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ORIGINAL RESEARCH—CLINICAL

Protein Phosphatase 1 Regulatory Subunit 3 Beta rs4240624 Genotype Is Associated With Gallstones and With Significant Changes in Bile Lipidome

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BACKGROUND AND AIMS: Gallstone disease (GSD) associates with significant morbidity and mortality. Decreased secretion of bile acids has been suggested as a driving factor for GSD. Recently, we linked the protein phosphatase 1 regulatory subunit 3 beta (*PPP1R3B)* rs4240624 genotype to decreased bile acid levels in bile. In this study, we investigated whether these individuals had an increased risk for GSD as well as the differences in the lipid composition of the gallbladder bile of these individuals compared to controls and patients with GSD. **METHODS:** Bile acids, cholesterol, and phospholipid levels in gallbladder bile samples were enzymatically measured in 46 patients (34 female, age 45.7 ± 9.8 years, BMI 41.3 ± 4.4 kg/m^2) who underwent elective laparoscopic Roux-en-Y gastric bypass. The lipidome of gallbladder bile was analyzed using high-performance liquid chromatography-mass spectrometry. Gallstone status was evaluated using abdominal ultrasonography before the surgery. **RESULTS:** The G allele of *PPP1R3B* rs4240624 was significantly associated with GSD in patients with obesity. We validated this association in the UK Biobank. Bile lipidomics demonstrated that 13 of the 17 minor lipid classes measured were higher in individuals with the G allele. The concentrations of bile acids, cholesterol, and phospholipids, as well as the cholesterol saturation index, were lower in patients with GSD than in those without gallstones. GSD had an effect similar to that of *PPP1R3B* genotype on minor lipids. **CONCLUSION:** The *PPP1R3B* rs4240624 genotype is associated with gallstones and with changes in gallbladder bile similar to those observed in patients with gallstones, suggesting that the *PPP1R3B* genotype contributes to the risk of gallstones by altering the bile lipidome.

Keywords: Gallstone disease; Bile; *PPP1R3B*; Lipidomics

Introduction

Gallstone disease (GSD) is a very common condition affecting up to 20% of adults in Western countries. Although most patients with GSD remain asymptomatic,

Abbreviations used in this paper: **CSI, cholesterol saturation index; DG, diacylglycerols; FDR, false discovery rate; GSD, gallstone disease; ICD, International Classification of Diseases; KOBS, Kuopio Obesity Surgery Study; LRYGB, laparoscopic Roux-en-Y gastric bypass; *PPP1R3B*, protein phosphatase 1 regulatory subunit 3 beta; SNP, single nucleotide polymorphism; TG, triglycerides.**

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some may experience significant morbidity and even mortality.[1] Although the pathogenesis of GSD is not completely understood, its development is considered multifactorial. The most important risk factors include age, female sex, obesity, physical inactivity, and metabolic syndrome.[2,3] The prevalence of GSD has increased alongside the increasing rate of obesity.[4] In addition, genetic factors explain approximately 25% of the risk of GSD.[5] Most genetic variants associated with GSD have been linked to genes involved in bile acid export/metabolism in the liver, including variants of ATP binding cassette subfamily B member 4, ATP binding cassette subfamily B member 11, ATP-binding cassette subfamily G member 5/8, and Sulfotransferase 2A1.[6] It is not surprising that the proteins encoded by these genes are involved in biliary lipid secretion. We recently demonstrated that G allele carriers of protein phosphatase 1 regulatory subunit 3 beta (*PPP1R3B*) rs4240624 had lower bile acid levels in their gallbladder bile than those with the A allele.[7] Lowered bile acid secretion has been suggested as a driving force for gallstone formation.[8,9] Thus, carriers of the *PPP1R3B* rs424064 genotype would be expected to be at risk of developing GSD. Therefore, we assessed the effect of the *PPP1R3B* rs424064 genotype on the risk of developing gallstones in the Kuopio Obesity Surgery Study (KOBS) cohort and validated the results in the UK Biobank cohort (UKBB). To substantiate these data, we assessed the biliary lipidome in carriers of the *PPP1R3B* rs424064 genotype and investigated whether the altered potential lipids were also present in the bile of patients with GSD.

Materials and Methods

Study Patients

KOBS cohort. The primary study group consisted of 46 individuals from the KOBS[10] cohort (12 males, 34 females, age: 45.7 ± 9.8 years, BMI: 41.3 ± 4.4 kg/m^2), that had gallbladder bile samples and information regarding gallstones available (Table 1).

Additionally, 261 individuals from the KOBS cohort who had the *PPP1R3B* rs4240624 genotype and gallstone information available and were used to evaluate the effect of the genotype on the risk of developing gallstones. This cohort consisted of 50 males and 141 females (age 48.2 ± 8.7 years, BMI 43.0 ± 5.1 kg/m^2, Table A1).

The study protocol was approved by the Ethics Committee of Northern Savo and followed the principles of the Declaration of Helsinki. Written informed consent was obtained from all study participants.

UK Biobank cohort. The UKBB database (application number approval 62,797) was used to validate our results regarding the association between the *PPP1R3B* genotype and gallstones. The UKBB includes European individuals, and diagnosis codes were used for the determination of diseases in this cohort. Genetic data is available from approximately 450,000 individuals aged 40–69 years, and the study participants were recruited throughout the UK between 2006 and 2014. The UKBB study received ethical approval from the North West - Haydock Research Ethics Committee (reference 16/NW/0274).[11]

Table 1. Characteristics of the Primary Study Cohort With Bile Acid Measurements and Gallstone Data Available

Gallstones	No n = 38	Yes n = 8	*P* Value
Sex (male/female)	11/27	1/7	.336
Age (y)	44.3 ± 8.7	52.6 ± 13.3	.314
BMI (kg/m^2)	41.4 ± 4.6	40.9 ± 3.7	.851
Total cholesterol (mmol/L)	4.6 ± .1.0	4.2 ± 0.9	.345
LDL cholesterol (mmol/L)	2.7 ± 0.9	2.2 ± 0.9	.260
HDL cholesterol (mmol/L)	1.2 ± 0.3	1.4 ± 0.5	.484
Triglycerides (mmol/L)	1.6 ± 0.8	1.4 ± 0.8	.637
Type 2 diabetes (%)	29	50	.248
Statin treatment (%)	21	25	.806
Fasting glucose (mmol/L)	6.9 ± 2.9	7.8 ± 3.6	.767
Fasting insulin (mU/L)	17.3 ± 8.8	15.2 ± 10.1	.467
ALT (U/L)	47 ± 30	39 ± 14	.873

Mann-Whitney U and Chi-square tests for statistics.
ALT, alanine aminotransferase; HDL, high-density lipoprotein; LDL, low-density lipoprotein.

We excluded withdrawn individuals and those without available genotypic data. We then propensity score-matched the individuals 1:1 to those without GSD, and those with GSD were matched to the nearest by sex and year of birth. The cases included those with registered inpatient International Classification of Diseases (ICD)-9 and ICD-10 codes for GSD (ICD-9: 574, ICD-10: K80), and the controls were those without GSD or related diagnoses (ICD-9: 574 and 575, ICD-10: K80, K81, and K82).

Characteristics of the individuals included in the UKBB cohort are presented in Table A2. Logistic regression was used to analyze the relationship between the *PPP1R3B* genotype and gallstones. Because *PPP1R3B* rs4240624 was not available in the UKBB, we used there single nucleotide polymorphism (SNP) at rs4841132, which is a proxy for complete linkage with rs4240624. However, it is important to note that with rs4240624 the minor allele is G and with rs4841132 it is A.[12]

Clinical and Laboratory Measurements

Plasma glucose, insulin, serum lipid, and lipoprotein lipid levels were determined as previously described.[13] The presence of gallstones was evaluated using ultrasonography before elective laparoscopic Roux-en-Y gastric bypass (LRYGB). Gallbladder puncture was performed using a 14-G needle during the LRYGB.

Bile Acid Measurements

Bile acid levels were measured in samples obtained by transhepatic gallbladder puncture during the LRYGB. Measurements were performed as previously described using a Nexera X2 UHPLC system (Shimadzu, Kyoto, Japan) coupled to a 5500 Qtrap mass spectrometer interfaced with an electrospray ion source (ABSciex, Toronto, Ontario, Canada).[14] Primary, secondary, tertiary, primary conjugated, and secondary conjugated bile acids were calculated based on the individual bile acids (taurolithocholate acid and secondary conjugated bile acids were not measured).

Bile acids, cholesterol, and phospholipids from the gallbladder bile samples were measured enzymatically, as described previously, using a Clariostar analyzer (BMG Labtech,

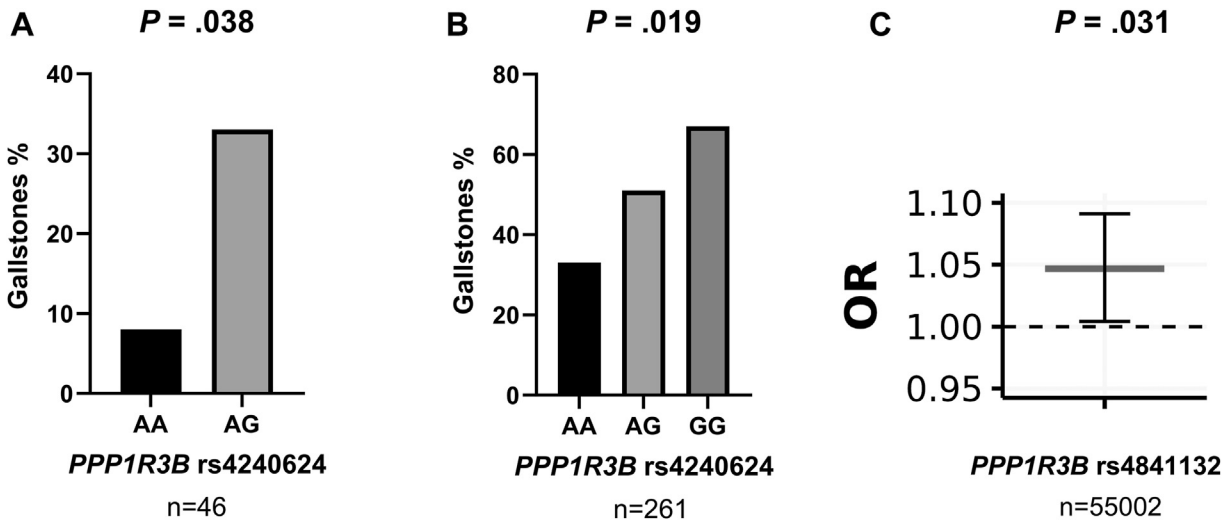

Figure 1. The prevalence of gallstones based on the *PPP1R3B* rs4240624 genotype in the original study cohort (n = 46) (A); in the larger study subset (n = 261) (B); and the association between *PPP1R3B* rs4841132 (complete linkage with rs4240624, but the minor allele is A) and gallstones in the UK Biobank cohort adjusted for birth year and sex (C). The Mann–Whitney U and Kruskal–Wallis test were used for the Kuopio Obesity Surgery Study (KOBS) cohorts, and logistic regression analysis for the UK Biobank cohort show the odds ratio (OR) and the whiskers show the 95% confidence interval.

Ortenberg, Germany).[15] Total lipid content and cholesterol saturation index (CSI) were calculated based on the critical tables by Carey.[16]

Lipidomics

Bile lipidomics was performed with high-performance liquid chromatography mass spectrometry at The Core Facility Metabolomics at UMC Amsterdam as recently described in detail.[17] A set of internal standards (Avanti Polar Lipids, Alabaster, AL, USA) was added to each sample after the sample workup and before data collection. The total amount of each lipid class was calculated by summing all individual lipid species belonging to this class.[17]

Genotyping

PPP1R3B rs4240624 was genotyped using TaqMan SNP Genotyping Assays (Applied Biosystems, Waltham, Massachusetts, USA), according to the manufacturer's protocol.

Statistical Analysis

Data are presented as mean ± SD. Prior to data analysis, lipidomic data were log-transformed to correct for skewed distributions. Differences between the study groups were evaluated by the $\chi 2$ test for categorical variables, and with Mann–Whitney U and Kruskal–Wallis independent samples tests for other variables. Spearman's rank correlation was used for the correlation analyses. The analyses were conducted using SPSS version 25 (IBM Inc., Armonk, NY, USA), R software version 3.6.0 (R Foundation for Statistical Computing, Vienna, Austria), and GraphPad Prism version 9.3.1 (GraphPad Software Inc., Boston, USA). A *P* value of less than .05 was considered statistically significant. False discovery rate (FDR) with the Benjamini, Krieger, Yekutieli two-stage set-up method with a desired FDR Q 5% was used to adjust for multiple comparisons in the lipidomic analyses.[18]

Results

The primary study cohort consisted of 46 individuals (12 males, 34 females, age: 45.7 ± 9.8 years, BMI: 41.3 ± 4.4 kg/m^2), who had gallbladder bile samples and information regarding gallstones available. In this group, 8 had gallstones. The characteristics of the study participants are shown in Table 1, demonstrating no differences between individuals stratified by GSD status.

PPP1R3B *rs4240624 Genotype Associated With Gallstones*

In the primary study group, the presence of the G allele of *PPP1R3B* rs4240624 was significantly associated with gallstones; 29% (10 out of 34) of those with the AA genotype and 71% (5 out of 7) of those with the AG genotype had GSD (P = .038). We also analyzed the association between the genotype and gallstones in a larger subset of the KOBS cohort (n = 261), where 33% of those with the AA genotype, 51% of those with the AG genotype, and 67% of those with the GG genotype had gallstones, (P = .019, Figure 1). When those with AG and GG genotype (only 2 had the GG genotype) were combined, 37% of those with the AA genotype and 51% of those with the AG/GG genotype had gallstones (P = .006, data not shown).

PPP1R3B *Genotype Associated With Gallstones in UK Biobank*

We validated the association between the *PPP1R3B* genotype and GSD in the UKBB. After propensity score matching, there were 27,501 individuals without gallstones and 27,501 with GSD. Most characteristics of the groups differed significantly, as shown in Table A2. Because *PPP1R3B* rs4240624 was not available in this cohort, we

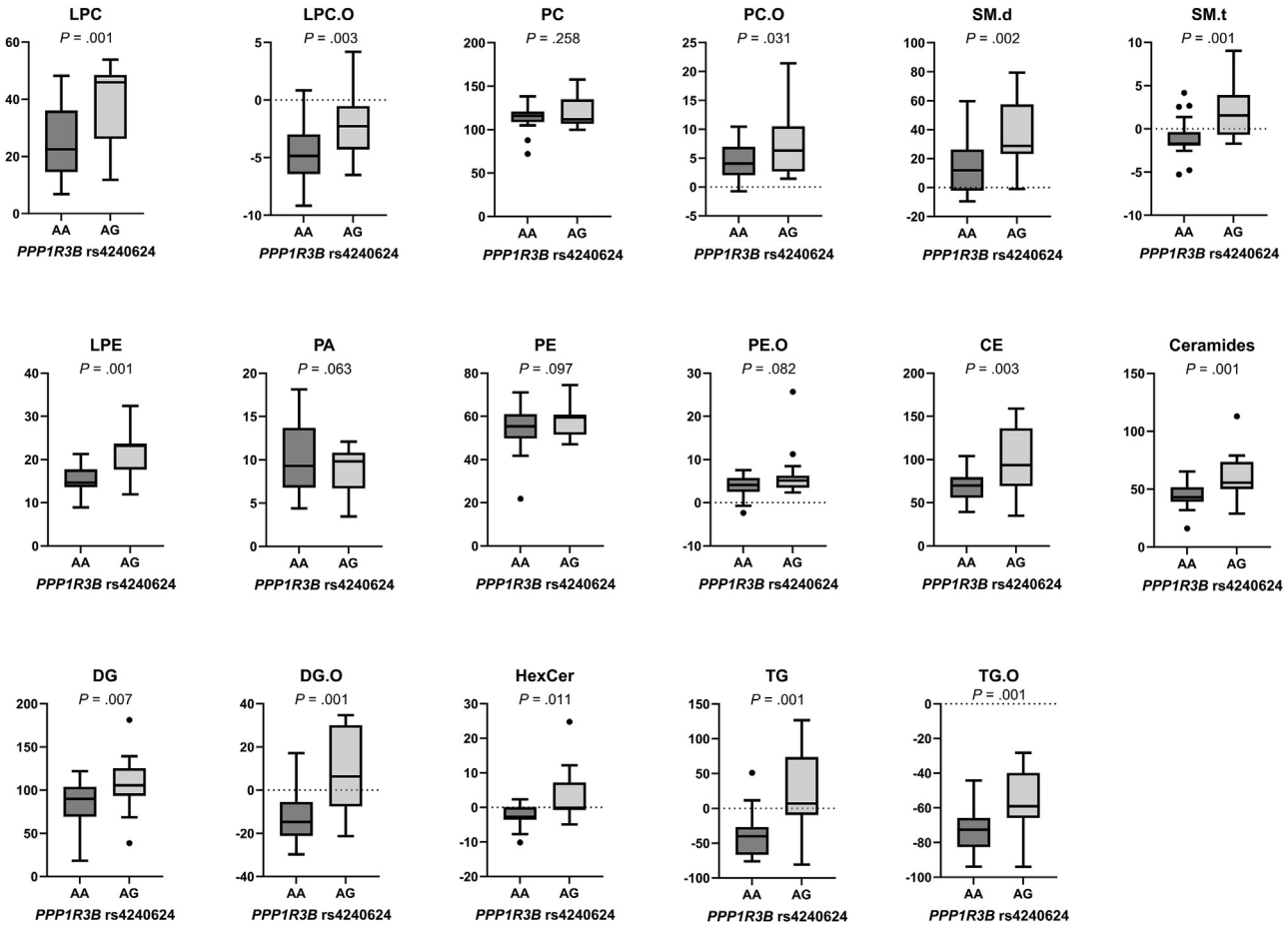

Figure 2. Logarithm-transformed total lipid levels in gallbladder bile based on *PPP1R3B* rs4240624 genotype. Panels demonstrate lyso-phosphatidylcholine (LPC), (lyso)alkyl-phosphatidylcholine; (LPC.O) phosphatidylcholine (PC), lyso-phosphatidylcholine (PC.O), sphingomyelin/ceramide phosphocholines (SM.d) and hydroxysphingomyelin (SM.t), lyso-phosphatidylethanolamine (LPE), phosphaditic acid (PA), phosphatidylethanolamine (PE), alkyl-phosphatidylethanolamine (PE.O), cholesterol esters (CE), ceramides, diacylglycerols (DG), alkyl-diacylglycerols (DG.O), hexosylceramide (HexCer), triglycerides (TG), alkyl-triglycerides (TG.O). The horizontal line indicates the median, the whiskers indicate the minimum and maximum values with the Tukey method, and the box borders indicate lower and upper quartiles. The false discovery rate-adjusted *P* value is shown.

used rs4841132 in our analyses. It is important to note that although rs4841132 is in complete linkage disequilibrium, the alleles are opposite (ie, the minor allele at rs4240624 is G and that at rs4841132 is A).[12] In the propensity score-matched analysis, *PPP1R3B* rs4841132 was associated with gallstones. In an unadjusted model, the odds ratio (OR) for GSD was 1.05 (95% confidence interval, CI 1.004–1.091, $P = .031$) and in the birth year- and sex-adjusted model the OR was similar at 1.05 (95% confidence interval, CI 1.004–1.091, $P = .031$), Figure 1.

Effect of PPP1R3B *rs4240624 Genotype on Gallbladder Lipidome*

We have previously shown that bile acid, cholesterol, and phospholipid levels were decreased in the bile of patients with the AG genotype of *PPP1R3B* rs4240624.[7] We also analyzed the effects on the biliary lipidome. We identified 1004 different individual lipids from 17 different lipid classes in the gallbladder bile. Surprisingly, despite a decrease in the main bile constituents, increases in 13 different lipid classes were observed (FDR-adjusted) in patients with the AG genotype, including lysophosphatidylcholines, diacylglycerols (DG), and triglycerides (TG) (Figure 2).

Major Lipids in Bile are Decreased in Those With Gallstones and Lipidomic Results Resembled Findings Regarding the PPP1R3B *rs4240624 Genotype*

To investigate whether the changes detected in the biliary lipidome of patients with the AG genotype of *PPP1R3B* rs4240624 mimic those observed in the bile of patients with GSD, we performed extended lipidomics in the gallbladder bile of both patients with and without gallstones. Total bile acids were significantly lower in those with gallstones

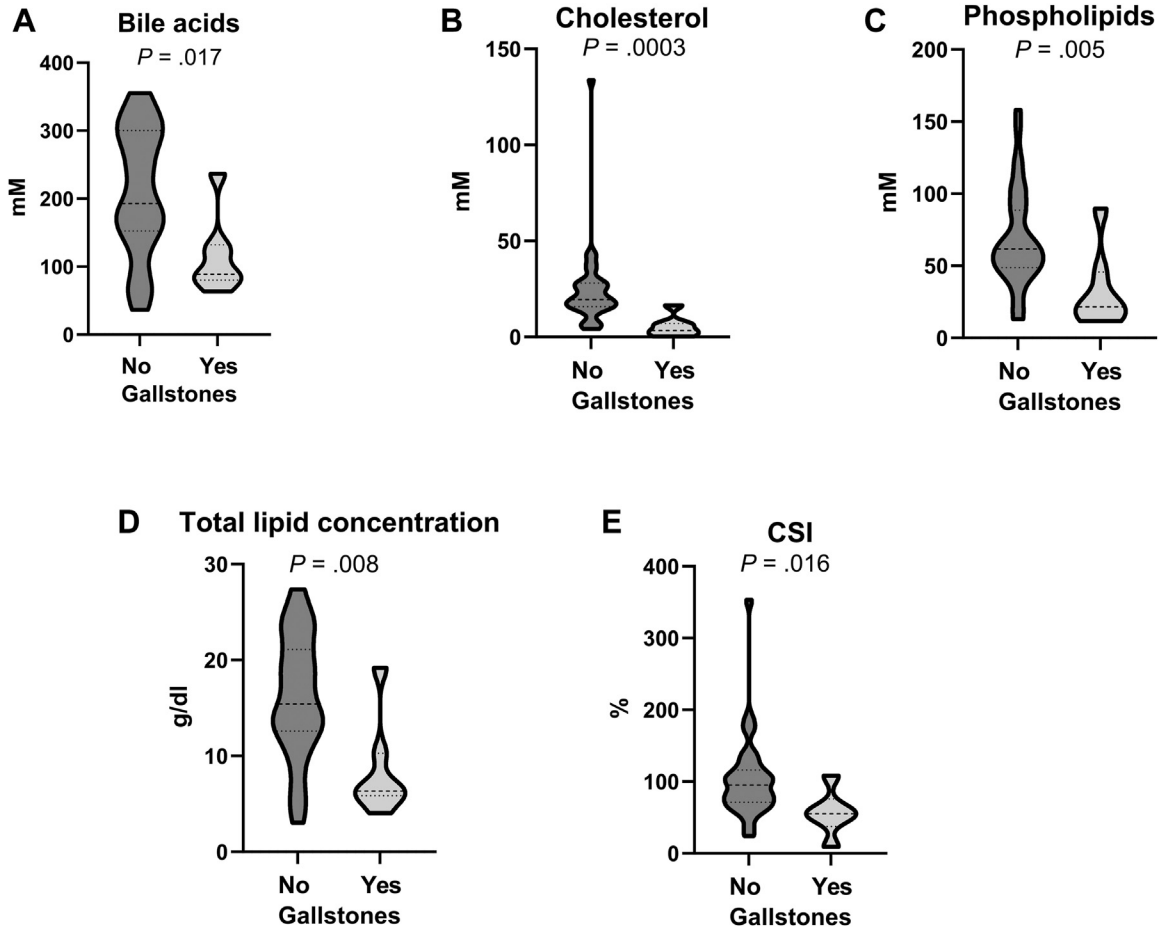

Figure 3. Basic lipid analyses from gallbladder bile based on the gallstone status are demonstrated here showing total bile acids (A) cholesterol (B) phospholipids (C) total lipid concentration (D) and cholesterol saturation index (CSI) (E) The horizontal bolded line indicates the median, the other horizontal lines indicate lower and upper quartiles, and the borders indicate the minimum and maximum values.

(211.0 ± 93.5 mM vs 115.2 ± 58.4 mM, $P = .017$). Furthermore, cholesterol (26.7 ± 25.3 mM vs 5.4 ± 5.5 mM, $P = .0003$) and phospholipid (72.5 ± 42.4 mM vs 32.3 ± 27.6 mM, $P = .005$) concentrations as well as total lipids (16.2 ± 6.4 g/dl vs 8.4 ± 5.1 g/dl, $P = .008$) were lower in those with GSD compared to those without GSD. Interestingly, the CSI was also lower in those with gallstones (102.9 ± 57.5% vs 56.5 ± 31.9%, $P = .016$) (Figure 3). Furthermore, ursodeoxycholic acid and some primary and secondary conjugated bile acids were decreased in the gallbladder bile (Table 2). Interestingly, the lipidome mimicked the changes observed in patients with the AG genotype of *PPP1R3B* rs4240624. Again, 13 of the 17 lipid classes were significantly elevated in patients with gallstones (when adjusted for multiple testing). For example, the lysophosphatidylcholines, DG, and TG levels in the gallbladder bile were significantly higher in patients with gallstones (Figure 4).

Discussion

In this study, we demonstrated that mutations in the *PPP1R3B* gene predispose patients to cholesterol gallstone formation. We also showed that the changes in the biliary lipidome found in the gallbladder bile of patients with SNP's in *PPP1R3B* mimic the changes observed in the gallbladder bile of patients with GSD.

In this study, patients with the G allele of the *PPP1R3B* rs4240624 genotype had gallstones more frequently, both in the original study subset and in a larger subset of the KOBS cohort (Figure 1). This variant has not been previously associated with GSD. We validated the association between the *PPP1R3B* genotype and GSD in the UKBB population. However, the association was quite modest. Since the patients in the primary cohort were all obese, we speculate that the UKBB association becomes stronger with a higher BMI. This could explain why this variant has not been identified as a risk variant for GSD, particularly in genome-wide association studies.[5,6]

At first sight, our data on gallbladder bile lipids are surprising because cholesterol hypersecretion is generally linked to GSD; however, we found that phospholipid, cholesterol, and total lipid concentrations together with CSI were lower in patients with GSD than in those without GSD. In addition, we observed lower concentrations of biliary bile

Table 2. Different Bile Acids in Gallbladder Bile Based on the Gallstone Status

Bile acids in bile (mM)	No (n = 38)	Yes (n = 8)	*P* Value
Primary BAs	0.023 ± 0.031	0.017 ± 0.021	.354
Cholic acid (CA)	0.013 ± 0.021	0.011 ± 0.015	.622
Chenodeoxycholic acid (CDCA)	0.010 ± 0.012	0.006 ± 0.007	.252
Secondary BAs	0.004 ± 0.004	0.003 ± 0.002	.310
Deoxycholic acid (DCA)	0.004 ± 0.004	0.003 ± 0.002	.310
Lithocholic acid (LCA)	0.0003 ± 0.0003	0.0002 ± 0.0001	.191
Tertiary BAs			
Ursodeoxycholic acid (UDCA)	0.001 ± 0.001	0.0002 ± 0.0004	**.044**
Primary conjugated BAs	69.8 ± 47.5	39.1 ± 37.5	.052
Glycocholic acid, (GCA)	28.5 ± 19.7	17.4 ± 17.3	.075
Taurocholic acid (TCA)	9.3 ± 5.8	3.6 ± 2.1	**.005**
Glycochenodeoxycholic acid (GCDCA)	26.6 ± 23.3	15.8 ± 19.3	.066
Taurochenoxycholic acid (TCDCA)	5.4 ± 3.6	2.2 ± 1.6	**.014**
Secondary conjugated BAs[a]	29.0 ± 15.7	15.1 ± 8.4	**.016**
Glycodehydrocholic acid (GDCA)	18.6 ± 10.7	11.6 ± 7.8	.064
Taurodeoxycholic acid (TDCA)	6.8 ± 4.5	2.1 ± 1.1	**.003**
Taurolithocholate (TLCA)	0.3 ± 0.2	0.1 ± 0.1	**.005**
Glycoursodeoxycholic acid (GUDCA)	2.9 ± 2.7	1.1 ± 0.9	**.040**
Tauroursodeoxycholic acid (TUDCA)	0.4 ± 0.3	0.1 ± 0.2	**.014**
Hyodeoxycholic acid (HDCA)	0.0002 ± 0.0002	0.00004 ± 0.0001	.160
7α-hydroxy-4-cholesten-3-one (C4)	0.001 ± 0.001	0.001 ± 0.002	.627

[a]Missing glycolithocholic acid (GLCA).
Mann-Whitney U test for statistics. Statistically significant values ($P < .05$) are bolded.

acids in patients with gallstones, which is in agreement with a previous report by Rudling et al., suggesting that decreased levels of biliary bile acids are a primary cause of GSD.[9]

CSI is usually thought to increase in patients with gallstones; thus, it is an important factor in the development of GSD. We do not have a conclusive explanation for this discrepancy, with our contrasting findings in patients with GSD. Importantly, many studies have measured the CSI in patients with symptomatic GSD. In our study, patients with GSD were asymptomatic, suggesting that stone formation occurred during a period when cholesterol secretion was possibly higher. This possibly explains our results about major lipids and CSI.

We also linked the *PPP1R3B* rs4240624 genotype to the gallbladder bile lipidome. We have previously shown that this genotype is associated with bile acid content in gallbladder bile.[7] Interestingly, the levels of many lipids were increased in those with the G allele, which was also associated with an increased prevalence of gallstones. As discussed earlier, we have no mechanistic explanation for the relationship between the *PPP1R3B* rs4240624 genotype and biliary bile salt secretion, but the correspondence to the effect of minor biliary lipids suggests that these play a direct role in the pathogenesis of GSD.

Only a few previous studies have investigated minor lipids (ie, lipids other than phospholipids and cholesterol) in gallbladder bile. Here, we report significant changes in the gallbladder lipid composition in patients with GSD. Specifically, the levels of many lipids were higher in patients with GSD than in those without gallstones. To our knowledge, few studies have evaluated the TG levels in the gallbladder bile of patients with GSD. However, Chinese researchers recently reported that those with GSD caused by pancreaticobiliary reflux had lower TG levels, suggesting that it was a result of gallstones.[19]

Recently, Haal et al. used the same lipidomic profiling methodology and found several significant differences between bariatric patients with gallstones and nonbariatric patients with gallstones. Interestingly, the levels of many lipid classes were higher in the nonbariatric group.[17] In contrast to the present study, they did not have access to a nongallstone group.

This study had some limitations. The study sample size was rather small, which may have affected the genetic analyses. However, in this study cohort, we demonstrated several significant differences in the gallbladder bile lipidome. In our KOBS cohorts there were more female than male. However, female sex is a known risk factor for GSD.[2,3] In addition, we were unable to collect gallstones for further analysis to explore the composition of stones, that is, to determine who had cholesterol or pigment stones. Furthermore, studies have shown that gallbladder emptying decreases in obese patients on low-fat low-calorie diets.[20,21] The majority of our study participants were on a very low-calorie diet for 12 weeks before surgery, which might have affected our results. However, we believe that diet does not have a different effect on gallbladder emptying in patients with GSD compared to those who do not have gallstones.

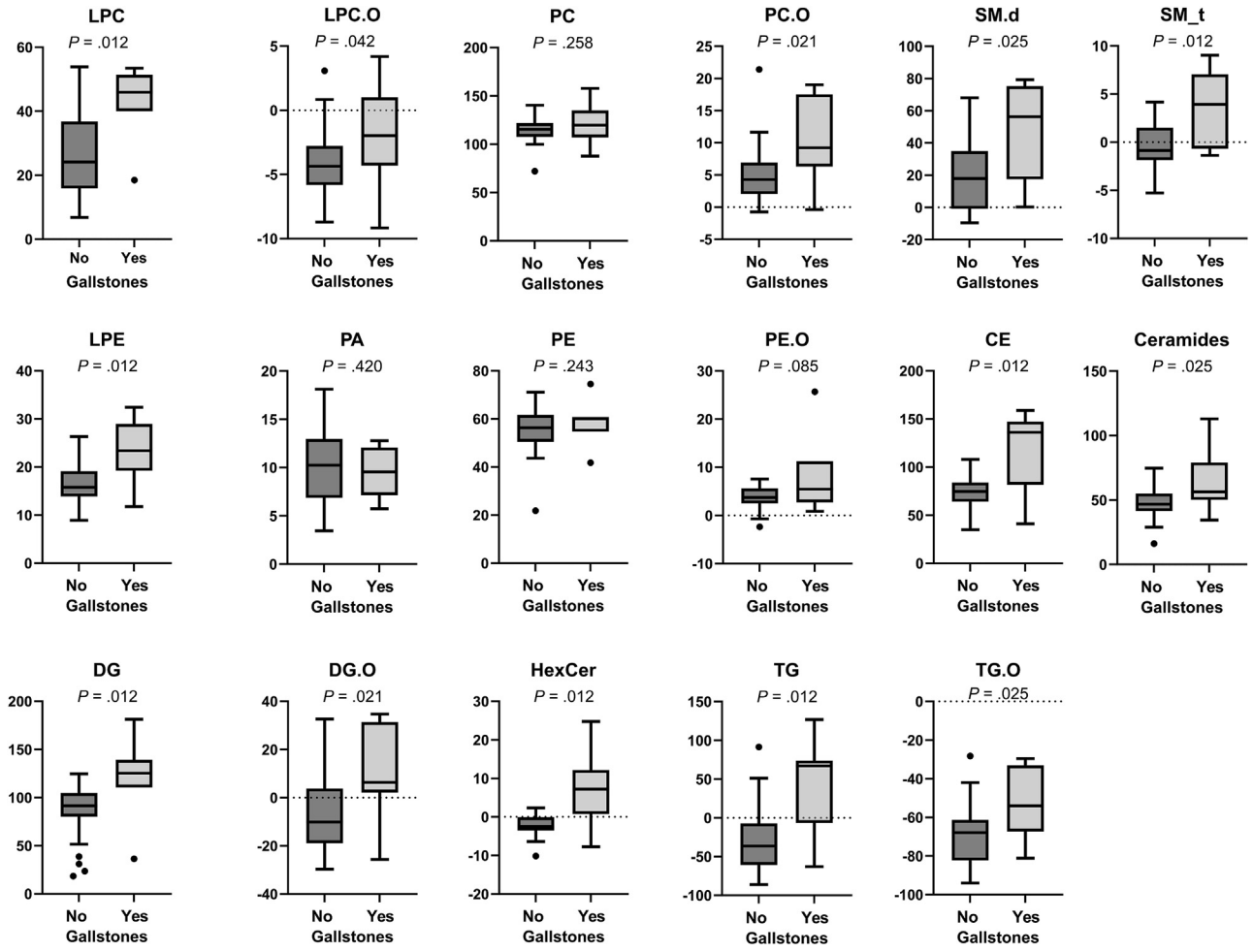

Figure 4. Logarithm-transformed total lipid levels in gallbladder bile based on the gallstone status. Panels demonstrate lyso-phosphatidylcholine (LPC), (lyso)alkyl-phosphatidylcholine (LPC.O), phosphatidylcholine (PC), lyso-phosphatidylcholine (PC.O), sphingomyelin/ceramide phosphocholines (SM.d), and hydroxysphingomyelin (SM.t), lyso-phosphatidylethanolamine (LPE), phosphaditic acid (PA), phosphatidylethanolamine (PE), alkyl-phosphatidylethanolamine (PE.O), cholesterol esters (CE), ceramides, diacylglycerols (DG), alkyl-diacylglycerols (DG.O), hexosylceramide (HexCer), triglycerides (TG), alkyl-triglycerides (TG.O). The horizontal line indicates the median, the whiskers indicate the minimum and maximum values with the Tukey method, and the box borders indicate lower and upper quartiles. The false discovery rate-adjusted *P* value is shown.

Conclusion

The *PPP1R3B* genotype was associated with the presence of gallstones in both the obesity surgery cohort and the UKBB. Furthermore, the genotype significantly affected the gallbladder bile lipidome, and changes in the lipidome were similar to those associated with gallstones.

Supplementary Materials

Material associated with this article can be found in the online version at https://doi.org/10.1016/j.gastha.2024.03.005.

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Ethical Statement:
This study protocol was approved by the Ethics Committee of Northern Savo. The UK Biobank cohort study received ethical approval from the North West - Haydock Research Ethics Committee (reference 16/NW/0274). The study followed the principles of the Declaration of Helsinki.

Data Transparency Statement:
The data supporting the findings of this study are available from the corresponding author upon reasonable request.

Reporting Guidelines:
STROBE.