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Sphingosine-1-Phosphate Signaling and Metabolism Gene Signature in Pediatric Inflammatory Bowel Disease: A Matched-case Control Pilot Study

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Goal: The aim of this study was to investigate gene expression levels of proteins involved in sphingosine-1-phosphate (S1P) metabolism and signaling in a pediatric inflammatory bowel disease (IBD) patient population.

Background: IBD is a debilitating disease affecting 0.4% of the US population. The incidence of IBD in childhood is rising. Identifying effective targeted therapies that can be used safely in young patients and developing tools for selecting specific candidates for targeted therapies are important goals. Clinical IBD trials now underway target S1PR1, a receptor for the pro-inflammatory sphingolipid S1P. However, circulating and tissue sphingolipid levels and S1P-related gene expression have not been characterized in pediatric IBD.

Methods: Pediatric IBD patients and controls were recruited in a four-site study. Patients received a clinical score using PUCAI or PCDAI evaluation. Colon biopsies were collected during endoscopy. Gene expression was measured by qRT-PCR. Plasma and gut tissue sphingolipids were measured by LC-MS/MS.

Results: Genes of S1P synthesis (SPHK1, SPHK2), degradation (SGPL1), and signaling (S1PR1, S1PR2, and S1PR4) were significantly upregulated in colon biopsies of IBD patients with moderate/severe symptoms compared with controls or patients in remission. Tissue ceramide, dihydroceramide, and ceramide-1-phosphate (C1P) levels were significantly elevated in IBD patients compared with controls.

Conclusions: A signature of elevated S1P-related gene expression in colon tissues of pediatric IBD patients correlates with active disease and normalizes in remission. Biopsied gut tissue from symptomatic IBD patients contains high levels of pro-apoptotic and pro-inflammatory sphingolipids. A combined analysis of gut tissue sphingolipid profiles with this S1P-related gene signature may be useful for monitoring response to conventional therapy.

Key Words: gene expression, inflammatory bowel disease, pediatric, sphingolipids, sphingosine-1-phosphate

INTRODUCTION

Inflammatory bowel disease (IBD) is a chronic inflammatory condition of the gastrointestinal (GI) system that can principally manifest as either ulcerative colitis (UC) or Crohn's disease (CD). Currently, IBD affects 0.3%–0.4% of the US population.[1] Recent epidemiological studies reviewing worldwide data demonstrate an increasing incidence of pediatric IBD, mostly due to increased incidence of CD.[2] IBD can have grave consequences in the pediatric population. In addition

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Abbreviations: ANOVA, non-parametric analysis of variance; CD, Crohn's Disease; C1P, ceramide-1-phosphate; CRP, C reactive protein; DHCer, dihydroceramide; DSS, dextran sodium sulfate; ESR, erythrocyte sedimentation rate; FDA, Food and Drug Administration; GI, gastrointestinal; H&E, hematoxylin & eosin; IBD, inflammatory bowel disease; IHC, immunohistochemistry; LC/MS/MS, liquid chromatography mass spectrometry; LPP, lipid phosphate phosphatase; LSM, least square means; MRM, multiple reaction monitoring; PCDAI, pediatric Crohn's Disease activity index; PUCAI, pediatric ulcerative colitis activity index; QIA, quantitative image analysis; qRT-PCR, quantitative reverse transcriptase polymerase chain reaction; S1P, sphingosine-1-phosphate; SGPP, sphingosine phosphate phosphatase; SPL, sphingosine phosphate lyase; S1PR, S1P receptor; TNBS, 2,4,6-trinitrobenzene sulfonic acid; UC, ulcerative colitis; WSI, whole slide imaging.

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to the symptoms of abdominal pain, weight loss, bloody diarrhea, and associated anemia that can affect patients of all ages, pediatric patients can also suffer from stunted growth, delayed puberty, and other extra-intestinal manifestations.[3–7] Inflammatory bowel disease presenting in infancy or early childhood can be associated with genetic abnormalities such as IL-10 deficiency or mutations in key genes involved in immune function, autophagy, or other innate immune processes. Present treatments for children including corticosteroids, anti-TNFα, and other immunosuppressive drugs may result in severe and long-term side effects. Thus, novel targeted therapeutic strategies are needed that can be administered safely and for long time periods to children with IBD.

Clinical IBD trials now underway target sphingosine-1-phosphate (S1P) receptor 1 (S1PR1). S1P is a pro-inflammatory sphingolipid found at high levels in the blood and present at lower levels in most tissues. S1P signaling controls many cellular activities including cell survival, differentiation, migration, and activation. Its actions are mediated in part by serving as a ligand for 5 G protein-coupled receptors (S1PR1-5) and by acting intracellularly on key transcriptional regulators including activation of the pro-inflammatory transcription factor NFκB and inhibition of epigenetic regulators of gene expression called histone deacetylases (HDACs). S1P signaling through S1PR1 controls lymphocyte egress from peripheral lymphoid organs, and S1PR1 antagonists prevent lymphocyte entry into inflamed tissues.

Evidence supporting the involvement of aberrant S1P signaling in IBD is primarily derived from preclinical models of colitis. Results of these studies suggest that inhibiting S1P production or interaction with S1PRs can effectively attenuate disease severity and colitis pathology in multiple different models of colitis.[8–13]

Steady-state levels of S1P are determined not only by its rate of synthesis by the sphingosine kinase enzymes (SPHK1 and SPHK2) but also by its rate of degradation. Once synthesized, S1P can be dephosphorylated by the S1P-specific phosphatases (SGPP1 and SGPP2) and by lipid phosphate phosphatases (LPPs) or irreversibly degraded by the enzyme sphingosine phosphate lyase (SPL), encoded by the SGPL1 gene.

Despite emerging data implicating the role of aberrant S1P signaling in preclinical models of IBD, clinical data linking S1P signaling and metabolism to the pathophysiology of IBD are sparse. Specifically, our knowledge of the impact of IBD on intestinal mucosal S1P-related gene expression is limited, and to our knowledge, no studies addressing this in a pediatric IBD population have been reported. Most importantly, we do not yet know how the sphingolipidome of the gut or the plasma might be affected by IBD nor how changes in the expression of genes of sphingolipid signalling and metabolism might contribute to such changes, particularly in a pediatric population, wherein these factors have not been studied.

In this pilot study, the focus was to address these questions. Our findings indicate a landscape of dynamic changes in S1P biosynthesis, turnover, and receptor signaling and an alteration in the ratio of S1P to ceramides in severe pediatric IBD patients. These findings could have significant biological and clinical consequences and suggest that further study of sphingolipid signaling and metabolism in pediatric IBD and clinical testing of Ozanimod in a pediatric IBD population are warranted.

MATERIALS AND METHODS

Patient Selection and Clinical Scoring

Patients were selected by a designated pediatric gastroenterologist from among a population of pediatric patients visiting the pediatric gastroenterology clinic at one of four enrollment sites. Inclusion criteria were patients 1–23 years of age undergoing colonoscopy and/or upper endoscopy for IBD, to rule out IBD, or for other medically indicated conditions. Patients with IBD and patients with no evidence of disease based on microscopic pathology reports were included in the study and assigned to the appropriate group (IBD or control). Exclusion criteria were IBD patients with colectomy and patients diagnosed with another form of colitis (e.g., immunodeficiency-associated). The control group consisted of patients without a prior gastroenterology diagnosis and found to have no evidence of gross pathology on endoscopic inspection or microscopic intestinal pathology on final biopsy report. The collaborating pediatric gastroenterologists and study coordinators selected the patients, obtained informed consent, and performed the endoscopies and biopsies. At the time of clinic evaluation, which occurred no more than one month before endoscopy, each IBD patient was given a pediatric ulcerative colitis activity index (PUCAI) or pediatric Crohn's disease activity index (PCDAI) score and was stratified into the following 3 IBD disease categories: 1) inactive/remission (PUCAI or PCDAI <10); 2) mild (PCDAI 11–30, PUCAI 10–34); and 3) moderate/severe (PCDAI >30, PUCAI >35).[14,15]

Sample Collection

For the gene expression analysis, only ascending colon biopsies were evaluated. We restricted our gene expression analysis to biopsies from ascending colon in order to reduce variations in gene expression associated with anatomical location. Ascending colon was chosen due to the availability of affected biopsies from this site. Diagnosis was confirmed by the pathologist at each collecting center. Biopsies were taken, placed immediately in RNAlater (Life Technologies, Carlsbad, CA), and stored at −80°C until processing. For tissue metabolite analysis, biopsies were taken from the cecum, ileocecal valve, transverse colon, descending colon, sigmoid colon, and the rectum. Site pathology scores were used to differentiate normal and active disease sites. Blood was collected by venipuncture into EDTA

tubes, placed on ice, and subjected to mild centrifugation at 4°C within 2 hours of collection to isolate plasma. Plasma was immediately frozen and maintained at −80°C until analysis.

Histology

Tissues embedded in paraffin were sectioned to 4 μm, mounted on glass slides, and stained with Mayer's hematoxylin and eosin (H&E) for pathologic analysis. Slides were scanned on an Aperio AT2 slide scanner (Leica Biosystems, Vista, CA) and viewed using ImageScope software (Leica Biosystems, Vista, CA). Regions of interest were selected and digitally captured by the pathologist (ADB).

Immunohistochemistry (IHC)

Paraffin blocks were sectioned and mounted on glass slides. All tissue sections were subjected to antigen retrieval which was performed for 45 minutes using a citrate buffer at pH 6.0 in a Decloaking Chamber (Biocare Medical, Concord, CA) at 125°C and 15 psi. Tissues were blocked with normal goat serum, then incubated with polyclonal rabbit antimurine SPL (1:1000)[16] primary antibodies overnight at room temperature in a humidified chamber, followed by a biotinylated goat antirabbit secondary antibody (1:1000). The Vectastain ABC Kit Elite Kit and a diaminobenzidine Peroxidase Substrate Kit (Vector Labs, Burlingame, CA) were used for amplification and visualization of signal, respectively. Slides were scanned as described above.

Quantitative Image Analysis and Density:Intensity Graphs of SPL IHC

The quantitative analysis of SPL expression is based on slides from normal and diseased cases stained with the antibody for mSPL. All slides were scanned and digitized using the Aperio ScanScope AT2 to capture digital whole slide imaging (WSI) using the 20X objective lens at 0.5 μm/pixel and stored in the Aperio Spectrum version 11, customized for laboratory workflow. Quantitative image analysis (QIA) was performed using Spectrum version 11, based on the Food and Drug Administration–approved (FDA) algorithms supplied by the manufacturer with modifications.[17] The digital whole slide imaging was analyzed using Aperio ImageScope software (https://www.leicabiosystems.com/digital-pathology/manage/aperio-imagescope/). The data for each slide were automatically stored in the Aperio Spectrum database. For accurate representation of the number of positive stained cells, a density:intensity graph[17] was created. After the algorithm was executed, the data were exported and analyzed. The data represent the frequency of cells in the annotation with a given intensity, binned in 240. Graphs of the data give a more detailed view of the intensity data than percent positive cells. To compare data from multiple annotations on the same axis, a line plot was used instead of a column-based graph.

Plasma and Tissue Sphingolipid Extraction

Frozen biopsied tissue samples (2–20 mg) were bead homogenized in 700 μL of 50 mM Tris-Cl buffer containing 0.25 M sucrose, 25 mM KCl, 0.5 mM EDTA, pH 7.4, using the FastPrep 120 cell disrupter (Thermo Fisher, Waltham, MA).

Plasma and tissue homogenates (100 μL) were spiked with 10 μL of internal standard mix and subsequently extracted with protocols described in reference (18). Internal standard mix was composed of sphingosine (d17:1, 3.5 μM final), ceramide (d18:1/C17:0; 9 μM final), D-*erythro*-sphingosine-d7-1-phosphate (3.2 μM final), ceramide-1-phosphate (d18:1/C12:0; 2.3 μM final), sphingomyelin (d18:1/C12:0, 2.5 μM final), and lyso-sphingomyelin (d17:1; 2.2 μM final) and were purchased from Avanti polar lipids (Alabaster, AL).

Liquid Chromatography Mass Spectrometry (LC-MS/MS) Detection of Sphingolipids

Detection system was composed of Agilent 1290 binary gradient ultra-high pressure chromatography system, coupled with Agilent 6490 triple quadrupole mass spectrometer. Sphingolipid metabolites were resolved on a Zorbax RRHD Eclipse Plus C18 column (2.1 × 50 mm; 1.8 micron) fitted with a precolumn composed of identical matrix. Samples were eluted from the column using a binary gradient composed of mobile phase A (2 mM ammonium formate and 0.2% formic acid in 18 mΩ water) and mobile phase B (1 mM ammonium formate and 0.2% formic acid in MS-grade methanol) at a flow rate of 1 mL/min. Initial composition was 75% B and was increased to 80%, 85%, and to 100% at 3, 3.1, and 10 minutes, respectively. Gradient was maintained at 100% B until 8.5 minutes and subsequently changed back to initial conditions until the end of the run at 10 minutes. Analysis was carried out using multiple-reaction-monitoring (MRM) mode. Specific MRM transitions and optimized conditions used for sphingolipid analysis are listed in the Supplementary Data section. For all compounds, the general source settings in the positive ionization modes were as follows: gas temperature 200°C; gas flow, 14 L min^{-1}; nebulizer 20 psi; sheath gas temperature 250°C; sheath gas flow 11 L min^{-1}; capillary voltage 3000 V; and nozzle voltage 0 V. The fragmentor voltage of 380 V and a dwell time of 15 minutes were used for all mass transitions, and both Q1 and Q3 resolutions were set to nominal mass unit resolution.

Colonic S1P-related Gene Expression

Gene expression analyses by qRT-PCR were conducted as we described previously.[19] Colonic biopsies were homogenized using a tissue homogenizer (Omni International, Kennesaw, GA) followed by total RNA extraction using the RNeasy Mini Kit (Qiagen, Valencia, CA) according to the manufacturer's protocol. RNA quality and quantity were analyzed by spectrophotometry using a Nanodrop (Thermo Fisher Scientific, Hanover Park, IL). Complementary DNA was synthesized

with the Superscript III kit (Life Technologies, Carlsbad, CA) according to the manufacturer's instructions. RT-PCR reactions were carried out in a 384-well optical plate on an ABI 7900HT Thermocycler (Applied Biosystems, Foster City, CA) in conjunction with PowerSYBR green (Life Technologies, Carlsbad, CA). Specific primers are listed in Table 1. The primer sets used were all tested using serial dilutions of complementary DNA to ensure efficiency in the 90%–110% range. All primers were rigorously tested and met MIQE criteria.[20] All qRT-PCR reactions were run in triplicate. All data were subsequently analyzed by the ΔCt method. The colonic mRNA expression levels of ten S1P-related genes were measured by qRT-PCR and normalized to the housekeeping gene *HPRT1*. Results compared among the three patient groups. These genes included the following: *SPHK1* and *SPHK2*; the two enzymes involved in the biosynthesis of S1P,[21] *SGPP1* and *SGPP2*; the two S1P-specific phosphatases;[22] *SGPL1*, which encodes the main enzyme responsible for S1P catabolism;[23] all 5 of S1P's cognate cell surface receptors *S1PR*1-5;[24] and *ORMDL3*, a protein inhibitor of sphingolipid biosynthesis that has been implicated as a genetic risk factor in IBD.[25]

Acquisition of Microarray Dataset and Data Processing

The dataset GSE10616 was retrieved from the Gene Expression Omnibus (GEO) database repository[26, 27]. This dataset is based on the platform of GPL5760 Affymetrix GeneChip Human Genome U133 Plus 2.0 Array and included colon tissues from pediatric patients: 11 controls, 14 CD, and 10 UC. All the colon samples were from ascending colon except 1 from a UC sample, which was from the rectum. The dataset GSE10616 was originally published by Kugathasan et al.[28] The GSE10616 dataset was analyzed using the GEO2R tool to identify differentially expressed mRNA between Controls and CD or Controls and UC.[9, 26, 27]

Statistical Analysis

Examination of the univariate distributions for each measure was tested using Shapiro–Wilks W-statistics, and log transformations were made to normalize skewed distributions. Descriptive statistics included means and standard deviations for continuous data and frequency distributions for categorical data. Generalized linear model procedure was used to calculate the least square means (LSM) of the controls and IBD patient groups stratified by disease severity; the differences in LSMs were asssessed. For the secondary analysis of association between outcomes and disease severity, a covariance model adjusted for effects of age, gender, medication use, and anatomical sites of the biopsied materials was used. Additionally, Pearson correlations analysis was performed to determine the associations between intestinal gene expression and plasma metabolites. A significance level of 0.05 was used for all statistical tests. The statistical analysis was performed using SAS version 9.4 software (SAS Institute, Cary, NC).

ETHICAL CONSIDERATIONS

This study was approved by the Institutional Review Boards (IRB) of UCSF Benioff Children's Hospital Oakland, UCSF Benioff Children's Hospital San Francisco, UT Southwestern Medical Center, and Lucile Packard Children's Hospital at Stanford. Informed consent from patients or their guardians was obtained, and tissues/plasma were handled and processed according to the guidelines outlined in the IRB protocol.

RESULTS

Table 1 shows the subject profile of pediatric IBD study participants. Owing to the small size of biopsied tissue samples, the samples generated from cohort 1 were used for the analysis of gut tissue S1P-related gene expression and plasma sphingolipid profiling. Biopsied materials collected from cohort 2 were utilized for analysis of gut tissue sphingolipid profiling. Our control cohort was composed of pediatric subjects who underwent diagnostic colonoscopy to rule out IBD and who were found to have no evidence of IBD or other significant pathology.

The first cohort was composed of 17 controls and 34 IBD patients, 19 subjects of which were diagnosed with UC, and the remaining subjects were diagnosed with CD. Gender composition and age differences between the two groups were not statistically significant (Table 1). Among the IBD patients, the numbers of subjects in each category of disease severity including remission (PUCAI or PCDAI <10), mild (PCDAI 11–30, PUCAI 10–34), and moderate/severe disease (PCDAI >30, PUCAI >35) were 16, 7 and 8, respectively. Approximately 20 of the 34 IBD patients were receiving IBD medications (e.g., remicade), whereas 4 of the 34 subjects were receiving steroid medications, and 3 of the 34 of the subjects were receiving antibiotics. All of the biopsied materials included in this analysis were obtained from ascending colon to avoid introducing differences in gene expression related to anatomical location in the gut.[29]

Table 2 shows the primer pair sequences used for detection of genes required for S1P synthesis (*SPHK1*, *SPHK2*, *ORMDL3*), dephosphorylation (*SGPP1*, *SGPP2*), and degradation (*SGPL1*). Because of high variability and differences in sample sizes, median, range, and 25–75% interquartile ranges are displayed as a box plot (Fig. 1).

Significant increases were observed in both genes involved in S1P biosynthesis (*SPHK1* and *SPHK2*) in IBD patients with moderate/severe symptoms as compared with healthy controls (Fig. 1). No significant differences in *SPHK1* expression were observed between controls and IBD patients in remission or with mild disease (Fig. 1). Comparison of *SPHK1* expression

TABLE 1: Study Participant Profile

	Cohort 1 (Gene Expression and Plasma Metabolomics)				Cohort 2 (GI tissue metabolomics)			
		IBD (N = 34)				IBD (N = 11)		
	Control (N = 17)	UC (N = 19)	CD (N = 15)	IBD Combined	Control (N = 6)	UC (N = 2)	CD (N = 9)	IBD Combined
Gender (F)	6	10	5	15	3	0	4	4
Age (yrs)	12.6 ± 1.4	15.6 ± 0.8	15.9 ± 1.0	15.8 ± 0.7[t]	14.2 ± 1.5	15 ± 1	14.2 ± 1.0	14.4 ± 0.8
Inflammatory markers								
CRP (mg/L)	1.4 ± 0.5 (N = 14)	2.4 ± 0.9 (N = 17)	7.1 ± 2.5 (N = 15)	4.8 ± 1.4 (N = 32)	<0.29 (N = 4)	<0.29 (N = 1)	<0.29 (N = 6)	<0.29 (N = 7)
ESR (mm/Hr)	10.3 ± 3.6 (N = 7)	19.8 ± 3.4 (N = 18)	21.7 ± 7.0 (N = 10)	20.6 ± 3.5 (N = 28)	4.2 ± 1.0 (N = 3)	14 (N = 1)	20.6 ± 3.3 (N = 5)	19.5 ± 2.9 (N = 6)
Severity Category (No. Subjects)[a]								
1 Remission		9	7	16			4	4
2 Mild		2	5	7			5	5
3 Moderate/Severe		5	3	8		2		2
Medication Use (No. of Subjects taking meds)[b]								
Antibiotics	1	0	3	3	0	0	1	1
Steroids	0	3	1	4	0	1	0	1
IBD Meds	0	11	10	21	0	2	9	11
Tissue Site (No. of tissue samples)[c]								
1. Cecum					1		2+	2+
2. Ileocecal Valve							1+	1+
3. Ascending colon	17	19	15	34				
4. Transverse						1+	1+	2+
5. Descending Colon					2		2+	2+
6. Sigmoid					3	1+	1+	2+
7. Rectum					4	2+	6+/1−	8+/1−

Results are expressed as mean ± SEM for continuous variables.

[a] Severity categories are defined as 1) remission (PUCAI or PCDAI <10), 2) mild (PCDAI 11–30, PUCAI 10–34), 3) moderate/severe (PCDAI >30, PUCAI >35).

[b] Study subjects on medication at the time of biopsy. Steroid use included use of prednisone. IBD meds included use of Remicade, Cimzia, Methotrexate, Azathioprine, Humira, and Lialda. One control subject was on amitriptyline, dicyclomine, fluoxetine, lisinopril, montelukast, and omeprazole.

[c] Among participants of the cohort 2, multiple tissue biopsies were taken from some of the subjects. Each biopsied sample was analyzed for disease pathology and active disease sites are noted with + and those that were benign are noted as −.

between controls and symptomatic (mild, moderate or severe) CD and UC groups showed that only symptomatic UC patients have significant increases in *SPHK1* expression relative to non-IBD controls (Fig. 1).

Median *SPHK2* expression levels were also higher among moderate/severe IBD participants as compared with non-IBD controls. Median *SPHK2* levels were similar among IBD patients in remission or with mild disease compared with controls, suggesting that *SPHK1* and *SPHK2* expression levels are specifically increased in moderate/severe IBD patients.

We next examined potential effects of disease severity and disease subtype on the expression levels of genes required for S1P degradation. As shown in Fig. 2, *SGPL1* gene expression was also significantly upregulated in IBD patients with moderate/severe disease but not in IBD patients in remission and with mild disease relative to controls (Fig. 2). In contrast to *SGPL1*, no significant differences in either *SGPP1* or *SGPP2* gene expression were observed. However, *SGPP2* expression trended higher in symptomatic UC patients relative to controls. Given the magnitude of the increase in *SGPL1* gene expression relative to changes in *SGPP2* expression, our data suggest that irreversible degradation of S1P may be an important component of the active IBD disease process.

To ascertain whether *SGPL1* gene expression elevation correlated with increased SPL protein expression in IBD colon tissues, immunohistochemistory (IHC) was performed on fixed colon tissues of control subjects (Fig. 3A), patients with moderate/severe (Fig. 3B–D), and quiescent IBD in remission (Fig. 3E). Moderate/severe IBD colons exhibited typical changes, including crypt regeneration with distorted crypts characteristic of regenerating epithelium (Fig. 3B), active cryptitis with infiltration of neutrophils (Fig. 3C) and abscess

TABLE 2: Primer Sequences Used for qRT-PCR

GENES	FORWARD PRIMER	REVERSE PRIMER	Fx
HPRT1	TGACACTGGCAAAACAATGCA	GGTCCTTTTCACCAGCAAGCT	HK
SPHK1	CCTTCACGCTGATGCTCACT	CGTTCACCACCTCGTGCAT	Synth
SPHK2	TAGGCTGGTAGGAGCAAGGA	TTTCCGGAAAGGGATCT	Synth
SGPP1	CGCTGGCAGTACCCTCTTAT	GAAGCCCGATGATGATGAAT	Dephos
SGPP2	CATTTGTGTTGGGACTGGTG	TATGACACACACGGGGAAGA	Dephos
S1PR1	CCTGCTTGAGCGAGGCTG	GAGAGCCTTCACTGGCTTCA	Rec
S1PR2	GCCTAGCCAGTTCTGTGAAAGCC	CCTGGACCTTGTTGGGGTTC	Rec
S1PR3	CCCATCTGGCATTCGAGCG	GAAAAAGGGCTCCTCCGTCG	Rec
S1PR4	CTCTCTGAGGCCAAGGGACA	ACCATCCACACGCAAGACTG	Rec
SGPL1	AGGCCCTAGGGGAAATGGAT	TCAGGAGTCCCTGAGATGGG	Degrad
ORMDL3	AGGATGAATGTGGGCACAGC	TACATGCCCATGTTGTGAATGA	Synth

Abbreviations: *HPRT1*, Hypoxanthine-guanine phosphoribosyltransferase; *SPHK1*, Sphingosine-1-phosphate kinase 1; *SPHK2*, Sphingosine 1-phosphate kinase 2; *SGPP1*, Sphingosine 1-phosphate phosphatase 1; *SGPP2*, Sphingosine 1-phosphate phosphatase 2; *S1PR1*, Sphingosine 1-phosphate receptor 1; *S1PR2*, Sphingosine 1-phosphate receptor 2; *S1PR3*, Sphingosine 1-phosphate receptor 3; *S1PR4*, Sphingosine 1-phosphate receptor 4, *SGPL1*, Sphingosine 1-phosphate lyase); *ORMDL3*, Sphingolipid Biosynthesis Regulator 3; Fx, Function; HK- house-keeping; Synth, Synthesis; Dephos, Dephosphorylation; Degrad, Degradation; Rec, Receptor

formation, with neutrophils filling and expanding the crypt lumen and interruption of epithelial continuity (Fig. 3D). Quiescent IBD colon tissues exhibited mild crypt drop out, edematous lamina propria, and plasma cell infiltrates without active colitis (Fig. 3E). As expected, SPL protein expression determined by IHC was high in the differentiated enterocytes of healthy colon crypts compared with the interstitium (Fig. 3F). An overall net increase in SPL expression was observed in the moderate/severe disease biopsies (Fig. 3G-I) compared with controls (Fig. 3F) and quiescent disease biopsies (Fig. 3J), but with varying expression depending on the degree of regeneration vs active inflammation. Interestingly, regenerating crypts were notable for higher SPL signal compared with healthy crypts (Fig. 3G). Areas of active cryptitis with evidence of neutrophil infiltration also stained positively for SPL (Fig. 3H). Similarly, SPL expression was evident in neutrophils present in crypt abscesses (Fig. 3I). Quiescent IHC samples with mild crypt dropout showed less intense SPL staining patterns (Fig. 3J). Chronic inflammatory cells, lymphocytes, and plasma cells also express SPL, and this is increased in the disease tissues compared with control. We next performed a quantitative analysis of SPL protein expression based on our IHC findings by scanning and digitalizing the IHC whole slide images, which were then subjected to quantitative image analysis to generate a density:intensity graph of SPL expression. As shown in Fig. 3K, SPL expression is shifted to the left in IBD, indicating more intense SPL staining in IBD compared with control samples. This pattern is also evident when results were analyzed by IBD disease subcategory (i.e., CD and UC), as shown in Fig. 3L.

We next examined the expression levels of all 5 S1PRs (Fig. 4). *S1PR5* expression was below the detection limit, consistent with its restricted expression in only a few cell types, including neuronal tissues and natural killer cells.[30, 31] A previous study showed that S1PR1 colocalizes with blood vessels in colonic tissue samples of patients suffering from IBD.[32] Median *S1PR1* gene expression levels among moderate/severe IBD patients were higher than non-IBD controls and IBD patients in remission (Fig. 4, panel A). No significant differences were noted between controls and IBD patients in remission or in the mild disease category (Fig. 4, panel A). The elevation of *S1PR1* was most prominent among symptomatic UC patients. Values among CD patients were similar to controls. S1PR2 is ubiquitously expressed and plays a critical role in exacerbation of inflammation and colon length shortening in response to TNBS challenge in mice.[9] *S1PR2* gene expression was highly upregulated in moderate/severe IBD relative to control and IBD patients in remission (Fig. 4, panel B). Again, levels in mild IBD were essentially unchanged relative to controls. Comparisons between control, UC, and CD patients trended higher in symptomatic (mild/moderate/severe) UC patients only (Fig. 4, panel B). *S1PR3* expression was similar between the groups analysed (Fig. 4, panel C). *S1PR4* is expressed predominantly in immune cells such as T cells and dendritic cells and is thought to modulate IL-17 production and IL-27 production.[33] As shown in Fig. 4D, median expression of *S1PR4* in moderate/severe IBD was also significantly upregulated relative to controls and IBD patients in remission. No differences were noted in IBD patients with mild disease compared with controls. Among the symptomatic IBD patients, only the UC subgroup showed significantly increased *S1PR4* expression (Fig. 4D). These data suggest that upregulation of both *S1PR2* and *S1PR4* is a dominant pathway that may serve as biomarkers of moderate/severe IBD.

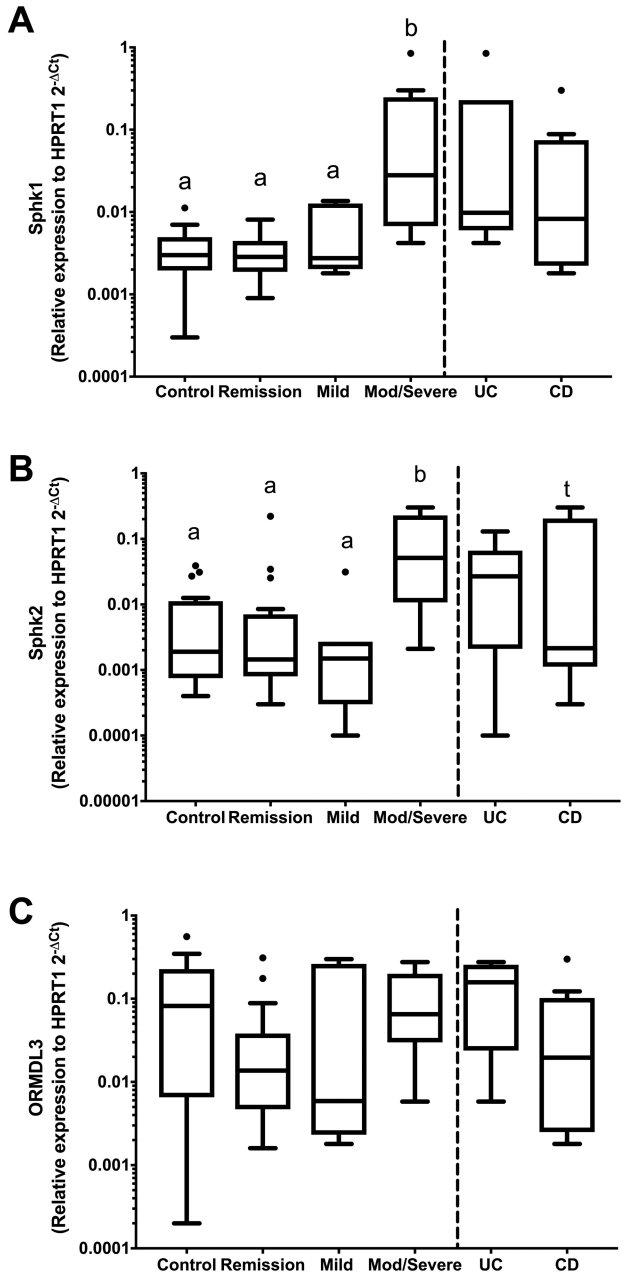

FIGURE 1. Inflammatory bowel disease (IBD) patients with moderate/severe disease severity express higher SPHK1 and SPHK2 expression in ascending colon. Gene expression levels of SPHK1, SPHK2, and ORMDL3 in biopsied ascending colon samples were quantified relative to HPRT1 gene expression by quantitative reverse transcriptase polymerase chain reaction (qRT-PCR), using the $2^{-\Delta\Delta CT}$ method. Results were plotted using a Tukey box plot. Whiskers in the Tukey box plot show the full range of values, with box depicting 25–75 interquartile ranges. The line within the box shows the group median. Grubbs test was used to identify outliers shown as individual values. Generalized estimated equation models were used to determine statistical differences between control (N = 17), IBD (UC+CD) patients in remission (N = 16), those with mild disease (N = 6), and those with moderate/severe disease (moderate/severe; N = 8). Results show that SPHK1 and SPHK2 expression levels were significantly ($P = 0.0001$) higher in pediatric IBD patients with moderate/severe symptoms as compared with non-IBD controls. To compare the expression levels of non-IBD controls with ulcerative colitis (UC) and Crohn's Disease (CD) patients, median values from symptomatic (mild+moderate/severe) UC and CD patient were compared. Differences between control, UC, and CD were analyzed by nonparametric analysis of variance (Kruskal-Willis) and post hoc tests were used to adjust for multiple comparisons. Although median expression levels of SPHK1 and SPHK2 were highest among UC patients, these differences were not statistically significant.

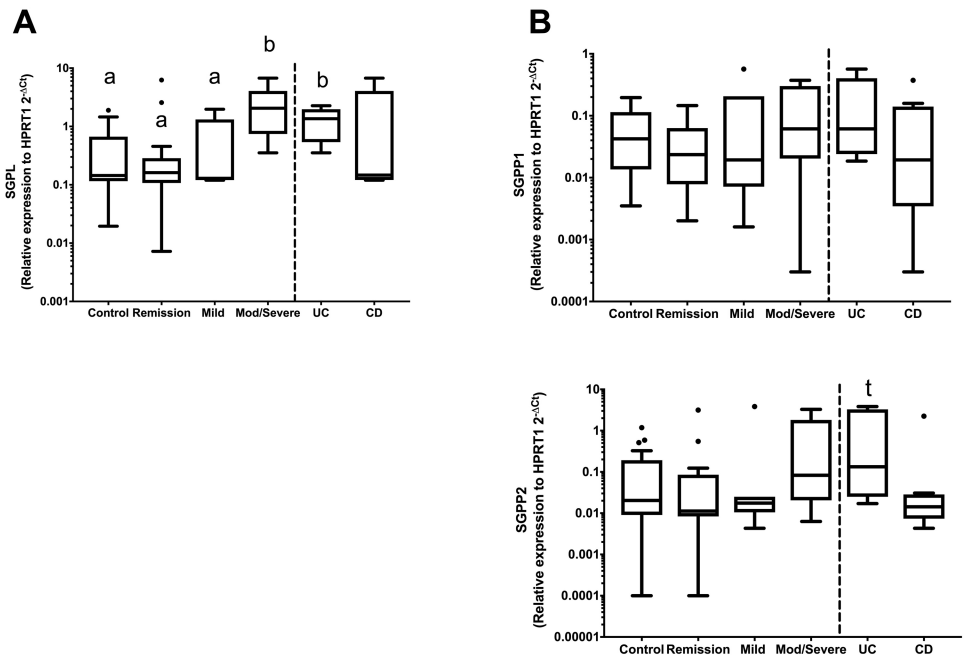

FIGURE 2. Significant increase in SGPL1 expression is associated with moderate/severe inflammatory bowel disease (IBD) and represents the major pathway for sphingosine-1-phosphate (S1P) removal. S1P can either be irreversibly degraded by sphingosine phosphate lyase (SPL), which is encoded by SGPL1 (A) or reversibly dephosphorylated by sphingosine phosphate phosphatases (Sgpp1 or Sgpp2) (B). Results show a significant 15-fold increase in median SGPL1 expression among pediatric IBD patients in moderate/severe disease category (N = 8; $P = 0.002$) relative to non-IBD controls (N = 17; Panel A). SGPL1 levels among IBD patients in remission (N = 16) or with mild disease (N = 7) remained similar to control values (A). In contrast to SGPL1, no significant differences in SGPP1 gene expression were noted (B). SGPP2 expression trended higher among ulcerative colitis (UC) patients (N = 7) relative to controls (N = 17; $P = 0.07$). Levels in Crohn's Disease (CD) patients (N = 8) remained unchanged relative to controls.

Kugathasan et al[28] performed a microarray experiment to identify candidate genes in a genome-wide association study performed in pediatric onset IBD (data accessible at NCBI GEO database,[26] accession GSE10616). Using this database, we queried differentially expressed genes involved in S1P synthesis, degradation, and signaling. Although not significant, *SPHK1*, *SGPP1*, *SGPP2*, *S1PR1*, *S1PR3*, and *ORMDL3* expression levels were increased in CD vs controls (Supplementary Table 1). However, we found that *SPHK1*, *SGPP2*, *SGPL1*, *S1PR1*, and *S1PR3* were all significantly increased in UC vs controls (Supplementary Table 2). The database analysis also revealed that *SPHK2* and *S1PR2* expression levels were significantly decreased in UC vs controls (Supplementary Table 2).

Given our finding that genes responsible for S1P synthesis (SPHK1, SPHK2) and degradation (SGPL1) are elevated in IBD, it became important to assess whether these changes perturbed plasma and colon sphingolipid profiles. To that end, plasma samples from each patient category were subjected to lipid extraction, separation, and sphingolipid profiling by tandem mass spectrometry. We focused our analysis on plasma levels of S1P, sphingosine, and ceramide metabolites (Supplementary Fig. 1). Median total ceramide concentration in the control plasma (N = 16) was 2.6 µM and was essentially unchanged compared with IBD patients in remission (2.8 µM; N = 15), patients with mild disease (3.1 µM; N = 6), or patients with moderate/severe disease (1.7 µM; N = 4). Median plasma sphingosine and S1P concentrations were also similar across all groups measured (Supplementary Fig. 1). Although colon tissues displayed significant sphingolipid remodeling at the level of the transcriptome, these changes were not reflected in the circulating levels of sphingolipids. The impact of these changes may be localized, or the contribution from the gut tissue to total circulating sphingolipids may be small.

Considering the above significant alterations in S1P-related gene expression in IBD colon tissues compared with controls, we next examined whether gut tissue sphingolipid profiles were perturbed in IBD. To address this, biopsied gut samples from cohort 2 were subjected to sphingolipid profiling by LC-MS/MS. As shown in Table 1, this cohort was age- and gender-matched and included 6 non-IBD controls and 11 IBD patients (UC = 2, CD = 9). Of the 11 IBD patients, 4 were in remission, 5 had mild disease, and 2 had moderate/severe disease. Mean C-reactive protein (CRP) values in controls and IBD patients in remission were all below detection limit. Among mild IBD patients (who were all CD patients) and severe IBD patients (who were all UC patients), mean CRP levels were elevated. Mean ESR levels were elevated in IBD patients compared with controls. In contrast to the cohort 1 samples, biopsied samples

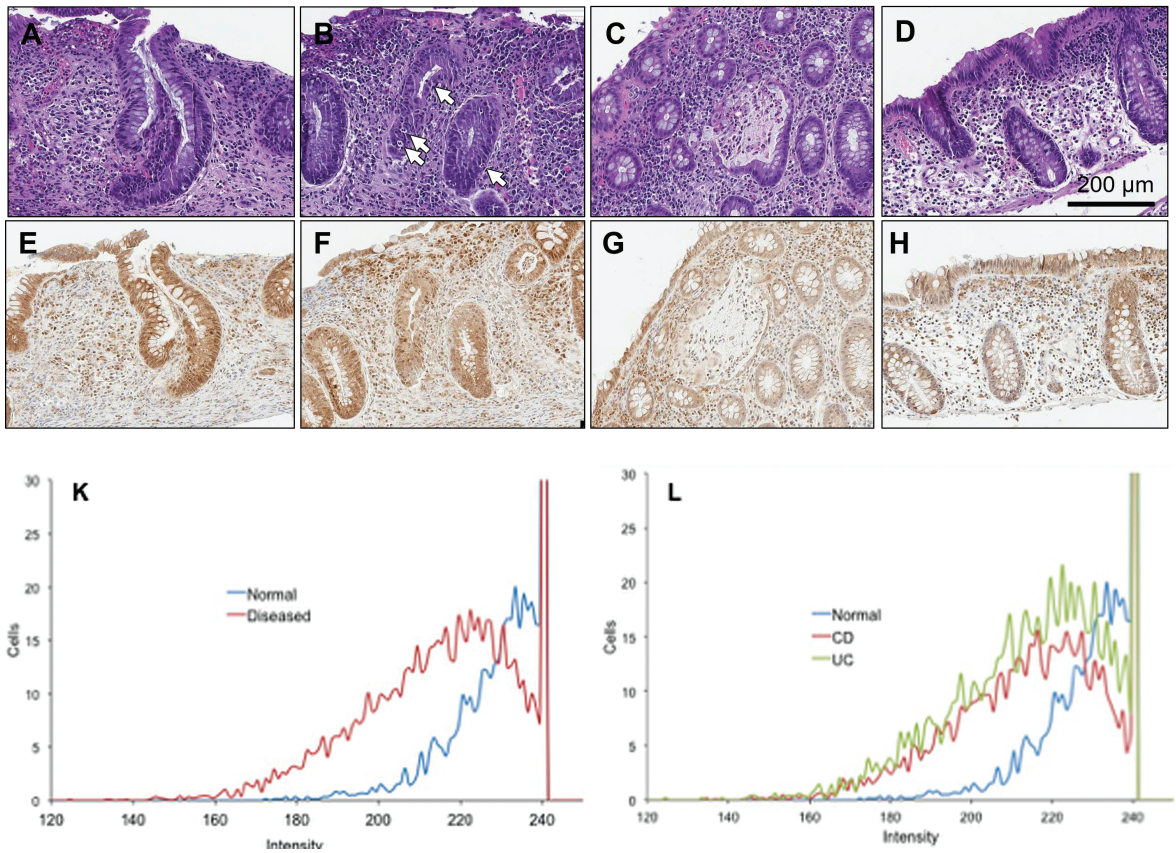

FIGURE 3. Sphingosine phosphate lyase (SPL) protein expression patterns in control colon and pediatric IBD colon (A–E). Colon tissue samples stained with hematoxylin & eosin (H&E). A, normal control pediatric colon biopsy. B, crypt architectural distortion, documenting chronic inflammation and regeneration with aberrant crypts. C, active cryptitis, neutrophils (arrows) infiltrate crypt epithelium. D, crypt abscess (center), neutrophils fill and expand the crypt lumen with disruption of the epithelial continuity. E, quiescent colitis, changes include mild crypt drop out, edematous lamina propria and patchy increased plasma cell infiltrates without active colitis. Serial sections of the same areas shown above stained with anti-SPL antibody (F–J) followed by secondary antibody and avidin-biotin linked hydrogen peroxide IHC. Chronic inflammatory cells, lymphocytes and plasma cells also express SPL, and this is increased in the disease tissues compared with control. Scale bar in panel A applies to all images (A-J). (K) Intensity of SPL staining based on quantitative image analysis. Straight red peak to the right of the image represents zero staining, and signals shifted leftward indicate increasing levels of staining intensity. Blue line is control. Red line is IBD (all categories). L, intensity of SPL staining separated by IBD disease category. Blue line is control. Red lines is CD. Green line is UC. Straight green peak to the right of the image represents zero staining.

from cohort 2 utilized for sphingolipid profiling were obtained from multiple different anatomical sections of the colon, as indicated in Table 1. Biopsied samples were analyzed for disease pathology, and active disease sites are noted with a plus sign (+) and those that were benign are noted with a minus sign (−). Statistical adjustments were made for both site of biopsy and pathology status, as described in methods.

Dihydroceramides (DHCer) are generated from de novo synthesis of sphingolipids from N-acylation of sphinganine. No significant changes in sphinganine were detected across all groups (data not shown). Group median and range of DHCer in tissues are shown in Fig. 5. Median DHCer concentrations increased in stepwise manner from control to remission, mild, and moderate/severe IBD groups (Fig. 5A). DHCers can be desaturated by DHCer desaturases (DES1 and DES2) to form ceramides. As shown in Fig. 5B, tissue ceramide content also increased with worsening IBD severity. Tissue ceramides increased stepwise from control levels to IBD patients in remission, mild, and moderate/severe groups. In contrast to the increase in ceramides, tissue levels of sphingosine were similar across all groups. (Fig. 5C) Consistent with increased SPL expression (Fig. 2), median tissue levels of S1P trended lower in the moderate/severe IBD patients when compared with controls. These results suggest that in moderate/severe IBD tissues, there is a significant increase in ceramide content as well as the ceramide/S1P ratio, both of which can have biological consequences that could impact the disease process.

Specific changes in the levels of DHCer, ceramide, and the pro-inflammatory ceramide metabolite ceramide-1-phosphate (C1P) species of varying acyl-chain lengths are displayed in the heatmap shown in Fig. 6. The metabolites are organized in rows. Columns represent each tissue specimen analyzed.

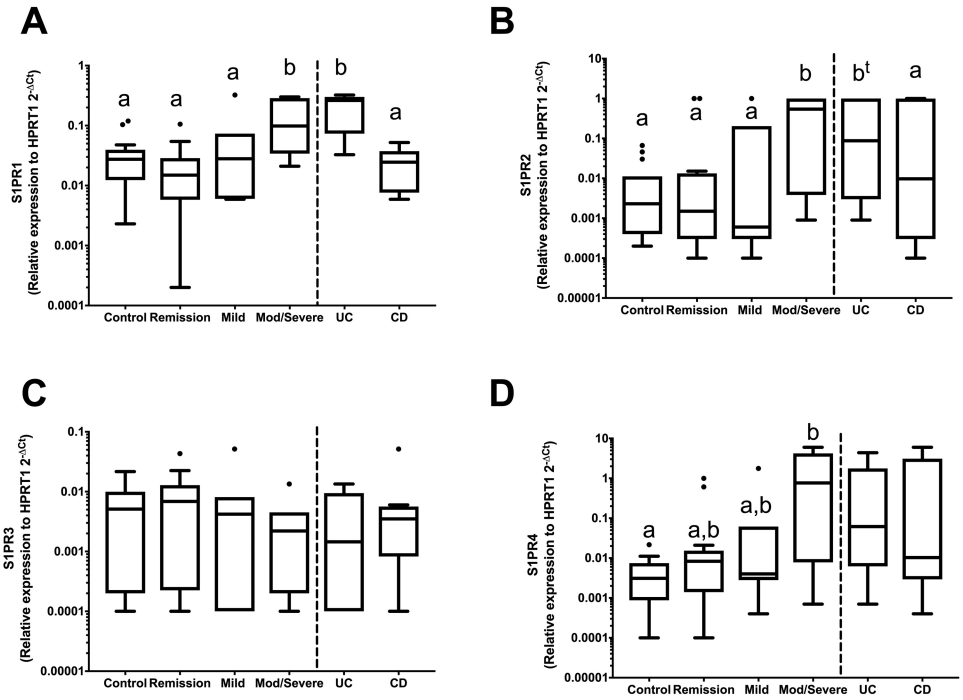

FIGURE 4. S1PR1, S1PR2, and S1PR4 gene expression levels are increased in colons of moderate/severe inflammatory bowel disease (IBD) patients. Panel A shows significant median S1PR1 gene expression increased by approximately 3.3-fold ($P = 0.05$) and 6.5-fold ($P = 0.002$) in pediatric moderate/severe IBD as compared with non-IBD controls and IBD patients in remission, respectively. Median expression of S1PR2 was also significantly upregulated in moderate/severe IBD by 235-fold ($P = 0.01$) and 360-fold ($P = 0.02$) relative to control and IBD patients in remission, respectively (B). Panel C shows no significant changes in S1PR3 expression across all groups examined. Panel D shows that median expression of S1PR4 in moderate/severe pediatric IBD was upregulated by 256-fold ($P = 0.001$) and 92-fold ($P = 0.01$) relative to controls and IBD patients in remission. These results suggest that upregulation of S1PR2 and S1PR4 are dominant signatures of severe IBD.

Metabolite data was median normalized and expressed as a fold-change over the group median, as indicated by color intensity shown in the legend (Fig. 6). Among IBD patients with moderate/severe disease severity, the most significant increases were observed in C14:0 DHCers and C16:0 DHCers. In contrast, C24:0 DHCers decreased relative to controls (Fig. 6). C20:0 and C22:0 DHCer species were also significantly elevated. Among the ceramide species, key pathological ceramide species for mitochondrial impairment and apoptosis, such as C14:0 and 16:0 ceramides were the most significantly increased relative to controls, (Fig. 6). In addition to these, other ceramide species (e.g., C18:1, C20:0, and C24:0) were also significantly increased in IBD patients relative to controls. Increases in DHCer and Cer suggest that both de novo and salvage routes of ceramide synthesis are increased in IBD colonic tissues. Analysis of C1P species showed divergent patterns of changes with C16:0 C1P species, increasing in the moderate/severe IBD patients (C24:0 C1P decreasing, see Fig. 6).

DISCUSSION

The major goal of the current work was to determine whether S1P signaling and metabolism are altered at the transcriptomic level or the metabolomic level—or both—in pediatric IBD. We also sought to establish whether any changes in the S1P pathway we identified correlate with disease severity. Our major finding is a marked and widespread upregulation in the expression of S1P-related genes in the colon tissues of pediatric patients with moderate/severe IBD compared with controls and IBD patients in remission or with mild disease. This included upregulation of genes responsible for S1P biosynthesis (*SPHK1* and *SPHK2*) and irreversible S1P degradation (*SGPL1*) and increases in the expression levels of *S1PR1*, *S1PR2*, and *S1PR4*. At the metabolomic level, colon tissues from patients with moderate/severe IBD exhibited lower S1P levels than controls. In addition, significant increases in DHCer, ceramide, and C1P levels were observed with moderate/severe IBD, resulting in a marked shift in the balance of ceramide/S1P. In contrast, no significant changes in the composition of the plasma sphingolipidome were observed.

We recently showed that *SPHK1* and *S1PR1* gene expression levels are elevated in colon tissues from IBD patients, whereas *SGPL1* levels were unchanged.[14] Pediatric patients were not included in that cohort, and the patients were not stratified by disease severity. To the best of our knowledge, only a few other clinical studies provide information on how disease severity impacts expression of key genes involved in S1P metabolism in pediatric IBD patients. A study by Huang

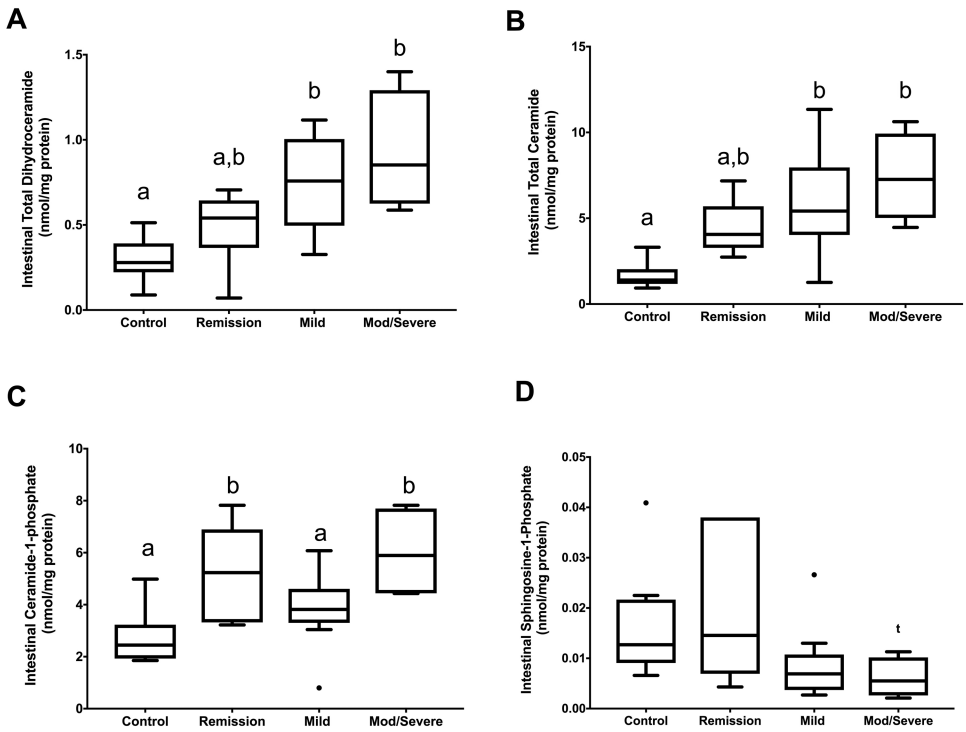

FIGURE 5. Intestinal dihydroceramide (DHCer), ceramide, and ceramide-1-phosphate (C1P) levels are significantly associated with worsening IBD symptoms. Tissue levels of DHCer increased as a function of worsening IBD severity (A). Box plot shows the median, range and 25th–75th percentiles. Median DHCer concentrations increased in stepwise manner from control values of 0.28 nmol/mg protein (N = 10) to 0.36 nmol/mg protein, 0.50 nmol/mg protein ($P = 0.004$) and to 0.63 nmol/mg protein (P = 0.004), in the remission, mild, and moderate/severe IBD groups, respectively (A). DHcer formed are further desaturated by DHCer desaturases (DES1 and DES1) to form ceramides. B, tissue ceramide content also increased with worsening IBD severity. Tissue ceramides increased from control values of 0.9 nmol/mg protein to 4.0 ± 0.6 (P = 0.1), 5.4 (P = 0.003), and 7.3 nmol/mg protein (P = 0.003) in IBD patients in remission, mild, and moderate/severe groups, respectively. In contrast to the increase in ceramides, tissue levels of sphingosine were similar across all groups. C, tissue levels of S1P trended lower in the moderate/severe pediatric IBD patients (0.006 nmol/mg tissue) as compared with controls (0.01 nmol/mg tissue; $P = 0.07$).

et al used gene expression omnibus datasets derived from 10 monozygotic twins discordant for UC and from inflamed colon biopsies obtained from 57 UC patients and uninflamed normal controls.[34] In that study, only SGPP2 gene expression was found to be significantly elevated in UC patients, but the report did not provide information on disease severity. Another study investigating histological differences between 6 UC patients and 7 normal controls revealed significant increases in SphK1 and COX-2 protein expression in the UC patients. However, the analysis did not examine other S1P-related proteins and did not provide additional clinical parameters.[12] French et al reported increased SphK1 protein expression in IBD patients (6 IBD patients compared with 3 controls).[35] More importantly, in that study colonic SphK1 protein expression correlated significantly with histopathological score.[35] In the current study, we investigated the expression of all 10 major genes involved in S1P metabolism and signaling. Due to limited amount of tissue sample available, it was not possible to quantify expression of other relevant genes including the S1P transporter *SPNS2* and *LPP1-3* genes. Nonetheless, our data corroborate previous reports showing *SPHK1* upregulation in IBD and demonstrate additional widespread activation of S1P-related genes in moderate/severe IBD colon tissues.

Our analysis of the GEO database GSE10616 revealed that in UC patients, *SPHK1*, *SGPP2*, *SGPL1*, and *S1PR1* expression levels were upregulated compared with controls (Supplementary Table 2). These results correlate with our own qRT-PCR results. However, the database analysis revealed that *SPHK2* and *S1PR2* expression levels were downregulated, findings that are in contradiction with our qRT-PCR results showing that *SPHK2* expression was increased in IBD moderate/severe patients and *S1PR2* expression was increased in UC patients (Supplementary Table 2, Figs 1B, and 4). We attribute these contradictory results to biological variability and differences in the representation of UC, CD, and methods of sample preparation between the 2 studies.[36,37] These findings suggest that S1P signaling and metabolism likely play an important role in IBD. Considering the established role of S1P in promoting inflammation through activation of STAT3 and NFκB,[19] it is likely that cytokine-mediated activation of these genes is part of the innate inflammatory response in IBD.

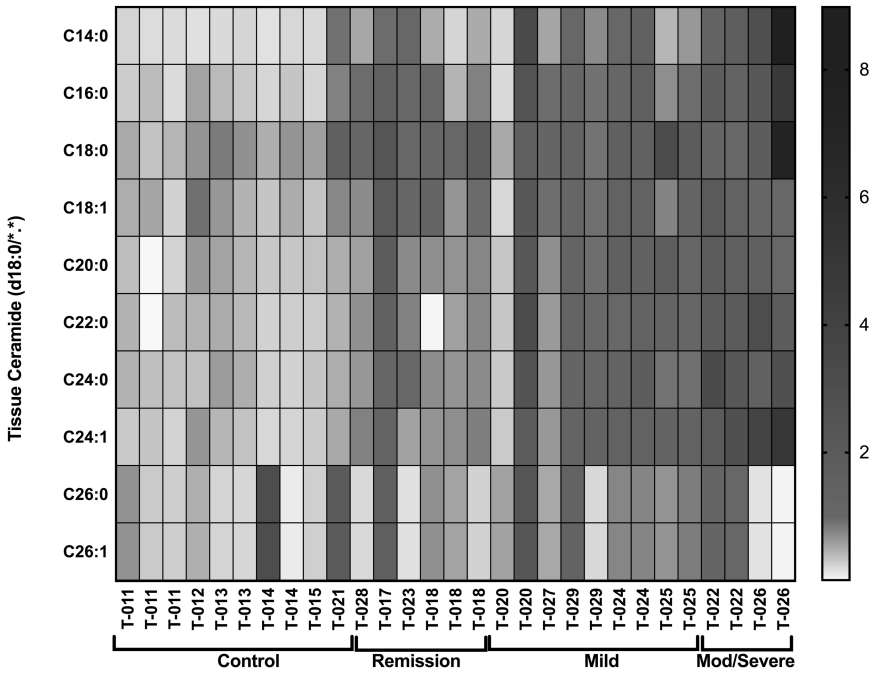

FIGURE 6. Alterations in dihydroceramide (DHcer), ceramide, and ceramide-1-phosphate (C1P) metabolites organized by increasing acyl-chain length and disease severity. Specific changes in DHCer, ceramide, and C1P species of varying acyl-chain lengths are displayed in the heatmap. Each of these metabolites is organized in rows. Columns represent each tissue specimen analyzed. Tissues values were first normalized by median values across all samples and expressed as a fold-change over group median as indicated by color intensity shown in the legend. Among IBD patients with moderate/severe disease severity, significant increases were observed in C14:0 DHCers (2.9-fold increase relative to controls; $P < 0.001$) and C16:0 DHCers (2.4-fold increase relative to controls; $P < 0.001$). C24:0 DHCers decreased by ~25% relative to controls ($P < 0.001$). C20:0 and C22:0 DHCer species were significantly elevated by ~2-fold ($P < 0.05$). Among the ceramide species, C14:0 and 16:0 ceramides were the most significantly increased by 14.7- and 7.0-fold relative to controls ($P < 0.05$), respectively. Other ceramide species (C18:1, C20:0, C20:4, and C24:0) were increased by >2-fold in IBD patients (mild+moderate/severe) relative to controls. C16:0 C1P levels increased in the moderate/severe IBD patients (3.3-fold; $P < 0.001$) and C24:0 C1P decreased by 50% ($P < 0.001$).

A previous study implicated the RS2872507 gene locus, which is associated with *ORMDL3* expression as a putative risk factor for CD.[38] ORMDL3 has been proposed as a negative regulator of serine palmitoyltransferase (SPT) enzymes that catalyze the initiation of de novo sphingolipid synthesis,[39] although direct in vivo evidence for ORMDL3 interaction with SPT enzymes has been recently been questioned.[40] Expression levels of ORMDL3 showed no significant changes between controls and IBD patients, regardless of disease severity or disease subtype (UC or CD) (Fig. 1).

Our histological findings and image quantification of SPL IHC in IBD patient tissues and control tissues demonstrate that SPL protein is upregulated in IBD tissues, which appears to be attributed to increased SPL expression in enterocytes of regenerating crypts and the influx of SGPL1-expressing immune cells. It is likely that increased tissue expression of sphingosine kinases and S1PRs can also be accounted for by a combination of increased endogenous epithelial expression and hematopoietic cell infiltration of inflamed tissues.

We previously reported that SPL is a critical regulator of colitis severity in mice and that lack of SPL expression in colonic epithelium predisposes to S1P accumulation, resulting in more severe colitis, cell transformation, and colitis-associated colon cancer.[19] These findings were found to be dependent upon activation of STAT3 signaling by S1P. Our current findings suggest that increased irreversible degradation of S1P may be a necessary compensatory mechanism to ensure that increased S1P synthesis does not result in sustained S1P accumulation, which could be deleterious and contribute to chronic inflammation. This model is consistent with our finding that S1P accumulation is characteristic of late stage colitis in mice (from our unpublished results). In that regard, our finding that S1P levels were not elevated in pediatric IBD and even trended lower than controls suggests that pediatric colon tissue may be more capable of vigorous crypt regeneration and associated SGPL1 upregulation than adult tissues. Considering the finding of enhanced cell transformation when SGPL1 is silenced, the upregulation of SPL in pediatric IBD might also explain why colitis-associated colon cancer rarely occurs in childhood.

Despite decreased steady-state levels of S1P, we observed significant transcriptional upregulation of *S1PR1*, *S1PR2*, and *S1PR4*. Expression of S1PR2 and S1PR4 is

mostly restricted to T cells, and S1PR1 and S1PR2 are expressed mostly in mast and macrophage innate immune cells.[41] S1PR2 appears to contribute to inflammation by activating NFκB, leading to increased TNFα expression, which in turn increases the expression ICAM-1 and VCAM-1 on endothelial cells.[42] S1PR2 is also a regulator of vascular permeability.[42] Importantly, S1PR2 signaling is required for epithelial cell extrusion, a critical process by which apoptotic epithelial cells are extruded from the epithelial membrane without compromising epithelial barrier integrity.[43] Thus, S1PR2 upregulation could represent a compensatory mechanism to protect the barrier in the face of inflammation-associated cell death. Our study also shows increased expression of S1PR4 in IBD tissues compared with control and IBD remission tissues. S1PR4 is mostly expressed on immune cells and was shown to affect dendritic cell function and Th17 cell differentiation in a mouse model of colitis.[44]

Metabolomic analysis of colon biopsies from IBD patients revealed significant differences in sphingolipid profiles, including elevated levels of DHCer, ceramide, and C16:0 C1P. In both CD and UC, increased levels of inflammatory cytokines such as TNF-α and interferon-γ have been observed. TNF-α is known to increase SPHK1 activity. TNF-α also increases ceramide levels through activation of both salvage (early) and de novo (late) synthesis pathways.[45] TNF-α-induced synthesis of C16:0 ceramide is essential for cellular apoptosis.[45] We observed significant increases in C14:0 and C16:0 DHCer and ceramides in biopsied samples obtained from moderate/severe IBD patients. Interestingly, C22:0 DHCer decreased by 25% in moderate/severe IBD patients. This may be due to the fact that both S1P and interferon-gamma decreases activity of CerS2, which preferentially incorporates longer chain acyl-CoAs (C20–C26).[46, 47]S1P directly binds an S1PR-like motif within CerS2 and inhibits its activity.[46] Importantly, ceramides can promote apoptosis and tissue damage, and the ratio of ceramide to S1P has been shown to be a critical factor in regulation of apoptosis.[48] Thus, elevated ceramide levels and an increased ceramide/S1P ratio could stimulate apoptosis, potentially challenging the ability of the tissue to maintain the gut mucosal barrier.

Although C1P is a known pro-inflammatory mediator, the role of C1P in IBD has not been well characterized. In our study, the total C1P concentrations increased in IBD, and we observed differential patterns of change with elevation of C16:0 and diminished levels of C24:0 C1P. C16:0 ceramide, which is more abundant than C24:0, is known to activate cytosolic phospholipase A2, which releases arachidonic acid required for eicosanoid generation, representing a pro-inflammatory effect.[49,50] Conversely, C1P can inhibit the metalloprotease TNF-converting enzyme (TACE), which cleaves pro-TNF to release the active inflammatory form.[51] Therefore, C1P could potentially contribute to both pro- and anti-inflammatory effects in IBD.

Our cumulative findings from colon tissue sphingolipid profiling demonstrate major shifts in the levels of DHCer, ceramides, and C1P and a shift in the S1P/ceramide ratio in the gut tissues of moderate/severe IBD patients. These shifts could have significant impact on inflammation and mucosal integrity by modulating apoptotic cell burden and the rate of apical cell extrusion.

In summary, we have observed profound transcriptional and metabolic alterations in sphingolipid metabolism in the colons of pediatric IBD patients with moderate to severe disease compared with those of healthy controls and IBD patients in remission. S1P and sphingolipids are known to influence the dynamics of mucosal homeostasis and inflammatory signaling. Our clinical data along with emerging preclinical study results implicate sphingolipids as critical factors in the complex inflammatory milieu of IBD. Importantly, our results suggest that this may be particularly true for pediatric IBD and that the efficacy of Ozanimod and other S1PR-targeted agents should be tested in the pediatric IBD population. Considering its safety record, Ozanimod could represent an important addition to the armamentarium of therapeutic agents for treatment of moderate to severe pediatric IBD. Our overall findings suggest that the role of sphingolipids as potential biomarkers, and therapeutic targets in pediatric IBD should be further explored.

SUPPLEMENTARY DATA

Supplementary data is available at *Inflammatory Bowel Diseases* online.

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