

Randomized Controlled Trial Substudy of Cell-specific Mechanisms of Janus Kinase 1 Inhibition With Upadacitinib in the Crohn's Disease Intestinal Mucosa: Analysis From the CELEST Study

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Background: Janus kinase (JAK) inhibition shows promise for treatment of patients with moderate to severe Crohn's disease. We aimed to provide mechanistic insights into the JAK1-selective inhibitor upadacitinib through a transcriptomics substudy on biopsies from patients with Crohn's disease from CELEST.

Methods: Seventy-four patients consented to this optional substudy. Ileal and colonic biopsies were collected during endoscopy at screening and week 12 or 16. RNA isolated from 226 samples was analyzed by RNAseq, with additional qPCR analysis. Additional biopsies from patients with Crohn's disease receiving anti-tumor necrosis factor (anti-TNF; n = 34) and healthy controls (n = 10) were used for qPCR. Single-cell RNAseq public profiles were used to evaluate treatment effects on specific cellular subsets, associations with endoscopic improvement, and indirect comparisons with the anti-TNF-treated cohort.

Results: In involved areas of mucosa with endoscopic remission after upadacitinib treatment, 1156 and 76 protein-coding genes were significantly regulated (false discovery rate < 0.05) at week 12/16 in colonic and ileal biopsies, respectively (60 overlapped), compared with baseline. Upadacitinib did not significantly affect transcriptomes of noninvolved intestinal areas. CELEST patients (mostly anti-TNF-refractory) showed baseline differences in gene expression compared with a separate cohort of biologic-naïve patients. Notably, upadacitinib reversed overexpression of inflammatory fibroblast and interferon- γ effector signature markers.

Conclusions: Upadacitinib modulates inflammatory pathways in mucosal lesions of patients with anti-TNF-refractory Crohn's disease, including inflammatory fibroblast and interferon- γ -expressing cytotoxic T cell compartments. This substudy is the first to describe the molecular response to JAK1 inhibition in inflammatory bowel disease and differential effects relative to anti-TNF treatment. (Clinical trial identifier: NCT02365649)

Key Words: Crohn's disease, JAK inhibitor, tumor necrosis factor, upadacitinib

Introduction

Crohn's disease is a chronic, remitting, and relapsing inflammatory disease that can affect any part of the intestinal tract and present with inflammation, fistulae, and/or stenosis. Therapies used to control disease activity and some of its complications include corticosteroids, immunosuppressants, and anti-tumor necrosis factor (anti-TNF), anti- $\alpha 4\beta 7$ and, more recently, anti-p40 (interleukin [IL]-12/IL-23) antibodies. Despite advances in biologic therapy, inadequate or loss of response remains a clinical challenge in >20% of patients.¹ Moreover, therapy is limited by adverse reactions and, in biologic agents, by immunogenicity. Novel therapies, specifically small molecules antagonizing different pathways involved in cell activation or recruitment, are being developed. Blocking the Janus kinase (JAK) family (JAK1, JAK2, JAK3, and tyrosine kinase 2), intracellular proteins associated with some cytokine receptors involved in inflammatory bowel disease

(IBD) pathogenesis, has become a focus of ulcerative colitis (UC) and Crohn's disease research.² Tofacitinib, an oral pan-JAK inhibitor (primarily targeting JAK1 and JAK3), was approved for the treatment of moderate to severe UC^{3,4} but failed to show efficacy in phase 2 studies in Crohn's disease.⁵

Recently developed JAK inhibitors have been designed with increased selectivity to a single JAK family member, with the aim of increasing efficacy and reducing side effects. Upadacitinib (ABT-494), an oral selective JAK1 inhibitor, is being investigated for the treatment of Crohn's disease. In the phase 2 CELEST study, upadacitinib demonstrated efficacy, inducing clinical and endoscopic remission in patients with Crohn's disease who had inadequate response/intolerance to an immunosuppressant or anti-TNF.⁶

This substudy of CELEST aimed to provide mechanistic insights into the effects of upadacitinib on the intestinal mucosa of patients with moderate to severe Crohn's disease, using

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RNA sequencing (RNAseq) from intestinal biopsies. The main objective was to identify molecular pathways modulated by JAK1 inhibition. A secondary objective was to identify the cellular subsets primarily regulated by upadacitinib. We also compared the upadacitinib-induced endoscopic remission signature with 2 independent cohorts consisting of healthy volunteers or patients with Crohn's disease who received anti-TNF treatment as standard of care to induce clinical and endoscopic remission. Overall, this substudy is the first to describe the molecular basis of response to JAK1 inhibition in patients with Crohn's disease and its differential effects relative to anti-TNF treatment.

Methods

Study Design and Upadacitinib Patient Recruitment

CELEST (NCT02365649) enrolled adults (age 18–75 years) with confirmed ileal, ileocolonic, or colonic Crohn's disease for ≥ 3 months, active disease (Crohn's Disease Activity Index [CDAI] of 220–450), an average daily liquid/very-soft-stool frequency ≥ 2.5 or daily abdominal pain score ≥ 2.0 , and evidence of mucosal inflammation (Simplified Endoscopic Score for Crohn's disease [SES-CD] ≥ 6 , or ≥ 4 for those with isolated ileal disease). At baseline (week 0), patients were randomized (1:1:1:1:1) to receive double-blind, 16-week induction oral treatment with the immediate-release formulation of upadacitinib at 3, 6, 12, or 24 mg twice daily, upadacitinib 24 mg once daily, or placebo. Patients were equally randomized for the follow-up ileocolonoscopy at week 12 or 16 (hereafter week 12/16), to evaluate optimal timing of endoscopic assessment for future studies. Ileocolonoscopies performed during screening and at week 12/16, for eligibility and for efficacy assessments, were centrally read blinded for patient data and time points.

Patients' randomization schedules were generated by an AbbVie system (WebRando) and loaded into a central interactive response technology (IRT) system (managed by a vendor external to AbbVie) ahead of enrollment. This included random sequences of treatment codes of prespecified length (block size) for each combination of the protocol-specified stratification factors to ensure balanced randomization across strata. Randomization was stratified by endoscopic disease severity (SES-CD < 15 and ≥ 15) and prior anti-TNF use. Study site personnel enrolled patients by accessing the IRT system, which centrally randomized patients who met the eligibility criteria into treatment groups and assigned the study drug to be dispensed based on the assigned treatment group. By using the central IRT system, the study site personnel, patients, and the sponsor study teams did not have access to the treatments assigned to the patients until the end of the study period, when the study database was locked and study data were analyzed.

Patients in the anti-TNF cohort were patients with Crohn's disease defined by active endoscopic disease at the time of inclusion (Global Crohn's Disease Endoscopic Index of Severity [CDEIS] ≥ 5 , or CDEIS ≥ 4 for ileal only disease) that had started anti-TNF treatment as standard of care. Healthy controls were patients with no history of IBD who were undergoing ileocolonoscopy for colorectal cancer screening and who presented no lesions at the time of examination. Patients in both cohorts consented to participating in the optional transcriptomics substudy with intestinal biopsies (Hospital Clinic Barcelona Ethics Committee, Barcelona, Spain; approval number: 2012/7956).

Study End Points

The primary outcome measure was to identify molecular pathways modulated by JAK1 inhibition. Secondary objectives were to identify the cellular subsets primarily regulated by upadacitinib and compare the upadacitinib-induced endoscopic remission signature with 2 independent cohorts consisting of healthy volunteers or patients with Crohn's disease who received anti-TNF treatment as standard of care to induce clinical and endoscopic remission.

Biopsy Processing

Participating patients had biopsies taken from the ileum and colon in the most significant areas of non-necrotic inflammation (involved areas) or healthy areas in the case of segments without inflammation (noninvolved) observed during endoscopy. Two or 3 biopsies per region were collected at screening (week 0) and at the end of induction (EOI) period (week 12/16), and these were placed in RNAlater RNA Stabilization Reagent (QIAGEN, Hilden, Germany) and stored at -80°C until RNA isolation. RNA was isolated using an RNeasy Kit (QIAGEN, Hilden, Germany) according to the manufacturer's instructions. RNA was quantified using a NanoDrop spectrophotometer (NanoDrop Technologies, Wilmington, DE, USA). The purity and integrity of the total RNA was assessed via 2100 Bioanalyzer (Agilent, Santa Clara, CA, USA). For quality control (QC) of RNA used in RNAseq, all RNA samples analyzed had an RNA integrity number ≥ 7.7 . Samples that passed QC ($n = 226$ samples) from 74 patients were sequenced (Supplementary Table S1).

RNAseq

Barcoded RNAseq libraries were prepared from total RNA using a TruSeq stranded mRNA kit (Illumina, San Diego, CA, USA) according to the manufacturer's instructions. Libraries were subjected to paired-end sequencing (50 bp) on a HighSeq-3000 platform (Illumina, San Diego, CA, USA) at the Genomic Service (Centro de Regulación Genómica, Barcelona, Spain). Quality filtering and adapter trimming was performed using Skewer version 2.2.⁸ Reads were mapped against the human reference genome using the STAR aligner version 2.5.2a. The genome used was GRCh38.p10, and gene annotation was based on Gencode version 27 (EMBL-EBI, Hinxton, UK). Read counts per gene were obtained using RSEM version 1.2.31 and the Ensembl GTF annotation file (EMBL-EBI, Hinxton, UK). Analyses were performed using the R (version 3.2.3) statistics package. Differential expression analysis was performed with the limma version 3.34.5 and edgeR version 3.20.6 packages, adjusting for batch (specifying a block argument for patient variable).

cDNA Synthesis and Real-Time PCR

The total RNA from biopsies that passed QC was transcribed to complementary DNA (cDNA) using a High-Capacity cDNA Archive RT kit (Applied Biosystems, Foster City, CA, USA) and was then used to perform real-time quantitative polymerase chain reaction (qPCR) in triplicate wells with a TaqMan Universal PCR Master Mix (Applied Biosystems, Foster City, CA, USA) containing the probe of interest and β -actin (TaqMan primers and probes; Applied Biosystems). Predesigned TaqMan Assays (Applied Biosystems, Foster City, CA, USA) for 19 different genes that are primarily expressed by individual cell types in the intestine (Supplementary Table

S2) were used. The PCRs were performed using an Applied Biosystems 7500 Fast Real-Time PCR detection system. Relative quantification (x) was calculated using the formula $x = 2^{-\Delta Ct} \times 1000$, where $\Delta Ct = Ct_{\text{target gene}} - Ct_{\beta\text{actin}}$.

Associations of Transcriptional Changes and Endoscopic Improvement

Transcriptional changes were analyzed separately for the ileum and colon and then compared with treatment efficacy measured by endoscopic improvements at the EOI period. The 2 end points used to assess endoscopic improvements in each of the ileal or colonic segments were endoscopic response (defined as a ≥ 2 -point reduction from baseline in the SES-CD of the respective ileal or colonic segment) and endoscopic remission (defined as an ulcer subscore ≤ 1 in the respective ileal or colonic segment) at week 12/16. Samples from all patients receiving upadacitinib at any of the study doses were pooled to achieve a sufficient number of samples achieving endoscopic response or remission to perform statistical analysis.

Identification of Cell Subsets Based on Transcriptional Changes

To link the transcriptional signatures induced on upadacitinib treatment with changes in specific cell types, publicly available single-cell RNA-sequencing (scRNAseq) data from human intestinal tissue were used.⁹ Smillie et al⁹ identified >40 different colonic cell subsets based on single-cell transcriptomes. In this study, these subsets were grouped into 4 epithelial populations (stem, enterocyte, M cell, and secretory cell), 4 myeloid cell types (macrophages, dendritic cells, monocytes, and mast cells), 4 lymphocyte cell types (including T and B cell subsets; cluster of differentiation 4 [CD4], CD8, regulatory T cells [Tregs], and cycling T cells), and 2 stromal subsets (fibroblast and endothelial cells; [Supplementary Table S3](#)).

Indirect Comparisons With External Cohorts of Anti-TNF-treated Patients and Healthy Volunteers

Two external cohorts were used for comparisons with the upadacitinib transcriptional changes. The first was from an observational prospective study of patients with active Crohn's disease who received anti-TNF treatment (infliximab or adalimumab) for 14 weeks as standard of care. All patients underwent clinical and endoscopic evaluation at weeks 0 and 14; biopsies were obtained when possible at both time points. [Supplementary Table S4](#) shows the baseline demographic, clinical, and endoscopic characteristics of the patients included in the anti-TNF cohort (colon samples, $n = 18$; ileal samples, $n = 16$). The second cohort consisted of healthy control individuals ($n = 10$) undergoing colonoscopy for gastrointestinal symptoms or for colorectal cancer screening who had a normal examination and no history of IBD. For these patients, tissue samples from the ileum and/or colon were collected and processed for RNA isolation and PCR analysis as described previously. Both studies were conducted at the Hospital Clinic Barcelona, Barcelona, Spain, and were approved by the institutional ethics committee.

Statistical Methodology

Baseline and disease characteristics were presented as median (range), and differences among treatment groups were

calculated by the Fisher exact test. Where CELEST data were pooled, upadacitinib- and placebo-treated patients were grouped separately. For 2-group comparisons, the Mann-Whitney-Wilcoxon test was used. All probability (P) values corrected for false discovery rate (FDR) < 0.05 were considered statistically significant. As some patients did not have biopsies at both time points that passed QC, only unpaired group analysis was performed.

Results

Seventy-four patients participated in this substudy. Demographic and baseline characteristics were generally balanced with no significant differences across treatment arms ([Table 1](#)). In total, 238 intestinal samples were collected from screening or week 12/16 ileocolonoscopies. Of these, 226 samples passed QC and RNAseq was performed ([Supplementary Table S1](#) shows the location of all intestinal samples sequenced). Principal component analysis using whole-transcriptome analysis of all 226 samples segregated colonic from ileal biopsies, regardless of involvement, in agreement with previous studies ([Supplementary Fig. S1](#)).¹⁰ Subsequently, we performed all further analyses of ileal and colonic data separately.

Endoscopic Remission After Treatment With Upadacitinib Is Associated With Transcriptional Changes in the Colon and Ileum

Endoscopic response and remission at week 12/16 with upadacitinib treatment resulted in the significant regulation (FDR < 0.05) of many genes in the colon compared with baseline at week 0 ([Table 2](#)). In the ileal samples, endoscopic remission, but not response, was associated with significant changes (FDR < 0.05) in gene expression. The number of samples used for each comparison is shown in [Table 2](#). Overall, gene regulation with upadacitinib treatment was more pronounced in the colon than in the ileum. In cases of endoscopic remission at the EOI period (week 12/16), 1156 protein-coding genes were significantly regulated (fold change [FC] > 1.5 ; FDR < 0.05) in colonic biopsies, whereas 76 genes were significantly regulated in ileal biopsies ([Table 2](#), and [Fig. 1A](#) and [B](#)). Of these 76 genes, 60 were similarly regulated in the colonic mucosa of patients who achieved endoscopic remission with upadacitinib. [Fig. 1C](#) shows the correlation of FC in colon vs FC in ileum for all 60 commonly regulated genes in patients achieving endoscopic remission at EOI with upadacitinib.

If a less-strict FDR arbitrary cutoff value was applied (FDR < 0.22 , to increase the number of differentially expressed genes for the comparison), a total of 1859 genes were changed at week 12/16 in ileal biopsies (FC > 1.5). Importantly, over half (58%) of the genes regulated in the colon were also significantly regulated in the ileum when using this less-strict statistical cutoff (data not shown), implying a shared response signature to upadacitinib despite large differences between the overall transcriptional signatures of colon and ileum ([Supplementary Fig. S1](#)).

In contrast to patients in the upadacitinib treatment group, no genes were significantly regulated at EOI in the placebo group, regardless of endoscopic response/remission status or disease location ([Table 2](#)).

Table 1. Demographic and Baseline Characteristics From Patients in the Upadacitinib Phase 2 Study With ≥ 1 Biopsy Sample Passing the Quality Control for Transcriptional Analysis

	Placebo (n = 11)	Upadacitinib (ABT-494)					P
		3 mg Twice Daily (n = 15)	6 mg Twice Daily (n = 13)	12 mg Twice Daily (n = 8)	24 mg Twice Daily (n = 14)	24 mg Once Daily (n = 13)	
Gender, F/M, n	8/3	5/10	6/7	3/5	9/5	7/6	0.338
Age, median years (range)	39.0 (21–56)	34.0 (19–56)	40.0 (27–76)	28.5 (20–56)	42.5 (20–65)	36.0 (23–57)	0.223
Disease duration, median years (range)	9.63 (1.9–33.1)	10.65 (2.4–34.0)	8.44 (2.2–46.4)	7.77 (4.5–25.0)	12.93 (1.1–33.7)	12.25 (4.6–36.3)	0.765
Disease location at baseline, ileal/ colonic/ileocolonic, n	2/0/9	4/2/9	3/3/7	0/2/6	3/2/9	2/5/6	0.528
Concomitant corticosteroids, n (%)	8 (72.7)	9 (60.0)	7 (53.8)	4 (50.0)	4 (28.6)	5 (38.5)	0.298
Prior anti-TNF use, n (%) ^a	11 (100)	15 (100)	12 (92.3)	8 (100)	14 (100)	13 (100)	0.608
Prior vedolizumab use, n (%)	4 (36.4)	3 (20.0)	6 (46.2)	6 (75.0)	6 (42.9)	7 (53.8)	0.195
CDAI, median (range)	265 (229–358)	266 (220–417)	307 (262–399)	301 (261–446)	272 (225–357)	292 (234–399)	0.345
SES-CD, median (range)	21 (4–37)	12 (4–32)	11 (6–35)	15 (6–38)	14.5 (5–29)	15 (4–31)	0.869
hsCRP, mg/L, median (range)	7.1 (0–68)	10.8 (1–53)	10.7 (3–72)	11.2 (1–117)	6.0 (1–54)	7.4 (2–53)	0.751
FCP, $\mu\text{g/g}$, median (range)	764 (126–9600)	1045.5 (63–6537)	1842 (315–4084)	2831.5 (1927–9566)	1744 (58–2087)	814 (84–9507)	0.368

^aAt least 1 anti-TNF biologic regimen. The P values for differences among groups were calculated by the Fisher exact test. Abbreviations: CDAI, Crohn's Disease Activity Index; F, female; FCP, fecal calprotectin; hsCRP, high-sensitivity C-reactive protein; M, male; SES-CD, Simplified Endoscopic Score for Crohn's disease; TNF, tumor necrosis factor.

Upadacitinib Does Not Significantly Affect the Transcriptomes of Noninvolved Intestinal Areas of Patients With Crohn's disease

To understand the potential effects of upadacitinib in noninvolved (healthy) areas of the intestine, we analyzed transcriptomes before and after patients received upadacitinib treatment. In total, 12 colonic and 7 ileal healthy biopsies were available at baseline, and 13 colonic and 6 ileal biopsies were available at week 12/16 from patients in the active treatment arms (all upadacitinib dose groups; [Supplementary Table S1](#)). Analysis of differentially expressed genes in noninvolved biopsies of patients with Crohn's disease receiving upadacitinib identified no significantly regulated genes at EOI (FC > 1.5; FDR < 0.05; [Table 2](#)).

Cell-specific Transcriptional Analysis Identifies the Main Subsets Affected by Upadacitinib Treatment in Crohn's Disease

Within the response signature to upadacitinib in the colon described by whole-biopsy RNAseq ([Fig. 1A](#)), 416 of 1156 genes could be assigned to ≥ 1 of the cell subsets defined by scRNAseq.⁹ In the ileum, 31 of 76 genes within the upadacitinib-responsive signature ([Fig. 1B](#)) were related to ≥ 1 of the cell subsets. Lists of the genes significantly regulated by upadacitinib that can be assigned to ≥ 1 cell lineage based on scRNAseq data are captured in [Supplementary Table S5A](#) and [S5B](#) (in the colon and ileum, respectively). To visualize gene expression changes induced by upadacitinib within each cell subset, we calculated the median normalized expression of each gene at week 0 and after upadacitinib treatment in endoscopic remitter and nonremitter patients based on the presence of ulcers in the ileum or colon at week 12/16 ([Fig. 2](#) and [Supplementary Fig. S2](#)).

The cell type with the largest number of genes significantly regulated by upadacitinib treatment in the colon was enterocytes (with overall increased expression in patients who achieve endoscopic remission), followed by a marked decrease in inflammatory monocytes, CD8+ T cells, endothelial cells, dendritic cells, and inflammatory fibroblast-related genes ([Supplementary Table S5A](#) and [Fig. 2](#)). Nonetheless, we could also assign numerous upadacitinib-regulated genes in the colon to all other defined subsets, including mast cells, all types of B and T lymphocytes, other fibroblast types, and various epithelial cell lineages besides enterocytes such as secretory (eg, goblet, enteroendocrine, and Tuft cells), M, stem, and transient amplifying (TA) cells ([Supplementary Table S5A](#) and [Fig. S2](#)). Similarly, with the enterocyte-associated signature, several genes in the secretory and stem/TA compartment were significantly upregulated with endoscopic remission. The only epithelial subset that showed a decrease in remitters was M cells (characterized by the expression of *TNFRSF11B*, a RANKL receptor, and the RANKL-induced gene *TNFAIP2*).

Within the ileal mucosa, most of the 31 significantly regulated genes were expressed by myeloid cells (primarily monocytes, followed by macrophages and dendritic cells) and stromal cells ([Fig. 2](#), [Supplementary Fig. S2](#), and [Supplementary Table S5B](#)).

Anti-TNF-refractory Patients Starting Upadacitinib Show Baseline Differences in Gene Expression Compared With Anti-TNF-naïve Patients

Next, we asked whether the cell-specific transcriptional changes observed in response to upadacitinib were unique to

Table 2. Number of Differentially Regulated Genes in Colonic and Ileal Biopsies at Week 12/16 of Treatment Compared with Week 0

	Colon			Ileum		
	Samples (week 0)	Samples (week 12/16)	FDR < 0.05	Samples (week 0)	Samples (week 12/16)	FDR < 0.05
Upadacitinib						
Endoscopic response	36	17	1203	30	8	0
Endoscopic remission	25	21	1156	25	14	76
Noninvolved	12	13	0	7	6	0
Placebo						
Involved	8	7	0	8	8	0

Endoscopic response is defined as a decrease in ≥ 2 points ($\Delta 2$) in the partial SES-CD score at weeks 12/16 compared with week 0 and endoscopic remission is defined as an ulcer subscore ≤ 1 at weeks 12/16. Changes in gene expression were also compared with noninvolved intestinal segments from patients receiving upadacitinib for 12/16 weeks. Differential gene expression was determined in involved intestinal segments of patients in the placebo group at weeks 12/16 compared with week 0, regardless of response. The numbers of samples included in each analysis fulfilling the different criteria are shown. Abbreviations: FDR, false discovery rate; SES-CD, Simplified Endoscopic Score for Crohn's disease.

JAK1 inhibition or could be driven by endoscopic remission regardless of the treatment received. To address this question, we assessed the expression patterns of a selected set of genes in patients with Crohn's disease who started anti-TNF treatment (infliximab or adalimumab, data pooled) as standard of care in our IBD unit and were treated for 14 weeks (Supplementary Table S4) and compared them with patients participating in the upadacitinib substudy. The selected transcriptional signature included 19 genes primarily expressed by well-defined cellular subsets within the intestinal mucosa (Supplementary Table S2).

The baseline global SES-CD (median, range) of patients receiving anti-TNF (10.0, 4–32) was similar to patients receiving upadacitinib (13.5, 4–38). Despite comparable endoscopic activity, patients who received upadacitinib experienced more refractory disease. Indeed, within the upadacitinib-treated groups, 98% of the patients had prior anti-TNF use and 45% had received vedolizumab (Table 1).

Baseline colonic and ileal expression of all 19 genes interrogated by qPCR is shown in comparison with tissue-matched biopsies from non-IBD control patients (Fig. 3 and Supplementary Fig. S3). Overall, the upadacitinib and anti-TNF cohorts showed significant upregulation in genes related to inflammatory fibroblasts (*CHI3L1*), neutrophils/monocytes/macrophages (*S100A8* and *OSM*), plasma cells (*DERL3*), and effector T cells (*IFNG*, *IL17A*, *GZMH*, and *TBX21*), and a significant downregulation of the Tuft cell marker *HTR3E* in the colon and ileum. The colonocyte marker *AQP8* is primarily expressed in the colon and was significantly downregulated in colonic baseline samples from both upadacitinib and anti-TNF cohorts compared with non-IBD controls. In addition to *CHI3L1*, we determined the expression of genes specific for different fibroblast subsets (eg, *THY1*, *COL3A1*, *SOX6*, *PDGFD*, and *PTGDR2*; Fig. 3 and Supplementary Table S5).

Patients with colonic involvement in the upadacitinib cohort also showed significant differences at baseline compared with those starting anti-TNF treatment (Fig. 3A and Supplementary Fig. S3A). Significant overexpression of fibroblast-expressed genes *CHI3L1*, *THY1*, and *COL3A1* (FDR < 0.001, < 0.01, and < 0.01, respectively) and neutrophil/monocyte/macrophage gene *OSM* (FDR < 0.05) was detected in the upadacitinib cohort at baseline. The enterocyte marker *AQP8* and the secretory goblet cell gene *RTNLB* had significantly lower expression in the upadacitinib group com-

pared with anti-TNF patients at week 0 (both FDR < 0.01), suggesting more severe disease in the upadacitinib cohort. Furthermore, the mast cell gene *TPSAB1* was significantly downregulated in the upadacitinib cohort compared with controls and the anti-TNF cohort. In contrast, *HDC* was significantly upregulated in the upadacitinib group vs controls and the anti-TNF cohort, whereas *SOX6* and *PDGFD* were significantly downregulated in the anti-TNF cohort compared with the upadacitinib cohort and controls. Besides showing differences in fibroblast signatures, patients starting upadacitinib treatment showed significant increases at inclusion in expression of *IFNG* and the cytotoxicity-related gene *GZMH* compared with the anti-TNF group. Overall, despite comparable baseline endoscopic scores at inclusion, these differences between cohorts suggest a more severe disease phenotype in the upadacitinib group.

Differences in the ileal biopsies from patients starting upadacitinib, or anti-TNF treatment, and non-IBD controls are shown in Fig. 3B and Supplementary Fig. S3B. In contrast to colonic samples, no significant differences were found at baseline between the ileal samples obtained from patients in the upadacitinib and anti-TNF cohorts, except for the mast cell gene *TPSAB1*, which was significantly downregulated in the upadacitinib cohort compared with controls and the anti-TNF cohort. Both cohorts showed similar upregulation of *CHI3L1*, *S100A8*, *OSM*, *IFNG*, *IL17A*, *DERL3*, *THY1*, and *COL3A1*, but expression of *SOX6*, *PTGDR2*, and *HTR3E* was significantly downregulated at baseline.

Upadacitinib Treatment Can Reverse Overexpression of Inflammatory Fibroblast Markers and Interferon- γ Effector Signatures in Anti-TNF-refractory Patients

Despite baseline differences, upadacitinib and anti-TNF treatment significantly downregulated acute inflammatory marker expression (*CHI3L1*, *OSM*, and *S100A8*) in colonic biopsies from patients who achieved endoscopic remission (Fig. 4). In addition, enterocyte and secretory cell genes *AQP8* and *RTNLB* were significantly recovered in patients responding to either treatment. Crypt fibroblast genes (*SOX6*, *PTGDR2*, and *PDGFD*) that were found to be significantly downregulated at inclusion in the anti-TNF group (Fig. 3) returned to control levels in patients responding to anti-TNF treatment (Fig. 4B). Similarly, expression of mast cell genes (*ADCYAP1* and *HDC*), which were significantly upregulated

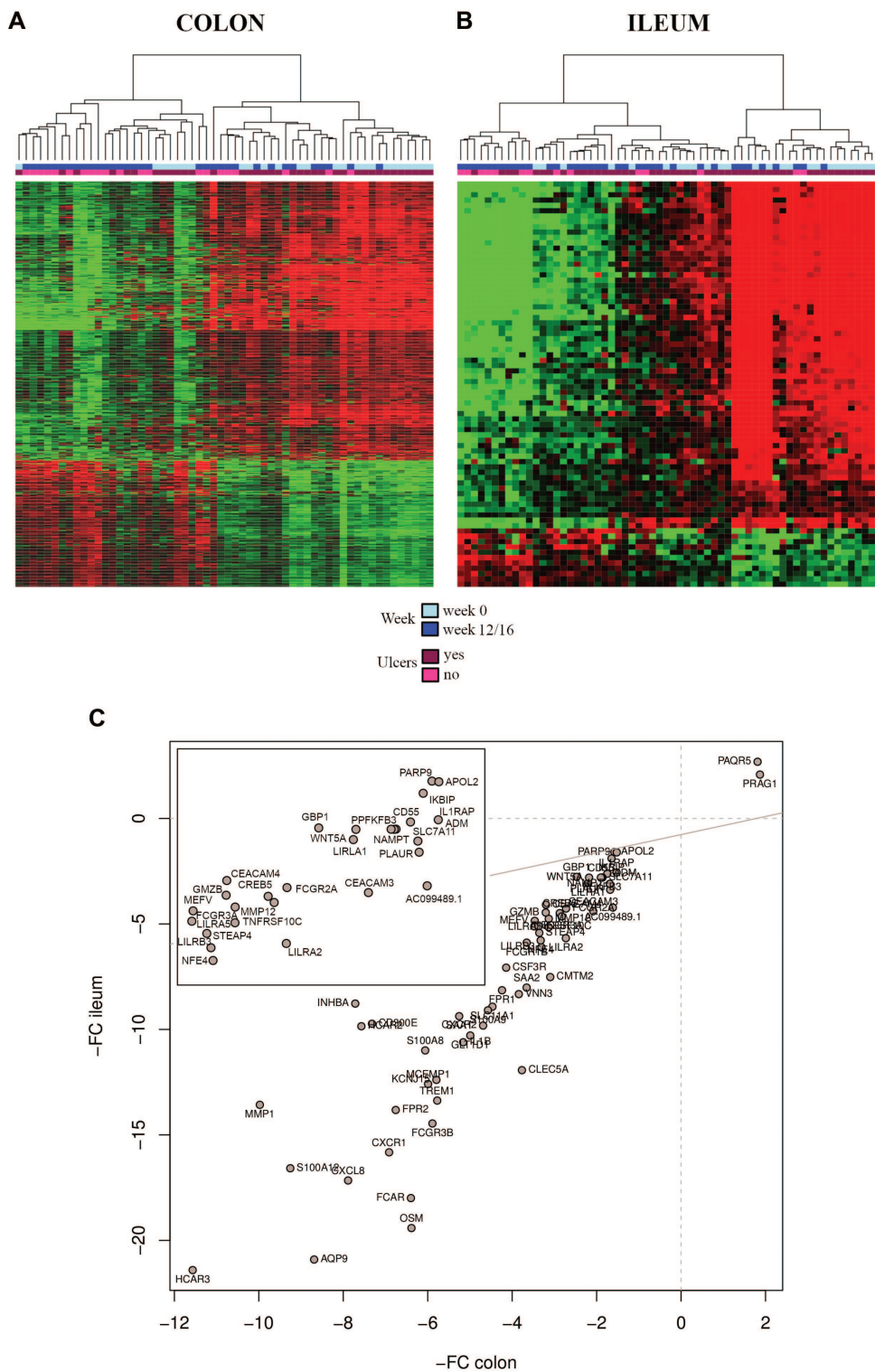


Figure 1. Effect of upadacitinib treatment on colonic and ileal transcriptional signatures. Heat map representations of differentially expressed genes (FDR < 0.05) at week 12/16 of upadacitinib treatment compared with week 0 in colonic (A) and ileal (B) biopsies. Presence or absence of ulcers is depicted on top of the heat map for each analyzed sample. High expression levels are shown in red and low expression levels in green. (C) Correlation of FC from week 0 to week 12/16 in the colon vs ileum for all genes significantly regulated in intestinal segments in patients achieving segmental endoscopic remission with upadacitinib. Abbreviations: FC, fold change; FDR, false discovery rate.

at week 0 in the upadacitinib cohort (Supplementary Fig. S3), normalized in patients who achieved endoscopic remission with upadacitinib (Supplementary Fig. S4A). Additional genes highly overexpressed in anti-TNF-refractory patients starting

upadacitinib, such as *THY1*, *COL3A1*, and the interferon (IFN)- γ signature genes *IFNG*, *TBX21*, and *GZMH*, were significantly downregulated in patients achieving endoscopic remission with upadacitinib (Fig. 4A). In contrast, in patients

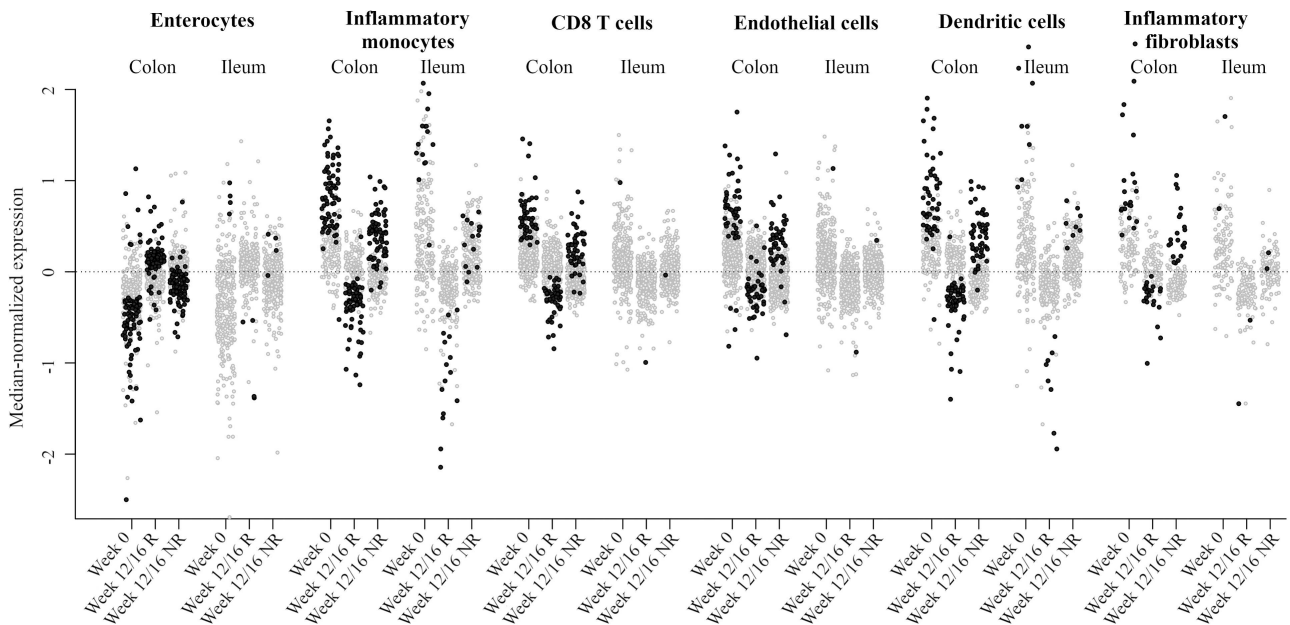


Figure 2. Effect of upadacitinib on cell-specific gene subsets. Transcriptional changes observed by RNA sequencing analysis of colonic and ileal biopsies in response to upadacitinib were assigned to different cell subsets. Each dot represents the median normalized expression of all samples at week 0 or at week 12/16 in endoscopic remitter and nonremitter patients. Genes that were significantly regulated in endoscopic R at week 12/16 relative to week 0 are shown in black. Abbreviations: CD8, cluster of differentiation 8; NR, nonremitter; R, remitter.

with endoscopic remission after anti-TNF or upadacitinib treatment, there were no significant changes in expression of the T-helper 17 (Th17) cell/innate lymphocyte cell (ILC)-3 gene *IL17A* or the Tuft cell-expressed gene *HTR3E* (Fig. 4). Of note, the mast cell gene *TPSAB1*, which was significantly downregulated at week 0 in the upadacitinib cohort, was further downregulated in upadacitinib remitters at week 12/16 (Supplementary Fig. S4A).

Changes in ileal expression of all 19 genes in the ileum of patients receiving upadacitinib or anti-TNF treatment are shown in Supplementary Fig. S5A and S5B. Besides significant downregulation of *OSM* and *S100A8* in remitters by anti-TNF and upadacitinib, few other significant effects were observed within the ileal mucosa.

Discussion

This study has provided evidence that JAK inhibition with upadacitinib is associated with significant transcriptional changes in the involved mucosa while not affecting noninvolved intestinal segments. By using whole-biopsy RNAseq analysis, a large response signature was identified, including genes involved in acute inflammation, epithelial regeneration, and tissue remodeling that are significantly regulated upon upadacitinib-induced segmental endoscopic remission. In contrast to the upadacitinib-treated group, no genes were significantly regulated at EOI regardless of endoscopic response/remission status or disease location in the placebo group. No baseline gene signature was identified that predicted endoscopic remission or clinical response to upadacitinib in CELEST; however, the cohort of individuals examined in this study may not be sufficiently powered to identify a predictive biomarker of drug response.

To investigate the transcriptional signatures from a complex tissue such as the intestinal mucosa, we overlapped the whole-genome transcription biopsy data with individual cell tran-

scriptomes.⁹ Traditionally, pathway analysis or cell deconvolution tools were used to explore transcriptional signatures of biopsies; however, these approaches come with several caveats.^{11–13} First, pathway analysis is performed on gene signatures derived from a mixture of cells present in biopsies which may erroneously infer pathways from genes expressed by different cellular subsets. Second, available curated pathways and deconvolution tools are predominantly based on data derived from peripheral blood cells or cell lines, which are not representative of the cell types present in the human intestinal mucosa. With the advent of single-cell transcriptomics, we are beginning to understand the complex gene regulation processes within the human intestine.^{9, 14, 15} When this study was designed, scRNAseq was unavailable. However, recently published single-cell transcriptomes can now be utilized to infer changes in specific cell types from our RNAseq data. In contrast to deconvolution, cells abundant in the intestinal mucosa but not in peripheral blood—and therefore not represented in available deconvolution matrices—could be explored. This approach proved to be useful to identify the main cellular subsets that respond to upadacitinib treatment and to discern the differences between patient populations that differ in refractoriness.

These data show that patients starting upadacitinib treatment in CELEST, most of whom were largely refractory to anti-TNF, presented with a unique molecular signature compared with patients naïve to anti-TNF treatment. We also show that markers of acute inflammation (*S100A8*, which encodes the calprotectin gene; *DERL3*, unique to the antibody-secreting plasma cell population; and *IL17A*, expressed by both Th17 effector cells and a subset of ILC3 cells) and intestinal epithelial genes (*RTNLB* and *HTR3E*) showed comparable deregulation in the 2 groups of patients at baseline. In contrast, despite having comparable endoscopic scores at inclusion, certain inflammatory pathways were overactivated with a characteristic inflammatory fibro-

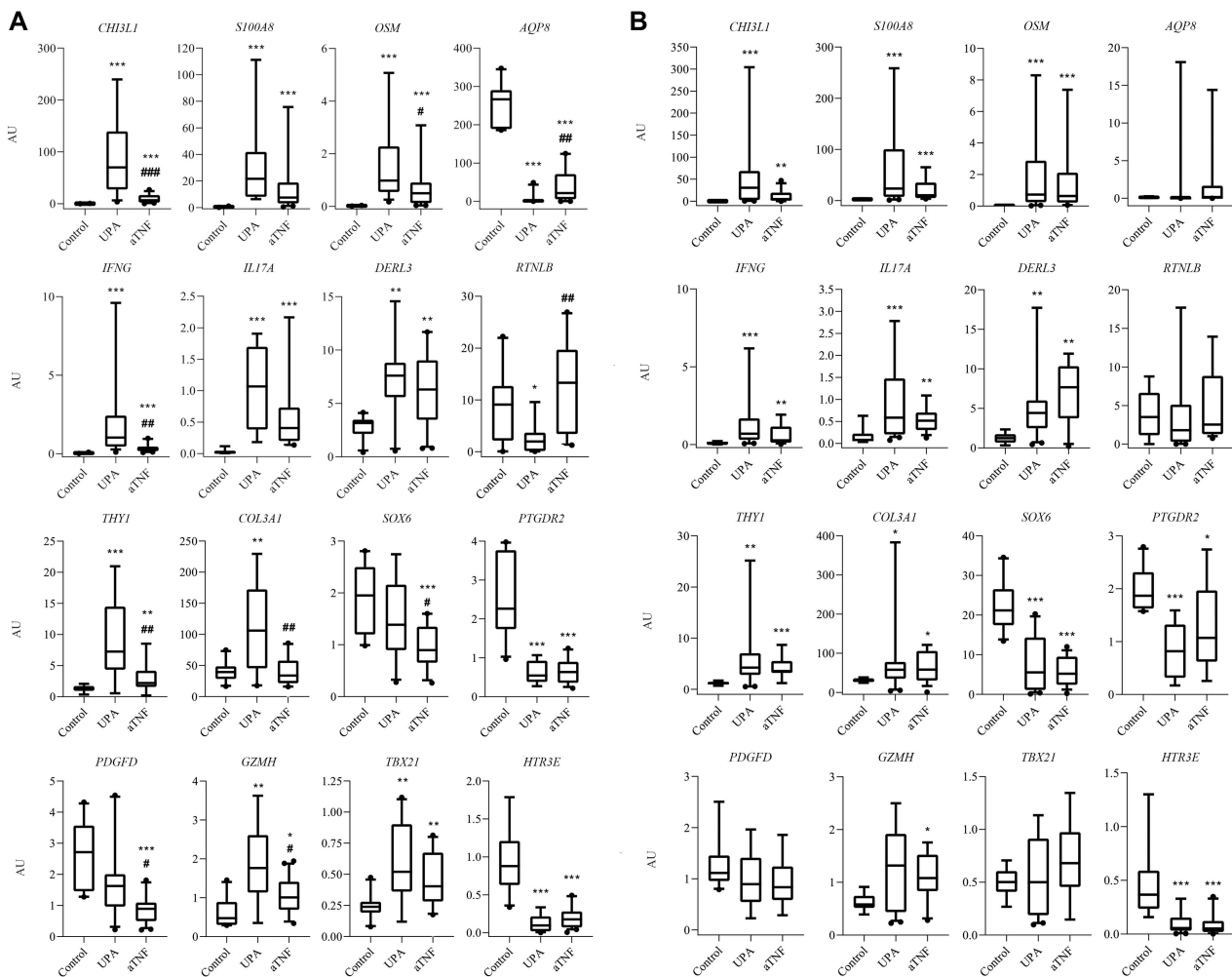


Figure 3. Transcriptional changes in cohorts of patients with Crohn's disease before starting upadacitinib or anti-TNF treatment. Cell-specific transcripts were determined at week 0 before starting upadacitinib ($n = 19$ – 24) or anti-TNF ($n = 19$ – 23) treatment in biopsies by real-time qPCR. Gene expression is also shown in intestinal biopsies from healthy controls ($n = 9$ – 10) for comparison. Box plots (whiskers represent 10th and 90th percentiles) representing baseline colonic (A) and ileal (B) gene expression are shown in AU relative to *ACTB* (β -actin) mRNA expression. *FDR < 0.05; **FDR < 0.01; ***FDR < 0.001 vs control (Mann–Whitney–Wilcoxon test corrected for FDR). *FDR < 0.05; **FDR < 0.01; ***FDR < 0.001 anti-TNF vs UPA (Mann–Whitney–Wilcoxon test corrected for FDR). Abbreviations: aTNF, anti-TNF; AU, arbitrary unit; FDR, false discovery rate; qPCR, quantitative polymerase chain reaction; TNF, tumor necrosis factor; UPA, upadacitinib.

blast and Th1/cytotoxic T lymphocyte signature that was differentially regulated in anti-TNF-experienced patients starting upadacitinib. In addition, these patients showed a further decrease in the enterocyte marker *AQP8* and significant overexpression of *OSM* and *HDC*, indicators of more severe inflammation. Interestingly, the tryptase gene *TPSAB1*, associated with mast cell degranulation, is significantly downregulated in patients in the anti-TNF-refractory cohort. This is contrary to the upregulation of other mast cell genes in this population, including *ADCYAP1* and *HDC*. Given that the transcriptional regulation of the *TPSAB1* gene after mast cell activation has not been studied, the significance of this differential regulation of mast cell genes in anti-TNF-refractory patients is unclear and beyond the scope of the current study.

These data support the view that patients with moderate to severe Crohn's disease who previously failed anti-TNF treatment may present with a differential signature. Identifying predictors of response using whole-biopsy transcriptomes has proven challenging, and this approach is unable to translate

into a clinically applicable marker. Genes such as *OSM* or plasma cell-related signatures may be upregulated among anti-TNF nonresponder populations.^{13, 16} Nonetheless, these are genes that may closely correlate with disease activity. Failure to respond to anti-TNF treatment may drive changes in the mucosal signature. A recent study identified IL-23R-expressing cells in the mucosa of patients who were exposed to and failed anti-TNF treatment and suggested that anti-TNF blockade expands an IL-23R T cell population in nonresponders.¹⁷ However, this cell subset could not be identified at baseline and, therefore, was not used as a predictor in that study.

We suggest that within the anti-TNF-refractory population, some patients overexpress a cellular and molecular signature—including the JAK1-dependent IFN- γ pathway—that responds to JAK1 inhibition. Inflammatory fibroblasts are sensitive to IFN- γ and express high levels of IL-6, IL-11, and *OSM* receptors,^{15, 18} all of which are dependent on JAK1 to relay their intracellular signals.¹⁹ In contrast to anti-TNF, upadacitinib can significantly regulate Th1/cyto-

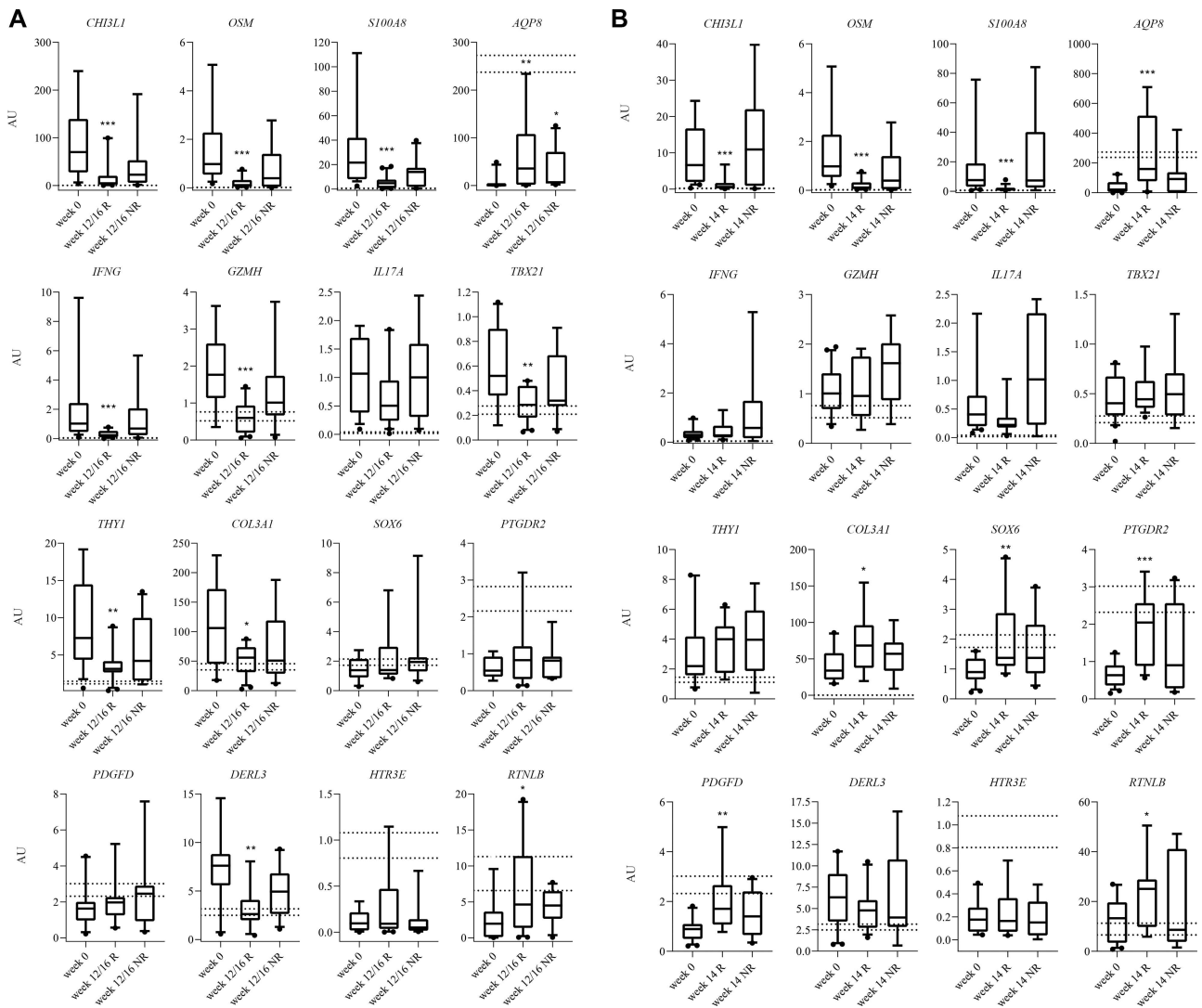


Figure 4. Upadacitinib and anti-TNF transcriptional changes in colonic Crohn's disease. Cell-specific transcripts were determined by real-time qPCR in colonic biopsies of patients with Crohn's disease at week 0 and at follow-up in remitters and non-remitters. Patients starting upadacitinib (A) or anti-TNF (B) were analyzed. Box plots (whiskers represent 10th and 90th percentiles) show gene expression in AU relative to *ACTB* (β -actin) mRNA expression. Dotted lines show the SEM for each gene in colonic biopsies from healthy controls. *FDR < 0.05; **FDR < 0.01; ***FDR < 0.001 compared with week 0 (Mann-Whitney-Wilcoxon test corrected for FDR). Upadacitinib: week 0, $n = 19$; remitters, $n = 21$; non-remitters, $n = 11$. Anti-TNF: week 0, $n = 20$ –21; remitters, $n = 15$; non-remitters, $n = 10$. Abbreviations: AU, arbitrary unit; FDR, false discovery rate; NR, nonremitter; qPCR, quantitative polymerase chain reaction; R, remitter; SEM, standard error of mean; TNF, tumor necrosis factor.

toxic T lymphocyte genes (eg, *IFNG*, *GZMH*, and *TBX21*), fibroblast genes (eg, *THY1* and *COL3A1*), and the plasma cell marker *DERL3*. Patients who achieved endoscopic remission in the upadacitinib and anti-TNF treatment cohorts showed marked downregulation of common inflammatory pathways, including *OSM*, *S100A8* (calprotectin), *CHI3L1* (inflammatory fibroblast), *ADCYAP1* (mast cell degranulation), and *HDC* (histamine synthesis), and significant upregulation of the enterocyte and goblet cell genes *AQP8* and *RTNLB*. Finally, anti-TNF but not upadacitinib treatment promotes recovery of crypt fibroblast genes (eg, *SOX6*, *PTGDR2*, and *PDGFG*). These observations were more evident in colon than ileal biopsies, although changes in *OSM* and *S100A8* expression were observed in both tissues.

These results suggest that although both upadacitinib and anti-TNF treatment reduce inflammation, they regulate both

overlapping and distinctive sets of genes involved in the colon and ileum. This may partially explain at a molecular level why upadacitinib is efficacious in anti-TNF-refractory patients. In the absence of validated clinical end points to evaluate improvements in fibrosis, demonstrating changes in pathways associated with the development or progression of fibrosis may provide insights into the potential impacts of therapies on this aspect of disease. In this respect, the observed impact of upadacitinib on multiple fibroblast types, including myofibroblasts and noninflammatory fibroblasts, is also of interest.

An important observation from this study is the lack of effects on noninvolved (healthy) segments of the colon and ileum of patients administered upadacitinib for 12/16 weeks. Despite the marked effects of upadacitinib on the mucosal transcriptional profiles of patients who responded to treatment, no significant transcriptional changes were detected

within healthy areas. As JAK1 signaling is upregulated in the inflamed mucosa, inhibition would be expected to have more dramatic effects within these areas. JAK1-dependent signaling is involved in many pathways, including Treg survival signals through the IL-2 receptor or epithelial homeostasis and anti-microbial responses in response to IL-10/IL-22. These data show no detectable effect of upadacitinib treatment on the healthy intestinal areas of patients with active disease, supporting the targeted effect of this treatment within the intestinal mucosal compartment.

Although the current study focused on upadacitinib in patients with Crohn's disease, we speculate that the effects seen in our study could be extrapolated to that of other JAK1 or JAK1/3 antagonists (eg, tofacitinib) in patients with Crohn's disease or UC. This is consistent with the efficacy of upadacitinib and tofacitinib in patients with moderate to severe UC,^{3,4,6} and the greater reduction of endoscopic inflammation in patients with Crohn's disease receiving tofacitinib vs placebo in a post hoc analysis—even though tofacitinib did not meet the primary end point of this phase 2 trial.^{5,20} However, JAK3-specific inhibitors may be associated with distinct effects compared with upadacitinib and other JAK1 inhibitors, and future research into the effects of nonoverlapping JAK pathways on IBDs may also be of interest.

Overall, data from the CELEST substudy support the hypothesis that upadacitinib modulates inflammatory pathways in patients with anti-TNF-refractory Crohn's disease. Upadacitinib targeted the inflammatory fibroblast and IFN- γ -expressing cytotoxic T cell compartment present in the mucosal lesions of this patient population. This study is the first to provide transcriptional signatures in JAK inhibitor-treated patients with Crohn's disease and improves our understanding of the mechanisms of action of this drug class in the context of intestinal inflammation.

Supplementary data

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

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Conflicts of interest

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