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ORIGINAL ARTICLE

State-of-the-Art Colorectal Cancer and Advanced Precancerous Lesion Screening: a Multitarget Stool DNA Test

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SUMMARY

Background: **Colorectal cancer (CRC) claims 900,000 lives per year. Colonoscopy offers reliable detection, but with low patient adherence rates. To significantly reduce CRC incidence and mortality, a more convenient screening measure for advanced precancerous lesions (APL) and CRC is urgently needed.**
Methods: **In this study, the clinical performance of a multitarget stool DNA (mt-sDNA) test combining fecal immunochemical test (FIT) with the analysis of genetic biomarkers by real-time PCR was evaluated in a cohort of 208 subjects.**
Results: **The mt-sDNA test showed a sensitivity of 84.2% for CRC (all stages) and 39.6% sensitivity for APL detection with a specificity of 91.5%. Within the APL group, high-grade dysplasia, characterized by the highest risk of further cancer progression, were detected with 75% sensitivity.**
Conclusions: **The mt-sDNA test represents a significant advancement for non-invasive detection of APL and CRC and bears great potential to enhance CRC prevention, incidence, and mortality.**
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INTRODUCTION

Colorectal cancer (CRC) is a global health concern, with a significant impact on morbidity and mortality. CRC is reported as the second leading cause of cancer-related deaths worldwide, with more than 1.9 million new cases following more than 930.000 cases of death in 2020 [1].
Early detection of CRC is crucial for successful treatment and reducing mortality. Patients diagnosed at early, localized stages have a five-year survival rate of > 90%, highlighting the importance of early identification

[2]. This applies even more regarding the identification of advanced precancerous lesions (APL) such as advanced adenomas (AA). With the highest probability among all colorectal polyps to develop into cancer and lesions, the importance of diagnosing APLs should not be underestimated [3].

Colonoscopy, the gold standard for CRC screening, has a high level of precision but sustains several limitations. It is perceived as an undesirable procedure due to its invasive nature and the involved preparation (e.g., use of laxatives), leading to low adherence rates ranging between 22 and 38% in the United States [4,5]. In addition, colonoscopy capacities have reached their limits, resulting in a backlog of colonoscopies [6]. To address these challenges, alternative, non-invasive tests for CRC and APL screening, with unique strengths but also some limitations, have emerged [5,6]. The most common non-invasive test, the fecal immunochemical test (FIT), has a limited sensitivity, especially in earlier staged CRC (stage I), ranging from 62.1% to 68.2% and an even lower sensitivity for APL, ranging from 23.3% to 31.5% (specificity of ≥ 90%) [7,8]. Further promising tests that are commercially available, under development, or in clinical testing phases combine FIT with the analysis of stool-based nucleic acids including RNA expression level, genetic somatic mutations, or aberrant methylation-based DNA [8-10].

Although some of these tests still have certain limitations such as being more expensive than colonoscopy, they show a higher sensitivity than FIT by detecting non-bleeding tumors and APL [5,11]. The investigational study by Dollinger et al. has demonstrated the potential of combining DNA biomarkers with quantification of occult blood in stool samples for the detection of CRC [9]. Besides verification of the previously reported data, our study aimed to confirm the clinical performance of the same biomarker panel in an independent cohort, additionally reporting an APL performance value for the first time.

MATERIALS AND METHODS

Study design

Stool samples were collected as part of the COLOFUTURE clinical study (DRKS-ID: DRKS00027888). The study was conducted according to Good Clinical Practice and the Declaration of Helsinki and was approved by local ethics committees. All subjects have given their written informed consent and underwent screening, diagnostic colonoscopy, or already had diagnosed colorectal lesions. The objective of the study was to determine the sensitivity and specificity for CRC (primary objective) and APL (secondary objective) with the Mainz Biomed colorectal cancer screening test.

Clinical classification

For clinical classification, colonoscopy was conducted and, if necessary, biopsies were taken according to national guidelines. CRC and APL categorization was obtained by at least two experienced pathologists, with disagreements resolved by consensus (Table 1).

Sample collection

Stool was collected in one SENTiFIT® pierceTube (SENTINEL CH. SpA, Milan, Italy) and in one DNA/RNA Shield™ Fecal Collection Tube (Zymo Research Corporation, USA).

Fecal immunochemical testing (FIT)

Hemoglobin quantification was performed with SENTiFIT® FOB Gold® test in combination with SENTiFIT 270 (SENTINEL CH. SpA, Italy) instrument, according to manufacturer's instructions.

Bead-based automated DNA extraction

DNA from stool samples stabilized in DNA/RNA Shield™ Fecal Collection tubes was extracted using the bead-based automated extraction kit according to manufacturer's instructions with slight modifications (Mainz Biomed Germany GmbH, Germany), on a KingFisher Apex™ instrument (Thermo Fisher Scientific, USA).

Quantitative real-time PCR

Quantification of human DNA (hDNA), somatic mutational analysis of *KRAS* (codon 12/13) and *BRAF* (codon 600/V600E/V600K) were performed using the ColoAlert Lab Kit Core II real-time PCR kit (Mainz Biomed Germany GmbH, Germany) according to manufacturer's instructions on the LightCycler® 480 System (Roche Diagnostics, Switzerland). Data were analyzed using LightCycler® 480 Software 1.5.1.62 (Roche Diagnostics, Switzerland).

Statistical analysis

Performance evaluation was conducted on a cutoff-based algorithm according to the manufacturer's recommendations, with a FIT cutoff value of ≥ 5 µg/g hemoglobin (SENTINEL CH. SpA, Italy) and with ≥ 1,000 pg/µL for absolute hDNA quantification (Mainz Biomed Germany GmbH, Mainz, Germany). Samples were scored positive, if at least one of the markers (FIT, hDNA, *KRAS*, or *BRAF*) was positive. Sensitivity, specificity, positive predictive value (PPV), and negative predictive value (NPV) including two-sided 95% Clopper-Pearson confidence intervals were calculated. Figures were created with GraphPad Prism 10.1.2 (GraphPad Software, USA).

RESULTS

Study population

This study examined a cohort of 208 subjects from eight study sites in Europe. Patients were classified into three groups according to colonoscopy results and, if relevant, to histological examination of respective lesions. Colonoscopies, including histological review, adhered

Table 1. Applied criteria for clinical classification.

Group	Diagnosis	Description
1	Colorectal cancer (CRC)	definite histological proof of an adenocarcinoma or any early carcinoma including intramucosal carcinoma, carcinoma in situ, and carcinoma in lamina propria. CRC staging was performed according to the American Joint Committee on Cancer (AJCC) cancer staging system [12]
2	Advanced precancerous lesions (APL)	definite histological proof of any advanced precancerous lesion including classical adenoma or sessile serrated lesions. Within this categorization, subjects were further hierarchically classified as follows: 1. Classical adenomas 1a) adenomas with high grade dysplasia ("dysplasia") 1b) adenomas ≥ 1.0 cm in size ("advanced") 1c) adenomas with significant villous/tubulo-villous features (≥ 25%) of any size ("villous") 2. Sessile serrated lesions 2a) sessile serrated lesions ≥ 1.0 cm in size ("lesion") 2b) sessile serrated lesions with cytological dysplasia of any size ("lesion dysplasia")
3	Control (normal colonoscopy)	all subjects with normal colonoscopy are assigned to the control group. This group also included subjects with hyperplastic polyps and excluded small adenomatous lesions

Table 2. Demographic information of the analyzed cohort.

	CRC n = 38	APL n = 53	Control n = 117	Total n = 208	Total %
Age (years)					
40 - 49	1	2	14	17	8.2%
50 - 59	11	13	42	66	31.7%
60 - 69	10	24	44	78	37.5%
70 - 79	14	12	14	40	19.2%
80 - 85	2	2	3	7	3.4%
Gender					
Female	15	17	61	93	44.7%
Male	23	36	56	115	55.3%
Body mass index (BMI)					
Underweight (< 18.5)	0	0	1	1	0.5%
Healthy weight (18.5 - 24.9)	18	16	47	81	38.9%
Overweight (25.0 - 29.9)	9	22	39	70	33.7%
Obese (≥ 30)	10	13	25	48	23.1%
N/A	1	2	5	8	3.8%

Table 3. Performance of the mt-sDNA test in comparison to colonoscopy and pathological diagnosis.

	CRC	APL	Combined (CRC + APL)
Sensitivity	84.21% (68.75, 93.98)	39.62% (26.45, 54.00)	58.24% (47.43, 68.50)
Specificity	91.45% (84.84, 95.83)	91.45% (84.84, 95.83)	91.45% (84.84, 95.83)
PPV	76.19% (60.55, 87.95)	67.74% (48.63, 83.32)	84.13% (72.74, 92.12)
NPV	94.69% (88.80, 98.03)	76.98% (69.08, 83.69)	73.79% (65.85, 80.74)

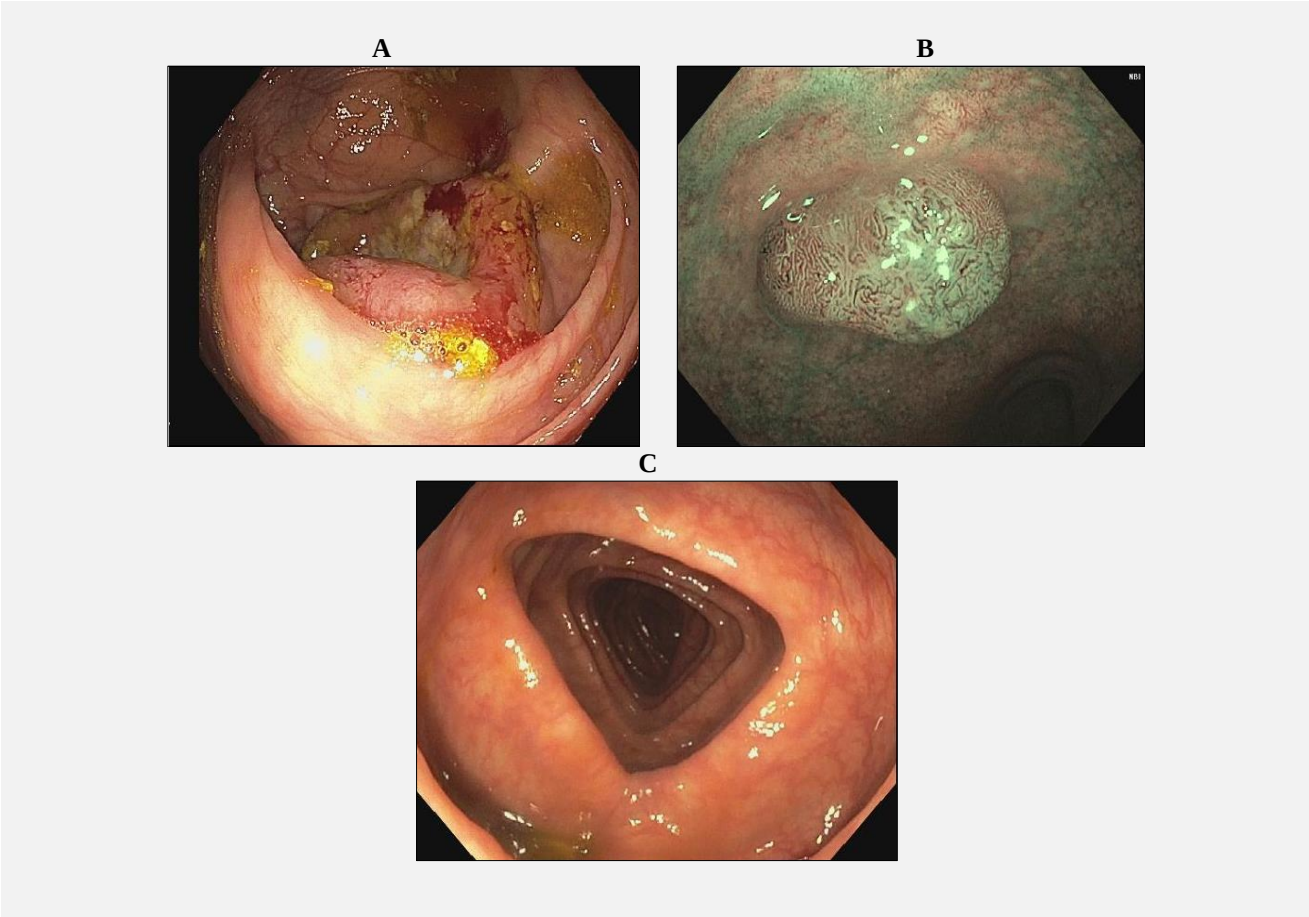

Figure 1. Exemplary colonoscopy images from subjects diagnosed with A) CRC (invasive adenocarcinoma of the right colon as seen with high-definition white light (HD - WL)), B) AA (tubulo-villous adenoma in the left colon with surface details enhanced by narrow band imaging (NBI)), and C) controls with no findings in colonoscopy (normal mucosa in the transverse colon (HD - WL).

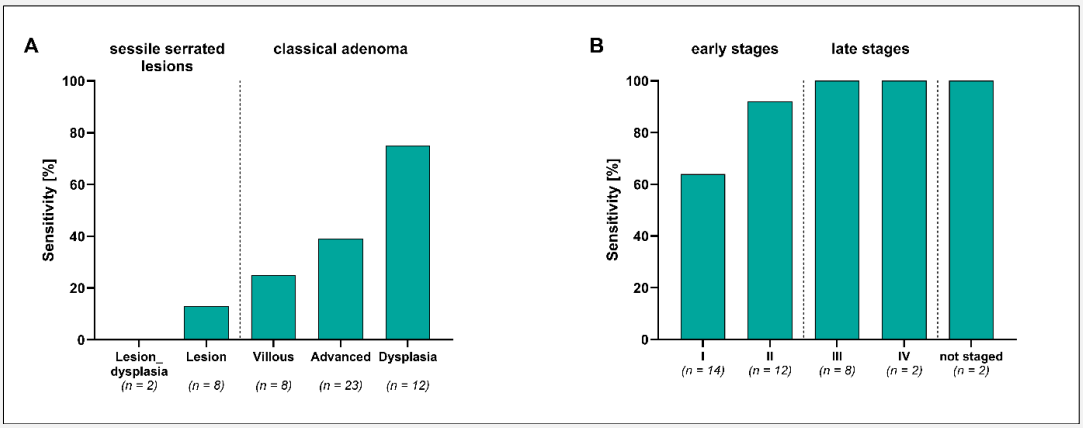

Figure 2. Sensitivity of the mt-sDNA test by pathological group for APL A) or CRC staging B).

Based on colonoscopy and pathological assessment, APL or CRC cases were grouped hierarchically into different categories or stages, and the sensitivity for each was calculated. For two CRC samples, there was no staging information available at the time of analysis (not staged).

to quality indicators described in the European guidelines with the final cohort comprising 38 CRC subjects (all stages), 53 APL and 117 control (no polypoid findings) subjects (Figure 1) [13]. The mean age was 62.2 years, ranging from 41 to 83 years (45% female, 55% male) (Table 2).

Mt-sDNA test shows high sensitivity and specificity for CRC and APL

To investigate the validity and diagnostic performance of the mt-sDNA test in the cohort, consisting of normal controls, APLs, and CRC, stool samples were analyzed for occult blood and genomic biomarkers, including absolute hDNA quantification and mutational analysis of *KRAS* and *BRAF*. Multitarget analysis enabled the detection of 32 out of 38 CRC samples, which corresponds to a sensitivity of 84.2%. Additionally, a PPV of 76.19% and a NPV of 94.69% were detected for the CRC group. Patients diagnosed with APL were detected with a sensitivity of 39.6%. Specificity against the negative control group was calculated at 91.5%. Performance data of the mt-sDNA test including 95% Clopper-Pearson confidence intervals are summarized in Table 3.

Mt-sDNA test detects early CRC stages with high sensitivity

Early detection of CRC and its precursor lesions is crucial to reduce CRC incidences and to improve survival rates. Therefore, sensitivity of the identified APL and CRC cases, classified by pathological group or CRC staging, was calculated. We were able to detect high-grade dysplasia (APL group) with a high sensitivity of 75%. Further, the mt-sDNA test not only detects cases in late CRC stages (III - IV) with a sensitivity of 100% but also the majority of the clinically important early-stage CRCs (Stage I: 64.3%; Stage II: 91.7%) (Figure 2).

DISCUSSION

Early detection of CRC and APLs is crucial for improving the overall prognosis for patients. The gold standard colonoscopy is still only poorly accepted by the screening population and its availability is limited in several countries. Further, the quantification of stool-based hemoglobin is recommended as non-invasive CRC screening test in many countries [14]. However, FIT only allows detection of limited subsets of bleeding CRC and APLs, with CRC sensitivity ranging from 50 - 83% [7,8]. In a recent screening trial with ~ 20,000 participants, the FIT sensitivity was even reduced (CRC: 67.3%; APL: 23.3%) [8]. Besides, DNA methylation or RNA expression levels of specific targets were investigated in combination with FIT leading to increased sensitivities for CRC and APL [8,10].

The herein described non-invasive mt-sDNA test combines FIT with the absolute quantification of hDNA and the somatic mutational analysis of the two oncogenes *KRAS* and *BRAF* in stool samples, showing comparable results regarding CRC and APL sensitivity and specificity [15-17]. Previously, the study by Dollinger et al. already demonstrated a promising performance of the same biomarker panel for the detection of CRC (84.6% sensitivity and 91.7% specificity) [9]. Based on this investigational study, the commercially available screening test ColoAlert (CE-IVD certified) was established. In our present study, these findings were verified in a prospective, multicentric, international study, which confirms the suitability of the selected biomarkers as a diagnostic approach. Since the mutations are quite common in early-staged CRC or even APL, analysis of the two oncogenes *KRAS* and *BRAF* could have contributed to identifying early cases such as APL, with a sensitivity of 39.6% [15-17]. Within APL high-grade dysplasia was detected with the highest sensitivity of 75%. This class is associated with an increased risk of further progression and is therefore of utmost importance to be detected [18]. In the present study, in contrast to the study by Dollinger et al., the group of adenomas was further subdivided, which helped to identify high-risk adenomas with the highest risk of developing into CRC [9, 18].

However, it must be taken into account that the study by Dollinger et al. used denaturing high performance liquid chromatography for genetic analysis and a guajak-based test for occult blood in stool, whereas in the present study advanced methodologies were employed [9]. Thus, the currently described configuration provides a reliable test that can easily be performed in standard diagnostic laboratories and is therefore suitable for high-throughput CRC screening.

Early detection remains a cornerstone of successful treatment outcomes, highlighting the need for accessible screening methods to address the low adherence rate of invasive screening measures. The test concept described here fulfills the requirements for a valuable screening concept, especially regarding the detection of early lesions and easy at-home collection with only a small amount of stool. Moreover, the test serves as a basis that could be further refined by additional targets, such as methylation markers or mRNA targets, to further improve the detection of CRC and APLs. A strength of this study was that sample specimen collection occurred in a realistic clinic scenario, as all subjects self-collected their stool samples at home, including the sample shipment logistics process to the clinical laboratory.

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(Stavanger, Norway); 1. Medizinische Klinik im Klinikum Passau (Passau, Germany); Praxis Dr. Aschenbeck (Berlin, Germany); MVZ für Gastroenterologie am Bayerischen Platz (Berlin, Germany); and Klinik für Innere Medizin I, Universitatsklinikum Ulm (Ulm, Germany).

Ethical Approval:
Stool samples were collected via the COLOFUTURE clinical study (DRKS-ID: DRKS00027888), conducted according to Good Clinical Practice and the Declaration of Helsinki and approved by local ethics committees.

Declaration of Interest:
LK, HM, FF, CS, PB, MS, AS, SG, AB, MK, and ME are employees of Mainz Biomed Germany GmbH and own stock or stock options of Mainz Biomed N.V. MD is the PI of the COLOFUTURE study and received study associated research funding from Mainz Biomed Germany GmbH.

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