Impact of the oral microbiota on oral mucositis in allogeneic hematopoietic stem cell transplantation

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Abstract

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Oral mucositis is a significant and debilitating complication of allogeneic hematopoietic stem cell transplantation. Though the pathophysiology of oral mucositis has been studied extensively, the precise mechanisms remain incompletely characterized, particularly in relation to the influence of the oral microbiota. The oral microbiota is recognized as one of the most diverse and complex microbial communities in the body, and understanding its impact on the development and severity of oral mucositis may improve patient outcomes. A prospective, non-interventional, single center study was conducted with longitudinal sampling of four distinct microbial communities in the oral cavity (saliva, gingival crevicular fluid, supragingival plaque, and subgingival plaque) in patients receiving allogeneic hematopoietic stem cell transplantation at four timepoints (baseline, days +14, +28, and +84 post-transplant). Calibrated examiners

scored oral mucositis severity using a validated instrument, the Oral Mucositis Assessment Scale, at days +7, +14, +21, +28, and +84. Shotgun metagenomic sequencing was used to characterize the oral microbiota. Microbial composition and mucositis scores were compared to examine potential microbial associations with mucositis severity. Three species of salivary bacteria at baseline (*Capnocytophaga sputigena*, *Prevotella nigrescens*, and an *Olsenella* species) were significantly enriched in patients with high mucositis scores at day +7, and three species of supragingival plaque bacteria at day +14 (*Capnocytophaga sputigena*, *Prevotella nigrescens*, and a *Granulicatella* species) were significantly enriched in patients with high mucositis scores at day +14. Age, sex, and conditioning intensity were identified as non-microbial factors correlated with mucositis severity at specific timepoints. Oral microbial diversity in saliva and supragingival plaque declined from baseline to day +14 but recovered in saliva by day +28 and in supragingival plaque by day +84. Results suggest that microbial factors may influence mucositis severity in patients receiving allogeneic hematopoietic stem cell transplantation. Further characterization of microbial dynamics is essential for future hypothesis-driven research to investigate the potential role of antibiotic and/or probiotic interventions in the prevention and management of oral mucositis.

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Dedication

To my beautiful fiancé, Jennifer, for her many years of unwavering love and support

Chapter 1. Introduction

1.1 Hematopoietic Stem Cell Transplantation

Hematopoietic stem cell transplantation (HSCT) is a potentially curative treatment for patients with hematologic malignancies, marrow failure disorders, and other immunodeficiency syndromes[1]. There are two primary types of HSCT: autologous and allogeneic. Autologous transplantation, used primarily in the treatment of lymphoma and multiple myeloma, involves transplantation of previously collected hematopoietic stem cells (HSCs) from the transplant recipient back into their own body to reestablish hematopoiesis after intensive chemo- or chemoradiation therapy[2]. In contrast, allogeneic HSCT (alloHSCT), used primarily in the treatment of leukemia, myelodysplasia, and marrow failure disorders, requires infusion of HSCs from a genetically matched donor's stem cells into a recipient's body[3,4].

1.1.1 Mechanisms and Complications

When available, a human leukocyte antigen (HLA)-matched sibling donor is the preferred source of stem cells for transplantation[5]. However, when this option is unavailable, alternative donor types include HLA-haploidentical related donors, HLA-matched unrelated donors, and cord blood. The curative potential of alloHSCT is due to an alloimmune targeting of the recipient's cancer cells by the donor-derived immune cells[6,7]. A perfect match (e.g., identical twins) would not permit any such graft-versus-tumor effect[8]. In contrast, a major mismatch can lead to severe graft-versus-host disease (GvHD) which could be fatal. GvHD occurs when the donor's grafted cells mount an immune response against the recipient's native tissues and organs. There are two distinct subtypes of GvHD: acute and chronic[9]. Acute GvHD is classified into two categories: classic (symptoms present ≤100 days post-HSCT) and late onset/recurrent/persistent (symptoms present >100 days post-HSCT)[10]. Irrespective of symptom onset, clinical features of acute GvHD

most frequently include skin (e.g., maculopapular rash, erythroderma), gastrointestinal (GI) tract (e.g., abdominal pain, nausea, vomiting), and liver involvement (e.g., hepatocellular injury leading to abnormal liver function tests)[11]. Other organ systems may also be involved including the lungs, kidneys, eyes, and oral cavity. Acute GvHD is graded on a scale of I-IV, and chronic GvHD is graded on a scale of 0-3 with higher numbers indicating greater severity in both[11,12]. Severe GvHD can result in skin damage, GI distress, liver dysfunction, pulmonary complications, susceptibility to infection, and death. HLA matching in alloHSCT is critical to transplant success and minimizing severe post-transplant events such as relapse, graft failure, GvHD, and death. An overview of alloHSCT can be found in **Figure 1**.

1.1.2 Conditioning Therapies

Successful infusion and engraftment of donor HSCs requires a form of conditioning therapy to prevent graft rejection by the recipient's native immune system[13,14]. In alloHSCT, conditioning is frequently achieved with myeloablative chemotherapy and/or irradiation to completely irradicate all immune and hematopoietic cells prior to infusion of donor HSCs. Myeloablation typically involves the use of chemotherapeutic agents, such as busulfan and cyclophosphamide, with or without high-dose total body irradiation (TBI). The precise conditioning regimen depends on patient characteristics, disease status, and other factors, but in general, higher intensity myeloablative regimens have been associated with decreased risk of disease relapse[15]. While this is favorable in treating the underlying cancer, higher intensity myeloablative regimens are also associated with greater treatment-related toxicities including oral mucositis, acute GvHD, secondary malignancies, and impaired development in children[16]. Newer treatment approaches, which include reduced-intensity regimens, are now widely used in older patients or those with comorbidities[13].

1.2 Oral Mucositis

Oral mucositis (OM) is a complication of cancer treatments including chemotherapy, radiotherapy, and HSCT[17–20]. It is characterized by pain, erythema, and ulceration of the oral mucosa. OM is not limited to the oral cavity but frequently extends to the oropharynx, esophagus, and entirety of the GI tract. OM is frequently described by patients as one of if not the most debilitating complication of HSCT[21,22]. In addition to pain, OM is associated with difficulty eating, swallowing, and speaking. Extensive GI mucositis can also be associated with nausea and vomiting which may exacerbate symptoms of OM. Patients experiencing severe OM will often require parenteral nutrition and pain management including intravenous opioid therapy. Impaired barrier function in immunocompromised patients is associated with increased risk of bacteremia, longer hospitalization, increased healthcare costs, and greater morbidity/mortality[23].

1.2.1 Pathophysiology

OM is fundamentally a process of destructive inflammation to the mucosal tissues lining the oral cavity. A five-phase model for OM development has been described as follows: 1) initiation, 2) signal amplification, 3) primary damage response, 4) ulceration, and 5) healing[17]. In the context of HSCT, initiation is driven by the cytotoxic effects of the myeloablative conditioning regimen. In myeloablative therapy, high doses of chemotherapy, sometimes in combination with TBI, is used to eliminate all cells, including cancerous ones, residing in the bone marrow. It has been proposed that OM develops in response to this therapy because it induces mucosal tissue damage and the generation of reactive oxygen species (ROS). This initiates a cascade of molecular events, and in particular, activation of nuclear factor-kappa B (NF-κB) serves as the central mediator of proinflammatory signaling. Active NF-κB will induce production of proinflammatory cytokines such as TNF-α and IL-1β, and these cytokines will amplify proinflammatory signaling,

ultimately leading to the activation of matrix metalloproteinases (MMPs)[17]. MMPs are proteolytic enzymes that degrade components of tissue such as subepithelial collagen (MMP1) and the epithelial basement membrane (MMP3), thus their recruitment will lead to the formation of painful ulcers (**Figure 2**). This complication can be especially dangerous in neutropenic patients as it provides an entryway for microorganisms of the oral cavity into the bloodstream. Oral ulcerations may be sustained or exacerbated by microorganisms as their presence continues to promote local inflammation.

In alloHSCT, OM begins within 5 to 10 days of myeloablation with severity of oral pain and ulcerations typically peaking 6 to 11 days post-HSCT[21]. Oral mucosal tissues will then take an additional 7 to 14 days to heal, while other oral complications such as salivary dysfunction and taste alteration may persist for weeks or months after transplant[24]. Predicting the severity of OM has been the objective of many research groups, but success has been limited. Intensity of the conditioning regimen, specific GvHD prophylactic protocols (e.g., methotrexate), and patient characteristics (e.g., BMI and genetic polymorphisms) have been linked to OM severity; however, these factors do not fully account for the diversity of patient response to myeloablation[21,25,26]. Despite progress, our understanding of OM pathophysiology is incomplete, and thus mechanistically based interventions that are fully effective remain elusive.

1.2.2 Assessment Instruments

In 1979, the World Health Organization (WHO) recognized the rapid increase in experimental cancer therapies under investigation and attempted to standardize reporting in clinical trials to allow for better comparison between studies[27]. The WHO handbook included grading scales for tissue and organ toxicities (e.g., hematologic, GI, renal, etc.). Within the GI system, they introduced grades for OM from Grade 0 to Grade 4. In the following years, many

groups published updated and validated instruments to assess OM; however, no single method has become a global standard. OM assessments can generally be divided into two categories: 1) Global Grading Scales: global scales assign a grade score (e.g., Grade 0, Grade 1, Grade 2, etc.) based on the presence or absence of OM defining characteristics such as erythema, ulceration, and patient impact. Higher grades reflect higher degrees of OM severity. These scales are simple, reproducible, and do not require extensive training to implement in a clinical setting. Scales with this design have been proposed by the WHO, the Radiation Therapy Oncology Group (RTOG), and the National Cancer Institute (NCI)[27–29]. 2) Morphologic Numerical Systems: morphologic systems create a more detailed picture by assigning numeric ratings of severity for individual mucositis characteristics (e.g., erythema, ulceration, etc.)[30,31]. Many systems of this type provide individual scores for oral anatomic sites (e.g., labial mucosa, buccal mucosa, tongue, etc.) which offers a higher level of detail than global scales while also allowing for generation of a composite score to report overall severity. These systems are highly effective in research settings but are more time intensive and require more extensive training and calibration between examiners. A summary of OM assessment instruments can be found in **Table 1**.

1.2.3 Prevention and Management

The Multinational Association of Supportive Care in Cancer (MASCC) and International Society of Oral Oncology (ISOO) have been publishing clinical practice guidelines that summarize the breadth of evidence for the prevention and treatment of OM since 2004[32]. Though extensive research in recent decades has improved our understanding of OM pathophysiology, patient management is still primarily focused on pain relief, dietary support, and secondary infection prevention. Basic oral care (brushing, flossing, and bland oral rinses) remains the primary recommendation based on expert opinion for preventing and/or treating OM, however, its efficacy

can be limited in moderate to severe cases. Other preventative measures and interventions such as anti-inflammatory agents (e.g., benzydamine), photobiomodulation, cryotherapy, and growth factor/cytokine therapy have shown promising results in preliminary studies, though some of these interventions have limited indications[32–37].

Increased understanding of OM pathophysiology in recent decades has improved outcomes for patients; however, our understanding of the factors influencing OM severity, particularly the role of the oral microbiota, remains incomplete[25,26,38–40]. Further characterization of the interplay between oral microbiota and OM pathophysiology may provide opportunities to decrease morbidity through future research.

1.3 The Human Microbiome

The human microbiome represents the entirety of microorganisms, microbial structural elements, and microbial metabolites found on or within the human body. It has emerged as an enormous topic of interest in recent decades by scientists and non-scientists alike[41]. Early estimates claimed that microbial cells, on average, outnumbered human cells about 10:1, however, more recent estimates place this ratio closer to 1:1 after a more accurate measurement of total human cells (particularly red blood cells)[42]. Regardless of the precise ratio, the human body is home to a vast number of microorganisms that play a significant role in health and disease. The role of pathogenic microorganisms has been under investigation since the development of the Germ Theory of Disease in the mid-1800's, but only in recent decades have we begun to appreciate the role of commensal and mutualistic microorganisms in human physiology. The term microbiota is used to describe the population of microorganisms that inhabit a specific environment such as the gut, skin, or mouth[41]. The specific composition of a given microbiota varies by site. GI flora is of particular interest as the gut microbiota plays a critical role in human health and disease. The gut

microbiota not only contributes to nutrient extraction, metabolism, and immunity, but also mediates the gut-brain axis thus impacting human neurophysiology and behavior[43]. The oral microbiota is another example in which complex interactions between polymicrobial communities and the host mediate processes of health and disease. For example, the presence of specific pathogens and the body's subsequent immune response drives the development of diseases like gingivitis and periodontitis[44,45]. In a healthy oral cavity, pathogen abundance is regulated by a combination of the host immune system and the presence of commensal and/or mutualistic microorganisms[46]. An imbalance of the microbiota favoring pathogenic species is known as dysbiosis and is a key concept explored in this study.

1.3.1 Gut Dysbiosis and Gastrointestinal Mucositis

Patients undergoing HSCT often experience disruptions to their gut microbiota throughout the course of treatment[47–50]. More specifically, studies have demonstrated that chemotherapy, radiation, and inpatient antibiotic use contributes to gut dysbiosis during hospitalization[51–53]. Microbial diversity has been closely linked with improved outcomes, and the heavy use of prophylactic and therapeutic antibiotics during neutropenia greatly diminishes this protective diversity. This allows for opportunistic growth of pathogenic bacteria. Furthermore, the cytotoxic effects of the conditioning regimen weaken barrier integrity in the gut thus increasing the risk for bacteremia and death[54–56]. Critically, gut dysbiosis has also been associated with the development and severity of GI mucositis[57–60]. These findings underpin the rationale to examine the oral microbiota and its potential relationship with OM. Efforts to offset gut dysbiosis by diet modification, prebiotic and probiotic administration, and fecal transplantation are all being evaluated to improve patient outcomes[61,62]. If oral microbial dysbiosis predicts or contributes to OM, variations of these strategies could be applied to the prevention and management of OM.

1.3.2 Oral Microbiota in Human Health and Disease

The oral microbiota is a diverse and complex population comprised of bacteria, microeukaryotes, archaea, and viruses[63]. Of these microbes, bacteria have been the most extensively characterized in the oral cavity. The healthy oral cavity harbors over 700 species of bacteria that are predominantly represented by the phyla *Firmicutes*, *Bacteroidetes*, *Proteobacteria*, *Fusobacteria*, and *Actinobacteria*[64–66]. Although great heterogeneity exists in microbial composition between individuals, the notion of a core oral microbiota, which represents the most commonly observed bacteria in healthy individuals, has been supported by several studies; genera of the core oral microbiota include *Streptococcus*, *Veillonella*, *Granulicatella*, *Gamella*, *Actinomyces*, *Corynebacterium*, *Rothia*, *Fusobacterium*, *Porphyromonas*, *Prevotella*, *Capnocytophaga*, *Nisseria*, *Haemophilis*, *Treponema*, *Lactobacterium*, *Eikenella*, *Leptotrichia*, *Peptostreptococcus*, *Staphylococcus*, *Eubacteria*, and *Propionibacterium*[65–69]. These bacteria interact with each other to form biofilm communities, and the precise composition of these communities not only varies between individuals, but also between the different geographical niches found within the host's oral cavity (e.g., labial mucosa, buccal mucosa, tongue, gingiva, teeth, etc.)[63,70–72]. The complex interplay between host and biofilm communities forms an ecosystem that preserves health at equilibrium. Parameters such as nutrient availability, environmental pH, and molecular signaling can promote the development of "healthy" biofilms which in turn maintains oral health[67–69,73].

Because the oral cavity is constantly exposed to the outside environment and prone to physiologic changes from within the host, an ecological shift (i.e., dysbiosis) may allow pathogens to proliferate and drive disease. Indeed, the link between oral microbial dysbiosis and conditions such as dental caries, gingivitis, periodontitis, and oral cancer is well appreciated[44,45,63]. Dysbiosis

can be the result of host mediated factors such as poor oral hygiene, carbohydrate rich diets, and immunosuppression, or the result of biofilm mediated factors such as genetic mutation and acquisition of virulence factors[70,74]. Regardless of the precipitating event, dysbiosis of the oral microbiota is characterized by an increase in the abundance of pathogenic species as compared to mutualistic or commensal species.

One of the earliest studies exploring oral microbiota in the context of chemotherapy associated stomatitis was completed in 1982 where the authors demonstrated a link between oral herpes simplex virus (HSV) and symptomatic oral ulcerations following cytotoxic chemotherapy[75]. In the mid-2010s, various groups demonstrated further links between oral microbiota and oral mucosal toxicities following cancer therapy[76,77]. Research in this space has flourished in the last ten years as DNA sequencing technologies have matured and financial barriers have decreased[78–81]. With respect to alloHSCT, five studies have investigated the relationship between the oral microbiota and OM in the last five years[82–86].

Starting in 2020, Shouval et al. characterized salivary microbiome behavior in 184 patients undergoing alloHSCT[82]. The investigators collected weekly saliva samples and assessed OM with the NCI grading scale for up to 34 days post-HSCT. Using 16S ribosomal RNA (rRNA) sequencing to profile oral bacteria, they demonstrated a statistically significant association between *Mycoplasma*, *Methylobacterium*, *Campylobacter*, and *Staphylococcus* presence at baseline and greater mucositis severity (NCI grades 3 and 4) at day +13 post-HSCT. Of the five studies, this investigation included the greatest number of participants and was the only study to report a metabolic pathway associated with OM development (e.g., the polyamine pathway).

Next, Laheij et al. conducted a similar analysis with 50 patients in 2022[84]. In this study, the investigators used the WHO grading scale, 16S rRNA sequencing, and collected weekly saliva

samples for up to four weeks post-HSCT followed by collections and assessments at days +90, +180, +365, and +540 to characterize long-term resilience of the oral microbiota. Patients who experienced OM (WHO grades 2-4) harbored greater abundance of *Actinobaculum*, *Lactobacillus*, and *Staphylococcus* compared to patients who did not experience OM (WHO grades 0-1) between two and three weeks post-HSCT. They also found that bacterial diversity dropped significantly in the weeks following transplant but recovered to pre-transplant levels by day +90.

Later in 2022, Bruno et al. recruited 30 patients to investigate the association between oral microbiota and the clinical course of OM. They used the WHO grading scale and oral cavity swabs of the bilateral buccal mucosa, alveolar mucosa, and dorsal tongue at three timepoints: pre-conditioning, onset of OM ulcers, and after OM ulcer healing. Bacteria were profiled with 16S rRNA sequencing. Thirteen patients in this study developed OM, and the investigators found an increase in the relative abundance of *Mycoplasma*, *Parvimonas*, and *Lactobacillus* from pre-transplant to the onset of ulcerative OM (WHO grade ≥2). Furthermore, the presence of *Porphyromonas* at pre-conditioning was positively correlated with ulcerative OM.

Kwiatkowski et al. conducted a pilot study in 2023 to analyze the salivary microbiome of patients undergoing auto- and alloHSCT[83]. Saliva samples were collected at pre-conditioning and days +3, +8, +15, and +21. These were paired with OM assessments using the WHO grading scale and 16S rRNA for sequencing. Unfortunately, only six alloHSCT patients were included, and no statistically significant associations were observed between oral microbiota and OM. The investigators reported a decrease in alpha diversity over time; however, they did not specify the timeframe and again, these changes were not associated with OM most likely due to their small sample size.

Finally in 2024, Faraci et al. published their prospective study correlating oral microbiota and OM severity in 17 pediatric patients[86]. Gingival swabs were collected at pre-conditioning, at engraftment, and day +30 or +100 post-HSCT. The WHO grading scale was used to assess OM severity, and 16S rRNA sequencing was used to characterize the microbiota. The investigators found a strong positive correlation between an increase in the relative abundance of *Fusobacterium* and the severity of OM. Furthermore, the presence of *Mycoplasma* at engraftment and *Prevotella oris* at day +30 demonstrated the strongest correlations with OM grade ≥2.

In summary, all but one of these studies showed positive correlations between OM and certain oral bacteria. The genera *Mycoplasma*, *Staphylococcus*, and *Lactobacillus* were each identified in more than one study. One consistent finding across all studies was the observation that oral microbiota diversity decreased in the immediate aftermath of HSCT. Microbial diversity recovered by day +90 to +100 in the two studies that sampled at those timepoints (Laheij et al. and Faraci et al.). Shouval et al. and Laheij et al. were the only two groups who demonstrated an association between loss of microbial diversity and the development of OM. Despite these findings, all five studies had evident limitations. First, every study used a global grading scale, either the WHO or the NCI scale, to assess OM. As mentioned, these instruments lack the specificity and detail offered by morphologic numerical systems. Next, every study used 16S rRNA sequencing to characterize the oral microbiota. While this method is a valuable tool for microbial community analysis, it offers limited taxonomic resolution often providing data only at the genus level. Other methods, such as shotgun metagenomic sequencing, could offer higher resolution at the species level. Species and subspecies resolution is important for future precision therapeutics. Finally, every study collected only one sample type from the oral cavity (e.g., saliva

or swab). The oral cavity is a complex environment with various niches harboring diverse microbial populations. A summary of these studies can be found in **Table 2.**

1.4 Thesis Overview

The goal of the study was to assess the impact of the oral microbiota on OM in patients receiving alloHSCT by taking longitudinal, multisite oral cavity samples, using a morphologic numerical system to assess OM severity, and employing shotgun metagenomic sequencing to characterize oral microbiota. There are two specific aims of this study: 1) to characterize the relationship between oral microbiota composition and OM severity following alloHSCT, and 2) to identify patterns of oral microbiota change and recovery up to three months post-transplant. We hypothesize that oral microbiota will predict the risk and severity of OM after allogeneic HSCT, and that oral microbial diversity will decline acutely following transplant but recover by the three-month post-transplant timepoint.

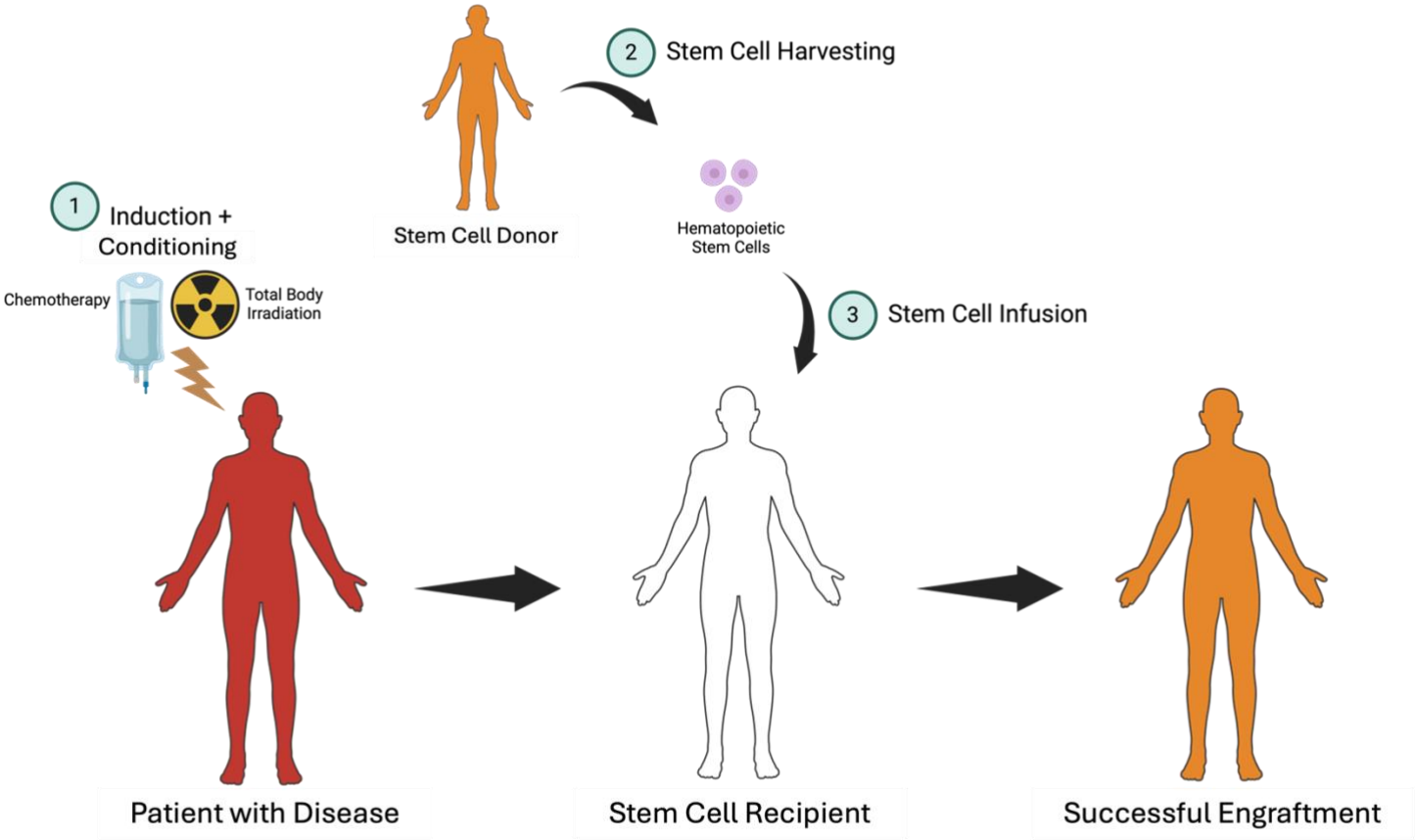

Figure 1. Overview of allogeneic hematopoietic stem cell transplantation
In alloHSCT, the patient is treated with one or more rounds of chemotherapy and/or total body irradiation (i.e., conditioning) for tumor cytoreduction and promotion of donor cell engraftment. Myeloablative regimens are intensive, typically requiring an extended hospital stay. In the meantime, hematopoietic stem cells are harvested from an allogeneic donor. These cells are then infused into the patient. Successful engraftment is determined with laboratory studies demonstrating recovery of blood cells post-transplant. Diagram created with BioRender.com.

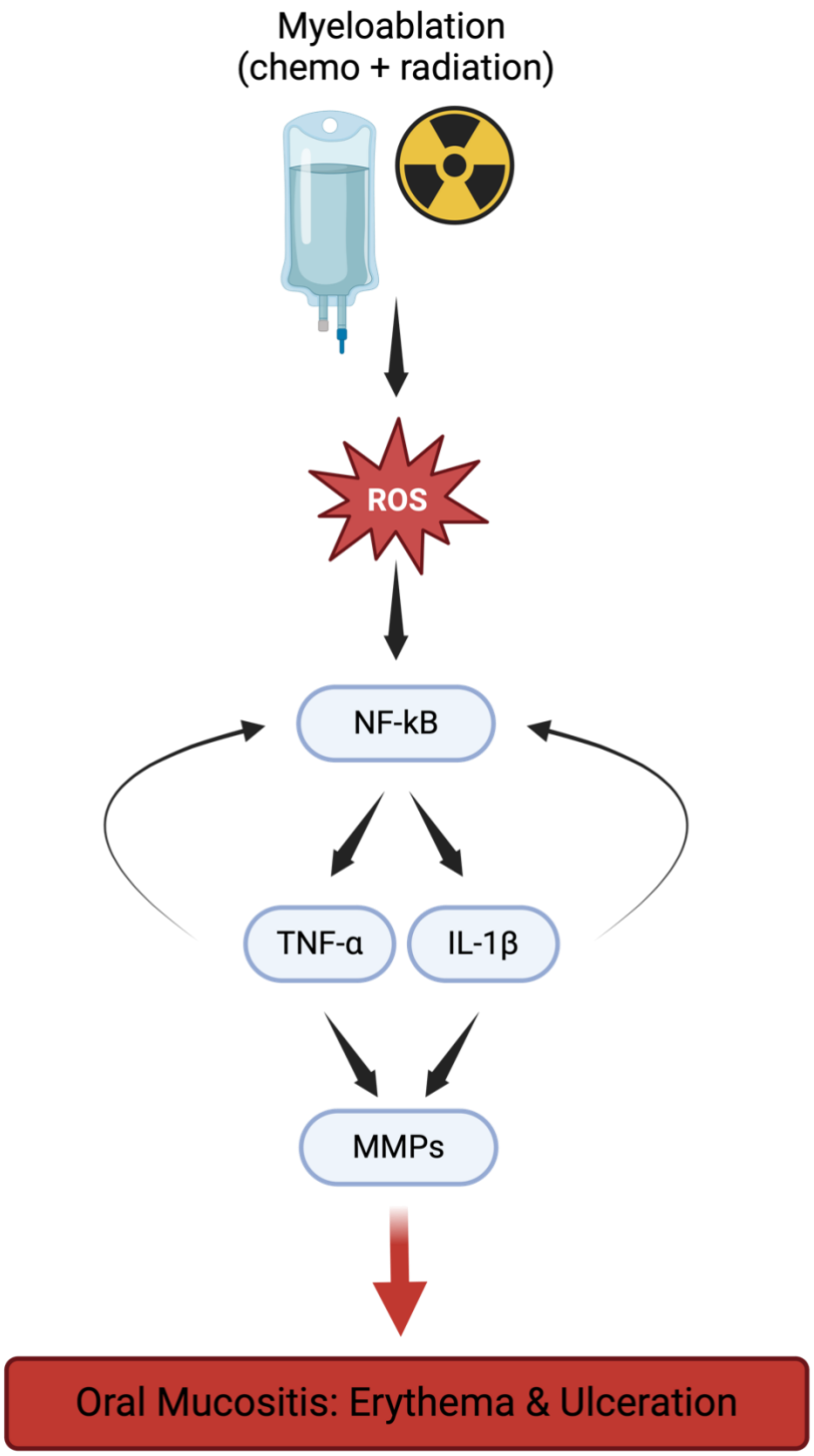

Figure 2. Molecular pathway of oral mucositis development
OM is initiated by the high dose chemotherapy and total body irradiation delivered during myeloablation. These will generate high concentrations of reactive oxygen species (ROS) in the oral epithelium. The presence of ROS will activate NF-κB which will in turn promote the production of proinflammatory cytokines such as TNF-α and IL-1β. These cytokines will amplify proinflammatory signaling ultimately generating matrix metalloproteins (MMPs) which will lead to the formation of erythema and ulceration. Diagram created with BioRender.com.

Table 1. Mucositis assessment instruments

		Year Published	Grade 0	Grade 1	Grade 2	Grade 3	Grade 4	Grade 5
Global Grading Scales	**WHO**	1979	No change	Soreness/erythema	Erythema, ulcers; can eat solids	Ulcers; requires liquid diet only	Alimentation not possible	N/A
	NCI	1982	N/A	Asymptomatic or mild symptoms; intervention not indicated	Moderate pain or ulcer that does not interfere with oral intake; modified diet indicated	Severe pain; interfering with oral intake	Life-threatening consequences: urgent intervention indicated	Death
	RTOG	1995	No change over baseline	Injection/may experience mild pain not requiring analgesic	Patchy mucositis that may produce an inflammatory serosanguinous discharge/ may experience moderate pain requiring analgesia	Confluent fibrinous mucositis/ may include severe pain requiring narcotic	Ulceration, hemorrhage, or necrosis	N/A

		Year Published	Sites	Categories	Scores	Analysis
Morphologic Numerical Systems	**Schubert et al.**	1992	Upper lip, lower lip, upper labial mucosa, lower labial mucosa, right buccal mucosa, left buccal mucosa, dorsal tongue, lateral tongue, ventral tongue floor of mouth, hard palate, soft palate, gingiva	Atrophy, erythema, hyperkeratosis, lichenoid, edema	0 = no change 1 = mild 2 = moderate 3 = severe	1) Total mucositis score
				Pseudomembrane, ulceration	0 = none 1 = ≤1cm^2 2 = 1cm^2 – 2cm^2 3 = ≥2cm^2	
	Sonis et al.	1999	Upper lip, lower lip, right cheek, left cheek, right ventrolateral tongue, left ventrolateral tongue, floor of mouth, soft palate/fauces, hard palate	Ulceration/pseudo membrane	0 = none 1 = <1cm^2 2 = 1cm^2 – 3cm^2 3 = >3cm^2	1) Mean mucositis score 2) Weighted mean mucositis score 3) Extent of mucositis score 4) Worst site score
				Erythema	0 = none 1 = not severe 2 = severe	

Table 2. Summary of relevant studies

Author, Year	Sample Size	Median Age (range)	Sex	Conditioning Regimen	Oral Sample Type	Oral Sample Timepoints	Mucositis Assessment Method	Mucositis Assessment Timepoints	Sequencing Method
Shouval et al., 2020	N = 184	58 (47-67)	Undisclosed	MAC: 76 RIC: 108	Saliva	Pre-conditioning then weekly up to day +34 (5 total)	NCI CTCAE v4.0	Same as oral cavity sampling	16S rRNA
Laheij et al., 2022	N = 50	52 (19-74)	26F 24M	MAC: 12 RIC: 38	Saliva	Pre-conditioning, weekly during hospitalization, then days +90, +180, +365, and +540 (8 total)	WHO	Same as oral cavity sampling	16S rRNA
Bruno et al., 2022	N = 30	50 (19-73)	14F 16M	MA: 12 RIC: 18	Buccal mucosa, alveolar mucosa, and dorsal tongue swab	Pre-conditioning, then onset of OM ulcers, and after OM ulcer healing (3 total)	WHO	Weekly during hospitalization	16S rRNA
Kwiatkowski et al., 2023	N = 6	34 (15-48)	2F 4M	MAC: 3 RIC: 3	Saliva	Pre-conditioning, then days +3, +8, +15, and +21 (5 total)	WHO	Same as oral cavity sampling	16S rRNA
Faraci et al., 2024	N = 17	6 (0.7-24.7)	Undisclosed	MAC: 16 RIC: 1	Gingival swab	Pre-conditioning, then at engraftment, and day +30 or +100 (3 total)	WHO	Daily during hospitalization	16S rRNA

MAC: myeloablative conditioning; RIC: reduced intensity conditioning

Chapter 2. Methods and Materials

2.1 Study Design and Patient Enrollment

A prospective, non-interventional, single-center study was completed at the Fred Hutchinson Cancer Center (FHCC) in Seattle, WA, USA between April 2023 and May 2024. Study protocol was approved by the FHCC's Institutional Review Board and conducted in accordance with the Declaration of Helsinki. Subjects were recruited from the FHCC Bone Marrow Transplant service during preparation alloHSCT. Inclusion criteria: ≥18 years of age at time of arrival to the BMT service, planned myeloablative alloHSCT at FHCC, ability to understand and communicate in English (to allow for informed consent). Exclusion criteria: platelet count <10,000/μL or absolute neutrophil count <500/μL within seven days before consent. Eligible patients were identified by the investigators through the FHCC electronic health record, presented with full details of the study by a BMT physician (AR), and allowed to ask clarifying questions related to the study before providing consent. Study and HIPPA consent forms were signed by the patient and investigators, filed within a locked cabinet in a locked office, and recorded online in the FHCC REDCap database. Participants were informed that consent could be withdrawn at any point within the study, for any reason, without impacting ongoing treatment. Our planned accrual goal was 50 evaluable patients, defined as those providing the baseline samples and undergoing alloHSCT. Ultimately, 56 patients were enrolled (four patients did not proceed to transplant, one died, and one withdrew consent).

2.2 Patient Sample Collection

Longitudinal microbial samples were obtained from distinct anatomic sites in the oral cavity by calibrated oral medicine specialists (HG, DD, GS, RA) both before and after alloHSCT. Four sample types (saliva, gingival crevicular fluid (GCF), supragingival plaque, subgingival

plaque) were collected in the Oral Medicine clinic at the FHCC immediately prior to standard pre-transplant oral evaluation in the course of clinical care. Microbial sample collections at day +14, +28, and +84 were limited to saliva and supragingival plaque to minimize risk of bleeding and/or infection. Day +84 samples were collected in the Oral Medicine clinic at the FHCC immediately prior to standard pre-departure oral evaluation in the course of clinical care. Microbial samples were collected at least 30 minutes after perioral exposures (e.g., eating, drinking, rinsing the mouth, performing oral hygiene, or capture of dental radiographs). OM assessments were completed at each study visit with additional weekly assessments completed during patient stay in the hospital. Sample collection and OM assessment details are included below, and a study timeline is outlined in **Figure 3**.

2.2.1 Saliva

Up to 5mL of saliva was collected by passive drooling into a sterile tube containing 5mL of sterile 95% ethanol. Patients were given 15 minutes to produce 5mL of saliva, and the total time of collection was recorded. If the patient was unable to produce 5mL, the precise volume was later measured using a sterile serological pipette. Once collected, the tube was pulse vortexed for 5 seconds to ensure homogenous mixture of saliva and ethanol. The tube was immediately placed on ice and transferred to -80°C for storage.

2.2.2 Gingival Crevicular Fluid

GCF samples were obtained from three teeth (one posterior and two anterior) using PerioPaper® Strips as described by manufacturer's protocol (Oraflow Inc., Smithtown, NY). Each tooth was isolated using sterile cotton rolls and thoroughly dried using a gentle stream of air. The PerioPaper® Strip was placed into the sulcus and held there for 30 seconds. The strips were then

placed into a sterile tube containing 200µL of sterile phosphate buffered saline, snap frozen on dry ice, and immediately transferred to -80°C. GCF samples were only collected at the baseline visit.

2.2.3 Supragingival Plaque

Supragingival plaque samples were collected using a sterilized periodontal scaler from the same three teeth as GCF (if possible). Samples contaminated with blood were discarded and reobtained from an adjacent tooth. Samples were transferred from the scaler to the tip of a sterilized plastic pick, and the pick was submerged into a sterile tube containing 500µL of sterile 95% ethanol. The pick was agitated until the clump of plaque fell off the instrument and into the ethanol. All supragingival plaque samples were pooled into the same tube. The tube was immediately placed on ice and transferred to -80°C for storage.

2.2.4 Subgingival Plaque

Subgingival plaque samples were collected with the same protocol as supragingival plaque samples, but with separate sterilized instrumentation to prevent cross contamination. Subgingival plaque samples were only collected at the baseline visit.

2.2.5 Mucositis Assessments

Mucositis assessments were completed at baseline, day +7, +14, +21 (if patient was still admitted to the hospital), +28, and +84 using the Oral Mucositis Assessment Scale (OMAS) as described by Sonis et al.[31]. A window of +/-2 days was used for each day +7 assessment, and a window of +/-3 days for all other timepoints. Total mucositis score (range 0-45) was defined as the sum of all scores (erythema and ulceration) across all sites as described by Schubert et al.[30]. The mucositis assessment form can be found in **Figure 4**.

2.3 Microbiota Sequencing

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 PE150 configuration. The median sequencing depth per sample was 15.6M read pairs. The ZymoBIOMICS® Microbial Community Standard (Zymo Research, Irvine, CA) was used as a positive control for each DNA extraction. The ZymoBIOMICS® Microbial Community DNA Standard (Zymo Research, Irvine, CA) was used as a positive control for each library preparation. Two of the investigators (healthy individuals on no medications and with no known oral or other medical problems) provided one saliva and one supragingival sample each, used as additional positive controls. 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. MetaPhlAn41 with default parameters was used for species-level taxonomic assignment[87].

2.4 Statistical Analysis

Potential predictors of mucositis were first investigated in univariate analysis (Chi-squared – with Fisher's exact test when appropriate – or Wilcoxon test) of the association between baseline clinical variables and total mucositis score. Salivary flow rate at the same or the closest preceding timepoint relative to mucositis assessment was also examined as a potential correlate of mucositis.

Samples were analyzed in three batches. Batch effect was evaluated using MMUPHin with adjustment for sample site and timepoint[88]. Microbiome analysis was performed using species-

level data; taxa relative abundances were visualized using genus-level data. Alpha diversity (i.e., within-sample diversity) was quantified by Shannon index which considers both richness and evenness[89]. Beta diversity (i.e., between-sample diversity) was quantified by Aitchison distance which uses centered log-ratio (CLR) transformed taxa abundances and accounts for compositionality of microbiome data[90]. Ordination was visualized by principal coordinate analysis using the first two axes and differences in microbiome composition were statistically examined using an adonis test with 999 permutations. Differential abundance analysis was performed using the Linear Models for Differential Abundance (LinDA) package[91]. LinDA analysis of microbiome compositional data fits linear regression models on CLR-transformed data and corrects the bias due to microbiota compositional effects. With its mixed-effects option, LinDA accounts for subject-specific factors as a random effect. We took advantage of this feature to analyze within-subject microbiome differences between oral sites. In these models, subject ID was included as a random effect. Species with a relative abundance of >0.01 in at least 10% of the samples were included in LinDA. In the analysis of microbiome predictors of mucositis, total mucositis score (≤ median vs. > median across all assessments at the given timepoint) was the outcome variable in LinDA, while microbiome data (species relative abundances) at the same or the closest preceding timepoint were included as predictors.

All analyses were pre-planned unless specified otherwise. No power or sample size calculation was performed. P values, and in case of multiple comparisons (e.g., LinDA) Benjamini-Hochberg-corrected P values (i.e., q values) < 0.05 were considered statistically significant[92]. R 4.2.0 was used for all analyses.

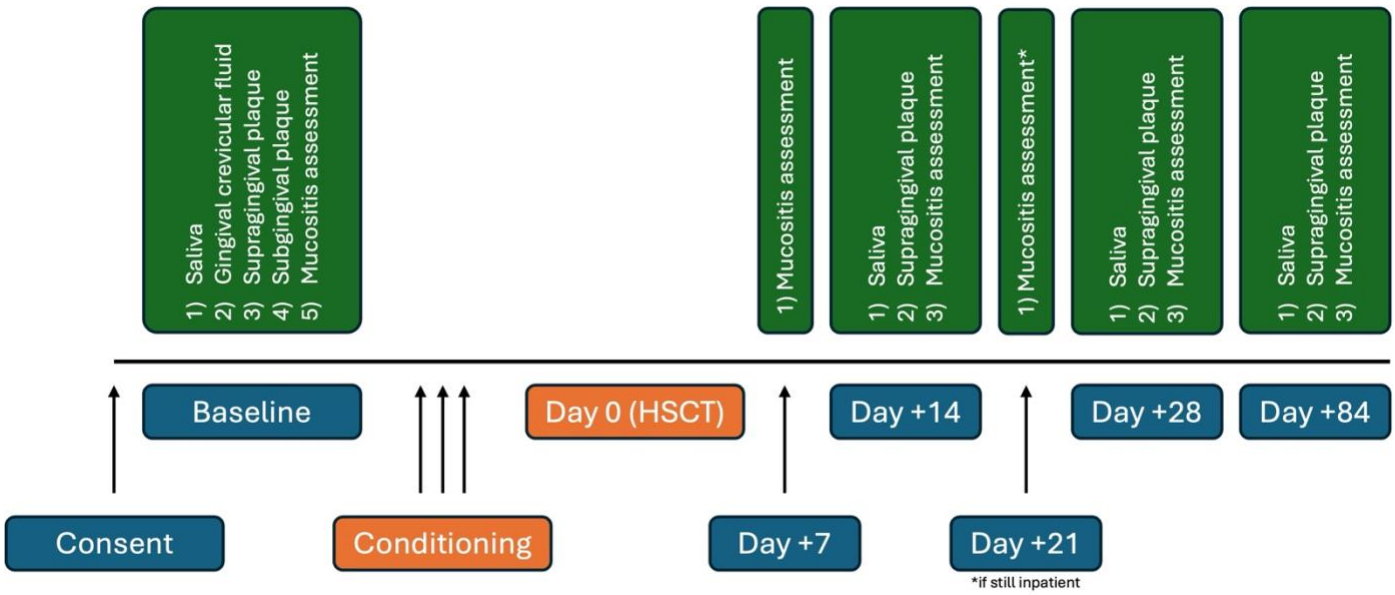

Figure 3. Sample collection timeline

Once consented, patients would complete their baseline collections prior to conditioning and transplant. GCF and subgingival plaque samples were only collected at this visit. After transplant, patients would receive one mucositis assessment at day +7. Saliva and supragingival plaque samples would be collected and a mucositis assessment would be completed at day +14, +28, and +84. If the patient were still admitted at day +21, another mucositis assessment would be completed at this timepoint.

MicOM Study
IRB #11089; Protocol #RG1123080
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Mucositis Assessment Form

Patient Name: _____ Patient ID: _____

Provider: _____ Date: _____

Location (circle one): Inpatient Outpatient

1) In the table, please circle one item per row based on the following parameters:

- Ulceration/Pseudomembrane
  - 0 = no lesion
  - 1 = <1cm^2
  - 2 = 1cm^2 – 3cm^2
  - 3 = >3cm^2
- Erythema
  - 0 = none
  - 1 = present, not severe
  - 2 = present, severe

(Reference: oxygenated blood)

Location	Ulceration/Pseudomembrane				Erythema		
Upper Lip	0	1	2	3	0	1	2
Lower Lip	0	1	2	3	0	1	2
Right Cheek	0	1	2	3	0	1	2
Left Cheek	0	1	2	3	0	1	2
Right Ventrolateral Tongue	0	1	2	3	0	1	2
Left Ventrolateral Tongue	0	1	2	3	0	1	2
Floor of Mouth	0	1	2	3	0	1	2
Soft Palate	0	1	2	3	0	1	2
Hard Palate	0	1	2	3	0	1	2

2) Is there oral infection present? Circle one: Yes No

3) Current oral pain level (scale 0-10): _____

4) Highest pain level in the last week (scale 0-10) and date: _____

5) Current difficulty swallowing (scale 0-10): _____

6) Most difficult swallowing in the last week (scale 0-10) and date: _____

7) Patient's tolerated diet:

☐ Normal ☐ Only soft, solid foods ☐ Only liquids ☐ No foods or liquids

8) Once complete, please group this form with the others until pickup by study team.

Figure 4. Mucositis assessment form

Chapter 3. Results

3.1 Patient Characteristics

A total of 56 patients were enrolled in this study. Four out of the 56 patients did not proceed to HSCT; thus, their data is not included in patient characteristics (**Table 3**), but their baseline samples were included in the analysis when appropriate. Of the remaining patients, 43 received myeloablative conditioning and 9 ultimately received a reduced-intensity regimen. Samples from both groups were included in the analysis. A summary of patient demographic and clinical characteristics is provided in **Table 3**.

3.2 Mucositis Assessments and Salivary Flow Rate

A total of 275 mucositis assessments were performed. Because many patients were discharged from the hospital between day +14 and +21, there were fewer day +21 assessments when compared to the other timepoints. Total mucositis score at different timepoints is shown in **Figure 5a**. Mucositis increased rapidly until day +7 followed by a slower rise and peak around day +14. From there, mucositis rapidly declined until day +21. Further decline occurred until day +28, though most patients did not experience complete resolution (i.e., total mucositis score of zero) in this timeframe. All mucositis scores were ≤ 3 by day +84. Of the 45 mucositis assessments completed at this timepoint, 7 patients (15.6%) had a score of 3, 4 patients (8.9%) had a score of 2, 9 patients (20%) had a score of 1, and 25 patients (55.6%) had a score of 0.

The relationship between baseline patient characteristics and total mucositis score was examined at different timepoints (**Figure 5b**). Several potential risk factors were identified based on the timepoint. Male sex was significantly associated with higher total mucositis scores at day +7 ($P = 0.01$), myeloablative conditioning with worse mucositis at day +14 ($P = 0.01$), older age with worse mucositis at day +21 ($P = 0.01$), and diseases other than acute leukemia with worse

mucositis at day +84 (P = 0.03). No clinical variable was associated with baseline or day +28 mucositis. Regarding day +14, conditioning intensity was collinear with donor type and graft source. Thus, although the latter two were also significantly associated with mucositis at day +14, but we selected only one (conditioning intensity) for multivariable analysis.

Although there was a visual trend for a decline in salivary flow rate after HSCT compared to baseline (especially at day +14), this trend did not approach statistical significance in a linear mixed model with patient number as a random effect, timepoint as the fixed effect, and salivary flow rate (mL/min) as the outcome variable (**Figure 5c**).

3.3 Alpha Diversity Analysis

A total of 455 samples were analyzed: saliva (N = 200), supragingival plaque (N = 199), and subgingival plaque (N = 56). Sample breakdown is shown in **Figure 6a**. The distribution of the 20 most abundant families among samples of each type is shown in **Figure 6c-e**. We first performed longitudinal analysis of within-subject diversity (i.e., alpha diversity) of saliva and supragingival microbiota using Shannon's index (**Figure 6b**). As the subgingival sample was only collected at baseline, its diversity could not be analyzed longitudinally. Both saliva and supragingival plaque microbiota lost their diversity from baseline to day +14. Saliva alpha diversity recovered by day +28, while recovery of supragingival alpha diversity occurred later, apparent at day +84. Saliva microbiota maintained a higher alpha diversity than supragingival plaque microbiota at all timepoints (Wilcoxon's P = 0.003, 0.03, $5x10^{-7}$, and 0.004 at baseline, day +14, day +28, and day +84, respectively). Finally, baseline alpha diversity was not different between subgingival and supragingival plaque (P = 0.62).

3.4 Oral Microbiota Comparison Between Sites

A total of 91 families and 1,037 species were identified among all sequenced samples. For batch effect analysis, we first performed permutational multivariate analysis of variance (PERMANOVA) on CLR-transformed species abundances. Sample type, sample timepoint, and batch number were included as explanatory variables. In this analysis, the largest fraction of microbiota variation was explained by sample type (R^2 = 0.07, adonis test with 999 permutations) and timepoint (R^2 = 0.02), while batch effect was minimal (R^2 = 0.008). Adjustment for batch number did not change these results significantly (R^2 for sample type, timepoint, and batch number 0.07, 0.02, and 0.004, respectively). These results argued against a significant batch effect.

At baseline, the overall microbiota composition was similar between subgingival and supragingival plaque (P = 1.00, adonis test with 999 permutations), but both were significantly different from saliva (P < 0.001) (**Figure 7a**). Baseline saliva microbiota composition in patients was significantly different from healthy controls (P = 0.009) (**Figure 7b**). Baseline supragingival microbiota composition in patients appeared to be different from healthy controls, but the difference did not reach statistical significance (P = 0.12, **Figure 7c**), possibly due to the small number of healthy control samples. The difference between saliva and supragingival plaque microbiota remained significant at all timepoints (**Figure 7d-f**). Differential abundance analysis was performed to identify under or overrepresented species when the overall microbiota composition was different between two groups. Considering the paired nature of these data (i.e., saliva and supragingival plaque samples from the same patient at a given timepoint), we implemented a mixed effect method (LinDA), with subject ID as a random effect. The results of this analysis are summarized in **Figure 8a-d**. At baseline, numerous *Actinomyces* species were differentially abundant in subgingival and supragingival plaque compared to saliva, while

Veillonella atypica was the dominant differentially abundant species of saliva. At days +14 and +28, some *Actinomyces* species (in addition to *Streptococcus sanguinis* and *Arachnia propionica*) were among differentially abundant taxa in supragingival plaque vs. saliva, while *Rothia mucilaginosa* was differentially abundant in saliva at these timepoints. No differentially abundant species were found between subgingival and supragingival plaque microbiota at baseline or between saliva and supragingival plaque microbiota at day +84.

3.5 Predictors of Oral Mucositis

Day +7 mucositis: In differential abundance analysis, three supragingival plaque species at baseline (*Capnocytophaga sputigena*, *Prevotella nigrescens*, and an *Olsenella* species) were significantly associated with day +7 mucositis (i.e., total mucositis score > median) (**Figure 9a**). No salivary or subgingival taxa were significant. In summary, predictors of day +7 OM were male sex and three supragingival plaque species at baseline.

Day +14 mucositis: Three salivary species at day +14 (*Capnocytophaga sputigena*, *Prevotella nigrescens*, and a *Granulicatella* species) were significantly associated with day +14 mucositis (**Figure 9b**). No subgingival or supragingival plaque taxa were significant. In summary, predictors of day +14 OM were myeloablative conditioning and three supragingival plaque species at day +14.

Day +21 and +28 mucositis: No significant correlates were found for day +21 or day +28 mucositis among species in salivary, subgingival plaque, or supragingival plaque samples at baseline, day +14, or day +28. Age was the only predictor of day +21 OM. There were no identified predictors for day +28 mucositis. Variability in mucositis at day +84 was too small for a meaningful differential abundance analysis.

Table 3. Patient characteristics

Total, N[1]	56
Sex Male Female Age (years) at transplantation (range)	 29 27 43 (20-60)
Underlying disease Acute leukemia MDS/MPN Non-Hodgkin lymphoma	 43 8 5
Conditioning regimen Myeloablative High-dose TBI-based Bu/Cy Flu/Bu Reduced intensity Flu/Mel/TBI (2-4 Gy) Flu/Cy/Thiotepa/TBI (4 Gy) Flu/Treosulfan/TBI (2 Gy)	 20 11 12[2] 3 4 2
Donor type HLA-matched sibling HLA-matched unrelated HLA-haploidentical Cord blood HLA-mismatched unrelated (1 antigen)	 13 27 4 7 1
Graft source Peripheral blood Cord blood	 45 7
GvHD prophylaxis Tacrolimus/Methotrexate PTCy-based Cyclosporine/MMF Cyclosporine/MMF/Sirolimus	 31 13 7 1
Hospitalization length in days (range)	26 (11-60)

[1]Four patients did not proceed to HSCT. [2]Four patients received additional TBI (4 Gy)
Bu: busulfan; Cy: cyclophosphamide; Flu: fludarabine; GvHD: graft-versus-host disease; HLA: human leukocyte antigen; MDS: myelodysplastic syndromes; MMF: mycophenolate mofetil; MPN: myeloproliferative neoplasms; PTCy: post-transplantation cyclophosphamide; TBI: total body irradiation

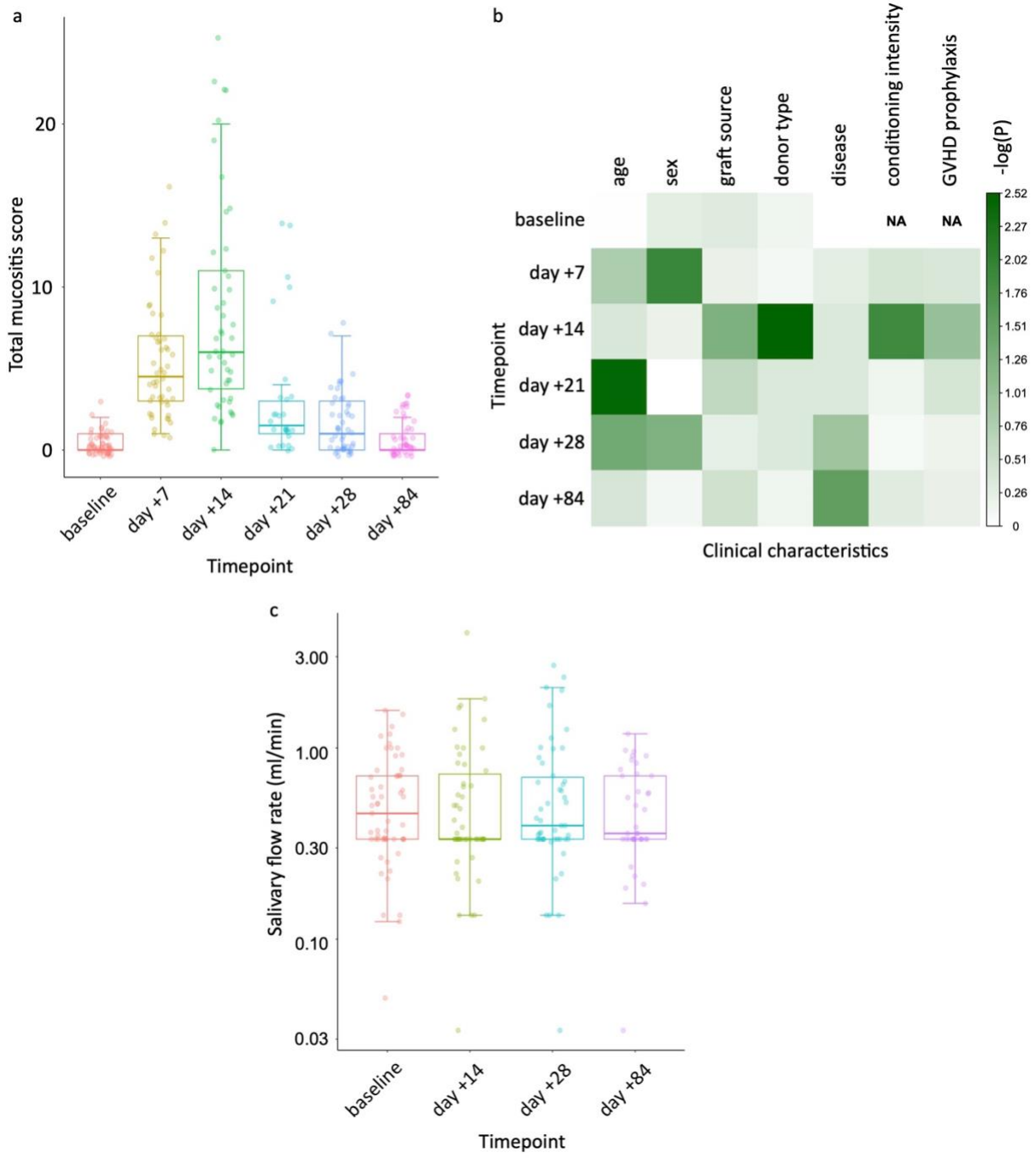

Figure 5. Total mucositis score, clinical predictors, and salivary flow rate

(**a**) Total mucositis score over time. Each circle represents an assessment. (**b**) Correlation plot showing P values along the color gradient for the association between baseline clinical variables and total mucositis score at different timepoints. (**c**) Salivary flow rate over time. Each circle represents a saliva sample collection. For panels (**a**) and (**c**), each box shows the median (horizontal middle line) and interquartile range at the corresponding timepoint. Whisker lines indicate non-outlier maximum and minimum values. A small jitter is included for better visualization.

NA: not applicable

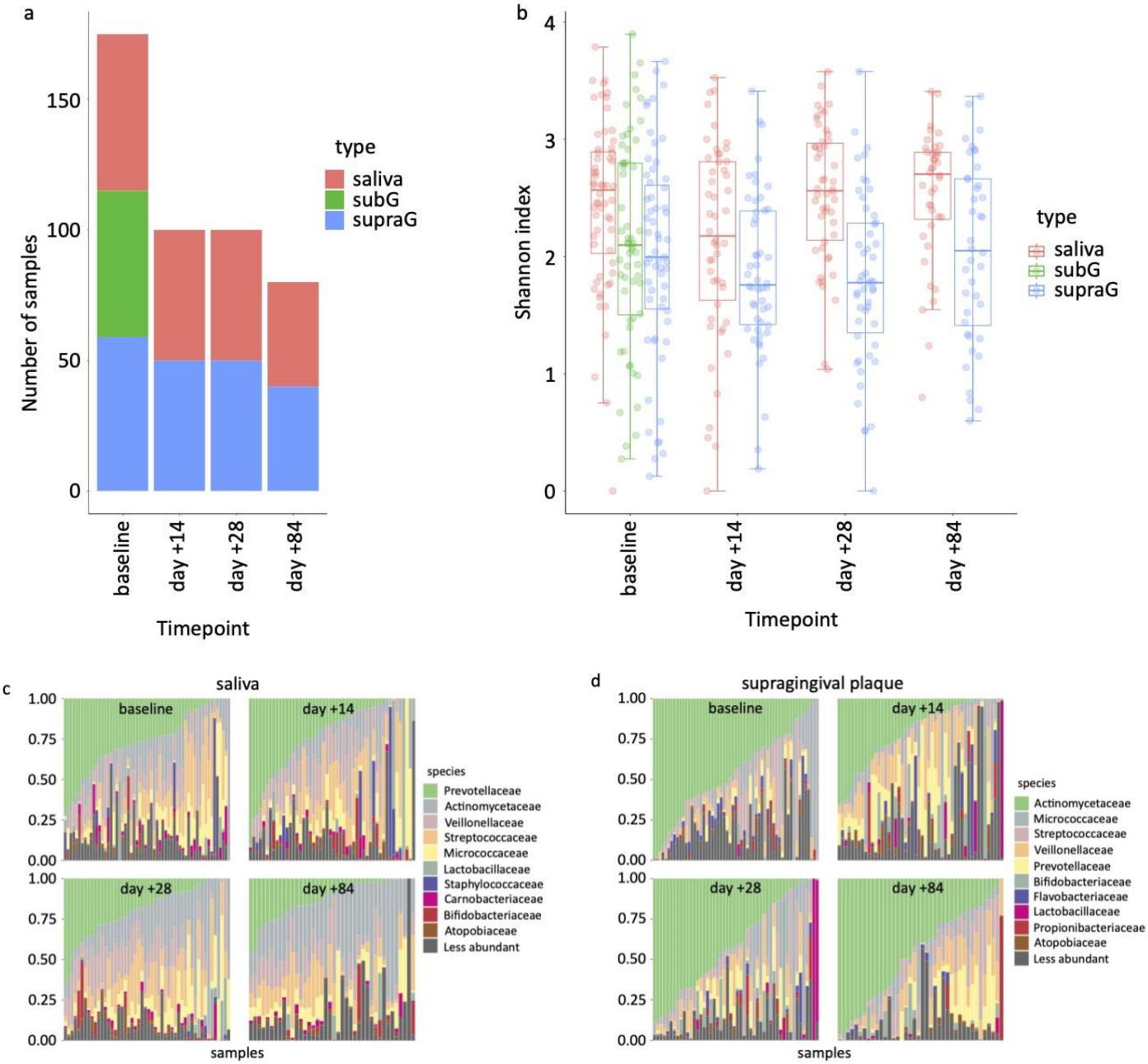

Figure 6. Sample breakdown and alpha diversity
(**a**) Sample breakdown. (**b**) Alpha diversity as measured by the Shannon's index. Subgingival samples were collected only at baseline. Each circle represents a sample. Each box shows the median (horizontal middle line) and interquartile range at the corresponding timepoint. Whisker lines indicate non-outlier maximum and minimum values. A small jitter is included for better visualization. (**c**-**e**) Relative abundance of the 20 most abundant families in salivary (**c**), supragingival plaque (**d**), and subgingival plaque (**e**) samples.
subG: subgingival plaque; supraG: supragingival plaque

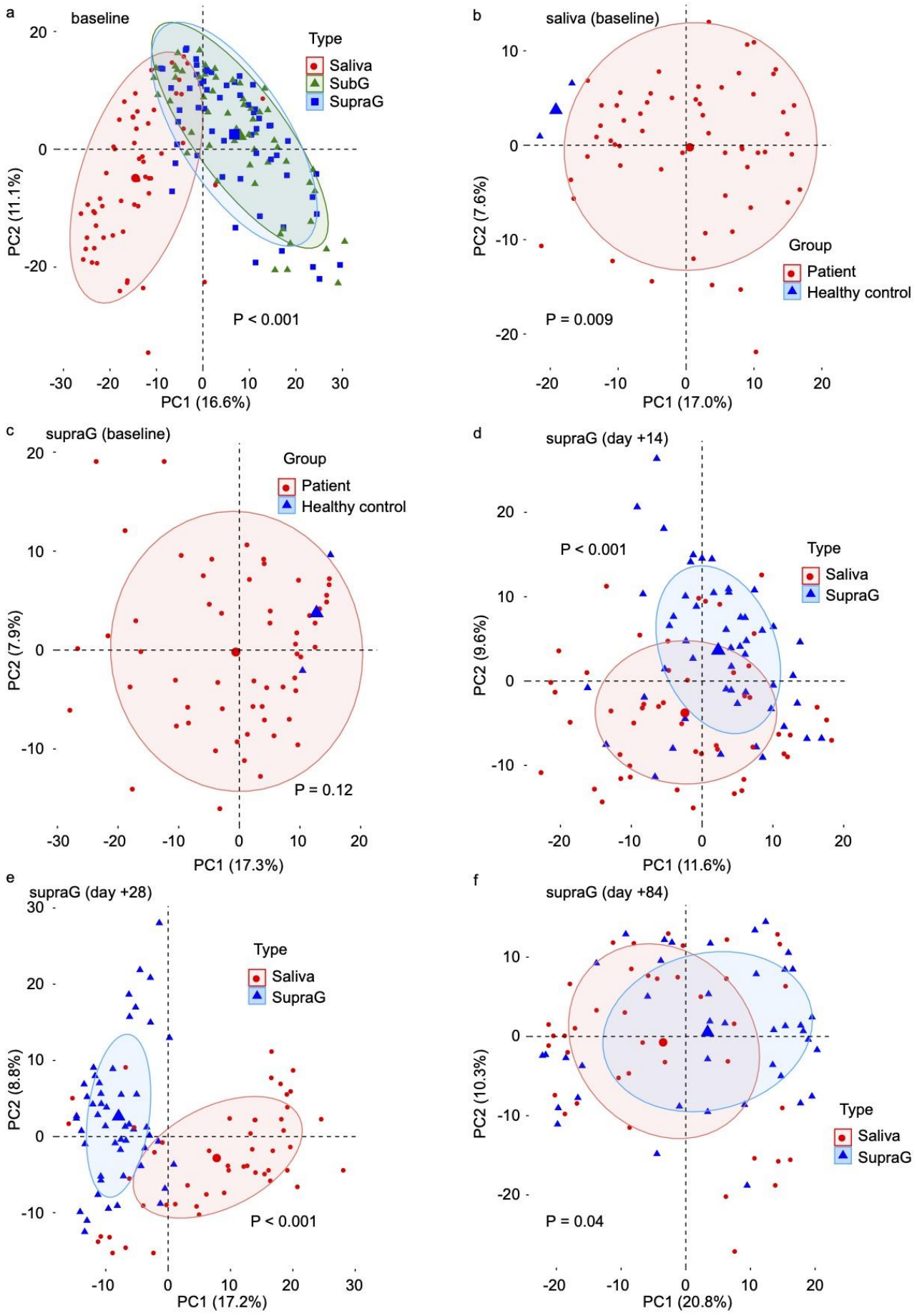
a
baseline
Type
Saliva
SubG
SupraG
P < 0.001
PC1 (16.6%)
PC2 (11.1%)
b
saliva (baseline)
Group
Patient
Healthy control
P = 0.009
PC1 (17.0%)
PC2 (7.6%)
c
supraG (baseline)
Group
Patient
Healthy control
P = 0.12
PC1 (17.3%)
PC2 (7.9%)
d
supraG (day +14)
P < 0.001
Type
Saliva
SupraG
PC1 (11.6%)
PC2 (9.6%)
e
supraG (day +28)
Type
Saliva
SupraG
P < 0.001
PC1 (17.2%)
PC2 (8.8%)
f
supraG (day +84)
Type
Saliva
SupraG
P = 0.04
PC1 (20.8%)
PC2 (10.3%)

Figure 7. Differences in microbiome composition between groups
Comparison is made between different oral sites in patients at baseline (**a**), baseline saliva from patients vs. healthy controls (**b**), baseline supraG from patients vs. healthy controls (**c**), day +14 saliva vs. supraG in patients (**d**), day +28 saliva vs. supraG in patients (**e**), and day +84 saliva vs. supraG in patients (**f**). Beta diversity and ordination visualized by principal coordinate analysis. Aitchison distance (using centered log-ratio transformed species abundances) was used to quantify the overall compositional difference between samples. The first two principal coordinates (PC1 and PC2) are shown. Numbers in parentheses indicate percent variation explained by the corresponding axis. P values are from an adonis test with 999 permutations. Each symbol represents a sample. The closer the two samples, the more similar their microbiome composition. The centroid of each cluster is shown by a larger symbol. 80% ellipses are shown. Ellipses could not be derived from healthy control samples due to sample size.
supraG: supragingival plaque

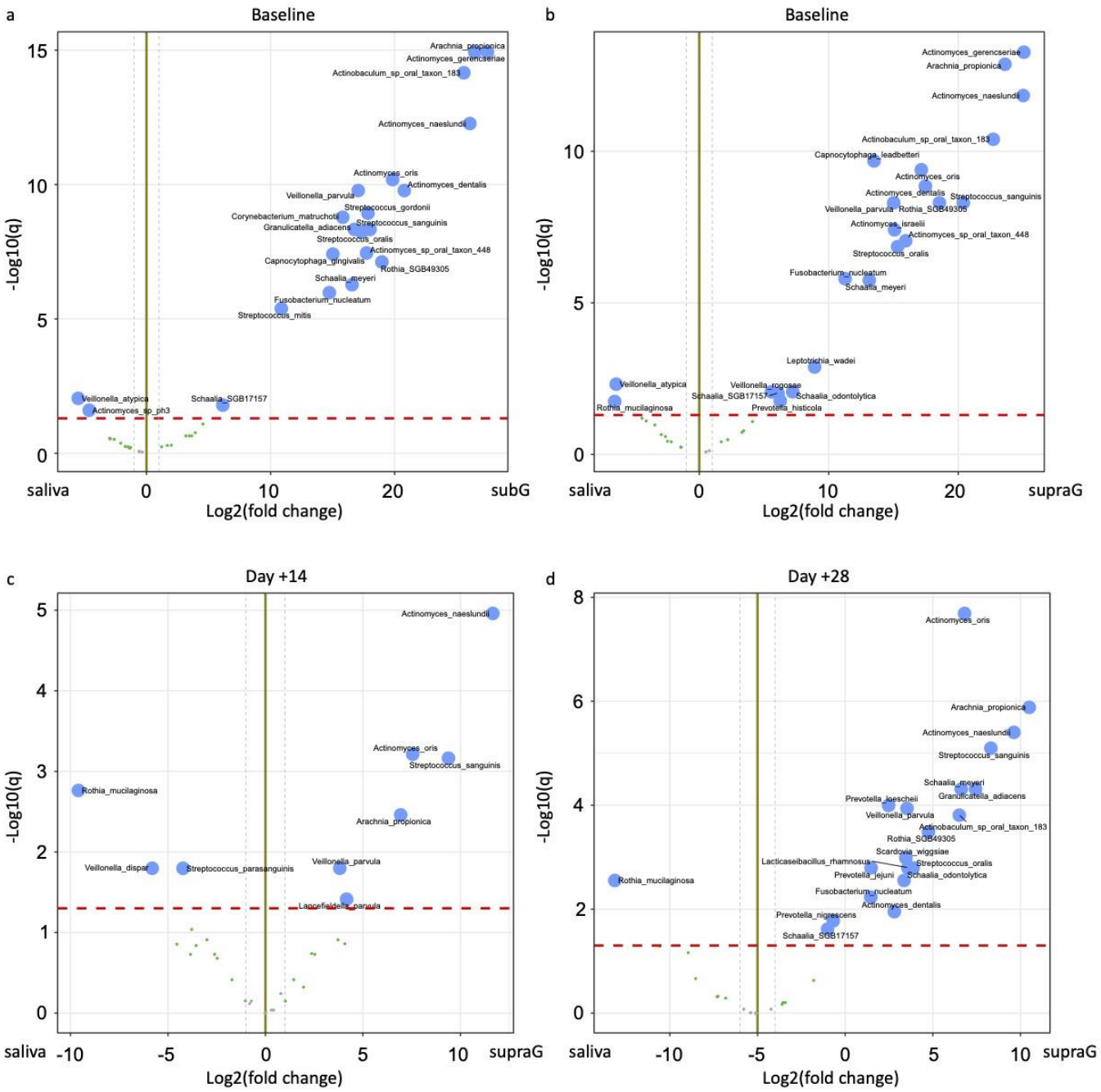

Figure 8. Differential abundance analysis of oral microbiota composition

Comparison is made between saliva and subG at baseline (**a**), saliva and supraG at baseline (**b**), saliva and supraG at day +14 (**c**), and saliva and supraG at day +28 (**d**). No differentially abundant species were found between saliva and supraG at day +84, thus no plot is shown. Each circle shows one species. Species to the right/left of the solid vertical line are more abundant in samples from the site indicated at the bottom of the plot on the right/left. The x axis shows the fold change in abundance. The y axis shows adjusted P values (q values), with circles above the red line representing species with differential abundance between the two sites.

subG: subgingival plaque; supraG: supragingival plaque

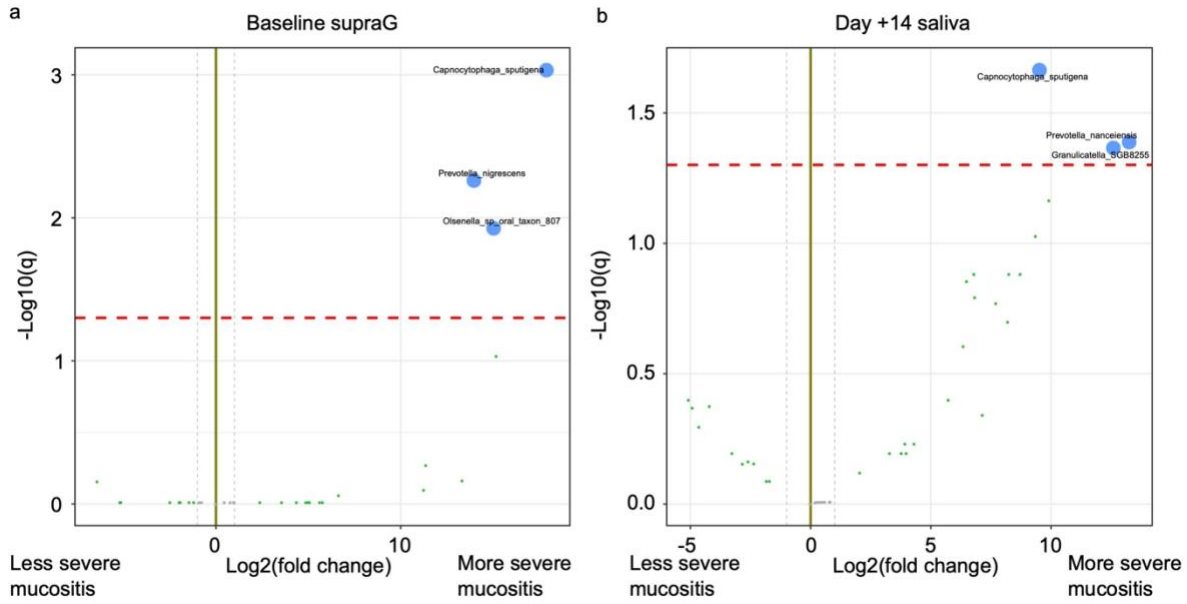

Figure 9. Microbiome correlates with oral mucositis

(**a**) Baseline supraG microbiome vs. day +7 OM. (**b**) Day +14 salivary microbiome vs. day +14 OM. Each circle shows one species. Species to the right/left of the solid vertical line are more abundant in patients with more/less severe OM. Severity was defined binarily (i.e., total mucositis score ≤ median vs. > median). The x-axis shows the fold change in abundance. The y-axis shows adjusted P values (q values), with circles above the red line representing species with differential abundance between the two sites.

supraG: supragingival plaque

Chapter 4. Discussion

In this prospective, non-interventional study, longitudinal, multisite oral cavity sampling was completed to examine the relationship between oral microbiota and OM severity in patients receiving alloHSCT. To our knowledge, this is the first time that multiple types of oral cavity samples were used to study this relationship along with a numerical OM grading system and shotgun metagenomic sequencing to characterize oral microbiota. With respect to the specific aims of the study, 1) three supragingival plaque species at baseline (*Capnocytophaga sputigena*, *Prevotella nigrescens*, and an *Olsenella* species) were significantly associated with day +7 OM (defined by total mucositis score > the median) and three salivary species at day +14 (*Capnocytophaga sputigena*, *Prevotella nigrescens*, and a *Granulicatella* species) were significantly associated with day +14 OM (**Figure 9a-b**); 2) oral microbial diversity in both saliva and supragingival plaque samples is reduced between baseline and day +14 and recovers by day +28 in saliva samples and by day +84 in supragingival plaque samples (**Figure 6b**). Similar to previous reports, we observed a peak in total mucositis score around day +14 followed by near-complete recovery by day +21 (**Figure 5a**)[18,30]. In addition to microbiota predictors, several clinical predictors of mucositis were identified including male sex for day +7 OM, myeloablative conditioning for day +14 OM, older age for day +21 OM, and diseases other than acute leukemia for day +84 OM (**Figure 5b**). While a reduction in salivary flow rate was observed between baseline and all other timepoints, this reduction did not reach statistical significance nor was it associated with OM severity at any timepoint (**Figure 5c**).

The overlap of two species, *Capnocytophaga sputigena* and *Prevotella nigrescens*, in both salivary and supragingival plaque samples associated with more severe OM is an important finding. Both species are gram-negative, facultatively anaerobic in the case of *Capnocytophaga*

sputigena, strictly anaerobic in the case of *Prevotella nigrescens*, and have been implicated in human infections ranging from periodontitis to bacteremia[93–98]. Both are considered commensal bacteria that can be found in the oral cavity but can act as opportunistic pathogens under certain conditions. For example, antibiotic use in our patients may have contributed to the decline in oral microbial diversity, and in the context of immunosuppression, these species were allowed to flourish and fill the newly vacated niche. Anaerobic, gram-negative bacteria are known to contribute to oral inflammatory processes through the production of endotoxins such as lipopolysaccharides (LPS) found in the bacterial cell walls[99]. When LPS is recognized by host immune cells through toll-like receptor 4 (TLR4), proinflammatory signaling pathways are initiated leading to the activation of NF-κB. As discussed previously, NF-κB is the key driver of proinflammatory signaling that ultimately leads to erythema and ulceration in the mouth, and this is one possible mechanism by which these species could exacerbate OM. The *Granulicatella* species found in our supragingival plaque samples is gram-positive, and various species within this genus have been associated with infections such as endocarditis and sepsis[100,101]. Species from the genus *Granulicatella* have been detected in all sites of the oral cavity[65]. The *Olsenella* species found in our saliva samples is gram-positive, strictly anaerobic, and was previously detected in subgingival and supragingival plaque samples[65]. Species from the genus *Olsenella* have been reported in cases of endodontic infection and pneumonia[102,103]. Furthermore, *Olsenella* belongs to the family *Atopobiaceae* which, alongside *Capnocytophaga sputigena*, was significantly enriched at engraftment and in day +30 gingival swab samples of patients who experienced WHO grade ≥2 OM in a previous study[86].

Salivary microbial composition of patients at baseline were significantly different than healthy controls which may suggest that AML and related blood disorders play a role in oral

microbial dysbiosis (**Figure 7b**). At baseline, microbial composition of patient supragingival and subgingival plaque were similar, but they both differed significantly from salivary microbial composition (**Figure 7a**). This reaffirms the notion that although plaque bacteria are shed into the saliva, sampling saliva alone cannot capture the entire diversity and abundance of the populations living in these environments. Lending more support to this idea, compositional differences between saliva and supragingival plaque were sustained across all timepoints throughout the study (**Figure 7c-f**). To further explore these differences, we conducted differential abundance analysis to identify overrepresented species in each environment (**Figure 8a-d**). *Actinomyces* was found to be overrepresented in baseline subgingival and supragingival plaque samples and *Veillonella atypica* overrepresented in baseline saliva samples. These findings are consistent with previous reports which show *Actinomyces* species to normally be found in subgingival and supragingival plaque and *Veillonella atypica* to normally be found in the throat, palatine tonsils, and dorsal tongue[65]. These species are part of the normal oral microbiota; however, their overgrowth has been associated with disease processes like dental abscess and periodontitis[104,105]. While these species were not associated with OM severity in our study, their differential abundance highlights important differences in bacterial composition between different oral environments. *Rothia mucilaginosa* was overrepresented in saliva samples at day +14 and day +28 while various species of *Actinomyces* continued to be overrepresented in supragingival plaque samples at those timepoints. *Rothia mucilaginosa* has been detected in nearly all sites of the healthy oral cavity, though less commonly in plaque biofilms, and while it is normally a commensal species, it has been associated with myriad oral and systemic disease processes in immunocompromised individuals[65,106]. *Streptococcus sanguinis* and *Arachnia propionica* emerged as overrepresented bacteria in supragingival plaque at day +14 and day +28. These species are typically associated

with healthy plaque biofilms, and future analyses may examine their role in protecting the oral cavity from the development of mucositis[63,65].

There are several analyses that are still in process from the data and samples generated by this study. Because we utilized shotgun metagenomic sequencing, these data allow for inference of functional microbial pathways using gene content. This analysis could give insight to the precise microbial genes and pathways involved in OM development and severity. Furthermore, GCF samples remain stored in -80°C, and these are an excellent resource for proteomic and metabolomic studies that can further elucidate molecular predictors of OM and contributors to its pathogenesis. Data related to antibiotic exposure has been collected and reviewed for accuracy. Antibiotic predictors of differentially abundant species will be analyzed in the future. Although antibiotics are doubtlessly a major cause of microbiome disruptions, we assumed in our analyses that they did not directly cause mucositis and are thus not a confounder in microbiota-mucositis associations; however, the use of certain antibiotics may be correlated with the enrichment of potentially pathogenic species. Future analyses could reveal classes of antibiotics that are associated with the enrichment of species found in patients with more severe OM. Finally, we plan to review data from allogeneic departure examination (near day +84) to determine if mucositis scores may have been impacted by mucosal inflammation unrelated to conditioning-induced mucositis (e.g., chronic GvHD). Data will also be collected from long-term follow-up visits to correlate sequencing data with the development of chronic GvHD.

There were several limitations to this study. First, some oral samples, especially salivary samples from patients with heavy mucosal damage, contained high quantities of host DNA thus diluting microbial DNA output and reducing sequencing depth. It is possible that we were unable to detect low abundance species in such samples. Deeper sequencing could identify other microbes

beyond bacteria (e.g., viruses). Here we report findings related to bacteria but also identified sequences of archaea and fungi which will be included in future analyses. Next, for safety purposes, we could not collect subgingival samples after transplant. This may have limited our ability to identify species that are only present in this environment. Finally, denser sampling and mucositis assessment schedules (i.e., two or three times a week) may have provided higher resolution data, but we maintained our schedule for patient comfort and logistical feasibility.

The findings in this study have potentially important implications related to mucositis risk, patient assessment, and future interventional studies. Baseline screening for bacterial species associated with more severe OM may be an effective way to identify patients who are at higher risk going into transplant. As sequencing technologies become more ubiquitous and affordable, oral cavity sampling could someday be incorporated into the routine oral examination that is already performed for each patient prior to transplant. Future studies may also explore interventions that can prevent or more effectively resolve OM when it occurs. For example, if one of these pathogenic bacteria are detected during screening, a thorough dental cleaning, possibly with topical antibiotics targeting gram-negative bacteria (e.g., aminoglycosides), could be trialed for OM prevention. If OM develops without having detected pathogenic bacteria at baseline, a less invasive, anti-infective protocol could be used (e.g., targeted antibiotic rinses) to accelerate ulcer healing. Furthermore, future studies may reveal the precise bacterial metabolites or molecular components that are contributing to OM. One possibility is LPS as discussed previously, but other novel substances may yet be undiscovered. Such a discovery could lead to the development of targeted therapeutics that either slow the proliferation of specific bacteria or sequester the pathogenic substance they create. Another strategy could focus on sustaining the healthy population of bacteria present pre-transplant to prevent the growth of pathogenic bacteria post-

transplant. Probiotic rinses or other oral hygiene interventions may be effective methods to accomplish this.

OM remains a challenging problem for both patients and providers. Especially as the number of HSCTs grows, it is imperative that we continue to elucidate the specific contributors and mechanisms to this treatment complication so that we may develop more effective preventative measures and/or therapeutics. The cross-disciplinary efforts of oncology, oral medicine, and microbiome sciences outlined in this thesis have advanced our understanding of OM and created a strong foundation for future projects in this field.

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