ORIGINAL RESEARCH

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Clinical Validation of a Multitarget Fecal Immunochemical Test for Colorectal Cancer Screening

A Diagnostic Test Accuracy Study

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Background: The fecal immunochemical test (FIT) is used in colorectal cancer (CRC) screening, yet it leaves room for improvement.

Objective: To develop a multitarget FIT (mtFIT) with better diagnostic performance than FIT.

Design: Diagnostic test accuracy study.

Setting: Colonoscopy-controlled series.

Participants: Persons (n = 1284) from a screening (n = 1038) and referral (n = 246) population were classified by their most advanced lesion (CRC [n = 47], advanced adenoma [n = 135], advanced serrated polyp [n = 30], nonadvanced adenoma [n = 250], and nonadvanced serrated polyp [n = 53]), along with control participants (n = 769).

Measurements: Antibody-based assays were developed and applied to leftover FIT material. Classification and regression tree (CART) analysis was applied to biomarker concentrations to identify the optimal combination for detecting advanced neoplasia. Performance of this combination, the mtFIT, was cross-validated using a leave-one-out approach and compared with FIT at equal specificity.

Results: The CART analysis showed a combination of hemoglobin, calprotectin, and serpin family F member 2–the mtFIT–to have a cross-validated sensitivity for advanced neoplasia of 42.9% (95% CI, 36.2% to 49.9%) versus 37.3% (CI, 30.7% to 44.2%) for FIT (P = 0.025), with equal specificity of 96.6%. In particular, cross-validated sensitivity for advanced adenomas increased from 28.1% (CI, 20.8% to 36.5%) to 37.8% (CI, 29.6% to 46.5%) (P = 0.006). On the basis of these results, early health technology assessment indicated that mtFIT-based screening could be cost-effective compared with FIT.

Limitation: Study population is enriched with persons from a referral population.

Conclusion: Compared with FIT, the mtFIT showed better diagnostic accuracy in detecting advanced neoplasia because of an increased detection of advanced adenomas. Moreover, early health technology assessment indicated that these results provide a sound basis to pursue further development of mtFIT as a future test for population-based CRC screening. A prospective screening trial is in preparation.

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Colorectal cancer (CRC) is a major contributor to cancer incidence and death worldwide (1). The stepwise transition from a colorectal precursor lesion, adenoma or serrated polyp, to CRC can take decades, leaving a window of opportunity for early detection and interception through screening (2, 3). To this end, many countries have implemented population-wide CRC screening programs, and most of these programs use a noninvasive fecal test followed by a colonoscopy for persons with a positive test result (4, 5). Reasons for this strategy are high participation rates, cost-effectiveness, limited burden for participants, and effective use of colonoscopy capacity (6, 7).

See also:

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Web-Only
Supplement

The fecal immunochemical test (FIT) detects human hemoglobin in feces (8). The FIT-based screening is effective in reducing CRC incidence and death (9). Nevertheless, although the sensitivity of FIT in 1 round of screening is high for CRC, the sensitivity for relevant precursor lesions, advanced adenomas (AA) and advanced serrated polyps (ASP), is much lower (10, 11). This underlines the clinical need for a noninvasive screening test with a higher sensitivity, with an equally high specificity as FIT, for relevant precursor lesions. Molecular screening tests, reflecting more aspects of the tumor biology and microenvironment, can potentially provide a solution (12). During the past decade, substantial efforts have been made to identify sensitive markers for the detection of advanced neoplasia (AN), which comprises CRC, AA, and ASP (13–16). The multitarget stool DNA test, which includes hemoglobin, DNA-mutation, and promoter-methylation markers, has been the most successful so far. This test showed a higher sensitivity for AA and ASP compared with FIT, albeit at a markedly lower specificity (14). However, this test is not widely adopted in programmatic screening because of its lower

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specificity, more complex sample logistics, and lack of cost-effectiveness compared with FIT (17). In population-based CRC screening programs, a high specificity is important to reduce the risk for undesirable overdiagnosis and overtreatment.

Protein tests, rather than DNA-based tests, may overcome these problems. Therefore, we previously embarked on mass spectrometry–based protein biomarker discovery in stool samples (18, 19). This yielded multiple combinations of protein biomarkers in stool with a higher sensitivity for AN than FIT, at equal specificity. Moreover, we provided proof of concept that these protein biomarkers are detectable in leftover FIT samples (18, 19).

On the basis of these findings, we aimed to further develop a biomarker panel with better diagnostic performance than FIT that would be suitable for programmatic screening. Therefore, we set out to use an antibody-based, multitarget FIT (mtFIT) rather than mass spectrometry, and leftover FIT samples rather than whole stool samples, consistent with the logistics of population-based CRC screening programs. This approach presents a major step forward toward an improved protein-based fecal biomarker test that is suitable for CRC screening.

METHODS

A detailed description of the methods is provided in the Supplement (available at Annals.org).

Study Population

Written informed consent was obtained from all persons. This study was done in compliance with the "Human Tissue and Medical Research: Code of Conduct for Responsible Use" formulated by the Federation of Dutch Medical Scientific Societies.

Stool samples consisted of leftover material in FIT collection devices (OC-Sensor, Eiken Chemical) from study participants within a screening population (study population 1) and a referral population (study population 2). Samples from the referral population were included to enrich for CRC cases. Persons were classified on the basis of their most advanced lesion, and clinical characteristics were retrieved (Table 1).

Study Population 1

The FIT samples were collected between 2009 and 2010 from 1038 participants in a Dutch colonoscopy-controlled screening trial (COlonoscopy or COlonography for Screening) (20). The most advanced lesions in this population were CRC (n = 8), AA (n = 94), ASP (n = 29), nonadvanced adenoma (n = 201), and nonadvanced serrated polyp (n = 51), next to persons without colorectal neoplasia (n = 655). All FIT samples were collected before colonoscopy.

Study Population 2

The FIT samples were collected between 2003 and 2014 from 246 participants in a Dutch colonoscopy-controlled referral population (18). The most advanced lesions in this population were CRC (n = 39), AA (n = 41), ASP (n = 1), nonadvanced adenoma (n = 49), and nonadvanced serrated polyp (n = 2), next to persons without colorectal neoplasia (n = 114). The FIT samples test samples were collected either at the Amsterdam University Medical Center, location VU University Medical Center (n = 174), or at Kennemer Gasthuis (n = 72). All samples were collected before colonoscopy, except for 9 samples from patients with CRC that were collected at least 2 weeks after the diagnostic colonoscopy and before surgery.

All persons from both study populations provided a single FIT sample, which was used for both FIT and mtFIT testing, allowing for paired analysis.

Table 1. Baseline Characteristics of Persons Included in this Study

Characteristic	Samples (n = 1284), n (%)
Sex	
Male	656 (51.1)
Female	628 (48.9)
Age	
<50 y	33 (2.6)
50–54 y	243 (18.9)
55–59 y	284 (22.1)
60–64 y	317 (24.7)
65–69 y	199 (15.5)
70–74 y	131 (10.2)
≥75 y	45 (3.5)
Unknown	32 (2.5)
Population type	
Screening	1038 (80.8)
Referral	246 (19.2)
Moment of collection	
Before colonoscopy	1275 (99.3)
After colonoscopy	9 (0.7)
Most advanced lesion	
Colorectal cancer	47 (3.7)
Advanced adenoma*	135 (10.5)
Advanced serrated polyp†	30 (2.3)
Nonadvanced adenoma	250 (19.5)
Nonadvanced serrated polyp	53 (4.1)
No colorectal neoplasia‡	769 (59.9)
Location	
Proximal (of splenic flexure)	255 (49.5)
Distal (of transverse colon)	258 (50.1)
Unknown	2 (0.4)
Stage of colorectal cancer	
I	10 (21.3)
II	11 (23.4)
III	11 (23.4)
IV	6 (12.8)
Unknown	9 (19.1)

* Advanced adenoma is an adenoma with at least 1 of the following characteristics: size ≥10 mm and/or >25% villous component and/or high-grade dysplasia.
† Advanced serrated polyp is a serrated polyp with at least 1 of the following characteristics: size ≥10 mm and/or any grade of dysplasia.
‡ Colorectal neoplasia is colorectal cancer, advanced adenoma, advanced serrated polyp, nonadvanced adenoma, and nonadvanced serrated polyp.

FIT Analysis

After collection, FIT samples were stored at −80 °C. Within several weeks from collection, hemoglobin concentrations were measured using the OC-Sensor DIANA automated analyzer

machine (Eiken Chemical), as reported previously (18–20). After measurement, samples were stored again at −80 °C until further use.

Protein Biomarkers

From 29 candidate protein biomarkers previously identified, on the basis of their complementarity and performance in multivariate analysis as well as biological considerations, 10 candidates (α-2-macroglobulin, calprotectin, C3 complement, hemoglobin, haptoglobin, hemopexin, lactotransferrin, myeloperoxidase, retinol-binding protein 4, and serpin family F member 2 [serpinF2]) were selected to develop antibody-based assays (**Supplement Table 1**, available at Annals.org) (18, 19).

Antibody-Based Fecal Protein Biomarker Assays

For these 10 candidate protein biomarkers, antibody-based assays were developed using industry-standard technology provided by Meso Scale Diagnostics. This technology allows for multiplex protein biomarker detection and quantification on the basis of electrochemiluminescence. Biobanked samples were analyzed in duplicates, and statistical analyses were done on mean protein concentrations. Meso Scale Diagnostics' SECTOR Imager 6000 plate reader was used to provide a quantitative result of each detected protein biomarker in leftover FIT samples. Results were analyzed with Discovery Workbench, version 4.0 software (Meso Scale Diagnostics) (18, 19, 21). A subset of the haptoglobin results was previously presented by Komor and colleagues (19).

Statistical Analysis

Statistical analyses were done in RStudio, version 1.1.453 (R Foundation for Statistical Computing) and included the rpart, pROC, and rms packages (22–26). In addition, IBM SPSS Statistics, version 25 was used.

Classification and regression tree (CART) analysis was done. This is a nonparametric regression method that contains nonlinear covariate effects and captures the interaction between covariates. With CART, the combination of complementing protein biomarkers that did best in classifying persons into case patients and control participants was identified. Control participants were defined as persons without colorectal neoplasia (n = 769), and case patients were defined as persons with AN (n = 212). The CART analysis defined both the optimal combination of protein biomarkers and the cutoff for these protein biomarkers to detect AN. As internal validation, a leave-one-out, cross-validated estimate of mtFIT sensitivity was determined to correct for possible overoptimism. The cross-validated results are presented throughout the manuscript. The cross-validated sensitivity of mtFIT was compared with the sensitivity of FIT at equal specificity using the McNemar test. Confidence intervals (Clopper–Pearson) were determined for the sensitivities observed, and concordant and discordant test results between mtFIT and FIT were evaluated.

Because the CART analysis was done using only persons without colorectal neoplasia as control participants (n = 769), we subsequently checked the performance of the algorithm after adding persons with nonadvanced lesions (n = 303) as control participants (n = 1072). Again, mtFIT was compared with FIT at equal specificity. Because the CART model was built using a study population without nonadvanced lesions, there was no need for additional cross-validation of the nonadvanced lesion predictions.

In addition, performance in each of the subcategories CRC, AA, and ASP, separately, was determined. To evaluate if AA specific characteristics (for example, high-grade dysplasia), age, or sex would affect mtFIT or FIT sensitivities for AAs, the χ^2 test for association with sensitivity was used for categorical variables, and the unpaired t test was used for continuous variables. P values less than 0.05 were considered statistically significant.

Early Health Technology Assessment

For the purpose of early health technology assessment, to assess the development potential of mtFIT, we compared projected long-term health outcomes and cost-effectiveness of mtFIT versus FIT in the context of the current Dutch FIT-based national screening program, using the externally validated Adenoma and Serrated pathway to Colorectal CAncer screening model (27, 28). For both tests, we simulated screening according to the Dutch program, that is, biennial screening between the ages of 55 and 75 years at 73% participation (29, 30). To obtain an indication of the potential cost-effectiveness of mtFIT, a threshold analysis was done. We assumed a willingness-to-pay threshold of €41 258 and $65 298 per life-year gained, corresponding to the Dutch and American gross domestic product per capita, respectively (31–33).

Role of the Funding Source

The funding sources had no role in the design, conduct, or reporting of the study or in the decision to submit the manuscript for publication.

RESULTS

Selection and Quantification of Protein Biomarkers

From 29 previously identified candidate protein biomarkers, 10 were selected for developing antibody-based fecal protein biomarker assays (**Supplement Table 1**) (18, 19). This was successful for 9 protein biomarkers, whereas for 1 protein biomarker, retinol-binding protein 4, assay development failed for technical reasons.

All 9 remaining protein biomarkers could be quantified in all 1284 FIT samples. All of these biomarkers showed significantly higher concentrations (P < 0.001) in samples from persons with CRC compared with those from persons without colorectal neoplasia (**Figure 1**). In addition, 8 of 9 protein biomarkers had significantly higher concentrations in samples from persons with AA compared with those from persons without colorectal neoplasia (P < 0.001 to 0.044). In contrast, samples from persons with ASP had protein biomarker concentrations similar to those from persons without colorectal neoplasia (**Figure 1**).

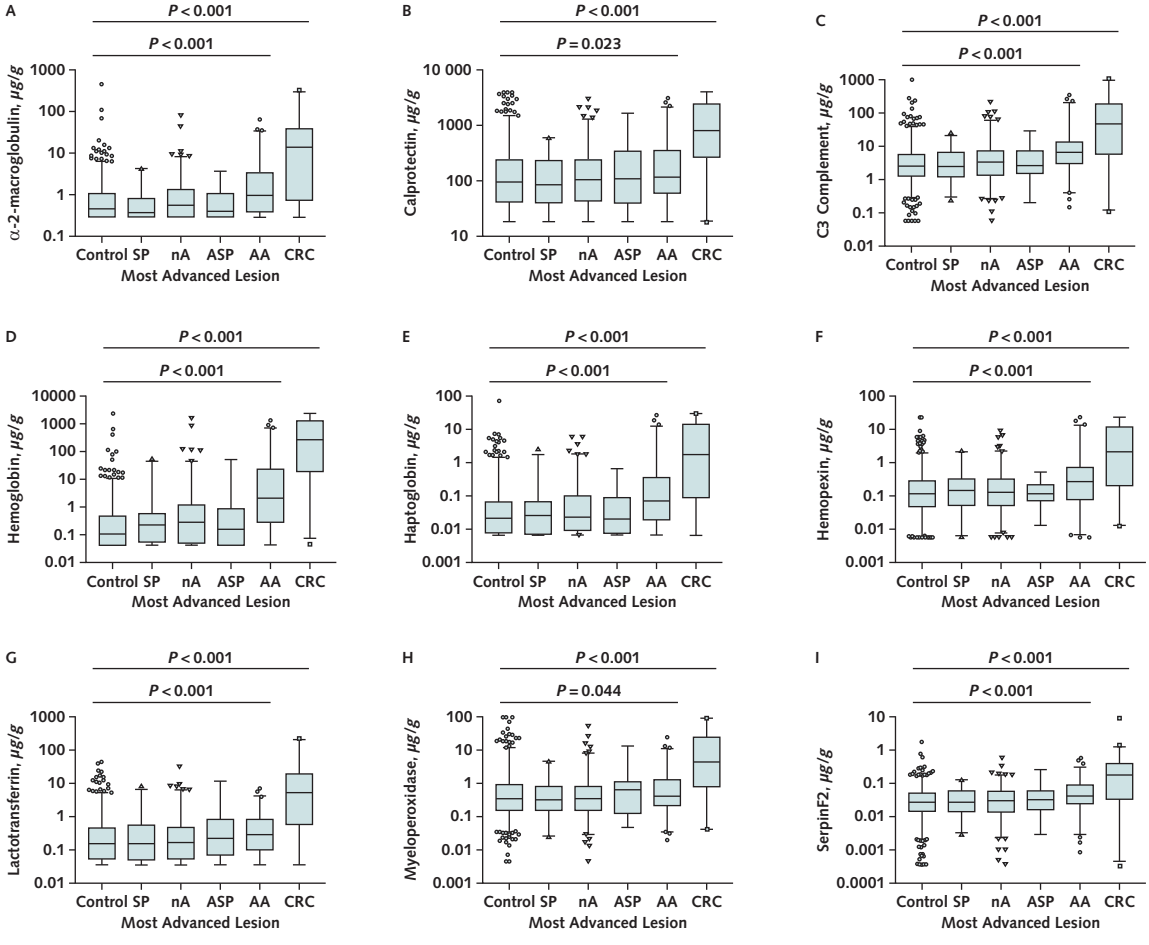
Selection of the Optimal Protein Biomarker Panel

The CART analysis yielded a combination of hemoglobin, calprotectin, and serpinF2, further called mtFIT,

to have the best diagnostic performance (**Supplement Figure 1** and **Supplement Table 2**, available at Annals.org). After leave-one-out cross-validation, at equal specificity of 96.6%, sensitivity for AN was 42.9% (95% CI, 36.2% to 49.9%) for mtFIT and 37.3% (CI, 30.7% to 44.2%) for FIT ($P = 0.025$) at a cutoff of 15.3 µg/g of feces (**Table 2**). This increase in cross-validated sensitivity was completely due to an increased sensitivity for AA (37.8% [CI, 29.6% to 46.5%] for mtFIT versus 28.1% [CI, 20.8% to 36.5%] for FIT; $P = 0.006$). The cross-validated sensitivity for CRC was 78.7% (CI, 64.3% to 89.3%) for mtFIT versus 80.9% (CI, 66.7% to 90.9%) for FIT ($P = 1.00$), and the cross-validated sensitivities of mtFIT and FIT for ASP were equal at 10.0% (CI, 2.1% to 26.5%) ($P = 0.48$) (**Figure 2** and **Table 2**).

Both FIT and mtFIT predicted 36 of 47 cases of CRC correctly, but 3 persons with CRC had discordant results. Two were FIT positive and mtFIT negative, and 1 vice versa (**Supplement Table 3**, available at Annals.org). In total, 16 persons with AA had a positive mtFIT result but a negative FIT result, whereas 3 persons with AA had a positive FIT result but a negative mtFIT result. The increased sensitivity for AA was not associated with sex or age (**Supplement**). The differences between the sensitivities for specific AA histologic characteristics (for example, tubular vs. villous histology and high-grade vs. low-grade dysplasia) were not statistically significant for both mtFIT and FIT. However, a significantly higher detection of AAs 10 mm or greater compared with AAs less than 10 mm by both FIT and mtFIT ($P = 0.003$ and

Figure 1. Differential abundance of 9 protein biomarkers in 1284 FIT samples.



Nine protein biomarkers were quantified in 1284 FIT samples. All protein biomarkers showed significantly higher concentrations in samples from persons with CRC compared with those from persons without colorectal neoplasia. Eight protein biomarkers showed significantly higher concentrations in samples from persons with AA compared with those from persons without colorectal neoplasia. In samples from persons with ASP, no significantly different concentrations were seen. The points outside the whisker boundaries (95% CI) are considered outliers (white circle = persons without colorectal neoplasia; triangle = persons diagnosed with a serrated polyp; reverse triangle = persons diagnosed with a nonadvanced adenoma; grey circle = persons diagnosed with an advanced adenoma; square = persons diagnosed with a colorectal cancer). AA = advanced adenoma; ASP = advanced serrated polyp; control = person without colorectal neoplasia; CRC = colorectal cancer; FIT = fecal immunochemical test; nA = nonadvanced adenoma; serpinF2 = serpin family F member 2; SP = nonadvanced serrated polyp.

Table 2. Sensitivity per Lesion Type of Cross-validated mtFIT Versus FIT at an Equal Specificity of 96.6%

Most Advanced Lesion*	Colonoscopy, *n*	Cross-validated mtFIT		FIT (15.3 µg/g >of Feces)		*P* Value
		Predicted Case Patients, *n*	Sensitivity (95% CI), %	Predicted Case Patients, *n*	Sensitivity (95% CI), %	
Advanced neoplasia	212	91	42.9 (36.2–49.9)	79	37.3 (30.7–44.2)	0.025
Colorectal cancer	47	37	78.7 (64.3–89.3)	38	80.9 (66.7–90.9)	1.00
Advanced adenoma	135	51	37.8 (29.6–46.5)	38	28.1 (20.8–36.5)	0.006
Advanced serrated polyp	30	3	10.0 (2.1–26.5)	3	10.0 (2.1–26.5)	0.48
		Predicted Control Participants, *n*	Specificity (95% CI), %‡	Predicted Control Participants, *n*	Specificity (95% CI), %‡	
Control participants (no colorectal neoplasia†)	769	743	96.6 (95.1–97.8)	743	96.6 (95.1–97.8)	–

FIT = fecal immunochemical test; mtFIT = multitarget FIT.
* Total equals 981 lesions.
† Colorectal neoplasia is defined as colorectal cancer, advanced adenoma, advanced serrated polyp, nonadvanced adenoma, and nonadvanced serrated polyp.
‡ Fixed specificity to enable comparison of mtFIT and FIT at an equal level. Therefore, there is no *P* value calculated for the control group.

P = 0.031, respectively) was seen (Supplement Table 4, available at Annals.org).

When persons with nonadvanced lesions were included as control participants in the data set, mtFIT specificity reduced from 96.6% to 94.6%. At equal specificity of 94.6%, sensitivity of mtFIT (42.9% [CI, 36.2% to 49.9%]) for AN remained higher compared with FIT (38.2% [CI, 31.6% to 45.1%]) (*P* = 0.066). This increase was again due to an increased sensitivity for AA (37.8% [CI, 29.6% to 46.5%] for mtFIT versus 29.6% [CI, 22.1% to 38.1%] for FIT; *P* = 0.022). Sensitivities for CRC remained not significantly different for both tests (78.7% [CI, 64.3% to 89.3%] for mtFIT versus 80.9% [CI, 66.7% to 90.9%] for FIT; *P* = 1.00). Sensitivities for ASP remained equal at 10% (CI, 2.1% to 26.5%) for both tests (*P* = 0.48) (Supplement Table 5, available at Annals.org).

Early Health Technology Assessment

On the basis of the cross-validated data, mtFIT screening compared with FIT screening was predicted to result in a 12% CRC incidence reduction and an 8% CRC mortality reduction (Supplement Tables 6 and 7 and Supplement Figure 2, available at Annals.org). The maximum cost per test, at which mtFIT screening would still be cost-effective compared with FIT screening, was estimated at double €59 or $84 for the Dutch or American willingness-to-pay threshold, respectively (Supplement Table 7).

DISCUSSION

Early detection and interception remain the stronghold for reducing CRC-related death. Of the early detection arsenal, FIT screening is considered the most cost-effective and is widely applied worldwide (4, 5). Yet, whereas FIT is very effective in detecting CRC, the sensitivity of FIT for high-risk precursor lesions is low, leaving room for improvement (10, 11). Therefore, we set out to develop a novel protein-based mtFIT that would outperform FIT in the detection of AN at an equally high specificity.

In theory, molecules from neoplastic cells or from the associated tumor microenvironment hold the most potential as diagnostic biomarkers to increase the yield of CRC screening. The multitarget stool DNA test involving hemoglobin, DNA-mutation, and promoter methylation markers has been the most successful approach so far. Indeed, this test has a higher sensitivity for high-risk precursor lesions than FIT (12, 14). Although this test has been widely adopted in the United States, in many other countries it has not yet substituted FIT-based programmatic screening. The main reasons are the more complex logistics, lower specificity, and lower cost-effectiveness than FIT (14, 17).

Previously, we discovered protein biomarkers that could aid in the detection of AN, 10 of which were selected for further validation in the current study (18, 19). Aiming to develop a test suitable for programmatic CRC screening, we took 2 critical steps: we moved away from mass spectrometry–based analysis to antibody-based assays and from whole stool samples to much smaller (leftover) FIT samples as input for the analysis. Antibody-based assays were successfully developed for 9 of the 10 selected candidate protein biomarkers. The CART analysis defined an optimal combination of 3 protein biomarkers (mtFIT) that have complementary diagnostic value for AN: hemoglobin, calprotectin, and serpinF2. The improved diagnostic accuracy for AN seemed to be completely due to a 34.5% relative improvement in AA sensitivity. The mtFIT detected 1 less CRC than FIT, yet this difference was not statistically significant (*P* = 1.00) (Table 2). Early health technology assessment was done to evaluate whether the results observed (that is, potential clinical effect and cost-effectiveness) would provide a sound basis to justify further clinical development, which we consider to be the case. Of note, early health technology assessment does not intend to provide cost-effectiveness data for health policy decision making. This should be based on large scale, prospective screening trials like the one in preparation (34).

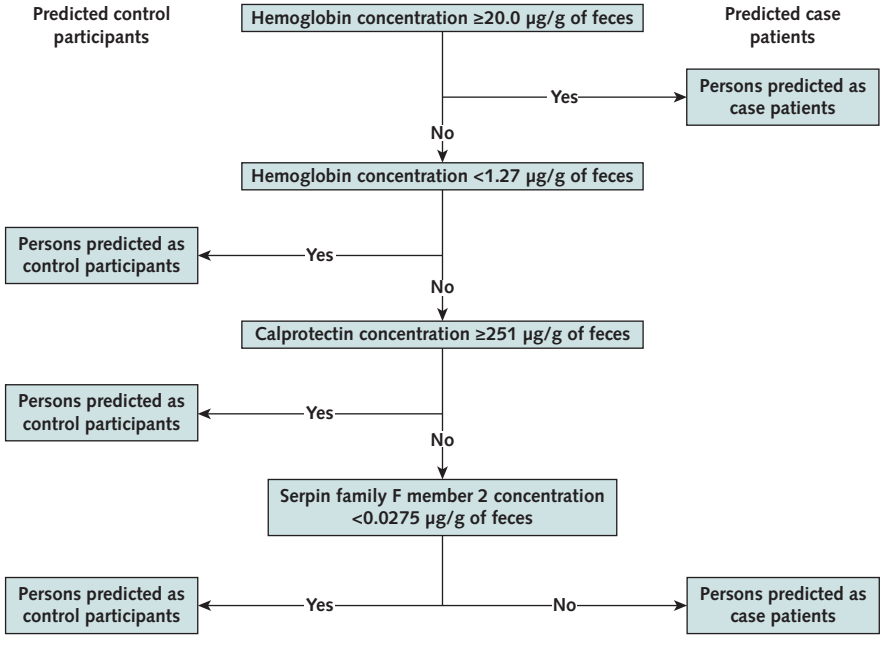
In the mtFIT, hemoglobin plays an important role, which should not come as a surprise given the success of

FIT-based screening and the inclusion of hemoglobin in the multitarget stool DNA test (14). However, because hemoglobin in mtFIT was used in combination with 2 other markers compared with hemoglobin as a single marker in FIT, while maintaining equal specificity for both tests, hemoglobin cutoffs used in mtFIT and FIT were not identical. The second biomarker in the mtFIT is calprotectin, a cytosolic leukocyte protein, which is abundant in neutrophils and disseminated in the tumor stroma from where it can reach the intestinal lumen (35, 36). Calprotectin is associated with inflammation. In fact, it has already been routinely used for monitoring disease activity in inflammatory bowel disease. Calprotectin has also been evaluated as a potential marker for early detection of CRC, but these studies remain inconclusive (37–41). In contrast to previous studies, in this study, calprotectin concentrations influenced the test outcome of only persons with an intermediate hemoglobin concentration (between 1.27 and 20.0 μg/g of feces). In this category of persons with an intermediate hemoglobin concentration, most true positives had an AA as the most advanced lesion. The mtFIT classified persons with intermediate hemoglobin concentrations and calprotectin concentrations above 251 μg/g of feces as control participants. The third biomarker is serpinF2, a serine protease inhibitor that has a role in balancing protein degradation, including fibrin (42–44). Serine proteases and their inhibitors also play a role in remodeling the tumor microenvironment (45). In addition, serpinF2 protein expression is increased in several types of cancer, including CRC (44, 46–48).

Sensitivity of mtFIT for ASP was as poor as that of FIT (10%). Most ASPs are defined based on their size being 10 mm or greater, yet mostly in absence of dysplasia. Interestingly, methylation markers in stool have been found to be able to improve the sensitivity of serrated polyps, and indeed the multitarget stool DNA test does show a higher sensitivity for serrated polyps (14, 49). In theory, by combining the mtFIT with methylation markers, sensitivity for ASP could be further improved. However, methylation markers cannot yet be reliably detected in small stool samples.

Although most samples in this study have been prospectively collected from a well-designed screening study, the study population was enriched with samples from a referral population to increase the number of cases, especially cases of CRC. This could be considered a limitation, although an increased sensitivity for AAs was also seen when looking at only the screening population (**Supplement Figure 3**, available at Annals.org). Furthermore, inherent to biomarker studies using retrospective collections, samples were frozen and thawed. Samples were frozen on collection and had 2 freeze–thaw cycles, 1 time to do the FIT analysis and a second time to do the mtFIT analysis. However, the hemoglobin levels as measured originally by FIT and those analyzed

Figure 2. Classification and regression tree model.



Persons were assigned as case patients or control participants on the basis of the proteins incorporated in the multitarget fecal immunochemical test. First, hemoglobin concentrations were used to assign predicted case patients (concentrations ≥20 μg/g of feces) and predicted control participants (concentrations <1.27 μg/g of feces). Thereafter, persons with hemoglobin concentrations between 1.27 and 20 μg/g of feces were assigned as predicted control participants if calprotectin was greater than 251 μg/g of feces. Finally, the remaining participants were assigned as predicted control participants (concentrations <0.0275 μg/g of feces) or predicted case patients (concentrations ≥0.0275 μg/g of feces) on the basis of serpin family F member 2 concentration.

by the mtFIT (that is, after an extra freeze-thaw cycle) showed a good correlation ($r^2 = 0.89$). This indicates that no substantial freeze-thaw effects have seemed to play a role (**Supplement Figure 4**, available at Annals.org). Nevertheless, a potential effect over time on the composition of proteins in the stool sample cannot be completely excluded. In the planned prospective screening study, this is a factor that can be controlled for.

In conclusion, this study provides clinical validation of a mtFIT with higher accuracy for detecting AN, in particular AA, compared with FIT. This enables early detection and interception at a premalignant stage rather than an early invasive stage, which could have a major effect on quality of life. Moreover, compared with FIT screening, mtFIT-based screening could lead to a further reduction in CRC incidence and death. The fact that, both logistically and health economically, mtFIT could be compatible with the current practice of FIT-based screening programs may largely facilitate future implementation of mtFIT. A prospective screening trial to further validate mtFIT within the context of the Dutch CRC screening program is in preparation (34).

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Reproducible Research Statement: *Study protocol:* Available from Dr. de Wit (e-mail, m.d.wit@nki.nl). *Statistical code and data set:* Available on request from Dr. de Wit (e-mail, m.d.wit@nki.nl).

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References

1. **Bray F, Ferlay J, Soerjomataram I, et al.** Global cancer statistics 2018: GLOBOCAN estimates of incidence and mortality worldwide for 36 cancers in 185 countries. CA Cancer J Clin. 2018;68:394-424. [PMID: 30207593] doi:10.3322/caac.21492
2. **Brenner H, Hoffmeister M, Stegmaier C, et al.** Risk of progression of advanced adenomas to colorectal cancer by age and sex: estimates based on 840,149 screening colonoscopies. Gut. 2007;56:1585-9. [PMID: 17591622]
3. **Kuipers EJ, Rösch T, Bretthauer M.** Colorectal cancer screening–optimizing current strategies and new directions. Nat Rev Clin Oncol. 2013;10:130-42. [PMID: 23381005] doi:10.1038/nrclinonc.2013.12
4. **Navarro M, Nicolas A, Ferrandez A, et al.** Colorectal cancer population screening programs worldwide in 2016: an update. World J Gastroenterol. 2017;23:3632-3642. [PMID: 28611516] doi:10.3748/wjg.v23.i20.3632
5. **Schreuders EH, Ruco A, Rabeneck L, et al.** Colorectal cancer screening: a global overview of existing programmes. Gut. 2015;64:1637-49. [PMID: 26041752] doi:10.1136/gutjnl-2014-309086
6. **Joseph DA, Meester RG, Zauber AG, et al.** Colorectal cancer screening: estimated future colonoscopy need and current volume and capacity. Cancer. 2016;122:2479-86. [PMID: 27200481] doi:10.1002/cncr.30070
7. **Zhong GC, Sun WP, Wan L, et al.** Efficacy and cost-effectiveness of fecal immunochemical test versus colonoscopy in colorectal cancer screening: a systematic review and meta-analysis. Gastrointest Endosc. 2020;91:684-697.e15. [PMID: 31790657] doi:10.1016/j.gie.2019.11.035
8. **Gies A, Cuk K, Schrotz-King P, et al.** Direct comparison of diagnostic performance of 9 quantitative fecal immunochemical tests for colorectal cancer screening. Gastroenterology. 2018;154:93-104. [PMID: 28958859] doi:10.1053/j.gastro.2017.09.018
9. **Chiu HM, Chen SL, Yen AM, et al.** Effectiveness of fecal immunochemical testing in reducing colorectal cancer mortality from the One Million Taiwanese Screening Program. Cancer. 2015;121:3221-9. [PMID: 25995082] doi:10.1002/cncr.29462
10. **Lee JK, Liles EG, Bent S, et al.** Accuracy of fecal immunochemical tests for colorectal cancer: systematic review and meta-analysis. Ann Intern Med. 2014;160:171. [PMID: 24658694]. doi:10.7326/M13-1484
11. **Imperiale TF, Gruber RN, Stump TE, et al.** Performance characteristics of fecal immunochemical tests for colorectal cancer and advanced adenomatous polyps: a systematic review and meta-analysis. Ann Intern Med. 2019;170:319-329. [PMID: 30802902]. doi:10.7326/M18-2390
12. **Dickinson BT, Kisiel J, Ahlquist DA, et al.** Molecular markers for colorectal cancer screening. Gut. 2015;64:1485-94. [PMID: 25994221] doi:10.1136/gutjnl-2014-308075
13. **Bosch LJ, Carvalho B, Fijneman RJ, et al.** Molecular tests for colorectal cancer screening. Clin Colorectal Cancer. 2011;10:8-23. [PMID: 21609931] doi:10.3816/CCC.2011.n.002
14. **Imperiale TF, Ransohoff DF, Itzkowitz SH, et al.** Multitarget stool DNA testing for colorectal-cancer screening. N Engl J Med. 2014;370:1287-97. [PMID: 24645800] doi:10.1056/NEJMoa1311194
15. **Issa IA, Noureddine M.** Colorectal cancer screening: an updated review of the available options. World J Gastroenterol. 2017;23:5086-5096. [PMID: 28811705] doi:10.3748/wjg.v23.i28.5086
16. **Rasmussen SL, Krarup HB, Sunesen KG, et al.** Hypermethylated DNA as a biomarker for colorectal cancer: a systematic review. Colorectal Dis. 2016;18:549-61. [PMID: 26998585] doi:10.1111/codi.13336

17. **Lansdorp-Vogelaar I, Goede SL, Bosch LJW, et al.** Cost-effectiveness of high-performance biomarker tests vs fecal immunochemical test for noninvasive colorectal cancer screening. Clin Gastroenterol Hepatol. 2018;16:504-512.e11. [PMID: 28733262] doi:10.1016/j.cgh.2017.07.011
18. **Bosch LJW, de Wit M, Pham TV, et al.** Novel stool-based protein biomarkers for improved colorectal cancer screening: a case-control study. Ann Intern Med. 2017;167:855-866. [PMID: 29159365]. doi:10.7326/M17-1068
19. **Komor MA, Bosch LJ, Coupé VM, et al.** Proteins in stool as biomarkers for non-invasive detection of colorectal adenomas with high risk of progression. J Pathol. 2020;250:288-298. [PMID: 31784980] doi:10.1002/path.5369
20. **de Wijkerslooth TR, de Haan MC, Stoop EM, et al.** Study protocol: population screening for colorectal cancer by colonoscopy or CT colonography: a randomized controlled trial. BMC Gastroenterol. 2010;10:47. [PMID: 20482825] doi:10.1186/1471-230X-10-47
21. **Debad JD, Glezer EN, Leland JK, et al.** Clinical and biological applications of ECL. In: Bard AJ, ed. Electrogenerated Chemiluminescence. Taylor & Francis; 2004:359-396.
22. **Friedman J, Hastie T, Tibshirani R.** Regularization paths for generalized linear models via coordinate descent. J Stat Softw. 2010;33:1-22. [PMID: 20808728]
23. **Robin X, Turck N, Hainard A, et al.** pROC: an open-source package for R and S+ to analyze and compare ROC curves. BMC Bioinformatics. 2011;12:77. [PMID: 21414208] doi:10.1186/1471-2105-12-77
24. **Therneau T, Atkinson B, Ripley B.** rpart: recursive partitioning and regression trees. R Foundation for Statistical Computing; 2016. Accessed athttps://cran.r-project.org/web/packages/rpart/index.html on 7 December 2020.
25. **Warnes GR, Bolker B, Bonebakker L, et al.** gplots: various R programming tools for plotting data. R package version 2.17.0. R Foundation for Statistical Computing; 2015. Accessed at https://cran.r-project.org/src/contrib/Archive/gplots/ on 7 December 2020.
26. **Wickham H.** ggplot2: Elegant Graphics for Data Analysis. Springer; 2016.
27. **Greuter MJ, Xu XM, Lew JB, et al.** Modeling the Adenoma and Serrated pathway to Colorectal CAncer (ASCCA). Risk Anal. 2014;34:889-910. [PMID: 24172539] doi:10.1111/risa.12137
28. **Lew JB, Greuter MJE, Caruana M, et al.** Validation of microsimulation models against alternative model predictions and long-term colorectal cancer incidence and mortality outcomes of randomized controlled trials. Med Decis Making. 2020;40:815-829. [PMID: 32845232] doi:10.1177/0272989X20944869
29. **National Institute for Public Health and the Environment, Ministry of Health, Welfare and Sport.** National monitoring of the Colorectal Cancer Screening Programme. Accessed at www.rivm.nl/en/national-monitoring-of-colorectal-cancer-screening-programme on 7 December 2020.
30. **Toes-Zoutendijk E, Portillo I, Hoeck S, et al.** Participation in faecal immunochemical testing-based colorectal cancer screening programmes in the northwest of Europe. J Med Screen. 2020;27:68-76. [PMID: 31645173] doi:10.1177/0969141319879712
31. **Hutubessy R, Chisholm D, Edejer TT.** Generalized cost-effectiveness analysis for national-level priority-setting in the health sector. Cost Eff Resour Alloc. 2003;1:8. [PMID: 14687420]
32. **CBS.** Regional key figures; national accounts 2010-2016. Accessed at www.cbs.nl/en-gb/figures/detail/82801ENG?q=Statistics%20Netherlands%20website on 7 December 2020.
33. **The World Bank.** GDP per capita (current US$)-United States 2021. Accessed at https://data.worldbank.org/indicator/NY.GDP.PCAP.CD?end=2019&locations=US&start=2019 on 7 December 2020.
34. **Gezondheidsraad.** Reactie staatssecretaris VWS op WBO: onderzoek naar een fecale eiwit biomarkertest in het BVO darmkanker. Accessed at www.gezondheidsraad.nl/documenten/adviezen/2020/04/14/reactie-staatssecretaris-vws-op-advies-wbo-onderzoek-naar-een-fecale-eiwit-biomarkertest-in-het-bvo-darmkanker on 7 December 2020.
35. **Osborn NK, Ahlquist DA.** Stool screening for colorectal cancer: molecular approaches. Gastroenterology. 2005;128:192-206. [PMID: 15633136]
36. **Urban CF, Ermert D, Schmid M, et al.** Neutrophil extracellular traps contain calprotectin, a cytosolic protein complex involved in host defense against Candida albicans. PLoS Pathog. 2009;5:e1000639. [PMID: 19876394] doi:10.1371/journal.ppat.1000639
37. **Røseth AG, Kristinsson J, Fagerhol MK, et al.** Faecal calprotectin: a novel test for the diagnosis of colorectal cancer. Scand J Gastroenterol. 1993;28:1073-6. [PMID: 8303210]
38. **Tibble J, Sigthorsson G, Foster R, et al.** Faecal calprotectin and faecal occult blood tests in the diagnosis of colorectal carcinoma and adenoma. Gut. 2001;49:402-8. [PMID: 11511563]
39. **Hoff G, Grotmol T, Thiis-Evensen E, et al.** Testing for faecal calprotectin (PhiCal) in the Norwegian Colorectal Cancer Prevention trial on flexible sigmoidoscopy screening: comparison with an immunochemical test for occult blood (FlexSure OBT). Gut. 2004;53:1329-33. [PMID: 15306594]
40. **Mari A, Baker FA, Mahamid M, et al.** Clinical utility of fecal calprotectin: potential applications beyond inflammatory bowel disease for the primary care physician. Ann Gastroenterol. 2019;32:425-430. [PMID: 31474787] doi:10.20524/aog.2019.0394
41. **Moris D, Spartalis E, Angelou A, et al.** The value of calprotectin S100A8/A9 complex as a biomarker in colorectal cancer: a systematic review. J BUON. 2016;21:859-866. [PMID: 27685906]
42. **Bedin C, Crotti S, Ragazzi E, et al.** Alterations of the plasma peptidome profiling in colorectal cancer progression. J Cell Physiol. 2016;231:915-25. [PMID: 26379225] doi:10.1002/jcp.25196
43. **Mosesson MW.** Fibrinogen and fibrin structure and functions. J Thromb Haemost. 2005;3:1894-904. [PMID: 16102057]
44. **Chen Y, Xie Y, Xu L, et al.** Protein content and functional characteristics of serum-purified exosomes from patients with colorectal cancer revealed by quantitative proteomics. Int J Cancer. 2017;140:900-913. [PMID: 27813080] doi:10.1002/ijc.30496
45. **Didiasova M, Wujak L, Wygrecka M, et al.** From plasminogen to plasmin: role of plasminogen receptors in human cancer. Int J Mol Sci. 2014;15:21229-52. [PMID: 25407528] doi:10.3390/ijms151121229
46. **Chan KY, Lai PB, Squire JA, et al.** Positional expression profiling indicates candidate genes in deletion hotspots of hepatocellular carcinoma. Mod Pathol. 2006;19:1546-54. [PMID: 16980951]
47. **Cavalcante Mde S, Torres-Romero JC, Lobo MD, et al.** A panel of glycoproteins as candidate biomarkers for early diagnosis and treatment evaluation of B-cell acute lymphoblastic leukemia. Biomark Res. 2016;4:1. [PMID: 26823978] doi:10.1186/s40364-016-0055-6
48. **Poersch A, Grassi ML, Carvalho VP, et al.** A proteomic signature of ovarian cancer tumor fluid identified by highthroughput and verified by targeted proteomics. J Proteomics. 2016;145:226-236. [PMID: 27222041] doi:10.1016/j.jprot.2016.05.005
49. **Bosch LJW, Melotte V, Mongera S, et al.** Multitarget stool DNA test performance in an average-risk colorectal cancer screening population. Am J Gastroenterol. 2019;114:1909-1918. [PMID: 31764091] doi:10.14309/ajg.0000000000000445

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