that NPC1L1, like the adenosine triphosphate-binding cassette transporter G5/8 (ABCG5/8), is not only expressed in human enterocytes, but also in hepatocytes, where NPC1L1 mediates the reuptake of cholesterol from the bile into the liver while ABCG5/8 has the opposite effect (Figure 1). Elevated levels of biliary cholesterol promote the formation of cholesterol gallstones, one of the most common gastrointestinal diseases. Therefore, treatment with ezetimibe might inhibit not just intestinal, but also hepatic NPC1L1-mediated uptake of cholesterol, causing an increase in biliary cholesterol content, and hence an increased risk of gallstone disease. In support of this, according to the package insert of Zetia (¼ ezetimibe) ezetimibe given to dogs (which like humans express NPC1L1 both in the intestine and in the liver) for 1 month increased the concentration of gallbladder cholesterol two- to four-fold, likely increasing the long-term risk of gallstone disease (http://www.merck.com/product/usa/pi_circulars/z/zetia/zetia_pi.pdf, accessed 27 March 2015). The formation of gallstones is a gradual process, and symptoms may take years or even decades to manifest. Consequently, this potential adverse effect of ezetimibe treatment might have been missed in the relatively short randomized controlled trials.

Because genetic variants are randomly assorted at conception, variants in genes that encode drug targets may mimic the long-term effects (and side effects) of the drug itself. To mimic the effect of ezetimibe treatment on NPC1L1 inhibition, we tested the hypothesis that genetic variation in NPC1L1 associated with low LDL cholesterol levels also was associated with reduced risk of IVD and with increased risk of symptomatic gallstone disease. We genotyped four common variants in NPC1L1, previously suggested to be associated with reduced LDL cholesterol levels, in two studies of the Danish general population totalling 67 385 participants, of whom 5255 developed IVD and 3886 developed symptomatic gallstone disease from 1977 through 2013.

**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. All participants were white and of Danish descent, as determined by the National Danish Civil Registration System.

**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 67 385 participants, of whom 5255 developed IVD (myocardial infarction and/or ischaemic stroke) and 3886 developed symptomatic gallstone disease. DNA was available on all participants, and lipid parameters were available on >98%. For additional information on study populations, endpoints, laboratory analysis and covariates, see Supplementary material online.

**Genotyping**

We genotyped four common variants (minor allele frequency >5%) in NPC1L1: −133A>G (rs11765562); −18C>A (rs41279633); L272L; C>G (rs2072183); and V1296V. For additional information on genotyping methods, see supplementary material online.

**Statistical analysis**

Stata SE 12 (Stata Corp., College Station, Texas) was used for all analyses. Chi-square tests evaluated Hardy–Weinberg equilibrium. To compare characteristics in individuals by disease status or genotype, Mann–Whitney U test or Kruskal–Wallis’ analysis of variance was used to compare continuous covariates, and Pearson’s $\chi^2$ test to compare categorical covariates. For trend tests, genotype or genotype combinations were coded 0, 1, 2, and so forth. For each variant, the genotype with the highest LDL cholesterol was used as the reference (coded 0). Cuzick’s test for trend, an extension of the Wilcoxon rank-sum test, was used to compare levels of continuous variables as a function of genotypes, individually and combined. From the four NPC1L1 variants that were individually associated with LDL cholesterol levels, we generated a combined, weighted genotype score based on the percentage reductions in LDL cholesterol compared with the reference genotype for the individual variants: −133A>G: AA = 0% (reference), AG = −0.1%,...
Results

Baseline characteristics by disease status are shown in Table 1. Risk factors for IVD or symptomatic gallstone disease were equally distributed among genotypes, although use of lipid-lowering therapy tended to be slightly less frequent in carriers of some LDL cholesterol-lowering alleles (see Supplementary material online, Table S2). In addition, genetic variants in NPC1L1 were not associated with plasma levels of bilirubin which were therefore unlikely to confound the results, or with other markers of liver function and inflammation (all P-trend: ≥0.10) (see Supplementary material online, Figure S1). All genotypes were in Hardy-Weinberg equilibrium (P-values ≥0.32); there was modest linkage disequilibrium between NPC1L1-18C>G and L272L C>G (R² = 0.60) (see Supplementary material online, Figure S2).

Plasma lipids, lipoproteins, and apolipoproteins

NPC1L1 genotypes were individually associated with stepwise decreases in total- and LDL cholesterol levels of up to 1.8% (0.11 mmol/L) for total cholesterol, and up to 3.3% (0.12 mmol/L) for LDL cholesterol, in homozygotes vs. reference genotypes; associations were similar in men and women (see Supplementary material online, Figure S3). Combined genotype scores were associated with stepwise decreases in total- and LDL cholesterol of up to 1.9% (0.11 mmol/L) and 3.5% (0.12 mmol/L), respectively, for individuals with a score of ≥5.0 vs. <2.0 (Figure 2; P-trend: 2 × 10⁻⁵ and 2 × 10⁻¹²). Associations between NPC1L1 genotypes and apolipoprotein B were generally concordant with those seen for total and LDL cholesterol, while genotypes, individually or combined, did not associate with plasma levels of triglycerides, HDL cholesterol, lipoprotein (a), or apolipoproteins A-I or –E (Figure 2 and see Supplementary material online, Figure S4).

Risk of IVD and symptomatic gallstone disease

Of the 67 385 participants, 5255 developed IVD (myocardial infarction and/or ischaemic stroke) and 3886 developed symptomatic gallstone disease from 1977 through 2013. In individuals with a genotype score of <2.0 through 2.0–2.9 to ≥5.0, there was a stepwise decrease in cumulative incidence of IVD, and a corresponding stepwise increase in cumulative incidence of symptomatic gallstone disease (Figure 3; P-trend: 0.005 and 0.01). The multifactorially adjusted HRs for IVD decreased stepwise with increasing genotype score to 0.82 (0.70–0.95) for individuals with a genotype score of ≥5.0 vs. <2.0 (Figure 4, middle column; P-trend: 0.004). The corresponding HRs for symptomatic gallstone disease increased stepwise with increasing genotype score to 1.22 (0.99–1.49) for individuals with a genotype score of ≥5.0 vs. <2.0 (Figure 4, right column; P-trend: 0.01). Corresponding stepwise results were similar for the individual genotypes, with the most pronounced effects for −18C>A which also had the largest effect on plasma levels of LDL cholesterol (see Supplementary material online, Figure S5).

Validation and sensitivity analyses

While genetic variants in NPC1L1 and ABCG8 affect both plasma and biliary cholesterol levels, genetic variants in LDLR/APOB/PCSK9 affect mainly plasma cholesterol levels. Therefore, to validate our results we compared the risk of IVD and gallstone disease caused by LDL cholesterol-lowering variants in NPC1L1 and ABCG8 with variants in LDLR/APOB/PCSK9. The multifactorially adjusted HRs for IVD decreased stepwise with increasing genotype score/allele count and decreasing LDL cholesterol levels to 0.88 (0.78–1.00) for ABCG8 and 0.37 (0.21–0.63) for LDLR/APOB/PCSK9 (Figure 4, middle column; P-trend: 0.009 and 0.04). Corresponding HRs for symptomatic gallstone disease were 2.47 (2.18–2.79) and 1.31 (0.48–3.57), respectively (Figure 4, right column; P-trend: 8 × 10⁻⁷ and 0.33).

A minority of individuals with gallstones have pigment/bilirubin gallstones for which bilirubin is a major risk factor. Therefore, to further rule out confounding by plasma bilirubin levels and hence propensity to develop pigment/bilirubin gallstones, we adjusted for bilirubin levels and UGT1A1 genotype, a major genetic determinant of plasma bilirubin levels associated with increased risk of gallstone disease. This did not change the results (see Supplementary material online, Figure S6 and data not shown). We also tested for interaction on

<table>
<thead>
<tr>
<th>Table 1</th>
<th>Baseline characteristics of participants</th>
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<td>Number of participants</td>
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<td>Hormone replacement therapy (%)</td>
<td>13</td>
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<tr>
<td>Lipid-lowering therapy (%)</td>
<td>7</td>
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Values are median and inter-quartile range or percentage.
*In women only.
*P < 0.001 vs. individuals with no event.
risk of symptomatic gallstone disease between NPC1L1 genotype and either plasma bilirubin levels (<20 vs. ≥20 μmol/L, corresponding to levels in Gilbert’s syndrome; P-interaction = 0.48) or UGT1A1 genotype (P-interaction = 0.09). Taken together, these data suggest that plasma bilirubin levels (and hence propensity to develop pigment/bilirubin gallstones) was unlikely to confound our results, and that NPC1L1 genotypes associate with risk of gallstone disease independent of plasma bilirubin levels.

To further test the robustness of our results, we conducted several sensitivity analyses. Dividing the genotype score at other cut points or using simple allele counts gave similar results (see Supplementary material online, Figure S7). A 1 unit increase in NPC1L1 genotype score was associated with HRs of 0.98 (0.96–1.00) for IVD and of 1.03 (1.01–1.05) for symptomatic gallstone disease (P-trend: 0.01 and 0.008). Excluding individuals on lipid-lowering therapy (97% statins), or additionally adjusting for this, gave similar results (see Supplementary material online, Figures S8 and S9). Moreover, stratifying the analyses by gender likewise gave similar results (see Supplementary material online, Figure S10).

Genetic variants in NPC1L1 and ABCG8 combined

NPC1L1 and ABCG8 transport cholesterol in opposite directions in the intestine and liver (Figure 1). To study a potential interplay between these transporters, we created a combined, weighted genotype score based on the percentage reductions in LDL cholesterol compared with the reference genotype (Supplementary material online, Table S1). P-values are for trend tests by Cuzick’s extension of a Wilcoxon rank-sum test. Lipoprotein (a) measurements were available on 25 429 participants. LDL, low-density lipoprotein; NPC1L1, Niemann-Pick C1-Like protein 1.

![Figure 2](http://www.eas-society.org/fileArchive/IMPROVE%20IT%20-%20PRESENTATION%202014-11-17.pdf, accessed 27 March 2015)

**Figure 2** Lipid and lipoprotein levels as a function of NPC1L1 genotypes, individually and combined. From the four NPC1L1 variants (−133 A>G, −18 C>A, L272L, V1296V), we generated a combined, weighted genotype score based on the percentage reductions in LDL cholesterol compared with the reference genotype (Supplementary material online, Table S1). P-values are for trend tests by Cuzick’s extension of a Wilcoxon rank-sum test. Lipoprotein (a) measurements were available on 25 429 participants. LDL, low-density lipoprotein; NPC1L1, Niemann-Pick C1-Like protein 1.

Short-term pharmacological vs. long-term genetic LDL reduction

In the IMPROVE-IT trial, the short-term time-averaged reduction in LDL cholesterol for participants treated with statins plus ezetimibe (10 mg) vs. statins alone was 0.41 mmol/L (15.8 mg/dL), and the corresponding reduction in risk of IVD was 6% (1–11%) (Figure 6, top) (http://www.eas-society.org/fileArchive/IMPROVE%20IT%20-%20PRESENTATION%202014-11-17.pdf, accessed 27 March 2015). Based on a proportional reduction in LDL cholesterol and risk of IVD,1 the corresponding risk reductions for a 0.41 mmol/L reduction in LDL cholesterol were 62% (30–103%) for the long-term genetic LDL reduction and 9% (8–10%) for short-term statin treatment (Figure 6, top). In the present study, the observed long-term genetic reduction in LDL cholesterol was 0.12 mmol/L (4.6 mg/dL), and the reduction in risk of IVD was 18% (5–30%) (Figure 6, bottom). The corresponding short-term reductions in risk of IVD for treatment with ezetimibe or statins were 1.8% (0.3–3.2%) and 2.6% (2.3–2.9%), respectively (Figure 6, bottom).

The median follow-up in IMPROVE-IT was 6 years, whereas in the genetic study the LDL reduction was presumably lifelong. For the same absolute reduction in LDL cholesterol, the reduction in risk of IVD was ~10-fold increased for genetic variants in NPC1L1.
compared with ezetimibe (Figure 6; 62%/6%); however, because ezetimibe was 3.42 times more potent than the genetic variants in reducing LDL cholesterol (0.41/0.12 mmol/L), the number of years on ezetimibe treatment necessary to obtain a similar reduction in risk of IVD would be \( \approx 17.5 \) years (6 years \( \times \) 3.42).

**Discussion**

The main finding of this study is that genetic variation in **NPC1L1** is associated with decreased levels of plasma LDL cholesterol, protecting against IVD, but increases the risk of symptomatic gallstone disease. These data suggest that genetic variation in **NPC1L1**, mimicking treatment with ezetimibe monotherapy, reduces the risk of IVD in a dose-dependent manner. However, they also raise the question whether long-term treatment with ezetimibe increases the risk of gallstone disease.

To our knowledge, this is the first study to suggest that genetic variation in **NPC1L1** is associated with opposing effects on risk of IVD and gallstone disease. It is also currently the largest study of combined IVD and symptomatic gallstone disease with DNA available. Our results were consistent across four different **NPC1L1** variants and showed a gene dosage effect for all variants. Results were similar when stratifying by gender and in several sensitivity analyses. To further validate our results, we compared the risk of IVD and symptomatic gallstone disease caused by variants in **NPC1L1** with the corresponding risks for variants in **ABCG8** and **LDLR/APOB/PCSK9**. While these variants all associated with a reduction in plasma levels of LDL cholesterol, only **NPC1L1** and **ABCG8** have been reported to associate with hepatobiliary cholesterol transport and biliary cholesterol levels, an important risk factor for gallstone disease. As expected from the known biological function, LDL cholesterol-lowering variants in **NPC1L1** (loss-of-function) and **ABCG8** (gain-of-function) were associated with a reduced risk of IVD, but with an increased risk of symptomatic gallstone disease. These associations became even stronger when combining variants in **NPC1L1** and **ABCG8** into a weighted genotype score. In contrast, LDL cholesterol-lowering variants in **LDLR/APOB/PCSK9** were associated with a reduced risk of IVD, but not with risk of symptomatic gallstone disease. Taken together, this suggests that these results for **NPC1L1** are real.

The mechanistic interpretation of the data presented here is straightforward. Genetically reduced activity of **NPC1L1** causes reduced uptake of cholesterol from both the intestine into enterocytes and from the bile into hepatocytes. Inhibition of intestinal cholesterol uptake leads to lower plasma levels of total and LDL cholesterol, reducing atherosclerosis and risk of vascular disease. Concomitantly, inhibition of the hepatic cholesterol reuptake from bile causes an increase in biliary cholesterol, biliary supersaturation with cholesterol, and an increased risk of cholesterol gallstones, the most common form of gallstones.9,12,13 This interpretation is in agreement with effects observed for gain-of-function variants in **ABCG8** in the present and previous studies.25

We found that common genetic variants in **NPC1L1** which were associated with a lifelong 3.5% (0.12 mmol/L) lower LDL cholesterol, conferred an 18% lower risk of IVD in the general population. These results are supported by results from a recent study on inactivating mutations in **NPC1L1** which associated with a 0.31 mmol/L (12 mg/dL) reduction in LDL cholesterol and a corresponding 53% reduction in risk of coronary heart disease.29 Assuming proportionality between LDL cholesterol and risk reduction, the predicted risk reduction associated with a 0.12 mmol/L reduction in LDL cholesterol—similar to that observed in our study—would be 20.5% (0.12 \( \times \) 53%/0.31 mmol/L) compared with 18% in our study. However, this study29 was unable to evaluate whether **NPC1L1** mutations lead to other phenotypic consequences, including risk of gallstone disease.

A key question is whether these long-term genetic effects can be extrapolated to patients treated with ezetimibe later in life. In the IMPROVE-IT trial (http://www.eas-society.org/fileArchive/IMPROVE%20IT%20-%20PRESENTATION%202014-11-17.pdf, accessed 27 March 2015), in which >18 000 patients with previous acute
coronary syndrome were randomly allocated to either ezetimibe 10 mg/dL plus a statin or statin alone, LDL cholesterol was reduced by 0.41 mmol/L in the ezetimibe/statin group vs. the statin group (http://www.eas-society.org/fileArchive/IMPROVE%20IT%20-%20PRESENTATION%202014-11-17.pdf, accessed 27 March 2015).

Although the average effect on LDL cholesterol lowering was more than three-fold greater than in our genetic study, the risk of IVD was only reduced by 6%, similar to the ≏9% expected from statin trials.1 Based on the observed reduction in LDL cholesterol and the corresponding reduction in risk of IVD in the IMPROVE-IT study with 6 years median follow-up, and the corresponding lifelong genetic effects observed in our study, we estimate that the time on ezetimibe treatment necessary to obtain the same risk reduction in IVD would be ≏17.5 years.

Whether these estimates can be extrapolated to risk of gallstone disease is at present unknown. In the IMPROVE-IT trial, patients treated with ezetimibe/statin did not have a higher risk of cholecystectomy or gallstone-related clinical symptoms than patients treated with statins alone (http://www.eas-society.org/fileArchive/IMPROVE%20IT%20-%20PRESENTATION%202014-11-17.pdf, accessed 27 March 2015). However, there are at least two important caveats. First, in IMPROVE-IT, ezetimibe was given in combination with a statin. Because statin therapy is associated with a reduced risk of gallstones in observational epidemiological studies,30,31 an increased risk...
of gallstone disease due to ezetimibe may have been countered by a protective effect of statins. Second, the median follow-up of 6 years in the IMPROVE-IT trial may have been too short to detect an increase in risk of symptomatic gallstones, a disease that may take decades to develop. Furthermore, in IMPROVE-IT, only 25% of the participants were women, known to have a considerably higher risk of gallstone disease than men. Finally, according to the package insert of Zetia, ezetimibe given as monotherapy to dogs (which like humans express NPC1L1 both in the intestine and in the liver) for 1 month increased the concentration of gallbladder cholesterol two- to four-fold, likely increasing the long-term risk of gallstone disease. Taken together, the present findings show that lifelong genetic LDL cholesterol lowering mimicking the effect of ezetimibe leads to a greater reduction in risk of IVD than 6 years treatment with ezetimibe later in life, and that an effect on increased risk of gallstone disease likely is masked in the IMPROVE-IT trial through concomitant statin treatment.

Intriguingly, a recent study found that ezetimibe indirectly increased ABCG8-mediated biliary cholesterol efflux in mice, suggesting an alternative mechanism by which ezetimibe could increase the risk of gallstone disease. However, results from these studies are difficult to translate to humans, because humans express NPC1L1 in both the intestine and liver, while mice do not express hepatic NPC1L1.

Study limitations

A limitation is that we did not have data on gallstone composition. A minority of gallstones may have been pigment/bilirubin stones, for which increased plasma bilirubin is a risk factor. However, our data suggested that bilirubin was not a confounder, and that NPC1L1 influences risk of gallstone disease independent of bilirubin levels. In the present study, four common variants previously associated with lowering of LDL cholesterol were used as genetic instruments to mimic the effect of ezetimibe. Of these, two were reported to be functional or linked to a functional variant. However, for Mendelian randomization studies such as the present, it is not a requirement that the genetic instruments used are functional per se, as long as they are likely in linkage disequilibrium with other variants in the same gene that have an effect on the intermediate phenotype (LDL cholesterol) similar to the drug effect. Finally, in Denmark, <1% of all individuals on lipid-lowering therapy use ezetimibe. This corresponds to fewer than 63 participants in our study and is unlikely to have influenced the results.

In conclusion, we show that genetic variation in NPC1L1, mimicking the effect of ezetimibe, is associated with a dose-dependent reduction in risk of IVD, with corresponding reductions in LDL cholesterol levels, but with a concomitant increase in risk of symptomatic gallstone disease. These findings provide reassurance that pharmacological inhibition of NPC1L1 by ezetimibe likely will reduce risk of IVD. However, they also raise the clinically important question whether long-term treatment with ezetimibe increases the risk of gallstone disease.

Supplementary material

Supplementary material is available at European Heart Journal online.

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