An international report on bacterial communities in esophageal squamous cell carcinoma

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Abstract

The incidence of esophageal squamous cell carcinoma (ESCC) is disproportionately high in the eastern corridor of Africa and parts of Asia. Emerging research has identified a potential association between poor oral health and ESCC. One possible link between poor oral health and ESCC involves the alteration of the microbiome. We performed an integrated analysis of four independent sequencing efforts of ESCC tumors from patients from high- and low-incidence regions of the world. Using whole genome sequencing (WGS) and RNA sequencing (RNAseq) of ESCC tumors from 61 patients in Tanzania, we identified a community of bacteria, including members of the genera \textit{Fusobacterium}, \textit{Selenomonas}, \textit{Prevotella}, \textit{Streptococcus}, \textit{Porphyromonas}, \textit{Veillonella} and \textit{Campylobacter}, present at high abundance in ESCC tumors. We then characterized the microbiome of 238 ESCC tumor specimens collected in two additional independent sequencing efforts consisting of patients from other high-ESCC incidence regions (Tanzania, Malawi, Kenya, Iran, China). This analysis revealed similar ESCC-associated bacterial communities in these cancers. Because these genera are traditionally considered members of the oral microbiota, we next explored whether there was a relationship between the synchronous saliva and tumor microbiomes of ESCC patients in Tanzania. Comparative analyses revealed that paired saliva and tumor microbiomes were significantly similar with a specific enrichment of \textit{Fusobacterium} and \textit{Prevotella} in the tumor microbiome. Together, these data indicate that cancer-associated oral bacteria are associated with ESCC tumors at the time of
diagnosis and support a model in which oral bacteria are present in high abundance in both saliva and tumors of some ESCC patients.

**KEYWORDS**
Africa, esophageal cancer, esophageal squamous cell carcinoma, *Fusobacterium*, microbiome

**What’s new?**
Striking geographic patterns in the global incidence of esophageal squamous cell carcinoma (ESCC), reflected most notably in disproportionately high incidence rates in parts of Africa and Asia, remain unexplained. A factor suspected of driving these patterns is the microbiome. Here, analysis of bacterial communities in ESCC tumors from nine countries across different regions of the world shows that bacteria linked to other gastrointestinal cancers are associated with ESCC. Moreover, composition of the saliva microbiome was correlated with composition of the ESCC tumor microbiome. Further research is needed to better understand the role of the oral microbiome in ESCC pathogenesis.
1 | INTRODUCTION

Esophageal cancer is the sixth most common cause of cancer-related death worldwide.\(^1\) There are two histologic subtypes of esophageal cancer with distinct biological characteristics, geographic distributions and risk factors.\(^2\) Esophageal adenocarcinoma is the most common histologic form of esophageal cancer in high-income countries and is associated with factors including gastroesophageal reflux disease, Barrett’s esophagus and obesity.\(^3,4\) By contrast, esophageal squamous cell carcinoma (ESCC) represents more than 90% of worldwide esophageal cancer cases and is the dominant histology in low-resource settings. In particular, there are two main regions where ESCC is endemic:\(^5\) the Asian esophageal cancer belt, extending from western/northern China to central and southeast Asia; and\(^6\) the eastern corridor of Africa, extending from Ethiopia to South Africa.\(^5,6\)

Emerging research has identified a possible association between poor oral health and ESCC. Studies from Asia, Europe, Latin America, Kenya and Iran have reported associations of ESCC with poor oral hygiene, chronic periodontal disease, dental decay and tooth loss.\(^7-16\) Recently, three parallel case-control studies in Kenya and Tanzania, conducted as part of the African Esophageal Cancer Consortium (AfrECC) and ESCCAPE (escape.iarc.fr) collaborations, reported possible associations of poor or infrequent oral hygiene with increased risk for ESCC in East Africa.\(^17,20\)

Alterations of the oral microbiome due to poor oral health is one proposed biological pathway that could explain the link between oral health and ESCC. Many bacterial genera associated with gastrointestinal cancers contain species that are traditionally associated with healthy or diseased oral microbiomes. For example, Helicobacter pylori was discovered to be associated with gastric cancers and mucosa-associated lymphoid tissue (MALT) lymphomas, indirectly by promoting gastric inflammation and directly by influencing cellular signaling.\(^21\) Similarly, bacteria of the genera Fusobacterium, Selenomonas and Prevotella are enriched in colorectal cancers\(^22,24\) and can be visualized invasively within tumor tissue.\(^25\) Fusobacterium, in particular, has been reported to promote carcinogenesis through the selective expansion or inhibition of certain classes of immune cells\(^26\) and may drive cellular proliferation by stimulating Wnt/β-catenin signaling.\(^27,28\) Other bacterial genera such as Porphyromonas, Campylobacter and Streptococcus have emerging associations with various human gastrointestinal cancers.\(^29-35\)

As part of ongoing investigation into the microbiome’s association with ESCC, we performed an integrated analysis of four independent sequencing efforts including ESCC tumors from patients from both high- and low-incidence regions of the world. In addition, we investigated the relationship between the microbiomes of matched ESCC tumors and saliva specimens in a subset of ESCC cases.

2 | MATERIALS AND METHODS

2.1 | Samples and sequencing efforts

The origin and number of samples is as follows: MUHAS Tanzania cohort (\(n = 61\)),\(^36\) UNC Project—Malawi cohort (\(n = 30\)).\(^37\) The Mutographs study (\(n = 210\))\(^38\) originated from patients in Golestan, Iran (\(n = 55\)), ESCCAPE case-control studies in Tanzania (\(n = 18\))\(^39\) and Kenya (\(n = 64\)),\(^17\) and patients in Shanxi, China (\(n = 71\)). While there are over 500 samples in the full Mutographs study, our investigation was limited to a subsample of 210 samples. TCGA ESCC (\(n = 36\)) and COAD samples (\(n = 51\)) have been previously described.\(^39,40\) The TCGA ESCC cohort includes tumors from patients in United States (\(n = 3\)), Ukraine (\(n = 3\)), Vietnam (\(n = 22\)) and Russia (\(n = 8\)), regions which have lower incidence of ESCC. Information on sample acquisition and sequencing for the UNC Project—Malawi, Mutographs, and TCGA patient samples can be identified in their relevant publications.\(^19,36-40\) Details on each sequencing cohort are also listed in Table 1.

We provide sequencing statistics on studied samples in Table S1. This table includes the following information for each sample: the total number of reads, the total number of reads that passed quality and complexity filtration during GATK-PathSeq analysis, the number of human-mapped reads, the number of reads mapped to the microbial reference database, and the number of unmapped reads.

2.2 | MUHAS Tanzania sample collection and sequencing

DNA and RNA were extracted from tumor samples using the Qiagen AllPrep kit. Saliva samples were collected in the Oragene DISCOVER ORG-500 tube and DNA was extracted using the Oragene PreipIT L2P extraction kit. Saliva samples were collected at the time of tumor collection. RNA integrity was assessed using an Agilent bioanalyzer, and DNA and RNA quantity was assessed by Nanodrop and PicoGreen.

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Table 1 | Sequencing effort characteristics

| Sequencing cohort            | Number of patients | References | Sequencing type                          |
|------------------------------|--------------------|------------|-----------------------------------------|
| MUHAS Tanzania               | 61                 | 36         | DNA: WGS                                |
|                              |                    |            | RNA: Human rRNA subtraction, random-hexamer priming |
| UNC Project—Malawi           | 30                 | 37         | RNA: Human rRNA subtraction, random-hexamer priming |
| Mutographs                   | 210                | 17,19,38   | DNA: WGS                                |
| TCGA                         | 36                 | 39         | DNA: WGS                                |
(Invitrogen) methods. DNA sequencing libraries were prepared with the KAPA Hyper Prep kit, and samples were sequenced to a target depth of ×60 coverage (for tumor) or ×30 coverage (for saliva) on an Illumina NovaSeq. RNA isolations with RIN > 7 were prepared with the KAPA Stranded RNAseq with RiboErase kit and sequenced on an Illumina HiSeq or NovaSeq to a target of 200 million 150 bp paired reads. Additional methodologic details can be found in the associated publication.36

2.3 Bacterial identification and quantification

GATK-PathSeq41 was used to conduct computational subtraction of human-mapping reads from input RNAseq and WGS datasets. GATK-PathSeq initially maps reads to a host reference database consisting of the human genome grch38 and various supplemental human reference sequences. Next, nonhuman reads are mapped against a comprehensive microbial database, and microbe read assignments are reported for further study.

For all analyses, we used the GATK-PathSeq “score,” which is roughly equivalent to read count. The GATK-PathSeq score is calculated by: (1) distributing a read count to multiple species if the read maps to multiple species (eg, if a read maps to two species, each species gets +0.5 of a read); and (2) normalizing the read count to genome length (only in WGS data). The score is calculated at the taxonomic level of the entry in the reference (usually a species or strain). The genus score is the sum of scores assigned to each species in that genus.

Bacterial abundance analyses and plotting were conducted in R (v3.5.1). To calculate relative abundance at a phylogenetic level (eg, phylum or genus), GATK-PathSeq results were filtered for taxa at the level, and relative abundance was calculated for each taxon as follows: (# of taxon reads)/(total # reads at the selected phylogenetic level). The rows of all bacterial abundance heatmaps are arranged according to the mean abundance across all samples. The sample order of relative abundance stacked barplots were determined based on Fusobacterium genus relative abundance except where noted. In Figure 2D, if any cohort contained more than 50 samples, 50 samples were randomly selected for plotting. The distribution of relative abundances of genera of interest in all samples can be found in Figure S2, where width of each violin represents the relative distribution of observed bacterial relative abundance for all patients in each patient cohort.

Jaccard distance between RNAseq and WGS data from each ESCC tumor was calculated in R based on bacterial genera with at least 1% relative abundance. The qualitative Jaccard index was used in this case because the comparison was between DNA and RNA analytes which would not be expected to be quantitatively identical.

2.4 Tumor-saliva similarity

Only tumor-saliva pairs from the MUHAS Tanzania cohort with at least 10 000 reads mapped to the bacterial superkingdom were analyzed for similarity (nге = 21). Bray-Curtis dissimilarity metrics between tumor-oral pairs were calculated using the R package vegan.42 Figure 3A presents the Bray-Curtis similarity (1 − Bray-Curtis dissimilarity), for each tumor-oral pair.

To determine the correlation between the relative abundance of specific genera between tumor and saliva microbiomes, common-abundant genera with at least 1% abundance in at least three tumor-saliva pairs were identified. This resulted in the identification of 16 common-abundant genera. Correlations represent a two-sided Pearson correlation coefficient. To determine tumor-saliva enrichment of common-abundant genera, the difference in relative abundance of each genus between each tumor-saliva pair was plotted (Figure 3C). For the relative abundance bar plots of tumor-saliva pairs (Figure 3D), bacterial genera that had been highlighted in previous figures are labeled.

2.5 Bacterial contaminant identification

We identified potential bacterial contaminants with two approaches. First, we used a bacteria genera blacklist identified by Salter et al and Poore et al43,44 as bacteria commonly present in negative-blank controls. Second, we implemented the Decontam program.45 Decontam works on the assumption that the relative abundance of contaminants should be inversely proportional to the library concentration, and thus incorporates information on library concentrations prior to pooling for sequencing. We ran Decontam on samples from the Mutographs cohort with an aggressive P value threshold of P < .2 and the “frequency” method. The code used to run Decontam can be found in running_decontam.Rmd. We then removed the blacklist and Decontam-called bacterial genera from each dataset, and regenerated Figure 2 as Figure S3B-E. The code used to do this analysis is present at Fig_S3_blacklist_and_decontam.Rmd.

We implemented Snippy (v4.4.5) (https://github.com/tseemann/snippy) for variant calling, and then FastTree46 for constructing phylogenetic trees of Fusobacterium isolates. We first ran snippy-multis, followed by rerunning snippy-core on samples with sufficient single nucleotide polymorphisms (SNPs) relative to the reference. We implemented Snippy only on Fusobacterium—high samples that had sufficient SNPs, resulting in four samples from MUHAS Tanzania and five from ESCCAPE Kenya. As the reference Fusobacterium strain, we used Fusobacterium nucleatum subspecies polymorphum (accession NZ_LN831027.1). We included WGS reads from genetically similar tumor-oral Fusobacterium pairs previously sequenced by Abed et al47 in each run of snippy-core.

2.6 Batch effects

Batch effects arise during separate collection, processing and sequencing of samples. In the context of microbiome studies, this will result in differences in the amount and identity of sequencing reads from contaminating genera. As noted above, because we do not have the controls necessary to concretely identify contaminating bacteria, we do not take action to remove contaminants. We acknowledge that batch effect may
FIGURE 1  Microbiome structure and composition of ESCC tumors. (A) Description of ESCC patients, and sample types, assessed in this study. ESCCAPE, Esophageal Squamous Cell Carcinoma African Prevention Research; Mutographs, Cancer Research UK Mutographs Project; TCGA, The Cancer Genome Atlas. (B) Bacterial burden of ESCC tumors for each patient cohort. Units are bacterial reads per million human reads as determined by GATK-PathSeq analysis. Each dot represents one sample. Analyte type (RNA or DNA) and tumor type (ESCC or COAD) are indicated by color. (C) Shannon diversity of ESCC tumors for each patient cohort. Shannon diversity was determined for each sample at the genus level based on genera that are at least 1% relative abundance. Each dot represents one sample. Analyte type (RNA or DNA) and tumor type (ESCC or COAD) are indicated by color. (D) Heatmap describing the relative abundance of the five top phyla sorted by average phylum relative abundance. Each column represents one sample. Rows represent the indicated phyla. Units are relative abundance. Samples from each cohort are WGS unless noted with “(RNA),” in which case they are RNAseq.
influence the relative abundance of non-contaminant bacteria (e.g., as a consequence of DNA or RNA extraction), meaning that there may be some minor quantitative differences as a result.

3 | RESULTS

3.1 | Study population

To evaluate the potential role of the host microbiota in ESCC, we investigated the microbiome of 299 ESCC specimens from patients in five different countries with a high incidence of ESCC. Specimens were collected through four independent sequencing efforts (Figure 1A). Specimens consisted of whole genome sequencing (WGS) and RNA sequencing (RNAseq) data from the tumor and saliva of 61 patients from Tanzania (the “MUHAS Tanzania” cohort), 36 RNAseq data from the tumors of 30 ESCC patients in Malawi (the “UNC Project—Malawi” cohort), and WGS from 208 additional samples of tumors from patients in high ESCC incidence regions, including specimens from ESCC patients in Tanzania (n = 18) and Kenya (n = 64) that were collected in the ESCCAPE studies (escape.iarc.fr) and specimens from ESCC patients in East Golestan, Iran (n = 55) and Shanxi, China (n = 71) that were sequenced as part of the Cancer Research UK Mutographs project (“Mutographs” cohorts).38 In addition, we analyzed WGS data of ESCC from The Cancer Genome Atlas,39 which includes a small number of tumors from patients in low-incidence geographic regions including the United States (n = 3), Ukraine (n = 3), Vietnam (n = 22) and Russia (n = 8) (the “TCGA” cohort). Patient characteristics are shown in Table 2.

3.2 | Bacterial populations are abundant and diverse in ESCC tumors

We used the metagenomic analysis tool GATK-PathSeq41 to process the RNAseq and WGS data. GATK-PathSeq uses a sequential mapping strategy to assign reads to human and microbial reference genomes, resulting in detailed information on sequencing reads of human and microbial origin (Figure S1A). We likewise used GATK-PathSeq to process WGS data sets from 50 colon adenocarcinoma (COAD) specimens available from TCGA40 for comparison, as there is strong evidence of microbial associations with COAD.22-25 The bacterial burden of ESCC tumors ranged from 10 to 1000 bacterial reads per million human reads, similar to numbers observed in TCGA COAD (Figure 1B). Furthermore, the Shannon diversity of bacterial populations at the genus level ranged from 2 to 3 (Figure 1C). By comparison, ESCC-associated bacterial communities are as diverse or more diverse than TCGA COAD. At the phylum level, ESCC bacterial populations generally consist of Firmicutes, Bacteroidetes, Proteobacteria, Actinobacteria and Fusobacteria (Figures 1D and S1B). Of note, the higher than expected abundance of the phylum Actinobacteria specifically in the TCGA ESCC samples is attributable, in particular, to a very high abundance of the genus Tetrasphaera (Figure S1C). This is evidenced by a
depressed Shannon diversity of Actinobacteria genera in these samples (Figure S1D) and may indicate contamination of the TCGA ESCC samples. Actinobacteria have been reported as a source of contaminating reads in TCGA gastrointestinal cancer samples.

3.3 Bacterial genera associated with carcinogenesis are observed at high relative abundance in ESCC tumors from Tanzania

To determine if bacteria with known associations with cancer are present in ESCC, we first analyzed the sequencing series of the 61 ESCC cases from the MUHAS Tanzania cohort with both WGS and RNAseq data. The paired WGS and RNAseq data from these tumors allowed investigation of bacterial communities at the DNA and RNA levels. Both WGS and RNAseq data revealed high relative abundance of bacterial genera previously associated with carcinogenesis in these ESCC tumors (Figure 2A,B). The high relative abundance of the Fusobacterium genus was particularly notable. Other bacterial genera of interest include Streptococcus, Porphyromonas, Campylobacter, Prevotella, Veillonella and Selenomonas, many of which have been associated with gastrointestinal malignancies alongside or independently of Fusobacterium.

FIGURE 2 Identification of bacterial genera associated with carcinogenesis. (A) Bacterial genera relative abundance of WGS data from the MUHAS Tanzania cohort. Each column represents a single sample. Samples are ordered by decreasing Fusobacterium relative abundance. Units are relative abundance of bacterial genus-mapping reads. Color indicates the genus and seven genera are specified. Only patients with GATK-PathSeq analysis from both RNAseq and WGS tumor data are plotted (n = 59). (B) Bacterial genera relative abundance of RNAseq data from the MUHAS Tanzania cohort. Each column represents a single sample. Here, column order is dictated according to the patient order in (2A). Units are relative abundance of bacterial genus-mapping reads. Color indicates the genus and seven genera are specified. Only patients with GATK-PathSeq analysis from both RNAseq and WGS tumor data are plotted (n = 59). Samples are ordered in the same order as (2A), which is by Fusobacterium genus relative abundance in the WGS data. (C) Jaccard index between RNAseq and WGS data of tumors from the MUHAS Tanzania cohort. For the “Paired by Sample” column, Jaccard indices were calculated only between the WGS and RNAseq data from the same tumor (n = 59 comparisons). For the “Random Pairs” column, Jaccard indices were calculated between all possible WGS-RNAseq pairs independent of patient of origin to represent the expected random distribution of Jaccard indices (n = 3481 comparisons). Jaccard index was calculated from relative abundance at the genus level based on genera that are at least 1% relative abundance. The width of the violin represents the relative proportion of comparisons with each Jaccard index, and lines indicate 25th, 50th and 75th percentiles. (D) Bacterial genera relative abundance of the remaining patient cohorts, including RNAseq and WGS data as indicated. Each column represents a single sample. Samples are ordered by decreasing Fusobacterium relative abundance within each patient cohort. Units are relative abundance of bacterial genus-mapping reads. Color indicates the genus and seven genera are specified. Here, if there were more than 50 samples in a patient cohort, 50 samples were randomly selected for visualization. RU, Russia; UA, Ukraine; USA, United States. All cohorts consist of WGS data, with the exception of the tumors from Malawi which are RNAseq (Number of samples plotted: UNC Project—Malawi 30; ESCCAPE Tanzania 18; ESCCAPE Kenya 50; Shanxi, China 50; Golestan, Iran 50; TCGA ESCC Vietnam 22; TCGA ESCC USA/UA/RU 14)
Next, we attempted to determine if similar bacterial genera were also present in ESCC from patients in high-incidence countries beyond Tanzania. Investigation of RNA sequencing data from patients in Malawi, WGS data from patients in Kenya, China and Iran, as well as from the independent ESCCAPE Tanzania patient group revealed pervasive evidence of similar bacterial genera in the tumors of these patients (Figures 2D and S2). To investigate if similar microorganisms were found in ESCC tumors from patients in low-incidence regions, we investigated WGS data from ESCC tumors originating from USA, Ukraine, Vietnam and Russia that were available through TCGA. While the number of ESCC tumors available from low-incidence regions was low and relies on a single sequencing effort, we found that the tumors of many of these patients contain similar bacterial genera (Figures 2D and S2). Colon cancers from the TCGA COAD cohort revealed evidence of *Fusobacterium*, as expected; however, these COAD samples were notable for much lower relative abundance of the other genera of interest when compared with ESCC tumors.

### 3.4 Assessing contamination

To understand if bacterial contaminants were influencing our results, we identified potential contaminating bacterial genera and assessed the impact of their removal. We identified potential contaminating

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**FIGURE 3** Association between synchronous saliva and tumor microbiomes in Tanzanian ESCC patients. (A) Bray Curtis Similarity comparing tumor-saliva pairs from patients in the MUHAS Tanzania cohort. Analysis was restricted to the 21 tumor-saliva pairs that contained at least 10,000 bacterial reads. This analysis was conducted at the genus level and using relative abundance. For the “Paired by Patient” column, Bray Curtis Similarity was calculated only between the tumor and saliva WGS data from the same patient. For the “Random Pairs” column, Bray Curtis Similarity was calculated between all possible tumor-saliva pairs independent of patient of origin to represent the expected random distribution of Bray Curtis Similarity (P = .0003, Wilcoxon rank sum test). (B) Correlation between the relative abundance of common-abundant bacterial genera in paired saliva and tumor WGS data. Analysis was restricted to the 21 tumor-saliva pairs that contained at least 10,000 bacterial reads. Common-abundant bacterial genera are bacterial genera that are at least 1% abundance in at least three tumor-saliva pairs—16 bacterial genera made this cutoff. Correlation represents a two-sided Pearson correlation. X-axis is the correlation coefficient and Y-axis is the correlation P value plotted on a log scale. (C) Enrichment of genera in the oral or tumor microbiome. Each row details one of the 16 common-abundant bacterial genera. Each row contains one data point per patient, for a total of 21 data points. The value of each point represents the difference in the relative abundance of the specified genus in the tumor and saliva microbiomes of one patient, with positive values indicating the genus is at higher relative abundance in a patient’s tumor. For example, if a genus is at a relative abundance of 0.7 (70%) in the tumor and 0.3 (30%) in the saliva of a patient, the plotted value for that genus and that patient is 0.4. Curves represent the distribution of this relative abundance difference across the tumor-saliva pairs, with dots indicating individual tumor-oral pairs. Vertical red lines indicate quartiles. (D) Relative abundance bar charts of tumor-saliva pairs. Analysis was restricted to the 21 tumor-saliva pairs that contained at least 10,000 bacterial reads. Units are relative abundance of bacterial genus-mapping reads. Color indicates the genus, and seven genera are specified. S, saliva; T, tumor.
genera in two ways: (1) bacteria that had been identified by Settler et al and Poore et al as potential contaminants present in "negative-blank" sequencing controls, and (2) bacteria that were identified by the Decontam algorithm as potential contaminants. An average of 1.58% of bacterial genus reads belonged to bacterial genera present in the blacklist (Figure S3A). We were able to implement the Decontam algorithm on the Mutographs samples (ESCAPE Tanzania; ESCAPE Kenya; Shanxi, China; and Golestan, Iran). In these samples, Decontam-called bacterial genera represented an average of 12.2% of bacterial genus reads (Figure S3A). We then assessed the impact of the removal of blacklist and Decontam-identified bacterial genera on our results and found that the removal of these bacterial genera only had a marginal effect that did not change our conclusions (Figure S3B-E).

While the identification of *Fusobacterium*, *Streptococcus*, *Porphyromonas*, *Campylobacter*, *Prevotella*, *Veillonella* and *Selenomonas* in multiple independent sequencing efforts reduces the likelihood they are contaminants, we implemented a secondary approach to rule out contamination. We focused on *Fusobacterium* in *Fusobacterium*-high samples from the MUHAS Tanzania and ESCAPE Kenya cohorts, reasoning that if *Fusobacterium* was a contaminant, it would be the same isolate (and thus genetically similar) in each sample within each sequencing effort. As a positive control, we included WGS data from two pairs of tumor and oral *Fusobacterium* isolates from colorectal cancers generated by Abed et al, who had found that these tumor and oral *Fusobacterium* pairs were genetically similar isolates. We found that while our approach groups the oral and tumor isolates as reported by Abed et al, *Fusobacterium* from patients in the MUHAS Tanzania (Figure S3F) and ESCAPE Kenya (Figure S3G) patient groups were genetically distinct within each sequencing effort, indicating that the *Fusobacterium* is not a sequencing contaminant.

3.5 | Evaluation of the association between saliva and tumor microbiomes in ESCC patients from Tanzania

We next investigated the similarity between the saliva and tumor microbiomes of ESCC patients. Paired tumor-saliva samples were only available from patients in the MUHAS Tanzania cohort; these paired tumor-saliva specimens were analyzed to evaluate bacterial abundance as a proxy for the oral microbiome.

We first assessed the similarity between paired saliva and tumor microbiomes with the Bray Curtis similarity index. To avoid potential confounding due to low bacterial read counts in some tumor samples, we limited these analyses to the 21 tumor-saliva pairs that contain appreciable microbial sequencing depth (at least 10 000 bacterial reads each). We found that the saliva and tumor microbiomes from the same patient in the Tanzanian samples are significantly more similar than random saliva-tumor pairs (*P* = .0003, Wilcoxon rank sum test) (Figure 3A). Next, we asked if there are bacterial genera whose relative abundance in the saliva correlates with their relative abundance in the tumor. For this analysis, we included only common-abundant bacterial genera with at least 1% relative abundance in at least three tumor-saliva pairs. The relative abundance of four bacterial genera (*Fusobacterium*, *Veillonella*, *Streptococcus* and *Porphyromonas*) are strongly correlated between tumor and saliva microbiomes, while other common-abundant bacterial genera were not (Figure 3B). To assess if any bacterial genera are preferentially enriched in the tumor microbiome relative to the saliva microbiome, we next calculated the difference in the relative abundance of the common-abundant bacterial genera between tumor-saliva pairs. Several genera including *Porphyromonas* and *Veillonella* were at higher relative abundance in the saliva, while *Prevotella* and *Fusobacterium* were enriched in the tumor microbiome (Figure 3C). Finally, the relative abundance of tumor-associated bacteria including *Fusobacterium*, *Prevotella*, *Selenomonas*, *Veillonella*, *Streptococcus* and *Campylobacter* are strikingly similar between the microbiomes of tumor and saliva pairs (Figure 3D).

4 | DISCUSSION

This report provides a comprehensive analysis of bacterial communities present in ESCC tumors from nine countries from different regions of the world, which were previously sequenced in four independent studies. We found traditionally oral, cancer-associated, bacterial genera in tumors from patients in Tanzania, Malawi, Kenya, China and Iran. These results provide evidence that these bacterial genera may be associated with ESCC in these high-incidence regions. We also identified similar bacterial genera in ESCC tumors from low-incidence regions, although this finding was based on a small sample size and only one sequencing cohort. Finally, in a sub-analysis of tumor and saliva pairs available from Tanzania, we demonstrated that the paired saliva and tumor microbiomes of ESCC patients were strikingly similar at the time of diagnosis; in particular, we identified a specific correlation between the saliva and tumor relative abundance of the bacterial genera *Fusobacterium*, *Veillonella*, *Streptococcus* and *Porphyromonas*, with *Prevotella* and *Fusobacterium* significantly enriched in the tumor microbiome. Altogether, these data support the hypothesis that there is an association between the saliva and tumor microbiomes of ESCC patients in Tanzania. Additional studies are necessary to confirm this finding in additional patient groups and to clarify the significance.

Many of the bacterial genera identified in this study have been previously implicated in the carcinogenesis of gastrointestinal cancers. For example, studies have found that oral microbiota including *Fusobacterium*, *Prevotella*, *Selenomonas*, *Veillonella*, *Streptococcus* and *Campylobacter* can be used to distinguish individuals with colorectal cancer from healthy controls, and that *Fusobacterium nucleatum* strains that colonize the oral cavity and tumors of patients with colorectal cancer are identical in some patients, raising the possibility that the oral cavity is a source of extra-oral cancer microbiota. Our group has previously shown that *Fusobacterium*, *Selenomonas* and *Prevotella* can be visualized invasively within colorectal tumors and liver metastases. *Fusobacterium nucleatum* has been previously identified in esophageal cancers and is associated with shorter overall survival. Members of the genus *Porphyromonas* have been previously observed invasively within ESCC tumors and have been
reported to promote oral squamous cell carcinoma through a variety of mechanisms.\textsuperscript{30,31} \textit{Campylobacter jejuni} has been reported to promote tumorigenesis in mice,\textsuperscript{32} and \textit{Streptococcus} species have been identified in human esophageal cancers.\textsuperscript{33} In addition, the striking association of \textit{Streptococcus bovis} with colorectal cancer has led to the recommendation that colonoscopy be performed upon detection of \textit{Streptococcus bovis} bacteremia or endocarditis.\textsuperscript{34,35} Oral commensal bacteria such as \textit{Veillonella} species have been previously implicated in pathogenesis of lung cancer.\textsuperscript{36} A prospective cohort of American patients\textsuperscript{37} and a study of Japanese patients\textsuperscript{38} likewise found that oral microbiome composition is associated with risk for development of esophageal cancer.

We found that bacterial genera including \textit{Fusobacterium}, \textit{Prevotella}, \textit{Selenomonas}, \textit{Veillonella}, \textit{Streptococcus} and \textit{Campylobacter} are pervasive in the microbiome of ESCC tumors from patients in high-incidence regions. Moreover, the bacterial composition of ESCC tumors is remarkably similar across countries in those high-incidence regions, raising the possibility that particular bacterial genera may be involved in ESCC carcinogenesis or that they may colonize tumors as a result of the common clinical presentation of patients with severe dysphagia. Notably, there are several alternative hypotheses that warrant mention. For example, it is possible that the ESCC-associated bacterial genera simply represent common members of the esophageal microbiome\textsuperscript{39} and that the microbial populations we observed in these cancers are not significantly different from those found in normal esophagus tissue. A limitation of our study is a lack of normal esophageal tissue from ESCC cases or healthy controls in these settings, which would allow us to address this possibility. Another possible explanation is that ESCC tumors provide a favorable niche in which these bacteria are sequestered, in the setting of malignant obstruction and allowed to colonize. Thus, it is plausible that ESCC-associated bacteria are not necessarily promoting ESCC carcinogenesis but rather represent passengers resulting from the sequestration of oral secretions proximal to an obstructing tumor. While the previous association of these bacterial genera with other cancers is consistent with the hypothesis that they influence carcinogenesis of ESCC, future studies are necessary to identify which, if any, direct influences these bacterial genera have upon ESCC carcinogenesis. Nevertheless, even if these bacterial genera do not have a role in increasing ESCC risk, but arise at the time of disease onset, they may have an important role to play as part of a biomarker for non-invasive early detection. Finally, a concern of all microbiome analyses is that observed bacteria can be a consequence of contamination at some step between tumor harvest and sequencing. However, our analysis of potential contaminants and the fact that we identify \textit{Fusobacterium}, \textit{Prevotella}, \textit{Selenomonas}, \textit{Veillonella}, \textit{Streptococcus} and \textit{Campylobacter} in four independently collected sequencing efforts indicates that these findings are unlikely due to contamination.

While this study focused on the presence of bacteria with ESCC in high-incidence regions, we found evidence of similar cancer-associated bacteria in tumors in patients from low-incidence regions (USA, Ukraine, Vietnam and Russia). A limitation of this assessment is the small sample size ($n = 36$) and reliance on a single TCGA cohort that likely contains contaminants.\textsuperscript{48} Regardless, this finding does not exclude the possibility that the microbiome could be a factor driving patterns of ESCC incidence. For example, it is possible that the prevalence of ESCC-associated bacteria in people could vary across regions, which in turn could drive these differing rates of ESCC incidence. This is an important topic for future study.

We found that the structure of synchronous paired tumor and oral microbiomes were strikingly similar, although in a limited group of 21 patients. It is possible that this similarity is driven by transient contact of saliva and its associated microbiome with the tumor (e.g., during swallowing or tumor extraction). However, we found that only four of 16 common-abundant bacterial genera correlate in abundance between the tumor and oral microbiomes, suggesting tumor-oral microbiome similarity is not driven exclusively by “in-trans” interactions between the saliva and tumor. We also found that genera including \textit{Prevotella} and \textit{Fusobacterium} are often specifically enriched in the tumor microbiome, supporting a model where specific oral bacterial preferentially colonize the tumor. A caveat of this study is that we infer oral bacterial populations from the saliva, despite diverse communities of bacteria throughout the oral cavity.\textsuperscript{57} However, we do observe \textit{Fusobacterium} in the saliva despite its general association with periodontal plaques,\textsuperscript{58} suggesting saliva is capable of detecting periodontal pathogens. Additionally, because the samples studied here are from patients with late-stage disease, it is possible that tumor-induced changes to upper-gastrointestinal physiology and dysphagia symptom-induced major dietary changes could themselves alter the oral microbiomes of these patients. The previous findings from the ESCCAPE studies in Kenya and Tanzania\textsuperscript{17,59} which found strong associations with dental staining (ORs > 10) and for which photographic validation studies suggest that most dental staining was not fluorosis, also point to a recent build-up of chromogenic bacteria. Studies of the oral microbiome of patients at earlier stages of ESCC and in prospective studies are necessary to address this possibility. We restricted our analysis to 21 tumor-saliva pairs that have a sufficient number of bacterial reads (at least 10,000). It is likely that excluded samples are not molecularly distinct from included samples but that the relatively low bacterial read counts in some tumors is simply reflective of low sequencing depth. Additional studies are necessary to understand the relationship between tumor and oral bacterial communities in additional patient groups in other high- and low- incidence regions.

Our observation of similar tumor and saliva microbiomes in ESCC patients is especially notable considering emerging evidence linking periodontal disease and poor oral health with increased risk of various cancers.\textsuperscript{17,59,60} This raises several important questions for future inquiry. It will be essential to determine if there is a difference in the oral prevalence of these identified cancer-associated bacteria between ESCC patients and non-patients earlier in the natural history of the disease, for example through comparisons of patients with esophageal squamous dysplasia and healthy controls. Because the prevalence of these bacteria may be associated with factors such as oral health, hygiene and diet, studies of the impact of these factors on the oral microbiome in the general population would inform whether the oral microbiome is on a pathway linking oral hygiene to ESCC risk and may have a role in prevention.
In conclusion, we show that cancer-associated, traditionally oral bacteria including the genera \textit{Fusobacterium}, \textit{Selenomonas}, \textit{Prevotella}, \textit{Streptococcus}, \textit{Porphyromonas}, \textit{Veillonella} and \textit{Campylobacter} are highly abundant within ESCC tumors from patients in regions with a high incidence of ESCC. We also show that there is a correlation between the genus composition of the saliva microbiome and the ESCC tumor microbiome of some ESCC patients. These findings will be foundational for future studies to understand if and how bacteria influence ESCC pathogenesis and to understand the role of the oral microbiome in this process. Finally, this study highlights the benefit of collaborative investigation to evaluate the international heterogeneity of this disease.

**AUTHOR CONTRIBUTIONS**

Conceptualization—Katherine Van Loon, Elia J. Mmbaga, Matthew Meyerson; Data curation—Jason Nomburg, Yulia Newton, Charles Vaske; Formal analysis—Jason Nomburg; Funding acquisition—Katherine Van Loon, Susan Bullman, James A. DeCaprio, Matthew Meyerson, Michael R. Stratton, Paul Brennan; Investigation—Jason Nomburg, Katherine Van Loon, Matthew Meyerson, Elia J. Mmbaga; Project Administration—Jason Nomburg, Katherine Van Loon; Resources—Dariush Nasrollahzadeh, Eric A. Collisson, Behnoush Abedi-Ardekani, Larry O. Akoko, Joshua R. Atkins, Geoffrey C. Buckle, Satish Gopal, Nan Hu, Bongani Kaimila, Masoud Khoshsia, Reza Malekzadeh, Diana Menya, Blandina T. Mmbaga, Sarah Moody, Gift Mulima, Beatrice P. Mush, Julius Mwaiseleage, Ally Mwanga, Yulia Newton, Dianna L. Ng, Amie Radenbaugh, Deogratias S. Rwakatema, Msiba Selelka, Joachim Schûz, Philip R. Taylor, Charles Vaske, Alisa Goldstein, Michael R. Stratton, Valerie McCormack, Paul Brennan, James A. DeCaprio, Matthew Meyerson, Elia J. Mmbaga, Katherine Van Loon; Software—Jason Nomburg, Katherine Van Loon; Supervision—Katherine Van Loon, Elia J. Mmbaga, Matthew Meyerson, James A. DeCaprio; Validation—Jason Nomburg; Visualization—Jason Nomburg; Writing—original draft—Jason Nomburg; Writing—review and editing—Jason Nomburg, Susan Bullman, Dariush Nasrollahzadeh, Eric A. Collisson, Behnoush Abedi-Ardekani, Larry O. Akoko, Joshua R. Atkins, Geoffrey C. Buckle, Satish Gopal, Nan Hu, Bongani Kaimila, Masoud Khoshsia, Reza Malekzadeh, Diana Menya, Blandina T. Mmbaga, Sarah Moody, Gift Mulima, Beatrice P. Mush, Julius Mwaiseleage, Ally Mwanga, Yulia Newton, Dianna L. Ng, Amie Radenbaugh, Deogratias S. Rwakatema, Msiba Selelka, Joachim Schûz, Philip R. Taylor, Charles Vaske, Alisa Goldstein, Michael R. Stratton, Valerie McCormack, Paul Brennan, James A. DeCaprio, Matthew Meyerson, Elia J. Mmbaga, Katherine Van Loon. The work reported in the paper has been performed by the authors, unless clearly specified in the text.

**ACKNOWLEDGEMENTS**

We thank Liu et al\textsuperscript{[27]} for providing access to sequencing data from ESCC patients in Malawi (dbGaP accession phs001448.v1.p1). Portions of this research were conducted using the O2 High Performance Computing Cluster, supported by the Research Computing Group at Harvard Medical School. We thank Aleksandar Kostic for his helpful discussions. We thank the teams from the MUHAS-ORCI-UCSF Cancer Collaboration, UNC-Malawi Project, Mutographs, ESCAPE, and TCGA for specimen and data collection.

**FUNDING INFORMATION**

National Institutes of Health, National Cancer Institute Cancer Center Administrative Supplement to Promote Cancer Prevention and Control Research in Low and Middle Income Countries, A119617, [CA0082629] to Katherine Van Loon. Susan Bullman received funding from NIH/NCI Grants: R00CA229984 and Cancer Center Support Grant P30 CA015704. Eric A. Collisson received funding from NIH/NCI Grants: U24CA210974, R01CA222862, R01CA227807, R01CA239604 and R01CA230263. This study was supported in part by the US Public Health Service grants R35CA232128 and P01CA203655 to James A. DeCaprio. Content does not reflect the views of the National Cancer Institute or National Institutes of Health. This work was supported by a Cancer Grand Challenges OPTIMISTICc team award (C10674/A27140) to Matthew Meyerson and a Cancer Grand Challenges Mutographs team award funded by Cancer Research UK [C98/A24032] to Michael R. Stratton and Paul Brennan. R21CA191965 supported the collection of ESCAPE Kenya tumors. The International Agency for Research on Cancer Section of Environment and Radiation supported the collection of ESCAPE Tanzania tumors. This study was supported in part by the Intramural Research Program of the National Institutes of Health, National Cancer Institute, Division of Cancer Epidemiology and Genetics.

**CONFLICT OF INTEREST**

Matthew Meyerson receives research support from Bayer, Janssen and Ono, has patents licensed to Bayer and Labcorp, is a consultant for Bayer, Interline and Isabl, and has equity in Interline and Isabl. James A. DeCaprio has received research support from Rain Therapeutics, Inc. James A. DeCaprio has served as consultant to Rain Therapeutics, Inc. and Takeda, Inc. Susan Bullman has consulted for GlaxoSmithKline and BioMx and is on the cancer program scientific advisory board for BioMx. Dianna L. Ng receives research funding from Cepheid, Inc., unrelated to this article. Eric A. Collisson received expert consulting fees arising from an unrelated matter from Nant Bio in 2021 and 2022. Charles Vaske is a shareholder of NantHealth. The other authors declare no conflict of interest exist.

**DATA AVAILABILITY STATEMENT**

Raw sequencing data for all samples are available as indicated in the relevant manuscripts. The microorganism-mapped, non-human reads from the MUHAS Tanzania samples are available on the SRA under BioProject PRJNA810436. Other data that support the findings of this study are available from the corresponding author upon request. All GATK-PathSeq output files and reproducible analysis and plotting R Notebooks are available. Zenodo: https://doi.org/10.5281/zenodo.4750577. GitHub: https://github.com/jnoms/ESCC_microbiome. Furthermore, all analysis and figures can be automatically reproduced through a series of Google Colab documents. Figure 1 and Supplementary Figure 1: https://github.com/jnoms/ESCC_microbiome/blob/main/collab/Figure1.ipynb. Figure 2 and Supplementary Figure 2: https://github.com/
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**SUPPORTING INFORMATION**

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

**How to cite this article:** Nomburg J, Bullman S, Nasrollahazadeh D, et al. An international report on bacterial communities in esophageal squamous cell carcinoma. Int J Cancer. 2022;151(11):1947-1959. doi:10.1002/ijc.34212