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Fecal microbiota transfer for refractory intestinal graft-versus-host disease — Experience from two German tertiary centers

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Abstract

Rationale: Steroid refractory graft-vs-host disease (sr-GvHD) represents a challenging complication after allogeneic hematopoietic cell transplantation (allo-HCT). Intestinal microbiota (IM) diversity and dysbiosis were identified as influencing factors for the development of acute GvHD. Fecal microbiota transfer (FMT) is hypothesized to restore IM dysbiosis, but there is limited knowledge about the significance of FMT in the treatment of sr-GvHD.

Objectives: We studied the effects of FMT on sr-GvHD in allo-HCT patients from two German tertiary clinical centers (n = 11 patients; period: March 2017 until July 2019). To assess safety and clinical efficacy, we analyzed clinical data pre- and post-FMT (day -14 to +30 relative to FMT). Moreover, IM were analyzed in donor samples and in a subset of patients pre- and post-FMT by 16S rRNA sequencing.

Results: Post-FMT, we observed no intervention-associated, systemic inflammatory responses and only minor side effects (5/11 patients: abdominal pain and transformation of peristalsis—each 3/11 and vomiting—1/11). Stool frequencies and volumes were significantly reduced [pre- vs post-FMT (d14): $P < .05$, respectively] as well as clear attenuation regarding both grading and staging of sr-GvHD was present upon FMT. Moreover, IM analyses revealed an increase of alpha diversity as well as a compositional shifts toward the donor post-FMT.

Conclusions: In our study, we observed positive effects on sr-GVHD after FMT without the occurrence of major adverse events. Although these findings are in line with

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Novelty Statement:

- Intestinal microbiota (IM) dysbiosis represents an important modulator of graft-versus-host disease (GvHD) and fecal microbiota transfer (FMT) is hypothesized to restore IM dysbiosis.
- Based on limited evidence about the FMT as rescue strategy in refractory GvHD, we examined one of the largest cohorts so far undergoing FMT and observed attenuation of GvHD as well as IM shifts upon FMT without the occurrence of major adverse events.
- Our results nicely reflect limited published data, but further randomized clinical studies are urgently needed to better define the clinical validity including mode of action.

published data on beneficial effects of FMT in sr-GvHD, further randomized clinical studies are urgently needed to better define the clinical validity including mode of action.

KEYWORDS

fecal microbiota transfer, graft-vs-host disease, human allogeneic hematopoietic cell transplantation, human intestinal microbiota

1 | INTRODUCTION

Acute graft-versus-host disease (GvHD), especially steroid-refractory (sr-)GvHD, is a potentially life-threatening complication after allogeneic hematopoietic cell transplantation (allo-HCT)[1,2] limiting its therapeutic success and prognosis.[3-5] Despite immense efforts, drivers of GvHD have not been completely understood and the overall incidence is still high, despite the introduction of novel treatment modalities (eg, Janus kinase, JAK inhibitors).[6] GvHD originates from activated, donor-derived T cells attacking host tissue; especially at body sites densely colonized by microorganisms such as the gastrointestinal tract (GIT).[7] Due to effects on immune regulation and mucosal homeostasis,[8-10] the intestinal microbiota (IM) have received considerable attention in the context of GvHD.[11-13] This microbial ecosystem is very susceptible to perturbations through diet or antibiotics[14] and represents an important modulator in the context of immunosuppression.[15-17] In fact, allo-HCT patients require intensive immunosuppressive and often also anti-infective therapies,[18,19] which predispose to dysbiotic shifts within the IM.[13,20] In line, diversity of the IM around the time of allo-HCT was linked to overall survival[21-23] as well as the development of acute GvHD.[20,24-26]

Fecal microbiota transfer (FMT) is considered to modulate and partially restore IM dysbiosis.[27] Today, FMT is already accepted as standard therapy for refractory and recurrent *Clostridioides difficile* infection (CDI)[28] resulting from diminished host colonization resistance of an injured host IM[29] and is currently investigated in many other disease areas associated with IM dysbiosis.[30] However, the mechanisms underlying the treatment benefits in different FMT indications remain elusive, and there are concerns about the peri-interventional and long-term safety,[31,32] especially in the context of a significant degree of immunosuppression.

Knowledge about efficacy and safety of FMT for the treatment of sr-GvHD is sparse.[33-39] In the case series presented here, we analyzed patients undergoing FMT as rescue therapy due to sr-GvHD of the GIT at two German tertiary clinical centers integrating our data in the limited body of evidence about application of FMT in GvHD.

2 | MATERIAL/METHODS

2.1 | Patients

We analyzed patients from two German university hospitals (Department of Internal Medicine III, Bonn and Department I of Internal Medicine, Cologne) that underwent FMT due to sr-GvHD of the GIT between March 2017 and July 2019. The diagnosis of sr-GvHD finally leading to FMT was made according to current guidelines.[40] FMT was performed within the context of an individualized treatment ("individueller Heilversuch") according to the German Medicines Act (*Arzneimittelgesetz, AMG*). FMT procedures and donor screening were performed as previously published.[41,42] A subset of patients from the Cologne cohort (K_001, K_003, K_005, K_006, K_008, K_009) contributed fecal samples for 16S rRNA sequencing after providing informed consent (ISI biobanking vote, No. 08-160). Clinical data for this study were collected retrospectively by chart review. The observational period for each patient was between day -14 before and day 30 after FMT and was divided into three main sub-periods for subsequent analyses: pre-FMT (day -14 to day -1), day of FMT (day 0), and post-FMT (day 1 to day 30)—with additional timepoints (i) at day 14 [post-FMT (d14)] due to a lack of consistent data within the whole post-FMT sub-period (d1-d30) and (ii) between day one and seven [post-FMT (d7)] for correlation analysis with IM alpha diversity. Final information regarding grading (overall) and staging (GIT-based) of acute GvHD (a-GvHD) was assessed as "best response" post-FMT based on available data. In case of missing data regarding output of fecal volumes, respective data were interpolated as following: stool frequency × 200 mL/defecation event [mL/d].

2.2 | FMT procedures

Handling of fecal material for and the specific procedure of FMT differed between the study sites.

Fecal material from donors was collected and processed as followed:

1. instant freezing (<3 hours after defecation) at −80°C up to the day of FMT with final careful thawing at room temperature (Bonn, n = 1) or fresh collection (less than 3 hours after defecation; Bonn, n = 1) of single related donors, respectively, and
2. as cryoconserved capsules manufactured as previously published[43] containing fecal material (all Cologne patients; single unrelated donor—n = 8, multiple unrelated donors—n = 1).

Fresh or thawed fecal material (less than 3 hours after removing from the freezer) was further processed for infusion (Bonn, n = 2) as previously published.[42,44]

TABLE 1 Patients' characteristics, FMT-related information, and adverse events (AE) as well as information about anti-infectives and immunosuppression for all patients as well as differentiated for patients of the Bonn and the Cologne cohort

			All	Bonn cohort	Cologne cohort
Patients	*- no.*		11	2	9
Female	*- no. (% of sub-group)*		2 (18.18)	0 (0)	2 (22.22)
Age	*- [years]; mean (range; SD)*		53.82 (30-76; 13.66)	51.5 (45-58; 9.19)	54.33 (30-76; 14.87)
Diagnosis leading to allo-HCT	*- no. (% of sub-group)*				
		AML (incl. Sec-AML)	6 (54.55)	2 (100)	4 (44.44)
		MDS	3 (27.27)	0 (0)	3 (33.33)
		T-PLL	1 (9.09)	0 (0)	1 (11.11)
		Thalassemia	1 (9.09)	0 (0)	1 (11.11)
Timepoint of FMT	*- interval since allo-HCT [d] - mean (range; SD)*		168.5 (44-642, 179.3)	120 (92-148, 39.6)	179.2 (44-642, 198.2)
Way of FMT application	*- no. (% of sub-group)*				
		Upper GIT	11 (100)	2 (100)	9 (100)
		- per oral (capsules)	9 (81.82)	0 (0)	9 (100)
		- naso-jejunal tube	2 (18.18)	2 (100)	0 (0)
FMT-related adverse events					
	Nausea	*FMT (d0) to d+7*	0	0	0
	Regurgitation	*- " -*	0	0	0
	Abdominal pain	*- " -*	3 (27.27)	1 (50)	2 (22.22)
	Vomiting	*- " -*	1 (9.09)	1 (50)	0
	Food intolerance	*- " -*	0	0	0
	Aspiration	*- " -*	0	0	0
	Aspiration pneumonia	*- " -*	0	0	0
	Changes in peristalsis	*- " -*	3 (27.27)	0	3 (33.33)
	Severe AE	*- " -*	0	0	0
	=> Overall (any AE)	*- " -*	5 (45.54)	1 (50)	4 (44.44)
Anti-infectives*					
	Antibiotics - no. (% of sub-group)				
		pre-FMT, d-14 to d-1	8 (72.73)	2 (100)	6 (66.67)
		FMT (d0)	0 (0)	0 (0)	0 (0)
		post-FMT, d+1 to d+30	7 (63.64)	2 (100)	5 (55.56)

(Continues)

TABLE 1 (Continued)

		All	Bonn cohort	Cologne cohort
	overall (d-14 to d+30)	10 (90.91)	2 (100)	8 (88.89)
Antivirals - no. (% of sub-group)				
	pre-FMT, d-14 to d-1	11 (100)	2 (100)	9 (100)
	FMT (d0)	9 (81.82)	0 (0)	9 (100)
	post-FMT, d+1 to d+30	11 (100)	2 (100)	9 (100)
	overall (d-14 to d+30)	11 (100)	2 (100)	9 (100)
Antimycotics - no. (% of sub-group)				
	pre-FMT, d-14 to d-1	11 (100)	2 (100)	9 (100)
	FMT (d0)	9 (81.82)	0 (0)	9 (100)
	post-FMT, d+1 to d+30	11 (100)	2 (100)	9 (100)
	overall (d-14 to d+30)	11 (100)	2 (100)	9 (100)
Immunosuppression*				
Ruxolitinib - no. (% of sub-group)				
	pre-FMT, d-14 to d-1	7 (63.64)	2 (100)	5 (55.56)
	FMT (d0)	6 (54.55)	1 (50)	5 (55.56)
	post-FMT, d+1 to d+30	7 (63.64)	2 (100)	5 (55.56)
	overall (d-14 to d+30)	7 (63.64)	2 (100)	5 (55.56)
Others - no. (% of sub-group)				
	pre-FMT, d-14 to d-1	11 (100)	2 (100)	9 (100)
	FMT (d0)	11 (100)	2 (100)	9 (100)
	post-FMT, d+1 to d+30	11 (100)	2 (100)	9 (100)
	overall (d-14 to d+30)	11 (100)	2 (100)	9 (100)

Note: Information about FMT-related AEs was recorded within the first 7 days after FMT (d0-d7). Regarding specific information about the use of anti-infectives and immunosuppression, data are given for the three predefined periods: pre-FMT (d-14 to d-1), day of FMT (d0), and post-FMT (d+1 to d+30) as well as for the overall investigation period (d-14 to d+30). Where applicable, numbers are given as means with range and standard deviation.

Abbreviations: AE, Adverse event; AML, Acute myeloic leukemia; d, Day; FMT, Fecal microbiota transfer; GIT, Gastrointestinal tract; MDS, Myelodysplastic syndrome; no., Number; SD, Standard deviation.; sec, Secondary; T-PLL, T-cell prolymphocytic leukemia.

*Detailed information about antibiotic and immunosuppressive therapy for each patient of the cohort is given in Table S1.

FMT procedures were conducted as followed:

1. infusion of prepared whole fecal solutions through a self-advancing naso-jejunal tube (Tiger 2™, Cook Medical, Bloomington, Indiana, USA) placed within the proximal jejunum (all Bonn patients) after bowel preparation and
2. cryoconserved capsules (n = 30 to n = 50 per FMT application; single application—n = 8, sequential application with a second cycle at d8—n = 1) were swallowed (period of ingestion: 2-3 days; all Cologne patients).

Regarding FMT by capsules, the first day of ingestion was defined as day of FMT (in case of consecutive FMT cycles—n = 1: the first day of the first cycle represents the day of FMT). Assessment of FMT-related adverse events (AE) was based on data within the first seven days after FMT (d0-d7).

If data from predetermined timepoints were not available, data of ±1 day were collected, respectively.

Safety of and clinical response to FMT were assessed as end points of this exploratory study. Clinical records were screened for any kind of AE, and clinical response was estimated through analysis of stool frequency (defined as defecation events per 24 hours). Additionally, we investigated LDH and bilirubin levels, markers of systemic inflammation (C-reactive protein—CRP, leucocyte, and neutrophile counts), and vital parameters (heart frequency, systolic blood pressure, and body temperature), as well as other routine clinical laboratory parameters (creatinine, alanine transaminase—ALT, aspartate transaminase—AST, serum protein, platelet counts, and hemoglobin).

2.3 | Microbiota analyses

From a subset of n = 6 individuals, patient feces and respective FMT donor material were collected at day 0 (pre-FMT) and day 7 (post-FMT) and stored at −80°C until DNA-extraction.

DNA was extracted using the FastDNA Spin Kit for Soil (MP Biomedicals, Solon, OH, USA) and used for sequencing of the V3-V4 region of the bacterial 16S rRNA gene.[45] 16S rRNA gene amplicons were purified using the Agencourt AMPure XP PCR Purification system (Beckman Coulter, Krefeld, Germany), processed (indexed, purified, normalized, and pooled), and sequenced in a 300-bp paired-end runs on the Illumina MiSeq.[46]

Sequencing data were processed using the DADA2 pipeline and QIIME version 2.[47,48] Quality profiles of the reads were analyzed. Reads were trimmed and processed by the QIIME DADA2 plugin with the denoise-paired option and standard parameters. Rarefaction curves were determined based on the feature table, and analysis of the relative proportion of each bacterial taxon was made after the data were rarefied at a depth of 6000 sequences per sample. Taxonomic classification was done by a Naïve Bayes classifier (sklearn),[49] trained on SILVA database release 132.[50] Microbiota analyses were carried out using R for Statistical Computing (version 3.6.1, R Foundation for Statistical Computing, Vienna, Austria).[51] The QIIME biom data were imported and diversity scores calculated using the phyloseq R package.[52] The beta diversity, in this case the weighted UniFrac distances between the samples, was visualized using principal coordinate analysis (PCoA), and the effect of the FMT on the beta diversity was tested by a permutational multivariate analysis of variance (PERMANOVA).

2.4 | Statistic data analysis

For clinical data description, median levels of all data available within the respective (sub-) periods were calculated. To unveil relevant differences, we compared levels pre-FMT with (i) post-FMT (d1-d30) and (ii) post-FMT (d14) by applying non-parametric Wilcoxon matched-pairs signed rank test. Associations were investigated performing linear regression analyses of median levels pre- vs post-FMT (d1-d30). For analyses regarding IM alpha diversity, respective data were compared [donor vs pre- vs post-FMT(d7)] by applying Tukey's multiple test-corrected post hoc analysis after performing one-way ANOVA for multiple testing analyzing differences between more than two sub-groups. Statistical differences were considered significant at *P*-values <.05.

For graphical display and analyses of part of data (clinical-based information and IM alpha diversity), GraphPad PRISM (GraphPad Software Inc, La Jolla, CA, USA) was applied.

3 | RESULTS

A total of 11 patients received FMT as an experimental treatment of sr-GvHD. Detailed demographic and procedural data are shown in Table 1. The main diagnosis leading to allo-HCT was acute myeloid leukemia (AML; n = 6, 54.55%). Most patients were male (n = 9, 81.82%) with a mean age of 53.8 years (range: 30-76). Considering the entire observation period (d -14 to d +30 relative to FMT), all patients received antiviral and antifungal systemic prophylaxis. Ten out of 11 patients also received antibiotics (pre-FMT 8/11, at FMT 0/11, and post-FMT 7/11 patients; detailed information about antibiotic and immunosuppressive therapy for each patient of the cohort is given in Table S1). Regarding any kind of antiviral and/or antifungal therapy, only on the day of FMT were 2/11 patients off treatment. Seven out of 11 patients received the Janus kinase (JAK) 1/2 inhibitor ruxolitinib prior to FMT, 6/11 at the day of FMT, and 7/11 post-FMT. Ten out of 11 patients were classified as refractory to steroids as well as 6/11 to Ruxolitinib; 2/11 were ruxolitinib dependent (Table S2).

The mean time between allo-HCT and FMT was 168.5 days (range: 44-642). In all cases, FMT was administered *via* the upper GI-route. The majority of patients received FMT by preprocessed, frozen FMT capsules (9/11); others received single infusions of whole fecal solution *via* a naso-jejunal tube (2/11). Regarding a-GvHD, most patients had a grading of III and a staging (GIT-based) of II-III at

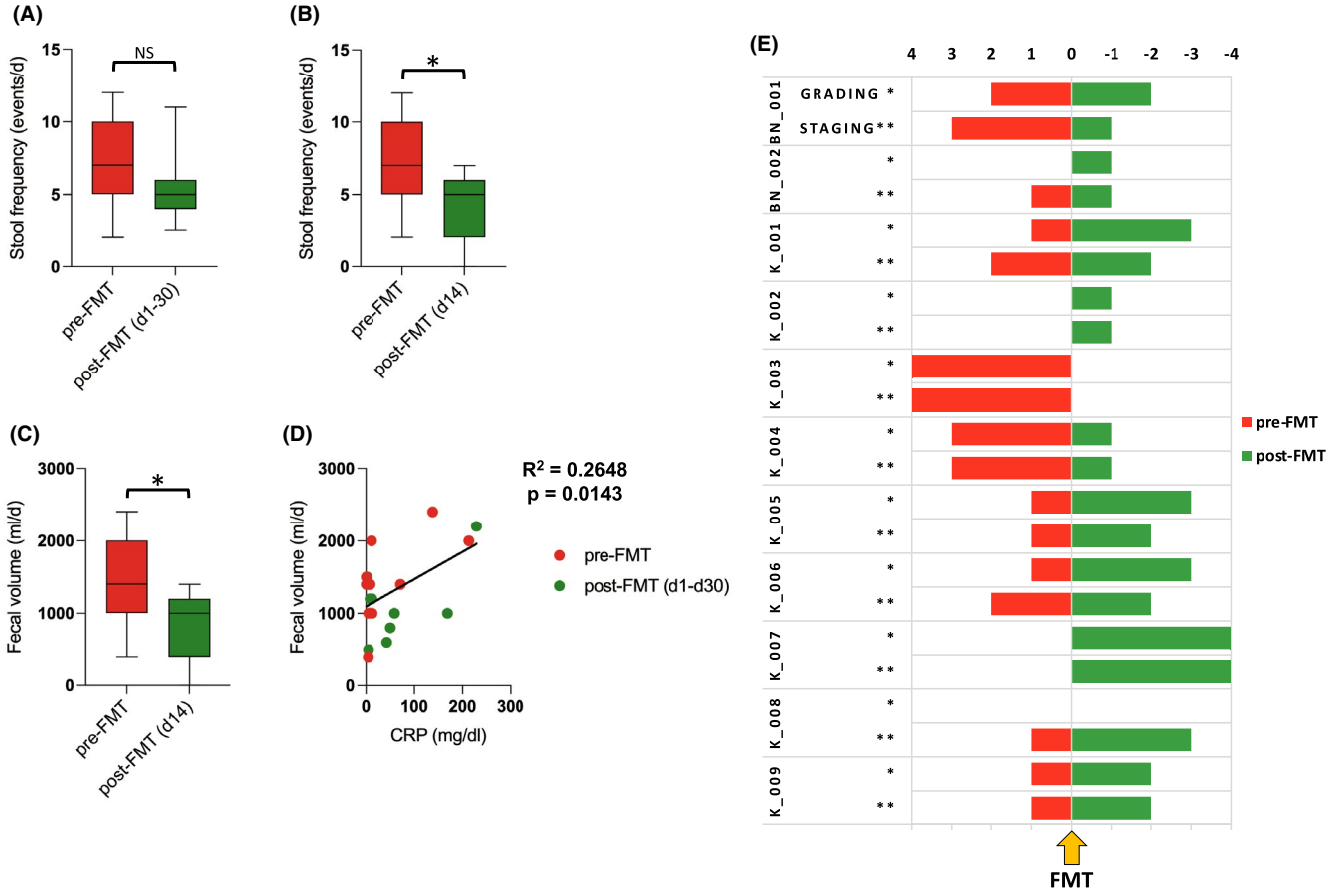

FIGURE 1 Stool frequency levels (A) pre- vs post-FMT (d1-30); stool frequency (B), and fecal volume (C) levels pre- vs post-FMT (d14) are shown. Additionally, linear regression analysis between fecal volume and CRP levels is shown with the respective regression line (D); statistical results of the linear regression analysis are displayed at the right upper quadrant of the diagram; data points indicate the relating sub-periods: pre-FMT, red and post-FMT (d14), green. Relative changes for each patient of the study regarding grading (overall) and staging (GIT-based) of a-GvHD within the observation period (pre-FMT vs post-FMT) are shown in reference to FMT (red bars indicate changes prior to and green bars after FMT, respectively) (E). FMT, Fecal microbiota transfer. Statistical significant results are indicated as followed: $P < .05$ (*); NS (not significant) [Colour figure can be viewed at wileyonlinelibrary.com]

TABLE 2 Biochemistry and clinical data for all patients regarding pre- and post-FMT (d1-30)

	Stool frequency [events/d]		Fecal volume [mL/d]		Creatinine [mg/dL]		Bilirubin [mg/dL]		ALT [U/L]		AST [U/L]		LDH [U/L]	
[median levels]	pre-FMT	post-FMT	pre-FMT	post-FMT	pre-FMT	post-FMT	pre-FMT	post-FMT	pre-FMT	post-FMT	pre-FMT	post-FMT	pre-FMT	post-FMT
BN_001	5	4	1000	800	0.5	0.57	0.315	0.24	-	39	29	20	393	391.5
BN_002	7.5	6	1500	1200	0.75	0.81	0.68	0.41	46	56	27	30	345	355
K_001	7.5	6	1400	1200	0.75	0.81	0.68	0.41	46	56	27	30	345	355
K_002	5	7.5	1000	1500	0.71	0.67	0.3	0.4	33	-	20	19	139	158
K_003	2	2.5	400	500	1.245	1.14	0.4	0.4	18	21	26	29	372	418
K_004	7	6	1400	1200	1.23	1.6	-	-	31	-	-	-	266	264
K_005	7	3	1400	600	1.07	1.24	0.9	1	138	57	46	33	312	402
K_006	7	5	1400	1000	0.6	0.66	0.8	0.4	-	-	29	21.5	243	229
K_007	10	11	2000	2200	0.8	0.75	0.8	0.7	47	26	27	27	317	358
K_008	12	5	2400	1000	0.44	0.79	21	24.4	19	19	20	21	60	71
K_009	10	5	2000	1000	0.58	0.68	0.2	0.2	14	13	15	14	199	153

Note: Numbers are given as median levels for the sub-periods.

Abbreviations: ALT, Alanine transaminase; AST, Aspartate transaminase; CRP, C-reactive protein; FMT, Fecal microbiota transfer; LDH, Lactate dehydrogenase.

the timepoint of FMT (Table S2; including also individual information about primary GvHD prophylaxis and the initial conditioning regime).

FMT-associated AEs[41,53,54] were mild and observed in 5/11 patients (Table 1) which included "abdominal pain" in 3/11, "vomiting" in 1/11, and "changes in peristalsis" in 3/11 patients. Severe AEs were not detected at any time. Within the observation period (between d+14 and d+30 post-FMT), one patient (K_008) died due to cytomegaly virus (CMV)-associated sepsis. This event was clearly assessed to be associated with the previous allo-HCT and subsequent immunosuppression, being further supported by a CMV-negative carrier status of the respective FMT donor.

Investigating data pre- vs post-FMT (d1-30) regarding stool frequency (Figure 1A) and fecal volume (not shown; Table 2, respectively) as main clinical correlate for the activity of GIT a-GvHD,[55,56] we detected trends toward decreased levels after the intervention. Individual responses to FMT regarding stool frequency are shown in Figure S1. Of note, at day 14 post-FMT, stool frequency (Figure 1B) and fecal volume (Figure 1C) were significantly decreased vs pre-FMT ($P < .05$, respectively). Moreover, we found fecal volume to be positively correlated with CRP [pre- vs post-FMT (d1-30): $R^2 = 0.2648$, $P = .0143$; Figure 1D]; for fecal frequency, we found similar associations [$R^2 = 0.2570$ $P = .016$; not shown]. Regarding other blood- and biochemical-based data (Table 2), we detected neither further differences between pre- vs post-FMT nor respective associations. In addition, biometrical data (systolic blood pressure, heart frequency, body temperature, and body weight) were also not affected during the procedure (data not shown).

Evaluating best response post-FMT regarding grading (overall) as well as staging (GIT-based) of a-GvHD, we documented attenuation for both (Figure 1E). The effect post-FMT seems to be stronger in those patients who did not profit from interventions prior to FMT.

3.1 | Intestinal microbiota changes

In addition to respective donor material, fecal samples from a subset of six patients were obtained also pre- and post-FMT for subsequent IM analysis. Regarding IM alpha diversity, numbers of detected IM members (related to detected OTUs) and Chao-1 index levels were significantly lower pre-FMT in allo-HCT patients compared to the donor ($P < .001$) and increased post-FMT (d7 vs d0: $P < .05$), but did not reach donor levels (d7 vs donor: $P < .01$; Figure 2A). Investigating IM compositional heterogeneity by beta diversity between the three groups (donor, d0 and d7), all three groups clustered separately (Figure 2B) and we observed a shift within the IM compositional heterogeneity post-FMT (d7) toward the donor in the PCoA analysis (PERMANOVA: R^2 0.26185, $P < .001$).

We further investigated IM alterations at the family level. As shown in Figure 2C, FMT led to an increase of Ruminococcaceae (mean abundance levels, respectively—pre-/d0 vs post-FMT/d7: 0.017 vs 0.088), as well as suppression of both Akkermansiaceae and Enterococcaceae (0.042 vs 0.016 and 0.288 vs 0.020) showing very low abundance (<0.01) and complete absence in donor samples, respectively. Moreover, we detected increases of Bacteroidaceae (0.011 vs 0.017) and Lachnospiraceae (0.064 vs 0.149) as well as a decrease of Veillonellaceae (0.165 vs 0.059) both displaying highest (0.284)/lowest (0.008) abundance in donor samples, respectively. Streptococcaceae, very low abundant in donor samples (<0.01), displayed a slight increase post-FMT (0.012 vs 0.018). Peptostreptococcaceae and Clostridiaceae 1, representing relevant abundance levels before FMT (0.019 and 0.054, respectively), displayed very low abundances post-FMT and in donor samples (<0.01, respectively). Rikenellaceae showed only relevant abundance levels in donor samples (0.030; pre- and post-FMT: <0.01, respectively). Interestingly, Lactobacillaceae increased post-FMT (0.064 vs 0.149)

CRP [mg/dL]		Protein [mg/dL]		Hemoglobin [g/dL]		Leucocyte count [G/L]		Platelet count [G/L]		Neutrophile count [G/L]		Lymphocyte count [G/L]	
pre-FMT	post-FMT	pre-FMT	post-FMT	pre-FMT	post-FMT	pre-FMT	post-FMT	pre-FMT	post-FMT	pre-FMT	post-FMT	pre-FMT	post-FMT
12.4	50.3	42	41	7.8	9.6	9.9	5.42	47	28.5	8.12	3.78	0.35	0.26
0.48	8.27	41.9	44.6	9.2	10	4.32	3.71	35	38	3.01	3.09	0.53	0.555
0.48	8.27	41.9	44.6	9.2	10	4.32	3.71	35	38	3.01	3.09	0.53	0.555
5.6	2.2	55	52	-	8.65	0.77	2.005	11	18.5	0.48	1.245	0.17	0.235
4.65	4.9	54.5	50	7.9	8.85	3.2	3.73	134.5	140	1.675	2.09	0.57	0.44
8.6	12.3	-	-	8.65	8.2	2.27	4.79	22	20	1.42	3.67	0.23	0.27
4.2	42.8	44.5	42	13.25	9.3	11.62	6.43	263	194	8.88	6.12	1.03	0.39
71.6	6.9	54	53	7.05	-	4.13	2.775	50	82	3.33	2.11	0.28	0.29
213.2	228.8	50	55	9.45	7.9	3.55	3.81	43	40	1.92	1.69	0.7	1.07
138.2	168.9	39	39	9.95	-	0.68	0.64	8	7	0.37	0.33	0.05	0.015
11.3	59.1	48	42	9.5	10.5	3.03	2.1	33	25	2.24	1.53	0.26	0.14

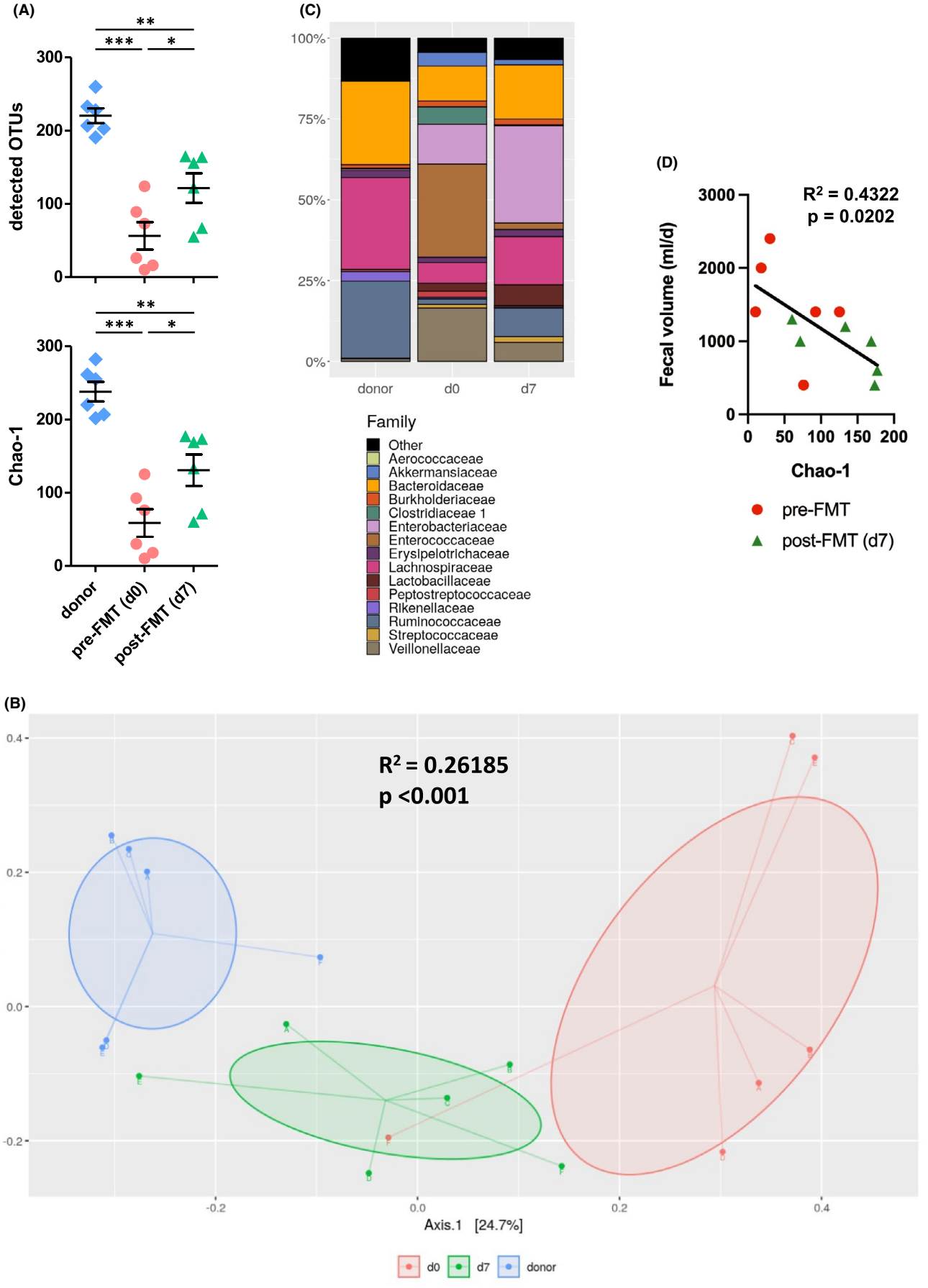

FIGURE 2 IM alterations of donor, pre-FMT (d0) and post-FMT (d7) samples of a subset of the study cohort (n = 6). Comparison between the groups is shown for detected OTUs (A, upper panel) and Chao-1 index levels (A, lower panel). Results of IM beta diversity analysis based on UniFrac PCoA analysis (display of first and second axis) is shown (B) with statistical results of the respective PERMANOVA analysis being displayed within the diagram. Distribution of the 15 most abundant IM family members between the sub-periods is shown as column chart (C). Additionally, linear regression analysis between Chao-1 index levels and fecal volume is shown with the respective regression line (D); statistical results of the linear regression analysis are displayed at the right upper quadrant of the diagram; data points indicate the relating sub-periods: pre-FMT, red and post-FMT (d7), green. FMT, Fecal microbiota transfer; OTU, Operational taxonomic unit; PERMANOVA, Permutational analysis of variance. Statistical significant results are indicated as followed: $P < .05$ (*), $P < .01$ (**) and $P < .001$ (***) [Colour figure can be viewed at wileyonlinelibrary.com]

although displaying lowest abundance in donor samples (0.024). A summary of relative changes of the 15 most abundant IM family members with results of the respective comparisons between pre- and post-FMT samples is given in Figure S2 (due to intraindividual variability and a low patient number, differences did not withstand FDR correction of *P*-values).

3.2 | Associations between IM alpha diversity and clinical parameters of a-GvHD

Of note, by further investigating associations between IM alpha diversity and clinical parameters of a-GvHD, we detected an inverse correlation of Chao-1 index levels with fecal volume [pre- vs post-FMT (d7): $R^2 = 0.4322$, $P = .0202$; Figure 2D] as well as with stool frequency [$R^2 = 0.4171$, $P = .0233$; not shown] in the course of FMT.

4 | DISCUSSION

Here, we report on the experiences of 11 patients undergoing FMT due to sr-GvHD of the GIT after allo-HCT at two academic transplant centers in Germany. FMT proved to be safe as no severe and only mild AEs were recorded. Clinical responses were documented by a significant reduction in stool frequency and fecal volume, as well as improvements regarding grading and staging (GIT-based) of a-GvHD post-FMT. Accompanying IM analyses confirmed a partial reconstitution of IM diversity explained by partial engraftment of donor IM.

By transferring a highly diverse IM community, FMT is considered to effectively influence and even restore IM dysbiosis.[27] In the light of published associations of the role of the IM in the onset and course of GvHD, application of FMT has become obvious in the context of GvHD; especially in patients not sufficiently responding to other rescue strategies in sr-GvHD.[57] Within a postallo-HCT setting, DeFilipp et al[58] performed third-party FMT with cryoconserved capsules shortly after neutrophile engraftment to investigate the safety and feasibility of the procedure as well as to determine whether restoration of a patient's injured, post-transplant IM diversity may improve the clinical outcome. Although the authors initially concluded that the procedure appeared to be feasible, safe, and associated with an increase in recipient IM diversity, two out of 18 patients subsequently developed acute GvHD of the GIT, with one patient also having concurrent bacteremia. Moreover, one patient died in the after-course of FMT in an allo-HCT context due to drug-resistant *E coli* bacteremia that was attributed to the intervention.[32]

4.1 | Case studies on FMT to treat GvHD

After a first case series was published in 2016,[34] additional cases[33,36,38] and preliminary data[35,37,39] regarding the use of FMT in sr-GvHD have been available (see Table 3). Until today, in total 16 cases were published (excluding data of meeting abstracts; range of patients per study: n = 1-8). All patients received initial allo-HCT due to hematological malignancies (mainly AML), and subsequent FMT was conducted due to steroid-dependent (n = 1) or sr-GvHD (n = 15). All studies[33,34,36,38] primarily investigated safety, but also assessed clinical efficacy. These studies used third-party fecal material (related and unrelated donors) of either a single universal donor,[33,34] or different donors for a single recipient.[36,38] The majority of patients received FMT via the upper GIT by naso-duodenal (-jeunal) tube (n = 12) as well as by preprocessed polymeric capsules (n = 1).[33] In one study, individuals received FMT via the lower GIT by colonoscopy (n = 3).[38] Most cases (n = 12) were treated by repeated FMT applications (2 up to 6 treatments per individual; single treatment—n = 4) with one study even using two consecutive cycles with repeated treatments.[33] FMT was performed between d+21 and d+174 after allo-HCT. In all studies, no severe AEs were observed during the reported observation periods. FMT-related AEs were mild and transient, and comorbid infections were not exacerbated after FMT,[34] also not after withdrawal of prophylactic antibiotics.[33] FMT-associated clinical responses ranged from general improvement of digestive symptoms (clinical/partial response: n = 11) to complete remission of GvHD (n = 5). Trying to integrate findings into a broader clinical context, Qi et al performed a retrospective analysis of allo-HSCT patients with sr-GvHD that did not receive FMT but were treated in the same center and at the same time. They could report improved progression-free survival, but no effect regarding overall survival.[36] However, these limited findings need to be validated in prospective, randomized trials.

Regarding specific IM alterations, FMT induced increases of IM diversity,[33,34,36,38] although IM diversity post-FMT was lower compared to donors.[34,36,38] Moreover, temporal IM dynamics seemed

TABLE 3 Summary of the already published cases of FMT application for GvHD treatment as well as information of preliminary data from published conference abstracts

Reference	Date of publication	Type of study	Number of individuals [N]	Diagnoses leading to allo-HSCT	Diagnoses leading to FMT	Design	FMT (Donor/graft)	FMT (Route of administration)
Kakihana K et al[34]	2016	Case Series	4	AML (n = 4)	steroid-resistant GvHD (n = 3); steroid-dependent GvHD (n = 1)	Pilot study; endpoints: 1) AE, 2) response	third-party (patient's spouse or a relative who passed the screening)	Upper GIT (naso-gastro-duodenal tube); repeated administration (max. 2 treatments)
Spindelboeck et al[38]	2017	Case Series	3	AML (n = 2), MDS (n = 1)	steroid-resistant GvHD (n = 3)	FMT procedures were performed on a compassionate use basis after individual (a priori) permission of the hospital board and obtaining informed consent	third-party with different donors for each patient (no data regarding status of relation)	Lower GIT (colonoscopy); repeated administration (up to 6 treatments)
Kaito S et al[33]	2018	Single Case Report	1	Ph-chromos. (pos)-ALL	steroid-resistant GvHD	N/D	third-party (related; sister of the patient)	Upper GIT (fecal material was processed and processed into polymeric capsules that were swallowed by the patient)
Qi X et al[36]	2018	Case Series	8	AML (n = 2); MDS (n = 2); CML (n = 1); ALL (n = 2); HAL (n = 1)	steroid-resistant GvHD (n = 8)	Pilot study; endpoints were Efficacy / safety; data were recorded until grade of GvHD, organ response, or OS reached a turning point (evaluation stopped at 90 days after the first FMT, or at any moment when the patient died or quitted)	Third-party (unrelated, female donors, n = 2)	Upper GIT (naso-gastro-duodenal tube); single (n = 4) and repeated administrations (2 treatments, n = 4)

to be linked to the gut condition as dominance of beneficial bacteria was related with response to FMT.[34] In addition, dominance of *Enterococcus* and increase of IM diversity correlated with frequency of diarrhea.[33] Post-FMT, dominance of *Escherichia*—being suspected to play a GvHD-promoting role[59,60]—were increased at the recurrence of acute GvHD.[34] In addition, recurrence of diarrhea post-FMT

Timepoint of FMT (after allo-HSCT)	Safety/adverse events	Main findings (clinical)	IM analyses	Main findings (IM)	Notes
d+92 (d+60 - d+174)	No severe AEs; potentially FMT-related AEs: mild and transient; case 4 developed hypoxia, paroxysmal atrial fibrillation, lower gastrointestinal bleeding, cholestatic liver damage, and transplant-associated thrombotic microangiopathy +fever 2 days after the second FMT; comorbid infections were not exacerbated in the peri-interventional course	Perceivable effects in all cases (complete response, CR - n = 3; partial response, PR - n = 1); steroid doses could be reduced post-FMT; after resuming antibiotics, FMT effect lasted (after initial response)	Phylogenetic analyses (16S rRNA sequencing; V1-2 region)	IM diversity did not fully recover after FMT (even with apparent clinical response); temporal microbiota dynamics seemed to be linked to the gut condition; dominance of beneficial bacteria were related with clinical response; dominance of E. coli was found before GvHD-relapse	In addition to IM analyses: FACS analyses of PBMC
d+37 - d+110	No severe AEs reported; no immediate procedure-related infections despite withdrawal of prophylactic antibiotics and no notion of an excess of infections after FMT	All 3 patients showed clinical response to FMT with reduced stool volumes that normalized with repeated interventions (up to 6 repeated FMTs); 1 patient displayed macroscopic improvement (colon histopathology); CR of GvHD (n = 2): at d+90 and d+110, respectively	Phylogenetic analyses (16S rRNA sequencing; V1-2 region)	Clinical effects were accompanied by IM improvement (increase of diversity and to some extent engraftment)	One patient failed follow-up after one FMT
2 cycles [1. cycle: 15 capsules at d+125-144 (4 timepoints); 2. cycle: 15 capsules at d+173-189 (3 timepoints)]	No specific data regarding safety; no severe AEs mentioned	Soon improvement of digestive symptoms after first FMT; in line with rising proportion of *Enterococcus* 4 weeks after the first administration of FMT, slight exacerbation of diarrhea and hemorrhagic stool were noticed	Phylogenetic analyses (16S rRNA sequencing; V1-2 region)	Dominance of Enterococcus and increase of IM diversity correlated with frequency of diarrhea; FMT-associated relevant increase of Lactobacillus (although very rare occurrence in the fecal graft) => speculation: IM diversity more relevant than engraftment; final IM composition: dominated by Bacteroides, Parabacteroides, Clostridium, Faecalibacterium, and Lactobacillus	Clinical response on GI-symptoms after FMT without influence on non-GI GvHD-manifestations (but: liver-GvHD was detected to no time)
d+80 (d+21 - d+369)	No severe AEs recognized	In a post hoc retrospective comparison with allo-HSCT patients and sr-GvHD (treated at the same time): effect regarding improved PFS, no effect regarding OS	Phylogenetic analyses (16S rRNA sequencing; only in n = 6 patients)	Pre-FMT: significantly higher level of Firmicutes and Enterococcaceae; post-FMT: significantly higher level of Bacteroidetes and Bacteroidaceae, Ruminococcaceae and Desulfovibrionaceae; beneficial bacteria (Bacteroides) showed dominance post-FMT; IM diversity significantly increased post-FMT (but post-FMT significantly lower than donor => incomplete reconstitution/ engraftment)	Until d+50 post-FMT 50% of patients died due to non-FMT-associated reasons

(Continues)

was associated with a re-expansion of *Enterococcus* spp.[33] Final IM composition displayed dominance of Bacteroides, Parabacteroides, Clostridium, and Faecalibacterium. Marked increases of *Lactobacillus* spp. shortly after each cycle of FMT—although with very rare occurrence in the initial fecal graft—were specifically recognized.[33]

TABLE 3 (continued)

Reference	Date of publication	Type of study	Number of individuals [N]	Diagnoses leading to allo-HSCT	Diagnoses leading to FMT	Design	FMT (Donor/graft)	FMT (Route of administration)
Shouval S et al[37]	2018	N/C	7	N/D	steroid-resistant GvHD (n = 6); steroid-dependent (n = 1)	single-arm pilot study; endpoints: 1) severe AE at d+28 after FMT, 2) GvHD response (CR or PR)	third-party (healthy, unrelated donors)	Upper GIT (30 frozen capsules over two consecutive days; courses could be repeated from the same or a different donor at the treating physician's discretion; total of courses, n = 15)
Van Lier YF et al[35]	2019	N/C	15	N/D	steroid-resistant and steroid-dependent GvHD	prospective, single-arm pilot study; endpoints: 1) safety and 2) efficacy of FMT for GvHD	third-party	Upper GIT (naso-duodenal tube; single infusion, n = 1)
Zhang F et al[39]	2019	N/C	1	N/D	steroid-resistant GvHD	Assessment of clinical phenotype and outcome and correlation with microbial profiles	third-party (different donors, n = 2)	Upper GIT (naso-duodeno-jejunal tube; repeated FMTs, n = 4)

Abbreviations: AE, Adverse event; AML, Acute myeloic leukemia; CR, Complete response; FMT, Fecal microbiota transfer; HAL, Hybrid acute leukemia; IM, Intestinal microbiota; LGI, Lower gastrointestinal tract; MDS, Myelodysplastic syndrome; N/C, Not concluded; N/D, No data available; OS, Overall survival; PFS, Progression-free survival; PR, Partial response; UGI, Upper gastrointestinal tract.

4.2 | Integrating own data

There is no consensus about the optimal route for FMT application with more or less invasive approaches *via* the upper and/or lower GIT.[27,41,61] This is reflected by different approaches of FMT application (naso-jejunal tube vs capsules) within the already published cases as it was also in our study due to differing center-based standards. Kaito et al[33] used encapsulated fecal material for FMT as done also in most of our patients (two patients of our cohort received whole fecal solutions). Arguments pro delivery of FMT by capsules are based on data of refractory CDI confirming less discomfort, higher safety but comparable efficacy compared to more invasive methods (tube and endoscopy).[62-64] Otherwise, as even sterile filtrates of fecal material (without viable bacteria) have shown efficacy in treating CDI,[65] a probable impact from non-viable bacterial metabolites[66] and other non-bacterial compounds in fecal material on the mode of action of FMT should be cleared in front of finally discarding the use of whole fecal solutions.

Our results of high safety figures are strong in line with observations in already published cases.[33,34,38] Moreover, it has been confirmed in patients within a general immunocompromised setting.[26,53,57,67-70] There, especially infectious AEs, have to be suspected, but are very unlikely to happen later than 28 days after FMT. Therefore, our data—although only based on a rather short observation period—further support the high safety of FMT in highly immunosuppressed individuals. One fatal event (CMV-associated sepsis) documented within the observation period was clearly assessed to be associated with the previous allo-HCT and subsequent immunosuppression. Moreover, sufficient prospective data are still pending in this context,[53] and rare events of fatal outcome[32] associated with previous FMT treatment highlight the unmet medical need of well-controlled prospective clinical studies. Albeit, clinical efficacy in our case series is supported by significant reductions of stool frequency and fecal volume that have to be considered relevant since stool frequency and the well-established collection of stool volumes for GvHD grading[71] represent obvious and subsumable clinical phenomenon of intestinal GvHD.[55,56] This seems to be even more important, as we only performed single FMT applications (except in one patient), which is in clear contrast to published cases where most individuals received repeated applications.[33,34,38] We conclude that FMT proved

Timepoint of FMT (after allo-HSCT)	Safety/adverse events	Main findings (clinical)	IM analyses	Main findings (IM)	Notes
N/D (d+39; IQR d+21-d+58 after GvHD diagnosis)	No severe AEs reported	CR (n = 2); follow-up: at median d+61 after FMT 3 of 7 patients were alive	Phylogenetic analyses (16S rRNA sequencing)	E. coli domination before with major reduction after FMT (n = 4); FMT led to introduction of new bacteria and an increase in bacterial diversity; severe courses of bacteriaemia could be ruled out to be associated with FMT (referring sequences could be found before FMT but not in donor material)	Published abstract; NCT 03 214 289
N/D	No severe AEs reported	In 6-month follow-up: CR (n = 11), "normalization of stool frequency and consistency" (n = 6), initial response and relapse after steroid taper (n = 5); durable response to FMT was associated with a better prognosis	Phylogenetic analyses (16S rRNA sequencing)	IM of CR patients resembled that of the donor the most post-FMT	Published abstract
N/D	N/D	FMT resulted in clinical recovery	Ultra-deep metagenomics sequencing and profiling of bacteriome and fungome; enrichment of fecal virus-like particles following by metagenomics sequencing and virome profiling	FMT altered the gut bacterial, fungal and viral communities simultaneously; gradual restoration of IM diversity; instant donor-derived fungi engraftment after single FMT; improved viral diversity; enhanced the ecological network of bacteria-fungi interactions post-FMT	Published abstract; authors proclaim first longitudinal data of intestinal mycobiome and virome after FMT in the setting of GvHD

to be safe with perceivable therapeutic effects being further supported by attenuation of grading (overall) and staging (GIT-based) of a-GvHD post-FMT in the majority of cases.

Our findings of significant positive correlation between fecal volume as well as stool frequency and CRP levels may reflect similar associations in inflammatory bowel disease where CRP levels represent one relevant marker of disease activity.[72,73] Moreover, the relevance of CRP alone or in combination with other markers for staging of GvHD has been an issue of ongoing investigations.[56,74-76]

In line with previous data, we detected massively decreased IM alpha diversity levels pre-FMT with partial restoration post-FMT which, however, did not reach donor levels,[33,34,36,38] potentially indicating incomplete engraftment of the donor IM.[34,36,38] Focusing on specific alterations, pre-FMT (GvHD disease state), we were able to confirm formerly detected high abundance levels of *Enterococcus*,[33,77,78] Enterobacteriaceae,[20] and Enterococcaceae.[36] Moreover, we found a bloom of *Lactobacillus* that—together with Enterococcaceae—showed very low abundances in donor samples, therefore hinting to represent GvHD-specific IM members.[20] In contrast to previous reports, post-FMT (intervention state) we detected further increasing abundances of Enterobacteriaceae formerly identified as potential GvHD-inducers.[24,79] Moreover, fitting to existing data,[33,34,36] we detected increases of Bacteroides, *Lactobacillus*, Faecalibacterium, and Ruminococcaceae post-FMT. Interestingly and also in contrast to formerly shown increases post-FMT,[33] we detected relevant abundance levels of Clostridium sensu stricto 1 post-FMT (displaying very low abundances in both pre-FMT and donor samples). In addition, we found *Escherichia-Shigella* to be increased post-FMT. Although *Shigella* spp. themselves represent relevant GI pathogens,[80] high similarities with *Escherichia coli* due to close relation of phenotype but also molecular-based markers impede proper discrimination.[81] Therefore, the real impact of each specific IM member remains elusive. Additionally, as *Escherichia* has been associated with GvHD itself[59,60] and with unfavorable courses,[34] our finding of increased abundance levels post-FMT may—at least partly—reflect findings of no complete resolution of GvHD symptoms after FMT in our cohort. This may also serve to explain why—although recognizing trends in our data—we could not detect correlations between stool frequency and IM alpha diversity as described before.[33] In the light of exposed IM alterations pre- and post-FMT, donor material (healthy

state) showed IM annotations referring to "others" with biggest fractions clearly reflecting the highest IM alpha diversity in donor samples. Moreover, we detected striking high abundances of Faecalibacterium, Ruminococcaceae UCG-002, and Subdoligranum with subsequently very low abundances of Akkermansia, Clostridium sensu strictu 1, *Enterococcus*, *Escherichia-Shigella*, Klebsiella, *Lactobacillus*, *Streptococcus*, and *Veillonella*. The specific finding of *Lactobacillus* displaying very low abundances within donor samples but showing relevant increases post-FMT in our cohort has also been acknowledged before[33] and gave rise to speculations if this represents an FMT-intrinsic effect going in line with the formerly stated relevance of *Lactobacillus* spp. in the context of GvHD pathogenesis.[20]

Finally, detected inverse associations between IM alpha diversity and clinical parameters (fecal volume and stool frequency) further support relevant functions of the IM in the context of a-GvHD in line with previous findings.[33]

Nowadays, ruxolitinib represents the standard of care for treatment of a-GvHD.[6,40] As most individuals within the observation period of this study were on treatment and only 2/11 patients did not receive any therapy during their postallo-HCT course (Table S2), specific effects of ruxolitinib could not be independently assessed within our cohort.

4.3 | Limitations of our study

Due to current legislative limitations by the *AMG* indicating fecal material for FMT as a drug with subsequent regulatory implications (good manufacturing process etc.), we were able to only collect and investigate FMT-related data retrospectively. In this context and due to a lack of clinical indications, we could not obtain biopsies for histopathological verification of FMT effects on mucosal restoration.[38] In addition, the short observation time after FMT and a rather small number of subjects represent another type of limitation. Regarding IM-based data and due to the fact that data of the former cases are almost entirely generated by amplification of the V1-2 region, while our data are based on V4 amplification, we have to state that some uncertainties about comparability between our and former data sets have to remain due to differences in phylogenetic analyses regarding the respective amplification of the hypervariable region(s) of the bacterial 16S rRNA gene.[82] We are aware of the general limitations of our IM-based data. Our case series is subject to significant bias based on its limited sample size and variation in internal (eg, immunological) as well as external (medication, interventions, diet etc.) factors known to influence the IM. Patients within an allo-HCT setting are frequently exposed to anti-infective therapy as it was also the case in the majority of our patients (see above). Especially the duration and amount of antibiotic therapy has been linked to the overall outcome after allo-HCT, but also to the onset and course of a-GvHD.[83-86] Of note, only two out of six patients (K_006 + K_008) received antibiotic therapy until d-1 pre-FMT; at the day of FMT (d0) 0/6 and until day 7 post-FMT, only 1/6 patients (K_008) received antibiotic therapy (see above and Table S1).

4.4 | Conclusion

Our study represents one of the largest cohorts of patients treated with FMT for acute sr-GvHD of the GIT so far. Of note, we confirmed FMT to be safe, even in heavily immunosuppressed patients after allo-HCT. Moreover, FMT induced perceivable therapeutic effects. Accompanying IM analyses mainly supported previously reported findings of increasing IM diversity and relevant microbial shifts post-FMT displaying associations with clinical parameters of a-GvHD.

Altogether, this adds important data to a still limited body of evidence about the relevance of FMT in the context of sr-GvHD. With our observations and published findings, we would like to stress the need for future prospective, randomized studies with sufficiently powered patient cohorts to prove clinical efficacy in a controlled setting and perform translational research to better define potential mode(s) of action.

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Nothing to declare.

CONFLICT OF INTEREST

The authors of this manuscript do not have to declare any conflicts of interest.

AUTHOR CONTRIBUTION

FG, TAWH, MJGTV, and MRCA involved in conceptualization and design; FG, BS, and MRCA involved in collection of patient derived data, and MRCA involved in fecal samples; FF and PH involved in establishing procedures/protocols and performing IM-based analysis; FG, BS, and MRCA analyzed clinical-based data, FF analyzed IM-based data, and FG, CST, FF, MJGTV, and MRCA involved in interpretation and integration of data; FG, CST, DW, MJGTV, and MRCA involved in writing of the manuscript; DW, CPS, PB, PH, CS, TAWH, and MJGTV supervised the study; all authors listed above proofread the final version of this manuscript.

DATA AVAILABILITY STATEMENT

In addition to the provided supplementary data of this manuscript, all data that support the findings of this study are available from the corresponding author upon reasonable request.

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SUPPORTING INFORMATION

Additional supporting information may be found online in the Supporting Information section.

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