Repeated fecal microbiota transplantations attenuate diarrhea and lead to sustained changes in the fecal microbiota in acute, refractory gastrointestinal graft-*versus*-host-disease

Acute graft-*versus*-host disease (aGvHD) is a serious complication of allogeneic hematopoietic stem cell transplantation (allo-HSCT).[1] Although aGvHD of any target organ represents morbidity, lower gastrointestinal (GI) tract involvement is complicated by high mortality.[2] Here, refractoriness to anti-GvHD therapies (e.g., nonresponse to corticosteroids after 5–7 days) is associated with one-year survival rates of less than 30%.[3,4] So far, there has been no satisfactory improvement in patient survival with refractory GvHD.[3,5]

Loss of intestinal bacterial diversity in GvHD, both in murine models as well as in patients after allo-HSCT, was reported by Jenq and colleagues.[6] Additionally, a relative shift toward *Enterococci* was recently observed in GI-aGvHD after allo-HSCT, with the degree of this Enterococci-dominant dysbiosis correlating to the severity of GI-aGvHD.[7] Recent data suggest that alterations of microbial metabolites have direct salutary effects on GvHD target tissues.[7,8] However, a causal relationship between microbial alterations and GvHD development or progression is still unproven.

Fecal microbiota transplantation (FMT) aims at restoration of a physiological microbiota by transferring normal fecal microorganisms to a patient's GI tract.[9] FMT is highly effective in *Clostridium difficile* infection (CDI), which is mainly mediated by the loss of the microbial colonization resistance to toxin-producing *Clostridium difficile* strains.[10]

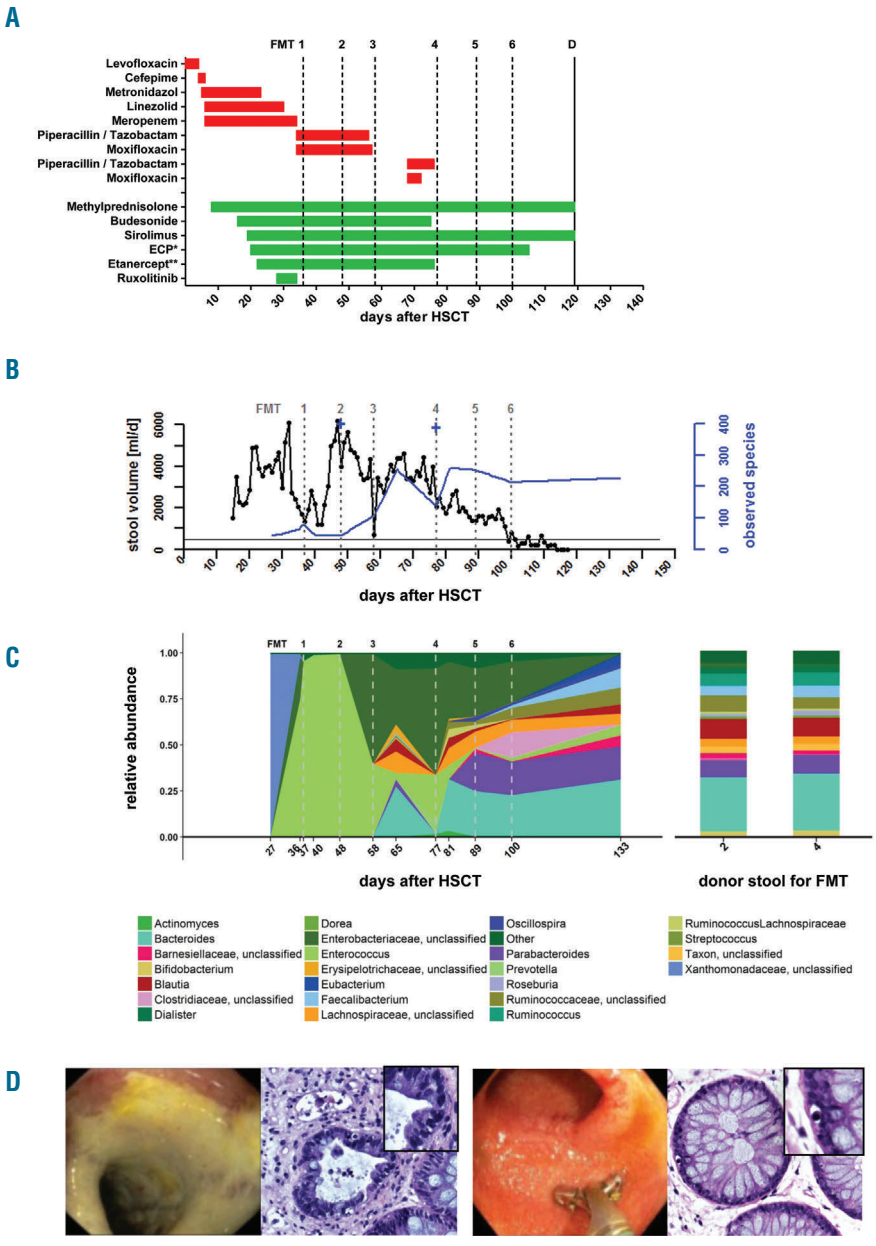

Figure 1. Characteristics of patient 1. A. Antibiotics (red) and immunosuppressants (green) given to patient 1 before, during and after the introduction of FMTs. ECP, extracorporeal photopheresis. In total, 15 ECP sessions (*) were applied and 13 doses (**) of etanercept 25 mg were given. Methylprednisolone was gradually reduced after FMT 3 (d+58) from 2 mg/kg/d to 1 mg/kg/d at d+111 and maintained beyond discharge. B. Longitudinal illustration of stool volumes [ml/day, black lines, left Y-axis] and richness [observed species, blue lines, right Y-axis] of patient 1 before, during and after six FMTs (grey dashed vertical lines). The horizontal black line represents 500ml of stool volume. Blue crosses represent the bacterial richness (number of observed species) in donor stools at FMT 2 and 4. Stool volumes peaked with 6200 ml at d+47 and substantially decreased to normal values after commencement of FMTs. After FMT 6, diarrhea subsided. C. Fecal microbiota analysis (16S rRNA gene analysis) of patient 1 before, during and after six FMTs (grey dashed vertical lines). Colors represent different taxa on the genus level according to their relative abundance (Taxonomic groups with an abundance less than 2% of the overall abundance are summarized as "Other"). Stool specimens were obtained either before/between FMTs or by aspiration during the colonoscopy before the FMT. Following the first three FMTs, there was no persistent colonization of the introduced stool microbiota (transient colonization after FMT 3). After FMT 4, colonization by donor stool is evident. D. Left panel: Patient 1 before and during FMTs. Endoscopy reveals severe ulcerative ileitis without evidence of any normal ileal mucosa. Histologically severe graft-versus-host disease was confirmed with partial loss of crypts with multiple apoptoses. At the top a completely destroyed crypt (20x magnification). Insert: higher magnification of apoptosis (40x). Right panel: Patient 1 at 37 days after FMT 6. Macroscopically regenerated ileal mucosa with a healed ulcer (center). Histologically mild graft-*versus*-host disease with a crypt with a single apoptosis on the left (9 o'clock) (20x magnification). Insert: higher magnification of apoptosis (40x).

Table 1. Clinical characteristics of three patients treated by FMT for refractory acute intestinal GvHD.

	Patient 1	Patient 2	Patient 3
Gender, age in years, ethnicity	male, 53, caucasian	female, 60, caucasian	female, 61, caucasian
Date of allo-HSCT	April 2015	January 2016	May 2014
Hematologic disease	MDS, RAEB-2, IPSS Intermediate-II	Secondary AML evolving from MDS	AML
Donor gender, relationship	female URD (HLA 9/10, HLA-DQB1 mismatch)	female matched URD (HLA 12/12)	male MSD (HLA 10/10)
Stem cell source	PBSC	PBSC	PBSC
Number of $CD34^+$ $x10^6$ content per kg recipient body weight	9.0	5.3	3.0
GvHD prophylaxis*	CsA, MMF	CsA, MMF	CsA, MMF
Onset of aGvHD (days after allo-HSCT; organ, stage)	+7; skin, 1	+10; skin, 2	+19; upper GI, 1
Onset of lower GI aGvHD (days after allo-HSCT)	+12	+11	+22
Diarrhea peak (days after allo-HSCT; volume)	+47; 6200 ml	+48; 3000 ml	+31; 1700 ml
aGvHD therapy prior to FMT (duration in days)			
Methylprednisolone	29	49	89
Budesonide	21	48	84
ECP	3 courses	7 courses	13 courses
Etanercept	5 doses	11 doses	12 doses
Ruxolitinib	7	–	–
Sirolimus	18	–	–
GvHD therapy at time of first FMT	Budesonide, ECP, Etanercept, Methylprednisolone, Sirolimus	CsA, Methylprednisolone	Budesonide, CsA, ECP, Methylprednisolone, MMF
Overall aGvHD grade[#] at time of first FMT	IV	IV	IV
Skin (Stage)	0-1	0	0
Liver (Stage)	0	0	0
Lower GI (Stage)	4	2	4
GI aGvHD at time of first FMT Endoscopic results	Ileum: Fibrinous ileitis (ileum intubated from FMT 2 onwards), Colon: hemorrhagic colitis, fibrinous ulcers	Ileum: Erosive Ileitis, Colon: right sided hemorrhagic colitis without erosions or ulcers	Ileum: Ulcerous ileitis, Colon: loss of normal vascular pattern, no erosions or ulcers.
Histologic grade according to Lerner	II	II	I
Number of FMTs	6	2	1
FMT donor relationship	unrelated donor	daughter	brother
Timepoint of first/last FMT (days after allo-HSCT)	+37 / +100	+61 / +76	+110 / n/a
GI GvHD outcome			
Clinical	Complete resolution of GI-GvHD, discharge on day +119	Complete resolution of GI-GvHD, discharge on day +95	GI-GvHD stage I
Endoscopic	Ileum: healed ulcer surrounded by normal mucosa, Colon: macroscopically normal	n/a	n/a
Histological (Lerner grade)	II	n/a	n/a
Infectious events after first FMT (days after allo-HSCT; pathogen)	~Bacteremia (+68; *E. coli*) ~UTI (+78; *E. coli*) ~UTI (+89; *E. coli*) ~CRBSI (+134; *E. coli*) ~Neutropenic sepsis (+176; *E. coli* and *K. pneumoniae subsp. pneumoniae*)	~ UTI (+80; *Pseudomonas sp.* and *K. pneumoniae subsp. pneumoniae*) ~Acute purulent pansinusitis with secondary meningitis (+129; *Candida sp*)	~UTI (+116; *Enterococcus*)
Overall outcome	Patient died from septicemia and multiorgan failure on day +177	Patient died from cerebral infarction on day +148	Patient died from cardiorespiratory failure on the basis of an organising pneumonia with alveolar damage on day +119

aGvHD: acute graft-*versus*-host disease; allo-HSCT: allogeneic hematopoietic stem cell transplantation; AML: acute myeloid leukemia; CRBSI: catheter-related bloodstream infection; CsA: cyclosporine A; *E. coli*: *Escherichia coli*; ECP: extracorporal photopheresis; FMT: fecal microbiota transplantation; GI: gastrointestinal; HLA: human leukocyte antigen (-A, -B, -C, -DRB1, -DQB1, -DPB1); IPSS: International Prognostic Scoring System; MMF: mycophenolate mofetil; MDS: myelodysplastic syndrome; MSD: matched sibling donor; n\a: not applicable; PBSC: peripheral blood stem cells; RAEB-2, refractory anemia with excess blasts-2; URD: unrelated donor; UTI: urinary tract infection; # Przepiorka D, Weisdorf D, Martin P, *et al.* 1994 Consensus Conference on Acute GvHD Grading. Bone Marrow Transplant 1995; 15:825; * none of the patients received antithymocyte globulin.

The few reports on its successful use in immunocompromised patients with CDI after allo-HSCT or solid organ transplantation as well as inflammatory bowel disease have described no relevant infectious complications.[11,12]

In view of the poor prognosis of patients with refractory GI-aGvHD and the limited therapeutic options, FMT might be a beneficial treatment modality in this challenging clinical situation.

We report three consecutive patients with severe cases of refractory GI-aGvHD following allo-HSCT that were associated with a highly dysbiotic stool microbiota. Repeated FMTs were associated with sustained improvement of GI-aGvHD.

The FMT procedures were performed on a compassionate use basis after individual (a priori) permission of the hospital board of the LKH Universitätsklinikum Graz and after obtaining informed consent. Publication was approved by the local institutional review board.

Donors (different persons for each patient) were healthy adult subjects. For details of donor screening and stool preparation please refer to the *Online Supplementary Methods* section. Homogenized stool solution (~250 ml) was instilled into the terminal ileum and caecum via the colonoscope. In patients 1+2 donors were maintained for repeated FMTs.

For details on the bacterial sequencing procedures, as well as for statistical analyses, please refer to the *Online Supplementary Appendix*.

The detailed patient data are compiled in Table 1. All patients received an allo-HSCT at our center for myelodysplastic syndrome or acute myeloid leukemia following a reduced intensity conditioning with fludarabin and melphalan. Onset of lower GI-aGvHD occurred on days +11 to +22 after allo-HSCT, and was confirmed histologically. Macroscopically in all patients, the right-sided colon and the terminal ileum turned out to be the main site of inflammation (Table 1, pictures of patient 1 in Figure 1D). Due to lack of therapeutic response, not only to corticosteroids (methylprednisolone, 2 mg/kg body weight) but also to other immunosuppressive strategies (Table 1, Figures 1A, S1A, S2A), with worsening of diarrhea, persistence or increase of inflammation and ulcerations in re-endoscopies and histological exams, FMT was performed as an experimental therapy.

Microbiota analysis by 16S rRNA gene sequencing before FMTs revealed a severely depleted microbiota in all patients, characterized by reduced microbial richness (i.e., number of observed species; patient 1: Figure 1B, C and patients 2 and 3: *Online Supplementary Figures S1B, C* and *S2B, C*). FMTs were performed one to six times between 37 to 110 days after allo-HSCT and after 4 to 6 lines of immunosuppressive treatments.

In patient 1, there was no significant or persistent improvement in bacterial richness by 16S rRNA gene analysis or diarrheal volumes after a single FMT (Figure 1B). Consequently, FMT was repeated at intervals of two to three weeks (cumulatively six FMTs). Antibiotics and budesonide were temporarily stopped prior to the fourth FMT (Figure 1A). Finally, clinical and endoscopic improvement was observed with stool volumes decreasing from initially > 6000 ml to 200-300 ml/day. In parallel, the donor microbiota engrafted in the patient's colon was associated with a substantial increase in microbial richness (Figure 1B and 1C). Total parenteral nutrition (TPN) was stopped at d+104, and anti-motility agents could be stopped at d+108. Complete remission from GI-aGvHD was reached on d+110. The patient could be discharged on d+119 without clinical signs of acute or chronic GI-GvHD. Immunosuppression with sirolimus was continued, and methylprednisolone was reduced to 0.7 mg/kg at d+168. The patient was re-hospitalized on d+176 due to community-acquired sepsis and died on d+177 despite intensive care measures. The observed duration of GI-GvHD remission in patient 1, therefore, was 9.6 weeks.

In patient 2, broad spectrum antibiotics and all immunosuppressants except methylprednisolone, cyclosporin A and mycophenolate mofetil, were discontinued before the first FMT performed on d+61. Four days after FMT 1, diarrhea disappeared completely but recommenced shortly thereafter, so a second FMT was conducted on d+76. After the first FMT, richness significantly improved to normal values (richness before FMT1: 82.1 ± 3.3; d+76, ahead of FMT2: 487.9 ± 9.8; adjusted $P<0.0001$; paired *t*-test) accompanied by complete resolution of symptoms after FMT 2 (*Online Supplementary Figure S1*). Total parenteral nutrition (TPN) was stopped at d+82, and anti-motility agents could be stopped at d+80. Methylprednisolone was reduced to 0.1mg/kg at d+130. The patient reached a complete remission from GI-aGvHD on d+90 and was free of complaints until her death from cerebral infarction on d+148. Thus, the observed duration of GI-GvHD remission was 8.3 weeks in patient 2.

In patient 3, diarrhea diminished transiently after FMT, and 16S rRNA gene sequencing on the day after FMT revealed a reconstituted microbiome (*Online Supplementary Figure S2*). However, diarrhea recommenced, accompanied by a decrease in bacterial richness. Patient 3 did not receive anti-motility agents, and TPN was maintained after FMT. Thus, GI-aGvHD remission was not reached.

This, together with a recent series from Kakihana *et al.*,[13] is a proof of concept for the treatment of multi-line refractory GI-aGvHD by FMT. Based on the finding of severe dysbiosis in aGvHD - signified by a loss of bacterial richness - and the potential of FMT to ameliorate dysbiosis, we performed FMT in three patients with grade IV aGvHD involving the lower GI tract after failure of established immunosuppressive therapies.

The likelihood of a steroid response to aGvHD depends on the primary organ involvement and the severity of GvHD manifestations. Severe involvement of the gastrointestinal tract has proven to be particularly difficult to treat, with a fatality rate of 70-100% in steroid-refractory cases.[3,4]

All three patients responded clinically to FMT with reduced stool volumes that normalized with repeated interventions. Endoscopic improvement could also be assessed in one patient. This coincided with the restoration of a significantly more diverse microbiome after one to six FMTs. A possible causative relationship of FMT in the reversal of severe intestinal dysbiosis and subsequent resolution of GI-aGvHD can therefore be hypothesized. It is notable that bacterial response to FMTs was transient and microbiome engraftment was only observed after repeated interventions. Sustained clinical improvement was seen despite reduction of concomitant immunosuppressive medications. However, due to the small patient number and the natural course of this high risk population with a tremendously high mortality, it is impossible to judge the medium and long-term effectiveness of this intervention as well as a possible effect on mortality reduction.

In these severely immunocompromised patients following allo-HSCT, concerns about infectious organisms transmitted through this procedure need to be addressed. Importantly, we did not observe any immediate proce-

dure-related infections despite the temporary withdrawal of prophylactic antibiotics to protect the transplanted microbiome. Temporal withdrawal of broad spectrum antibiotics might be a prerequisite of successful FMT since some antibiotics are associated with perturbation of gut microbial composition and increased GvHD-related mortality.[14] Several infectious events occurred during the follow-up of the patients (Table 1). On the basis of our data, we cannot firmly exclude that infections with intestinal pathogens were induced by FMT. Due to the long-term immunosuppressive therapy, such infections are common in the natural course of patients with HSCT and (refractory) aGvHD, and we did not note an excess of infections after FMTs. Infections are a major complication in patients undergoing HSCT with a cumulative incidence rate of ~80% at day 180, as recently described in a randomized phase III trial.[15] The occurrence of GvHD was significantly associated with even higher rates of bacterial and fungal infections during GvHD therapy.[4,15,16] No bacteriemias or systemic infections occurred immediately after FMTs. However, we observed one bacteraemia ten days after FMT, and the death of one patient nine days after FMT with respiratory failure without bacteraemia. Since we would expect infections causally linked to FMT earlier, we do not attribute these events to FMT. Patient 2 died of septicemia, but since this infectious complication occurred 77 days after the last FMT, we do not assume it to be FMT-related. Rather, we believe that early initiation of FMT therapy could possibly spare unnecessary immunosuppression and related subsequent infectious complications.

In conclusion, we show that altering the intestinal microbiota by FMT is an attractive novel treatment approach for patients with refractory GI-aGvHD. Well-designed prospective studies are warranted to confirm these promising results in order to overcome the unmet clinical need of effective therapies for refractory GI-aGvHD and, thus, substantially improve the prognosis of these patients.

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