

Understanding pathophysiology of gallstone disease: a multi-omic analysis focused on women with obesity

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Abstract

Background

Gallstone disease has a complex, multifactorial pathophysiology. Both the gut microbiome and the liver have been implicated as potential contributors. Because obesity is associated with alterations in gut microbial composition and is itself a risk factor for gallstone formation, we investigated the relative roles of the gut microbiome, the liver, and adipose tissue in gallstone disease in female patients with obesity.

Methods

From the BARIA cohort, 108 consecutive female patients with obesity were included, of whom at baseline 39 had ultrasound proven gallstones (cases) whereas 69 did not have gallstones (controls). Fecal shotgun metagenomics, untargeted fasting plasma metabolomic analysis and bulkRNAseq transcriptome analyses of liver, visceral (VAT) and subcutaneous (SAT) biopsies (taken during surgery) were analyzed.

Results

Especially VAT showed differences in expression of 57 genes, whereas liver gene expression revealed differences only after secondary analysis. In contrast to other publications, in liver “classic” ABC transporter gallstone genes were not identified in this cohort. Instead, the cholesterol transporter ABCG1 popped up, and was decreased in patients with gallstone disease. Plasma metabolome showed increase of primary and secondary bile acids in patients with gallstone disease, possibly a consequence of decreased bile acid secretion in the bile.

Conclusions

The results of our study suggest VAT instead of the liver as a potential driver for gallstone formation.

Introduction

Gallstone disease (GSD) is common worldwide. Up to twenty percent of the adult population develops gallstones and up to 20% of these individuals develop symptoms(1, 2). Etiological factors include cholesterol crystallization, impaired gallbladder motility and excess bilirubin. Approximately 80% of gallstones are cholesterol stones, whereas the remaining 20% are pigment or mixed stones. Risk factors for GSD include female sex, specific medication (e.g., estrogens, GLP1-R agonists), obesity, metabolic syndrome, hemolytic anemia, prolonged fasting, rapid weight loss and bariatric surgery.

Genetic factors account for approximately 25% of individual susceptibility. (3)

GSD is a heterogeneous disease, and its exact pathophysiology remains incompletely understood. Whereas pigment gallstones are primarily caused by hemolysis, the mechanisms underlying cholesterol gallstone formation are multifactorial.

Cholesterol crystallization is often preceded by an increased cholesterol saturation index (CSI) in bile, reflecting supersaturation of cholesterol. Genetic variants in genes involved in cholesterol transport (ABCG5, ABCG8, ABCB4) and bile acid metabolism (CYP7A1, ABCB11, SLC10A2, HNF4A, and SERPINA1) have been associated with gallstone disease, as they influence hepatic secretion of biliary cholesterol (3–5) and thereby alter bile composition. A reduced bile acid concentration in bile is another contributor to an increased CSI (6). Gut microbiome can influence bile acid metabolism via conversion of primary bile acids into secondary bile acids (7–9). In patients with gallstones, higher overall concentrations of fecal bile acids and decreased microbial diversity were observed, identifying the genera *Roseburia* and *Oscillospira* as biomarkers for gallstone disease (10). However, this was not confirmed in another study (11).

Prevalence of obesity and GSD has been rising for the last decades and obesity is an important risk factor for gallstone disease (12, 13). Mendelian randomization studies found a causal association between elevated BMI and increased risk of symptomatic gallstone disease (14). Obesity has also been associated with altered bile metabolism and gut microbiome and recently the G allele of PPP1R3B rs4240624, associated with lower bile acid levels, was significantly associated with gallstone disease in patients with obesity (15).

Another important risk factor for gallstone development is female sex. Women have approximately twice the risk of developing gallstones compared with men (1). Estrogen receptor expression may be one of the underlying mechanisms (16), which is supported by the observation that estrogen therapies, including oral contraceptives, further increase the risk of gallstone formation (17). Furthermore, sex differences in lipid metabolism and bile acid plasma metabolites have been reported in humans, underscoring the importance of sex-specific research (18).

Gallstone disease is a complex, multifactorial condition, and studying specific populations is essential to better understand its underlying mechanisms. Therefore, the aim of this study is to focus on female patients with severe obesity to gain deeper insight into the mechanisms involved in gallstone formation using a multi-omics approach. We compared fasting plasma metabolites, gut microbiome composition, and bulk RNA-sequencing–based gene expression in liver and adipose tissue between female patients with obesity with and without gallstones.

Methods

Participants

For the current study, we included 108 female patients with obesity and with available data on gallstone presence or disease from the BARIA cohort in the Netherlands. For details regarding complete study

protocol with recruitment, data selection and metabolic workup of the BARIA cohort, see van Olden et al (19). Although the BARIA cohort consists of patients undergoing bariatric surgery, the present study focused on the cross-sectional data obtained before and at the day of surgery and not on the possible influence of weight loss and altered anatomy after bariatric surgery.

Baseline clinical characteristics, fasting blood samples and fecal samples were collected, including a transabdominal gallbladder ultrasonography to determine the presence of gallstones. Tissue biopsies of subcutaneous and visceral adipose tissue, jejunum and liver were obtained during scheduled bariatric surgical procedure. Datasets from 108 participants in the BARIA cohort fit the above-mentioned criteria.

This study was performed in accordance with the Declaration of Helsinki and was approved by the Academic Medical Center Ethics Committee of the Amsterdam University Medical Center (METC 2015_357). All patients provided written informed consent.

Data and sample collection and preparation

At the baseline visit participant characteristics were recorded and fasting blood samples were collected. Fasting plasma was sent to Metabolon (Morrisville, NC, USA) for analysis using untargeted metabolomics via ultra-high-performance liquid chromatography coupled with tandem mass spectrometry (LC-MS/MS). Patients were also instructed to collect fecal samples either on the day of their scheduled surgical procedure or one day prior. These samples were promptly frozen at -70°C. Total genomic DNA was extracted from the fecal samples and shotgun metagenomic sequencing was conducted to study the fecal microbiome. Finally, during the bariatric surgery the surgeon collected liver and adipose tissue biopsies. RNA extraction and bulk RNAseq gene expression analysis were then performed to obtain transcriptomic data from these tissues.

Study outcomes and definitions

Primary outcomes of this study were differences in plasma metabolites, gut microbiome composition, and bulk RNA seq based gene expression in the liver, subcutaneous fat, and visceral fat tissue between patients with or without gallstones. We defined presence of gallstones as gallstones present on ultrasound of the gallbladder during outpatient clinic, weeks before surgery or previous (laparoscopic) cholecystectomy (LC) for symptomatic gallstone disease. Secondary outcomes were clinical characteristics and subgroup analysis comparing the three subgroups: patients without gallstones, patients with gallstones on ultrasound, and patients with previous LC. Diabetes mellitus type 2 and hypertension were registered if patients were treated with drugs for these conditions. Dyslipidemia was defined as the use of lipid lowering drugs or if any of the following preoperative laboratory results were observed: HDL < 0.9 mmol/l, LDL $\geq$ 5 mmol/l, total cholesterol $\geq$ 6.5 mmol/l, or triglycerides: $\geq$ 5 mmol/l.

Statistical analysis

Standard descriptive statistics were used to analyze baseline clinical characteristics. Data for the continuous variables that followed a normal distribution were analysed using the unpaired t-test. Categorical data and nonparametric data were analysed using the Fisher's exact test. Normally distributed data were presented as mean and standard deviation or as proportions. Non-parametric data was presented by median and interquartile range. These analyses were performed with R version 4.4.1 and two-sided P-values < 0.05 were considered statistically significant.

Metagenome, transcriptome and metabolome analyses

Bulk RNAseq transcriptomic reads of liver, subcutaneous fat, and visceral fat tissue samples were analyzed as described previously (20, 21) In short, paired-ended reads were trimmed and cropped with trimmomatic v0.38. High-quality reads were then mapped against the GRCh38 human genome assembly with kallisto v0.46.0 (options: `--bias`, `-b 100`, and `--rf-stranded`).

Gut microbiome reads were trimmed and quality filtered with fastp v0.23.2 (option `--detect_adapter_for_pe`) (22), after which reads were used to determine community composition with Kraken v2.1.2 (23), using the PlusPF-16 index (version 20230605) that includes RefSeq Archaea, Bacteria, Viruses, Plasmid, Human, Protozoa, and Fungi (<https://benlangmead.github.io/aws-indexes/k2>). Ecological measures were determined with the vegan R package v2.6-6.1¹⁰ and the phyloseq R package v1.48.0¹¹. Differential abundance analyses were performed using the DeSeq2 R package v1.44.0 using Benjamini-Hochberg adjustment for multiple testing. Metabolites concentrations were analysed per sub-pathway.

Results

Study population

Data of 108 female patients with morbid obesity (BMI > 35 kg/m²) was included, of which 69 patients did not have gallstones and 39 had evidence for gallstones. Table 1 shows the baseline characteristics for the total population and for the patients with and without gallstones separately. There were no significant differences in patient characteristics between groups. Mean age was 45 ± 10.6 years and mean BMI was 39.6 ± 4.2 kg/m². Among the patients with gallstones, 21 patients had previous cholecystectomy, and 18 patients had gallstones on ultrasound. Patient characteristics were not different between both groups.

RNAseq based transcriptome

Liver

Transcriptomic analysis of liver tissue without selection of genes did not show differences in gene expression between patients with or without gallstones. However, to improve sensitivity we performed a subset analysis of the unadjusted gene expression targeted on specific genes associated with gallstone

formation, including genes from the ABCG (liver/canaliculi cholesterol transport) and ABCB (phospholipid and bile salt transport) families. ABCG1 expression was significantly lower among gallstone patients compared to controls ($p = 0.039$) (Supplementary Fig. 1).

These secondary analyses comparing controls, patients with previous LC and patients with gallstones on ultrasound showed differences in gene expression of 15 genes between these subgroups (Supplementary Fig. 2). Expression of the uncharacterized *AC008695.1* gene was different in two comparisons: expression was elevated in controls compared to patients with previous LC ($q\ 0.01-0.001$, $lfc\ 3.0$), and expression was decreased in patients with previous LC compared to patients with gallstones on ultrasound ($q\ 0.001-0.0001$, $lfc\ 3.0$). In the subset analysis for specific genes associated with gallstone formation, ABCG1 expression was decreased significantly

Visceral adipose tissue (VAT)

Differential expression of visceral adipose tissue transcriptome revealed differences in 57 genes between patients with gallstones and those without. Most significant positive correlations were seen in *SCUBE1* ($q\ 0.001-0.01$, $LFC\ 1.0$), *NRP2* ($q\ 0.001-0.01$, $LFC\ 0.2$), *NPR3* ($q\ 0.001-0.01$, $LFC\ 0.4$), *ALDH4A1* ($q\ 0.001-0.01$, $LFC\ 0.2$), of which expression was increased among gallstone patients. Strongest negative correlation was for *CA3* ($q\ 0.01-0.05$, $LFC\ -1.0$) (Table 2). An overview of all 57 genes can be found in the Supplementary material Fig. 3.

Moreover, when a subgroup analysis was done compared against controls without gallstones, patients with previous LC and patients with gallstones on ultrasound reported differences in VAT gene expression. Genes with differential expression in more than one comparison are shown in Fig. 1.

Subcutaneous adipose tissue (SAT)

Neither primary analysis of controls and gallstone patients, nor subset analysis of three groups found differences in expression among genes in subcutaneous adipose tissue.

Gutmicrobiome analyse

Fecal metagenomic analysis did not show differences in species richness nor alpha diversity between patients with or without gallstones. In addition, principal coordinate analysis (PCoA) of the metagenomics samples also did not show significant differences in beta-diversity between patients with or without gallstones either (PERMANOVA $p > 0.05$). Differential abundance analysis using ANCOM-BC did not show significant differences in gutmicrobiome composition.

However, subgroup analysis comparing the three groups controls, patients with previous LC and patients with gallstones on ultrasound reported difference in beta-diversity as calculated with PCoA (permanova 0.04, axis 1 12.1%, axis 2 8.6%). Two bacterial species were significantly different between both groups (adjusted $P \leq 0.05$ and $\log_2$ fold change ≤ -1 or ≥ 1). *Shigella flexneri* was more abundant in patients with

gallstones on ultrasound in both comparison with patients with previous LC (q 0.001–0.001, lfc – 2.0) and patients without gallstones (q 0.001–0.001, lfc – 1.5) and *Shigella boydii* was more abundant in patients with gallstones on ultrasound compared to patients without gallstones (q 0.01–0.05, lfc – 1.0).

Plasma metabolomics

Untargeted plasma metabolomics revealed that 13 metabolites differed between patients with or without gallstones. Direct comparison showed that Beta-cryptoxanthin, phenylacetylglutamine and phenylacetylglutamate were more abundant in patients without gallstones compared to gallstone patients. In contrast, plasma mannitol, sorbitol and bile acids glycochenodeoxycholate, glycocholate, taurocholate and taurochenodeoxycholate and glycodeoxycholate 3-sulfate, glycolithocholate, glycooursodeoxycholate were more abundant among gallstone patients (Fig. 2).

Secondary analysis compared differences between controls, patients with previous LC and patients with gallstones on ultrasound. Subgroup analysis of patients with gallstones on ultrasounds compared to patients with previous LC (Fig. 3) showed that 17 α -hydroxypregnenolone 3-sulfate was elevated in patients with LC (coef 1.0, q 0.01–0.05). Cholic acid glucuronide was more abundant in patients with ultrasound gallstones (coef – 1.0, q 0.01–0.05). Docosadioate (C22-DC) is increased in controls compared to LC group. Taurocholate, taurochenodeoxycholate, glycocholate, glycochenodeoxycholate glucuronide (1), glycochenodeoxycholate 3 – sulfate, glycochenodeoxycholate, glycooursodeoxycholate and glycolithocholate were elevated in patients with previous LC compared to patients without gallstones. Compared to patients with gallstones on ultrasound, 1 – palmitoleoyl – 2 – linoleoyl – GPC (16:1/18:2)* and cholic acid glucuronide is more abundant in patients without gallstones. Mannitol/sorbitol, taurocholate, taurochenodeoxycholate, glycocholate and glycochenodeoxycholate were elevated in patients with ultrasound gallstones when compared to patients without gallstones.

Discussion

The present study is the first one to provide a multi-omics approach, including plasma metabolome, fecal metagenome and transcriptome of adipose tissue and liver, specifically focused on gallstone disease in women with severe obesity. The results indicate that differences in gene expression of in particular visceral adipose tissue (VAT), in combination with changes in liver tissue, might contribute to altered hepatic lipid metabolism in patients with gallstones. Subsequent subgroup analysis of fecal microbiome revealed that potentially bile-sensitive species were more abundant in patients with gallstones. Additionally, fasting plasma primary and secondary bile acids were elevated in patients with gallstones.

Bile and gallstone-related research has predominantly focused on the liver, and several genes have been implicated in incident gallstone disease, including *ABCG5* and *ABCG8*.(3, 24). Inactivation of either of these two genes results in a lipid disorder characterized by excessive accumulation of sterols in the bloodstream (25). Interestingly, our primary analysis of liver transcriptome did not show any differences in gene expression between groups. However, subgroup analysis in patients with previous LC compared

to patients without gallstones did show decreased expression of *AC008695.1*, which is enhanced in immune cells, though its function remains unknown. Additionally, *IGHV1-69*, an immunoglobulin gene (26), demonstrated a log fold change of approximately 2.5 in the LC group compared to patients without gallstones. This gene has been associated with chronic lymphocytic leukemia (CLL) and is linked to lipid peroxidation and inflammation in that context (26).

Interestingly, our targeted gene analysis to liver-specific genes as reported in literature did not identify the classic gallstone related genes; *ABCG5/G8*, *ABCB4* or *ABCB11*. In contrast *ABCG1*, a cholesterol exporter involved in the reverse cholesterol transport pathway, was found to be less abundant in cases (patients with gallstones) compared to controls. Indeed in mice, *ABCG1* is critical for maintaining cholesterol homeostasis (27). Notably, *ABCG1* in mice mobilizes cholesterol and disruption of *ABCG1* has been shown to result in significant accumulation of cholesterol esters, triglycerides and phospholipids in hepatocytes and macrophages (28). Thus, decreased liver *ABCG1* in patients with gallstones suggests altered hepatic lipid metabolism and accumulation as a possible contributor to altered bile composition.

In line, in our study we observed several differentially expressed genes in visceral adipose tissue (VAT) were identified that offer new insights into gallstone formation, especially given the scarcity of clear liver-specific gene findings. No differences in distinct inflammatory pathways between groups were evident in VAT transcriptomic analysis while VAT inflammatory pathways are activated in the vast majority of females with obesity and apparently other pathways dominate the pathogenesis of gallstone formation. This observation of VAT gene expression suggests a novel angle to explore gallstone formation through adipose tissue mechanisms, rather than hepatic pathways. Although carbonic anhydrase 3 (CA3) is primarily studied in the liver, we found VAT CA3 expression increased in patients without gallstones, adding complexity to its role in adipose tissue regulation. One study in rats reported increase of CA3 during liver adipogenesis as response on a high fat diet, and inhibitors of CA3 to reduce liver fat accumulation, indicating possible influencing of (hepatic) lipid metabolism by CA3 (29). Another interesting finding is the role of *FFAR3* (free fatty acid receptor 3, also termed GPR41) in VAT. A recent study in mice also demonstrated that GPR41 plays a crucial role in the anti-obesity effects and improvement of hepatic steatosis by stimulating the lipid catabolism pathway (30) and GPR41 is activated by microbially produced short-chain fatty acids (31). The variability in VAT gene activation across individuals further supports the idea that VAT could serve as an important driver in metabolic processes related to gallstone formation.

Finally, upon fecal microbiome analysis an increased abundance of *Shigella* was found in patients with gallstones, suggesting a potential role in gallstone formation. A study by Kose et al. (2018) using metagenomics to investigate the bacterial composition of pigmented and cholesterol gallstones identified the presence of *Shigella flexneri*, a bile-sensitive bacterium. The study highlighted the expression of the PhoQ regulatory protein in *Shigella*, which is closely associated with the PhoP regulon. Together, the PhoP-PhoQ system has been implicated in bacterial bile resistance, enabling bacteria to survive in the hostile bile environment (32). This bile resistance mechanism, previously observed in

Salmonella spp., may explain *Shigella*'s survival and potential involvement in gallstone pathogenesis. Further investigation is warranted to explore the interaction between *Shigella* and adipose tissue in relation to inflammation and gallstone formation. Interestingly, *S. flexneri* also metabolizes phenylalanine, of which phenylacetylglutamine is a metabolite. Phenylacetylglutamine was elevated in controls, whereas *S. flexneri* was increased in patients with gallstones (33).

When we analyzed differences in fasting plasma metabolites, we observed that primary and secondary bile acids were elevated in patients with gallstones. These findings corroborate with our previously published results (21). We demonstrated elevated glycochenodeoxycholate in plasma of gallstones patients, which has been associated with hepatocellular cholestasis (34). Other bile acids, including chenodeoxycholic acid, have been identified as potential markers for cholesterol gallstones (35). The concentration of bile acids in the gallbladder of gallstone patients is decreased (6) and our study found elevated bile acids in plasma. Either an unknown process in the liver results in lower bile acid concentration in the gallbladder, or the uptake from plasma bile acids to the liver is impaired. Further research is necessary to clarify this mechanism.

Contrary with our previously published paper on gallstones during follow-up after surgery, in the current study we found no significant differentially expressed genes between both groups in subcutaneous adipose tissue. We speculate that (beyond sample size) VAT may have a specific role during pronounced weight loss after surgery. As stated above, our VAT analysis result implicate a potential role in gallstone formation via altered metabolic activity.

A strength of our study is that we confirmed gallstones by ultrasound at baseline (that is before surgery) thus underscoring the validity of our findings. A limitation of this study is that fecal metagenomic analysis was done with a different method than in our previous study (Ancombc vs DESeq2). The Ancombc package is a relatively new package for analysing differential abundance and correlation analysis for microbiome data specifically (36). Other limitations are the sample size which precluded further subgroup analyses, and by selecting only females we cannot extrapolate our results to gallstone formation in severe obese males.

In conclusion, in this study we aimed to increase homogeneity in our population by focusing on women with obesity. Our main findings suggest an association between differences in VAT gene expression related to lipid metabolism and gallstone formation and increased plasma bile acids in female gallstone patients. Identifying the underlying mechanisms of gallstone formation remains a complex quest complicated by its multifactorial etiology. Our study underscores the importance of further research towards a possible causal association between visceral adipose tissue activity and gallstone formation in female patients with obesity.

Declarations

CONFLICT OF INTEREST STATEMENT

M. Nieuwdorp is co-founder and member of the Scientific Advisory Board of Caelus Pharmaceuticals and Advanced Microbiota Therapeutics, the Netherlands. M.N. is also on the board of directors of Diabeter Netherlands BV. However, none of these bear any relevance to the content of this current manuscript. None of these are directly relevant to the current paper. There are no patents, products in development or marketed products to declare. The other authors declare no conflict of interest.

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AUTHOR CONTRIBUTIONS

JH, AKG and VG wrote and edited the manuscript. PJ. performed data pre-processing, analysis and gave expert opinions. JH, MG, SH, OA, AM, DPS, SB, R.F, YA.and LB. recruited the patients and collected the data. FB and MN gave expert opinions and helped with the study design. J.H. is the guarantor.

DATA AVAILABILITY STATEMENT

The datasets generated during and/or analysed during the current study are available from the corresponding author on reasonable request.

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Tables

Tables 1 and 2 are available in the Supplementary Files section.

Figures

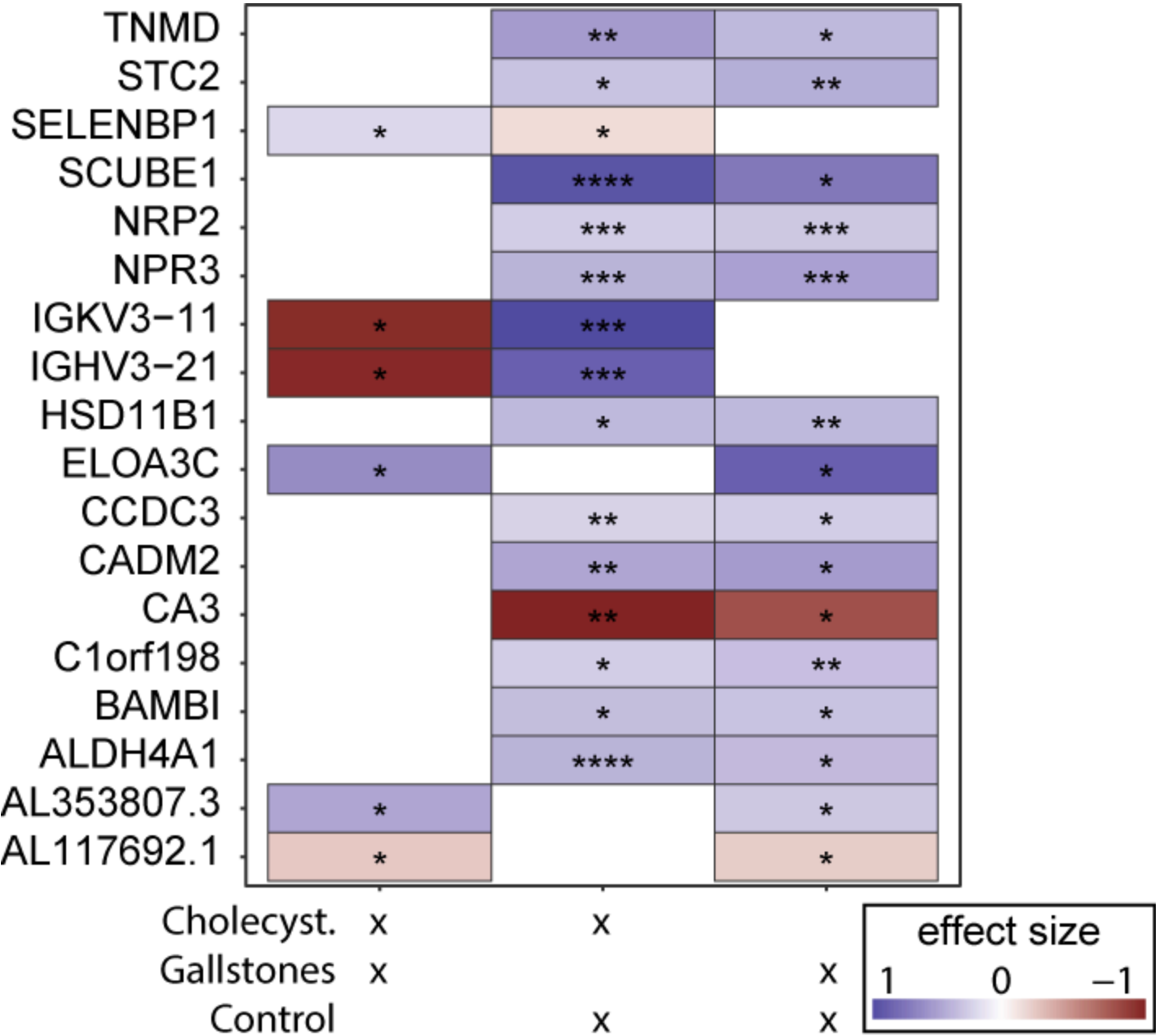


Figure 1

Subgroup analysis of differential expression in visceral adipose tissue.
Expression different in more than one comparison

* between (q, 0.01, 0.05); ** between (q, 0.001, 0.01); *** between (q, 0.0001, 0.001).

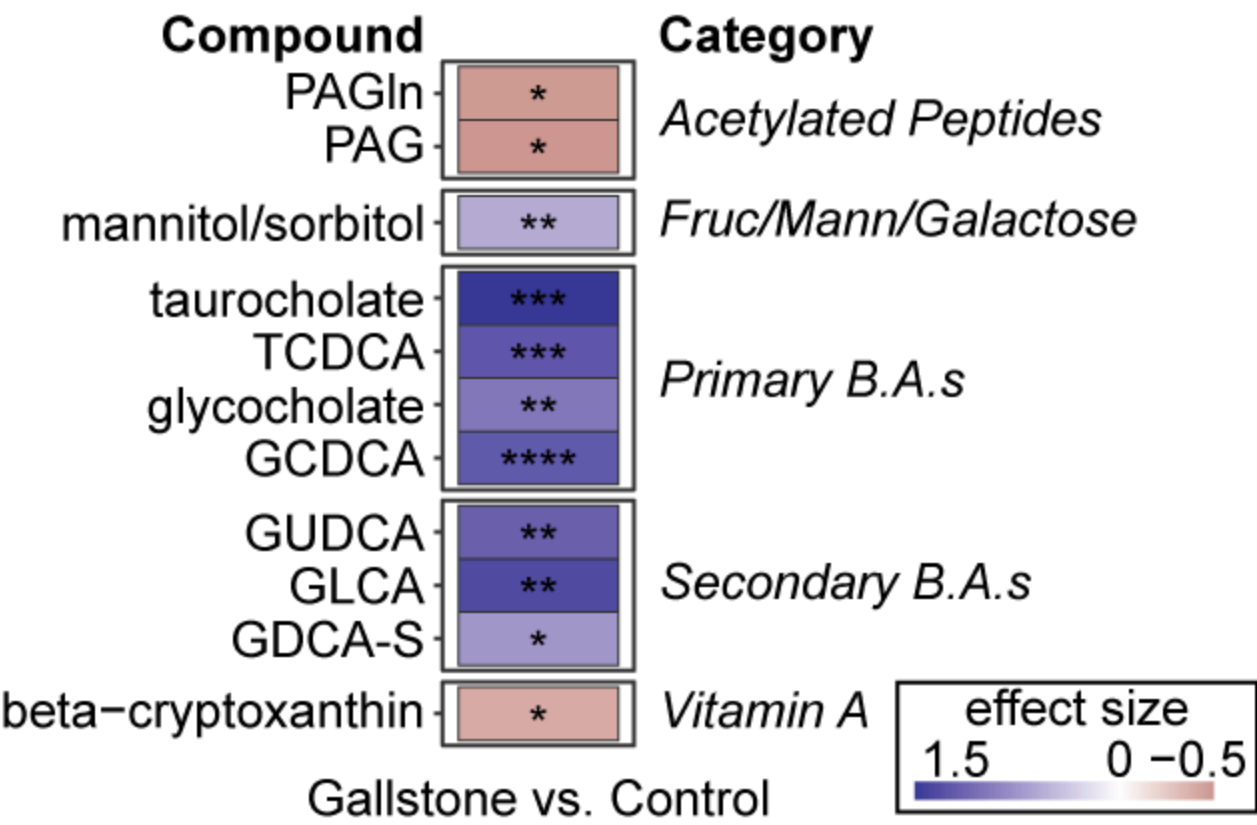


Figure 2

Plasma metabolites differences in primary analysis, between gallstone group and controls.

* between (q, 0.01, 0.05); ** between (q, 0.001, 0.01); *** between (q, 0.0001, 0.001).

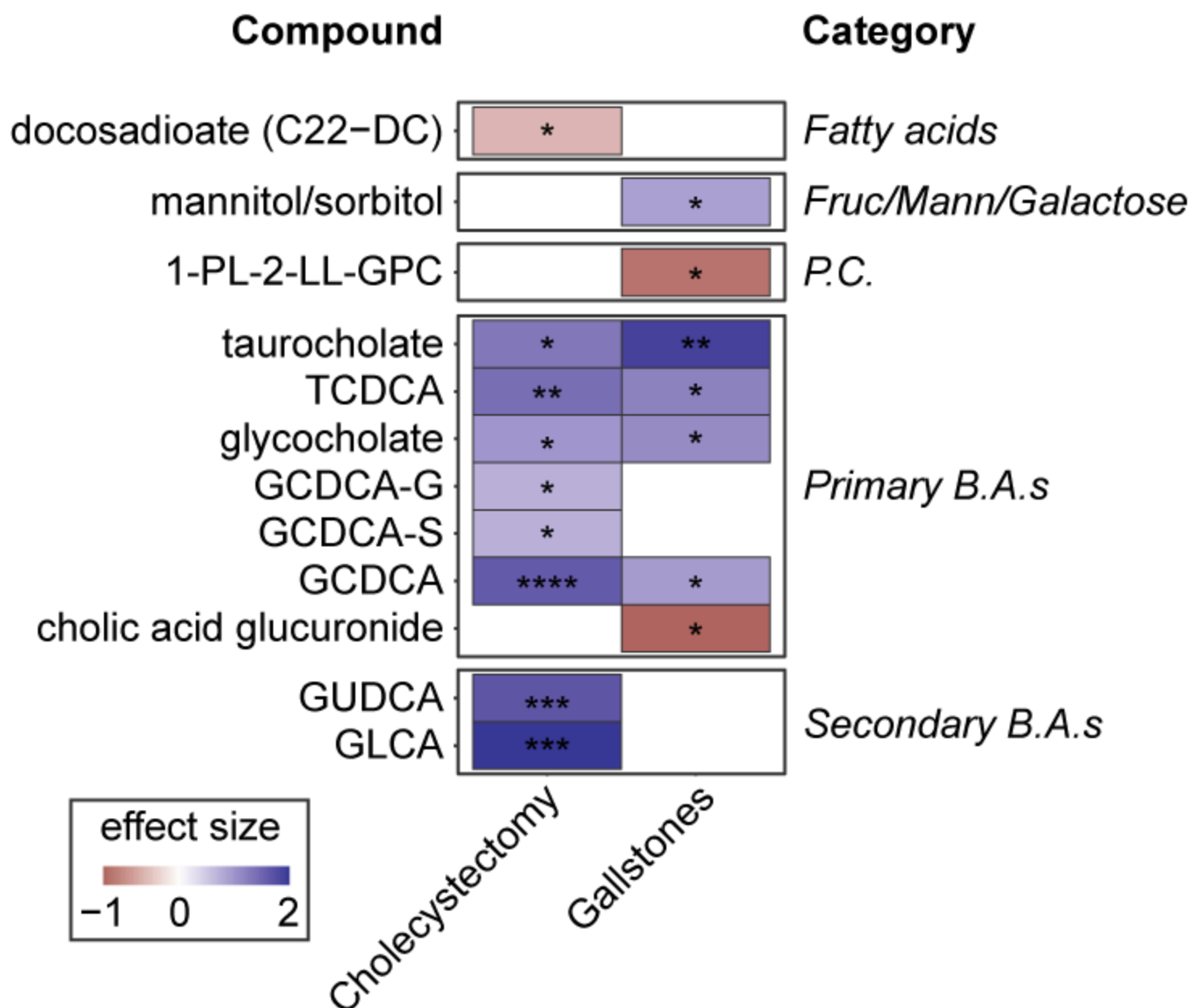


Figure 3

Plasma metabolites differences in subgroup analysis, LC and ultrasound gallstones

* between (q, 0.01, 0.05); ** between (q, 0.001, 0.01); *** between (q, 0.0001, 0.001).

Supplementary Files

This is a list of supplementary files associated with this preprint. Click to download.

- [Table1.xlsx](#)
- [Table2.xlsx](#)
- [SupplementaryMaterialv3.docx](#)