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REVIEW

Biomarkers for detecting colorectal cancer non-invasively: DNA, RNA or proteins?

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Abstract

Colorectal cancer (CRC) is a global problem affecting millions of people worldwide. This disease is unique because of its slow progress that makes it preventable and often curable. CRC symptoms usually emerge only at advanced stages of the disease, consequently its early detection can be achieved only through active population screening, which markedly reduces mortality due to this cancer. CRC screening tests that employ non-invasively detectable biomarkers are currently being actively developed and, in most cases, samples of either stool or blood are used. However, alternative biological substances that can be collected non-invasively (colorectal mucus, urine, saliva, exhaled air) have now emerged as new sources of diagnostic biomarkers. The main categories of currently explored CRC biomarkers are: (1) Proteins (comprising widely used haemoglobin); (2) DNA (including mutations and methylation markers); (3) RNA (in particular microRNAs); (4) Low molecular weight metabolites (comprising volatile organic compounds) detectable by metabolomic techniques; and (5) Shifts in gut microbiome composition. Numerous tests for early CRC detection employing such non-invasive biomarkers have been proposed and clinically studied. While some of these studies generated promising early results, very few of the proposed tests have been transformed into clinically validated diagnostic/screening techniques. Such DNA-based tests as Food and Drug Administration-approved multitarget stool test (marketed as Cologuard®) or blood test for methylated septin 9 (marketed as Epi proColon® 2.0 CE) show good diagnostic performance but remain too expensive and technically complex to become effective CRC screening tools. It can be concluded that, despite its deficiencies, the protein (haemoglobin) detection-based faecal immunochemical test (FIT) today presents the most cost-effective option for non-invasive CRC screening. The combination of non-invasive FIT and confirmatory invasive colonoscopy is the current strategy of choice for CRC screening. However, continuing intense research in the area promises the emergence of new superior non-invasive CRC screening tests that will allow the development of improved disease prevention strategies.

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Core tip: Numerous biomarkers detectable in non-invasively collected samples of stool, colorectal mucus, blood, urine, saliva and exhaled air have been investigated to develop new tests for colorectal cancer (CRC) early detection and screening. Promising results are often reported, but it is difficult to achieve the right balance between technical complexity, cost and diagnostic performance of the new tests. Today the combination of non-invasive faecal immunochemical test and confirmatory invasive colonoscopy remains the CRC screening strategy of choice. However, on-going intense research promises the emergence of new superior non-invasive screening tests that will allow the development of improved prevention strategies for these malignancies.

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INTRODUCTION

Colorectal cancer (CRC) is currently the third most frequently diagnosed cancer worldwide. The global incidence for 2018 is estimated at 1801000 new cases, and the number of CRC-related deaths for this period is 861700[1]. Although the highest CRC incidence continues to be observed in economically developed Western countries, it is now rapidly increasing in other parts of the world[2]. Sporadic CRC development can take decades and is in most cases characterised by a slow progression from aberrant crypt formation in the colonic mucosa to benign polyps that may give rise to early cancer, then gradually evolving to invasive and metastasising advanced neoplasms (Figure 1)[2-4]. These pathogenetic features make CRC one of the most preventable and often curable malignancies. However, disease curability entirely depends on its early detection, which is not straightforward as clinical symptoms usually emerge only when CRC is already advanced. The latter factor warrants the necessity of active population screening for CRC, and it has been well proven that screening saves lives[2].

Full colonoscopy is regarded as the gold standard diagnostic technique for colorectal tumour detection[5], and it has become a very popular method for primary CRC screening[6-8] in the United States. One apparent reason for this trend is that diagnostic colonoscopy is usually combined with the simultaneous removal of detected polyps and functions as both a diagnostic and preventive procedure clearly reducing mortality from CRC[9]. Nonetheless, colonoscopy is an expensive and invasive technique that requires unpleasant bowel preparation and occasionally causes serious complications[10]. Moreover, its sensitivity is not perfect, with polyps sometimes missed[11], the latter problem often depending on the operator's skills[12]. Although colonoscopy as the final (confirmatory) diagnostic step is undisputable, its use in primary CRC screening remains questionable as indiscriminate application of this method inevitably results in frequent negative outcomes and a large health economic burden[13]. In theory, the global introduction of non-invasive tests employing biomarker analysis to select patients that really require endoscopy could dramatically reduce the numbers of unnecessary colonoscopies. Unfortunately, none of the existing non-invasive tests successfully combine high diagnostic sensitivity and specificity with technical simplicity and low cost, the key characteristics of an ideal screening modality. This paper provides a brief overview of the current state of the area encompassing biomarker-based non-invasive tests for CRC detection.

SOURCES OF MATERIAL FOR NON-INVASIVE CRC BIOMARKER TESTING

CRC development is an extraordinarily complex process driven by multiple genetic,

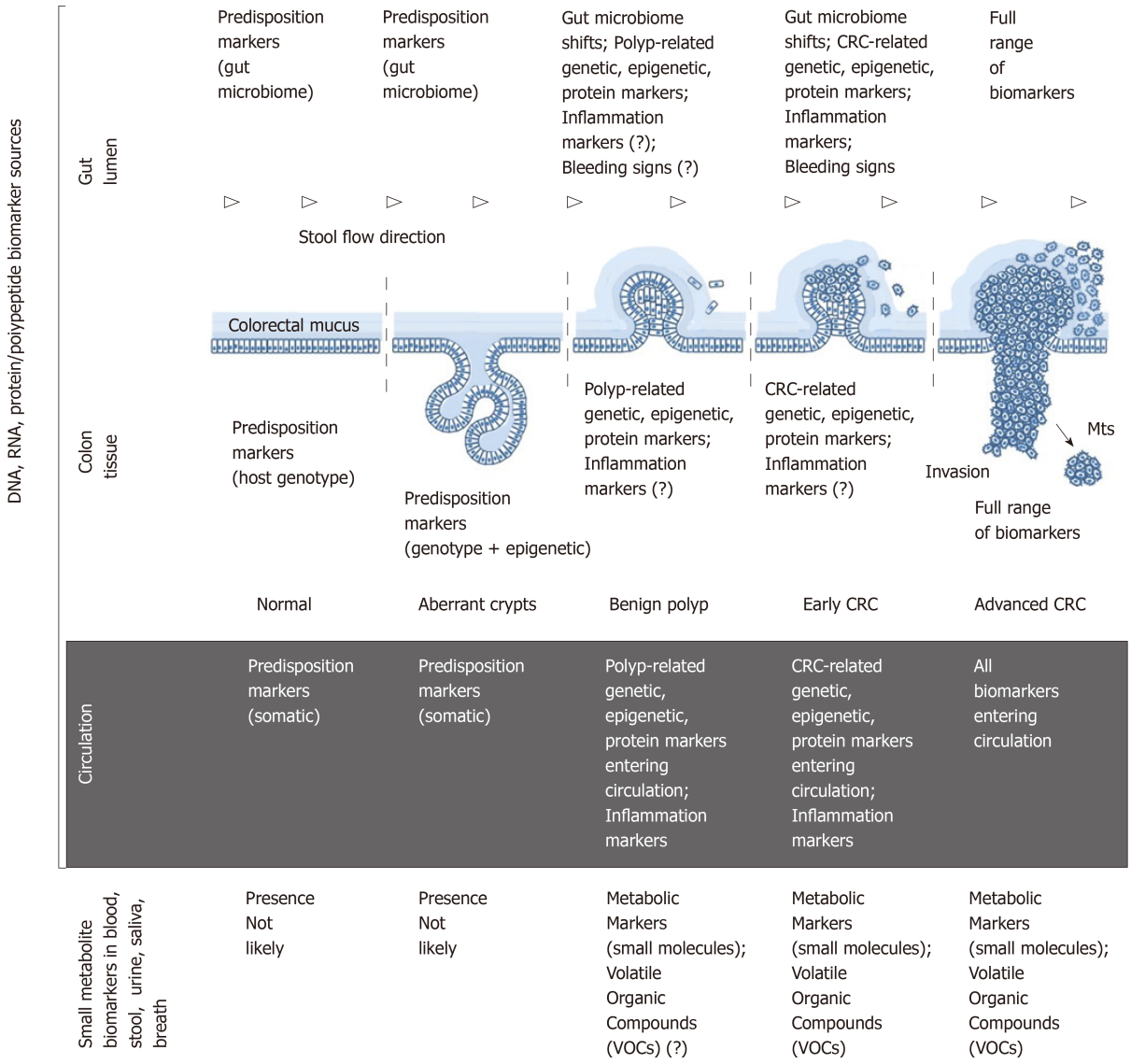

Figure 1 Colorectal cancer pathogenesis and sources of potential diagnostic biomarkers at different stages of colorectal cancer development. CRC: Colorectal cancer.

epigenetic, metabolic and immune alterations at the host level and influenced by numerous environmental factors[4,14,15]. Despite intense research, precise mechanisms of CRC development remain largely obscure[4,14,15]. Genome-targeting investigations, especially genome-wide association studies, have revealed a highly complex pathogenetic landscape comprising multiple alternative cascades of molecular events that may eventually result in cancer[4,16]. This complexity leads some investigators to a hardly satisfactory conclusion that "each patient's CRC is genetically and epigenetically unique"[4]. Nevertheless, colorectal tumours frequently have common molecular patterns that are diagnostically relevant and will be considered below.

The series of morphological events accompanying CRC development is presented in Figure 1. This sequence involves numerous associations with various types of biomolecules that can be characterised as biomarkers. The ideal biomarkers for CRC can be defined as substances that satisfy the following criteria: "(1) Are measured easily and inexpensively to identify a patient's cancer; (2) Identify a patient's prognosis to improve treatment outcome; and (3) Predict a patient's response to a specific treatment"[15]. This paper is focused only on the first category, *i.e.*, diagnostic biomarkers of CRC that can be sampled and tested non-invasively.

Figure 1 outlines the main sources of CRC biomarkers in relation to disease stages. From the morphological point of view, it is obvious that (1) colon tissue; (2) gut lumen; (3) blood/lymph circulation are the main sources of CRC-associated DNA, RNA and protein/polypeptide biomarkers associated with the host; (4) moreover, specific pattern shifts in small metabolite molecules derived from CRC-affected metabolic pathways constitute an additional group of post-metabolic markers that can

be analysed by metabolomics techniques[17,18]; and (5) CRC-associated gut microbiome changes[19] deserve to be considered as a separate category of diagnostic markers of non-human origin.

Normal and neoplastic colon tissue

Colonic epithelium is the site of neoplastic growth initiation. After that CRC progresses within the colonic wall until advanced stages of the disease, hence pre-malignant and malignant colon tissues are certainly the richest biomarker sources[4]. However, invasive biopsies are required for sampling tissue. Therefore, CRC markers detectable in tissue samples are not discussed here.

Gut lumen

Colonic epithelium is the key element of the gastrointestinal barrier between host tissues and microbiota-rich colon contents. Until recently it was presumed that all host cells exfoliated or migrated from the surface of the colonic epithelium were immediately incorporated in the faecal matter. According to this simplistic notion, it seemed to be logical that analysing naturally excreted stool samples constitutes the only perfectly non-invasive approach to investigating CRC biomarkers. It should, however, be stressed that stool is a complex mixture of microbiota-dominated faecal matter and occasional fragments of colorectal mucus secreted by goblet cells of the colonic epithelium. While the prevailing faecal component of stool entirely belongs to the environment, colorectal mucus is host-derived. The two-layered structure and functional significance of the mucus overlaying colonic epithelium have been elucidated only during the last decade[20,21], and it is now clear that colorectal mucus rather than faecal matter is the main receptacle of all cells and biomolecules released from either normal or malignant epithelium[22,23]. Intrarectal collection of colorectal mucus had demonstrated high informativeness of this substance[22,23], which was shown to accept CRC-generated malignant colonocytes exfoliated from tumour surface and transport them distally alongside stool flow without incorporating them into faeces (Figure 1)[20,23]. Biomarker-rich colorectal mucus essentially serves as a border between well oxygenated colonic epithelium and anaerobic gut lumen. Our group has recently developed a simple technique for non-invasive sampling of this mucus[24-26], the analysis of which may constitute a very convenient alternative to stool-based tests.

Blood/lymph circulation

Blood-derived biomarker analysis is another area of significant interest in the context of CRC detection since blood collection is regarded as a practically non-invasive procedure. It is evident that a wide range of CRC-associated biomarkers can be detected in the circulating blood and lymph of patients with these malignancies, but lymph collection cannot be performed with minimal invasiveness. For this reason, only biomarkers measurable in blood will be discussed below. In the modern literature the term "liquid biopsy" is often applied to this group of biomarker-based techniques[27]. Nevertheless, despite the easiness of blood sampling and the availability of numerous analytical techniques for biomarker detection in human plasma or serum, the presence of cancer biomarkers in blood may or may not be associated with CRC. Malignancies of other sites should always be excluded if this approach is considered for CRC screening.

Post-metabolic biomarkers

The use of metabolomics for revealing CRC-specific changes in patterns of low molecular weight metabolites has recently become another area of active exploration[28]. This new approach can potentially employ a wider range of biological samples comprising blood, stool, colorectal mucus, urine, saliva and exhaled breath, thus bringing about additional diagnostic options.

Gut microbiome changes associated with CRC

Recent research has revealed that specific changes in gut microbiome composition may be associated with the development of CRC[19]. In this context stool samples are usually investigated quantitatively for the presence of particular types of bacteria.

The limited choice of sample sources for non-invasive testing creates obvious problems. Collecting gut-derived samples looks preferable, but stool samples, albeit containing cells and molecules originating from the colonic mucosa (*i.e.*, colorectal mucus fragments), are usually dominated by the presence of abundant microbiota-rich faecal matter that often interferes with analytical procedures employed for host-related biomarker detection. A recently described analysis of non-invasively collected colorectal mucus presents a very interesting alternative; however, this approach is new and requires further testing. On the other hand, blood collection is very

straightforward and easy to standardise, but molecular changes detectable in blood (or plasma/serum) samples are not necessarily gut-specific. Finally, although the use of easily collectable materials (urine, saliva or exhaled air) is extremely attractive, the presence of CRC-specific biomarkers in such samples remains to be adequately explored. The sources of biological material characterised above may contain several types of diagnostic biomarkers that are discussed in the next section.

BIOMARKERS ASSOCIATED WITH CRC DEVELOPMENT

The story of non-invasively detectable CRC markers started due to a 1967 publication by Greegor, describing his observation of the frequent presence of occult blood in stool samples collected from patients with CRC[29]. That important discovery resulted in the development and prolonged use of the haemoglobin-recognising faecal occult blood test (FOBT) as the only non-invasive test for CRC detection. The situation had changed considerably in 1992, when a publication by Sidransky *et al*[30] described K-ras gene mutation detection in stool samples obtained from CRC patients and shifted the focus of attention to molecular markers. The area of CRC biomarker research has since exponentially expanded with thousands of papers published, but many initially promising findings failed to transform into clinically relevant diagnostic approaches. The purpose of this paper is to briefly outline the present status of non-invasive biomarkers proposed for detecting asymptomatic CRC. Only the most impressive and clinically relevant observations related to the main groups of these biomarkers (proteins/polypeptides, DNA, RNA, small metabolites, microbiome changes) are highlighted in the text below. However, numerous other markers that demonstrated promise in the context of CRC detection are presented in comprehensive Tables 1, 2, 3, 4 and 5. As it was impossible to cover all relevant studies, restrictions had to be applied when the Tables were prepared. Publications describing very small studies or reporting negative results were omitted. Likewise, only papers related to CRC, but not adenoma detection, were included since in most cases diagnostic sensitivity of biomarker tests for adenomatous polyps correlates with that for CRC. In addition, the necessity of non-invasive detection of colorectal polyps is still a debatable question, as the proportion of adenomas likely to progress to malignancy is relatively small, whereas the vast majority of these lesions (especially small polyps) never give rise to CRC[134,135].

Protein markers

Protein biomarkers considered in CRC early detection and screening are listed in Table 1. Historically, the use of haemoglobin detection in stool for non-invasive CRC detection can be regarded as the most popular approach in terms of population screening. Indeed, the traditional guaiac FOBT was almost exclusively employed for this purpose for several decades, and was attractive due to its simplicity and low cost. Although this test has insufficient sensitivity, it can be credited for saving many human lives[2,136,137]. Nevertheless, the outdated FOBT is now being replaced by the faecal immunochemical test (FIT) characterised by a much higher sensitivity. In a recent comprehensive review on FIT, Gies *et al*[31] discussed numerous studies of varying sizes and reported sensitivities between 66% and 74% and specificity levels between 84% and 95% when numbers of analysed CRC cases and controls were over 50. Table 1 also shows that M2-pyruvate kinase (M2-PK) is a relatively well-studied stool marker of CRC[32,33]; however, FIT performs better and remains considerably more popular. Other stool tests, including metalloproteinase 9 (MMP9)[34] and multimarker protein panels (see Table 1) have been investigated, but these tests have not been clinically accepted so far. It is also intriguing that in a recent small study, our group compared 24 protein biomarkers in non-invasively collected samples of colorectal mucus and concluded that haemoglobin, tissue inhibitor of metalloproteinase 1, M2-PK, peptidyl arginine deiminase 1, C-reactive protein and MMP9 could reliably detect CRC[138].

Blood (or plasma/serum) testing for CRC-associated proteins has been employed by many research groups (Table 1), but most of those studies produced relatively modest results. Among single protein markers detectable in the serum only CA11-19 marker protein[36], cysteine-rich 61 protein of the CCN family (Cyr 61)[38], B6-integrin[39] and trefoil factor 3 (TFF3)[36] can be regarded as promising. A number of protein panels were also examined; however, analysing multiple proteins is usually more technically complex and expensive. Impressive test sensitivity and specificity values (98.7% and 94.8%, respectively) were reported for combined testing for lectins DC-SIGN and DC-SIGNR by Jiang *et al*[42] in 2014, but these results remain to be confirmed in larger studies. Although blood collection is simple and easy to standardise, protein

Table 1 Non-invasive protein (including cytokine) biomarkers used for colorectal cancer detection

Study setting	Sample type	Marker type	Biomarker(s)	Sensitivity (or its range)	Specificity (or its range)	Ref.
Screening (reviewed)	Stool	Protein	Haemoglobin (FIT)	66.0%-74.0%	84.0%-95.0%	[31]
Case-control (reviewed)	Stool	Protein	M2-PK	68.0%-93.0%	70.0%-97.5%	[32,33]
Case-control	Stool	Protein	MMP 9	89.30%	91.20%	[34]
Case-control	Stool	Protein panel	Complement C3, Lactotransferrin, Haemoglobin subunit α1 and Haptoglobin	71.00%	95.00%	[35]
Case-control	Serum	Protein	CA11-19	98.00%	84.00%	[36]
Case-control	Serum	Protein (cytokine)	MIC-1 (GDF15)	43.80%	96.70%	[36]
Case-control	Serum	Protein (cytokine)	IL-6	28.0%-89.5%	46.0%-94.0%	[37]
Case-control	Serum	Protein (cytokine)	IL-8	70.00%	91.00%	[36]
Case-control	Serum	Protein (cytokine)	Growth-related gene product β1	56.10%	95.30%	[36]
Case-control	Serum	Protein	Cyr61	83.00%	97.00%	[38]
Case-control	Serum	Protein	B6-integrin	69.80%	100.00%	[39]
Case-control (reviewed)	Serum	Protein	TIMP-1	52.0%-85.0%	60.0%-95.0%	[40]
Case-control	Serum	Protein	RBP4	74.90%	81.70%	[36]
Case-control	Serum	Protein	THBS2	64.90%	87.10%	[36]
Case-control	Serum	Protein	TFF3	74.20%	94.80%	[36]
Case-control	Serum	Protein	COL3A1	98.80%	69.10%	[36]
Case-control	Serum	Protein	COL10A1	63.00%	85.00%	[36]
Case-control	Serum	Protein	AZGP1	55.80%	85.00%	[36]
Case-control	Serum	Protein	Angiopoietin-2	79.30%	82.40%	[36]
Case-control	Serum	Protein	Kininogen	63.60%	65.90%	[36]
Case-control	Plasma	Protein	Melanotransferrin	48.20%	92.50%	[36]
Case-control	Serum	Protein panel	RBP4 and CEA	80.80%	91.20%	[36]
Case-control	Serum	Protein panel	TFF3 and CEA	89.40%	87.80%	[41]
Case-control	Serum	Protein panel	sDC-SIGN and sDC-SIGNR	98.70%	94.80%	[42]
Case-control	Serum	Protein panel	IGFBP-3 and CEA	75.00%	90.00%	[43]
Case-control	Serum	Protein panel	AZGP1, CEA and CA19-9	67.50%	82.50%	[36]
Case-control	Serum	Protein panel	IGFBP2, DKK3 and PKM2	73.00%	95.00%	[36]
Case-control	Plasma	Protein panel	BAG4, IL6ST, VWF, EGFR and CD44	73.00%	90.00%	[44]
Case-control, prospective	Serum	Protein panel	CEA, hs-CRP, CYFra21-1 and Ferritin	60.0%-70.0%	81.0%-89.0%	[45]

FIT: Faecal immunochemical test.

biomarkers of CRC present in stool or colorectal mucus currently look more diagnostically reliable than those detectable in blood.

An additional advantage of using protein biomarkers for CRC detection is defined by the fact that their immunochemical detection can be easily presented as point of care (POC) tests, which are already available for FIT[139].

DNA and mRNA markers

This sub-section briefly discusses studies on CRC detection using DNA and mRNA markers that are listed in Table 2.

Gene mutations, especially those of *K-Ras* and *APC* genes, were the first CRC-associated genetic markers assessed with the purpose of developing new non-invasive modalities for CRC early detection and screening. Regrettably, it soon became clear

Table 2 Non-invasive DNA, messenger RNA and long non-coding RNA biomarkers used for colorectal cancer detection

Study setting	Sample type	Marker type	Biomarker(s)	Sensitivity (or its range)	Specificity (or its range)	Ref.
Screening	Stool	DNA mutation panel	3 *K-ras* mutations, 10 *APC* mutations, 8 *p53* mutations, microsatellite instability marker BAT-26 and long DNA marker	51.60%	94.40%	[46]
Case-control	Stool	Panel including DNA mutation, DNA methylation, DNA amount and protein testing	*K-ras* mutation, methylation of *Vimentin* (*VIM*), *BMP3, NDRG4* and *TFPI2* genes, DNA measurement by *β-actin* assessment and HemoQuant test for haemoglobin	78.0%-85.0%	85.0%-90.0%	[47]
Screening	Stool	Panel including DNA mutation, DNA methylation, DNA amount and protein testing	*K-ras* mutation, *BMP3* and *NDRG4* promoter methylation, DNA measurement by *β-actin* assessment and test for haemoglobin (FIT)	92.30%	86.60%	[48]
Case-control	Stool	Methylated DNA	*BMP3* gene	51.0%-84.0%	90.0%-100.0%	[49]
Case-control	Stool	Methylated DNA	*CDKN2A* gene	20.0%-40.0%	84.0%-100.0%	[49]
Case-control	Stool	Methylated DNA	*ECAD* gene	65.20%	88.00%	[49]
Case-control	Stool	Methylated DNA	*FBN1* gene	72.00%	93.30%	[49]
Case-control	Stool	Methylated DNA	*GATA 4/5* gene promoter	42.9%-71.0%	84.0%-95.0%	[49,50]
Case-control	Stool	Methylated DNA	*HLTF* gene	20.0%-37.5%	90.0%-92.6%	[49]
Case-control	Stool	Methylated DNA	*HIC1* gene	42.30%	98.00%	[49]
Case-control	Stool	Methylated DNA	*HPP1* gene	71.20%	57.10%	[49]
Case-control	Stool	Methylated DNA	*ING1b* gene	73.70%	95.00%	[49]
Case-control	Stool	Methylated DNA	*ITGA4* gene	40.00%	96.80%	[49]
Case-control	Stool	Methylated DNA	*MGMT* gene	33.9-55.1%	52.0%-100.0%	[49]
Case-control	Stool	Methylated DNA	*NDRG4* gene promoter	53.0%-92.0%	89.1%-100.0%	[49-51]
Case-control	Stool	Methylated DNA	*P16INK4A* gene	71.70%	86.00%	[49]
Case-control	Stool	Methylated DNA	*PHACTR3* gene	55.0%-66.0%	95.0%-100.0%	[49]
Case-control	Stool	Methylated DNA	*RASSF2* gene	45.30%	94.70%	[49]
Case-control	Stool	Methylated DNA	*SDC2* gene	81.10%	93.30%	[52]
Case-control	Stool	Methylated DNA	*SEPT9* gene	20.0%-84.8%	80.0%-94.5%	[49]
Case-control	Stool	Methylated DNA	*SFRP1* gene	26.4%-89.0%	86.0%-95.5%	[49]
Case-control	Stool	Methylated DNA	*SFRP2* gene	32.1%-94.2%	54.0%-100.0%	[49,51]
Case-control	Stool	Methylated DNA	*SPG20* gene	80.2%-89.0%	99.0%-100.0%	[49,51]
Case-control	Stool	Methylated DNA	*SNCA* gene	83.90%	75.00%	[49]
Case-control	Stool	Methylated DNA	*TFPI2* gene	63.3%-92.0%	79.0%-100.0%	[49-51]
Case-control	Stool	Methylated DNA	*TP53* gene	56.30%	100.00%	[49]
Case-control	Stool	Methylated DNA	*Vimentin (VIM)* gene	32.6%-86.0%	82.0%-100.0%	[49-51]
Case-control	Stool	Methylated DNA	*WIF1* gene	19.3%-60.4%	96.7%-99.4%	[49]
Case-control	Stool	Methylated DNA	*XAF1* gene	55.90%	52.00%	[49]
Case-control	Stool	Methylated DNA panel	*BMP3* and *NDRG4* genes	98.00%	90.00%	[49]
Case-control	Stool	Methylated DNA panel	*MGMT* and *XAF1* genes	73.50%	52.00%	[49]
Case-control	Stool	Methylated DNA panel	*MGMT-B* and *SFRP2* genes	88.30%	91.20%	[49]
Case-control	Stool	Methylated DNA panel	*RASSF1A* and *SFRP2* genes	75.00%	89.40%	[51]

Case-control	Stool	Methylated DNA panel	*SNCA* and *FNB1* genes	84.30%	93.30%	[53]
Case-control	Stool	Methylated DNA panel	*Vimentin (VIM)* and *SFRP2* genes	92.50%	91.20%	[53]
Case-control	Stool	Methylated DNA panel	*AGTR1*, *WNT2* and *SLIT2* genes	74.0%-78.0%	88.0%-89.0%	[49,50]
Case-control	Stool	Methylated DNA panel	*ECAD*, *MGMT* and *P16INK4A* genes	72.00%	88.00%	[49]
Case-control	Stool	Methylated DNA panel	*ITGA4*, *SFRP2* and *P16INK4A* genes	70.00%	96.80%	[49]
Case-control	Stool	Methylated DNA panel	*MGMT*, *CDKN2A* and *hMTH1* genes	55.00%	63.00%	[49]
Case-control	Stool	Methylated DNA panel	*MGMT*, *MLH1* and *Vimentin (VIM)* genes	75.00%	86.50%	[49,51]
Case-control	Stool	Methylated DNA panel	*SFRP2*, *HPP1* and *MGMT* genes	93.70%	77.10%	[49]
Case-control	Stool	Methylated DNA panel	*WIF-1*, *ALX-4* and *Vimentin (VIM)* genes	25.00%	98.00%	[49]
Case-control	Stool	Methylated DNA panel	*Vimentin (VIM)*, *OMSR* and *TFPI2* genes	86.70%	87.60%	[49]
Case-control	Stool	Methylated DNA panel	*SFRP2*, *GATA4/5*, *NRDG4* and *Vimentin (VIM)* genes	96.40%	65.00%	[49]
Case-control	Stool	Human DNA content	Total human DNA content	66.00%	89.80%	[54]
Case-control	Bowel Lavage Fluid	Methylated DNA panel	*miR-124-3*, *LOC386758* and *SFRP1* genes	82.00%	79.00%	[55]
Case-control	Intrarectally collected colorectal mucus	Human DNA content	Total human DNA content	60.40%	94.80%	[56]
Case-control	Serum/plasma	Methylated DNA	*ALX4* gene	23.0%-90.7%	72.5%-100.0%	[57]
Case-control	Serum/plasma	Methylated DNA	*APC* gene	57.0%-86.5%	86.0%-92.1%	[57]
Case-control	Plasma	Methylated DNA	*CDH1 (E-cadherin)* gene	60.00%	84.00%	[55]
Case-control	Serum/plasma	Methylated DNA	*SDC2* gene	87.0%-90.7%	72.5%-95.2%	[36,57]
Case-control	Serum/plasma	Methylated DNA	*SEPT9* gene	47.1-95.6%	81.0%-96.7%	[36,57-62]
Case-control	Serum/plasma	Methylated DNA	*SFRP2* gene	54.0%-69.4%	40.0%-98.7%	[57,63]
Case-control	Plasma	Methylated DNA	*THBD (Thrombomodulin)* gene	70.70%	80.30%	[51]
Case-control	Serum/plasma	Methylated DNA	*TPEF* gene	65.0%-81.0%	69.0%-90.0%	[57]
Case-control	Serum/plasma	Methylated DNA	*VIM (Vimentin)* gene	59.0%-90.7%	72.5%-93.0%	[57]
Case-control	Plasma	Hypomethylated DNA	LINE-1 transposable DNA element	65.80%	90.00%	[36]
Case-control	Serum/plasma	Methylated DNA panel	*IKFZ* and *BCAT1* genes	62.1%-95.0%	92.0%-95.0%	[36,57]
Case-control	Serum	Methylated DNA panel	*SEPT9* and *SDC2* genes	86.50%	92.10%	[64]
Case-control	Serum/plasma	Methylated DNA panel	*APC*, *MGMT*, *RASSF2A* and *WIF-1* genes	86.50%	92.10%	[57]
Case-control	Plasma	Methylated DNA panel	*ALX4*, *BMP3*, *NPTX2*, *RARB*, *SDC2*, *SEPT9* and *VIM* genes	90.70%	72.50%	[63]
Case-control	Serum	ALU115 DNA content	Free ALU115 DNA content	69.20%	99.10%	[36]
Case-control	Serum	DNA integrity	ALU247/115 DNA integrity index	73.10%	97.30%	[36]
Case-control	Serum	Free DNA content	ALU-based cell-free DNA	64.50%	98.90%	[36]

Case-control	Whole blood	mRNA expression	*TSPAN8* gene	83.60%	58.20%	[36]
Case-control	Whole blood	mRNA expression	*LGALS* gene	82.10%	61.20%	[36]
Case-control	Whole blood	mRNA expression	*COL1A2* gene	73.10%	59.70%	[36]
Case-control	Whole blood	mRNA expression	*CEACAM6* gene	65.70%	61.20%	[36]
Case-control	Whole blood or serum	mRNA expression	*SALL4* gene	85.9%-96.1%	85.7%-95.0%	[65,66]
Case-control	Whole blood	mRNA expression panel	*TSPAN8* and *LGALS4* genes	92.50%	67.20%	[36]
Case-control (CRC and high-risk adenomas in the case group)	Whole blood	mRNA expression panel	*LGALS4, CEACAM6, TSPAN8* and *Col1A2* genes	75.00%	87.00%	[67]
Case-control	Whole blood	mRNA expression panel	*CEA, EpCAM, CK19, MUC1, EGFR* and *C-Met* genes	87.00%	85.00%	[68]
Case-control	Whole blood	Long non-coding RNA expression	NEAT1 variant 1	69.00%	79%	[36]
Case-control	Whole blood	Long non-coding RNA expression	NEAT1 variant 2	70.00%	96.00%	[36]
Case-control	Serum	Long non-coding RNA expression	BLACAT1	83.30%	76.70%	[69]
Case-control	Plasma	Long non-coding RNA expression panel	ATB and CCAT1	82.00%	75.00%	[70]
Case-control	Plasma	Long non-coding RNA expression panel	91H, PVT-1 and MEG3	82.80%	78.60%	[71]
Case-control	Serum	Long non-coding RNA expression panel	LOC285194, RP11-462C24.1 and Nbla12061	68.30%	86.90%	[72]

FIT: Faecal immunochemical test; CRC: Colorectal cancer.

that using gene mutations alone does not achieve satisfactory levels of diagnostic sensitivity. One demonstrative study evaluating this approach in a representative colonoscopy screening group concluded that the sensitivity of a panel comprising 21 DNA alterations (point mutations in *K-ras*, *APC* and *p53* genes, microsatellite instability marker BAT-26 deletions and long DNA assay) was only slightly above 50%[46].

The relatively disappointing diagnostic performance of mutation-based assays stimulated the search for CRC-related epigenetic changes, in particular aberrant hypermethylation of CpG islands usually located in gene promoter regions[140]. Gene-specific DNA methylation in stool was extensively investigated (Table 2), and several genes, including *BMP3*, *NDGR4*, *septin 9* (*SEPT9*), *SFRP2, SPG20, TFPI2* and *vimentin* (*VIM*) were shown to have diagnostic sensitivities between 50% and 92% at specificities between 80% and 100% for CRC detection (see recent reviews by Liu *et al*[49], Lam *et al*[50] and Rasmussen *et al*[51]). However, the reproducibility of these results was often problematic, and attempts to combine multiple methylated genes in panels were undertaken to increase assay reliability. It is remarkable that high CRC detection sensitivity and specificity values could be achieved by combining methylation testing for *BMP3* and *NDRG4*[49] or *VIM* and *SFRP2*[53] genes, but these results need to be corroborated. The Colosure™ test detecting methylated *VIM* in stool was the first methylation-based commercial test for CRC[141]. This diagnostic product was marketed in the USA but has recently been replaced by a more efficient multimarker Cologuard® test considered later in this sub-section.

Table 2 demonstrates that in the context of CRC diagnostics, DNA methylation markers detectable in blood attract at least as much attention as similar markers in stool. Although investigations of different groups often produce conflicting results, it is now apparent that *SEPT9* methylation detection is the best studied option amongst these blood tests[57]. This test has recently been commercialised and regulated for clinical application as Epi proColon® 2.0 CE[142], but its use appears to be limited to opportunistic CRC screening[57]. Moreover, DNA methylation analysis in biological samples is relatively laborious (especially for multimarker panels) and difficult to present in POC format. These factors limit diagnostic potential of this approach. In addition, Table 2 shows that samples of stool, blood, bowel lavage fluid and colorectal mucus were also tested for total and ALU-based DNA quantification, DNA integrity

assessment, examination of gene expression and long non-coding RNA expression. However, none of these assays could provide sufficiently high values for diagnostic sensitivity and specificity.

It is now becoming clear that tests involving DNA markers tend to perform better only when markers of different types are combined. Long-term research projects led by a United States company, Exact Sciences, allowed the design of a multitarget stool test that demonstrated high levels of sensitivity and specificity for CRC detection. An early version of this test that included *K-ras* mutation, methylation of *VIM, BMP3, NDRG4* and *TFPI2* genes, DNA measurement by *β-actin* assessment and the HemoQuant test for haemoglobin achieved diagnostic sensitivity between 78% and 85% at specificity between 85% and 90% in a case-control study[47]. It is remarkable that this test performed significantly better when directly compared with the test for methylated *SEPT9* in plasma (similar to Epi proColon)[143]. The multitarget test was then simplified, and its final version includes only determination of *K-ras* mutation, *BMP3* and *NDRG4* promoter methylation, DNA measurement by *β-actin* assessment and FIT. Screening application of this test in a large study produced CRC detection sensitivity of 92.3% at a specificity of 86.6%[48], which makes this assay the best among all available tests involving DNA markers. The test was approved by the United States Food and Drug Administration in 2014 and is now marketed as Cologuard®. However, this test, which can be regarded as an enhanced version of FIT, requires stool collection, remains technically complex, with a multistep analytical procedure required[144], and is very expensive at over $600.

MicroRNA markers

MicroRNAs (a sub-class of small non-coding RNA molecules) were discovered and characterised during the last decade of the XX century. Since that time, it was established that microRNAs are important regulators of gene expression intimately involved in the pathogenesis of many diseases including cancer[145]. As many of them are associated with the presence of colorectal tumours, it was suggested that microRNA determination in stool or blood samples may provide a new diagnostic modality for CRC early detection and screening[73]. MicroRNA variants investigated as potential CRC markers are listed in Table 3. Several published studies that used stool sample analysis highlight miR-21 as the best-studied marker of this type, but do not show outstanding sensitivity and specificity values[73]. MiR-451 and miR-223 detectable in stool produced high sensitivity and specificity values in a small study[75]; however, these markers looked less impressive in other studies, when combined with other microRNAs[73,76]. It is impossible to exclude that these discrepancies may be associated with either technical problems or different ethnic composition of the studied patient groups since clinical studies providing material for microRNA analyses were performed mostly in East Asia.

Table 3 also indicates that microRNA markers of CRC were intensely investigated in blood. Hitherto most of these studies produced modest or inconsistent results. Again, miR-21 was assessed by many groups, and conflicting results were published. Although very high diagnostic sensitivity (96.6%) and specificity (97.8%) values were reported by Ng *et al*[80] for miR-139-3p, which was shown to be downregulated in the serum of CRC patients, this finding remains to be confirmed. Combinations of microRNA markers detectable in plasma or serum were also tested as diagnostic panels. Among these panels (Table 3), combinations of downregulated miR-144-3p, miR-425-5p and miR-1260b[88] and upregulated miR-19a, miR-19b, miR-15b, miR-29a, miR-335 and miR-18a[90] demonstrated sensitivity and specificity levels exceeding 90%.

In addition, it should be noted that a recent small study has revealed that quantification of miR-21 in saliva samples resulted in CRC detection with 97% sensitivity and 91% specificity[93]. However, these highly intriguing results remain to be corroborated.

Although microRNAs constitute a group of promising CRC biomarkers, further research in this relatively new area is needed to establish clinically valid diagnostic techniques using these markers. The relative technical complexity of laboratory procedures used in microRNA analysis (RNA extraction, reverse transcription and qPCR analysis) and the necessity of careful assay optimisation and standardisation[146] should also be taken into account when the diagnostic potential of this interesting approach is considered.

Volatile organic compounds (VOC) and small metabolite biomarkers

Metabolomics is a new discipline that focuses on evaluating a wide variety of endogenous metabolites produced by the organism[17,18,28]. These metabolites can serve as late stage biomarkers of either normal physiological or pathophysiological events, and cancer metabolome is defined as the entire suite of low molecular weight (< 1500 Da) cancer-specific metabolites[17]. Interestingly, some of these metabolites are VOC-s

Table 3 Non-invasive microRNA biomarkers used for colorectal cancer detection

Study setting	Sample type	Marker type	Biomarker(s) and detection methods	Sensitivity (or its range)	Specificity (or its range)	Ref.
Case-control	Stool	MicroRNA	miR-18a, upregulated	61.00%	69.00%	[73]
Case-control	Stool	MicroRNA	miR-20a, upregulated	55.00%	82.00%	[73]
Case-control	Stool	MicroRNA	miR-21, upregulated	56.0%-86.0%	73.0%-81.1%	[73,74]
Case-control	Stool	MicroRNA	miR-92a, upregulated	72.00%	73.00%	[73]
Case-control	Stool	MicroRNA	miR-106a, upregulated	34.00%	97.00%	[73]
Case-control	Stool	MicroRNA	miR-135b, upregulated	78.00%	68.00%	[73]
Case-control	Stool	MicroRNA	miR-144*, upregulated	74.00%	87.00%	[73]
Case-control	Stool	MicroRNA	miR-221, upregulated	62.00%	74.00%	[73]
Case-control	Stool	MicroRNA	miR-223, upregulated	77.00%	96.00%	[75]
Case-control	Stool	MicroRNA	miR-451, upregulated	88.00%	100.00%	[75]
Case-control	Stool	MicroRNA panel	miR-223 and mir-92a, both upregulated	97.00%	75.00%	[73]
Case-control	Stool	MicroRNA panel	miR-17-93 cluster and miR-135b, all upregulated	74.00%	79.00%	[73]
Case-control	Stool	MicroRNA panel	miR-144-5p, miR-451a and miR-20b-5p, all upregulated	66.00%	95.00%	[76]
Case-control	Plasma	MicroRNA	miR-17-3p, upregulated	64.00%	70.00%	[73,77]
Case-control	Plasma	MicroRNA	miR-18a, upregulated	73.10%	79.10%	[77]
Case-control	Plasma	MicroRNA	miR-20a, upregulated	46.00%	73.40%	[73,77]
Case-control	Serum/plasma	MicroRNA	miR-21, upregulated	65.0%-91.4%	74.4%-95.0%	[73,77-79]
Case-control	Plasma	MicroRNA	miR-24, downregulated	78.40%	83.80%	[77]
Case-control	Plasma	MicroRNA	miR-29a, upregulated	69.00%	89.10%	[77]
Case-control	Serum/plasma	MicroRNA	miR-29b, downregulated	61.4%-77.0%	72.5%-75.0%	[77]
Case-control	Plasma	MicroRNA	miR-92, upregulated	89.00%	70.00%	[77]
Case-control	Serum/plasma	MicroRNA	miR-92a, upregulated	65.5%-84.0%	71.2%-82.5%	[73,77]
Case-control	Plasma	MicroRNA	miR-96, upregulated	65.40%	73.30%	[73,77]
Case-control	Plasma	MicroRNA	miR-106a, upregulated	74.00%	44.40%	[77]
Case-control	Serum	MicroRNA	miR-139-3p, downregulated	96.60%	97.80%	[80]
Case-control	Serum	MicroRNA	miR-139a-5p, upregulated	76.70%	88.00%	[81]
Case-control	Plasma	MicroRNA	miR-155, upregulated	58.20%	95.00%	[73]
Case-control	Plasma	MicroRNA	miR-182, upregulated	78.00%	91.00%	[82]
Case-control	Serum	MicroRNA	miR-194, downregulated	72.00%	80.00%	[77]
Case-control	Serum	MicroRNA	miR-196b, upregulated	63.00%	87.40%	[84]

Case-control	Plasma	MicroRNA	miR-200c, upregulated	64.10%	73.30%	[77]
Case-control	Serum	MicroRNA	miR-210, upregulated	74.6%-88.6%	73.5%-90.1%	[77,79]
Case-control	Plasma	MicroRNA	miR-221, upregulated	86.00%	41.00%	[73,77]
Case-control	Plasma	MicroRNA	miR-320a, downregulated	92.80%	73.10%	[77]
Case-control	Serum	MicroRNA	miR-338-5p, upregulated	76.30%	92.50%	[84]
Case-control	Serum	MicroRNA	miR-372, upregulated	81.90%	73.30%	[77]
Case-control	Serum	MicroRNA	miR-375, downregulated	76.90%	64.60%	[77]
Case-control	Plasma	MicroRNA	miR-423-5p, downregulated	91.90%	70.80%	[77]
Case-control	Plasma	MicroRNA	miR-506, upregulated	76.80%	60.70%	[85]
Case-control	Plasma	MicroRNA	miR-601, downregulated	69.20%	72.40%	[77]
Case-control	Plasma	MicroRNA	miR-760, downregulated	80.00%	72.40%	[77]
Case-control	Serum	MicroRNA	miR-1290, upregulated	70.10%	91.20%	[86]
Case-control	Plasma	MicroRNA	miR-4316, upregulated	76.80%	75.00%	[85]
Case-control	Plasma	MicroRNA panel	miR-19a and miR-19b, both upregulated	78.60%	77.40%	[77]
Case-control	Serum	MicroRNA panel	miR-21 and miR-92a, both upregulated	68.00%	91.20%	[73,77]
Case-control	Plasma	MicroRNA panel	miR-29a and miR-92a, both upregulated	83.00%	84.70%	[73,77]
Case-control	Plasma	MicroRNA panel	miR-200c and miR-18a, both upregulated	84.60%	75.60%	[36,77]
Case-control	Plasma	MicroRNA panel	miR-223 and miR-92a, both upregulated	76.00%	71.00%	[73]
Case-control	Plasma	MicroRNA panel	miR-320d, downregulated; miR-1290, upregulated	81.20%	90.70%	[87]
Case-control	Plasma	MicroRNA panel	miR-431 and miR-139-p3, both upregulated	91.00%	57.00%	[77]
Case-control	Plasma	MicroRNA panel	miR-601 and miR-760, both downregulated	83.30%	69.10%	[73,77]
Case-control	Plasma	MicroRNA panel	miR-19a, miR-19b and miR-15b, all upregulated	78.60%	79.20%	[77]
Case-control	Plasma	MicroRNA panel	miR-24, miR-320a and miR-423-5p, all downregulated	92.80%	70.80%	[36,77]
Case-control	Plasma	MicroRNA panel	miR-144-3p, miR-425-5p and miR-1260b, all downregulated	93.80%	91.30%	[88]
Case-control	Serum	MicroRNA panel	miR-145, downregulated; miR-106a and miR-17-3p, upregulated	78.50%	82.80%	[73,77]
Case-control	Plasma	MicroRNA panel	miR-409-3p, upregulated; miR-7 and miR-93, downregulated	82.00%	89.00%	[73,77]

Case-control	Plasma	MicroRNA panel	miR-18a, miR-21, miR-22 and miR-25, all upregulated	67.00%	90.00%	[89]
Case-control	Serum	MicroRNA panel	miR-23a-3p, miR-27a-3p, miR-142-5p and miR-376c-3p, all upregulated	89.00%	81%	[36]
Case-control	Plasma	MicroRNA panel	miR-29a, miR-92a, upregulated; miR-601, miR-760, downregulated	83.30%	93.10%	[77]
Case-control	Serum	MicroRNA panel	miR-21, miR-29, miR-92, miR-125, miR-223, all upregulated	84.70%	98.70%	[78]
Case-control	Plasma	MicroRNA panel	miR-19a, miR-19b, miR-15b, miR-29a, miR-335, miR-18a, all upregulated	91.00%	90.00%	[90]
Case-control	Plasma	MicroRNA panel	miR-21, let-7g, upregulated, mir-31, mir-92a, miR-181b, miR-203, downregulated	96.00%	81.00%	[73]
Case-control	Plasma	MicroRNA panel	miR-103a-3p, miR-127-3p, miR-151a-5p, miR-17-5p, miR-181a-3p, miR-18a-5p, miR-18b-5p, all upregulated	76.90%	86.70%	[91]
Case-control	Plasma	Exosomal MicroRNA panel	miR-27a, miR-130a, both upregulated	82.50%	75.00%	[92]
Case-control	Saliva	MicroRNA	miR-21, upregulated	97.00%	91.00%	[93]

that are present in the gas phase of various excreted biological materials and can potentially be used for detecting malignancies including CRC[99]. The outcomes of metabolomic studies on CRC detection are summarised in Table 4. Remarkably, very impressive results (with CRC detection sensitivity reaching 97% at 99% specificity) were achieved by Sonoda *et al*[97], when dog scent judgment was applied to faeces and exhaled breath samples for discriminating between CRC patients and controls. Unfortunately, it is not realistic to expect that this natural phenomenon could constitute a reliable diagnostic tool. Hence, advanced Electronic Nose technologies are being developed and tested for CRC detection (Table 4) alongside widely used combinations of gas chromatography (GC) and mass spectrometry (MS)[18,94,99]. The latter approach, albeit regarded as the technical gold standard, is complex, costly and unsuitable for population screening. This point is especially important because most of the numerous studies applying metabolomic approaches to detecting CRC-related metabolites (non-VOC-s) in biological substances use various versions of MS (Table 4). Although some of the studies listed in Table 4 produced sensitivity and specificity values above 90% for CRC detection[102,109,113,116,125], cost and complexity issues remain major obstacles to the introduction of these assays into routine clinical practice. In this context, the use of electronic noses sensing CRC-associated VOC-s appears to be more promising, especially in view of CRC detection sensitivity and specificity both reaching 95% in a recent study by Zonta *et al*[98].

Markers of CRC-associated changes in gut microbiome

The structure of the gastrointestinal tract engenders permanent interactions between its epithelial tissue and luminal microbiota, thus significant microbial impact in colorectal carcinogenesis appears to be likelier than in any other neoplasia. Steadily accumulating evidence indicates a pivotal role for the gut microbiome in influencing the development of CRC[19]. It is now believed that bacterial effects predisposing to CRC include impacts in gut surface barrier disruption, induction of colonic inflammation, direct genotoxic action against epithelial cells and dysbiosis leading to CRC-promoting shifts in gut microflora composition and the colonic microenvironment[19,147]. These advances prompted interest in evaluating gut microbiome shifts as possible diagnostic markers for CRC[148]. The results of several recent studies (presented in Table 5) show that alterations in gut microbiome composition can potentially serve as non-invasive diagnostic markers for this disease.

Table 4 Non-invasive volatile organic compounds and small metabolite biomarkers used for colorectal cancer detection

Study setting	Sample type	Marker type	Biomarker(s) and detection methods	Sensitivity (or its range)	Specificity (or its range)	Ref.
Case-control	Stool	VOCs	Hydrogen sulphide, Dimethylsulphide, Dimethyldisulphide, *mlz* 90 - detected by selected ion flow tube (SIFT) mass spectrometry (MS)	72.00%	78.00%	[94]
Case-control	Stool	VOCs	Propan-2-ol, 3-methylbutanoic acid - detected by gas chromatography (GC) and MS	87.90%	84.60%	[95]
Case-control	Stool	VOCs	Methyl mercaptan (increased) and hydrogen (decreased) – detected by GC	90.00%	57.70%	[96]
Case-control	Stool	VOCs	Pattern recognition technique - canine scent judgment	97.00%	99.00%	[97]
Case-control	Stool	VOCs	Pattern recognition technique (eNose Cyranose® 320)	85.00%	87.00%	[94]
Case-control	Stool	VOCs	Pattern recognition technique (SCENT A1)	95.00%	95.00%	[98]
Case-control	Urine	VOCs	Ion mobility spectroscopy technology (FAIMS)	88.00%	60.00%	[99]
Case-control	Urine	VOCs	Ion mobility spectroscopy technology (FAIMS)	63.00%	63.00%	[100]
Case-control	Urine	VOCs	Pattern recognition technique (eNose applied)	78.00%	79.00%	[99]
Case-control	Breath	VOCs	Pattern recognition technique - canine scent judgment	91.00%	99.00%	[97]
Case-control	Breath	VOCs	Acetone (increased), ethyl acetate (increased), ethanol (decreased) and 4-methyl octane (decreased) detected by GC-MS	85.00%	94.00%	[99]
Case-control	Breath	VOCs	Nonanal, decanal, 4-methyl-pentanone, 2-methylbutane, 4-methyloctane, 4-methylundecane, 2-methylpentane, methylcyclopentane, cycloxehane, methylcyclohexane, trimethyldecane-1,2-pentadiene, 1,3-dimethylbenzene, 1,4-dimethylbenzene – detected by GC-MS	86.00%	83.00%	[99]
Case-control	Stool	Magnetic resonance spectra	Magnetic resonance spectra patterns	85.20%	86.90%	[101]
Case-control	Stool	Small metabolites	Acetate – detected by proton magnetic resonance spectroscopy (PMRS)	94.70%	92.30%	[102]

Case-control	Stool	Small metabolites	Succinate – detected by PMRS	91.20%	93.50%	[102]
Case-control	Serum	Aromatic carboxylic acids	Benzoic acid – detected by CE-time of flight (TOF) MS	89.00%	82.00%	[103]
Case-control	Serum	Fatty acids	GTA-446 – detected by flow injection analysis MS	83.30%	84.80%	[104]
Case-control	Plasma	Amino acid metabolites	L-kynurenine – detected by high-performance liquid chromatography (HPLC)	85.20%	100.00%	[105]
Case-control	Plasma	Fatty acids	Decanoic acid – detected by CE-TOFMS	87.80%	80.00%	[106]
Case-control	Serum	Multiple metabolites	38 metabolites detected by GC-MS	85.00%	86.00%	[107]
Case-control	Serum	Phospholipids (sphingomyelins and phosphatidylcho-lines)	SM (34:1), PC (34:1), PC (34:2), PC (36:4), PC (36:2), PC (36:3) - detected by MS	♂77.3%; ♀80.8%	♂92.4%; ♀85.9%	[108]
Case-control	Serum	Unsaturated free fatty acids (panel)	C16:1, C18:3, C20:4, C22:6, all downregulated – detected by MS	93.80%	92.20%	[109]
Case-control	Serum	Amino acids (panel)	8 amino acids – detected by LC-MS/MS	65.00%	95.00%	[110]
Case-control	Serum	Amino acids, fatty acids, carbohydrates	13 metabolites – detected by LC-MS/MS	96.00%	80.00%	[111]
Case-control	Serum	Metabolite panel	2-hydroxy-butyrate, aspartic acid, kynurenine, cystamine – detected by GC-MS	83.10%	81.00%	[112]
Case-control	Serum	Lipid metabolites (panel)	Palmitic amide, oleamide, hexadecaneodioic acid, octadecanoic acid, eicosatrienoic acid, LPC(18:2), LPC(20:4), LPC(22:6), myristic acid, LPC(16:0) – detected by ion cyclotron resonance MS	98.10%	100.00%	[113]
Case-control	Serum	Panel of hydroxylated polyunsaturated ultra long-chain fatty acids	C28H46O4, C28H48O4 and C28H50O4, all downregulated – detected by LC-MS/MS and nuclear MR	75.00%	90.00%	[114]
Case-control	Serum	Multiple metabolites (panel)	11,14-eicosadienoic acid, 12a-hydroxy-3-oxocholadienic acid, 12-ketodeoxycholic acid, 12-keto-tetrahydro-leukotriene B4, 13-cis-retinoic acid, 1b-hydrocholic acid, 1-methylhistamine, 1-monopalmitin, 2,3-dihydroxybutanoic acid, 24-hydroxycalcitriol – detected by GC-TOFMS and UPLC-QTOFMS	83.70%	91.70%	[115]

Case-control	Plasma	Amino acids, fatty acids, carbohydrates	8 metabolites – detected by CT-TQMS	99.30%	93.80%	[116]
Case-control	Plasma	Choline-containing phospholipids (panel)	Total saturated lysophosphatidyl-cholines (LPCs), 18:2 LPC and sphingosylphosphorylcholine – detected by LC-MS/MS	88.30%	80.00%	[117]
Case-control	Plasma	Choline-containing phospholipids (panel)	Total saturated lysophosphatidyl-cholines (LPCs) and the difference between 18:2 LPC and 18:1 LPC – detected by LC-MS	82.00%	93.00%	[118]
Case-control	Dried blood	Amino acids and acylcarnitines (panel)	C16, Arg, C4/C8, C5/C3, Val, Phe/Tyr, Ala, C4/C3 – detected by direct infusion MS	81.20%	83.90%	[119]
Case-control	Urine	Polyamines	N1, N12-diacetylspermine – detected by ELISA	75.80%	96.00%	[120]
Case-control	Urine	Polyamines and amino acid metabolites	N1, N12-diacetylspermine and kynurenine – detected by LC-MS	80.00%	80.00%	[121]
Case-control	Urine	Amino acids and acetoacetate (panel)	Alanine, glutamine, aspartic acid and acetoacetate – detected by PMRS	87.50%	91.30%	[122]
Case-control	Urine	Nucleosides (panel)	5-hydroxymethyluracil and 8-oxo-7,8-dihydroguanine – detected by UPLC-MS/MS	78.60%	75.00%	[123]
Case-control	Urine	Nucleosides (panel)	Cytidine, 3-methylcitidine, 1-methyladenosine, 2-deoxyguanosine, adenosine, inosine – detected by HPLC-MS/MS	69.00%	98.00%	[124]
Case-control	Urine	Metabolite panel	Citrate, Hippurate, p-cresol, 2-aminobutyrate, myristate, putrescine and kynurenate - detected by UPLC-QTOFMS	97.50%	100%	[125]
Case-control	Urine	Nucleosides (panel)	Adenosine, N4-acetylcytidine, cytidine, guanosine, inosine, 1-methyladenosine, 1-methylguanosine, 1-methylinosine, 2-methylguanosine, 2,2-methylguanosine, N6-methyladenosine, uridine, 3-methyluridine+5-methyluridine, pseudouridine – detected by reverse phase HPLC	76.90%	90.40%	[126]

Case-control	Urine	Nucleosides (panel)	Adenosine, N4-acetylcytidine, cytidine, guanosine, inosine, 1-methyladenosine, 1-methylguanosine, 1-methylinosine, 2-methylguanosine, 2,2-methylguanosine, N6-methyladenosine, 5-methyluridine, pseudouridine, uridine – detected by column switching HPLC	71.00%	96.00%	[127]

One remarkable common feature of all the studies listed in Table 5 is the obligatory presence of *Fusobacterium nucleatum* (*F. nucleatum*) as one of the components of all tested panels. Indeed, *F. nucleatum*, an anaerobic oral commensal, is now identified as a pathogenetic factor contributing to multiple disorders comprising among others inflammatory bowel disease and CRC[19,148,149]. This interesting diagnostic approach is being actively investigated; however, further studies are necessary to firmly establish the value of the gut microbiome in non-invasive CRC detection.

NON-INVASIVE BIOMARKER TESTING USE IN CRC SCREENING TODAY AND FUTURE CHALLENGES

The existing plethora of potential non-invasive approaches to CRC detection briefly reviewed in this paper looks impressive in terms of numbers, but often disappointing in terms of outcome. Most of the published results clearly fail to transform into diagnostic or screening tests that would be highly sensitive and specific, simple to perform and not associated with excessive cost. As a matter of fact, the choice of available biomarker-based tests practically used for CRC screening remains strictly limited. Today FIT is by far the most popular option[2,9,31] owing to its relative simplicity and affordability. The recently introduced and widely advertised multitarget Cologuard® stool test or Epi proColon test targeting *SEPT9* methylation in plasma, albeit approved for clinical use, are technically complex and prohibitively expensive. Comparative studies addressing the health economics of CRC screening have demonstrated that the multitarget stool test, being more cost-effective that no screening, is significantly less cost-effective when compared to the FIT or invasive endoscopic testing[150-152]. Likewise, methylated SEPT9 detection in plasma samples[153] is clearly less cost-effective than the FIT. Considering a unit cost of $8 for the FIT (sampling kit and analysis only), Lansdorp-Vogelaar *et al*[154] concluded that a biomarker-based test that detects CRC with higher levels of sensitivity and specificity (up to 100%) should never be more expensive than $57 to be cost-effective. These estimates seem to indicate that in practical terms the FIT is currently the most cost-effective test for non-invasive CRC screening. Other authors argue that a highly specific non-invasive biomarker with an improved sensitivity for advanced adenomas (that progress to CRC) would probably be cost-effective at higher threshold costs[155], but the $600 price tag currently attached to Cologuard® is obviously excessive.

In any case, it is apparent that the FIT is not a perfect screening test. Its specificity reaching 95% is sufficiently high to be deemed satisfactory, but the sensitivity of this test remains relatively modest[31]. There is, however, an opinion that repeated FIT testing with one-year intervals may compensate for the lack of sensitivity[12]. Moreover, accurate identification of individuals with different levels of CRC risk could lead to creating objective approaches to risk stratification and personalised screening[12,155,156].

The effectiveness of a screening strategy is defined not only by screening test performance characteristics, but also by screening participant adherence[12]. One additional practical problem in CRC screening programmes employing faecal tests is insufficient screening uptake[157,158] that often results from participants' reluctance to collect stool samples[159,160]. The use of non-invasively collected colorectal mucus samples[24,138] in FIT-like tests can help solve this problem, but this new approach remains to be thoroughly evaluated, and this will require large comparative randomised trials that usually take several years to complete[155]. The existing

Table 5 Non-invasive faecal bacterial biomarkers used for colorectal cancer detection

Study setting	Sample type	Marker type	Biomarker(s)	Sensitivity (or its range)	Specificity (or its range)	Ref.
Case-control	Stool	Bacterial	*Fusobacterium nucleatum*	54.0%-92.8%	79.8%-91.0%	[128-131]
Case-control	Stool	Bacterial	*clbA*-positive bacteria	56.4%	81.5%	[131]
Case-control	Stool	Bacterial panel	*Fusobacterium nucleatum*, *Bacteroides clarus*, *Roseburia intestinalis* and *Clostridium hathewayi*	92.8%	79.8%	[130]
Case-control	Stool	Bacterial panel	*clbA*-positive bacteria and *Fusobacterium nucleatum*	84.6%	63.1%	[131]
Case-control	Stool	Bacterial panel	Ratio of *Fusobacterium nucleatum* to *Bifidobacterium*	84.6%	92.3%	[132]
Case-control	Stool	Bacterial panel	Combination of ratios of *Fusobacterium nucleatum* to *Bifidobacterium* and *Fusobacterium nucleatum* to *Faecalibacterium prausnitzii*	90.0%	90.2%	[132]
Case-control (CRC and adenomatous polyps in the case group)	Stool	Bacterial panel	*Fusobacterium nucleatum*, *Enterococcus faecalis*, *Streptococcus bovis*, *Enterotoxigenic Bacteroides fragilis*, and *Porphyromonas* spp	91.4%	93.5%	[133]

CRC: Colorectal cancer.

combination of the FIT and confirmatory colonoscopy is the strategy of choice today, and its further optimisation is currently regarded as the main factor in improving CRC screening effectiveness.

The present strong position of the FIT as the test of choice for non-invasive CRC screening will certainly be temporary as this test has one intrinsic deficiency that is impossible to eliminate. The FIT detects blood, which is shed but not produced by tumours, and bleeding may not occur in some CRC patients. For this reason, FIT sensitivity will never approach 100%, and it is likely that this target will become achievable only when a screening test employing CRC-specific biomarker(s) is developed. As no single biomarker detectable in all colorectal tumours has been identified so far, multitarget strategies combining either multiple markers of the same type or different assays (such as Cologuard®) emerge as CRC screening options advocated by some experts. However, these complex assays usually require sophisticated laboratory equipment and are laborious and expensive. Although future technological advances can help in eliminating these deficiencies, the search for more reliable and easily detectable single CRC biomarkers should continue.

It can be expected that rapid progress in cancer biomarker research accompanied by accelerated development of new non-invasive tests promises forthcoming breakthroughs in CRC screening and prevention of this disease.

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