Pilot randomized trial to assess the effects of an antimicrobial mouthwash on the esophageal and gastric microbiome

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The incidence of esophageal adenocarcinoma (EAC) has risen nearly 10-fold over the past half century, yet the reasons for this dramatic change are largely unknown. During this same time period, the gastrointestinal microbiome has undergone a significant shift with the advent of antibiotics and the progressive decline in Helicobacter pylori infection rates. This raises the possibility that the upper gastrointestinal microbiome may play a key role in the development of BE and EAC. The esophageal microbiome in patients with reflux esophagitis or Barrett's esophagus (BE, the precursor to EAC) is heavily populated with Gram-negative bacteria, which may drive a chronic inflammatory, pro-neoplastic state. Also observed is an increased abundance of Fusobacterium nucleatum; work by our team has shown that F. nucleatum may directly contribute to colon carcinogenesis, and we suspect that this species may have similar effects in promoting esophageal neoplasia. We have shown that there is a relatively high abundance of F. nucleatum in the gastric cardia, and in animal models chronic inflammation in the gastric cardia leads to the development of Barrett's esophagus and EAC. Thus, we speculate that the upper gastrointestinal microbiome plays a key role in the development of BE and EAC.

The esophageal microbiome is very similar to the oral microbiome; both contain an abundance of anaerobes as well as a high ratio of Firmicutes to Bacteroidetes. The microbiome of the upper gastrointestinal tract is presumably heavily influenced by migration of bacteria from the mouth via swallowed secretions. Therefore, it stands to reason that altering the oral microbiome can directly impact the microbiome of the esophagus and gastric cardia. Chlorhexidine has broad antimicrobial effects, and chlorhexidine-based mouthwashes have been used clinically for many years in the treatment of gingival and other oral diseases. Chlorhexidine mouthwash effectively reduces anaerobes and F. nucleatum in particular in the mouth. It also has anti-inflammatory effects (likely due to reductions in pro-inflammatory bacteria), and protects against mucositis in chemotherapy patients. We therefore propose to perform a randomized open-label pilot study to assess the effects of chlorhexidine mouthwash on the microbiome of the gastric cardia. The results of this study provide valuable information as to whether altering the oral microbiome can serve as a novel chemopreventive intervention to reduce the risk of esophageal adenocarcinoma.

This is a randomized, open-label pilot study to assess whether treatment with chlorhexidine mouthwash can alter the esophageal and gastric cardia microbiome.

We will enroll a total of 20 subjects. Patients scheduled for upper endoscopy for clinical indications will be randomized to 2 weeks of either no treatment or use of twice daily chlorhexidine mouthwash leading up to the endoscopy.

The hypotheses to be tested are:

1. Treatment with chlorhexidine mouthwash will significantly reduce the absolute abundance of F. nucleatum in saliva.
2. Subjects treated with chlorhexidine mouthwash will have significantly lower absolute abundance of F. nucleatum in the gastric cardia, compared to subjects who receive no treatment.

We will enroll patients scheduled for upper endoscopy by their treating physician and who meet the inclusion and exclusion criteria. There will be 2-3 study visits, at Day 0 and Day 14, and for

those subjects in the mouthwash arm, a final visit 1-2 weeks following the Day 14 visit. Subjects will be required to fast overnight prior to the visits on Days 0 and 14.

Day 0:
• Collect baseline demographic information, clinical history, medication use, and smoking history. Record height, weight, and waist and hip circumference.
• Administer a Reflux Assessment Questionnaire and a Food Frequency Questionnaire
• Saliva collection and oral swab
• Take baseline photographs of teeth (mouthwash arm only)
• Distribution of chlorhexidine mouthwash (mouthwash arm only)

Day 14:
• Medication reassessment
• Food Frequency Questionnaire
• Adverse event assessment
• Take photographs of teeth
• Saliva collection and oral swab (prior to endoscopy)
• Upper endoscopy (with esophageal and gastric cardia brushings and biopsies for study purposes) Optional dental cleaning visit (mouthwash arm only; 1-2 weeks after the Day 21 visit):
• Dental examination and cleaning
• Provide recommendations for follow-up dental care

Treatment arm assignment:
Subjects will be randomized to either twice daily chlorhexidine mouthwash or no treatment for 14 days. We will perform blocked randomization stratified by the presence of gastro-esophageal reflux (as determined by the Reflux Assessment Questionnaire) as well as by regular PPI use.

Chlorhexidine mouthwash:
Subjects assigned to the mouthwash arm will use a 0.12% chlorhexidine gluconate mouthwash (Peridex™; 3M ESPE Dental Products, St. Paul, MN). Subjects will be instructed to rinse 15 mL twice daily for 30 seconds and then spit. The mouthwash rinse will be performed in the morning and evening after brushing their teeth.

Subjects from both arms will be instructed to continue regular oral health care (brushing and flossing) per usual practice, although use of other mouthwashes or rinses will be prohibited during the study period.

Concomitant medications:
Use of other mouthwashes or rinses will be prohibited during the study period.
Any subject who takes antibiotics or immunosuppressive medications (including steroid inhalers or nasal sprays) during the study period will be excluded from final analyses.

Photographs of Teeth:
A potential side effect of chlorhexidine mouthwash is tooth staining. For subjects assigned to the mouthwash arm, we will take digital photographs of the teeth at Visits Day 0 and Day 14. The photographs will be used to help assess whether there has been tooth staining.

Subjects in the mouthwash arm will be offered an optional dental cleaning (below) regardless of any evidence of tooth staining as assessed by the photographs.
No identifiable facial features will be visible on the images.

Dental examination and cleaning:
Staining of teeth and dental hardware is a known side effect of chlorhexidine mouthwash. The expected incidence of staining after 14 days of use is difficult to predict, although it is possible that some of the subjects in the study will have staining.
For the subjects in the mouthwash arm, we will take digital photographs of the mouth at Day 0 and then again at Day 14 to assess staining.
These subjects will be offered an optional, free dental cleaning, 1-2 weeks after the Day 14 visit. During this visit we will perform a dental exam and cleaning. Based on the results of the exam, we will provide recommendations for any additional dental care that may be indicated.

Specimen collections:
At the Day 0 visit we will collect saliva and also swab the inside of the mouth (cheek, tongue, hard palate, and gingiva).
This will be repeated at the Day 14 visit prior to the upper endoscopy.
During the endoscopy, the following samples will be taken for study purposes: 2 brushings (1 from normal appearing esophagus (3 cm above the gastroesophageal junction) and 1 from the gastric cardia); and 2 biopsies (one each from the esophagus and gastric cardia).

Microbiome and tissue analyses:
F. nucleatum. We will quantitatively assess absolute abundance of F. nucleatum from specimens via qPCR of the FadA adhesin gene.

16S rRNA gene sequencing. At the end of the study, we will perform batched DNA extraction performed using the Qiagen DNeasy Blood and Tissue Kit (Qiagen, Valencia, CA). We will perform PCR targeting the V3 and V4 hypervariable variable regions of the 16S rRNA gene with 347f/803r primers derived from the human microbiome project. We will pool and purify samples with the QIAquick PCR kit and perform library quantification using a KAPA Library Quantification Kit (Kapa Biosystems, Wilmington, MA).

Sequencing of the 16S rRNA gene V3 and V4 regions will be performed at the New York Genome Center using the Illumina MiSeq 300PE platform (Illumina, San Diego, CA).
Based on taxonomic assignments from the 16S sequencing analyses, we will calculate relative abundance of genera and species identified in the samples. We will subsequently assign each of these taxa as Gram-negative or not Gram-negative, based on our previously assembled reference list. We will then sum the relative abundances of each of the Gram-negative taxa to calculate the relative abundance of Gram-negative bacteria for each sample.

Inflammatory Cytokines, Stem Cell Proliferation, and Intestinalization. We will perform qRT-PCR on tissue biopsies to assess for levels of NF-B, IL-1, IL-6, and IL-8. In order to assess whether Gram-negative bacteria induced inflammation may lead to BE, we will also perform qRT-PCR and IHC (with available antibodies) of markers of intestinal progenitor cell activation (Lgr5, Notch, Cck2r, Dclk1, Bmp4) and intestinalization (Cdx2, Sox2, Tff2, Tff3).

Statistics:
The primary outcome will be the absolute abundance of F. nucleatum. To address the hypothesis that chlorhexidine mouthwash reduces the absolute abundance of F. nucleatum in the mouth, we will assess within individual differences in subjects treated with chlorhexidine and in those who received no treatment.

We will calculate mean and standard deviation for the within-individual change in F. nucleatum for the treated and untreated arms. These two means will then be compared using t-tests. For the primary analysis will use data from the saliva samples, and will then perform secondary analyses with data from the oral swab samples.

We will then address the hypothesis that subjects treated with chlorhexidine have lower abundance of F. nucleatum in the gastric cardia compared to those who did not receive treatment. We will calculate the mean absolute abundance of F. nucleatum in the cardia of the treated and untreated groups, and compare means using t-tests. We will then analyze data in a similar fashion from esophageal brushings in secondary analyses. All analyses will be 2- sided. For the primary analyses, statistical significance will be defined as $p < 0.05$.

We will perform numerous additional secondary analyses as part of this pilot study. For each subject and sample collected we will calculate the relative abundance of Gram-negative bacteria (%GNB). We will then make the same comparisons described above, with %GNB as the outcome. For these analyses we will use Wilcoxon rank sum tests instead of t-tests.

Additional 16S sequencing analyses. For secondary analyses related to the microbiome, sequences will be grouped into operational taxonomic units (OTUs) of 97% similarity. Using these OTU definitions, we will estimate the microbial diversity of a sample by calculating distance measures based on various metrics, including the Shannon diversity index (alpha diversity), and Unifrac. This includes adjusting for the number of taxa detected for their relative abundance (proportions). We will align a representative set of OTU sequences and assign taxonomy based on the most recent Greengenes OTU database. We will phylogenetically assess phyla-level differences in relative abundance between groups, as well as exploratory analyses of the relative abundance of individual taxa, using the BenjaminiHochberg method to adjust for multiple hypothesis testing. We will normalize data with the R packages phyloseq and DESeq2. We will perform Principal Coordinate Analysis (PCA) and Hierarchical clustering of specimens based on phyla-level phylogeny or count-based distance metrics to compare groups of samples. Finally, we will perform PICRUSt analyses to estimate and compare the functional metagenomic compositions of the samples using the 16S data.

Other secondary analyses. We will compare the treated and untreated groups in terms of levels of the above prespecified inflammatory cytokines and markers of stem cell proliferation and intestinalization. We will also review the pre- and post- photographs of the mouth of the mouthwash arm subjects to determine the proportion of subjects who developed staining. The individual assessing the images will be blinded as to which images are pre- or post- treatment. We will use t-tests and Wilcoxon rank sum tests to compare continuous data and Fisher exact

tests for categorical data between groups. All analyses will be 2-sided, and statistical significance will again be defined as $p<0.05$.

Sample Size Considerations:
There is no published data on which to base power calculations for this study.
Prior studies have demonstrated that chlorhexidine mouthwash significantly reduces F. nucleatum in the mouth. However, these studies used older techniques to quantify F. nucleatum, and the results cannot be readily translated to expected findings using qPCR.

A sample size of 20 subjects (10 per arm) has 83% power (assuming alpha=0.05) to detect a 1.3 SD difference in mean F. nucleatum between the groups, which likely represents a biologically meaningful difference. As this is a pilot study, we believe that in the event no significant reduction in F. nucleatum is observed, we will still obtain valuable data from the secondary analyses that will assist in the design of future studies aimed at altering the microbiome to reduce the risk of BE and EAC.

We anticipate that 10% of enrolled subjects will be excluded, removed, or lost to follow-up. We therefore plan to enroll 22 subjects.