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Microbiota-derived butyrate dampens linaclotide stimulation of the guanylate cyclase C pathway in patient-derived colonoids

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Abstract

Background & Aims: Disorders of gut-brain interaction (DGBI) are complex conditions that result in decreased quality of life and a significant cost burden. Linaclotide, a guanylin cyclase C (GCC) receptor agonist, is approved as a DGBI treatment. However, its efficacy has been limited and variable across DGBI patients. Microbiota and metabolomic alterations are noted in DGBI patients, provoking the hypothesis that the microbiota may impact the GCC response to current therapeutics.

Methods: Human-derived intestinal organoids were grown from pediatric DGBI, non-IBD colon biopsies (colonoids). Colonoids were treated with 250 nM linaclotide and assayed for cGMP to develop a model of GCC activity. Butyrate was administered to human colonoids overnight at a concentration of 1 mM. Colonoid lysates were analyzed for cGMP levels by ELISA. For the swelling assay, colonoids were photographed pre- and post-treatment and volume was measured using ImageJ. Principal coordinate analyses (PCoA) were performed on the Bray–Curtis dissimilarity and Jaccard distance to assess differences in the community composition of short-chain fatty acid (SCFA) producing microbial species in the intestinal microbiota from pediatric patients with IBS and healthy control samples.

Key Results: Linaclotide treatment induced a significant increase in [cGMP] and swelling of patient-derived colonoids, demonstrating a human in vitro model of linaclotide-induced GCC activation. Shotgun sequencing analysis of pediatric IBS patients and healthy controls showed differences in the composition of commensal SCFA-producing bacteria. Butyrate exposure significantly dampened linaclotide-induced cGMP levels and swelling in patient-derived colonoids.

Conclusions & Inferences: Patient-derived colonoids demonstrate that microbiota-derived butyrate can dampen human colonic responses to linaclotide. This study supports incorporation of microbiota and metabolomic assessment to improve precision medicine for DGBI patients.

KEYWORDS
butyrate, DGBI, guanylate cyclase C, linaclotide, microbiota, organoid

1 | INTRODUCTION

Disorders of gut-brain interaction (DGBI), including irritable bowel syndrome (IBS) and functional constipation (FC), are complex conditions that result in decreased quality of life and a significant cost burden for both the patient and the healthcare system. Indeed, the impact on quality of life is comparable to diseases such as inflammatory bowel disease, diabetes, and end-stage renal disease.[1,2] It is estimated that IBS alone is responsible for 3.6 million annual physician visits and consumes $1.7 billion in healthcare resources.[3,4] Both adults and children are affected by DGBI and it has been proposed to impact 25% of the population within the United States.[5,6] However, the etiology of DGBIs is complex and multifactorial likely representing a nexus of genetic predisposition, environmental triggers, and psychosocial augmentation. All DGBIs share a combination of cardinal features including motility disturbance, visceral hypersensitivity, altered immune function, modified gut microbiota, and disorders of gut-brain interactions.[7–9] However, DGBIs have no identifiable imaging or laboratory abnormalities and rely solely on clinical diagnosis. Therefore, improved understanding of DGBIs is required to guide new therapeutic approaches to prevent or treat disease.

The guanylin cyclase C (GCC) receptor is expressed on the luminal side of enterocytes throughout the gastrointestinal tract and upon ligand-mediated activation leads to synthesis of intracellular cyclic guanosine monophosphate (cGMP).[10,11] cGMP acts as a second messenger in several vital cellular processes including maintenance of gut fluid homeostasis. Thus, pharmaceutical targeting of this pathway regulates intestinal fluid movement and relieves constipation, one of the most common DGBIs.[12,13] Since these discoveries, cGMP signaling has been linked to all of the DGBI cardinal features representing a promising target for directed therapy.[11,14–16] Currently, two FDA approved medications, linaclotide and plecanatide, target the GCC receptor and have been approved for treatment of IBS and constipation. However, the efficacy of these drugs has been limited and variable across DGBI patients. In a linaclotide clinical trial, patients showed a modest improvement in clinical symptoms and disease outcomes.[17] Unfortunately, 40%–70% of patients failed to respond to treatment.[18] Similar results were noted with linaclotide trials in pediatric patients, although these studies are ongoing.[19] Despite initial promising results, further investigation into factors that may impact linaclotide efficacy are required.

The intestine harbors trillions of commensal microbes, collectively called the microbiota, which interact with enterocytes and play a significant role in regulating intestinal health. Beyond engagement of pattern recognition receptors, the microbiota can produce small molecule intermediates, or metabolic by-products called metabolites, which influence host cells. Short-chain fatty acids (SCFAs), including butyrate, derive from the breakdown of dietary fibers by microbial species and are among the most abundant microbial metabolites present within the intestine. Butyrate is a major energy source for colon enterocytes and is essential for regulating host immunity, intestinal barrier function, and vital homeostatic functions of enterocytes.[14,20,21] Interestingly, the intestinal microbiome of patients with DGBIs, especially IBS and constipation, is altered compared to healthy individuals.[22,23] Furthermore, it has been shown that specific alterations in the microbiome and microbial-derived metabolites occur with IBS and may influence gastrointestinal function, thereby provoking the hypothesis that the microbiota may impact the effectiveness of current DGBI therapeutics.[24,25] By employing DGBI patient-derived intestinal colonoids, we show that linaclotide treatment increases intracellular cGMP production and enhances colonoid swelling. These data are consistent with previous studies of GCC receptor agonism in healthy human-derived colonoids and suggest that patients with DGBIs have an intact and functional GCC pathway.[26–29] In addition, metagenomic analysis of the intestinal microbiota from pediatric patients indicates differences and highly variable proportions of SCFA-producing species within DGBI pediatric patients. Remarkably, butyrate treatment suppressed linaclotide-induced cGMP production and luminal swelling, indicating that microbial-derived factors may regulate intestinal responsiveness to linaclotide treatment. Collectively, these data reveal that colonoids can be used to assess whether alterations in the microbiota may influence the efficacy of DGBI treatments that may account, in part, for the disparity in clinical patient outcomes. Furthermore, assessing microbiota composition and intestinal metabolites in DGBI patients may allow for the design of more effective personalized therapeutic interventions.

Key points

- Lumen active medications may be influenced by bacterial-derived metabolites.
- Altered microbiome and bacterially-derived short chain fatty acids may affect linaclotide activity and efficacy in DGBI patients.
- Microbiome analyses may assist in designing personalized patient management.

2 | MATERIALS AND METHODS

2.1 | Patient samples

Human-derived intestinal sigmoid biopsies were acquired prospectively from pediatric patients at Cincinnati Children's Hospital Medical Center, Cincinnati, Ohio that were originally recruited

as potential controls for the pediatric inflammatory bowel disease (IBD) Specimen Repository (IRB: 2011-2285). Selected patients had histologically normal colon tissue, as confirmed by a staff pediatric pathologist. These patients were undergoing endoscopy to rule out IBD because of non-specific gastrointestinal symptoms (Table 1) and had no biochemical evidence of ongoing systemic inflammation. Patients were not selected based on ongoing therapeutics, which were most commonly laxatives or proton pump inhibitors (Table 1) and had no previous exposure to linaclotide.

2.2 | Colonoid cultures

Human colon spheroids were generated from colonic crypts as previously described.[30] Briefly, histologically normal sigmoid biopsies (2–4) were collected as described in Section 2.1. Biopsies were minced and digested in 2 mg/mL Collagenase I solution (ThermoFisher) for 20 min, with vigorous pipetting every 10 min. Colon crypts were filtered through a 70-μm strainer and pelleted by centrifugation. The pellet was resuspended in 120 μL cold Matrigel (Corning) and plated in 40 μL Matrigel droplets in a 24-well tissue culture plate with IntestiCult Organoid Growth Medium (StemCell) supplemented with 10 μM Y-27632 (Tocris Bioscience) and 10 μM SB 431542 (Selleckchem). After establishment of spheroids, media was switched to organoid growth media (50% advanced DMEM/F12 media supplemented with 10 mM HEPES, 2 mM L-glutamate, 50% L-WRN conditioned media, 1x N2 supplement, 1x B27 supplement, 50 ng/mL EGF, 10 μM Y-27632, and 10 μM SB 431542). Culture media was changed every 2–3 days.

2.3 | Linaclotide stimulation, butyrate exposure

Colonoids were exposed to 1 mM sodium butyrate (Sigma-Aldrich) over 22 h 3 days after passage. Exposed and unexposed colonoids were then stimulated with 250 nM linaclotide (ThermoFisher) for 1 h and immediately lysed for cGMP assay as described below. Butyrate and linaclotide were all dissolved in warmed media.

2.4 | Cyclic GMP ELISA

cGMP ELISA (Cayman chemical) was used to measure the intracellular concentration of cGMP following manufacturer instructions. Briefly, colonoids were chemically lysed by adding 0.1 M HCl solution for 20 min and then mechanically lysed using a TissueLyser II (Qiagen) for 3 min at 30 Hz. Samples were centrifuged for 10 min, lysates were extracted, and the reaction neutralized with ELISA buffer. Samples were plated on pre-coated plates, developed with Ellman's reagent, and absorbance read at 410 nm. cGMP concentrations were normalized to total protein concentration that were determined by BCA assay (ThermoFisher).

TABLE 1 Clinical summary of patients. Sigmoid biopsies were collected from patients diagnosed with a DGBI using a combination of ROME questionnaire, chart review, or existing diagnosis. Eosinophilic gastrointestinal disorders, celiac disease, and inflammatory bowel disease were excluded by endoscopic and histologic findings.

Patient demographics				Clinical and endoscopic findings				Histologic findings		
ID	Age	Sex	Race	Primary symptom	BMI (percentile)	Upper endoscopy	Lower endoscopy	Upper GI	Colon	Clinical diagnosis
1	17	F	White	Weight loss	18.4 (14th)	Normal	One small sessile polyp	Mild chronic gastritis	Inflammatory pseudopolyp	Functional dyspepsia
2	18	M	White	Nausea	32.1 (96th)	Esophageal edema	Normal	Normal	Normal	Functional dyspepsia
3	14	F	White	Abdominal pain	19.6 (56th)	Normal	Normal	Chemical gastropathy	Normal	Functional constipation
4	18	F	White	Abdominal pain	20.4 (37th)	Normal	Normal	Normal	Normal	IBS-U
5	16	F	White	Diarrhea	18.3 (15th)	Normal	Normal	Normal	Normal	IBS-D
6	16	M	White	Abdominal pain	26.1 (93rd)	Normal	Terminal ileum-patchy edema/erythema	Chemical gastropathy	Normal	IBS–U

Abbreviations: IBS-D, IBS-diarrhea predominant; IBS-U, IBS undetermined.

2.5 | Swelling assay

Colonoids were passaged, plated, and treated as described above. For each patient, 10 colonoids were selected from a single well, per condition specified (four wells per patient, one well per condition—vehicle, butyrate only, linaclotide only, both treatments). The same 10 colonoids were measured in their pre-treatment phase (3 days post passage, time 0), and re-measured at their post-treatment phase (time 23–24 h). Using the EVOS XL Core Imaging System (ThermoFisher) microscope, brightfield images were taken of colonoids suspended in Matrigel pre- and post-treatment. Images were stitched together using ImageJ Stitching plugin included with the basic FIJI ImageJ package.[31] Colonoids were measured using a combination of oval selection and the interactive wand 2D plugin tool.[30] Colonoid swelling was extrapolated from 2-D light microscopy.[26,32–34] The measured area was converted to volume using $4/3\pi\left[\sqrt{(\text{area}/\pi)}\right]^3$ and volumes were normalized to their respective pre-treatment volumes and reported as relative growth. Settings were kept the same for capturing images during the full course of the experiment.

2.6 | Stool collection

Patients with IBS were recruited from Cincinnati Children's Hospital Medical Center (CCHMC) gastroenterology clinics. Participants 11–18 years of age and meeting the ROME IV criteria for IBS, but negative on testing for celiac disease and inflammatory disorders, were enrolled in the IBS cohort.[35] Patients were excluded if they were taking a probiotic or on antibiotics, receiving formula as the sole source of nutrition, or had a diagnosis of another gastrointestinal disorder. Healthy control (HC) samples were obtained from CCHMC patients participating in the ENvISION trial (clinicaltrials.gov identifier NCT04131504) who were free of IBS (and IBD) based on a negative screening using the ROME IV diagnostic questionnaire. Stool kits containing a sterile, self-sealing container with 2 mL of ethanol as a preservative were sent to participants along with detailed instructions for collection and delivery. Samples were collected by participants and then shipped to CCHMC and frozen at −80°C until sequencing. All samples were sequenced within 1 year of biospecimen collection.

2.7 | Microbial DNA extraction, sequencing, and bioinformatic processing

DNA was extracted from 0.1 g of stool using the PowerFecal DNA isolation kit (MO Bio Laboratories) per the manufacturer recommendations. DNA samples were diluted to 200 ng/mL and sequencing libraries generated using the Nextera XT protocol (Illumina). Sequencing was performed on an Illumina NovaSeq 6000 machine using 150-bp DNA paired end (PE) reads to a depth of approximately 4G base pairs per sample. Raw sequence data were de-multiplexed and converted to fastq format for downstream processing using the Illumina program bcl2fastq. Taxonomic and functional profiling was performed using the bioBakery3 metagenomics workflow.[36] Kneaddata (version 0.10.0) was used to remove reads failing the default quality control, those <90 bp in length, or those mapping to the human reference database. Taxonomic profiling was performed using MetaPhlAn3 (version 3.0.7) under the default settings.[37] Species with a relative abundance less than $1e^{-5}$ were removed prior to statistical analysis.

2.8 | Statistical analysis

For the GCC activity assays and the swelling assays, Student's paired two-tailed *t*-test was used to assess statistical significance. Data were expressed as mean ± standard error of the means. *p*-values of <0.05 were deemed to be statistically significant. Testing was performed using Prism version 7.0 (GraphPad Software). Ordinations of the first two principal coordinate analysis (PCoA) axes were used to examine differences in the community composition of SCFA producing microbial species between IBS and HC participant samples. SCFA producing species retained in the species abundance matrix included: *Prevotella* spp., *Ruminococcus* spp., *Bifidobacterium* spp., *Bacteroides* spp., *Clostridium* spp., Streptococcus spp., *Anaerostipes* spp., *Akkermansia muciniphila*, *Faecalibacterium prausnitzii*, *Eubacterium rectale*, *Eubacterium hallii*, and *Roseburia intestinalis*. PCoA was performed using the ordinate function in phyloseq (version 1.36.0) on the Bray–Curtis dissimilarity and Jaccard distance after subsampling to the lowest observed read depth (2,693,802 reads). PERMANOVA as implemented by the adonis2 function in the vegan package (version 2.5.7) was used to obtain the proportion of variance explained and to test for differences in the distances/dissimilarities.[38]

3 | RESULTS

3.1 | Patient characteristics

Patients presented to CCHMC for DGBI symptoms such as abdominal pain, nausea, and constipation. Sigmoid intestinal biopsies were taken from patients undergoing upper endoscopy and colonoscopy. IBD, eosinophilic gastrointestinal disorders, celiac disease, and peptic ulcer disease were ruled out and there were no signs of systemic chronic diseases at baseline. Patients' ages ranged from 14 to 18 years with a mean age of 16.6 years. Patient's macro and microscopic findings on colonoscopy were normal as concluded by a pediatric gastroenterology attending at the time of the scope and confirmed by a pediatric pathologist. Gender-related patterns were not observed in response to either linaclotide or butyrate. Colonoids derived from the two male subjects had an average response to both compounds. The female colonoids were split between both high and low responsiveness. The patients' final clinical diagnosis and additional summary information is included in Table 1 and was based on a combination of chart review and ROME questionnaire filled out by the patient at least a week following colonoscopy.[7,35]

3.2 | Linaclotide increases cGMP levels in colonoids derived from pediatric DGBI patients

Linaclotide, by acting directly on the GCC pathway and thus some of the canonical features of DGBI, was expected to be a widespread therapeutic for DGBIs. However, the effectiveness and its overall ability to ameliorate DGBI symptoms remains inconsistent. Therefore, to determine whether DGBI patient intestinal samples were able to respond to linaclotide, patient-derived sigmoid colonoids were generated from pediatric DGBI patients and treated with linaclotide (Figure 1A). Linaclotide exposure induced robust cGMP production in six patient-derived colonoids (Figure 1B,C), indicating that DGBI patient-derived colonoids respond to linaclotide treatment, consistent with previous data employing healthy control patient-derived enteroids.[26] One patient-derived colonoid sample with low basal cGMP levels (0.0001 pmol/μg) did not exhibit significantly increased cGMP with linaclotide (Figure S1). Thus, while the quality of a colonoid derivation and baseline cGMP activity might impact responsiveness, colonoids derived from DGBI patients generally retain the ability to activate the GCC pathway.

3.3 | Linaclotide treatment expands the size of patient-derived colonoids

After confirming activity of the GCC response in DGBI-derived colonoids, we next investigated whether the downstream outcome of increased cGMP occurred in patient-derived colonoids. Increased cGMP levels lead to a net movement of intracellular fluid into the lumen of the gut. In a colonoid model, this would present as overall growth of the colonoid or swelling. Thus, DGBI patient-derived colonoids were treated with linaclotide and assessed for changes in colonoid size. Interestingly, patient-derived colonoids exhibited a significant increase in colonoid size following linaclotide treatment, compared to vehicle stimulated controls (Figure 2A,B). These findings are consistent with previous studies, and confirms that linaclotide stimulation of the GCC pathway induces luminal fluid exchange that may impact intestinal motility and improve constipation.[26,39,40] Furthermore, this finding supports that DGBI-derived colonoids exhibit fluid transport into the lumen in response to linaclotide stimulation.

3.4 | The intestinal microbiota from pediatric DGBI patients exhibit differences in SCFA-producing bacterial species

DGBI patients are characterized as having altered microbiota composition compared to the microbiota of healthy individuals.[41–45] In some studies, IBS patients have been noted to have variations in the proportions of SCFA-producing bacterial species compared to healthy patients.[43,46] Therefore, shotgun sequencing was employed to investigate differences in the community composition of SCFA-producing bacteria between DGBI patients and healthy controls using our pediatric cohort. While these analyses demonstrated variability within groups, differences in the community composition of SCFA-producing bacterial species that included *Prevotella* spp., *Ruminococcus* spp., *Bifidobacterium* spp., *Bacteroides* spp., *Clostridium* spp., *Streptococcus* spp., *Anaerostipes spp*, *A. muciniphila*, *F. prausnitzii*, *E. rectale*, *E. hallii*, *R. bromii*, *R. intestinalis* was observed between DGBI and healthy control samples for both the Bray–Curtis dissimilarity (Figure 3A, $R^2=0.04$, $F=2.20$, $p=0.011$) and Jaccard distance (Figure 3B, $R^2=0.03$, $F=1.73$, $p=0.013$). These findings provoked the hypothesis that SCFAs or SCFA-producing bacteria in the intestine may impact patient responses to linaclotide.

3.5 | Butyrate inhibits linaclotide-induced responses in patient-derived colonoids

To next determine whether the response to linaclotide may be altered due to production of SCFAs, patient-derived colonoids were exposed to the SCFA butyrate then treated with linaclotide. Consistent with earlier data, linaclotide treatment induced cGMP production in patient derived colonoids, and butyrate exposure alone had minimal effects on baseline cGMP levels (Figure 4A). However, cGMP induction was significantly blunted in linaclotide-treated samples that were exposed to butyrate (Figure 4A). To further characterize whether microbiota-derived butyrate could affect linaclotide responses, the colonoid swelling assay was performed. Consistent with reduction in intracellular cGMP levels, butyrate exposure significantly decreased colonoid size following linaclotide treatment (Figure 4B). In addition, the dampening effect of butyrate on linaclotide-induced luminal swelling was consistent across multiple DGBI patient-derived samples (Figure 4C). Taken together, these data indicate that microbiota-derived butyrate can potently suppress linaclotide induction of the GCC pathway and limit fluid secretion into the intestinal lumen, thus potentially reducing the efficacy of the drug on treating DGBI symptoms.

4 | DISCUSSION

DGBIs represent a significant health challenge in pediatric gastroenterology. These conditions are common, difficult to characterize, and extremely problematic to treat. Currently, diagnosis relies on excluding other chronic conditions such as inflammatory bowel disease, celiac disease, eosinophilic esophagitis, peptic ulcer disease, gallbladder conditions, and infections. Once these conditions are precluded, a clinical scoring platform such as the ROME questionnaire can be used to segregate the condition further, in hopes that better characterization leads to more individualized treatment. However, current therapeutics fail to ameliorate symptoms in a majority of pediatric patients, thus further investigation into mechanisms that may influence DGBI treatments is necessary. In this study, we demonstrate that patient-derived colonoids can

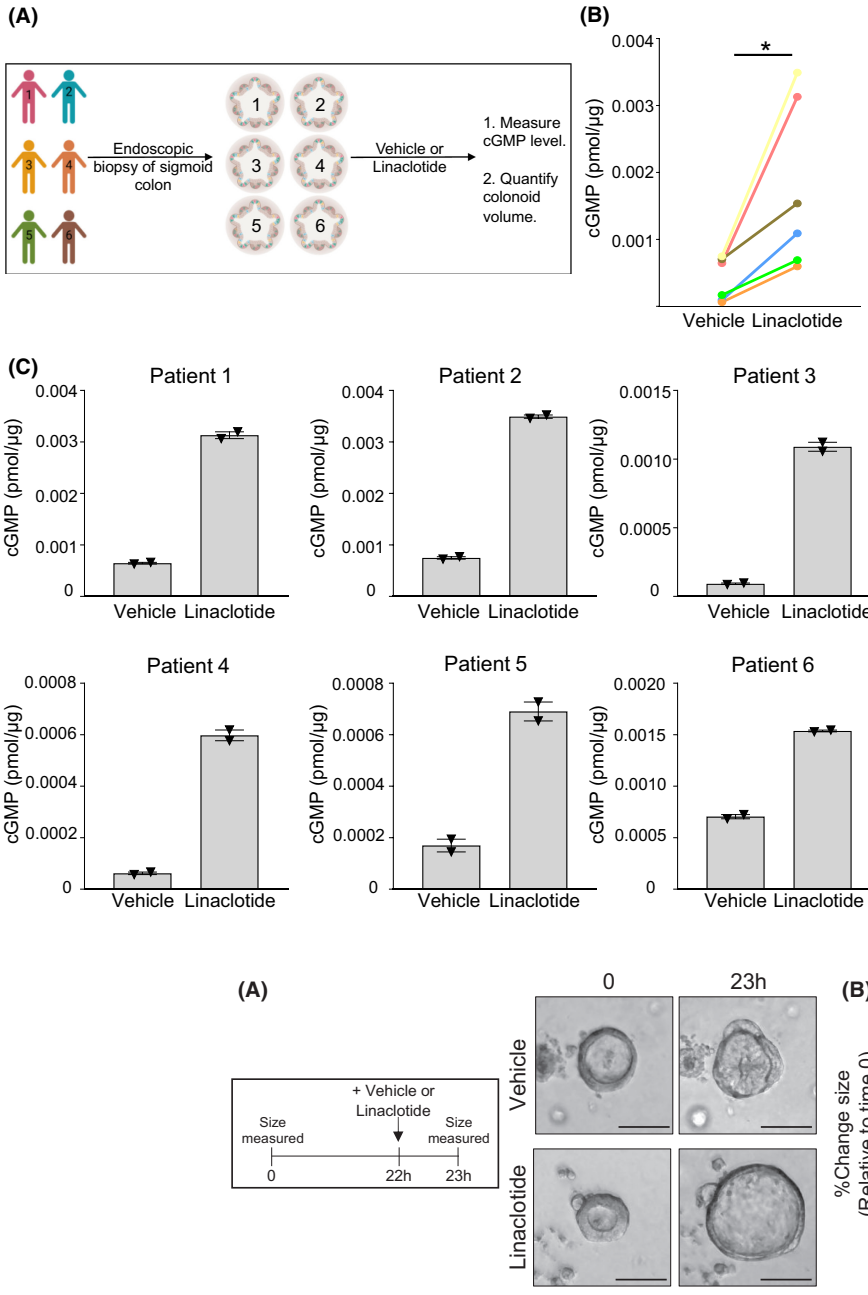


FIGURE 1 Linaclotide increases cGMP levels in colonoids derived from pediatric DGBI patients. (A) Experimental design. (B) Mean cGMP concentration by ELISA per µg protein in six pediatric patient-derived colonoids following linaclotide stimulation (250 nM). (C) cGMP concentration for each patient represented in (A) and (B). Two technical replicates for each treatment per patient. $^{*}p < 0.05$.

FIGURE 2 Linaclotide treatment expands the size of patient-derived colonoids. (A) Representative colonoid images pre (time 0) and post 1 h vehicle or 250 nM linaclotide stimulation. Scale bar, 100 µm. (B) Change in colonoid size following vehicle or linaclotide treatment, relative to time 0, 10 colonoids per condition. Data are representative of three independent experiments. $^{**}p < 0.001$.

be used to assess the effects of bacteria-derived metabolites on the linaclotide induction of human GCC activity. By employing this model, we have identified that the microbiota-derived metabolite butyrate dampens linaclotide response in human colonocytes, suggesting that butyrate may play a role in the diverse efficacy of these medications (Figure 4D). Microbiome analyses from a pediatric population with IBS demonstrated differences, as well as significant variability, in the community composition of SCFA-producing bacterial species compared to healthy control samples. This degree of variability in SCFA-producing bacterial species, and the effect of SCFAs on GCC activity shown here may help explain the discordant efficacy of linaclotide in clinical use. Furthermore, varied patient responsiveness to other GCC targeted therapeutics may also relate to microbiota composition, so incorporation of microbiota and metabolomic assessment is likely essential to improve precision medicine for DGBI patients.

The GCC pathway was first discovered as a target for heat stable *Escherichia coli* enterotoxin that leads to profound secretory diarrhea and has become an attractive goal for constipation therapeutics. This pathway is critical for homeostatic intestinal function and defense against enteric infection, highlighting the necessity for this pathway in intestinal health.[47–51] In addition, the second

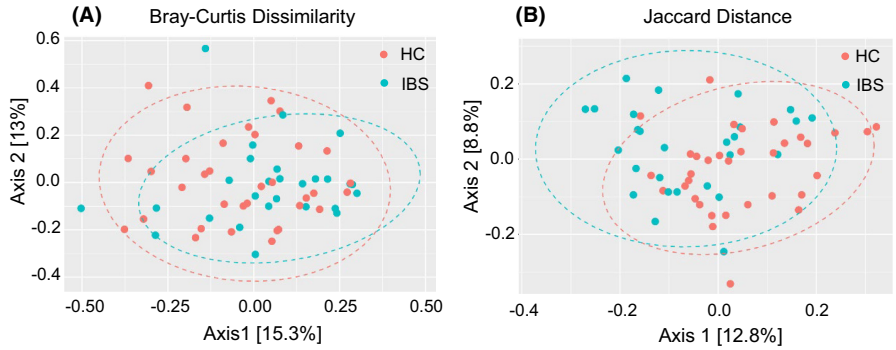
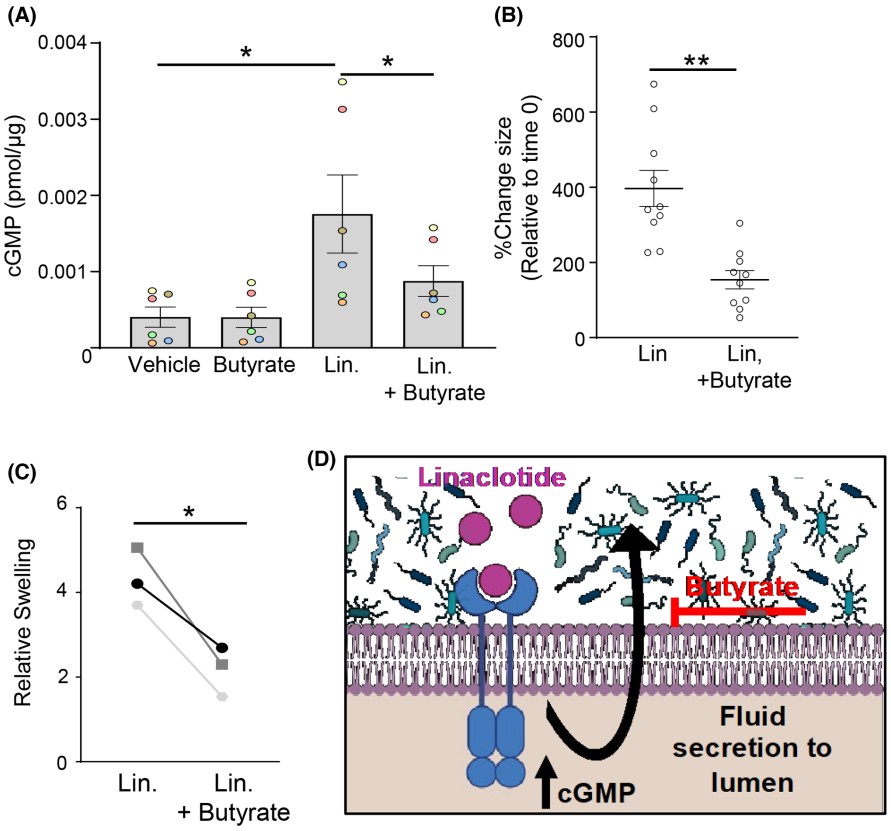


FIGURE 3 The intestinal microbiota from pediatric DGBI patients exhibit differences in SCFA-producing bacterial species. (A) Principal coordinate analysis (PCoA) performed on the Bray–Curtis Dissimilarity and (B) PCoA performed on the Jaccard Distance. Samples collected from $n=34$ healthy controls (HC) and $n=27$ pediatric IBS patients. Plots reflect reads retained for the following commensal bacterial populations predicted to produce SCFAs in the intestine: *Prevotella* spp., *Ruminococcus* spp., *Bifidobacterium* spp., *Bacteroides* spp., *Clostridium* spp., *Streptococcus* spp., *Anaerostipes spp*, *A. muciniphila*, *F. prausnitzii*, *E. rectale*, *E. hallii*, *R. bromii*, *R. intestinalis*. Results from PERMANOVA demonstrated differences in the Bray–Curtis dissimilarity ($R^2=0.04$, $F=2.20$, $p=0.011$) and Jaccard distance ($R^2=0.03$, $F=1.73$, $p=0.013$) between DGBI and HC samples.

FIGURE 4 Butyrate inhibits linaclotide-induced responses in patient-derived colonoids. (A) cGMP concentration from six different patient-derived colonoids exposed to vehicle or 1 mM butyrate (time 0), then stimulated after 22 h with 250 nM linaclotide for 1 h. Each point represents an individual patient. Data represent mean ± SEM. (B) Change in colonoid size following linaclotide treatment ± butyrate, relative to time 0, 10 colonoids per condition. (C) Average change in colonoid size for three different patients following linaclotide treatment ± butyrate, relative to time 0. (D) Butyrate produced by commensal bacteria dampens activation of the GCC pathway in human colonic epithelial cells in response to linaclotide treatment. $^*p<0.05$, $^{**}p<0.001$.



messenger, cGMP, has been suggested to be involved in peripheral nerve modulation.[52] This feature is especially appealing as a possible therapeutic for DGBIs as the pain/visceral hypersensitivity component of this disease is exceedingly difficult to manage. Unfortunately, since its release, the efficacy of linaclotide has been found to be limited and variable. In recent adult studies of IBS patients, response rates based on abdominal pain and constipation scores ranged widely and found to be low.[53] It is also important to note that the clinical relevancy of these indices remains controversial and has only been validated in a single study.[54] Furthermore, an independent randomized controlled study also demonstrated a low response rate ranging from 19% to 32% in composites scores of abdominal pain and constipation.[55] Collectively, systematic and meta-analysis reviews similarly indicate poor efficacy of linaclotide treatment.[56–58] However, DGBI patient-derived colonoids retain the ability to respond to linaclotide and activate intracellular cGMP production and luminal fluid exchange in vitro as noted in our study. Therefore, DGBI patients may not exhibit intrinsic defects in the GCC pathway, but instead external or environmental factors may impact cellular responsiveness to linaclotide.

The microbiome represents a diverse community of trillions of microorganisms harbored within the lumen of the gut. These microorganisms form an intricate ecosystem that continuously interacts with host intestinal cells. Therefore, the composition of the microbiome can have drastic effects on the host and has been associated with several chronic human diseases.[59] The microbiome of patients with IBS and constipation have been shown to be less diverse and have an overrepresentation of disruptive commensal bacterial communities and pathobionts.[43,45,60,61] Among these, the *Firmicutes/Bacteroidetes* ratio was reported to be increased in an IBS patient cohort, with an over-representation of *Clostridial* species, suggesting an elevated abundance of SCFA-producing bacteria.[62–65] However, a recent study of fecal metabolomics found that SCFAs are lower in another IBS cohort, highlighting the potential complexity of the microbiome and its effects.[25] Our study includes microbiome analysis of a cohort of 27 IBS pediatric patients. Within this cohort, we found differences in the variation of bacterial communities associated with SCFA metabolism between DGBI and healthy control patient samples (Figure 3). There was also extensive variability which may also contribute to the disparate efficacy of linaclotide clinically. Indeed, exposure of the SCFA butyrate blunted cGMP production and lumen fluid exchange following linaclotide treatment (Figure 4D), suggesting that the microbiota composition and metabolite production can influence linaclotide effectiveness. Microbiome composition was not included in the cohort used for functional patient-derived colonoid analyses (Table 1). Therefore, a limitation is that individual colonoid responses cannot be related to levels of SCFA-producing bacteria for these patients. However, based on our findings, this would be an interesting prospective approach for future study. Endoscopy is rarely performed in pediatric patients that do not have symptoms, thus limiting access to colonoids from pediatric "normal" patients. Statistically significant clinical differences were not observed between high versus low responders in the described patient-derived colonoids, so clinical diagnosis alone may not predict response magnitude. However, a larger cohort will help elucidate whether there is a pattern between level of linaclotide/butyrate responsiveness, clinical diagnosis, metabolites, and composition of the microbiota.

Patient-derived organoids are becoming common models for translational science and building blocks for drug studies and individualized medicine. Currently, no standardized colonoid model exists as a platform for drug development; however, several groups have commenced trials to evaluate clinical interventions. *Skovdahl* et al. utilized patient-derived colonoids maintained under hypoxic conditions, to better simulate intestinal physiology, as a model to evaluate the effectiveness of IBD therapeutics.[66] While X–Y plane measurements employed in the swelling assay are reflective of colonoid size, colonoids are 3-D structures and future work can employ 3D visualization of each colonoid using confocal Z-plane intervals. By improving, and better mirroring the physiologic state, organoids can be a powerful tool to individualize medicine. As part of our study, we created a model to test the impact of microbiome-derived metabolites on colon enterocyte directed therapies. We confirmed that the linaclotide-mediated effect on the GCC pathway was retained in colonoids derived from patients with a diagnosed DGBI. Further, butyrate exposed colonoids exhibited dampened responsiveness to linaclotide treatment as evidenced by reduce intracellular cGMP production and colonoid swelling (Figure 4D). These data suggest that patients with an overrepresentation of SCFA-producing bacteria may experience reduced efficacy to GCC pathway drugs. While not directly tested in our study, increased concentrations, or doses of linaclotide may be needed to overcome this effect and, thus, analysis of a patient's microbiome/metabolome could help direct therapeutic dosing. Although the mechanisms by which butyrate dampens linaclotide treatment remain unclear, the impact of butyrate on intestinal health and disease is well-studied. Beneficial effects of butyrate relate to anti-inflammatory properties, maintaining barrier homeostasis, and energy metabolism.[20] In contrast, butyrate has also been noted to impact gut motility and visceral sensitivity in rodents, although its overall influence remains controversial.[67,68] Additionally, butyrate and other SCFAs have been implicated in the function of the gut-brain axis.[69,70] While formal connections between butyrate and DGBIs remain to be determined, it is plausible that SCFAs play a significant role in DGBI pathogenesis.

Our study highlights the impact of microbiota on the efficacy of human therapeutics. Specifically, we show that butyrate, a common microbiota-derived metabolite, dampened the linaclotide-mediated effect on human enterocytes. These data provide a feasible explanation as to why linaclotide, despite targeting a promising pathway involved in the pathogenesis of DGBIs, has variable levels of efficacy. Collectively, our study emphasizes the importance of including microbiota and metabolite analyses as a factor in assessing treatment prognosis.

AUTHOR CONTRIBUTIONS

Theresa Alenghat, Alejandro Velez Lopez, Amanda Waddell, Neha Santucci, Daniel Castillo, and Nicholas J. Ollberding designed the studies and analyzed the data. Alejandro Velez Lopez, Amanda Waddell carried out organoid experiments. Amanda Waddell, Simona Antonacci, Emily M. Eshleman, and Lee A. Denson provided technical and clinical expertise. Theresa Alenghat, Emily M. Eshleman, and Alejandro Velez Lopez wrote the manuscript.

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CONFLICT OF INTEREST STATEMENT

The authors have no competing interests.

DATA AVAILABILITY STATEMENT

The data that support the findings of this study are available from the corresponding author upon reasonable request.

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SUPPORTING INFORMATION

Additional supporting information can be found online in the Supporting Information section at the end of this article.

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