Independent Validation of a Tissue Systems Pathology Assay to Predict Future Progression in Nondysplastic Barrett's Esophagus: A Spatial-Temporal Analysis

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INTRODUCTION: **An automated risk prediction assay has previously been shown to objectively identify patients with nondysplastic Barrett's esophagus (NDBE) who are at increased risk of malignant progression. To evaluate the predictive performance of the assay in 76 patients with NDBE of which 38 progressed to high-grade dysplasia/esophageal adenocarcinoma (progressors) and 38 did not (nonprogressors) and to determine whether assessment of additional (spatial) levels per endoscopy and/or multiple (temporal) time points improves assay performance.**

METHODS: **In a blinded, nested case-control cohort, progressors and nonprogressors were matched (age, sex, and Barrett's esophagus length). All random biopsy levels from the baseline endoscopy (spatial samples) and all available previous endoscopies back to 10 years before progression (temporal samples) were assayed. Because the 1:1 ratio of progressors to nonprogressors does not reflect the real-world Barrett's population, negative and positive predictive values were adjusted for prevalence.**

RESULTS: **Seventy-six patients (58 men), mean age of 63 ± 9 years, were studied. A high-risk score was associated with a prevalence-adjusted annual progression rate of 6.9%. The assay identified 31% of progressors when assessing a single biopsy level from the baseline endoscopy. Sensitivity increased to 50% and 69% in spatial and temporal analyses, respectively, while specificity remained at 95%.**

DISCUSSION: **The assay identified a significant subset of NDBE patients who progress at a rate comparable with published estimates for expert-confirmed low-grade dysplasia. Assessing additional spatial and temporal biopsies increased the predictive accuracy, allowing for identification of most future progressors. Additional studies will evaluate the predictive performance of the assay in low-prevalence settings.**

SUPPLEMENTARY MATERIAL accompanies this paper at http://links.lww.com/CTG/A389, http://links.lww.com/CTG/A390, and http://links.lww.com/CTG/A391

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INTRODUCTION

In Barrett's esophagus (BE), the squamous epithelium that usually lines the esophagus is replaced by columnar epithelium. This is of clinical relevance because BE is a known precursor of esophageal adenocarcinoma (EAC), a cancer with a rising incidence worldwide and a poor 5-year survival rate of 20% when diagnosed at a symptomatic stage (1–3). Nondysplastic BE (NDBE) progresses in a stepwise process to low-grade dysplasia (LGD), then high-grade dysplasia (HGD), and finally to EAC, with the histopathological diagnosis of dysplasia being the current best predictor of progression (4,5). Periodic endoscopic surveillance with biopsies is recommended by gastrointestinal societies to identify dysplasia and EAC early when amenable to endoscopic eradication therapy (EET) (5–7). However, interobserver variability between pathologists in diagnosing dysplasia, biopsy sampling error, and a debatable cost-effectiveness calls endoscopic surveillance into question (8–10). In addition, the inability to accurately risk stratify BE patients without histologic dysplasia limits the effectiveness of surveillance because NDBE patients represent the majority of the BE population. An objective biomarker assay that can risk stratify BE patients independently from histological diagnosis may overcome these limitations and would allow for individualized risk-tailored management. Although costly and invasive endoscopies could be

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reduced in patients with low risk of progression, more frequent surveillance or even preventive EET could be considered in those at high risk of progression to HGD/EAC.

Recent work demonstrated that a tissue systems pathology assay (TissueCypher Barrett's Esophagus Assay) accurately risk stratifies NDBE patients into low, intermediate, or high risk of progression to HGD/EAC within 5 years. This multivariable risk prediction assay quantifies 9 protein-based biomarkers and nuclear morphology *in situ* in esophageal biopsies (11,12). After multiplexed fluorescent labeling of tissue sections, the slides are imaged by whole slide fluorescence scanning. Automated image analysis software extracts quantitative digital features that capture the expression and localization of the 9 biomarkers and morphology. Finally, a multivariable classifier integrates the quantitative features to produce a risk score that ranges from 0 to 10 and risk classes (low, intermediate, or high risk) for progression to HGD/EAC within 5 years. This computational pathology approach has been demonstrated to objectively identify NDBE patients who are at increased risk of incident progression independent from other clinical risk factors (13,14). Moreover, recent findings suggest that this risk prediction assay may capture a field effect because BE patients with prevalent HGD/EAC were discriminated from patients with no evidence of HGD/EAC (15). Although the most recent of these studies evaluated the spatial distribution of the assay results in a subset of the evaluated patients (14), the spatial distribution and temporal distribution of the assay results in NDBE patients have not been well characterized.

The spatial variability and temporal variability of biomarkers in the BE segment have been shown to be high (16), although there is limited literature available. Spatial variability can be assessed by evaluating biopsies from different levels in the esophagus taken according to the Seattle protocol, and temporal variability can be studied by testing biopsies taken at multiple surveillance endoscopies (17,18). The spatial and temporal variation of risk stratification biomarkers may affect both the ideal sampling technique and the recommended surveillance intervals after testing. Biomarkers that exhibit high spatial variability across a long BE segment may benefit from extensive sampling or collection methods such as brushes to maximize the probability of detecting high-risk expression patterns. Furthermore, consistent high-risk results at multiple endoscopic time points in patients who progress to EAC potentially increase the predictive power of a biomarker, as has been recently demonstrated for expert-confirmed LGD (19). In addition, evaluation over time allows for estimation of the predictive window of a biomarker.

The aims of this study were to evaluate the predictive performance of the risk prediction assay in an independent cohort of NDBE patients who progressed to HGD/EAC (progressors) compared with those who did not (nonprogressors) and to assess whether the accuracy of the assay increases if multiple biopsy levels and multiple endoscopies are tested. We conducted a nested case-control study which used NDBE patients from the Amsterdam ReBus cohort, a community-based BE cohort using the most stringent inclusion criteria.

METHODS

Population and setting

The Amsterdam ReBus cohort consists of BE patients who progressed to HGD/EAC (progressors) during endoscopic surveillance and those who never showed progression (nonprogressors) during endoscopic follow-up (20). Progressors were referred from community centers to 3 tertiary referral centers in the Netherlands for endoscopic work-up of early BE neoplasia. Using the nationwide network and registry of histopathology and cytopathology in the Netherlands (PALGA database), previous surveillance endoscopies were identified, and subsequently, all original endoscopy reports, pathology reports, and formalin-fixed paraffin-embedded blocks were retrieved. The PALGA database has nationwide coverage since 1991, archiving all pathology reports in the Netherlands (21).

Nonprogressors were identified from a retrospective BE surveillance registry in 10 community hospitals in the Amsterdam region, which was initiated in 2003 and updated from 2011 onward. To be eligible for the ReBus cohort, patients were required to have had a baseline (BL) endoscopy demonstrating columnar-lined esophagus with specialized intestinal metaplasia on subsequent histological examination, minimum 2 years' endoscopic surveillance before the endpoint of the study (diagnosis of HGD or EAC in progressors) and maximum stage T1 disease at time of progression (progressors), and a progression diagnosis based on an endoscopic resection or 2 subsequent endoscopies with endoscopic progression (progressors). The biopsy protocol was consistent with the Seattle protocol. Data elements collected were age, sex, segment length, collection date, and original diagnosis of every biopsy specimen block, presence or absence of hiatal hernia, and survival times after diagnoses.

The risk prediction assay was run on each specimen in a blinded manner without knowledge of the clinical outcome (nonprogressor or progressor) or other clinicopathologic information. Risk prediction test results were reported to an outside consultant statistician who followed an *a priori* statistical analysis plan. The study was reviewed by the institutional ethics committee of the Academic Medical Center, Amsterdam. The institutional biobank review committee of the Academic Medical Center officially approved the ReBus biobank.

Study cohort

Patients were selected from the Amsterdam ReBus cohort. An endoscopy was selected for each patient that was 2–5 years before the HGD/EAC endpoint for progressors and at least 5 years before the last surveillance endoscopy in nonprogressors. This selected endoscopy was termed the "baseline" endoscopy for each patient. This enabled assessment of the predictive accuracy of the assay in progressors with time to progression of 2–5 years, which is within the known 5-year predictive window of the assay and also excluded patients with prevalent HGD/EAC. Inclusion criteria for both progressors and nonprogressors were a BL endoscopy containing biopsies from at least 2 endoscopic levels across the BE segment and pathologic diagnosis of NDBE at all biopsied endoscopic levels. The spatial distribution of assay results was assessed at the BL endoscopy. Biopsies from all available endoscopies before the BL endoscopy but no more than 10 years before HGD/EAC endpoint were analyzed in progressors. The temporal distribution of assay results was assessed by evaluating results at the BL and pre-BL endoscopies. In nonprogressors, no additional endoscopies before the BL were analyzed. Progressors and nonprogressors were matched based on sex, age ($\pm$5 years), and the maximal length of the BE segment ($\pm$2 cm).

Risk prediction testing

The risk prediction assay was run on each biopsy specimen at Cernostics CLIA-certified clinical laboratory (CLIA#: 39D2110302, Pittsburgh, PA) according to established standard operating

Table 1. Baseline characteristics of the study population (n = 76)

	Progressors (n = 38)	Nonprogressors (n = 38)	*P* value
Age, yr, median (IQR)	64 (57–71)	62 (53–71)	0.012
Male, n (%)	31 (82)	27 (71)	NS
Barrett's segment length, cm, median (IQR)	7 (5–8)	6 (5–8)	NS
Hiatal hernia, n (%)	27 (71)	30 (79)	NS
Time in study, yr,[a] median (IQR)	3.2 (2.3–4.3)	6.1 (5.5–7.2)	<0.001
Spatial characteristics			
Levels per endoscopy, n, median (IQR)	2 (2–3)	3 (2–3)	NS
Total number levels, n	107	102	NS
% Required levels,[b] n, median (IQR)	90 (66.7–100)	70.1 (65–100)	NS
Temporal characteristics			
Levels per endoscopy, n, median (IQR)	1 (1–2)	NA	
Total number levels, n	120	NA	

P-values are from the Wilcoxon signed-rank test for continuous variables and the McNemar test for categorical variables.
IQR, interquartile range; NA, not available; NS, not significant.
[a]Time from baseline endoscopy to progression (progressors) or last endoscopic follow-up (nonprogressors).
[b]Percentage of levels tested as required per Seattle protocol (4q every 2 cm).

procedures. All assay parameters were prespecified and locked, including the 9 protein-based biomarkers that were labeled and imaged in biopsies, the 15 image analysis features that were extracted from the images, scaling parameters, the risk classifier model, and cutoffs, as defined in previous studies (13,15). The testing process to generate the risk score and risk class is summarized in the Supplemental Information (see Supplementary Digital Content 3, http://links.lww.com/CTG/A391).

Statistical analyses

Continuous variables with a normal distribution were described with mean and SD. Median and interquartile range were used to describe continuous variables with a skewed distribution. The paired *t* test was used to compare progressors *vs* nonprogressors for normally distributed continuous variables, the Wilcoxon signed-rank test for non-normally distributed continuous variables, and the McNemar test for categorical variables.

Kaplan-Meier (KM) curves were used to graphically represent the risk of progression to HGD/EAC in the 3 risk classes determined by the assay. Hazard ratios with 95% confidence intervals (CI) were calculated from Cox regression, and the log-rank test was used to evaluate the equality of progression curves of the 3 risk groups from KM analysis. Sensitivity (proportion of progressors scored high risk by the assay), specificity (proportion of nonprogressors scored low or intermediate risk), negative predictive value (NPV, proportion of patients scored low risk who did not progress to HGD/EAC), and positive predictive value (PPV, proportion of patients scored high risk who progressed to HGD/EAC) were calculated based on progression to HGD/EAC within 5 years. NPV, PPV, and proportions of patients scoring low, intermediate, and high risk were adjusted for prevalence assuming 5-year prevalence of 5.07%, based on previously reported progression rates from ND, indefinite for dysplasia, and LGD to HGD/EAC (4). The variability in temporal and spatial distribution of risk prediction assay results was estimated by a linear mixed model as the SD of the random effects for endoscopy time points within patients and residuals within endoscopy time points, respectively.

RESULTS

Patients

BL characteristics of the study population and spatial and temporal endoscopies are summarized in Table 1. Seventy-six patients (38 progressors and 38 nonprogressors) with a BL endoscopy with biopsies taken at multiple levels met the inclusion criteria (Figure 1 for exclusions). Despite matching for age within a range of ±5 years, progressors were slightly but significantly older compared with nonprogressors (64 *vs* 62 years, mean difference of 1.3 years [95% CI 0.4–2.3]; $P = 0.008$). There was no significant difference in BE segment length or sex between matched case-control sets. All patients in the study had long-segment Barrett's. The median time from BL endoscopy to the HGD/EAC endpoint was 3.2 years (interquartile range 2.3–4.3) in progressors, and the time from BL endoscopy to last follow-up was 6.1 years (interquartile range 5.5–7.2) in nonprogressors.

Predictive performance of the risk prediction assay

Evaluation of single and multiple spatial levels. KM analysis of the most distal biopsy level (closest to gastroesophageal junction) from the BL endoscopy demonstrated that the risk prediction assay could distinguish between progressors and nonprogressors (Figure 2a). Patients who scored high risk were 3.2× (95% CI 1.6–6.5; $P = 0.0032$) more likely to progress to HGD/EAC than patients who scored low risk (Figure 2a). Sensitivity (proportion of progressors scoring high risk) and specificity (proportion of nonprogressors scoring low or intermediate risk) of the assay at 5 years were 30.4% and 95.0%, respectively (Table 2). Evaluation of the highest scoring of all additional spatial biopsy levels from the BL endoscopy significantly increased the detection rate of progressors by 63.5% (from 30.4% to 49.8%; $P = 0.016$). Specificity remained 95.0% irrespective of the number of tested levels at BL

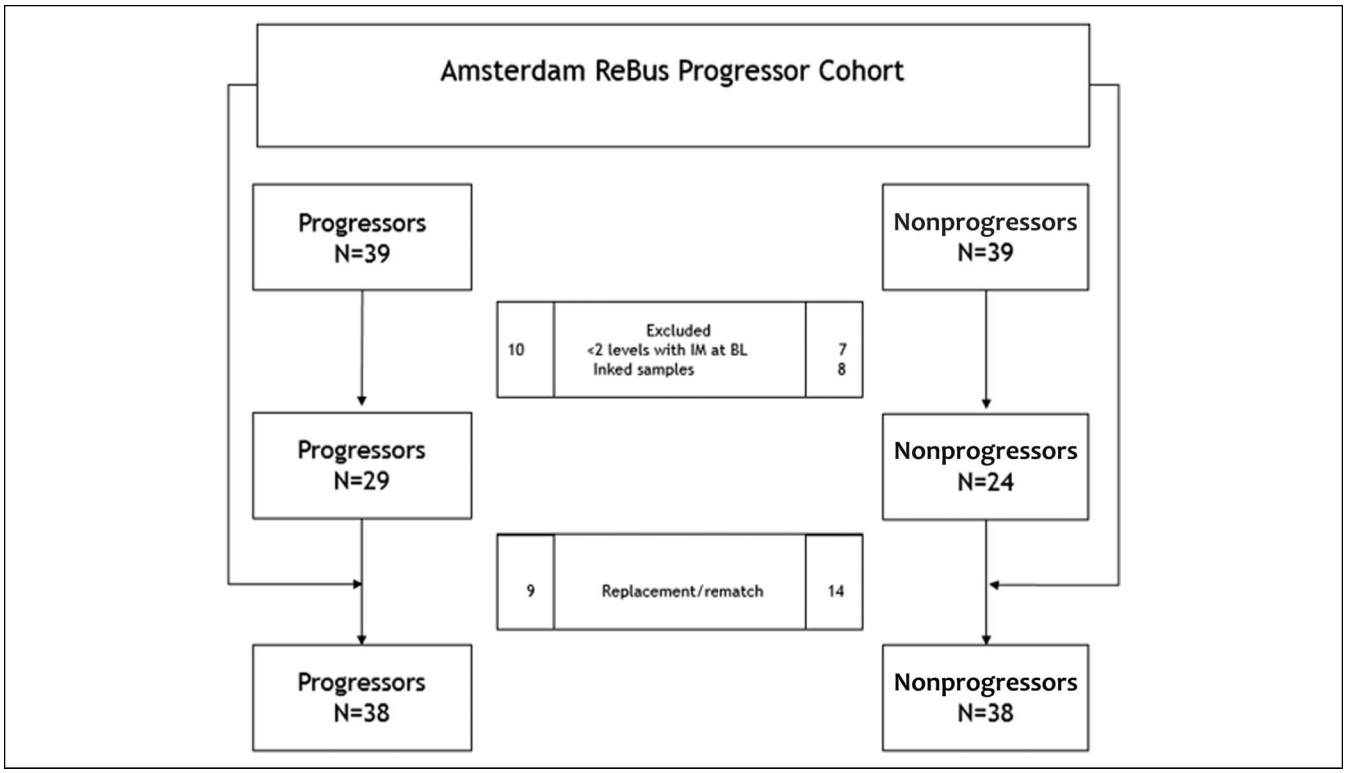

Figure 1. Flowchart of the included progressor and nonprogressor patients originating from the Amsterdam ReBus progressor cohort. Ten progressors and 15 nonprogressors had to be excluded after the first analysis. To replace the lost participants and maintain the number of matched case-control sets as high as possible, the source population (Amsterdam ReBus cohort) was screened again. Nine progressors and 14 nonprogressors meeting the predefined matching criteria were identified/rematched, leading to the final number of 38 matched case-control sets. BL, baseline; IM, intestinal metaplasia.

because no additional nonprogressors scored high risk in additional spatial biopsy levels. When evaluating all BL spatial biopsy levels, patients who scored high risk were 5.5× (95% CI 2.7–11.4; $P \leq 0.0001$) more likely to progress than patients who scored low risk (Figure 2b).The prevalence-adjusted positive (PPV) and negative (NPV) values for prediction of progression within 5 years were 34.6% and 97.7%, respectively, indicating an annual progression risk of 6.9% in NDBE patients scoring high risk. The NPV and PPV were adjusted for prevalence to prevent overestimation of the predictive accuracy of the assay in this case-control cohort. Linear mixed model analysis estimated that the SD of continuous risk scores in biopsies from separate endoscopic levels was 1.22 (Table S2, Supplementary Digital Content 2, http://links.lww.com/CTG/A390).

Evaluation of additional time points. Twenty-nine progressors (76%) had additional endoscopies tested pre-BL/before progression (as summarized in Table 2). Evaluation of the highest scoring of all biopsies from the BL and pre-BL (i.e., temporal) endoscopies led to an additional, although not statistically significant increase of the detection rate by 37.6% (from 49.8% to 68.5%). Specificity, PPV and NPV were not calculated because biopsies from pre-BL endoscopies were not evaluated in nonprogressors. KM analysis of the highest scoring biopsy from all available BL and pre-BL endoscopies showed that patients who scored high risk were at 7.0-fold (95% CI 3.3–14.8; $P \leq 0.0001$) increased risk of progression within 5 years *vs* patients who scored low risk (Figure 2c). The SD of continuous risk scores at temporal endoscopies was 0.41 (Table S2, Supplementary Digital Content 2, http://links.lww.com/CTG/A390).

Independent validation of the risk prediction assay. Biopsy specimens from 20 patients (13 progressors and 7 nonprogressors) were previously evaluated in a development study (13), whereas 56 patients were exclusively tested in the current study. The risk prediction assay was evaluated in this subset of patients to independently validate the predictive performance of the test in NDBE patients. The BL characteristics were comparable between the previously and newly tested participants, with the exception of age (Table S1, Supplementary Digital Content 1, http://links.lww.com/CTG/A389). The risk prediction assay provided significant risk stratification in the subset of 56 new patients who were not previously tested in an earlier study (Figure 3). When evaluating the highest scoring biopsy from the BL endoscopy, which the spatial analysis described above indicated is the optimal way to use the assay, NDBE patients who scored high risk were 5.9x (95% CI 2.4–14.4; $P < 0.0001$) more likely to progress to HGD/EAC within 5 years compared with patients who scored low risk. The sensitivity and specificity of the assay in predicting progression within 5 years were 43.8% and a specificity of 96.8%, respectively. The PPV and NPV were 42.0% and 97.5%, respectively, indicating an annual progression risk of 8.4% in NDBE patients scoring high risk. Representative images of the 9 biomarkers in nondysplastic specimens from progressor and nonprogressor patients and assay workflow are shown in Figures 4 and 5.

Table 2. Risk prediction assay performance metrics in single level, spatial, and temporal analyses

	BL endoscopy				BL and pre-BL endoscopies	
	Single level (most distal)		Spatial (highest scoring)		Temporal (highest scoring)	
	Progressors, n = 38	Nonprogressors, n = 38	Progressors, n = 38	Nonprogressors, n = 38	Progressors, n = 38	Nonprogressors, n = 38
Low risk, n	22	34	13	29	10	29
Intermediate risk, n	4	3	6	8	4	8
High risk, n	12	1	19	1	24	1
Sensitivity,[a] %	30.4		49.8		68.5	
Specificity,[a] %	95.0		95.0		NA	
5-yr PPV, %	24.6		34.8		NA	
5-yr NPV, %	96.6		97.7		NA	

PPV/NPV adjusted for high-grade dysplasia/esophageal adenocarcinoma prevalence of 5.07%/5 years.
BL, baseline; NA, not available because temporal endoscopies were not tested in NP; NPV, negative predictive value; PPV, positive predictive value.
[a]Metrics calculated from Kaplan-Meier based on progression to high-grade dysplasia/esophageal adenocarcinoma within 5 years.
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DISCUSSION

In this blinded, independent validation study, we demonstrate that an objective, automated risk prediction assay is able to identify patients with NDBE who progress to HGD/EAC at an annual rate of 6.9%. The assay was able to identify incident progressors on NDBE biopsies; i.e., before any morphological changes associated with dysplasia. This is the fourth study to independently validate the ability of this risk prediction assay to risk stratify patients with BE, and it provides an additional level of evidence to support clinical adoption of the assay. The study expands on previous findings that sampling multiple endoscopic levels across the Barrett's segment further increases the detection rate for patients at high risk of incident progression.

The 6.9% annual progression rate in NDBE patients scoring high risk with the assay is comparable with the progression rate in expert confirmed LGD in European studies (19,22) and even higher than the progression rate of expert-confirmed LGD in US studies (23,24). Since this risk prediction assay is able to identify both incident and prevalent progression (13,15,25), patients scoring high risk should be referred to a specialist with expertise in BE for a high-quality imaging endoscopy to exclude any visible lesions. After excluding any visible lesions, a management approach similar to the approach for confirmed LGD should be considered, which includes shorter surveillance intervals or EET.

In our study, the assay identified approximately half of all patients who progressed to HGD/EAC at a nondysplastic stage. Applying this risk prediction assay on all NDBE cases would thus allow prophylactic treatment of 50% of all cancer progressors and significantly reduce the risk of the remaining patients. In addition

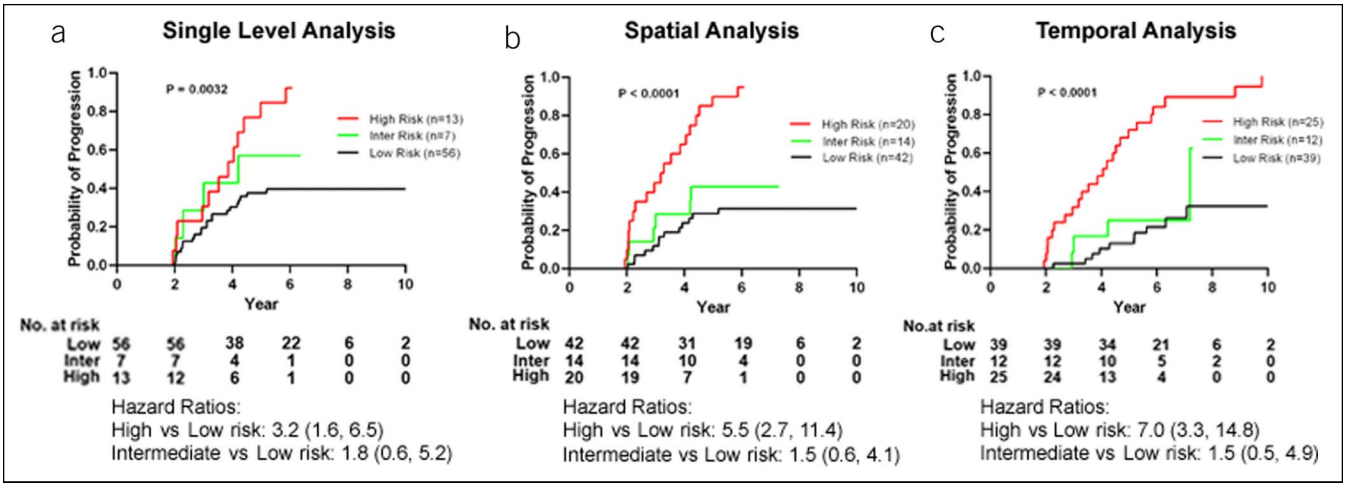

Figure 2. Assessment of additional spatial and temporal information improves the performance of the risk prediction assay. Kaplan-Meier analysis of probability of progression to high-grade dysplasia/esophageal adenocarcinoma in nondysplastic Barrett's esophagus patients scored low, intermediate, and high risk by the risk prediction assay based on assessment of (**a**) a single biopsy level (most distal) from baseline (BL) endoscopy; (**b**) the highest scoring of all available spatial biopsy levels from BL endoscopy; and (**c**) the highest scoring of all available spatial biopsy levels from BL and pre-BL endoscopies. *P*-values are from log-rank tests evaluating the equality of progression curves of the 3 risk groups.

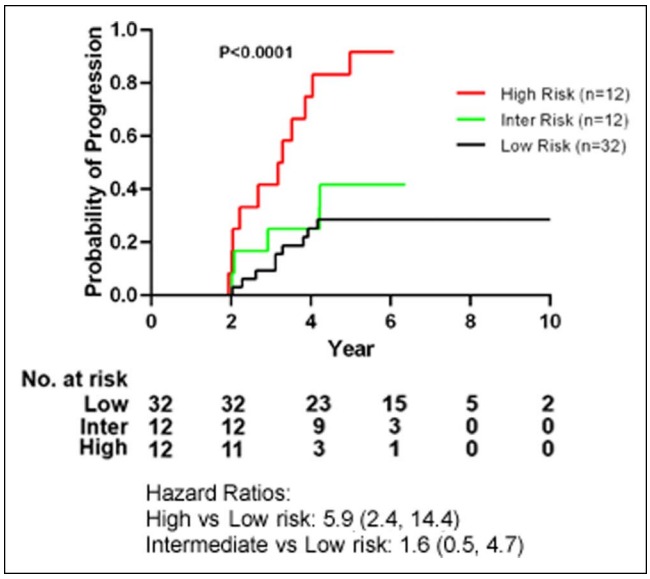

Figure 3. Independent validation of the risk prediction assay in nondysplastic Barrett's esophagus patients. Kaplan-Meier analysis of probability of progression to high-grade dysplasia/esophageal adenocarcinoma in the 56 previously untested patients scored low, intermediate, and high risk by the assay. The 56 patients (25 progressors and 31 nonprogressors) have not been tested in previous studies (i.e., the 20 patients tested in the earlier validation study (13) were excluded from this analysis).

to a recent study (14), we demonstrate a persistent high specificity in parallel with increasing sensitivity while testing multiple levels, thus patients will not be harmed (for example by a false-positive high-risk score) by testing additional levels. A recent study used Markov decision modeling and simulation to evaluate the cost-effectiveness of care guided by this risk prediction assay *vs* the current standard of care from the perspective of a large US health care system (26). Use of EET in patients scoring high risk with the assay, in parallel with extension of surveillance intervals to 5 years in patients scoring low risk to 5 years, was predicted to (i) be cost-effective within 5 years, (ii) reduce the progression to HGD, EAC, and EAC-related deaths by 51.7%, 47.1%, and 37.6%, respectively, and (iii) reduce the use of endoscopies by 16.6%, even at 75% adherence by physicians to the assay-guided care.

In contrast to most published biomarker studies, our study evaluated the impact on predictive accuracy of testing multiple separate levels per endoscopy and multiple endoscopies over time. The risk prediction assay identified significantly more progressors when multiple endoscopic levels across the Barrett's segment were tested compared with a single endoscopic level, indicating that when the assay is used clinically, all available biopsies from an endoscopy should be submitted for testing. This can be performed without increasing cost to the health care system because the assay is billed once for each endoscopy encounter, independently of the number of specimens submitted for testing. The predictive performance of assay may be further improved by applying the assay to esophageal specimens acquired through tools such as brushes and sponges that sample a larger area of the Barrett's segment. The assay also identified more progressors when additional time points before the BL endoscopy were assessed, although the increase in assay sensitivity was not statistically significant. Compared with adding additional spatial

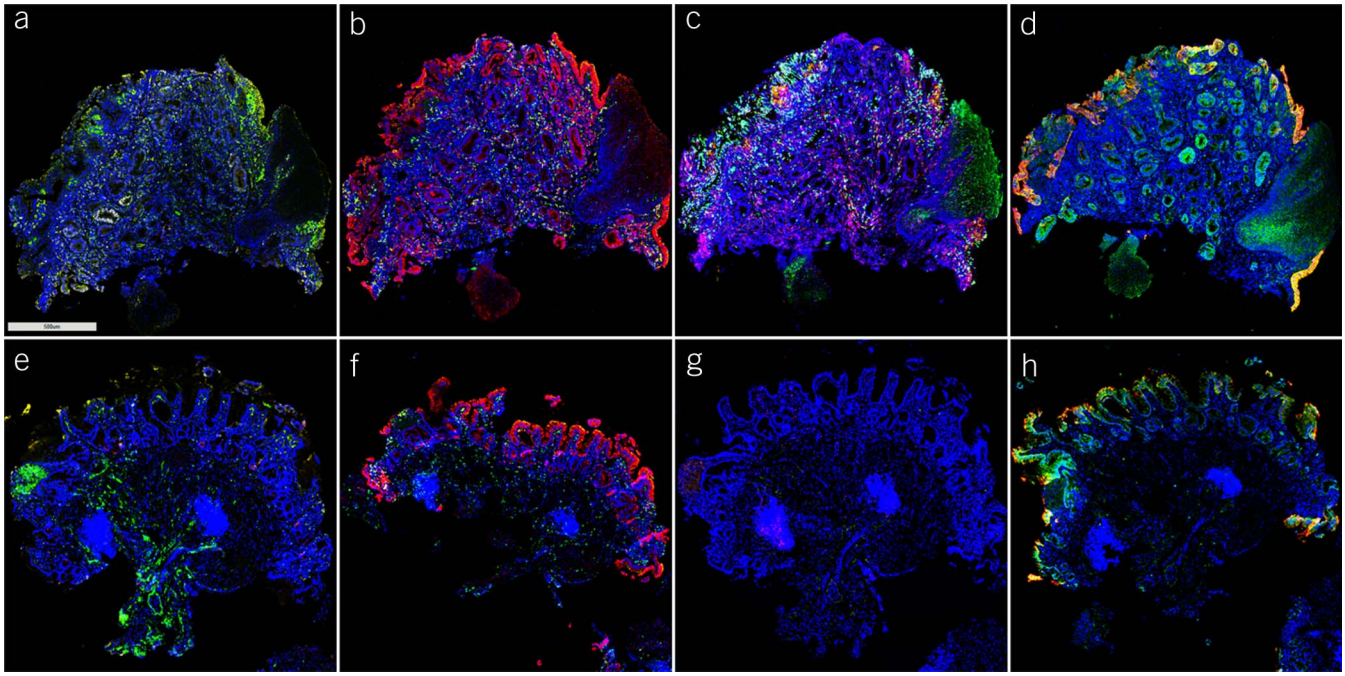

Figure 4. Representative images of biomarkers labeled in nondysplastic Barrett's esophagus (NDBE) biopsies. Panels (**a**–**d**) show a biopsy from a 66-year-old man with 8-cm Barrett's esophagus (BE) segment and NDBE who was diagnosed with esophageal adenocarcinoma 3.9 years later (progressor). This biopsy was scored 10/high risk by the risk prediction assay. Panels (**e**–**h**) show a biopsy from a 69-year-old man with 11-cm BE segment and NDBE who had 6.1 years' surveillance data showing no disease progression (nonprogressor). This biopsy was scored 5.4/low risk by the risk prediction assay. (**a** and **e**) p16-green, AMACR-red, and p53-yellow; (**b** and **f**) CD68-green and COX-2-red; (**c** and **g**) HIF-1α-green and CD45RO-red; (**d** and **h**) HER2-green and K20-red. Hoechst labeling of nuclei is shown in blue in all panels.

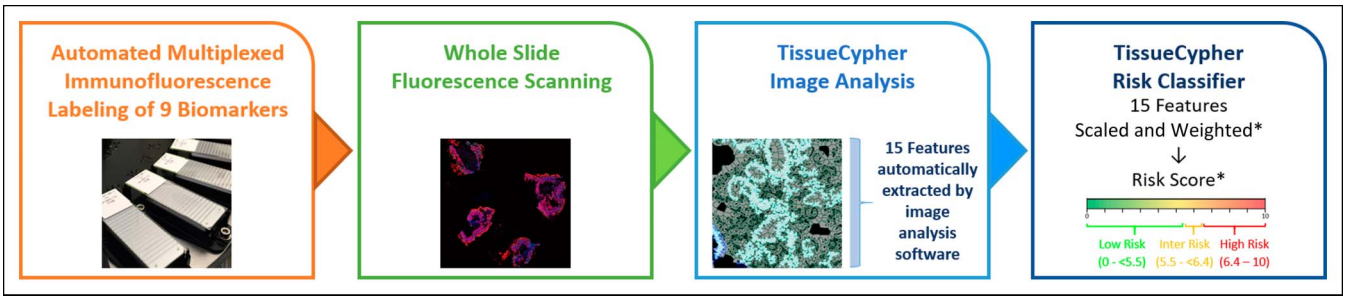

Figure 5. Overview of the risk prediction assay. The risk score and risk classes are calculated from the scaled and coefficient–weighted sum of 15 features (quantitative image analysis measurements) that are extracted automatically from whole slide images of 9 protein-based biomarkers and nuclear morphology in tissue slides that have been labeled by multiplexed immunofluorescence. In the first step, serial sections of formalin-fixed paraffin-embedded Barrett's esophagus biopsies are fluorescently immunolabeled for p16, AMACR, p53, HER2, K20, CD68, COX-2, HIF1α, and CD45RO, plus Hoechst. The labeled slides are imaged by whole slide fluorescence scanning that generates image data on each labeled biomarker and nuclei. The whole slide images are then analyzed by automated image analysis software that extracts 15 predefined features*, which are measures of the 9 protein-based biomarkers and morphometric-based biomarkers. In the final step, the 15 features are scaled using center and scale parameters, and then weighted by coefficients*. The weighted sum of the 15 scaled features is scaled* to produce a continuous risk score (0–10), and cutoffs are applied to classify patients for risk of progression, as follows: risk class = low if scaled score 0–<5.5, intermediate if scaled score 5.5 < 6.4, and high if scaled score 6.4–10. *All scaling parameters and coefficients were derived and locked in the initial training study (13).

samples, the addition of testing multiple time points before the BL endoscopy did not significantly increase the number of progressors identified by assay. Since we enforced a minimum window between BL sampling and progression, we only tested endoscopies before BL (hence longer before progression), which may be outside of the predictive window of the assay. Logically, the effect of prospectively using the assay on sequential temporal samples should be more pronounced. Since the assay was developed to predict progression within 5 years, it may be valuable to repeat the test at the interval guided by the assay result.

The following limitations need to be discussed. First, the retrospective selection of patients is vulnerable to selection bias. However, the risk prediction assay was performed in a blinded manner. In addition, the retrospective design allowed us to stringently select nondysplastic BL samples at least 2 years before progression, which significantly reduces the probability of prevalent dysplasia or cancer in the tested samples. Second, a 1:1 ratio of progressors to nonprogressors as described in this case-control study does not reflect a real-world Barrett's population and may overestimate the predictive ability of the assay. To address this, as in previous studies, the NPV and PPV values were adjusted for HGD/EAC prevalence. Third, because of the retrospective design, it cannot be guaranteed that the number of tested biopsy levels represents the entire Barrett's segment because endoscopists tend to undersample particularly in long-segment Barrett's (27). However, we only included cases in which endoscopies had at least 50% of levels sampled every 2 cm as recommended per Seattle protocol. Our current results indicate an even higher detection rate of progressors would be achieved with improved sampling quality according to the Seattle protocol. Finally, additional time points were only assessed in patients who progressed; however, the assay retained high specificity (95%) when additional biopsies from the BL endoscopy were assessed.

In summary, the results of this independent blinded validation study indicate that NDBE patients scoring high risk with this automated risk prediction assay progress to HGD and/or cancer within 2–5 years at a similar rate to BE patients with expert-confirmed LGD. This high-risk subset of NDBE patients may benefit from either an intensified surveillance program or may even be considered for preventive endoscopic ablation. The predictive performance of this assay can be increased by assessment of additional spatial and temporal specimens.

CONFLICTS OF INTEREST

Guarantor of the article: Jacques J.G.H.M. Bergman, MD, PhD.

Specific author contributions: N.F.F. provided administrative, technical and material support, acquired data, analyzed and interpreted data, conceived and designed the study, reviewed and revised the manuscript, and approved the final draft submitted. K.K. provided administrative, technical and material support, acquired data, analyzed and interpreted data, reviewed and revised the manuscript, and approved the final draft submitted. E.A.B. provided administrative, technical and material support, acquired data, analyzed and interpreted data, reviewed and revised the manuscript, and approved the final draft submitted. K.S. provided administrative, technical and material support, acquired data, analyzed and interpreted data, reviewed and revised the manuscript, and approved the final draft submitted. Y.Z. analyzed and interpreted data, reviewed and revised the manuscript, and approved the final draft submitted. R.E.P. analyzed and interpreted data, reviewed and revised the manuscript, and approved the final draft submitted. R.J.C.-T. provided administrative, technical and material support, acquired data, analyzed and interpreted data, supervised the study, reviewed and revised the manuscript, and approved the final draft submitted. J.J.G.H.M.B. provided administrative, technical and material support, analyzed and interpreted data, supervised the study, conceived and designed the study, reviewed and revised the manuscript, and approved the final draft submitted.

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Potential competing interests: E.A.B. and K.S. have ownership interest (stock options) in Cernostics, Inc., R.J.C.-T. has ownership interest (stock, stock options, and patents) in Cernostics, Inc., and Y.Z. is a consultant to Cernostics, Inc. J.J.G.H.M.B. is the recipient of

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Study Highlights

WHAT IS KNOWN

- ✓ An objective risk prediction assay has been shown to identify nondysplastic Barrett's patients who are at increased risk of progression.
- ✓ Spatial and temporal variability may affect the performance of a biomarker.

WHAT IS NEW HERE

- ✓ The assay identified nondysplastic Barrett's patients who progress at a rate comparable with patients with expert confirmed LGD.
- ✓ Assessing biopsies from additional spatial levels and time points increased the performance of the assay and identified most future progressors.

TRANSLATIONAL IMPACT

- ✓ Applying the assay to esophageal specimens acquired through tools such as brushes and sponges that sample a larger area of the Barrett's segment may further improve the performance of the assay.

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