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ORIGINAL RESEARCH—CLINICAL

Analysis of Fecal, Salivary, and Tissue Microbiome in Barrett's Esophagus, Dysplasia, and Esophageal Adenocarcinoma

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BACKGROUND AND AIMS: Esophageal adenocarcinoma (EAC) incidence has risen dramatically in the Western countries over the past decades. The underlying reasons are incompletely understood, and shifts in the esophageal microbiome have been postulated to increase predisposition to disease development. Multiple factors including medications, lifestyle, and diet could influence microbiome composition and disease progression. The aim of this study was (1) to identify a feasible method to characterize the tissue-associated microbiome, and (2) to investigate differences in the microbiome of saliva, esophageal tissue, and fecal samples by disease state and validate with 2 external cohorts. **METHODS:** Forty-eight patients (15 Barrett's esophagus [BE], 4 dysplasia, 15 EAC, and 14 healthy) were enrolled in this cross-sectional study (Munich cohort). Demographics, epidemiologic and clinical data, medications, smoking, and alcohol consumption were assessed. 16S rRNA Gene sequencing was performed on saliva, tissue biopsy and fecal samples. PAXgene fixation was used as a novel methodology. Microbial community alpha- and beta-diversity, as well as microbial composition at phylum and genus level, were characterized for this cohort and compared with 2 external cohorts: New York cohort and Cooperative Health Research in the Augsburg Region cohort. **RESULTS:** We first established PAXgene fixation is a feasible method for microbiome analysis and utilized it to identify a distinct microbial shift in tissue biopsies from patients with EAC, whereas overall microbial diversity in salivary and fecal samples did not differ significantly between disease states. Our findings were similar in a reanalysis to those from a US cohort that used a standardized fresh frozen biopsy collection protocol (New York cohort, N = 75 biopsies). Nevertheless, we could not distinguish German Munich cohort patients from a German population-based cohort (Cooperative Health Research in the Augsburg Region cohort, N = 2140 individuals) when fecal bacterial profiles were compared between both cohorts. In addition, we used data integration of diagnosis and risk factors of patients and found associations with microbiome alterations. **CONCLUSION:** Sample collection and microbiome analysis are indeed feasible and can be implemented into clinical routine by an easy-to-use biopsy protocol. The presence of BE and EAC together with epidemiologic factors can be associated with alterations of the salivary, tissue, and fecal microbial community in an easy-to-use data integration concept. Given a possible role of the microbiome in BE and EAC, it will be important in future studies to take tissue-specific microbial communities and individual taxa into account in larger prospective studies.

Keywords: Barrett Esophagus; Esophageal Adenocarcinoma; Microbiome; Dysbiosis

Introduction

Esophageal adenocarcinoma (EAC) arises from progression of Barrett's esophagus (BE), an intestinal metaplasia of the distal esophagus, and low-/high-grade dysplasia (DP). The incidence of EAC is increasing in Western countries, representing the eighth most prevalent cancer and

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Abbreviations used in this paper: **BE, Barrett's esophagus; DP, dysplasia; EAC, esophageal adenocarcinoma; KORA, Cooperative Health Research in the Augsburg Region; NY, New York; TLR4, toll-like-receptor 4; zOTU, zero-radius operational taxonomic unit.**

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the sixth most common cause of cancer-related deaths.[1] The rapid increase in EAC incidence points to the influence of environmental factors in its pathogenesis. None of the known epidemiologic risk factors, such as gastro-esophageal reflux disease, smoking, high-fat diet intake, and obesity, when separately considered, fits chronologically to the increased incidence of EAC.[2] Intriguingly, EAC incidence has risen with the advent of antibiotics and the decline in *Helicobacter pylori* infection rates, suggesting a potential role of the microbiome in disease manifestation and progression at the esophagogastric junction.[2] Mechanistically, BE progression is associated with the infiltration of CD11b + Gr1 + myeloid cells,[3] and it was shown that these cells also respond to bacterial lipopolysaccharide, a component of the outer membrane of Gram-negative bacteria.[4] The esophageal microbiome of BE and EAC patients is furthermore characterized by a general increase in Gram-negative bacteria.[5] We investigated the fecal microbiome driven by our recent findings in the L2-IL1β mouse model of BE, where high-fat diet led to DP independent of obesity by changing the gut microbiome and consequently the inflammatory microenvironment.[6] We investigated the salivary microbiome based on reports of similarity between the esophageal and oropharyngeal microbiome, suggesting saliva as a possible and readily accessible biomarker for pathologies of the esophagus.[7,8] The microbiome may summarize several changes occurring in the organism that promote cancer, representing thus a comprehensive factor as a potential biomarker or even causal factor or therapeutic target.

Thanks to the rapid advancement of high-throughput DNA sequencing technologies and bioinformatics analysis tools, the study of microbiome structure and function has become possible. Studies to date focused their research in defining the local esophageal tissue microbiome and revealed associations between microbiome and BE.[9] However, biopsy sampling from esophagus represents an invasive process. As such, in addition to the local tissue-associated microbiome, we sought to describe the corresponding salivary and fecal microbiome of these patients. To date, there is no standardized and established sample collection protocol to interrogate the esophageal microbiome. The preanalytic sample collection (brushing, fresh frozen, paraffin fixation) have not been taken into account as a potential bias factor. Indeed, the identification of patients at earlier stages of the disease (BE) might improve disease prognosis and reduce EAC-associated mortality. Therefore, an easy procedural protocol for the identification of putative disease-associated microbiome signatures that could aid improved disease prognosis and disease management is of great interest in the clinical settings.

Utilizing the prospective BarrettNET cohort described previously,[10] we present in this cross-sectional study the implementation of a clinical workflow to analyze integrated clinical-pathological (diagnosis), epidemiologic (smoking, alcohol, reflux), and microbiome profiling in saliva, tissue, and feces from patients with EAC and its precursor lesions. Using 16S microbiome profiling, we characterize disease-associated microbiota in PAXgene-fixed biopsies during disease progression and compare the data to a frozen biopsy collection of a US cohort of BE and EAC patients (New York [NY] cohort) as well as to the Cooperative Health Research in the Augsburg Region (KORA) population-based cohort in Germany to elucidate the feasibility of our protocol to detect possible associations among microbiome, phenotype, and risk factors in BE and its neoplastic progression.

Methods

Study Design

This study was a case-control cross-sectional study including patients diagnosed with BE, BE-associated DP, EAC, and control patients with no history of BE or advanced associated pathologies (control/healthy). All subjects underwent a planned upper endoscopy for clinical reasons, including the control patients. Patients were approached to participate in the study according to the regulations of the ethics committee (#5428/12) and gave written informed consent. They provided epidemiologic information and biospecimens including tissue biopsies for histopathological assessment and microbiome characterization and saliva and fecal samples for microbiome characterization. This study was carried out in the Department of Gastroenterology, Interdisciplinary Endoscopy, Klinikum rechts der Isar, from March 2017 to February 2018 as part of the BarrettNET study, a Germany-wide multicenter prospective cohort study of BE patients.[10] In the present study, the previously reported inclusion and exclusion criteria of BarrettNET[10] were adopted, and additional criteria such as antibiotic use in the last 6 weeks, chronic inflammatory bowel disease, diarrhea, vegetarian or vegan eating habits, and diseases of liver and gallbladder were considered. By the time of inclusion, the diagnosis of BE, DP, and EAC is confirmed by a pathologist based on the previous patient's surveillance visits. After histologic evaluation of tissue biopsies, patients were divided into subgroups based on diagnosis, according to the most advanced histologic evaluation. The patient-related epidemiologic information was acquired in the form of a comprehensive questionnaire containing questions on demographics, lifestyle factors, and health.[10] The information was subsequently registered in the online BarrettNET database. The epidemiologic, histopathological, and microbiome information was merged for integrated analysis (see Supplemental Section: "Data Integration"). Finally, the results of our study (Munich cohort) were compared with the results of 2 reference cohorts: NY cohort and KORA cohort. The steps of this work are visualized in Figure A1.

Sample Collection

Saliva, feces, and biopsies were collected in a cross-sectional study at the timepoint of inclusion for microbiome characterization. The collection procedures of biopsies using the PAXgene system were reported previously.[10] Two to six forceps biopsies per patient were sampled: 1× inconspicuous esophageal mucosa; 1–4× biopsies from suspected Barrett, dysplastic, or neoplastic tissue, if present; and 1× inconspicuous cardia mucosa. The PAXgene Tissue System, a formalin-free preparation system for biopsy tissue fixation and stabilization, enables histopathologic and biomolecular analysis from tissue biopsy,[11,12] which allowed us to perform both

histologic tissue evaluation and DNA extraction for microbiome characterization from each biopsy sample.[11] Snap freezing in liquid nitrogen, which is the most popular method used for molecular analytical purposes to date, may damage tissue morphology and antigenicity. It requires high methodical and logistical effort and is cost-intensive, making its integration into the clinical routine difficult. The hematoxylin and eosin-stained PAXgene-fixed biopsy images from the present cohort are shown in Figure A2.

Saliva and fecal material were self-sampled by the patients and shipped to the clinic laboratory responsible for human material storage, as previously reported.[10]

Saliva samples were collected using the STARTEC SalivaGene Collector (STARTEC Molecular GmbH) in DNA stabilizer. For this purpose, a saliva collection kit was provided, including a SalivaGene Collection tube containing 150 mg of dry stabilization buffer, disposable gloves, an illustrated description of the collection procedure, as well as a shipment box. Patients were instructed to collect 2 mL of saliva in the SalivaGene Collection tube followed by gentle shaking to dissolve the dry DNA stabilization buffer (STRATEC Molecular GmbH, 2018). To facilitate the collection, patients were advised to rub their cheeks from the outside and press them against their teeth to ease saliva secretion (STRATEC Molecular GmbH, 2018). The sample collection procedure was conducted at home or in the clinic prior to endoscopy. Patients were requested to remain fasted and refrain from eating, drinking, smoking, or chewing gum for at least 30 minutes prior to saliva provision according to the manufacturer's instructions. Samples were subsequently shipped to the clinic human sample biobank and were stored at −80 °C.

Fecal specimens were self-sampled by patients using the STARTEC Stool collection tube. Patients received a stool collection kit, including a stool collection tube with integrated spatula and 8 mL of stool DNA stabilizer reagent, disposable gloves, an illustrated description of the collection procedure, as well as a shipment box. In addition to the written and illustrated information, patients received verbal instructions for the collection procedure, the optimal stool sample size, and shipping possibilities. Patients were requested to collect fecal samples in a clean stool collection tube and homogenize it briefly by shaking. The sampling procedure was conducted mostly at home or at the clinic if patients were hospitalized. Samples were shipped to the clinic human sample biobank and were stored at −80 °C.

Metagenomic DNA Extraction From Saliva, Tissue Biopsies, and Fecal Samples

DNA was extracted from patient fecal samples by bead-beating followed by a modified version of the protocol by Godon et al.[13] In brief, a volume of 600 μL of DNA stabilizing solution (STARTEC Biomedical, Germany) was added to the fecal aliquots in a 2-mL screw-cap polypropylene micro-centrifuge tube containing sterile 500 mg of silica beads (0.1 mm in diameter; BioSpec Products) and kept on ice. A volume of 250 μL of 4 M guanidine thiocyanate, 0.1 M Tris (pH 7.5), and 500 μL of 5% N-lauroyl sarcosine, 0.1 M phosphate buffer (pH 8.0), fecal suspensions was vortexed and incubated at 70 °C for 1 hour with shaking. Mechanical disruption via bead beating was performed using a FastPrep-24 bead beater (MP Biomedicals) supplied with a 24 × 2-mL cooling adaptor 3 times each for 40 seconds at a speed of 6.5 m/s. An amount of 15 mg of polyvinylpolypyrrolidone (Sigma Aldrich) was added as a polyphenol adsorbent, and the suspension was centrifuged for 3 minutes at 15,000×g at 4 °C. The supernatant was recovered in a new 2-mL tube and further centrifuged for 3 minutes at 15,000×g at 4 °C. To remove bacterial RNA, a volume of 2 μL of RNAse (10 mg/mL) was added to 500 μL of clear supernatant and incubated at 37 °C for 30 minutes with constant shaking. Finally, the genomic DNA was purified using the NucleoSpin gDNA Clean-up kit (Macherey-Nagel) following the manufacturer's instructions. Concentration and purity of the extracted DNA was determined using the NanoDrop spectrophotometer ND-1000 (ThermoFisher Scientific), and samples were stored at 4 °C during library preparation and at −20 °C for long-term storage. Extraction of DNA from PAXgene-fixed biopsy samples was performed using a PAXgene Tissue DNA Kit (PreAnalytiX) according to manufacturer's instructions. DNA from saliva samples was extracted using the DNA Mini kit (Qiagen), following the manufacturer's instructions for purification of DNA from saliva stabilized in RNAprotect Saliva Reagent (STARTEC Biomedical, Germany).

High-Throughput 16S rRNA Gene Amplicon Sequencing

High-throughput 16S rRNA gene sequencing and downstream analysis were performed as previously described.[14,15] V3-V4 regions of the 16S rRNA gene were amplified through 25 cycles for fecal and saliva samples and through 15 cycles for tissue biopsies, using the previously described 2-step protocol[16] and the primer pair 341F-785R.[17] Polymerase chain reaction purification was performed using the AMPure XP system (Beckman-Coulter). A total of 124 samples were sequenced in paired-end modus (PE275) using a MiSeq system (Illumina, San Diego, CA) according to the manufacturer's instructions and 25% (v/v) PhiX standard library. 16S rRNA Gene sequencing resulted in an average total number of 33,867 ± 20,750 demultiplexed reads per sample. An average of 12,349 ± 10,732 raw reads per sample remained after filter trimming and removal of chimeras. The large variation can be explained by the huge variation in community complexity in different sample types (fecal, tissue, and saliva). Rarefication curves verify that all included sample contained more than 3000 reads after quality filtering (Figure A3). A table of zero-radius operational taxonomic units (zOTUs) is constructed by considering all reads before any quality filtering. zOTUs Are valid operational taxonomic units that provide the maximum possible biological resolution compared with conventional 97% OTUs. Since using 97% identity may merge phenotypically different strains with distinct sequences into a single cluster, zOTUs are found to be superior to conventional OTU clusters.[18,19] Downstream analysis was performed in the R programming environment using Rhea (https://lagkouvardos.github.io/Rhea/).[15] Alpha- and beta-diversity analyses, as well as analysis at taxon level, were performed.

Microbiome Profiling

The provided raw sequencing data were processed using an integrated microbial next generation sequencing platform.[14]

Sequences were demultiplexed, trimmed to the first base with a quality score <3, and then paired. Sequences with less than 300 and more than 600 nucleotides and paired reads with an expected error >3 were excluded from the analysis. Remaining reads were trimmed by 10 nucleotides on each end to avoid gastric cardia (GC) bias and nonrandom base composition. Data were analyzed as described in detail previously.[20] Downstream analysis was performed in the R programming environment using Rhea. Rarefaction curves were used to assess sequencing depth and to eliminate low-quality reads, zOTUs were normalized, and percentage relative abundance was computed. At this step, one fecal sample from the total 124 samples sequenced was excluded from the downstream analysis due to insufficient sequencing depth. Alpha-diversity was assessed on the basis of species richness and Shannon effective diversity.[21] Beta-diversity was computed based on generalized UniFrac distances.[22,23] Visualization of the multidimensional distance matrix was performed by multidimensional scaling plot. A permutational multivariate analysis of variance using distance was performed in each case to determine if the separation of groups is significant, as a whole and in pairs. Next, taxonomic classification of zOTUs at higher taxonomic levels (kingdom, phyla, class, order, family, and genus) was performed. Group comparisons of OTUs or taxonomies were performed comparing all groups using the nonparametric Kruskal-Wallis rank sum test. Pairwise comparisons of more than 2 groups were performed using Mann-Whitney test. The obtained pairwise test significance values are corrected for multiple testing using the Benjamini-Hochberg method. Finally, metadata and taxonomic variables were correlated.

Reference Cohort (1): NY Cohort

This cohort was used in a case-control study of patients without or with a diagnosis of BE, BE-associated DP (low grade and high grade), and EAC at a single academic medical center (Columbia University Medical Center, New York, NY). Analysis of salivary and mucosal tissue microbiome from these patients has been previously reported.[5,24] Biopsies of the squamous esophagus as well as GC were sampled (N = 75 biopsies). Tissue biopsies were snap-frozen upon collection. After DNA extraction, 16S rRNA gene sequencing was performed by amplification of the V4 hypervariable ribosomal RNA region using primers 515F and 806R.[25] Tissue biopsies were stratified to healthy, BE, DP, and EAC as done for the Munich cohort.

Reference Cohort (2): KORA Cohort

KORA is a longitudinal population-based cohort study (Cooperative Health Research in the Augsburg Region) in southern Germany focused on cardiometabolic health, especially diabetes. Detailed study design and methods have been published previously.[26] In the present manuscript, we included analysis of fecal samples from KORA (n = 2140), fecal samples from patients with malignant neoplasms of digestive organs (n = 23), and samples from patients with other types of cancer (n = 199). The collection of fecal samples, DNA extraction, and amplification of the V3-V4 hypervariable region of the 16S rRNA gene using primers 341F-ovh and 785r-ovh are as previously described.[20] The pooled amplicons were sequenced using the Illumina HiSeq platform (Illumina, San Diego, CA) with 2 × 250-bp paired-end sequencing.

Results

Compilation of a Representative Patient Cohort to Analyze the Salivary, Tissue Biopsy, and Fecal Microbiome in BE, DP, and EAC

To study salivary, tissue biopsy-associated, and fecal microbiota in a small representative cohort demonstrating BE to EAC progression, we implemented a novel procedural protocol to improve procedural convenience within a clinical endoscopy unit setting with a translational research focus. Thus, we included patients from the previously described BarrettNET[10]; the mean age of this small cross-sectional cohort was 62.02 years, ranging from 33 to 80 years, and the gender distribution was 82.74% male and 17.26% female. Demographic characteristics; body mass index; smoking, alcohol, reflux history; and medication intake were analyzed to provide an overview of the distribution of characteristics between the diagnosis groups (Table A1). The epidemiologic and clinical patient information is described in detail in the Supplemental Section (see Supplemental Section: "Patient Epidemiological and Clinical Information").

We compared the microbial community structure of fecal, saliva, and tissue biopsy samples in all enrolled subjects. Community diversity (both species richness and Shannon effective counts) differed between the 3 sample types (Figure 1A and B). Expectedly, fecal samples were characterized by highest community diversity as calculated by Shannon effective counts (201.11 ± 75.40), followed by tissue biopsy samples (184.88 ± 89.48) (Figure 1B). Salivary microbiota showed the lowest community diversity (93.56 ± 46.21) (Figure 1B). Beta-diversity analysis showed significant clustering of fecal, saliva, and mucosal tissue samples, emphasizing the presence of distinct microbial communities in different sample types (Figure 1C). Bacteroidetes and Firmicutes were the most dominant bacterial phyla in the fecal samples. On the other hand, the microbial community of the mucosal tissue samples was mainly dominated by Bacteroidetes, Firmicutes, and Proteobacteria, while the microbial community of the saliva samples was dominated by Firmicutes and Actinobacteria (Figure 1D). Surprisingly, we observed that Bacteroidetes and Proteobacteria were not more dominant than Actinobacteria in the saliva samples, which could be explained by our pre-analytical sample collection protocol.

In summary, these data demonstrate that the establishment of our novel translational concept of collecting nonfrozen biopsies, fecal samples, and saliva samples within the clinical routine in combination with a detailed questionnaire for epidemiologic analysis is feasible and allows distinct microbiome analysis.

Tissue Biopsy Microbiome Reflects Disease State in the Munich Cohort and Shows Similar Patterns in the NY Cohort

Disease-associated microbial community alterations were only observed in the microbiome profiles obtained

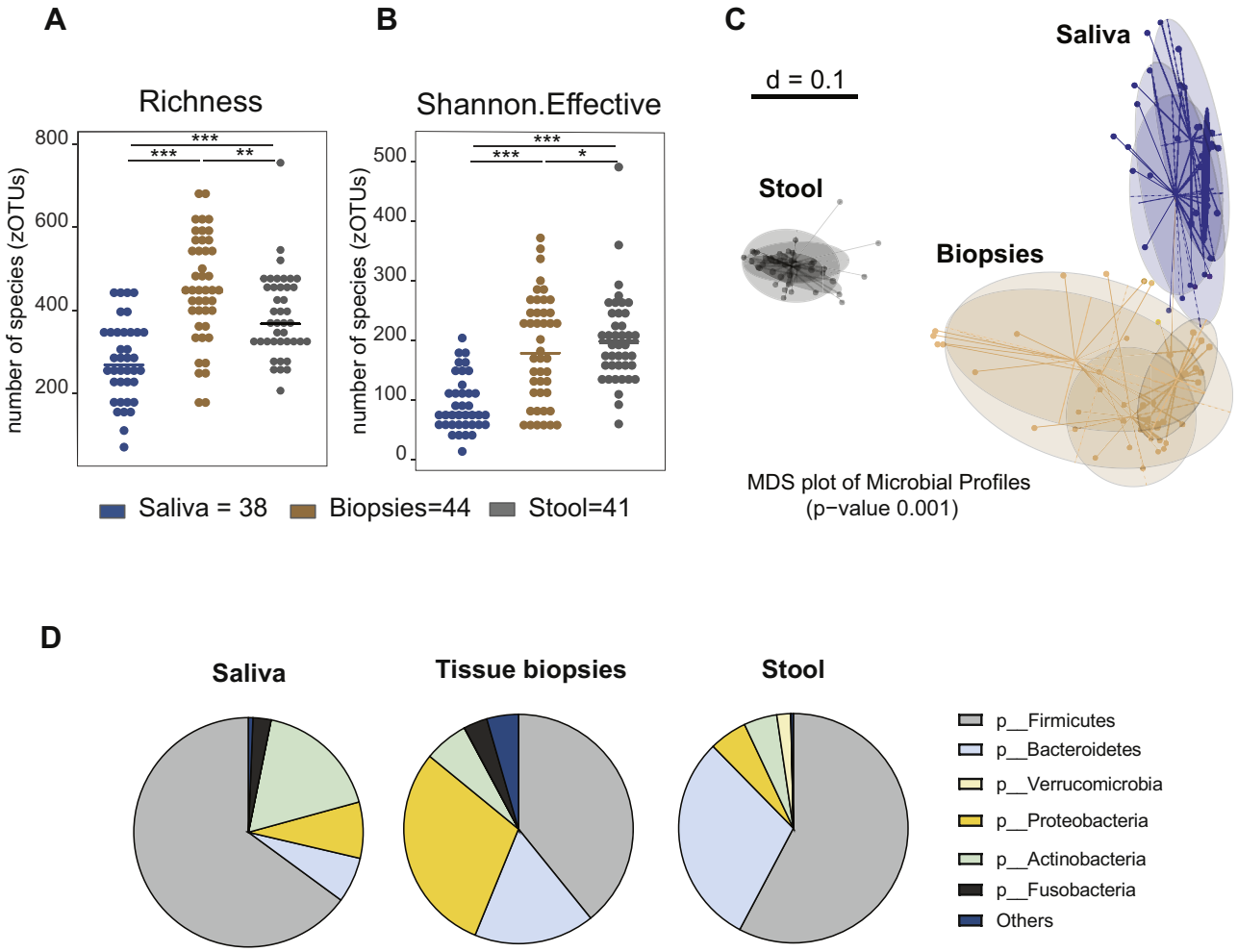

Figure 1. Global bacterial community structure differed between feces, saliva, and mucosal tissues. (A) Alpha-diversity measured by community species richness and (B) Shannon effective number of species, showing a reduced diversity metrics in saliva samples compared with biopsies and stool samples. (C) Beta-diversity comparison: multidimensional scaling (MDS) plot of all samples stratified by sample type, for which the Permutational Multivariate Analysis of Variance (PERMANOVA) test detected a significant separation of study groups in terms of bacterial community composition. Each point represents the microbiota composition of one sample. (D) Taxonomic comparison at the phylum level. $^{*}P \leq .05$, $^{**}P \leq .01$, $^{***}P \leq .001$.

from PAXgene-fixed tissue samples and were not reflected in the stool or saliva samples. This was visualized by performing alpha-diversity and beta-diversity analyses. Alpha-diversity measured by richness showed significantly lower number of species in patients with EAC than in patients with DP and BE (Figure 2A). However, no significant difference in alpha-diversity was observed as measured by Shannon effective number of species analysis (Figure 2B). Beta-diversity analysis showed significant separation of microbial profiles based on disease state, with EAC patients showing the highest interindividual variation in their microbiome profiles (Figure 2C). Pairwise comparisons of microbial profiles showed significant separation between healthy patients and all patients during different states of disease progression (healthy vs BE [$P = .028$], healthy vs DP [$P = .0345$], healthy vs EAC [$P = .024$], and between BE and EAC [$P = .024$]).

To test the plausibility of our PAXgene-fixed biopsy results and to check for an international and intercohort comparison, we compared the data from the Munich cohort to those from the NY cohort. In the NY cohort, biopsies from GC and squamous epithelium of the esophagus (SE) were sampled by a so far gold standard of preservation by snap freezing. The biopsies were stratified to healthy, BE, DP, and EAC as done for the Munich cohort. The healthy group included 23 biopsies (10 from GC and 13 from SE), the BE group 15 biopsies (8 from GC and 7 from SE), the DP group 23 biopsies (15 from GC and 8 from SE), and the EAC group 14 biopsies sequenced (6 from GC and 8 from SE). In the NY cohort, 16S rRNA gene sequencing was achieved by amplification of the V4 region of the 16S gene compared with amplification of the V3-V4 region in the present study (Munich cohort). We reanalyzed the NY data set using our in-house implemented bioinformatic tools, integrated

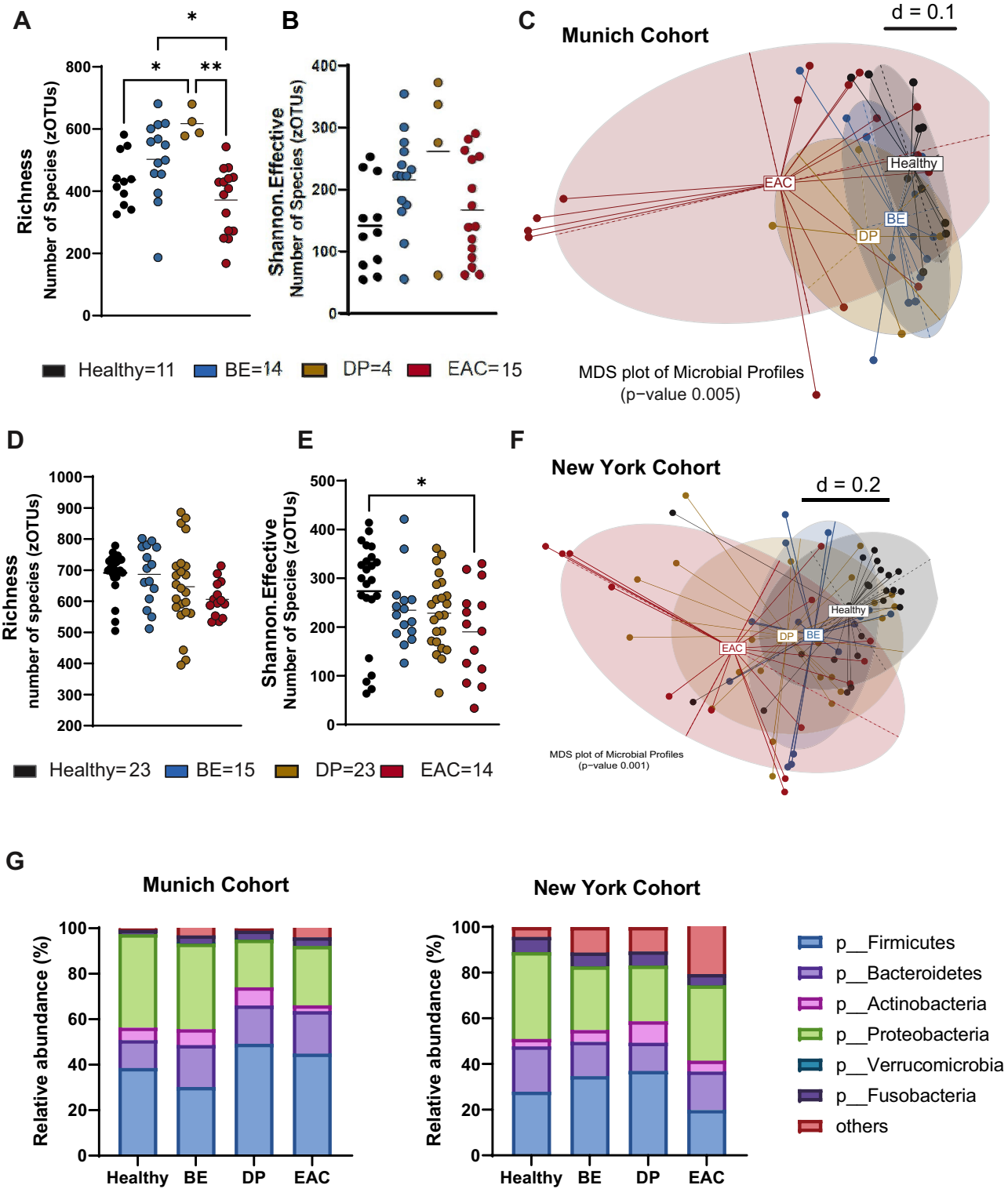

Figure 2. Tissue biopsy-associated microbiome reflects disease progression. (A, B) Alpha-diversity analysis measured by community species richness and Shannon effective in Munich cohort. Significance is calculated by Mann-Whitney test. *$P \leq .05$, **$P \leq .01$, ***$P \leq .001$. (C) Beta-diversity analysis of mucosal bacterial community profiles in Munich cohort showing multidimensional scaling (MDS) plot of tissue biopsies stratified by disease state. Each point represents the microbiota composition of one sample. (D, E) Alpha-diversity analysis measured by community species richness and Shannon effective in NY cohort. (F) Beta-diversity analysis of mucosal bacterial community profiles in NY cohort showing multidimensional scaling (MDS) plot of tissue biopsies stratified by disease state. Each point represents the microbiota composition of one sample. (G) Taxonomic composition at phylum level in Munich and NY cohorts. BE, Barret's esophagus; DP, dysplasia; EAC, esophageal adenocarcinoma; NY, New York.

microbial next generation sequencing platform and Rhea pipeline,[14,15] and show here the results of this reanalysis.

Alpha-diversity measured by richness number of species in the NY cohort showed no significant decrease with disease progression, in contrast to the Munich cohort (Figure 2D). However, in the NY cohort, alpha-diversity measured by Shannon effective number of species showed a significant decrease of community diversity in EAC compared with the healthy group (Figure 2E).

Similar to the Munich cohort, significant separation of microbial profiles based on disease state was observed in the NY cohort as shown by beta-diversity analysis (Figure 2F).

Taxonomic classification at phylum level in Munich cohort showed a decrease of the phylum of Actinobacteria in EAC compared with all other groups and an increase of the phylum of Firmicutes in EAC compared with BE. On the other hand, in the NY cohort, the phylum of Bacteroidetes showed a significant decrease in EAC and DP compared with healthy, and the phylum of Fusobacteria a decrease in EAC compared with healthy (Figure 2G). Taxonomic classification at genus level in both cohorts showed various significant differentially abundant genera between the disease states (Figure A4). Despite the differences in the preservation method and sequencing, these data show in terms of alpha- and beta-diversity comparable changes in mucosal microbiota composition in response to disease progression in both cohorts.

EAC Biopsy-Associated Dysbiosis is Only Weakly Reflected in Stool and Saliva Samples

To investigate the shift in structure and composition of oral and gut microbial communities, we performed the same analyses in salivary and stool samples from all enrolled subjects. No significant differences in community alpha-diversity (Richness and Shannon effective number of species) were observed (Figure 3A and C), and beta-diversity analysis showed no separation of microbial profiles from patients with different disease phenotypes in either sample types (Figure 3B and D). We used discriminative linear discriminant analysis effect size analysis to identify differentially abundant genera between patients with BE and patients with the most severe phenotype. Discriminative analysis based on fecal samples between the 2 groups did not result in significantly discriminative taxa (data not shown). However, linear discriminant analysis effect size analysis based on saliva samples resulted in a list of differentially abundant genera between patients with BE and patients with EAC (Figure 3E and F).

The Stool Microbiome Does Not Differ Between Munich and KORA Cohorts

Ideally a biomarker could distinguish healthy controls from diseased patients. To look at the distribution of fecal microbial community profiles in patients with BE-associated pathologies compared with other large-scale population-based cohorts, we integrated fecal microbial profiles of the Munich cohort in another population-based cohort study from Germany (KORA cohort) with N = 2140 individuals. Our analysis showed a homogeneous distribution of all individuals based on their fecal microbial profile with no distinct clustering of cancer cases from the KORA or the Munich cohort (Figure 4). Furthermore, fecal microbial profiles in patients with malignant neoplasms of digestive organs (ICD10: C15-C26) and other cancer cases showed no distinct clustering (Figure 4).

Epidemiologic Risk Factors for EAC Correlate With Microbiome Alterations

As a proof of principle concept, we integrated epidemiologic risk factors into our study design. Several risk factors exist for BE and EAC including obesity, tobacco smoking, and gastrointestinal reflux disease and might influence the gut, saliva, and tissue microbiome composition. For the current preliminary analysis, we stratified the samples based on patient diagnosis and factors like (1) smoking, (2) alcohol consumption, and (3) presence of reflux symptoms. After stratifying based on the above characteristics, 8 groups were compared with each other. For example, here we show the outcome of the beta-diversity analyses (Figure 5). The analyses showed not only significant differences between disease states as mentioned previously but also single differences within the disease state dependent on smoking, alcohol consumption, or reflux. Nevertheless, most differences were found between the disease states. This demonstrates that future studies with large number of patients should include these parameters in the analysis to exclude confounding factors.

Discussion

In this study, we established a methodology of profiling the human microbiome in biospecimens originating from esophageal/cardia biopsies, saliva, and stool from patients with BE-associated pathologies and controls in a routine clinical setting. This methodology may allow to better include the analysis of the microbiome as one potential disease-altering factor in future clinical studies. Such a standardized collection, analysis, and most importantly, integration with clinical data seem to be crucial for the analysis of the microbiome as a potentially important tool for clinical decision-making or risk evaluation and may be utilized in larger clinical studies. Most importantly, we provide evidence that tissue biopsy-associated microbiome has a close association with the disease state of patients compared with saliva or fecal microbiome, which will place the focus on microbiota role on tissue mucosa in the future.

Utilizing our novel methodology, we observed a drop in richness number of species in EAC compared with other phenotypes, but no difference in Shannon effective number of species between the phenotypes. In NY cohort analysis,

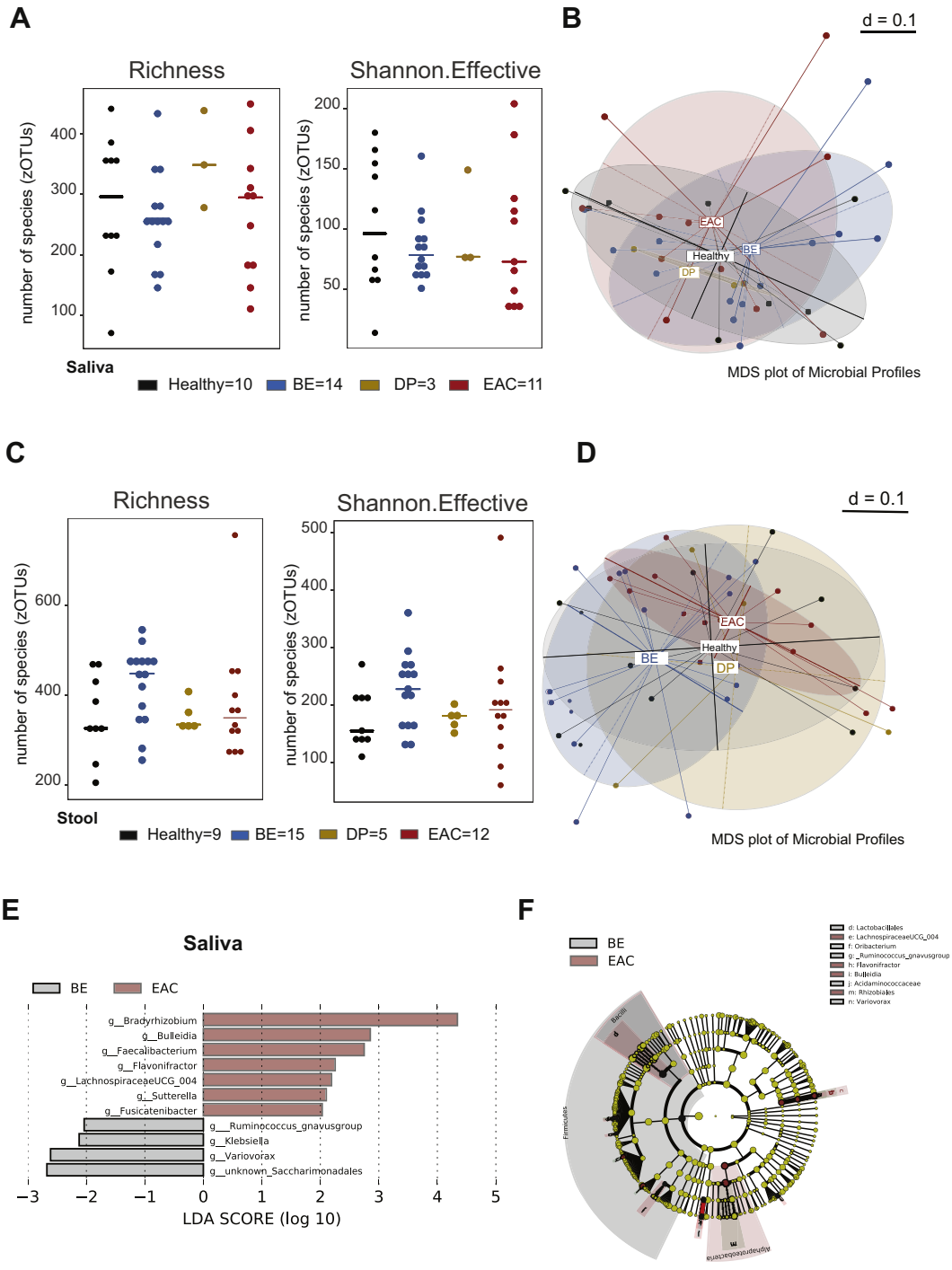

Figure 3. EAC mucosal-associated dysbiosis is only weakly reflected in stool and saliva samples. (A) Alpha-diversity analysis measured by community species richness and Shannon effective number of species in saliva samples. (B) MDS plot of microbial profiles of saliva samples stratified by disease state. (C) Alpha-diversity analysis measured by community species richness and Shannon effective number of species in stool samples. (D) MDS plot of microbial profiles of stool samples stratified by disease state. (E) Comparison of relative abundance of bacterial genera between salivary microbiome in patients with BE and that in patients with EAC using linear discriminant analysis effect size (LEfSe) in saliva. Taxa meeting an linear discriminant analysis significant threshold 2 are shown. (F) Cladogram of differentially abundant genera in salivary microbiome of patients with BE or with EAC. BE, Barret's esophagus; DP, dysplasia; EAC, esophageal adenocarcinoma.

we found no significant drop in richness number of species with disease progression but a significant drop in Shannon effective number of species in EAC compared with the healthy group. Although the results of Munich and NY cohorts are not identical, they suggest a decline in community diversity with disease progression (alpha-diversity). Second,

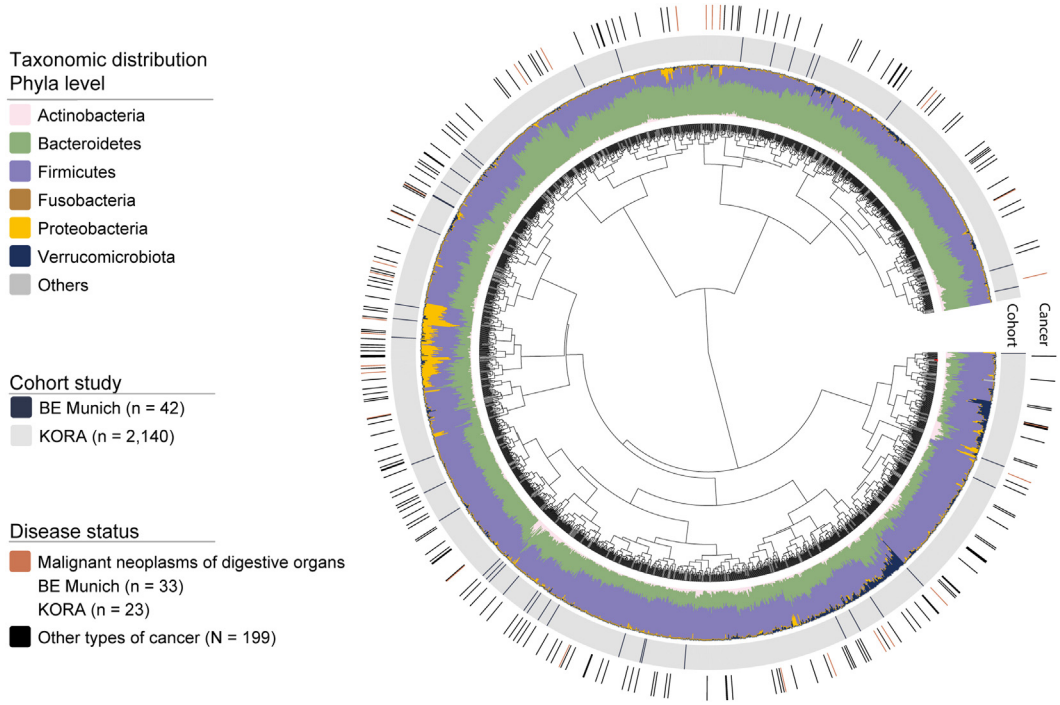

Figure 4. Integration of patients and controls of the Munich cohort in KORA population study cohort with a focus on individuals with gut-related cancer. Beta-diversity of the fecal microbiota in Munich and KORA cohorts. The dendrogram shows similarities between microbiota profiles based on generalized UniFrac distances between 42 Munich and 2140 KORA subjects represented by individual branches. Individual taxonomic composition at the phylum level is shown as stacked bar plots around the dendrogram. Bars in the outer part of the figure indicate disease status: first ring, in the outer part of the figure, indicates the cohort study (blue, BE Munich; grey, KORA); second ring shows cancer cases (orange, malignant neoplasms of digestive organs; grey, other cancer types). BE, Barrett's esophagus.

in both cohorts, we revealed distinct microbial profiles between phenotypes (beta-diversity). At genus level, there were only single taxa showing same trends in both Munich and NY cohorts. This is certainly partly due to the different preservation methods (snap freezing in liquid nitrogen and PAXgene) and different primers used in both cohorts. The fact that, in both cohorts, shifts in microbiota composition were found dependent on patient's diagnosis stresses that the esophageal microbiota is strongly associated with the phenotype. In addition, it confirms both preservation methods are feasible to identify the microbiome.

With the characterization of tissue biopsy microbiome, we revealed the mucosa-associated microbiome. It is suggestive that mucosa-associated microbiota is in direct contact with the mucosal cells and may thus interact directly with the mucosal cells through surface proteins, metabolites, and interaction with immune cells. For *Fusobacterium nucleatum*, there are hypothesis about a direct interaction with the mucosal colon cells in cancer promotion. It is assumed that *Fusobacterium* promotes cell proliferation through binding of its fusobacterium adhesin A protein to E-cadherin and through binding of its lipopolysaccharides on toll-like-receptor 4 (TLR4) and then oncogenic expression of microRNA.[27–29] The mechanisms mediated by lipopolysaccharide and TLR4 could be also of importance for EAC progression, since a higher number of Gram-negative bacteria and higher expression of TLR4 have been found in EAC carcinogenesis.[5,30]

While the tissue biopsy-associated microbiome somewhat reflected disease progression, this was only weakly reflected in stool and saliva samples in this small cohort. A comparison of BE and EAC gut microbiome profiles with a large population-based cohort (KORA) did not reveal any usage of the fecal microbiome as a BE or cancer biomarker, with the limitation of a small sample size of the Munich cohort compared with the KORA cohort. The data show the heterogeneity of fecal microbial profiles in patients with the same disease status/cohort origin.

We also integrated the patient's epidemiologic information and diagnosis with the microbiome data. Although the numbers are too small to draw major conclusions, we observed that the diagnosis itself, even in smaller groups, had an important impact on microbiome composition, whereas single risk factors have less impact. These findings emphasize that the microbial community in the esophagus is highly associated with the diagnosis and not with the exposure to single risk factors, proposing that either the disease itself causes alterations on the microbiome or that single risk factors do not stand alone in perturbations of the microbiota. Certainly, to clarify the impact of single risk factors within the diagnosis groups and to exclude confounding factors, larger cohorts will have to be analyzed.

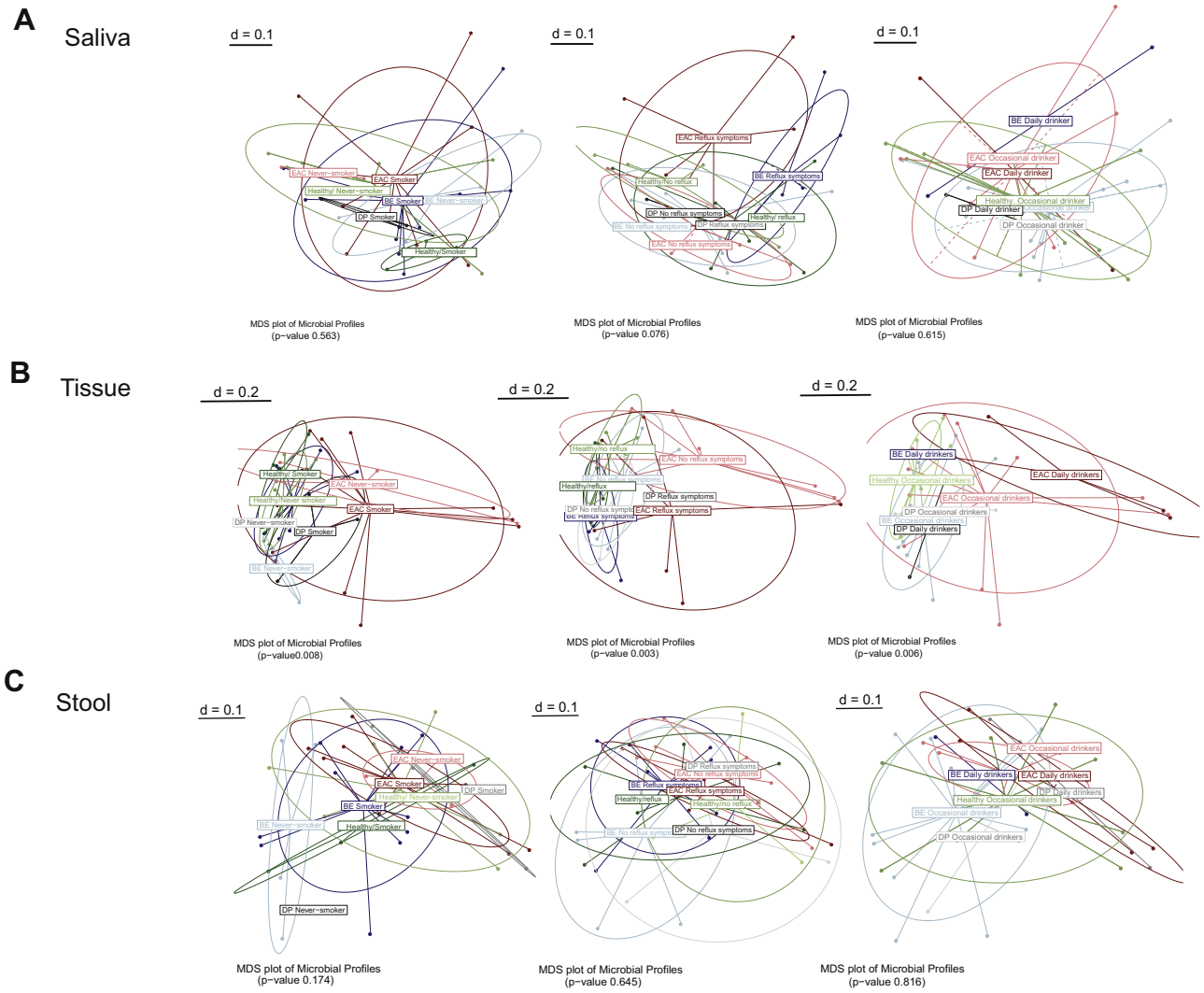

Figure 5. Risk factors for EAC correlate with microbiome diversities. Beta-diversity visualized by multidimensional scaling (MDS) of microbial profiles of (A) saliva, (B) tissue biopsies, and (C) stool from patients simultaneously subdivided by diagnosis and smoking status; diagnosis and alcohol consumption; diagnosis and presence of reflux symptoms. BE, Barret's esophagus; DP, dysplasia; EAC, esophageal adenocarcinoma.

Our study provides some strengths, which include the fact that we included patients with EAC, whose microbiome composition has been poorly investigated so far in comparison to patients with BE and gastrointestinal reflux disease. Other strengths of this study were the stringent exclusion criteria (such as exclusion of patients with antibiotics use in previous 6 weeks, diarrhea, and vegetarian eating habits); the acquisition and integration of comprehensive epidemiologic data, all of which may interact with the microbiome; the histologic confirmation of diagnosis by certified gastrointestinal pathologists; the standardized sampling and preanalytical workflow; as well as DNA quality and the bioinformatical statistical analyses used.

Limitations of this study were the low number of analyzed samples and the low number of patients diagnosed with DP. Furthermore, the diagnosis of chronical gastritis among control patients represents a study limitation because gastritis may have implementations in microbiome composition and diversity of esophagus. However, the number of healthy patients with *H. pylori* colonization was equal to the number of patients without *H. pylori* colonization. Difficulties with inclusion of healthy controls without contraindications were encountered due to the invasiveness of the examination and strict indications for an upper endoscopy in Germany. Hence, only patients with prolonged or severe abdominal pain or reflux symptoms were eligible for an esophagogastroduodenoscopy in the first place.

It remains unclear if changes of the microbiome are contributing to cancer development or if it is the tumors' topography which is favorizing the colonization of certain taxa.[31] Snider et al[5] showed that different sampling locations such as SE, BE, or cardia have similar biota diversity and composition. This suggests that the tissue structure, at least in noncancerous tissue similar to cancerous tissue in its glandular structure, is not decisive for the microbiota community. The results of our study may open new doors

for future findings to elucidate the role of distinct microbiomes in EAC. Therefore, prospective large-cohort human studies are needed to identify the alternations of microbiome at different progression stages and rest stages of disease and to validate possible biomarkers.

Supplementary Materials

Material associated with this article can be found in the online version at https://doi.org/10.1016/j.gastha.2022.04.003.

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Data Transparency Statement:
Data, analytic methods, and study materials will be made available to other researchers through personal contact due do ethical regulations on human data handling.