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Microbiome alteration via fecal microbiota transplantation is effective for refractory immune checkpoint inhibitor–induced colitis

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AST, WM, and H A-S contributed to clinical data collection, and analysis.
Z-DJ, HLD contributed to donor's stool and manuscript review and revision.
TMH, TH, CS, RE-H, AB, IF, LM, VC, MH, MOT, DP, SSW, EH, C-CC, and RRJ completed stool studies and analyzed/interpreted data.
BS and ERP completed multiplex immunofluorescence studies and analyzed/interpreted data
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YW has consulted for AzurRx pharma, Tillotts Pharma, Sanarentero.
RRJ is on the scientific advisory board for Seres Therapeutics, Inc., has consulted for Ziopharm Oncology and Microbiome Dx, and holds patents licensed to Seres Therapeutics, Inc.
The other authors declare no competing interests.

Data and materials availability
Human 16S rRNA and whole DNA shotgun sequencing data have been uploaded to Sequence Read Archive (SRA) under citation accession PRJNA803517. Any additional data needed to evaluate the conclusions in the paper are present within the paper and/or the Supplementary Materials.

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Abstract

Immune checkpoint inhibitors (ICIs) target advanced malignancies with high efficacy but also predispose patients to immune-related adverse events, such as immune-mediated colitis (IMC). Standard treatment strategies include halting ICI therapy and administering immunosuppressants; these in turn may compromise cancer outcomes. Given the association between gut bacteria with response to ICI therapy and IMC risk and severity, fecal microbiota transplantation (FMT) represents a feasible way to manipulate microbial composition in patients with a potential clinical benefit for IMC. Here we present the largest case series to date of 12 patients with refractory IMC that underwent FMT from healthy donors as salvage therapy. All 12 patients had grade 3 or 4 ICI-related diarrhea or colitis that failed to respond to standard first-line (corticosteroids) and second-line immunosuppression (infliximab or vedolizumab). Ten patients (83%) achieved symptom improvement after FMT, including seven patients (58%) who had a complete clinical response. Three patients required repeat FMT, two of which had no subsequent response. At the end of the study period, 92% achieved IMC clinical remission. 16S rRNA sequencing of patient stool samples revealed that compositional differences between FMT donors and IMC patients before FMT were associated with a complete response after FMT. Comparison of pre- and post-FMT stool samples in patients with complete responses showed significant increases in alpha diversity, as well as increases in the abundances of *Collinsella* and *Bifidobacterium*, which were significantly depleted in FMT responders before FMT. Histologically evaluable complete responders also had decreases in select immune cell subtypes, including CD8+ T cells, in the colon post-FMT. This study validates FMT as an effective treatment strategy for IMC and gives insights into the unique microbial signatures that may play a critical role in FMT response.

One Sentence Summary:

FMT effectively treats IMC in patients by significantly altering baseline microbial diversity and composition.

INTRODUCTION

Immune checkpoint inhibitor (ICI) therapies are novel oncologic treatment strategies that promote an antitumor response by impairing inhibitory T-cell pathways. ICIs enhance immune-mediated responses to malignancies by bypassing checkpoints including cytotoxic T-lymphocyte-associated protein 4 (CTLA-4), programmed cell death protein 1 (PD-1), and programmed death-ligand 1 (PD-L1). This checkpoint blockade enhances immunity by dampening normal protective immune tolerance mechanisms or extending T cell effector

responses and thus can sometimes trigger unwanted immune-related adverse events (irAEs) that may involve almost any organ system(1, 2).

The gastrointestinal (GI) tract is one of the organ systems that is most frequently affected. GI toxicities can vary in severity from mild disease to aggressive life-threatening clinical presentations that require immediate intervention. Milder cases of immune-mediated colitis (IMC) are managed supportively, while vigorous selective immunosuppression is implemented in aggressive disease in addition to withholding ICI therapy. FMT has also been reported to be beneficial in cases of IMC refractory to immunosuppression(3).

The gut microbiota comprises an assemblage of organisms, including resident bacteria, viruses, and fungi, and can vary with diet, lifestyle, and other environmental factors(4). Dysbiosis, or a change in the gut microbiota including loss of beneficial microbes and/or expansion of pathogens, has been linked to cancer pathogenesis and impaired responses to cancer therapies(5-8). Certain gut bacterial signatures have been linked to ICI therapy responses, and others to the risk for developing IMC. Modulation of the gut microbiome in mice via FMT from cancer patients resulted in substantial modulation of anti-tumor responses to ICI therapy(9-11). Similarly, specific bacterial taxa may modulate ICI-related toxicity: *Bacteroidetes* and *Bifidobacterium* have been reported to be associated with a lower risk of IMC, while *Faecalibacterium*, *Clostridia*, and *Escherichia* may pose an increased risk(12). Two early-phase studies in melanoma patients in whom ICI therapy had previously failed recently demonstrated that FMT derived from patients who had responded to ICIs could result in cancer responses (13, 14). Additional studies of microbiome manipulation with the goal of increasing the efficacy and reducing the toxicity of cancer therapies may result in the development of microbiome optimization strategies as an integral part of cancer treatment regimens.

Herein, we document a case series of patients with IMC that were refractory to both first-line corticosteroids and standard second-line selective immunosuppression therapy (infliximab or vedolizumab) and were subsequently treated with FMT as third-line therapy. We describe the changes that were observed clinically, endoscopically and histologically while also comparing microbial differences through analyses of gut microbiota composition.

RESULTS

Patient baseline characteristics.

All 12 patients in our cohort were White; 75% were male, with a median age of 56 years (IQR 45-67 years) at the time of ICI initiation (Table 1). Half of the patients had received treatment with ICI for genitourinary cancers (50%); the majority had stage IV disease (75%). With respect to ICI therapy, CTLA-4 blockade, PD-1 or PD-L1 blockade, and combination therapy with CTLA-4 blockade and PD-1 or PD-L1 were used in 1, 6, and 5 patients, respectively.

IMC characteristics and initial treatment.

The median time from ICI initiation to the onset of adverse GI symptoms was 71 days (IQR 14-586 days) (Table 2). All patients in our cohort had CTCAE grade 3 or 4

diarrhea; 50% also had CTCAE grade 3 or 4 colitis. On initial endoscopy at IMC onset, 50% had ulcerative inflammation. Ten patients (83%) were found to have chronic active inflammation on histologic examination. Each patient received but did not respond to systemic corticosteroids, which were given for a median duration of 68 days, followed by second-line selective immunosuppression with vedolizumab (median, three infusion doses) or infliximab (median, 2.5 infusion doses). Nearly all patients (92%) required hospitalization for IMC, with a median hospital stay of 15 days.

FMT salvage therapy and clinical outcomes.

FMT was given an average of 89 days following IMC symptom onset and achieved 83% symptom improvement at a median duration of 14 days (IQR 9-16 days) after treatment. Nearly half (42%) of the patients achieved both endoscopic and histologic remission at last endoscopic follow-up. Of the 12 patients in our cohort, three received additional FMT for partial responses and two achieved complete responses since. There were no FMT-related complications at 7 days and 30 days, respectively, in our entire sample. Four patients required additional immunosuppressant treatment for IMC after FMT. At the end of the study period, 92% had achieved clinical remission of their IMC. With respect to cancer progression, three patients (25%) had experienced cancer progression by the time of FMT. Four patients (33%) underwent non-ICI cancer therapy after FMT, and three later succumbed to their underlying malignancy. Stool samples were collected from 9 of 12 patients within 24 hours prior to FMT (pre-FMT; day 0). Each patient also provided a post-FMT stool sample (n=12, range: day 10 to 120; median: day 28). Aliquots of donor FMT product (n=12) were also stored for independent evaluation. Three patients received a second therapeutic FMT after the first FMT did not successfully resolve their IMC symptoms. Information regarding available samples collected at specific time points from individual patients is provided in Supplementary Table 1.

Baseline patient microbial composition predicts IMC FMT response.

We compared the microbiome composition of pre-FMT stool samples using 16S rRNA sequencing from complete response (CR) patients with those from non-CR patients and healthy controls, represented by FMT donors. No significant differences in microbial alpha diversity were seen between CR and non-CR patients or FMT donors (including second FMTs for two non-CR patients), as measured by the inverse Simpson's index (Fig. 1a). We also examined stool samples from controls with pre-FMT samples from CR and non-CR patients using principle coordinate analysis (PCoA) to visualized beta diversities of baseline samples (Fig. 1b). Donor stools were distinct from the stools of CR patients (PERMANOVA, $P = 0.01$), while differences between donor and non-CR patient stool samples were not statistically significant ($P = 0.4$), nor were there significant differences between CR and non-CR patients (Fig. S1; $P = 0.8$). Differential abundance analyses revealed that two bacterial genera, *Bifidobacterium* and *Collinsella*, were depleted in CR patients at baseline compared to non-CR patients (Fig. 1c) and were also depleted in CR patients compared to FMT donors (Fig. 1d). In contrast, several other genera were enriched in CR patients (Fig. 1c). Overall, our analyses of pre-FMT microbial compositions from IMC patients revealed that CR patients were more dissimilar to FMT donors than

non-CR patients, and CR patients' stool samples were characterized by low abundances of *Bifidobacterium* and *Collinsella*.

Next, we evaluated the effects of FMT on the fecal microbiota composition of individual IMC patients. We evaluated beta diversity of pre-FMT, post-FMT, and donor samples for 4 CR and 3 non-CR patients where post-FMT samples were available, utilizing samples collected on day 30 +/− 20 after FMT as post-FMT samples (Fig. 2a). Interestingly, while all 4 CR patients became more similar to their FMT donor when comparing pre- and post-FMT samples, 2 of 3 non-CR patients actually became less similar to their FMT donor (Figs. 2b and 2c).

Select bacterial taxa are associated with IMC FMT response.

Our results suggested that success of FMT for treating IMC may depend to an extent on whether the FMT procedure results in a microbiota composition that is more similar to that of the donor. Consistent with this, we found that in CR patients, the FMT procedure consistently resulted in increases in alpha diversity, while 2 of 3 non-CR patients actually experienced reductions in alpha diversity (Fig. 3a). The compositions of stool samples collected at each time point for all individual patients are presented in Fig. S2.

To identify specific bacterial taxa that could be mediating a benefit in CR patients, we examined for genera that were statistically changed following FMT in these patients using a paired analysis. We found that *Collinsella*, *Bifidobacterium*, *Family XIII AD3011 group*, and *Coprococcus* were all enriched in CR patients following FMT, while *Tyzerella* was reduced (Fig. 3b and 3c). Interestingly, we had already found that pre-FMT samples from CR patients are depleted in *Collinsella* and *Bifidobacterium* (Fig. 1c-d). Together, these results suggest that reduced *Collinsella* and *Bifidobacterium* can potentially identify FMT candidates who are then more likely to experience increases in these bacterial subsets from the FMT procedure. We further validated these results using real-time quantitative PCR (Fig. S4); *Bifidobacterium* was enriched post-FMT in CR patients while *Collinsella* was more heterogenous and could not be recapitulated using real-time qPCR. To gain potential insights into taxonomic resolution of these associations beyond the Genus level, we performed whole DNA shotgun sequencing of a subset of samples that had sufficient biomass to be evaluated. We found that most *Bifidobacteria* in samples from this cohort belonged to *Bifidobacterium dentium*, and most *Collinsella* belonged to *Collinsella intestinalis* (Fig. S3), with the exception of Patient 1 who experienced a CR and harbored *Bifidobacterium longum, Bifidobacterium pseudocatenulatum* and *Collinsella aerofaciens* post-FMT.

We were interested in asking if any clinical features of patients (Tables 1 and 2) were associated with the taxa of interest identified by 16S rRNA sequencing at baseline prior to undergoing FMT. We examined for correlations between taxa abundances and clinical features that were continuous (Fig. 4a) or categorical (Fig. 4b). We found that relative abundances of *Collinsella* and *Coprococcus*, which increased after FMT in all CR patients, were inversely correlated with duration of colitis and diarrhea symptoms (Fig. 4a). Most clinical characteristics that were not related to IMC symptomatology (i.e. age, sex, ICI type, cancer stage) did not show significant associations with bacterial taxa of interest.

Collectively, these data suggest that some clinical features may explain baseline differences in abundances of taxa associated with FMT response.

Changes to the gut immune environment following administration of FMT.

Finally, we asked if effects of FMT therapy were associated with changes in immune infiltrating populations or epithelial proliferation in the colon. We performed multiplex immunofluorescence (IF) on formalin-fixed paraffin-embedded (FFPE) colon tissue samples collected from IMC patients in the cohort who had paired samples collected before and after FMT, including 2 CR and 2 non-CR patients. Quantification of the IF images showed that total CD3+ and CD3+CD8+ cells decreased in both CR patients post-FMT but not in both non-CR patients (Figs. 5a and 5b); Representative IF images from select patients are shown in Fig. 5c. These data suggest that complete response of IMC by FMT therapy could be mediated by loss of total lymphocytes (CD3+) and cytotoxic T cells (CD3+CD8+). Additional markers, including FoxP3+ regulatory T cells (CD4+ FoxP3+), B cells (CD20+), NK cells (CD56+), epithelial cells (CK+), and proliferating cells (Ki67+) showed no clear patterns (Fig. S5).

DISCUSSION

ICI has revolutionized the management, outcomes and overall survival of patients with many types of malignancies. However, IMC can cause significant morbidities with a wide range of severity(15-17). The mainstay of medical treatments for moderate to severe IMC is limited to immunosuppressive agents e.g. corticosteroids, infliximab, and/or vedolizumab, as well as reports of success with administering interleukin- (IL) 12/23 blockade(18), IL-6 receptor antagonist therapy(19), and Janus kinase inhibition(20) for refractory disease cases. Given the increasing recognition that patients with longer disease courses of colitis disease may have improved cancer response rates(21), therapeutic options are needed that can avoid abrogating beneficial effects of ICI, such as FMT. In this case series, which is an extension of our previous 2-patient case series(22), we demonstrated a high clinical response rate of 83% to FMT in treating refractory IMC with only mild adverse events. In addition, we also described microbiome features associated with favorable responses to FMT.

Gut dysbiosis, or an imbalance in the normal gut flora, may alter host responses, promote a chronic inflammatory state, and impact a variety of cancer outcomes, including cancer development, progression, and response to cancer therapeutic agents(23-26). Unique differential gut microbial signatures have been identified among responders versus non-responders to ICI therapy, as well as among those with a predisposition for developing IMC. Modulation of the gut microbiome in gnotobiotic mice via FMT from cancer patients alters anti-tumor immunity and response to ICIs. Prospective clinical trials have also raised the possibility that microbiome modulation improves cancer responses among melanoma patients who previously did not respond to ICI therapy after receiving FMT from melanoma patients who responded(10, 11, 13). Additionally, novel microbe-based adjuvants for enhancing ICI therapy are emerging as suitable strategies for augmentation of select cancers(27). Relatedly, it has been hypothesized that targeting specific gut bacterial taxa may abrogate ICI-related toxicity(12, 28, 29). FMT has been speculated

to be effective in patients with ICI-induced enterocolitis that is refractory to standard-of-care immunosuppressive therapy which is further supported by the current study with a larger sample analysis. Future prospective clinical trials (https://clinicaltrials.gov/ct2/show/NCT03819296) will provide more evidence in measuring the efficacy and safety of FMT as a therapeutic.

FMT for the treatment of refractory IMC represents a novel approach to a common and detrimental side effect of ICI therapy. We presented data that suggests the microbiome of IMC patients can be manipulated by FMT and changes in the composition of the intestinal microbiome may confer a health benefit to recipients. Paired analyses of patient stool samples showed a significant increase in alpha diversity in responders. This result is consistent with those of numerous published reports that suggest that greater microbial diversity is typically associated with better overall health and that patients with a less diverse gut microbiome often have related inflammatory conditions(30). Additionally, we observed a significant difference in the beta diversity of donor stool and pre-FMT stool in CR patients, indicating that patients who ultimately responded to FMT had greater dysbiosis at baseline. This association could be due to the greater ability of FMT-derived bacteria to engraft in dysbiotic hosts. An alternative, non-exclusive explanation is that in a subset of patients with refractory IMC, microbiome dysbiosis drives colitis pathophysiology, while in other patients, the microbiome is not a significant contributor. Additionally, IMC may be a driver of host dysbiosis as host genetics also influence microbial composition(31-33). Further studies identifying if the hosts have genetic predispositions to inflammation may also inform whether a patient would benefit from FMT as a treatment for IMC.

Two genera of bacteria, *Bifidobacterium* and *Collinsella*, were depleted in CR patients prior to FMT and then increased substantially after a successful response. Members of these genera belong to the phylum *Actinobacteria* and are anaerobic, gram-positive, non-motile, non-sporulating rods that commonly reside in the gastrointestinal tracts of humans and other mammals(34). Many species of the genus *Bifidobacterium* (ex. *B. longum*, *B. bifidum*, *B. breve*, and *B. infantalis*) are currently in use as probiotic supplements that have been reported to alleviate gastrointestinal inflammation(35, 36). In pre-clinical models, introducing *Bifidobacteria spp*. has led to improved tumor responses to anti-PD-1 or oxaliplatin therapy and mitigated anti-CTLA-4 induced colitis(29, 37). These effects seem to be at least partially modulated through the increased presence of regulatory T cells or secretion of the anti-inflammatory cytokine IL-10(38). It is of note that we did not see changes in regulatory T cells in our immune cell evaluation of colon tissue between CR and non-CR IMC patients (Fig. S5).

Less is known about *Collinsella* in the context of IMC. Some studies have found that *Collinsella* bacteria are associated with inflammatory states(39, 40). However, *Collinsella aerofaciens* has been previously shown to be more abundant in patients with metastatic melanoma who respond favorably to anti-PD-1 therapy; the same study found numerous species of the *Bifidobacterium* genus that were also associated with anti-PD-1 efficacy in both patients and mice(11). In ulcerative colitis patients who had been treated with FMT, *Bifidobacteriaceae* and *Coriobacteriaceae* (which contain *Bifidobacterium* and *Collinsella*, respectively) showed increased abundance in patients who achieved clinical remission(39).

Our results suggest that *Bifidobacterium* and *Collinsella* species may protect some patients against developing IMC and that repleting these bacteria can protect against or reduce the severity of IMC. Prior studies demonstrate that *Collinsella* has been shown to alter gut physiology, induce expression of IL-17 network cytokines and affect T cell mediated responses(41, 42). We also identified additional potentially beneficial bacteria, including *Coprococcus* and *Family XIII AD3011 group*, which were found to be decreased in patients with inflammatory bowel disease, suggesting that their recovery may reduce gut inflammation(43, 44). Conversely, *Tyzerella*, which had lower abundance in CRs after FMT, was recently found to be enriched in the ileal mucosal biopsies of Crohn's disease patients compared to those of healthy controls(45). Further research is still needed to determine how these taxa contribute to IMC disease phenotypes.

IMC is caused in large part by stimulation of the host immune system by ICI treatment. Gut commensals modulation of the immune system has been well described (26, 46-51) but more studies are needed in the context of IMC. Our analyses of IMC patient colon biopsies showed a marked decrease in both total lymphocytes and CD8+ T cells in complete responders post-FMT, suggesting FMT-mediated reduced inflammation in complete responders. This also leads to changes in the ratios of regulatory T cells to cytotoxic T cells, which may also have played a role in disease mediation. Multiplex immunofluorescence profiling of additional immune cells and epithelial markers revealed trends that may lead to FMT-mediated mechanisms of action in IMC treatments. Additional studies are necessary to confirm results and explore pathways of interest within these cell subtypes.

Our study is limited by its retrospective nature, small sample size, and lack of a control arm to appropriately measure the impact of FMT on colitis outcomes. However, FMT is not yet established or recommended as a standard treatment of choice for refractory IMC. This is offered as compassionate therapy to patients at our institution. Additionally, the gut microbiome is highly sensitive to a variety of factors including but not limited to antibiotic use, medications, diet, smoking, and demographic, oncologic, immunologic, and geographic factors, all of which are beyond the scope of our case series. We used real-time qPCR to validate findings from 16S rRNA sequencing and found that we could recapitulate the results for *Bifidobacterium*; *Collinsella* samples proved to be more heterogenous and low initial abundance in IMC patient samples could have contributed to poor amplification efficiency. It is of note that correlation analyses showed discrepancies between the relative abundance from 16S rRNA sequencing and fold change from real-time qPCR. It could be that *Collinsella* qPCR primers do not well-amplify all species of *Collinsella*, or could alternatively be amplifying non-*Collinsella* species. Corroborating this possibility, we found that for *Bifidobacterium*, abundances quantified by 16S and qPCR generally showed reasonable correlations (R=0.81, Fig S4C), while abundances for *Collinsella* were not as well-correlated (R=0.54, Fig S4D).

To our knowledge, this is the largest series to demonstrate the utility of gut microbial manipulation via FMT in the management of ICI toxicity in advanced cancer patients. Importantly, our study showed that FMT was generally well-tolerated in these patients. Furthermore, we have been able to provide preliminary data to suggest that initial gut

dysbiosis and increased alpha diversity after FMT may be favorable in predicting FMT-related efficacy for IMC management. These observations may serve as a foundation to inform the design of larger prospective placebo-controlled studies to evaluate the therapeutic efficacy of targeting the gut microbiome to avoid and/or treat ICI gut toxicities and ensure continuance of cancer care.

In summary, manipulation of the gut microbiota through FMT represents a promising approach to the treatment of IMC in patients who are otherwise refractory to first- and second-line standard immunosuppressive treatment. In addition, our studies identified a potential role for FMT-mediated changes in select immune cells that mediate IMC severity. Larger prospective studies will be needed to fully characterize the efficacy and safety of this strategy, as well as to better identify microbiome parameters that can prognosticate IMC severity and FMT response. In our case series, we found minimal adverse events attributable to FMT. We expect that FMT will become a novel approach to treat patients with IMC at earlier stages of presentation, as an addition or alternative to standard treatments, which could, in turn, be guided by insights from this study into unique microbial signatures that may play a role in the therapeutic IMC response to FMT.

MATERIALS AND METHODS

Study design and methods.

We determined the impact of FMT from healthy donors on the outcome of patients with refractory IMC. We identified 12 patients at The University of Texas MD Anderson Cancer Center who met the following inclusion criteria: 1) adult cancer patients aged 18 years and older; 2) diagnosis of IMC with diarrhea and/or colitis related symptoms (e.g. bleeding, pain, mucus in the stool); 3) lack of response to or recurrence with ≥ grade 2 GI symptoms following first- and second-line standard therapy for IMC and 4) absence of active infection, as well as no clinical indication for antibiotics at the time of FMT. Patients with concurrent gastrointestinal infection were excluded from this case series. First line of therapy refers to weight-based systemic corticosteroids (1-2 mg/kg) with a taper course. Second line of therapy refers to biologics- vedolizumab and/or infliximab. FMT was performed under individual compassionate Investigational New Drug applications that had been approved by the Institutional Review Board (IRB) and authorized by the United States Food and Drug Administration (FDA) between June 2017 and April 2020. Data follow up of this study ended December 2021.

Clinical and oncological data.

Baseline demographic data (including age, sex, and race), oncology variables (cancer type and stage and ICI therapy type), and IMC characteristics (including severity grade of diarrhea and colitis, medical treatment received, and outcomes) were extracted from institutional electronic medical records and pharmacy databases. IMC severity at different timepoints was measured using the Common Terminology Criteria for Adverse Events (CTCAE) version 5.0. The use of antibiotics after FMT was also collected as a potential factor that affects microbiome composition. Medical treatments of IMC were categorized as non-immunosuppressive or immunosuppressive. Treatment response is defined as clinical

improvement of symptoms of IMC with a lower CTCAE grade or remission to CTCAE grade 1 or lower until last follow-up. Recurrence refers to onset of active clinical symptoms (CTCAE) of IMC after initial response or remission post medical treatment. Refractory IMC refers to the persistent colitis symptoms despite above-mentioned therapy for a minimum duration of 2 weeks. These patients were routinely taken off immunosuppressants after FMT treatment. Immunosuppressant regimen was resumed if FMT was ineffective. Cancer status at IMC onset and last follow-up after FMT was classified as stable, remission, or disease progression. Patients' vital status and survival at last follow-up were also recorded.

Endoscopic and histological evaluation.

Data on symptoms as well as endoscopic evaluation were collected before and after FMT as available usually within 1 week prior and 2 months window post treatment. Endoscopic findings included mucosal ulcerations, non-ulcer inflammation (erythema, exudate, loss of normal vascularity, and atrophy), or a normal appearance. Histological patterns were graded as acute colitis, chronic active colitis, microscopic colitis, or normal. Endoscopic and histological classification criteria have been previously described[11]. Endoscopic and histological remissions were assessed as secondary outcome, which were defined as endoscopic resolution of ulceration or non-ulcer inflammation and resolution of active histological inflammation, respectively.

Fecal Microbiota Transplantation (FMT).

FMT treatment was performed following an institutional protocol. Once an eligible patient had been identified, an individual compassionate Investigational New Drug application was requested from the IRB and approved by the FDA before treatment. Patients were consented to an individual IND and IRB protocol on separate occasion at the time of indication. Healthy donor stool samples were provided by the stool bank of The University of Texas School of Public Health (Dr. Herbert DuPont's laboratory). Donors completed screening questionnaires and underwent laboratory testing, as required by FDA regulations (supplemental material). FMT was performed via colonoscopy after routine colon cleansing with polyethylene glycol, with delivery of 50 grams of donor stool in liquid form to the right colon. Patients were observed for 1 hour in the endoscopy unit before being discharged. Clinical symptoms and side effects were monitored daily for the first 7 days and again at 30 days. Response after FMT and duration of response were recorded. Complete response (CR) to FMT was defined as CTCAE grade ≤ 1 of GI symptoms within 30 days after FMT. A partial response to FMT was defined as a reduction in the CTCAE grade of GI symptoms that did not meet the criteria of CR after first FMT or recurrent symptoms within 6 weeks that necessitated a second FMT. Non-responders were those who had persistent, unchanged symptoms within 30 days despite FMT. Extra aliquots of donor stool samples, as well as patient stool samples collected at time points before and after FMT, were stored for subsequent analysis.

DNA extraction and 16S rRNA sequencing for microbiome analyses.

Genomic DNA was isolated from stool samples using the commercially available QIAamp DNA stool mini kit (Qiagen) according to the manufacturer's protocol, modified to include an additional lysis step via bead-beating. One 3.2-mm steel bead, 150 mg of zirconium

beads, and lysis buffer were added into each tube containing pre-weighed stool samples for DNA isolation. Stool samples underwent bead-beating for 8 min (two repetitions of 4 min) at 3800 rpm. The V4 region of the 16S rRNA gene was amplified by PCR from 100 ng of each extracted and purified genomic DNA using 515 forward and 806 reverse primer pairs. The QIAquick gel extraction kit (Qiagen) was used to purify the amplicon pool and sequenced on the Illumina Miseq sequencer platform using a 2 x 250-bp paired-end protocol. Paired-end reads were de-multiplexed using QIIME2(52), merged and de-replicated using VSEARCH(53), de-noised with UNOISE 3(54), and classified using mothur(55) with the Silva database version 138(56). Alpha diversity and weighted UniFrac beta diversity(57) were quantified using QIIME2.

Whole genome shotgun sequencing.

Genomic DNA was extracted from IMC patient stool in the same manner as 16S rRNA sequencing (see above). Libraries for shotgun sequencing preparation were constructed using Illumina DNA Prep Kit (Illumina), according to manufacturer's protocol. The final libraries were loaded into the NovaSeq 6000 platform (Illumina) and sequenced 2×150 bp paired-end read, resulting in ~5 Gb per sample. NovaSeq raw reads are filtered by their phred quality score less than or equal to 15 by vsearch. Bacterial taxonomic alignment and pathway analyses were done by HUMAnN 3.0(58).

Real-time quantitative PCR.

Remaining genomic DNA used previously in the 16S rRNA sequencing was aliquoted and normalized to 10ng with ultra-pure, nuclease-free water for use in real-time qPCR assay. qPCR was performed as previously described (59). Briefly, real-time PCR was carried out in 96-well optical plates on QuantStudio Flex 6 RT-PCR (Thermo Fisher) and KAPA SYBR FAST 2X Master Mix (Applied Biosystems). Primers used in this assay have been previously described elsewhere: *Bifidobacterium* (60)and *Collinsella* (61). The PCR conditions included one initial denaturing step of 10 min at 95°C and 35 cycles of 95°C for 15 sec and 55°C (*Bifidobacterium*) and 60°C (*Collinsella*) for 1 min. Melting-curve analysis was performed after amplification to improve amplification specificity. 16S rRNA gene sequences were amplified from total fecal DNA using the primers 926F (5′-AAACTCAAAKGAATTGACGG-3′) and 1062R (5′-CTCACRRCACGAGCTGAC-3′) as an endogenous control.

Multiplex Immunofluorescence.

Multiplex immunofluorescence (mIF) staining was performed using similar methods and reagents that have been previously described(62). Briefly, 4 μm-thick formalin fixed, paraffin embedded (FFPE) tissue sections were automated staining system Leica BOND-RX (Leica Microsystems, Buffalo Grove, IL) against: cytokeratin (clone AE1/AE3, dilution 1:50, Dako, Santa Clara, CA), CD3 [clone D7A6E (AM), dilution 1:100, Cell Signaling Technology, Danvers, MA], CD8 (clone C8/144B, dilution 1:25, ThermoFisher Scientific, Waltham, MA), CD4 (Clone EPR6855, dilution 1:200, Abcam, Cambridge, MA), CD56 (clone 123C3, dilution 1:25, Dako), FOXP3 (clone D2W8E, dilution 1:100, Cell Signaling Technology), CD20 (clone L26, dilution 1:50, Dako), and Ki67 (clone MIB-1, dilution 1:100, Dako). All the markers were stained in sequence using their respective fluorophore

containing in the Opal 7 kit (catalogue #NEL797001KT; Akoya Biosciences, Waltham, MA) and the TSA fluorophore Opal Polaris 480 (#FP1500001KT, Akoya Biosciences). Stained slides were scanned using the multispectral microscope, Vectra Polaris 3.0.3 imaging system (Akoya Biosciences), under fluorescence and low magnification at 10x. Following scanning, a pathologist selected representative regions of interest (each ROI, 0.63 mm^2) per sample using the phenochart 1.0.9 viewer (Akoya Biosciences)(63). ROIs were analyzed using the InForm 2.8.2 image analysis software (Akoya Biosciences). Marker colocalization was employed to identify different cellular phenotypes and quantified as number of cells/mm^2 in mucosa and in submucosa compartment. Data were consolidated using the R studio 3.5.3 (Phenopter 0.2.2 packet, Akoya Biosciences).

Statistical analysis.

For 16S rRNA sequencing differential abundance analyses, bacterial genera were ranked by median abundance and variance and analyzed using Mann-Whitney test; the highest 20 features were analyzed using DESeq2(64) and corrected for the false discovery rate using the Benjamini-Hochberg method(65). Paired abundance testing was performed using the Wilcoxon test after logit transformation. The details of our computational analysis pipeline have been described previously(3). The Spearman correlation analysis was performed between continuous clinical parameters and relative abundances of microbiome as well as for comparisons between relative abundance data generated from 16S rRNA sequencing, whole genome shotgun sequencing, and real-time qPCR. The differences between discrete values in clinical parameters and microbial relative abundances were analyzed using the DESeq2. P-values were adjusted using FDR. Data were visualized using volcano plots, correlation analyses, and heatmaps created in R Studio 3.5.3. Any additional analyses, including Student t-tests and tests for normality between groups, were performed using GraphPad Prism version 8.0 (GraphPad Software).

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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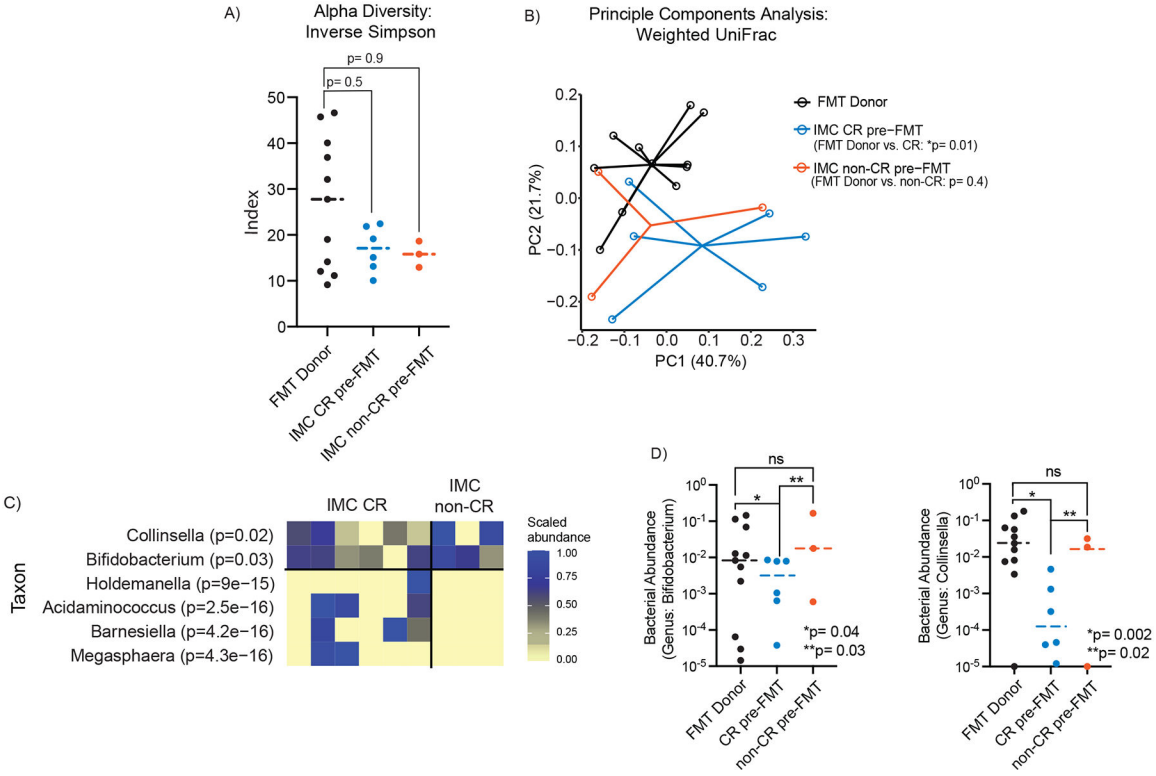

Fig. 1: Comparisons of alpha and beta diversity of baseline pre-FMT samples from IMC patients treated with FMT and healthy individuals.

(A) Alpha diversity by inverse Simpson index on samples from healthy individuals (FMT donors), pre-FMT samples from Complete Response (CR), and pre-FMT samples from non-Complete Response (non-CR) patients, compared using Mann-Whitney test. **(B)** Principle coordinates analysis (PCoA) of FMT donors (n=11), CR (n=6), and non-CR (n=3) patient samples; significance was assessed with PERMANOVA statistical testing. Axis percentage labels represent variance explained. **(C)** Differentially abundant bacterial genera in samples collected pre-FMT from CR compared with non-CR using DESeq2 with adjustment for multiple comparisons using the Benjamini-Hochberg method. **(D)** Differentially abundant bacterial genera in samples collected pre-FMT CR compared with samples from healthy FMT donors, analyzed as in (C).

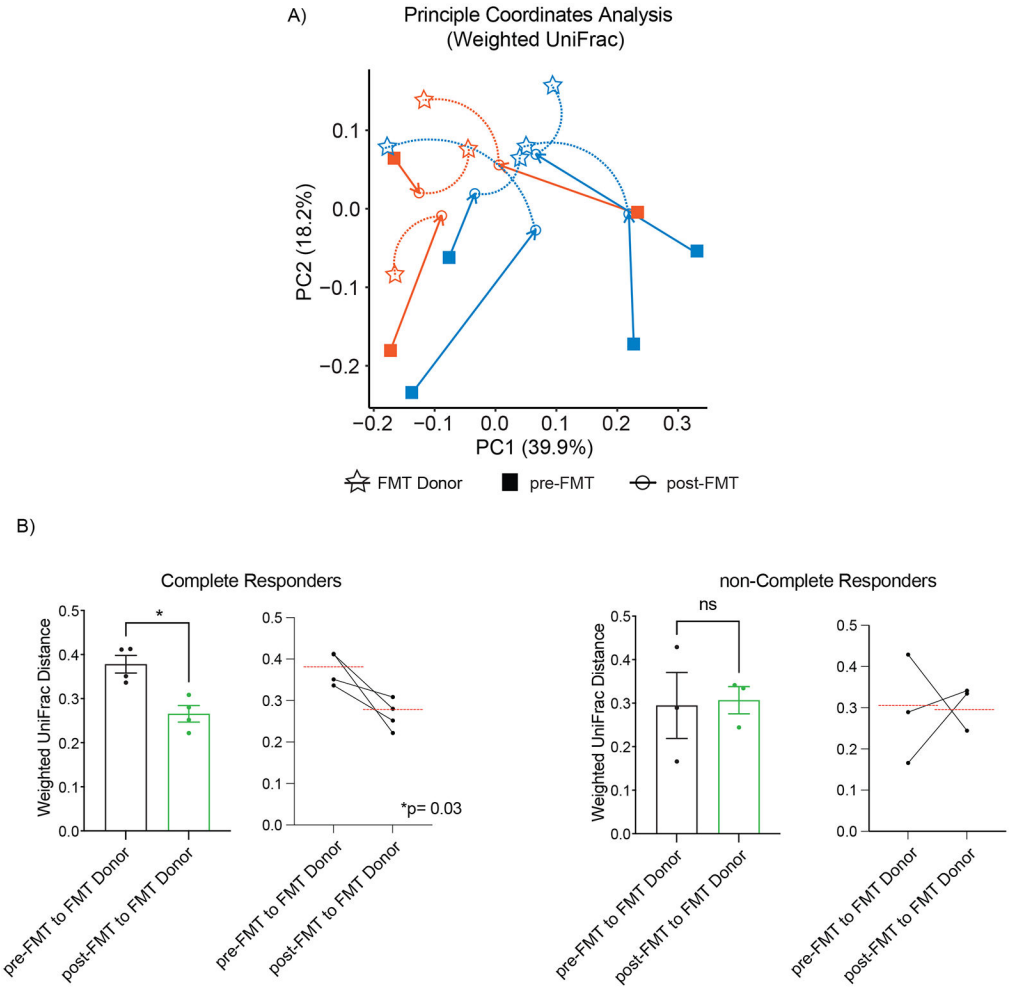

Fig. 2: Changes in beta diversity predict IMC response to FMT.

(A) Principle coordinates analysis (PCoA) of samples from Complete Response (CR; blue), and non-Complete Response (non-CR; orange) patients are shown. Pre-FMT (square), post-FMT (Day 30 +/− 20; circle), and corresponding FMT donor sample (star) for each patient are indicated in the figure. Solid lines indicate linked pre- and post-FMT samples, while dashed lines indicate linked post-FMT and FMT donor samples. Axis percentage labels represent variance explained. **(B)** Beta diversity distances were quantified using weighted UniFrac values comparing pre- and post-FMT samples against FMT donor samples in CR and non-CR IMC patients. Data are shown using both bar and paired dot plots. Comparisons were analyzed using Mann-Whitney statistical test.

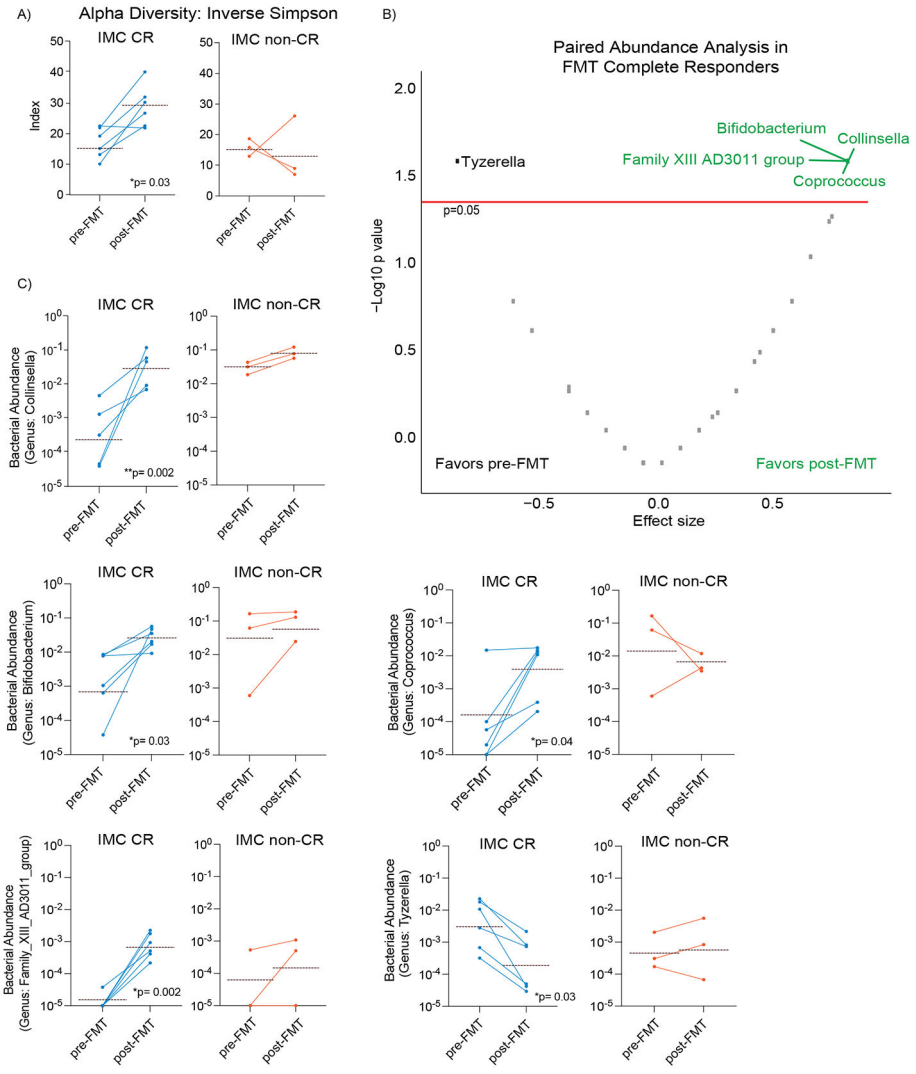

Fig. 3: Increases in alpha diversity and specific bacterial taxa are associated with FMT response.

(A) Comparison of alpha diversity by inverse Simpson index pre-FMT (baseline) and post-FMT (Day 30 +/− 20) in Complete Response (CR) and non-Complete Response (non-CR) IMC patients using paired Wilcoxon statistical test. **(B)** Volcano plot of differentially abundant bacterial genera comparing pre-FMT and post-FMT samples from CR patients using paired Wilcoxon statistical test with adjustments for multiple comparisons (Benjamini-Hochberg method). **(C)** Relative abundance of significantly changed bacterial genera identified in (B) quantified in paired samples for CR and non-CR patients.

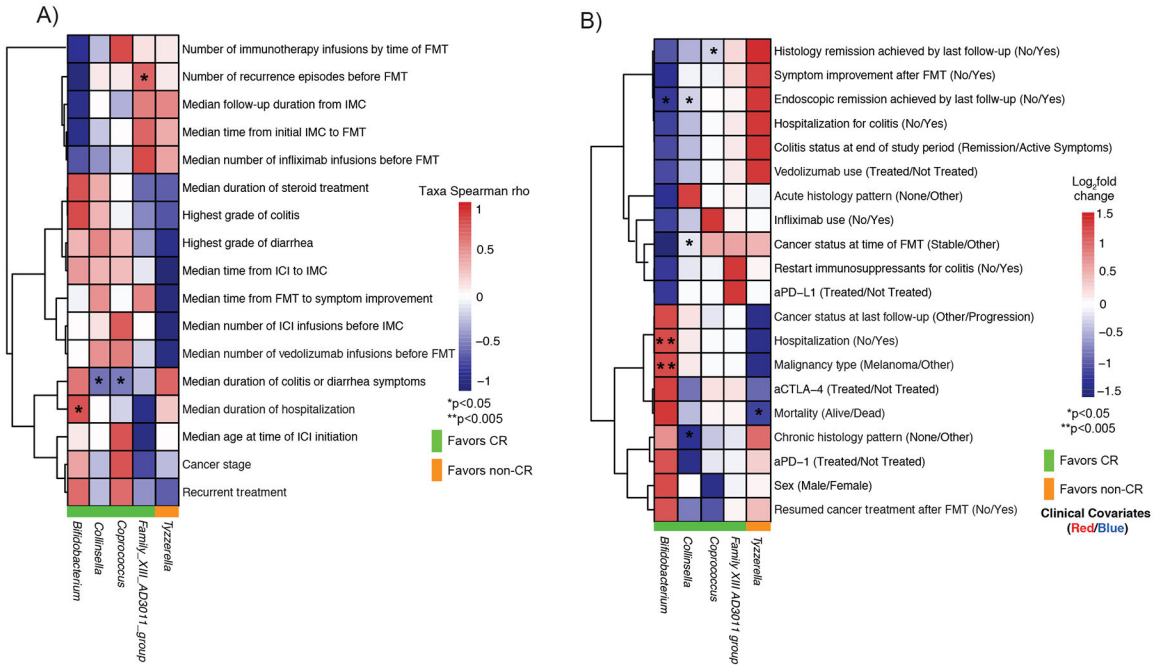

Fig. 4: Associations of select taxa with pre-FMT clinical features of IMC patients.

(A) Spearman's rank correlation of clinical variables with bacterial taxa pre-FMT. (B) Binary clinical associations using DESeq2. Log2fold change represents effect size and directionality is indicated in parentheses next to each clinical feature (red/blue). Previously identified taxa of interest (Fig.3) are present at the bottom of each heatmap based on response to FMT therapy (CR, green; non-CR, orange). All available clinical features were considered (see Tables 1 and 2). Significance was assessed using calculated values normalized to each sample mean and scaled for visualization using heatmaps generated in R.

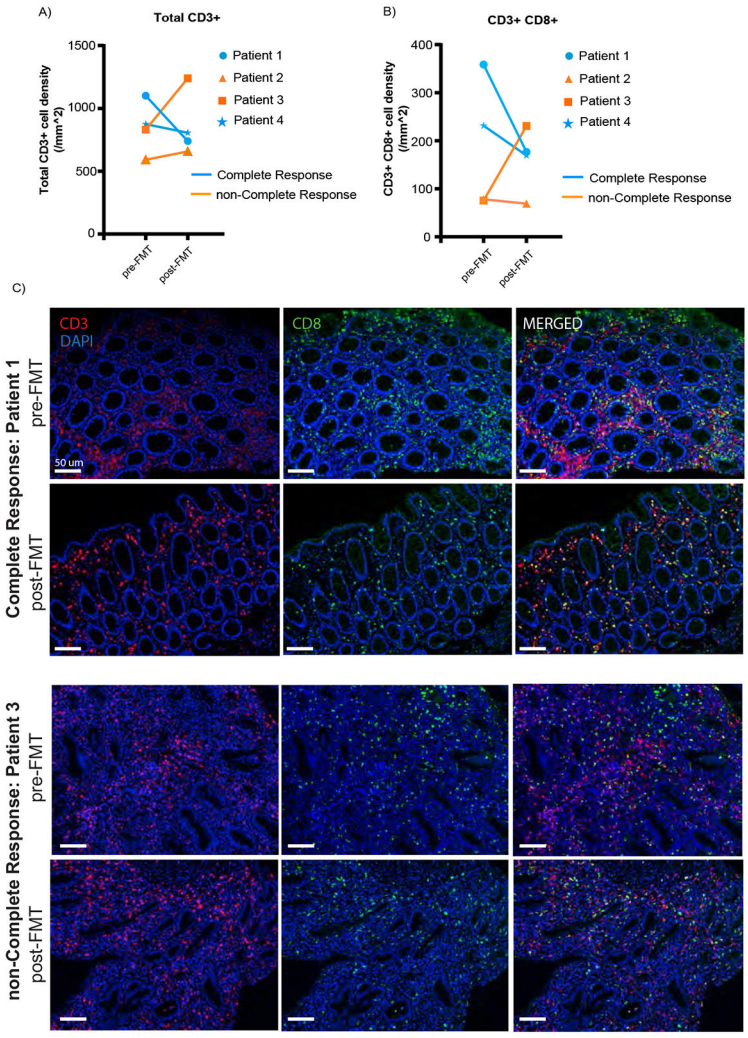

Fig. 5: Immune infiltrate changes in FMT treated IMC patients.

Multiplex immunofluorescence quantification of (A) total CD3+ and (B) CD3+ CD8+ T cells. Total area of colonic epithelium and stroma of IMC patients were used to quantify changes in immune cells. Individual patients are depicted using shapes. (C) Representative immunofluorescent images depicting CD3+ & DAPI, CD8+ & DAPI, and merged for IMC patients treated with FMT. Scale bars represent 50 um. P- values not reported due to small sample size.

Table 1:

Patients' baseline characteristics (n = 12)

Characteristic	Data (n=12)
Median age at time of ICI initiation – years (IQR)	56 (45-67)
Male sex – no. (%)	9 (75)
White race – no. (%)	12 (100)
Cancer type – no. (%)	
Genitourinary	6 (50)
Melanoma	3 (25)
Gastrointestinal	1 (8)
Head and neck	1 (8)
Hematological cancer	1 (8)
Cancer stage –no. (%)	
Stage I-II	2 (17)
Stage IV	10 (83)
Checkpoint inhibitor type – no. (%)	
aCTLA-4	1 (8)
aPD-(L)1	6 (50)
Combination	5 (42)
Median number of ICI infusions before IMC – no. (IQR)	9 (1-25)
Immunotherapy stopped because of IMC– no. (%)	12 (100)
Median follow-up duration from IMC – months (IQR)	22 (3-44)

ICI: immune checkpoint inhibitor; CTLA-4: cytotoxic T-lymphocyte-associated protein 4; PD-(L)1: programmed cell death protein (ligand) 1; IMC: immune- mediated colitis; IQR: interquartile range.

Table 2:

Characteristics and outcome of immune-mediated colitis (n = 12)

Characteristic	Data (n=12)	CR (n=7)	Non-CR (n=5)
Median time from ICI to IMC – days	71 (14-586)	69 (14-189)	171 (47-586)
Highest grade of diarrhea – no. (%)			
3 or 4	12 (100)	7 (100)	5(100)
Highest grade of colitis – no. (%)			
1 or 2	6 (50)	4 (57.1)	2 (40)
3 or 4	6 (50)	3 (42.9)	3 (60)
Initial endoscopic findings – no (%)			
Ulcers	6 (50)	4 (57.1)	2(40)
Non-ulcer inflammation	3 (25)	2 (28.6)	1(20)
Normal	3 (25)	1 (14.3)	2(40)
Initial histology findings – no (%)			
Active inflammation	2 (16.7)	1 (14.3)	1 (20)
Chronic active inflammation	9 (75)	6 (85.7)	3 (60)
Normal	1 (8.3)	0	1 (20)
Hospitalizations – no. (%)	11 (92)	7 (100)	4 (80)
Median duration of hospitalization – days	15 (5-63)	12 (5-31)	7 (0-30)
Treatment of GI adverse events – no. (%)			
Steroid	12 (100)	7 (100)	5 (100)
Infliximab/vedolizumab added	12 (100)	7 (100)	5 (100)
Median duration of steroid treatment – days	68 (46-93)	70 (46-117)	81 (36-130)
Median number of infliximab infusions before FMT – no	1 (0-4)	0 (0-4)	2 (0-4)
Median number of vedolizumab infusions before FMT – no	3 (2-4)	3 (2-4)	3 (2-4)
FMT characteristic and outcome			
Median time from initial IMC to FMT– days	89 (58-386)	100 (71-305)	78 (58-386)
Median duration of colitis or diarrhea symptoms-- days	57 (1-235)	125 (1-235)	31 (1-72)
Symptom improvement after FMT – no (%)	10 (83)	7 (100)	3 (60)
Median time from FMT to symptom improvement– days	14 (9-16)	10 (0-20)	14 (5-14)
FMT-related complications within 7 days –no (%)	0	0	0
FMT-related complications within 30 days –no (%)	0	0	0
Cancer status at the time of FMT –no (%)			
Remission	3 (25)	1 (14.3)	2 (40)
Stable disease	7 (58.3)	5 (71.4)	2 (40)
Progression	2 (16.7)	1 (14.3)	1 (20)
Required selective immunosuppressants for recurrent or refractory colitis after FMT – no (%)	4 (33.3)	2 (28.5)	2 (40)
Resumed cancer treatment after FMT – no (%)	4 (33)	2 (28.5)	2 (40)
Endoscopic remission achieved by last follow-up – no (%)	5 (42)	5 (71.4)	0

Histology remission achieved by last follow-up –no (%)	3 (25)	3(42.9)	0
Colitis status at the end of the study period			
Clinical remission – no (%)	11 (92)	6 (85.7)	5 (100)
Persistent symptoms – no (%)	1 (8)	1 (14.3)	0
Cancer status at last follow-up –no (%)			
Remission	3 (25)	1 (14.3)	2 (40)
Stable disease	3 (25)	1 (14.3)	2 (40)
Progression	6 (50)	5 (71.4%)	1 (20)
Mortality– no. (%)	3 (25)	2 (28.5)	1 (20)

ICI: immune checkpoint inhibitor; FMT: fecal microbiota transplantation; IMC: immune-mediated colitis; IQR: interquartile range.

Six patients received both infliximab and vedolizumab before FMT.

All three patients who died were in remission or had responded.