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Adipose tissue, bile acids, and gut microbiome species associated with gallstones after bariatric surgery.

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Short title: Associations with gallstones after bariatric surgery.
Abstract

Several risk factors are associated with gallstone disease after bariatric surgery, but the underlying pathophysiological mechanisms of gallstone formation are unclear. We hypothesize that gallstone formation after bariatric surgery is induced by different pathways compared to gallstone formation in the general population, since postoperative formation occurs rapidly in patients who did not develop gallstones in preceding years. To identify both pathophysiological and potentially protective mechanisms against postoperative gallstone formation, we compared the preoperative fasting metabolome, fecal microbiome, and liver and adipose tissue transcriptome obtained before or during bariatric surgery of obese patients with and without postoperative gallstones. In total, 88 patients were selected from the BARIA longitudinal cohort study. Within this group, 32 patients had postoperative gallstones within two years. Gut microbiota metagenomic analyses showed group differences in abundance of 41 bacterial species, particularly abundance of Lactobacillaceae and Enterobacteriaceae in patients without gallstones. Subcutaneous adipose tissue transcriptomic analyses revealed four genes that were suppressed in gallstone patients compared to patients without gallstones. These baseline gene expression and gut microbiota composition differences might relate to protective mechanisms against gallstone formation after bariatric surgery. Moreover, baseline fasting blood samples of patients with postoperative gallstones showed increased levels of several bile acids. Overall, we revealed different genes and bacteria associated with gallstones than those previously reported in the general population, supporting the hypothesis that gallstone formation after bariatric surgery follows a different trajectory. Further research is necessary to confirm the involvement of the bile acids, adipose tissue activity, and microbial species observed here.

Key words: gallstone formation, conjugated bile acids, BARIA study, Lactobacillaceae, subcutaneous adipose tissue, visceral adipose tissue, transcriptomic, metabolomics, metagenomics, gallstone disease.
Introduction

Worldwide, an increasing number of bariatric surgeries are performed, as it is the most effective treatment leading to sustainable weight loss in patients with morbid obesity (1). Nevertheless, the rapid weight loss after these procedures is a risk factor for gallstone formation in about one third of patients after bariatric surgery (2-4). While most patients remain asymptomatic, approximately 8-15% of patients require cholecystectomy. The relationship between bariatric surgery and gallstone disease has been widely investigated. However, the underlying pathophysiological mechanisms leading to gallstone formation are still not completely identified. Factors associated with gallstone formation in the general population include decreased secretion of bile acids, hypersecretion of cholesterol, rapid phase transitions of cholesterol in bile leading to the precipitation of cholesterol crystals, and impaired gallbladder motility with hypersecretion of mucus (5). Furthermore, gut microbiome has also been suggested as one of the drivers of cholesterol gallstone formation and can influence bile acid metabolism via conversion of primary bile acids into secondary bile acids in the gut (6-9).

A study in mice showed that intestinal flora imbalance can affect bile acid and cholesterol metabolism, which was associated with gallstone formation (10). In patients with gallstones, higher overall concentrations of fecal bile acids and decreased microbial diversity were found, identifying the genera *Roseburia* and *Oscillospira* as biomarkers for gallstone disease (11). However, a more recent study did not observe significant differences between patients with asymptomatic gallstones disease and healthy controls (12). Since studies report contradictory results, the relationship between gut microbiome composition and gallstones is not well understood (13). Finally, multiple variants in genes involved in cholesterol metabolism (such as *ABCG5*, *ABCG8*, and *CYP7A1*) and bile acid metabolism (for example variants in *SLC10A2*, *HNF4A*, and *SERPINA1*) are associated with gallstone disease (14, 15). This indicates that regulation of specific liver genes and intestinal uptake of bile acids are involved in gallstone formation.

However, the actual contribution of cholesterol metabolism, bile acids, and genetic factors in gallstone formation in patients after bariatric surgery has yet to be clarified. In fact, in a recent study comparing bile and gallstone composition of gallstone patients after bariatric surgery to gallstone patients in the general population, differences were found in the total levels of specific lipid classes: cholesterelester, phosphatidic acid, alkyl-phosphatidylcholine, alkyl-phosphatidylethanolamine, and especially triglyceride were
significantly lower in bariatric gallstone patients (16). It seems that the formation of gallstones in bariatric patients follows a different trajectory than in patients without previous bariatric surgery. This is likely based on the changed gastrointestinal anatomy after bariatric surgery and the following rapid weight loss that is accompanied by a decrease of adipose tissue mass and pronounced alterations of glucose, lipid, and bile acid metabolism. Bariatric surgery also leads to changes in composition of gut microbiota and derived metabolites. Nevertheless, most obese patients undergoing bariatric surgery do not develop gallstones despite having several risk factors. We hypothesized that apart from risk factors, other mechanisms might exist that protect against gallstone formation after bariatric surgery. Investigating patients just before and after bariatric surgery might reveal new and possibly protecting pathways or metabolic processes against gallstone disease in this patient population. The aim of the present study was to compare preoperative plasma metabolites, gut microbiome composition, and genetic expression in liver and adipose tissue of patients without gallstones after bariatric surgery to patients with gallstones after bariatric surgery. The findings can potentially increase the predictability of gallstone formation in bariatric patients and can be used in future studies to further unravel the pathological mechanisms involved in gallstone formation and/or prevention after bariatric surgery.
Materials and methods

Study design and population

Patients for the present study were selected from the BARIA study, a longitudinal cohort study in bariatric surgery patients using a systems biology approach to investigate gut microbial, immunological and metabolic markers in relation to obesity. Inclusion criteria for the BARIA study were: age 18 to 65 years, a BMI of ≥ 40 kg/m² or ≥ 35kg/m² in combination with an obesity related condition, such as diabetes mellitus type 2 or hypertension, and scheduled to undergo a Roux-en-Y gastric bypass or omega-loop gastric bypass. The study protocol and metabolic workup of the BARIA study were described previously (17). In short, clinical characteristics, fasting blood samples, and fecal samples are collected prior to the scheduled surgery. Preoperative samples also include tissue biopsies of adipose tissue and the liver, which are obtained during the surgical procedure. After bariatric surgery, ultrasonography of the gallbladder is performed at the follow-up moments at 1 and 2 years. For the present study, data from the first 106 participants in the BARIA study were used. Exclusion criteria were: no postoperative ultrasound of the gallbladder (n=16; either not performed or previous cholecystectomy), missing preoperative data on the metabolome, transcriptome, and metagenome (n=2), and preoperative symptoms of gallstone disease (n=0). In total, 88 patients were included in the analyses. None of these patients used oral bile acids or postmenopausal estrogens. 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. All patients provided written informed consent.

Data and sample collection and preparation

Before surgery, patients were asked to visit the hospital within a maximum of three months before bariatric surgery was scheduled. During this visit baseline characteristics were gathered and fasting blood was collected. Plasma samples were shipped to METABOLON (Morisville, NC, USA) for performing analysis using ultra high-performance liquid chromatography coupled to tandem mass spectrometry (LC-MS/MS) untargeted metabolomics. Second, patients were asked to collect fecal samples on the day of the scheduled surgical procedure, or one day before. These samples were immediately frozen at -80 °C. Total fecal genomic DNA was extracted and shotgun metagenomic sequencing was performed to analyze the fecal microbiome. At
last, biopsies of liver and adipose tissue were collected during the bariatric surgical procedure by the surgeon. Transcriptomics from the liver and adipose tissue was obtained via RNA extraction and gene expression analysis. A more detailed description of these procedures is included in the Supplementary Material and illustrated in Figure S1. Postoperatively, during follow-up visits at one and two years after bariatric surgery (n=32 and n=45, respectively), an ultrasound of the gallbladder was performed by a trained physician to detect the presence of gallstones or sludge. During these visits, weight loss and the presence of symptomatic gallstone disease or the need for cholecystectomy were registered.

Study outcomes and definitions

Primary outcomes of this study were abundance in metabolites, gut microbiome composition, and gene expression in the liver, subcutaneous adipose tissue, and visceral adipose tissue before bariatric surgery. Secondary outcomes were clinical characteristics and possible pathways involved in gallstone formation. Patients with and without gallstones detected after surgery were compared. The gallstone positive group comprised patients with gallstones or sludge present on an ultrasound of the gallbladder which was performed after bariatric surgery or patients that underwent a cholecystectomy for symptomatic gallstone disease after bariatric surgery. 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: high-density lipoprotein <0.9 mmol/l, low-density lipoprotein ≥5 mmol/l, total cholesterol ≥6.5 mmol/l, or triglycerides: ≥5 mmol/l.

Statistical analysis

Standard descriptive statistics were used to analyze baseline clinical characteristics. Data for the continuous variables followed a normal distribution and were analysed using the unpaired t-test. Categorical data was analysed using the Chi square test. Data were presented as mean and standard deviation or as proportions, respectively. These analyses were performed in IBM SPSS statistics (version 26, Armonk, New York) and two-sided P-values <0.05 were considered statistically significant.
Metagenome, transcriptome and metabolome analyses

Paired-end reads of liver, visceral adipose tissue, and subcutaneous adipose tissue transcriptomes were first trimmed and cropped using trimmomatic v0.38 with the following settings: HEADCROP:6 SLIDINGWINDOW:4:15 MINLEN:50 (18). The resulting read sets were then mapped using kallisto v0.46.0 against the GRCh38 assembly of the human genome with sequence bias correction, 100 bootstrap samples (options --bias, -b 100, and --rf-stranded) (19). For gut microbiome data, fecal microbial DNA sequencing reads were quality trimmed using fastp v0.23.1, and subsequently removed human reads and determined microbial population profiles with the MEDUSA pipeline (20,21). MEDUSA used bowtie v2.4.0 to align reads to the reference databases and yielded read count tables (22).

Ecological measures

Richness, evenness, and alpha diversity were calculated using the vegan R package v2.5-7 (23). All principal coordinates analyses (PCoA) were done with the phyloseq R package v1.36.0 and used a Bray-Curtis distance matrix constructed from compositionally transformed read tables (24). The sole exception to this was the gut microbiome data, for which read counts were converted by calculating centered log-ratios. Significance levels were calculated using the adonis function from the vegan R package v2.5-7 and were adjusted for sex (23).

Differential expression and abundance analysis

For both the transcriptomics and microbiome data, differential abundance analyses were performed using the the DESeq2 R package v1.32.0 using Wald significance testing and parametric fitting. DESeq2 p-values were adjusted using independent hypothesis weighting as implemented in the IWH R package v1.20.0 and Ignatiadis et al (21,25). In the case of the transcriptomics data, pathway enrichment analysis used the enrichR R package v3.0 (26). For the metabolomics data, differences in metabolite concentrations between the two participant groups were calculated using the Wilcoxon signed-rank test, of which p-values were adjusted for multiple testing using the Benjamini-Hochberg procedure (27). Metabolites concentrations were analysed per sub-pathway as described in Supplementary Table 1.
Results

Out of 88 included patients, 32 (36.4%) had gallstones or sludge after surgery. Eleven patients underwent a cholecystectomy for symptoms of gallstone disease after a mean of 9.6 ± 4.8 months after bariatric surgery (range 5 to 20 months after surgery). The ultrasound showed gallstones in three of these patients. Unfortunately the ultrasound results of the other eight patients were lost due to bankruptcy of the hospital.

Among the remaining 21 patients, three had sludge and 18 patients had gallstones on ultrasounds performed at one year after surgery in two patients and at two years in 19 patients.

Table 1 shows the baseline characteristics for the total population, and for patients with and without gallstones. The mean age was 46.4 ± 9.8 years and most patients were female (77.3%). The BMI before surgery (SD) was 39.6 (3.8) kg/m². Significant differences at baseline were only found for albumin (mean difference 1.96 g/l; 95%CI 0.07-3.84, p=0.04) and folic acid (mean difference 4.81 nmol/l; 95%CI 1.20-8.43, p=0.01). Although there was a trend upon more protein intake in patients without gallstones (mean difference -0.67 gram, 95%CI -1.32-0.04; p=0.06), dietary intake before surgery did not significantly differ between groups.

Furthermore, 82 patients (93.2%) underwent Roux-en-Y gastric bypass surgery, whereas six patients (6.8%) underwent an omega loop gastric bypass. Histologic assessment of the liver biopsies taken during surgery showed that steatosis (>33%) was present in 11 out of 88 patients and steatohepatitis was present in 12 out of 88 patients. The prevalence of liver pathology did not differ between patients with and without gallstones.

However, data on weight loss at one year after surgery showed that, although not significant (mean difference in percentage total weight loss -2.67; 95%CI -5.52-0.17, p=0.06), there was a trend upon more weight loss in patients with gallstones. Moreover, patients with gallstones had a significantly lower BMI both one and two years after surgery, compared to patients without gallstones (mean difference 1.63 kg/m²; 95%CI 0.03-3.24; p=0.046 and 1.80 kg/m²; 95%CI 0.00-3.60; p=0.049, respectively).

Fecal gut microbiota analyses

Analysis of 88 fecal samples showed no differences in species richness according to the observed and Chao1 indeces (Wilcoxon signed-rank test, p=0.72 and p=0.54, respectively), nor in alpha diversity as determined
with the Shannon Index (Wilcoxon signed-rank test, p=0.87) and the Inverse Simpson Index (Wilcoxon ranked-sum test, p=0.93). In addition, no difference was found in beta-diversity as calculated using Bray-Curtis distances (Permanova, p=0.995). However, differential abundance analyses at the level of bacterial species using DeSeq2 with independent hypothesis weighting revealed 41 bacterial species that were significantly differently abundant between groups (adjusted p ≤ 0.05 and log2 fold change ≤-1 or ≥1) (21,25).

In patients with gallstones, Bacteroides intestinalis, Finegoldia magna, Ruminococcus gnavus and Prevotella buccalis were more abundant than in patients without gallstones. In patients without gallstones, higher abundance of 37 bacterial species was observed, of which the majority were members of the Lactobacillaceae (12 species) and Enterobacteriaceae (7 species), as illustrated in Figure 1.

**Liver, visceral adipose, and subcutaneous adipose tissue RNA seq**

Transcriptomic analysis of liver tissue revealed a significant increased expression of four genes in patients with gallstones compared to patients without gallstones: TEX14, MPPED1, GREB1 and AC005666.1 (Figure 2). These genes are involved in different pathways regulating cell division (Figure S2). Moreover, in subcutaneous adipose tissue differential expression of 13 genes was observed. Of these genes, nine were upregulated in patients with gallstones (ALB, APOA1, TAT, TRPV5, CYP4F2, CTSE, HMGCS2, MOGAT2, and ALDOB) and four in patients without gallstones (DRP2, MT1A, SFRP5, and ANGPTL7). Finally, in visceral adipose tissue, two genes were significantly more often expressed in patients with gallstones. The (most relevant) pathways involved are shown in Figures 2, S3 and S4.

**Metabolites**

Untargeted plasma metabolomics revealed over 700 different metabolites. Direct comparison of patient groups showed higher concentrations of several plasma metabolites particularly among secondary bile-acids in patients with gallstones (Wilcoxon signed-rank test with Benjamini-Hochberg adjustment, p ≤0.05). Compared to patients without gallstones, the bile acids glycochenodeoxycholate 3-sulfate, glycochenodeoxycholate glucuronide, glycocholate, glycodeoxycholate 3-sulfate, glycohyocholate, glycolithocholate sulfate, taurochenodeoxycholic acid 3-sulfate, and taurolithocholate 3-sulfate were increased (Figure 3).
Discussion

This study is the first to relate differences in metabolic activity of subcutaneous and visceral adipose tissue to the presence of gallstones in patients after bariatric surgery. Of the 88 included patients, 56 did not have gallstones at follow-up one (n=2) or two (n=54) years after bariatric surgery. Fecal microbiome analysis in these patients revealed species that might act protective against gallstone development. On the other hand, transcriptomic analysis of adipose tissue showed that altered lipid (cholesterol) metabolism might contribute to gallstone development after bariatric surgery. Moreover, several sulfated bile acids were higher concentrated in patients with gallstones.

Most cases of gallstone presence in this study were detected within the first year after bariatric surgery, as was also reported by Wanjura et al. (28). The prevalence of patients forming gallstones rather decreases in the second year after surgery. In patients with gallstones after bariatric surgery, we observed a higher abundance of Ruminococcus gnavus, a microbe that was recently identified as a biomarker for gallstones (9). Further, fecal metagenomic shotgun sequencing revealed higher abundance of Lactobacillaceae and Enterobacteriaceae in patients without gallstones. Interestingly, Klebsiella pneumoniae (Enterobacteriaceae) and Lactobacillaceae are able to produce microbial ethanol (29,30). Exogenous alcohol consumption in turn is associated with a decreased risk of gallstone formation (31). Thus, the abundance of the mentioned species as a possible protection factor against gallstones might be related to the endogenous ethanol production of these bacteria.

Moreover, anaerobic bacteria such as lactobacilli produce bile salt hydrolase (BSH) (32). BSH deconjugates bile acids in the small intestine and plays a role in bile acid mediated signaling pathways, which regulate lipid absorption, glucose metabolism, and energy homeostasis. Lactobacillaceae are therefore studied as possible cholesterol lowering probiotics (33, 34,). A recent study in mice reported gut microbiome enriched in Desulfovibrionales, also acting via BSH, as an important factor in the development of gallstones (35). In contrast, we found higher abundance of Desulfovibrio sp. in patients without gallstones after bariatric surgery. Future studies should verify these findings in humans both in the general population and in patients after bariatric surgery.

Next, adipose tissue was analysed. Adiposity is a known risk factor for gallstone formation in both men and women, but not all severely obese patients develop gallstones (36, 37). Most studies have focused on the
quantity of adipose tissue, for example on abdominal circumference or on visceral fat as measured on imaging scans (38). In visceral and subcutaneous adipose tissue of patients with gallstones, we identified metabolic pathways involved in inflammatory response and lipid metabolism, including cholesterol and fatty acid metabolism. Furthermore, among other genes involved in steroid synthesis, expression of APOA1, involved in adipocyte cholesterol efflux, was increased in subcutaneous adipose tissue of patients with gallstones.

Interestingly, up to 50% of cholesterol in obese patients is stored as free cholesterol in the adipose tissue, which is the state of cholesterol when excreted via bile (39-41). During the phase of rapid weight loss after bariatric surgery, the total mass of adipose tissue is reduced, possibly resulting in the release of a large amount of free cholesterol to the liver. We speculate that the increased release of cholesterol from adipose tissue induces a transient cholesterol hypersecretion in bile, resulting in supersaturated bile prone to cholesterol crystal formation and gallstone formation. Subsequently, the elevated gene expression of genes involved in tissue regulation in patients without gallstones, might indicate a more adaptive tissue state as a protective mechanism against gallstone formation during weight loss after bariatric surgery. Furthermore, the absence of liver genes traditionally associated with gallstone disease strengthens the hypothesis that gallstone formation after bariatric surgery follows a different trajectory compared to gallstone formation in the non-bariatric population (14, 16, 42, 43).

Third, increased plasma levels of conjugated bile acids were observed in patients with gallstones, which is in line with previous studies and might be a consequence of lower excretion of bile acids into the gallbladder (44, 45). Interestingly, most of the bile acids we observed were sulfated. The sulfation process of bile acids takes place in the liver and makes bile acids more water soluble (32, 46). At last, it should be mentioned that patients with gallstones had elevated plasma folic acid levels, which has previously been associated with cholesterol and lipid metabolism in mice(47-49).

Several limitations to this study should be addressed. First, the group of gallstone patients was heterogeneous since no ultrasound was performed before bariatric surgery and some of these patients may have already had asymptomatic gallstones before surgery (50). This number is probably limited given the fact that none of the included patients had symptoms of gallstone disease and/or cholecystectomy prior to bariatric surgery.

Besides, a previous study reported that asymptomatic gallstones do not seem to be associated with changes in
microbiome composition (12). Nevertheless, literature on this topic is inconclusive and future research to clarify the role of microbiome in gallstone disease is needed. Since multiple different mechanisms might be involved in gallstone formation, we would recommend for future studies to separately analyze subgroups of patients with pre-existing gallstones prior to bariatric surgery, patients who did not develop gallstones after surgery, and patients who did develop gallstones after bariatric surgery.

Second, the differences in microbiome, bile acids and adipose tissue were all assessed before the bariatric surgery procedure, but were not assessed postoperatively. Therefore the results cannot be used to make statements on the effect of bariatric surgery on these metabolic parameters. However, this study does provide insight in possible protective mechanisms for gallstone formation after bariatric surgery by investigating associations between absence of gallstones in the first two years after bariatric surgery and baseline data.

Future research should continue to explore the potential role of microbial species as protecting factors against gallstone formation and the involvement of adipose tissue in gallstone development. Assessment of changes in fecal microbiome, plasma metabolome, and tissue transcriptome induced by bariatric surgery is needed to identify the pathological pathways leading to gallstone formation. Eventually, this information can help to predict which patients are likely to develop or stay free of gallstone disease after bariatric surgery.

At last, our sample size was relatively small, whereas a larger sample size can increase power and generalization of findings.

In conclusion, the present study observed higher abundance of Lactobacillaceae and Enterobacteriaceae in patients without gallstones as a possible protective factor for gallstone presence in the first two years after bariatric surgery. Furthermore, in patients with gallstones pathways involved in cholesterol and inflammation metabolism in subcutaneous and visceral adipose tissue were identified, suggesting a potential role of adipose tissue, lipid, and cholesterol metabolism in gallstone development after bariatric surgery. Yet, it should be mentioned that the exact pathogenesis of gallstone formation remains to be clarified, both in the general population and bariatric surgery population. The results of this study provide guidance and focus for future prospective research, which are needed to further explore and verify these findings.
Conflicts of interest: For all authors, none are declared.

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Data availability statement: Raw data were generated at Amsterdam University Medical center. Derived data supporting the findings of this study are available from the corresponding author [MG] on request with the permission of the principal investigators of the BARIA study.
References


Table 1. Clinical characteristics of 88 included patients.

<table>
<thead>
<tr>
<th></th>
<th>Total population n=88</th>
<th>With gallstones n=32</th>
<th>Without gallstones n=56</th>
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<tr>
<td>Age - years</td>
<td>46.4 ± 9.8</td>
<td>44.4 ± 9.9</td>
<td>47.5 ± 9.6</td>
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<tr>
<td>Female gender - n</td>
<td>68 (77.3)</td>
<td>25 (78.1)</td>
<td>43 (76.8)</td>
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<tr>
<td>Weight before surgery - kgs</td>
<td>125.6 ± 18.4</td>
<td>123.6 ± 17.1</td>
<td>126.6 ± 19.1</td>
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<td>Body Mass Index before surgery - kg/m²</td>
<td>39.6 ± 3.8</td>
<td>39.3 ± 3.8</td>
<td>39.9 ± 3.8</td>
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<tr>
<td>Percentage TWL one year after surgery⁴</td>
<td>34.6 ± 6.5</td>
<td>36.3 ± 6.3</td>
<td>33.6 ± 6.4</td>
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<td>Body Mass Index one year after surgery - kg/m²</td>
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<td>26.6 ± 3.4</td>
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<td>Body Mass Index two years after surgery - kg/m²</td>
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<td>28.6 ± 4.0</td>
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<tr>
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<td>11 (34.4)</td>
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<td>10 (31.3)</td>
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<td>273.6 ± 87.1</td>
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<tr>
<td>Creatin - μmol/l</td>
<td>6.4 ± 5.7</td>
<td>5.4 ± 4.6</td>
<td>6.9 ± 6.2</td>
</tr>
<tr>
<td>Sodium - mmol/l</td>
<td>70.7 ± 16.6</td>
<td>71.4 ± 22.8</td>
<td>70.4 ± 12.0</td>
</tr>
<tr>
<td>Potassium - mmol/l</td>
<td>140.5 ± 1.8</td>
<td>140.1 ± 19</td>
<td>140.5 ± 1.8</td>
</tr>
<tr>
<td>Magnesium - mmol/l</td>
<td>4.1 ± 0.3</td>
<td>4.1 ± 0.3</td>
<td>4.1 ± 0.2</td>
</tr>
<tr>
<td>Calcium corrected - mmol/l</td>
<td>0.8 ± 0.1</td>
<td>0.8 ± 0.1</td>
<td>0.8 ± 0.1</td>
</tr>
<tr>
<td>Total protein - mmol/l</td>
<td>23.1 ± 0.1</td>
<td>23.0 ± 0.1</td>
<td>23.0 ± 0.1</td>
</tr>
<tr>
<td>Albumin*- μg/l</td>
<td>443.3 ± 44.4</td>
<td>431.2 ± 24.0</td>
<td>450.0 ± 5.0</td>
</tr>
<tr>
<td>ALP - U/l</td>
<td>81.2 ± 19.2</td>
<td>79.7 ± 16.6</td>
<td>82.0 ± 20.6</td>
</tr>
<tr>
<td>AST - U/l</td>
<td>34.8 ± 32.8</td>
<td>32.1 ± 18.1</td>
<td>36.3 ± 38.7</td>
</tr>
<tr>
<td>ALT - U/l</td>
<td>26.7 ± 10.7</td>
<td>24.1 ± 6.1</td>
<td>28.2 ± 12.3</td>
</tr>
<tr>
<td>Total bilirubin - μmol/l</td>
<td>34.9 ± 21.2</td>
<td>31.6 ± 15.4</td>
<td>36.7 ± 23.8</td>
</tr>
<tr>
<td>Total cholesterol - mg/dl</td>
<td>8.3 ± 3.5</td>
<td>8.6 ± 3.6</td>
<td>8.2 ± 3.5</td>
</tr>
<tr>
<td>LDL - mmol/l</td>
<td>4.9 ± 1.1</td>
<td>4.9 ± 1.2</td>
<td>4.9 ± 1.1</td>
</tr>
<tr>
<td>HDL - mmol/l</td>
<td>3.1 ± 1.0</td>
<td>3.1 ± 1.1</td>
<td>3.1 ± 1.0</td>
</tr>
<tr>
<td>Triglycerides - mmol/l</td>
<td>1.2 ± 0.4</td>
<td>1.2 ± 0.4</td>
<td>1.2 ± 0.3</td>
</tr>
<tr>
<td>Ferritin - µg/l</td>
<td>16.4 ± 8.3</td>
<td>13.2 ± 4.4</td>
<td>18.1 ± 9.4</td>
</tr>
<tr>
<td>Vitamin B12 - pmol/l</td>
<td>319.1 ± 177.9</td>
<td>340.5 ± 257.2</td>
<td>306.9 ± 111.1</td>
</tr>
<tr>
<td>Vitamin D - nmol/l</td>
<td>53.9 ± 26.5</td>
<td>54.9 ± 29.6</td>
<td>53.3 ± 24.8</td>
</tr>
</tbody>
</table>

Data are shown as mean ± standard deviation or number (percentages).

N: number; TWL: total weight loss; mmol: millimole; l: liter; mg: milligrams; μmol: micromole; g: grams; dl: deciliter; µg: microgram; nmol: nanomole; ALP: alkaline phosphatase; GGT: γ-glutamyl transferase; AST: aspartate aminotransferase; ALT: alanine aminotransferase; LDL: low-density lipoprotein; HDL: high-density lipoprotein.

# Except TWL and BMI one and two years after surgery, all other variables are measured before bariatric surgery.

* Mean difference 1.96 g/L, 95% CI 0.07-3.84, p=0.04.

** Mean difference 4.81 nmol/l, 95% CI 1.20-8.43, p=0.01.
Intestinal microbiota composition in patients with and without gallstones after bariatric surgery

A. Significantly differentially expressed bacteria

The first four species are more abundant in patients with gallstones (grey). The following 37 species were more abundant in patients without gallstones (red).

B. Taxonomy of negatively associated bacteria

For example, of the 37 species that were more abundant in patients without gallstones, 12 were members of the *Lactobacillaceae*, and 7 belonged to the *Enterobacteriaceae*.

Figure 1. Intestinal microbiota composition in patients with and without gallstones after bariatric surgery. A. Significantly differentially expressed bacteria. The first four species are more abundant in patients with gallstones (grey). The following 37 species were more abundant in patients without gallstones (red). B. Taxonomy of negatively associated bacteria. For example, of the 37 species that were more abundant in patients without gallstones, 12 were members of the *Lactobacillaceae*, and 7 belonged to the *Enterobacteriaceae*. 
Figure 2. Differentially expressed genes in the liver, subcutaneous and visceral adipose tissue of individuals with and without gallstones.
Figure 3. Plasma metabolites with different concentrations between patients with and without gallstones. Bile acids are increased in patients with gallstones after p-value adjustment per sub-pathway.
Credit Author Statement:

M.S.S. Guman: Investigation, Project administration, formal analysis, writing – original draft, Visualization

J.B. Hoozemans: Project administration, writing – original draft, Visualization

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P.A. de Jonge: Methodology, formal analysis, Visualization

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