



# Circulating molecular biomarkers for screening or early diagnosis of colorectal cancer: which is ready for prime time?

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**Abstract:** According to recent statistics, colorectal cancer (CRC) is a frequent disease, the second most frequent malignancy in women and the third most common malignant disease in men, respectively. Although reinforced emphasis on CRC screening by means of immunochemical fecal occult blood test, colonoscopy or sigmoidoscopy has contributed to decrease cancer-related deaths, alternative diagnostic tests would be needed for establishing earlier and more potentially effective treatments. Innovative diagnostic techniques have recently emerged, some of which hold promises for screening and/or early CRC detection. Recent evidence suggests that the so-called “liquid biopsy”, conventionally defined as detection and quantification of circulating tumor cells (CTCs) and cancer-related nucleic acids in peripheral blood, may allow earlier diagnosis of CRC combined with lower invasiveness and less patient inconvenience, higher throughput, faster turnaround time, inferior usage of healthcare resources and relatively low cost. Encouraging data have emerged from trials based on CTCs detection, though the sensitivity of the current diagnostic techniques is still perhaps insufficient for enabling early CRC diagnosis. Among the various biomarkers that can be detected with liquid biopsy, *SEPT9* methylation displays good diagnostic performance and relatively high cancer detection rate (between 57–64% in patients with CRC stages 0–I), which would make this test a promising tool for population screening, alone or in combination with other conventional diagnostic investigations. Encouraging evidence has also been recently published for *BCAT1/IKZF1* methylation. Regarding microRNA (miRNAs), the available evidence highlights that the combination of some of these biomarkers rather than the assessment of a single miRNA alone would enable efficient identification of early CRCs, though widespread clinical application is still challenged by a number of preanalytical, analytical and clinical issues.

**Keywords:** Colorectal cancer (CRC); circulating tumor DNA (ctDNA); circulating tumor cells (CTCs); early diagnosis; microRNAs

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## State-of-the-art in colorectal cancer (CRC) screening

CRC is a very frequent disease, accounting for as many as 1.85 million of new cases each year, and thus being currently considered the second most frequent malignancy in women

after breast cancer and the third most common malignant disease in men after lung and prostate cancers (1). Several lines of evidence now attest that the prognosis of this cancer is highly dependent on early diagnosis, whereby the 5-year cumulative survival rate decreases from 90% when CRC

is diagnosed at an early stage, to ~15% in patients with advanced, metastatic disease (1).

Although reinforced emphasis placed on CRC screening has indeed contributed to ameliorate its otherwise unfavorable biologic course, the vast majority of patients with this malignant disease can only be diagnosed after symptoms onset, when cancer growth has already seriously jeopardized treatment success and life expectancy (2,3). Despite its well-known, inherent limitations (4), the immunochemical fecal occult blood test (iFOBT) is still considered the most cost-effective analysis for CRC screening around the world. The widespread use of this test is endorsed (in people aged 45 years or older) by the American Cancer Society (ACS) (5). Rather similar indications are provided by other national and international scientific organizations, which are almost concordant to agree on the cost-effectiveness of screening subjects aged between 50–75 years, by means of iFOBT, colonoscopy or flexible sigmoidoscopy (6).

The evidence that iFOBT screening contributes to lower the overall number of deaths for CRC is now generally taken for granted, though recent evidence seems to contradict this widespread perception, at least for what concerns all-cause deaths and certain cancer sites. Shaukat *et al.* for example, recently published the results of the Minnesota Colon Cancer Control Study, in which over 46,000 participants aged between 50–80 years were randomized to receive either no screening or annual or biennial iFOBT testing (2). At the end of 30-year follow-up, colorectal-cancer mortality was reduced by ~30% with annual screening [relative risk (RR), 0.68; 95% confidence interval (95% CI), 0.56–0.82] and ~20% with biennial screening (RR, 0.78; 95% CI, 0.65–0.93), but no significant decline was noted for all-cause mortality (annual screening; RR, 1.00 and 95% CI, 0.99–1.01; biennial screening: RR, 0.99; 95% CI, 0.98–1.01). Most notably, similar results were nearly concomitantly published by Nishihara *et al.*, who explored the effectiveness of endoscopy for preventing CRC mortality in over 88,000 participants of the Nurses' Health Study and the Health Professionals Follow-up Study (7). At the end of 22-year follow-up, CRC mortality was considerably reduced by ~40% [hazard ratio (HR), 0.59; 95% CI, 0.45–0.76] with screening sigmoidoscopy and by ~70% (HR, 0.32; 95% CI, 0.24–0.45) with screening colonoscopy, respectively. Nevertheless, a sub-analysis of the data revealed that screening sigmoidoscopy was ineffective to lower the number of deaths due to proximal colon cancer (HR, 1.04; 95% CI, 0.73–1.48).

Importantly, Welch and Robertson have recently highlighted that the decline of all-cause and cancer-related mortality recently observed in patients with colorectal malignancies cannot be entirely explained by adherence to population screening, whereby multiple other factors may have played a role (8). Therefore, even without openly denying the unquestionable effectiveness of strategies currently used for CRC screening (i.e., iFOBT, colonoscopy or sigmoidoscopy), the still elevated mortality for CRC and the significant risk of false negative test results, lead the way to developing alternative diagnostic tests, which may allow to establish an earlier and thus more potentially effective treatment (9). Innovative diagnostic techniques have recently been proposed, such as video capsule endoscopy, liquid biopsy, analysis of multitarget-stool DNA or analysis of volatile organic compounds in exhaled air (10,11), but none of these approaches seems ready for prime time. Nevertheless, encouraging data are emerging for some molecular blood biomarkers, whose clinical usefulness will be discussed in the following parts of this article.

### Liquid biopsy: current evidence and future perspectives

The blood of patients with different forms of cancer may theoretically contain a variable number of circulating tumor cells (CTCs), cell-derived vesicles (such as exosomes) and cell-free nucleic acids released from the tumor itself. The term “liquid biopsy” was originally used for identifying CTCs in peripheral blood, but was then extended to detection and quantification of circulating tumor DNA (ctDNA) and microRNAs (miRNAs). Even if blood-based assays are those most widely used, ctDNA could be detected in many other biological fluids such as pleural effusion, ascites, saliva, urine, stool and cerebrospinal fluid. Therefore, the term “liquid biopsy” is now used for characterizing a broad set of tests used for analysis of tumor-derived biomarkers isolated from biological fluids of cancer patients (12).

Since CRC is often the worse consequence of evolving precancerous lesions (e.g., adenomas), screening is mostly focused on early detection of these pathologies. The use of liquid biopsy not only would be useful for early diagnosing CRC, but also may carry many other advantages, including lower invasiveness and less patient inconvenience (compared with colonoscopy and/or sigmoidoscopy), higher throughput, faster turnaround time, minor usage of healthcare resources and relatively low cost (12,13).

Since malignant cells follow a continuous and dynamic evolution in the bloodstream, paralleling cancer growth and spread, another advantage of liquid biopsy is the possibility to monitor cancer genome and molecular properties in real time, thus enabling rapid detection of somatic mutations that may impact the clinical course of colorectal malignancies (14).

Several clinical applications of liquid biopsy have been investigated in CRC, ranging from early disease detection to their usage as prognostic or predictive tools, for monitoring disease or for studying the mechanisms involved in development of drug resistance (15). Nevertheless, the use of this diagnostic strategy is still limited in clinical practice. The insufficient level of standardization for isolation, detection and quantification of nucleic acids is perhaps one of the most important causes for its narrow usage (16). Moreover, further prospective studies, including large sample sizes, would be needed to clearly demonstrate the clinical effectiveness or superiority of this technique over the current armamentarium of conventional diagnostic tests (17).

### CTCs

CTCs are essentially tumor-derived cells, in particular epithelial cancer cells, which shed into peripheral blood by passive cell shedding or active regulated process (18). Epithelial-to-mesenchymal transition (EMT) is the highly dynamic process by which a sub-population of primary cancer cells acquires increased motility and invasiveness potential (19). CTCs have been identified in several types of cancers, as a single CTC or in aggregates ( $\geq 2$  CTCs), named “clusters” or “tumor microemboli” (20). It has been demonstrated that CTCs clusters are more prone to form metastases than single CTC (21), due to enhanced likelihood of “survival” (22). In fact, most CTCs released in the bloodstream early succumb for combined influence of mechanical and environmental injuries such as shear forces, oxidative stress and immune system response. The half-life of CTCs in the circulation is hence extremely narrow, typically comprised between 1–2 h (23).

In studies on breast cancer patients, some subset of CTCs with tumor-initiating capacity have been demonstrated (24,25). Since the number of circulating CTCs is usually low, ranging between 1 and 10 cells per 10 mL, perhaps even lower in early stage cancers (26), CTC enrichment and detection are two essential steps for accurate quantification. On the other hand, contamination is a major source of

diagnostic problems, since most detection systems tend to capture also a variable number of leukocytes (12). CTCs enrichment can be achieved by assessing the expression of epithelial surface specific proteins or by targeting specific physical features such as size or density of cancer cells (27). As regards detection, several approaches (immunological, molecular and functional techniques) have been developed. Nevertheless, the only technology that has been approved so far by the US Food and Drug Administration (FDA) for CTC enumeration in patients with metastatic breast, prostate and CRC is CellSearch (Janssen Diagnostics, Raritan, NJ, USA), which is based on anti-epithelial cell adhesion molecule (EpCAM) antibodies (28).

What has clearly emerged from recent literature data is that the methods for enrichment, detection and characterization of CTCs lack sensitivity, specificity and reproducibility (29). Some technological breakthroughs hold promises, but they will need careful evaluation and verification before being implemented in routine clinical practice. Abouleila *et al.* have recently developed an interesting approach, based on use of live single cell mass spectrometry integrated with microfluidics-based cell enrichment, for assessing differences in metabolomic profile between CTCs originating from different cancer groups (30). Su *et al.* have also developed an integrated microfluidic chip device that is capable to easily and automatically enrich and identify CTCs from blood of CRC patients (31). Although these preliminary results seem promising (the enrichment efficiency for CTCs was reported to be as high as 70%), further validation studies would be needed.

Although the prognostic value of CTC in early stages of CRC has already been proven by a large number of clinical studies (17,32), their role for screening and early detection remains controversial (33). The still inefficient diagnostic performance has been principally attributed to evidence that the number of CRC patients who will test positive for CTCs in early stages of cancer is perhaps too low to achieve sufficient sensitivity with currently available techniques (15). Baek *et al.* have recently explored the clinical significance of CTCs assessment in early CRC detection (34). Although sensitivity and specificity for distinguishing CRC patients ( $n=88$ ) from a small cohort of healthy controls ( $n=31$ ) were 75% and 100%, with a corresponding area under the curve (AUC) of 0.91, the authors did not carried out a sub-analysis for addressing the diagnostic performance of CTCs in early CRC stages (i.e., I and II). Nevertheless, encouraging results were published by Tsai and colleagues (35), who

assessed CTCs in 182 healthy controls, 111 patients with precancerous lesions and 327 patients with stages I–IV CRC. The sensitivity, specificity and AUC of CTCs testing were 76.6%, 97.3% and 0.84 for differentiating patients with precancerous lesions from healthy subjects, and 86.9%, 97.3% and 0.88 for distinguishing CRC patients from healthy subjects. Other studies will be obviously needed to validate the possible use of CTCs for early diagnosis of CRC.

### ctDNA

#### General considerations

The term ctDNA is conventionally used for identifying the portion of circulating free DNA (cfDNA) comprised only by nucleic acid fragments originating from tumor cells. ctDNA is thought to principally originate from apoptosis and necrosis of cancer cells in tumor microenvironment (36), though active release by living and circulating cancer cells has also been suggested. The assessment of cfDNA integrity index (DII), defined as the ratio of longer to shorter DNA fragments, is often exploited as surrogate marker for distinguishing cfDNA released by necrosis or apoptosis. The rationale behind this assumption is that DNA originated from necrotic cells varies in size, whilst DNA released from apoptotic cells is uniformly truncated into fragments shorter than 200 bp. Although DII is frequently increased in patients with cancer, including those with CRC, thus enlightening that necrosis may be the prevalent source of ctDNA, we and other groups have convincingly shown that predominance of one tumor cell mechanism over others may depend on specific cancer identity and also varies throughout cancer development (37).

Along with DII assessment, cfDNA can be used for detecting genetic and epigenetic CRC-specific abnormalities, whereby two of the most investigated ctDNA markers entail *KRAS* mutations and *SEPT9* promoter methylation. In a study comparing data of a cfDNA assay with CRC tumor-tissue analysis we could demonstrate that both *KRAS* mutation analysis and *SEPT9* methylation assay are characterized by a high degree of concordance in both blood and cancer tissue, thus supporting the hypothesis that cfDNA assessment may represent a valuable alternative to tissue analysis for both genetic and epigenetic tumor characterization (38). Interestingly, we also showed that transition of DNA harboring the epigenetic alteration into the circulation is more consistent than transition of DNA harboring a *KRAS* mutation in early CRC stages. Aberrant

methylation of *SEPT9* gene can thus be considered a sensitive and specific biomarker for early non-invasive diagnosis of CRC, which may theoretically be exploited for screening purposes. Unlike these findings, the overall sensitivity of *KRAS* ctDNA analysis for detecting CRC was found to be low, ranging between 3–63%, and this has been explained with evidence that mutations in *KRAS* may only be present in a minority of CRC patients (39). This preamble explains why *KRAS* status is mostly assessed for prognostic purposes, for monitoring tumor burden and for predicting chemo/immunotherapy response in CRC, but not for early diagnosing CRC. In particular *KRAS* status may predict response to established epidermal growth factor receptor (EGFR) inhibitors, whereby mutant *KRAS* is associated with resistance to anti-EGFR monoclonal antibody immunotherapy. An informative update on the role of *SEPT9* methylation and other methylated genes in early CRC diagnosis is summarized in the follow paragraphs of this article.

#### SEPT9 methylation

The *SEPT9* gene encodes a protein called septin-9, which is involved in a vast array of biological pathways such as cytoplasm division, cell polarization, vesicle transport and membrane reconstruction. This protein also acts as tumor suppressor, since it actively regulates cell growth and prevents uncontrolled cellular division. The methylation of *SEPT9* at specific CpG islands in the promoter region is accompanied by gene silencing, with consequent loss of cancer suppressor activity. Due to the proven role of *SEPT9* downregulation in pathological progression from benign to malignant lesions in colorectal tissue, the company Epigenomics AG (Frankfurt Prime Standard: ECX; Frankfurt, Germany) has commercialized the first blood-based real-time PCR *SEPT9* methylation assay nearly 10 years ago, which is available as commercial CE-IVD test throughout Europe with the name Epi proColon 1.0. (40). In 2011 the same company launched a second-generation test (Epi proColon 2.0), characterized by enhanced diagnostic performance and featuring some notable technical advancements (i.e., lower reagents, fewer handling steps and shorter turnaround time), which would be aimed at facilitating its use in routine diagnostics (41).

The *SEPT9* methylation assay is an essentially qualitative test, which can be interpreted as positive or negative according to application of different algorithms. Positive test results can hence be defined by one positive count out of three PCRs (1/3 algorithm), one positive count out

of two PCRs (1/2 algorithm), two positive counts out of three PCRs (2/3 algorithm), or one positive count out of one PCR (1/1 algorithm). To date, 25 independent trials, mostly case-control or cohort studies, have investigated the diagnostic performance of *SEPT9* gene methylation assay for CRC detection by using either research assays or Epi proColon kits (42). The sensitivity and specificity of the assay were found to be largely dependent on study design, sample size, study population and type of test, cumulatively ranging between 48.2–95.6% and 79.1–99.1%, respectively (42). Sixteen of these studies reported separated analyses for detection rate of *SEPT9* methylation assay in polyps, adenoma and early stage cancers, as shown in Table 1 (43–58). As predictable, the highest detection rate was achieved for both stage I and stage II, by applying the 1/3 algorithm. The overall diagnostic sensitivity of the 1/3 algorithm ranged between 37.1–84.0% in stage I CRC, and between 57.1–100% in stage II CRC. The overall diagnostic sensitivity of the 2/3 algorithm ranged between 17.1–66.7% in stage I CRC, and between 53.3–92.8% in stage II colorectal malignancies. The Prospective Evaluation of Septin 9 Performance for Colorectal Cancer Screening (PRESEPT) is the only screening study based on an average-risk population that has been performed so far. Song and coauthors compared data obtained in this study with those resulting from fecal immunochemical test (FIT) and FIT-DNA testing in the same population (42), demonstrating that the *SEPT9* methylation assay displays lower diagnostic sensitivity (68.0% versus 79.0% for FIT and 92.3% for FIT-DNA test, respectively) and lower diagnostic specificity (80.0% versus 94.0% for FIT and 86.6% for FIT-DNA test, respectively) (59,60). The diagnostic performance of blood *SEPT9* assay in asymptomatic population screening was hence seemingly lower than that of both FIT and FIT-DNA tests. Nevertheless, the *SEPT9* assay displayed a substantial better compliance than FIT testing.

Along with screening of average-risk population, the diagnostic performance of the *SEPT9* assay was also investigated as part of opportunistic screening in high-risk population. In a large population study, including four northern Chinese hospitals and using the SensiColon test, *SEPT9* assay displayed 76.6% sensitivity and 95.9% specificity for cancer identification, averaging a remarkable cancer detection rate as high as 25.8% in asymptomatic subjects (58). In another recent opportunistic screening study based on the Epi proColon 2.0 CE kit, *SEPT9* assay displayed 75.1% sensitivity and 95.1% specificity for

opportunistic CRC detection (55). Notably, the cancer detection rate was as high as 57% in CRC stage 0, 64% in CRC stage I and 88% in CRC stage II, thus potentially making it a valuable tool for population screening, alone or in combination with other more conventional diagnostic investigations.

### Other methylated genes

Beside *SEPT9* methylation, which has unquestionably been the largest abnormality that has been investigated so far for screening and/or early diagnosis of CRC, the clinical significance of other methylated epigenetic biomarkers has been recently addressed.

In 2014, Mitchell and collaborators used a pipeline combining gene expression, targeted DNA methylation and genome-wide DNA methylation analyses for identifying a panel of methylated genes in CRC patients (61). Based on low background in plasma of normal subjects (3.5% and 4.9% respectively), the two genes *BCAT1* and *IKZF1* were found to be suitable for assay development and optimization, and were finally selected as putative biomarkers (62). In a 218-person case/control study, combined analysis of these two genes exhibited a 77% cumulative diagnostic sensitivity for detecting CRC, associated with detection rate of 50% in stage I and 68% in stage II, respectively (63). The same combination of methylated genes was then assessed in a larger prospective clinical trial, including over 3,900 subjects (64). This study confirmed the highly positive detection rate in both stage I and II CRC (38% and 69%, respectively). In the same cohort of patients Symonds *et al.* showed that a test based on detecting methylated *BCAT1/IKZF1* DNA in blood (positivity threshold of 10 µg Hb/g) displayed comparable diagnostic sensitivity (62% versus 64%) and specificity (92% for both) with FIT for early detecting CRC (65). In these studies, the diagnostic sensitivity of biomarkers combination for detecting colorectal adenomas was comprised between 6–9% (64,65).

In 2015 Lin *et al.* used a high-resolution, genome-wide approach for demonstrating that the promoters of *AGBL4*, *FLII* and *TWIST1* genes are hypermethylated in over 80% of CRCs, and that these epigenetic modifications could be reliably detected in plasma DNA samples. The sensitivity of any of the three genetic abnormalities in cfDNA for detecting CRC (including also stages I or II cancers) was as high as 90% (66). In another retrospective study, Lee *et al.* analyzed plasma samples of 243 patients with stage I and II sporadic CRC, 276 healthy individuals and 64 patients with colorectal adenomas using methylation-

**Table 1** Sensitivity of *SEPT9* methylation assay in early stage colorectal cancer

Study	Year	Country	Type of study	Kit used	Algorithm	Polyps	Adenoma	Stage I	Stage II
Ahliquist et al. (43)	2012	USA	Case-control	Epiopro colon 1.0	1/3	-	13.6% (3/22)	57.1% (4/7)	57.1% (4/7)
Church et al. (44)	2014	USA, Germany	Prospective	Epiopro colon 1.0	1/2	-	8.8% (46/523)	36.4% (8/22)	57.1% (8/14)
Ding et al. (45)	2015	China	Case-control	Epiopro colon 2.0	2/3	-	-	50.0% (1/2)	53.3% (16/30)
deVos et al. (46)	2009	Germany	Case-control	Epiopro colon 1.0	1/3	-	-	52.6% (10/19)	75.0% (30/40)
					2/3			26.3% (5/19)	60.0% (24/40)
Grützmann et al. (47)	2008	Germany	Case-control	Research assay	2/3	11.8% (6/51)	-	50.0% (11/22)	69.4% (25/36)
He et al. (48)	2014	China	Case-control	Epiopro colon 2.0	2/3	-	-	35.7% (5/14)	81.0% (17/21)
Jin et al. (49)	2015	China	Case-control	Epiopro colon 2.0	2/3	20.7% (35/169)	27.4% (23/84)	66.7% (12/18)	82.6% (19/23)
Johnson et al. (50)	2014	USA	Prospective	Epiopro colon 1.0	1/3	-	-	61.5% (16/26)	80.0% (16/20)
Kang et al. (51)	2014	China	Case-control	Epiopro colon 2.0	2/3	-	-	48.0% (12/25)	82.6% (19/23)
Li et al. (52)	2015	China	Case-control	Epiopro colon 2.0	1/1	-	-	40.0% (2/5)	65.5% (19/29)
Lofton-Day et al. (53)	2008	USA	Case-control	Research assay	1/1	-	-	30.0% (6/20)	56.3% (18/32)
Ørntoft et al. (54)	2015	Denmark	Case-control	Epiopro colon 2.0	1/3	-	14.3% (3/21)	37.1% (13/35)	91.4% (32/35)
					2/3		0% (0/21)	17.1% (6/35)	74.3% (26/35)
Song et al. (55)	2016	China	Case-control	Epiopro colon 2.0	1/3	26.4% (23/87)	37.2% (42/113)	64.3% (27/42)	87.6% (92/105)
					2/3	9.2% (8/87)	26.5% (30/113)	54.8% (23/42)	82.9% (87/105)
Tóth et al. (56)	2012	Hungary	Case-control	Epiopro colon 2.0	1/3	-	-	84.0% (21/25)	100.0% (14/14)
					2/3			60.0% (15/25)	92.9% (13/14)
Warren et al. (57)	2011	USA, Russia	Case-control	Epiopro colon 1.0	1/3	-	-	71.4% (5/7)	90.3% (28/31)
Wu et al. (58)	2016	China	Case-control	Research assay	1/1	5.2% (6/116)	9.8% (21/214)	64.9% (24/37)	72.7% (48/66)

specific PCR (67). A panel of methylated genes, including *APC*, *MGMT*, *RASSF2A* and *Wif-1*, displayed 86.5% diagnostic sensitivity and 92.1% diagnostic specificity. Notably, sensitivity was still as high as 86.5% for detecting early stage CRC, and approximated 75% for detecting adenomas. The detection rate of adenoma reported in this study is indeed the highest overall, and could not be replicated in other independent studies. *SFRP2*, *ITGA4* and *GATA5* are three additional methylated genes, for which some preliminary data were published in patients with colorectal adenoma. In a small case-control study, Zhang *et al.* reported a detection rate for precancerous lesions approximating 40% for *SFRP2*, 43% for *GATA5* and 40% for *ITGA4*, respectively. The combination of *SFRP2* and *GATA5* displayed acceptable predictive efficiency for detecting colorectal adenomas (odds ratio, 3.35; 95% CI, 1.29–8.71) (68).

### Circulating miRNAs

MiRNAs, small-non-coding single-strand stable RNAs containing approximately 20–24 nucleotides, play an essential role in post-transcriptional regulation of eukaryotic genes, so that they have become the most studied non-coding RNAs (ncRNAs) (69). These small molecules are actively involved in a kaleidoscope of both physiological and pathological cellular processes, thus including malignant transformation. Both plasma and serum are suitable sample matrices for investigating miRNAs expression (70,71), though miRNAs can be theoretically assayed in all body fluids, since it is universally considered a relatively stable molecule (72). The leading techniques for studying miRNAs expression include quantitative reverse transcription-PCR (qRT-PCR), microarrays and next-generation sequencing (NGS) (73).

A number of meta-analyses have attempted to summarize the potential clinical usefulness of circulating miRNAs in early CRC detection (74–78), all converging to the conclusion that the diagnostic performance could be substantially magnified by simultaneously detecting more than a single miRNA.

In 2014, Wang and colleagues carried out a sub-analysis of 47 CRC studies (out of a total number of 107 studies in gastrointestinal malignancies), concluding that single-miRNA assays (29 studies) had lower performance for diagnosing CRC compared to multiple-miRNAs assays (18 studies) (AUC, 0.79 versus 0.89) (74). Sensitivity, specificity and AUC were as high as 81%, 84% and 0.89 in

the meta-analysis published by Zeng *et al.*, which included 24 studies from 19 different articles, totaling 1,558 CRC patients and 1,085 controls (75). In keeping with earlier findings, multiple miRNAs testing displayed higher diagnostic accuracy (AUC, 0.92; 84% sensitivity and 87% specificity) than assessment of a single miRNA (AUC, 0.84; 78% sensitivity and 78% specificity). In another meta-analysis including 103 studies and totaling 3,124 CRC patients and 2,579 healthy individuals, Yan *et al.* calculated an overall AUC of 0.86 for miRNA testing, reiterating the concept that multiple miRNAs testing may be more effective than analysis of single miRNA (AUC, 0.92 versus 0.81; sensitivity, 85% versus 72%; specificity, 86% versus 77%) (77). In 2017, Carter *et al.* performed another meta-analysis based on 34 studies with 3,454 CRC patients and 2,556 healthy controls (78). In their statistical analysis the authors could identify 17 miRNAs that were upregulated, along with 14 miRNAs which were instead downregulated in CRC patients. A four-miRNA signature, including miR-29a, miR-92a, miR-601 and miR-760, displayed a remarkably high diagnostic performance (AUC, 0.94) for identifying CRC patients, thus persuading the authors to conclude that this four-miRNA signature could even be tested as screening tool in CRC. Nevertheless, an important shortcoming that characterizes all these previous meta-analyses is that the diagnostic efficiency of miRNAs has not been specifically assessed in patients with early CRC stages, whilst patient data have been pooled and analyzed altogether.

As regards other studies, Kanaan *et al.* demonstrated that an eighth miRNAs panel (miR-532-3p, miR-331, miR-195, miR-17, miR-142-3p, miR-15b, miR-532, and miR-652) was capable to efficiently distinguishing patients with precancerous lesions from healthy controls, displaying an AUC of 0.87 (79). In the study of Zheng *et al.*, including 307 patients with CRC, 164 with colorectal adenoma and 226 healthy controls, a miRNA panel composed of miR-19a-3p, miR-223-3p, miR-92a-3p and miR-422a exhibited good diagnostic accuracy for distinguishing colorectal adenoma from CRC (AUC, 0.89) (80). Fang *et al.* investigated the expression levels of miR-24, miR-320a and miR-423-5p in plasma samples of 130 healthy controls and in those of 223 patients with colorectal diseases (111 with CRC, 59 with adenoma, 24 with colorectal polyps and 29 with inflammatory bowel disease). The sensitivity of miR-24, miR-320a and miR-423-5p for detecting early stage CRC was 77.8%, 90.7%, and 88.9%, respectively. The combination of the three miRNAs displayed a cumulative

AUC of 0.94 (81). Vychytilova-Faltejskova *et al.* also used a four-miRNAs panel (miR-142-5p, miR-23a-3-p, miR-27a-3p, miR-376c-3p) for distinguishing patients with CRC (n=203) from healthy subjects (n=100), and could demonstrate that this approach efficiently identifies all stages (AUC, 0.92), as well as early stages (I and II) CRC (AUC, 0.88) (82).

Among the miRNAs that have been individually assessed, miR-21 is perhaps the most studied, since is strongly up-regulated in CRC patients and has hence been proposed as both diagnostic and prognostic biomarker (83). In particular, Kanaan *et al.* reported that plasma miR-21 assessment was an effective strategy for distinguishing CRC patients from healthy controls with 90% sensitivity, 90% specificity and 0.91 AUC (84). In the meta-analysis of Zhang and colleagues (85), which included 14 studies and totaled 1,598 patients, the pooled sensitivity, specificity and AUC of circulating miR-21 for diagnosing CRC were found to be as high as 76%, 81% and 0.81. In a subsequent meta-analysis, based on 9 studies and totaling 746 CRC patients and 476 healthy controls, the pooled sensitivity, specificity and AUC of miR-21 for detecting CRC were 72%, 85% and 0.87, respectively (86). The subsequent meta-analysis of Peng *et al.*, including 16 studies and involving 1,270 CRC patients and 944 controls (87), revealed that the overall sensitivity, specificity, and AUC of miR-21 for predicting CRC cancer were 64%, 85% and 0.85, respectively. Unfortunately, no specific subset analysis was conducted for assessing the diagnostic performance of miR-21 in the early stage CRC in any of these meta-analyses.

With the purpose of investigating whether miR-21 could be a useful biomarker for distinguishing benign disease from early stages CRCs, our group has earlier studied miR-21 expression in 76 CRC patients and in 20 patients with benign polyps (88). Notably, no significant differences in plasma levels of miR-21 could be observed between early stages CRC patients (stage I and II, n=33) and patients bearing benign polyps (P=0.85). In line with these findings, Kumarswamy *et al.* showed that miR-21 expression may be enhanced in a vast array of non-neoplastic diseases (89), but it can also be up-regulated in patients with inflammatory diseases (90), and its reliable detection can be impaired by sample hemolysis (91) or by contamination with platelet miRNA (92), all factors contributing to lowering its predicated diagnostic performance.

MiR-92a and miR-29a are other interesting molecules, which were found potentially useful for early CRC

detection. More specifically, Huang *et al.* (93) showed that these two miRNAs can efficiently discriminate patients with advanced adenomas from healthy controls with an AUC of 0.77 for miR-29a and 0.75 for miR-92a, respectively. The combination of these two miRNAs yielded even better diagnostic performance, displaying an AUC of 0.88 (83% sensitivity and 85% specificity) for detecting CRC, and an AUC of 0.773 (73% sensitivity and 80% specificity) for identifying patients with advanced adenomas. Interesting evidence has then been recently published by Zanutto *et al.* (94), who studied plasma miRNAs expression in 60 subjects positive to FIT, and then extended their analysis to an internal validation cohort (n=201) and to a large external validation cohort (n=1,121 cases). Overall, a relevant number of miRNAs were found to be significantly deregulated in the first phase of the study (two miRNAs for CRC and six miRNAs for adenomas). In multivariate analysis including sex and age, the AUCs of these signatures were found to be 0.64, 0.67 and 0.68 for low-grade adenoma, high-grade adenoma and CRC, respectively.

In conclusion, although the interesting data emerged from several studies have highlighted the potential clinical usefulness of measuring circulating miRNAs for improving diagnosis and care of CRC patients (94,95), some aspects remain largely undefined, such as diagnostic efficiency and standardization of analytical techniques, lack of a reference standard, increased expression in non-malignant diseases, impact of preanalytical variables, as well as the real possibility of translating this innovative diagnostic approach from the bench to the bedside (96).

## Conclusions

Accumulating evidence suggests that the so-called “liquid biopsy”, envisioned as detection and quantification of CTCs and other cancer-related nucleic acids in peripheral blood, may allow earlier diagnosis of CRC combined with lower invasiveness and less patient inconvenience, higher throughput, faster turnaround time, inferior usage of healthcare resources and relatively low cost. Encouraging data have been reported in trials based on CTCs detection, though the sensitivity of the current diagnostic techniques is still perhaps insufficient for enabling early diagnosis of CRC. Among the various biomarkers that can be detected by means of liquid biopsy, *SEPT9* methylation displays good diagnostic performance and relatively high cancer detection rate (between 57–64% in patients with CRC stages 0–I), which would make this test a promising tool to be tested

in population screening, alone or in combination with more conventional diagnostic investigations. Promising evidence has also been recently published for *BCAT1/IKZF1* methylation. Regarding miRNAs, the combination of some of these biomarkers rather than the assessment of a single miRNA alone seems more effective for efficiently identifying early CRCs, though widespread clinical application is still challenged by a number of preanalytical, analytical and clinical issues.

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## Footnote

*Conflicts of Interest:* The authors have no conflicts of interest to declare.

*Ethical Statement:* The authors are accountable for all aspects of the work in ensuring that questions related to the accuracy or integrity of any part of the work are appropriately investigated and resolved.

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