Gastro Hep Advances 2022;1:299–312

ORIGINAL RESEARCH—CLINICAL

Enterohepatic, Gluco-metabolic, and Gut Microbial Characterization of Individuals With Bile Acid Malabsorption

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BACKGROUND AND AIMS: Bile acid malabsorption (BAM) is a debilitating disease characterized by loose stools and high stool frequency. The pathophysiology of BAM is not well-understood. We investigated postprandial enterohepatic and gluco-metabolic physiology, as well as gut microbiome composition and fecal bile acid content in patients with BAM. **METHODS:** Twelve participants with selenium-75 homocholic acid taurine test–verified BAM and 12 healthy controls, individually matched on sex, age, and body mass index, were included. Each participant underwent 2 mixed meal tests (with and without administration of the bile acid sequestrant colesevelam) with blood sampling and evaluation of gallbladder motility; bile acid content and microbiota composition were evaluated in fecal specimens. **RESULTS:** Patients with BAM were characterized by increased bile acid synthesis as assessed by circulating 7-alpha-hydroxy-4-cholesten-3-one, fecal bile acid content, and postprandial concentrations of glucose, insulin, C-peptide, and glucagon. The McAuley index of insulin sensitivity was lower in patients with BAM than that in healthy controls. In patients with BAM, colesevelam co-administered with the meal reduced postprandial concentrations of bile acids and fibroblast growth factor 19 and increased 7-alpha-hydroxy-4-cholesten-3-one concentrations but did not affect postprandial glucagon-like peptide 1 responses or other gluco-metabolic parameters. Patients with BAM were characterized by a gut microbiome with low relative abundance of bifidobacteria and high relative abundance of *Blautia*, *Streptococcus*, *Ruminococcus gnavus*, and *Akkermansia muciniphila*. **CONCLUSION:** Patients with BAM are characterized by an overproduction of bile acids, greater fecal bile acid content, and a gluco-metabolic profile indicative of a dysmetabolic prediabetic-like state, with changes in their gut microbiome composition potentially linking their enterohepatic pathophysiology and their dysmetabolic phenotype. ClinicalTrials.gov number NCT03009916.

Keywords: Glucose Metabolism; Gut Microbiota; Pathophysiology; Prediabetic

Bile acid malabsorption (BAM) is an underdiagnosed disease. In a systematic review, BAM was found in one-third of patients suffering from diarrhea-predominant irritable bowel syndrome known to affect around 5% of the general population.[1,2] The main symptoms of BAM are high frequency of bowel movements, abdominal pain, loose stools, fecal urgency, and fecal incontinence,[3] making it a socially debilitating disease with severe consequences for the individual patient as well as societies. Current treatments to decrease stool frequency and to firm the stools—including conventional antidiarrheals such as loperamide and codeine

Abbreviations used in this paper: **AUC, area under the curve; BAM, bile acid malabsorption; BMI, body mass index; bsAUC, baseline-subtracted AUC; C4, 7 alpha-hydroxy-4-cholesten-3-one; CCK, cholecystokinin; C_{max}, maximum concentration; CTRL, healthy controls; DPP-4, dipeptidyl peptidase 4; FGF19, fibroblast growth factor 19; FXR, farnesoid X receptor; GBEF, gallbladder ejection fraction; GIP, glucose-dependent insulinotropic polypeptide; GLP-1, glucagon-like peptide 1; SeHCAT, selenium-75 homocholic acid taurine test; T_{max}, time of maximum concentration.**

Most current article

2772-5723
https://doi.org/10.1016/j.gastha.2021.12.007

and bile acid sequestrants—are often ineffective and associated with side effects,[3–6] and a better understanding of BAM pathophysiology and detailed phenotyping of patients with BAM are needed to develop better treatment modalities.

Under normal conditions, 95% of postprandially released bile acids are reabsorbed in the small intestine (primarily in the terminal ileum) and return to the liver via the portal vein in the so-called enterohepatic circulation of bile acids.[7,8] In BAM, the spillover of bile acids is larger than 5%, and the excess bile acids in the colon irritate the colonic mucosa, thereby causing osmotic-induced fluid accumulation causing the abovementioned symptoms.[3,8,9] Whether the increased spillover of bile acids to the colon is caused by a reduced capacity to reabsorb bile acids in the small intestine or a reduced negative feedback inhibition of bile acid production is being debated.[10] Negative feedback inhibition by bile acids may involve the nuclear farnesoid X receptor (FXR) in the small intestine, which induces secretion of fibroblast growth factor 19 (FGF19) known to inhibit hepatic bile acid synthesis.[7] Importantly, bile acids are known to interfere with gluco-metabolic pathways and have been proposed to be involved in the regulation of gluconeogenesis, glycogen synthesis, insulin sensitivity, and secretion of insulin.[11,12] In addition, bile acids stimulate the secretion of the glucose-lowering and satiety-promoting gut hormone glucagon-like peptide 1 (GLP-1) through activation of Takeda G protein–coupled receptor 5 (TGR5) in the basolateral membrane of GLP-1–producing enteroendocrine L cells.[13–15] Despite the many well-described links between bile acid biology and glucose metabolism, a gluco-metabolic characterization of patients with BAM has not been carried out.

Here, we provide an in-depth enterohepatic and gluco-metabolic phenotyping of well-defined patients with BAM as compared with carefully sex-, age-, and body mass index (BMI)–matched healthy controls (CTRLs). We performed mixed meal tests (with and without co-administration of the bile acid sequestrant colesevelam) and evaluated postprandial gallbladder emptying, circulating bile acids and FGF19, bile acid synthesis, indices of insulin sensitivity, pancreatic endocrine function, postprandial gut and pancreatic hormonal responses as well as gut microbiome composition and fecal bile acid content.

Methods

Ethics

The study was approved by the Scientific-Ethics Committee of the Capital Region of Denmark (registration no. H-15004394) and registered at clinicaltrials.gov (NCT03009916). All participants gave verbal and written consent after receiving written and verbal information about the study. All authors had access to the study data and reviewed and approved the final article.

Study Participants

Participants with selenium-75 homocholic acid taurine (SeHCAT) test–verified moderate-to-severe primary/idiopathic BAM were recruited via local gastroenterology departments and practicing gastroenterologists as well as from the Department of Clinical Physiology and Nuclear Medicine, Copenhagen University Hospital – Herlev and Gentofte, Hellerup, Denmark (performing SeHCAT tests). All individuals were diagnosed within a year from inclusion. Before the study, all participants with BAM had tried a variety of treatments, for example, cholestyramine, colesevelam, opioids, and loperamide. CTRLs individually matched on sex, age, and BMI were recruited among previous healthy study participants consenting to be contacted for future studies. In addition to ± SeHCAT test–verified diagnosis of moderate-to-severe primary/idiopathic BAM, the inclusion criteria for both groups were as follows: age 18–70 years; Northern European descent; BMI >23 kg/m^2 and <35 kg/m^2; fasting plasma glucose <6.5 mmol/L; glycated hemoglobin A1c <48 mmol/mol; normal hemoglobin; and informed and written consent. The key exclusion criteria for both groups were liver and kidney disease, gastrointestinal disease (other than BAM for the patients with BAM), previous intestinal resection, cholecystectomy or any major intra-abdominal surgery, first-degree relatives with diabetes, and recent or active malignant diseases. Women of childbearing potential had to use medical contraception for inclusion.

Study Design

We adopted a prospective, observational, case-control study design to compare postprandial enterohepatic and gluco-metabolic physiology, gut microbiome composition, and fecal bile acid content between patients with BAM and carefully matched CTRLs. Furthermore, we used a randomized, placebo-controlled, double-blind, crossover design to determine how the bile acid sequestrant colesevelam affects postprandial enterohepatic and gluco-metabolic physiology in the 2 groups. On separate days, in a randomized order, (randomizer.org was used for randomization) all participants underwent 2 mixed meal tests with double-blind single-dose administration of colesevelam and placebo.

Experimental Procedures

The study was performed at Center for Clinical Metabolic Research, Copenhagen University Hospital – Herlev and Gentofte, Hellerup, Denmark. After inclusion, participants with BAM were instructed not to take any medication for their BAM symptoms for a period of 72 hours before each of the 2 experimental days. Each participant received equipment and instructions for collecting stool samples, which they collected at home the day before or in the morning before the experimental days and immediately stored in their private freezer. The stool sample was transported to the clinical research facility in a cooler bag with ice, and on arrival, it was immediately stored at -80 °C. On experimental days, the participants arrived at the clinical research facility after a 10-hour overnight fast. The participants were comfortably positioned in a semirecumbent

position in a hospital bed and were instructed to relax. A catheter was inserted into an antecubital vein, and the arm was heated by a heating pillow for arterialization of the venous blood. At time 0 minute, a 200-mL liquid mixed meal (Nutricia, Danone, Allerød, Denmark) containing 1260 kJ (36.8-g carbohydrate, 11.6-g protein, and 12.0-g lipid) mixed with 1.5-g acetaminophen dissolved in 50-mL water (for evaluation of gastric emptying) was orally administered together with colesevelam (1875 mg) or indistinguishable placebo tablets (containing 330 mg of lactose monohydrate, 335 mg of potato starch, 12 mg of gelatine, 3.5 mg of magnesium stearate, and 31.5 mg of talc). During the administration, only an unblinded staff member was in the room. Apart from mixing and administering the liquid mixed meal, the unblinded staff member was not involved in the study. Blood samples were drawn at time −30, −15, 0, 10, 20, 30, 45, 60, 75, 90, 120, 150, 180, 210, and 240 minutes. Ultrasonic imaging (GE Healthcare, Waukesha, WI) of the gallbladder was conducted at times −30, 30, 60, 120, and 240 minutes. For the analysis of glucose, tubes coated with sodium fluoride were used and centrifuged at bedside at 7400 *g* and room temperature for 30 seconds. For the analysis of gastrin, cholecystokinin (CCK), glucagon, glucose-dependent insulinotropic polypeptide (GIP), GLP-1, and FGF19, blood was collected in ice-cooled tubes with ethylenediaminetetraacetic acid and a dipeptidyl peptidase 4 inhibitor. For the analysis of acetaminophen and lipid profiles, blood was collected in tubes with lithium heparin. For the analysis of insulin, C-peptide, bile acids, and 7-alpha-hydroxy-4-cholesten-3-one (C4), blood was collected in dry tubes and left to coagulate for 15 minutes at room temperature before centrifugation. EDTA, lithium heparin, and dry tubes were centrifuged at 2900 *g* and 4 °C for 15 minutes, and plasma/serum was stored at −20 °C or −80 °C until further analysis.

Analyses

Plasma concentrations of glucose were measured using the YSI 2300 STAT glucose analyzer (Xylem Inc, Yellow Springs, OH). Serum concentrations of insulin and C-peptide were measured with a 2-sited sandwich immunoassay using direct chemiluminescent technology (Siemens Healthcare A/S, Ballerup, Denmark) for the ADVIA Centaur XP. Plasma concentrations of CCK and gastrin were measured by specific radioimmunoassays as previously described.[16,17] Plasma concentrations of total GLP-1, total GIP, and glucagon were measured with specific radioimmunoassays as previously described.[18–20] Plasma concentrations of FGF19 were measured using the Quantikine enzyme-linked immunosorbent assay human FGF19 assay, as previously described.[21] Plasma concentrations of acetaminophen were measured by photometry at 670 nm (VITROS, Ortho-clinical Diagnostics, Rochester, NY). Serum concentrations of bile acids were measured by liquid chromatography tandem mass spectrometry as previously described.[22] Total bile acid concentration was calculated as the sum of all bile acids (cholic acid [CA], chenodeoxycholic acid [CDCA], hyocholic acid, deoxycholic acid, lithocholic acid [LCA], ursodeoxycholic acid, and hyodeoxycholic acid) in their unconjugated forms and glycine- or taurine-conjugated forms. Serum C4 was measured by liquid chromatography tandem mass spectrometry as previously described.[23] Plasma concentrations of total cholesterol were measured by reflection photometry (VITROS, Ortho-Clinical Diagnostics). Analysis of bile acids in fecal samples was measured by quantitative liquid chromatography mass spectrometry as previously described.[24] DNA from fecal samples was extracted using the Bead-Beat Micro AX Gravity Kit (A&A Biotechnology, Gdynia, Poland) as per the manufacturer's instructions. Purity and DNA concentration were determined by using the NanoDrop 1000 Spectrophotometer (Thermo Fisher Scientific, MA) and Varioskan Flash (Thermo Fisher Scientific, MA), respectively. A 16S rRNA gene amplicon library was constructed by amplifying the 16S rRNA gene with a unique molecular identifier containing multiple forward and reverse primers (Table A5). Polymerase chain reaction (PCR) conditions for the amplification were as follows: 95 °C for 5 minutes, 2 cycles of 95 °C for 20 seconds, 48 °C for 30 seconds, 65 °C for 10 seconds, 72 °C for 45 seconds, and a final extension at 72 °C for 4 minutes. A second PCR step was then performed to barcode PCR amplicons with the following conditions: 95 °C for 2 minutes followed by 33 cycles of 95 °C for 20 seconds, 55 °C for 20 seconds, 72 °C for 40 seconds, and a final extension at 72 °C for 4 minutes. After each PCR reaction, PCR amplicons were cleaned up using SpeedBeads magnetic carboxylate (obtained from Sigma Aldrich, MO). The size of barcoded PCR products (approximately 1500 bp) was checked by 1.5% agarose gel electrophoresis. A sequencing library from pooled barcoded PCR products was prepared by following the ligation sequencing protocol SQK-LSK110 (Oxford Nanopore Technologies, Oxford, UK) and loaded on the R9.1.4 flow cell for 72 hours using GridIONX5 (Oxford Nanopore Technologies, Oxford, UK).

Data Analysis Workflow for 16S rRNA Gene Sequencing

Nanopore sequencing software GridION (Oxford, UK), version 21.02.5, (https://nanoporetech.com) was used for data collection. Base calling and demultiplexing of sequencing data were performed by ONT's Guppy, version 4.5.2 (https://nanoporetech.com). Nanofilt, version 2.7.1,[25] was then used for filtering and trimming of demultiplexed sequences (minimum = 1300 bp, maximum = 1600 bp, q score ≥ 10). Taxonomy assignment was conducted using parallel_assign_taxonomy_uclust.py script of Quantitative Insights into Microbial Ecology (Qiime) 1, version 1.8.0.[26] Greengenes database, version 13.8,[27] was used as a reference database.

Calculations and Statistical Analysis

Baseline values of parameters measured in blood are defined as the mean of the 3 values obtained in the fasted state before meal consumption (time points −30, −15, and 0 minutes) except for bile acids, C4, FGF19, and gallbladder volume (GV), for which only one baseline value was available. Postprandial responses were evaluated by the maximum plasma/serum concentration (C_{max}), time to C_{max} (T_{max}), and area under the curve (AUC) calculated using the trapezoidal rule. If baseline values differed between the 2 groups on placebo or colesevelam days, baseline-subtracted AUC was also evaluated. GV during meal tests was calculated by the ellipsoid method based on ultrasound-assessed longitudinal and cross-sectional diameters.[28] To evaluate postprandial gallbladder motility, the area under the gallbladder ejection fraction (GBEF)–time curve was evaluated. The GBEF at

specific time points was calculated using the formula $100 \times (GV_{baseline} - GV_{timepoint})/GV_{baseline}$. Homeostatic model assessment for insulin resistance (HOMA-IR) was calculated using the HOMA2 Calculator (Version 2.2.3 [LIB 2.2.3] Diabetes Trial Unit, University of Oxford, Oxford, UK). For evaluation of peripheral insulin sensitivity, the Matsuda index was calculated using the formula $10{,}000/\sqrt{([glucose_{baseline} \times insulin_{baseline}]/[glucose_{mean} \times insulin_{mean}])}$. For evaluation of beta cell function, the insulinogenic index (IGI) was calculated $(insulin_{30\ min} - insulin_{baseline})/(glucose_{30\ min} - glucose_{baseline})$ in addition to the disposition index (DI) (Matsuda index × IGI). The McAuley index, which has strong accuracy for detecting insulin resistance and for discriminating metabolic syndrome from nonmetabolic syndrome subjects,[29,30] was calculated using the following formula: $McAuley = \exp(2.63 - 2.28 \ln[insulin_{fasting}] - 0.31 \ln[triglycerides_{fasting}])$. Results are reported as means with 95% confidence intervals (CIs) unless otherwise stated. Paired 2-sided Student's *t* tests were used to analyze differences between the 2 groups regarding demography, HOMA-IR, Matsuda index, IGI, DI, and McAuley index. Data were analyzed by repeated measures 2-way analysis of variance (ANOVA) in a linear mixed-effects model using group and treatment as fixed effects and subjects as a random effect. The interaction term was used to estimate the treatment effect (placebo vs colesevelam) between groups. The assumptions of a Gaussian distribution of residuals and homogeneity of variances were assessed visually by drawing histograms, residual plots, and probability plots. If assumptions could not be met, continuous variables were transformed using appropriate transformations. We chose to calculate type III sums of squares for the fixed effects. Between-group differences on the placebo days were tested using the ANOVA post hoc test (applying the mean-square residual) and Bonferroni adjustment for multiple comparisons, for each analysis. A 2-sided *P* value of .05 was used to indicate significant differences. In case of different baseline values between groups on only one type of the study day (colesevelam/placebo), a comparison of pooled baseline data was made using a paired 2-sided Student's *t* test. Statistical analyses were performed using GraphPad Prism (8.4.3) for Windows/Mac (GraphPad Software, San Diego, CA) and R (3.6.2) for Windows/Mac (R-Studio, Boston, MA). We chose to power the study based on the postprandial GLP-1 AUC as we hypothesized that BAM-associated changes in the enterohepatic circulation of bile acids and colesevelam's interaction with these might be reflected in changes in the activation of TGR5 in enteroendocrine L cells and, thus, changes in postprandial responses of this glucose-lowering and satiety-promoting gut-derived incretin hormone. Based on a power of 80%, a significance level of 5%, and a standard deviation of postprandial GLP-1 plasma responses (AUC) from previous experiments,[31] we included 12 participants in each group to detect a relevant difference of ∼1 standard deviation between groups and between meal tests with colesevelam and placebo, respectively. For the gut microbiome analysis, QIIME 2,[32] version 2020.6.0, combined with R packages (phyloseq, ggplot2, and vegan) was used. All samples were rarified to a uniform sequencing depth of 1300 near full-length 16S rRNA gene amplicon reads per sample using QIIME2. Alpha diversity was calculated as observed species (summarized to the L7 level) followed by a Wilcoxon rank-sum test. Principal coordinate analysis plots were generated using binary Jaccard distance and weighted Bray-Curtis dissimilarity metrics, and permutational multivariate analysis of variance was performed to determine differences between groups, and *P* values were adjusted by Benjamin-Hochberg correction. The differential abundance of microbial taxa between the patients with BAM and CTRLs was tested with the linear discriminant analysis effect size.[33]

Table 1. Clinical Characteristics of Individuals With BAM and CTRLs

Parameter	BAM (n = 12)	CTRL (n = 12)	*P*
Sex (male/female)	6/6	6/6	
Age (y)	39.1 (13.2)	39.3 (15.6)	.882
BMI (kg/m^2)	28.4 (4.2)	29.1 (3.9)	.157
Height (cm)	179.0 (12.8)	177.3 (12.5)	.309
Weight (kg)	91.3 (17.7)	92.0 (18.0)	.690
HbA1c (mmol/mol)	31.4 (3.7)	31.0 (2.8)	.765
Total cholesterol (mmol/L)	4.8 (1.0)	4.9 (1.0)	.686
Bilirubin (μmol/L)	14.8 (9.2)	12.8 (7.5)	.267
TSH (IU/L)	2.0 (1.0)	1.9 (0.7)	.870
ALT (U/L)	43.8 (27.7)	33.3 (12.8)	.261
Albumin (g/L)	44.2 (2.6)	43.8 (3.1)	.601

Data are presented as means with standard deviations in parentheses. *P* values are from paired 2-sided Student's *t* tests.
ALT, alanine aminotransferase; HbA1c, hemoglobin A1c; TSH, thyroid-stimulating hormone.

Results

Characteristics of Participants

Twelve individuals with BAM (3 individuals with moderate BAM and 9 individuals with severe BAM) and 12 CTRLs matched on BMI, age, and sex with no differences between the groups on weight, thyroid-stimulating hormone, total cholesterol, or hemoglobin A1c were included (Table 1). No participants dropped out or were excluded after inclusion, and none of the participants reported any side effects or harm during the study.

Gallbladder Motility, Circulating Bile Acids, FGF19, C4, and Fecal Bile Acid Content

GV in the fasted state and the AUC for GV during the meal test tended to be greater in patients with BAM vs CTRLs (9.5 cm^3 [95% CI −0.14; 19] $P = .053$ and 1772 min × cm^3 [95% CI −33.6; 3578] $P = .055$, respectively). No difference in the GBEF (assessed as the AUC and maximum GBEF) was observed between the groups (Table 2). A single dose of colesevelam decreased the GV AUC in both groups and increased the GBEF AUC in the BAM group (Table A4).

Patients with BAM and CTRLs exhibited similar baseline as well as postprandial concentrations of circulating total bile acids, and no differences in postprandial responses were seen (Table 2 and Figure 1). Single-dose colesevelam co-administered with the meal reduced the postprandial AUC for total bile acids in the BAM group, but not in the CTRLs (Table A2). Compared with the CTRL group, patients

Table 2. Gallbladder Volume and Motility, Plasma Concentrations of Total Bile Acids, FGF19, and C4 During Liquid Mixed Meal Test As Well As Fecal Content of Bile Acids in Individuals With BAM and CTRLs

Parameter	BAM (n = 12)	CTRL (n = 12)	Δ	*P*
Gallbladder				
Baseline (cm^3)	43 (26; 59)	33 (23; 43)	9.5 (−0.14; 19)	.053
AUC (min × cm^3)	7622 (4453; 10,790)	5849 (3967; 7732)	1772 (−33.6; 3578)	.055
EF AUC (min × %)	4943 (1429; 8456)	3843 (−1622; 9307)	1100 (−4371; 6571)	.999
EF_{max} (%)	62.3 (52.9; 71.9)	54.3 (33.6; 75)	8 (−10.7; 26.6)	.581
Total bile acids				
Baseline (μmol/L)	2.6 (1.4; 3.9)	2.1 (0.98; 3.2)	0.51 (−0.88; 1.9)	.717
C_{max} (μmol/L)	7.3 (4.8; 9.7)	6.6 (3.9; 9.3)	0.67 (−1.9; 3.2)	.999
T_{max} (min)	98 (59; 136)	94 (40; 148)	3.3 (−56; 63)	.999
AUC (min × μmol/L)	1076 (753; 1399)	886 (604; 1169)	190 (−130; 509)	.304
FGF19				
Baseline (pmol/L)	120 (68; 173)	138 (72; 204)	−18 (−65; 29)	.687
C_{max} (pmol/L)	328 (194; 463)	326 (212; 441)	1.7 (−115; 118)	.999
T_{max} (min)	193 (161; 225)	190 (158; 222)	2.5 (−49; 54)	.999
AUC (min × nmol/L)	47 (30; 64)	45 (30; 60)	2.3 (−9.3; 13.9)	.999
C4				
Baseline (nmol/L)	108 (46; 169)	41 (20; 64)	68 (31; 101)	**.001**
C_{max} (nmol/L)	135 (61; 208)	72 (37; 107)	63 (28; 97)	**.001**
T_{max} (min)	105 (29; 181)	210 (163; 257)	−105 (−184; −26)	**.011**
AUC (min × μmol/L)	24 (11; 37)	13 (6; 20)	11 (4; 18)	**.003**
bsAUC (min × μmol/L)	−1.7 (−6.3; 3)	3 (0.3; 5.7)	−4.7 (−11.7; 2.4)	.224
Fecal total bile acids (nmol/g)	1799 (646; 2952)	519 (782; 857)	−1280 (−2385; −175)	**.027**

Data are presented as means with 95% CIs. *P* values are from ANOVA post hoc test and Bonferroni adjustment for multiple comparisons. Delta values refer to the difference between the BAM group and CTRLs.
P values < .05 are indicated with bold font.
bsAUC, baseline-subtracted area under the curve.

with BAM exhibited greater baseline concentrations of taurine-conjugated CA on placebo days but not on colesevelam days. In the pooled baseline analysis, the difference between patients with BAM and CTRLs persisted ($P = .003$) (Table A2). For LCA, the baseline concentration was significantly greater in the CTRL group vs the BAM group on colesevelam days, but this difference did not persist in the pooled baseline analysis ($P = .172$) (Table A2). Administration of colesevelam decreased the AUC of taurine-conjugated CA and LCA and glycine-conjugated CDCA statistically significantly in the BAM group only (Table A2). Baseline concentrations of FGF19 were similar in the 2 groups, and the 2 groups exhibited similar postprandial responses of FGF19 (Table 2 and Figure 1). Single-dose colesevelam co-administered with the meal significantly reduced T_{max} (−80 min [95% CI −132; −28], $P = .004$) for FGF19 in patients with BAM. In the CTRLs, colesevelam significantly reduced C_{max}, (−130 pmol/L [95% CI −246; −13], $P = .030$), T_{max} (−84 min [95% CI −136; −32], $P = .003$), and AUC for FGF19 compared with placebo (Table A4). Compared with CTRLs, patients with BAM were characterized by significantly greater baseline plasma concentrations of C4; however, on colesevelam days, baseline C4 concentrations were not statistically significantly different, neither in the pooled analysis ($P = .058$). The differences on baseline concentrations of C4 resulted in a statistically significant greater AUC for C4, whereas the postprandial response as assessed by the baseline-subtracted AUC was similar to that of CTRLs (Table 2 and Figure 1). Compared with placebo, administration of colesevelam shortened T_{max} (−98 min [95% CI −177; −18], $P = .017$) of C4 in CTRLs (data not shown) and decreased the AUC for C4 in patients with BAM (Table A4).

The BAM group exhibited higher concentrations of total bile acids in their feces than the CTRL group (Table 2).

Gastric Emptying, Gastrin, CCK, GIP, and GLP-1

There was no difference between the groups in terms of the gastric emptying rate as assessed by the plasma concentrations of acetaminophen (Table A1 and Figure 2). Administration of a single dose of colesevelam decreased the AUC of acetaminophen in the CTRL group (Table A3). No differences in baseline or postprandial gastrin plasma concentrations were observed between the groups (Tables A1 and A3). There were no differences in plasma concentrations of CCK between the groups, but administration of a single dose of colesevelam increased the AUC in both the CTRL group and in the BAM group (Table A3). No differences in plasma concentrations of GIP or GLP-1 were seen between the groups, neither following colesevelam or placebo (Tables A1 and A3).

Glucose, Insulin, C-peptide, Glucagon, and Gluco-metabolic Indices

Baseline concentrations of C-peptide were statistically significantly higher in the BAM group than those in CTRLs,

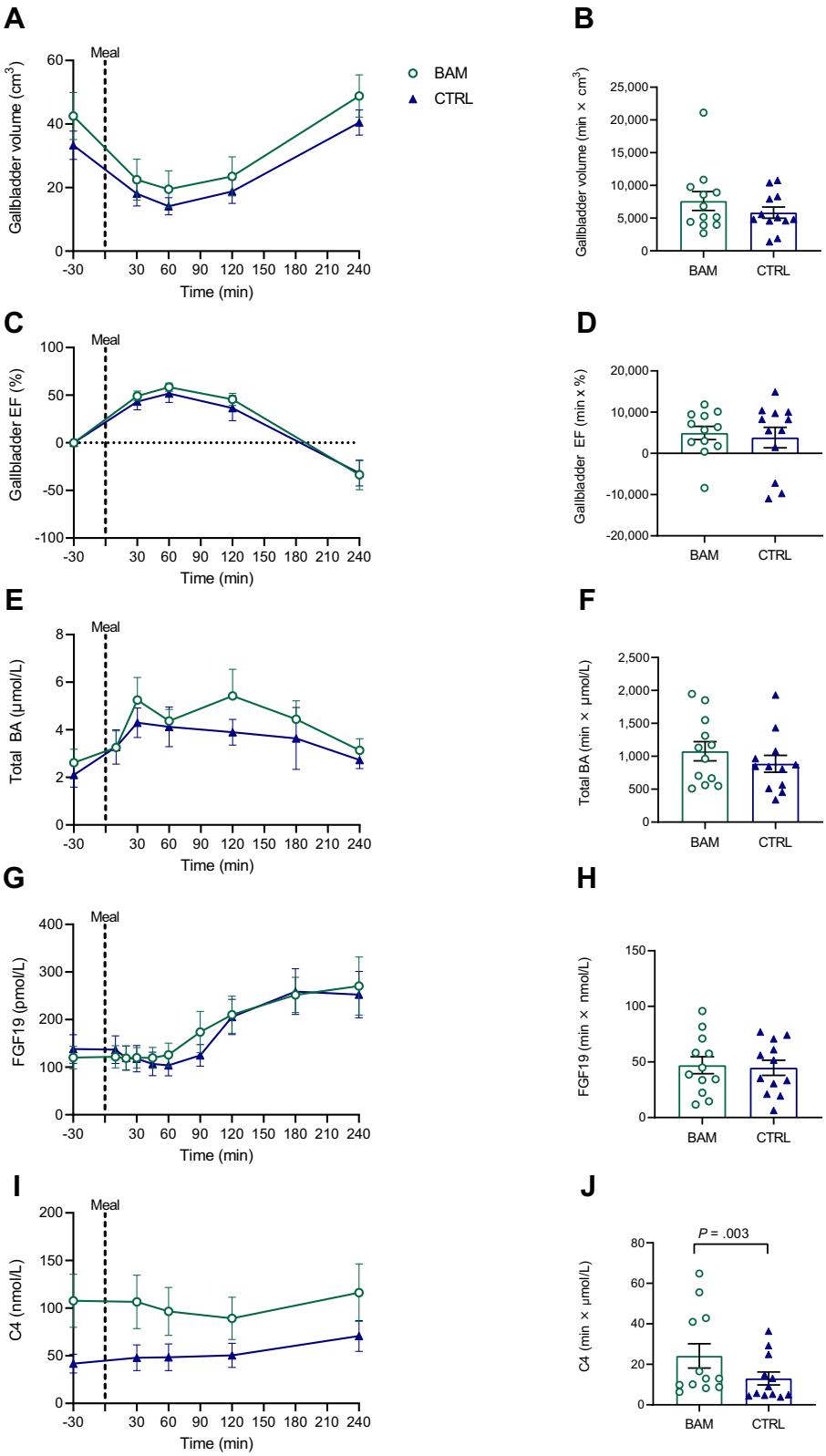

Figure 1. Gallbladder volume (A) and ejection fraction (EF) (C) and plasma concentrations of total bile acids (BA) (E), FGF19 (G), and C4 (I) during a 30-minute baseline period followed by a 240-minute liquid meal test in 12 individuals with BAM and 12 matched controls (CTRLs). Corresponding AUCs are also provided (B, D, F, H, and J). Data are presented as means ± standard error of the mean.

whereas baseline concentrations of glucose, insulin, and glucagon were similar in the 2 groups (Table 3 and Figure 3). Postprandial plasma excursions of glucose, insulin, C-peptide, and glucagon were statistically significantly larger in the BAM group than those in the control group (Table 3 and Figure 3). Administration of a single dose of colesevelam did not induce any changes in postprandial glucose, insulin, C-peptide, or glucagon in any of the groups. The McAuley index of insulin sensitivity amounted to 6.2 (95% CI 5.6; 6.8) and 7.1 (95% CI 6.4; 7.9) in the BAM group and CTRLs, respectively, with a statistically significant difference between the groups of 0.98 (95% CI

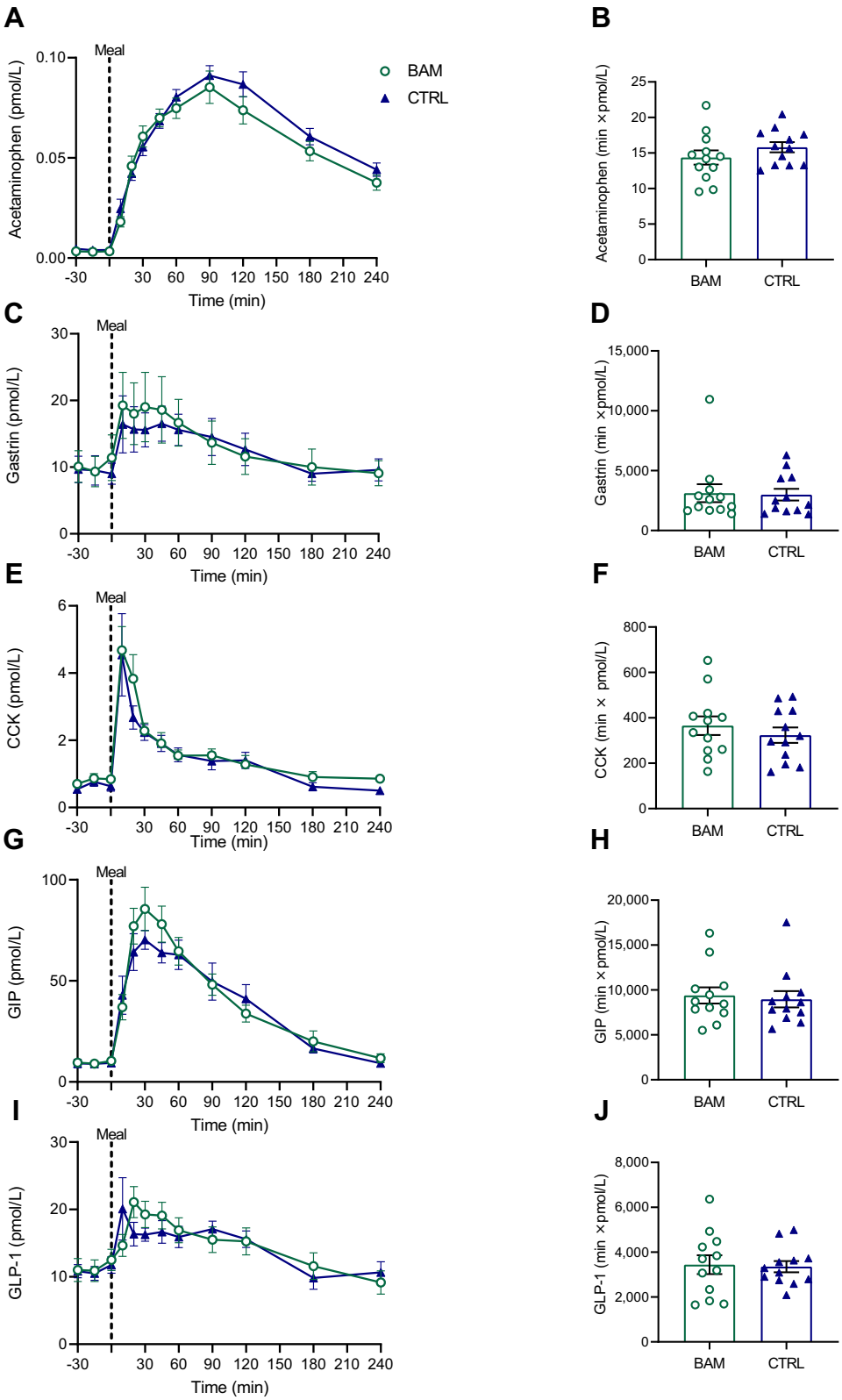

Figure 2. Plasma concentration of acetaminophen (A), gastrin (C), CCK (E), GIP (G), and GLP-1 (I) during a 30-minute baseline period followed by a 240-minute liquid meal test in 12 individuals with BAM and 12 matched controls (CTRLs). Corresponding AUCs are also provided (B, D, F, H, and J). Data are presented as mean ± standard error of the mean.

0.03; 1.9) ($P = .044$) (Table 3). The BAM group had a HOMA-IR of 1.5 (95% CI 1.3; 1.7), and the CTRL group had a HOMA-IR of 1.2 (95% CI 0.9; 1.4), with no statistically significant difference between the groups ($P = .079$) (Table 3). The Matsuda index of insulin sensitivity amounted to 4.2 (95% CI 2.9; 5.6) and 5.9 (95% CI 4.2; 7.6) in the patients with BAM and CTRLs, respectively, with a mean group difference of 1.7 (95% CI −0.19; 3.5) ($P = .074$). No differences in the IGI or DI were observed between the groups (Table 3).

Table 3. Serum/Plasma Concentrations of Glucose, Insulin, C-peptide, and Glucagon During Liquid Mixed Meal Test in Individuals With BAM and CTRLs and Specific Gluco-Metabolic Indexes

Parameter	BAM (n = 12)	CTRL (n = 12)	Δ	*P*
Glucose				
Baseline (mmol/L)	5.2 (5.0; 5.5)	5.1 (4.8; 5.5)	0.07 (−0.11; 0.26)	.668
C_{max} (mmol/L)	7.5 (7.2; 7.8)	6.9 (6.5; 7.3)	0.60 (0.11; 1.1)	**.018**
T_{max} (min)	40 (28; 52)	33 (20; 46)	7 (−32; 45.7)	.999
AUC (min × mmol/L)	1371 (1300; 1443)	1313 (1237; 1389)	58.2 (26.9; 89.4)	**.001**
Insulin				
Baseline (pmol/L)	88.2 (62.9; 113.6)	73.8 (48.8; 98.8)	14.4 (−18.7; 47.6)	.566
C_{max} (pmol/L)	900 (650; 1150)	649 (367; 902)	251 (20.7; 522)	.071
T_{max} (min)	37 (25; 49)	31 (25; 37)	6 (−13; 24)	.861
AUC (min × nmol/L)	63.7 (47.6;79.9)	42.2 (29.9; 54.5)	21.5 (10; 33)	**.001**
C-peptide				
Baseline (pmol/L)	644 (516; 771)	502 (347; 658)	141 (1.9; 280)	**.047**
C_{max} (pmol/L)	2337 (1969; 2705)	1767 (1306; 2227)	571 (227; 914)	**.003**
T_{max} (min)	48 (33;63)	51 (34; 68)	−3 (−20; 14)	.999
AUC (min × nmol/L)	307 (259; 356)	229 (176; 283)	78 (60; 95)	**<.001**
bsAUC (min × nmol/L)	153 (125; 181)	109 (71; 147)	44 (14; 74)	**.006**
Glucagon				
Baseline (pmol/L)	10.5 (5.9; 15.2)	8.5 (4.7; 12.4)	2 (−0.8; 4.8)	.174
C_{max} (pmol/L)	20.5 (13.3; 27.7)	15.3 (10; 20.7)	5.2 (0.3; 10)	**.036**
T_{max} (min)	22 (19; 24)	38 (12; 64)	−17 (−43; 9)	.251
AUC (min × pmol/L)	2764 (1588; 3941)	2061 (1273; 2849)	703 (250; 1156)	**.004**
Insulin sensitivity indices				
McAuley index	6.2 (5.6; 6.8)	7.1 (6.4; 7.9)	0.98 (0.03; 1.9)	**.044**
HOMA-IR	1.5 (1.3; 1.7)	1.2 (0.90; 1.4)	−0.33 (−0.7; 0.04)	.079
Matsuda index	4.2 (2.9; 5.6)	5.9 (4.2; 7.6)	1.7 (−0.19; 3.5)	.074
IGI	2.7 (2.1; 3.4)	2.1 (0.45; 3.8)	−0.66 (−2.5; 1.2)	.452
DI	10.5 (7.4; 13.6)	14 (2.6; 25.4)	3.5 (−9.9; 16.9)	.579

Data are presented as means with 95% CIs. *P* values are from ANOVA post hoc test and Bonferroni adjustment for multiple comparisons. Delta values refer to the difference between the BAM group and CTRLs.
P values < .05 are indicated with bold font.
bsAUC, baseline-subtracted area under the curve.

Gut Microbiome

The number of observed species in the gut microbiome of patients with BAM and CTRLs was not different (Figure 4C), but beta diversity analysis using both Jaccard similarity (presence/absence) and Bray-Curtis dissimilarity (weighted) metrics showed that the gut microbiome of the 2 groups clearly differed (Figure 4B). Thus, linear discriminant analysis effect size analysis showed that BAM subjects were characterized by higher relative abundance of several taxa, most prominent for *Blautia*, *Streptococcus*, *Dorea*, *Ruminococcus gnavus*, and *Akkermansia*, and lower relative abundance of *Clostridiaceae* members (Figure 4D).

Discussion

Here, we show that individuals with BAM, compared with carefully sex-, age-, and BMI-matched healthy controls, are characterized by an overproduction of bile acids (assessed by circulating C4), greater fecal bile acid content, and a dysmetabolic prediabetic-like state with elevated postprandial plasma concentrations of glucose, insulin, C-peptide, and glucagon. Furthermore, we provide novel information on the gut microbiota composition in patients with BAM showing clear differences from CTRLs with higher relative abundance of the taxa *Blautia*, *Streptococcus*, *Ruminococcus gnavus*, and *Akkermansia muciniphila*, potentially linking their enterohepatic pathophysiology and their dysmetabolic phenotype.

Despite carefully matching on sex, age, and BMI, the relatively low number of participants limits our ability to detect smaller and subtle differences between the 2 groups or between sexes within the groups, which, nevertheless, may be important for BAM pathophysiology. Thus, any lack of differences between the 2 groups should be interpreted with caution, whereas the careful matching adds robustness to the observed differences. The high number of parameters investigated may be considered overly multifaceted, but we believe that the explorative nature of the study aiming at a thorough pathophysiological characterization of BAM justifies the broad scope of endpoints. In addition, we used Bonferroni adjustment for multiple comparisons known as a conservative adjustment method. Even though multiple parameters were evaluated, the study could have benefited from even more measures such as circulating amino acids and liver fat content, which potentially could have provided

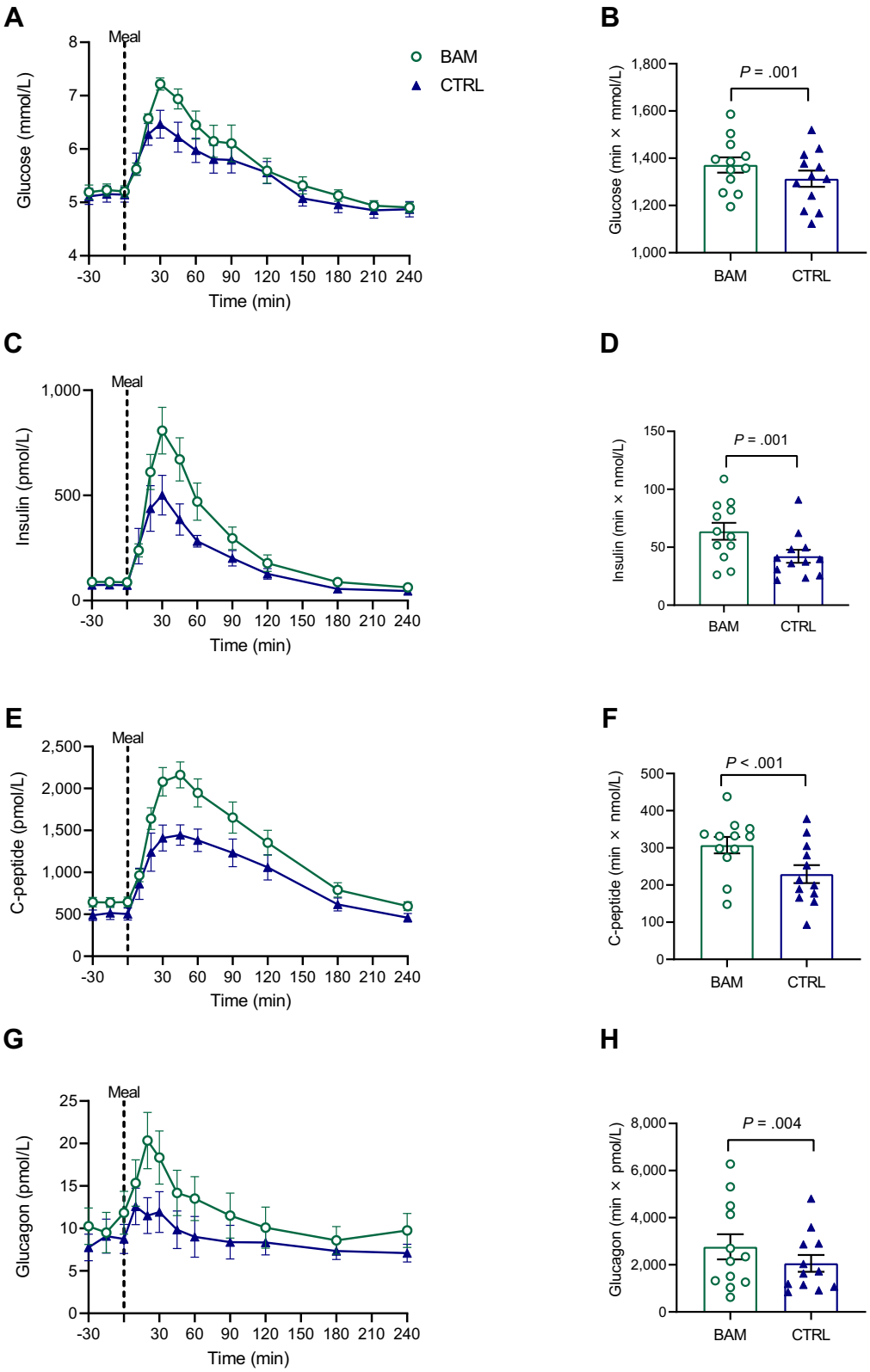

Figure 3. Plasma/serum concentrations of glucose (A), insulin (C), C-peptide (E), and glucagon (G) during a 30-minute baseline period followed by a 240-minute liquid meal test in 12 individuals with BAM and 12 matched controls (CTRLs). Corresponding AUCs are also provided (B, D, F, and H). Data are presented as means ± standard error of the mean. *P* values are from the multiple comparisons test with Bonferroni's correction.

data-driven explanations of the observed pathophysiologic characteristics of BAM. The most important strength of the present study is the dedicated and well-characterized group of patients with SeHCAT-verified diagnosis of severe-to-moderate BAM combined with careful sex, age, and BMI matching of CTRLs. As the present study is the first to provide a gluco-metabolic characterization of individuals with BAM, comparison with other similar investigations is not possible. However, the straightforward study design and the well-established and simple methodology used to describe

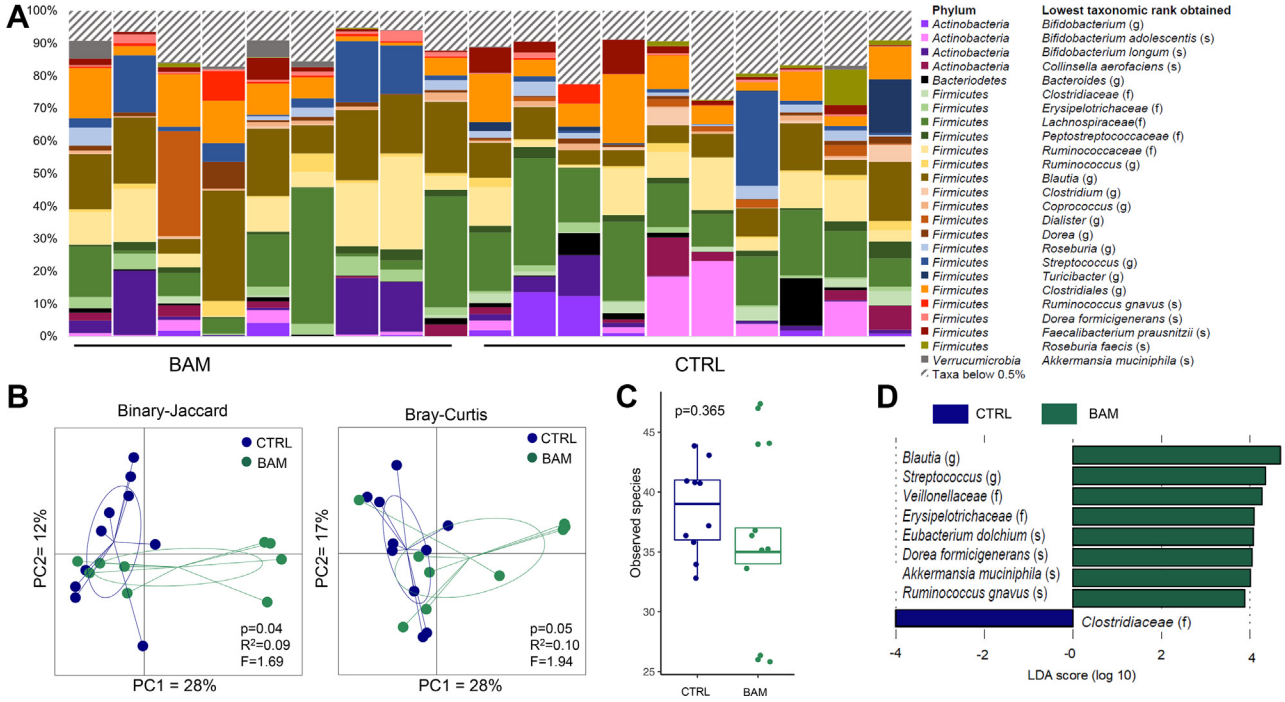

Figure 4. (A) Gut microbiome composition of individuals with BAM and controls (CTRLs) (relative abundance, taxa <0.5 summarized as "remaining", f, family; g, genus; s, species). (B) The gut microbiome composition differs between subjects with BAM and CTRLs as determined by Jaccard distance (presence/absence) and Bray-Curtis dissimilarity (weighted) metrics (PERMANOVA was performed to determine differences between groups; *P* values were adjusted by Benjamin-Hochberg correction). (C) No difference in the number of observed species in the gut microbiome between subjects with BAM and CTRLs (Wilcoxon rank-sum test). (D) Differential abundance analysis was carried out by linear discriminant analysis (LDA) effect size (LefSe) between the BAM and CTRL group (f, family; g, genus; s, species); only taxa found to be differential abundant are shown. The gut microbiome of subjects with BAM and CTRLs was determined using near-full-length 16S rRNA gene amplicon sequencing.

glucose metabolism in our patients with BAM should make it easy to test the reproducibility of the present findings.

Fracchia et al[34] analyzed bile-rich duodenal fluid sampled during intravenous ceruletide infusion (inducing contraction of the gallbladder) in the fasting state in 13 individuals with SeHCAT-verified idiopathic BAM and 23 CTRLs with normal bowel function and found higher percentage bile acid values and lower percentage phospholipid values in patients with BAM. Fracchia et al's findings support the notion that increased amount of bile in the upper small intestine may play a pathophysiological role in BAM. In the present study, we did not sample duodenal fluid, but we provide detailed information on postprandial responses of circulating bile acids and their composition. We show higher plasma concentrations of taurine-conjugated CA in the fasting state of patients with BAM than in CTRLs, whereas no other clear differences in circulating bile acids were observed in the fasting state. Differences in postprandial plasma concentrations of bile acids were also not observed between the 2 groups. Interestingly, however, single-dose colesevelam co-administered with the test meal reduced the postprandial AUC for total bile acids in the BAM group (driven by reductions in concentrations of taurine-conjugated CA, LCA, and glycine-conjugated CDCA), whereas postprandial plasma bile responses were less affected by colesevelam in CTRLs. We speculate that the pronounced effect of colesevelam on postprandial plasma concentrations of bile acids in patients with BAM may reflect a greater amount of bile acids in the small intestinal lumen and, hence, a better basis for bile acid sequestration. In line with this speculation, we observed a tendency to a larger baseline GV and AUC for GV in the BAM group, perhaps explaining the higher percentage of bile acids in the duodenal fluid from patients with BAM analyzed by Fracchia et al.[34] As expected, and previously described in individuals with diarrhea-predominant irritable bowel syndrome,[35] we observed a greater fecal bile content in patients with BAM than in CTRLs.

We observed greater plasma concentrations of C4 in patients with BAM vs CTRLs, corroborating observations by Borup et al and Brydon et al showing higher plasma concentrations of C4 in patients with BAM than in patients suffering from diarrhea due to miscellaneous causes.[36,37] These findings point to greater bile acid synthesis in BAM and support the notion of a reduced negative feedback inhibition of bile acid production in BAM.[10] In general, bile acids regulate their own synthesis via negative feedback by binding to the FXR in the small intestine and in the liver.[38,39] In the enterocytes of the small intestine, FXR activation induces expression and secretion of the hormone FGF19,

which via the hepatic receptor complex FGFR4/β-Klotho suppresses bile acid synthesis.[40,41] In the present study, circulating FGF19 concentrations in the fasting as well as in the postprandial state were similar in patients with BAM and CTRLs, but single-dose colesevelam administered with the mixed meal test reduced postprandial plasma FGF19 responses in CTRLs only. The lack of effect of colesevelam in the BAM group may indicate a greater amount of unbound bile acids in these patients, perhaps reflecting greater amounts of intraluminal bile acids than in CTRLs.

Collectively, the abovementioned findings are not conclusive but point to an overproduction of bile acids in patients with BAM, as suggested in previous studies,[10] providing support for the notion of a reduced negative feedback inhibition of bile acid production (vs a reduced capacity to reabsorb bile acids in the small intestine) playing a predominant pathophysiological role in BAM.

Because loss of cholesterol occurs indirectly via fecal excretion of bile acids (a phenomenon that is potentiated by bile acid sequestrants for the treatment of hypercholesterolemia[42]), we found it surprising that the BAM group did not exhibit lower fasting plasma concentrations of total cholesterol. Furthermore, as bile acids via activation of TGR5 in the basolateral membrane of enteroendocrine L cells induce secretion of the glucose-lowering and satiety-promoting gut-derived hormone GLP-1,[13–15] we were surprised not to see increased postprandial plasma GLP-1 responses in our patients with BAM. We have previously shown that gallbladder emptying in response to intravenous CCK administration results in GLP-1 secretion (CCK is not a direct stimulus for GLP-1 secretion[43]) without meal ingestion.[14,44] Nevertheless, the strongest secretory GLP-1 stimulus comes from ingested macronutrients (primarily from carbohydrates),[31,45] and we speculate that potential differences in bile acid–induced GLP-1 secretion between patients with BAM and CTRLs may be overshadowed by the robust meal-induced GLP-1 responses observed in the present study. Increased bile acid–induced activation of the FXR, known to inhibit GLP-1 secretion, may also influence the observed postprandial GLP-1 responses.[46] Finally, general small intestinal malabsorption of bile acids in patients with BAM may prevent activation of the basolateral TGR5 and, thus, influence the overall postprandial GLP-1 response. It is also interesting that the enhanced colonic exposure of bile acids in the BAM subjects, assessed by a greater fecal loss of bile acids, did not result in increased fasting GLP-1 concentrations.[47–49] As expected, colesevelam increased the postprandial CCK response in both groups,[50] perhaps—together with the colesevelam-induced interruption of the enterohepatic circulation of bile acids and FGF19 secretion—contributing to the reduced gallbladder refilling observed during meal tests with concomitant single-dose colesevelam administration.[51]

To our knowledge, gluco-metabolic characteristics of patients with BAM have not been reported previously. As bile acids and their derived effects, for example, induction of GLP-1 release, have been associated with several metabolic effects of potential benefit for individuals with obesity-related dysmetabolism, we anticipated a similar or even healthier metabolic profile in patients with BAM vs matched CTRLs. However, we found the opposite: patients with BAM exhibited a dysmetabolic prediabetic-like phenotype with larger postprandial responses of glucose, insulin, C-peptide, and glucagon compared with CTRLs. Furthermore, the McAuley index, which is considered a solid measure of insulin resistance and other features of the metabolic syndrome in the general population,[29,30] was found to be significantly reduced in the BAM group with values under the defined cutoff of 6.3 indicating impaired insulin sensitivity.[30] Similar tendencies were observed for HOMA-IR and the Matsuda index.[52] Collectively, these data suggest that individuals with BAM have disturbances in their gluco-metabolic homeostasis resembling individuals with metabolic syndrome and/or prediabetes. Future research should further explore the dysmetabolic phenotype of patients with BAM and their risk of type II diabetes and cardiovascular disease.

As mentioned previously, BAM subjects were characterized by greater fecal content of bile acids than CTRLs. Bile acids that escape jejunal reabsorption and end up in the colon are toxic to many gut microbes, leading to membrane damage, thereby influencing gut microbiome structure.[53,54] This is also clearly reflected in the present study, where the gut microbiome in BAM subjects differed from CTRLs. In a cohort of 11 BAM subjects (7 subjects with moderate and 4 subjects with severe BAM), Jeffrey et al[55] also observed a skewed gut microbiome relative to healthy controls, but only in the 4 subjects with severe BAM, precluding determination of changes in specific bacterial species. Generally, Gram-negative gut bacteria are considered more resistant to bile than Gram-positive bacteria,[53] and this is also partly reflected in the present study, where the patients with BAM were characterized by a lower relative abundance of the Gram-positive *Clostridiaceae* family but also an increased level of the Gram-positive *Blautia*, *Streptococcus*, *Dorea*, and *Ruminococcus*. The finding that the relative abundance of *Blautia* is increased in BAM subjects was surprising to us, as this genus generally is found to be rather sensitive to the bactericidal effects of certain bile acids,[54,56] while *Dorea* species on the other hand have been suggested to be involved in colonic cholic acid dihydroxylation, offering a likely explanation why this taxon is increased in patients with BAM.[57] Interestingly, abundance of the Gram-negative *A muciniphila* was increased in our patients with BAM. In vitro, most bile acids inhibit the growth of *A muciniphila*,[58] but it can be speculated that BAM-induced colonic mucus secretion may provide a higher substrate availability for the mucin-degrading *A muciniphila*.[59,60] *A mucinphila* has also been shown to be negatively associated with type II diabetes.[60] *Blautia* has been positively associated with visceral fat accumulation, which is known to be a part of metabolic risk factors such as increased blood glucose, hypertension, and other metabolic syndrome–related comorbidities.[61] *Ruminococcus gnavus* has been shown to produce

an inflammatory polysaccharide, which may play a role in the pathophysiology of Crohn's disease,[62] and thus, it can be speculated that the increased relative abundance of this bacterium in our patients with BAM might contribute to the pathophysiology of BAM and play a role in metabolic dysregulation.[63] Both *Blautia* and *R. gnavus* have been positively associated with type II diabetes,[60] and the increased relative abundance of these genera in patients with BAM may link the enterohepatic and the gluco-metabolic pathophysiology of BAM described in this study.

In conclusion, our findings support previous observations pointing to increased bile acid synthesis as a predominant feature of BAM pathophysiology and provide novel evidence of BAM as a dysmetabolic and prediabetes-like state with a specific gut microbiome composition that may link their enterohepatic pathophysiology and their dysmetabolic phenotype.

Supplementary Materials

Material associated with this article can be found in the online version at https://doi.org/10.1016/j.gastha.2021.12.007.

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Received November 11, 2021. Accepted December 14, 2021.

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Acknowledgments:
The authors thank all study participants and acknowledge the experimental and laboratory assistance from Sisse M. Schmidt and Inass Al-Nachar at Center for Clinical Metabolic Research, Copenhagen University Hospital – Herlev and Gentofte, and laboratory technicians at University of Copenhagen. Some of the data reported here were presented at the 79th Scientific Sessions of the American Diabetes Association in 2019.

Authors' Contributions:
Martin L. Kårhus, Andreas Brønden, David P. Sonne, and Filip K. Knop contributed to study concept and design. Martin L. Kårhus, Andreas Brønden, and Lukasz Krych contributed to data collection. Martin L. Kårhus, David P. Sonne, Martin Thomasen, Anne-Marie Ellegaard, Jens J. Holst, Jens F. Rehfeld, Oscar Chávez-Talavera, Anne Tailleux, Bart Staels, Tina Vilsbøll, Dennis S. Nielsen, Lukasz Krych, Lars O. Dragsted, Andreas Brønden, and Filip K. Knop contributed to analysis and interpretation of data. Martin L. Kårhus, David P. Sonne, Anne-Marie Ellegaard, Andreas Brønden, and Filip K. Knop contributed to drafting of the manuscript. Martin L. Kårhus, David P. Sonne, Martin Thomasen, Anne-Marie Ellegaard, Jens J. Holst, Jens F. Rehfeld, Oscar Chávez-Talavera, Anne Tailleux, Bart Staels, Tina Vilsbøll, Dennis S. Nielsen, Lukasz Krych, Lars O. Dragsted, Andreas Brønden, and Filip K. Knop contributed to revision of the manuscript. MLK and FKK contributed to obtainment of funding.

Conflicts of Interest:
The authors disclose no conflicts.

Funding:
The study was supported by unrestricted grants from the Novo Nordisk Foundation and the A. P. Møller Foundation for Advancement of Medical Sciences.

Ethical Statement:
The corresponding author, on behalf of all authors, jointly and severally, certifies that their institution has approved the protocol for any investigation involving humans or animals and that all experimentation was conducted in conformity with ethical and humane principles of research.

Data Transparency Statement:
Individual participant data will not be shared, but will be available on reasonable request provided adherence to General Data Protection Regulation.