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

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SUPPLEMENTARY FIGURE LEGENDS:

Figure S1: Details of patient 2:

A:

Antibiotics (red) and immunosuppressants (green) given to patient 2 before, during and after the introduction of FMTs. ECP, extracorporeal photopheresis. In total, 16 ECP sessions (*) were applied and 11 doses (**) of etanercept 25mg were given.

B:

Longitudinal illustration of stool volumes [ml/day, black lines, left Y-axis] and richness [observed species, blue lines, right Y-axis] of patient 2 before, during and after two 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 from both FMTs.
Stool volumes peaked with 3000 ml at d+48 and substantially decreased after commencement of FMTs to normal values. After commencement of FMTs diarrhea subsided.

C:

Fecal microbiota analysis (16S rRNA gene analysis) of patient 2 before, during and after two 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 application. Introduction of FMTs lead to colonization by donor stool. However, analyses reveal changes of the bacterial composition without interventions after FMT 2 suggesting that the transplanted microbiome is not stable over time. A

substantial reappearance of *Enterococcus* was observed eight days after FMT 2.

Figure S2: Details of patient 3:

A:

Antibiotics (red) and immunosuppressants (green) given to patient 3 before, during and after the introduction of FMT. ECP, extracorporeal photopheresis. In total, 13 ECP sessions (*) were applied and 12 doses (**) of etanercept 25mg were given.

B:

Longitudinal illustration of stool volumes [ml/day, black lines, left Y-axis] and richness [observed species, blue lines, right Y-axis] of patient 3 before, during and after FMT (grey dashed vertical lines). The horizontal black line represents 500ml of stool volume.

Stool volumes peaked with 1650 ml at day +31.

C:

Fecal microbiota analysis (16S rRNA gene analysis) of patient 3 before, during and after one FMT (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 application. FMT lead only to a transient increase in bacterial richness and suppression of *Enterococci* that again emerged 7 days after FMT. Accordingly, bacterial richness decreased to pre-FMT values 7 days after FMT (see panel B).

SUPPLEMENTARY METHODS:

DNA Isolation and PCR Amplification

DNA was extracted from stool samples using the Magnapure Bacterial DNA Kit (Roche) following the recommended procedures. DNA concentration was measured by picogreen fluorescence. The variable V1–V2 region of the bacterial 16S rRNA gene was amplified with PCR from 50 ng DNA using oligonucleotide primers 16s_515_S3_fwd: GATTGCCAGCAGCCGCGGTAA and 16s_806_S2_rev : GGACTACCAGGGTATCTAAT. Bacterial 16S rRNA was amplified with the Mastermix 16s Complete PCR Kit (Molzym, Bremen, Germany). The first PCR reaction product was subjected to a second round of PCR with primers fusing the 16s primer sequence to the A and P adapters necessary for Ion Torrent sequencing while additionally including a molecular barcode sequence to allow simultaneous multiplexing of up to 96 samples. PCR products were subjected to agarose gel electrophoresis and the band of the expected length (330nt) was excised from the gel and purified using the QiaQick (Qiagen, Hilden, Germany) gel extraction system. DNA concentration of the final PCR product was measured by picogreen fluorescence.

Sequencing

Amplicons from up to 60 samples were pooled equimolarly and subjected to emulsion PCR using the Ion Torrent One Touch 2.0 Kit according to the manufacturer´s protocols. After emulsion PCR the beads were purified on Ion ES station and loaded onto Ion Torrent 318 chips for sequencing. Sequencing reactions were performed on Ion Torrent PGM using the Ion 400BP Sequencing Kit running for 1082 flows (all reagents from Thermo Fisher Scientific, MA, USA). Sequences were split by barcode and transferred to the Torrent suite server. Unmapped bam files were used as input for bioinformatics.

Bioinformatics and Phylogenic Analysis

All sequences were initially trimmed by a sliding window quality filter with a width of 20nt and a cutoff of Q20. Reads shorter than 100 nucleotides and reads mapping to the human genome were removed using DeconSeq.(1) The remaining reads were subjected to error correction using the Acacia tool (version 1.52b159)(2) leading to error correction of 10-20% of reads. Subsequently PCR chimeras were removed by usearch (version 6.1.544) algorithm in de-novo and reference based settings.(3) The final sequence files were then analyzed by QIIME 1.8 workflow scripts.(4) OTU search was performed using the parallel_pick_open_reference_otus workflow script and the greengenes 13_8 reference database.(5)

Statistical analysis and visualization

OTUs were visualized as OTU tables, bar charts, and PCoA plots using the QIIME core microbiome script. Groupings supplied in the mapping file were tested for statistical significance using the QIIME implementation of the Adonis test and significance of individual bacterial strains was determined by the Kruskal-Wallis test. LEfSe analysis was performed to detect statistically relevant strains in several of the study groupings.(6)

Further analysis and visualizations were performed with custom R scripts (R 3.2.4). Number of observed species (depth of 8000 sequences per sample) was tested for significance by paired t-tests with FDR adjustment to account for multiple comparisons. Graphics were created using ggplot2 (version ggplot2_2.1.0) based on the relative abundance values at genus level calculated during bioinformatical microbiome analysis using QIIME.

Preparation of stool for fecal microbiota transplantations:

In patients 1 and 2 the stem cell and the stool donor were different persons, in patient 3 both stem cells and stool were provided of the patient´s brother.
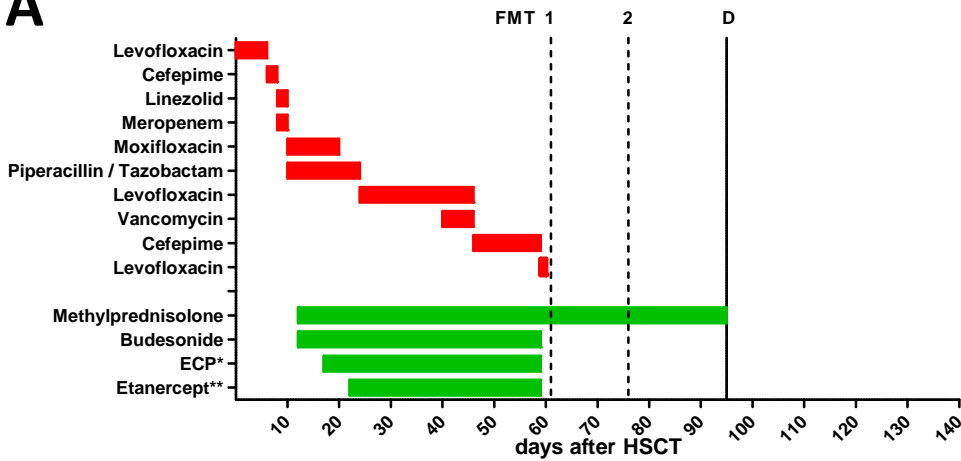
Donors were healthy adult subjects screened to exclude infectious and other possible transmittable

diseases according to standard guidelines.(7) Stool of a single bowel movement not older than 6 hours and weighing 50 to 150 g was diluted with sterile saline (~200-300 ml) and homogenized within a blender. The mixture was then filtered to remove debris. A total of 200 to 300 ml of the resulting suspension was placed into 20ml syringes and applied within 1 hour through a colonoscope. Storage from preparation to application (<1h) was done at room temperature
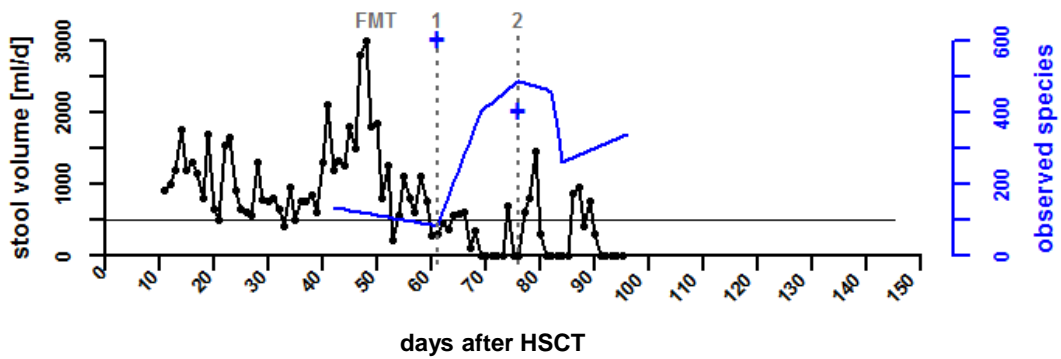
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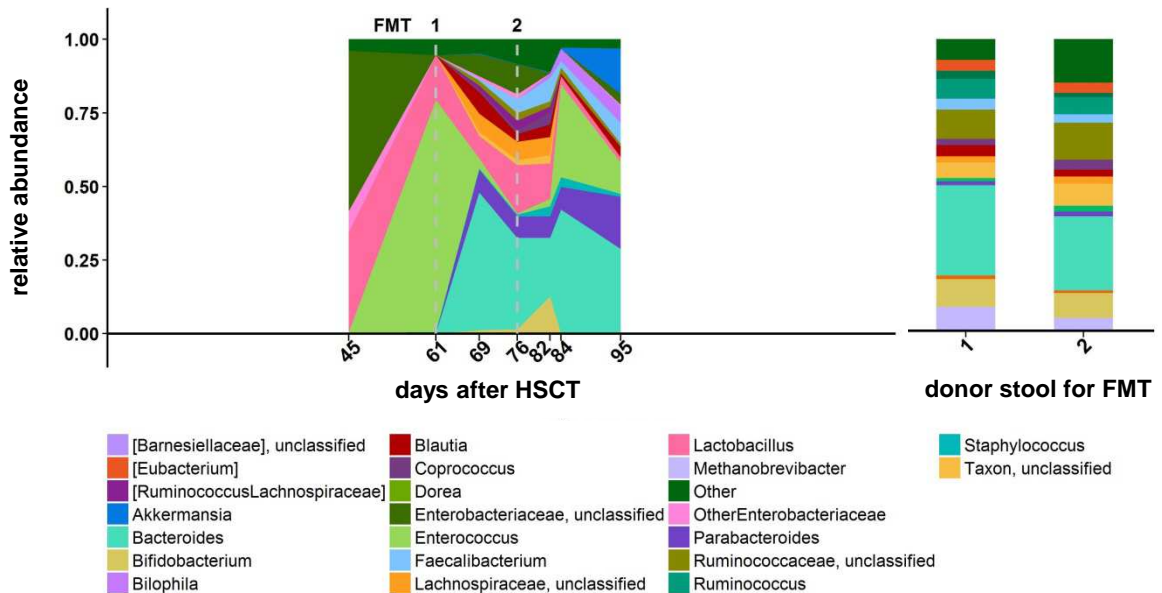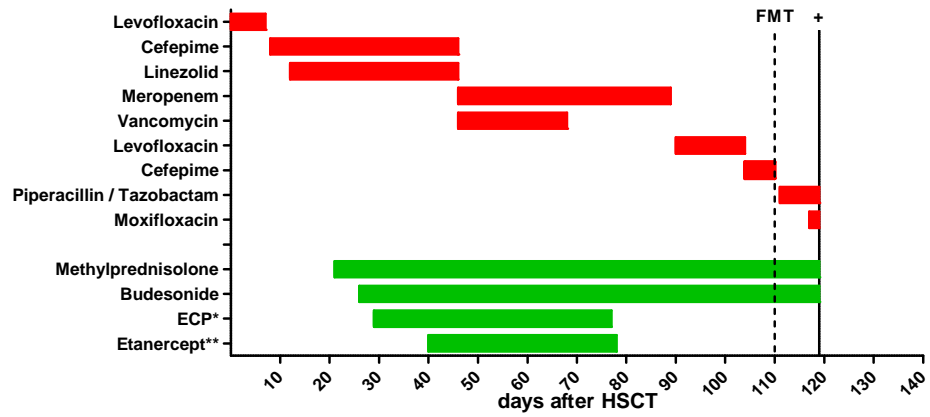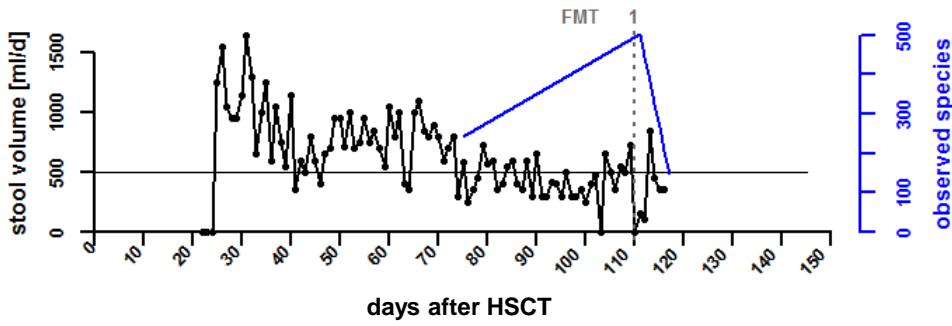
Figure S1

Figure S2

A

B

C