Dental plaque microbiota following allogeneic hematopoietic cell transplantation and risk of chronic graft-*versus*-host disease

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Supplementary material

Supplementary Methods

Standard pre-transplant dental examination at Fred Hutchinson Cancer Center
The primary purpose of the standard pre-transplant dental examination is to prevent oral complications of alloHCT with a particular focus on recognizing and eliminating any sources of possible opportunistic infection[1]. The protocol for this examination includes a comprehensive review of dental and medical history, a head and neck examination (i.e., palpation of structures such as the lymph nodes, salivary glands, sinuses, muscles of mastication, and the temporomandibular joints), dental radiography (e.g., a panoramic radiograph with supplemental bitewing and/or periapical radiographs or a full mouth series), oral mucosal examination, caries assessment, and periodontal evaluation[1,2]. Patients are also counseled on proper oral care throughout the transplant timeline (i.e., proper techniques for brushing, flossing, and rinsing if necessary)[3,4]. This examination is typically completed 2-4 weeks prior to transplantation, and upon completion, a full dental treatment plan is completed (i.e., cleaning, restoration, root canal therapy, extraction, etc.) with enough time for recovery prior to transplant. Dental treatment planning is consistent with the Multinational Association of Supportive Care in Cancer/International Society of Oral Oncology (MASCC/ISOO) and the European Society for Blood and Marrow Transplantation (EBMT) Position Paper on Basic Oral Care for hematology-oncology patients and hematopoietic stem cell transplantation recipients[4].

Dental cleanings in our center include dental prophylaxis, scaling and root planing, and periodontal maintenance based on a patient's periodontal status (which is determined by an Oral Medicine specialist working closely with a registered dental hygienist who performs periodontal evaluation as part of all pre-transplant dental evaluations). Dental cleaning is performed to decrease risk for oral source infection in the peri-transplant period and are treatment planned for patients who are judged to benefit from periodontal intervention (e.g., those with gingival inflammation/infection in relation to local plaque and calculus accumulation, periodontal pocketing ≥4mm, or chronic unmanaged periodontal disease). Dental cleaning is performed by a registered dental hygienist using an ultrasonic scaler, supplemented with hand instrumentation. High volume suction is utilized to minimize risk for aspiration. Anti-infective protocols, including antibiotic prophylaxis and pre-operative rinsing with chlorhexidine gluconate, are utilized when patients have an absolute neutrophil count (ANC) <1.0, if a Hickman line or Port-au-Cath is in place, and/or if the patient has cardiac indications for antibiotic prophylaxis based on American Heart Association guidelines for prevention of infective endocarditis. Antibiotic choice and timing are based on the same AHA guidelines[5,6]. Local anesthesia is utilized when scaling and root planing is performed or when patient comfort would otherwise limit calculus removal. Oral sedation with a one-time pre-treatment dose of a benzodiazepine is used in a limited capacity for patients with high levels of dental anxiety (in coordination with WA State Law related to oral sedation)[7]. Oral hygiene instruction is also performed by a registered dental hygienist to help optimize local plaque control following dental procedures.

Mucositis assessment

Mucositis assessments were performed by 4 calibrated oral medicine specialists using the Oral Mucositis Assessment Scale (OMAS)[8] at baseline, day +7, day +14, day +21 (if the patient was still in the hospital), day +28, and day +84. OMAS evaluates 9 intraoral sites (upper and lower lip, left and right buccal mucosa, left and right ventrolateral tongue, floor of mouth, and hard and soft palates) for severity of ulceration/pseudomembrane (range 0-3) and erythema (range 0-2). Total mucositis score (range 0-45) at each timepoint, defined as the sum of the scores (ulceration/pseudomembrane and erythema) across all sites[9], was used as a quantitative continuous variable estimating mucositis severity at the corresponding timepoint.

Sample collection and processing

Samples were collected after at least 30 minutes of no oral intake or oral hygiene. Up to 5 mL of saliva was collected by passive drooling into a sterile tube containing 5 mL of sterile 95% ethanol. The procedure was stopped after a maximum of 15 min. The tube was pulse vortexed for 5 seconds to ensure homogenous mixture of saliva and ethanol before storage at -80°C. Plaque samples were taken from 3 teeth and transferred from the scaler to the tip of a sterilized plastic pick. Samples contaminated with blood were discarded and taken again from an adjacent tooth. The pick was submerged into a sterile tube containing 500 µL of sterile 95% ethanol and agitated until the clump of plaque fell off the instrument into ethanol. All 3 plaque samples from the same patient were pooled into the same tube. Samples were transferred to -80°C on the day of collection. The use of 95% ethanol for storing oral samples has been validated and allows long-term DNA preservation at room temperature[10,11].

Microbiota sequencing and pathway analysis

DNA was extracted using the ZymoBIOMICS®-96 MagBead DNA Kit (Zymo Research, Irvine, CA). Sequencing libraries were prepared using the Illumina® DNA Library Preparation Kit (Illumina, San Diego, CA) following the manufacturer's protocol and with unique dual-index 10 bp barcodes with Nextera® adapters. All libraries were pooled in equal abundance and the final pool was quantified using qPCR and TapeStation® (Agilent Technologies, Santa Clara, CA). The final library was sequenced on an Illumina NovaSeq 6000 using a S2-300 flow cell and a PE150 configuration. The ZymoBIOMICS® Microbial Community DNA Standard (Zymo Research, Irvine, CA) was used as a positive control for each library preparation. Multiple negative controls (i.e., blank extraction control, blank library preparation control) were included to assess the level of bioburden carried by the wet-lab process. The criteria for the negative controls for a pass run and findings in the present study are summarized in the Table below:

Metric	Criteria	Result
Extracted DNA concentration	<0.5 ng/uL	Negative extraction control #1 = 0.146 ng/uL Negative extraction control #2 = 0.29 ng/uL
Sublibrary DNA concentration	<3 ng/uL	Sublibrary- negative extraction control #1 = 0.5 ng/uL Sublibrary- negative extraction control #2 = 0.8 ng/uL
Read count	<100,000	Negative extraction control #1 = 856 reads Negative extraction control #2 = 51 reads

Raw paired-end metagenomic sequence reads were quality-processed using the integrated pipeline provided in KneadData v.0.12.0. This sequence-level procedure included two main steps: (*i*) removal of reads mapped to the human reference genome GRCh37 (hg19) using Bowtie2 v.2.4.5[12] and (*ii*) removal of adapter sequences and low-quality reads using Trimmomatic v.0.39[13] with default settings. Output files consisting of surviving paired and orphan reads were concatenated and used as input to MetaPhlAn4[14]. MetaPhlAn4 with default parameters was used for species-level taxonomic assignment. MetaPhlAn4 uses a set of species-level genome bins (SGBs)[15] as primary taxonomic units and accurately profiles their presence and abundance in metagenomes. The latest version (version 4) of MetaPhlAn uses a database containing ~5.1 million unique clade-specific marker genes for 21,978 existing SGBs and 4,992 yet-to-be-characterized SGBs (defined solely based on metagenome-assembled genomes).

Pathways and gene family abundances were profiled using HUMAnN v3.6[16]. HUMAnN's tiered search occurs in 3 phases. First, it identifies community species using MetaPhlAn and its clade-specific marker genes. Then, it maps KneadData-processed reads against the pangenomes of the identified species using Bowtie2[36]. Finally, it aligns unmapped reads to a comprehensive, non-redundant protein database (EC-filtered UniRef90) using DIAMOND[17]. HUMAnN performs read-count-based quantification of the microbial gene families and functional pathways present within each sample on both per-species and community-level basis. Pathways were annotated using MetaCyc v24.0 definitions[18] and gene families using UniRef90 definitions[19]. HUMAnN's default reads per kilobase values for gene family and pathway abundances were normalized to copies per million (CPM).

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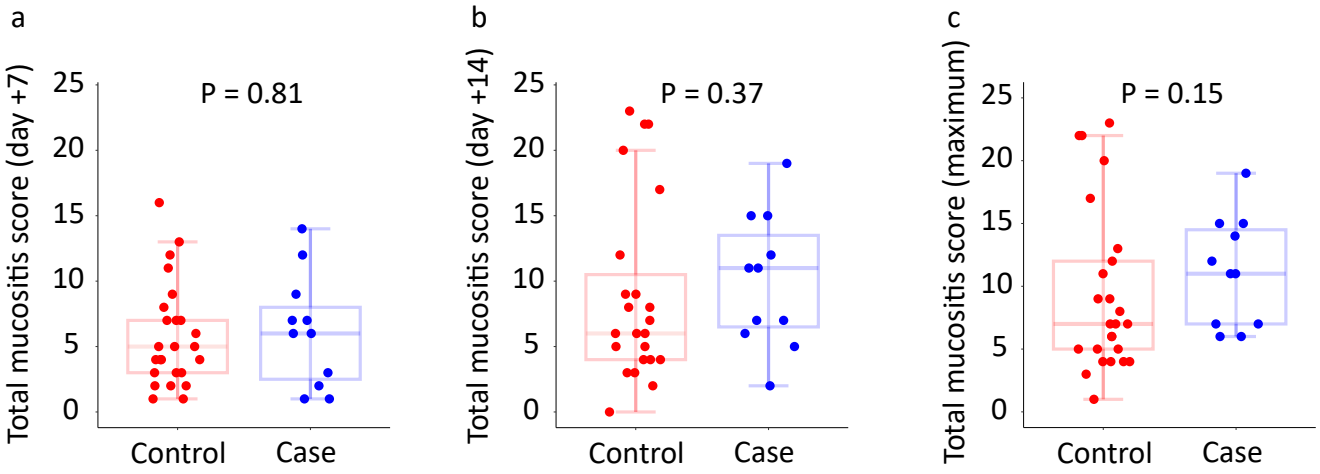
a
Total mucositis score (day +7)
25
20
15
10
5
0
P = 0.81
Control
Case
b
Total mucositis score (day +14)
25
20
15
10
5
0
P = 0.37
Control
Case
c
Total mucositis score (maximum)
25
20
15
10
5
0
P = 0.15
Control
Case

Fig. S1: Oral mucositis severity and chronic GVHD
Comparison between cases and controls for oral mucositis severity, using total mucositis score on the OMAS scale, at day +7 (**a**), day +14 (**b**), and maximum of the two (**c**). P values are from a Wilcoxon's test. Each box shows the median (horizontal middle line) and interquartile range. Whisker lines indicate non-outlier maximum and minimum values. A small jitter is included for better visualization. Cases and controls are defined as patients with moderate/severe and mild/no chronic GVHD, respectively.

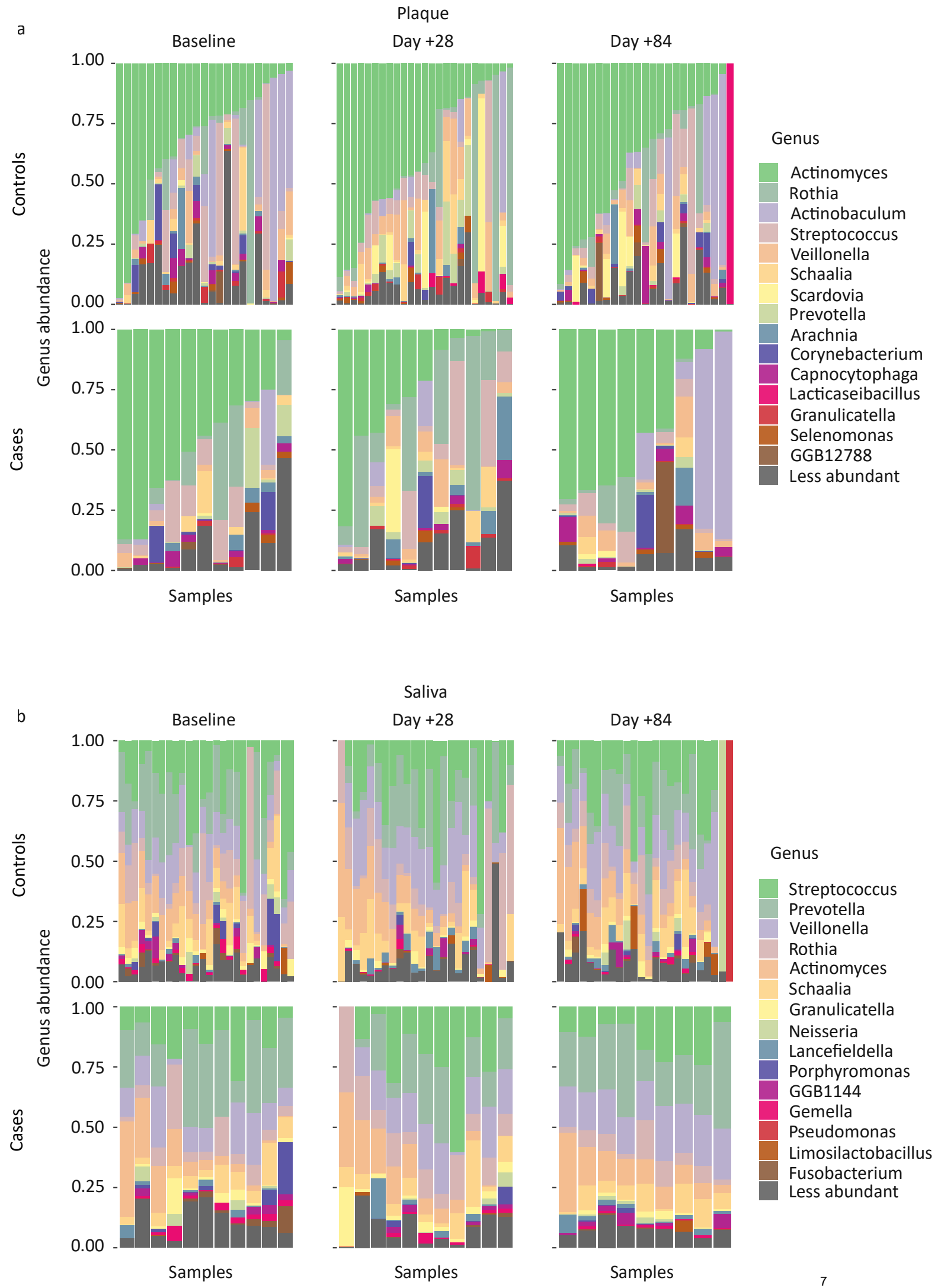
a
Plaque
Baseline
Day +28
Day +84
Controls
Cases
Genus abundance
1.00
0.75
0.50
0.25
0.00
Samples
Genus
Actinomyces
Rothia
Actinobaculum
Streptococcus
Veillonella
Schaalia
Scardovia
Prevotella
Arachnia
Corynebacterium
Capnocytophaga
Lacticaseibacillus
Granulicatella
Selenomonas
GGB12788
Less abundant
b
Saliva
Baseline
Day +28
Day +84
Controls
Cases
Genus abundance
1.00
0.75
0.50
0.25
0.00
Samples
Genus
Streptococcus
Prevotella
Veillonella
Rothia
Actinomyces
Schaalia
Granulicatella
Neisseria
Lancefieldella
Porphyromonas
GGB1144
Gemella
Pseudomonas
Limosilactobacillus
Fusobacterium
Less abundant

Fig. S2: Microbiota composition

(**a**) Relative abundance of the 15 most abundant genera in baseline (left), day +28 (middle), and day +84 (right) supragingival plaque microbiota among controls (top) and cases (bottom). (**b**) Relative abundance of the 15 most abundant genera in baseline (left), day +28 (middle), and day +84 (right) salivary microbiota among controls (top) and cases (bottom). Each column indicates a sample. The bars show relative abundances of different genera. Cases and controls are defined as patients subsequently developing moderate/severe and mild/no chronic GVHD, respectively.

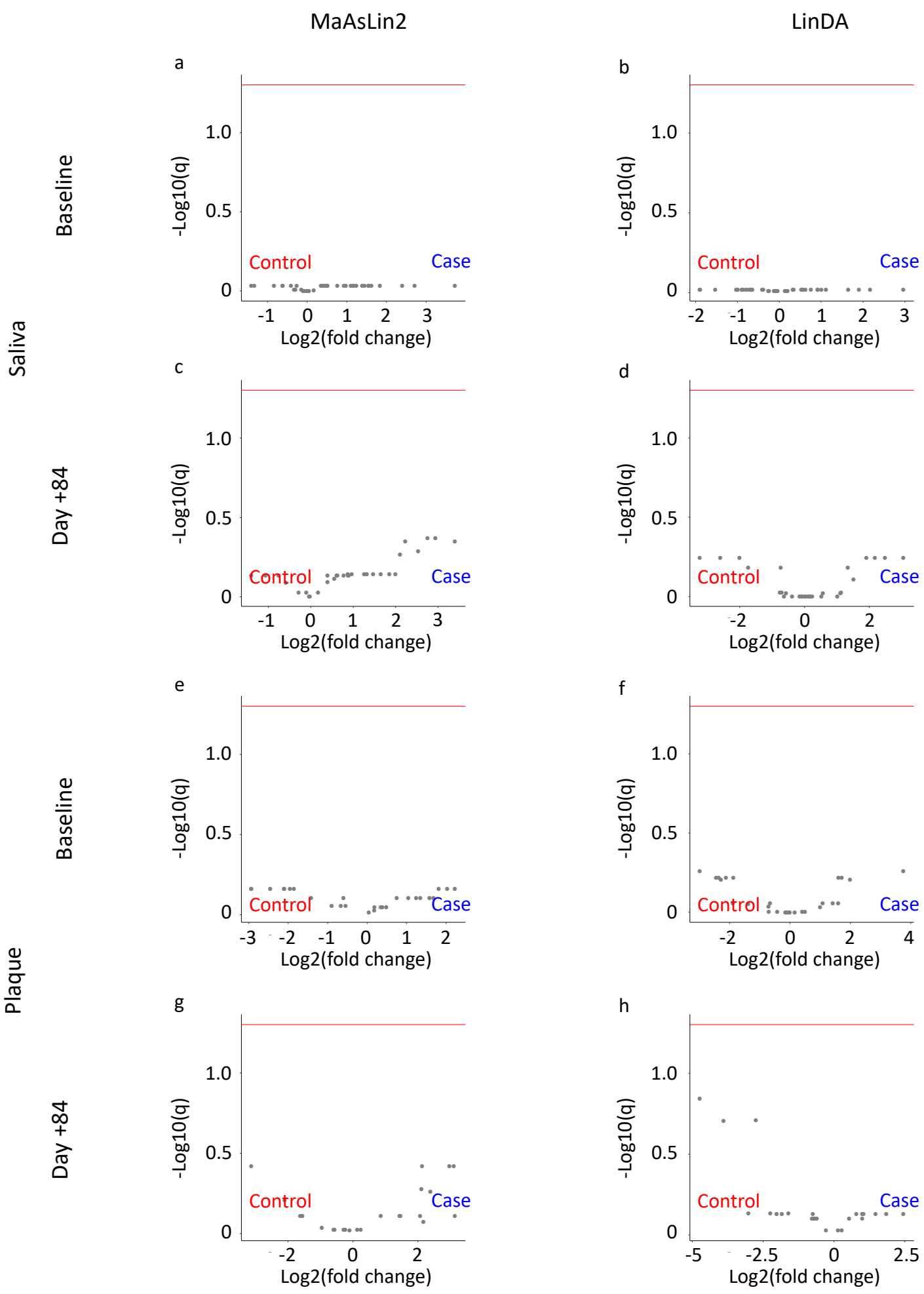
MaAsLin2
LinDA
Saliva
Plaque
Baseline
Day +84
a
b
c
d
e
f
g
h
-Log10(q)
Log2(fold change)
Control
Case

Fig. S3: Differential abundance analysis for salivary and plaque microbiota at baseline and day +84

(**a-d**) Volcano plots showing the results of differential abundance analysis for salivary microbiota at baseline (panels a-b) and day +84 (panels c-d). (**e-h**) Volcano plots showing the results of differential abundance analysis for supragingival plaque microbiota at baseline (panels e-f) and day +84 (panels g-h). Results from MaAsLin2 are shown on the left (panels a, c, e, and g); those from LinDA are shown on the right (panels b, d, f, and h). Each point shows a species. Points above the horizontal line (-log10(0.05) = 1.30 and corresponding to q = 0.05) are statistically significant. Species to the right (left) are enriched among cases (controls). To derive q values, P values were corrected using the Benjamini-Hochberg method. Cases and controls are defined as patients with moderate/severe and mild/no chronic GVHD, respectively.

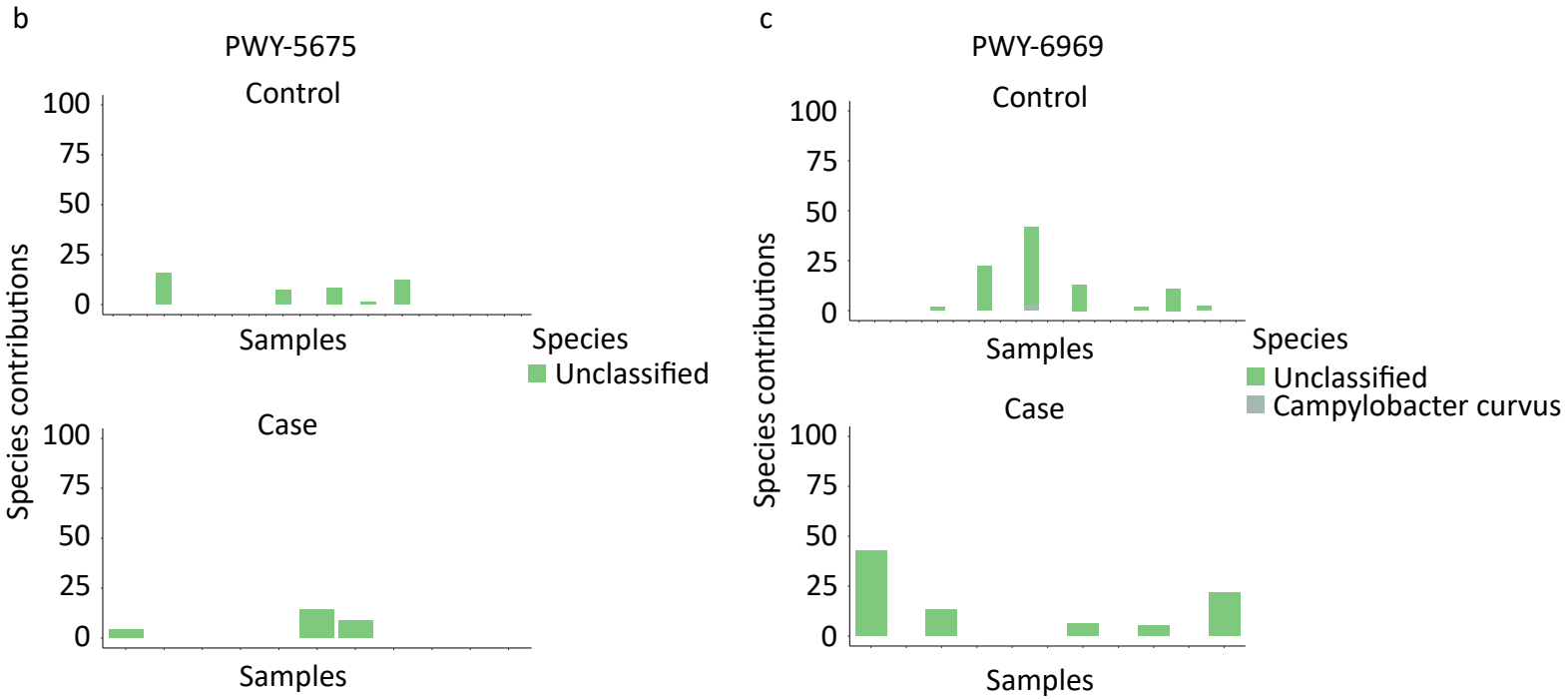
a
PWY0-1479
Control
Case
Species contributions
Samples
100
75
50
25
0
Species
Unclassified
Neisseria elongata
Klebsiella oralis
Neisseria sp oral taxon 014
Neisseria macacae
Haemophilus parainfluenzae
Eikenella corrodens
Neisseria sicca
Neisseria mucosa
Morococcus cerebosus
Haemophilus sp C1
Neisseria cinerea
b
PWY-5675
Control
Case
Species contributions
Samples
Species
Unclassified
c
PWY-6969
Control
Case
Species contributions
Samples
Species
Unclassified
Campylobacter curvus

Fig. S4: Species contributions to the other enriched pathways in Figure 4a
Of the 5 pathways enriched in day +28 plaque microbiota of cases (Fig. 4a), species contributions to two (PWY0-1477 and GOLPDLCAT-PWY) are shown in Fig. 4b-c. Species contributions to the other 3 pathways are shown here. Each panel shows data for 1 pathway. HUMAnN's pathway abundance tables (normalized to copies per million) include the number of copies of the entire pathway of interest assigned to each species. These numbers were used to quantify species contributions. Each column is a sample. The top panels show controls and the bottom panels show cases. Cases and controls are defined as patients with moderate/severe and mild/no chronic GVHD, respectively.

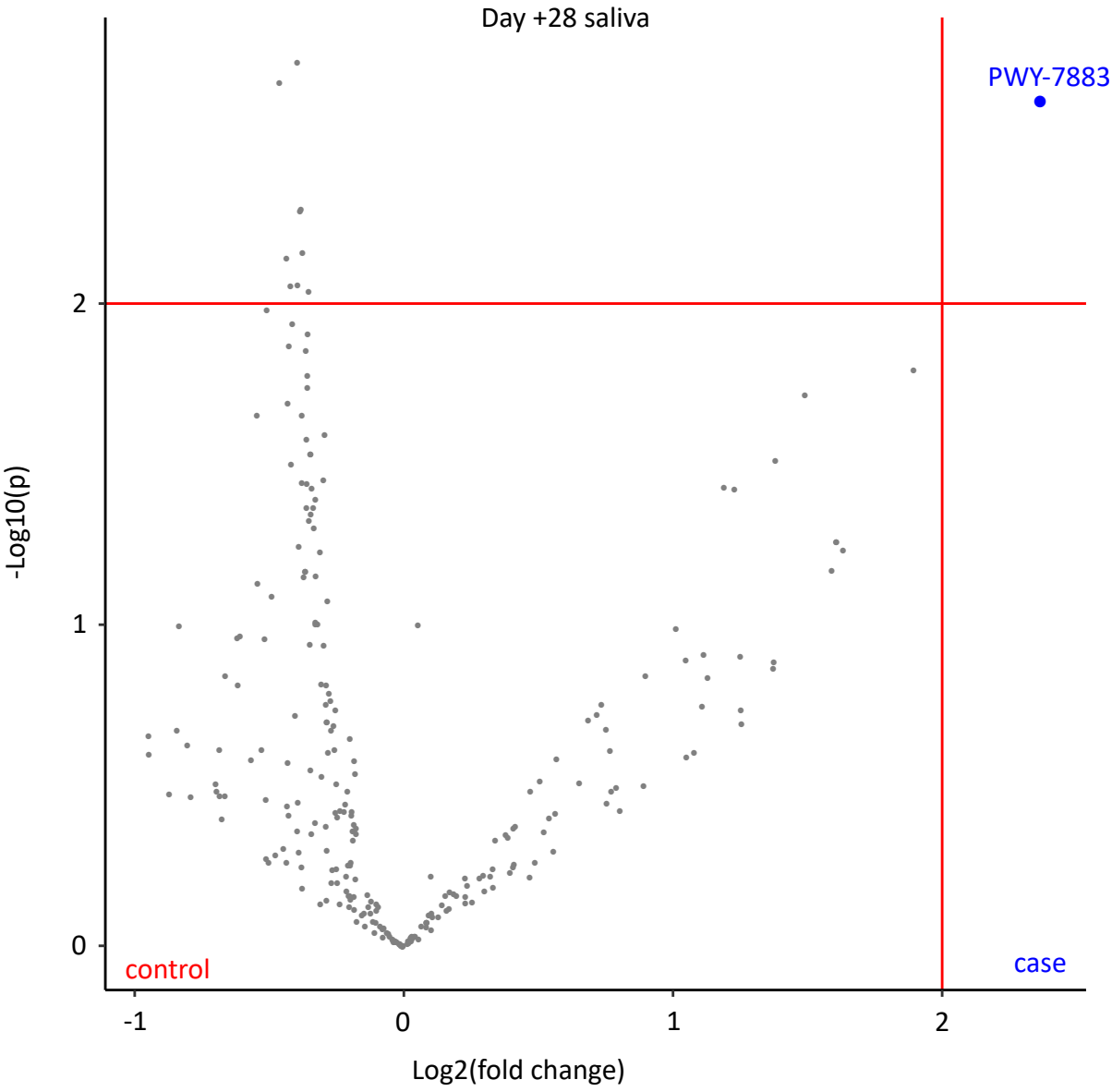
Day +28 saliva
PWY-7883
2
1
0
-Log10(p)
control
case
-1
0
1
2
Log2(fold change)

Fig. S5: Microbiota functional pathways in day +28 saliva and chronic GVHD
Volcano plot showing the results of differential abundance analysis for day +28 salivary microbiota using MaAsLin2. Each point shows a pathway. Points above the horizontal red line (-log10(0.01) = 2 and corresponding to P = 0.01) are statistically significant. Species to the right (left) are enriched among cases (controls). The vertical red line narrows down the enriched species to those with more substantial enrichment, defined as having more than two-fold abundance among cases. Cases and controls are defined as patients with moderate/severe and mild/no chronic GVHD, respectively.

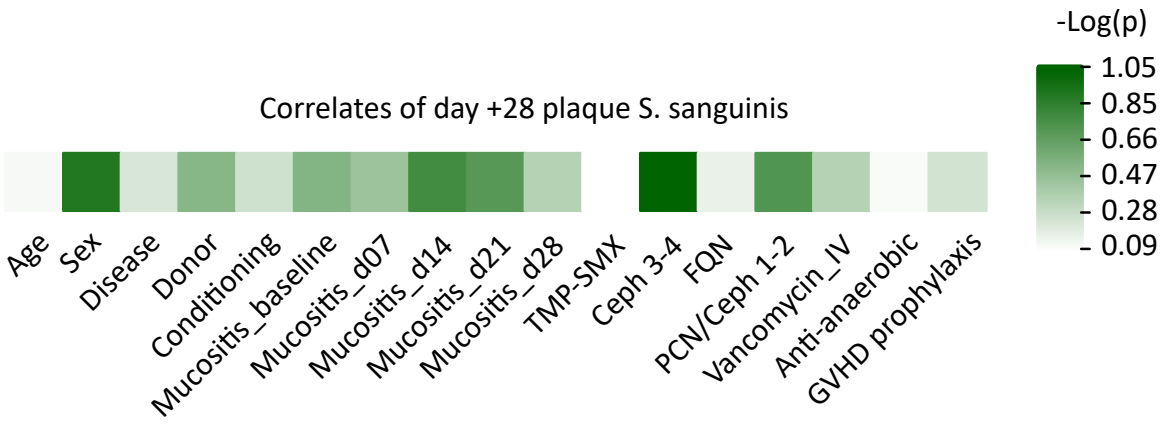
Correlates of day +28 plaque S. sanguinis
-Log(p)
1.05
0.85
0.66
0.47
0.28
0.09
Age
Sex
Disease
Donor
Conditioning
Mucositis_baseline
Mucositis_d07
Mucositis_d14
Mucositis_d21
Mucositis_d28
TMP-SMX
Ceph 3-4
FQN
PCN/Ceph 1-2
Vancomycin_IV
Anti-anaerobic
GVHD prophylaxis

Fig. S6: Baseline and early post-transplant clinical correlates of day +28 plaque *Streptococcus sanguinis*

Correlation plot showing log10-transformed P values along the color gradient for the association between baseline and early post-transplant clinical variables and *Streptococcus sanguinis* relative abundance in day +28 supragingival plaque. None of the variables reached statistical significance. A Wilcoxon's test was used for the categorical variables and Spearman's correlation test for continuous variables. Disease: acute leukemia vs. others. Donor type (multicollinear with graft source): HLA-matched donor vs. others. Conditioning: High-dose TBI-based vs. chemotherapy-based. Mucositis severity at each timepoint was derived from the quantitative OMAS score (total mucositis score). Antibiotic exposures are binary (0: not exposed; 1: exposed) and defined for the interval between day -7 and day +28. Ceph 1-2: first or second generation cephalosporins; Ceph 3+: third or higher generation cephalosporins; FQN: fluoroquinolones; PCN: penicillins; TMP-SMX: trimethoprim-sulfamethoxazole