A non-endoscopic sampling device to sample the oesophageal microbiota: a case-control study

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Summary

Background

The strongest risk factor for oesophageal adenocarcinoma (OAC) is reflux disease, and the rising incidence coincides with the eradication of *Helicobacter pylori*, both of which may alter the oesophageal microbiota. We aimed to profile the microbiota at different stages of Barrett's carcinogenesis and investigate the Cytosponge™ as a minimally invasive tool for sampling the oesophageal microbiota.

Methods

16S rRNA gene amplicon sequencing was performed on 210 oesophageal samples from 86 patients representing the Barrett's progression sequence (normal squamous controls, non-dysplastic and dysplastic Barrett's oesophagus, and OAC), relevant negative controls and replicates on the Illumina MiSeq platform. Three different oesophageal sampling methods were compared for microbial DNA yield (qPCR), diversity and community composition: fresh frozen tissue, fresh frozen endoscopic brushings and the Cytosponge™ device.

Findings

There was decreased microbial diversity in OAC tissue compared to normal control patients as measured by the observed OTU richness, Chao estimated total richness and Shannon diversity index (all $p < 0.01$). *Lactobacillus fermentum* was enriched in OAC ($p = 0.028$), and lactic acid bacteria dominated the microenvironment in 7 (47%) of 15 OAC cases. Comparison of oesophageal sampling methods showed that the Cytosponge™ yielded more than ten-fold higher quantities of microbial DNA in comparison to endoscopic brushes ($p < 0.001$) or biopsies

(p<0·0001) using qPCR. The Cytosponge™ samples contained the majority of taxa detected in biopsy and brush samples, but were enriched for genera from the oral cavity and stomach, including *Fusobacterium, Megasphaera*, *Campylobacter*, *Capnocytophaga* and *Dialister.* The Cytosponge™ detected decreased microbial diversity in patients with high grade dysplasia in comparison to controls as measured by the observed OTU richness, Chao estimated total richness and Shannon diversity index (all p<0·05).

Interpretation

Alterations in microbial communities occur in the lower oesophagus in Barrett's carcinogenesis, which can be detected at the pre-invasive stage of high grade dysplasia using the novel Cytosponge™.

Funding

Introduction

Oesophageal adenocarcinoma (OAC) is an aggressive malignancy with poor outcomes that generally develops from a pre-malignant columnar epithelium called Barrett's oesophagus. The incidence of OAC has increased six-fold in the Western world during the past three decades.[1] Both Barrett's and OAC are thought to develop in response to chronic acid reflux in the lower oesophagus, which precipitates inflammation and mucosal injury over time.[2] Reflux disease has increased with the obesity epidemic and altered eating habits in the Western world, and central adiposity may also influence carcinogenesis through the release of adipokines.[3] Epidemiologic evidence also suggests that the rising incidence of OAC coincides with the eradication of *Helicobacter pylori,* which may alter the composition of microbiota and promote bacterial overgrowth.[4] Furthermore, reflux disease is treated with antacid medications such as proton pump inhibitors, which have profound effects on gastric acidity and may affect the gastro-oesophageal microbiota.[5]

There is growing evidence linking abnormal changes in the microbiota, or dysbiosis, with human cancer. One of the best described examples is colon carcinoma, in which gastrointestinal microbiota have been shown to promote carcinogenesis in the setting of colonic inflammation.[6-8] Recent evidence has also linked *Fusobacterium nucleatum* to colon carcinoma through an altered tumour immunoenvironment, but without associated colitis.[9,10] The oesophagus has far fewer bacteria than the colon; nevertheless alterations in the microbiota may occur in reflux oesophagitis and Barrett's oesophagus.[11-13] However, the role of the microbiota in Barrett's carcinogenesis is not clearly defined and there is currently no clinical reference standard for sampling the oesophageal microbiota. One of the challenges in studying the oesophageal

microbiota is that endoscopy is a relatively invasive test that provides only a focal sampling of the microbiota in biopsy samples and a slightly larger surface area with endoscopic brushings. Minimally invasive methods for sampling the oesophageal microbiota could be clinically useful for detection and risk stratification of patients with Barrett's oesophagus.

Here we propose the Cytosponge™ as a non-endoscopic cell-sampling device that can collect a representative sample of cells along the length of the oesophagus.[14,15] The device consists of a spherical mesh compressed within a gelatine capsule and attached to a string. Once swallowed, the capsule dissolves and the Cytosponge™ expands in the patient's stomach before being withdrawn on a string through the patient's mouth. We have previously shown that this device is a safe, acceptable method for diagnosing Barrett's oesophagus, with promising accuracy and cost-effectiveness characteristics.[14-16] The goal of the current study was to provide a comprehensive description of the microbiota in the different pathogenic stages of OAC using 16S rRNA gene amplicon sequencing and to test the feasibility of the Cytosponge™ to detect changes in the microbiota occurring in Barrett's oesophagus and high grade dysplasia.

Methods

Study design and participants

Endoscopic biopsies, brushes, Cytosponge™ samples and throat swabs were collected from patients with a diagnosis of non-dysplastic Barrett's oesophagus (n=24), high grade dysplasia (n=23) or control patients (n=20) with symptoms of reflux or dyspepsia enrolled in the Barrett's Oesophagus Screening Trial (BEST2) at five UK hospitals. Matched endoscopic biopsies were taken from an area of Barrett's oesophagus and proximal normal squamous oesophagus. Endoscopic brushings were taken from an area of normal squamous oesophagus only. In patients with a diagnosis of OAC, tissue samples from the tumour and matched normal squamous oesophagus (n=19) were collected from six UK hospitals participating in Oesophageal Cancer Clinical and Molecular Stratification (OCCAMS) for the International Cancer Genome Consortium (ICGC). The tissue samples collected in the OCCAMS/ICGC study included endoscopic biopsies, endoscopic mucosal resection specimens and surgical biopsies after oesophagectomy. Sampling from oesophagectomy specimens was performed with a sterile scalpel blade (cutting down to submucosa) within one hour of surgical resection. All samples were flash frozen in liquid nitrogen and stored at -80°C except the Cytosponge™ samples, which were preserved in BD SurePath™ liquid at 4°C. Table 1 displays patient demographics and the oesophageal samples sequenced. The number of patients recruited at each participating hospital centre and additional clinicopathologic data for the OAC patients is provided in the web appendix (pages 2–3).

The patient inclusion criteria included age between 20 and 90 years, no current infection or antibiotics, no prior chemotherapy treatment, and no pathologic findings on endoscopy except

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where Barrett's oesophagus or OAC was documented. Acid-suppressant medication was taken regularly by 23 (100%) of 23 dysplastic cases, 22 (92%) of 24 Barrett's cases, 12 (71%) of 17 OAC cases (two not reported) and 15 (75%) of 20 controls. All patients were fasting overnight prior to endoscopy or surgery. As part of routine perioperative procedure, OAC patients who underwent oesophagectomy received prophylactic intravenous antibiotics at the time of surgery, up to six hours before the research samples were obtained. The very close timing of this perioperative antibiotic exposure should not critically affect microbial community composition profiles as 16S rRNA gene amplicon sequencing detects both live and dead bacterial cells cross-sectionally (see Supplementary text).[17] One patient with Barrett's oesophagus and two patients with high grade dysplasia reported taking a course of antibiotics within the past month.

Ethical approval was obtained from the National Research Ethics Services Cambridgeshire Research Ethics Committee on behalf of all hospital centres in the BEST2 trial (REC 10/H0308/71, July 7, 2011) and the OCCAMS/ICGC trial (REC 07/H0305/52 and 10/H0305/51, February 7, 2010). Written informed consent was obtained from all subjects prior to the collection of samples and recording clinical information.

Table 1. Patient demographics and oesophageal samples

	Control (n=20)	Barrett's (n=24)	Dysplasia (n=23)	Cancer (n=19)	p-value
		Patient diagnosis			
Median Age (Range)	57 (29–86)	67.5 (53–79)	65 (50–82)	70 (44–79)	**0·001**
Male Sex (%)	7 (35)	16 (67)	19 (83)	15 (79)	**0·006**
Caucasian Ethnicity (%)	19 (95)	24 (100)	23 (100)	16 (89), 1 NR	0·089
Antacid usage (%)	15 (75)	22 (92)	23 (100)	12 (71), 2 NR	**0·011**
Samples sequenced/passed QC					
Cytosponge™	**20**/20	**24** /24	**23** /23		
Brush (squamous only)	**19**/19	**19** /19			
Tissue	**19**/16	Barrett's: **24** /17		Cancer: **19** /15	
		Squamous: **24**/15		Squamous: **19** /15	

NR=not recorded, QC=quality control as described in Methods.

Procedures

DNA extraction and 16S rRNA gene amplicon sequencing

Cytosponge™ samples were vortexed and centrifuged to pellet cellular debris (215 g for 5 min), and the residual supernatant was used for microbial DNA extraction after further high speed centrigugation (14,000 g for 10 min). DNA was isolated from all oesophageal samples using the Precellys Soil DNA Kit (Peqlab Ltd.). The 16S rRNA gene was amplified using primers for the V1-V2 region: 27F 5'AATGATACGGCGACCACCGAGATCTACAC TATGGTAATT CC AGMGTTYGATYMTGGCTCAG and 338R 5'CAAGCAGAAGACGGCATACGAGAT NNNNNNNNNNNN AGTCAGTCAG AA GCTGCCTCCCGTAGGAGT, where Illumina adapter sequences are at the 5' end, and the N string is a unique barcode. The majority of samples had two barcoded replicates to ensure reproducibility (labelled "A" or "B" in the web appendix, page 4). The reaction conditions were 98°C for two minutes, 25 cycles of 98°C for 30 s, 50°C for 30 s, 72°C for 90 s, and extension at 72°C for five minutes. Negative controls were kit reagents or nuclease free water, and underwent 45 amplification cycles. All samples were amplified in duplicate and pooled to minimize PCR bias and maximize yield. The PCR products

were concentrated using ethanol precipitation and quantified using a Qubit 2.0 Fluorometer prior to sequencing on the MiSeq Illumina platform using 2 x 250 bp read length. 16S rRNA gene amplicon sequencing data has been deposited in the European Nucleotide Archive under accession number ERP005191: http://www.ebi.ac.uk/ena/data/view/ERP005191.

Analysis of 16S rRNA gene amplicon *sequence data*

The 16S rRNA gene amplicon sequence analysis was performed using mothur.[18] The Miseq standard operating procedure[19] was followed with the exception of chimera checking, which was performed with chimera.perseus, and unique sequences were removed using the split.abund and remove.seqs commands prior to building the distance matrix. Contaminant OTUs were defined as having greater proportional abundance in negative controls, alongside previous evidence that these OTUs were derived from genera that are common contaminants,[20], and these reads were removed using remove.seqs (3434 OTUs defined as contaminants from 5757 total OTUs). Comparisons between replicate samples (using Metastats[21] as implemented in mothur) revealed no significant differences so replicates were pooled using the Linux sed command to maximize the number of reads per sample, and samples with fewer than 550 reads and/or Good's coverage less than 95% were removed using remove.groups. The Good's coverage estimator[22] is used to assess what proportion of the total OTUs present within a given sample are detected in the sequencing results, and thus gives an indication of how thorough the sampling has been at the chosen sequencing depth. For measures of diversity that are sensitive to the sequencing depth, random sub-sampling was performed at the lowest number of reads per sample using the sub.sample command in mothur. Data were sub-sampled at 631 reads for the analysis of tissue samples (median Good's coverage 96.36%, range 92.08%–99.37%), 656 reads for matched

tumour-normal pairs (median Good's coverage 97.41%, range 95.27%–98.78%), 631 reads for

the comparison of different sampling methods (median Good's coverage 96.51%, range 91.13%–

99.84%), and 19303 reads for the analysis of Cytosponge™ samples (median Good's coverage

99.83%, range 99.70%–99.95%). When determining the shared genera between Cytosponge™

samples, biopsies and brushes a cut-off of 0.0001% proportional abundance was used to focus on

the more abundant OTUs that are less likely to be susceptible to errors introduced by

subsampling (below 0.0001% there were less than 27 reads supporting each OTU across all the

samples). A second cut-off value of 0.1% was chosen arbitrarily to show the similarity between

sample types and is a common cut-off used in the literature. Sequence identity was confirmed at

the species level where possible by carrying out NCBI BLAST analysis on representative

sequences using MegaBLAST.[23]

Quantitative PCR for overall bacterial abundance

Quantitative PCR for the 16S rRNA gene was performed using SYBR Green I Master Mix

(Roche) on the LC480 LightCycler 480 II (Roche), in triplicate. The reaction conditions were

95°C for five minutes, 40 cycles of 95°C for 15 s, 60°C for 30 s and 72°C for 90 s, and a melt

curve. The primer sequences were 331F 5'TCCTACGGGAGGCAGCAGT and 797R

5'GGACTACCAGGGTATCTAATCCTGTT.[24]

Statistical Analysis

The nonparametric Kruskal-Wallis test and Dunns multiple comparisons post-test were used for

comparisons between diagnostic groups in Graphpad Prism version 6. Within Graphpad, the

Wilcoxon signed rank test was used for analyses involving matched tumour-normal pairs and the

Friedman test and Dunns post-test were used for analyses of matched samples from endoscopic biopsies, brushes and the Cytosponge™. Linear discriminant analysis effect size (LEfSe)[25] was used within mothur to compare differences in proportional abundance of microbial taxa. All sequencing reads were included for the composition analysis using LEfSe. LEfSe ranks OTUs in order that it considers these taxa to be most likely to explain differences between microbial communities using linear discriminant analysis (LDA) to estimate effect size. A full explanation of the statistical approaches employed in LEfSe can be found in the original paper by Segata *et al.*[25] Within mothur, the Bray-Curtis calculator was used to describe the dissimilarity between communities by taking into account both the overlap in OTUs that are present between samples and the proportional abundance of those OTUs in each sample. Using dissimilarity information calculated with the Bray-Curtis calculator, the parsimony test and the analysis of molecular variance (AMOVA) test were used to indicate significantly different clustering between microbiota profiles from the different diagnostic groups. IBM SPSS Statistics Version 24 was used to analyse patient data (ANOVA for mean age and Fisher's Exact test for sex, ethnicity and antacid usage). A significant p-value was defined as less than 0·05.

Role of the funding source
The funding sources had no role in the study design, analysis, interpretation of data or writing of the report.

Results

To investigate whether the development of OAC was associated with dysbiosis, we performed 16S rRNA gene amplicon sequencing on tissue samples from patients with OAC (n=19), Barrett's oesophagus (n=24) and normal control patients (n=19, table 1). Patients with OAC and Barrett's were older (p=0·001) and were predominantly male (p=0·025) in comparison to the controls, which is consistent with the known epidemiology of this disease.[26] The majority of samples had two barcoded replicates to ensure reproducibility (web appendix, page 4), and negative controls from every DNA extraction step underwent additional PCR cycles to identify contaminant organisms. After filtering the sequencing data and removing contaminant sequences, the average number of reads for tissue samples was 6649 (median 3064) and the proportion of reads that were sub-sampled for diversity analyses was 9.5% (for 631 reads cut-off) and 9.9% (for 656 reads cut-off). Fourteen tissue samples did not meet quality criteria and were excluded from the analysis, leaving 16 controls, 17 Barrett's and 15 OAC samples. Five phyla accounted for the majority of sequencing reads in the dataset: *Firmicutes* (59·9%), *Bacteroidetes* (15·1%), *Proteobacteria* (12·8%), *Actinobacteria* (5·8%) and *Fusobacteria* (5·4%). 1060 operational taxonomic units (OTUs) were identified and classified as belonging to 345 different genera.

We used linear discriminant analysis effect size (LEfSe), a metagenomic biomarker discovery method, to identify microbial taxa that differed significantly between controls, Barrett's and OAC samples. At the phylum level, the Barrett's samples contained a higher proportional abundance of *Proteobacteria* (mean 18·6%, median 14·8%, p=0·017) compared to controls (mean 8·5%, median 8·2%) and OAC samples (mean 7·6%, median 3·9%). The control samples

were enriched for several taxa at the family level, including the Gram-negative, anaerobic *Veillonellaceae* (p=0·012, overall proportional abundance 5·3%) and microaerophilic *Campylobacteraceae* (p=0·00038, overall proportional abundance 0·2%), the Gram-positive, anaerobic *Lachnospiraceae* (p=0·012, overall proportional abundance 1%) and *Erysipelotrichaceae* (p=0·0021, overall proportional abundance 0·4%), and the Gram-positive, facultative anaerobic *Carnobacteriaceae* (p=0·038, overall proportional abundance 1·6%) and *Actinomycetaceae* (p=0·0019, overall proportional abundance 0·8%, figure 1A). Significant genera within these families included *Veillonella* (p=0·002, overall proportional abundance 3.8%), *Megasphaera* (p=0·0027, overall proportional abundance 0.3%), *Granulicatella* (p=0·037, overall proportional abundance 1.6%), *Actinomyces* (p=0·0022, overall proportional abundance 0.8%), *Solobacterium* (p=0·012, overall proportional abundance 0.3%) and *Campylobacter* (p=0·0004, overall proportional abundance 0.2%, figure 1B). In OAC samples, the Gram-positive, anaerobic *Coriobacteriaceae* was enriched at the family level (p=0·01, overall proportional abundance 1·9%), but there were no significant genera identified within this family. At the species level, the Gram-positive, facultative anaerobic *Lactobacillus fermentum* was enriched in OAC with mean proportional abundance of 0·6% (median 0·009%) compared to 0·01% (median 0%) in Barrett's and 0·004% (median 0%) in controls (p=0·028). Sequence identity was confirmed where possible using NCBI BLAST. One Barrett's sample contained a high proportional abundance of *Helicobacter pylori* sequences (>99%).

Typically, OAC samples clustered away from controls in a Bray-Curtis cluster dendrogram (p=0·002, parsimony test), emphasizing the difference in community structure (figure 2A). The Bray-Curtis algorithm describes the dissimilarity between communities by taking into account

both the overlap in OTUs that are present and the proportional abundance of those OTUs. Samples that have fewer overlapping OTUs and OTUs with less similar proportional abundances will cluster separately, and this differential clustering was further demonstrated by principal coordinate analysis (p=0·001, AMOVA test, web appendix, page 1). The microbial communities of 7/15 OAC samples were dominated by the Gram-positive order *Lactobacillales*. Of the seven patients with a high proportion of acid-tolerant *Lactobacillales*, six were taking antacid medication. Five OAC samples contained high proportional abundance of *Streptococcus* (69% to 98%) and two samples contained high proportional abundance of *Lactobacillus* (87% and 92%). NCBI BLAST showed that the representative species were *Streptococcus pneumoniae/mitis, Streptococcus salivarius/vestibularis, Streptococcus parasanguinis, Lactobacillus gasseri*, and *Lactobacillus helveticus/suntoryeus/gallinarum*. Although relatively abundant in the acidic stomach environment, such a high proportional abundance of *Lactobacillus* was an unexpected finding in the oesophagus. When we examined matched normal and tumour tissue for the two patients with high *Lactobacillus* we found that this genus dominated the lower oesophagus regardless of disease state, (figure 2B). Gram-positive rods were visualized in areas of ulceration in tumour PS003 with a high proportional abundance of *Lactobacillus* (figure 2C).

Alpha diversity refers to the species diversity within a given environment and includes the number of species ("richness") and the proportion of those species ("evenness") within the microbial community. Three indices of alpha diversity, observed OTU richness (p=0·0012, figure 3A), the Chao estimate of total OTU richness (p=0·0004, figure 3B), and the Shannon diversity index (p=0·0075, figure 3C), showed that diversity was lower in OAC samples compared to controls. In comparison to Barrett's samples, the OAC samples showed a decrease

in observed OTU richness and the Chao estimate, but not the Shannon diversity index. Thirteen OAC patients had matched normal squamous tissue sampled proximal to the tumour, and in these patients there was no difference in OTU richness between the normal and tumour tissue (p=0·9065, figure 3D). Similarly, there was no difference for the Chao estimate (p>0·999) or the Shannon index (p=0·6355). Furthermore, there was no difference in overall bacterial abundance between matched normal squamous and tumour tissue (p=0·782, figure 3E). These results suggested that the decreased microbial diversity was pervasive throughout the lower oesophagus in OAC and independent of the absolute quantity of oesophageal bacteria.

We performed additional analyses to ensure that the differences observed were not due to sex, age or acid suppression. We repeated the diversity analysis with male patients only (excluded 10 controls, 4 Barrett's and 3 cancer cases), and the results were consistent, with decreases in diversity in cancer samples compared to controls for the observed OTU richness (p=0·0029), the Chao estimate of total OTU richness (p=0·0017), and the Shannon diversity index (p=0·0070). We repeated the diversity analysis with patients 60 years and older (excluded 9 controls, 2 Barrett's and 2 cancer samples), and the results showed a similar trend, which was significant for the observed OTU richness (p=0·0448), the Chao estimate of total OTU richness (p=0·0288), but not the Shannon diversity index (p=0·0892). We also performed a subgroup analysis for age within each diagnostic subgroup (using median age as a cut-off within each group) and there were no significant differences in diversity for younger vs. older patients within any of the subgroups. When we excluded patients who were not taking acid suppression (4 control patients, 2 Barrett's patients, 4 cancer patients) or unknown (1 cancer patient) the results were similar, with decreased diversity in OAC samples compared to controls, as evidenced by the observed

OTU richness (p=0·0065), the Chao estimate of total OTU richness (p=0·0033), and the Shannon diversity index (p=0·0202).

Given that the decrease in microbial diversity in OAC appeared widespread throughout the oesophagus, we questioned whether the Cytosponge™ could be a useful tool to sample the microbiota along the entire length of the oesophagus and upper GI tract. Fifteen patients with Barrett's oesophagus and 16 controls had Cytosponge™ samples that underwent 16S rRNA gene amplicon sequencing with matched endoscopic biopsies and brushes taken from an area of normal squamous oesophagus. A subset of these patients also had swabs of their posterior pharynx to analyse the similarities and differences between the oesophageal and oral microbiota (n=13). Overall 1455 OTUs were identified and mapped to 381 genera. Using a cut-off of 0·0001% overall proportional abundance (138 genera classified), 84·1% of genera were shared between Cytosponge™ samples, biopsies and brushes, and 83·6% were shared between Cytosponge™ samples and throat swabs. A stricter cut-off of 0·1% overall proportional abundance (41 genera classified) showed supporting reads for 100% of genera in all sample types, indicating overlap in community membership between the oral cavity, oesophagus and gastric cardia.

Although the majority of microbial taxa overlapped between sample types, the proportional abundances differed. At the phylum level, the Cytosponge™ samples contained a higher proportional abundance of *Tenericutes* in comparison to the other sample types using LEfSe (p=4·7x10^{-5}, overall proportional abundance 0.2%). At the genus level, the Cytosponge™ samples contained greater proportional abundances of *Fusobacterium* (p<0·0001, overall

proportional abundance 2%), *Megasphaera* (p<0·0001, overall proportional abundance 1.8%), *Campylobacter* (p<0·0001, overall proportional abundance 1.7%), *Capnocytophaga* (p=0·00058, overall proportional abundance 0.7%) and *Dialister* (p<0·0001, overall proportional abundance 0.2%). In keeping with these findings, principal coordinate analysis using the Bray-Curtis algorithm demonstrated that the Cytosponge™ samples clustered away from the throat swabs, endoscopic biopsies and brushes (p<0·001, AMOVA test, figure 4A). The throat swabs clustered distinctly from all the other sample types as well (p<0·001). There was no difference in clustering between biopsies and brushes on the PCoA plot (p=0·459).

As expected due to increased sampling surface area, quantitative PCR of overall bacterial abundance showed the quantity of microbial DNA isolated from Cytosponge™ samples was greater than matched biopsies and brushes (n=20 patients, p<0·0001, figure 4B). After sub-sampling to normalize for sequencing depth there was a decrease in observed OTU richness (p=0·0104, figure 4C) and the Chao estimate of total OTU richness (p=0·0156, figure 4D) in endoscopic brush samples, but no difference for the Shannon index (p=0·5968, figure 4E). To translate our findings to the setting of early detection, we tested the utility of the Cytosponge™ to detect changes in microbial diversity in patients with high grade dysplasia (n=23). The average number of reads for Cytosponge™ samples was 40753 (median 40821), and the proportion of reads that were sub-sampled was 47% (for 19303 reads cut-off). The observed OTU richness was decreased in high grade dysplasia compared to controls (p=0·0147, figure 5D), as was the Chao estimate of total OTU richness (p=0·023, figure 5E) and the Shannon index (p=0·0085, figure 5F). There was a trend towards decreased diversity in Barrett's oesophagus, which was only significant for the Shannon index. In general, the Cytosponge™ samples showed

more homogenous results for microbiota composition between diagnostic groups at the phylum and family levels, suggesting that the fraction of microbiota sampled from the area of Barrett's was diluted by the copious bacteria sampled from the rest of the oesophagus, oral cavity and stomach. Despite this, three genera were identified that distinguished controls from the other sample types using LEfSe: *Dialister* (p=0·027, overall proportional abundance 0.3%), *Schlegelella* (p=0·016, overall proportional abundance 0.1%) and unclassified *Prevotellaceae* (p=0·047, overall proportional abundance 1.3%).

Discussion

Our sequencing data revealed decreased microbial diversity and altered community composition in OAC. Interestingly, OAC patients appeared to have this reduced diversity regardless of whether cancerous or normal oesophageal tissue was sampled. The genera that were decreased in proportional abundance in OAC included Gram-negative (*Veillonella, Megasphaera* and *Campylobacter*) and Gram-positive taxa (*Granulicatella*, *Atopobium*, *Actinomyces* and *Solobacterium*). There was significantly increased proportional abundance for *Lactobacillus fermentum* in OAC compared to control patients and Barrett's oesophagus, and there was a high proportional abundance of acid-tolerant *Lactobacillales* (*Lactobacillus* and *Streptococcus*) in a subset (7/15) of OAC samples. To translate our findings to the setting of early detection, we investigated the utility of the Cytosponge™ device for sampling the oesophageal microbiota in Barrett's oesophagus and high grade dysplasia. The Cytosponge™ had high microbial DNA yield and detected significantly decreased diversity in high grade dysplasia compared to control patients.

Lactobacillales, or lactic acid bacteria, are so named for their ability to produce lactate from the fermentation of carbohydrates and survive under harsh acidic conditions.[27] Their resilience to low pH may enable *Lactobacillus* and *Streptococcus* to thrive in the tumour niche in a subset of OAC patients, and production of lactic acid by these bacteria could further acidify the microenvironment. Lactic acid fermentation can also produce noxious byproducts such as hydrogen peroxide that directly inhibit the growth of competitor bacteria and enable *Lactobacillales* to dominate the lower oesophagus. Given the altered microbial composition in OAC samples, it would be interesting to correlate microbiota data with expression and activity of Toll-like receptors, particularly TLR2, given the increased proportional abundance of Gram-positive genera in a subset of cancer samples. Other authors have investigated TLR expression in Barrett's carcinogenesis and found overexpression of TLRs 1, 2, 4, 6 and 9 in human OAC samples[28-31] and TLRs 1-3, 6, 7 and 9 in a rat reflux model.[32]

While the microbial community structure differed significantly in OAC in our study, there was only a modest reduction in diversity in Barrett's oesophagus and no genera were identified that discriminated between controls and Barrett's, or Barrett's and OAC. It is possible that very low abundance genera may be difficult to detect in oesophageal biopsies given the low microbial DNA yield, and notably some pathogens have been shown to cause overt disease while only accounting for a low proportional abundance of the total microbiota, such as *Clostridium difficile,*[33,34] *Citrobacter rodentium* and *Fusobacterium* spp.[35] Similarly, Amir *et al.* were unable to identify any taxa that differentiated between controls (n=15) and Barrett's (n=6) or oesophagitis (n=13) using LEfSe.[5] In contrast, Yang *et al.* reported that Gram-negative bacteria were significantly enriched in Barrett's (n=10) and reflux oesophagitis (n=12) compared to

controls (n=12).[11] The main limiting factor of these microbiota studies is the relatively small

sample size and inter-individual variation in microbiota composition. Another limitation is that

while LEfSe is useful for biological interpretation of metagenomic data, it does not correct for

multiple comparisons so there is a risk of false discovery (p-value, $\alpha =0\cdot05$). The inclusion of

appropriate negative controls and replicate samples is also paramount for low microbial biomass

samples to facilitate removal of contaminant OTUs that may also lead to false discovery,[20] and

this was a major strength of our study. We also imposed strict quality control criteria, resulting in

the exclusion of 14 tissue samples with low sequencing read numbers and Good's coverage

estimates. The difficulty in obtaining good quality sequencing data from oesophageal samples

highlights the potential utility of the Cytosponge™ device, which samples a larger surface area.

Our results suggest that it is feasible to sample the oesophageal microbiota using the

Cytosponge™, and the device detected the majority of genera present in endoscopic biopsies and

brushes. The high microbial DNA yield collected by the Cytosponge™ reflects sampling the

entire length of the oesophagus as well as the proximal stomach and oral cavity as it is

withdrawn. The throat swabs showed similarities in community membership between the oral

cavity and oesophagus, but the proportional abundances differed as reflected by distinct

clustering in principal coordinate analysis. Despite dilution from sampling the upper GI tract, it

was still possible for the Cytosponge™ to detect a decrease in diversity and community

composition between normal squamous controls and high grade dysplasia. Similar to the

Cytosponge™, Fillon *et al.* described a minimally invasive oesophageal string test to sample the

microbiota in a paediatric population.[36] The oesophageal string test detected comparable

microbial composition to matched oesophageal biopsies but required the patients to remain in

hospital overnight with the string secured to their cheek. Alternatively, the Cytosponge™ is a convenient test that can be administered in a General Practitioner's office with the supervision of a trained nurse, and takes only 5-7 minutes to complete.[14,37] The Cytosponge™ can also provide histologic data for inflammatory pathologies such as candidal oesophagitis, herpes oesophagitis and eosinophilic oesophagitis.[38] Our initial results using the Cytosponge™ are promising, and future test development should focus on longitudinal sampling of the microbiota to monitor for changes in microbial diversity over time in a larger cohort of patients. Further research should also examine the role of diet, dysphagia and other external influences on the oesophageal microbiota.

Research in context
Evidence before this study
Epidemiologic evidence suggests that the rising incidence of oesophageal adenocarcinoma (OAC) coincides with the obesity epidemic, gastrooesophageal reflux disease and eradication of *Helicobacter pylori* using antibiotics and acid suppression therapy – all risk factors that are capable of altering the gastrooesophageal microbiota. Three studies with small numbers of patients have shown modest alterations in the microbiota in Barrett's oesophagus and oesophagitis using 16S rRNA gene amplicon sequencing. However, studies using culture-independent methods to profile the oesophageal microbiota in OAC or high grade dysplasia are currently lacking. One explanation for the scarcity of oesophageal microbiota studies is the challenge of endoscopic sampling and low microbial DNA yield. Based on prior publications, we hypothesized that the novel Cytosponge™ could be an effective tool to sample the microbiota along the entire length of the oesophagus.

Added value of this study
This study provides a comprehensive characterisation of the microbiota at different stages of the Barrett's progression sequence using 16S rRNA gene amplicon sequencing, and compares OAC and Barrett's cases to normal control patients. We found decreased microbial diversity in OAC tissue compared to controls with enrichment of acid-tolerant bacteria such as *Lactobacillus fermentum.* The microbial diversity was reduced in the lower oesophagus regardless of whether cancerous or normal oesophageal tissue was sampled within the same patients. We further translated our findings to the setting of early detection using the Cytosponge™ to sample the microbiota in Barrett's oesophagus and high grade dysplasia. We showed that the Cytosponge™ collected high microbial DNA yield and detected decreased diversity in the pre-invasive stage of high grade dysplasia.

Implications of all the available evidence
Alterations in microbial communities occur in the lower oesophagus in Barrett's carcinogenesis, which are possible to detect using the minimally invasive Cytosponge™. Our findings are potentially applicable to early disease detection, and future test development should focus on longitudinal sampling of the microbiota to monitor for changes in microbial diversity in a larger cohort of patients.

Environmental Science and Analysis Service (RESAS). The authors declare no conflicts of interest.

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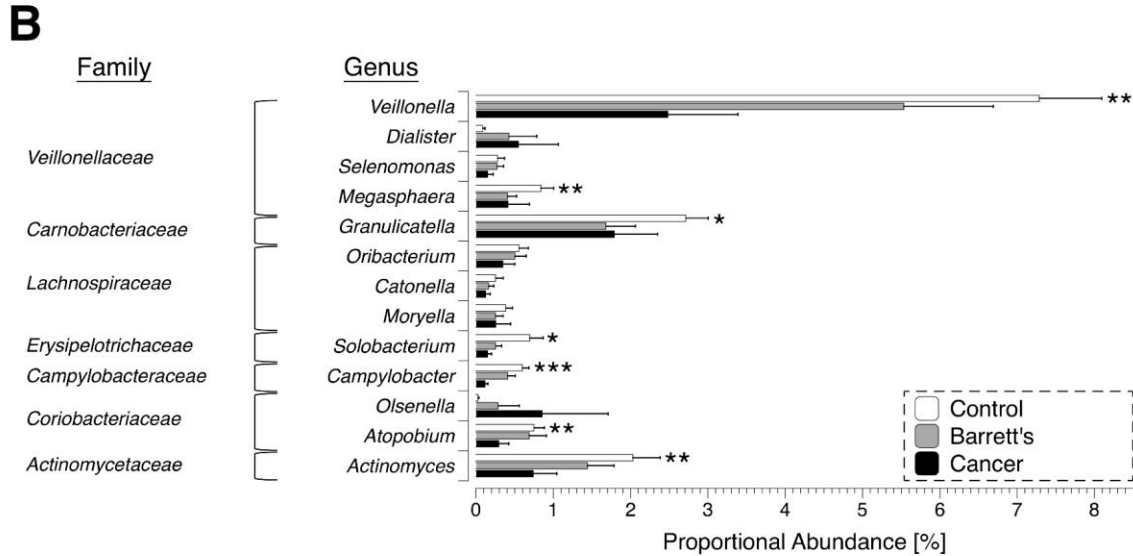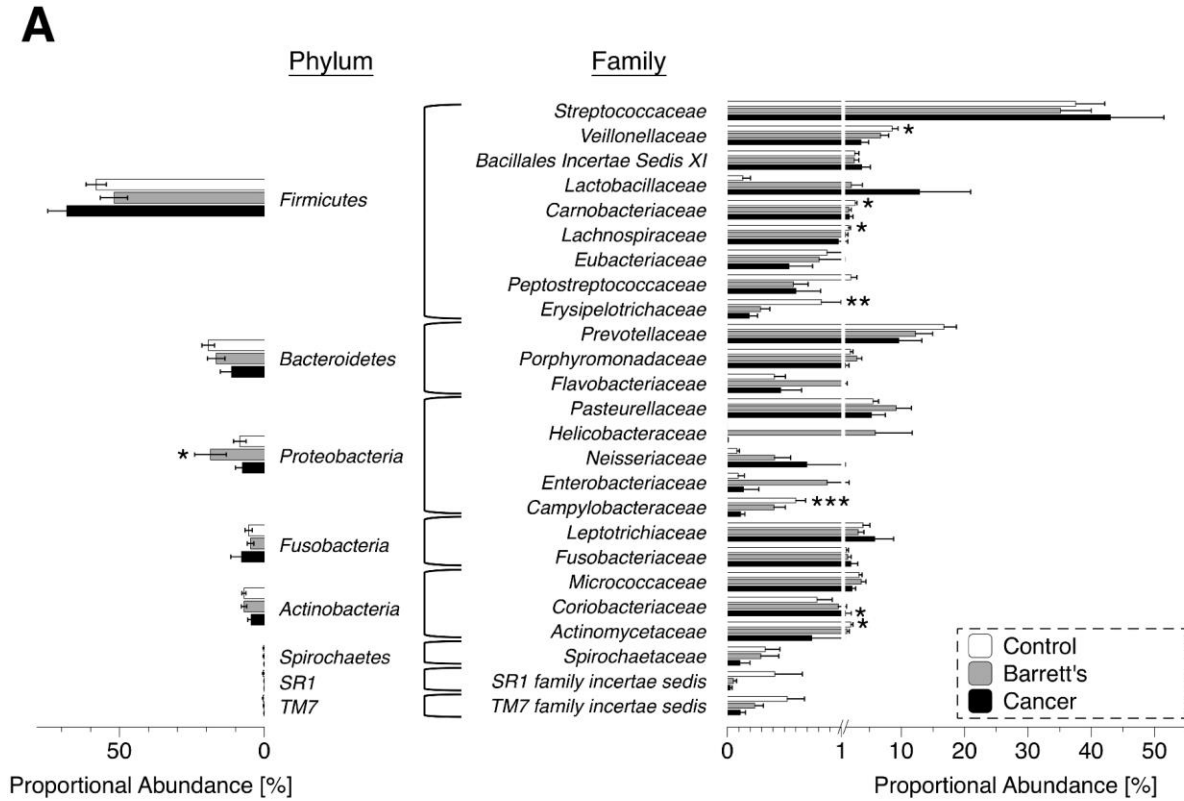
Figure 1. Proportional abundance of microbial taxa differs in Barrett's carcinogenesis. (A) Mean proportional abundance of the 8 most prevalent phyla and 25 most prevalent families in tissue samples for normal control patients (n=16), Barrett's (n=17) and OAC (n=15), significant differences calculated using LEfSe (* p<0·05, ** p<0·01, *** p<0·001). Error bars are standard error of the mean. (B) Mean proportional abundance of representative genera from significantly enriched families identified in A. Only genera with overall proportional abundances greater than 0·1% are included.
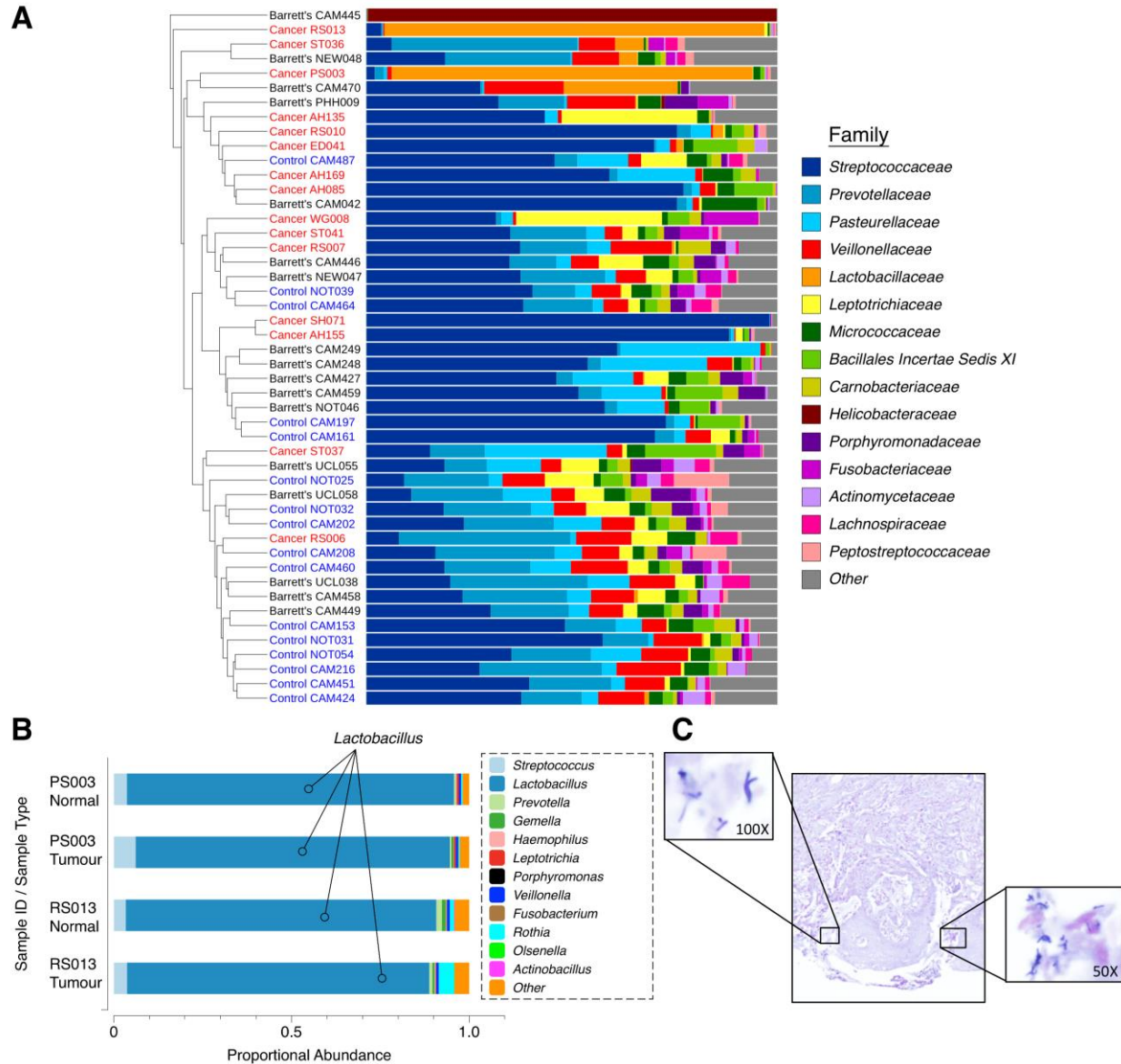
Figure 2. Microbial community composition is altered in OAC. (A) The OAC and control patient groups largely cluster away from each other in this Bray-Curtis cluster dendrogram (p=0·002, parsimony test), but there is no significant difference in clustering for Barrett's oesophagus. Microbial composition is illustrated at the family level for each tissue sample. Data were sub-sampled at 631 reads per sample. (B) *Lactobacillus* is the dominant taxon in two tumour samples (PS003 and RS013) and matched normal squamous tissue. (C) Tumour sample PS003 stained with haematoxylin and eosin, scanned at 20X magnification, imaged at 5% digital zoom. Inserts show Gram stain with Gram-positive rods, imaged at 50% or 100% digital zoom.
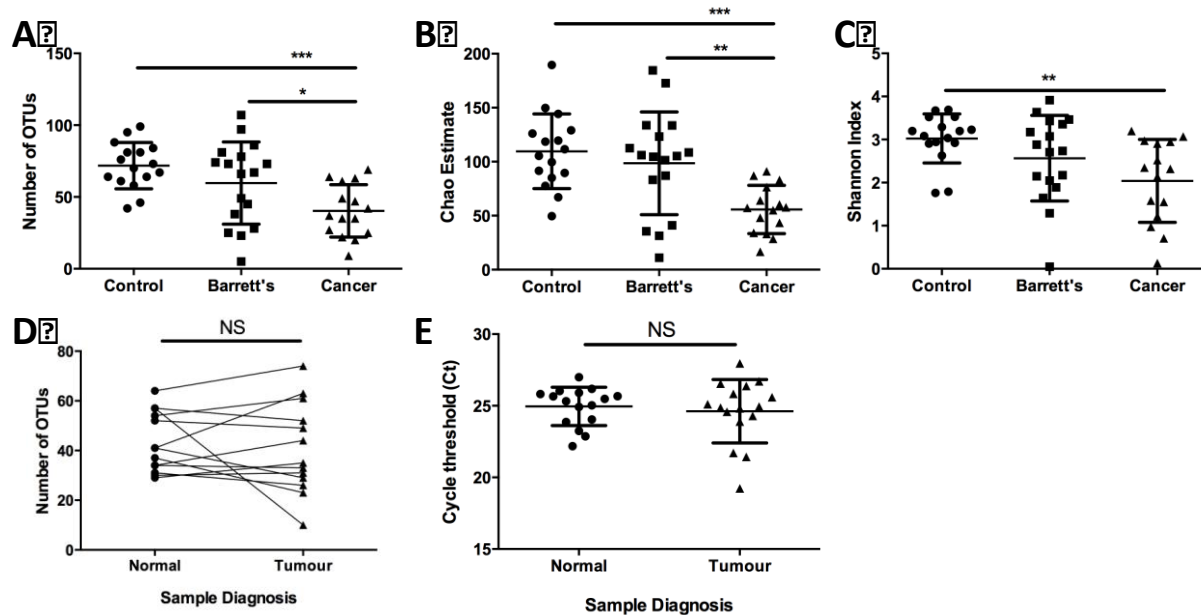
Figure 3. Alpha diversity is decreased throughout the lower oesophagus in OAC. (A) Observed richness of bacterial operational taxonomic units (OTUs), (B) the Chao estimate of total OTU richness, and (C) the Shannon diversity index are shown for tissue samples from normal controls (n=16), Barrett's (n=17) and OAC (n=15) patients. Statistical significance calculated using Kruskal-Wallis test and Dunns multiple comparisons post-test (* p<0·05, ** p<0·01, *** p<0·001). Data were sub-sampled at 631 reads per sample. (D) Observed richness of bacterial OTUs for paired normal and tumour tissue samples 13 patients (26 samples), Wilcoxon signed rank test. Data were sub-sampled at 656 reads per sample. (E) Overall bacterial abundance using 16S rRNA gene qPCR in matched tumour and normal squamous tissue from 16 patients (32 samples), Wilcoxon signed rank test. Error bars represent standard deviation.
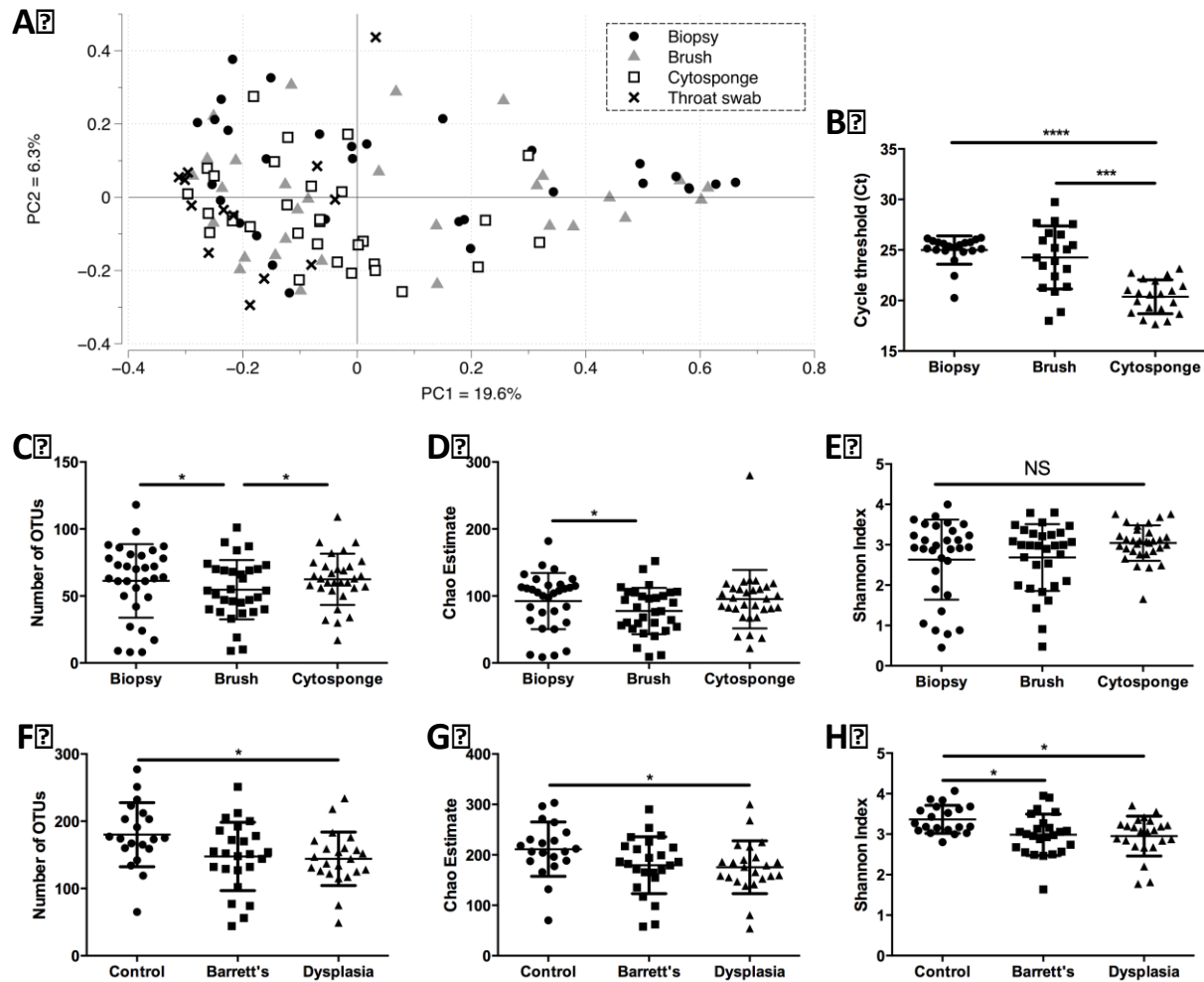
Figure 4. The Cytosponge™ has high microbial DNA yield and detects decreased alpha diversity in high grade dysplasia (A) Principal coordinate analysis using the Bray-Curtis algorithm for matched endoscopic biopsies, brushes and Cytosponge™ samples (n=31 patients) and 13 throat swabs from a subset of these patients. The first axis (PC1) accounts for 19·6% of the sample variance and the second axis (PC2) accounts for 6·3% of the variance. Data were sub-sampled at 631 reads per sample. (B) Overall bacterial abundance using 16S rRNA gene-based qPCR in matched endoscopic biopsies, brushes and Cytosponge™ samples (n=20 patients), Friedman test and Dunns multiple comparisons post-test (* $p<0·05$, ** $p<0·01$, *** $p<0·001$, **** $p<0.0001$). (C) The observed diversity of bacterial OTUs, (D) the Chao estimate of total OTU richness, and (E) the Shannon diversity index for matched endoscopic biopsies, brushes and Cytosponge™ samples (n=31 patients), Friedman test and Dunns multiple comparisons post-test. Data were sub-sampled at 631 reads per sample. (F) Observed richness of bacterial operational taxonomic units (OTUs), (G) the Chao estimate of total OTU richness, and (H) the Shannon diversity index for Cytosponge™ samples taken from normal squamous controls (n=20), Barrett's (n=24) and high grade dysplasia (n=23) patients. Statistical significance calculated using Kruskal-Wallis test and Dunns multiple comparisons post-test. Data were sub-sampled at 19303 reads per sample. Error bars represent standard deviation.