A Multimodal Stool RNA, FIT, and Machine Learning Concept for the Detection of Advanced Precancerous Lesions and Colorectal Cancer

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ABSTRACT

Accurate and effective early detection of colorectal cancer and advanced precancerous lesions (APL) is still a challenge. The purpose of this study was to evaluate the clinical performance of a novel, noninvasive multimodal stool RNA (mm-stRNA) test that combines five human messenger RNA (mRNA) biomarkers and a fecal immunochemical test (FIT) in a machine learning (ML)–generated algorithm for the sensitive detection of APLs and colorectal cancers. For this purpose, stool samples from 265 subjects (34 colorectal cancers, 68 APLs, and 163 controls) were evaluated as part of the eAArly DETECT study, a US multisite study with subjects suspected to have at least one APL or colorectal cancer, as well as average-risk individuals. FIT was evaluated with clinical positivity thresholds of 5 μg hemoglobin (Hb)/g of stool and 17 μg Hb/g. RNA was isolated from stabilized stool and analyzed for the expression levels of five mRNA biomarkers. Lab data were analyzed using an ML-generated algorithm that was developed in a stratified split-sample design and then applied as a locked model to the full 265-subject cohort. The mm-stRNA test achieved 97.1% sensitivity for colorectal cancer and 83.8% sensitivity for APLs, with 95.7% specificity. When applying the cutoff levels of 5 μg Hb/g versus 17 μg Hb/g, FIT sensitivity was 76.5% versus 70.6% for colorectal cancer and 45.6% versus 36.8% for APL, with a specificity of 84.0% versus 90.8%, respectively. The mm-stRNA approach seemed to have substantially improved performance compared with existing tests, but results need to be replicated in an independent prospective cohort.

Prevention Relevance: This study evaluates a noninvasive stRNA test–FIT that markedly improves the detection of APLs and early colorectal cancers compared with FIT alone. By enabling the removal of high-risk lesions before malignant transformation, this approach could substantially reduce colorectal cancer incidence and mortality in population-based screening programs.

Introduction

Colorectal cancer is the third most common cancer and the second leading cause of cancer death worldwide, representing a global challenge (1). Effective colorectal cancer screening and early detection of colorectal cancer and advanced precancerous lesions (APL) are therefore essential to address this challenge. In 2021, only 59% of all adults aged 45 or older in the United States participated in colorectal cancer screening (2). Two predominant screening options for colorectal cancer are colonoscopy and the fecal immunochemical test (FIT). Colonoscopy, with its high sensitivity and specificity, is considered the current gold standard in colorectal cancer screening but is an invasive procedure and is often associated with poor acceptance rates in the general screening population (3). FIT is noninvasive and therefore is more widely accepted for programmatic screening but has poor sensitivity for early colorectal cancer and APL detection (4). Therefore, highly sensitive noninvasive screening options are urgently needed to increase screening participation rates. In the last decade, stool-based DNA tests combined with FIT have emerged, the first of which received its initial FDA approval in 2014 (5). Today, a second-generation multifactorial stool DNA-FIT and an RNA-FIT are reported to be more sensitive in identifying colorectal cancer compared with FIT alone, but sensitivity for detecting APLs remains relatively low (6–8). Blood-based tests for colorectal cancer screening are now either commercially available or in development (9, 10). However, blood-based tests are reported to be inferior in detecting early colorectal cancer stages and especially APLs compared with multifactorial stool DNA-FIT and RNA-FIT (6, 9–11).

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In a previous 102-patient study including 78 colorectal cancer stage I–III and 24 advanced adenomas (AA), as well as 32 healthy controls, a panel of stool-derived messenger RNA (mRNA) biomarkers [*CEACAM5*, *ITGA6*, metastasis-associated in colon cancer 1 (*MACC1*), *PTGS2*, and *S100A4*] was evaluated. Sensitivities of 75% and 89% for AA and colorectal cancer, respectively, were observed at ≥95% specificity, with further improvements when combined with FIT (12).

Recent studies have established an association between these biomarkers and colorectal cancer, as well as tumor-associated cellular processes, including inflammation, proliferation, and metastasis. In this context, high expression and hypermethylation in the promoter region of *CEACAM5* have been observed in colorectal cancer cells and can be associated with colorectal cancer or adenomas, suggesting its potential use as a noninvasive diagnostic marker (13). The enzyme cyclooxygenase-2, encoded by the *PTGS2* gene, serves as the rate-limiting factor in prostaglandin synthesis and plays a crucial role in regulating inflammation. In general, inflammation significantly contributes to the risk of developing colorectal cancer. Therefore, the prostaglandin-endoperoxide synthase 2 pathway promotes tumor progression, and overexpression is associated with an early event in colorectal cancer development (14). The *ITGA6* gene encodes the protein integrin α6. This protein serves as the α6 subunit of integrins, which are essential cell surface proteins consisting of both an alpha and a beta chain. Each alpha chain can pair with various beta chains, leading to the formation of different integrin complexes. The selective loss of this integrin chain in the intestinal epithelium, along with the disruption of hemidesmosomes, leads to chronic colitis and the development of invasive adenocarcinomas (15). Additionally, the integrin α6 subunit transcript promotes tumor cell proliferation and has been found to be upregulated in the majority of colorectal cancers at the transcript level (12). *MACC1* is a key prognostic biomarker that promotes cancer proliferation, migration, invasion, and metastasis, and it has emerged as a prognostic biomarker for tumor progression and metastasis, independent of tumor stage (16). *S100A4*, a key member of the *S100* protein family, plays a crucial role in promoting tumor progression and metastasis. Its molecular mechanisms vary across different malignant tumors. Given its involvement in cancer development, *S100A4* expression is emerging as a promising biomarker for early cancer detection and metastasis prediction (17). Based on the findings of Herring and colleagues (12) and other relevant studies, these five biomarkers were identified as potential candidates for a novel noninvasive stool-based diagnostic test.

Although prior studies, including the work by Herring and colleagues (12), have shown the potential of stool-derived mRNA biomarkers for detecting colorectal cancer and APLs, these findings were limited by small sample sizes, retrospective designs, frozen samples, and a lack of representation across lesion subtypes including sessile serrated polyps. Building on these findings, the present study aims to further investigate the identified mRNA targets in an independent, US-based cohort, with a larger number of APLs, including more diverse subtypes. Importantly, this study also included a subset of average-risk individuals that are representative of the colorectal cancer screening population and compares the performance of two clinical positivity thresholds for FIT.

By combining the mRNA biomarkers and FIT in a machine learning (ML)–generated algorithm, this strategy enables the detection of the full diversity of APL types and molecular pathways at earlier stages of the progression of colorectal cancer that have historically been missed by other DNA- and RNA-based tests. Unlike DNA-based tests that primarily detect somatic mutations or methylation changes, mRNA biomarkers reflect active gene expression changes that occur during tumor initiation and progression. Resulting improvements in sensitivity for early-stage colorectal cancer and APLs compared with existing individual FIT (at two different clinical thresholds), as well as existing DNA- and RNA-based tests, have the potential to maximize the preventative impact of screening programs and significantly reduce colorectal cancer incidence and mortality (13). Early detection enables curative treatment and removal of APLs before malignant transformation and contributes to improvements in the overall cost effectiveness of colorectal cancer screening programs (18).

Materials and Methods

Study design

Stool samples used in this study were collected from subjects as part of the Institutional Review Board (IRB)–approved eAArly DETECT study (NCT06864338); central IRB approval was received from Advarra IRB.

In total, 265 subjects (34 colorectal cancer, 68 APLs, and 163 controls) were examined in this study. The study cohort consisted of average-risk subjects ($n = 119$, composed of 14 APLs, 76 normal subjects, and 29 non-APL subjects) representing the screening population, as well as subjects who were suspected to have at least one advanced precancerous lesion or colorectal cancer (identified with a positive noninvasive screening test and/or colonoscopy but before the initiation of any treatment procedure). This "diagnostic"-enriched population subset was composed of 146 subjects, including 34 colorectal cancer subjects, 54 APL subjects, 35 normal subjects, and 23 non-APL subjects.

Inclusion and exclusion criteria for both the screening and diagnostic groups are described in detail in Supplementary Table S1. Controls included those "normal subjects" with negative colonoscopy findings or histopathologic review and "non-APL subjects," referring to the detection of adenomas that are not considered precancerous (**Table 1**). APLs were defined according to standard high-risk criteria, including adenomas with carcinoma *in situ* or high-grade dysplasia of any size, villous adenomas (≥25% villous component), adenomas ≥1 cm in size, and serrated lesions ≥1 cm or with cytologic dysplasia, as summarized in **Table 1**.

This first phase of eAArly DETECT was designed as an observational feasibility study to enable the development and initial evaluation of the multimodal stool RNA (mm-stRNA) test, an ML-generated algorithm incorporating FIT and five mRNA markers, rather than to formally test a predefined hypothesis. Consequently, no a priori power calculation was performed. Target enrollment of approximately 250 subjects, including at least 30 colorectal cancer and 60 to 70 APL cases, was chosen based on the expected case yield from participating sites and to ensure sufficient numbers for algorithm training and preliminary estimation of sensitivity and specificity with acceptable precision.

A table summarizing subject allocation to clinical outcome groups across all sites is provided in Supplementary Table S2. Details about the representativeness of study participants are shown in Supplementary Table S3. All subjects gave their written informed consent and underwent a screening colonoscopy, were referred for diagnostic colonoscopy, or already had a diagnosed colorectal lesion. The study was performed at 21 different US specialized gastroenterologic study sites and was conducted according to the study protocol and in compliance with Good Clinical Practice, the ethical principles stated in the Declaration of Helsinki, and other applicable regulatory requirements. The primary study objective was to evaluate the feasibility and test optimization of an mm-stRNA test for the early detection of colorectal cancers and APLs. The gender distribution of evaluable subjects was 51.3% female and 48.7% male; the average age was 60.3 years. Subjects who did not meet the inclusion/exclusion criteria, who did not provide a stool sample, who did not undergo a colonoscopy, who discontinued the examination, or for whom no analysis results were available were excluded from the final analysis. Clinical test performance was determined in comparison with the gold standard of colonoscopy and pathology results with a central pathology review (**Table 1**).

FIT

FIT was processed in accordance with the manufacturer's instructions using the SENTiFIT FOB Gold test (Sentinel Diagnostics CH. SpA) and stool samples collected in one SENTiFIT pierceTube (Art. No.: 1156188; Sentinel Diagnostics CH. SpA). The clinical threshold for a positive result for this test is set at 5 μg hemoglobin (Hb)/g of stool. The assessment of the clinical performance of FIT was also performed at 17 μg Hb/g stool.

mRNA isolation

Total RNA was isolated from stool samples collected in a DNA/RNA Shield Fecal Collection Tube using the bead-based automated extraction kit (Art. No.: PG007090; Mainz Biomed) according to the manufacturer's instructions with slight modifications.

Quantitative real-time PCR

Targeted host-specific biomarker amplification was performed by quantitative real-time PCR (qRT-PCR) using TaqMan gene expression assays (Applied Biosystems) on a LightCycler 480 System (Roche Diagnostics). Data were analyzed using LightCycler 480 Software 1.5.1.62 (Roche Diagnostics). Normalization of mRNA expression levels was performed by ΔCP value calculation between biomarkers of interest and one or more housekeeping genes.

ML-generated algorithm

To develop and evaluate a combined FIT and stool mRNA diagnostic algorithm, we used a quantitative ML platform (Emerge, Liquid Biosciences) based on evolutionary computing. The platform is designed as a scalable, unbiased methodology to produce transparent algorithms based on mathematical relationships from complex data, without any prior assumptions other than the patient selection criteria used in the studies yielding data for analysis. The software identifies both key variables and their mathematical relationships associated with outcomes of interest.

The full dataset ($N = 265$; 34 colorectal cancers, 68 APLs, and 163 controls) was randomly divided, using stratified randomization by clinical outcome category (colorectal cancer, APL, non-APL, and normal) and risk group (average-risk screening vs. diagnostic/high-risk) into three distinct (nonoverlapping) subsets of approximately equal size (training, selection, and test). The training data were used to derive an ensemble of candidate algorithms. Performance metrics of sensitivity and specificity were then evaluated on the selection subset to select a final algorithm. The selected algorithmic model was then locked prior to any further analysis and was validated on the held-out test set in a fully blinded manner to obtain unbiased performance estimates. Sensitivity for colorectal cancers and APLs was calculated as the number of correctly classified colorectal cancer or APL cases divided by the total number of colorectal cancer or APL cases, using colonoscopy findings as the reference gold standard. Specificity was calculated as the percentage of correctly classified negatives (including both "healthy" and non-APL groups as negative). High consistency of training and selection performance was observed. The detailed distribution of average-risk screening and diagnostic/high-risk subjects and of colorectal cancer, APL, non-APL, and normal controls across the three subsets is also provided in **Table 1**. The accuracy values reported in Supplementary Table S4 summarize the performance of the selected mm-stRNA model within the training, selection, and blinded test subsets during model development and are distinct from the pooled sensitivity and specificity estimates in **Table 2**, which are derived by applying the locked algorithm to the full 265-subject cohort. Across internal optimization runs, the same core set of inputs—namely the FIT result and the predefined panel of five stool RNA biomarkers—was repeatedly selected in high-performing models, indicating a stable feature set. Although the final algorithm was derived from one third of the dataset, in light of the nature of this study's intent being proof of principle and limitations on sample numbers, the locked algorithm was applied to the entire pooled dataset to

Table 1. Clinical classification of study subjects and allocation across model development subsets ($n = 265$).

Section	Category/subgroup	Subset	CRC	APL	Normal	Non-APL	Total
Clinical classification	CRC, all stages (I–IV)	—	34	—	—	—	34
	Stage I	—	11	—	—	—	11
	Stage II	—	9	—	—	—	9
	Stage III	—	6	—	—	—	6
	Stage IV	—	4	—	—	—	4
	APL, total	—	—	68	—	—	68
	Adenoma with carcinoma *in situ*/high-grade dysplasia, any size	—	—	12	—	—	12
	Adenoma with villous growth pattern (≥25%), any size	—	—	12	—	—	12
	Adenoma ≥1 cm in size	—	—	34	—	—	34
	SSL ≥1 cm in size	—	—	8	—	—	8
	SSL with cytologic dysplasia	—	—	1	—	—	1
	Traditional serrated adenoma ≥1 cm in size	—	—	1	—	—	1
	Non-APL, total	—	—	—	—	52	52
	1–2 adenomas and/or SSLs, <1 cm in size	—	—	—	—	45	45
	≥3 adenomas and/or SSLs, <1 cm in size	—	—	—	—	7	7
	Normal subjects, total	—	—	—	111	—	111
	Negative inclusion nonneoplastic findings on histopathology	—	—	—	35	—	35
	No findings on colonoscopy, no histopathologic review	—	—	—	76	—	76
Allocation across model development subsets	Screening (average-risk)	Training	0	3	30	10	43
		Selection	0	6	20	9	35
		Test	0	5	26	10	41
		Total	0	14	76	29	119
	Diagnostic/high-risk	Training	11	20	8	8	47
		Selection	12	17	17	8	54
		Test	11	17	10	7	45
		Total	34	54	35	23	146
	All subjects	Training	11	23	38	18	90
		Selection	12	23	37	17	89
		Test	11	22	36	17	86
		Total	34	68	111	52	265

Abbreviation: CRC, colorectal cancer.
Staging information was unavailable for four colorectal cancer subjects.

increase the precision of performance estimates. We report sensitivity and specificity numbers of the algorithm for the entire pooled dataset. The multistage design of random partitioning, blinded testing, and model locking provides the statistical framework used to prevent overfitting and provide generalizable performance.

Results

To evaluate the clinical performance of the mm-stRNA test, stool samples from 265 subjects collected in the eAArly DETECT study were evaluated for FIT alone at the two clinical positivity thresholds and the mm-stRNA test. For the mm-stRNA test, the ML-generated algorithm incorporated the results of FIT and the relative quantification of the five mRNA markers. Applying the ML-generated algorithm to the entire study cohort, our mm-stRNA concept showed a sensitivity of 97.1% for colorectal cancers and 83.8% for APLs compared with FIT performance at a 5 μg Hb/g clinical positivity threshold, which had a sensitivity of 76.5% for colorectal cancers and 45.6% for APLs, whereas FIT performance at a 17 μg Hb/g clinical positivity threshold had a sensitivity of 70.6% for colorectal cancers and 36.8% for APLs. In the combined group of advanced colorectal neoplasia, the mm-stRNA test reached 88.2% sensitivity. The overall specificity of the mm-stRNA test was found to be 95.7% (**Table 2**). Sensitivity and specificity for the final algorithm were comparable across the training, selection, and blinded test subsets (see Supplementary Table S4), supporting the robustness of the model and justifying the additional presentation of pooled performance estimates for the overall cohort.

In addition to the overall sensitivity, APL and colorectal cancer samples were also analyzed by APL category or colorectal cancer stage, as described in **Table 1**, and clinical performance was again compared with FIT at both 5 and 17 μg Hb/g clinical positivity thresholds.

Sensitivities are provided by the APL subgroup for the entire cohort (both screening and diagnostic): high-grade dysplasia 75% (FIT 17 μg Hb/g), 75% (FIT 5 μg Hb/g), and 100% (mm-stRNA); villous features ≥25% 41.7% (FIT 17 μg Hb/g), 50% (FIT 5 μg Hb/g), and 100% (mm-stRNA); and

Table 2. Performance of the mm-stRNA test and two FIT cutoffs for colorectal cancers, APLs, and the combined colorectal cancer + APL (advanced colorectal neoplasia) group.

	Sensitivity		
Category	**mm-stRNA**	**FIT (5 μg/g) cutoff**	**FIT (17 μg/g) cutoff**
CRC (95% confidence interval)	97.1% (84.7%–99.9%)	76.5% (58.8%–89.3%)	70.6% (52.5%–84.9%)
APL (95% confidence interval)	83.8% (72.9%–91.6%)	45.6% (33.5%–58.1%)	36.8% (24.4%–49.3%)
CRC + APL (95% confidence interval)	88.2% (80.4%–93.8%)	55.9% (45.7%–65.7%)	48% (38%–58.2%)
	Specificity		
Normal + non-APL (95% confidence interval)	95.7% (91.4%–98.3%)	84% (77.5%–89.3%)	90.8% (85.5%–94.8%)

Abbreviation: CRC, colorectal cancer.

adenoma ≥1 cm 29.4% (FIT 17 μg Hb/g), 41.2% (FIT 5 μg Hb/g), and 82.4% (mm-stRNA). The combined group of sessile serrated lesions (SSL) ≥1 cm, SSL with cytologic dysplasia, and traditional serrated adenomas ≥1 cm were at 10.0% (FIT 17 μg Hb/g), 20.0% (FIT 5 μg Hb/g), and 50.0% (mm-stRNA); see **Fig. 1**.

For the mm-stRNA assay, diagnostic colorectal cancer stagewise sensitivities were 90.9% (stage I), 100% (stages II–IV), and 100% for the unknown colorectal cancer stages (overall diagnostic colorectal cancer sensitivity 97.1%) with 95.7% specificity. For colorectal cancer, stagewise sensitivity with FIT at 5 μg Hb/g was as follows: stage I was 54.5%, stage II was 88.9%, stage III was 83.3%, stage IV was 100%, and unknown was 75% (overall diagnostic colorectal cancer sensitivity 76.5%) with 84% specificity. For colorectal cancer, stagewise sensitivity with FIT at 17 μg Hb/g was as follows: stage I was 45.5%, stage II was 77.8%, stage III was 83.3%, stage IV was 100%, and unknown was 75% (overall diagnostic colorectal cancer sensitivity 70.6%) with 90.8% specificity.

Considering only the screening group ($n = 119$; 14 APLs, 76 normal subjects, and 29 non-APL subjects) for the APLs, we were able to draw initial conclusions about the mm-stRNA test concept's prospective screening capabilities. In this group, the mm-stRNA assay demonstrated a sensitivity of 78.6% with 94.3% specificity. In comparison, FIT sensitivity was 14.3% with 92.4% specificity at 17 μg Hb/g positivity threshold, and 21.4% sensitivity with 85.7% specificity at 5 μg Hb/g positivity threshold.

Analyses by risk category were prespecified as exploratory. Due to the limited number of intraluminal APLs in the average-risk screening subset ($n = 14$), this subgroup was not powered to provide precise estimates of sensitivity, and results should be interpreted descriptively.

Additionally, an analysis investigating the mm-stRNA test performance in younger age groups was performed. In the presented study population, 17% of our cohort ($n = 45$; 6 colorectal cancers, 6 APLs, 25 normal subjects, and 8 non-APL subjects) were aged ≤49 years. In this subgroup, FIT at a 17 μg Hb/g positivity threshold achieved 83.3% colorectal cancer sensitivity, 50% APL sensitivity, and 87.9% specificity, whereas at a 5 μg Hb/g FIT positivity threshold, it achieved 83.3% colorectal cancer sensitivity, 66.7% APL sensitivity, and 81.8% specificity. In the same age group, the mm-stRNA assay reached a sensitivity of 83.3% for colorectal cancers and 100% for APLs, with a 93.9% specificity.

When comparing the mm-stRNA performance with the individual FIT performance, the mm-stRNA test showed improved sensitivity and specificity not only for the detection of colorectal cancers but also, in particular, for the detection of APLs, underlining the high potential of the mm-stRNA assay to detect curable stages in its early forms (**Fig. 1**).

Discussion

Generally, mRNA biomarkers originate from colonocytes shed in the gastrointestinal tract, offering a uniform sample of tissue detectable in stool. These biomarkers may provide a strong signal in the form of a unique signature, allowing for the detection of various precancerous changes leading to tumor development (19). Recently, the use of mRNA as biomarkers has proven more advantageous compared with other conventional approaches (6). Certain mRNA biomarkers can be deregulated early in disease processes, allowing earlier detection compared with DNA mutations that may appear later and can be static over time (12). In the context of colorectal cancer screening, specific mRNA biomarkers enable the detection of early changes in gene expression levels and therefore enhance the sensitivity for APLs, which is critical for early intervention and could lead to a reduction in colorectal cancer incidence and mortality.

Our findings are consistent with previous studies demonstrating the utility of mRNA biomarkers for the early detection of colorectal cancer. For instance, Barnell and colleagues observed a similar trend in the early detection of APLs using a stool-based mRNA panel combined with FIT in an algorithm. In their development study that also used average-risk and enriched cohorts, their approach observed 62% sensitivity for APLs, albeit using different mRNA targets (20). This represents a significant improvement over existing FIT or DNA-FIT (24% and 42% sensitivity, respectively; refs. 5, 21). It is

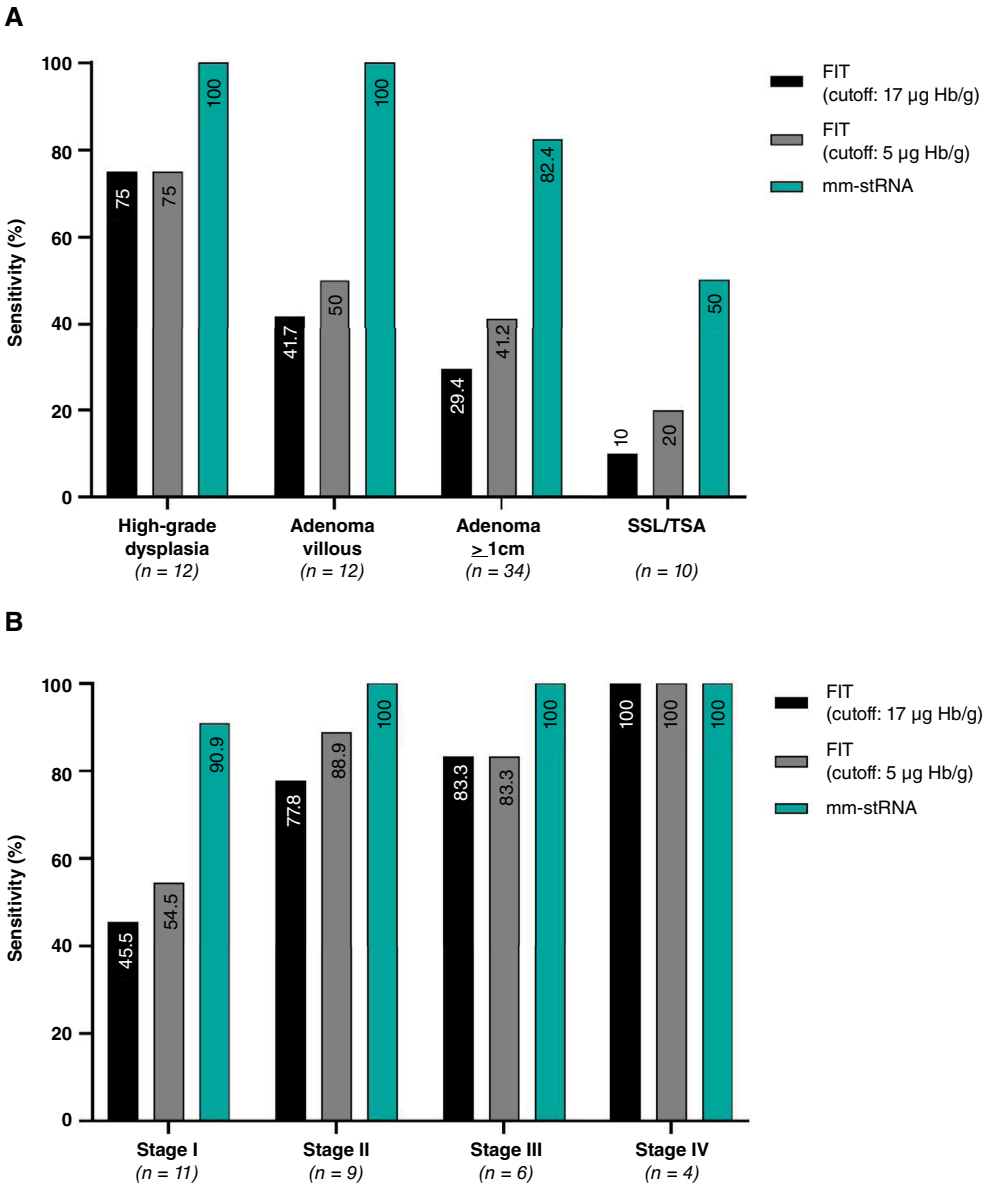

Figure 1.
Comparative performance of FIT vs. the mm-stRNA assay across APL subgroups and colorectal cancer stages. **A,** Comparison of APL subgroups for the individual FIT performances and the mm-stRNA approach. SSL/TSA = SSL, including SSL > 1 cm and SSL with cytologic dysplasia/traditional serrated adenoma (TSA). APL samples were analyzed by FIT using the cutoff values of 5 and 17 µg Hb/g. **B,** Comparison of colorectal cancer sensitivity by stage for individual FIT performances and the mm-stRNA approach. Colorectal cancer samples were analyzed by FIT using the cutoff values of 5 and 17 µg Hb/g.

important to note that when the mRNA panel was evaluated in a large, prospective, average-risk cohort (CRC-PREVENT study), the observed sensitivity for APLs decreased to 46% (6). Accordingly, a decrease in APL sensitivity may be observed when the mm-stRNA approach described here is assessed in an average-risk colorectal cancer screening population. Such a decline in sensitivity, however, is unlikely to be explained solely by the change from an enriched to an average-risk cohort because sensitivity and specificity are mathematically independent of disease prevalence. More plausibly, it reflects differences in study design, biomarker and model selection, and/or a shift in the spectrum of disease within the case group, for example, a higher proportion of very early-stage or smaller lesions in the large average-risk cohort. Our feasibility study data described here demonstrated encouraging clinical test performance for all colorectal cancer stages using the mm-stRNA test concept. Notably, we also observed very high sensitivity for APLs including the subgroup of APLs that were limited to participants enrolled in the screening group. In a direct comparison with the FIT performance alone, the increase in sensitivity for both colorectal cancer and, in particular, for APLs is substantial. Importantly, our study compared the performance of two FIT clinical positivity thresholds: the manufacturer's recommendation of 5 µg Hb/g stool and the cutoff of 17 µg Hb/g, which is used in many US-based screening programs and as a benchmark for FIT performance in large average-risk screening cohorts (6, 7, 21). The use of 5 µg Hb/g as a comparator in our study represents a clinical positivity threshold with higher sensitivity for early colorectal cancers and APLs but underscores the improved

sensitivity and specificity of our investigational mm-stRNA test in detecting clinically actionable lesions. Our findings also suggest that current commercial US-based FIT thresholds may underestimate the true potential of FIT when combined with molecular biomarkers, and they highlight the potential clinical benefit of adopting lower thresholds in multimodal stool-based strategies. In real-world settings, the ability to detect a higher proportion of APLs while maintaining high specificity could translate into a meaningful reduction in colorectal cancer incidence and mortality through earlier preventive intervention.

In the context of this evaluation of the mm-stRNA test, the use of a lower FIT cutoff as a comparator ensures that the observed performance gains of the mm-stRNA test are not merely a result of comparison with a low-sensitivity FIT benchmark but rather reflect a true diagnostic enhancement over existing competitive FIT-based approaches.

It is important to note that the average-risk screening subset contained only a small number of APLs (14/119), and the study was not powered to provide precise sensitivity estimates in this group. Because the screening subset included no colorectal cancer cases and only 14 APLs, it was not feasible to construct a fully independent test subset composed solely of average-risk participants; instead, both risk groups were represented in all three ML subsets, and screening-specific performance was evaluated exploratorily by applying the locked algorithm to the screening subgroup only. The overall cohort included a partially enriched diagnostic/high-risk population contributing the majority of APLs (54/68), which likely reflects a somewhat different disease spectrum compared with asymptomatic average-risk screening populations. As a result, the pooled APL performance is dominated by the diagnostic/high-risk subset and should not be interpreted as a definitive estimate of screening accuracy for population-based programs.

The observed APL prevalence in the average-risk screening subgroup (11.8%) is at the upper end of what has been reported in large colonoscopy-based screening cohorts and likely reflects the specific mix of sites and local referral patterns in this feasibility phase; these prevalence figures are therefore not intended to be extrapolated to the general population. Consistent with this, prevalence-dependent measures such as positive and negative predictive values cannot be directly inferred from our cohort, and confirmation of APL performance in a larger, prospectively recruited average-risk screening population is required.

This is a proof-of-principle study with limitations. The sample size and partial enrichment of disease cases were determined pragmatically, without a formal hypothesis-driven power calculation, which is typical for feasibility studies but limits the certainty with which sensitivity estimates can be generalized. The partially enriched design was implemented to ensure enough colorectal cancer and APL cases for algorithm development. Accordingly, our primary accuracy metrics were sensitivity and specificity, which are prevalence-independent and therefore remain interpretable in an enriched feasibility cohort. Translation to a true average-risk screening setting is achieved not by reestimating sensitivity and specificity but by combining these measures with the disease prevalences in the target population to obtain context-specific positive and negative predictive values.

To mitigate the risk of model overfitting and circularity, we employed a three-way data partition with a locked algorithm: Candidate models were generated on the training subset, a single model was selected based on performance in the independent selection subset, and this locked model was then evaluated once on a blinded test subset before being applied to the pooled cohort. This approach reduces the likelihood that the observed superiority of the mm-stRNA concept over FIT is an artifact of reusing the same data for both model construction and evaluation. We were not able to conduct a full formal perturbation analysis (e.g., bootstrap-based stability of feature importance scores) across large numbers of resampled datasets. We therefore acknowledge the lack of a comprehensive resampling-based stability assessment as a methodologic limitation. Nonetheless, the consistent selection of the same core feature set (FIT plus the five stool RNA markers) across internal optimization runs, together with the similar sensitivity and specificity observed in the training, selection, and blinded test subsets (Supplementary Table S4), provides empirical evidence for robustness within the constraints of this feasibility dataset. In summary, the current findings should be interpreted as exploratory and hypothesis-generating and as evidence of comparative performance versus FIT, rather than as definitive measures of population-level screening accuracy. It should be emphasized that these findings are not intended to be interpreted as definitive evidence of screening performance in a general population setting. Given the low prevalence of colorectal cancers in average risk individuals, a partial enrichment strategy was needed to gain sufficient sample sizes in our disease group. Despite these constraints, the observed confidence intervals around the mm-stRNA sensitivity estimates are relatively narrow and consistently higher than those of FIT, supporting a robust feasibility signal that warrants confirmation in a larger, homogeneous average-risk cohort. To overcome the limitations of our study, future research is warranted to evaluate the mm-stRNA concept in a larger colorectal cancer screening cohort to confirm its performance. To address this need, the second phase of our eAArly DETECT study has been initiated in the United States with a larger cohort of average-risk individuals (eAArly DETECT, NCT06864338). In summary, our feasibility data address the central problem outlined in the introduction: the need for a highly sensitive, noninvasive colorectal cancer screening option that can meaningfully improve early detection while encouraging participation. By combining our selected stool mRNA biomarkers with FIT and an ML algorithm, the mm-stRNA approach demonstrated substantially higher sensitivity for APLs and early-stage colorectal cancer than FIT alone at both clinical positivity thresholds while maintaining high specificity. Although confirmation in a larger, average-risk cohort is warranted, the presented results support the mm-stRNA test as a practical next-generation screening concept that could be implemented alongside

existing programs to improve screening rates, detect clinically treatable diseases earlier, and ultimately deliver the preventive impact that current strategies have not yet fully achieved.

Data Availability

The data that support the findings of this study are not publicly available due to institutional and patient privacy restrictions. Restrictions apply to the availability of these data, which can be accessed only under a data use agreement. Deidentified data are available from the corresponding author upon reasonable request and subject to approval by the responsible institutions.

Authors' Disclosures

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Authors' Contributions

R.S. Bresalier: Conceptualization, supervision. **M.R. Eidens:** Conceptualization, data curation, formal analysis, writing–original draft, writing–review and editing. **L. Krammes:** Conceptualization, resources, data curation, formal analysis, investigation, visualization, writing–review and editing. **N. Nolan:** Conceptualization, formal analysis, writing–original draft, writing–review and editing. **P. Lilley:** Data curation, software, formal analysis, writing–review and editing. **D.K. Turgeon:** Supervision, investigation, writing–review and editing.

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Note

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