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ORIGINAL RESEARCH

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# Quantification of very late xerostomia in head and neck cancer patients after irradiation

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## Abstract

**Objective:** Radiation therapy (RT) for head and neck cancer (HNC) can result in severe xerostomia, or the subjective feeling of dry mouth. Characterizing xerostomia is critical to designing future clinical trials investigating how to improve HNC patients' quality of life (QoL). Few studies have investigated the very late (>5 years post-RT) effects of RT for HNC. We undertook preliminary studies quantifying very late xerostomia.

**Methods:** Six adults who underwent RT for HNC at least 5 years prior and reported xerostomia were enrolled. Five healthy adults without a self-reported history of HNC or xerostomia were enrolled as controls. All participants completed three validated surveys to measure xerostomia-related QoL. Salivary production rates were measured and compositional analysis of the saliva and oral microbiome was completed.

**Results:** The QoL survey scores for the HNC participants were significantly worse as compared to the control participants. The HNC participants produced less unstimulated saliva ($p = .02$) but not less stimulated saliva. The median salivary mucin significantly higher in HNC participants than in control participants ($p = .02$). There was no significant difference between the pH, amylase, or total protein. Microbiome analysis revealed alpha diversity to be significantly lower in the HNC participants.

**Conclusion:** In the survivors of HNC who suffer from late toxicities, multiple means of measuring toxicity may be useful. We found that in patients with radiation-induced xerostomia over 5 years after therapy, not only were the QoL surveys significantly worse, as expected, but other measurements such as mucin and oral microbiome diversity were also significantly different.

**Level of evidence:** 3.

KEYWORDS
head and neck cancer, quality of life, radiation therapy, xerostomia

## 1 | INTRODUCTION

Radiation therapy (RT) for head and neck cancer (HNC) can result in severe xerostomia, or the subjective feeling of dry mouth. Dry mouth can lead to difficulty speaking or swallowing, decreased dental health, and diminished quality of life (QoL). Despite modern radiation techniques, xerostomia continues to affect over 40% of HNC patients.[1] Characterizing xerostomia is critical to designing future clinical trials investigating how to improve HNC patients' QoL.

Xerostomia can be measured multiple ways, including through surveys designed to detect QoL changes from xerostomia and the associated symptoms including dysphagia. Another, possibly more objective, method of quantifying xerostomia can be via salivary measurements. As expected, radiation can result in decreased salivary flow rate both shortly after RT and several years later.[2,3] Interestingly, differences in salivary composition, including mucin levels, are associated with more severe xerostomia, despite similar amounts of saliva.[4] Mucin including MUC5B is produced by the submandibular glands and coat and protect the oral mucosa, contributing to the sense of oral moisture at baseline between meals.[4–6] Amylase is produced by both the parotid and submandibular glands and is one of the most prevalent proteins in saliva providing both enzymatic and antibacterial activity.[7]

Hyposalivation after radiation for HNC has been shown to change the microorganisms present in the mouth.[8,9] There is increasing interest in understanding how changes in the oral microbiome are a cause or an effect of xerostomia, as high-throughput next generation sequencing techniques become more available.[10]

While the short-term changes of saliva during and after radiation have been investigated, very few studies have investigated the very late effects of RT for HNC, including the impact of very late xerostomia and the alterations in the oral microbiome.[11] We undertook preliminary studies quantifying xerostomia as a very late toxicity of RT in HNC patients for use in a planned clinical trial.

## 2 | MATERIALS AND METHODS

### 2.1 | Patient enrollment

This clinical trial was approved by the University of Wisconsin's Institutional Review Board and informed consent was obtained from all participants. Six adults who underwent radiation for HNC at least 5 years prior and reported xerostomia with subjective saliva production at 80% or lower than pre-RT baseline in follow up were enrolled. Data on radiation treatment was collected. Five healthy adults without a self-reported history of HNC or xerostomia were enrolled as controls. Participants with HNC had two study visits at least 6 months apart, healthy controls had one study visit. Planned enrollment was for 10 participants with xerostomia after radiation for HNC, however this was re-evaluated due to COVID-19 pandemic.

### 2.2 | QoL questionnaires

All participants completed three validated tools to measure xerostomia-related QoL: University of Michigan Xerostomia-Related QoL (XeQOL), the Visual Analogue Scale (VAS), and MD Anderson Dysphagia Inventory (MDADI) at each study visit. The XeQOL is a validated patient-reported 15-item assessment scale with 4 domains: physical functioning, pain/discomfort, personal/psychologic functioning, and social functioning.[12] Higher scores indicate increased xerostomia burden.[12] The MDADI is a 20-item questionnaire designed for evaluating the impact of dysphagia on the QOL of patients with HNC.[13] A lower scale represents worse symptoms of xerostomia and worse QoL.[13] The VAS xerostomia questionnaire is an 8-item questionnaire that provides a validated measure of the perception of dry mouth, with a lower scale representing less symptoms.[14]

### 2.3 | Salivary collection and analysis

Salivary production rates were measured under unstimulated and stimulated saliva collection conditions as previously described.[15,16] Briefly, unstimulated saliva was collected over 5 min via the passive drool method–participants allowed saliva to pool in the mouth and gently guided saliva into a saliva collection aid attached to a cryovial. Stimulated saliva was collected over 5 min during which participants chewed inert gum base to the pace of a metronome (70 beats per minute) while expectorating saliva into a cryovial.[17] Cryovials were weighed before and after salivary collection, with the difference in weight representing amount of saliva.

Salivary composition analysis examined qualitative aspects of saliva previously found to change following radiation treatment: salivary pH, total protein, amylase, and mucins (MUC5B).[2,4,7,18–20] Salivary pH was measured using Plastic pH Indicator Strips (ThermoFisher Scientific, Waltham, MA, USA) at 10 s after test strip exposure. Enzyme-linked immunosorbent assays were used to quantify total protein (Thermo Fisher Scientific #23225, Waltham, MA, USA), amylase (Salimetrics #1–1902, Carlsbad, CA, USA), and mucin (LSBio #LS-F22609, Seattle, WA, USA).

### 2.4 | Microbiome analysis

Saliva was pelleted using centrifugation at 1000 × *g* for 15 min at 4°C, pellets had DNA extracted using the DNeasy 96 PowerSoil Pro QIAcube HT Kit (Qiagen, Hilden, Germany). DNA concentration was verified fluorometrically using either a Qubit dsDNA HS Assay Kit or a Quant-iT PicoGreen dsDNA Assay Kit (ThermoFisher Scientific, Waltham, MA, USA). Microbiome in salivary pellets was analyzed using a 16S rRNA amplicon sequencing method, performed at the University of Wisconsin Biotechnology Center at Madison, WI as described previously.[21] Briefly, samples were prepared in a manner similar to the Illumina's 16S Metagenomic Sequencing Library Preparation Protocol, Part #15044223 Rev. B (Illumina Inc., San Diego, CA, USA), modified

with previously described region-specific primers (underlined sequences) that had 0 or 6 random nucleotides $(N)_{0/6}$ and the Illumina adapter overhang nucleotide sequences 5′ of the gene-specific sequences.[22] The modifications are as detailed: the 16S rRNA gene V3/V4 variable region was amplified with fusion primers (forward primer 341f: 5′-ACACTCTTTCCCTACACGACGCTCTTCCGATCT$(N)_{0/6}$ *CCTAC GGGNGGCWGCAG*-3′, reverse primer 805r: 5′-GTGACTGGAGTTCA GACGTGTGCTCTTCCGATCT$(N)_{0/6}$ *GACTACHVGGGTATCTAATCC*-3′). After initial amplification, reactions were cleaned using AxyPrep Mag PCR clean-up beads (Axygen Biosciences, Union City, CA, USA). In the subsequent polymerase chain reaction, Illumina dual indexes and sequencing adapters were added using forward primer 5′-ATGATA CGGCGACCACCGAGATCTACAC[5555555555] ACACTCTTTCCCTACA CGACGCTCTTCCGATCT-3′, and reverse primer 5′-CAAGCAGAAGA CGGCATACGAGAT[7777777777]GTG ACTGGAGTTCAGACGTGTG CTCTTCCGATCT-3′ (where bracketed sequences are custom unique dual indexes of 10 base pairs). After polymerase chain reaction, reactions were again cleaned with AxyPrep Mag PCR clean-up beads. The quality and quantity of the finished libraries were assessed using an Agilent DNA 1000 kit (Agilent Technologies, Santa Clara, CA, USA) and Qubit dsDNA HS Assay Kit (ThermoFisher Scientific), respectively. Libraries were pooled in an equimolar fashion and appropriately diluted before sequencing. Paired-end, 300-base-pair sequencing was performed using the Illumina MiSeq Sequencer and a MiSeq 600-base-pair (v3) sequencing cartridge. Images were analyzed using the standard Illumina Pipeline, version 1.8.2.

Microbiome analysis was performed using Quantitative Insights Into Microbial Ecology (QIIME2) version 2, as described.[23] Illumina sequencing reads were first denoised and quality filtered using the program DADA2.[24] Following this, the sequence variants, equivalent to operational taxonomic units, were aligned and masked using MAFFT[25] and the phylogenetic tree of the amplicon sequence variants created using FastTree.[26] Taxonomy was assigned using a Bayesian classifier–Scikit-learn based on a pretrained Silva database curated to the exact 16S amplicon region. Alpha rarefaction curves using Shannon and Simpson indices were calculated, with a rarefaction upper limit of median depth/sample count and the alpha diversity between different treatments will be compared using Wilcoxon rank sum test. Beta-diversity was calculated, and ordination plots were generated using Bray–Curtis and Jaccard indices and weighted and unweighted UniFrac on amplicon sequence variant data leveled, according to the lowest sample depth.

### 2.5 | Statistical analysis

Data are reported as mean +/− SD or median with range. Calculations were performed using GraphPadPrism v9.3.1 software (La Jolla, CA, USA). Comparisons between groups were made using unpaired t-test, Mann–Whitney test or Kruskal-Wallis test.

## 3 | RESULTS

Six participants with HNC and self-reported xerostomia were enrolled. The median age was 65 (range 58–70), other characteristics displayed in Table 1. Radiation dosing is detailed in Table S1. Five participants with no prior diagnosis of HNC and no reported xerostomia were enrolled as the controls. The median age of the controls was 60 (range 33–62).

The average XeOLS for the HNC participants was significantly worse as compared to the healthy participants (Figure 1A $p = .006$). The average VAS and MDADI scores were also significantly worse than healthy participants (Figure 1A, $p = .002$ for each). There was no significant difference in QoL scores between visits for HNC patients. All scores are reported in Table S2.

The HNC participants had an average unstimulated saliva of 0.13 ml/minute for both visits (SD 0.17 ml/min) and the control participants produced an average unstimulated saliva of 0.57 ml/min (SD 0.92 ml/minute, $p = .02$). Three of the 6 HNC participants were unable to produce any unstimulated saliva. The HNC participants produced an average of 0.42 ml/min (SD 0.32 ml/minute) stimulated saliva, as compared to 0.92 ml/minute (SD 0.64 ml/minute) stimulated saliva from the controls ($p = .24$). The amount of unstimulated or

**TABLE 1** Head and neck cancer diagnoses, curative therapies, and the treatment completion dates of the 6 patients enrolled in our study

| Participant | Diagnosis | Treatment | Treatment completion |
|---|---|---|---|
| 1 | Stage IV (T3N2cM0) squamous cell carcinoma of the right base of tongue, p16+ | Definitive chemoradiation 70 Gy with weekly cetuximab | 2/2014 |
| 2 | Stage IVA (T3N2cM0) squamous cell carcinoma of the right base of tongue, p16+ | Definitive chemoradiation 70 Gy with weekly cisplatin | 3/2014 |
| 3 | Stage IVA (T1cN2cM0) squamous cell carcinoma of the right base of tongue, p16+ | Definitive chemoradiation 70 Gy with weekly cisplatin | 12/2014 |
| 4 | Stage IVA (T3N2bM0) squamous cell carcinoma right tonsil and RMT, p16+ | Definitive chemoradiation 70 Gy with weekly cisplatin | 7/2014 |
| 5 | Stage II (T2N0M0) squamous cell carcinoma right buccal mucosa | Adjuvant radiation 60 Gy | 9/2014 |
| 6 | Stage IVA (TxN2aM0) squamous cell carcinoma of unknown primary | Definitive radiation 64.8 Gy | 7/2011 |

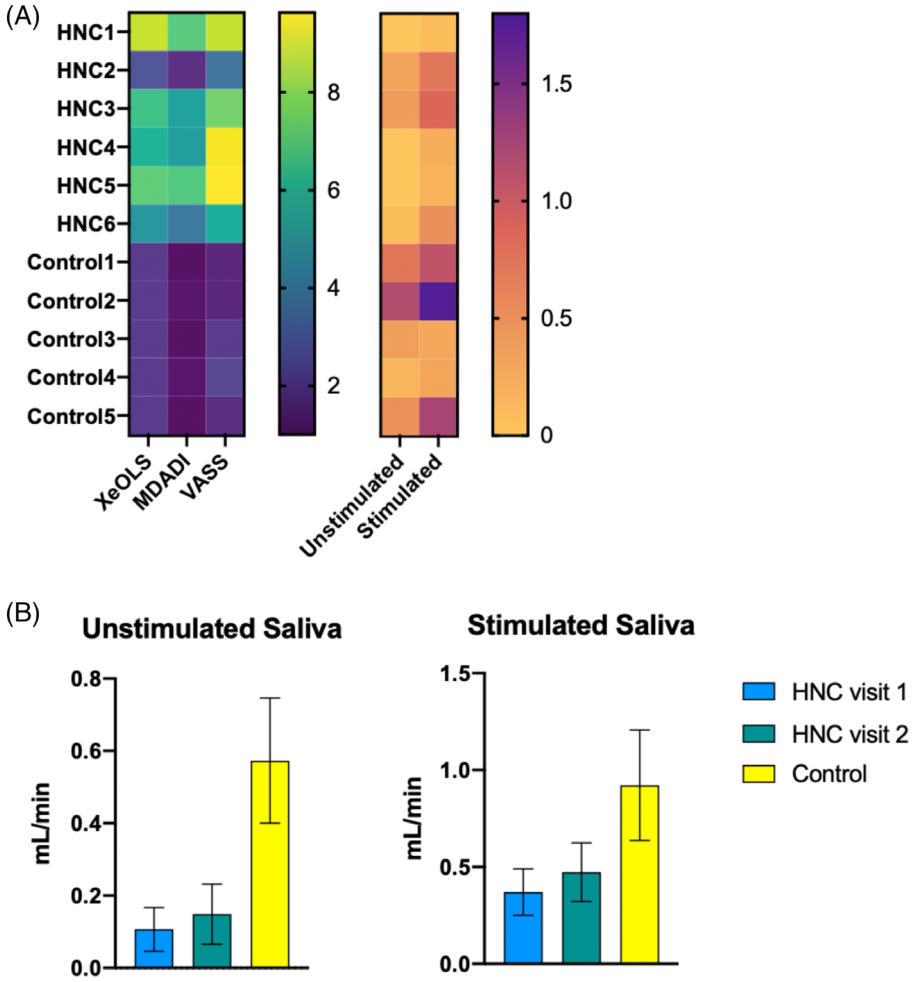


**FIGURE 1** (A) Heat map of xerostomia Quality of Life (QoL) survey scores (University of Michigan Xerostomia-Related Quality-of-Life [XeQOL], the Visual Analogue Scale [VAS], and MD Anderson Dysphagia Inventory [MDADI]) and salivary amount per patient. All QoL scores were scaled to a 0–10 scale with lower scores representing better quality of life. Each of the three QoL surveys were significantly different between the head and neck cancer (HNC) participants and the control participants (XeQOL $p = .006$, VAS $p = .002$, MDADI $p = .002$). Amount of saliva is presented in mL/minute for both stimulated and unstimulated collections. Three of the six patients (HNC 1, 4, and 5) were unable to produce any unstimulated saliva. (B) Graph of the average amount of stimulated and unstimulated saliva produced by HNC and control participants. The HNC participants had an average unstimulated saliva of 0.13 ml/min (SD 0.17 ml/min), controls had average unstimulated saliva of 0.57 ml/min (SD 0.92 ml/minute). The HNC participants produced an average of 0.42 ml/min (SD 0.32 ml/min) stimulated saliva, as compared to 0.92 ml/min (SD 0.64 ml/min) stimulated saliva from the controls. Neither amount of unstimulated or stimulated saliva was significantly different between HNC and control participants

stimulated saliva was not significantly different between HNC participant visits (Figure 1B).

The salivary composition was also investigated. As three of six HNC participants were unable to produce unstimulated saliva, we focused our analysis on stimulated saliva. The median amylase was 151.2 U/ml (SD 138.8 U/ml) for HNC participants in both visits, 101 U/ml (SD 69.2 U/ml) for control participants ($p = .66$, Figure 2A). The median mucin was 2886 ng/ml (SD 1275 ng/ml) for HNC participants in both visits, 201.1 ng/ml (SD 271.6 ng/ml) for control participants ($p = .02$, Figure 2B). The median total protein was 3943 pg/ml (SD 2748.5 pg/ml) for HNC participants in both visits, 1708 pg/ml (SD 594.2) for control participants ($p = .08$, Figure 2C). The median pH for HNC participants over both visits stimulated saliva was 6.4 (SD 0.5) and unstimulated saliva was 6.0 (SD 0.6). The median pH for control participants stimulated saliva was 6.5 (SD 0.3) and unstimulated saliva was 6.3 (SD 0.4). There was no significant difference between the pH for either stimulated or unstimulated saliva.

Microbiome analysis revealed the Shannon Index, measuring alpha diversity, to be significantly lower in the HNC participants as compared to controls (median of 3.5 vs. 4.2, $p = .04$, Figure 2D). The microbial communities of the control participants formed a sub-cluster within the principle coordinate analysis (PCoA) plot without clustering separately from the HNC samples–the beta diversity was not significantly different (Figure 2E). Additionally, as expected, beta diversity between the two timepoints for HNC was not different. The microbial profile did have some differences in genus between the two

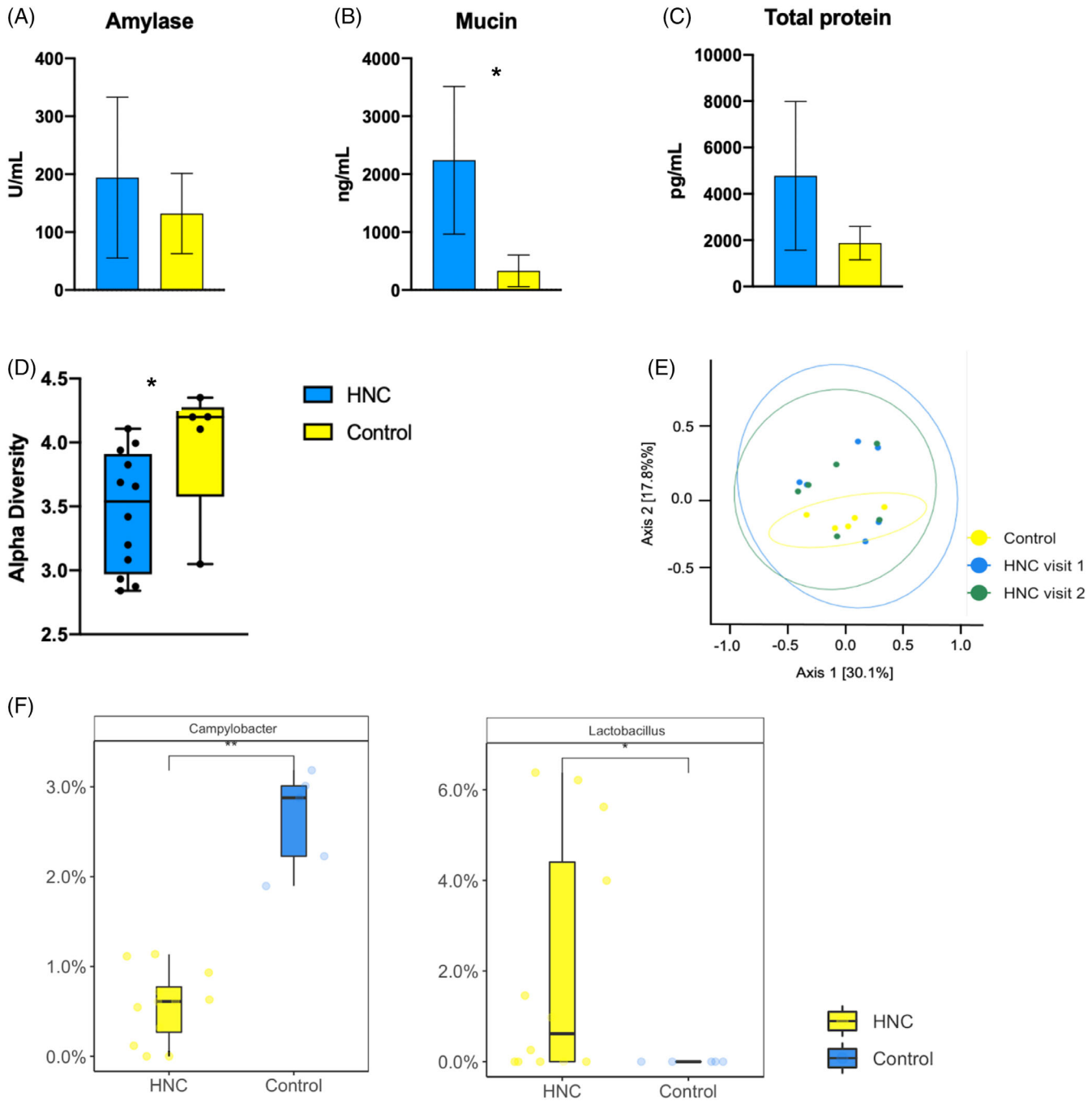


**FIGURE 2** (A) The mean amylase was 194.2 U/ml (SD 138.8 U/ml) for head and neck cancer (HNC) participants, 132.2 U/ml (SD 69.2 U/ml) for control participants ($p = .37$). (B) The mean mucin was 2241 ng/ml (SD 1275 ng/ml) for HNC participants, 330 ng/ml (SD 271.6 ng/ml) for control participants ($p = .03$). (C) The mean total protein was 4109.9 pg/ml (SD 2748.5 pg/ml) for HNC participants, 1544.6 pg/ml (SD 594.2) for control participants ($p = .07$). The mean pH for HNC participants stimulated saliva was 6.3 (SD 0.5) and unstimulated saliva was 5.8 (SD 0.6). (D) The Shannon Index was significantly lower in the HNC participants as compared to controls (3.5 vs. 4.2, $p = .04$). (E) Principal Coordinate Analysis (PCoA) based on the microbial community profiles using Bray-Curtis distance matrices show no significant difference between the beta diversity of the HNC and control participants. (F) The relative abundance of Campylobacter was significantly decreased ($p = .002$), while the relative abundance of Lactobacillus was significantly increased ($p = .045$), all other genera had no significant difference. $^*p < .05$, $^{**}p < .01$

participant groups. The relative abundance of Campylobacter was significantly decreased ($p = .002$), while the relative abundance of Lactobacillus was significantly increased in HNC participants ($p = .045$, Figure 2F). All other genera had no significant differences (Figure S1).

## 4 | DISCUSSION

In the survivors of HNC who suffer from late toxicities years after therapy, multiple means of measuring toxicity may be useful. We

found that in patients with radiation-induced xerostomia over 5 years after therapy, not only were the QoL surveys significantly worse, as expected, but other measurements such as mucin and oral microbiome diversity were also significantly different.

Interestingly, despite significantly worse QoL survey scores, only a trend toward decreased saliva in HNC participants as compared to control participants was seen. This is likely due in part to the small numbers of our study, with 11 total participants. Additionally, there were large amounts of variation in salivary production among participants in the same group, as shown in Figure 1. All control participants were able to produce unstimulated saliva, while three of the six HNC participants could not–and these three did tend to have worse QoL scores. However, as shown in Figure 1, there is no clear correlation between QoL scores and amount of saliva produced.

We found that HNC patients had significantly higher levels of salivary mucin, even years after radiation treatment, however the amounts of amylase and total protein were similar between groups. Previous studies have found a non-significant trend for MUC5B to decrease in the months after radiation, likely due to damage to salivary gland cells.[4,7] The more concentrated salivary mucin we saw is consistent with impaired water transport regulation seen after radiation.[27] This altered level of mucin may contribute to difficulty swallowing, given the importance of mucin in providing oral lubrication, contributing to HNC patients dysphagia scores.[6] There was additionally a trend toward increased total protein, which has been shown to increase in concentration after radiation.[2,28,29] The lack of significance may be due to our small study numbers. Amylase follows a similar pattern of increasing concentration in the months after radiation.[7] We found amylase to return to similar levels in HNC and controls, years after radiation. pH has been shown to be decreased both during and 2 years after radiation treatment[18,19] however we did not find significantly lower pH in our HNC participants. Interestingly, we did find increased abundance of the acidophilic bacteria Lactobacillus, consistent with prior studies.[8–10] Lactobacillus is found in high levels in patients who underwent radiation, particularly in those with low-salivary rates.[8] The exact role of Lactobacillus in xerostomia is unclear–whether the acidic, dry oral environment after radiation results in higher populations of Lactobacillus or if a larger population of Lactobacillus contributes to the acidic environment and xerostomia.[8,9,30] We also found a decrease in overall alpha diversity of the microbiome; prior work by Kumpitsch et al. found a trend but no significant decrease.[10] Alpha diversity describes the distribution of species abundances in a specific sample. The decrease in alpha diversity five or more years after radiation suggests that RT may result in chronic microbiome changes including the diminished presence of certain species. This is the first evidence that the oral microbiome of HNC patients remains altered, even years after RT. The relationship between the subjective report of xerostomia and the altered microbiome is complex, but our research suggests that oral microbiome should be considered in future studies of late toxicities after RT. We are currently examining the changes in the oral microbiome and salivary composition in a clinical trial, along with the use of mesenchymal stromal cells to treat radiation-induced xerostomia.

The treatment of radiation-induced xerostomia is currently supportive in nature. Patients are encouraged to increase water consumption, consume specially prepared food, utilize salivary substitutes or attempt to stimulate salivary production through the use of parasympathomimetic drugs, organic acids, chewing gum, or sugar-free mints.[31–34] These interventions are not curative, failing to address the root cause of xerostomia. There are some curative therapies being investigated, including salivary gland transfer and stem cell injection. Adult stem cells are the ultimate source for replenishment of salivary gland tissue. Marrow-derived mesenchymal stromal cells (i.e., mesenchymal stem cells, MSCs) are a viable cell-based therapy for xerostomia.[35] We are currently conducting a first-in-human clinical trial investigating autologous bone marrow derived MSCs as a treatment for chronic radiation-induced xerostomia.[36]

Our study does have several limitations, the main one being the small number of participants. Additionally, our participants and controls were not matched based on age and sex. However, this is a preliminary study into very late xerostomia, with future research planned. The differences seen for Lactobacillus and Campylobacter were the most significant comparisons out of the 15 major genera studied, without Bonferroni adjustment made. Future studies should further investigate our exploratory analysis of the genera, and investigate the association of mucin with the altered microbiome in HNC patients with very late xerostomia. We also did not investigate participants' dental health, which can be a significant toxicity from radiation and contributes to QoL. Future research should incorporate dental information, alcohol and tobacco use, and fungal studies.

Here we present a preliminary study investigating the late effect of radiation induced xerostomia, the first to examine QoL related to xerostomia and swallowing function, salivary quantity, salivary composition, and microbiome in patients over 5 years after RT. Our data on various quantifications of late xerostomia suggests that the submandibular glands, primarily responsible for unstimulated salivary production, may be a large factor in symptomatic xerostomia. This prompted our investigation into restoring the submandibular salivary function using mesenchymal stromal cells.[36] Our study provides insight into the late toxicities of radiation for HNC. While small, this study supports the need for further research into late xerostomia and curative therapies. Future investigations should utilize multiple means to quantify xerostomia and QoL.

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## CONFLICT OF INTEREST

The authors have no conflicts of interest to declare.

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## SUPPORTING INFORMATION

Additional supporting information can be found online in the Supporting Information section at the end of this article.

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