SUPPLEMENTAL MATERIALS

Regulatory Approval

The study was approved by Mayo Clinic Institutional Review Board (#17-004639), and the study was registered with ClinicalTrials.gov #NCT03270085. All authors had access to the study data and reviewed and approved the final manuscript.

Identification and Eligibility of Participants

Using natural language processing of medical records of patients evaluated at Mayo Clinic, Rochester, MN, we identified potential candidates with symptoms consistent with Rome III criteria for IBS with predominant diarrhea; some patients had previously undergone measurement of biochemical parameters suggestive of BAD. Baseline measurements of fasting serum C4 and fecal BAs were required for eligibility to enter the study: fasting serum C4 >52.5ng/mL, total 48h fecal BA >2337µmoles/48h, or total 48h fecal BA >1000µmoles/48h plus primary fecal BAs >4%, or primary fecal BAs >10%.[1] The diagnosis of BAD is based on elevated total and individual fecal BAs. Previous evaluations have demonstrated that increased colonic transit alone is not sufficient to increase fecal BAs.[2]

Participants and Characterization of Patient Symptoms at Screening

Participants were females or males, 18-65 years of age. Females of childbearing potential had a negative pregnancy test before initiation of medication. Stool 48-hour collections (for BAs) and fasting serum samples (for serum C4) were collected during baseline before treatment to determine eligibility, or had these measurements completed in the past (and included in the medical record).

All participants completed a validated bowel disease questionnaire (BDQ, corresponding to Rome criteria) and the Hospital Anxiety and Depression Scale (HAD) to ensure they had

symptoms consistent with IBS-D based on Rome III criteria, and that they had no significant uncontrolled affective disorder based on HAD score >11.[3,4] Daily diary was utilized to measure bowel function and stool consistency based on Bristol Stool Form Scale (BSFS).[5-8]

Exclusions Based on Concomitant Medications or Illnesses

Within the first week prior to the baseline period or during the subsequent 4 weeks during the study period, the following drugs were prohibited: agents that alter gastrointestinal transit [including opioids, narcotics, anticholinergics, tricyclic antidepressants, serotonin and norepinephrine reuptake inhibitors (SNRI) antidepressants], analgesic drugs [including opiates, non-steroidal anti-inflammatory drugs (NSAIDs), COX-2 inhibitors], and medications that could interfere with the interpretation of the study. However, birth control pills, estrogen replacement therapy, and thyroxine replacement were permitted.

Female subjects who were pregnant or breast-feeding, patients with prior abdominal surgery (except appendectomy), and patients with known chronic liver disease or history of elevated AST/ALT 2.0X upper limit of normal were excluded.

Colesevelam

Colesevelam was purchased in 625-mg tablet form (Welchol®, Daiichi Sankyo, Inc., Parsippany, NJ) through the Mayo Clinic Research Pharmacy. Participants took 1875mg (3 tablets, 625mg each) of the medication orally, twice daily, with lunch and supper for at least 28 days (with a maximum of 35 days to accommodate for participant schedules or weekends).

Birth Control

Because colesevelam can interfere with absorption of other medications, subjects taking hormonal contraceptives for birth control were instructed to use a double barrier method (such as a condom or diaphragm) with a spermicide.

Daily Bowel Function Assessment

During the study, participants completed a daily diary to record their bowel functions for each bowel movement including consistency [Bristol Stool Form Scale (BSFS):[5] 1-hard lumps, 2-lumpy sausage, 3-cracked sausage, 4-smooth sausage, 5-soft lumps, 6-mushy and 7-watery]. These diaries have been previously shown to document responsiveness to treatment of bowel dysfunctions.[6-8]

Serum 7α-hydroxy-4-cholesten-3-one (C4)

The blood samples were stored in a -70°C freezer, until batch analysis for serum C4 values in the Mayo Clinic Department of Laboratory Medicine and Pathology.

Measurement of serum 7α-C4 is a validated method for detecting BAD. In head-to-head comparisons with the ^{75}SeHCAT retention test, increased serum C4 had sensitivity of 90% and specificity of 79% in diagnosing BAD,[9] where shorter retention half-time of ^{75}SeHCAT is associated with increased level of C4; and it had 98% negative predictive value and 74% positive predictive value for diagnosis of BAD.[10] Based on the method published from our laboratory,[11,12] adapted from Galman et al.,[13] we used HPLC/tandem mass spectrometry to measure serum C4 to evaluate BA synthesis rate.

Fecal Bile Acid Measurement

Using HPLC/tandem mass spectrometry, we have adapted a method used with serum samples for fecal BA measurement.[14] Single stool samples obtained at baseline and at the end of treatment were prepared for assay by HPLC/MS by methanol extraction, and results were presented as concentration (that is μmol/g stool). We have previously shown that the methanol extraction delivers the BAs that are bound to the colesevelam in the stool samples.[15]

Approximately 100mg of each stool homogenate was incubated with 2mL of methanol for 30 minutes to extract the BAs.

RNA Isolation Methods

Rectosigmoid biopsies were preserved in a solution of RNALater and stored at −80°C. Total RNA was purified using the miRNeasy mini kit (Qiagen, Valencia, CA), including on-column DNAse treatment to remove genomic DNA. RNA quality was assessed by Agilent Bioanalyzers. Thirty-three of 36 samples had RNA Integrity Numbers (RIN) greater than 7.0. The remaining three had RNA Integrity Numbers (RIN) >6.4.

RNA Sequencing and RNA Analysis

The methods used[16-19] and selection of genes of interest[20,21] are summarized below. Mucosal gene expression was estimated as reads per kilo base per million mapped reads (RPKM), which is calculated by the total read counts per million (RPM) to normalize for sequencing depth; the RPM values are then corrected for the length of the gene, in kilobases (RPKM). In order to compare effects in the two treatment groups, we estimated within each participant the ratio of the mucosal gene expression post-/pre-treatment, and then compared the two treatment groups.

Analysis of Genes of Interest in Bile Acid Diarrhea

Based on a prior RNA-sequencing study[20] and a study based on RT-PCR of rectosigmoid mucosa in IBS-D patients compared with controls which showed increased mRNA expression of *GUC2AB*, *PDZD3*, and *PR2Y4* and decreased expression of *CLDN1* and *FN1*,[21] we explored effects of colesevelam and placebo on mucosal biopsies obtained in the patients who consented to undergo flexible sigmoidoscopy and biopsies at baseline and at end of treatment. The genes of special interest were: *GUCA2B*, *P2RY4*, *PDZD3*, and *VIP* (neurotransmitters and ion channels);

SLC10A2, FGFR4, KLB, TGR5 [*GPBAR1*] and *NR1H4* (genes for IBAT transporter, bile acid synthesis and the bile acid receptors FXR and TGR5); *FN1, RBP2, TFF1* (related to mucosal repair and cell adhesion), *CCL20* and *C4BP4* (chemokine-related), *IFIT3* and *TNFSF15* (immune function), *CLDN1, OCLN* and *ZO-1* (tight junction protein).

RNA Sequencing

RNA libraries were prepared using 200ng of total RNA according to the manufacturer's instructions for the TruSeq Stranded mRNA Sample Prep Kit (Illumina, San Diego, CA). The concentration and size distribution of the completed libraries were determined using an Agilent Bioanalyzer DNA 1000 chip (Santa Clara, CA) and Qubit fluorometry (Invitrogen, Carlsbad, CA).

Libraries were sequenced following Illumina's standard protocol using the Illumina cBot and HiSeq 3000/4000 PE Cluster Kit, yielding approximately 42 million to 77 million fragment reads per sample. The flow cells were sequenced as 100 X 2 paired end reads on an Illumina HiSeq 4000 using the HiSeq 3000/4000 sequencing kit and HiSeq Control Software HD 3.4.0.38 collection software. Base-calling was performed using Illumina's RTA version 2.7.7.
A link to the Mayo Clinic Department of Laboratory Medicine and Pathology website is included here: http://intranet.mayo.edu/charlie/genome-analysis-core-rst/customer-resources/acknowledging-the-core/

RNA Analysis

The raw RNA sequencing paired-end reads for the samples were processed through the Mayo RNA-Seq bioinformatics pipeline, MAP-RSeq version 3.0.2.[16] Briefly, MAP-RSeq employs the very fast, accurate and splice-aware aligner, STAR,[17] to align reads to the reference human genome build hg38. Gene and exon expression quantification were performed using the

Subread package[18] to obtain both raw and normalized (RPKM – Reads Per Kilobase per Million mapped reads) reads. Using the raw gene counts report from MAP-RSeq, genes that are differentially expressed between the groups were assessed using the bioinformatics package EdgeR 2.6.2.[19] Genes that were found different between the groups were reported along with their magnitude of change (log2 scale) and level of significance (False Discovery Rate, FDR <5%). Canonical pathway analysis was performed using the Ingenuity pathway analysis software IPA (Ingenuity® Systems, www.ingenuity.com). Biological functions and diseases information within the IPA software were used to investigate the canonical pathways of interest.

Random Allocation

Equal BMI distribution was included because patients with BAD have higher BMI than patients with chronic diarrhea without BAD. This randomization sequence remained with a research pharmacist who assigned patients and distributed treatment or placebo. Random allocation was performed by numbered medication containers held in the research pharmacy. Both treatment and placebo were encapsulated, and appeared identical to ensure blinding. Research staff and research participants were all blinded throughout the study. Participants were enrolled by the study staff [study coordinators, nurses, and medical research fellow (PV)]. Assigned medications were provided to participant by the research pharmacist and Clinical Trials Research Unit nurses.

Statistical Power and Analysis

Based on our previous open-label study, the proposed sample size (15 per group) has ~80% power (2-sided α=0.05) to detect clinically relevant effect sizes in stool frequency and form, fecal BAs and serum C4 [expressed as detectable difference (Δ)], as shown in Supplemental Table 1. Consistent with intention-to-treat principles and the statistical analysis

plan, the patient response outcome data from one patient (who discontinued intervention for personal reasons) in one of the study arms were imputed using the average result for all participants in the two arms of the patient data. In order to compensate for this imputation, the number of degrees of freedom was reduced by one for all the analyses conducted on the patient responses.

Data from 15 participants in each treatment group were analyzed for all end points except mucosal gene expression, which was available for 8 of 15 participants in the colesevelam group and 9 of 15 participants in the placebo group.

Given the sample sizes and observed skewed distribution of several parameters, the data are summarized as median and interquartile range (IQR), and the analysis is based on non-parametric rank sum tests with baseline used as covariate when such measurements were available (e.g., for colonic transit at 24 hours, serum C4 and FGF19, fecal BA excretion, stool number and stool consistency). Patient reports of fecal consistency and numbers of bowel movements each day were averaged for the 7-day baseline period and the 28-day treatment period. Differences of least square means were used to compare the changes in study parameters between the placebo and colesevelam arms. Spearman correlation was used to compare the change in primary BAs (%) to the two primary outcome measures (stool frequency and consistency) in the two treatment groups. Unpaired t test or Mann-Whitney test (for non-parametric data) were used to compare the ratio of post-/pre-treatment mucosal expression of genes of interest in the two groups.

Nominal p value was utilized to assess the mucosal gene expression. Within each of the clinical outcomes and mucosal gene expression evaluations, no correction for multiple

comparisons was conducted since we assessed a single pathophysiology mechanism and did not rely on multiple outcomes to assess a single mechanism.

Correlations between changes in fecal bile acid excretion and stool frequency or consistency in placebo and colesevelam groups were analyzed using rank Spearman correlation, except for the analysis between fecal bile acid excretion and stool frequency which was analyzed using a non-linear analysis based on $y=1/(x + a)$, where a represents the intercept term.

Supplemental Materials References

1. Vijayvargiya P, Camilleri M, Chedid V, Carlson P, Busciglio I, Burton D, Donato L. Analysis of fecal primary bile acids detects increased stool weight and colonic transit in patients with chronic functional diarrhea. Clin Gastroenterol Hepatol 2019;17:922-929.e2.

2. Shin A, Camilleri M, Vijayvargiya P, Busciglio I, Burton D, Ryks M, Rhoten D, Lueke A, Saenger A, Girtman A, Zinsmeister AR. Bowel functions, fecal unconjugated primary and secondary bile acids, and colonic transit in patients with irritable bowel syndrome. Clin Gastroenterol Hepatol 2013;11:1270-1275.

3. Talley NJ, Phillips SF, Wiltgen CM, Zinsmeister AR, Melton LJ 3rd. Assessment of functional gastrointestinal disease: the bowel disease questionnaire. Mayo Clin Proc 1990;65:1456-1479.

4. Zigmond AS, Snaith RP. The hospital anxiety and depression scale. Acta Psychiatr Scand 1983;67:361-370.

5. Heaton KW, Radvan J, Cripps H, Mountford RA, Braddon FE, Hughes AO. Defecation frequency and timing, and stool form in the general population: a prospective study. Gut 1992;33:818-824.

6. Andresen V, Camilleri M, Busciglio I, Grudell A, Burton D, McKinzie S, Foxx-Orenstein A, Kurtz CB, Sharma V, Johnston JM, Currie MG, Zinsmeister AR. Effect of 5 days linaclotide on transit and bowel function in females with constipation-predominant irritable bowel syndrome. Gastroenterology 2007;133:761-768.

7. Manini ML, Camilleri M, Goldberg M, Sweetser S, McKinzie S, Burton D, Wong S, Kitt MM, Li YP, Zinsmeister AR. Effects of velusetrag (TD-5108) on gastrointestinal transit and bowel function in health and pharmacokinetics in health and constipation. Neurogastroenterol Motil 2010;22:42-49, e7-8.

8. Oduyebo I, Camilleri M, Nelson AD, Khemani D, Nord SL, Busciglio I, Burton D, Rhoten D, Ryks M, Carlson P, Donato L, Lueke A, Kim K, Rossi SJ, Zinsmeister AR. Effects of NGM282, an FGF19 variant, on colonic transit and bowel function in functional constipation: a randomized phase 2 trial. Am J Gastroenterol 2018;113:725-734.

9. Sauter GH, Münzing W, von Ritter C, Paumgartner G. Bile acid malabsorption as a cause of chronic diarrhea: diagnostic value of 7alpha-hydroxy-4-cholesten-3-one in serum. Dig Dis Sci 1999;44:14-19.

10. Brydon WG, Nyhlin H, Eastwood MA, Merrick MV. Serum 7 alpha-hydroxy-4-cholesten-3-one and seleno-homocholyltaurine (SeHCAT) whole body retention in the assessment of bile acid induced diarrhoea. Eur J Gastroenterol Hepatol 1996;8:117-123.

11. Camilleri M, Nadeau A, Tremaine WJ, Lamsam J, Burton D, Odunsi S, Sweetser S, Singh R. Measurement of serum 7alpha-hydroxy-4-cholesten-3-one (or 7alphaC4), a surrogate test for bile acid malabsorption in health, ileal disease and irritable bowel syndrome using liquid chromatography-tandem mass spectrometry. Neurogastroenterol Motil 2009;21:734-e43.

12. Donato LJ, Lueke A, Kenyon SM, Meeusen JW, Camilleri M. Description of analytical method and clinical utility of measuring serum 7-alpha-hydroxy-4-cholesten-3-one (7aC4) by mass spectrometry. Clin Biochem 2018;52:106-111.

13. Gälman C, Arvidsson I, Angelin B, Rudling M. Monitoring hepatic cholesterol 7alpha-hydroxylase activity by assay of the stable bile acid intermediate 7alpha-hydroxy-4-cholesten-3-one in peripheral blood. J Lipid Res 2003;44:859-866.

14. Tagliacozzi D, Mozzi AF, Casetta B, Bertucci P, Bernardini S, Di Ilio C, Urbani A, Federici G. Quantitative analysis of bile acids in human plasma by liquid chromatography-electrospray tandem mass spectrometry: a simple and rapid one-step method. Clin Chem Lab Med 2003;41:1633-1641.

15. Camilleri M, Acosta A, Busciglio I, Boldingh A, Dyer RB, Zinsmeister AR, Lueke A, Gray A, Donato LJ. Effect of colesevelam on faecal bile acids and bowel functions in diarrhoea-predominant irritable bowel syndrome. Aliment Pharmacol Ther 2015;41:438-448.

16. Kalari KR, Nair AA, Bhavsar JD, O'Brien DR, Davila JI, Bockol MA, Nie J, Tang X, Baheti S, Doughty JB, et al. MAP-RSeq: Mayo Analysis Pipeline for RNA sequencing. BMC Bioinformatics 2014;15:224.

17. Dobin A, Davis CA, Schlesinger F, Drenkow J, Zaleski C, Jha S, Batut P, Chaisson M, Gingeras TR. STAR: ultrafast universal RNA-seq aligner. Bioinformatics 2013;29:15-21.

18. Liao Y, Smyth GK, Shi W. The Subread aligner: fast, accurate and scalable read mapping by seed-and-vote. Nucleic Acids Res 2013;41:e108.

19. Robinson MD, McCarthy DJ, Smyth GK. edgeR: a bioconductor package for differential expression analysis of digital gene expression data. Bioinformatics 2010;26:139-140.

20. Camilleri M, Carlson P, Acosta A, Busciglio I, Nair AA, Gibbons S, Farrugia G, Klee E. RNA sequencing shows transciptomic changes in rectosigmoid mucosa in patients with irritable bowel syndrome-diarrhea: a pilot case-control study. Am J Physiol 2014;306:G1089-G1098.

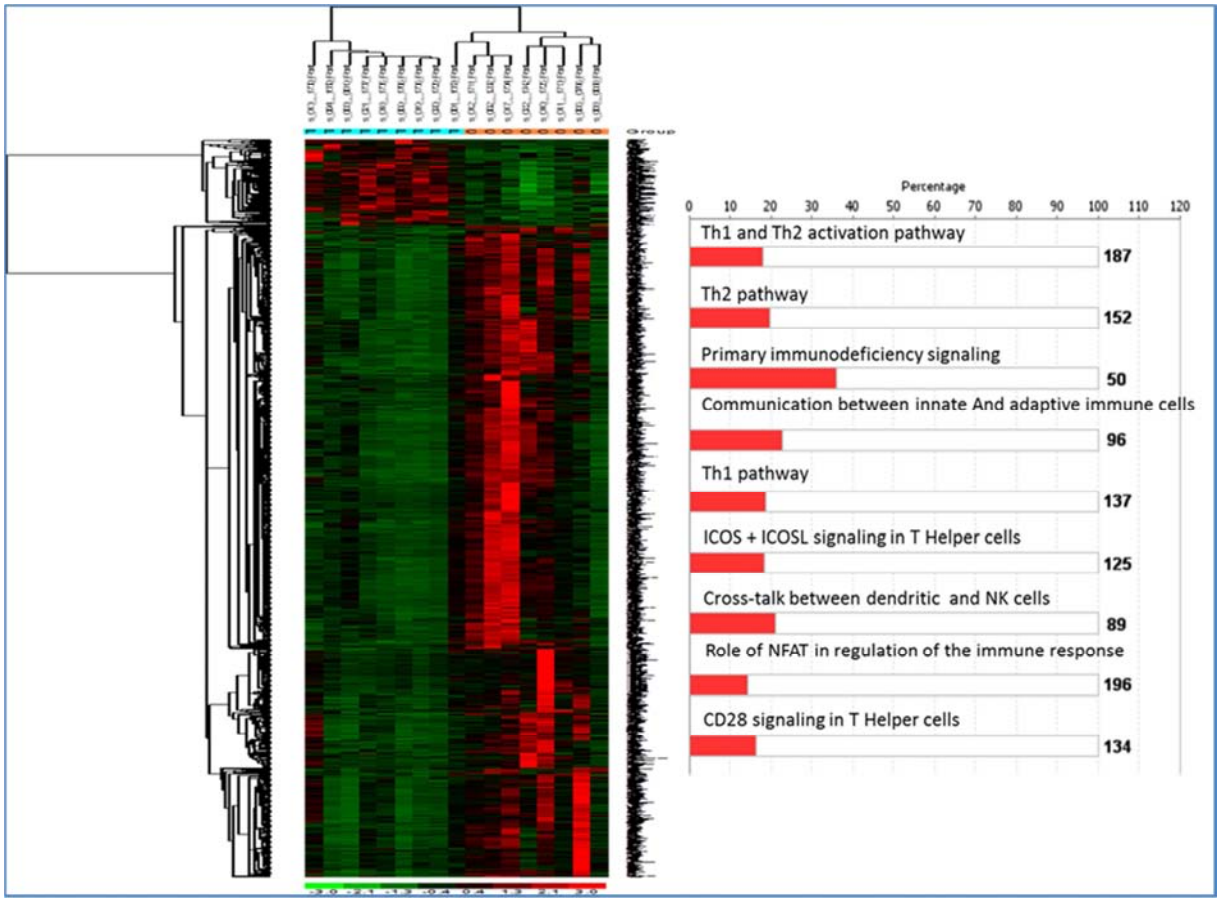
21. Camilleri M, Carlson P, Acosta A, Busciglio I. Colonic mucosal gene expression and genotype in irritable bowel syndrome patients with normal or elevated fecal bile acid excretion. Am J Physiol Gastrointest Liver Physiol 2015;309:G10-G20.

Supplemental Table 1. Statistical Power

Response	**Mean (SD)**	**Detectable difference with n=15 per group at 2-sided α level of 0.05 and 80% power**
#Stools/week	15.1 (6.6)	7.00
Stool form, BSFS (1-7 scale)	4.4 (1.0)	1.06
Total fecal BA excretion µmol/48h	3496 (2456)	2650
Serum C4 ng/mL	72.3 (4.3)	45

BSFS=Bristol Stool Form Scale; BA=bile acid

Supplemental Figure 1. RNA-sequencing showing heat map (left side) comparing participants randomized to colsevelam (orange) and placebo (cyan) as well as canonical pathway analysis (right side). Note that the vast majority of the differentially expressed genes are associated with immune functions.



NEED TO KNOW

Background: Many patients with IBS-diarrhea (IBS-D) have increased bile acid (BA) synthesis or excretion. Colesevelam has been reported to improve bowel function, consistent with luminal BA sequestration, in these patients.

Findings: In a randomized trial, colesevelam increased delivery of total and secondary BAs to stool, hepatic BA synthesis, and colon expression of genes that regulate BA, farnesoid X, and GPBAR1 receptors.

Implications for patient care: Colesevelam might be used to treat patients with IBS-D, but larger studies are needed to determine its efficacy.