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

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Abstract

Microbiota disruptions have been associated with short-term complications after allogeneic hematopoietic cell transplantation (alloHCT). However, only a few studies have examined the relationship between dysbiosis and chronic graft-*versus*-host disease (cGvHD), the main long-term immunologic toxicity of alloHCT. Considering the role of oral microbiota in systemic inflammatory diseases, we evaluated whether oral microbiota at day 28 post HCT corresponding to clinical recovery from the acute events after transplantation is associated with subsequent cGvHD. Shotgun metagenomic sequencing of 207 saliva and supragingival plaque samples collected longitudinally at baseline (pre-conditioning), day +28, and day +84 from 37 patients (11 with subsequent moderate/severe cGvHD) revealed a significant association between day +28 plaque microbiota composition and cGvHD. Two orthogonal statistical approaches demonstrated *Streptococcus sanguinis* and *Prevotella loescheii* in day +28 plaque to be associated with cGvHD. Metagenome-based functional analysis identified 4 microbial metabolic pathways associated with future cGvHD, 2 of which were highly attributed to *S. sanguinis*. These pathways – ethanolamine utilization and glycerol metabolism – increase bacterial fitness by providing an alternative carbon/nitrogen source and improving survival in inflamed tissues. Our findings propose a novel mechanism by which the early post-transplant dental biofilm may contribute to cGvHD months later, offering a potential target for early prophylactic intervention.

Introduction

Allogeneic hematopoietic cell transplantation (alloHCT) is a potentially curative treatment for many patients with hematologic malignancies. Hematopoietic cells obtained from a donor are infused to the patient following a conditioning regimen – typically consisting of one or more chemotherapeutic drugs with or without total body irradiation – varying in intensity from non-myeloablative to reduced-intensity to myeloablative. The immunologic attack mounted by the new immune system against allogeneic tumor antigens mediates the graft-*versus*-tumor effect, ultimately leading to eradication of cancer and cure in successful cases. Despite its high curative potential, alloHCT is associated with several short- and long-term toxicities. Common short-term toxicities include oral and intestinal mucositis related to conditioning-induced damage to epithelial tissues, infections, and acute graft-*versus*-host disease (aGvHD). The prototype chronic toxicity of alloHCT is chronic GvHD (cGvHD), a systemic inflammatory condition with significant morbidity that occurs in 20-40% of alloHCT recipients despite immunosuppressive prophylaxis.[1] In addition to various toxicities to host tissues, alloHCT is commonly associated with disruptions to the intestinal and oral microbiota.[2-4] Most of these disruptions occur in the first few weeks after alloHCT due to heavy antibiotic use to prevent and treat infections.

Prevention and treatment of cGvHD are currently based on the use of immunosuppressive medications which increase the risk of infections, among other short- and long-term toxicities. Known risk factors for cGvHD include donor/recipient HLA mismatch, use of peripheral blood as the

stem cell source, female-to-male transplant, older patient age, and acute GvHD (aGvHD).[5-8] Other than aGvHD, none of these risk factors can be modified post transplantation. If modifiable clinical or laboratory features predictive of higher risk for cGvHD based on each patient's experience in the early post-transplant period could be identified, personalized preventative strategies may be developed to prevent cGvHD in high-risk patients.

Growing evidence suggests that early post-transplant disruptions to the microbiota (a.k.a. dysbiosis), especially the colonic microbiota, influence the risk for aGvHD.[9-13] Fewer studies have examined the potential involvement of the gut microbiota in cGvHD pathogenesis,[14-17] and even fewer have focused on the oral microbiota.[18-20] The contribution of oral microbiota to systemic inflammatory diseases such as rheumatoid arthritis (RA) is increasingly recognized. Oral bacteria can reach distant organs and cause inflammation via two pathways: (i) direct hematogenous spread from the mouth;[21,22] and (ii) translocation to the gut[23,24] via the swallowed saliva or the lymphatics.[20,25] In the setting of alloHCT, oral mucositis resulting from transplant conditioning facilitates bacterial translocation through mucosal breaks into the circulation. Indeed, oral streptococci are among the most common causes of bacteremias after alloHCT.[26] In addition, in a murine cGvHD model where mice with oral dysbiosis (induced by ligature) were used as allograft recipients, oral enterococci migrated to the draining cervical lymph nodes, activating local antigen-presenting cells and increasing alloreactive T-cell proliferation.[20]

To advance this knowledge and examine the potential role of the oral microbiota in cGvHD pathogenesis in humans, we focused on supragingival plaque microbiota at day +28 of alloHCT in patients who received myeloablative conditioning. We made these choices because: (i) Day +28 represents an early post-engraftment timepoint when most acute toxicities of conditioning have resolved, antibacterial antibiotics have been discontinued, and enteral intake has been re-established.[27] New biofilm with potentially different microbiota composition from baseline has started to form by this point;[27] (ii) supragingival plaque can serve as a reservoir of pathogens for the subgingival plaque where the species are closer to the gingival mucosa and can more easily reach the submucosa;[28] (iii) most patients receiving myeloablative conditioning develop oral mucositis,[29,30] increasing the likelihood of systemic bacterial translocation. We assumed that the accumulation of putative pathogens in the supragingival plaque would partly mirror the evolution of the subgingival plaque where microbiota-host interaction may have implications for subsequent cGvHD. Thus, we chose supragingival plaque, hereafter referred to simply as plaque, as the focus of our microbiota analysis. In addition, we sequenced the salivary microbiota as a control. Longitudinal samples at two additional timepoints (baseline and day +84) were included to permit assessment of microbiota dynamics and evaluation of whether day +28 findings extend to earlier or later timepoints.

The specific hypothesis we tested in this work was whether the composition of plaque microbiota at day +28 is associated with subsequent risk for cGvHD. Using shotgun metagenomic sequencing, we find *Streptococcus sanguinis* and *Prevotella loescheii* to be associated with future moderate or severe cGvHD. Metagenome-based functional analysis identifies two microbial metabolic pathways increasing bacterial virulence and fitness, as putative mechanisms for *S. sanguinis* contribution to cGvHD pathogenesis.

Methods

Study design and procedures

We used samples from our umbrella protocol of longitudinal multi-site oral samples combined with mucositis assessment data, a prospective single-center collaborative study between HCT and Oral Medicine teams at Fred Hutchinson Cancer Center (FHCC; Seattle, WA, USA). The protocol was approved by FHCC's Institutional Review Board (#RG1123080). (See Figure 1A for a summary of the protocol.) The recruitment period was between March 2023 and April 2024. All patients provided written informed consent, and study procedures were in accordance with the Declaration of Helsinki. The only eligibility criteria were age >18 years and a myeloablative alloHCT. Day 0 was defined as the day of stem cell infusion. The baseline visit was performed by an Oral Medicine specialist during the standard transplant workup and before the initiation of conditioning. Details of the standard pre-transplant Oral Medicine evaluation in our center are provided in the *Online Supplementary Methods*. Baseline samples were collected during this visit. We refer most patients without a recent dental cleaning for cleaning following the baseline visit. Supragingival plaque is thoroughly removed during the cleaning visit. For the present work, we selected the subset of patients with saliva and plaque samples at baseline, day +28, and day +84. Patients with relapse before cGvHD or non-GvHD death by one year post transplant were not included.

Chronic graft-*versus*-host disease was diagnosed and graded according to the National Institutes of Health (NIH) Consensus Criteria.[31] Cases were defined as patients developing moderate or severe cGvHD by one year post transplant; controls were defined as those with no or mild GvHD. Details of mucositis assessment and sample collection/processing are provided in *Online Supplementary Methods*. The microbiota was profiled to the species level using shotgun metagenomic sequencing. (See the *Online Supplementary Methods* for details.)

Statistical and bioinformatic analysis

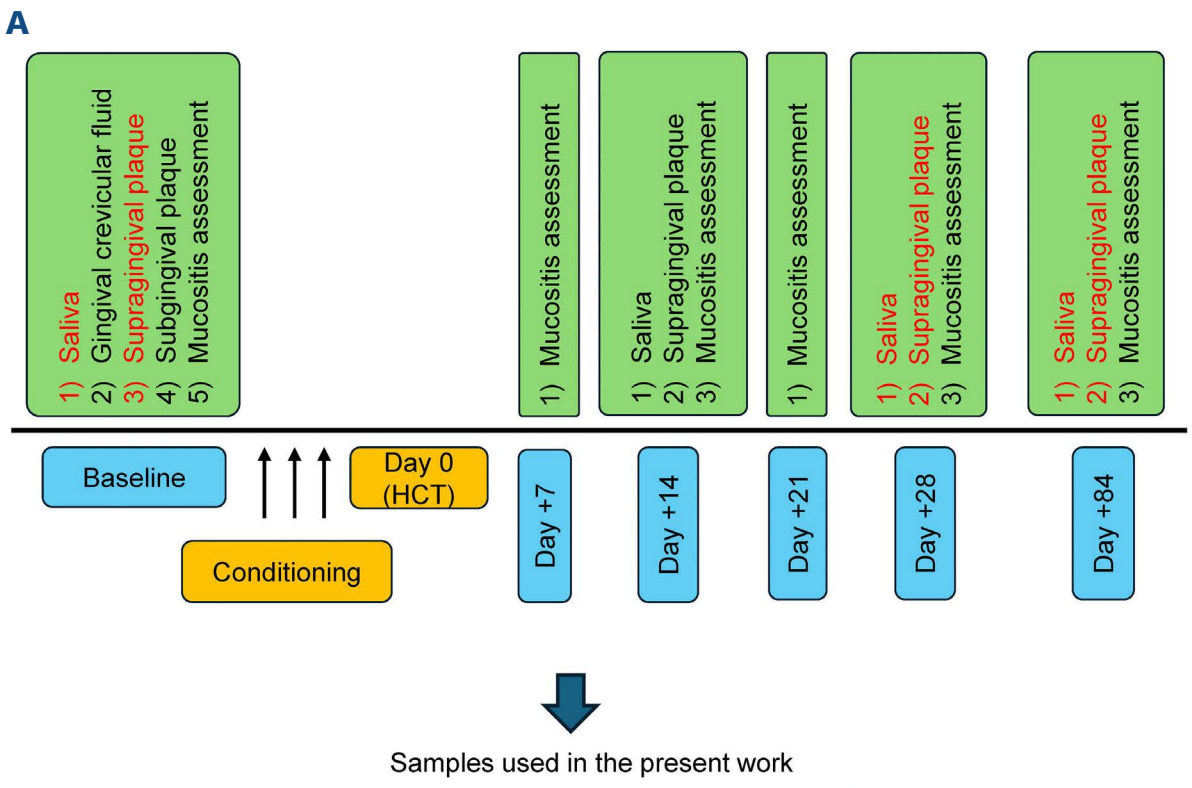
All eligible patients and their baseline, day +28, and day +84 saliva and plaque samples were included, with no power or sample size calculation. Microbiota analysis was performed

using species-level data. Within-sample microbiota diversity (i.e., alpha diversity) was quantified by Shannon index.[32] Between-sample microbiota diversity (i.e., beta diversity) was quantified by Aitchison distance.[33] Ordination was visualized by principal co-ordinate analysis using the first two axes and differences between cases and controls in the overall microbiome composition were statistically examined using an adonis test with 999 permutations. To identify differentially abundant species between cases and controls, two orthogonal approaches were used: (i) general linear model-based Microbiome Multivariable Association with Linear Models (MaAsLin2),[34] using species relative abundance and prevalence thresholds of 1% and 10%, respectively. Other than a stricter significance threshold of 0.05 than the software's default for Benjamini-Hochberg corrected *P* values[35] (q values), default parameters were used; and (ii) LinDA,[36] which fits linear regression models on centered log-ratio transformed data and corrects the bias due to compositional effects. To identify differentially abundant pathways between cases and controls, CPM-normalized pathways were first filtered using relative abundance and prevalence thresholds of 0.01% and 10%, respectively, and then analyzed using MaAsLin2. A *P* value threshold of 0.01 and effect size (i.e., enrichment coefficient) threshold of 2 were used to identify the pathways most likely relevant for the species-cGVHD associations observed.

Continuous variables were compared between the groups using a Wilcoxon's test. Correlations were evaluated using a Spearman's correlation test. All analyses were performed in R 4.2.0.

Results

Thirty-seven eligible patients were included (Table 1), including 11 cases (moderate or severe cGvHD) and 26 controls (no or mild cGvHD). The only significant difference between the groups was a higher proportion of female to male transplants among cases (*P*=0.02). The only death event was a cGvHD-related death at ten months post transplant. A total of 207 samples were profiled and analyzed (Figure 1A). Antibacterial antibiotic exposures between day -7 and day +28 are shown in Figure 1B. Fifteen (41%) patients developed grade II-IV acute GvHD. Five of these patients also developed cGvHD (cases). The remaining 6 patients with cGvHD (cases) did not have a history of grade II-IV aGvHD. The median time to onset of cGvHD was six months (range: 3-12 months). In 4 patients with cGvHD, only one organ was involved, while in the remaining 7 patients, multiple organs were involved. Overall, the most commonly affected organ with cGvHD was the mouth (9 patients), followed by the skin (5 patients), eyes (4 patients), and genitalia (4 patients). The median number of involved organs was 3 (range: 1-4).



	Baseline	Day +28	Day +84
Saliva	37 (11 cases, 26 controls)	35 (11 cases, 24 controls)	33 (9 cases, 24 controls)
Plaque	34 (11 cases, 23 controls)	36 (11 cases, 25 controls)	32 (9 cases, 23 controls)

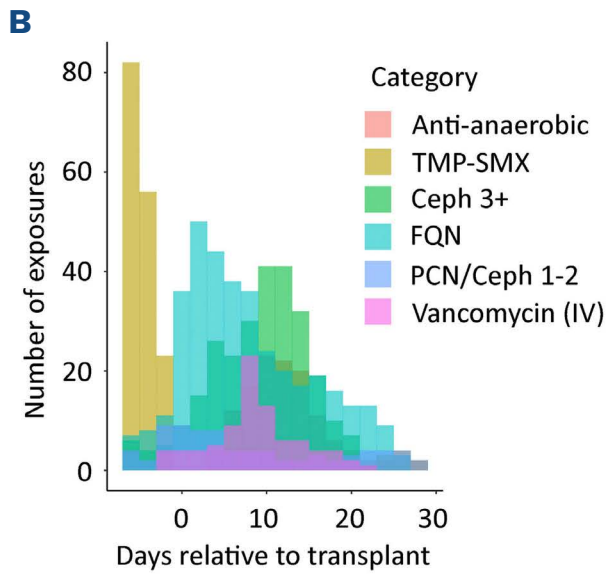


Figure 1. Study schema and antibiotic exposures. (A) Study schema for the umbrella protocol. Samples used in the present analysis are shown in red. (B) Antibacterial antibiotic exposures between days -7 and +28. The 6 most commonly used antibiotics are shown. Ceph 1-2: first or second generation cephalosporins; Ceph 3+: third or higher generation cephalosporins; FQN: fluoroquinolones; HCT: hematopoietic cell transplant; PCN: penicillins; TMP-SMX: trimethoprim-sulfamethoxazole.

Day +28 plaque microbiota is associated with the risk of future chronic graft-*versus*-host disease

A recent study reported a higher risk for cGvHD in patients with more severe oral mucositis in the first month after alloHCT.[20] Consistent with previous work by others, oral mucositis in our patients peaked in the second week after transplantation.[37] Therefore, we compared cases and controls for total mucositis score (derived from the comprehensive OMAS scoring system) at days +7 and +14 separately. No significant differences were observed between cases and controls in these comparisons (*Online Supplementary Figure S1A, B*). However, the maximum total score (over days +7 and +14) was higher among cases, though not statistically significantly (*P*=0.15) (*Online Supplementary Figure S1C*).

The number of high-quality microbial reads was >0.5M for all samples, with a median of 13.2M (mean 15.2M). A total of 452 species were identified (plaque: 373 species; saliva: 381 species). The distribution of the 15 most abundant genera in samples of each type at each timepoint, grouped by cases and controls, is shown in *Online Supplementary Figure S2*. No significant differences were found between cases and controls in saliva or plaque microbiota diversity at baseline (plaque: *P*=0.83; saliva: *P*=0.18) or at day +28 (plaque: 0.08; saliva: *P*=0.11). At day +84, there was no difference in plaque microbiota diversity between cases and controls (*P*=0.74), but salivary microbiota diversity was higher in cases compared to controls (*P*=0.02) (Figure 2A-F). There was a significant difference in microbiota composition

Table 1. Patient characteristics.

Characteristic	Cases	Controls	Total	*P*
Total, N	11	26	37	-
Sex, N (%) Male Female	 5 (45) 6 (55)	 10 (38) 16 (62)	 15 (41) 22 (59)	0.73
Median age, years (range)	43 (20-60)	41 (24-55)	43 (20-60)	0.40
Female to male transplant, N (%)	5 (45)	2 (8)	7 (19)	0.02
Underlying disease, N (%) Acute leukemia Other	 8 (73) 3 (27)	 22 (85) 4 (15)	 30 (81) 7 (19)	0.40
Conditioning regimen, N (%) Cy/TBI, 12 Gy Flu/Cy/TBI, 12-13.2 Gy Flu/Thiotepa/TBI, 13.2 Gy Bu/Cy Flu/Bu4 Flu/Bu4/TBI, 4 Gy Flu/Cy/Thiotepa/TBI, 4 Gy	 5 (46) 0 0 4 (36) 2 (18) 0 0	 10 (38) 1 (4) 1 (4) 4 (15) 4 (15) 3 (12) 3 (12)	 15 (41) 1 (3) 1 (3) 8 (22) 6 (16) 3 (8) 3 (8)	1.00
Donor type, N (%) HLA-matched sibling HLA-matched unrelated Cord blood	 6 (55) 5 (45) 0	 5 (19) 17 (65) 4 (15)	 11 (30) 22 (59) 4 (11)	0.07
Graft source, N (%) Peripheral blood Cord blood	 11 (100) 0	 22 (85) 4 (15)	 33 (89) 4 (11)	0.30
GvHD prophylaxis, N (%) Tacrolimus/methotrexate PTCy-based Cyclosporine/MMF	 10 (91) 1 (9) 0	 17 (65) 5 (19) 4 (15)	 27 (73) 6 (16) 4 (11)	0.36
Grade II-IV acute GvHD, N (%) Onset by day +28	6 (55) 2	9 (35) 4	15 (41) 6	0.30
Use of total parenteral nutrition, N (%)	3 (27)	14 (54)	17 (46)	0.17

Cases and controls are defined as patients with moderate/severe and mild/no chronic graft-*versus*-host disease (GvHD), respectively. Some of the percentages do not sum up precisely to 100% due to rounding. *P* values were derived from a Fisher's exact test for categorical variables and a Wilcoxon's test for continuous variables. Bu: busulfan; Cy: cyclophosphamide; Flu: fludarabine; HLA: human leukocyte antigen; MMF: mycophenolate mofetil; PTCy: post-transplantation cyclophosphamide; TBI: total body irradiation.

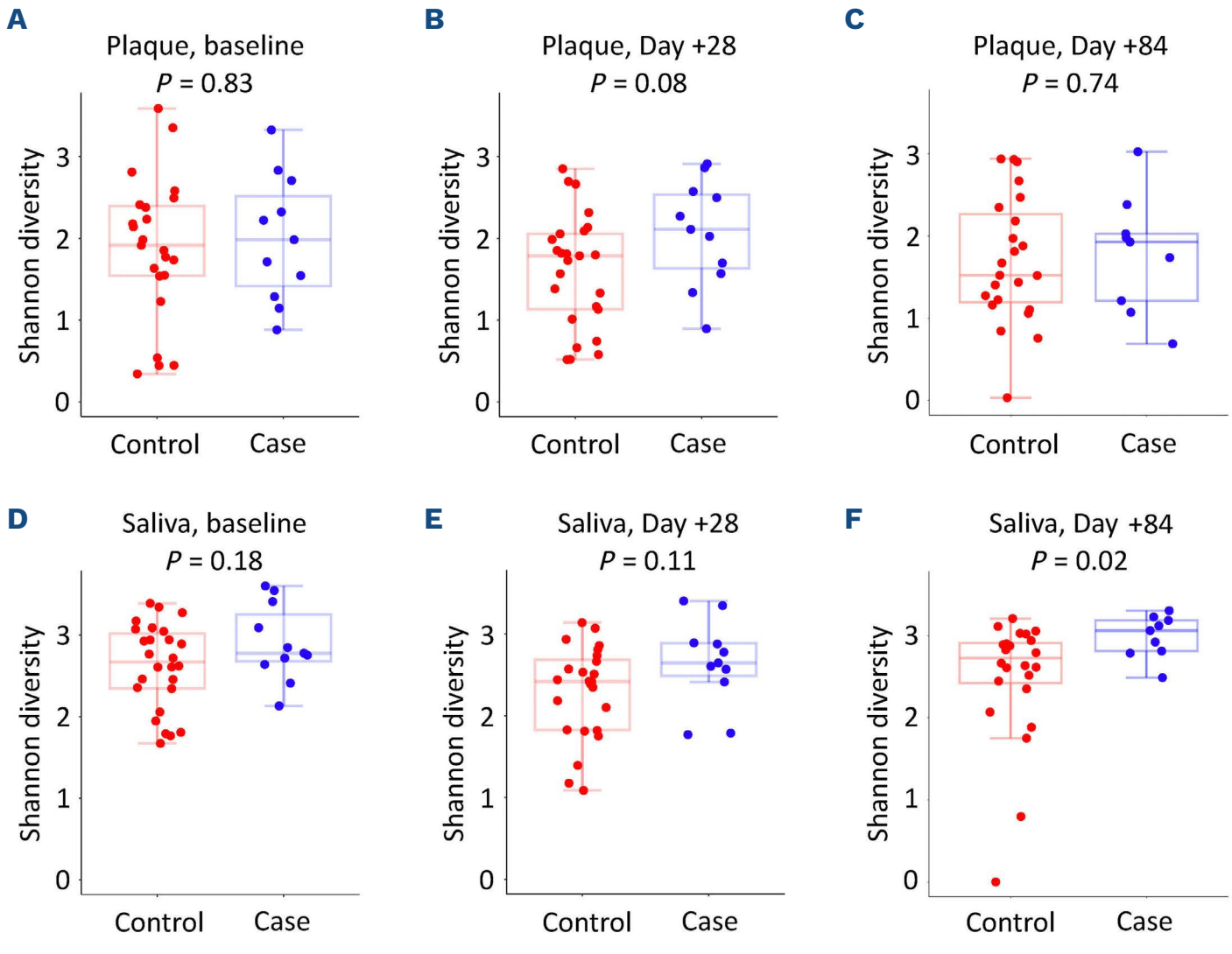


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Figure 2. Microbiota composition and chronic graft-*versus*-host disease. (A-F) Alpha diversity as measured by the Shannon's index, compared between cases and controls at baseline (supragingival plaque in A; saliva in D), day +28 (supragingival plaque in B; saliva in E), and day +84 (supragingival plaque in C; saliva in F). *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. (G-L) Principal co-ordinate analysis by applying principal component analysis on species-level Aitchison distances. Supragingival plaque microbiota is compared between cases and controls at baseline (G), day +28 (H), and day +84 (I); Salivary microbiota is compared between cases and controls at baseline (J), day +28 (K), and day +84 (L). The first two principal co-ordinates (PC1 and PC2) are shown, with numbers in parentheses indicating percent variation explained by the corresponding axis. *P* values are from an adonis test with 999 permutations. Cases and controls in all panels are defined as patients with developing moderate/severe and mild/no chronic graft-*versus*-host disease (GvHD), respectively.

between cases and controls for day +28 plaque (*P*=0.003) but not for baseline (*P*=0.57) or day +84 plaque (*P*=0.43) or for saliva at any timepoint (baseline: *P*=0.57; day +28: *P*=0.24; day +84: *P*=0.18) (Figure 2G-I). In differential abundance analysis using MaAsLin2 to identify enriched and depleted species in day +28 plaque microbiota that drove the overall microbiota compositional difference between cases *versus* controls, two species were enriched in cases (q < 0.05) (Figure 3A): *S. sanguinis* and *P. loescheii*. Differential abundance analysis using LinDA identified the same 2 species as the most significantly enriched species in day +28 plaque microbiota of cases, but also *Schaalia odontolytica* and *Granulicatella*_SGB8255 (q < 0.05 for all 4 species) (Figure 3B). Since *S. sanguinis* and *P. loescheii* were strongly associated with cGvHD in both models (Figure 3C, D), we focused on these 2 species in further analyses. *Online Supplementary Figure S3* shows the results of differential abundance analysis at baseline and day +84; no differentially abundant species were identified in either site at either timepoint.

Metagenome-based functional analysis identifies a putative mechanism for microbiota effects on chronic graft-*versus*-host disease risk

So far, our data demonstrated an association between

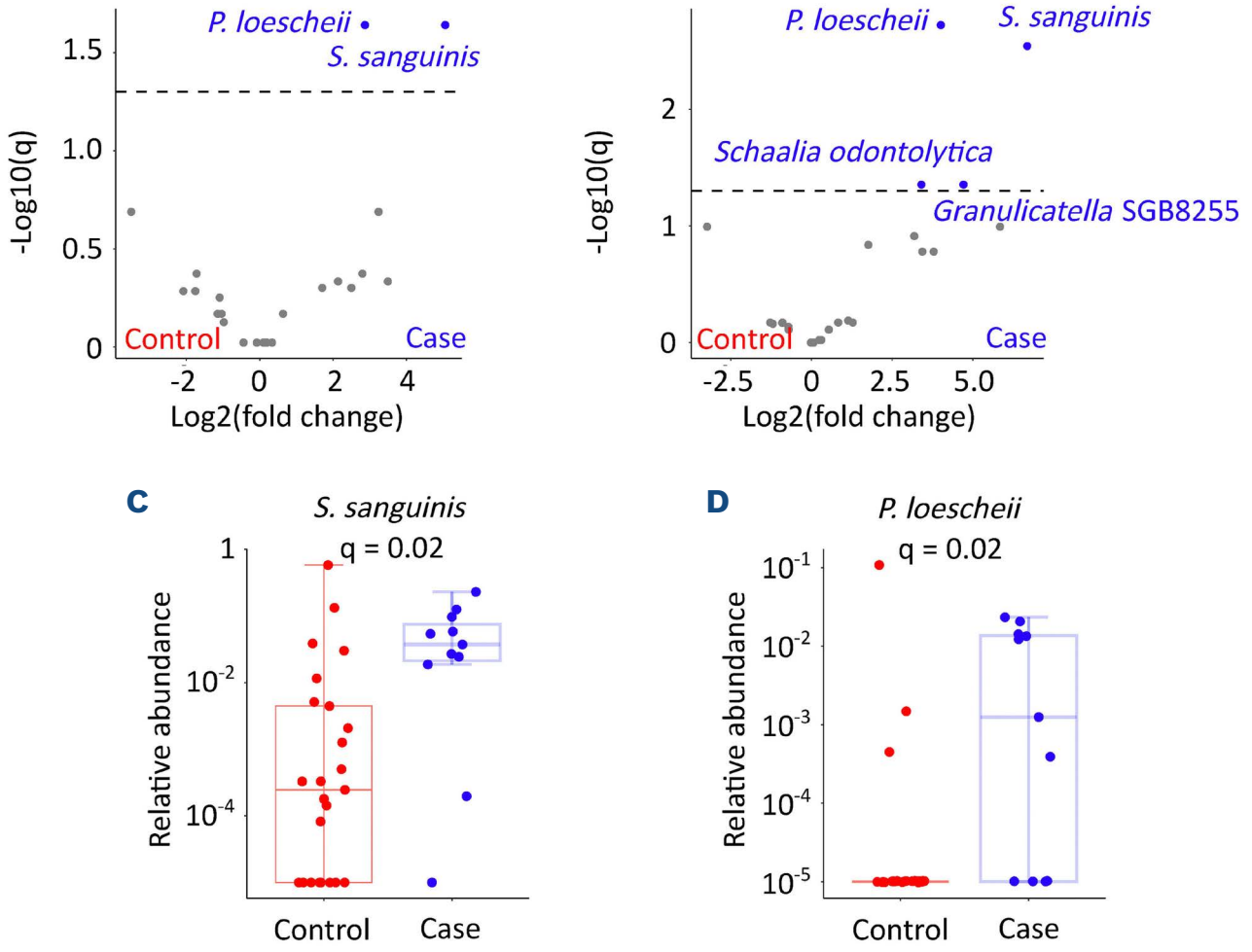
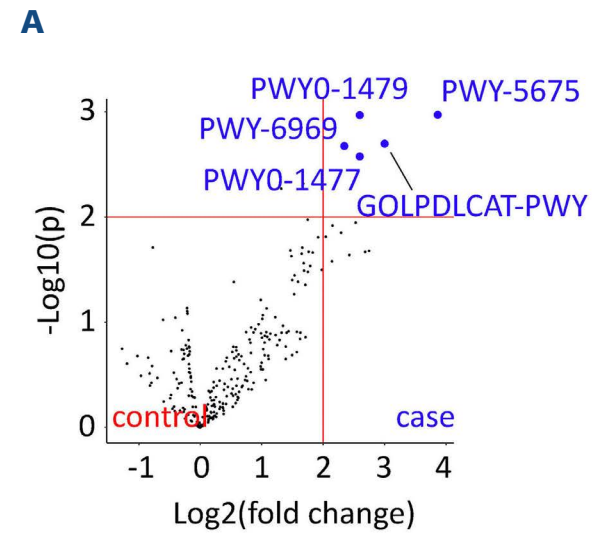
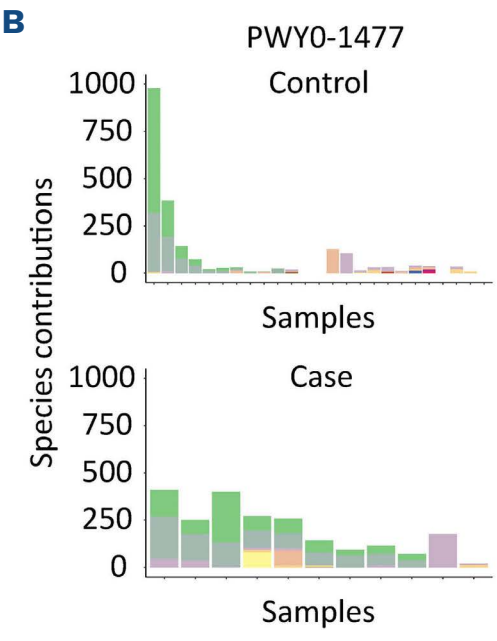
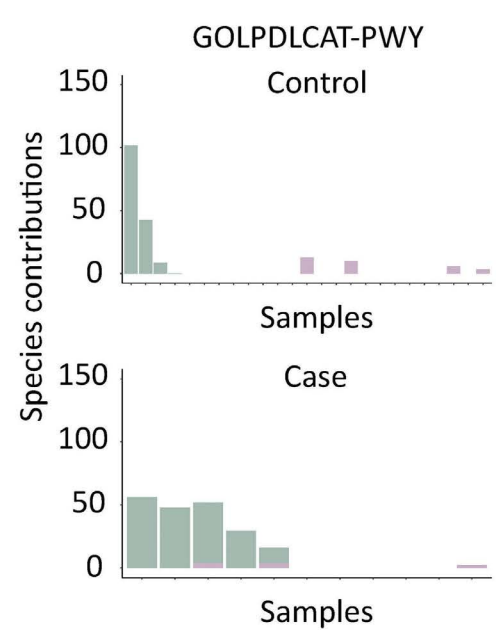
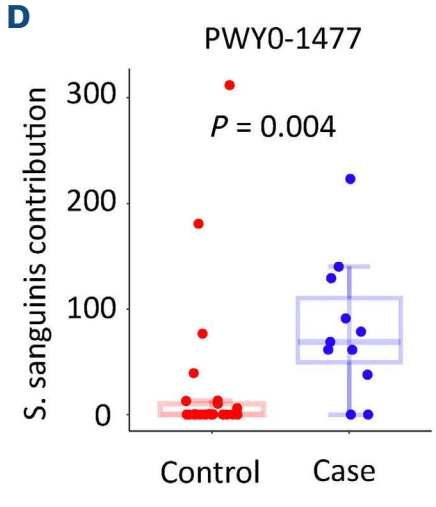


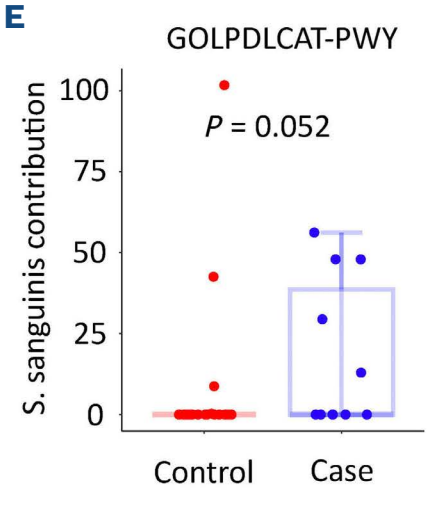
Figure 3. Differential abundance analysis for day +28 plaque microbiota. (A and B) Volcano plots showing the results of differential abundance analysis for day +28 plaque microbiota using MaAsLin2 (A) and LinDA (B). Each point shows a species. Points above the horizontal line (-log10(0.05) = 1.30 and 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. (C and D) Comparison between cases and controls for the relative abundance (in day +28 plaque microbiota) of the 2 species identified by both MaAsLin2 and LinDA. *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 graft-*versus*-host disease, respectively.

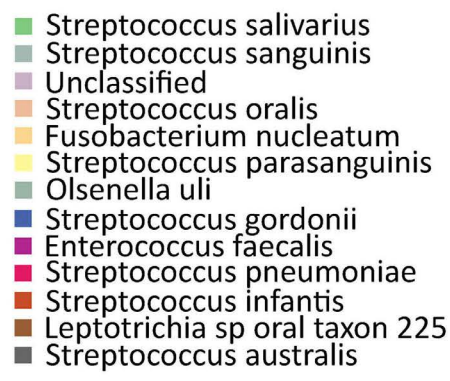


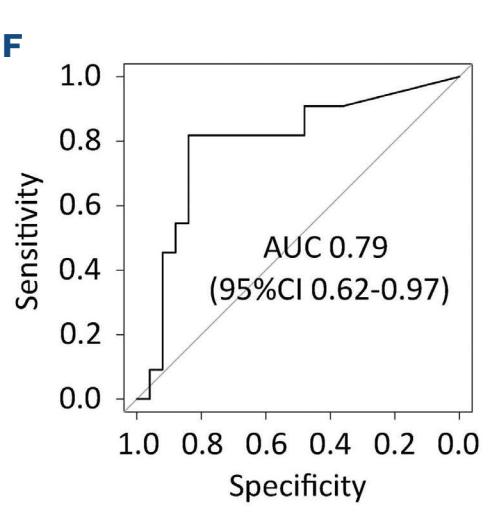


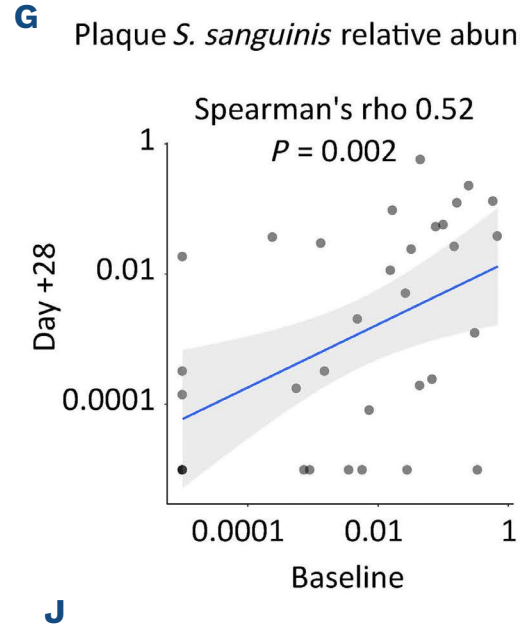


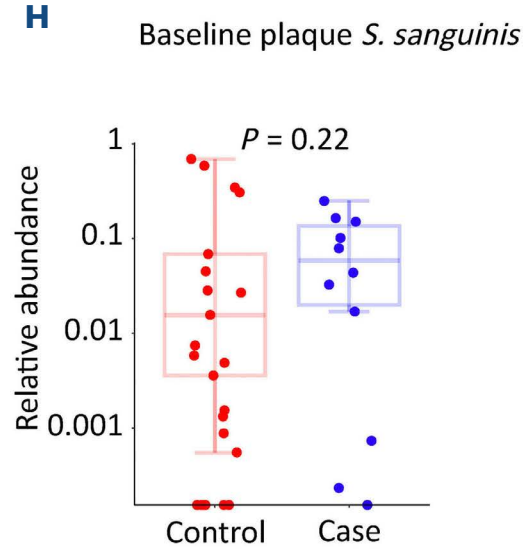


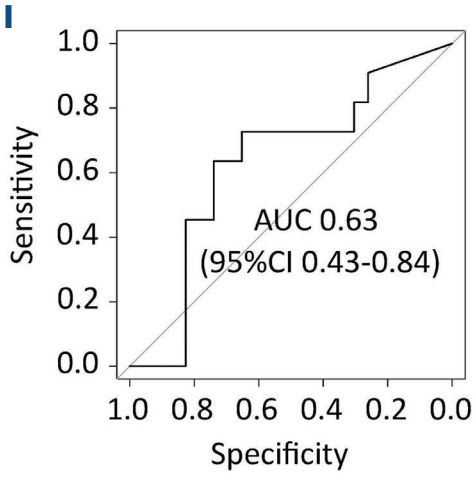


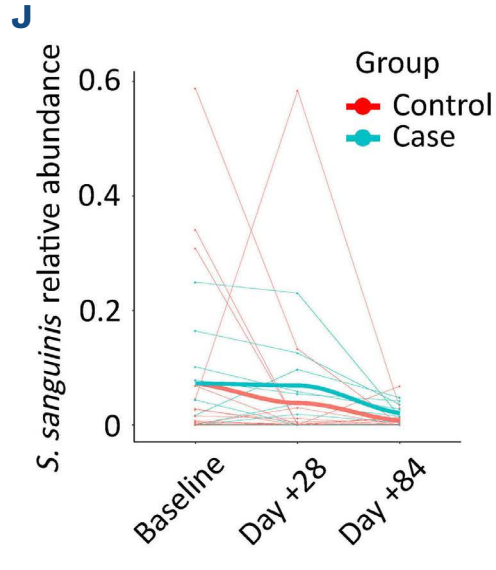












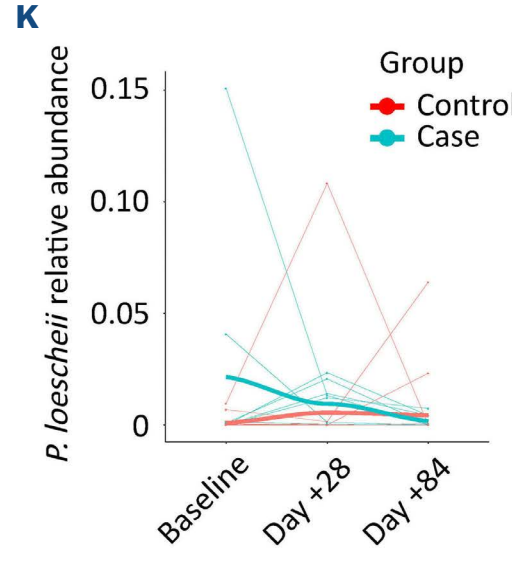


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Figure 4. Microbiota functional pathways and chronic graft-*versus*-host disease. (A) Volcano plot showing the results of differential abundance analysis for day +28 plaque microbiota using MaAsLin2. Each point shows a pathway. Points above the horizontal 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. (B and C) Two enriched pathways (among cases) in panel (A) were found in *S. sanguinis*. All species contributions to these pathways are shown. 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. *S. sanguinis* made the second top contributor to PWY0-1477 and the only classified contributor to GOLPDLCAT-PWY. (D and E) Contribution of *S. sanguinis* to the 2 pathways in panels B and C. (F) Receiver operating curve characteristic analysis for day +28 plaque *S. sanguinis* relative abundance predicting case *versus* control. The area under the curve (AUC) and its 95% Confidence Interval (CI) are shown. (G) Correlation between baseline and day +28 plaque *S. sanguinis* relative abundance in the same patient. The Spearman's correlation coefficient and its corresponding *P* value are shown. (H) Comparison between cases and controls for the relative abundance of *S. sanguinis* in baseline plaque microbiota. (I) Receiver operating curve characteristic analysis for baseline plaque *S. sanguinis* relative abundance predicting case *versus* control. The AUC and its 95%CI are shown. (J) *S. sanguinis* dynamics. Relative abundances of this species at each timepoint is shown, with samples from the same patient connected. A loess smoother is added for each cohort as a thicker line. (K) *P. loescheii* dynamics. Relative abundances of this species at each timepoint is shown, with samples from the same patient connected. A loess smoother is added for each cohort as a thicker line. Each box in panels (D, E and H) 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. *P* values in these panels are from a Wilcoxon's test. Cases and controls are defined as patients with moderate/severe and mild/no chronic graft-*versus*-host disease (GvHD), respectively.

day +28 plaque microbiota, especially *S. sanguinis* and *P. loescheii*, and future cGvHD. Next, we evaluated whether metagenome-based functional analysis can identify putative mechanisms for this novel association. HUMAnN-based functional analysis identified 5 pathways enriched in day +28 plaque (Figure 4A): PWY0-1477 (ethanolamine utilization), GOLPDLCAT-PWY (superpathway of glycerol degradation to 1,3-propanediol), PWY-5675 (nitrate reduction V [assimilatory]), PWY0-1479 (tRNA processing), and PWY-6969 (TCA cycle V). Per-sample species attributions of PWY0-1477 and GOLPDLCAT-PWY, the only pathways with *S. sanguinis* attribution, are shown in Figure 4B and C. *S. sanguinis* was the second most significant contributor to PWY0-1477 (Figure 4D) and the only classified contributing species to GOLPDLCAT-PWY (Figure 4E). *P. loescheii* had no contribution to any of the enriched pathways. Species contributions to the other enriched pathways are shown in *Online Supplementary Figure S4*. Together, these data suggest PWY0-1477 and/or GOLPDLCAT-PWY pathways as putative mechanisms by which *S. sanguinis* in day +28 plaque may increase the risk of subsequent cGvHD. The area under the curve (AUC) in receiver operating characteristic curves analysis for day +28 plaque *S. sanguinis* to predict subsequent cGvHD was 0.79 (95% Confidence Interval [CI]: 0.62-0.97) (Figure 4F), suggesting its potential value as a predictive biomarker. Importantly, the enriched pathways in the plaque were not enriched in the saliva (*Online Supplementary Figure S5*), supporting niche specificity of our findings.

S. sanguinis abundance in day +28 plaque depends on patient-intrinsic and post-transplant factors

Next, we asked whether specific patient or transplant characteristics including antibiotic exposures and mucositis severity were predictive of day +28 plaque *S. sanguinis* abundance. None of the variables analyzed were significant (*Online Supplementary Figure S6*). However, *S. sanguinis* abundance in baseline plaque was moderately correlated with *S. sanguinis* abundance in day +28 plaque from the same patient (Spearman's correlation coefficient 0.52, *P*=0.002) (Figure 4G). As patients without recent dental cleaning (27 patients; 73%) underwent thorough cleaning including plaque removal following baseline research sample collection, day +28 plaques represented *de novo* biofilm formation. Therefore, the higher abundance of a given species in day +28 plaque suggests a patient-specific tendency to accumulate more of that species (consistent with individuality of plaque microbiota[38]) combined with post-transplant factors (e.g., diet, oral hygiene) as other determinants. Importantly, although baseline and day +28 plaque *S. sanguinis* correlated, baseline levels were not associated with cGvHD (*P*=0.22) (Figure 4H) and had little predictive value (AUC 0.63, 95% CI: 0.43-0.84) (Figure 4 I). These findings suggest that day +28 plaque *S. sanguinis* abundance is personalized and depends on (unknown) post-transplant variables.

The dynamics of *S. sanguinis* and *P. loescheii* from baseline to day +28 to day +84 are shown in Figure 4J-K. *S. sanguinis* abundance declined between baseline and day +28 in the cohort without cGvHD but remained stable in the cGvHD cohort. Between days +28 and +84, the abundance of this species declined in both cohorts, while remaining higher in the cGvHD cohort.

Discussion

The microbiota has been repeatedly associated with short- and long-term toxicities of alloHCT. Here, we used our detailed clinical database of longitudinal multi-site oral mucositis assessments associated with a biorepository

including longitudinal salivary and dental plaque samples to examine whether the plaque microbiota at the time of recovery from the early phase of alloHCT (i.e., day +28) was associated with subsequent cGvHD. We used saliva as a control for sample type. For comparison purposes, samples of both types at baseline and day +84 were also included. We demonstrated that only the day +28 plaque microbiota was associated with cGvHD. Specifically, enrichment of day +28 plaque in *S. sanguinis* and *P. loescheii* was associated with a higher risk for future cGvHD. Metagenome-based functional analysis revealed two metabolic pathways increasing bacterial fitness as potential mechanisms for *S. sanguinis* association with cGvHD.

The role of oral microbiota in systemic inflammatory conditions has been established, most compellingly in RA.[39] Consistent with our findings in cGvHD, recent studies in RA point to streptococci as the main potential microbial contributor to pathogenesis. Streptococci are abundant members of the dental plaque, with *S. sanguinis* being a pioneer species and an early colonizer of the biofilm.[40] Through adherence to the enamel, *S. sanguinis* permits other species to colonize the biofilm, forming a complex structure.[41,42] Streptococci are enriched in the oral cavity of patients with RA and their poorly degraded cell wall peptidoglycan-polysaccharide polymers can induce proinflammatory cytokine production and arthritis in mice.[43] Oral mucosal breaks seem to be necessary for pathogenicity of oral bacteria in patients with RA, a process facilitated by periodontitis, which is particularly common in these patients.[44] Oral streptococci, citrullinated by neutrophils in the periodontal tissues, enter the bloodstream through a fragile mucosal barrier and activate inflammatory monocytes found in inflamed RA synovia. In addition, these bacteria stimulate B cells to make anti-citrullinated protein antibodies with cross-reactivity against citrullinated host autoantigens found in target joints.[45] Conditioning-related oral mucositis after myeloablative alloHCT can play the same role as periodontitis in patients RA by facilitating bacterial translocation. Although the association between mucositis severity and cGvHD risk did not reach statistical significance in our patients, there was a clear trend, consistent with the finding of moderate-to-severe oral mucositis as an independent risk factor for cGvHD in a recent study with a larger sample size (230 patients).[20] Whether plaque *S. sanguinis* is citrullinated in these patients and can be found in the circulation or cGvHD target organs is worthy of further research.

In a murine alloHCT model, and the only published mechanistic study of oral microbiota connection with cGvHD, ligature-induced oral dysbiosis and periodontitis exacerbated cGvHD. Expansion of the Enterococcaceae family in the mouth and its translocation to cervical lymph nodes activated antigen-presenting cells, promoting the expansion of donor-derived inflammatory T cells and worsening of cGvHD. Importantly, ligature removal or antibiotic therapy reversed the process, confirming causality.[20] In the companion human study reported along with this mechanistic murine work, buccal swab microbiota profiling of 31 alloHCT patients (16 with cGvHD) using short-amplicon sequencing showed a tendency for differences between the two groups in their family-level microbiota compositional changes from pre- to post-transplant timepoints. Using shotgun metagenomic sequencing which permitted species level resolution, we here found a significant association between day +28 plaque *S. sanguinis* and cGvHD. Associations between the oral microbiota and other post-alloHCT outcomes (e.g., survival, immune reconstitution, aGvHD, relapse of the underlying disease) have been demonstrated in recent work.[3,4,46]

Our sequencing platform also informed on microbial gene content and functional pathways, allowing us to identify a potential mechanism for our finding. The ethanolamine utilization pathway, enriched in patients with cGvHD, was strongly attributed to *S. sanguinis*. The main source of ethanolamine in humans is diet and cell membranes (of both microbiota and the host). Some bacteria, with *Salmonella typhimurium* being the most extensively studied species, can break down ethanolamine into acetaldehyde and ammonia; the latter can be used as a non-fermentable source of carbon and/or nitrogen.[47] This ability confers a fitness advantage in inflamed tissues by reducing the competition with bacteria that rely entirely on fermentative metabolism.[48] As a plausible model, we hypothesize that ethanolamine utilization in *S. sanguinis* increases its likelihood of surviving in the bloodstream and reaching GvHD target tissues where it can either trigger *de novo* pathogenesis or worsen pre-existing inflammation. Further expansion of *S. sanguinis* in tissues affected by cGvHD due to ethanolamine release from dying cells creates a vicious cycle leading to more damage. The other pathway enriched in patients with cGvHD was the superpathway of glycerol degradation to 1,3-propanediol. In *in vitro* assays, glycerol metabolism using this pathway increased the competitive fitness of *S. sanguinis* against other streptococci.[49] Other than an ability to form biofilm *in vitro* and being present in dental plaque,[50] there is little literature on *P. loescheii,* the other species associated with cGvHD in our analysis. Our pathway analysis was not informative with regard to this species. Further research is needed to validate our observation for *P. loescheii* and the potential mechanisms involved.

Limitations of the present work include its small sample size and single-center nature. Validation in other cohorts will be important to support the value of day +28 plaque *S. sanguinis* as a biomarker for cGvHD risk and a precision target for preventative intervention. Ultimately, demonstration of causality will require mechanistic research, either via a randomized trial showing decreased cGvHD risk by decreasing *S. sanguinis* abundance or controlled murine experiments. Whether *S. sanguinis* reaches cGvHD target

tissues or stimulates the immune system locally (e.g., in draining cervical lymph nodes) cannot be determined from our work. In addition, we did not find the etiology of enrichment for *S. sanguinis* in day +28 plaque. Although we found evidence for patient-specific tendency to accumulate more *S. sanguinis* (consistent with individuality of plaque microbiota), diet, oral hygiene, and salivary biochemical composition are among other possible determinants of plaque microbiota composition that we did not collect data on. Thorough dietary and oral hygiene surveys and biochemical analysis of saliva could help identify patients more likely to experience expansion of *S. sanguinis* early after alloHCT. Lifestyle modification or simple oral health interventions in such patients might help reduce the risk of cGvHD.

In conclusion, we show an association between day +28 plaque microbiota composition and cGvHD risk. Using shotgun metagenomic sequencing, we identified 2 candidate species and 2 metabolic pathways as potential mechanisms of pathogenesis. If validated in other cohorts, these findings could support simple topical interventions targeting the oral microbiota as a novel non-immunosuppressive approach to cGvHD prophylaxis. cGvHD is a leading cause of long-term morbidity after alloHCT, thus more effective prevention could significantly improve the quality of life in transplant survivors.

Disclosures

AR has received consulting fees from Seres Therapeutics and serves as a member of an Emmes Data and Safety Monitoring Board, both outside the scope of the present study. All the other authors have no conflicts of interest to disclose.

Contributions

HG, DRD, ML and AR designed the study. HG, ME and AR collected the data. HG, GS, RA, DRD and AR conducted the study. AR and SSM analyzed the data. HG and AR wrote the manuscript. All authors read and approved the final version of the manuscript for publication.

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Data-sharing statement

The sequencing data reported in this paper are available from NCBI Sequence Read Archive (SRA) BioProject ID PRJNA1190407. De-identified patient-level data will be available from the corresponding author upon reasonable request.

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