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Multitarget Stool DNA Test Performance in an Average-Risk Colorectal Cancer Screening Population

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INTRODUCTION: **We set out to evaluate the performance of a multitarget stool DNA (MT-sDNA) in an average-risk colonoscopy-controlled colorectal cancer (CRC) screening population. MT-sDNA stool test results were evaluated against fecal immunochemical test (FIT) results for the detection of different lesions, including molecularly defined high-risk adenomas and several other tumor characteristics.**

METHODS: **Whole stool samples (n = 1,047) were prospectively collected and subjected to an MT-sDNA test, which tests for *KRAS* mutations, *NDRG4* and *BMP3* promoter methylation, and hemoglobin. Results for detecting CRC (n = 7), advanced precancerous lesions (advanced adenoma [AA] and advanced serrated polyps; n = 119), and non-AAs (n = 191) were compared with those of FIT alone (thresholds of 50, 75, and 100 hemoglobin/mL). AAs with high risk of progression were defined by the presence of specific DNA copy number events as measured by low-pass whole genome sequencing.**

RESULTS: **The MT-sDNA test was more sensitive than FIT alone in detecting advanced precancerous lesions (46% (55/119) vs 27% (32/119), respectively, $P < 0.001$). Specificities among individuals with nonadvanced or negative findings (controls) were 89% (791/888) and 93% (828/888) for MT-sDNA and FIT testing, respectively. A positive MT-sDNA test was associated with multiple lesions ($P = 0.005$), larger lesions ($P = 0.03$), and lesions with tubulovillous architecture ($P = 0.04$). The sensitivity of the MT-sDNA test or FIT in detecting individuals with high-risk AAs (n = 19) from individuals with low-risk AAs (n = 52) was not significantly different.**

DISCUSSION: **In an average-risk screening population, the MT-sDNA test has an increased sensitivity for detecting advanced precancerous lesions compared with FIT alone. AAs with a high risk of progression were not detected with significantly higher sensitivity by MT-sDNA or FIT.**

SUPPLEMENTARY MATERIAL accompanies this paper at http://links.lww.com/AJG/B323, http://links.lww.com/AJG/B324

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INTRODUCTION

Colorectal cancer (CRC) is the third most common cancer and is one of the leading causes of cancer-related mortality (1). Although CRC incidence is increasing, a decrease in mortality has been observed due to the implementation of CRC screening and improved therapies (2,3). CRC screening not only leads to a reduction in morbidity and mortality but also to a lower CRC incidence when neoplastic lesions can be detected and removed at a premalignant stage (2). Therefore, CRC screening is considered cost-effective or even cost-saving (4).

Currently, colonoscopy is used for opportunistic screening programs in the United States, whereas in Europe, fecal immunochemical test (FIT) is the first choice for CRC screening in invitation-based screening programs. Both are considered cost-effective. Yet, FIT has suboptimal sensitivity for CRC (average 79%) and advanced adenomas (AAs; average 31%), precursor lesions with an increased risk of progression (5,6).

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We and others have shown that molecular stool tests have the potential to outperform FIT (7–9). We identified aberrant *NDRG4* CpG promoter hypermethylation as a stool biomarker for the early CRC detection (7), after which it was incorporated in an FDA-approved multitarget stool DNA (MT-sDNA) test (Cologuard; Exact Sciences, Madison, WI). This test contains multiple DNA targets (*NDRG4* and *BMP3* hypermethylation and *KRAS* mutations) and fecal hemoglobin. This MT-sDNA test was clinically validated in stool samples from almost 10.000 average risk individuals, where it detected up to 42% of AAs and 92% of CRCs. These sensitivities provide higher single-application sensitivity than a commercial FIT for both CRC and AA (10). Although single-application MT-sDNA specificity is lower than FIT as generally used in annual population screening, guidelines in the United States (11) recommend MT-sDNA screening every three years which has similar specificity to 3 annual FIT applications.

Although most studies focus on the detection of CRC rather than precursor lesions, it is evident that most room for improving screening tests lies in more sensitive adenoma detection (12,13). AAs, defined by histopathological criteria of size, presence of villous architecture, and/or high-grade dysplasia, have been considered to be at higher risk of progressing to carcinoma than non-AAs (14). Detection of AAs is therefore used as an important intermediate endpoint in evaluating screening. However, only about 5% of all adenomas will ever progress to cancer (15–17); thus, ideally, the interventional screening target should be even more focused. Our research group established an alternative molecular-defined intermediate endpoint based on specific DNA copy number aberrations (gain of 8q, 13q, and 20q; loss of 8p, 15q, 17p, and 18q) that distinguish adenomas at low risk of progression from adenomas with a high risk of progression and later stage cancers with a sensitivity of 78% and specificity of 78% (18). These specific DNA copy number aberrations were found in 25% of the AAs and also occurred in 3% of non-AAs (19). Moreover, in functional studies, it was observed that only adenoma organoids that carried chromosomal instability were able to progress to invasive carcinomas (20). These studies indicate a role of copy number alterations in predicting the risk of progression to CRC (16,21).

In this study, we evaluated the performance of an MT-sDNA test, analyzing aberrant *NDRG4* and *BMP3* promoter hypermethylation, *KRAS* mutations, and hemoglobin, in archived stool samples from a Dutch average-risk primary screening population. Moreover, we compared the sensitivity of MT-sDNA and FIT for detecting AAs with a high risk and a low risk of progression.

MATERIALS AND METHODS

Study population

Between June 2009 and July 2010, a total of 6,600 asymptomatic individuals in the regions of Amsterdam and Rotterdam, aged 50–75 years, were randomly selected by the regional municipal administration registrations and invited for primary colonoscopy screening. The protocol of this population-based screening pilot (COlonoscopy or COlonography for Screening [COCOS] trial) has been described in detail previously (22). The results on participation and diagnostic yield of this trial have been published (23). In short, participation with CT colonography was significantly better than with colonoscopy, but colonoscopy identified significantly more advanced neoplasia per 100 participants (7,8) than did CT colonography (1,6). The diagnostic yield for advanced neoplasia per 100 invitees was similar for both strategies (1.5 per 100 invitees for colonoscopy and 2.0 per 100 invitees for CT colonography). The trial was registered in the Dutch Trial Register: NTR1829 (http://www.trialregister.nl). At the time of the trial, the Netherlands did not have a population-based CRC screening program, and invitees were screening-naive. Invitees who had had a full colonic examination in the previous 5 years (complete colonoscopy, CT colonography, and/or double-contrast barium enema) were excluded from the screening trial. Invitees planned for surveillance colonoscopy (personal history of CRC, colonic adenomas, or inflammatory bowel disease) and individuals with end-stage disease and a life expectancy of less than 5 years were also excluded. Ethical approval was obtained from the Dutch National Health Council (2009/03WBO, Hague, the Netherlands).

Screening participants allocated to the colonoscopy arm of the COCOS trial (n = 1,426) and willing to undergo colonoscopy were invited to collect 1 sample FIT (OC-Sensor; Eiken Chemical, Tokyo, Japan) and 1 whole stool sample before their screening colonoscopy and before the start of laxative treatment. Spontaneously passed stool samples were collected from 1,047 participants who completed colonoscopy and had a valid FIT test. Participants were verbally instructed at the screening center or at home. Screening participants who agreed to participate gave written informed consent. The results on the diagnostic accuracy of the FIT have been published (5). For FIT with threshold 50 ng/mL, sensitivities were 35% for detecting AA and 88% for CRC with 91%–93% specificity.

Histology

Excised lesions were assessed by 1 of 2 expert gastrointestinal pathologists, 1 in each center as described before (23). Lesions were categorized as non-neoplastic, serrated polyp, adenoma, or carcinoma (24). Dysplasia was defined as either low grade or high grade. Advanced precancerous lesions refer to both AA and advanced serrated polyps (ASPs). An AA was defined as an adenoma ≥ 10 mm or an adenoma with villous histology (≥25% villous) and/or high-grade dysplasia of any size. An ASP was defined as a serrated or hyperplastic polyp ≥1 cm and/or a serrated polyp with low- or high-grade dysplasia. Advanced neoplasia included ASP, AA, and/or carcinoma.

We adhered to previous histological assessments (23) in the current study. Because the high sensitivity of the MT-sDNA test to detect high-grade dysplastic lesions (10) could not be confirmed in this study, lesions from patients with a single high-grade dysplastic lesion were rereviewed for the current study. Because the tissue slides as used for the previous histological assessment (23) were not available, new slides were used for these revisions.

Colonoscopy

All colonoscopies were performed according to the standard quality indicators defined by the Society of Gastrointestinal Endoscopy and recorded on DVD (25). Details of the procedure have been published (23). The most important quality indicators were qualifications and colonoscopy experience of the endoscopist (>500), adequate bowel preparation (Ottawa bowel preparation score <11), colonoscope withdrawal time (>6 minutes), and completeness of the colonoscopy (26). All colonoscopies were performed by endoscopists with experience of more than 1,000 colonoscopies, the median Ottawa bowel preparation score was 5 (interquartile range 3–8), the median net withdrawal time was 10 minutes (interquartile range 8–15 minutes), and the cecal intubation rate was 99% (5,27).

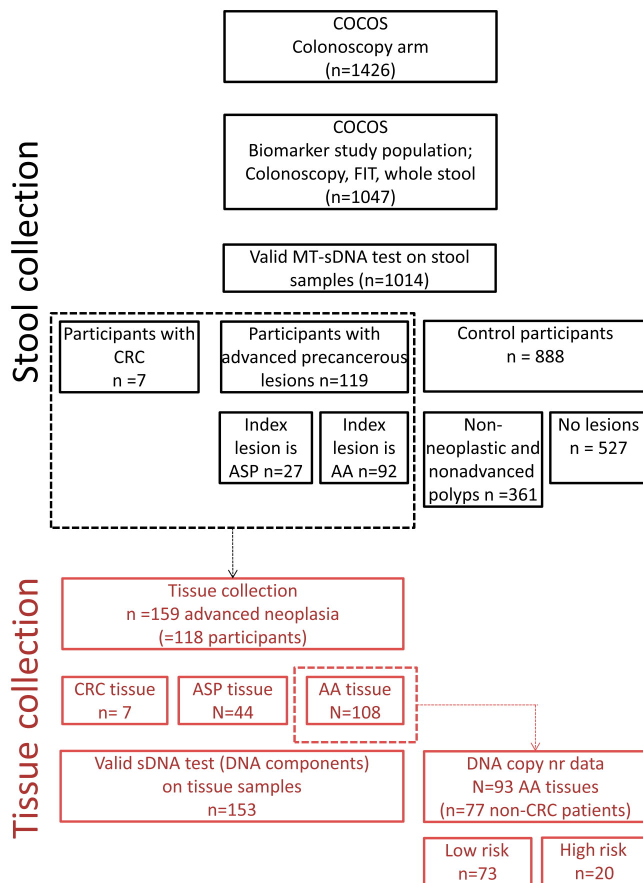

Figure 1. Flowchart of the study design. The upper part indicates the stool collection derived from the colonoscopy arm of individuals who participated the COCOS trial. Only individuals who had a complete colonoscopy, who had a valid FIT result, and who provided a whole stool sample were included in the biomarker study population. Tissues were collected from participants with an advanced neoplasia, e.g., CRC, AA, and/or ASP, of which the numbers and performed analyses are indicated in the lower part. AA, advanced adenoma; ASP, advanced serrated polyp; COCOS, COlonoscopy or COlonography for Screening; CRC, colorectal cancer.

FIT

FIT (OC-Sensor, Eiken Chemical, Tokyo, Japan) was performed by the screening participants before their preparation for screening colonoscopy. Details of the procedure have been published (5). Quantitative FIT values were available for the current study. Cutoff levels of 50 (FIT50), 75 (FIT75) and 100 (FIT100) ng hemoglobin (Hb)/mL of buffer, corresponding with 10, 15, and 20 μg Hb/g of feces, respectively, were considered as regular cutoff levels for calling a FIT positive or negative.

Stool samples and MT-sDNA test

All stool samples for DNA marker analysis were collected and processed according to a uniform standard operational procedure and analyzed at Exact Sciences Laboratory (Madison, WI). Single spontaneous stool samples were collected into a provided collection container. Stool stabilization buffer (Exact Sciences) was added to the stool sample by the screening participants immediately after defecation. The samples were processed in the laboratory with a final stool:buffer w/v ratio of 1:7 within 72 h after defecation, separated into aliquots, and stored at −80 °C until use. Extraction and analysis of stool DNA was performed as described before (10).

The DNA components were measured with quantitative molecular assays for aberrantly methylated *BMP3* and *NDRG4* promoter regions and 7 point mutations in *KRAS* (2 groups of mutations in the KRAS gene in codon 12 [referred to as KRAS1] and codon 13 [referred to as KRAS2]) (10). *ACTB* was used as a reference gene for human DNA quantity, both for the methylation assay and for the mutation assay (10). Fecal human hemoglobin levels were available from the previously measured FIT (OC-Sensor) values (5), and these values were used to calculate the final MT-sDNA result. As this assay differs from that is included in the MT-sDNA test as described before (OC FIT-CHEK; Polymedco, Cortlandt, NY [10]), the hemoglobin levels measured by OC-Sensor were recalculated to OC FIT-CHEK values for use in the MT-sDNA analytic algorithm (data not shown). Quantitative measurements of each marker (DNA and hemoglobin) were incorporated into this validated, prespecified logistic-regression algorithm, and a regression score (MT-sDNA score) was calculated, with a value of 183 or more, indicating that the MT-sDNA test result was positive (10) (see Supplementary Methods and Results, Supplementary Digital Content 1, http://links.lww.com/AJG/B323 and Figure 1, Supplementary Digital Content 2, http://links.lww.com/AJG/B324). Laboratory testing was performed without knowledge of the results of either the comparator FIT or clinical findings.

Tissue samples

Collection, storage, and use of patient-derived tissue and data were performed in compliance with the Code for Proper Secondary Use of Human Tissue in The Netherlands Dutch Federation of Biomedical Scientific Societies (28). Formalin-fixed paraffin-embedded tissue blocks from advanced neoplasia from patients with a valid MT-sDNA test were requested through the Dutch National Tissue Portal in close collaboration with the Dutch nationwide network and registry of histopathology and cytopathology in the Netherlands PALGA (29,30). DNA was isolated by a column-based method (QIamp DNA microkit; Qiagen, Hilden, Germany) as described before (31), with few adaptations for small lesions. These adaptations included a 5-day incubation period with lysis buffer (ATL buffer, QIAmp; DNA micro-kit, Qiagen) and freshly added (once every day) proteinase K (10 μL of 20 ng/μL) (32). Concentrations and purity were measured on a Nanodrop ND-1000 spectrophotometer (Nanodrop Technologies, Wilmington, DE). Tissue samples from a total of 159 advanced neoplasia from 118 individuals (often two or more advanced neoplasia present in 1 patient) were retrieved for molecular analysis.

MT-sDNA test on tissue samples Tissue DNA yields were adequate for analysis in 158 of 159 samples and were assayed for *KRAS* mutations aberrant *NDRG4* and *BMP3* methylation and *ACTB as* described before (33). Successful analysis was obtained for 153 samples.

Low-pass whole genome sequencing A total of 96 AA tissue samples from non-CRC patients were available for low-pass whole genome sequencing and genomewide DNA copy number analysis as described before (19,34). The DNA copy number data are available in the European Genome-Phenome Archive (EGA; https://www.ebi.ac.uk) under EGAS00001002952/EGAD00001004078. Three AA samples from this EGA archive were excluded in the current study because they either belonged to patients who have a CRC as most advanced lesion or were derived from different blocks from the same tumor.

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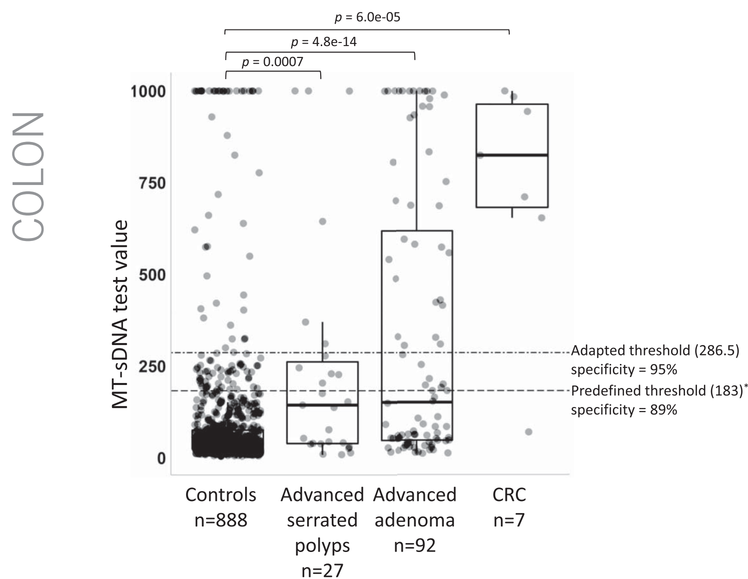

Figure 2. Boxplots showing sDNA test values in the COCOS biomarker population. Boxplots show first quartile, median, third quartile, and range of sDNA test values. Dots represent individual data points. The horizontal dashed lines indicate the predefined threshold for a positive test results as reported before (*) [ref Imperiale et al., *NEJM*, 2014], and the adapter threshold as defined in the current study using a fixed specificity of 95%. COCOS, COlonoscopy or COlonography for Screening.

AAs with at least 2 of 7 cancer-associated events (gain of 8q, 13q, and 20q; loss of 8p, 15q, 17p, and 18q) are defined as high-risk adenomas (18,19). Individuals with at least 1 high-risk adenoma are defined as high risk. When DNA copy number analysis of 1 or more AAs was missing from an individual with otherwise low-risk AAs, the individual was excluded from further analyses on molecular high- vs low-risk individuals.

Study design

The study design is described in detail below (Figure 1).

The following study endpoints were investigated

1. The sensitivity and specificity of the MT-sDNA and FIT using previously defined thresholds; sensitivity was defined for CRC, advanced precancerous lesions (AA, ASP, and high-grade dysplastic lesions) and non-AAs. Specificity was defined for different control groups (with and without non-neoplastic and/or nonadvanced polyps).
2. The sensitivity of the MT-sDNA test with FIT using an equal fixed specificity of 95%; sensitivity was defined and compared between the 2 tests for CRC and advanced precancerous lesions (AA, ASP, and high-grade dysplastic lesions).
3. Stool test results in relation to different tumor characteristics, number of lesions, size, location, morphology, histology, and dysplasia
4. Stool test results in relation to molecular-defined low- vs high-risk AAs.

Data analyses

Statistical analyses were performed using the computing environment R version 3.4.4, including the packages pROC, ggplot2, and epiR (35–37).

All screening participants who underwent a successful colonoscopy had a valid FIT result and who provided a stool sample appropriate for MT-sDNA testing were included in the analysis. The most advanced colorectal epithelial lesion was used to categorize screening participants for MT-sDNA analysis. The categories were controls (including screening participants without colorectal neoplasia and screening participants with non-AA, non-ASPs, and other non-neoplastic lesions), advanced precancerous lesions (including ASP and AA), and CRC. In case of multiple equivalent advanced precancerous lesions (2 or more AAs or ASPs), the largest was designated as the index lesion. In case of co-occurrence of an ASP and AA with the same size, an AA was considered more advanced than an ASP. In addition, molecular low-risk and high-risk AAs, as determined by DNA copy number aberrations (see the section on DNA copy number analysis), were considered as alternative intermediate endpoints.

Test characteristics were evaluated for advanced precancerous lesions as a group because there were only 7 cancers present in the study cohort. Receiver operating characteristic (ROC) analyses were used to evaluate the overall performance of the MT-sDNA test and FIT to discriminate advanced neoplasia from controls by calculating the area under the ROC curve (AUC). To test the statistical significance of the difference between the AUCs of the multitarget DNA test vs FIT, the DeLong test with 2000 bootstrap replications was used.

95% confidence intervals (CIs) for sensitivity and specificity measures were calculated using an exact binomial test. Given the fact that the prevalence of CRC is relatively small in the general population, a high specificity of the test is necessary to reach a high enough positive predictive value and reduce the number needed to screen as much as possible. Hence, a fixed and equal specificity of 95% was used to statistically compare the sensitivity of the MT-sDNA test with the sensitivity of FIT, for which test cutoffs were determined by ROC analysis. To test the statistical significance of the difference of the sensitivity at 95% specificity, the McNemar test was used.

Evaluating sDNA or FIT results in relation to the number of lesions per individual or tumor characteristics was performed by the χ^2 test or Fisher exact test, in case of low expected counts in the contingency table. DNA marker results in stool samples were compared with those in matched tissue samples by Pearson correlation tests. Comparing the sensitivity of the MT-sDNA test and FIT in high-risk vs low-risk AA was performed using the Fisher exact test.

RESULTS

Study population

Whole stool samples for MT-sDNA analysis, FIT results, and colonoscopy results were available for 1,047 individuals, accounting for 73% (1,047/1,426) of individuals who underwent colonoscopy within the COCOS trial. Next to missing FIT results and unsuccessful colonoscopy, some participants were not willing to collect whole stool samples or not enough stool volume was obtained for future analysis. MT-sDNA testing was successful in 1,014 of 1,047 evaluated samples (97%). The average age in these 1,014 individuals was 60 years (range 49–75), and 51% (520/1,014) was men. No differences in age or sex were found between evaluable and nonevaluable individuals. CRC was present in 7 evaluable individuals (0.7%). Advanced precancerous lesions were detected in 119 individuals (12%), of which 92 had AA and 27 had with ASP as index lesion. Nonadvanced lesions were

Table 1. Sensitivity and specificity at predefined cutoffs for the MT-sDNA test (threshold 183) and FIT (threshold 50, 75, and 100)

Most advanced finding	Total nr	MT-sDNA test		FIT50		MT-sDNA test vs FIT50	FIT75		MT-sDNA test vs FIT75	FIT100		MT-sDNA test vs FIT100
		Positive test (nr)	Sensitivity (95% CI)	Positive test (nr)	Sensitivity (95% CI)	Statistical difference, *P*	Positive test (nr)	Sensitivity (95% CI)	Statistical difference, *P*	Positive test (nr)	Sensitivity (95% CI)	Statistical difference, *P*
CRC	7	6	85.7% (42.0–100.0)	7	100% (59.0–100.0)	1.0	6	85.7% (42.0–100.0)	1.0	6	85.7% (42.0–100.0)	1.0
Advanced precancerous lesions	119	55	46.2% (37.0–55.6)	32	26.9% (19.2–35.8)	0.00001	27	22.6% (15.1–31.6)	0.0000008	27	22.6% (15.1–31.6)	0.0000008
AA	92	44	47.8% (37.3–58.5)	30	32.6% (23.2–43.2)	0.0005	25	27.2% (18.4–37.4)	0.00004	25	27.2% (18.4–37.4)	0.00004
ASP	27	11	40.7% (22.4–61.2)	2	7.4% (0.9–24.3)	0.02	2	7.4% (0.9–24.3)	0.02	2	7.4% (0.9–24.3)	0.02
High-grade dysplasia	22	8	36.4% (17.2–59.3)	5	22.7% (7.8–45.4)	0.2	4	18.2% (5.2–40.3)	0.2	4	18.2% (5.2–40.3)	0.2
Nonadvanced adenoma	191	33	17.3% (12.2–23.4)	21	11% (6.9–16	0.04	13	6.8% (3.7–11.4)	0.0001	9	4.7% (2.2–8.8)	0.000003

Most advanced finding	Total nr	MT-sDNA test		FIT50		MT-sDNA test vs FIT50	FIT75		MT-sDNA test vs FIT75	FIT100		MT-sDNA test vs FIT100
		Positive test (nr)	Specificity (95% CI)	Positive test (nr)	Specificity (95% CI)	Statistical difference, *P*	Positive test (nr)	Specificity (95% CI)	Statistical difference, *P*	Positive test (nr)	Specificity (95% CI)	Statistical difference, *P*
Control individuals, including non-neoplastic and nonadvanced polyps[a]	888	97	89.1% (86.8–91.1)	60	93.2% (91.4–94.8)	0.0003	37	95.8% (94.3–97.0)	0.00000000004	25	97.2% (95.9–98.2)	0.0000000000000009
Control individuals, including non-neoplastic polyps[b]	697	64	90.8% (88.4–92.9)	39	94.4% (92.4–96.0)	0.004	24	96.6% (94.9–97.8)	0.0000002	16	97.7% (96.3–98.7)	0.0000000002
Control individuals, no lesions found at colonoscopy	527	31	94.1% (91.8–96.0)	28	94.7% (92.4–96.4)	0.7	16	97.0% (95.1–98.3)	0.007	12	98.0% (96.1–98.8)	0.0003

AA, advanced adenoma; ASP, advanced serrated polyp; CI, confidence interval; CRC, colorectal cancer; FIT, fecal immunochemical test; MT-sDNA, multitarget stool DNA.
[a]Including 191 nonadvanced adenoma, 125 nonadvanced serrated polyps, 45 other non-neoplastic lesions, and 527 negative findings with colonoscopy.
[b]Including 125 serrated polyps, 45 other non-neoplastic lesions, and 527 negative findings with colonoscopy.

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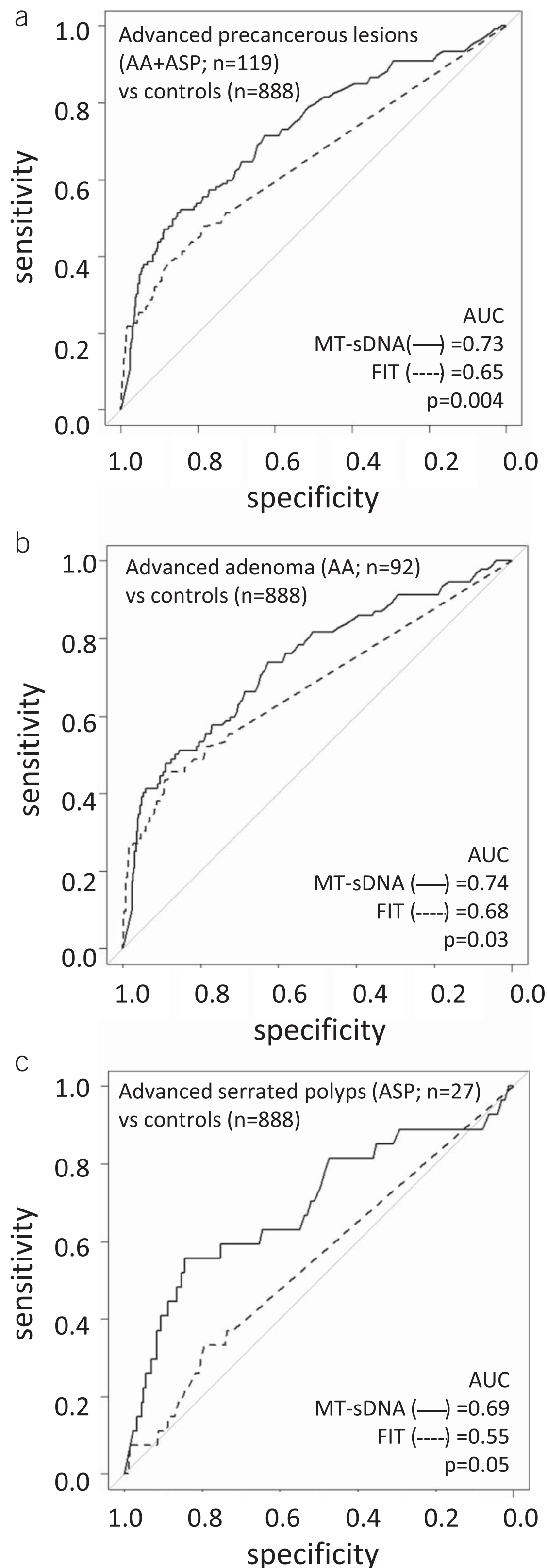

Figure 3. Receiver operating characteristic (ROC) curves of the sDNA test vs FIT on advanced precancerous lesions (**a**) subdivided into advanced adenoma (**b**) and advanced serrated polyps (**c**). The continuous and dashed lines in the ROC curves show the performance of sDNA and FIT, respectively. AUC, area under the curve; FIT, fecal immunochemical test.

detected in 361 individuals (36%), of which 191 had non-AAs, 125 serrated polyps, and 45 other non-neoplastic polyps. In 527 individuals (50%), no lesions were detected (Figure 1).

Multitarget molecular analysis of stool samples

The MT-sDNA test values in individuals with ASP, AA, and CRC were significantly higher than in those from control individuals ($P = 0.0007$, $P = 4.8e\text{-}14$ and $P = 6.0e\text{-}05$, respectively; Figure 2). Using the same threshold previously defined for the clinically validated MT-sDNA test (10) for a positive test result, 6 of 7 individuals with CRC (sensitivity of 85.7%; 95% CI 42.0–100) and 55 of 119 individuals with advanced precancerous lesions (sensitivity of 46.2%; 95% CI 37.0–55.6) tested positive. Of the 92 individuals with AA, 44 were detected (sensitivity of 47.8%; 95% CI 37.3–58.5). Of the 27 individuals with ASP, 11 were detected (sensitivity of 40.7%; 95% CI 22.4–61.2) (Table 1).

For the 888 individuals with non-AAs, serrated polyps, other non-neoplastic polyps, or negative results, specificity of the MT-sDNA test was 89.1% (95% CI, 86.8–91.1). For the 527 individuals with negative findings (no lesions found at all), specificity was 94.1% (95% CI 91.8–96.0) (Table 1).

MT-sDNA test compared with FIT

FIT50 was positive in all 7 individuals with CRC. Because the number of individuals with CRC was too small to perform relevant statistical analysis, we further focused on individuals with advanced precancerous lesions, i.e., AA and ASP. Sensitivity for AA and ASP using FIT50 was 32.6% (95% CI 23.2–43.2) and 7.4% (95% CI 0.9–24.3), respectively, with specificity of 93.2% (95% CI 91.4–94.8). For the 527 individuals with negative findings (no lesions found at all), specificity was 94.7% (95% CI 92.4–96.4). Sensitivity and specificity results for FIT50, FIT75, and FIT100 are shown in Table 1. In addition, the MT-sDNA test detected 24 of 119 advanced precancerous lesions that were not detected by FIT50 (20.2%), whereas FIT50 detected only 1 of 119 advanced precancerous lesions that was not detected by the MT-sDNA test (0.8%) ($P < 0.001$). The MT-sDNA test detected 10 ASPs that were not detected by FIT50, whereas FIT50 detected only 1 ASP that was not detected by the MT-sDNA test ($P = 0.02$). The specificity of single-application FIT was higher than the MT-sDNA test among individuals without advanced colorectal neoplasia (93.2% vs 89.1%, respectively, $P = 0.0003$), but equal with the MT-sDNA test for individuals with negative findings only, e.g., no colorectal lesion of any type (94.7% vs 94.1%, respectively, $P = 0.7$).

When comparing the overall performance of the MT-sDNA test and FIT, i.e., with no predefined cutoffs, the performance of the MT-sDNA test was significantly better than FIT alone for discriminating individuals with advanced precancerous lesions from individuals with non-neoplastic or negative findings (an ROC AUC of 0.73 for the MT-sDNA test vs an ROC AUC of 0.65 for FIT; $P = 0.004$) (Figure 3). When comparing the sensitivity of the MT-sDNA test with FIT using a fixed specificity of 95% (adapted threshold of 268.5 for MT-sDNA and 65.5 for FIT), significantly more advanced precancerous lesions were detected with the MT-sDNA test than with FIT alone (35% vs 25% sensitivity, respectively [$P = 0.01$). AAs were detected more often with the MT-sDNA test compared with FIT alone (39% vs 30% sensitivity, respectively ($P = 0.06$) (Table 2). ASPs were also detected with a higher sensitivity than by FIT alone (22% vs 7%,

Table 2. Sensitivity and specificity at equal fixed specificity of 95%

Most advanced finding	Total nr	Multitarget molecular test[a] Positive test (nr)	Multitarget molecular test[a] Sensitivity (95% CI)	FIT[a] Positive test (nr)	FIT[a] Sensitivity (95% CI)	Multitarget molecular test vs FIT Statistical difference, *P*
CRC	7	6	85.7% (42.0–100.0)	7	100% (59.0–100.0)	1.0
Advanced precancerous lesions	119	42	35.3% (26.8–44.6)	30	25.2% (17.7–34.0)	0.01
AA	92	36	39.1% (29.1–49.9)	28	30.4% (21.3–40.9)	0.06
ASP	27	6	22.2% (8.6–42.3)	2	7.4% (0.9–24.3)	0.2
High-grade dysplasia	22	5	22.7% (7.8–45.4)	5	22.7% (7.8–45.4)	1.0

Most advanced finding	Total nr	Multitarget molecular test[a] Positive test (nr)	Multitarget molecular test[a] Specificity (95% CI)	FIT[a] Positive test (nr)	FIT[a] Specificity (95% CI)	Multitarget molecular test vs FIT Statistical difference, *P*
Control individuals, including non-neoplastic and nonadvanced polyps[b]	888	45	94.9% (93.3–96.3)	45	94.9% (93.3–96.3)	1.0
Negative findings with colonoscopy	527	15	97.2% (95.3–98.4)	19	96.4% (94.4–97.8)	0.5

AA, advanced adenoma; ASP, advanced serrated polyp; CI, confidence interval; CRC, colorectal cancer; FIT, fecal immunochemical test.
[a]Adapted threshold to reach a fixed specificity of 95%; sDNA 286.5; FIT 65.5.
[b]Including 191 nonadvanced adenoma, 125 nonadvanced serrated polyps, 45 other non-neoplastic lesions, and 527 negative findings with colonoscopy.

respectively), but this difference did not reach statistical significance.

Test results in relation to AA characteristics

In total, 119 individuals had at least 1 advanced precancerous lesion (AA or ASP). Of those, 36 had 1 single AA and no other (non-) AA, ASP, or CRC. The MT-sDNA test, using the previously reported cutoff of 183, was more often positive in individuals with multiple advanced precancerous lesions compared to individuals with only 1 advanced precancerous lesion (69% vs 38%, respectively, $P = 0.005$). No statistically significant differences were observed for FIT50 in detecting individuals with multiple or 1 advanced precancerous lesion (38% vs 23%, respectively, $P = 0.18$; Table 3).

For the 36 patients who had only 1 single advanced precancerous lesion (AA or ASP), test results could be well evaluated for their association with tumor size, location, histology (presence of villous architecture), dysplasia (presence of high-grade dysplasia), and morphology. A positive MT-sDNA test result was associated to larger lesions ($P = 0.03$) and lesions with tubulovillous architecture ($P = 0.04$), whereas a positive FIT50 result did not show any significant associations for these tumor characteristics. Both MT-sDNA and FIT50 detected polypoid lesions with a higher rate than sessile or flat lesions (both tests detected 7/13 [54%] polypoid lesions). Although FIT50 did not detect sessile, flat, or serrated (ASP) single lesions, MT-sDNA detected 2/18 (11%), 2/5 (40%), and 4/11 (36%) of these lesions, respectively. Six patients had a single high-grade dysplastic advanced precancerous lesion (1 ASP and 5 AAs). Of these 6 lesions, 1 was 1 cm in size, and 5 were smaller (3–5 mm). None of them were positive for the MT-sDNA test or for FIT50. All details are provided in Table 3.

Test results in low- and high-risk AAs

DNA copy number analysis was unsuccessful in 15 evaluated AAs. This was due to (i) too little DNA, (ii) poor quality of DNA, and/or (iii) noninterpretable DNA copy analyzes. DNA copy number aberrations were identified successfully in 93 of 108 evaluated AAs. In total, 20 of 93 AA lesions (from 77 non-CRC patients) were defined as high risk (22%). From 6 (8%) of these 77 individuals, DNA copy number analysis of 1 or more AAs was missing. They were excluded from further analyses on high- or low-risk individuals. From the remaining 71 individuals, 19 had at least 1 HR adenoma (27%) and therefore considered high risk. The MT-sDNA test, using the previously reported cutoff of 183, was positive for 10 of 19 high-risk individuals (sensitivity of 53%, 95% CI 29–76) vs 24 of 52 low-risk individuals (sensitivity of 46%, 95% CI 32–61) ($P = 0.8$). FIT50 was positive for 9 of 19 high-risk individuals (sensitivity of 47%, 95% CI 24–71) vs for 18 of 52 low-risk individuals (sensitivity of 35%, 95% CI 22–49) ($P = 0.4$). Both FIT75 and FIT100 were positive for 8 of 19 high-risk individuals (sensitivity of 42%, 95% CI 20–67) vs for 14 of 52 low-risk (sensitivity of 27%, 95% CI 16–41) ($P = 0.3$). Thus, none of MT-sDNA, FIT50, FIT75, or FIT100 showed significantly different sensitivities to detect individuals with high-risk adenomas vs individuals with low-risk adenomas.

DISCUSSION

Accuracy in detecting early CRCs and precancerous lesions is one of the major determinants of success of a CRC screening program. Based on extensive cost-effectiveness evaluation, many organized screening programs use FIT as a triage test to select individuals for colonoscopy (38). Although detection of blood in stool has shown to be successful in reducing mortality from CRC (39–42), senstivity can still be improved. In this study, we evaluated an MT-sDNA test that previously showed to have great potential to improve on FIT (10), in an average-risk primary screening population in the Netherlands.

Sensitivity is an important attribute of cancer-screening tests and crucial for screening programs to reduce death from CRC. However, given the low prevalence of CRC in the general population, a high specificity of the test is important to reach a high positive predictive value and reduce the number of participants

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Table 3. Stool test results in relation to different tumor characteristics

	MT-sDNA test			FIT50			FIT75			FIT100		
	Negative	Positive	*P* value	Negative	Positive	*P* value	Negative	Positive	*P* value	Negative	Positive	*P* value
No. of advanced precancerous lesions (n = 119 individuals)												
1 (n, %)	54 (62)	33 (38)	**0.005**	67 (77)	20 (23)	0.18	70 (80)	17 (20)	0.27	70 (80)	17 (20)	0.27
>1 (n, %)	10 (31)	22 (69)		20 (63)	12 (38)		22 (69)	10 (31)		22 (69)	10 (31)	
Size (n = 36 single lesions)												
<10 mm (n, %)	12 (92)	1 (8)	**0.03**	12 (92)	1 (8)	0.4	12 (92)	1 (8)	0.4	12 (92)	1 (8)	0.4
≥10 mm (n, %)	13 (57)	10 (43)		17 (74)	6 (26)		18 (78)	5 (22)		18 (78)	5 (22)	
Location (n = 36 single lesions)												
Left-sided (n, %)	18 (72)	7 (28)	0.7	18 (72)	7 (28)	0.08	19 (76)	6 (24)	0.1	19 (76)	6 (24)	0.1
Right-sided (n, %)	7 (64)	4 (36)		11 (100)	0 (0)		11 (100)	0 (0)		11 (100)	0 (0)	
Morphology (n = 36 single lesions)												
Polypoid (n, %)	6 (46)	7 (54)	**0.02**	6 (46)	7 (54)	**0.0004**	7 (54)	6 (46)	**0.002**	7 (54)	6 (46)	**0.002**
Sessile (n, %)	16 (89)	2 (11)		18 (100)	0 (0)		18 (100)	0 (0)		18 (100)	0 (0)	
Flat (n, %)	3 (60)	2 (40)		5 (100)	0 (0)		5 (100)	0 (0)		5 (100)	0 (0)	
Histology (n = 36 single lesions)												
Tubular (n, %)	10 (91)	1 (9)	**0.04**	10 (91)	1 (9)	0.06	10 (91)	1 (9)	0.15	10 (91)	1 (9)	0.15
Tubulovillous (n, %)	7 (50)	7 (50)		8 (57)	6 (43)		9 (64)	5 (36)		9 (64)	5 (36)	
Serrated (n, %)	7 (88)	1 (13)		8 (100)	0 (0)		8 (100)	0 (0)		8 (100)	0 (0)	
Hyperplastic (n, %)	1 (33)	2 (67)		3 (100)	0 (0)		3 (100)	0 (0)		3 (100)	0 (0)	
Dysplasia (n = 36 single lesions)												
Low grade (n, %)	16 (67)	8 (33)	0.16	17 (71)	7 (29)	0.29	18 (75)	6 (25)	0.3	18 (75)	6 (25)	0.3
High grade (n, %)	6 (100)	0 (0)		6 (100)	0 (0)		6 (100)	0 (0)		6 (100)	0 (0)	

FIT, fecal immunochemical test; MT-sDNA, multitarget stool DNA.
P values were calculated using the χ^2 test or Fisher exact test in case of low sample number per cell.
Bold values denote statistical significance at the $P < 0.05$ level.

subjected to a follow-up colonoscopy. To compare the single-application sensitivity of a new screening test with a comparator, equal specificity for both tests is required, although when a new screening test has different recommended screening intervals, the effect of interval will also impact the comparison. In the current study, we evaluated the single-application performance of the MT-sDNA test and compared it with single-application performance of FIT alone with a fixed high specificity of 95% for both tests. In this setting, the MT-sDNA test showed 10% absolute increase in sensitivity in detecting advanced precancerous lesions compared with FIT (25%–35%). This is of importance as the best opportunity for a CRC screening test to be more cost-effective than FIT is by a higher detection rate for advanced precancerous lesions, rather than an increased detection of CRC (12,13). The MT-sDNA test had a significantly higher sensitivity for multiple lesions, larger lesions, and lesions with tubulovillous architecture. In contrast to an earlier report (10), in our study, high-grade dysplasia was not detected with higher sensitivity. Histological revisions of these lesions revealed that 4 of them did not show high-grade dysplasia in newly used slides, suggesting that the size of the previously observed focus of high-grade dysplasia might have been small in these lesions. The fact that the lesions themselves were small (3–10 mm) and the focus of high-grade dysplasia might also have been small could explain why the MT-sDNA did not detect them. In addition, the lower sensitivity could also be related to the small number (n = 6) of the high-grade lesions in our study.

ASPs were detected with significant higher sensitivity by the MT-sDNA test (40.7%) compared with FIT (7.4%). In line with the knowledge that serrated lesions have high frequencies of DNA methylation and CpG Island Methylator Phenotype (43,44), we found that the single components *NDRG4* and *BMP3* methylation of the MT-sDNA tests showed significant increased values in ASPs as compared to controls, but not for *KRAS* mutations or FIT (data not shown). These data align with the previous observation that the current MT-sDNA test is more sensitive for serrated lesions than FIT (10).

No correlations were found between the MT-sDNA test in stool and the values of the isolated DNA components as measured in matched tissue samples. Although such a one-to-one comparison cannot be made as other factors, polyclonality of CRC and sampling issues, size, and other tumor characteristics also play a role in marker detection, this finding underlines the complexity of molecular marker detection in stool. It is often not clear whether the signal stems from the tumor itself, its microenvironment, or both. Besides, the transit time and stability of the markers in the gut are unknown factors as well. Moreover, our

results suggest that the hemoglobin component in the MT-sDNA test is of major value and lends support to the idea of improving FIT by adding the detection of molecular markers to hemoglobin, rather than replacing it (9,45,46).

As it will take at least 10 years to reach the endpoint in CRC screening, intermediate endpoints are used as an alternative for evaluating the performance of a screening test and the overall effect of a screening program. Recently, it has been published that patients with AA have an increased risk in developing CRC (47). AA, defined by size and histology of adenomas, is the most widely used intermediate endpoint. This definition has been adopted since 1987 (48); however, only a minority of AAs show histologic or molecular features associated with high risk of progression (19). As a result, performance of screening tests may be substantially underestimated, when evaluated against AAs as intermediate endpoint. Therefore, we investigated the MT-sDNA test performance, when considering the molecular high-risk features, i.e., specific DNA copy number aberrations in AAs associated with high risk of progression (18,19,49). DNA copy number alterations themselves are very challenging to measure in stool samples where a high background of normal and/or bacterial DNA is present; hence, alternative molecular stool tests are required to detect high-risk lesions. The MT-sDNA test that was used in the current study did not identify individuals with high-risk lesions better when compared to individuals with only low-risk lesions. This is not an unexpected finding as the MT-sDNA test includes 2 DNA methylation markers. DNA methylation is an early event in the development of (pre)neoplastic lesions, even before the onset of DNA copy number aberrations (50), and hence, DNA methylation markers in stool are expected to detect early lesions. This is exemplified by the fact that the specificity of the MT-sDNA test was equal to FIT when only individuals without any lesion were included in the control group, but was significantly lower when individuals with nonadvanced lesions were included in the control group. Yet, future molecular markers that do correlate with high-risk adenomas could further improve its performance.

The current study is the first to validate the MT-sDNA test for CRC screening, in an average-risk population outside the United States. Moreover, we are the first to investigate whether the MT-sDNA test, as well as FIT, can detect AAs with a high risk of progression with a higher sensitivity than AA with low risk. A limitation to this study is the low number of patients with CRC because we used an invitational average-risk screening population.

In conclusion, the MT-sDNA test combining DNA methylation and mutation markers with hemoglobin has a significantly increased sensitivity for advanced precancerous lesions than FIT alone at equal specificity, indicating its potential for application in population screening for CRC.

CONFLICTS OF INTEREST

Guarantor of the article: Gerrit A. Meijer, MD, PhD.
Specific author contributions: L.J.W.B. and V.M., contributed equally to this work. L.J.W.B., V.M., E.D., B.M.B., M.v.E., B.C., and G.A.M.: study concept and design. L.J.W.B., V.M., S.M., K.L.J.D., S.T.v.T., E.M.S., T.R.d.W., C.R., and M.J.D.: acquisition of data. L.J.W.B., V.M., and B.C.: interpretation of data. L.J.W.B. and V.M.: drafting of manuscript. All authors: critical revision. L.J.W.B. and V.M.H.C.: statistical analysis. E.J.K. and G.A.M.: obtained funding. B.M.B. and G.A.M.: study supervision.
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