Original research

Liquid biopsy to identify Barrett's oesophagus, dysplasia and oesophageal adenocarcinoma: the *EMERALD* multicentre study

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ABSTRACT

Background There is no clinically relevant serological marker for the early detection of oesophageal adenocarcinoma (EAC) and its precursor lesion, Barrett's oesophagus (BE).

Objective To develop and test a blood-based assay for EAC and BE.

Design Oesophageal MicroRNAs of BaRRett, Adenocarcinoma and Dysplasia (*EMERALD*) was a large, international, multicentre biomarker cohort study involving 792 patient samples from 4 countries (NCT06381583) to develop and validate a circulating miRNA signature for the early detection of EAC and high-risk BE. Tissue-based miRNA sequencing and microarray datasets (n=134) were used to identify candidate miRNAs of diagnostic potential, followed by validation using 42 pairs of matched cancer and normal tissues. The usefulness of the candidate miRNAs was initially assessed using 108 sera (44 EAC, 34 EAC precursors and 30 non-disease controls). We finally trained a machine learning model (XGBoost+AdaBoost) on RT-qPCR results from circulating miRNAs from a training cohort (n=160) and independently tested it in an external cohort (n=295).

Results After a strict process of biomarker discovery and selection, we identified six miRNAs that were overexpressed in all sera of patients compared with non-disease controls from three independent cohorts of different nationalities (miR-106b, miR-146a, miR-15a, miR-18a, miR-21 and miR-93). We established a six-miRNA diagnostic signature using the training cohort (area under the receiver operating characteristic curve (AUROC): 97.6%) and tested it in an independent cohort (AUROC: 91.9%). This assay could also identify patients with BE among patients with gastro-oesophageal reflux disease (AUROC: 94.8%, sensitivity: 92.8%, specificity: 85.1%).

Conclusion Using a comprehensive approach integrating unbiased genome-wide biomarker discovery and several independent experimental validations, we have developed and validated a novel blood test that might complement screening options for BE/EAC.

Trial registration number NCT06381583.

WHAT IS ALREADY KNOWN ON THIS TOPIC

⇒ There are no non-invasive biomarkers for early detection of oesophageal adenocarcinoma (EAC) and its precursor lesions, the second most lethal gastrointestinal malignancy. A blood-based test would complement the screening options available and likely improve patient outcomes.

WHAT THIS STUDY ADDS

⇒ We have developed and independently validated the robustness of a blood-based test named O*E*sophageal *M*icroRNAs of Ba*RR*ett, *A*denocarcinoma and *D*ysplasia ('*EMERALD*') for the early detection of both EAC and Barrett's oesophagus precancerous lesions in the largest multicentric biomarker cohort to date, an effort that involved institutes from four countries.

HOW THIS STUDY MIGHT AFFECT RESEARCH, PRACTICE OR POLICY

⇒ The *EMERALD* blood test could enrich the toolkit of methods available for EAC screening. Successful implementation of the *EMERALD* assay would be expected to improve patient outcomes. Although the model may support a more cost-effective approach with 5 yearly *EMERALD* screening, a head-to-head study would provide definitive evidence to guide clinical practice.

INTRODUCTION

Oesophageal adenocarcinoma (EAC), the second most lethal gastrointestinal malignancy after pancreatic cancer, is linked to chronic exposure to gastro-oesophageal reflux disease (GERD) and the development of Barrett's oesophagus (BE).[1–3] The progression from BE to low-grade dysplasia (LGD), high-grade dysplasia (HGD) and ultimately EAC unfolds slowly over approximately 20 years (1%–3% risk year).[4–6] This extended time frame theoretically offers ample opportunities for

cancer prevention. However, fewer than 20% of patients with BE receive a diagnosis before they are diagnosed with EAC.[7–9] Therefore, most EAC cases are diagnosed de novo, bypassing the window for preventive interventions.[10 11] Moreover, the lethality of EAC is further explained by its rapid progression from a localised stage to regional and distant metastases because the oesophageal anatomy, devoid of a serosa but rich in a dense lymphatic network, offers minimal resistance against the rapid and early spread of cancer.[12]

EAC represents a public health threat: its incidence has risen dramatically since the 1980s, and despite advances in therapy, the overall 5-year survival has remained below 20%.[13–15] These observations, coupled with evidence for the cost-effectiveness of endoscopic screening,[16–20] have led to recommendations for endoscopy for patients with persistent GERD or risk factors for BE and EAC.[21–25] While early detection of EAC alone would reduce mortality but not incidence, early detection of BE, followed by BE surveillance and treatment for LGD/HGD, can halt the progression to EAC with low rates of recurrence.[26–28] However, the current reliance on endoscopy has limitations that include its invasive nature, costs and potential discomfort, contributing to poor patient adherence to screening programmes.[29] Given the prevalence of GERD and the growing concern over EAC, new clinical strategies complementing current guidelines could be highly beneficial. A minimally invasive approach, such as a liquid biopsy targeting both precancerous lesions and early-stage EAC, may improve patient compliance. This study aimed to address this need by developing a diagnostic model of EAC and its precursor lesions, leveraging state-of-the-art machine learning (ML) driven by biological and clinical data.

ML involves identifying patterns within data and fitting models to the endpoint of interest.[30] Ensemble classifiers combine multiple weak learners (ie, the boosting procedure) to achieve higher accuracy.[30] To enhance the accuracy of these models, stacking involves creating a 'meta-model' on the predictions of the baseline models.[30] Importantly, models like XGBoost and AdaBoost prioritise accuracy alongside interpretability, enabling analysis of biomarker importance with techniques like SHAP values analysis.[30]

MicroRNAs (miRNAs), non-coding single-stranded RNAs regulating gene expression and various cellular processes, have been involved in EAC pathogenesis.[31] Due to their stability in body fluids and disease specificity, circulating miRNAs are potentially promising candidates for developing non-invasive liquid biopsies.[32] Individual biomarkers alone are often not discriminative enough for cancer detection. Therefore, biomarker panels are created to combine multiple biomarkers and increase the test's performance by virtue of ML approaches. Previous reports described potential circulating miRNAs for the diagnosis of EAC, but these studies lacked a systematic and comprehensive biomarker discovery approach or have not validated these biomarkers in multiple independent patient cohorts with adequate statistical power.[33 34] In addition, most of these studies have focused essentially on a single or handful of biomarkers, which has resulted in limited sensitivities and specificities.[33 34]

In this research effort, we leveraged ML and circulating miRNAs to develop a liquid biopsy assay diagnostic for EAC and BE. By integrating a systematic genome-wide biomarker discovery and clinical validation approach in more than 750 tissue and blood specimens from multiple independent patient cohorts with EAC, HGD, LGD, BE and healthy subjects from five countries (USA, UK, Ireland, Italy, Netherlands), we have identified, developed, and established a novel liquid biopsy assay ('*EMERALD*'—OEsophageal MicroRNAs of BaRRett, Adenocarcinoma and Dysplasia) to complement EAC screening and prevention. We conclude that, pending future prospective validation, our non-invasive circulating miRNA-based signature could potentially be transformative in the clinic and improve survival outcomes.

MATERIALS AND METHODS

Study populations

This study analysed data from 792 patients from publicly available miRNA expression datasets (N=134) and four prospectively collected independent clinical cohorts (N=658, figure 1). One clinical cohort was comprised entirely of histological biospecimens, and three were based on blood for the development, training and independent evaluation of the liquid biopsy assay (online supplemental table 1).

The in silico discovery phase used expression data from The Cancer Genome Atlas (TCGA) and Gene Expression Omnibus (accession number: GSE164560). It was designed to identify biomarkers differentially expressed between EAC tissue and normal oesophageal mucosa (N=134) and then select those demonstrating a statistically significant increase across disease stages (analysis of variance (ANOVA) $p<0.05$) from patient-matched normal mucosa to LGD, HGD, and EAC (N=32). We further excluded miRNAs lacking significant expression differences between EAC tissues (Stage I-III) and patient-matched normal mucosa (N=42 each) from a separate clinical cohort (Radboud University Medical Center, Netherlands). The remaining miRNAs were examined in our 'development cohort' (N=108), encompassing serum specimens from 51 patients with EAC/HGD, 27 with BE/LGD and 30 non-disease controls (NDCs) (Norton Thoracic Institute at St. Joseph's Hospital and Medical Center, Phoenix, Arizona, USA and Baylor University Medical Center, Dallas, Texas, USA).

The diagnostic assay was developed in the 'training cohort' (N=160), which included 96 patients with EAC/HGD (Veneto Institute of Oncology IOV-IRCCS, Padova, Italy) and 64 NDCs. Finally, the blood-based assay was externally and independently tested in the 'testing cohort' (N=306), which included 125 patients with EAC/HGD, 98 BE/LGD and 74 NDCs (Queen's University Belfast, UK and the National Cancer Registry Ireland, Ireland, for the FINBAR study (Factors INfluencing the Barrett's Adenocarcinoma Relationship); The Johns Hopkins Hospital, Baltimore, Maryland, USA; Translational Genomics Research Institute, Phoenix, Arizona, USA; 9 excluded after quality control). Full details for study populations are in Supplementary Methods.

All individuals diagnosed with EAC, HGD, LGD or BE were considered cases. All individuals who received a negative endoscopic evaluation of the foregut during BE screening were considered NDCs.[35] The presence of dysplasia was confirmed by a second pathologist with expertise in oesophageal diseases.[21 36]

Study design

This study was an international, multi-institutional, retro-prospective, multiphase biomarker study covering EDRN phases I, II and III (Early Detection Research Network), STARD (Standards for Reporting Diagnostic accuracy studies) and TRIPOD-AI (Transparent Reporting of a multivariable prediction model for Individual Prognosis or Diagnosis-Artificial Intelligence) compliant (both as supplementary) and included both tissue-based and the blood-based biospecimens. Briefly, EDRN phase I is intended to discover the biomarkers associated with the condition of interest (BE/EAC, in this case), EDRN phase

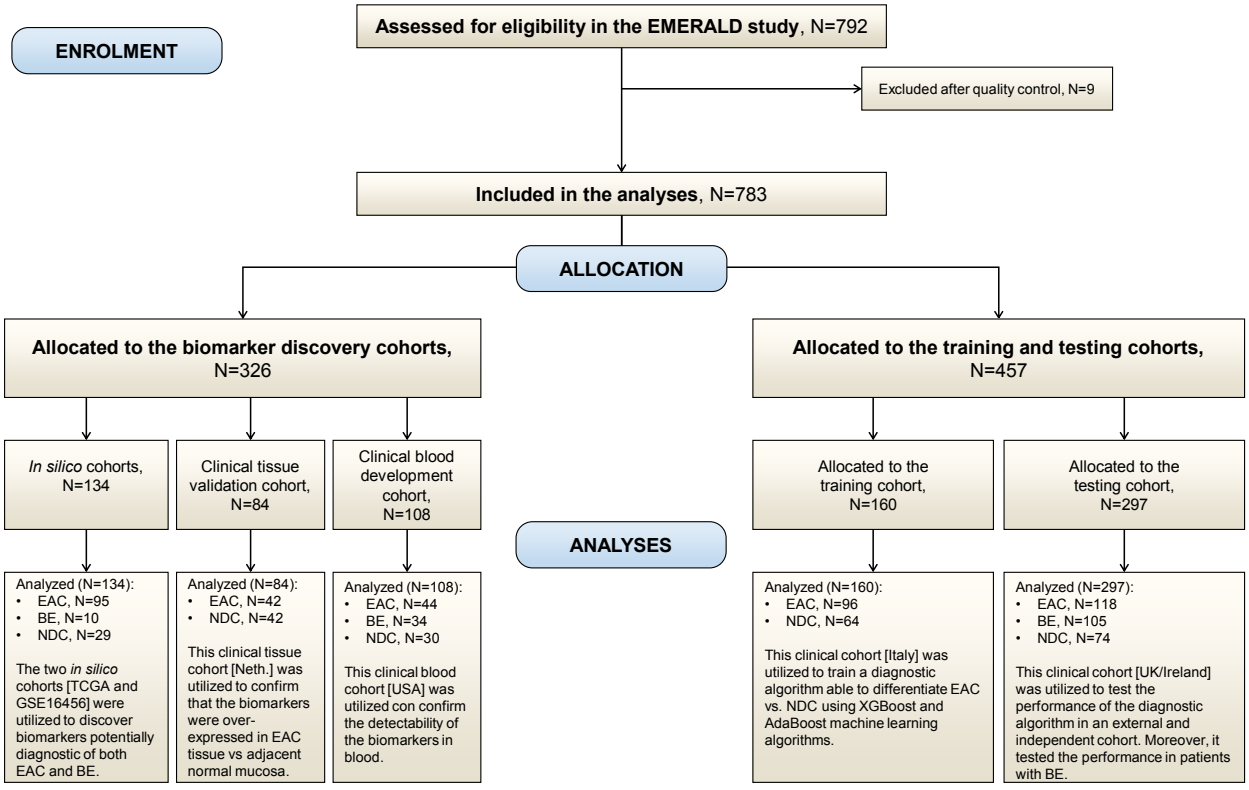


Figure 1 CONSORT diagram for study cohorts allocation. BE, Barrett's oesophagus; CONSORT, Consolidated Standards of Reporting Trail; EAC, oesophageal adenocarcinoma; EMERALD, Oesophageal MicroRNAs of BaRRett, Adenocarcinoma and Dysplasia; NDC, non-disease control; TCGA, The Cancer Genome Atlas.

II is intended to develop a diagnostic assay for the condition of interest, and EDRN phase III tests the performance of the assay in an independent and external cohort (online supplemental figure 1). Three independent tissue-based cohorts were used to discover biomarkers with diagnostic potential for EAC and BE and prioritise the biomarkers with the highest diagnostic potential (phase I). The serum biomarker panel was finalised in the blood-based biomarker 'development cohort'. Subsequently, we used a two-level ML approach to train an EAC/BE risk-score formula ('*EMERALD*') on qRT-PCR data from the training cohort (N=160, phase II). Finally, the model was fully locked and then tested in an independent, non-overlapping, external testing cohort (N=306, phase III).

Assay

Tissue samples were collected therapy-naïve, placed in RNAlater immediately after surgery and stored at −80°C. Whole blood samples were collected primarily before treatment, centrifuged at 3000 g for 10 min within 12 hours after collection and stored in RNase-free Eppendorf tubes at −80°C.

RNA was isolated from tissue and serum using the RNeasy Mini and miRNeasy Serum/Plasma Kits, respectively (Qiagen, Valencia, California, USA). RNA was then reverse-transcribed using the TaqMan MicroRNA Reverse Transcription Kit (Applied Biosystems). Real-time PCRs were conducted using MicroRNA Assay Kits and TaqMan Universal Master Mix II using QuantStudio 6 Flex Real-Time PCR System (Applied Biosystems). The expression of miRNAs was normalised against U6, a commonly used endogenous control (Ambion, Austin, Texas, USA), and the data were $2^{-\Delta Ct}$ transformed. Further details are in Supplementary Methods.

Statistical approaches

Candidate biomarkers were selected in the TCGA dataset based on the following criteria: $\log_2$(fold-change) >1 (EAC/HGC vs NDCs), a Benjamini-Hochberg-adjusted $p<0.00001$, an individual candidate area under the receiver operating characteristic curve (AUROC) >70%, and an average miRNA expression level higher than the median of all differentially expressed miRNAs. This phase was adequately powered (power=0.94) to detect a twofold change ($\rho=200\%$) at a false discovery rate of 5% under conservative specifications of the depth of coverage for the transcript ($\lambda_0=30$ times) and coefficient of variation in expression between samples (CV=0.5).

In all qPCR experiments, expression levels were compared using two-sided Student's t-tests for paired comparisons and ANOVA for comparisons between multiple groups. A $p<0.05$ was considered statistically significant. The AUROCs with 95% CI were computed by the method of DeLong, with optimal cut-off thresholds determined by Youden's index. The odds ratios (ORs) of disease as a function of the *EMERALD* score were computed with restricted cubic spline curves.

The final diagnostic model, named *EMERALD*, involved two independently trained ML models using six candidate biomarkers. The two algorithms used, XGBoost and AdaBoost, are popular models that employ a sequential iterative boosting strategy from weaker learners (decision trees for XGBoost and decision stumps for AdaBoost). Multivariate logistic regression

was used to derive a formula to predict EAC risk from the two models. This model was fully locked for independent and external testing.

We employed a Markov model to simulate five cohorts of patients with chronic GERD, all aged 45 years, undergoing one of five screening options: endoscopy every 10 years, *EMERALD*-based screening every 5 years, 3 years or 1 year, vs no screening. The model uses a cycle time of 1 year and runs for 30 years, allowing one to observe early detection, disease prevention, and disease stage anticipation (stage shift). Quality-adjusted life-years (QALYs) and costs serve as the primary outcome measures. The model incorporates the possibility of progression to BE, dysplastic BE, and, ultimately, EAC. The natural history of EAC, the compliance with screening, the treatment outcomes, and the associated costs were derived from the literature or, when unavailable, were internally derived.[37–40] Full details on the Markov model assumptions are presented in Supplementary Methods and online supplemental table 2. All analyses were performed in R.

RESULTS

Characteristics of patients with EAC, LGD, and HGD, and healthy subjects

The characteristics of the study participants are summarised in online supplemental table 1. There were no significant differences in the distribution of age and sex between cases and NDCs in any cohort. Tissue and serum specimens from patients with EAC, HGD, LGD and BE, as well as healthy subjects used in this study, were primarily collected prior to surgery or chemotherapy treatment. Overall, our study included 783 individual patient-derived specimens, of whom 134 were from in silico studies, 84 were from a tissue cohort (42 vs 42) and 565 were from the blood cohorts (258 EAC, 139 BE and 168 NDCs). All participants labelled as NDCs underwent upper endoscopic examination to exclude foregut diseases.

Identification of candidate miRNAs

In the first part of the tissue-based phase, we interrogated the TCGA miRNA expression dataset to identify candidate biomarkers that can distinguish patients with EAC from healthy subjects. We initially identified 22 differentially expressed miRNAs based on differential gene expression and significance level (figure 2A). We then ranked them based on AUROC values and selected only the candidates that demonstrated a discriminative AUROC value of >70% and, finally, excluded the miRNAs with a low expression level. After this initial selection, we identified a pool of 14 candidate biomarkers of potential diagnostic interest (figure 2B). Applying the unweighted pair-group Ward-D2 method for unsupervised clustering, only the cancer status was co-segregated with unsupervised clustering, while other clinical characteristics (biological sex, stage, histological differentiation and race) did not (figure 2C). Next, we tested whether these biomarkers were also differentially expressed during the malignant progression from BE to LGD, HGD and EAC. Using a public dataset (GSE16456) of 32 tissue biospecimens (6 EAC, 5 HGD and 5 LGD with corresponding patient-matched normal mucosa), we assessed the miRNA expression levels of these 14 candidates, quantified with microarrays (as opposed to sequencing). We observed a statistically significant trend of progressively increasing expression from normal mucosa towards EAC (ANOVA, $p<0.05$) for 10 of the 14 biomarkers. Because the overall aim of this study was to develop a biomarker signature capable of detecting EAC and its precursor lesions, four miRNAs were excluded (hsa-miR-135b-5p, hsa-miR-196a-1-5 p, hsa-miR-335-3 p and hsa-miR-15b-5p, online supplemental figure 2).

Transitioning from *in silico* analyses to RT-qPCR, we evaluated the expression levels of the remaining 10 candidates in a clinical cohort of 42 EAC patients with patient-matched adjacent normal tissue specimens. We confirmed the robustness of the in silico predictions by verifying that nine were significantly over-expressed in EAC tissues also in the first clinical cohort of our study ($p<0.05$, two-sided paired Student's t-tests; online supplemental figure 3). Therefore, after the exclusion of one miRNA (hsa-miR-17-5 p), nine biomarker candidates were given full consideration for their ability to differentiate EAC from NDCs and were therefore carried over to the blood-based phase of our study.

In summary, the tissue-based discovery phase employed a systematic approach to identify a panel of nine candidate miRNAs independently associated with both EAC and its precursor lesions across three quantification methods (sequencing, microarray and RT-qPCR) in three independent cohorts.

Development of the circulating miRNA panel

On transitioning our tissue-based discovery phase into a blood-based assay, we sought to confirm whether the nine tissue-derived candidate miRNAs could be measured in blood and if these were also upregulated in the serum collected from EAC patients. In our development cohort (N=108; 78 cases vs 30 NDCs), three miRNAs (hsa-miR-181a-5p, hsa-miR-181b-5p and hsa-miR-196b-5p) had an expression level below the detection limit (average cycle threshold >35) and were excluded. The remaining six miRNAs (hsa-miR-106b-5p, hsa-miR-146a-5p, hsa-miR-15a-5p, hsa-miR-18a-5p, hsa-miR-21-5 p and hsa-miR-93-5 p) were abundant in blood and could be carried over to the subsequent phases of our study (model training and independent testing). Interestingly, these biomarkers all demonstrated the potential to discriminate cases versus NDCs, with AUROC values ranging from 62.1% to 80.8% (figure 3). More importantly, we observed that five of these biomarkers were significantly upregulated in the EAC/HGD vs NDC pair-wise comparison and in patients with BE/LGD compared with NDCs, supporting their potential as non-invasive biomarkers of EAC precancerous lesions ($p<0.05$). Four of these biomarkers were expressed at similar levels in BE/LGD and EAC/HGD, potentially indicating that they represent neoplastic processes that occur early during oesophageal malignant transformation and may be of diagnostic potential for both EAC and its precursor lesions. To assess the cancer-specificity of our six selected miRNAs, we compared their blood expression levels in two large and independent multi-cancer datasets (GSE113486, N=580, of whom N=100 NDCs and N=480 individuals with 1 of 12 cancers; and GSE113740, N=1334, of whom N=1033 NDCs and N=301 with 1 of 12 cancers). Differential gene expression analysis revealed virtually no statistically significant differences in miRNA levels in blood between individuals with various cancers and their respective controls (online supplemental figure 4). This corroborates the high EAC/BE specificity for the miRNAs that were selected during the discovery phase of this study.

Development and independent testing of a circulating miRNA signature

Next, we assessed the expression of these six miRNAs in the training cohort (N=160, 96 EAC patients, of whom 23 had stage 0 disease, pT_{is}; 64 NDCs). In this cohort, too, all six miRNAs were

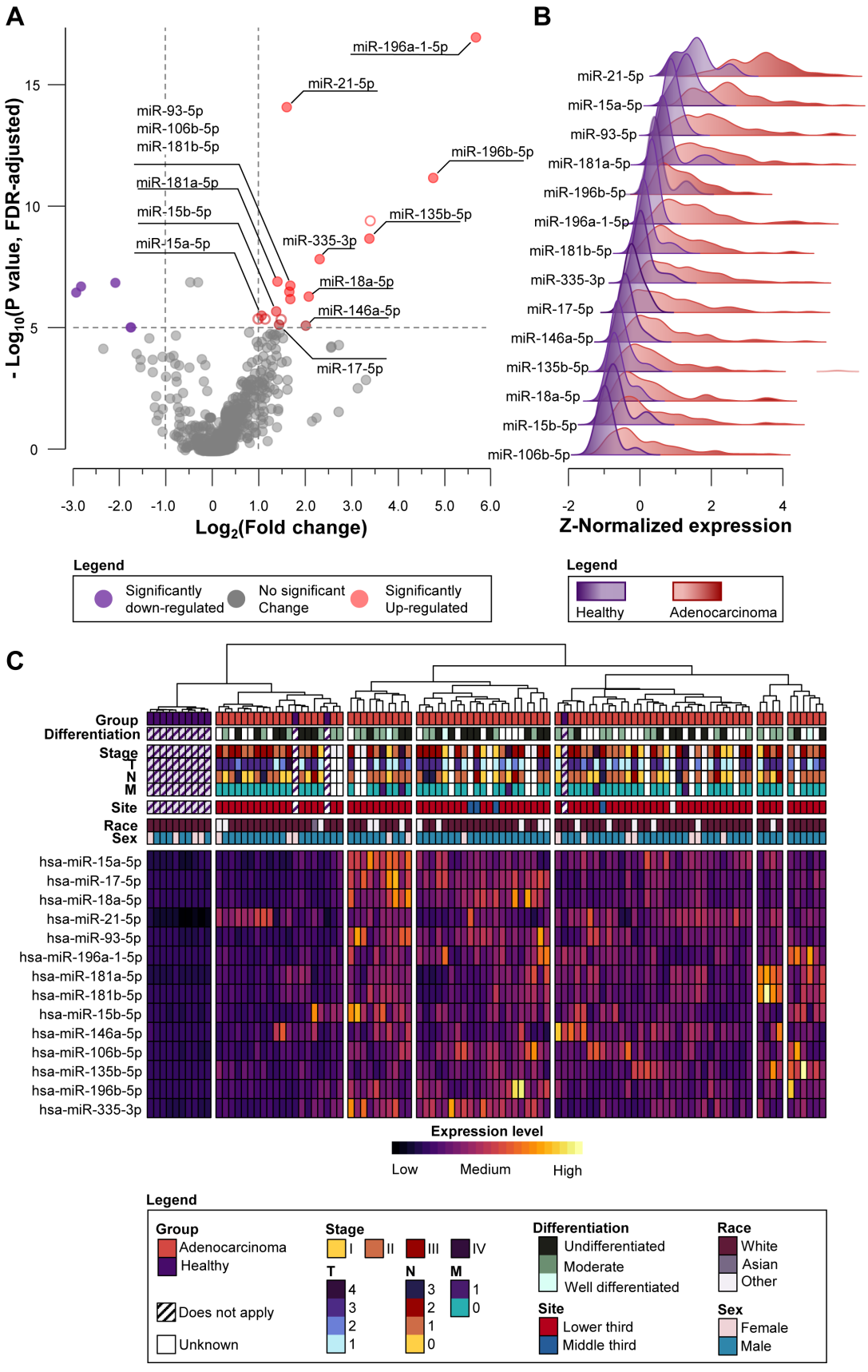


Figure 2 In silico discovery and prioritisation of candidate miRNAs. (A) Volcano plot of differentially expressed microRNAs between cases and controls using TCGA miRNA expression dataset. Colour grading follows significance. 22 miRNAs were differentially expressed and 14 miRNAs (identified as fully coloured) were prioritised as our initial candidates for further analysis. (B) Ridgeline plot of the initial pool of 14 microRNAs; (C) A heatmap with unsupervised clustering illustrates the expression levels of the 14 candidate miRNAs in the TCGA miRNA expression dataset. FDR, false discovery rate; TCGA, The Cancer Genome Atlas.

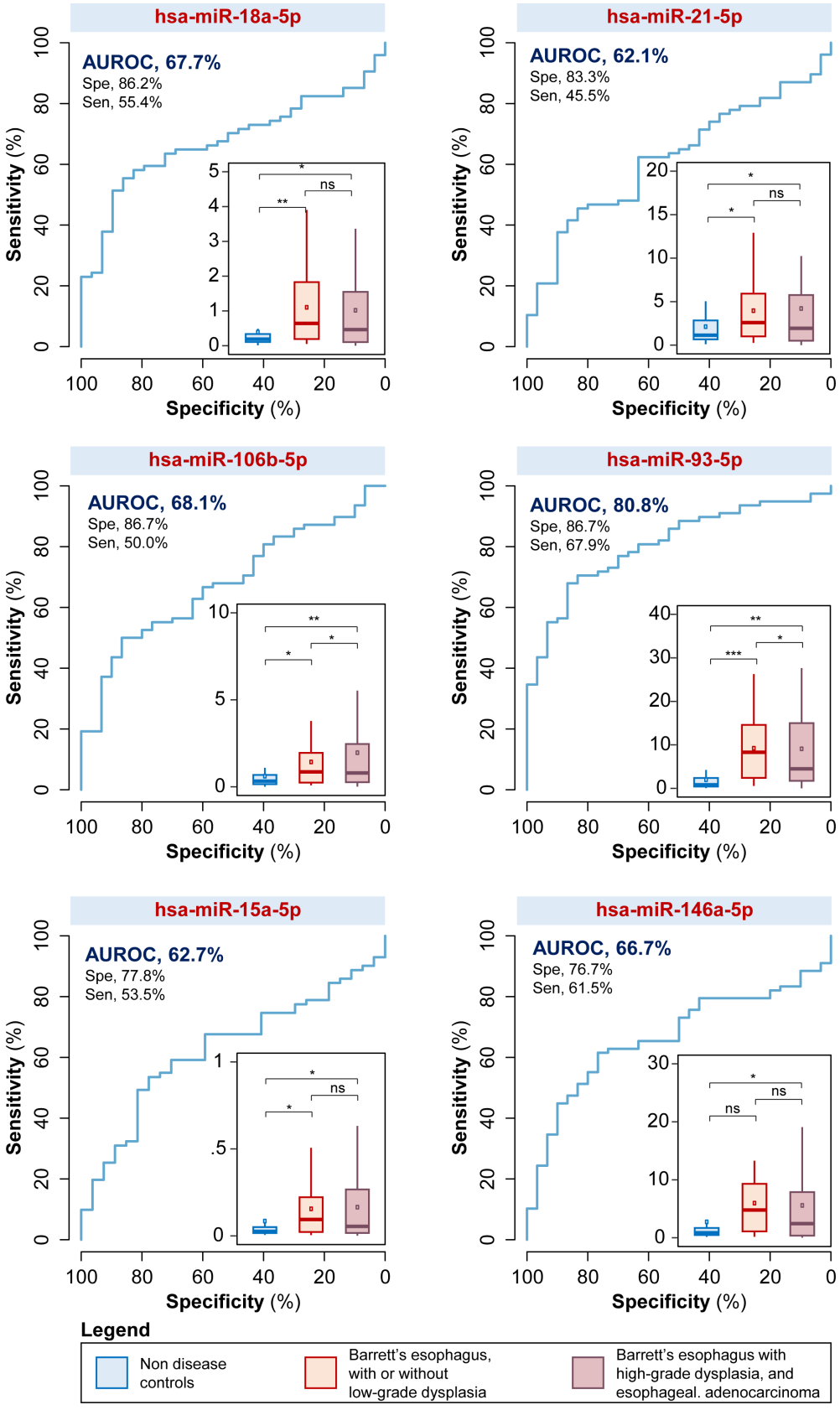
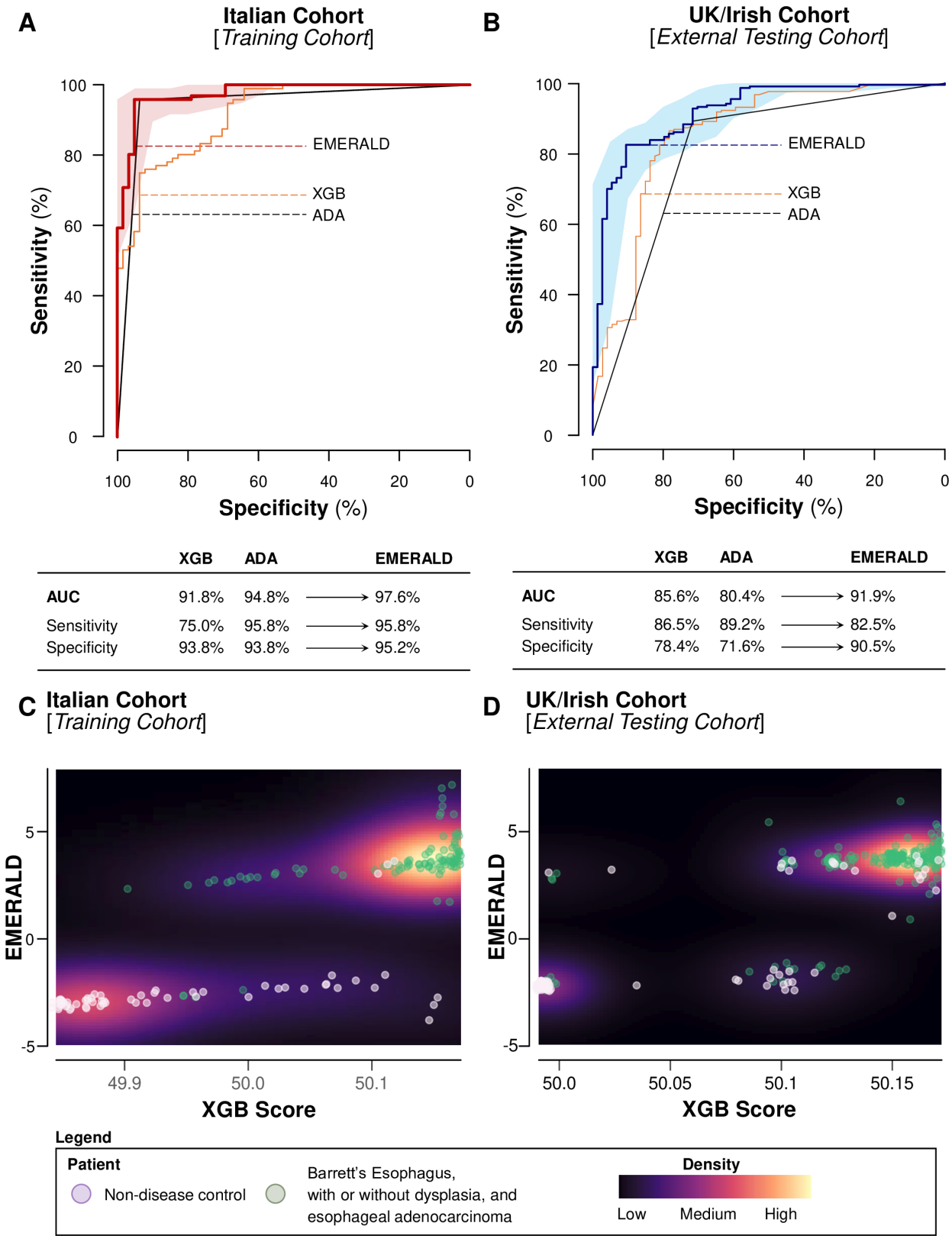


Figure 3 Assessment of the six miRNAs diagnostic potential in the US (development) cohort. Six circulating miRNAs were significantly upregulated in cases (EAC, HGD, LGD and BE) compared with NDCs and demonstrated AUROC values of diagnostic interest, ranging from 62.1% to 80.8%. In subgroup analysis (inserts), all candidate miRNAs demonstrated a significant differential expression between NDCs (blue) versus EAC/HGD (maroon), five for NDCs versus BE/LGD (orange) and two for BE/LGD versus EAC/HGD (*p<0.05, **p<0.01, ***p<0.001; two-sided Student's t-tests). AUROC, area under the receiver operating characteristic curve; BE, Barrett's oesophagus; EAC, oesophageal adenocarcinoma; HGD, high-grade dysplasia; LGD, low-grade dysplasia; NDC, non-disease controls; ns, not significant; Sen, sensitivity; Spe, specificity.



	XGB	ADA	EMERALD
AUC	91.8%	94.8%	→ 97.6%
Sensitivity	75.0%	95.8%	→ 95.8%
Specificity	93.8%	93.8%	→ 95.2%

	XGB	ADA	EMERALD
AUC	85.6%	80.4%	→ 91.9%
Sensitivity	86.5%	89.2%	→ 82.5%
Specificity	78.4%	71.6%	→ 90.5%

Figure 4 Establishment and external testing of the six circulating-miRNA signature (A) AUROC and performance metrics of the two first-level classifiers (XGB: XGBoost, orange; ADA, AdaBoost, black) and the resulting stacked model (*EMERALD*) in the Italian (training) cohort. (B) AUROC and performance metrics of the two first-level classifiers (XGB: XGBoost, orange; ADA, AdaBoost, black) and the resulting stacked model (*EMERALD*) in the UK/Irish (testing) cohort; (C, D) Density-scatter plots: the background colour gradient is a density plot and highlights the areas where most observations are encountered, without knowledge of them being cases and controls. The dots (green for cases and pink for NDCs) represent the scatter plot. The same patterns can be seen in both the Italian (training, C) and UK/Irish (testing, D) cohorts, with two clusters where cases and controls appear to separate (top right and bottom left, respectively). AUROC, area under the receiver operating characteristic curve; EMERALD, Oesophageal MicroRNAs of BaRRett, Adenocarcinoma and Dysplasia.

Table 1 Statistical evaluations of the *EMERALD* test for differentiating cases from controls in the training and testing cohorts

	Training cohort (N=160)			External and independent testing cohort (N=297)		
Area under the receiver operating characteristic curve (95% CI)	**97.6% (95.5%–99.6%)**			**91.9% (88.3%–95.5%)**		
	No.	**No. detected**	**Sensitivity (95% CI)**	**No.**	**No. detected**	**Sensitivity (95% CI)**
Oesophageal adenocarcinoma and Barrett's oesophagus, with or without dysplasia	96	92	95.8% (89.8% to 98.4%)	223	184	82.5% (77.0% to 86.9%
EAC/HGD	96	92	95.8% (89.8% to 98.4%)	125	105	84.0% (76.6% to 90.4%)
BE/LGD*	--	--	--	98	79	80.6% (71.7% to 87.2%)
	No.	**No. negative**	**Specificity (95% CI)**	**No.**	**No. negative**	**Specificity (95% CI)**
Non-disease controls, all	64	61	95.2% (87.1% to 98.4%)	74	67	90.5% (81.7% to 95.3%)
Non-disease controls, with long-standing or refractory heartburn†	--	--	--	47	44	93.6% (82.8% to 97.8%)
Non-disease controls, with dysphagia, dynophagia or regurgitation†	--	--	--	39	37	94.9% (83.1% to 98.6%)
Non-disease controls, with epigastralgia†	--	--	--	51	50	98.0% (89.7% to 99.7%)

*The training cohort enrolled several Tis but not BE/LGD.
†The training cohort did not include symptom-specific data other than the indication for BE screening.
BE, Barrett's oesophagus; EAC, oesophageal adenocarcinoma; EMERALD, Oesophageal MicroRNAs of BaRRett, Adenocarcinoma and Dysplasia; HGD, high-grade dysplasia; LGD, low-grade dysplasia.

significantly upregulated in the serum of patients compared with NDCs ($p<0.05$, two-sided Student's t-tests; online supplemental figure 5). We then trained a stacked ML model (XGBoost+AdaBoost, online supplemental figure 6A) based on ΔCt values. The resulting classifier, *EMERALD*, was based on the contributions from both XGBoost and AdaBoost. The most important contributors to both models were hsa-miR-106b-5p, hsa-miR-93-5 p and hsa-miR-18a-5p (online supplemental figure 6B,C for XGBoost; online supplemental figure 6D,E for AdaBoost). This approach allowed the stratification of patients in the training cohort into high and low-risk groups based on Youden's index (positivity threshold=3.403). As a result, the blood-based test achieved a high performance in distinguishing EAC patients from NDCs with an AUROC of 97.6% (95% CI 95.5% to 99.6%), with a corresponding sensitivity of 95.8% and a specificity of 95.2% (figure 4A). Importantly, the assay's sensitivity for Tis was 95.7% (95% CI 79.0% to 99.2%), 100.0% for stage I (95% CI 100% to 100%) and 92.0% for stage II EAC (95% CI 75.0% to 97.8%).

To independently test the diagnostic accuracy and performance of our *EMERALD* liquid biopsy, we evaluated the signature's robustness in an external testing cohort. Among the 297 serum samples included in the testing cohort (118 EAC, 105 BE, 74 NDCs), we observed substantial replicability of our training efforts, where the six-miRNA signature maintained a strong ability to distinguish patients from NDCs, with an AUROC value of 91.9% (figure 4B) and corresponding sensitivity and specificity values of 82.5% and 90.5%, respectively (table 1), including sensitivity of 85.6% for EAC (95% CI 78.1% to 90.8%) and 85.2% for non-dysplastic BE (95% CI 76.4% to 91.2%). More interestingly, the key hypothesis of our machine-learning approach was that combining two algorithms would enhance assay robustness. The density plots (figure 4C,D, respectively) confirm this, as the distribution of cases and NDCs in both training and testing cohorts closely resemble each other. Two distinct clusters emerge in both cohorts—one enriched with cases (green) in the top-right corner and another enriched with NDCs (pink) in the bottom-left corner.

It is noteworthy that while the training cohort primarily consisted of patients with early stage or in situ EAC, the testing cohort also included a significant number of patients with precancerous lesions (BE, LGD, or HGD). Despite this compositional difference, patients with either EAC or precancerous lesions consistently displayed higher *EMERALD* values compared with NDCs in both cohorts (figure 5A,B, respectively). Furthermore, we investigated whether higher *EMERALD* scores correlated with a greater likelihood of oesophageal disease. Interestingly, in both cohorts, the ORs of having the disease progressively increased with higher *EMERALD* values (figure 5C,D, respectively). The overall trend of the spline curves supports the reproducibility of the assay across both cohorts despite their differences.

Discriminatory capacity among controls with GERD-related symptoms

All NDCs met the criteria for BE screening, and their endoscopic evaluation was negative (chronic GERD or risk factors for BE and EAC[21–25]). Next, we evaluated the model's performance according to symptom clusters. In the testing cohort, 63.5% of the NDCs had long-standing, persistent, or treatment-refractory heartburn, with a few having reflux oesophagitis (figure 6A). The results were promising, demonstrating that *EMERALD* values were higher in cases than controls (figure 6B, $p<0.0001$ for all comparisons, Student's t-tests). There was no statistically significant difference between GERD patients with versus without reflux oesophagitis ($p=0.79$) and between BE/LGD versus EAC/HGD ($p=0.45$). Indeed, the circulating six-miRNA signature maintained a robust discriminatory power for the aggregate measure of oesophageal cases versus symptomatic controls (N=270; AUROC=95.0%, 95% CI 92.2% to 97.9%) and, most importantly, it distinguished premalignant lesions from symptomatic controls (n=145; AUROC: 94.8%, 95% CI 91.3% to 98.2%; figure 6C). In addition, the performance of the six-miRNA signature was superior to individual miRNAs when discriminating EAC/HGD from symptomatic controls

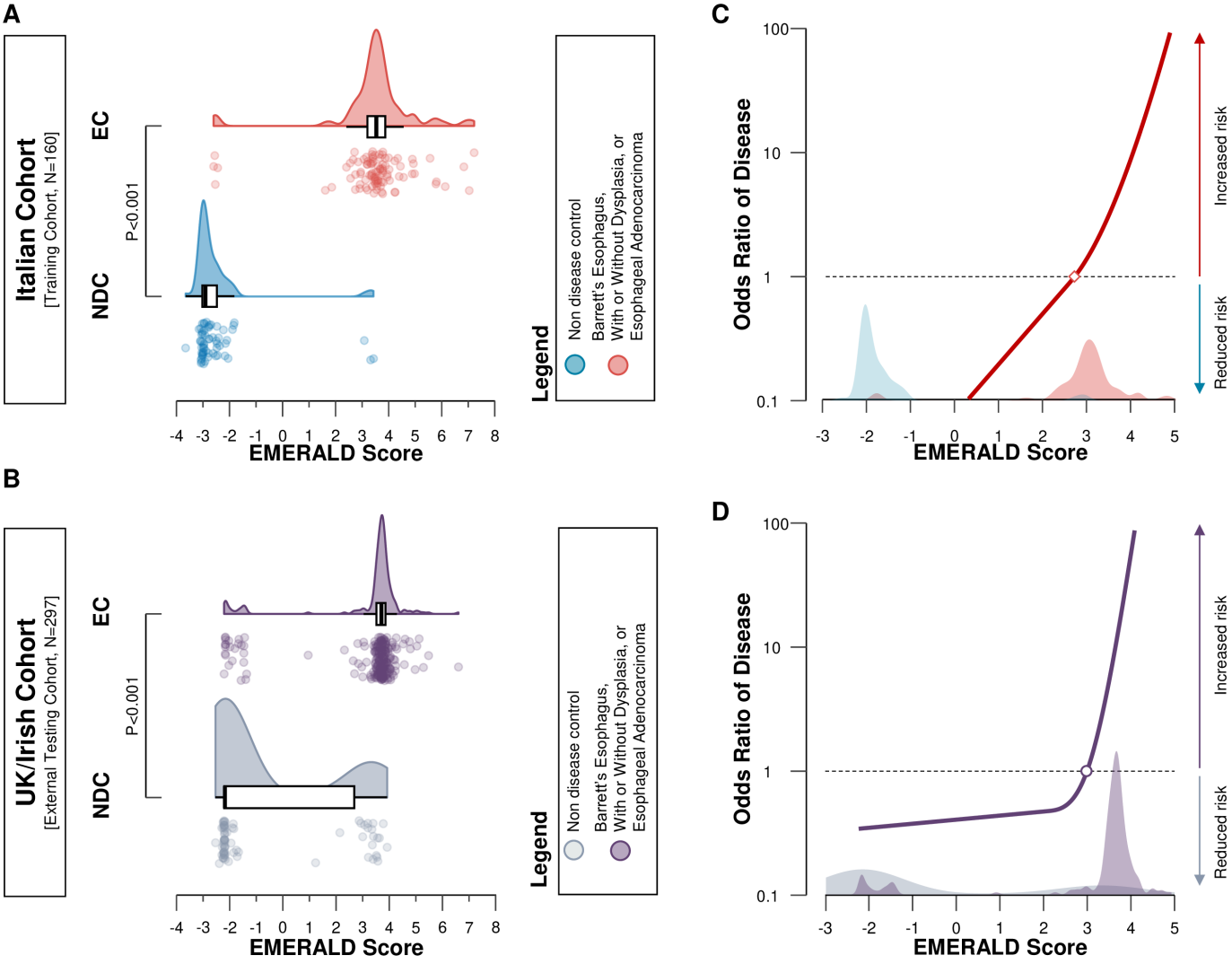


Figure 5 Evaluation of the diagnostic performance of the six-circulating-miRNA signature. (A, B) Raincloud plots with super-imposed box and whisker plots demonstrating the distribution of EMERALD values between cases and controls in the Italian (training, A) and UK/Irish (testing, B) cohorts; (C, D) ORs for the presence of a compound endpoint of EAC, HGD, LGD or BE with restricted cubic splines in the Italian (training, C) and UK/Irish (testing, D) cohorts. BE, Barrett's oesophagus; EAC, oesophageal adenocarcinoma; EC, Oesophageal cases; HGD, high-grade dysplasia; LGD, low-grade dysplasia; NDC, Non-disease controls.

(N=172, figure 6D), achieving an AUROC value of 95.3% (95% CI 92.2% to 98.3%). Similar results were observed among those who reported symptoms other than heartburn, including epigastralgia (online supplemental figure 7A–C) or dysphagia, odinophagia, and regurgitation (online supplemental figure 7D–F). Finally, to assist clinicians in prioritising endoscopic evaluations, we conducted a multinomial multivariate logistic regression analysis. This analysis used the same biomarkers to predict, among EMERALD-positive subjects, the 'high-risk' (EAC/HGD) and the 'low-risk' (BE/LGD) positive subjects. This analysis identified high-risk and low-risk subgroups within *EMERALD*-positive subjects, providing valuable information for risk stratification (online supplemental figure 8). Collectively, these data confirmed the diagnostic significance of our non-invasive, six-miRNA signature and highlighted its ability to separate symptomatic patients with premalignant or malignant diseases from those without.

Finally, using the sensitivity and specificity calculated in the testing cohort, we constructed a Markov-based decision model to simulate the course of events for five cohorts of patients, aged 45 at simulation start, undergoing no screening, endoscopy-based screening every 10 years, or *EMERALD*-based screening every 5 years, 3 years or 1 year for 30 years (figure 7A). The results of our cost-effectiveness analysis support that both an endoscopy-first approach and a non-invasive approach would lead to a stage-shift effect, where an increase in early-stage diagnoses would be observed after a few years, with a subsequent decrease in the number of late-stage diagnoses (figure 7B–E), which would result in a reduction in mortality (figure 7F). While the costs of a more compliant and non-invasive approach would initially surpass the costs of a less compliant endoscopy-first approach (especially at more frequent intervals of testing), the overall costs would be in favour of a non-invasive programme after 15 years, justified by a reduction in the number of patients with more advanced-stage and more expensive diagnoses (figure 7G), with a corresponding increase in QALYs (figure 7H). Finally, we estimated the cost-effective frontier to be in favour of a non-invasive, *EMERALD*-based approach at 5-year intervals (figure 7I), which would reduce the number of cancer-attributable deaths with a stage-shift effect for a lower price than other intervals of testing (Supplementary Table 3).

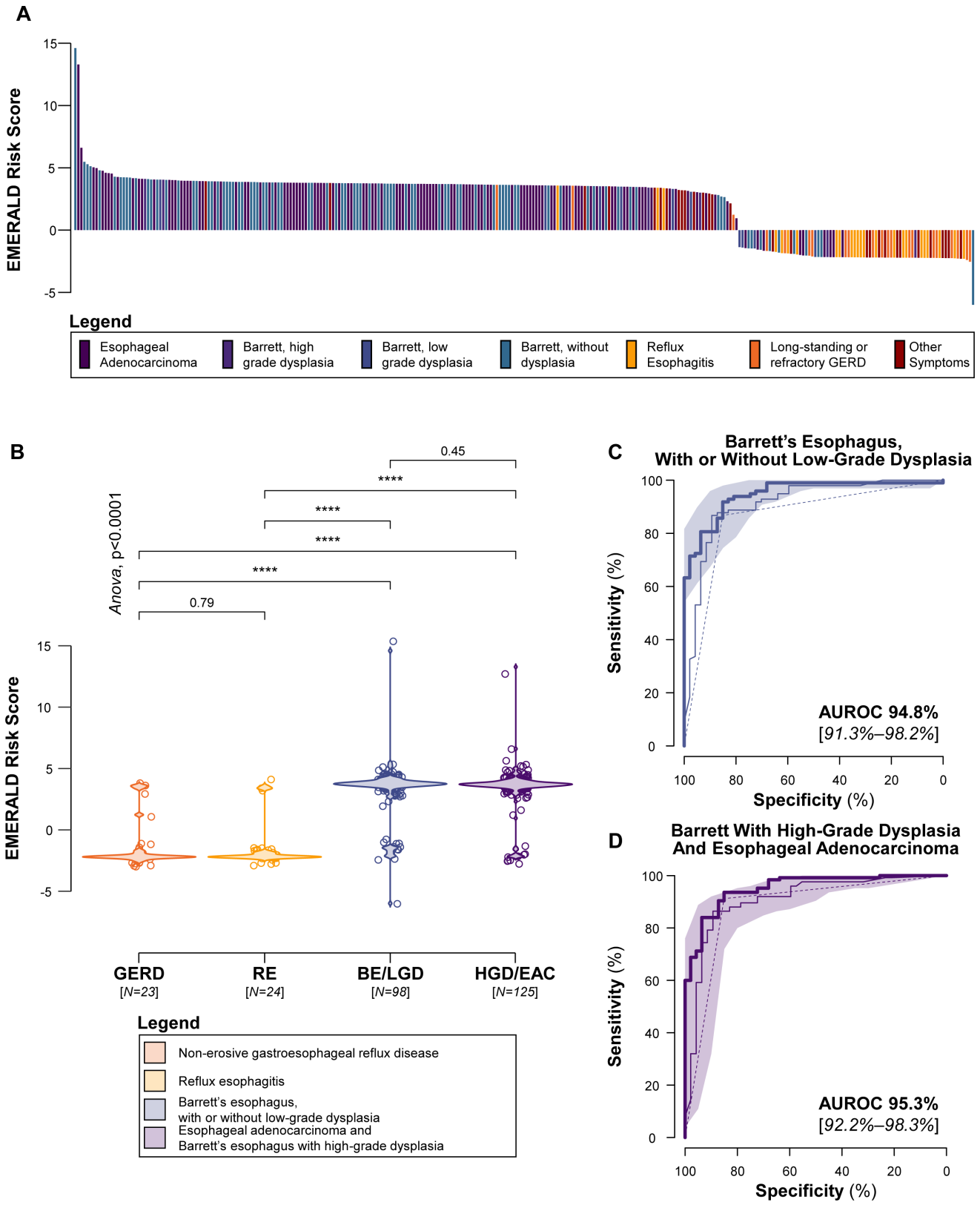


Figure 6 Evaluation of the *EMERALD* assay among symptomatic controls with GERD. (A) Waterfall plot of the *EMERALD* values in the UK/Irish (testing) cohort, with controls sublabelled by the presence of symptoms; (B) Violin plot of *EMERALD* values according to the presence of GERD-related symptoms, with and without reflux oesophagitis versus cases with BE or LGE and cases with EAC or HGD (****p<0.0001; two-sided Student's t-tests). (C, D) AUROCs of the two first-level classifiers (XGB: thin line; ADA, dashed line) and the stacked model (thick line with 95% CIs) in a subanalysis of the UK/Irish (testing) cohort which only included symptomatic controls versus BE cases, with or without LGD (C) and cases with either HGD or adenocarcinoma (D).AUROC, area under the receiver operating characteristic curve; BE, Barrett's oesophagus; EAC, oesophageal adenocarcinoma; EMERALD, Oesophageal MicroRNAs of BaRRett, Adenocarcinoma, and Dysplasia; GERD, gastro-oesophageal reflux disease; HGD, high-grade dysplasia; LGD, low-grade dysplasia; RE, reflux oesophagitis.

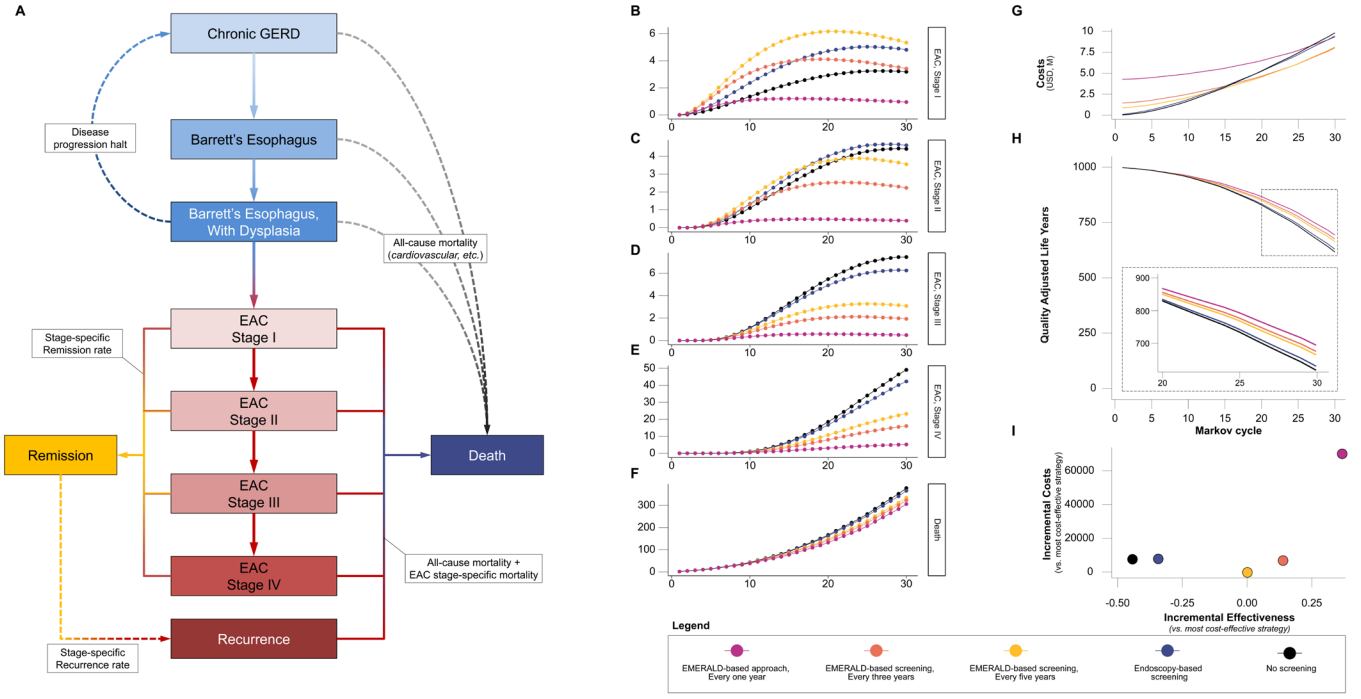


Figure 7 Markov simulation. (A) Markov model structure: for each strategy, the Markov chain assumes a progression through pre-malignant disease, malignant disease, cancer recovery, recurrence, and, eventually death. Mortality can be from any cause (cardiovascular, other cancers, etc) or stage-specific mortality rates. Early detection of Barrett's oesophagus with dysplasia is assumed to trigger its eradication. EAC diagnosis, whether due to symptoms, endoscopic screening uptake, or non-invasive screening uptake, is assumed to trigger treatment. Costs and utility values are derived from the literature or, if unavailable, from internal estimates. The starting condition of each individual in the Markov chain is a 45-year-old with chronic GERD who is followed up for 30 years or until death. Five screening strategies are tested: non-invasive, *EMERALD*-based screening at 45% compliance every 5, 3 or 1 year(s), endoscopy-based screening at 10% compliance every 10–15 years, or no screening. (B) Number of stage I EAC diagnoses per screening strategy. (C) Number of stage II EAC diagnoses per screening strategy. (D) Number of stage III EAC diagnoses per screening strategy. (E) Number of stage IV EAC diagnoses per screening strategy. (F) Number of deaths from all causes (both EAC and non-EAC related) per screening strategy. (G) Annual costs associated with each screening strategy. (H) Quality-adjusted life-years per year by screening strategy, with a call-out box to demonstrate the effects of different screening strategies. (I) Incremental costs and effectiveness of each screening strategy against the most cost-effective screening strategy and interval. EAC, oesophageal adenocarcinoma; GERD, gastro-oesophageal reflux disease.

DISCUSSION

This multicentre study is the first to successfully develop and externally validate a liquid biopsy for the noninvasive diagnosis of EAC and BE (with and without dysplasia). We employed a multistep approach with multiple patient cohorts to ensure our final assay could capture the full spectrum of the disease, ranging from GERD to EAC. Leveraging ML and rigorously selected biomarkers, the *EMERALD* assay was developed and independently tested in two large cohorts. The assay effectively distinguished BE with and without dysplasia (LGD/HGD), as well as EAC, from control groups, including non-disease controls and chronic GERD patients with and without oesophagitis. This offers a valuable addition to the existing screening options for this aggressive and deadly cancer. Furthermore, using a Markov model, we estimated that implementing the *EMERALD* assay at a 5-year interval could offer the most cost-effective strategy to reduce EAC mortality and incidence.

Once a rare cancer, EAC has become the dominant form of oesophageal cancer in developed countries.[13 14] While treatment advancements have modestly improved survival rates, half of patients die within a year of their diagnosis.[15] A key limitation of the current approach lies in its reactive nature, focusing only on EAC. A more strategic approach would prioritise identifying individuals at high risk, those with BE.[16–20] Though largely benign, BE represents a precursor lesion for EAC. Early detection and intervention at this premalignant stage, with dysplasia surveillance and treatment, can interrupt the disease's natural history and prevent it.[26–28] The current screening strategies could benefit from additional, non-invasive options. The Cytosponge represents such an option, detecting the trefoil factor 3 through a patient-swallowed sponge that captures oesophageal epithelial cells.[41] In a pragmatic, multicentric trial, it demonstrated encouraging patient interest rates (39% expressing interest), with high sensitivity for BE (80%–90%, depending on the BE segment length) and specificity (92%).[42 43] A blood-based test expands the arsenal of screening tools by offering a readily repeatable, minimally invasive approach that might improve overall screening participation.

MiRNAs have long been recognised as promising non-invasive biomarkers due to their structural stability and abundance in circulation.[44] Studies examined the diagnostic potential of serum miRNAs in various cancers.[45] However, few attempted evaluation of the circulating miRNAs in EAC and BE, and most included analysis of single miRNAs or biomarkers not discovered in a comprehensive manner.[46] Not surprisingly, the diagnostic potential of these individual miRNA markers was limited, and the miRNA panels were constructed with biased criteria or lacked adequately powered clinical validation cohorts. Nonetheless, these studies highlighted the potential of circulating miRNAs and set the stage for more comprehensive studies.[33 34]

Interestingly, three of the biomarkers included in the *EMERALD* assay (miR-15a-5p, miR21-5p and miR-93-5 p) were previously identified as potential biomarkers for the non-invasive diagnosis of BE/EAC by others, too.[47–49] In this multicentre study, we first analysed independent cohorts of EAC tissues to identify the most consistently overexpressed miRNAs in EAC and HGD patients, hence ensuring their clinical relevance. Subsequently, we developed and validated a circulating miRNA signature by applying rigorous bioinformatic and statistical algorithms in multiple cohorts to validate the circulating biomarker panel. To the best of our knowledge, this is the largest cohort of patients with EAC, HGD, LGD, BE and healthy subjects, to date, to establish and validate such an approach. Nonetheless, we would like to acknowledge that our study was retrospective in nature, and future prospective studies will allow a more rigorous assessment of this blood-based test. Staging information was not available for patients with EAC from the FINBAR study (from which most of the testing samples were derived). Therefore, while we cannot absolutely confirm the stage-specific sensitivity derived from the training cohort, we do not expect a large gap in sensitivity values between early-stage and late-stage EAC, given the stability of all other sensitivity values for all other elements of the disease spectrum, including BE, LGD and HGD. The nature of our BE patient cohorts, primarily consisting of patients with long-segment BE, also restricts the analyses to this patient population. Therefore, future studies should investigate the correlation between *EMERALD* scores and BE lengths (including short-segment BE), and in those with gastric intestinal metaplasia. Additionally, geographical differences within the testing cohort could potentially introduce some bias, although the large sample size of the study minimises the impact of such factors. Finally, the *EMERALD* blood-based test is intended for diagnostic purposes and presently may not distinguish between BE cases that are poised to progress to EAC and those that are not, like the Cytosponge. However, other tools are available to predict the risk of progression and may be used with *EMERALD* once the blood-based test returns positive results.[50]

In conclusion, we have developed a non-invasive blood assay for the early detection of EAC and its precursor lesions using a comprehensive biomarker discovery approach and successfully validated its robustness using independent multicentre cohorts. Our *EMERALD* assay has the potential to transform liquid biopsy-based cancer and high-risk precancer screening of EAC patients in the future.

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Contributors JM and AM are the 'guarantors' who had full access to the study's data. They took responsibility for the integrity of the data and the accuracy of the data analysis. Conceptualisation: JM, AM, ST, XW and AG. Data curation: JM, AM, FG, ST and AG. Formal analysis: JM, AM, FG and AG. Funding acquisition: XW and AG. Investigation: JM, AM, FG and AG. Methodology: JM, AM, FG, ST, XW and AG. Project administration: MS, TW, YC, TB, IDN, HGC, XW and AG. Resources: MS, TW, LI, YC, TB, IDN, HGC, XW and AG. Software: JM, AM, ST, FG, XW and AG. Supervision: MS, LI, TT, AHZ, SJM, HGC, XW and AG. Validation: JM, AM, MS, MJS, AHZ, SJM, HGC, LAA and AG. Visualisation: AM and AG. Writing–original draft: AM and AG. Writing–review/edits: JM, AM, FG, ST, SJM, TW, LI, TT, YC, TB, IDN, MJS, AHZ, SJM, HGC, LAA, XW and AG.

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Patient consent for publication Consent obtained directly from patient(s).

Ethics approval This study involves human participants and was approved by the Institutional Review Boards of each participating institution (Baylor University, Johns Hopkins Hospital, National Cancer Registry Ireland, Queen's University Belfast, Radboud University, St. Joseph's Hospital, Translational Genomics Research Institute and Veneto Institute of Oncology; Supplementary Methods and Supplementary Table 1), conducted in accordance with the Declaration of Helsinki. All participants provided written informed consent. Participants gave informed consent to participate in the study before taking part.

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at publication via a signed data access agreement and at the discretion of the investigators' approval of the proposed use of such data.

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