Accurate Nonendoscopic Detection of Barrett's Esophagus by Methylated DNA Markers: A Multisite Case Control Study

Prasad G. Iyer, MD, MSc[1], William R. Taylor, BS[1], Michele L. Johnson, PTA, CCRP[1], Ramona L. Lansing, RN[1], Kristyn A. Maixner, APRN, CNP[1], Lois L. Hemminger, APRN[2], Frances K. Cayer, CCRP[2], Tracy C. Yab, MBA[1], Mary E. Devens, CCRP[1], Seth W. Slettedahl, MS[3], Brendan T. Broderick, BS[3], Douglas W. Mahoney, MS[3], Maria C. McGlinch, BS[1], Calise K. Berger, BS[1], Patrick H. Foote, BS[1], Maria Giakomopoulos, PhD[4], Hatim Allawi, PhD[4], Thomas C. Smyrk, MD[5], Kenneth K. Wang, MD[1], David A. Katzka, MD[1], Herbert C. Wolfsen, MD[2], James A. Burke, MD[6], David A. Ahlquist, MD[1] and John B. Kisiel, MD[1]

INTRODUCTION: **Nonendoscopic Barrett's esophagus (BE) screening may help improve esophageal adenocarcinoma outcomes. We previously demonstrated promising accuracy of methylated DNA markers (MDMs) for the nonendoscopic diagnosis of BE using samples obtained from a capsule sponge-on-string (SOS) device. We aimed to assess the accuracy of these MDMs in an independent cohort using a commercial grade assay.**

METHODS: **BE cases had ≥ 1 cm of circumferential BE with intestinal metaplasia; controls had no endoscopic evidence of BE. The SOS device was withdrawn 8 minutes after swallowing, followed by endoscopy (the criterion standard). Highest performing MDMs from a previous study were blindly assessed on extracted bisulfite-converted DNA by target enrichment long-probe quantitative amplified signal (TELQAS) assays. Optimal MDM combinations were selected and analyzed using random forest modeling with *in silico* cross-validation.**

RESULTS: **Of 295 patients consented, 268 (91%) swallowed the SOS device; 112 cases and 89 controls met the pre-established inclusion criteria. The median BE length was 6 cm (interquartile range 4–9), and 50% had no dysplasia. The cross-validated sensitivity and specificity of a 5 MDM random forest model were 92% (95% confidence interval 85%–96%) and 94% (95% confidence interval 87%–98%), respectively. Model performance was not affected by age, gender, or smoking history but was influenced by the BE segment length. SOS administration was well tolerated (median [interquartile range] tolerability 2 [0, 4] on 10 scale grading), and 95% preferred SOS over endoscopy.**

DISCUSSION: **Using a minimally invasive molecular approach, MDMs assayed from SOS samples show promise as a safe and accurate nonendoscopic test for BE prediction.**

SUPPLEMENTARY MATERIAL accompanies this paper at http://links.lww.com/AJG/B545, links.lww.com/AJG/B546, links.lww.com/AJG/B547, http://links.lww.com/AJG/B548, http://links.lww.com/AJG/B549

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INTRODUCTION

Esophageal adenocarcinoma (EAC) is a lethal cancer with increasing incidence in the West over the past 3 decades. Most EAC presents symptomatically at late stage with poor survival rates (1). Presymptomatic early-stage EAC, by contrast, has substantially improved outcomes (2). Barrett's esophagus (BE) is the only known precursor and strongest risk factor for EAC (3). Dysplasia detected during endoscopic surveillance of BE can be effectively ablated by endoscopy which reduces risk of subsequent EAC (4,5). This has led to recommendations for BE screening in those with multiple risk factors, followed by endoscopic surveillance for detecting prevalent and incident dysplasia or EAC (6–8).

The most commonly used method for BE screening is sedated endoscopy. Despite recommendations for screening, the uptake of endoscopy in gastroesophageal reflux disease patients remains low (10%–20%) (9). Although unsedated transnasal endoscopy is

[1]Division of Gastroenterology and Hepatology, Mayo Clinic, Rochester Minnesota, USA; [2]Division of Gastroenterology and Hepatology, Jacksonville, Florida, USA; [3]Division of Biomedical Statistics and Informatics, Mayo Clinic, Rochester Minnesota, USA; [4]Exact Sciences, Madison, Wisconsin, USA; [5]Division of Anatomic Pathology, Mayo Clinic, Rochester Minnesota, USA; [6]Division of Family Medicine, Mayo Clinic Health System, Austin, Minnesota, USA. **Correspondence:** Prasad G. Iyer, MD, MSc. E-mail: iyer.prasad@mayo.edu.

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recommended as an option for screening, given favorable comparative effectiveness (10), tolerability, accuracy (11), and cost data (12), it must be performed by an endoscopist, and uptake remains low (13).

Nonendoscopic methods to detect BE are being developed using esophageal cell capture devices in combination with biomarkers. Investigators have targeted a protein marker (trefoil factor 3) alone (14), or in combination with other markers such as microRNAs (15). Others (16,17) have shown promising early results using methylated DNA markers (MDMs) on samples obtained via a capsule sponge or inflatable balloon devices in smaller studies.

In a previous report (18), we identified MDM candidates for BE detection by unbiased next-generation DNA sequencing discovery (using reduced representation bisulfite sequencing), biologically validated these MDMs on whole esophageal brushings, and pilot tested the most promising MDMs for the nonendoscopic diagnosis of BE using DNA extracted from cytology samples taken from a 25-mm sponge-on-string (SOS) device (EsophaCap, Capnostics, Doylestown, PA). In that study, we compared 2 configurations (10 and 20 pores per inch [ppi]) of the 25-mm SOS device, concluding that the 10 ppi configuration was better tolerated with equivalent DNA yield. In that small proof-of-concept study (including 19 BE cases: 10 of whom had dysplasia, and 20 controls), a 2 MDM model (*VAV3* and *ZNF682*) assayed by quantitative methylation-specific PCR demonstrated excellent sensitivity and specificity for nonendoscopic BE detection.

To build on these findings, further study was needed in an independent cohort with a larger number of nondysplastic BE cases, which are more common in the population. In addition, the small sample size in the pilot study did not allow for robust selection of additional potentially informative markers or cross-validation of the results.

In this current study, we aimed to first assess the accuracy of promising MDMs for nonendoscopic BE detection, in a large independent multicenter cohort, using the 25-mm 10 ppi SOS device and a recently validated commercial grade assay: target enrichment long-probe quantitative amplified signal (TELQAS) (19). Second, we sought to establish a BE prediction algorithm with a reduced MDM panel using robust cross-validation methods. Finally, we intended to confirm the safety and tolerability of the SOS device.

METHODS

This study was approved by the Mayo Clinic Institutional Review Board and registered on clinical trials.gov (NCT 02560623). All authors had access to the study data after unblinding and reviewed and approved the final study report.

Patient identification and recruitment

Patients were recruited at the Mayo Clinic, Rochester, Minnesota, Mayo Clinic, Jacksonville, Florida and Mayo Clinic Health System, Austin, Minnesota (a rural community medical center). BE cases included those undergoing endoscopic surveillance or therapy of BE-related neoplasia. Using criteria more stringent than previous trials (14), BE was defined as the presence of at least 1 cm of circumferential esophageal columnar mucosa with intestinal metaplasia (IM) without dysplasia on histology. This was performed to reduce misclassification bias, given the relatively early stage of marker selection in this case control study. In the presence of dysplasia, BE was defined as the presence of at least 1 cm of columnar mucosa with IM, given their higher risk of progression. IM was defined as the presence of goblet cells on hematoxylin and eosin (H&E) stains.

All histology was reviewed by expert gastrointestinal pathologists. Patients with a history of BE ablation were excluded for several reasons. First, previous data show that ablation may alter the tissue DNA methylation profile in comparison with that of pretreatment BE (16). Second, in this study, MDMs were selected for BE prediction in a high-risk group, and not postablation surveillance. Previous endoscopic resection of focal dysplasia was not a contraindication because residual BE tissue left *in situ* was not expected to have undergone architectural or chemical alteration.

Controls were defined as subjects without endoscopic evidence of BE who were undergoing clinically indicated endoscopy. Those with a history of gastric neoplasia or surgery for esophageal or gastric neoplasia were excluded to avoid confounding from potential residual field cancerization, known to alter methylation (20).Given the potential for mechanical injury related to sponge withdrawal, those with uninvestigated dysphagia, eosinophilic esophagitis, and untreated achalasia were excluded, as were patients on active anticoagulation and patients with cirrhosis (with potential for varices and coagulopathy).

SOS administration

Patients swallowed the encapsulated sponge with a few sips of water and were given the option of topical pharyngeal anesthesia with lidocaine spray before or after swallowing the capsule. The expanded sponge was pulled with the attached cord 8 minutes after swallowing the capsule; this time period allowed for dissolution of the gelatin/vegetable capsule shell and expansion of the polyurethane foam sphere (25 mm diameter, 10 ppi).

Patients completed a tolerability assessment (rating pain, choking, gagging and anxiety separately, and overall tolerability on a 1–10 visual Likert scale) immediately after sponge retrieval (see Supplementary Material, Supplementary Digital Content 4, http://links.lww.com/AJG/B548). All patients then underwent endoscopy (the criterion standard) within 24 hours.

Endoscopic assessment post-SOS administration

Endoscopy was performed under standard conscious sedation or monitored anesthesia care (per clinical indications) by consultant gastroenterologists by using standard endoscopes (Olympus, Center Valley, PA). Esophageal landmarks were confirmed, and a determination of the presence or absence of BE and/or esophagitis (Los Angeles classification) was made, with confirmatory and surveillance (in BE cases) biopsies taken for histological evaluation. Multiple photographs documenting landmarks and pathology were obtained during endoscopy, and all endoscopic procedures were video recorded. Videos were reviewed by a single expert investigator (P.G.I.) to make the final study classifications of case or control status. A mucosal injury score (ranging from 1, no trauma, to 6, perforation) was scored from video recordings. In those with no endoscopic evidence of BE, 2 research biopsies were taken from the top of the gastric folds. H&E stained slides of these biopsies were reviewed by an expert gastrointestinal pathologist (T.T.W.) for the presence of cardia IM. A research coordinator called all participants 7 days later to assess for any complications or adverse events. Patients were also asked whether they preferred the SOS procedure or sedated endoscopy at this call.

Participants recruited to the study were classified into the following 3 groups: "cases" met the *a priori* established case definition, "controls" met the *a priori* control definition, and "indeterminates"

who met the *a priori* indeterminate definition. Beause patients in this latter group are likely to be encountered in a population at risk for BE, these were not excluded but were studied and reported separately after the primary analysis was performed on cases and controls.

The indeterminate group included patients with any of the following prespecified justifications: (i) *Erosive esophagitis* obscures the presence of BE in up to 15% of cases (21), and repeat endoscopy is recommended to exclude BE after proton pump inhibitor treatment, (ii) *non circumferential ≤ 1 cm* columnar segments with IM without dysplasia increased the potential for misclassification bias in the diagnosis of BE, (iii) *SOS device failure* was defined as dwell time < 5 minutes or tether detachment (given inadequate mucosal sampling because of the incomplete capsule expansion and reduced DNA yield after prolonged gastric acid exposure in case of tether detachment needing endoscopic removal), (iv) some gastroesophageal junction (*GEJ*) *cancers lacked visible BE* mucosa, (v) *eosinophilic or infectious esophagitis* might have altered methylation, and (vi) patients met endoscopic criteria for BE but *lacked histologic* IM.

SOS processing and assays

After withdrawal, the sponge was placed in a vial containing 20 mL of a cell preservative buffer (PreservCyt; Cytyc Corporation, Marlborough, MA) and agitated to dislodge cells (at speed 10 for 1 minute) once received in the laboratory. This step was repeated with an additional 20 mL aliquot of PreservCyt for a total of 40 mL. The cells were pelleted by centrifugation of the sample (1200 G for 10 minutes), the pellet then lysed in 1 mL of buffer (Puregene Buccal Cell Kit; Qiagen, Germantown, MD), and DNA was extracted following the manufacturer's directions. After bisulfite conversion (Zymo Research, Irvine, CA), the samples were assayed by a commercial grade assay, the recently validated TELQAS assay (19), a novel modification to the FDA-approved quantitative allele-specific target and signal amplification assay (22). For the TELQAS assay, 12 cycles of multiplex PCR preamplified the MDMs and the reference gene in the bisulfite-converted DNA. Using synthetic multiplex DNA controls, multiplex PCR efficiencies for all MDMs and the reference gene were confirmed to be ~100%. PCR products were diluted, and 10 μL of the diluted amplicons were used in the assay for MDM detection. MDM strands numbers were determined by comparing the crossing point of the target genes with the standard curves for each gene. Methylated *B3GALT6* was used as a reference for total DNA. Marker selection was informed by performance in the pilot SOS 1 trial. Four additional BE markers (*ARHGEF4*, *LRRC4*, *ZNF671*, and *ZNF781*) were included from earlier tissue and brushing validation studies (18). Assays were performed by laboratory personnel who were blinded to all clinical data.

Statistical analysis

Sample size justification. The sample size was selected to limit the width of the 95% confidence interval (CI) of the sensitivity and specificity for detecting BE to< ±10%. Assuming a sensitivity of 85% at a specificity of 90%, 100 subjects per group (i.e., subjects with and without BE) provided CI widths no larger than ±7% for sensitivity and ±6% for specificity. Under the same assumptions for the point estimates of sensitivity and specificity, the bounds of ±10% would be maintained for group sizes as low as 60 subjects per group.

Analysis. The distribution of each individual MDM in association with BE case and control status was first displayed using box plots and standardized by the TELQAS product of *β3GALT6* in each sample. The diagnostic accuracy of individual MDMs was summarized as the area under the curve (AUC) with corresponding 95% CIs.

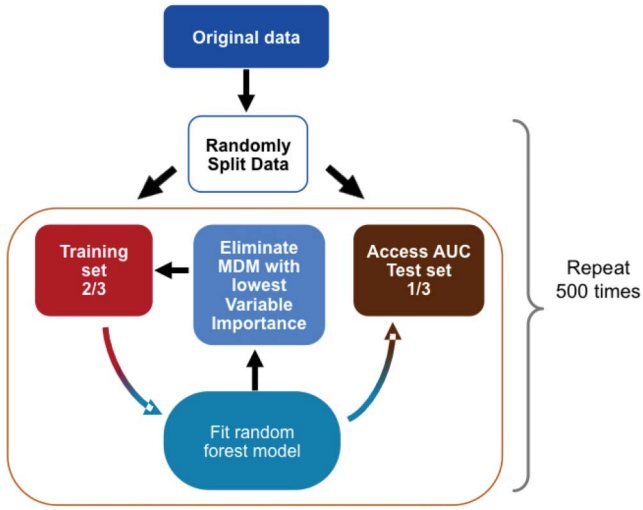

Figure 1. Schematic representation of a cross-validation analysis for the development of the reduced 5 marker MDM panel. The original data set was randomly sampled into training and test sets in 2:1 proportions. Random forest models with the default of 500 trees were fit to the training set data and applied to the test set data. The entire process was repeated 500 times. MDM, methylated DNA marker.

Random forest (rForest) regression (23) was used to model the relationship between the panel of MDMs and BE status because it has been shown to provide superior generalizability and predictive accuracy in test data sets compared with logistic regression with minimal concerns of overfitting (24,25). To reduce the number of MDMs, a backward elimination process was used to create a reduced rForest MDM model and is depicted in Figure 1. The original data set was randomly sampled into training and test sets in 2:1 proportions. rForest models with the default of 500 trees were fit to the training set data and applied to the test set data. The entire process was repeated 500 times. The variable importance statistic as measured by the mean decrease in predictive accuracy was calculated for each MDM, and the lowest performing MDM was eliminated sequentially (23). This elimination process was iterated until a panel of 5 MDMs remained or if the AUC in the test set decreased by more than 10% from the previous iteration, similar to previously described methods (26). MDMs that remained after elimination across the 500 training-test splits were summarized and used to select the final reduced rForest model. The choice of 5 MDMs was set *a priori* to minimize the number of triplex TELQAS assays to 2 (5 MDMs with one normalizing sequence [*B3GALT6*]) that will be used in a subsequent prospective community screening study (NCT 03961945).

The effect of clinical factors (age, sex, BE length, and presence of dysplasia) on the discrimination accuracy of the reduced rForest model was investigated by comparing stratified AUC values, and the association of the predicted probability of BE with BE segment length was investigated using Spearman correlations.

Secondary analyses included measurement of the predictive accuracy of the cross-validated model in the indeterminate group. We also conducted a sensitivity analysis to assess how patients with < 1 cm circumferential BE & GEJ cancers lacking BE (both included with cases) and erosive esophagitis (included with controls)

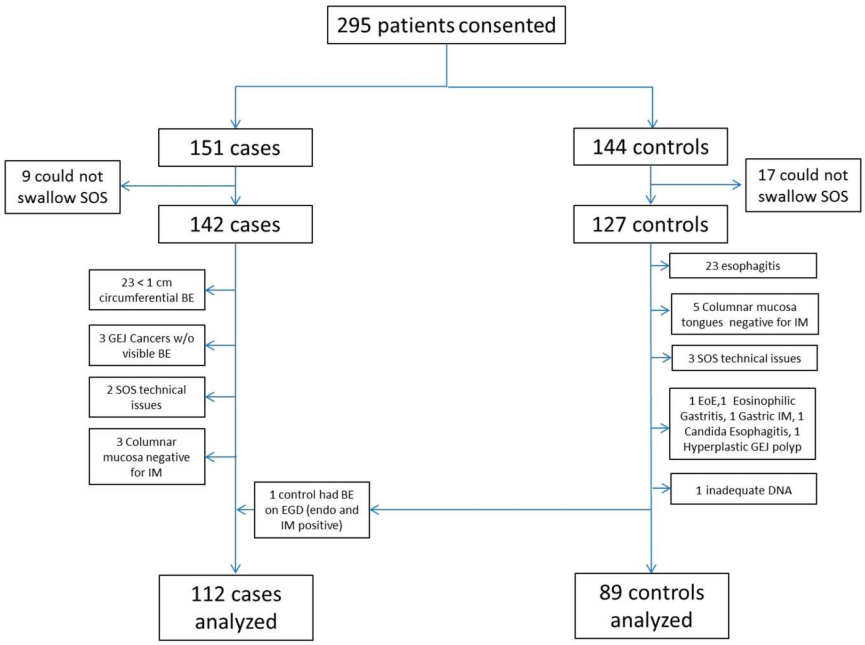

Figure 2. Patient recruitment and flow in the study. Cases with < 1 cm circumferential BE, GEJ cancers without visible BE, columnar mucosa negative for IM, technical sponge-on-string issues and controls with esophagitis, and other inflammatory or neoplastic conditions of the esophagus or stomach were placed in the indeterminate category. BE, Barrett's esophagus; EoE, eosinophilic esophagitis; GEJ, gastroesophageal junction; IM, intestinal metaplasia.

might influence the model performance. For secondary analyses, nominal *P* values were reported.

RESULTS

Two hundred and ninety five participants were consented. Of these, 268 (91%) successfully swallowed the SOS device. Using the criteria listed above, 112 were classified as BE cases, 89 as controls, and 67 as indeterminate. Details of participant flow are outlined in Figure 2. Five patients (7%) had technical issues with the SOS administration. These included either SOS retention < 5 minutes because of intolerance, leading to premature withdrawal before full expansion of the sponge (4 participants) or tether detachment (1 patient, leading to gastric retention 45 minutes before endoscopic removal).

Controls underwent endoscopy for the evaluation of chronic reflux or follow-up of previously diagnosed esophagitis (46, 52%), abdominal pain or dyspepsia (14, 16%), gastrointestinal bleeding (5, 6%), diarrhea (5, 6%), iron deficiency anemia (4, 5%), or miscellaneous reasons (15).

Baseline characteristics of all participants are outlined in Table 1. Most cases were men, with a history of smoking, whereas women constituted most controls. Eleven (10%) BE cases had short segment BE (<3 cm). Approximately 50% of BE cases had no dysplasia.

SOS administration and withdrawal was well tolerated, with an overall median (interquartile range) tolerability score of 2 (0, 4) on a scale of 0–10, where 0 is extremely well tolerated and 10 is the worst tolerated: 248 (94%) participants stated that they would choose the SOS test again for BE detection and preferred the SOS test to endoscopy. The SOS test was performed initially in Rochester by physicians and later in 116 participants by a nurse, following a process of education and supervision in 15 participants. All SOS tests in Jacksonville were performed by a nurse practitioner. Success rates were comparable between physicians and nurses. On endoscopy, there was either no trauma (injury score of 1) in 171 (64%) participants or superficial mucosal abrasions without bleeding (injury score of 2) in 97 (36%).

Several individual MDMs tested were highly discriminant for BE detection. Methylation intensity (in a color-coded display) of all MDMs (at 95% specificity in controls) is displayed in Figure 3.

Table 1. Baseline clinical characteristics of BE cases and controls

	BE cases N = 112	Controls N = 89	*P* value
Median age (IQR)	66 (57, 72)	59 (49, 66)	<0.001
Male sex, N (%)	92 (82)	42 (47)	<0.001
Median maximum BE length cm (IQR)	6 (4, 9)	NA	
Median circumferential BE length cm (IQR)	4 (2, 8)	NA	
BE histology at the time of SOS, N (%)			
No dysplasia	54 (49)	NA	
Indefinite dysplasia	20 (18)		
LGD	15 (14)		
HGD/EAC	23 (19)		
BMI (IQR)	31 (27, 35)	29 (24,32)	0.123
Ever smokers (current or ex-smokers), N (%)	69 (62)	45 (51)	0.116
Esophagitis, N (%)	6 (5)	0 (0)	0.999

BE, Barrett's esophagus; BMI, body mass index; EAC, esophageal. adenocarcinoma; IQR, interquartile range; HGD, high-grade dysplasia; LGD, low-grade dysplasia.

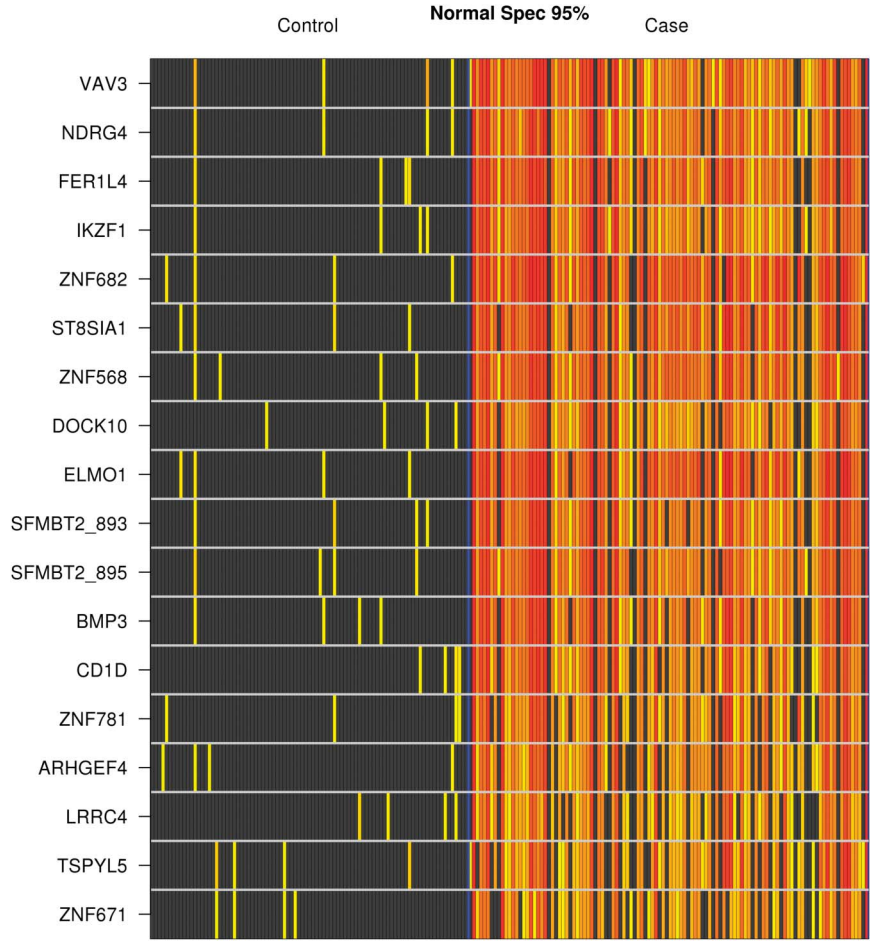

Figure 3. Methylation intensity of candidate MDMs assayed on SOS samples in BE cases and controls. Increasing intensity of yellow-red color spectrum indicates methylation strand counts in decile values above the 95th percentile values for the control group of each MDM (rows) in SOS samples from each case and control (columns). Black boxes indicate values falling below the 95th percentile in controls. MDM, methylated DNA marker; SOS, sponge-on-string.

Using the cross-validated backward elimination approach, a reduced 5 marker rForest model was selected for BE prediction. This panel included *VAV3*, *ZNF682*, *NDRG4*, *FER1L4*, and *ZNF568*, all of which were present in at least 75% of the models after backward elimination (see Table 1, Supplementary Digital Content 5, http://links.lww.com/AJG/B549). Notably, *VAV3* and

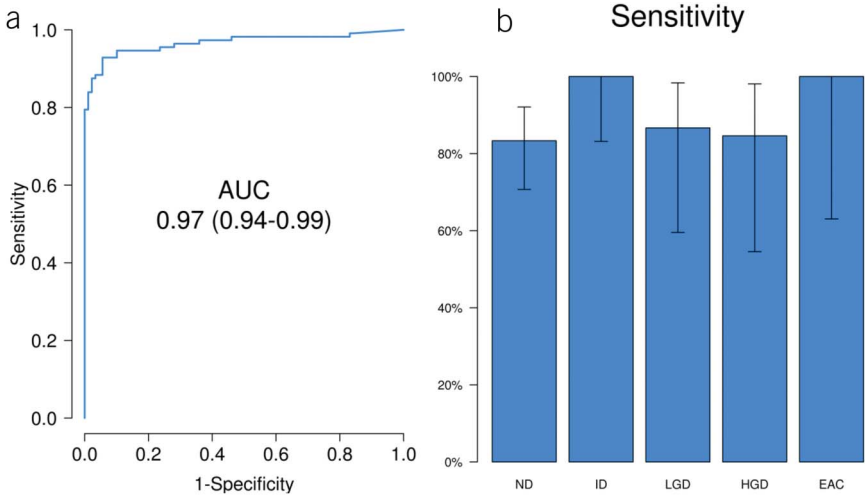

Figure 4. High discrimination of MDM panels for the nonendoscopic detection of BE when assayed on sponge-on-string samples. (**a**). receiver operating curve displaying the cross-validated performance characteristics of the reduced random forest MDM panel (**b**). Sensitivity for the diagnosis of BE by dysplasia grade, in the reduced 5-marker cross-validated model. BE, Barrett's esophagus; MDM, methylated DNA marker.

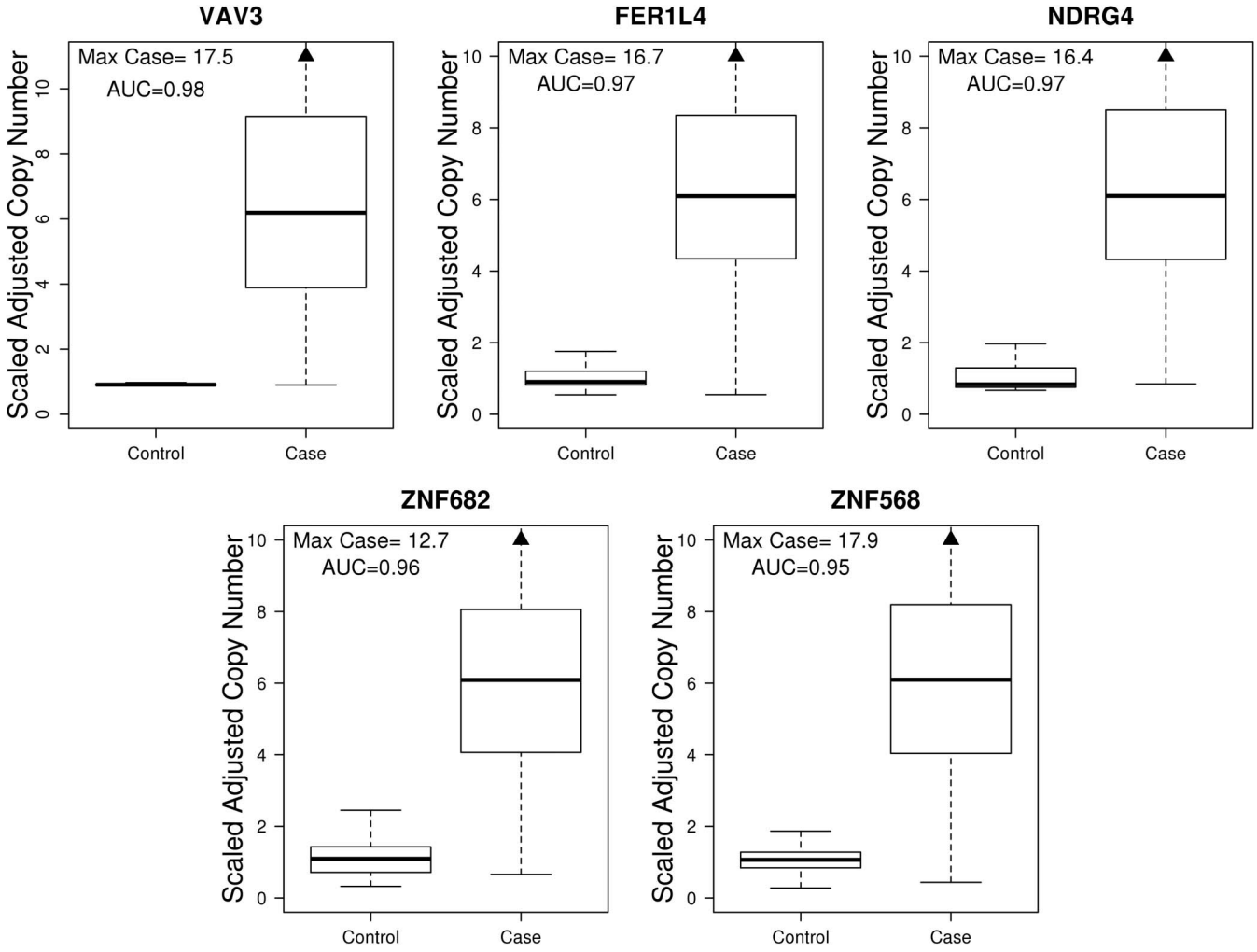

Figure 5. MDM levels in cases and controls and corresponding areas under the curve (AUCs) of 5 MDMs in the reduced 5-marker cross-validated model, assayed on sponge-on-string samples for BE detection. Scaled adjusted copy number is calculated from the quantitative MDM TELQAS product from each sample standardized to *β3GALT6* product from the same sample and scaled by division of the standard deviation from all samples. MDM, methylated DNA marker.

ZNF682 (the best-performing markers from the SOS 1 pilot study) (18) were also present in the reduced model. The sensitivities of this reduced rForest model for BE prediction were 93% (95% CI, 86%–97%) at 90% specificity (82%–95%) and 90% (83%–95%) at 95% specificity (89%–99%) with an overall AUC of 0.97 (93%–99%) (Figure 4a). Marker levels of the 5 MDMs in the cross-validated model are displayed in Figure 5 (remaining MDM marker levels are shown in Figure 1, Supplementary Digital Content 1, http://links.lww.com/AJG/B545).

The association of dysplasia grade with the sensitivity of the rForest BE prediction model is depicted in Figure 4b. The cross-validated sensitivity for BE without dysplasia was 89% (95% CI: 77%–96%) compared with 95% (95% CI; 85%–99%) for BE with any grade of dysplasia. This difference was not statistically significant ($P = 0.16$). The cross-validated AUCs for the rForest model were also not significantly different when stratified by age (by the median, 63 years), sex, body mass index, or smoking status (see Table 1, Supplementary Digital Content 5, http://links.lww.com/AJG/B549). As seen in Figure 6, the predicted probability of BE correlated with BE segment length ($P < 0.0001$). Four short segment, nondysplastic BE cases were not detected by the model.

The predicted probability of BE and test positivity rates (at 95% specificity) for the reduced rForest model in the indeterminate participants are shown in Table 2. The model was positive in 57% of patients with < 1 cm nondysplastic BE and in 26% of patients with esophagitis. All 3 patients with early GEJ adenocarcinomas without visible BE were positive.

A secondary analysis reclassifying all patients with esophagitis (23) as controls and patients with < 1 cm circumferential BE (23) and those with GEJ cancers (3) as cases (applying the reduced rForest model at a preset 95% specificity) yielded a test sensitivity of 87% at a specificity of 90% for BE prediction.

H&E sections of cardia biopsies revealed IM of the cardia (without dysplasia) in 6 individuals, of whom 3 were in the indeterminate group (because of esophagitis), 1 case (initially recruited as a control), and 2 controls. Marker levels in these 2 controls and 1 case were comparable with those without IM of the cardia (see Figure 2, Supplementary Digital Content 2, http://links.lww.com/AJG/B546). Of note, in 33 controls, only squamous epithelium was seen, likely because of the sampling error during endoscopy. Controls with IM of the cardia were classified as controls in the primary analysis.

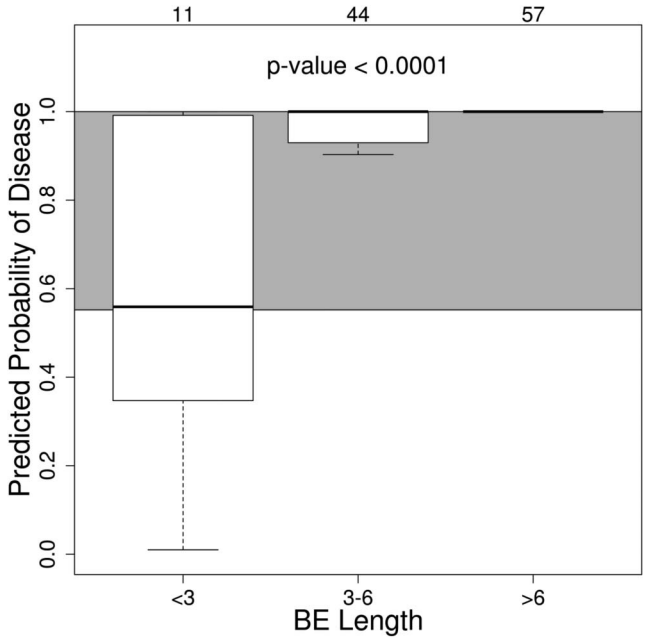

Figure 6. Impact of BE segment length on predicted probability of BE using the reduced 5-marker cross-validated model. BE, Barrett's esophagus.

Patients testing positive and negative with SOS and EGD for BE are also summarized as per the STARD guidelines for reporting diagnostic accuracy studies (see Figure 3, Supplementary Digital Content 3, http://links.lww.com/AJG/B547).

DISCUSSION

In this multisite case control study, we have further confirmed promising results from our pilot study (18). In the present study, we were able to identify a highly discriminant and cross-validated model with 5 MDMs (including the 2 best-performing MDMs from the preceding pilot study) to accurately predict BE status in at-risk individuals, using a commercial grade assay. We also established the safety and tolerability of the EsophaCap device for BE detection in a second larger cohort.

For clinical application, several features are desirable in a minimally invasive BE prediction tool. These include high accuracy, safety, tolerability, and cost-effectiveness (27). To apply this tool widely, BE biomarkers must also be scalable for automated high-throughput testing, eliminating assay operator dependence. Sampling should also minimize operator dependence. MDM assay by TELQAS on DNA obtained by the SOS device meets these requirements. All nonendoscopic devices used in BE screening studies have been safe. MDMs have desirable marker characteristics, given their quantitative nature, scalability of their measurement assay, and the absence of need for pathology interpretation. However, the use of protein markers, such as trefoil factor 3, for the nonendoscopic detection of BE is limited by the need for subjective interpretation of immunohistochemistry by pathologists and the challenge of developing a high throughput and scalable assay (14). MDMs for BE detection have been described by other investigators in smaller proof-of-concept studies (16,17). This is the largest case control study to demonstrate the potential of MDMs for BE prediction in a multisite cohort. Nonendoscopic esophageal sampling devices include compressed polyurethane sponges (17,18,28) and inflatable balloons (16). The balloon device reported by Moinova et al. requires inflation, withdrawal to 5 cm above the GEJ and subsequent inversion into a shell (by deflation) to prevent squamous contamination, increasing susceptibility to operator dependence in sample acquisition.

Most cases of BE in the community do not have dysplasia. However, treatment of BE is recommended only for those harboring dysplasia or carcinoma (6). Hence, it is critical that nonendoscopic BE prediction tests detect not only nondysplastic BE but BE cases with dysplasia and EAC as well. Hence, we incorporated markers discriminant for both dysplastic and nondysplastic BE in our discovery and marker selection cohorts. The tertiary care status of 2 recruitment sites led to a higher proportion of dysplastic BE cases than prevalent in the community. Although MDM levels in dysplastic BE were higher than those with nondysplasia BE, we reassuringly did not see a statistically significant difference between the sensitivity for dysplastic and nondysplastic BE. Similar to other trials (14,16,17) reporting lower sensitivity for shorter nondysplastic BE segments, all BE cases missed in this study had short segments with no dysplasia. This could reflect either under-sampling of short noncircumferential BE segments by a single pass of the SOS through a dilated lower esophagus, and/or dilution of BE-associated MDMs among total DNA which includes sampling from a greater amount of proximal squamous epithelium.

It is notable that controls were younger and more likely to be women (compared with BE cases who are older and men). This imbalance is not unique to our study and has been seen in all other minimally invasive biomarker BE screening trials (14,16,28). However, model accuracy was not influenced by age or sex. Similarly, BE cases also had a higher prevalence of smoking than

Table 2. Median (Q1-Q3) predicted probability of BE and positivity rates (at 95% specificity) using the 5 MDM cross-validated model by the criteria for indeterminate classification for BE status

Reason for Indeterminate status	N	Median BE Probability	Q1	Q3	Positivity, %
BE < 1 cm noncircumferential	23	0.758	0.099	0.956	56.5
Esophagitis	23	0.076	0.019	0.550	26.1
SOS failure	5	0.166	0.113	0.403	25
GEJ cancer only without visible BE	3	1.000	1.000	1.000	100
Biopsy negative for BE	9	0.372	0.010	0.538	22.2
Gastric IM, eosinophilic gastritis	2	0.120	0.120	0.120	0

BE, Barrett's esophagus; GEJ, gastroesophageal junction; IM, intestinal metaplasia; MDM, methylated DNA marker; SOS, sponge on a string.

controls, and we did not observe any association of smoking status with the BE model accuracy. Others have associated elevated methylation in squamous epithelial control samples from the current or former smokers and attempted to overcome this problem by sampling with invertible balloons to reduce squamous contamination (16).

Our definition of BE cases was comparable with and more inclusive than that used by other investigators, who have excluded patients with <3 cm of circumferential BE (14). Some patients recruited as cases had only subcentimeter islands or an irregular Z line on study endoscopy. This was primarily because of the misclassification of BE length on endoscopy reports from outside institutions, a common problem which is well described in the literature (29). The cross-validated BE prediction model was positive in 57% of these patients. In distinction to previous reports, we considered dysplastic BE of any length (≥1 cm of columnar mucosa with IM) as a case, given their higher risk of progression. Patients with esophagitis may be diagnosed with BE, in up to15% of cases after treatment; hence, they were analyzed in the indeterminate group to avoid misclassification bias, particularly at this early phase of test development (21,30). We observed a 26% rate of MDM positivity in these patients. Given the requirement of IM for BE diagnosis in US guidelines (6), we also placed patients with esophageal columnar metaplasia without IM into the indeterminate category. Consistent with studies reporting that 30%–40% of these patients have IM on subsequent endoscopy (31), the cross-validated model was positive in 33% of these patients. Reassuringly, however, even after secondary analysis that reclassified most indeterminates as cases and controls, discrimination with the reduced MDM panel remained high and within the CIs of the rForest model.

Safety of the capsule sponge devices is well documented (32). Tether detachment has been reported with the Cytosponge device in both UK and US trials and was noted in 1 patient in this study. The EsophaCap has been used in 2 other studies with more than 250 patients (17,33), without reports of detachment. Hence, the overall rate of detachment with the EsophaCap is acceptable. Additional technical modifications are ongoing to reduce this risk further.

This study has several strengths. In addition to a large sample size, participants were prospectively recruited at 3 sites: 2 tertiary care centers (in different geographic regions of the United States) and 1 community hospital, increasing generalizability of the results. SOS administration was successfully performed by nonphysicians in most participants. Case and control definitions were made *a priori*. Assays were run in a blinded fashion. Both markers identified in the pilot study (*VAV3* and *ZNF682*) were selected by the modeling process and cross-validated with 3 other MDMs (*NDRG4*, *FER1L4*, and *ZNF568*) in this study. Importantly, we were able to apply robust statistical modeling techniques specifically selected to reduce the risk of overfitting. The use of bootstrap sampling to train and test the rForest model is anticipated to increase the generalizability of our results when applied to independent validation data sets (25).

Some limitations should be acknowledged. Given the deliberate change in assay platform to a commercial grade assay, the cutoffs established for *VAV3* and *ZNF682* in the SOS 1 study could not be applied to the present data set. Although there was no evidence of a significant effect of IM of the cardia on marker levels, this was limited by missed sampling of the cardia in a third of controls. Approaches to improve sensitivity in those with short segment BE without dysplasia need to be developed, such as improved sampling, assessing methylation levels in short BE segments, and optimizing assay performance. Finally, we acknowledge that this study has been conducted in a selected population of mostly long-segment BE with a higher proportion of dysplasia than that commonly encountered in the population, in an enriched population of dysplastic BE cases. This was performed to reduce the risk of misclassification bias and ensure the detection of dysplastic BE. Studies to assess the performance characteristics (sensitivity and specificity) and positive and negative predictive values of the BE detection test in a screening primary care population, with lower BE prevalence and dysplasia rates, are logical next steps. Such studies are being initiated (NCT 03961945). Of note, secondary analysis, including most of the indeterminate subjects as cases (with short segment BE) and controls, showed continued reasonable assay performance.

In summary, we have developed additional evidence for a minimally invasive molecular strategy for BE prediction in an at-risk population. This approach will require further refinement using optimized sample collection and assay methods to establish firm MDM cutoff values in an independent sample set. We anticipate that such a test will be best performed in the office setting, in patients with risk factors, with positive tests being followed by endoscopy for confirmation of diagnosis.

CONFLICTS OF INTEREST

Guarantor of the article: Prasad G. Iyer, MD, MSc.

Specific author contributions: Study concept and design: P.G.I., S.W.S., D.W.M., D.A.A. Acquisition of data: W.R.T., M.L.J., R.L.L., K.A.M., T.C.Y., J.A.S., M.E.D., C.K.B., P.H.F., T.C.S. Analysis and interpretation of data: P.G.I., W.R.T., S.W.S., B.T.B., D.W.M., D.A.A., J.B.K. Drafting of the manuscript: P.G.I. Critical revision of the manuscript for important intellectual content: P.G.I., W.R.T., S.W.S., D.W.M., K.K.W., D.A.K., D.A.A., H.C.W., J.B.K. Obtained funding, study supervision: P.G.I., D.A.A., J.B.K.

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Study Highlights

WHAT IS KNOWN

- ✓ BE screening is currently recommended by GI societies in those with multiple risk factors.
- ✓ Endoscopy (sedated or unsedated transnasal) is the current standard for BE diagnosis but is invasive and not widely applicable.
- ✓ Nonendoscopic minimally invasive approaches combining esophageal cell collection devices and biomarkers are promising approaches amenable to wider application.
- ✓ We have previously reported promising MDMs for the nonendoscopic prediction of BE in a pilot study.

WHAT IS NEW HERE

- ✓ In a multisite independent case control study, several MDMs assayed on SOS samples using a commercial grade assay were highly discriminant for BE (using endoscopy as gold standard).
- ✓ A reduced 5 MDM panel for BE prediction, with an AUC of 0.97, was developed using a robust *insilico* cross-validation approach.
- ✓ The SOS device was safe and well tolerated in this independent cohort of patients.

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