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Methylated Septin9 identified patients with colorectal carcinoma and showed higher sensitivity than conventional biomarkers in detecting tumor

Sabine Leerhoff[a], Arnold Raem[b], Ernst-Wolfgang Kolbe[a], Laura Schulz[a1], Kirsten Borchers[a], Thomas Köhler[c], Günther Winde[a], Carmen Kirchner[a]

[a]Department of General and Visceral Surgery, Thoracic Surgery and Proctology, Ruhr University Bochum, Klinikum Herford, Schwarzenmoorstr. 70, 32049 Herford, Germany

[b]arrows biomedical Deutschland GmbH, Heisenbergstr. 1, 48149 Muenster, Germany

[c]Department of Anaesthesiology and Intensive Care Medicine, Klinikum Halberstadt, Gleimstr. 5, 38820 Halberstadt, Germany

[1] Present address: Bruederkrankenhaus St. Josef Paderborn, Husenerstr.46, 33098 Paderborn, Germany

Corresponding author
Prof. Dr. med. Guenther Winde
Department of General and Visceral Surgery, Thoracic Surgery and Proctology, Ruhr University Bochum, Klinikum Herford, Schwarzenmoorstr. 70, 32049 Herford, Germany:
prof.winde@klinikum-herford.de
+49 5221 941283

Authors´ Email addresses:

sabine.leerhoff@klinikum-herford.de

raem@arrows-biomedical.com

ernst-wolfgang.kolbe@klinikum-herford.de

laura.schulz@ruhr-uni-bochum.de

thomas.koehler@ruhr-uni-bochum.de

kirsten.borchers@ruhr-uni-bochum.de

prof.winde@klinium-herford.de

carmen.kirchner@klinikum-herford.de

Highlights

- Non-invasive surveillance of CRC by commercial assays for m*SEPT9*, CEA, CA19-9.
- PreOP sensitivity of m*SEPT9* for colorectal carcinoma 57.1%.
- PreOP sensitivity of combined m*SEPT9*, CEA, CA19-9 65.9%.
- Continuous m*SEPT9* decrease postOP (3 months).
- Consistent positive m*SEPT9*: 65% patients had distant metastasis, 25% tumour remnants.

Abstract

Introduction

It is worth noting the limitations in sensitivity of the existing biomarkers carcinoembryonic antigen (CEA) and carbohydrate antigen (CA 19-9) in detection of colorectal cancer (CRC). In our study, we address the performance of the liquid biopsy biomarker "methylated septin 9" (*mSEPT9*) in the detection and disease surveillance of CRC.

Materials and Methods

The monocentric prospective survey encompassed 120 patients diagnosed with CRC who underwent planned curative resection between December 2018 and December 2020. Blood samples were collected from the participants preoperatively as well as at 7 days, 6 weeks, and 3 months postoperatively. The presence of *mSEPT9*, CEA, and CA 19-9 was detected using the pro Epi Colon® 2.0 CE test, Elecsys® CEA, and Elecsys® CA19-9 electrochemiluminescence immunoassay, respectively.

Results

In the preoperative setting, *mSEPT9* demonstrated superior capability in identifying patients with CRC compared to CEA and CA 19-9, with detection rates of 57%, 32%, and 18% respectively. Combining all three biomarkers increased the overall sensitivity to 66% preoperatively. In considering UICC stage and T-status, *mSEPT9* exhibited higher sensitivity across all stages in comparison with conventional tumor markers, and 84% of patients with metastases were identified preoperatively through *mSEPT9*. Tumor recognition after surgery was achieved with the sensitivity of 71 % and specificity of 91%.

Conclusions

We recommend using *mSEPT9* as a non-invasive diagnostic tool for the ongoing monitoring of patients with CRC. The sensitivity and specificity exhibited by *mSEPT9* in recognition of tumor after surgery, highlights its particular potential for monitoring of CRC patients.

Keywords: colorectal cancer, clinical prospective study, non-invasive diagnosis, disease surveillance, liquid biopsy, metastases.

Abbreviations

CA 19-9, carbohydrate antigen 19-9; CEA, carcinoembryonic antigen; CRC, colorectal carcinoma; FOBT, fecal occult blood test; *mSEPT9*, methylated septin 9 gene; R1, microscopic residual tumor; R2 macroscopic residual tumor; UICC, Union Internationale Contre le Cancer.

Introduction

Colorectal carcinoma (CRC) poses a significant challenge in the field of oncology and is recognized as one of the most prevalent forms of malignancy. The burden of CRC is substantial, with approximately 60,000 new cases diagnosed annually and nearly 25,000 deaths attributed to CRC in Germany alone each year [1]. Despite significant advancements in the treatment of colorectal carcinoma CRC [2] such as the utilization of neoadjuvant chemotherapies [3, 4], immunotherapy [5], and surgical techniques [6], an advanced stage at the time of diagnosis is still associated with very poor prognosis. Addressing this issue urgently calls for effective diagnostic tools that are non-invasive and well-tolerated by patients. Current common screening methods for CRC include the fecal occult blood test (FOBT), flexible sigmoidoscopy, and colonoscopy. The FOBT is a widely available screening method that detects the presence of hemoglobin in stool through enzymatic or immunological means. However, its sensitivity and specificity can vary and exhibit inconsistency, which limits its utility as a reliable method for CRC detection. On the other hand, colonoscopy is considered the gold standard for diagnosis, but it is an invasive procedure that often leads to patient discomfort, resulting in lower compliance rates. Post-surgical monitoring for CRC typically involves the use of imaging techniques like computed tomography (CT) scans, along with the measurement by conventional tumor markers such as carcinoembryonic antigen

(CEA) and carbohydrate antigen 19-9 (CA 19-9). However, these tools have limitations when it comes to early recurrence detection and identifying small lesions. The accuracy of CT scans in distinguishing between postoperative findings and tumor recurrence is not always certain, potentially leading to the oversight of early recurrences or small lesions. Furthermore, the increase in tumor markers like CEA and CA 19-9 can also be observed in benign diseases, compromising their specificity for detecting CRC [7, 8]. These limitations underline the need for additional diagnostic tools for identifying and effectively monitoring CRC. The emergence of liquid biopsy has opened up completely new possibilities in the field of diagnostics. Abnormal DNA methylation is a common occurrence in cancer development. In particular, hypermethylation of the septin9 gene is frequently observed in colorectal cancer (CRC). Consequently, the detection of methylated septin9 (*mSEPT9*) in peripheral plasma has emerged as a promising approach for the minimally invasive diagnosis of CRC across all stages [9].

SEPT9 ("HPA: SEPTIN9") is a member of the GTP (guanosine triphosphate)-binding protein family. It plays a significant role in crucial cellular processes including vesicle trafficking, apoptosis, and cell division [10]. In the context of carcinogenesis, tumor cells associated with CRC release *mSEPT9* into the peripheral blood. This characteristic allows for the relatively easy and more frequent detection of *mSEPT9* both before, during, and after surgical interventions. Detecting *mSEPT9* in the blood can potentially aid in identifying and monitoring patients with CRC. A systematic review focusing on the analysis of circulating tumor DNA in CRC diagnosis revealed that *SEPT9* hypermethylation was among the most accurate candidate markers. It demonstrated high sensitivity, with reported rates of up to 100%, and strong specificity, at rates of up to 97% for late-stage CRC [7]. However, recently published meta-analyses calculated pooled sensitivities of 66-69% and specificities of 91-92% [10-12].

Keyfactors, that influence the diagnostic accuracy of *mSEPT9* testing, seem to be the algorithm used for detection (1/3 algorithm vs. 2/3 algorithm) [7, 8], as well as age and tumour stage [12-17].

The *mSEPT9* biomarker has demonstrated superior sensitivity and specificity in the primary detection of CRC compared to well-established screening methods such as FOBT and the biomarkers CEA and CA19-9 [10, 13, 15, 17]. It has been proposed that combination of *mSEPT9* with FOBT yielded a high sensitivity of up to 94% in detecting CRC - however, at the expense of lower specificity, which was reported to be around 68% raising the prospect of a higher likelihood of false-positive results [18].

Methylated *SEPT9* has demonstrated its significance beyond diagnostic screening for CRC by also playing a valuable role in disease monitoring and predicting therapeutic efficacy. Fu et al. have observed that the persistence of plasma *mSEPT9* positivity after surgery was strongly associated with impending recurrences or metastases within one year, with 100% sensitivity [19]. Other researchers have reported a high sensitivity of 92.2% in patients with liver metastasis, which showed a significant decrease one week after undergoing simultaneous or staged liver surgery [20]. Patients who tested negative for *mSEPT9* in both the pre- and postoperative settings exhibited better survival rates compared to those who tested positive. In cancer patients, elevated levels of *mSEPT9* after surgery were associated with significantly higher rates of new metastases and mortality [21, 22].

While the determination of *mSEPT9* in peripheral blood is not currently standardized in Europe for use in screening or monitoring CRC, it is noteworthy that the *U.S. Food and Drug Administration (FDA)* has approved *mSEPT9* as a diagnostic tool [23].

In the present study, our objective was to contribute to further evaluation of *mSEPT9* for monitoring CRC early after surgery. We compared the diagnostic value of *mSEPT9* with the conventional tumor markers CEA and CA 19-9 in both the preoperative and postoperative

settings for patients with colorectal tumors in relation to TNM stage, UICC stage, and the presence of tumor remnants.

Materials and Methods

The study was approved by the Ethics Committee of the Heart and Diabetes Center Bad Oeynhausen, Germany (# 2018-368, 25/07/2018) and was designed as a prospective monocentric study. It was registered at the German Clinical Trials Register (DRKS00022787) and was accredited by the DKG (ST-D469). Informed consent was obtained from each patient, indicating their voluntary agreement to participate and provide their clinical data and blood samples for the study.

Blood samples were taken from December 2018 to December 2020 at the Department of General and Visceral Surgery, Thoracic Surgery and Proctology of the Ruhr University Bochum at Klinikum Herford.

This study included patients diagnosed with CRC who were scheduled for curative operative treatment. The decision for surgical intervention was made following discussion and consensus in an interdisciplinary tumor conference. The study included a representative population in terms of sex and age. However, we did not conduct detailed analyses specifically focusing on sex- or age-based factors in this particular stage of the study.

Patients with other forms of cancer such as lung, mammary, or prostate carcinoma were excluded from the study, as were patients with chronic gastritis, esophagitis, and non-rheumatic arthritis in order to avoid falsified results (see manufacturer´s guideline).

Blood samples were collected from the study participants during routine pre-surgery evaluations and as part of guideline-based aftercare. To prevent damage to the blood cells from shear forces, Venofix® Safety Gauge 21 needles from B. Braun SE (Melsungen, Germany), were used for the venipuncture procedure. In order to preserve the cell-free DNA

in the blood samples, cell-free DNA BCT® tubes from pluriselect (Leipzig, Germany) were utilized. These tubes were filled with 10 ml of blood from each participant, placed in a protective container, and then shipped within four days at the latest. Processing and analysis of the *mSEPT9* level of blood samples were performed by *arrows biomedical Deutschland GmbH* (Epi Colon® 2.0 CE Plasma quick Kit (M5-02-001), Epi proColon® 2.0 CE PCR Kit (M5-02-002), Epi proColon® 2.0 CE Control Kit (M5-02-003); Epigenomics AG, Berlin, Germany) [24, 25]. The analysis of the samples was performed using the Roche LightCycler® 480 Instrument II (Roche Deutschland Holding GmbH, Grenzach-Wyhlen, Germany). A positive result for *mSEPT9* was logged when at least two out of the three PCR replicates yielded a positive signal (2/3-algorithm).

The analysis of CEA and CA 19-9 was performed at the Institute of Laboratory Medicine at the Johannes Wesling Klinikum Minden (Roche cobas® e 801 high throughput immunochemistry module, Elecsys® CEA and Elecsys® CA19-9 electro-luminescence assay (ECLIA), all manufactured by Roche Diagnostics International Ltd). For CEA analysis, test results equal to or above 5.2 µg/l were defined as pathological, indicating abnormal levels. Similarly, for CA 19-9, a value equal to or above 34 U/ml was considered pathological. We conducted the statistical analysis of the data with SPSS 15.0, a widely used statistical software package. The Student t-test was used to compare mean values of two groups. P-values less than 0.05 were considered statistically significant, indicating a high level of confidence in the observed results. Confidence intervals of 95% were used to estimate the precision and reliability of the findings. In the text and diagrams, percentages are rounded to the nearest whole percent.

Results

Our study included a total of 120 patients, of which 54 were female and 66 were male. The determination of the sex of the patients was based on the information provided by the patients

themselves. The age of the participants ranged from 27 to 90 years, with an average age of 67 +/- 12 years. The average age for males was 67.3 years, while for females it was 65.7 years. The median age of the entire group was found to be 65 years. There was no statistically significant difference in age between males and females (p-value: 0.39). The average age of patients who tested negative for *mSEPT9* was 64.5 (+/- 13.1) years, while the average age of patients with a positive *mSEPT9* status was 68.2 (+/- 11.1) years (p-value: 0.26). Additional patient characteristics are described in Table 1 below.

Table 1 Disease characteristics related to number of patients

Characteristic	Number of patients	Percentage (%)
UICC stage		
I	33	27.5
II	31	25.8
III	31	25.8
IV	25	20.8
Primary tumor category		
T0	5	4.2
T1	11	9.2
T2	20	16.7
T3	56	46.7
T4	26	21.7
Tx	2	1.7
Region node category		
N0	67	55.8
N1	30	25.0
N2	22	18.3
N3	1	0.8
Distant metastasis		
M0	95	79.2
M1	25	20.8
Lymphatic invasion		
L0	83	69.2
L1	37	30.8
Vascular invasion		
V0	89	74.2
V1	31	25.8
Perineural invasion		
Pn0	98	81.7
Pn1	22	18.3
Surgical margin		
R0	111	92.5
R1	3	2.5
R2	4	3.3
N/A	2	1.7
Histopathological grade		
G1	6	5.0
G2	77	64.2
G3	37	30.8
Tumor localization		
Caecum	3	2.5
Ascending colon	30	25.0
Transverse colon	8	6.7
Descending colon	6	5.0
Sigmoid colon	18	15.0
Rectum	55	45.8

Methylated *SEPT9*, CEA, and CA 19-9 were measured at different time points throughout the study. The number of patients included in the analysis preoperatively, and at seven days, six weeks and three months postoperatively was as follows: 119, 118, 107 and 98 (*mSEPT9*), 118, 117, 105, 98, (CEA) and 118, 117, 105, 98 (CA19-9). Four patients died (two from causes not related to the disease). Additionally, some patients refused to have blood samples taken for testing during the COVID-19 pandemic, while others were unavailable for follow-up due to reasons such as relocation. Invalid test results were excluded from the analysis. Among the 119 patients included in the study, 68 patients had positive *mSEPT9* results preoperatively, indicating a sensitivity of 57% (Figure 1). In comparison, the CEA sensitivity was found to be 32%, (elevated in 38 out of 118 patients), the CA 19-9 sensitivity was 18% (elevated in 21 out of 118 patients). Seven days after tumor resection, 49% of the patients (58 out of 118) showed a positive result for *mSEPT9*. This percentage decreased to 26% (28 out of 107 patients) six weeks after surgery, and further dropped to 24% (24 out of 98 patients) after three months (Figure 1). These findings indicate that the proportion of patients with positive *mSEPT9* test results decreased over time following tumor resection, while the percentage of patients with negative test results increased.

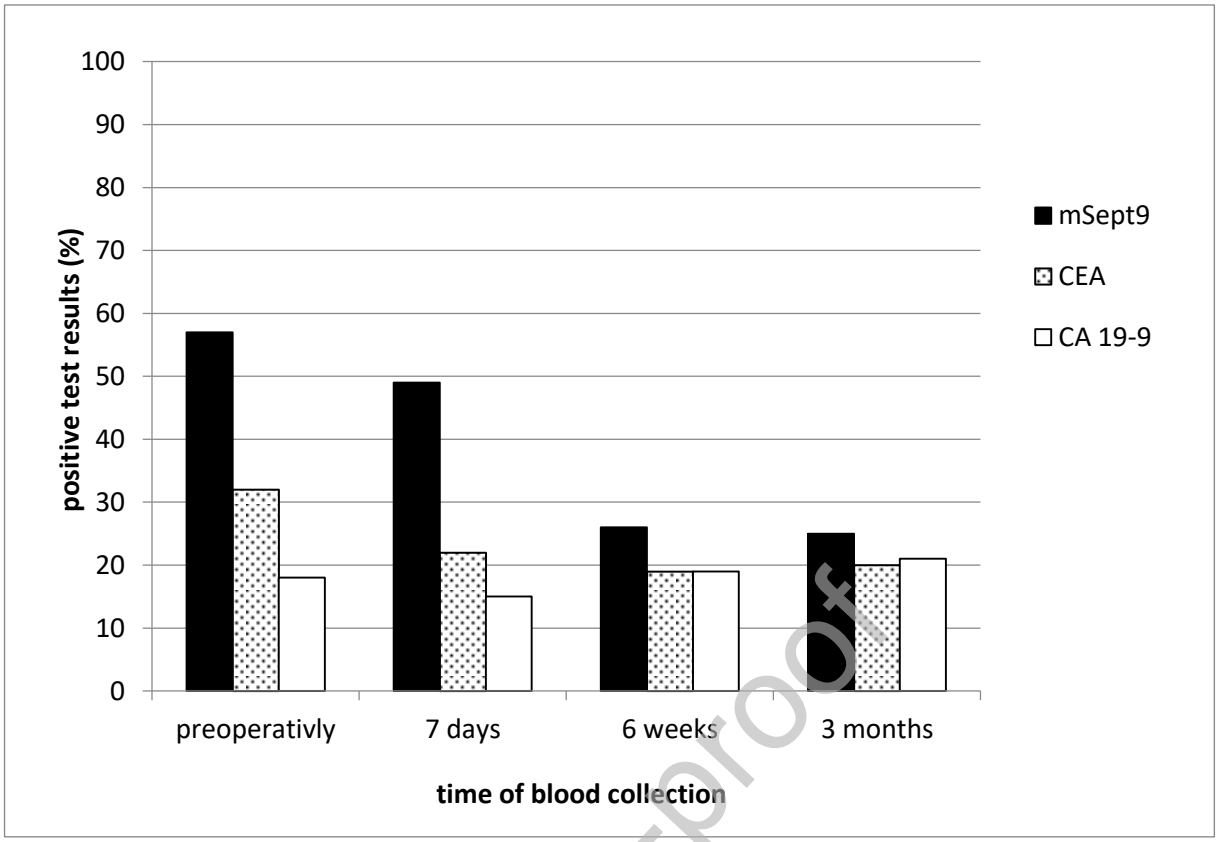


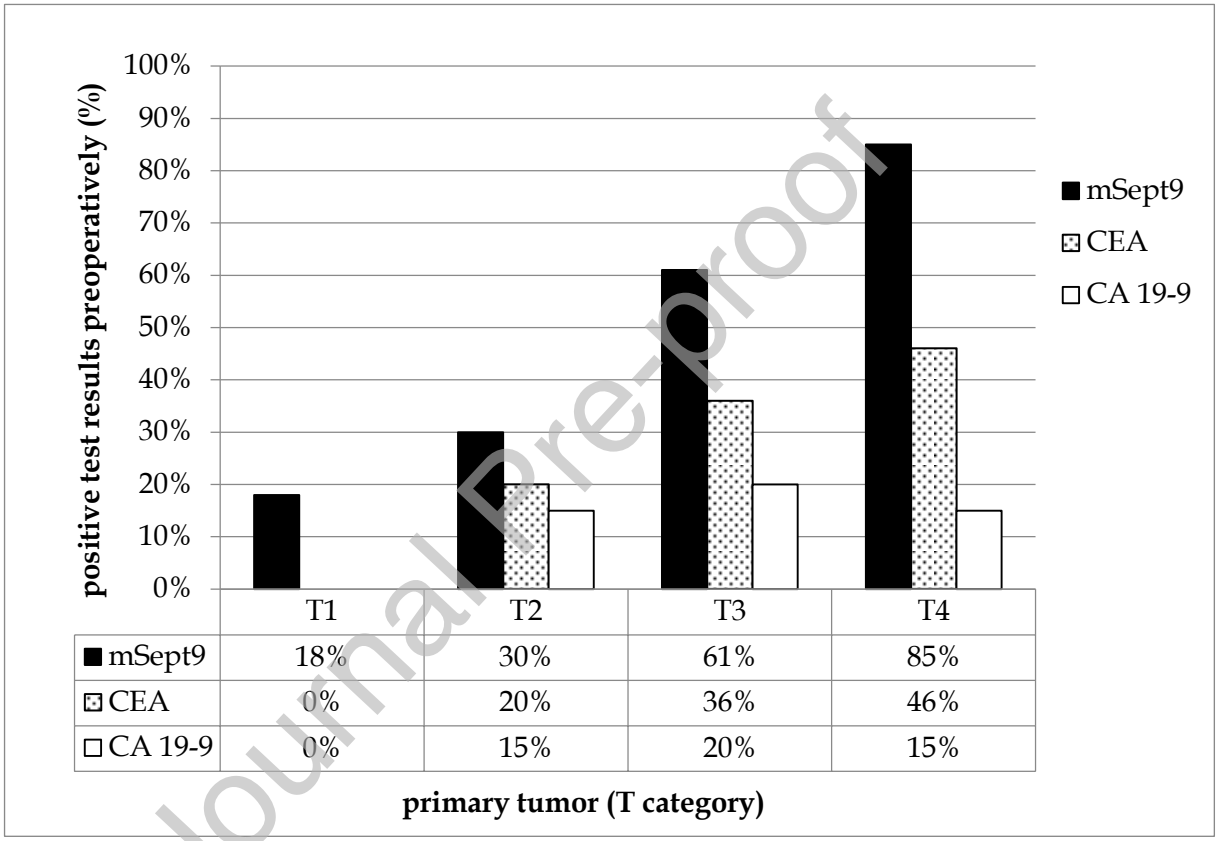
Figure 1: Sensitivity (percentage of positive test results for CRC patients) for *mSEPT9*, CEA and CA 19-9 at different points in time: Preoperatively, then at seven days, six weeks and three months after tumor resection.

CEA decreased to 22% (26 of 117 patients) with positive test results seven days after surgery, CA 19-9 to 15% (17 of 117 patients) (see Figure 1). Six weeks after surgery, CEA was positive in 19% (20 of 105 patients), CA 19-9 also in 19% (20 of 105 patients)). Three months after surgery, CEA was positive in 20% (20 of 98 patients) and CA 19-9 in 21% (21 of 98 patients).

In summary, our study found that *mSEPT9* had a higher detection rate for colorectal tumors compared to conventional tumor markers CEA and CA 19-9. This difference was statistically significant ($p<0.00$). However, no statistically significant difference was observed between CEA and CA 19-9 (p: 0.169). By combining all three biomarkers, the sensitivity for preoperative detection increased to 66% if either *mSEPT9*, CEA, or CA 19-9 were elevated.

T-Stage

Regardless of the T-stage, *mSEPT9* showed a higher detection rate for patients compared to CEA and CA 19-9 (**Error! Reference source not found.**). Specifically, in advanced tumor stages (T4), *mSEPT9* exhibited a sensitivity of 85% (22 out of 26 patients), outperforming CEA with a sensitivity of 46% (12 out of 26 patients) and CA 19-9 with a sensitivity of 15% (4 out of 26 patients).



	T1	T2	T3	T4
mSept9	18%	30%	61%	85%
CEA	0%	20%	36%	46%
CA 19-9	0%	15%	20%	15%

Figure 2: Sensitivity (percentage of preoperative positive test results for CRC patients) for *mSEPT9*, CEA and CA 19-9 depending on patient T-status (T4, n=26; T3, n=56; T2, n=20; T1, n=11).

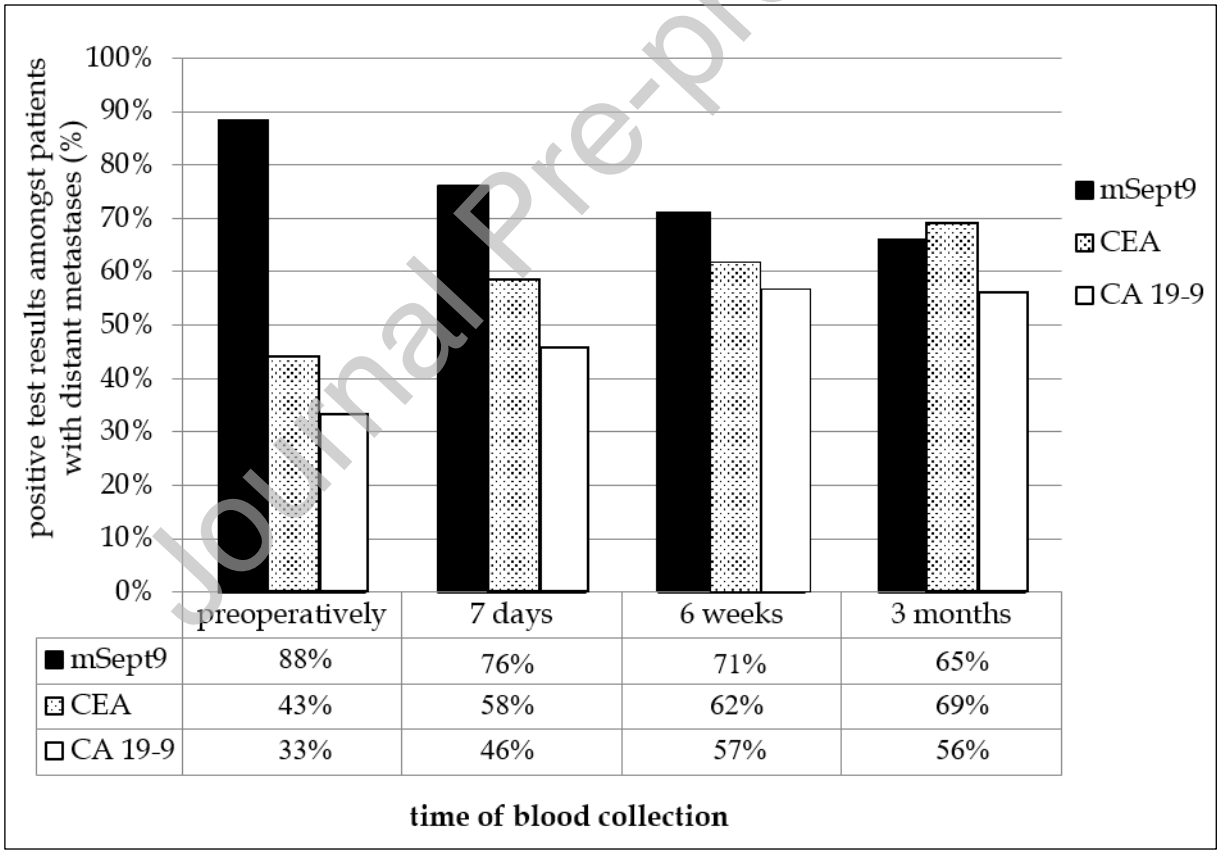
Nodal stage

In patients with infiltrated lymph nodes (n=53), as observed in the preoperative histopathological examination, *mSEPT9* had a positive result in 64% of patients (34 out of

53), CEA showed a positive result in 36% of patients (19 out of 53), and CA 19-9 had a positive result in 19% of patients (10 out of 53).

Distant metastases

In patients with known distant metastases (n=25), *mSEPT9* showed higher sensitivity compared to conventional tumor markers at all points in time (Figure 3). Preoperatively, *mSEPT9* had a sensitivity of 88% (21 out of 24 patients with metastases), which was significantly higher than the sensitivity of CEA or CA19-9 ($p<0.00$). Seven days after resection, the difference in sensitivity was statistically significant only when compared to CA19-9 ($p<0.00$), while no significant difference was observed in comparison with CEA.

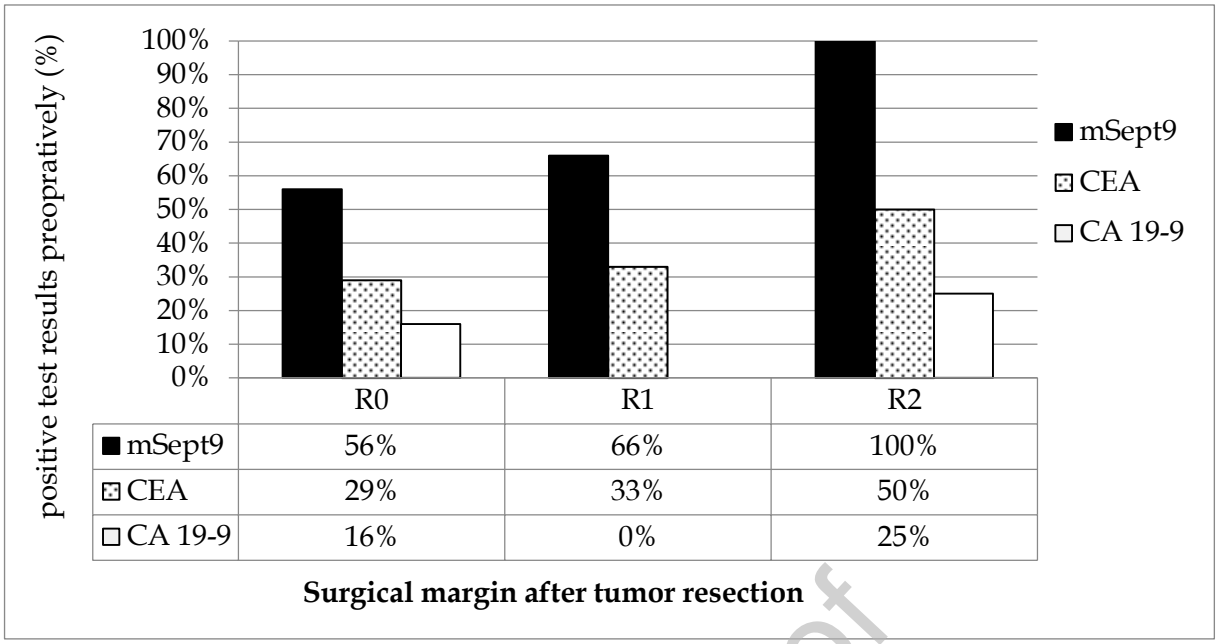


	preoperatively	7 days	6 weeks	3 months
mSept9	88%	76%	71%	65%
CEA	43%	58%	62%	69%
CA 19-9	33%	46%	57%	56%

Figure 3: Detection rate of patients with distant metastases by *mSEPT9*, CEA and CA 19-9 preoperatively and after tumor resection at 7 days, 6 weeks and 3 months.

R-status

Out of the 118 patients who underwent tumor resection, 111 patients had a complete tumor resection with no detectable tumor remnants (R0). Three patients had microscopic tumor remnants (R1), indicating the presence of residual tumor cells at a microscopic level. Additionally, four patients had macroscopic tumor remnants (R2), indicating visible tumor tissue after the resection. Among the patients who had a later R0 resection (n=111), the percentage of *mSEPT9*-positive test results decreased from 56% preoperatively (62 of 111 patients) to 16% three months after surgery (18 of 111 patients) (Figure 4). Similarly, CEA levels decreased from 29% preoperatively to 13% three months after surgery, CA19-9 levels decreased slightly from 16% preoperatively to 15% three months after surgery. Among the patients with R1 status (n=3), the share of *mSEPT9*-positive test results was 33% (one of three patients) at both six weeks and three months after resection. In contrast, none of the patients with R1-resection had elevated levels of CEA or CA19-9 at either six weeks or three months after resection. Among the patients with R2 status (n=4), all patients had *mSEPT9*-positive test results at all time points. CEA was elevated in 50% of the cases at six weeks after surgery (two of four patients) and in 75% of the cases at three months after surgery (three of four patients). CA19-9 was elevated in 25% of the cases at six weeks after surgery (one of four patients) and in 75% of the cases at three months after surgery (three of four patients).



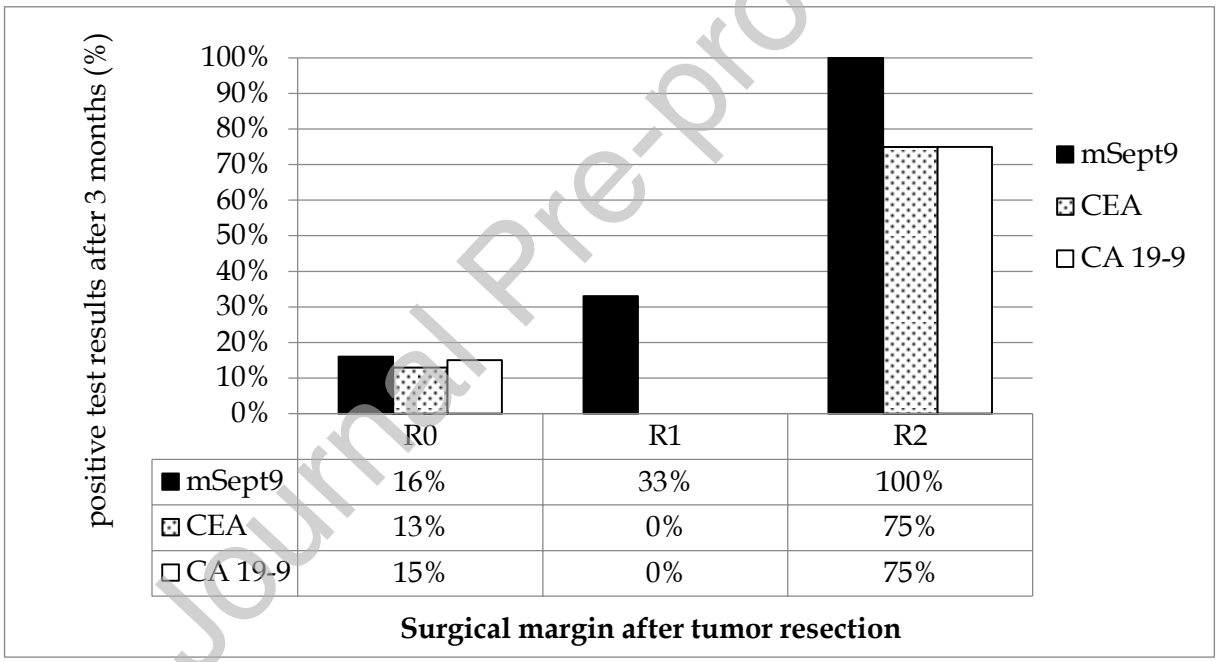
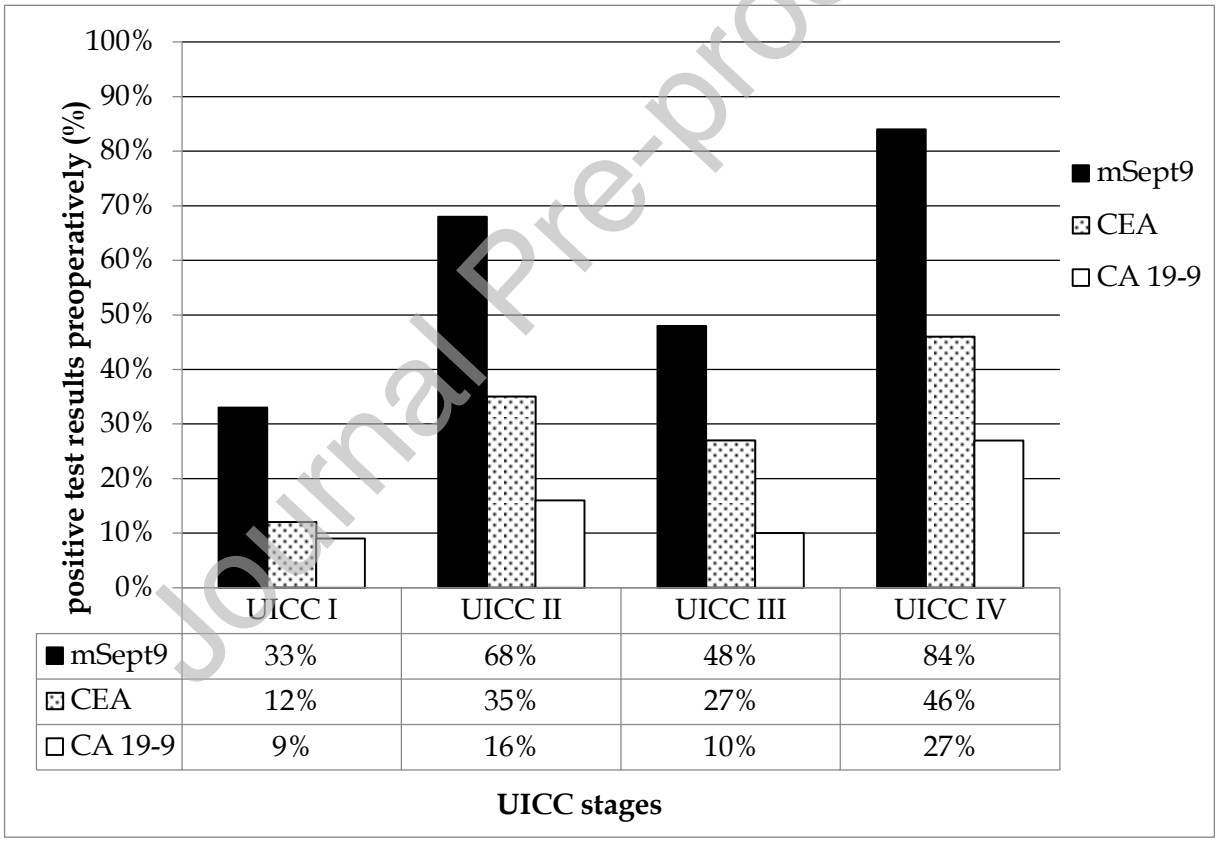


Figure 4: Positive test results for mSEPT9, CEA and CA 19-9 depending on tumor remnants at 3 months after tumour resection (bottom), and for the same patients preoperatively for comparison (top). R0=complete resection, R1=microscopic tumor remnants, R2=macroscopic tumor remnants.

UICC stage

In terms of sensitivity broken down into UICC stages, *mSEPT9* showed the highest sensitivity in UICC stage IV, with 84% of patients (21 out of 25) having positive test results (Figure 5). In UICC stage III, 48% of patients (15 out of 31) were *mSEPT9*-positive, while in UICC stage II, 68% of patients (21 out of 31) were *mSEPT9*-positive. In UICC stage I, 33% of patients (11 out of 33) had positive *mSEPT9* results. Comparatively, *mSEPT9* demonstrated higher sensitivity at all UICC stages compared to conventional tumor markers. Specifically, in UICC stage II, *mSEPT9* was able to detect nearly double the number of patients (68%) compared to CEA (35%) and more than four times the number of patients compared to CA 19-9 (16%).



	UICC I	UICC II	UICC III	UICC IV
mSept9	33%	68%	48%	84%
CEA	12%	35%	27%	46%
CA 19-9	9%	16%	10%	27%

Figure 5: Sensitivity (percentage of preoperatively positive test results for CRC patients) for *mSEPT9*, CEA and CA 19-9, depending on UICC stage of disease (UICC I, n=33; UICCI I, n=31; UICC III, n=31; UICC IV, n=25).

Tumor location

No significant difference was observed when analyzing patients based on tumor location. Among patients with colon carcinoma, 58% (38 out of 65 patients) had a positive *mSEPT9* status. Similarly, among patients with rectal carcinoma, 55% (30 out of 55 patients) had positive results for *mSEPT9*.

Presence of tumor after surgery

Follow-up data on the M-stage of the patients were either generated in-house or provided from the treating physicians and have been used to calculate preliminary values for sensitivity and specificity of *mSEPT9* in relation to the presence of tumor after surgery (Table 2).

After three months, 17 of initially 25 patients with M1 status and 74 of the 95 patients who had initially been classified M0 were available for *mSEPT9* testing.

Eight M1 patients and 14 former M0 patients were not available for follow-up or had invalid *mSEPT9* tests. No up-dated information on the stage of disease was available for seven former M0 patients: These patients have not been included in the analysis.

Thirty-five patients with preoperatively negative *mSEPT9* results remained *mSEPT9* negative and M0. Three patients with preoperatively negative *mSEPT9* results had a positive *mSEPT9* test three months after surgery, and were still classified M0.

Two M0 patients with preoperatively positive *mSEPT9* results remained *mSEPT9* positive and were again classified M0. Seven M0 patients with preoperatively positive *mSEPT9* results remained *mSEPT9* positive and received a chemotherapy, indicating worsening of the disease. Twenty six patients with preoperatively positive *mSEPT9* results had a conversion to negative *mSEPT9* test results three months after surgery and were still classified M0. One patient with

preoperatively positive *mSEPT9* results had a conversion to a *mSEPT9* negative test result three months after surgery with diagnosed renal cell carcinoma (Table 2).

The calculated sensitivity and specificity of *mSEPT9* three months after removal of the primary tumor were 72 % (18 positive *mSEPT9* tests out of 25 patients with known metastases or other evidence of tumor cells) and 91% (61 negative tests results out of 65 patients with proven M0 status: 35 preoperatively negative, 26 with conversion from *mSEPT9* positive to *mSEPT9* negative), respectively.

Table 2 Preliminary estimation of sensitivity and specificity of *mSEPT9* in relation to detection of metastases, remnant or secondary tumor or potential tumor recurrence after surgery; *mSEPT9+: mSEPT9* positive test result, *mSEPT9-*: *mSEPT9* negative test result, n.v.: non-valid test, n.a.: patient not available.

	3 months postOP			
Status of disease	*mSEPT9+*	*mSEPT9-*	n.v.	n.a.
M1 (preOP as well as 3 months postOP) (25 patients) Secondary tumor (renal cell carcinoma (1 patient) Adjuvant chemotherapy (7 patients)	11 7	6 1		8
M0 3 months postOP (87 patients)	5	61	1	20
Sensitivity	**72%**			
Specificity		**91%**		

Discussion

A recent study conducted in Spain reported findings similar to ours, with a detection sensitivity of 55.6% for colorectal carcinoma using *mSEPT9* in a small group of 32 patients [26]. Other studies have shown a wide range of detection sensitivities for *mSEPT9* in colorectal carcinoma, varying between 36.6% and 95.6% [27]. Meta-analyses have suggested that the diagnostic sensitivity of *mSEPT9* may be higher among Asians compared to Whites *mSEPT9*W [12, 16], which could contribute to the wide variation in results observed. Another

factor that may contribute to relatively low sensitivities is the use of the 2/3-algorithm, which we also employed in this study. Song et al. demonstrated that using the 1/3 algorithm for *mSEPT9* detection in blood could improve sensitivity but also increase the false positive rate in terms of specificity [16]. Since our study was designed without a control group, no false positive results were observed. For the commercially available Epi proColon 2.0 test, which utilizes the 2/3 algorithm, a false positive rate of less than 3% has been reported [12].

In our study, the combination of *mSEPT9*, CEA, and CA19-9 markers preoperatively demonstrated the highest sensitivity (66%), suggesting that it as a supplement for current diagnostic tools. Other studies that explored the combination of *mSEPT9* with fecal immunochemical testing (FIT) showed potential further increased sensitivity for CRC detection compared to *mSEPT9* alone (e.g. 89%-98% vs. 73%-77% [15, 27]). Similarly, the combination of *mSEPT9* with FOBT and CEA has been investigated [13]. Currently discussed is whether FOBT, in particular, can enhance sensitivity in early-stage CRC, but this has yet to be confirmed [13, 15].

In our study, we found no significant difference in the mean age between *mSEPT9*-positive and *mSEPT9*-negative patients, which is consistent with the observations made by Song et al. [16] They also reported that there was no age-related difference in the sensitivity of *mSEPT9*-based detection of CRC. However, they did observe a trend towards a higher false positive rate in healthy subjects above 60 years compared to younger individuals [16]. Since our study did not include a control group, we cannot offer further insights or comments on these specific findings.

Our study population, with its average age of 67 years and wide age range, included six patients with hereditary non-polyposis colorectal cancer (HNPCC) as the probable tumor origin. Mauri et al. discussed in a review that the number of CRC patients younger than 50 years is increasing, particularly in industrialized countries, with approximately 50% of these

patients having no known inherited predispositions. This suggests the presence of other factors that contribute to the development of colorectal carcinoma, which are not yet fully understood [28]. The adoption of a "Westernized lifestyle," for example, has been shown to have led to a steady increase in the incidence of colorectal carcinoma in the Chinese population [29].

In our analysis, we found that *mSEPT9* had improved sensitivity of approximately 85% in patients at an advanced tumor stage (T4), UICC stage IV, or with distant metastases. This strongly suggests that *mSEPT9* may have a higher diagnostic value in patients with a high tumor burden, particularly in terms of metastases.

We have calculated preliminary values for the sensitivity (72 %) and specificity (91%) of *mSEPT9* testing based on the results observed in our study three months after removal of the primary tumor. Thus, only nine percent in the group of patients classified M0 had positive m*SEPT9* results three months after surgery (Table 2). In contrast, 65% of M1 patients had positive mSEPT9 results three months after surgery (Figure 3, Table 2). Based on our study results, we expect a high detection rate for remaining tumor or reoccurrences and at the same time an acceptable risk of false positive diagnoses. The timely detection of tumor recurrence in patients with colorectal carcinoma is of critical importance, as a significant proportion of patients, ranging from 30% to 50%, may experience tumor recurrence following initial treatment [33]. The study by Fu et al. demonstrating 100% sensitivity and specificity (two of 16 patients) for prediction of tumor recurrence using *mSEPT9* during a one-year follow up period is promising: Their findings suggest that if *mSEPT9* remains positive 7-14 days after surgery, it could serve as a reliable indicator of future recurrence [19]. They also found a strong correlation between *mSEPT9* status and tumor recurrences during long-term follow-up.

It has been reported that simultaneous resection of the primary tumor and synchronous liver metastases led to a significant 923-fold reduction in quantitative levels of *mSEPT9* [20].

These findings support the potential utility of *mSEPT9* as a marker for monitoring tumor burden and response to treatment in advanced colorectal carcinoma patients [16]. Indeed, multiple studies have reported a positive correlation between the stage of disease and the sensitivity of *mSEPT9* [12-14, 16, 17], although there may be differing individual results [17, 27].

Our observation of the greatest increase in sensitivity of *mSEPT9* between stage I and stage II (Duke's scale) colorectal carcinoma is interesting and consistent with findings from other studies, e.g., Sun et al. This trend may suggest a potential association between *mSEPT9* sensitivity and the deficiency in mismatch repair (MMR) and microsatellite instability (MSI), [17], which are also reported to occur mainly in the early stages of CRC [30]. The finding that 68% of patients in UICC II stage could be positively detected preoperatively with *mSEPT9* highlights the potential value of this new diagnostic marker even at lower tumor stages. and could also be important for patients with intention for long-term curative treatment.

Moreover, quantitative levels have proven to be associated with UICC stage and TNM category [8, 31]. We were able to show that *mSEPT9* had better sensitivity than CEA or CA19-9 in patients with tumor remnants (R1 or R2 status) as well as higher UICC stage.

A study by Ma et al. also suggests that increased quantitative levels of *mSEPT9* measured after surgery may serve as an indicator of tumor recurrence in colorectal cancer patients. The findings indicated that patients with higher levels of *mSEPT9* after surgery have a higher risk of developing new metastases and increased mortality rates [22]. A study by Jin et al. provided evidence that elevated levels of *mSEPT9* can be detected eight months prior to radiologic imaging. [32].

Adjuvant chemotherapy may induce a change in *mSEPT9* status from positive to negative after therapy, suggesting a potential correlation between the effectiveness of chemotherapy on

residual tumor load and *mSEPT9* levels [32]. In our study adjuvant chemotherapy was associated with positive *mSEPT9* results. Assumingly, the time point was too early to detect an onset of the treatment.

We believe that this novel biomarker has the potential to be a valuable asset in assessing the success of tumor resection. Quantitative measurement of *mSEPT9* levels could offer additional information regarding treatment effectiveness and the risk of tumor recurrence. However, more studies are needed to establish specific cut-off values in this field.

The limitations of our study should be acknowledged. First, the follow-up period of only three months may not be sufficient to detect early metastases, and longer-term follow-up would be valuable. Additionally, the limited number of patients with remnant tumors or metastases may affect the generalizability of the findings. Another limitation is the absence of a healthy control group for comparison. Despite these limitations, the main strength of our study lies in its contribution to the evaluation of *mSEPT9* as an additional follow-up marker in patients with advanced stages of CRC, particularly as a tool for early recognition of remaining tumor after surgery.

In summary, our study provides additional evidence that *mSEPT9* potentially should play a relevant role in monitoring of patients with CRC: The calculated sensitivity of *mSEPT9* in detection of metastases, tumor remnants and potential tumor recurrence could provide vital clues to the persistence of the disease in clinical praxis. Due to the high specificity, possible false-positive diagnoses are minimized. The ease of carrying out *mSEPT9* testing further enhances its potential as a standard monitoring tool. Recent studies have highlighted an increase in the rate of locally advanced rectal cancer (from 58% to 79%), and a tripling of metastases (from 3% to 9%), potentially attributed to delayed diagnoses during the Covid-19 pandemic [34, 35]. This highlights the need for comprehensive screening strategies that include high-sensitivity non-invasive biomarker detection for advanced stages of CRC.

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Author contributions

Leerhoff, S.: investigation, formal analysis, visualization, writing the original draft;

Raem, A.: investigation, methodology;

Kolbe, E.W.: formal analysis;

Schulz, L.: investigation, validation, writing the original draft;

Borchers, K.: writing the original draft, review, editing;

Köhler, T.: investigation, supervision;

Winde, G.: conceptualization, resources, review;

Kirchner, C.: writing, review, project administration.

Declaration of interests

☒The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

☐The authors declare the following financial interests/personal relationships which may be considered as potential competing interests: