

ORIGINAL RESEARCH—CLINICAL

Development and Clinical Validation of a Blood Test for Early Detection of Colorectal Adenomas and Cancer for Screening and Postpolypectomy Surveillance



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BACKGROUND AND AIMS: There is a lack of convenient, sensitive, noninvasive strategies for screening and surveillance for colorectal neoplasia. An assay combining the results of circulating epithelial cells (CECs) and somatic mutations of cell-free DNA adjusting for age/sex using a unique algorithm is evaluated in patients requiring colonoscopy. **METHODS:** A prospective single-site 458-subject study (asymptomatic: 43% screening/43% surveillance, enriched with 65 symptomatic subjects undergoing colonoscopy) was conducted. The test analyzed CECs and somatic mutations. The probability of advanced neoplasia (advanced adenoma [AA] and CRCs) was determined by logistic regression methods adjusted for expected CRC incidence rate, prior history of AA, and patient age and sex on a training subset. A linear predictor was developed to generate a score scaled from 0 to 100. The test performance was evaluated on an independent set of subjects using pre-specified algorithms and cut point. **RESULTS:** Based on a pre-defined clinical threshold and predictive model derived from the training set (n = 232), analysis of an independent asymptomatic validation set (n = 194) yielded 89% (lower exact one-sided 95% confidence interval [CI]: 80%) specificity and 100% (95% CI: 37%)/78% (95% CI: 61%) sensitivity for detection of CRC/AA. In a secondary analysis, excluding surveillance subjects, the 97-subject screening cohort yielded 91% (95% CI: 79%) specificity and CRC/AA sensitivity at 100% (95% CI: 37%)/83% (95% CI: 56%, 87% for advanced neoplasia 95% CI: 64%). Significant associations ($P < .0001$) were detected between FirstSight scores and adenoma size, number, and ordinal increasing pathology classification. **CONCLUSION:** A multimodal blood test that included CECs and somatic mutations with adjustment for age and sex demonstrated high sensitivity for the diagnosis of advanced colorectal neoplasia. The resulting score captures prognostic information for CRC progression of index adenoma size and number and has the potential to enable stratification of patients for screening or postpolypectomy surveillance colonoscopy.

Keywords: Circulating Epithelial Cells; Colorectal Cancer; Adenoma; ctDNA Mutations

Introduction

The American Cancer Society (ACS) estimates that in 2020, 145,600 people will be diagnosed with colon cancer and 53,200 will die from this disease.¹ Most of the people diagnosed with colon cancer are expected to be those who do not follow the ACS, United States Preventive Services Task Force, and other professional society recommendations for screening.² Tragically, colon cancer is largely preventable with timely removal of adenomatous polyps using colonoscopy. The National Polyp Study demonstrated that endoscopic removal of adenomatous polyps results in a 60% decrease in incidence and a 53% reduction in mortality from colorectal cancer (CRC).^{3–5}

The 2 primary aims of CRC screening are to detect and remove adenomatous polyps to prevent cancer and to detect cancer in earlier stages when survival is increased, and intervention is less costly. As noted by the Surveillance, Epidemiology, and End Results Program, detection of cancer in earlier stages has improved 5-year survival rates.⁶ In addition, timely and risk-based surveillance after polypectomy is critical as described in recent guidelines.⁷ Unfortunately, a goal by the National Colorectal Cancer Roundtable to reach a screening target of 80% by 2018 has not been achieved.⁸ As reported in the recent ACS-published guideline update, current approved noninvasive screening tests include a fecal DNA test and a blood test for detection

Abbreviations used in this paper: AA, advanced adenoma; ACS, American Cancer Society; AN, advanced neoplasia; CEC, circulating epithelial cell; cfDNA, cell-free DNA; CI, confidence interval; EpCAM, epithelial cellular adhesion molecule; FIT, fecal immunochemical testing; VA, Veteran Affairs.

Most current article

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of Septin 9 gene methylation as well as various versions of fecal immunochemical testing (FIT) for blood.^{9,10} Combining the fecal DNA and FIT tests into a single assay result has greatly improved overall test results for cancer; however, these tests exhibit low sensitivity for precancerous adenomas and may suffer from reduced compliance because of the need for stool sampling.^{9,11}

New technologies have shown promise for improving noninvasive testing performance and convenience by making use of cancer-associated markers in peripheral blood. A microfluidic method to detect rare circulating colorectal epithelial cells shed from tumors and precursor lesions has been reported to have high specificity and sensitivity for all colon tumor stages in adult subjects.^{12,13} The method uses an anti-epithelial cellular adhesion molecule (EpCAM)-functionalized supported lipid bilayer to capture epithelial cells including circulating tumor cells. The cells are further confirmed with differential immunofluorescent staining and imaging. Of particular importance, this method appears to greatly increase precursor adenoma detection.¹⁴ Circulating cell-free DNA (cfDNA) has also been used to identify DNA mutations that have known associations with colon cancer.¹⁵ As reported here, these methods have been combined using a unique algorithm including subject age, sex, and prior screening history, to yield a single predictive result, the FirstSight test. The performance of this combination was evaluated in a large group of adult subjects requiring colonoscopy.

Materials and Methods

Institutional Review Board

The study protocol was reviewed and approved by the Stanford University's Institutional Review Board (IRB 6, Registration #4947) for clinical utility, statistical methodology, and ethical considerations in compliance with the Declaration of Helsinki. Written informed consent was obtained from all study participants.

Study Design and Participants

This was an exploratory, prospective, single-center, clinical study where neither the predictive algorithm nor clinical thresholds were predefined before the study start. Subjects scheduled for colonoscopy were enrolled from 2018 to 2020 at the Veteran Affairs Palo Alto Healthcare System. The study included both subjects at average risk and those scheduled for surveillance colonoscopies. To increase statistical power, the study population was enriched for subjects with CRC and advanced adenoma (AA) via the inclusion of symptomatic and referral participants (the "enrichment group"). The enrichment group consisted of subjects scheduled for colonoscopy for symptoms such as hematochezia or abdominal pain, FIT positive, resection of previously identified polyps, or suspected CRC based on imaging or other clinical findings. [Figure 1](#) shows clinical sample enrollment workflow for sample collection depicting three different types of subject enrollments for the clinical study.

Statistical analyses involving logistic regression and weighted maximum likelihood estimation methods were utilized

to account for enrichment of the study with CRC and adenoma subjects and enable approximations to prevalence rates expected in the US population. Exclusion criteria consisted of subjects with personal history of CRC, colorectal surgical resection, familial CRC syndrome, inflammatory bowel disease, or a history of any type of cancer in the past 5 years.

Average risk screening is the intended use population but has low prevalence of CRC and high-risk adenomas. Surveillance and enriched populations allow the use of case-cohort study designs for algorithm development purposes. Each study cohort was selected to minimize study biases by using predefined inclusion and exclusion criteria, use of a single site and acceptance of all subjects who met criteria. Details of subject allocation are described in [Supplementary Materials](#).

Blood Sample Collection and Preanalytics

Three tubes of venous blood were drawn from each subject before the scheduled colonoscopy. Blood was first collected in 2 Cell-Free DNA BCT tubes (Streck, La Vista, NE) before filling a third ethylenediaminetetraacetic acid Vacutainer tube (BD Biosciences) to prevent any skin cell contamination of the circulating epithelial cell (CEC) assay. A Streck cell preservative was added on blood collection and mixed well by gently inverting the tube. Blood samples were deidentified, and tubes were assigned unique barcodes, placed in a validated vibration-resistant transportation box, and shipped at room temperature. On arrival in the laboratory, the blood samples were rejected if grossly hemolyzed or clotted. Two aliquots (2.5 mL each) of blood with preservative from the ethylenediaminetetraacetic acid tube were processed for CEC assay while circulating cell-free DNA was extracted from plasma in the Cell-Free DNA BCT tubes following the previously published protocol.¹⁵

FirstSight Multimodal Assay Development

CEC Assay. The CEC assay followed previously described processes which include CEC capture from whole blood using microfluidic chips with the anti-EpCAM antibody coated on layers of anti-fouling lipids, air-foam release of captured cells, immunofluorescent staining, microscopy image capture, and artificial intelligence-enabled CEC identification and quantification.^{15,16} An antibody to EpCAM on the microfluidic chip provides capture of epithelial cells, in general, whereas tetramethylrhodamine (red) is used for cytokeratin 20 to image gastrointestinal epithelial cells specifically, fluorescein isothiocyanate (green) for the lymphocyte common antigen (CD45), and 4',6'-diamidino-2-phenylindole (blue) for nucleus counterstain.

Cell-Free, Circulating Tumor DNA Mutational Assay. cfDNA was extracted from blood plasma, and a library was prepared.¹³ Next-generation sequencing was performed using a gene panel which included 10 hot spot regions in 7 genes (KRAS, TP53, APC, PIK3CA, BRAF, FBXW7, and NRAS). These mutations are prevalent in colorectal adenocarcinomas.¹⁷ In brief, analytical validation was performed as per recently published American College of Medical Genetics and Genomics and Association for Molecular Pathology guidelines and established the limit of detection, sensitivity, specificity, accuracy, and reproducibility using 126 gold-standard reference samples, healthy donor samples verified by whole-exome

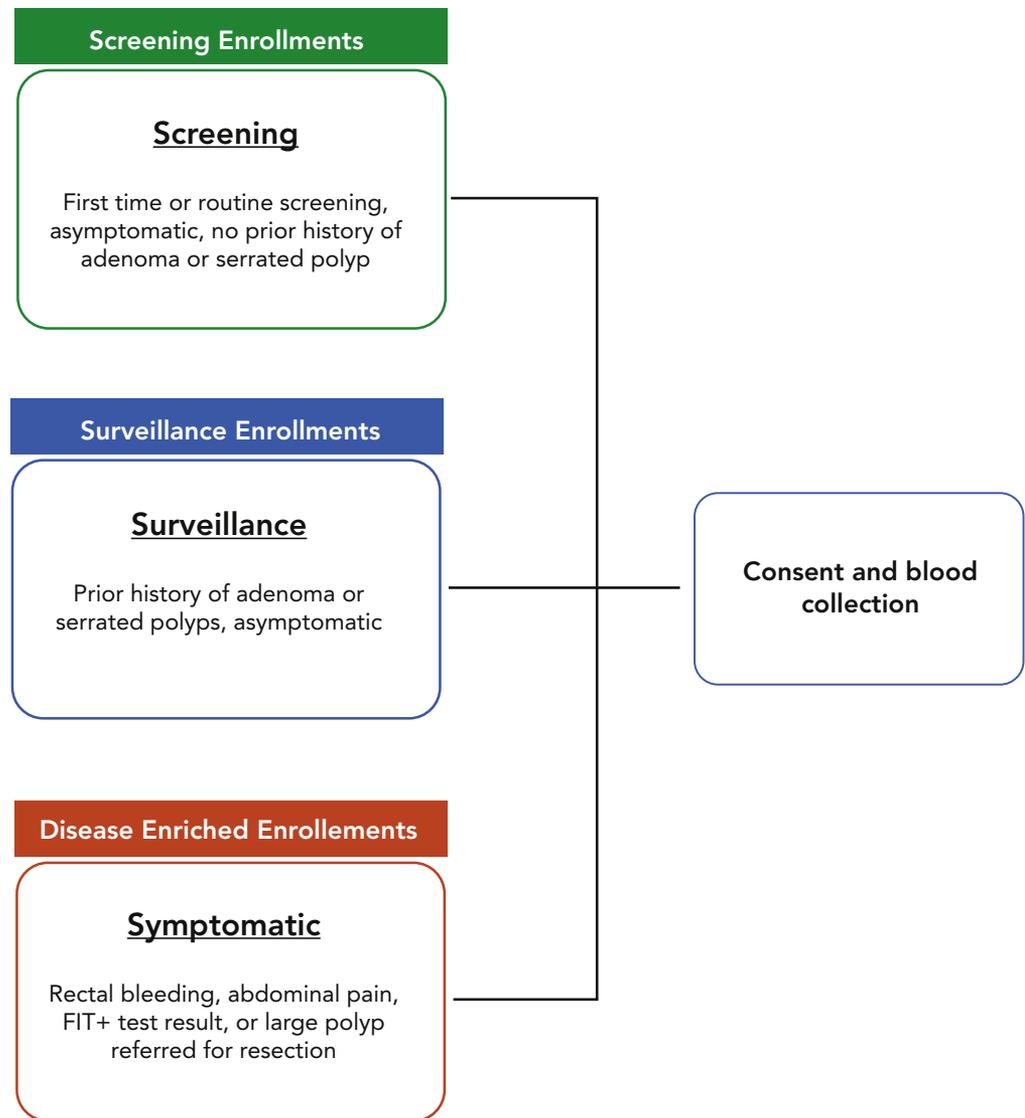


Figure 1. Clinical sample enrollment workflow for sample collection depicting 3 different types of subject enrollments for the clinical study.

sequencing by an external College of American Pathologists reference laboratory and cell lines with known variants. The assay detects variant allele frequencies of $\geq 0.1\%$ for single-nucleotide variants.

Reference Method

All study participants received a colonoscopy within 1 month after blood samples were collected. When suspicious lesions were identified during colonoscopy, they were removed and biopsied, and histopathology on all colorectal lesions was performed. In cases with multiple lesions, subjects were classified based on their most advanced or largest neoplasm. Colonoscopy and histopathology results served as reference.

Test Design

The workflow of the FirstSight multimodal assay is depicted in the [Figure A6](#). The assessment uses a CellMax proprietary formula to combine 4 elements into a simple weighted linear model. These are the quantitative measurements of circulating gastrointestinal epithelial cell signals, the mutational profiles of

cfDNA for 7 genes (bottom panel in the figure), with the subject’s CRC incidence rate (age and sex), as well as an indicator of prior history of AAs (yes or no). The formula yields an adenoma/cancer score (the FirstSight score), scaled between 0 (low risk of CRC or AA) and 100 (high risk of CRC or AA). To assess the contribution of the biomarkers to the prediction of AAs or CRC, nominal and ordinal logistic regressions were used to model the disease state (advanced neoplasia (AN) vs healthy) as a function of biomarker measurements, expected CRC incidence rate, derived from patient age and sex, and prior history of AA. Overall estimates of sensitivity and specificity, predictive values, receiver operating characteristic curve statistics, associated confidence intervals, scores, and standard errors were obtained along with estimates for each disease status.

Biomarker identification and algorithm development were performed on a training set of 232 samples using a simple logistic model for detection of CRC and AAs (together as AN) vs healthy participants, which were defined as either a subject with no findings on colonoscopy or with only non-neoplastic findings on pathology. Biomarker selection and algorithm development are described in [Supplementary Materials](#).

Results

Study Cohort and Disease Characteristics

A total of 706 subjects at the Veteran Affairs (VA) hospital scheduled for colonoscopy were enrolled in the period of November 2018 to February 2020. One hundred forty-nine participants (21%) were excluded primarily early in study because of low blood volume collected or predefined age for enrollment.

An additional number of 99 subject samples were excluded because of blood clotting, hemolysis, or quality control failure in different steps of the assay. The final cohort for analysis included blood samples from 458 participants. The training set included samples from 232 subjects, and the validation set included samples from 226 participants. Study population makeup and enrichment sources are shown in [Table 1](#). Because the study was conducted in a VA hospital, a majority (97%) of the subjects were male and the mean age was 66.5 years. However, subjects in each age group are distributed evenly between training and validation sets.

Model Development and Validation

The 458 samples were divided using stratified randomized sampling into a nonoverlapping training set of 232 participants, which comprised 99 screening, 100 surveillance, and 33 enrichment subjects, and the 226-subject validation set comprised 97 screening, 97 surveillance, and 32 enrichment subjects. The composition of the study population with respect to demographics, colonoscopy indications, and clinical and pathological features is summarized in [Table A1](#).

Logistic regression was performed on the training data using a prespecified set of parameters, and the resulting regression coefficients were used to develop a linear predictor. The FirstSight score was derived by scaling the resulting linear predictor onto a scale from 0 to 100. The cut point of 53 for the FirstSight score was selected to achieve approximately 90% specificity. The same algorithm, predictor, and cut point were then used to assess the validation group.

Sensitivity and Specificity

Sensitivity and specificity determinations for the validation group and various subsets are shown in [Table 2](#). For

Table 1. Study Cohort Characteristics

Study cohort by indication			
Screening	Surveillance	Enrichment group	Total
196	197	65	458
Enrichment group by symptom			
Rectal bleeding	Abdominal pain	FIT+	Referral
27	6	30	2

the entire 226-subject validation group, 89% (exact one-sided lower 95% confidence interval [CI]: 82%) specificity and 100% (exact one-sided lower 95% CI: 61%) and 78% (exact one-sided lower 95% CI: 63%) sensitivity for CRC and AAs, respectively, were achieved. For the validation subset of 97 screening participants, the test specificity was 91% (exact one-sided lower 95% CI: 79%), and the sensitivity was 100% (exact one-sided lower 95% CI: 37%) or 83% (exact one-sided lower 95% CI: 56%) for detection of CRC or AA, respectively. The test performance for the 97 validation surveillance participants was slightly lower, at 87% (exact one-sided lower 95% CI: 72%) specificity and 73% (exact one-sided lower 95% CI: 49%) sensitivity for the detection of AA (there were no CRC cases in the surveillance group).

Correlation of Size and Number of Adenomas With FirstSight Scores

As indicated in [Table 3](#), a significant association between the multimodal FirstSight score and adenoma size was detected (likelihood ratio $\chi^2 = 45.0$, $P < .0001$), as well as for the number of adenomas (likelihood ratio $\chi^2 = 87.6$, $P < .0001$) and ordinal increasing pathology classification (Cuzick trend test $z = 63.5$, $P < .0001$). [Table 3](#) shows the mean size of index adenomas and the mean number of adenomas for each progressive disease category against its mean score. These results suggest that the scores can provide predictive information for adenoma size, number, and disease prognosis. In a more detailed analysis, [Figure A1](#) displays the distribution of FirstSight scores as a function of index polyp size. There is a significant ($P < .0001$) linear relationship between FirstSight scores and index polyp sizes. Based on this model, the optimal clinical threshold for detection of CRC or AA corresponds to the FirstSight score that provides a predicted index polyp size of 5.4 or larger and therefore may be a predictor of index polyp size as well as the probability of AA or CRC. This is further demonstrated in [Figure 2](#) where the FirstSight score is plotted against disease pathology classification (based on colonoscopy or biopsy results). The figure also shows that using a cutoff of 53, cancer and higher-risk adenomas can be clearly distinguished from lesser conditions.

A predictiveness curve ([Figure 3](#)) displays the estimated probability of AN as a function of the percentile of the FirstSight score and further demonstrates the clinical utility of the test. The lower quartile of participants has an estimated risk of AA or CRC less than 10%. In contrast, the upper quartile of participants has an estimated risk of AA or CRC greater than 72%.

Additional results, including receiver operator curves (training, validation, asymptomatic validation, and asymptomatic, average-risk screening validation cohorts), are shown in [Figures A2–A5](#). The sensitivity and specificity for each disease category and exact one-sided lower bound 95% CIs are shown for the $N = 194$ asymptomatic subjects (excluding enrichment), $N = 226$ all validation,

Table 2. Validation of Average Risk Subgroup Results by Disease Status

97 asymptomatic screening subjects

Disease category	Positive	Total	Sensitivity	Exact one-sided lower 95% CI
CRC	3	3	100%	37%
AA	10	12	83%	56%
Advanced neoplasia	13	15	87%	64%
Adenomas <1 cm, no high-grade dysplasia, and <25% villous features	17	48	35%	24%
	Negative	Total	Specificity	Exact one-sided lower 95% CI
Non-neoplastic findings	12	12	100%	78%
Negative findings	19	22	86%	68%
Negatives	31	34	91%	79%

97 asymptomatic surveillance subjects

Disease category	Positive	Total	Sensitivity	Exact one-sided lower 95% CI
CRC	NA	NA	NA	NA
AA	11	15	73%	49%
Advanced neoplasia	11	15	73%	49%
Adenomas <1 cm, no high-grade dysplasia, and <25% villous features	27	52	52%	40%
	Negative	Total	Specificity	Exact one-sided lower 95% CI
Non-neoplastic findings	7	8	88%	53%
Negative findings	19	22	86%	68%
Negatives	26	30	87%	72%

194 asymptomatic screening (97) and surveillance (97) subjects

Disease category	Positive	Total	Sensitivity	Exact one-sided lower 95% CI
CRC	3	3	100%	37%
AA	21	27	78%	61%
Advanced neoplasia	24	30	80%	64%
Adenomas <1 cm, no high-grade dysplasia, and <25% villous features	44	100	44%	36%
	Negative	Total	Specificity	Exact one-sided lower 95% CI
Non-neoplastic findings	19	20	95%	78%
Negative findings	38	44	86%	75%
Negatives	57	64	89%	80%

and the entire study cohort N458 (Tables A2–A4). Negative predictive value and positive predictive value estimates for the N = 194 asymptomatic subjects' validation results are also shown with AA and CRC prevalence based on the literature (Table A2).⁹

Discussion

Colorectal carcinogenesis is now understood to be a continuum of malignancy rather than dichotomous premalignant and malignant stages.¹⁸ Recent studies have

Table 3. Correlation of Mean Size and Number of Adenomas With FirstSight Scores

Clinical outcome	# of subjects	Index adenoma size (mm) mean (95% CI)	Number of adenomas mean (95% CI)	FirstSight score mean (95% CI)
Negative colonoscopy	101	-	0	42.2 (41.4, 43.0)
Non-neoplastic findings	52	3.5 (3.0, 4.0)	1.8 (1.5, 2.2)	42.4 (39.5, 45.3)
Nonadvanced adenoma	225	4.8 (4.6, 5.0)	3.1 (2.8, 3.3)	54.6 (50.9, 58.3)
Advanced adenoma	66	17.5 (14.3, 20.7)	4.7 (3.9, 5.5)	64.2 (57.1, 71.3)
Colorectal cancer	14	34.5 (27.8, 41.3)	3.7 (1.2, 6.2)	74.2 (63.4, 85.0)

The cut point for FirstSight was 53.47.

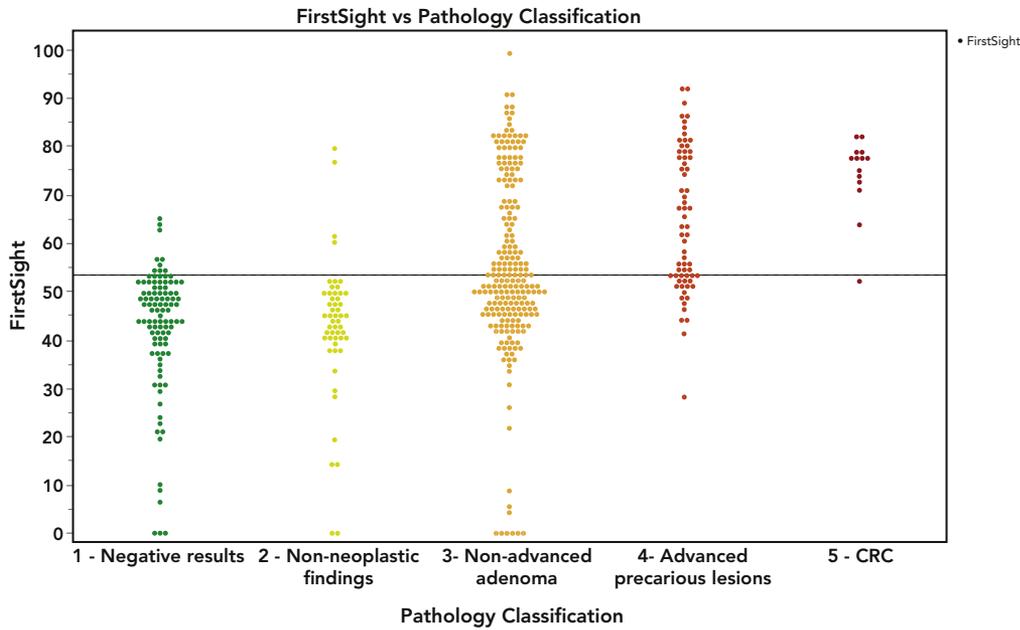


Figure 2. Distribution of FirstSight scores by pathology based on colonoscopy or biopsy results. A horizontal line indicates the cut point of 53.47.

described the role of stromal architecture and immune contexture as well as early adenoma genetic variation that results in adenoma cell mobility separate from size or villous features.^{19,20} A subset of early adenomas (“bad actors”) may harbor this more aggressive dysplastic feature.^{21–23} The National Polyp Study demonstrated that colonoscopic removal of all adenomatous polyps, regardless of size, resulted in an approximately 60% decrease in incidence and a 53% reduction in mortality from CRC.^{4,24–26} However, 31% of eligible subjects avoid colonoscopy examination.²⁷ Therefore, there remains a compelling need for

an accurate and reliable noninvasive test to encourage compliance to testing.

Noninvasive alternatives to colonoscopy are recognized in screening guidelines.¹¹ However, these test methods have significantly inferior AA detection. For example, fecal immunochemical tests have demonstrated a sensitivity of 80% for the detection of CRC but only 28% for the detection of adenomas.²⁸ Although the United States Food and Drug Administration-approved stool DNA test has demonstrated a sensitivity of 92.3% for the detection of CRC, the assay only achieves 42.2% sensitivity for AAs and is associated

Predictiveness Curve for FirstSight™

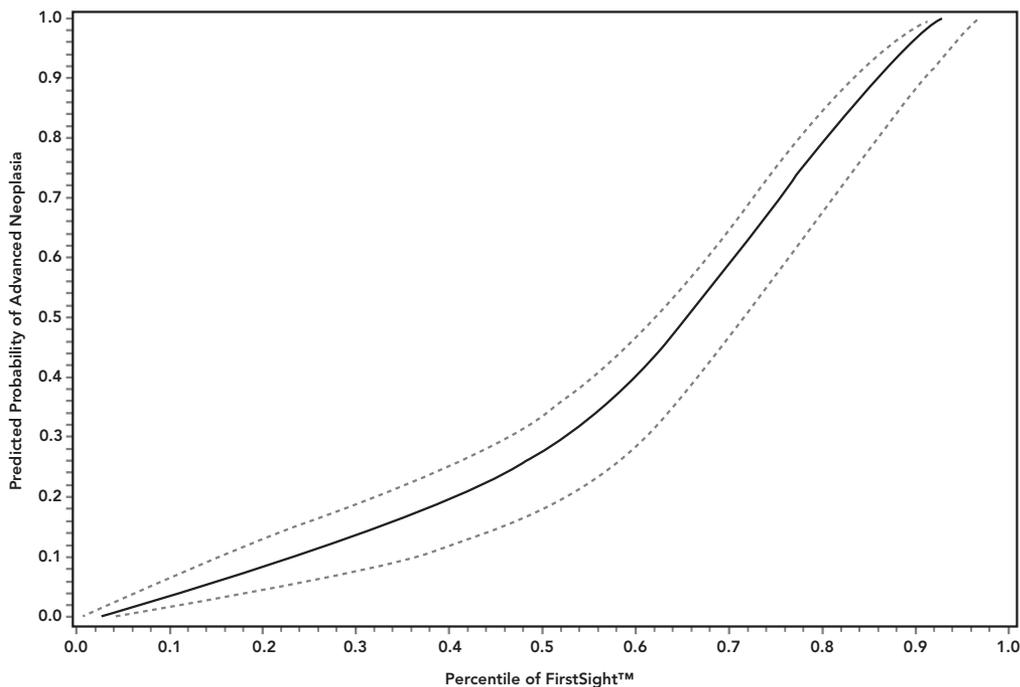


Figure 3. The predictive-ness curve for the FirstSight score for subjects in this study.

with an adherence rate of only 71% among a population of Medicare beneficiaries.^{9,29}

The FirstSight test is a multimodal blood test that targets molecular and cellular markers associated with the adenoma-carcinoma continuum of disease. Including subject age, sex, and history, both intrinsic and extrinsic adenoma factors are expected to be represented in the resulting FirstSight score. The test targets relevant mutations in cfDNA and detects rare CECs (disseminated from dysplastic or hyperplastic regions of the colon). These disseminated cells may be reflective of extrinsic factors in CRC development and harbingers of subsequent metastasis.^{19,30,31} Samples were analyzed from 458 subjects requiring colonoscopy. A training subset of 232 subjects was used for the development of an algorithm by multivariate logistic regression. Application of the final algorithm to a second, validation subset of 226 subjects demonstrated that the blood test appears to have high sensitivities for both CRC (100%) and AAs (78%) while retaining high specificity (89%). The quantitative nature of the FirstSight score has the potential to enable risk assessment of subjects for screening or postpolypectomy surveillance colonoscopy and therefore to be more understandable and accessible to patients and clinicians, or, if used with appropriate thresholds, categorical classification.

The study has several limitations. One limitation is the small number of subjects in the study. A second limitation is the post hoc separation of sample sets to discern score performance of average risk and surveillance subject groups. A third limitation of the study is the recruitment of primarily older male subjects from a single VA hospital. Single-institution studies may not reflect the range of preanalytical variation generally found in multi-institution studies. VA hospitals are also known to have higher adenoma detection rates (as much as 40%) than other institutions.³² These differences could introduce bias in relatively small studies which have limited statistically significant split analyses of screening vs surveillance populations. Finally, the presented predictiveness curve is for the subjects in the study rather than an average risk screening cohort. Finally, the study aggregates all adenomas less than 10 mm with less than 25% villous content into one category. Given the imperfect nature of adenoma size as a predictor of malignancy and the continuum of malignancy of the adenoma-carcinoma pathway, there may be value in discerning differential risk adenoma subsets in this catch-all category.

With respect to the performance differences observed between the screening and surveillance subgroups of the validation cohort, a possible explanation is mixed disease history where some surveillance subjects may have been unaware of previously resected higher-risk AA. Such unaccounted history would not be included in the model, potentially resulting in a reduced score.

Several factors are important for considering novel CRC screening solutions as described in this report. First, the significant and troubling increase in CRC incidence

combined with the substantial fraction of noncompliance for colonoscopy for both average risk patients and post-polypectomy patients argues for innovation in this setting.¹¹ Second, the decrease in incidence and mortality associated with removal of all adenomas by colonoscopy should encourage an objective of equipoise between colonoscopy and noninvasive testing strategies. The objective should not only be to identify cancer, but the detection of AAs as well. Third, size estimates of adenomas and estimates of requisite villous content are inaccurate and therefore introduce technical variation that should be accommodated in uncertainty estimates.

We describe here a novel noninvasive multimodal blood-based assay that analyzes CEC and cfDNA for somatic mutations and integrates history of AAs and the known risk factors of age and sex relevant for CRC (colon and rectum Surveillance, Epidemiology, and End Results survival rates by time since diagnosis, 2000–2017, National Institutes of Health National Cancer Institute, 20 April 2021). The assay is significantly correlated with adenoma size and number, both of which provide key prognostic information for the transition from adenoma to adenocarcinoma. Optimization and prospective validation of the FirstSight test in larger, multisite clinical settings are underway.

Supplementary Materials

Material associated with this article can be found in the online version at <https://doi.org/10.1016/j.gastha.2021.11.004>.

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Authors' Contributions:

Shai Friedland, Drew Watson, and Rui Mei contributed to study concept and design. Shai Friedland, Jennifer Pan, Zulfiqar Gulzar, Alexander Atkins, Pratyush Gupta, Jr-Ming Lai, and Huangpin Hsieh contributed to acquisition of data. Huangpin Hsieh, John J. Sninsky, Rui Mei, and Drew Watson contributed to drafting of the manuscript. Ashish Nimgaonkar, Samir Gupta, and John J. Sninsky contributed to critical revision of the manuscript for important intellectual content. Drew Watson contributed to statistical analysis. Yu Chen and Stephen Su contributed to technical or material support. Rui Mei, Huangpin Hsieh, and Zulfiqar Gulzar contributed to study supervision. All authors had access to the study data and reviewed and approved the final manuscript.

Conflicts of Interest:

Shai Friedland, Jennifer Pan, Yu Chen, Ashish Nimgaonkar, and Samir Gupta disclose no conflicts. Drew Watson, Zulfiqar Gulzar, Alexander Atkins, Pratyush Gupta, Jr-Ming Lai, Huangpin Hsieh, Stephen Su, John J. Sninsky, and Rui Mei are employees of CellMax Life.

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Ethical Statement:

The corresponding author, on behalf of all authors, jointly and severally, certifies that their institution has approved the protocol for any investigation involving humans or animals and that all experimentation was conducted in conformity with ethical and humane principles of research.

Data Transparency Statement:

Data, analytic methods, and study materials will be made available to other researchers on request.