Minimally-invasive Detection of Barrett's Esophagus and Barrett's Esophagus Related Dysplasia/Carcinoma by a Sponge on String Device

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PI : Prasad G. Iyer MD MSc,

Co-Investigators: David Ahlquist MD, David A Katzka MD, Herbert Wolfsen, MD, Cadman Leggett, MD, Eduardo Antpack, MD, Francisco Ramirez, MD, Allon Kahn, MD, John Kisiel, MD, Arvind Trindade, MD, Vani Konda, MD, Grace Dosanjh, MD

Michele Johnson, Ramona Lansing, Rachel Rhody, Erin Gibbons, Frances Cayer, Karen Doering, Seth Slettedahl, Douglas Mahoney, Kelli Burger, Mary Devens, William Taylor, Pooja Bhakta, Molly Stewart, Daniel Kim, Kristen Garcia, Amanda O'connor

Background

Barrett's esophagus (BE) is the strongest risk factor for and only known precursor for esophageal adenocarcinoma (EAC), a lethal malignancy with poor survival (<20% at 5 years) when detected after the onset of symptoms. (1) The incidence of esophageal adenocarcinoma has increased by almost 600% in the last three decades in the population.(2) BE progresses to EAC through a step-wise pathway from no dysplasia, to low grade dysplasia (LGD) to high grade dysplasia (HGD) to carcinoma.

This metaplasia to dysplasia to carcinoma sequence has prompted several national gastroenterology societies to recommend screening for BE in high risk subjects with multiple risk factors followed by endoscopic surveillance (depending on the grade of dysplasia) to detect the development of dysplasia or carcinoma at an early stage.(3-5) Endoscopic treatments of LGD, HGD and early carcinoma have been developed and shown to be effective in reducing the incidence of carcinoma and improving survival in BE subjects.(6-9)

Screening for BE is currently performed using conventional sedated endoscopy (sEGD) which reveals the replacement of the normal squamous lining of the esophagus by metaplastic columnar epithelium in subjects with BE. However sedated endoscopy is expensive with both direct and indirect costs and not suitable for widespread application. It is also associated with potential complications.(10) Other techniques such as unsedated transnasal endoscopy (uTNE) have comparable accuracy to sEGD with lower cost, but continue to be poorly regarded as a widely applicable tool by providers.(11-13) Despite adequate access to the uTNE device the utilization of uTNE by referring physicians remains limited.(14) The absence of accurate risk stratification tools to determine BE risk and target screening efforts are additional limitations to a widely applicable BE screening.(10)

Endoscopic detection of dysplasia is currently performed using four quadrant random biopsies every 1-2 cm of the BE segment in addition to careful inspection of the BE segment with high resolution white light imaging and advanced imaging techniques. While this has been

recommended by GI societies (3-5), the compliance with these recommendations amongst practicing gastroenterologists remains poor.(15) Indeed compliance decreases with increasing BE segment length leading to increasing rates of missed dysplasia. Other challenges with dysplasia detection in BE include the spotty distribution of dysplasia in BE(16) which leads to sampling error, poor inter-observer agreement amongst pathologists while grading dysplasia and the relatively poor sensitivity of current surveillance strategies in detecting prevalent dysplasia or carcinoma.(17) The utility of advanced imaging techniques in the community remains unclear with only a third of practicing gastroenterologists reporting use routinely in BE surveillance.(18)

Recently a sponge on a string device has been studied in BE screening.(19) This device consists of a polyurethane foam sponge compressed in a vegetable material derived capsule, attached to a string. The capsule is swallowed by the patient. The shell of the capsule dissolves in the gastric fluid releasing the foam device as a sphere which is then pulled out with the attached string, providing brushing/cytology samples of the proximal stomach and esophagus. Biomarker studies can then be performed on these samples to detect BE. Two large multicenter studies have been performed in the United Kingdom with such a device using trefoil factor 3 (a protein specific to BE epithelium) detected on immunohistochemistry as a BE marker, demonstrating the feasibility, safety and accuracy of this approach. (19, 20) The sensitivity and specificity of this marker in the detection of BE has been reported to be 73% and 94% for BE segments of > 1 cm in circumferential length. Additionally this capsule sponge device has been used safely in a study conducted at Mayo Clinic Rochester in subjects with eosinophilic esophagitis.(21)

Methylated DNA markers specific to BE epithelium (with and without dysplasia) have been described by us and others.(22) We have identified several methylated DNA markers which are highly specific and sensitive for the detection of BE and BE related dysplasia/carcinoma (see preliminary data section).

An investigational capsule sponge device (SOS: EsophaCap) is currently available in the United States in two sizes (25 mm and 30 mm size) and two porosity configurations (10 pores per inch [ppi] and 20 pores per inch). There is currently no data on the optimal size and pore configuration in terms of DNA yield, patient tolerability and safety on this device.

The aims of our Phase 1 pilot study are:

1. Compare patient acceptance and tolerability with the 25 mm 10 ppi and 25 mm 20 ppi configurations capsule sponge devices.
2. Compare presence and degree of mucosal abrasions following swallowing and withdrawal of capsule sponge, as determined on endoscopy with the 25 mm 10 ppi and 25 mm 20 ppi configurations) capsule sponge devices
3. Compare DNA yield obtained from esophageal cytology specimens obtained with the 25 mm 10 ppi and 25 mm 20 ppi and capsule sponge devices.
4. Explore sensitivity and specificity of DNA methylation markers in detecting BE and BE related dysplasia.

Phase 2 Aims:

Following successful completion of Phase 1, we will proceed with Phase 2 of the study. The aims of this phase will be:

1. Assess the sensitivity and specificity of a panel of DNA methylation markers in the non-endoscopic detection of BE using a capsule sponge device.
2. Assess the sensitivity and specificity of a panel of DNA methylation markers in the non-endoscopic detection of BE related dysplasia/carcinoma using a capsule sponge device.
3. Estimate the degree of missed dysplasia during routine surveillance endoscopy with random endoscopic biopsies, by utilizing follow up intensive endoscopic surveillance and capsule sponge examination.
4. Explore the utility of demographic, anthropometric and circulating biomarker risk factors in predicting the presence of BE and BE related dysplasia.

The inclusion and exclusion criteria, study procedures and biomarker assays will be identical to those described in Phase 1. All subjects will undergo the capsule sponge assessment followed by the clinically indicated endoscopy as described in Phase 1. All studies will be conducted using a single size sponge with the same porosity configuration selected from Phase 1. Study procedures, testing and follow up will be similar to that in Phase 1. Recruitment will be monitored and a future interim analysis may be conducted.

In addition to Mayo Clinic Rochester, subjects will also be recruited at Mayo Clinic Jacksonville; Mayo Clinic Arizona; Mayo Clinic Health Systems – Austin; Mayo Clinic Health Systems – Mankato; Northwell Health in Manhasset, NY; and Baylor University Medical Center in Dallas, TX. Enrollment is closed at University of Colorado, Denver – Anschutz Medical Campus in Aurora, CO.

Dr. Herbert Wolfsen will be site PI at Mayo Clinic Jacksonville. Dr. Francisco Ramirez will be site PI at Mayo Clinic Arizona. Dr. Eduardo Antpack, MD will be the site PI at Mayo Clinic Health Systems – Austin. Dr. Grace Dosanjh will be the site PI at Mayo Clinic Health Systems – Mankato. Dr. Arvind Trindade will be site PI at Northwell Health. Dr. Vani Konda will be the site PI at Baylor University Medical Center.

Preliminary Data:

- Identification and initial feasibility testing of methylated DNA markers for BE diagnosis.

We aimed to prospectively assess the accuracy of methylated BMP3 and NDRG4 to identify BE using endoscopic biopsies (Phase 1) and brushings from the whole esophagus and cardia to simulate non-endoscopic sampling devices (Phase 2).

Methods: Cases with and controls without BE were recruited prior to endoscopy. BE cases had >1cm of circumferential columnar mucosa with confirmed intestinal metaplasia; controls had no BE endoscopically. In Phase 1, biopsies were obtained in cases from BE, gastric cardia ((GC); 1

cm below Z-line) and squamous epithelium ((SE); >2 cm above BE) and in controls from GC (as for BE) and SE (5 cm above Z-line); then promptly frozen. Biopsy samples were processed as a batch, and assayed in blinded fashion. In Phase 2, specimens were obtained using a high capacity endoscopic cytology brush (Hobbs Medical, Stafford Springs CT); the cardia, BE (in cases), and full esophageal length were brushed to simulate a swallowed sponge sampling device. Following DNA extraction and bisulfite treatment, methylation on target genes was assayed by quantitative allele-specific real-time target and signal amplification. *β*-actin was also quantified as a marker for total human DNA.

Results: We prospectively studied 100 subjects. Phase 1: Among 40 BE cases and 40 controls: median age was 65 (quartiles 55-77) and 54 (37-69) and men comprised 78% and 48%, respectively. Median BE length was 6 cm (range 3-10). Median levels of methylated markers were substantially higher (34-600 times) in BE than in adjacent SE and GC or than in normal SE and GC (Table 1). In contrast to methylated markers, *β*-actin distributions were similar across tissue groups. Both marker levels increased with BE length and age, $p<0.001$ whereas only NDRG4 increased significantly with presence of dysplasia (none (19), low grade (9), high grade (11); $p=0.003$). Factors not significantly affecting marker levels included sex and inflammation. Phase 2: Among 10 BE cases and 10 controls, median age was 64 (59-70) and 66 (49, 71) and men comprised 80 and 30% respectively. Median BE length was 2 cm (range 1-4). Discrimination of BE by markers was extraordinary with AUC of 1.0 for NDRG4 and 0.99 for BMP3; levels were >100 times higher in cases than controls (Figure 1).

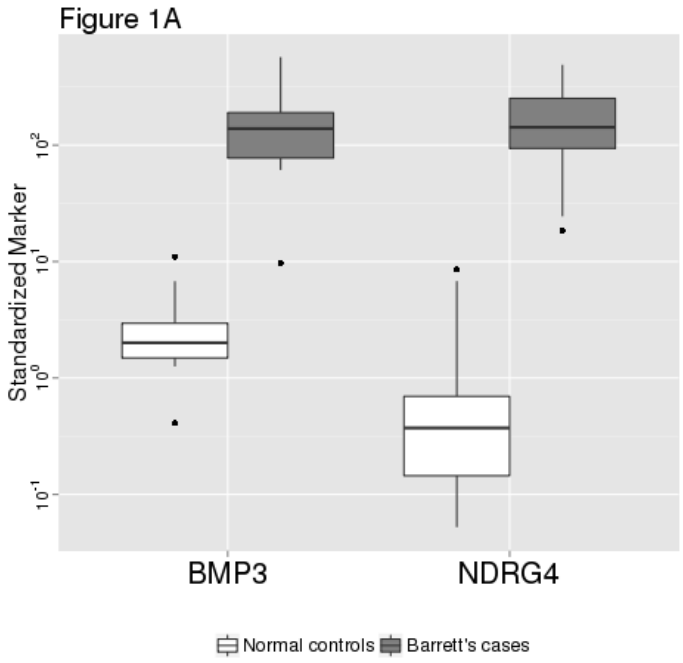
Conclusions: Selected methylated DNA markers highly discriminate BE from normal GC and SE, both in biopsy and brushed specimens, and hold promise for non-endoscopic screening applications.

Table 1: Marker levels (copy numbers of markers adjusted for beta actin) for BMP3 and NDRG4 biopsies from BE cases (cardia, Barrett's, squamous) and controls (cardia, squamous).

	BMP3		NDRG4	
	Normal controls	Barrett's cases	Normal controls	Barrett's cases
Squamous Q1, Q3 P90, P95	0.8 0.3, 2.2 7.0, 23.0	5.6 0.7, 14.8 25.5, 50.3	1.0 0.5, 2.7 5.0, 13.7	4.9 1.5, 10.9 32.0, 64.1
BE Q1, Q3 P90, P95		300.2 137.1, 659.5 1083.1, 1219.0		390.6 146.6, 763.5 921.8, 1006.6
Cardia Q1, Q3 P90, P95	0.5 0.3, 1.9 10.3, 16.4	8.2 2.8, 40.3 190.7, 431.5	2.3 1.0, 6.3 13.1, 15.4	11.5 5.0, 48.3 116.7, 345.0
Composite Q1, Q3 P90, P95	1.3 0.4, 3.8 10.0, 15.3	131.4 67.1, 242.7 402.9, 417.9	2.3 1.1, 5.3 8.1, 12.5	136.5 68.9, 272.3 344.0, 383.3

Pvalue	<0.0001	<0.0001

Figure 1: Marker levels of BMP3 and NDRG4 in brushings (cardia + whole esophagus) in BE cases and controls.



- **Detection of Barrett's Dysplasia by Assay of Methylated DNA Markers on Whole Esophageal Brushings: A Prospective Feasibility Study**

Molecular markers may aid in detection of Barrett's esophagus (BE) and surveillance of BE-related dysplasia by either endoscopic or non-endoscopic methods. Assay of methylated DNA markers accurately detects BE on whole esophageal brushings (Gastroenterology 2014;146:S148), but it is less clear whether later methylation events occur and can be targeted to discriminate emergent dysplasia. We aimed to (1) identify and validate novel methylated DNA markers for BE dysplasia, (2) test the feasibility of candidate markers for detection of BE dysplasia from whole-esophageal brushings.

Methods: *Discovery & Validation.* Using whole methylome bisulfite sequencing on DNA from BE tissues with no dysplasia, low grade dysplasia (LGD), high grade dysplasia (HGD) or adenocarcinoma (EAC) (18 specimens per group), we identified candidate markers to separate BE with from BE without dysplasia. The top 63 candidate markers were validated by methylation-specific PCR assay in independent tissues including BE without dysplasia, BE- LGD, and BE-HGD (30-36 specimens per group). The best 12 validated markers were selected for blinded analyses in the subsequent clinical feasibility study. *Feasibility Testing on Esophagus Brushings*: Consenting BE subjects scheduled for endoscopic BE surveillance or endoscopic assessment of BE related cancers underwent whole esophageal brushings using a high capacity cytology brush

(Hobbs Medical, Stafford Springs, CT) with circumferential sampling from the cardia through the full esophageal length (BE + squamous mucosa) to simulate a swallowed sponge-on-string device. Following DNA extraction and bisulfite treatment, methylation on target genes was assayed by methylation-specific PCR or quantitative allele-specific real-time target and signal amplification. Marker levels were normalized to *β-actin* (marker for total human DNA).

Results: *Discovery & Validation Study*. The 12 aberrantly methylated genes that best discriminated BE with from BE without dysplasia (e.g. areas under ROC curve 0.86-0.97) were selected for feasibility testing, including *DIO3, MAX20.218, CD1D, T-SPYL5, ZNF568, ST8SIA1, ELMO1, ELOVL2, BMP3, NDRG4, HUNK*, and CDKN2A;. *Feasibility Study on Brushings.* 39 subjects were studied with a median age was 69 (28-94) years, 74% were males, and median BE length was 4 (1-14) cm; 18 had no dysplasia and 21 had dysplasia (9 LGD, 7 HGD, and 5 EAC (4 asymptomatic early stage). A 3 marker set (*DIO3, MAX20.218, NDRG4)* at 95% specificity detected 78% of LGD, 71% of HGD, 100% of EAC and 81% of all dysplasia (Figure 2). This study demonstrates that selected methylated DNA markers discriminate BE without from BE with dysplasia.

Conclusions: Combinations of such markers appear feasible for detection of BE dysplasia when assayed from brushed samples of the whole esophagus. Test optimization and further clinical studies are warranted.

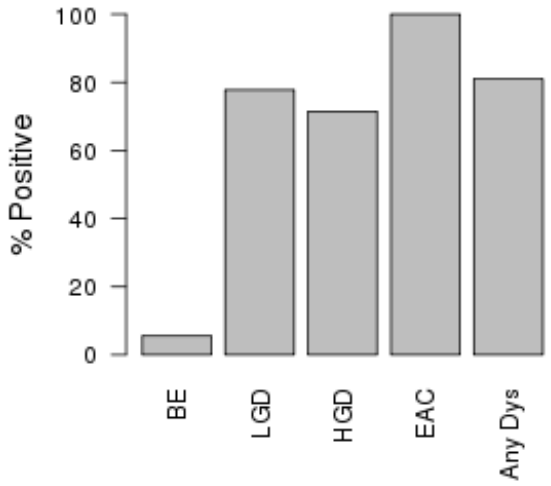


Figure 2 : Positivity rates of a 3-marker panel (*DIO3, MAX20.218, NDRG4)* **in tissue DNA from BE subgroups without dysplasia and with different severities of dysplasia.**

Methods:

Study Design.

We will conduct this study in two phases: Phase 1 and Phase 2. Phase 1 will consist of the currently submitted study wherein the most optimal sponge on string (SOS) design will be chosen based on patient acceptance, tolerability, mucosal irritation and DNA yield, using a randomized factorial design pilot trial. Patients will first undergo the SOS test followed by clinical endoscopy. Following completion of the trial, outcomes (specified above) will be assessed and the most optimal capsule sponge (SOS) configuration will be chosen for a larger Phase 2.

Phase 1 (pilot) and Phase 2:

Participants: Patients with and without known BE will be recruited from the Esophageal, Barrett's esophagus and general gastroenterology clinics in Mayo Clinic, Rochester (Phase I and 2), Mayo Clinic Health Systems – Austin, Mayo Clinic Health Systems – Mankato, Mayo Clinic Florida, Mayo Clinic Arizona, Northwell Health, and Baylor University Medical Center,. (Phase 2 only). Patients with known BE will be recruited to enrich the sample with BE subjects and those with BE related dysplasia/carcinoma. Potential cases declining to participate in the capsule sponge procedure or those not eligible due to exclusion criteria b.3. below will have the option to participate in the esophageal brushings portion of the study.

Inclusion criteria:

a. Subjects with known BE.
    1. Patient between the ages 18 – 90.
    2. Patients with a BE segment ≥ 1cm in maximal extent endoscopically.
    3. Histology showing evidence of intestinal metaplasia with or without presence of dysplasia.
    4. Undergoing clinically indicated endoscopy.
b. Subjects without known evidence of BE
    1. Undergoing clinically indicated diagnostic endoscopy.

Exclusion criteria:

a. Subjects with known BE.

    1. Patients with prior history of ablation (photodynamic therapy, radiofrequency ablation, cryotherapy, argon plasma coagulation). *Patients with history of endoscopic mucosal resection alone will not be excluded.*
    2. Patients with history of esophageal resection for esophageal carcinoma.

b. Subjects with or without known evidence of BE (on history or review of medical records).

    1. Pregnant or lactating females.
    2. Patients who are unable to consent.
    3. Patients with current history of uninvestigated dysphagia (this does not apply to the brushings only portion of the study).
    4. History of eosinophilic esophagitis, achalasia.
    5. Patients on oral anticoagulation including Coumadin, Warfarin.

6. Patients on antiplatelet agents including Clopidogrel, unless discontinued for at least three days prior to the sponge procedure.
7. Patients on oral thrombin inhibitors including Dabigatran and oral factor X a inhibitors such as rivaroxaban, apixaban and edoxaban, unless discontinued for at least three days prior to the sponge procedure.
8. Patients with history of known varices or cirrhosis.
9. Patients with history of esophageal resection for esophageal carcinoma.
10. Patients with congenital or acquired bleeding diatheses.
11. Patients with a history of esophageal squamous dysplasia.
12. Patient has known carcinoma of the foregut (pancreatic, bile duct, ampullary, stomach, or duodenum) within 5 years prior to study enrollment.

Study procedures:

Participants will report fasting for ≥ 6 hours for the study.

A. Pre-procedure assessment:
   a. Baseline height, weight, hip, and waist circumference will be measured by the research coordinator.
   b. A validated questionnaire assessing reflux symptoms (RSQ) will be completed by the subject.
   c. At the Mayo Clinic sites only, 10 ml of blood will be obtained via venipuncture for measurement of circulating biomarkers (cytokines, adipokines and methylated DNA markers) of BE risk. Blood will be processed and separated into serum, plasma, and buffy coat for subsequent analysis for BE biomarkers.

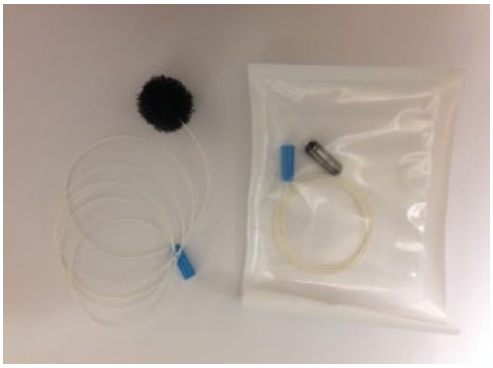


Figure 1 : 25 mm sponge, intact and expanded

   d. Pregnancy test will be obtained for women of child bearing potential.

B. Capsule sponge assessment:

For phase 1 subjects will be randomized using concealed allocation to one of two capsule sponges: 25 mm 10 ppi, 25 mm 20 ppi. All phase 2 subjects will receive the same single size and porosity sponge (porosity to be determined after analysis of Phase 1).

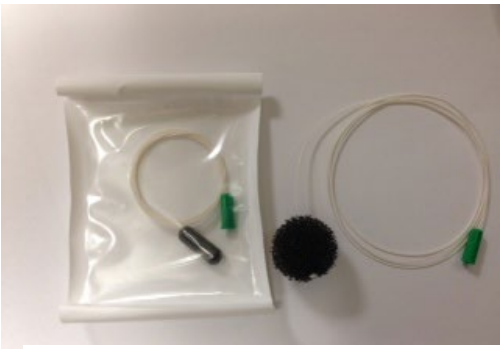


Figure 2 : 30 mm sponge, intact and expanded

   a. Participants will be seated upright and asked to swallow the capsule sponge to which they have been randomized to, with sips of water. The pharynx will then be anesthetized with Benzocaine 20% aerosol

spray (Topex). After an interval of 5-10 min, the capsule sponge will be pulled out using steady traction using the string attached to the capsule sponge. As detailed in another Mayo IRB approved protocol (IRB 11-006429). The sponge will then be placed in a preservative solution and transferred to the laboratory for storage and subsequent analysis.

b. Following completion of the capsule sponge study participants will fill out a tolerability questionnaire which allows patients to grade of discomfort during the procedure on a Likert scale of 0-10. 10 representing the "worst experience" and 0 the "best experience." This scale allows a comprehensive and individual assessment of the degree of pain, choking, gagging, and anxiety experienced during the procedure. Participants will also be asked if they would have the procedure again (yes/no).

C. Endoscopic assessment:

a. All participants will undergo a diagnostic clinically indicated sedated endoscopy within 24 hours of the Capsule sponge test. This will be performed in one of the clinical endoscopy units at Mayo Clinic Rochester, Mayo Clinic Florida, Mayo Clinic Arizona, Mayo Clinic Health Systems – Austin, Mayo Clinic Health Systems - Mankato, Northwell Health, and Baylor University Medical Center.

b. Patients will receive sedation (using a combination of intravenous midazolam and Demerol) or monitored anesthesia care as clinically appropriate and undergo clinically indicated endoscopy with standard endoscopic equipment.

i. The following landmarks will be identified, recorded (in centimeters from the incisors) and photographed: squamo-columnar junction, gastroesophageal junction (GEJ) (defined as top of the gastric folds with the esophagus partially deflated) and the diaphragmatic hiatus. Endoscopically suspected BE will be defined as the presence of ≥ 1cm of columnar mucosa above the GEJ. Targeted clinical biopsies will be obtained to confirm a diagnosis of BE. Presence of intestinal metaplasia with goblet cells will be a pre-requisite for the diagnosis of BE (histologically confirmed BE).

ii. The presence or absence of esophagitis will be recorded. If present, esophagitis will be graded as per the LA classification.

iii. The presence of any trauma from the passage of the sponge will be assessed and recorded photographically. This will be defined as:
   1. No evidence of trauma
   2. Superficial mucosal abrasion without bleeding
   3. Superficial mucosal tear abrasion with minimal oozing similar to that from biopsy.
   4. Deep mucosal abrasion without bleeding.
   5. Deep mucosal abrasion with greater than minimal oozing.
   6. Bleeding requiring endoscopic therapy.

iv. In those with known BE, clinical surveillance biopsies (with or without endoscopic mucosal resection if clinically indicated for visible lesions) will be obtained as per current clinical recommendations: four quadrant biopsies every 1-2 cm. All clinical histology will be read by GI pathologists as per standard practice at Mayo Clinic Rochester, Mayo Clinic Florida, Mayo Clinic Arizona, or Mayo Clinic Health Systems. Slides or scanned slides from Northwell Health and Baylor University Medical Center

will be sent to Mayo Clinic Rochester GI study Pathologist to confirm Barrett's Esophagus and dysplasia grade.

v. Endoscopic brushings will be obtained from the Barrett's mucosa in BE cases, and from the squamous mucosa in control subjects. Brushings will be obtained using a high capacity cytology brush (Hobbs Medical) to enable detection of methylation biomarkers of dysplasia. 1 brush will be used for every 5 cm of Barrett's epithelium in BE case subjects. In control subjects, 1 brush will be used to sample the cardia and the distal 5cm of the esophageal squamous mucosa. These brushings will be stored at -80^{o}C for identification of additional biomarkers.

vi. Two research biopsies will be obtained from the gastroesophageal junction in controls for identification of methylated DNA biomarkers from the GEJ. One will be frozen and stored at -80^{o}C and one will be formalin fixed, paraffin embedded.

vii. The diagnostic part of the procedure will be video recorded and stored in a de-identified fashion for subsequent review.

viii. Patients who are biomarker positive (for dysplasia related DNA methylation markers) but negative for dysplasia, as well as those biomarker negative but positive for dysplasia on clinical surveillance histology will be offered a research endoscopy (funded by the study) after biomarker analysis is complete, for additional endoscopic surveillance to detect prevalent dysplasia which may have been missed at the initial study endoscopy as well as assess for sensitivity of the marker panel. In addition to surveillance biopsies (paid for by the study), sponge procedure will be repeated and research esophageal brushings using a high capacity endoscopic brush (Hobbs Medical) will be obtained for detection of methylated biomarkers of dysplasia.

ix. An equal number of biomarker negative (for dysplasia related DNA methylation markers) who are negative for dysplasia on clinical surveillance histology will also be offered a research endoscopy (funded by the study) In addition to surveillance biopsies (paid for by the study), sponge procedure will be repeated, and research esophageal brushings using a high capacity endoscopic brush (Hobbs Medical) will be obtained for detection of methylated DNA biomarkers of dysplasia.

x. If the visit 2 research procedure coincides with a participants clinically indicated upper EGD and procedures that are ordered cannot be done on the Clinical Research Unit, (i.e. radiofrequency ablation, endoscopic mucosal resection, esophageal dilation, endoscopic ultrasound) the research portion of the procedure will be done during the clinically indicated upper endoscopy. These research procedures will include the sponge on a string procedure, esophageal brushings, and surveillance biopsies (all paid for by research) as stated above.

D. Post procedure assessment.

   a. Patients will be called by the research coordinator at 7 days following the study (capsule sponge and endoscopy) to assess for any complications or adverse effects including bleeding, pain, dysphagia, need for seeking medical care and loss of work.

Assessment of Safety:

The Principal Investigator will monitor all adverse events and serious adverse events and they will be reported in IRB continuing reviews or immediately as appropriate. All complications and the endoscopy complication score will be reviewed every month by the PIs and continuation of the study will be assessed on this basis.

Biomarker assays:

a. Methylated DNA marker assessment:

Following histologic confirmation, tissue DNA will be extracted in usual fashion and bisulfite-treated. Methylation on target genes shown in our previous study to have >150-fold higher tissue levels in Barrett's Esophagus compared to esophageal squamous mucosa (*BMP3, NDGR4, TFPI2, and HPP1*) will then be quantified in blinded fashion by methylation-specific PCR. Novel markers for BE-related dysplasia and cancer identified by whole methylome sequencing will be quantified in blinded fashion on DNA extracted from cytologic brushings using bisulfite treatment and assay methods we have described previously. (23)

b. Levels of circulating biomarkers: Serum levels of Leptin, cytokines : IL6, 8, 10, 12p7,and methylated DNA markers will be measured. Assays will be performed using commercially available enzyme immunoassay kits. Unutilized samples will be stored for subsequent biomarker analysis.

Sample size and Statistical analysis:

Phase1: Detect ideal size and porosity for tolerability and DNA yield

Table 1

Sponge Diameter	25mm		30mm	
Pores Per Inch	10	20	10	20
BE	5	5	5	5
No BE	5	5	5	5

A factorial design will be used to compare porosity of sponge 25 mm 10 pores per inch (PPI) vs. 25 mm 20 PPI, and Barrett's Esophagus vs. normal esophagus. Tolerability will be measured using a tolerability score as well as level of mucosal abrasion (scale 0-6, 6=worst). The assumed standard deviation for the tolerability score of 2.2 was obtained from Sami, et al.. Using a two-sided test of significance of 5% and 80% power, we can detect a minimal shift in tolerability score of at least 2. For mucosal abrasion score, a rough estimate of 1 standard deviation (s.d. ≈ range of data / 4) will be used and assuming a two-sided test of significance of 5% we have 86% power to detect a difference in mean mucosal abrasion score of at least 1. DNA yield will be measured using wild type Beta-actin. Using a two-sided test of significance of 5% we have 82% power to detect a fold change difference of 50%. All above power calculations are based on detection of main effect comparisons with n=20 per group. The ability to detect interactions between main effects will be very limited with the given sample size. Recruitment will continue until 40 individuals have successfully completed the sponge procedure using the

25 mm sponge. We anticipate 25% will decline swallowing sponges after they have consented, therefore the total number of people we anticipate recruiting will be set at 60.

Phase 2: For the detection of Barrett's Esophagus, sample size justification will be based on minimizing the width of the 95% confidence interval of the sensitivity for detecting Barrett's Esophagus with a fixed specificity of 90%. From table 2 below, based on various assumed sensitivities, the largest confidence interval width for the proposed sample size is less than ±9%. Similarly for detection of dysplasia among Barrett's Esophagus patients, the largest width for the proposed sample size is less than ±12. These confidence widths are reasonable for this initial feasibility study. We also intend to perform multivariable modeling using machine learning techniques to evaluate a minimum of 14 methylation markers. Using the rule of thumb for machine learning of 10-15 events per marker, a minimum of 140 to 210 cases and an equal number of controls is required (total 280 to 420 patients) to account for assay failures and indeterminate histological findings on endoscopic exam. We anticipate enrolling up to 1000 participants.

In addition, clinically archived FFPE tissue from up to 10 cases will be used to assess methylated DNA marker levels in biopsies versus the sponge.

Due to the known dysplasia miss rate with routine surveillance endoscopy, we hypothesize that there will be undetected or missed dysplasia in BE patients with a negative surveillance histology. This imperfection in the endoscopic "gold" standard will bias the resulting accuracy estimates of methylation markers in a multitude of ways. There are several approaches in the literature to address this issue; all with their own caveats. The three approaches that will be investigated are latent class analysis, Bayesian estimation, and discrepant resolution. We have hence inflated the sample size estimates in table 2 to correspond with a 30% prevalence of undetected dysplasia in those with no dysplasia on conventional surveillance endoscopy to ensure that we have 50 true BE subjects without dysplasia.

Table 2

	BE vs. No BE				BE no dysplasia vs. BE w/dysplasia			
Confidence Level	0.95	0.95	0.95	0.95	0.95	0.95	0.95	0.95
Sensitivity	0.75	0.80	0.85	0.90	0.75	0.80	0.85	0.90
Confidence bands	0.085	0.078	0.070	0.059	0.105	0.097	0.087	0.073
N no BE/BE without dys/BE with dys	100	100	100	100	65	65	65	65

Statistical analysis

Phase 1: Tolerability score and level of mucosal abrasion will be summarized as median and interquartile range. Analysis of variance will be used to detect statistically significant differences in tolerability score and level of mucosal abrasion between different levels of sponge porosity and size. Failure/complication rate will be summarized as the percentage of the group total. Comparisons between sponge porosity and size for failure/complication rate will be analyzed using the Chi-squared test.

Phase 1 and 2: Marker specificity will be defined as the 90th percentile value in subjects without BE. Several markers will be combined based on the coefficients from a logistic regression model using subjects with BE vs subjects without BE as the response. This approach is used as they represent the broadest spectrum of disease and any marker combination that fails to discriminate between these two subgroups would not be of future value. The specificity of the combined markers will then be set equal to the 90th percentile in non-BE subjects. The corresponding sensitivity of individual markers as well as combined markers will be estimated with 95% confidence intervals.

Marker specificity will be defined as the 90th percentile value in subjects with BE and no dysplasia. Several markers will be combined based on the coefficients from a logistic regression model using BE subjects with dysplasia vs subjects without dysplasia as the response. This approach is used as they represent the broadest spectrum of disease and any marker combination that fails to discriminate between these two subgroups would not be of future value. The specificity of the combined markers will then be set equal to the 90th percentile in BE subjects without dysplasia. The corresponding sensitivity of individual markers as well as combined markers will be estimated with 95% confidence intervals.

The association of demographic (age, gender, race), anthropometric (body mass index, waist hip ratio) and circulating biomarker levels (leptin, interleukins) with a BE diagnosis will be assessed using univariate and multivariable logistic regression analysis.

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