Transcriptome analysis in acute gastrointestinal graft-*versus* host disease reveals a unique signature in children and shared biology with pediatric inflammatory bowel disease

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Received: October 10, 2022.
Accepted: January 25, 2023.
Early view: February 2, 2023.

https://doi.org/10.3324/haematol.2022.282035

Abstract

We performed transcriptomic analyses on freshly frozen (n=21) and paraffin-embedded (n=35) gastrointestinal (GI) biopsies from children with and without acute acute GI graft-*versus*-host disease (GvHD) to study differential gene expressions. We identified 164 significant genes, 141 upregulated and 23 downregulated, in acute GvHD from freshy frozen biopsies. *CHI3L1* was the top differentially expressed gene in acute GvHD, involved in macrophage recruitment and bacterial adhesion. Mitochondrial genes were among the top downregulated genes. Immune deconvolution identified a macrophage cellular signature. Weighted gene co-expression network analysis showed enrichment of genes in the ERK1/2 cascade. Transcriptome data from 206 ulcerative colitis (UC) patients were included to uncover genes and pathways shared between GvHD and UC. Comparison with the UC transcriptome showed both shared and distinct pathways. Both UC and GvHD transcriptomes shared an innate antimicrobial signature and FCγ1RA/CD64 was upregulated in both acute GvHD (log-fold increase 1.7, *P*=0.001) and UC. Upregulation of the ERK1/2 cascade pathway was specific to GvHD. We performed additional experiments to confirm transcriptomics. Firstly, we examined phosphorylation of ERK (pERK) by immunohistochemistry on GI biopsies (acute GvHD n=10, no GvHD n=10). pERK staining was increased in acute GvHD biopsies compared to biopsies without acute GvHD (*P*=0.001). Secondly, plasma CD64, measured by enzyme-linked immunsorbant assay (n=85) was elevated in acute GI GvHD (*P*<0.001) compared with those without and was elevated in GVHD compared with inflammatory bowel disease (n=47) (*P*<0.001), confirming the upregulated expression seen in the transcriptome.

Introduction

Acute graft-*versus*-host disease (GvHD) is the greatest barrier to a successful hematopoietic stem cell transplant (HSCT), with mortality rates of up to 50% in advanced cases.[1] Gastrointestinal (GI) GvHD is particularly challenging to treat and carries a higher mortality rate when compared to acute skin GvHD.[2] Steroids are the mainstay of acute GvHD therapy, but treatment failure is frequent, and toxicities are common.[3] Knowledge gaps in the pathophysiology of acute GvHD are main reasons for poor outcomes, especially in steroid-refractory acute GI GvHD.[4] Several research studies have identified the need to study target organ (skin, intestine or liver) biology in humans at the time of GvHD to identify targetable pathways, but also acknowledge logistic challenges of obtaining target organ tissue for research.[5] As a result, most GvHD studies use blood biomarkers or animal models to construct the biology of acute GvHD, and these studies currently guide prophylaxis, classification, treatment, and prognosis.

Studies of target organ biology in pediatric inflammatory bowel disease (IBD) may provide us with a template to further study pediatric acute GvHD. Transcriptome analyses of intestinal biopsies in pediatric ulcerative colitis (UC) have identified a reduction in epithelial mitochondrial genes and associated energy production pathways, a novel observation.[6] Upregulated gene signatures in pediatric UC are enriched for integrin signaling ($P<1.08\times10^{-12}$) and the TNF pathway ($P<9.9\times10^{-93}$), aligning with current therapeutic approaches.[6] Epithelial gene signatures in

Crohn's disease identified upregulation of expression of the antimicrobial gene dual oxidase (*DUOX2*) along with decreased expression of lipoprotein APO1.[7] The combination of *DUOX2* upregulation and *APO1* downregulation was associated with Proteobacteria and *Firmicutes* expansion, increased oxidative stress and increased severity of gut inflammation in pediatric Crohn's disease.[7] These findings are collectively directing individualized therapeutic approaches and driving further understanding of IBD in children.

Transcriptome analyses of peripheral blood mononuclear cells in non-human primates at the time of acute GvHD onset have identified aurora kinases as novel pathways which could be targeted to ameliorate manifestations of GvHD.[8] Furthermore, a recent epithelial transcriptomic study of 22 adults with acute GI GvHD identified important associations and clues to mechanisms of disease but was limited by extraction of RNA from formalin-fixed paraffin-embedded (FFPE) tissue, in which RNA is commonly degraded or chemically altered, raising the possibility that important findings could be overlooked.[9] We performed a study of transcriptome analyses of intestinal biopsies collected and frozen at the time of clinical diagnosis of acute GI GvHD and compared these findings to patients who underwent endoscopy for routine clinical indications pre or post HSCT and did not have evidence of colitis or acute GI GvHD. We also compared these results to a previously established cohort of patients with UC in which transcriptomic data was performed on freshly frozen GI biopsies. Finally, we performed transcriptomic analyses on 35 allogeneic HSCT patients with (n=20) and without acute GI GvHD (n=15) using FFPE tissue to augment our small sample size of our prospective study and further to highlight similarities and differences in differential gene expression using different tissue sources.

Methods

Patient enrollment for prospective study

The Cincinnati Children's Hospital Medical Center (CCHMC) Institutional Review Board approved this study. Patients were eligible if they were aged 2 years and older, pre- or post-allogeneic HSCT and were scheduled for a lower GI endoscopy at CCHMC for clinical indications such as diarrhea, hematochezia, and abdominal pain, or for routine screening. Additional details are described in the *Online Supplementary Appendix*. Clinical grading of acute GvHD was done using the modified Glucksberg criteria.[10]

Patient selection for formalin-fixed paraffin-embedded tissue RNA sequencing

Pediatric allogeneic HSCT patients with available FFPE biopsies were selected and separated into two cohorts: patients with acute GI GvHD at the time of biopsy (n=20) and patients without acute GI GvHD at the time of biopsy and at any time clinically (n=15). All patients had consented to our institutional tissue biorepository where clinical samples may be used for future unspecified research. Demographic information was collected retrospectively. Details of transcriptome analyses for both cohorts are described in the *Online Supplementary Appendix*.

Weighted gene co-expression network analyses

A weighted gene co-expression network analyses (WGCNA) was performed to generate gene modules.[11] Details are described in the *Online Supplementary Appendix*. Data from freshly frozen biopsies was used for these analyses.

Immune deconvolution

We performed a cell type deconvolution to estimate cell subset proportions using CIBERSORT, a versatile computational method, to provide an estimation of the abundances of member cell types in a mixed cell population using gene expression data.[12] The statistical significance between cell populations in acute GI GvHD and no GvHD was determined by *t*-test. Data from freshly frozen biopsies was used for these analyses.

Immunohistochemistry for pERK

Samples of 20 separate and de-identified FFPE GI biopsies (acute GI GvHD n=10, post-HSCT biopsies without acute GI GvHD n=10) were stained for pERK. Additional details are described in the *Online Supplementary Appendix*.

Plasma CD64

Cryopreserved plasma samples from 85 HSCT recipients (acute GI GvHD n=30, no GvHD n=30, acute skin GvHD n=25) were obtained from our institutional biorepository where consecutive patients are consented for future research and compared with 47 children with IBD and 42 non-IBD controls. Details are described in the *Online Supplementary Appendix*.

Comparison with ulcerative colitis

We had access to data from 206 UC patients who underwent high-throughput RNA sequencing of intestinal biopsies prior to treatment, from the Predicting Response to Standardized Pediatric Colitis Therapy cohort, which originally included 428 UC patients from 29 pediatric gastroenterology centers in North America.[7] Transcriptomic data from these 206 UC patients were included in our study for analyses to uncover genes and pathways shared between acute GI GvHD and IBD. Data from freshly frozen biopsies was used for these comparative analyses between acute GI GvHD and IBD as IBD biopsies were also freshly frozen. For this comparative cohort of pediatric UC,[6] all samples were rectal biopsies.

Results

Twenty-one patients underwent endoscopy pre (n=5) or post BMT (n=16) for routine clinical indications and met study criteria between 2017-2019. The median age of patients was 13.5 years (range, 4.5-25 years). Patient demographics and details of GvHD including stage and additional organ involvement are described in the *Online Supplementary Table S1*. Each patient contributed a single lower GI biopsy sample for RNA sequencing and no patient had multiple samples submitted for transcriptomics. In all but one patient, lower GI biopsies were obtained at diagnosis of acute GI GvHD, prior to treatment. In one patient, biopsy was obtained after treatment was initiated with ruxolitinib to follow disease response. Nine patients had clinical symptoms of acute GI GvHD, and the diagnosis was confirmed with biopsies reviewed by an experienced pathologist. Three of the nine patients had steroid-re-

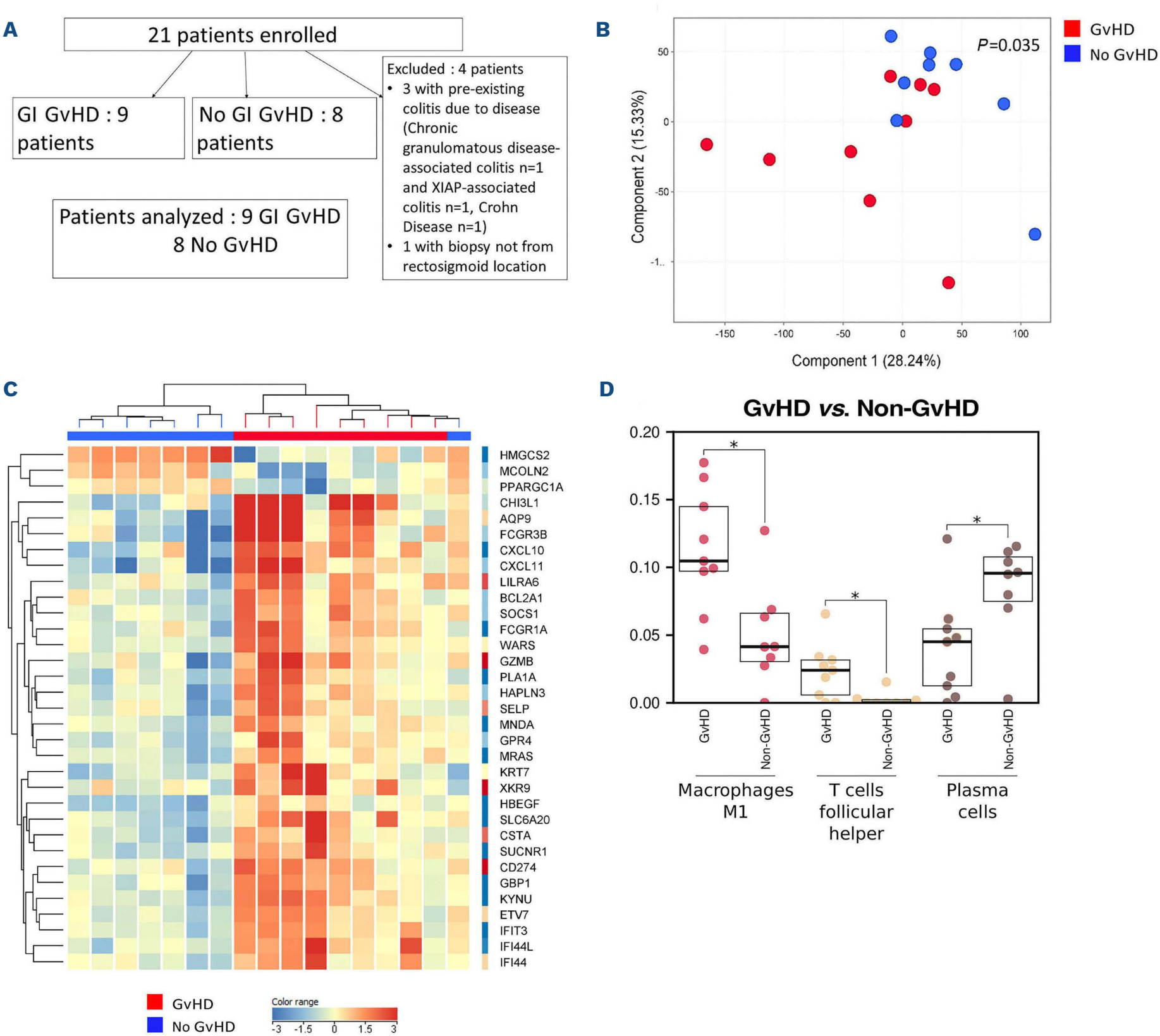

Figure 1. Results of freshly frozen gastrointestinal biopsy analyses. (A) Study profile showing number of patients enrolled on the prospective study number of patients excluded from analyses and final number of patients included for RNA sequencing analyses. (B) Principal component analysis of 9 patients with gastrointestinal (GI) graft-*versus*-host disease (GvHD) and 8 patients without acute GI GVHD using 14,229 protein-coding genes that passed expression filtering. PCA PC1 value between groups were compared and were significantly different (*t*-test *P*=0.035). (C) Unsupervised hierarchical clustering analysis using the top up- and downregulated genes in patients with acute GvHD compared to patients without GvHD from freshly frozen GI biopsies. (D) Immune deconvolution showing significant cell types in acute GI GvHD compared to patients without acute GvHD. *P<0.05.

sponsive acute GI GvHD while the rest had steroid refractory acute GI GvHD. Eight patients did not have acute GI GvHD at the time of the intestinal biopsy or subsequently during their clinical course. Of these eight patients without acute GI GvHD, six patients did not have any abnormality on their biopsies, one patient had changes consistent with mild CMV infection and one patient's pre-transplant biopsy had intestinal changes reflective of brincidofovir treatment. All biopsy samples were obtained from the rectum except one patient who was subsequently excluded from our analyses (Figure 1A).

Four patients were excluded from analyses (Figure 1A). The final analyses included nine patients with acute GI GvHD and eight patients without acute GI GvHD.

Paraffin-embedded GI biopsies were available for 35 allogeneic HSCT patients who underwent endoscopy post HSCT

Table 1. Top 20 upregulated genes from freshly frozen gastrointestinal biopsies within the core acute gastroinstestinal graft-*versus*-host disease gene signature with false discovery rate-adjusted *P* value <0.05.[13]

	Genes	Adjusted *P* value (GvHD *vs.* no GvHD) Freshly obtained biopsies	Log fold change	Adjusted *P* value (GvHD *vs.* no GvHD) Paraffin-embedded biopsies	Log fold change	Function
1	*CHI3L1*	0.0001	4.5	$2.10x10^{-12}$	3	Participates in tissue remodeling, Th2 response, M2 differentiation, bacterial adhesion/invasion, mediating AKT1 signaling and IL-8 production in colonic epithelial cells
2	*AQP9*	0.0005	4.3	0.0012	1.7	Water channel protein regulates permeability to water and other solutes; induced in sepsis
3	*MTRNR2L8*	0.02	3.9	0.1	0.7	Antiapoptotic factor
4	*MMP3*	0.003	3.7	$1.49x10^{-5}$	2	Metabolism of extracellular matrix, wound repair
5	*SERPINA3*	0.0005	3.4	$1.26x10^{-5}$	2	Inhibitor of cathepsin G, a neutrophil serine protease
6	*MMP7*	0.006	3.4	0.07	0.9	Participates in wound healing, and regulating defensins in intestinal mucosa
7	*IDO1*	0.003	3.3	0.08	0.9	Modulates T-cell behavior by pericellular catabolism of tryptophan; expressed in dendritic cells, monocytes, and macrophages
8	*FCGR3B*	0.003	3.2	0.1	0.8	Trap for immune complexes in the peripheral circulation
9	*TNFRSF6B*	0.02	3.2	nd	na	Regulatory role in suppressing Fas L- and LIGHT-mediated cell death
10	*MMP10*	0.003	3.2	0.1	0.7	Metabolism of extracellular matrix, wound repair
11	*ACKR4*	0.01	3.2	nd	na	Binds dendritic cell- and T-cell-activated chemokines including CCL19/ELC, CCL21/SLC, and CCL25/TECK
12	*CXCL11*	0.002	3.1	0.06	1	Chemotactic for interleukin-activated T cells
13	*KRT7*	0.001	3.1	0.003	1.4	Epithelial cytokeratin
14	*MARCO*	0.004	3.1	0.06	1	Expressed in macrophages, pattern recognition receptor, binds bacteria for removal
15	*S100A8*	0.008	3	0.02	1	Cell cycle progression and differentiation
16	*DUOXA2*	0.04	2.9	0.3	0.5	Steroid metabolic processes and antimicrobial responses in IBD
17	*TM4SF4*	0.03	2.9	0.7	0.2	Regulation of cell development, activation and growth
18	*FPR2*	0.03	2.9	0.0005	1.7	Neutrophil chemotactic factor
19	*CXCR2*	0.004	2.8	0.01	1.3	Mediates neutrophil migration to sites of inflammation
20	*CLEC4E*	0.01	2.6	0.002	1.5	Recognizes damage-associated molecular patterns of abnormal self and pathogen-associated molecular patterns of bacteria and fungi

Comparison of top upregulated genes of freshly frozen gastrointestinal (GI) biopsies from paraffin-embedded GI biopsies also shown. GvHD: graft-*versus*-host disease; IBD: inflammatory bowel disease; na: not analyzed; nd: not detected.

for evaluation of diarrhea. Fifteen patients had no evidence of acute GvHD while 20 patients had clinical, and biopsy proven acute GI GvHD. Patient demographics and details of acute GvHD including clinical stages and organs involved in addition to the gut are described in the *Online Supplementary Table S2*. Of the 15 patients with no acute GI GvHD, one patient was positive for CMV by tissue polymerase chain reaction while the remaining 14 had no abnormalities identified on histopathology. A lower GI biopsy was obtained in all patients at onset of acute GI GvHD symptoms, except for one patient included in the freshly frozen biopsy cohort who was on ruxolitinib and underwent a lower GI biopsy to follow-up on disease response. Apart from this above-mentioned patient on ruxolitinib, all remaining patients were not on treatment for acute GI GvHD at the time of lower GI biopsy.

Differential expression analyses of freshly frozen gastrointestinal biopsies

A primary principal component analysis performed across reasonably expressed 14,229 protein-coding genes shows distinct patient clustering between those with and without

Table 2. Top 20 downregulated genes from freshly frozen gastrointestinal biopsies within the core acute gastrointestinal graft-*versus*-host disease gene signature with false discovery rate-adjusted *P* value <0.05.

	Genes	Adjusted *P* value (GvHD *vs.* no GvHD) Freshly obtained biopsies	Log fold change	Adjusted *P* value (GvHD *vs.* no GvHD) Paraffin-embedded biopsies	Log fold change	Function
1	*GPR15*	0.03	-2.5	0.5	-0.4	Chemokine receptor
2	*HMGCS2*	0.001	-2.2	0.2	-0.6	Mitochondrial enzyme that catalyzes the first reaction of ketogenesis
3	*NPY1R*	0.03	-1.7	0.1	-0.5	Stimulation of food intake
4	*MCOLN2*	0.007	-1.7	0.4	-0.3	Regulation of chemokine secretion and macrophage migration
5	*YBX2*	0.04	-1.3	0.1	-0.7	Regulation of the stability and/or translation of germ cell mRNA
6	*PPARGC1A*	0.02	-1.3	0.006	-0.8	Metabolic reprogramming in response to dietary availability through coordination of the expression of a wide array of genes involved in glucose and fatty acid metabolism
7	*B3GNT8*	0.03	-1.2	0.009	-0.8	Protein glycosylation and elongation of specific branch structures of multiantennary N-glycans
8	*C9orf24*	0.04	-1.2	0.1	-0.6	Plays a role in cyclogenesis
9	*MTRNR2L12*	0.01	-1.2	0.7	-0.2	Antiapoptotic factor, associated with Hirschsprung's disease
10	*ENPP1*	0.002	-1.1	0.1	-0.3	Extracellular ATP metabolism and insulin signaling
11	*SLC36A1*	0.04	-0.9	0.09	-0.4	Symporter activity and L-alanine transmembrane transporter activity
12	*AMACR*	0.03	-0.9	0.3	-0.3	Bile acid synthesis
13	*CDC14A*	0.02	-0.9	0.04	-0.4	Cell cycle control
14	*KCNJ2*	0.04	-0.9	0.6	-0.2	Potassium transport
15	*TMCC3*	0.04	-0.9	0.09	-0.4	Protein Coding gene, function unknown
16	*FSD1L*	0.04	-0.7	0.6	0.1	Protein Coding gene, function unknown
17	*PPP2R3A*	0.04	-0.7	0.02	-0.5	Inhibition of cell growth and division
18	*DMTN*	0.009	-0.7	nd	na	F-actin-binding activity that induces F-actin bundles formation and stabilization
19	*PANK1*	0.03	-0.7	0.1	-0.4	Pantothenate kinase activity
20	*SPATA24*	0.02	-0.7	0.8	-0.1	Cytoplasm movement

Comparison of top downregulated genes of freshly frozen gastrointestinal (GI) biopsies from paraffin-embedded GI biopsies also shown.[13] GvHD: graft-*versus*-host disease; na: not analyzed; nd: not detected.

GI GvHD, indicating distinct transcriptomic mechanisms driving disease (PCA PC1 value difference *P*=0.035) (Figure 1B). We identified 164 genes that were significantly differentially expressed (false discovery rate [FDR] <=0.05 and fold change [FC] >=1.5) in acute GI GvHD including 23 downregulated and 141 upregulated genes (*Online Supplementary Dataset S1* showing the indicative FDR *P* value, and $\log_2$ fold change). Genes with the highest fold change are shown in Figure 1C as a heatmap showing the normalized expression of a specific gene per patient, and the complete heatmap of the 164 genes are shown in the *Online Supplementary Figure S1.*

Differential expression analyses of paraffin-embedded gastrointestinal biopsies

Eight hundred and fifty-seven genes were up regulated and 485 were downregulated from the paraffin-embedded GI biopsy dataset (FDR<=0.05 and FC>=1.5, *P*<0.05]. *Online Supplementary Figure S2* shows the heatmap of protein-coding genes, log-transformed FPKM values with an adjusted *P*<0.05, and a FC=3. The complete gene list is presented in the *Online Supplementary Dataset S1*.

The top 40 differentially expressed genes in acute GI GvHD from freshly frozen biopsies are shown in Tables 1

Table 3. Top 10 upregulated GO term molecular functions in acute gastrointestinal (GI) graft-*versus*-host disease from freshly frozen GI biopsies and comparison values from paraffin-embedded GI biopsies.

Upregulated GO term molecular function	FDR B&H *P* value Freshly frozen GI biopsies	FDR B&H *P* value Paraffin-embedded GI biopsies
GO:0050786_RAGE receptor binding	$7.04x10^{-4}$	0.011
GO:0001664_G-protein-coupled receptor binding	$4.45x10^{-3}$	0.0007
GO:0004175_Endopeptidase activity	$5.50x10^{-3}$	0.01
GO:0019864_IgG binding	$5.50x10^{-3}$	0.0006
GO:0035325_Toll-like receptor binding	$8.09x10^{-3}$	0.009
GO:0042379_Chemokine receptor binding	$1.03x10^{-2}$	0.0005
GO:0008237_Metallopeptidase activity	$1.13x10^{-2}$	nd
GO:0060089_Molecular transducer activity	$1.33x10^{-2}$	nd
GO:0005124_Scavenger receptor binding	$1.33x10^{-2}$	nd
GO:0038187_Pattern recognition receptor activity	$1.37x10^{-2}$	nd

GO: gene ontology; FDR: false discovery rate; B&H: Benjamini-Hochberg procedure; nd: not detected.

Table 4. Top 10 upregulated pathways in acute gastrointestinal (GI) graft-*versus*-host disease of genes from freshly frozen GI biopsies and comparison pathway *P* values from paraffin-embedded GI biopsies.

Upregulated pathways	FDR B&H *P* value Freshly frozen biopsies	FDR B&H *P* value Paraffin-embedded biopsies
REACTOME_1269310_Cytokine signaling in immune system	$4.43x10^{-7}$	KEGG_04060 $4.3x10^{-14}$
REACTOME_1269314_Interferon gamma signaling	$2.83x10^{-6}$	0.9
BIOCARTA_M5885_Ensemble of genes encoding ECM-associated proteins including ECM-affiliated proteins, ECM regulators and secreted factors	$5.98x10^{-6}$	0.05
REACTOME_1457780_Neutrophil degranulation	$8.31x10^{-6}$	0.001
REACTOME_1269312_Interferon alpha/beta signaling	$1.66x10^{-5}$	0.9
REACTOME_1270258_Activation of matrix metalloproteinases	$3.46x10^{-5}$	0.05
REACTOME_1269203_Innate immune system	$8.81x10^{-5}$	nd
KEGG_122191_NOD-like receptor signaling pathway	$1.87x10^{-4}$	KEGG_04621 0.19
REACTOME_1269547_Chemokine receptors bind chemokines	$2.06x10^{-4}$	$6.4x10^{-7}$
KEGG_1474301_IL-17 signaling pathway	$1.39x10^{-2}$	nd

FDR: false discovery rate; B&H: Benjamini-Hochberg procedure; nd: not detected

and 2 with FC and *P* values of same genes from the paraffin-embedded GI biopsy dataset for comparison. There was upregulated of genes involved in the regulation of key immune effectors, e.g., macrophages and T cells, including CHI3L1 and AQP9.[13] Similarly, genes related to the host response to microbes, including CHI3L1, AQP9, and CLEC4E were up regulated in acute GI GvHD.[13] We also saw differential expression of genes related to cell migration and chemotaxis, including *CXCR2*.[13] Genes downregulated in acute GI GvHD including several genes related to metabolism of nutrients, including *HMGCS2, NPY1R, PPARGC1A* and *AMACR* from the freshly frozen GI biopsies but these were not significantly downregulated in the paraffin-embedded GI biopsy cohort.[13] Of note, we did not observe any aurora kinase gene expression in our dataset from both cohorts, regardless of statistical significance.

Molecular functions

The top 10 upregulated molecular functions from freshly frozen biopsies are shown in Table 3. Comparison values from the paraffin-embedded biopsies are also shown in

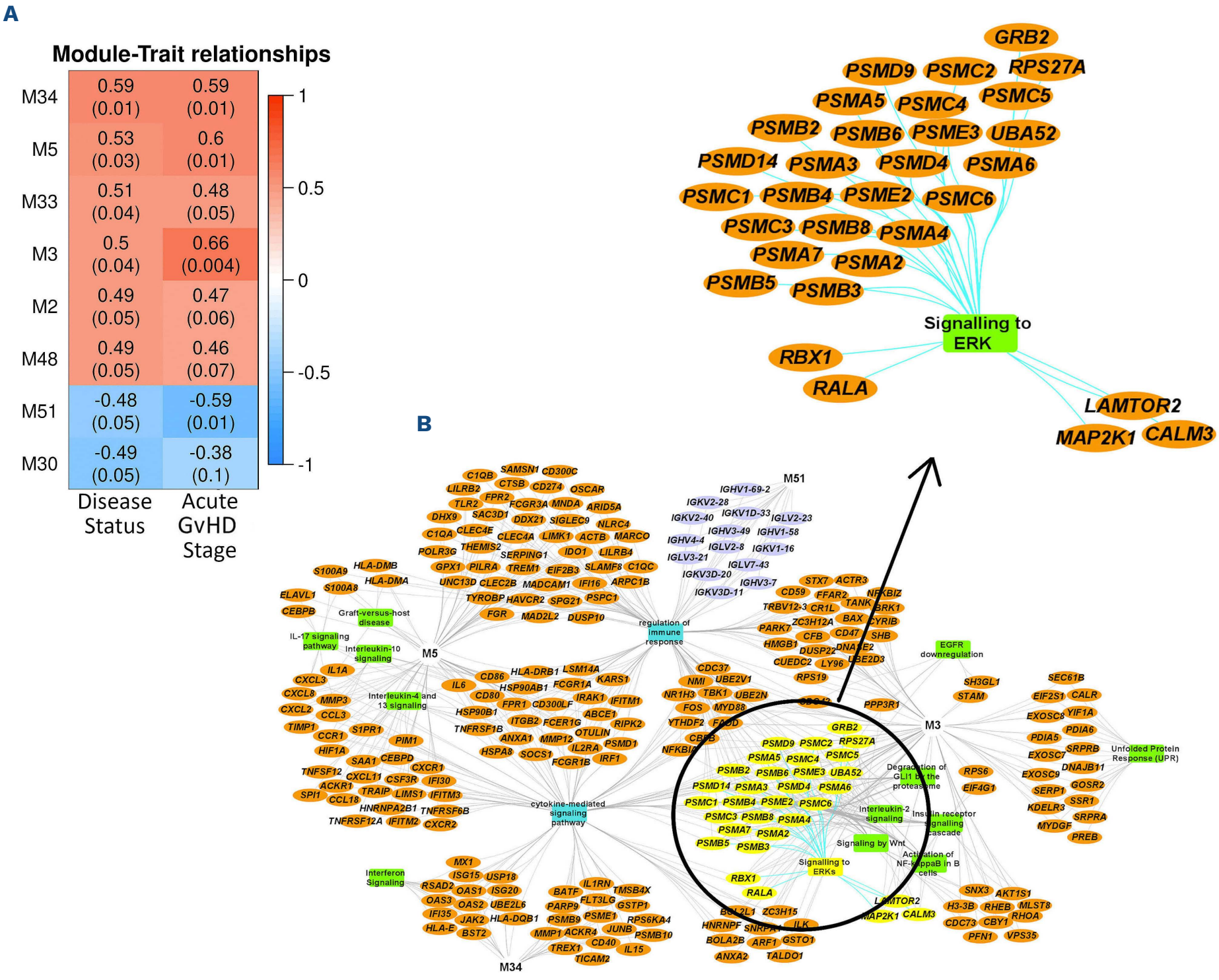

Figure 2. Results of weighted gene co-expression network analyses of freshly frozen gastrointestinal biopsies. (A) Heat map generated by the weighted gene co-expression network analyses showing statistically significant gene modules correlated with the disease status (acute gastrointestinal [GI] graft-*versus*-host disease [GvHD] present or absent) and stage of GI GvHD as previously described in the *Online Supplementary Table S1* (stage 1, 2, 3 and 4 GI GvHD) analyzed as stage 1-2 *vs*. stage 3-4. Four modules, namely, M34, M5, M3, and M51 were significantly correlated with the presence of acute GI GvHD and acute GI GvHD stage. (B) Functional enrichment analyses demonstrating enrichment of genes for the ERK signaling pathway (denoted in yellow) included in the M3 gene module (from Figure 5) and shown separately as a magnified insert below. The circular nodes represent WGCNA gene modules. Orange and purple colored nodes are genes that are upregulated or downregulated respectively in GvHD. The rectangle nodes are enriched biological processes and pathways.

Table 3 showing overlap between the first five upregulated molecular functions. Functions related to the host response to microbial pathogens were upregulated, including pattern recognition (RAGE-receptor binding, scavenger receptor binding), as were functions of innate immunity (e.g., toll-like receptor binding). Analysis of biological processes showed similar findings. The top 10 upregulated biological processes in acute GI GvHD from freshly frozen biopsies are shown in the *Online Supplementary Table S3* with comparisons from the paraffin-embedded biopsies also shown, confirming the importance of host defense against microbes, but also highlighting an interferon-driven inflammatory response, also seen in the pathway analysis from freshly frozen GI biopsies (Table 4).

Immune deconvolution

We observed significant enrichment of M1 macrophages and follicular helper T cells in patients with acute GI GvHD compared to patients without acute GI GvHD. Plasma cells were enriched in patients without acute GI GvHD compared to patients with acute GI GvHD (Figure 1D).

Weighted gene co-expression network analysis and ERK signaling

We further investigated biologically significant changes in gene expression with a WGCNA that avoids focusing on one or a small number of differentially expressed genes and examines changes that occur throughout a pathway or network. Figure 2A shows a heat map showing modules generated in the WGCNA grouped by presence or

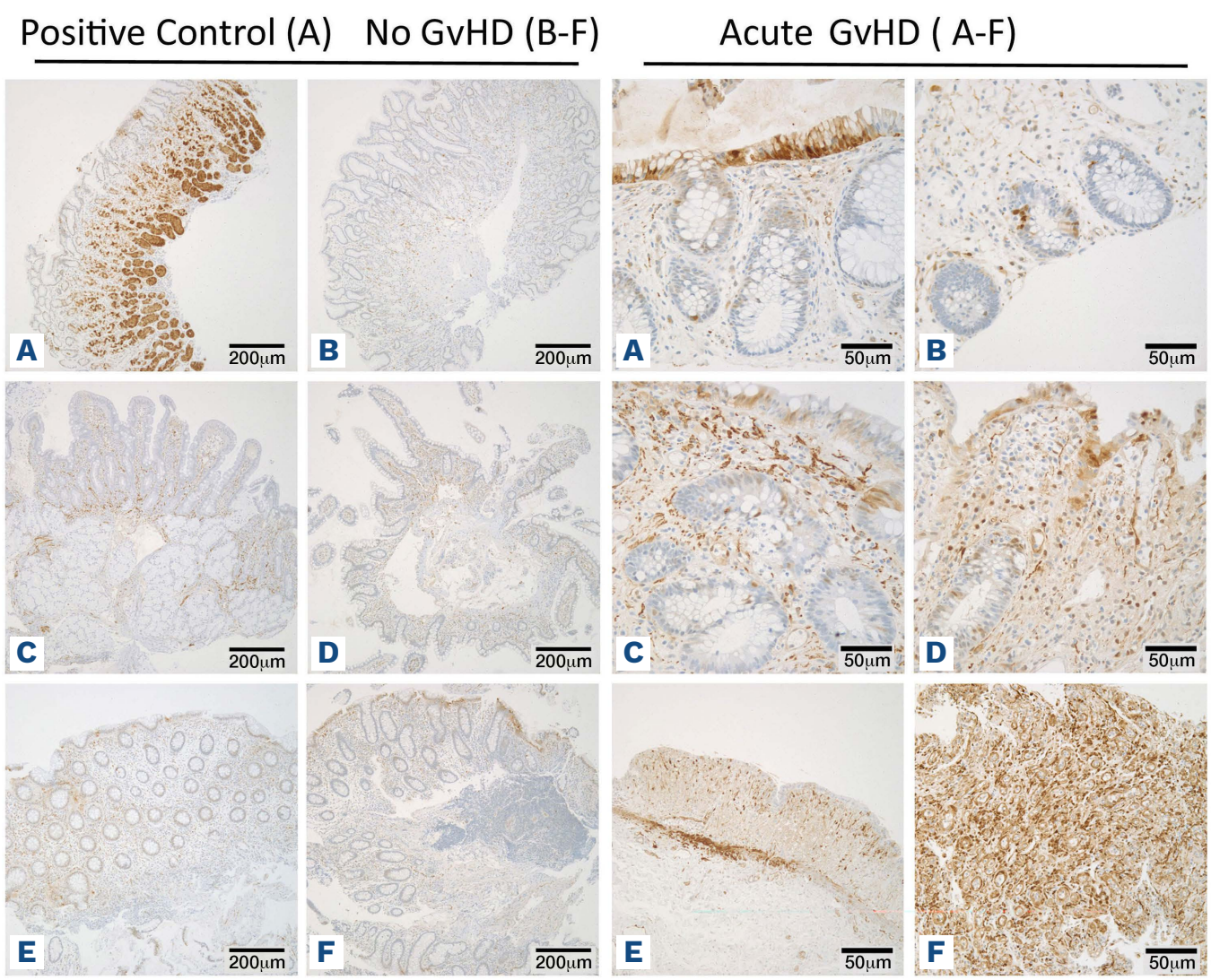

Figure 3. pERK expression by immunohistochemistry in ten separate acute gastrointestinal (GI) graft-*versus*-host disease (GvHD) and ten not acute GI GvHD biopsies. *No GvHD disease: immunohistochemical stain for pERK in human control tissues (left and center):* (A) gastric body (positive control): pERK is strongly expressed in the acid producing oxyntic glands in the gastric body as expected; (B) gastric antrum: weak normal pERK staining observed; (C) duodenum: weak normal pERK staining observed; (D) small intestine: weak normal expression in lamina propria neurites and stromal cells is seen throughout; (E) small intestine: weak normal pERK staining as described in (D); (F) colon: weak focal to patchy expression, is seen in colonic surface mucosal cells. Formalin-fixed paraffin-embedded (FFPE) tissue 4-micron-thick immunohistochemistry tissue sections, 100x magnification. *Acute GI GvHD: immunohistochemical stain for pERK in human GvHD colonic tissue sections as follows (right)*: (A) grade 1 GvHD: note patchy but strong surface expression and focal expression in crypts, including adjacent to apoptotic figures (B); (C) grade 2 GvHD: note patchy surface and crypt expression with increased superficial density and staining of lamina propria neurites and stromal cells; (D) grade 3 GvHD: strong pERK surface expression on epithelium in addition to lamina propria; (E) grade 3-4 GvHD: strong pERK surface expression on epithelium and lamina propria; (F) grade 4 GvHD: diffuse strong pERK expression on surface epithelium. Scattered positive capillary endothelial cells positive in all grades, most notably in grade 4 GvHD ulcer bed with granulation tissue vascularity. FFPE tissue 4-micron thick immunohistochemistry sections, 400x magnification. Grades of GvHD are pathological gradings.

absence of acute GI GvHD and stages of acute GI GvHD. The most significant difference was seen in the M3 module, comprised of about 800 genes expressed differentially in the presence of acute GI GvHD compared with no acute GI GvHD (*P*=0.04), and in higher stages of acute GI GvHD compared with lower stages of acute GI GvHD (*P*=0.004) (*Online Supplementary Dataset S2*). Functional enrichment analysis of module M3 (Figure 2B) showed upregulation of genes in the ERK signaling pathway in acute GI GvHD compared to patients without acute GI GvHD.

pERK expression on intestinal biopsy specimens

We sought to verify ERK pathway upregulation using immunohistochemistry staining of GI biopsies from children with and without GvHD. We found greater pERK expression on epithelial cells of biopsies in patients with acute GI GvHD compared to HSCT patients without GvHD (Figure 3). The median percent positive pixel count for pERK was 46% (range, 13-54%) in acute GI GvHD compared to 14% (range, 10-17%) in no GI GvHD (*P*=0.001), supporting our transcriptomic findings (Figure 4).

Shared genes, biological processes, and pathways between graft-*versus*-host disease and ulcerative colitis

We wanted to compare differentially regulated genes in UC and acute GI GvHD, as successful therapeutic strategies in UC might be applicable to acute GI GvHD if biology is similar. One hundred and twenty-nine (91%) of the 141 genes upregulated in acute GI GvHD were also upregulated in UC, and 17 (17/23, 74%) genes downregulated in acute GI GvHD were also downregulated in UC (Figure 5A, C).

Figure 5B highlights similarities in key pathways and functions between acute GI GvHD and UC. Important upregulated biological processes common to both include responses to microbes, cytokines and innate immune system responses. Notable upregulated pathways common to both diseases include interferon pathway signaling, neutrophil activation and responses to microbes.

Differential expression of genes unique to graft-*versus*-host disease compared to ulcerative colitis

Twelve genes were upregulated, and six genes were downregulated in acute GI GvHD but not in UC (*Online Supplementary Table S4)*. Notable genes upregulated in acute GI GvHD only are related to macrophage function (*MARCO, CXCL16*), metabolism (*CA8*), signaling (*COTL1, FAM195B*), cell adhesion (*TM4SFL*) and response to infection (LYAR). Genes downregulated in acute GI GvHD only include a chemokine receptor (*GPR15*), transcriptional regulators (*ZBTB38, YBX2*) and nucleotide metabolism (*ADAL*).[13] These genes are involved in the innate immune system, macrophage activation and chemokines signals expressed on macrophages

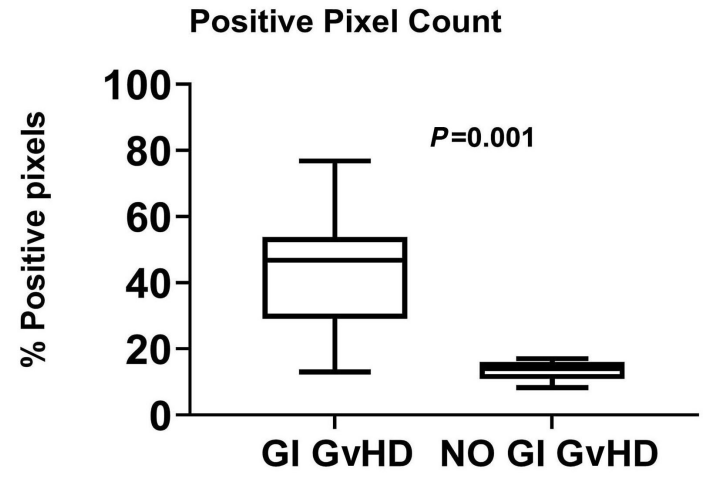

Figure 4. Percent positive pixel count of pERK staining in acute gastrointestinal graft-*versus*-host disease biopsies and no graft-*versus*-host disease biopsies. Graphs represent median values of percentage of positive pixels in each group with *P* values generated by the Mann Whitney U test. GvHD: graft-*versus*-host disease; GI: gastrointestinal.

Plasma CD64

FCγR1A also known as CD64, is expressed on neutrophils and monocytes, and is upregulated by mediators of inflammation such as interferon-γ, G-CSF, infection, or tissue injury.[14] Plasma CD64 is elevated in new onset IBD and is a marker of intestinal inflammation.[15] Additionally, plasma CD64 and neutrophil CD64 expression have been strongly correlated in IBD.[16] Elevated surface expression of CD64 on neutrophils has been associated with loss of responses to steroids and infliximab in IBD.[17] In our data, FCγRA1 gene expression was upregulated in acute GI GvHD (log fold increase 1.7, *P*=0.001) and in UC (FC increase =5.8, $P=4.18x10^{-23}$).[6] As CD64 is associated with mucosal inflammation, steroid and infliximab responsiveness in pediatric IBD, we wanted to study CD64 in acute GI GvHD to confirm our transcriptome findings.

Patient demographics are shown in the *Online Supplementary Table S5* (GvHD) and *Online Supplementary Table S6* (IBD and non-IBD controls). Median plasma CD64 was 222 ng/mL (range, 35-411 ng/mL) in patients with acute GI GvHD compared to 76 ng/mL (range, 0-360 ng/mL) in patients without acute GvHD, 72 ng/mL (range, 11-208 ng/mL) in patients with isolated skin GvHD, 72 ng/mL (range, 27-174 ng/mL) in patients with IBD and 29 ng/mL (range, 11-72 ng/mL) in non-IBD controls (*P*<0.001; Figure 5D).

Discussion

We report the first pediatric transcriptome analyses of intestinal tissue involved in acute GI GvHD using freshly frozen GI biopsies. We demonstrate a unique gene signature in acute GI GvHD with differential expression of genes which have been previously described as important in GvHD such as *IDO1*,[18] *CXCL10*[19] and *Granzyme B*.[20] We observed upregulation of biological processes involved in antimicrobial responses supporting a role for the intestinal

microbiome modulation in acute GI GvHD. Additional biological processes involved in acute GI GvHD included responses to cytokines, specifically interferons and innate immune responses. Important upregulated pathways included interferon-γ signaling and neutrophil degranulation. We added 35 paraffin-embedded GI biopsies to compare our findings from the freshly obtained GI biopsies. We notably observed that transcriptomics from paraffin-embedded GI biopsies did not identify relevant genes such as *FCγR1A* or pathways such as ERK, shown to be signifi-

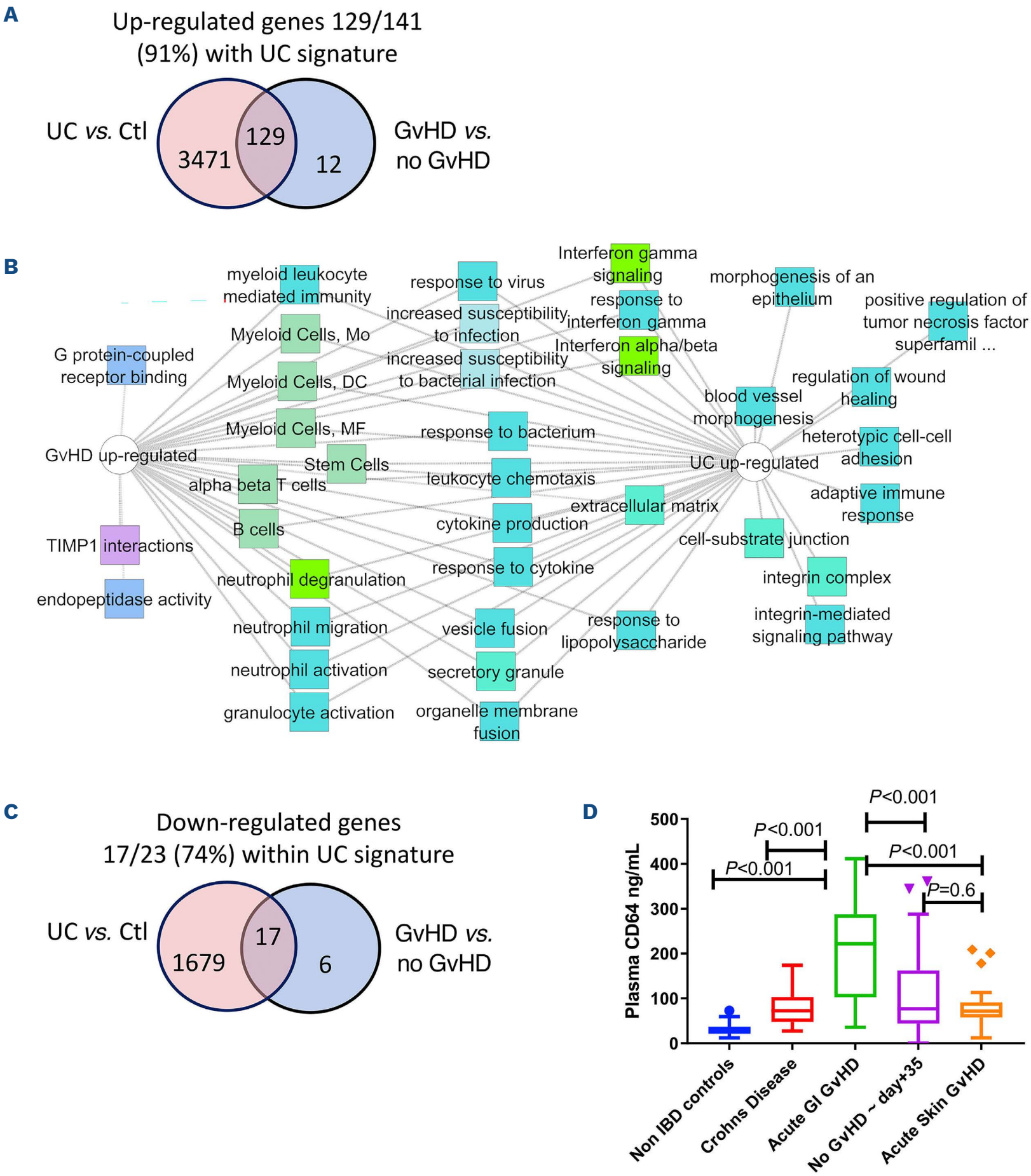

Figure 5. Comparison between transcriptomics and biomarkers in ulcerative colitis and acute gastrointestinal graft-*versus*-host disease. Venn diagram shows shared upregulted (A) and (C) downregulated genes in patients with acute graft-*versus*-host disease (GvHD) and ulcerative colitis (UC). Shared upregulated pathways in acute GvHD and UC are shown in (B). (D) Plasma CD64 levels (ng/mL) in non-inflammatory bowel disease (IBD) controls (Ctl) (n=42), IBD (n=47), acute gastrointestinal (GI) GvHD (n=30), hematopoietic stem cell transplant patients without GvHD (n=30) and acute isolated skin GvHD (n=25).

cantly upregulated in acute GI GvHD in correlative studies. However, we also found several areas of overlap of key genes, biological processes, pathways and molecular functions, lending strength to our observations from data obtained from freshly frozen GI biopsies. Notably, *CHI3L1* upregulation was observed strongly in transcriptomics from both cohorts. *CHI3L1* is of particular interest given its roles in neovascularization, macrophage recruitment, and bacterial adhesion.[21-25] There are limited prior data on the role of CHI3L1 in HSCT,[26] but one report identified elevated plasma CHI3L1 in patients with a very high HSCT comorbidity index,[27] suggesting that CH13L1 could be a pro-inflammatory marker. Several anti-CHI3L1 therapeutics are being studied to alter CHI3L1-induced effector responses, such as monoclonal anti-CHI3L1 (FRG), kasugamycin, and inhibitors of CHI3L1 phosphorylation in the context of SARS-COV2,[28] and these could potentially be studied in acute GI GvHD in future clinical trials.

Abnormalities of several mitochondrial genes have been described in acute GI GVHD including *GPR43*,[29] *SIRT3*,[30] *NLRP6*[31] and *Signal 3*,[32] none of which were detected in our dataset. However, the second most downregulated gene HMGCS2 in our dataset from freshly frozen GI biopsies is a mitochondrial enzyme that catalyzes the first reaction of ketogenesis.[33] Recent work has demonstrated a potential link of HMGCS2 with the butyrate metabolism,[34] which is of interest, given data regarding butyrate depletion in acute GI GvHD[35] and testing of strategies to optimize intestinal butyrate to decrease acute GvHD incidence. Patients with UC had substantial suppression of all 13 electron transport mitochondrial-encoded genes (*Complex I, III, IV*, and *V*), *PPARGC1A* (*PGC1*α), and epithelial *MMP* demonstrating colonic mitochondriopathy.[6] *PPARGC1A* was similarly significantly downregulated in acute GI GvHD.

The *Online Supplementary Table S7* shows comparisons between notable genes described in our study from both cohorts with published adult transcriptome data.[9] Important similarities between the two include highest differential expression of *CHI3L1*, upregulation of IDO1 and significant downregulation of HMGCS2.[9] Our study supports an important role for macrophages in the pathogenesis of acute GI GvHD, similar to findings in the adult study.[9] However, we also observed several differences in our findings compared to the adult study including lack of differential expression of *AQP8*, *CCL18* and *ITLN1*, which could be due to differences in analysis platform, limiting direct comparisons.

Gene expression profiles of T cells collected at the time of acute GvHD in non-human primates demonstrated aurora kinase A pathway as a possible druggable target.[8] We did not observe aurora kinase A pathway involvement in our data, likely due to the differences in sample tissue used for the description of gene expression profiles, as our samples were not enriched specifically for T cells.

Our findings demonstrated a significant overlap between differentially regulated genes and pathways in pediatric GI GvHD and IBD. Expression of the antimicrobial dual oxidase gene (*DUOX2*) was the highest differentially expressed gene associated with an expansion of Proteobacteria in UC[6] and was also upregulated in pediatric GI GvHD. Expression of apolipoprotein A1 (*APOA1*) was downregulated and predicted 6-month steroid-free remission in IBD, when combined with microbial abundance.[7] We did not observe downregulation of *APOA1* in pediatric acute GI GvHD but saw upregulation of apolipoprotein L1, which in turn binds to APOA1, perhaps resulting in similar biological effects.

Our findings of upregulation of *DUOX2* and downregulation of butyrate metabolism in acute GI GvHD complement current work and understanding of the intestinal microbiome in acute GI GvHD. In UC, an abnormal increase of antimicrobial dual oxidase 2 expression was detected in association with an expansion of *Proteobacteria*.[7] Unlike our comparison cohort of UC patients, we did not perform concurrent stool microbiome analyses to complement our findings, but independent studies have shown lower microbial diversity along with increased relative abundance of *Proteobacteria* and *Enterobacteriaceae* in acute GI GvHD.[36, 37] We have also previously shown that lower fecal butyrate is observed in acute GI GVHD.[38] Efforts are underway to increase intestinal butyrate in adults and pediatrics with various prebiotics such as fructo-oligosaccharide and potato-based starch.[39, 40] Additionally, several microbiome-targeted approaches such as fecal microbiome transplant and judicious use of antibiotics are being studied.

We also saw upregulation of FCγR1A, also known as CD64, in pediatric acute GI GvHD, a known marker of neutrophil activation which predicts responses to steroids and infliximab in pediatric IBD[15,16] CD64 has also been previously described in skin biopsies of patients with acute skin GvHD.[41] Additional experiments demonstrated elevation of plasma CD64 in acute GI GvHD, an independent confirmation of findings observed in our transcriptome analysis. Moreover, plasma CD64 levels were strikingly comparable in patients with acute skin GvHD, allogeneic HSCT recipients without GvHD, and UC and all three groups had higher CD64 levels compared to non-IBD controls. Elevations of plasma CD64 levels in allogeneic HSCT patients without any GvHD compared to non-IBD controls was notable, suggestive of subclinical but widespread tissue injury. CD64 elevation in acute GI GvHD is higher than what is observed in UC despite shared transcriptomic profiles. Plasma CD64 is also elevated in acute GI GvHD compared to acute skin GvHD. Collectively, these findings suggest that plasma CD64 could be a specific and unique marker to study further in acute GI GvHD.

The integrin-mediated signaling pathway, is upregulated in both pediatric GI GvHD and UC, which is of relevance as ve-

dolizumab, an α4β7 integrin blocker is used in both pediatric IBD and acute GI GvHD.[42,43] We observed upregulation of pathways involved in activation of matrix metalloproteinases 1, 3, 7 and 10 (MMP) in GvHD (*Online Supplementary Dataset S1*). MMP are expressed in healthy intestinal tissue to assist in the degeneration and remodeling of the extracellular matrix and the basement membrane, leading to improved mucosal barrier function.[44] MMP□1 (collagenase□1) expression correlates with the severity of UC.[45] Additional shared pathways include a wound healing signature and antimicrobial responses in addition to cytokines and neutrophil activation, further emphasizing rationale for microbiome modulation in both diseases.

We also observed differential expression of genes in acute GI GvHD which are not observed in UC. The biological processes that determine differences in alteration of gene expression in acute GI GvHD and UC is unclear, but a topic of great interest. Macrophages are implicated in pathophysiology of both entities, but it is possible that specific macrophages are involved, based on tissue specific trafficking signaling unique to acute GI GvHD. It is also possible that our small sample size of acute GI GvHD patients did not achieve statistical significance for additional genes which might contribute to biological differences between acute GI GvHD and UC. A pediatric study of transcriptome analyses of biopsies in patients with UC reported the following significant cell types infiltrating biopsies, by immune deconvolution: active dendritic cells (DC), B cells, $CD4^+$ naive T cells, conventional DC, memory B cells, plasma cells, Th1 cells, and monocytes. In contrast, M1 macrophages and follicular helper T cells were identified as significant cell types in acute GI GvHD further suggesting important differences in biology of acute GI GvHD and UC, despite many shared genes and pathways.

We observed upregulation of the ERK signaling pathway in our study, a novel finding in pediatric acute GI GvHD. Additionally, pERK expression was detected on immunohistochemistry in ten de-identified intestinal biopsies of acute GI GvHD, further validating our findings. There is preclinical evidence of the role of the ERK pathway in acute GvHD in murine and adult allogeneic transplant studies.[46-49] Phosphorylation of ERK1/2 and STAT-3 have been shown as important events during T-cell activation in GvHD in murine studies.[48] Single-cell analysis of ERK1/2 phosphorylation in murine T cells suggested that *ex vivo* MEK inhibition inhibited alloreactivity.[47] Low dose trametinib inhibited ERK1/2 phosphorylation and prolonged survival of GvHD mice and attenuated GvHD symptoms and pathology in the gut and skin.[49] Administration of selumetinib in a major histocompatibility complex major- and minor-mismatched murine model delayed the onset of GvHD-associated mortality without affecting myeloid engraftment.[49] Lastly, phosphorylation of ERK1/2 in T and B cells was analyzed by flow cytometry in 20 adult allogeneic-transplant recipients and occurrence of acute GvHD was associated with phosphorylation of ERK1/2 in CD4 T cells at day 30, which was suppressed by *ex vivo* exposure to trametinib at clinically achievable concentrations.[46] Our findings complement this body of evidence on ERK upregulation and acute GvHD.

Our study has several strengths. This is the first transcriptomic analysis of freshly frozen GI biopsies in children with acute GI GvHD. The findings of this study lend themselves to several translational applications, namely targeting the intestinal microbiome, optimizing intestinal butyrate in patients to prevent acute GI GvHD, and paving the way for future macrophage-directed treatments. ERK pathways may be targeted in future studies to treat acute GI GVHD. CHI3L1 is another therapeutic target for acute GI GvHD, with the availability of monoclonal antibodies targeting this in SARS-COV2.[28] We also show a possible role for CD64 as a marker of acute GI GvHD, which may be studied in larger scale studies prospectively as a biomarker of acute GI GvHD. However, our study also has several limitations. Obtaining biopsy material for research from ill children is challenging, and so our sample size is small, and we have no biopsies from healthy children available for comparison, limiting some analyses. In order to address this limitation, we augmented our cohort with 35 allogeneic HSCT patient GI biopsies retrospectively and observed considerable overlap between findings but also observed important differences including lack of expression of *DUOAX2* and lack of involvement in the ERK pathway from paraffin embedded biopsies, suggesting important variability of these two approaches. Our patient population is heterogenous with inclusion of differing strengths of conditioning regimens and acute GvHD prophylaxis, inherent to pediatric studies likely contributing to important inter-patient variability. We tried to account for variables which could influence our results. Location of the GI biopsy sample could influence results and we therefore only chose rectal biopsies and excluded the single patient from the freshly frozen biopsy cohort in whom the biopsy was not from the rectum. We attempted to choose patients for analyses whose biopsies were obtained at diagnosis of acute GI GvHD, however one patient in the freshly frozen biopsy cohort underwent endoscopy after an established diagnosis of acute GI GvHD. Further, prior treatments for acute GI GvHD at the time of GI biopsy could influence results but we obtained almost all biopsies at diagnosis of GvHD with only one patient being on treatment before biopsy was obtained. Lastly, we excluded patients with pre-existing colitis due to their underlying disease to reduce additional confounding variables which could influence our results. Our previous work has shown the role of human milk in influencing markers of intestinal inflammation[50] but no patient in our cohort was actively breastfeeding, reducing the influence of diet relevant to pediatric studies. In addition, while no patients were on prebiotics before or at the time of lower GI biopsy, there

could be additional influences of diet in this study which we cannot comment on as we did not take a detailed dietary history. Our controls underwent endoscopy for clinical symptoms and while the majority had no abnormal findings on histopathology, one patient in the freshly frozen biopsy cohort and one patient from the paraffin-embedded biopsy cohort tested positive for cytomegalovirus from the tissue biopsy, which could have also influenced our results. A more uniform assessment in the future could be enabled if patients are selected with the same underlying diagnosis, similar preparative and acute GvHD prophylactic regimens and enrolling age-matched controls with detailed dietary histories for both cohorts. Furthermore, similar to our current approach, obtaining biopsies in all patients at diagnosis of acute GI GvHD from the same location of the lower intestinal tract and eliminating patients with pre-existing colitis due to their underlying disease will streamline assessments further. Our experiments on plasma CD64 are preliminary and larger scale research is required for confirmation of our findings. Additionally, as we chose patients based on the incidence of acute GvHD in the CD64 experiment, there could be a selection bias.

In summary, our study has several clinical implications including justification of shared therapeutics with pediatric IBD and identification of important similarities with adult acute GI GvHD.

Disclosures

No conflicts of interest to disclose.

Contributions

PK collected intestinal biopsy samples, interpreted the data and wrote the manuscript. DL, AB and ASN enrolled patients on study and collected intestinal biopsies. KL and BL processed intestinal biopsy samples. NL extracted RNA from intestinal biopsy samples. RK, YH, AJ and SG assisted with bioinformatic analyses of data. DL and JK assisted with IHC interpretation of intestinal biopsies. ML performed CD64 ELISA experiments in HSCT patients. PM contributed CD64 data in IBD patients. LAD provided RNA sequencing data from patients with ulcerative colitis and assisted with study design. SMD oversaw the entire study and assisted with data interpretation and edited the manuscript critically. All authors reviewed the manuscript critically for edits.

Acknowledgements

We would like to acknowledge patients, families, and the clinical staff at Cincinnati Children's Hospital Medical Center who generously participated in this work.

Funding

P30 DK078392 Digestive Diseases Research Core Center in Cincinnati; U01 DK095745 (LAD).

Data-sharing statement

Full gene lists may be found in the Online Supplementary Appendix. RNA sequencing datasets were deposited in GEO (GSE168116 & GSE215068).

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