GRAFT-VERSUS-HOST DISEASE

Donor fecal microbiota transplantation ameliorates intestinal graft-versus-host disease in allogeneic hematopoietic cell transplant recipients

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Disruption of the intestinal microbiota occurs frequently in allogeneic hematopoietic cell transplantation (allo-HCT) recipients and predisposes them to development of graft-versus-host disease (GvHD). In a prospective, single-center, single-arm study, we investigated the effect of donor fecal microbiota transplantation (FMT) on symptoms of steroid-refractory or steroid-dependent, acute or late-onset acute intestinal GvHD in 15 individuals who had undergone allo-HCT. Study participants received a fecal suspension from an unrelated healthy donor via nasoduodenal infusion. Donor FMT was well tolerated, and infection-related adverse events did not seem to be related to the FMT procedure. In 10 of 15 study participants, a complete clinical response was observed within 1 month after FMT, without additional interventions to alleviate GvHD symptoms. This response was accompanied by an increase in gut microbial α-diversity, a partial engraftment of donor bacterial species, and increased abundance of butyrate-producing bacteria, including Clostridiales and *Blautia* species. In 6 of the 10 responding donor FMT recipients, immunosuppressant drug therapy was successfully tapered. Durable remission of steroid-refractory or steroid-dependent GvHD after donor FMT was associated with improved survival at 24 weeks after donor FMT. This study highlights the potential of donor FMT as a treatment for steroid-refractory or steroid-dependent GvHD, but larger clinical trials are needed to confirm the safety and efficacy of this procedure.

INTRODUCTION

Allogeneic hematopoietic cell transplantation (allo-HCT) is a curative treatment for many hematological malignancies, but its therapeutic success is limited by anti-host immune responses leading to graft-versus-host disease (GvHD). Individuals with GvHD, particularly steroid-refractory GvHD, have a poor prognosis (*1*). There is a need for therapies that dampen alloreactivity, preferably without further compromising the immune system. The intestinal microbiota might offer an attractive target for therapeutic intervention.

The extensive interplay between the intestinal microbiota and mucosal immune system is essential to preserve homeostasis and mediate colonization resistance in the human gut (*2*–*4*). Recent studies have shown that this delicate balance is commonly disrupted in patients receiving an allo-HCT, which negatively affects transplant outcome (*5*–*7*). Dysbiosis of the gut microbiota is observed to precede GvHD (*8*–*12*). Pretransplant conditioning regimens, including remission-induction chemotherapy and total body irradiation, combined with prophylactic or therapeutic use of antibiotics, gradually decrease gut microbial diversity and induce a shift in the composition of the enteric microbial community of allo-HCT recipients. More specifically, microbial domination by Gammaproteobacteria or Lactobacillales classes is associated with pulmonary complications and bacteremia (*13*, *14*), whereas the presence of bacterial genera from the Clostridiales order, including *Blautia*, are linked to lower relapse rates, decreased GvHD mortality, and better overall survival (*15*–*18*). The beneficial effect of Clostridia bacteria could be explained by their ability to produce butyrate, one of the gut microbiota–derived short-chain fatty acids that act as an important energy source for intestinal epithelial cells. A study in mice showed that administration of 17 rationally selected strains of high-butyrate–producing Clostridia mitigated intestinal GvHD, although the underlying mechanism remains to be elucidated (*19*).

Fecal microbiota transplantation (FMT) was introduced as effective therapy for recurrent *Clostridium difficile* infections (*20*), and its use has rapidly been extended to treat inflammatory conditions of the intestine (ulcerative colitis and Crohn's disease) and beyond (*21*). Studies involving allo-HCT recipients have shown that autologous or donor FMT could restore microbial diversity and eradicate antibiotic-resistant bacteria and, despite the immunocompromised status of allo-HCT patients, this procedure appeared safe (*22*–*25*). In addition, a number of case reports have suggested that repeated donor FMT could resolve intestinal GvHD (*26*–*29*). Here, we report results of a prospective, single-arm study in which 15 patients with steroid-resistant or steroid-dependent intestinal GvHD received a single FMT from a healthy donor via nasoduodenal infusion.

RESULTS

Characteristics of study participants

Seventeen allo-HCT recipients received a donor FMT. Fifteen patients were evaluable for treatment response, with two patients excluded from follow-up because they received additional GvHD medication

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within 3 days after donor FMT and, therefore, response to donor FMT could not be assessed (Fig. 1). Stool samples were collected at set time points after donor FMT to study the composition of the gut microbiota. For one patient (P05), follow-up gut microbiota analysis could not be performed. This patient had hyperacute, rapidly progressive grade IV GvHD upon inclusion in the study. Donor FMT did not reverse the course of disease, and he died 1 week after donor FMT.

Patients received an allo-HCT for acute myeloid leukemia, myelodysplastic syndrome, Hodgkin's lymphoma, non-Hodgkin's lymphoma, or myeloproliferative disorder (Table 1 and table S1). GvHD prophylaxis consisted of cyclosporin and mycophenolic acid except for P12 who received methotrexate instead of mycophenolic acid after a busulfan-based conditioning regimen. All patients had clinically diagnosed grade II to IV acute or late-onset acute GvHD of the gut that was confirmed by histological evaluation of gut biopsies (*30*). Serum biomarkers for acute GvHD REG3α (regenerating islet-derived protein 3α) and ST2 (suppression of tumorigenicity 2) (*31*, *32*) obtained at baseline were significantly increased in study participants when compared to healthy controls (Mann-Whitney *U* test: $P = 0.013$ and $P = 0.0017$, respectively) (Fig. 2). Six patients were refractory to steroid treatment (P05, P10, P11, P14, P16, and P17), whereas the other participants had steroid-dependent GvHD, characterized by long-term reliance on steroid treatment and persistent recurrence of diarrhea and/or fecal incontinence upon tapering of steroid therapy. Consequently, median time between allo-HCT and donor FMT was generally shorter for steroid-refractory patients compared to steroid-dependent patients (3.3 versus 9 months, respectively). In addition to systemic steroids, most (93%) of the patients were administered at least one other local (budesonide) or systemic (cyclosporin, mycophenolate mofetil, methotrexate, ruxolitinib, sirolimus, or tacrolimus) immunosuppressant drug at the time of donor FMT. A detailed medication history for each patient is provided in Fig. 3 and table S1. Two patients (P12 and P17) received topical steroid therapy for cutaneous GvHD at the time of donor FMT. All patients received infection prophylaxis that included antibiotic (trimethoprim/sulfamethoxazole, amoxicillin, pentamidine, ceftriaxone, or imipinem/cilastatine), antiviral (valaciclovir or aciclovir), and, in some cases, antifungal (fluconazole, posaconazole, or voriconazole) medication. This prophylaxis was continued during donor FMT. Four participants had comorbid infections at the time of donor FMT: two patients (P11and P14) received ganciclovir for cytomegalovirus colitis, and two patients (P10 and P16) were treated for *C. difficile* infection with vancomycin and metronidazole, respectively. In P16, metronidazole was stopped shortly after donor FMT.

Donor FMT was well tolerated by allo-HCT recipients

Patients received a bowel lavage 2 hours before donor FMT, according to study protocols in our hospital (*20*), with the rationale to provide enough space for the new gut microbiota. Subsequently, a homogenized fecal suspension from one of the two healthy fecal donors was slowly administered (10 cm^3/min) via nasoduodenal infusion. Side effects reported by patients on the day of donor FMT included discomfort of the nasoduodenal tube, transient abdominal distention, and cramps (table S2). Four patients experienced nausea, and one patient regurgitated directly after FMT, but no other complications were seen. All side effects resolved spontaneously within hours.

Five patients developed an infection within the first month after donor FMT (table S2). These included otitis media (P09, 18 days after FMT) and, in two patients (P12 and P16), uncomplicated cystitis (7 and 19 days after FMT). One patient (P11) was diagnosed with pneumonia 5 days after donor FMT. *Staphylococcus aureus* and *Klebsiella pneumoniae* were cultured from sputum, but neither pathogen was detectable in the donor fecal sample. The patient was successfully treated with piperacillin/tazobactam. Another patient (P02) was diagnosed with *Escherichia coli* sepsis (14 days after FMT), originating from the urinary tract. *E. coli* was cultured from blood

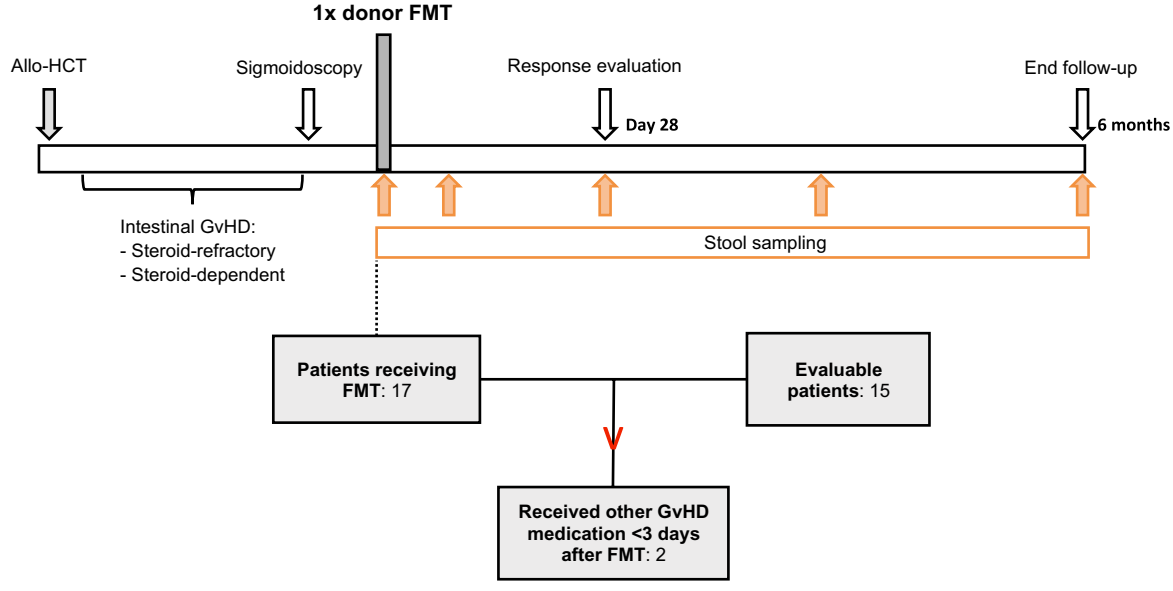

Fig. 1. Study design. Seventeen allo-HCT recipients (age 18 years and older) with biopsy-proven, steroid-refractory or steroid-dependent, acute or late-onset acute intestinal GvHD received a single-donor FMT via nasoduodenal infusion. Fifteen patients were evaluable for treatment response; two subjects received other GvHD medication within 3 days after donor FMT and were therefore excluded from further evaluation. Fecal samples were collected before donor FMT (baseline) from donors and patients and from patients at 1, 4, 12, and 24 weeks after donor FMT.

Table 1. Characteristics of study participants. GvHD staging was performed according to internationally accepted criteria (*56*, *57*). FMT, fecal microbiota transplantation; GI, gastrointestinal; GvHD, graft-versus-host disease; HCT, hematopoietic cell transplantation.

Characteristic	Value
Median age (range), years	57 (20–72)
Sex, female/male, *n*	10/5
FMT indication, steroid refractory/dependent, *n*	6/9
Median time between HCT and FMT (range), months	7 (1.6–49.9)
Diagnosis, *n*	
Acute myeloid leukemia	8
Myelodysplastic syndrome	3
Hodgkin's lymphoma	1
Non-Hodgkin's lymphoma	1
Myeloproliferative disorder, myelofibrosis	2
Graft source, *n*	
Peripheral blood stem cells	14
Bone marrow	0
Cord blood	1
Donor, *n*	
Matched unrelated	13
Matched related	2
Conditioning, *n*	
Reduced intensity	12
Myeloablative	3
GI GvHD stage at start of FMT, *n*	
I	7
II	3
III	3
IV	2
GI GvHD treatment other than systemic steroids at start of FMT, *n*	
Local therapy	8
Systemic therapy	12
GvHD involvement of other organs at time of FMT, *n*	
Skin	3
Liver	1

and urine and was also detected by 16*S* ribosomal RNA (rRNA) sequencing in the patient's fecal sample before FMT but not in the donor fecal sample. The patient recovered after successful antibiotic treatment with ceftriaxone. None of the subjects developed a viral or fungal infection in the first 28 days after donor FMT.

Diarrhea resolution after donor FMT in two-thirds of individuals with GvHD

Three of six patients with steroid-refractory GvHD and seven of nine patients with steroid-dependent GvHD showed a complete response, defined as complete resolution of GvHD symptoms on day 28 after donor FMT. None of these patients had intensification of existing therapy or initiation of new therapy to treat GvHD in those first 4 weeks after FMT (Fig. 3, A and B). Five participants were classified as nonresponders, with no improvement of symptoms (Fig. 3C). Treatment response was associated with a lower stage of gastrointestinal GvHD at the time of FMT (Mann-Whitney *U* test: $P = 0.044$). Four patients had a coexisting infectious gastroenteritis at the time of donor FMT. Of those, two subjects (P11 and P16) responded to donor FMT therapy, whereas two others (P10 and P14) did not. In the complete response group, improvement of symptoms (normalization of stool frequency and consistency and reduction of abdominal cramps) was observed between weeks 1 and 2 after donor FMT. After a complete response, tapering of immunosuppressant drug therapy was initiated and successfully continued in six individuals showing a complete response throughout the follow-up period of 6 months (Fig. 3A and fig. S1). In four other individuals undergoing a complete response, GvHD symptoms returned upon tapering of immunosuppressant drug therapy. These patients were categorized as complete responders with secondary failure. All of them received antibiotic treatment shortly after donor FMT in addition to standard antibiotic prophylaxis, either as prophylaxis during neutropenia or to treat concurrent infections (Fig. 4A). Four of five nonresponders died from complications of GvHD during follow-up. Complete response to donor FMT was associated with a better prognosis (Mantel-Cox test: $P = 0.0068$) (Fig. 4B).

Increased gut microbiota α-diversity in allo-HCT recipients responding to donor FMT

Bacterial composition was determined and α-diversity metrics were calculated for stool samples collected from participants before donor FMT (baseline) and at 1, 4, 12, and 24 weeks after donor FMT and from the donors using 16*S* rRNA sequencing. Stool samples were excluded from analysis if patients had received new GvHD medication before stool collection. Consequently, for most nonresponding patients, only baseline and 1-week stool samples could be included because, in the absence of clinical improvement, most received other GvHD medication in the weeks after donor FMT.

GvHD patients responding to donor FMT started with higher baseline gut microbial diversity (Fig. 5A). α-Diversity, depicted as the number of observed bacterial species (left), the Shannon index (which accounts for the abundance and evenness of the species present; middle), and the phylogenetic diversity (assessing the α-diversity from a higher taxonomic rank; right) were highest in the complete response group at baseline, before donor FMT. With resolution of diarrhea, diversity of stool samples increased after donor FMT in patients with a complete response and complete response with secondary failure, reaching values close to that of the donor. We assessed the engraftment of donor FMT by determining whether bacteria in the follow-up stool samples from study participants were either already present in the patient's baseline sample (patient origin), derived from the donor sample (donor origin), detected in both samples (shared), or detectable in neither donor nor patient's baseline sample (unknown origin). In all patients, the relative contribution of patient-derived species to the total composition of their fecal microbiota decreased (Fig. 5B). Instead, bacteria of donor origin expanded in individuals with a complete response suggesting successful engraftment of donor FMT; in nonresponding patients, the contribution of bacteria of unknown origin increased. At baseline,

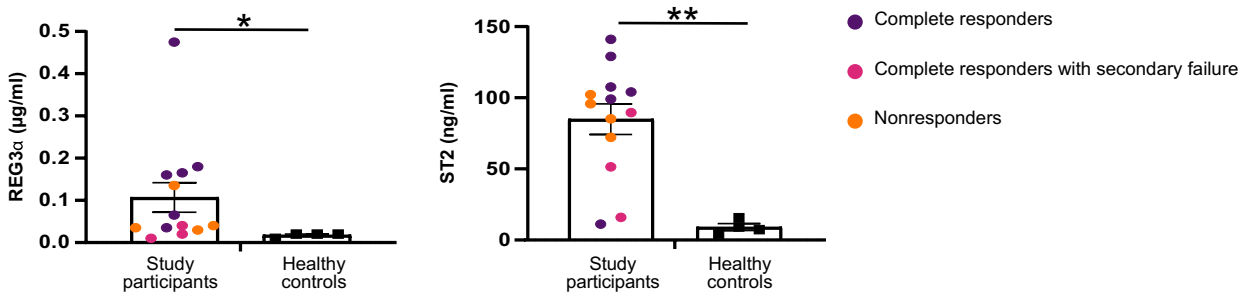

Fig. 2. Serum concentrations of GvHD biomarkers. Serum concentrations of REG3α and ST2, biomarkers of GvHD, at baseline for complete responders ($n = 6$), complete responders with secondary failure ($n = 3$), nonresponders ($n = 4$), and healthy individuals ($n = 4$) are shown. Data are means ± SEM. *$P < 0.05$ and **$P < 0.01$, Mann-Whitney U test.

the gut microbiota of those with a complete response had the closest resemblance to that of the donor compared to those complete responders with secondary failure or nonresponders (Jaccard similarity index, Fig. 5C), although this was not statistically significant. Engraftment of donor fecal bacterial species further increased resemblance of the composition of the recipient's gut microbiota to the donor's gut microbiota 1 week after FMT.

Next, we investigated which host factors, such as fucosyltransferase 2 (FUT2) secretor status, might have influenced engraftment of the donor fecal transplant bacterial species. FUT2 secretors have at least one functional *FUT2* allele that enables the production of the FUT2 enzyme, whereas nonsecretors do not. FUT2 is essential for epithelial fucosylation and the secretion of ABO blood group antigens into body fluids and into the gut lumen, where they serve as a carbohydrate source for commensal bacteria (*33*–*35*). FUT2 secretor status has been suggested as one of the genetic factors determining gut microbiota diversity (*36*). We hypothesized that a FUT2 secretor genotype might enable engraftment of donor FMT bacterial species. Therefore, we visualized secretor status by epithelial staining for antigen H (*37*), the backbone of ABO antigens, on gut biopsies from our patients with GvHD (Fig. 5D). From the nine complete responders with an available gut biopsy, eight had a positive staining for expression of epithelial antigen H and were thus classified as secretors. Two nonresponders were also secretors, whereas the other two nonresponders had a nonsecretor genotype.

Greater abundance of beneficial bacteria in donor FMT responders

We then investigated the particular gut microbiota composition over time of each allo-HCT recipient administered a donor FMT. Figure 6A depicts the individual genus composition of the donor fecal samples and of all evaluable patient stool samples. Donor 2 was used for all patients except P01. Stool samples from donor 2 (Fig. 6A, top row) showed a consistent composition over the course of the study. In some patients (P02 and P10), antibiotic therapy eradicated microbial diversity. In P02, microbial diversity recovered slowly after cessation of antibiotic therapy. To examine whether donor FMT introduced bacteria that have been associated with a superior outcome after allo-HCT (*13*, *15*, *17*), we checked the abundance of intestinal species including *Blautia*, Clostridiales, and predicted butyrate–producing bacteria (Fig. 6B). Complete responders and complete responders with secondary failure tended to have a greater initial relative contribution of butyrate producers and *Blautia* as compared to nonresponders. Baseline *Blautia* abundance in responders was comparable to that of donor fecal samples and further increased after successful donor FMT. In addition, the abundance of Clostridiales and butyrate producers gradually increased in both complete responders and complete responders with secondary failure, reaching similar values as in the donor fecal samples. In contrast, the abundance of these bacteria remained stable or declined in the nonresponders.

DISCUSSION

Here, we found that restoration of gut microbial diversity via a single-donor FMT could resolve steroid-refractory and steroid-dependent GvHD of the intestine. FMT was well tolerated by all participants, and the infection-related adverse events that did occur were unlikely to be related to the FMT. The results of this single-arm trial merit further research into the potential of donor FMT for treating steroid-refractory and steroid-dependent intestinal GvHD.

The immunocompromised status of allo-HCT recipients warrants a cautious approach toward the infusion of a high load of donor-derived micro-organisms into these patients. The use of stringent donor screening protocols is of utmost importance, as transfer of multidrug-resistant organisms into immunocompromised patients could have fatal consequences (*38*). Safety concerns have been the reason to exclude immunocompromised patients from most donor FMT clinical trials, and therefore, data on the safety of FMT in these patients are limited. A systematic review of clinical studies involving 303 immunocompromised patients concluded that there was no additional risk to using donor FMT as treatment for *C. difficile* infection in immunocompromised patients compared to immunocompetent individuals (*39*). The small studies that have reported on the use of donor FMT in the setting of GvHD did not attribute the serious adverse events that occurred, such as bacteremia, to the donor FMT procedure (*26*, *40*). In our study, five participants developed an infection after donor FMT. Given the seemingly unrelated sites of infection, the long interval between donor FMT and infection, or the absence of the causative pathogen in patient or donor stool samples, we deemed these infections unrelated to donor FMT in our cohort.

Donor FMT has been demonstrated to induce a lasting change in the microbial composition of the intestine in a number of patients, including those undergoing allo-HCT (*23*). In our study, most (67%) of the allo-HCT recipients with GvHD showed a complete response to donor FMT. A single FMT dose was sufficient to allow successful tapering of immunosuppressant drug therapy in six study participants. For four complete responders, immunosuppressant tapering resulted in a return of GvHD symptoms. All of these individuals with secondary failure after a complete response received antibiotics shortly after donor FMT, and this may have interfered with a lasting

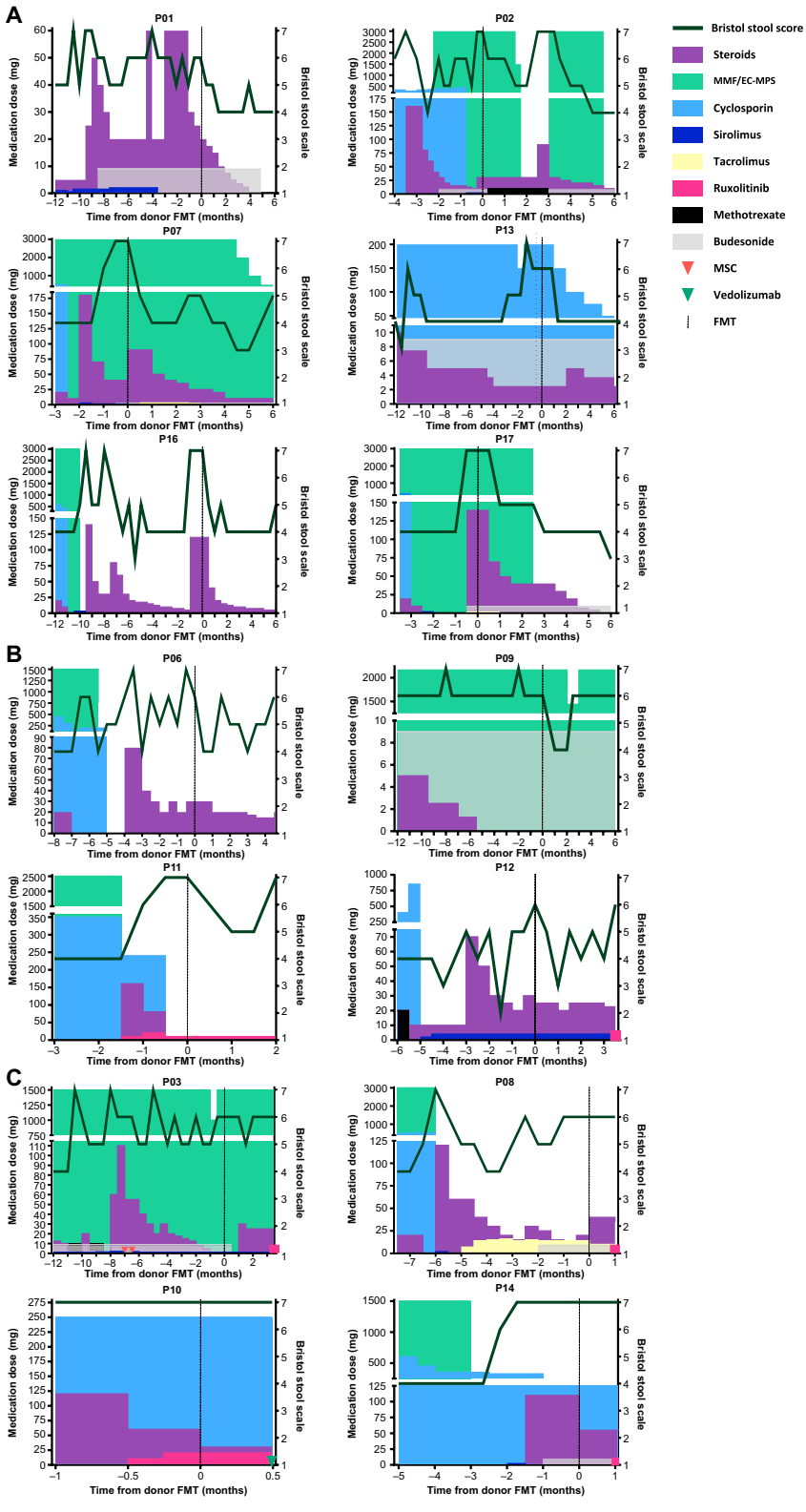

Fig. 3. Individual timelines of GvHD symptoms and immunosuppressive medication before and after FMT. Individual data for complete responders (**A**), complete responders/secondary failure (**B**), and nonresponders (**C**) are depicted. Histograms show the dosage of prescribed immunosuppressant drugs (left *Y* axis) and Bristol stool scale (dark green line) as a measure of intestinal GvHD symptoms (right *Y* axis) for each study participant indicated by their number, starting from 12 months before donor FMT or from the time of allo-HCT. Data were collected from medical records and scored every 2 weeks. Bristol stool scale between 3 and 5 was referenced as healthy. EC-MPS, enteric-coated mycophenolate sodium; FMT, fecal microbiota transplantation; MMF, mycophenolate mofetil; MSC, mesenchymal stromal cells.

response in these patients. The detrimental effects of antibiotic use on gut microbiota diversity in allo-HCT recipients have previously been described (*9*, *12*, *17*, *41*, *42*). In particular, the use of antibiotics in the first week after FMT was associated with treatment failure in our patients, but in the absence of daily stool sampling, it is not possible to relate changes in gut microbial diversity during that first week to either the donor FMT or antibiotics. In two complete responders (P02 and P16) who received antibiotic treatment within the first 3 weeks after donor FMT, gut microbial diversity recovered after cessation of therapy and relapse of GvHD symptoms did not occur. Another factor to take into account was the diet of study participants. In this clinical trial, we did not collect accurate data on the dietary intake of study participants, but diet has been implicated in FMT outcome and endurance of response (*43*).

Other determinants of successful engraftment of the donor fecal microbiota in recipients have yet to be defined. We used a single fecal donor for 14 of the 15 evaluable patients participating in this study. This donor was proven to have a stable gut microbiota composition throughout the complete course of the study (Fig. 6A, top row), demonstrating that patient-intrinsic factors are important determinants for the efficacy of donor FMT. Our previously published data studying glucose metabolism and donor FMT showed that lower baseline bacterial diversity was predictive of treatment response (*44*). In addition, in patients with persistent *C. difficile* infection, whose gut microbial diversity was drastically diminished after multiple rounds of antibiotics, donor FMT efficiently reestablished a diverse and stable gut microbiota (*45*). In contrast, data from the present clinical trial imply that providing more "space" in the microbial community before FMT does not necessarily translate into better engraftment of donor bacterial strains. Patients with higher baseline gut microbial diversity were more likely to respond to donor FMT. It has been reported that intestinal microbiota resist allowing new (donor) strains to inhabit the gut, unless these strains are closely related to species already present in the individual's gut microbial population (*46*). This could explain the superior response of patients with GvHD who had the highest baseline diversity, and it raises the question of whether those with lower microbial diversity at baseline could benefit from multiple FMT doses instead of just one. Of note, a meta-analysis of FMT in a variety of other inflammatory diseases suggested that serial FMTs

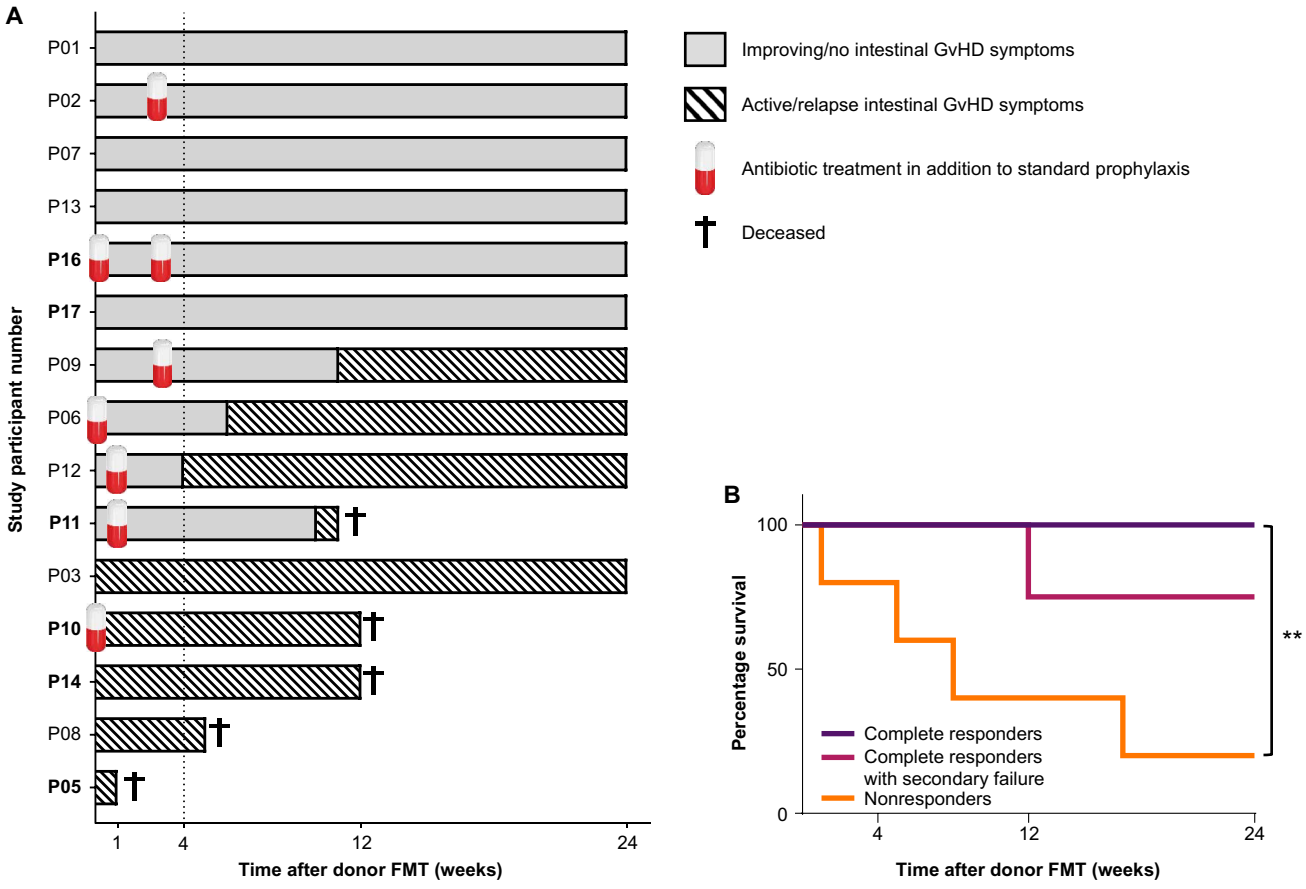

Fig. 4. Clinical course and survival after donor FMT. (**A**) Clinical response to donor FMT in 15 allo-HCT recipients with GvHD indicated by their study participant number. Participants with steroid-dependent GvHD are shown in regular font; those with steroid-refractory GvHD are marked in bold. Antibiotics were administered to P02 (intravenous ceftriaxone for 7 days), P16 (oral metronidazole for 5 days and oral nitrofurantoin for 5 days), P09 (oral amoxicillin for 2 days), P06 (oral ciprofloxacin for 170 days), P12 (oral nitrofurantoin for 7 days), P11 (intravenous piperacillin/tazobactam for 7 days), and P10 (vancomycin intravenously for 3 days). (**B**) Kaplan-Meier survival curves indicating the percentage of allo-HCT recipients who survived at 24 weeks after donor FMT. $^{**}P < 0.01$, Mantel-Cox test.

do not increase efficacy (*47*), but this remains to be confirmed in patients with GvHD. One could also speculate that microbial diversity reflects overall disease severity, which inherently affects chances of recovery. Nonresponding patients in this study had a higher stage of intestinal GvHD than did the patients who did respond to donor FMT.

A less studied factor that may affect FMT engraftment may be the FUT2 secretor status of donor FMT recipients. Activity of the FUT2 enzyme in "secretors" results in fucosylation of intestinal epithelium that mediates adherence of donor bacterial strains and the secretion of ABO blood group antigens that are used by commensals as an energy source. The secretor phenotype is most prevalent in the Caucasian population (~80%) (*48*) and has been suggested to be a genetic determinant of gut microbiota diversity (*36*, *49*), although this hypothesis is still under debate (*50*, *51*). It has been reported that FUT2 nonsecretors are more susceptible to gastrointestinal infections, inflammatory bowel disease, and bacteremia after allo-HCT (*52*–*54*). At the same time, healthy nonsecretors tend to carry higher loads of bacteria belonging to the *Blautia* genus (*36*). The large majority (77%) of our study participants had a secretor phenotype. Although this study was underpowered to correlate this phenotype to FMT outcome, we observed that 50% of nonresponding donor FMT recipients had a secretor phenotype, which is lower than expected in this group of Caucasian allo-HCT recipients. Response to donor FMT was associated with an increase in donor-derived bacteria and an increase in beneficial bacteria such as the butyrate-producing bacteria, Clostridiales and *Blautia*, indicative of reestablishment of a healthy gut microbiota.

A limitation of our study is the small and heterogeneous group of participants that prohibits drawing any definite conclusions. In addition, the question of whether donor FMT by itself induced complete resolution of GvHD or whether it merely helped to boost a spontaneous recovery remains unanswered by this single-arm trial. The latter seems unlikely given the long-term reliance on prednisolone in most patients with recurrent flares of GvHD symptoms upon tapering of immunosuppressant drug therapy. The high mortality among steroid-refractory and steroid-dependent patients with GvHD, which was also observed in our trial, underlines the urgent need to identify factors that enhance gut microbiota engraftment and determine effectiveness of donor FMT. Larger prospective cohorts are needed to confirm the safety and efficacy of donor FMT in allo-HCT recipients and to determine the optimal frequency, source, and timing of donor FMT in those with steroid-refractory or steroid-dependent intestinal GvHD.

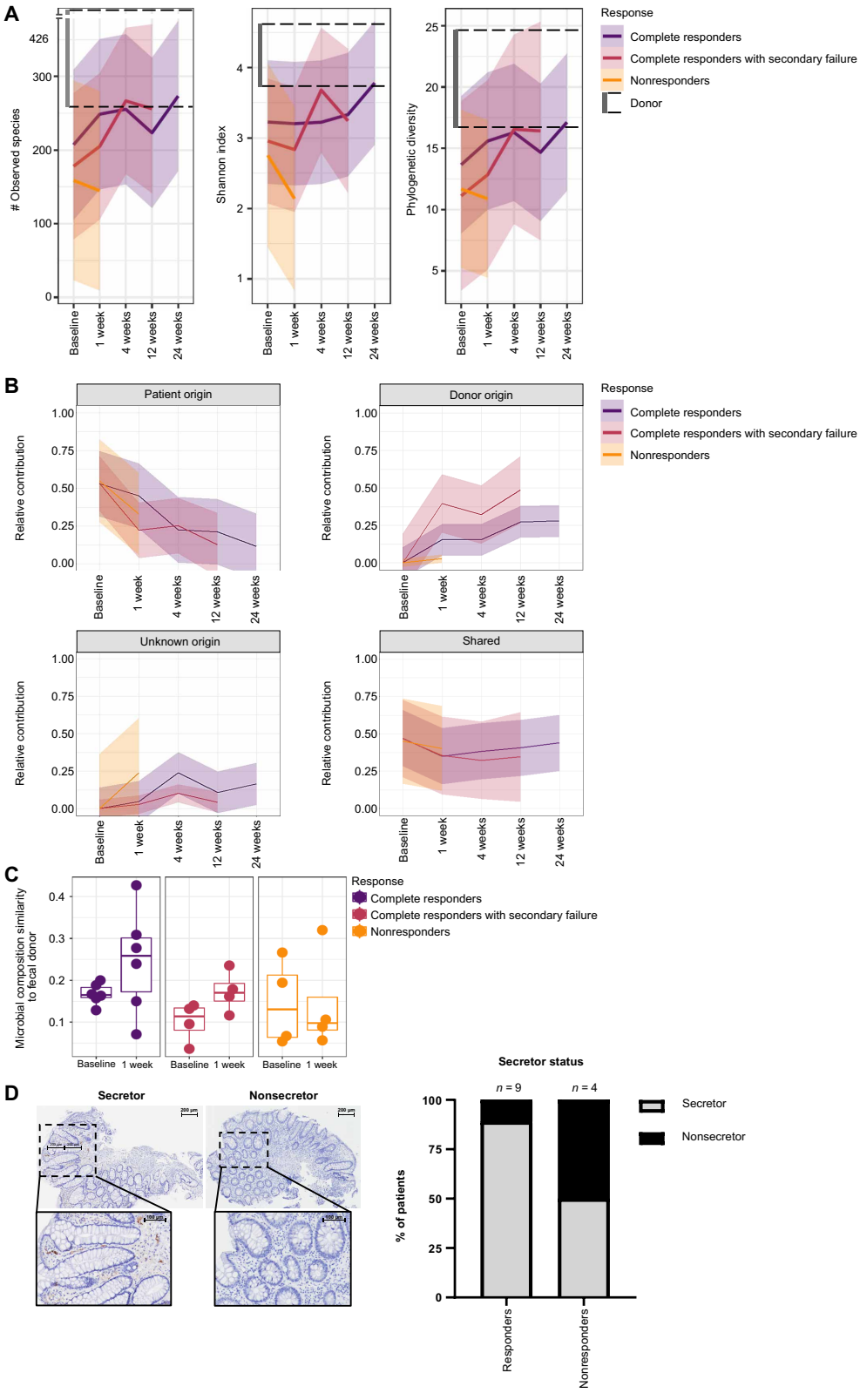

Fig. 5. Microbial α-diversity, microbial engraftment, and FUT2 secretor status. (**A**) Microbial α-diversity of study participant stool samples at baseline and 1, 4, 12, and 24 weeks after donor FMT. Microbial α-diversity is depicted as the number of observed bacterial species (left), the Shannon diversity index (middle), and phylogenetic diversity (right). The gray dashed lines span the range of α-diversity detected in the donor fecal samples showing the highest and lowest microbial α-diversity detected in the donor fecal samples. Colored solid lines depict mean values; shading marks the SDs for microbial α-diversity of study participant stool samples. (**B**) Gut microbial engraftment plots show the relative contribution of bacteria originating from the patient, the fecal donor, shared by both patient and donor, or an unknown source after donor FMT in complete responders ($n = 6$), complete responders with secondary failure ($n = 4$), and nonresponders ($n = 4$). Colored solid lines depict mean values; shading marks SDs. (**C**) Box plots indicate similarity (Jaccard index) of the gut microbial composition of study participants to that of their fecal donor at baseline and at 1 week after donor FMT. (**D**) Representative gut biopsies from study participants who were either a FUT2 secretor or nonsecretor stained for epithelial antigen H expression. Histogram shows aggregate results for the 13 study participants for antigen H expression. Statistical analysis (ANOVA) for (A) to (C) did not reveal significant differences within or between groups.

MATERIALS AND METHODS

Study design

This study was a single-center, single-arm, nonrandomized intervention trial to evaluate the safety and efficacy of a single-donor FMT in allo-HCT recipients (age 18 years and older) with steroid-refractory or steroid-dependent, acute or late-onset acute intestinal GvHD (ISRCTN14530574, Fig. 1). Sample size was not formally calculated because this study was only to determine safety of FMT in this particular group of patients with intestinal GvHD who had no other treatment options. Steroid dependence was defined as repeated recurrence of intestinal GvHD during steroid tapering; steroid-refractory GvHD was defined as intestinal GvHD that did not respond to prednisolone therapy (2 mg/kg per day for acute GvHD), showed no improvement in overall grade of GvHD within 7 days, or showed progression of at least one grade within the first 72 hours after starting high-dose prednisolone treatment. Participants were excluded in the case of an ileus or signs of diminished bowel passage, admission to the intensive care unit or vasopressive dependency at the time of inclusion (critically ill patients), or known food allergy to peanuts, wheat, tree nuts, shellfish, fish, milk, sesame, chickpeas, or eggs. Sigmoid

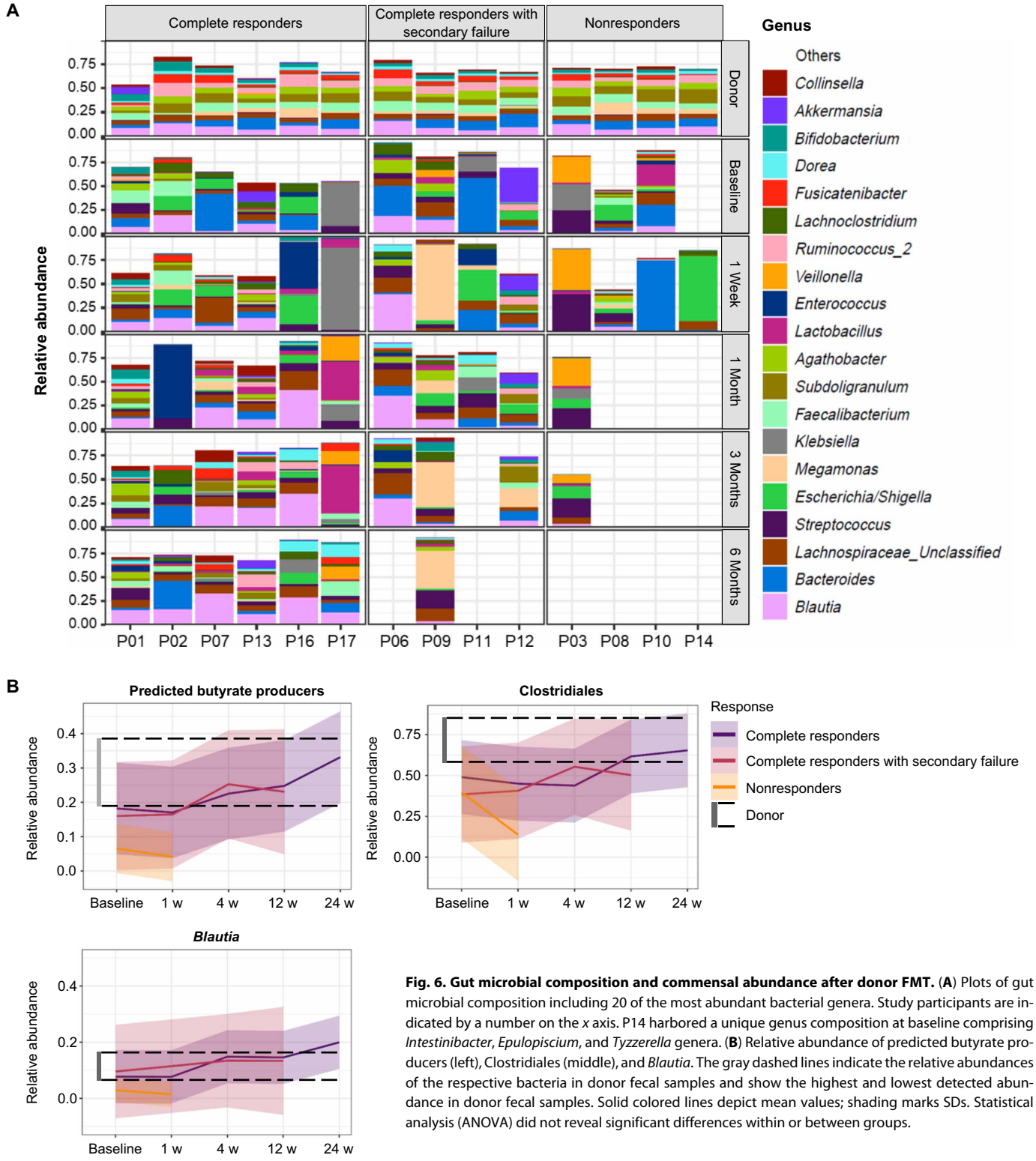

Fig. 6. Gut microbial composition and commensal abundance after donor FMT. (**A**) Plots of gut microbial composition including 20 of the most abundant bacterial genera. Study participants are indicated by a number on the *x* axis. P14 harbored a unique genus composition at baseline comprising *Intestinibacter*, *Epulopiscium*, and *Tyzzerella* genera. (**B**) Relative abundance of predicted butyrate producers (left), Clostridiales (middle), and *Blautia*. The gray dashed lines indicate the relative abundances of the respective bacteria in donor fecal samples and show the highest and lowest detected abundance in donor fecal samples. Solid colored lines depict mean values; shading marks SDs. Statistical analysis (ANOVA) did not reveal significant differences within or between groups.

colon biopsies were taken before donor FMT to confirm intestinal GvHD diagnosis by histological assessment and to exclude other diagnoses as underlying causes of diarrhea. If needed, participants received a platelet transfusion before nasoduodenal tube placement to reach platelet counts above 50×10^9/liter to safely perform tube placement for delivery of the donor FMT. Follow-up lasted for up to 6 months. Seventeen patients received a donor FMT, and two subjects were excluded from follow-up because they received other GvHD medication within 3 days after donor FMT and were therefore not evaluable. Written informed consent was obtained from all

participants. The study was approved by the Institutional Review Board and conducted at the Amsterdam UMC, Academic Medical Center, Amsterdam, The Netherlands, in accordance with the Declaration of Helsinki.

Donor selection and FMT procedure

Healthy volunteers (male/female, <60 years of age) with a Western diet were selected as FMT donors according to internationally accepted criteria (*55*). To prevent transmission of multidrug-resistant organisms, health care workers were excluded from donation. A questionnaire was used to screen donors for risk factors for potentially transmissible diseases, (family) history of bowel or metabolic diseases, and antibiotic use. Subsequent screening of donor fecal samples excluded donors carrying viral pathogens (rota-, adeno-, sapo-, noro-, astro-, entero-, and parechoviruses), parasites (including *Blastocystis hominis* and *Dientmoeba fragilis*), *C. difficile* and enteropathogenic bacteria (including *Campylobacter*, *Salmonella*, *Shigella*, and *Yersinia* species), shiga toxin–producing *E. coli*, extended-spectrum β-lactamase–producing enterobacteriaceae, vancomycin-resistant enterococci, and methicillin-resistant *S. aureus*. In parallel, blood was screened for antibodies against HIV; human T cell lymphotropic virus types 1 and 2; hepatitis A, B, and C; cytomegalovirus; Epstein-Barr virus; *Treponema pallidum*; *Strongyloides stercoralis*; and *Entamoeba histolytica*. In total, two donors were used: donor 1 (female, 31 years) to treat patient P01, and donor 2 (male, 28 years) for the other participants. Patients received a bowel lavage with 1 liter of macrogol solution (Klean-Prep/Moviprep, Norgine) at least 2 hours before FMT according to study protocols in our hospital (*20*). Fresh donor feces (>50 g) was collected on the day of transplantation, diluted with sterile saline (0.9%), stirred, and filtered in a laminar flow cabinet under normoxic conditions to obtain a fecal suspension of 300 to 500 ml. This solution was slowly administered (10 cm^3/min) via a nasoduodenal tube within 6 hours after feces collection.

Clinical response and safety assessment

The primary efficacy end point was complete response to treatment, defined as complete resolution of GvHD symptoms on day 28 after donor FMT. Improvement of GvHD by at least one grade defined a partial response. If there was no improvement of clinical grade GvHD (stable disease) or progression with at least one grade 1 month after fecal transplantation, participants were classified as nonresponders. GvHD grading was performed according to internationally accepted criteria (*56*, *57*). Patients were followed up for 6 months, and in case of a complete response, tapering of immunosuppressive medication was initiated at day 28. Complete responders were subcategorized as complete responders with secondary failure in case of reoccurrence of symptoms upon taper of immunosuppressants. Safety of the procedure was assessed by recording all (serious) adverse events reported by the subject or observed by the investigator in the first 4 weeks after FMT.

Fecal microbiota composition

Fecal samples were collected before donor FMT (baseline) from donor and patient and, subsequently, from all patients at 1, 4, 12, and 24 weeks after donor FMT and stored at −20°C until further analysis. DNA was extracted and purified from nonthawed feces samples using a repeated bead-beating protocol in combination with Maxwell RSC Whole Blood DNA Kit (method 5) (*58*). 16*S* rRNA gene amplicons were generated using a single-step polymerase chain reaction (PCR) protocol targeting the V3-V4 region (*59*). PCR products were purified using Ampure XP beads, and purified products were equimolar pooled. The libraries were sequenced using a MiSeq platform using V3 chemistry with 2 × 251 cycles. Forward and reverse reads were length trimmed at 240 and 210, respectively, and amplicon sequencing variants were inferred and merged using DADA2 V1.5.2 (*60*). Taxonomy was assigned using DADA2 implementation of the Ribosomal Database Project classifier (*61*) and SILVA (*62*) 16*S* ribosomal database V128. Microbiota data were further analyzed and visualized using phyloseq (*63*). Predicted butyrate–producing genera were aggregated according to those listed by Haak *et al.* (*13*).

Serum collection and ST2 and REG3α measurements

Heparinized blood samples were collected from all patients except patients P05 and P11. Serum was obtained by centrifugation of whole blood and stored at −80°C until further analysis. ST2 and REG3α serum concentrations were measured by enzyme-linked immunosorbent assay (ELISA) from R&D Systems (Human ST2/IL-33R Quantikine kit) and MBL International [Ab-Match ASSEMBLY Human PAP1 (REG3α) kit], respectively. ELISAs were performed according to the manufacturer's instructions. Standards and samples were run in duplicate; patient samples were diluted 1:20 for ST2 and 1:10 for REG3α ELISA. Absorbance was measured with a VersaMax microplate reader (Molecular Devices).

FUT2 secretor status assessment

Formalin-fixed paraffin-embedded gut biopsies from 13 patients (P05 and P11 not included) were obtained and prepared for histological analysis following institutional protocols. Tissue sections (3 μm) were heated at 60°C for 20 min, followed by heat-induced antigen retrieval in PT link (Dako, Glostrup, Denmark) with EnVision FLEX Target Retrieval Solution, Low pH (Dako). Single-staining immunohistochemistry for blood group H antigen was performed using an EnVision Peroxidase/DAB Detection System kit (Rabbit/Mouse K5007, Dako, Glostrup, Denmark) and using an automated immunohistochemistry robot (Autostainer, Dako). Endogenous peroxidase activity was inhibited by EnVision FLEX Peroxidase-Blocking Reagent, and serum-free protein block (Dako) was applied to prevent nonspecific background staining, before staining with mouse anti-human blood group H antigen (Thermo Fisher Scientific, clone 97-I, diluted 1:250) for 1 hour. A horseradish peroxidase–conjugated anti-mouse secondary antibody (Dako), followed by the nonpermeable chromogen 3,3′-diaminobenzidine (DAB), was used to detect antigen H staining. Sections were counterstained with Mayer's hematoxylin to visualize background. High-resolution digital images of the sections were generated through an automated digital slide-scanning robot (ScanScope CS, Aperio).

Stained sections were scored in a blinded and independent manner by three scientists with experience in immunohistochemistry. Biopsies were labeled positive in case of any epithelial antigen H staining, whereas no staining was visible in negative biopsies. In one case, scores were not unanimous and it was decided to categorize this biopsy as scored by most of the evaluators.

Statistical analysis

Data are represented as individual values with mean ± SEM or SD. Statistical significance was determined with Mann-Whitney *U* test, log-rank (Mantel-Cox) test, or analysis of variance (ANOVA) using GraphPad Prism software and R.

SUPPLEMENTARY MATERIALS

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Fig. S1. Steroid dosing for complete responders over time.
Table S1. Individual characteristics of study participants.
Table S2. Adverse events.
Table S3. Individual data of α-diversity metrics.
Table S4. Individual engraftment data of fecal bacterial species.
Table S5. Individual data of the microbial composition similarity to the fecal donor.
Table S6. Individual data of the relative abundance of predicted butyrate producers, Clostridiales, and *Blautia*.

View/request a protocol for this paper from *Bio-protocol*.

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Donor fecal microbiota transplantation ameliorates intestinal graft-versus-host disease in allogeneic hematopoietic cell transplant recipients

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FMT fires up treatment of GvHD

Allogeneic hematopoietic cell transplantation (HCT) is a beneficial treatment for hematological malignancies. However, HCT can lead to graft-versus-host disease (GvHD), which affects various organs including the gut. Fecal microbial transplantation (FMT) from allogeneic donors has successfully treated intestinal disorders such as *Clostridium difficile* infection and ulcerative colitis. van Lier *et al.* conducted a single-arm clinical trial to investigate whether allogeneic FMT could ameliorate symptoms of intestinal GvHD in 15 HCT recipients. Within a month of treatment, intestinal GvHD resolved and gut microbial diversity was restored in 10 of 15 study participants. Although confirmation is required in larger trials, allogeneic FMT may be a promising treatment for intestinal GvHD.