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Review

Towards novel non-invasive colorectal cancer screening methods: A comprehensive review

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Simple Summary: Since the 70s, a variety of colorectal cancer (CRC) screening programs have been adopted throughout the world with the aim of reducing the mortality rate of one of the leading cancer-related causes of death in the world. However, currently employed techniques present numerous shortcomings which negatively affect early-stage CRC detection, thus urging us to consider new and improved alternatives. Among the cited shortcomings are invasiveness and cultural stigma surrounding certain sample collection techniques, both of which negatively affect screening compliance. For this reason, many of the viable alternatives collected and described in this review aim to achieve good diagnostic performance while minimizing patient stress and discomfort. This text should serve as a guiding light for healthcare providers specialized in preventive medicine in the continuous pursuit of improved patient care.

Abstract: Colorectal cancer (CRC) is one of the leading cancer-related causes of death in the world. Since the 70s, many countries have adopted different CRC screening programs which has resulted in a decrease in mortality. However, current screening test options still present downsides. The commercialized stool-based tests present high false-positive rates and low sensitivity, which negatively affects the detection of early stage carcinogenesis. The gold standard colonoscopy has low uptake due to its invasiveness and the perception of discomfort and embarrassment that the procedure may bring.

In this review, we collected and described the latest data about alternative CRC screening techniques that can overcome these disadvantages. Web of Science and PubMed were employed as search engines for studies reporting on CRC screening tests and future perspectives. The searches generated 555 articles, of which 93 titles were selected. Finally, a total of 50 studies, describing 14 different CRC alternative tests, were included. Among the investigated techniques the main feature that could have an impact on CRC screening perception and uptake was the ease of sample collection. Urine, exhaled breath and blood-based tests promise to achieve good diagnostic performance (sensitivity of 63-100%, 90-95%, 47-97%, respectively) while minimizing stress and discomfort for the patient.

Keywords: colorectal cancer screening; test; alternative; non-invasive; CRC; review;

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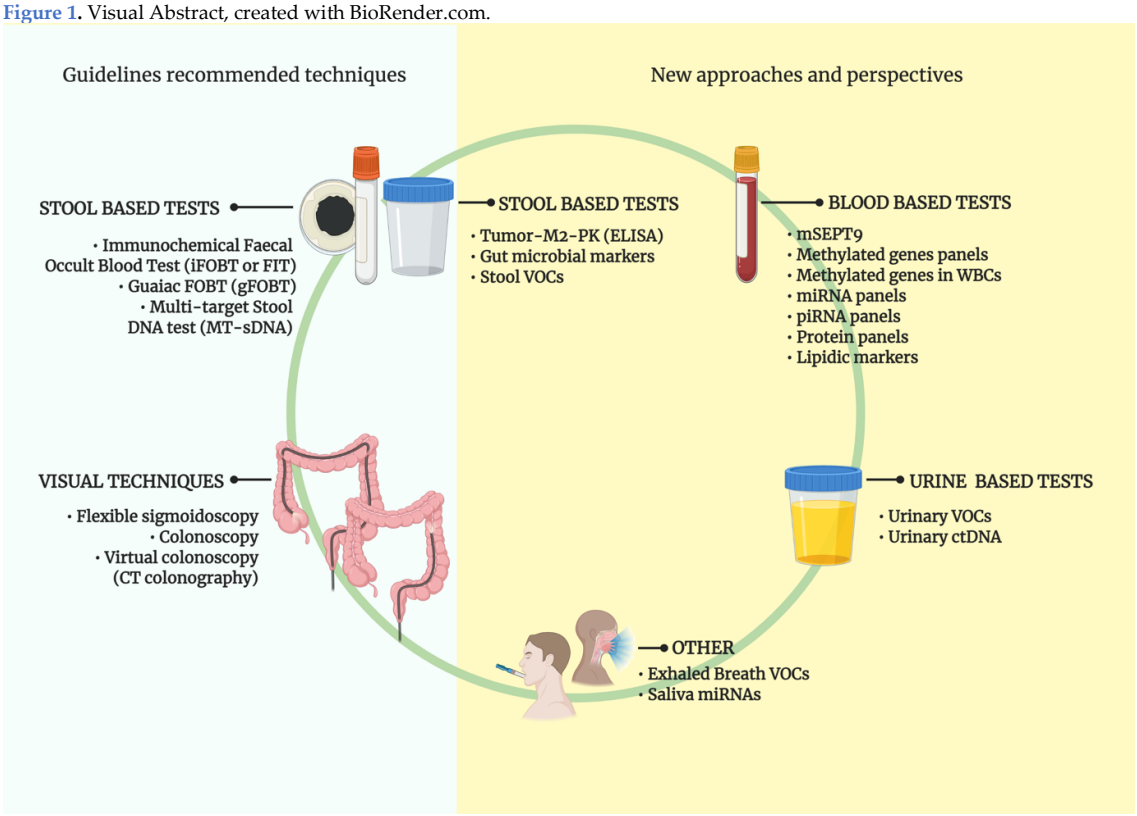
Figure 1. Visual Abstract, created with BioRender.com.



1. Introduction

From its introduction in the 70s, colorectal cancer (CRC) screening is developing and evolving at a dramatically fast pace, with many new studies revealing potential markers for early diagnosis of CRC. Current CRC screening options, suggested by international guidelines, can be classified as either stool-based or imaging tests. The stool-based tests' principle is that of detecting bleeding or shedding of neoplastic cells in patients' stool. On the other hand, the aim of imaging tests is to directly visualize colonic polyps and cancers [1].

For a population between 45 and 80 years of age, the European guidelines for quality assurance in colorectal cancer screening and diagnosis recommend Immunochemical FOBT (iFOBT or FIT), Guaiac Fecal Occult Blood Test (gFOBT), flexible sigmoidoscopy (FSIG) and colonoscopy as current gold standard tests for screening [2]. Within the considered population (45-80) at least the 60-64 should be included due to highest incidence and mortality. While these guidelines recognize newer screening technologies such as computed tomography (CT) colonography, stool DNA testing and capsule endoscopy as emerging possibilities, they do not recommend using them for screening the average-risk population. The American Cancer Society guidelines recommend the same tests, starting at the age of 45 until the age of 75. Nevertheless, they also indicate the Multi-target Stool DNA test (MT-sDNA) and CT colonography (virtual colonoscopy) as possible options for the average-risk population [3].

However, the above-mentioned tests may present certain down sides (high false-positive rates and low sensitivity for stool-based tests; invasiveness and the need for bowel

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preparation which negatively affects the compliance for colonoscopies). In fact, the aim of this review is to describe alternative techniques in the field of CRC screening which may facilitate sample collection (eg. blood, urine and breath-based tests) thereby positively affecting compliance or heighten sensitivity and lower false-positive rates. Because of these reasons, we believe it is important to give the reader an overview of the most promising studies on this topic.

In general, the principles of evidence-based medicine would require studies such as randomized controlled trials (RCTs) presenting mortality outcomes as the gold standard in order to demonstrate the efficacy of screening tests and preventive interventions. However, because RCTs are often not feasible, also observational studies, such as case-control designs, have been used to assess the effectiveness of colorectal cancer screenings [4] and are consequentially reported in this literature review.

2. Materials and Methods

As illustrated in **Figure 2**, in Web of Science and PubMed studies reporting on colorectal cancer screening tests and future perspectives were searched with following inclusion criteria: I. articles written in English, II. articles from the last 5 years III. articles should include original research describing colorectal cancer screening studies or tests proposed for future screening. An exception was made to point III. in order to include meta-analyses and systematic reviews since they present the advantage of comparing many different studies all together to come to highly reliable conclusions. This was especially useful in some cases in which the amount of data recognized as possibly useful was excessively large. Furthermore, certain articles that were added after the initial research (as reported at the end of this section) did not respect the time restriction in point II.

The tests included in the guidelines have been excluded from our literature search as they are already in use and well described. An exception to this point was made in order to illustrate the Food and Drug Administration (FDA)-approved Cologuard® MT-sDNA, seen as this test is still not widely utilised in common practice as other non-invasive tests of the same kind (eg. FIT) are, and it is not yet recommended by the official European guidelines as first line screening test.
New imaging technologies were also excluded from our literature review. Though they prove to be promising in the field of CRC detection, due to their low cost-effectiveness - as reported by Thayalasekaran *et al*. [5] - no significant evidence supporting their use in large population-based screening programs was found.

These searches generated 555 articles, of which 93 titles were selected. Of these, 39 abstracts initially fulfilled the inclusion criteria and were further analyzed, 12 were added as sources. As a result, a total of 51 articles were read, and of these 10 were excluded. The tests cited in each of the remaining articles were also searched individually resulting in the addition of 9 new papers. Finally, a total of 50 studies, describing 14 different CRC diagnostic tests – which are summed up in **Table 1** – were included.

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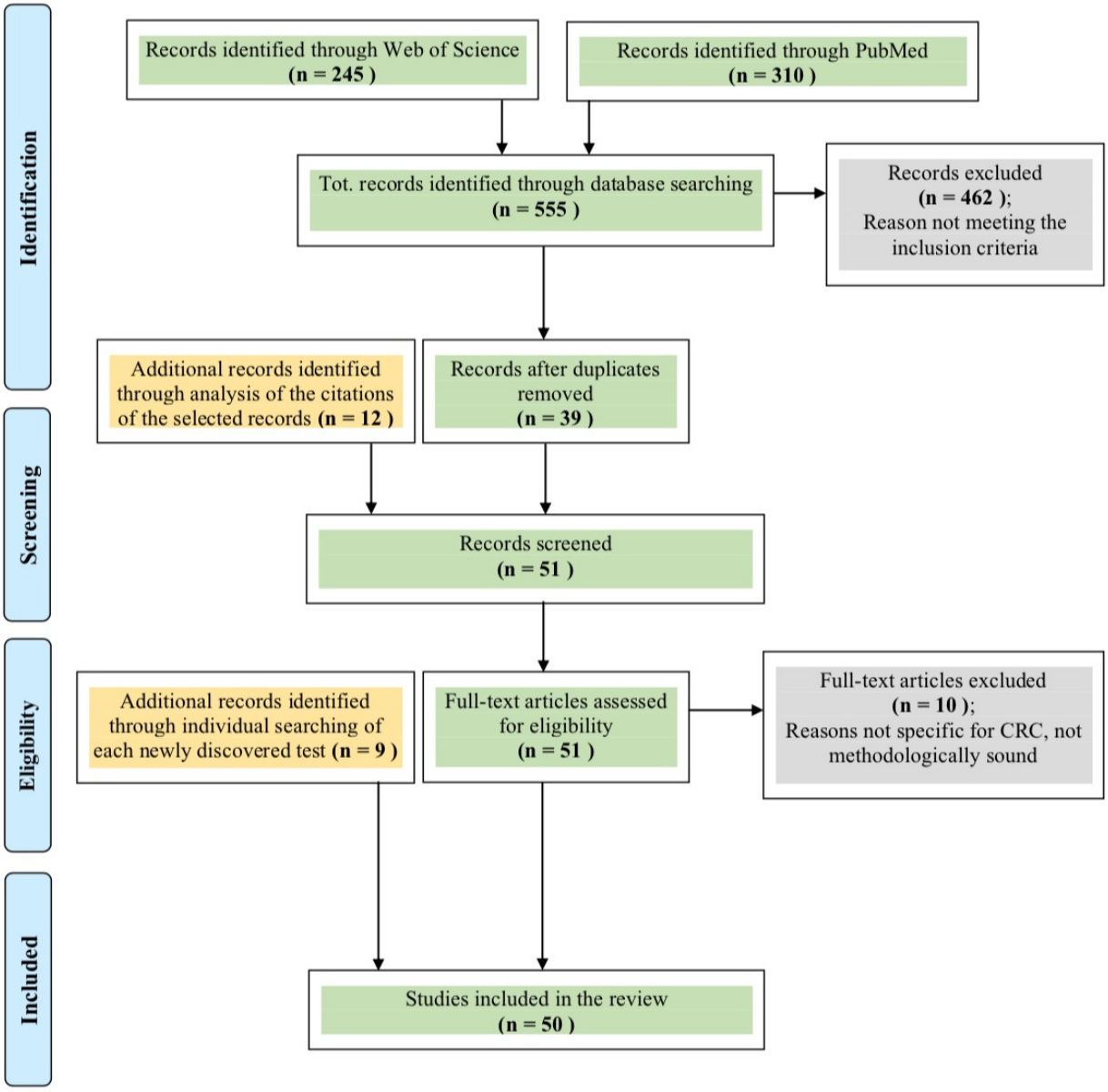
Figure 2. Methods



Adapted from: Moher D, Liberati A, Tetzlaff J, Altman DG, The PRISMA Group (2009). *P*referred *R*eporting *I*tems for *S*ystematic Reviews and *M*eta-*A*nalyses: The PRISMA Statement. PLoS Med 6(7): e1000097. doi:10.1371/journal.pmed1000097

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Table 1. Review summary.

		CRCs vs controls			AAs** vs controls	
Guidelines recommended techniques*		**Sensitivity**	**Specificity**		**Sensitivity**	
Colonoscopy		0.95	0.86		0.75-0.95	
gFOBT		0.70	0.92		0.07-0.24	
FIT		0.74	0.96		0.08-0.24	
MT-sDNA		0.92	0.87		0.17-0.42	
New approaches and perspectives	**Authors**	**AUC range**	**Sensitivity range**	**Specificity range**	**AUC range**	**Sensitivity range**	**Specificity range**
Blood based tests							
mSEPT9	Potter, 2014 Wu, 2016 Chen, 2017 Song, 2017 Xie, 2018	0.76-0.87	0.47-0.82	0.80-0.96	NA	0.09-0.59	NA
Methylated genes PANELS	Bartak, 2017 Rasmussen, 2017 Freitas, 2018 Young, 2019	0.86-0.97	0.74-0.97	0.72-0.97	0.94 (Bartak only)	0.89 (Bartak only)	0.86 (Bartak only)
Methylated genes in WBCs	Boonsongserm, 2019	0.72-0.98	0.30-0.90	0.96-0.98	NA	NA	NA
miRNA PANELS	Vychytilova-Faljejskova, 2016 Yan, 2016 Carter, 2017 (Wang, 2012) Sazanov, 2017 Guo, 2018 Herreros-Villanueva, 2019 Zanutto, 2020	0.68-0.96	0.65-0.89	0.26-0.93	0.91-0.95	0.94-0.95	0.85-0.90
piRNA PANELS	Yin, 2019 Mai, 2020 Wang, 2020	0.88-0.90	0.86-0.89	0.65-0.94	NA	NA	NA
Protein PANELS	Chen, 2017 Bardwaj, 2017 (Zhang, 2016) (Pengjun, 2013) Bardwaj, 2020 Loktionov, 2020 (Jiang, 2014)	0.75-0.99	0.56-0.99	0.80-0.99	0.60 (Chen only)	0.80-0.90 (Chen only)	0.22-0.32 (Chen only)
Lipidic markers	Zang Y., 2016 Zhang L. 2017	0.93 (Zang Y. only)	0.85 (Zang Y. only)	0.89 (Zang Y. only)	NA	NA	NA
Stool based tests							
Tumor-M2-PK	Zaccaro, 2017 Dabbous, 2018 Che Alhadi, 2020	0.71-0.92	0.63-1.00	0.40-1.00	NA	0.20 (Che Alhadi only)	0.54 (Che Alhadi only)
Gut microbial markers	Yu, 2017 Eklof, 2017 Amitay, 2017 Grobbee, 2020	0.72-0.84	0.56-0.69	0.77-0.81	NA	0.20-0.31 (Eklof only)	NA
Stool VOCs	Bond, 2016 Wang, 2017 Song, 2018 Ishibe, 2018 Bond, 2019 Zonta, 2017 Bosch, 2020	0.76-0.82	0.27-0.95	0.58-0.95	NA	0.17-0.33 (Bosch only)	0.88-0.95 (Bosch only)
Urine based tests							
Urinary VOCs	Arasaradnam, 2014 Widlak, 2018 Mozdiak, 2019 Deng, 2016 Kim, 2019	0.67-0.98	0.63-1.00	0.42-0.95	0.54-0.61 (Mozdiak only)	NA	NA
Urinary ctDNA	Xiao, 2015 Song, 2020	0.96 (Song only)	0.73-0.91	0.82-0.85	NA	NA	NA
Other:							
Exhaled Breath VOCs	Wang, 2014 Altomare, 2020 Van Keulen, 2019	0.84-0.98	0.90-0.95	0.64-0.93	NA	NA	NA
Saliva miRNAs	Sazanov, 2017	NA	0.97	0.91	NA	NA	NA

*Chyke Doubeni, Tests for screening for colorectal cancer. In: UpToDate, Post, TW (Ed), UpToDate, Waltham, MA, 2020. [6]

**AAs: advanced adenomas

Blood-based tests

DNA Methylation

Methylation involves the covalent addition of a methyl group to a protein, DNA, or another molecule. DNA methylation plays an important role in the development of cancer by changing the gene expression.

In particular, in cancer, DNA is generally hypomethylated and presents focal hypermethylation areas. These areas are often found in the promoter regions of tumor

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suppressor genes leading to their epigenetic silencing. Moreover, generalized hypomethylation in the gene bodies leads to genomic instability.

With regards to CRC, when comparing colorectal cancer cells with normal colorectal epithelial cells, it has been found that more than 10% of the protein-coding genes are differentially methylated [7-8].

ctDNA methylation - SEPT9 Methylation assays

One of the genes for which methylation has been linked to CRC development is SEPT9.

Various SEPT9 gene methylation assays have been developed based on the assumption that the risk of CRC development can be assessed by identifying the degree of DNA methylation of the promoter region of the *SEPT9* gene in peripheral blood. The DNA that is searched in this blood-based test is called circulating tumor DNA (ctDNA) because, as the name suggests, cancer cells release it into the peripheral blood from necrotic and apoptotic processes during carcinogenesis [9]. Moreover, it is also actively released from exosomes.

At present, there is only one *SEPT9* methylation assay kit already approved by the FDA as a valid alternative to CRC screening tests that have already been included in guidelines. This kit is to be used on adults, age 50 or above, at average risk for CRC.

This kit, called Epi proColon® 2.0, uses a real-time polymerase chain reaction (PCR) with a fluorescent hydrolysis probe for the detection of specific methylation in the *SEPT9* DNA target [10-11].
The study that led the FDA to approve this test was the PRESEPT study, that started in June 2008 and finished in April 2010 [12]. This study was performed in a screening setting of an average-risk population, ranging from 50 to 75 years old. The reported sensitivity was 48.2% (using a 1/2 algorithm). The sensitivity increased to 68.2%, with a reduction in specificity to 80%, in a later study by Potter et al. who performed triplicate PCRs (1/3 algorithm) using samples from the same study [13]. In 2016, the FDA approved the Epi proColon®, based on the data presented in the PRESEPT study with a 1/3 algorithm [9].

The necessary number of PCR tests to be performed in order to get a positive result is the basis on which the choice of which algorithm to employ is made.

The possible algorithms from which to choose from are the following: 1/3 algorithm, employed when one positive count out of three PCRs is needed; 1/2 algorithm, employed when one positive count out of two PCRs is needed; 2/3 algorithm, employed when two positive counts out of three PCRs are needed and 1/1 algorithm, employed when one positive count out of one PCR is needed [9].

Out of the studies that met our initial criteria, 4 of them - conducted in China and Taiwan between 2016 and 2018 - proved the efficacy of *SEPT9* Methylation assays tests. Some of these also employ kits different from the Epi proColon®. These are listed in **Table A1 (Appendix)** [14-17].

These studies demonstrated a variable sensitivity (ranging between 47% and 82%) and specificity (ranging between 81% and 95.9%). They also showed a good sensitivity range for early stages disease (stage I and II) around approximately 60% for stage I and 70% for stage II.
In general, these results appear to be comparable to the ones obtained by the PRESEPT study using Epi proColon® and confirm the feasibility of *SEPT9* Methylation assays test

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as useful options to screen CRC patients.

In **Table 2** the focus lays on the efficacy of *SEPT9* when combined with other commonly used tests such as FIT or carcinoembryonic antigen (CEA).

Table 2. Efficacy of *SEPT9* in combination with FIT or CEA for CRC detection.

SEPT 9 combined with other commonly used tests	Model(s)	Sensitivity	Specificity	Significant outcomes and possible limitations	Author/year
SEPT9 + FIT	Chi-square test or Fisher's exact test were used to analyze differences in sensitivity. ROC curve evaluated the diagnostic accurance. Comparison among the methods of FIT, mSEPT9, and the combination were evaluated by AUC. Two-side P-value <0.05 was considered statistically significant. [SAS statistical software, version 9.4]	84.1%	62.2%	• The combination of mSEPT9 with FOBT further improved the AUC value, reaching 0.807 (95%CI 0.752–0.863). The overall sensitivity was 86.0% for colon and 80.7% for rectum, 100.0% for stage I, 82.6% for stage II, 88.9% for stage III, and 50.0% for stage IV. It aslo reached 85.7% for patients with regional lymph node metastasis, 83.3% for patients with distant metastasis, and 82.4% for patients with vascular and neural infiltration. • However, the combination of the two tests caused a decline in specificity [62.2% (50.8–72.4%)].	Xie et al. 2018
SEPT9 + FIT	Data from sensitivity and specificity were used to plot the ROC curve. Because most cycle threshold (Ct) values from normal controls were not detected in the PCR reaction the Ct value was set to 45 (the maximal number of PCR cycles ran in the assay) for those not detected normal controls to plot the curve. The authors noted that this limitation led to the lack of specificity data points for Ct values >45. Therefore, no data were plotted above certain percentage for 1-specificity (the x axis) in the ROC curves.	94.4%	NA	The combination of SEPT9 with FIT exhibited high sensitivity (94.4%) and the combination of SEPT9, FIT, and CEA increased the sensitivity from 76.6% (SEPT9 alone) to 97.2%. Instead, the combination of FIT + CEA showed no significant difference with SEPT9 alone. The authors concluded that because the contribution of CEA was limited, SEPT9+FIT alone might be the optimal strategy in CRC opportunistic screening.	Wu et al. 2016
SEPT9 + CEA		86.4%	NA		
SEPT9 + CEA + FIT		97.2%	NA		

While the PRESEPT study investigated the efficacy of Epi proColon® in a population-based mass screening, the other mentioned authors studied *SEPT9* methylation assays in an opportunistic screening setting. This type of screening occurs when potential subjects willingly undergo examinations or tests because of illness or discomfort or, even if without symptoms or complaints, request to be screened early for CRC outside of a current screening program. This model presents the disadvantage of only taking into consideration patients who seek medical care, leaving others out of the program. Nevertheless, it still represents a crucial diagnostic and preventive tool.

All these recent studies were conducted in Asian populations, causing the risk of producing misleading results when referring to other ethnicities.
Song et al. addressed this issue comparing their findings observed on a Chinese population to the results obtained by Potter et al. [13] on Caucasian and African American populations in 2014.
The false positive rate of m*SEPT9* assay was similar among Chinese and Caucasian populations in every age group, whereas the African American group between 50 and 59 years of age exhibited a significantly higher false positive rate than the other two ethnicities.
Also, the older population, over 60 years of age, exhibited higher false positive rates than the younger population in Chinese and Caucasian populations. Furthermore, the overall detection rate showed a significant difference between the study conducted by Song, where it was 83.8%, and Potter's one on African American and Caucasian populations, where it was of the 68.2%.
However, even though a 1/3 algorithm was employed in both studies, the considered kits presented key differences: the PRESEPT study adopted the Epi proColon® assay while Song's study adopted the Epi proColon® 2.0 assay. For this reason, the author concluded that these observations alone are not enough to affirm whether there is a difference between Chinese and other ethnicities. Therefore, this topic needs further investigation.

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[15]

In conclusion, different studies demonstrated a variable sensitivity of the FOBT tests, ranging from 33% to 79% [18-19]. The sensitivity of Epi proColon® 2.0 and the commercialized m*SEPT9* assay, did not differ from the most widely used Fecal immunochemical tests (FIT), which has a sensitivity up to 79% at 94% specificity [18-20]. When taking into consideration the fact that FOBT tests have a low compliance in terms of screening uptake [21] and many factors can lead to a false-positive result such as inflammation, infections, ulcers, and hemorrhoids, the m*SEPT9* assay – which is not affected by those factors – could be considered superior, in terms of detection rate, to the fecal test. However, because of the higher cost-effectiveness, FIT remains the first choice among diagnostic tests for CRC screening.
It is also notable that different studies found that m*SEPT9* assay sensitivity was further enhanced when it was combined with CEA or FIT. Consequentially, a combined MS-9 DNA blood test and FIT/CEA may help to achieve a higher detection rate of CRC and may represent a valid option for screening.

DNA methylation in White Blood Cells (WBCs)

As mentioned before, DNA methylation can be found in circulating cells in peripheral blood and some evidences show that these cells can either be circulating tumor cells or white blood cells (WBCs) [22].
A 2019 study by Boonsongsermt *et al.* [23] in Bangkok investigated the changes in DNA methylation of peripheral blood mononuclear cells (PBMCs) among normal individuals and CRC cases using a methylation microarray.
The cohort included 32 CRC patients and 57 normal controls, both investigated directly by colonoscopy. *PLOD1* and *MMP9* were selected to assess the DNA methylation of the WBCs from CRC patients using real-time methylation-specific PCR. *MMP9* showed high diagnostic efficacy with 90.63% sensitivity, 96.49% specificity and high Positive Predictive Value (PPV) (93.33%), and Negative Predictive Value (NPV) (93.22%). On the other hand, the *PLOD1* methylation test showed high specificity (97.92%) but low sensitivity (30%). Moreover, neither of these methylation changes were found to correlate with tumor grade or stage. Although this study was limited by its small sample size, it still paves the way for future screening setting studies.

Panels of methylated genes

SEPT9 only represents one of the many genes associated with CRC to present methylation alterations. Different indeed, are the studies that take into consideration genes that could be used as diagnostic tools in the detection of CRC and different are the approaches that can be used to analyze CRC specific methylation among which we remember the methylation affinity-based isolation, the methylation-specific restriction enzyme digestion, and the chemical modification of cytosine with sodium bisulphite conversion [24-26].

Four studies, exploring the feasibility of panels of methylated genes as CRC screening tests, are summarized in **Table A2 (Appendix)** [26-29].

These studies demonstrated a varying sensitivity (ranging between 73.9% and 96.6%) and specificity (ranging between 72.5% and 97.3%) for different gene panels.
Notably, the panel studied by Bartak *et al.* - constituted by SFRP1, SFRP2, SDC2, and PRIMA1 gene promoters – showed 89.2% sensitivity and 86.5% specificity (AUC: 0.937, 95% CI: 0.885 to 0.989) for adenoma detection, which represents a crucial feature of any

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good CRC screening test.
Another significant result was achieved by Freitas *et al.*, using a panel including MGMT, RASSF1A and SEPT9, that was able to identify tumors at any disease stage with similar efficiency (sensitivity of 100%, 94.2%, 95.9% for stage I/II, stage III and stage IV, respectively) with PPV 91.5% (AUC= 0.97).

Because the disease prevalence in those screening-like cohorts is low, in general large sample sizes are essential and this poses a significant logistical and economic barrier to the correct assessment of a reliable model [24].
Notably in some of these studies, despite the fact that a very large screening population was recruited, the final sample was small, constituting a limitation to the statistical reliability of these results. Nonetheless, some of these panels revealed to have very high detection rates and may be worth further exploration in larger screening setting studies.

RNA

As mentioned before, methylation plays a variety of roles in cancer, changing the regulation of gene expression. The (epi)genetic alterations that drive the transformation from normal colon epithelium into adenocarcinoma can affect noncoding RNAs and mRNAs as well [30] and therefore, investigating them may represent a valuable option for the early diagnosis of CRC.

miRNA
MicroRNAs are small and non-protein-coding RNAs, molecules of 21-25 nucleotides long, that regulate gene expression and exhibit important regulatory functions related to cell differentiation, development, and growth. There is evidence that the levels of some miRNAs are altered in cancers such as CRC and that miRNAs regulate the cancer-promoting RAS gene [31]. This is why many authors and studies have focused on finding miRNAs useful in the early detection of CRC.
For example, a meta-analyses incorporating 103 studies with a total of 3124 CRC patients and 2579 healthy individuals, performed by Yan *et al.* [32] in 2016, found that miRNAs have a good performance in detecting CRC with 76.9% sensitivity (95%CI 0.733– 0.802), 80.6% specificity (95% CI 0.781 – 0.829) and AUC 0.857. With subgroup analysis and meta-regression, they also proved how multiple miRNAs seem to be more favorable than single miRNA (sensitivity 0.853 > 0.718, specificity 0.860 > 0.772, AUC: 0.918 > 0.813,). They also noticed how comparing samples of plasma, blood, tissue, and feces, miRNAs obtained from serum samples were more powerful for detecting CRC, particularly in Asians.
Another recent meta-analyses, published in 2017 by Carter *et al.* [33] based on a search result of 34 articles, found a total of 31 miRNAs to be either upregulated (n=17) or downregulated (n=14) in CRC cases as compared to controls. In this analysis 14 studies identified panels of dysregulated miRNAs and the highest AUC, 0.943, was identified using a panel of 4 miRNAs (miR-29a, -92a, -601, -760), with 83.3% sensitivity and 93.1% specificity (Wang *et al.*, 2012). They found that the overall sensitivity and specificity of 28 individual miRNAs in the diagnosis of CRC were both 76% (95% CI 0.72-0.80), indicating good discriminative ability of miRNAs as biomarkers for CRC.

In the vast amount of studies investigating miRNAs as CRC biomarkers we further selected five up-and-coming studies from the last 5 years, which are listed in **Table A3 (Appendix)** [34-38].

Noticeably, in the study conducted by Sazanov *et al.* [35], microRNA-21 was found to be expressed in saliva as well and showed higher diagnostic efficacy than miR-21 expression

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in the plasma, with a sensitivity of 97% and specificity of 91%, at confidence interval of reference values 0.65 to 2.49 (P > 0.95). Even with the small sample size these results are important, considering that a saliva-based test is a non-invasive and cheap procedure, perfectly suitable in a screening setting.

As suggested by several authors, miRNA definitively represent a potential specific biomarker for CRC detection. However, there are currently no relevant diagnostic products widely available for clinical use, meaning that additional research is warranted to implement these markers for clinical use in a screening setting.

piRNA

piRNAs or Piwi-Interacting RNAs represent a novel class of small non-coding RNA molecules expressed in animal cells. They form RNA-protein complexes with a subset of Argonaute proteins of the piwi-family and these complexes are mostly involved in the epigenetic and post-transcriptional silencing of transposable elements and other spurious or repeat-derived transcripts [39]. Recent studies have shown that the expression of piRNAs is frequently deregulated in different types of cancers including CRC [40]. Because of their small size, high stability, and ease of detection, piRNAs represent a strong diagnostic and predictive biomarker [41] with high potential as CRC screening tools.

The results of a subgroup of the initially selected works which take into consideration the use of piRNAs as CRC screening tests are listed below, **Table A4 (Appendix)** [42-44].

Protein Panels

The number of protein molecules indicated by the literature as possible CRC markers in blood is wide. However, only two are currently the main blood-based biomarkers available to detect CRC patients: carcinoembryonic antigen (CEA) and carbohydrate antigen 19-9 (CA19-9).

CEA is a high-molecular-weight glycoprotein and it is expressed in embryonic tissue as well as colorectal malignancies. CEA is particularly useful when used as a prognostic factor (poor prognostic factor for resectable CRC, cancer progression and recurrence after surgery) but when used as a tool for early detection in a screening setting its sensitivity is low because its levels are strictly related to the tumor stage. Moreover, CEA is not specific for CRC, but a higher level can be caused by liver disease, pancreatitis, Inflammatory Bowel Disease (IBD) and other malignancies. On the other hand, CA19-9 antigen is even less sensitive and specific for CRC, while it represents a highly reliable marker for the detection of pancreatic and biliary malignancies [45-46].

These observations point to the need for new molecules with higher reliability in the early detection of colorectal cancer. As previously mentioned, the number of markers recognized as possibly useful for this task is large, for this reason the following section will also include evidences collected through systematic reviews. These works present the advantage of comparing many different studies concomitantly to come to highly reliable conclusions.

Particularly, Bhardwaj *et al.* [47] published the PRISMA systematic review in 2017 based on 36 studies - which unfortunately were performed in a clinical and not in a screening setting - ranging from 23 to 512 cases of CRC and a number of proteins included in the signature ranging from 3 to 13. What they found is that, among the 21 studies that performed some form of validation, the best diagnostic performance was reported by Zhang *et al.* [48] for a panel including CA199, CA242, CA125, CA153 and CEA, which showed to have 94% sensitivity, 98% specificity and AUC 0.988. They also investigated

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studies that did not use any form of validation, and among these the best diagnostic performance was found for a combination of inflammatory markers (IL-8, MMP-2, TNF-a) found by Pengjun *et al.* [49], which presents 96% sensitivity, 99% specificity and AUC 0.996.

The same author performed a two-stage study in 2020 [50], aiming to measure 275 protein markers. They used the proximity extension assay (PEA) in plasma samples with a discovery set, which included 98 newly-diagnosed CRC patients and 100 age-and-gender-matched healthy controls screened with colonoscopy. Moreover, an independent external validation set was also used. This set included 56 CRC patients and 102 healthy controls, recruited from a pool of 945 participants of a true screening study. They found a 12 markers algorithm (including AREG, CEA, GZMB, ITGAV, KRTI9, MCP1, PON3, TR, MASP1, RARRES2, S100A4 and TRAP), particularly promising in the detection of early stage CRC, that in the validation set showed 61% sensitivity and 80% specificity, with an AUC= 0.75 (95% CI 0.67–0.84), comparable with the above mentioned Epi proColon 2.0.
Moreover, the potential of AREG as an excellent diagnostic tool was also studied by Chen *et al.* in the BLITZ study, which is illustrated in **Table 3** [51]. In this two-stage design study in China with a discovery set and a validation set including 7,197 participants, they studied a panel made up of 4 protein markers (GDF-15, AREG, FasL, Flt3L) + serum level of TP53 autoantibody. Their results showed for this panel sensitivities of 66.7% and 56.4% and specificities of respectively 80% and 90%, also comparable to the diagnostic performance of Epi proColon® 2.0. However, it presented limited diagnostic efficacy in detecting advanced adenomas, with an AUC of 0.60 (95% CI, 0.52–0.69).

Table 3. BLITZ study: findings on the blood based GDF-15, AREG, FasL, Flt3L and serum TP53 autoantibody panel as feasible option for CRC detection [50].

	Target population	Study design/setting	Exclusion criteria	N. of cases	N. of controls	Sensitivity	Specificity	Sensitivity for adenomas	Significant outcomes and possible limitations
GDF-15, AREG, FasL, Flt3L	A total of 7,197 participants have been recruited. Samples were collected from two study populations: the validation set consisted of prediagnostic samples from a large cohort of participants attending screening colonoscopy, therefore representing the target population for CRC screening. Both CRC and its precursors were included in the validation set.	China. Two-stage design with a discovery set and a validation set, defined in such a way that it represents a true screening setting (BLITZ study). An algorithm based on GDF-15, AREG, FasL, Flt3L and TP53 autoantibod was constructed. From November 2005 to September 2014.	Exclusion criteria: 1) missing plasma samples; 2) blood taken after screening colonoscopy; 3) inflammatory bowel disease or previous CRC; 4) insufficient bowel preparation (only for individuals with no significant findings at screening colonoscopy); 5) incomplete colonoscopy (only for individuals with no significant findings at screen- ing colonoscopy)	147	107	At 80% specificity: 63.4% (95%CI: 48.8%–82.9%). At 90% specificity: 53.6% (95%CI: 26.8%–70.7%)	cutoff value	At 80% specificity: 23.6% (95%CI: 15.1–38.7). At 90% specificity:18.9% (95%CI: 8.5–27.4)	In the validation set, the AUC of this five-marker algorithm were 0.82 (95% CI, 0.74–0.90) for detecting CRC, with a comparable diagnostic performance compared to the plasma mSEPT9, the only US FDA approved blood-based test for CRC screening. However, the panel presented limited diagnostic efficacy in detecting advanced adenomas, with an AUC of 0.60 (95% CI, 0.52–0.69). Also the diagnostic performance was still inferior to diagnostic performance of FIT and also a multi-target stool DNA test (CologuardTM). Limitations include the small sample size of CRC included in the validation set, despite the very large screening population recruited, reflecting the very low prevalence of CRC in a true screening population.
GDF-15, AREG, FasL, Flt3L + TP53 autoantibody						At 80% specificity: 66.7% (95%CI: 48.7%–82.1%). At 90% specificity: 56.4% (95% CI: 38.4%–71.8%)	cut off value	At 80% specificity: 31.7% (95%CI: 18.3–45.1). At 90% specificity: 22.0% (95%CI: 13.4–35.4)	

Finally, Loktionov *et al.* [52] completed another review in 2020 based on various works of research investigating CRC associated proteins. Even if most of the studies produced modest results, the authors pointed out some promising molecules to be used in a screening setting that are worth a mention: CA11-19, TFF3 (Nikolau *et al.*, 2018), Cyr61 (Song Y. *et al.*, 2017), and B6-integrin (Bengs *et al.* 2019). These molecules are to be intended as single protein markers also because, as the author noted, panels of proteins are more technically complex and expensive to realize. Despite this, the review still highlights the results obtained by Jiang *et al.* (2014) with a panel composed of lectins DC-SIGN and DC-SIGNR that showed a sensitivity of 98.7% and a specificity of 94.8%. Moreover, this panel has also been pointed out by the Nikolau *et al.*'s review (mentioned above) which, in 2018, compared 51 studies and found CA11-19, DC-SING and DC-SIGNR, and IL8 to be interesting diagnostic biomarkers.

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When looking at this data it is evident how further research conducted in larger screening setting studies could pave the way for new efficient tests available for the clinical routine. At this moment, the current evidence is still insufficient when comparing protein biomarkers with the cost-effective and already widespread FIT.

Combination of protein and genes panels

Omitted from our review because it is a non-CRC specific cancer screening test, but still described because of its relevance as feasible future cancer screening tool, is CancerSEEK. In 2018, Cohen *et al.* developed this blood test that detects multiple types of cancer, including CRC, by combining the detection of circulating free DNA (cfDNA) and protein biomarkers (including CA-125 and CEA) that are released by tumors.
This test works with an algorithm that weighs the protein and DNA data collected from the blood in order to detect patients who are likely to have a tumor.

Preliminary performance of the test was evaluated in a set of 1005 individuals with known cancers who were compared with 812 healthy controls.
Tumor type and location influenced the accuracy of the prediction: the highest accuracy was achieved for colorectal cancer. For lung cancer instead it was the lowest. In particular, specificity of the test was over 99% in 8 cancer types: ovarian, liver, stomach, pancreatic, esophageal, colorectal, breast, and lung. Sensitivity ranged from approximately 98% in ovarian and liver cancer and 33% in breast cancer, with a sensitivity of about 70% for the remaining cancers. Moreover, the tissue of origin was correctly identified in approximately 80% of patients.
Although the false-positive rate was low in the trial, it would be expected to be higher in the real-world setting when the test is applied to a healthy population without known diagnosis of cancer. In fact the authors weighted the results for the actual incidence in the United States and estimated a sensitivity of 55% among the eight cancer types.
In conclusion, the authors stated that multi-analytic tests, such as this one, are not meant to replace other non-blood-based screening tests - such as those for colorectal cancer – but are meant to provide additional information that could help identify and diagnose patients who are at higher risk of having a malignancy [53-54].

Lipidic markers

In recent years there has been a growing interest in lipids as potential biomarkers in numerous clinical conditions and lipidomic studies represent a new important tool in monitoring CRC patients. In clinical practice, lipid status is estimated based on serum concentrations of total cholesterol, HDL, LDL and triacylglycerols. However, other currently available techniques, for instance mass spectrometry, may provide a more detailed insight into the structure and function of these molecules as well as into the lipid profile of CRC patients [55].

Often, these lipid alternations in patients with CRC have been studied and investigated as prognostic factors or markers for late stage disease but modest are the results obtained when looking at these molecules as screening test tools. China has been the setting for a few promising studies focused on free fatty acids (FFAs) and their products as potential biomarkers to screen CRC patients: they took place in Beijing and Zhanjiang (Guangdong), respectively.
Zhang Y. [56] performed a two stage study based on a training set, which included 59 CRC patients and 69 healthy controls. Moreover, a validation set was also used. This set included 80 CRC patients, 55 Benign Colorectal Diseases (BCD) patients and 116 healthy controls in which the levels of Serum Unsaturated Free Fatty Acids were evaluated using Mann-Whitney U tests to compare patients and controls. The results showed excellent diagnostic performance for a pool of four Unsaturated FFAs (C16:1, C18:2, C20:4, C22:6)

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with 84.6% sensitivity, 89.4% specificity and AUC 0.926.
Along this direction, Zhang L. [57] investigated Poly-unsaturated fatty acid (PUFA) metabolites, inflammatory mediators that can affect progression and treatment of cancer. Ultra-high-performance liquid chromatography tandem mass spectrometry (UPLC-MS/MS) was used to assess their levels in a cohort of 25 CRC patients and 10 healthy controls. Of the 158 PUFA and metabolites studied, they found the following abnormal changes in CRC patients: of 2, 3- dinor-8-iso-PGF2α, 19-HETE and 12-keto-LTB4 from arachidonic acid and significant lower levels of 9-HODE and 13-HODE from linoleic acid. So far, the results obtained by studying these molecules are modest but this data could be taken into consideration for the set-up of larger screening setting studies that could lead to the implementation of lipidomic profile as an important tool for CRC detection.

Stool-based tests

Multitarget stool DNA (MT-sDNA) test

As mentioned before, the American Cancer Society guidelines already recommend the Multitarget Stool DNA test as a feasible option to screen the average-risk population, for this reason it was omitted from our literature search. Nonetheless, we decided to dedicate a section to the FDA-approved MT-sDNA test, called Cologuard®, seen as this test is still not widely utilised in common practice as other non-invasive tests of the same kind (eg. FIT) and it is not yet recommended by the official European guidelines as first line screening test.
Cologuard® is a molecular assay for aberrantly methylated BMP3 and NDRG4 promoter regions, mutant KRAS, and β-actin - which is used as a reference gene for DNA quantity – combined with an immunochemical assay for human hemoglobin.

The study that granted the FDA approval was a cross-sectional study conducted by Imperiale at al. in 90 different areas throughout the United States and Canada, from June 2011 through November 2012. It enrolled 9989 asymptomatic individuals between the ages of 50 and 84 years who were considered to be at average risk for colorectal cancer and who were scheduled to undergo screening colonoscopy. It compared the MT-sDNA test with FIT only and the results were generated with the use of a logistic-regression algorithm, with positive score threshold of 183 or more considered to be positive.
DNA testing showed 92.3% sensitivity for CRC and 42.4% for advanced adenomas (AAs). FIT showed 73.8% sensitivity for CRC ($P = 0.002$) and 23.8% for AAs ($P<0.001$).
Moreover, polyps with high-grade dysplasia and serrated sessile polyps measuring 1 cm or more were detected with a rate of respectively 69.2% and 42.4%. However, DNA testing showed lower specificity than FIT: 86.6% compared to FIT's 94.9% specificity among patients with nonadvanced or negative findings ($P<0.001$) and 89.8% compared to FIT's 96.4%, among those with a negative colonoscopy result ($P<0.001$).
These results show that the MT-sDNA test, in asymptomatic persons at average risk for colorectal cancer, detects significantly more cancers than FIT. Therefore, as the authors suggested, being a noninvasive test with a high single-application sensitivity for curable-stage cancer, Cologuard® may provide a suitable option for persons who prefer noninvasive testing, although with lower specificity [58].

Tumor M2-PK

Pyruvate kinase isoenzyme type M2 (M2-PK) is a pyruvate kinase isoform present in differentiated tissues, such as lung tissue, fat tissue, the retina, pancreatic islets and highly proliferating cells like fibroblasts, lymphocytes, embryonic cells, adult stem cells

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and colonocytes, and it is up-regulated in many types of tumor [59-60]. Usually pyruvate kinase isoenzymes in their active form in healthy tissues are tetramers. Tumor cells instead contain high levels of – almost inactive - dimeric M2-PK which for this reason has been named "Tumor M2-PK". Signal metabolites in tumor cells influence the ratio between M2-PK tetramer and dimer. This regulation is crucial because M2-PK plays a role in cell cycle progression and supports anabolism and tumor growth in several contexts [59,61]. Concerning colorectal cancer, researchers found that the HPV-16 E7 protein, which concurs with the k-RAS gene in cell transformation, interacts with PKM2, inducing and stabilizing the tumor form of M2-PK [62].

Because colonocytes are shed into the gut lumen, t-M2-PK can be detected in stool samples by an enzyme-linked immunosorbent assay (ELISA), making fecal determination of this isoenzyme a useful test for early detection of CRC. Its evaluation gave very encouraging results in pilot studies in 2004 and 2006 [62-64], paving the way for more studies. Among the papers we initially selected, 3 cited t-M2-PK as a promising tool for CRC detection and are therefore described in **Table A5 (Appendix)** [65-67].

Gut microbiota as CRC screening tool

The gut is the home of a large microbial community containing bacteria, fungi, and viruses for a total number that may reach 100 trillion. This flora live in a mutualistic relationship with his host, filling many roles: it enhances the epithelial defense against pathogens, accelerates the maturity of the immune system, protects the local homeostasis, and it also shows endocrine functions. Particularly, human gut microorganisms ferment dietary fiber into short-chain fatty acids - which are subsequently absorbed -, promote the synthesis of vitamin B and vitamin K and it is involved in the metabolization of bile acids, sterols, and xenobiotics. These aspects clearly show how the dysregulation of the gut microbiota can affect the immune response and play a role in the development of inflammatory and autoimmune conditions [68-71].

According to studies on twins and relatives, it is estimated that the heritability of CRC is only 12–35% and genetic predisposition syndromes for CRC only account for a minority of CRC cases [72]. This relatively low level of heritability of CRC reflects the importance of environmental factors and among them the role of microorganisms has been increasingly recognized over the years.

Findings show that the gut microbiota of CRC patients is different compared with the gut microbiota of healthy individuals, containing higher richness of species, a lower quantity of potentially protective bacteria and increased presence of procarcinogenic bacteria such as Bacteroides, Escherichia, Fusobacterium, Peptostreptococci and Porphyromonas [72-74].

For this reason, in **Table A6 (Appendix)** [74-77] we described four recent studies aiming to identify CRC patients using PCR to analyze bacteria in the stool sample of individuals undergoing colonoscopy or FIT.
Among them, Elkof *et al.* [75] had the most promising results: *F. nucleatum* identified CRC patients with 69,2% sensitivity and 76,9% specificity.

Volatile organic compounds (VOCs)

Stool VOCs

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In the recent past there has been an increasing interest in the human volatilome as potential non-invasive diagnostic biomarker in clinical medical practice. VOCs can be defined as the spectrum of volatile organic chemicals originating from (patho)physiological metabolic processes in the human body and detectable in a large range of secretions. A 2014 review by de Lacy Costello *et al.* reported 1765 volatile compounds to appear in exhaled breath (n=872), saliva (n=359), blood (n=154), milk (n=256), skin secretions (n=532) urine (n=279), and feces (n=381) in apparently healthy individuals [78-79].
Lately, different studies have evaluated the usability of VOCs present in the headspace of feces as diagnostic tools for gastrointestinal diseases. Particularly in those diseases in which microbiota alterations occur such as colorectal carcinoma in which, as mentioned above in this review, microbial agents are considered to play a significant etiological role.

Currently, VOC detection techniques can be divided into two different categories: chemical analytical techniques for the quantitative and qualitative detection of individual VOCs and pattern-based techniques, using electronic devices containing an array of different VOC sensors that compare the total set of gases with a pattern recognition algorithm. On one hand Gas chromatography-mass spectrometry (GC-MS), which is a chemical analytical technique, is considered the ongoing gold standard in VOC detection [85] but on the other hand, VOC sensors are getting more and more relevance because they are inexpensive, often portable, and easy to use [89]. This is why findings as the ones listed in **Table A7 (Appendix)** could represent the next frontier for large population-based screening programs [80-88].

Breath VOCs

For the convenience of the acquisition of samples, VOCs are gaining importance in cancer screening studies. These studies use stool and different secretions such as exhaled breath, aiming to find smart sensory tests that are able to facilitate the diagnosis and increase screening adherence in the population. For example, Wang *et al.* [90], collected exhaled breath of 20 CRC patients and 20 healthy controls in their study in China, in 2014. They used solid-phase microextraction methods including gas chromatography/mass spectrometry (SPME-GC/MS) to assess the participants' VOC pattern. The mean age of the patients in the cancer group was 58.1 years, with a standard deviation of 14.2 years. The team used the statistical methods of principal component analysis (PCA) and partial least squares discriminant analysis (PLS-DA) to process the final data. Significant differences were found in VOCs in the exhalations of CRC patients compared to the VOCs in the exhalations of healthy controls. In fact, CRC patients presented higher levels of cyclohexanone, 2,2-dimethyldecane, dodecane, 4-ethyl-1-octyn-3-ol, ethylaniline, cyclooctylmethanol, trans-2-dodecen-1-ol, and 3-hydroxy-2,4,4-trimethylpentyl 2-methylpropanoate and significantly lower levels of 6-t-butyl-2,2,9,9-tetramethyl-3,5-decadien-7-yne ($P < 0.05$).
More recently, in a case–control study in Italy, Altomare *et al.* [91] studied the breath print of 83 patients with colorectal cancer and 90 non-cancer controls collected using Gas chromatography–mass spectrometry. They used ROC curve analysis to discriminate the ability of each VOC in detecting colorectal cancer, and finally cross-validated the results by the leave-one-out method and applying stepwise logistic regression analysis. 14 VOCs (tetradecane, ethyl- benzene, methylbenzene, acetic acid, 5,9-undecadien- 2-one, 6,10-dimethyl (E), decane, benzaldehyde, benzoic acid, 1,3 bis(1-metiletenil) benzene, decanal, unidenti- fied compound T22_75, dodecane, 2-ethyl-1-hexanol and ethanone, 1[4-(1-methylethenyl)phenyl]) were found to have significant discriminatory ability in detecting patients with colorectal cancer. The model presented an AUC of 0.979 and further cross-validation of the model resulted in a true predictive value for colorectal

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cancer of 93% overall, 90% sensitivity and 93% specificity. Moreover, the reliability of the breath analysis was maintained no matter the cancer stage with 86% sensitivity and 94% specificity for early stage disease.

Also, the above-mentioned pattern-based techniques are being explored in breath VOCs detection. We see this in Van Keulens' *et al.* 2019 multicentered study in the Netherlands [92]. Their study was carried out on adult colonoscopy patients and evaluated exhaled volatile organic compounds using an electronic nose. This device, named Aeonose (The eNose Company) is a portable, battery-powered device, that contains three metal-oxide sensors with different material properties that create a patient "breathprint". Inhaled air is also filtered to prevent contamination of the e-nose by environmental VOCs bacteria or viruses and the analysis takes 15 minutes of which the patient breathes into the device for 5.
In the study 511 breath samples were collected with 70 CRC patients and 125 heathy controls. Training models for CRC and AAs had an AUC of 0.76 and 0.71 and blind validation resulted in an AUC of 0.74 and 0.61 respectively. Ultimately the final models that the authors found for CRC and AAs showed an AUC of 0.84 (sensitivity 95% and specificity 64%) and 0.73 (sensitivity and specificity 79% and 59% respectively).

From these results it can be concluded that analysis of breath VOCs could represent an effective and convenient screening method for the disease, particularly when a device is capable of providing a binary answer (cancer/no cancer) to eventually direct to further work-out.

Urinary tests

Urinary VOCs

Over the years the study of urinary volatilome has also been gaining importance. There are two methods currently available to differentiate between healthy controls and CRC patients: mass spectrometry (MS) and nuclear magnetic resonance spectroscopy (NMR) [93]. The method which is used more often is currently Ion Mobility Mass Spectroscopy (FAIMS), which is based on physical properties rather than chemical properties [94]. Particularly, it uses the differences in the electric field dependence of ionized chemical mobilities to separate chemical components. One of its advantages is that, unlike other similar analytical techniques, it can work at atmospheric pressure and room temperature [94].

Field Asymmetric Ion Mobility Mass Spectroscopy (FAIMS)-based studies:

In 2014 in the UK Arasaradnam *et al.* [95] investigated the VOC signature of 133 subjects (83 CRC patients and 50 healthy controls), with a mean age of the CRC patients of 60 years (standard deviation of 17 years), and 64% of males. Simultaneously with CRC diagnosis urine was collected and headspace analysis was conducted using FAIMS. Fisher Discriminant Analysis was used to process the data, which demonstrated that the VOC profiles of CRC patients differed from the healthy controls with 88% sensitivity and 60% specificity. This result is lower compared to the gold standard colonoscopy, but it is comparable with current fecal stool testing including the gFOBT and FIT.

In 2018 Widlak *et al.* [96] conducted a large single-center, prospective and blinded study on a subset of 562 patients with matching urine and stool samples (FIT and faecal calprotectin) who were included for final statistical analysis from an initial population of 1850 patients meeting criteria for inclusion. They used a commercial gas analysis

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instrument based on ion mobility spectroscopy (FAIMS) to analyze VOCs emanating from the urine samples. The results showed that the sensitivity and specificity for CRC using FIT was 80% and 93%, respectively and for urinary VOCs it was 63% (95% CI 0.46-0.79) and 63% (95% CI 0.59-0.67), respectively. Notably, for CRC patients who were FIT-negative (false negatives), the addition of urinary VOCs to FIT resulted in a sensitivity of 97% (95% CI 0.90-1.0) and specificity of 72% (95% CI 0.68-0.76). The authors concluded that, when applied to a FIT-negative group, urinary VOCs improve CRC detection and can be considered a promising second-stage test to complement FIT in the detection of CRC.

In another study settled in the UK during 2019, Mozdiak *et al.* [97] analyzed the urine of 163 FOBT+ patients from a screening population using field asymmetric ion mobility spectrometry (FAIMS) and gas chromatography coupled with ion mobility spectrometry (GC–IMS). The collected data was analysed using a machine learning algorithm and the results showed a high test accuracy for differentiating CRC from control with a high degree of separation. Using GC–IMS AUC was 0.82 (0.67–0.97) with sensitivity 80% (95% CI 0.44–0.97) and specificity 83% (95% CI 0.63–0.95). Using FAIMS AUC was 0.98 (0.93–1) with 100% sensitivity (0.74–1) and 92% specificity (0.62–1). However, even though the separation of CRC patients from the normal controls was high, when CRC cases were grouped with adenomas the accuracy dropped significantly (AUC range 0.83–0.92) and the independent grouping of adenoma and controls was poor with AUC range 0.54–0.61, using both modalities.

When looking at these results we should notice some limitations: current studies are limited to case control and cohort studies, not taking in consideration a real screening setting. Also, due to their complex interaction in the gut, external factors such as diet and medications play a role in VOCs profiling (88) and they are often not deeply analysed before proceeding into the CRC-control differentiation. Nonetheless, these studies highlight the potential of FAIMS in analyzing Urinary VOCs for CRC detection.

Urine nuclear magnetic resonance (NMR)-based studies:

In 2016, Deng *et al.* [98] conducted a prospective study in Shanghai where they evaluated a novel urine-based metabolomic diagnostic test for colonic adenoma detection (PolypDx™) on 1000 Chinese patients undergoing a colonoscopy. This test was originally developed and validated on a Canadian Cohort [99].
Particularly, the PolypDx™ prediction algorithm utilizes concentrations of three key metabolites in urine sample - which is determined by one-dimensional nuclear magnetic resonance (NMR) analysis - and participant clinical features (age, sex, and smoking history) to compute prediction results as positive (adenoma detected) or negative (adenoma not-detected). They calculated an AUC of 0.717 and a sensitivity and specificity of 82.6% and 42.4% respectively. If we compare these results to the fecal-based tests the specificity is lower. However, we should consider that these fecal tests were designed to detect colorectal cancer, and not all polyps. MTI's urine-based test is instead designed to detect adenomatous polyps which, as the authors notice, makes it appropriate to serve as a population-based screening tool for CRC.

An analysis of effectiveness and cost-effectiveness of PolypDx™ test every 2 years, in comparison to screening strategies such as colonoscopy every 10 years, annual gFOBT, and annual FIT, was performed in 2018 by Barichello *et al.* [100]. They found that despite the higher cost (incremental cost-effectiveness ratio (ICER) at $46,783 vs. $51,616, $29,568 and $31,008 respectively) the metabolomics-based urine screening strategy compared with the other techniques was the most effective method. It was found to be correlated with a reduction in mortality by 41% and a gain of 0.13 life-years per person (vs.

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reduction by 25% and 0.08 life-years gained, reduction by 15% and 0.04 life-years gained, and reduction by 36% and 0.11 life-years gained, respectively) and, in conclusion, a cost-effective strategy.

In 2019, another study by Kim *et al.* [101]. explored the potential of urine NMR metabolomics. Urine samples from 92 patients with colorectal neoplasia and 156 healthy controls were collected and analyzed. The team, using the Orthogonal Projections to Latent Structures Discriminant Analysis (OPLS-DA) model, found a metabolomics profile consisting of taurine, alanine, and 3-aminoisobutyrate to be a good discriminator for CRC patients with an AUC of 0.823, 0.783 and 0.842 respectively. The sensitivity and specificity for diagnosing pre-invasive colorectal neoplasia was 96.2% and 95%, respectively, revealing the urine-NMR metabolomics to be a high potential screening tool for accurate diagnosis of pre-invasive CRC.

Urinary Circulating tumor DNA (ctDNA)

Circulating tumor DNA molecules can be found in various body fluids and CRC, notoriously, has a high tumor cell loss factor into the peripheral blood from necrotic and apoptotic cancer cells that occurs during carcinogenesis. While ctDNA has been extensively studied in serum only a couple of studies focus on their presence in different bodily fluids to distinguish between CRC and healthy subjects.

In 2015, Xiao *et al.* [102]. explored the use of methylated NDRG4 gene as a candidate biomarker in urine and stool for diagnosis of CRC. They collected DNA samples from 84 patients and, using nested methylation-specific PCR and denaturing high-performance liquid chromatography (DHPLC), found the *mNDRG4* gene to be present in colorectal carcinoma tissue, paracarcinoma tissues, stool, blood, and urine. The sensitivity and specificity of methylated *NDRG4* gene expression was analyzed and compared with 16 age-matched healthy controls. In stool, the positive detection rate was 76.2% and in urine 72.6%. Considering the convenience of the acquisition of urine samples, the team collected samples from an additional group of 76 patients with CRC. The positive detection rate of methylated *NDRG4* was 72.4% (55/76) in this cohort.

Moreover, a recent study by Song *et al.* [103] evaluated the sensitivity of total ctDNA recovered from urine and its clinical relevance in diagnosing metastatic CRC. The total DNA quantities in urine specimens of 150 CRC patients were prospectively examined in serial samplings during treatment and the team found tumor and urine specimens' molecular profiles to have 90% concordance. Having established a cutoff of 8.15 ng for elevated total DNA, mCRC patients were compared to healthy volunteers, and were identified with sensitivity 90.7% and specificity 82.0%. Even if this study did not focus on early CRC diagnosis it points out how urine total ctDNA could represent a promising diagnostic tool to evaluate in future studies.

4. Discussion

This review identified a large number of recent studies exploring non-invasive techniques for diagnosis of CRC. The feature of non-invasiveness acquires fundamental importance when considering recent statistics regarding screening uptake. In the US, around 30–50% of individuals eligible for CRC screening never begin the process and more than half of individuals presenting abnormal results in their initial screening do not complete follow-up investigations [21].

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European data in the field is much more fragmented due to the high difference in screening programs between countries. However, statistics show that the overall participation is 49.5% (range 22.8%–71.3%) in countries adopting FIT and 33.2% (range 4.5%–66.6%) in countries adopting gFOBT, whereas the desirable uptake rate in the EU Guidelines is >65% [104, 2].

In sight of this, the ability of these new approaches to achieve an early diagnosis while minimizing stress and discomfort for the patient is noteworthy. In particular, urinary VOCs, exhaled breath VOCs and saliva miRNAs seem to be the most promising techniques in terms of ease of collection. An important aspect to consider in VOCs-based tests (including stool VOCs-based tests) is that some exploit electronic devices which use pattern recognition techniques. This technology is portable and inexpensive, making screening simpler and more applicable on a wider scale.

However, even though a number of these studies have reported promising diagnostic performance -comparable to FIT - validation in studies conducted in true screening settings (in an average risk population not undergoing opportunistic screening) would be essential.

Being cost-effectiveness a fundamental characteristic of a good screening program, further research should be dedicated to the cost-effective nature of the previously compiled list of alternative screening techniques with respects to the gold-standards FIT and colonoscopy.

5. Conclusions and future perspectives

To our knowledge, this review represents the most recent and complete summary of the novel non-invasive screening techniques available in the field of CRC detection. Our observations indicate that, to validate the feasibility of most of the alternative tests presented, further studies will be necessary.

Nevertheless, techniques that achieved approval from national agencies for drug regulation such as the American FDA, subsequent to studies conducted in true screening settings, appear to be the most reliable. Among them, the blood-based *SEPT9* gene methylation assay is the most promising one, achieving 68.2% sensitivity and 80% specificity using the Epi proColon® kit and up to 82% sensitivity and 95.90% specificity using different kits. In general, gene methylation assays proved themselves to be an interesting diagnostic tool in the field of CRC screening: different methylated gene panels exhibited up to 97% sensitivity and specificity.

Among the other blood based tests described, non-protein-coding RNAs, particularly miRNAs, presented good discriminative ability (up to 89% sensitivity and 93% specificity for detecting CRC and up to 95% sensitivity and 90% specificity for detecting AAs) and therefore could be considered to be included in screening programs.

Given that higher adherence rates might be achieved in blood-based compared to stool-based tests, they promise to become good alternatives for CRC screening in the US and Europe as well.

Similarly, the Multitarget stool DNA (MT-sDNA) test Cologuard®, already recommended by the American Cancer Society guidelines, represents another feasible option that could catch on in Europe seen as it presents higher sensitivity than FIT (92.3%).

As already mentioned in the discussion section, stool, urinary and exhaled breath VOCs that exploit portable and inexpensive electronic devices based on pattern recognition could make screening simpler and more applicable on a wider scale.

However, validation in studies conducted in true screening settings will be essential to determine wether these techniques already present efficacy and features needed to supersede the already in-place screening tests.

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In conclusion, there are many alternatives that may be worth looking into in the context of true screening programs in order to establish if the ongoing goldstard techniques (FIT and gFOBT) still represent the most accurate tests available at the current moment to screen the avarage risk population. A possible approach to avoid exposing a hypothetical target population to higher false negative rates could be to combine the test object of the study to FIT. This method showed positive results, for example, for the *mSEPT9* + FIT combination which enhanced the sensitivity compared to FIT alone.

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Appendix A:
Tables A1 –A7

Table A1. Efficacy of *SEPT9* methylation assays based tests for CRC detection.

mSEPT9 Kit	Target population	Study design/setting	Inclusion/exclusion criteria	Algo-rithm	N. of cases	N. of controls
A new mSEPT9 Assay (in comparison with Epi Pro Colon 2.0)	• 1031 inpatients and outpatients. • 10 mL blood samples • All subjects received colo--noscopy to confirm diagnosis. • The median age was 60.	• China. • Prospective randomized, single-blind study. Opportu--nistic screening program. • From February, 2014 to April, 2015.	Patients and samples with incomplete informations (sample number, sex, age, and clinical diagnosis), patients with other cancer history, or any chemotherapy or radiotherapy, women who were pregnant were excluded.	1 of 3	504	527
Abbott MS-9 DNA blood test method	• 60 subjects from a sample of 2456 patients who underwent colonoscopy. • 15 mL blood samples. • All subjects received MS-9 DNA blood test, iFOBT and a combination of the two tests. • The median age was 66.	• Taiwan. • Prospective randomized trial. • From July 1st, 2012 to December 31st, 2013.	Patients with previous CRC, other gastrointestinal tract malignancy, incomplete colonoscopy study, or refused the MS-9 DNA blood test, iFOBT, adenoma, hyperplastic polyps, and negative finding during the colonoscopy were excluded.	1 of 3	51	9
Epi Pro Colon 2.0	• 1225 inpatients and outpatients. • 10 mL blood samples. • All subjects received colonoscopy to confirm diagnosis. • The median age was 60.	• China. • Prospective, single-blind study. • Opportunistic screening program.	Subjects who received chemotherapy, radiotherapy, or surgical intervention before the blood draw and colonoscopy were excluded.	1 of 3	527	590
Methylated Human SEPT9 Gene Detection Kit (Tellgen Corporation)	• 248 subjects. • 3 mL blood samples. • All cases were confirmed by pathological diagnosis. • The median age was 66 and the majority (73.4%) aged at least 60 years.	• China. • Prospective. • From October 1st, 2016 to January 31st, 2018.	• Only subjects who simultaneously performed CEA, Ca-199, and mSEPT9 examinations and patients with complete informations (sex, age, pathological type, tumor stage, and metastasis status) were enrolled . • Patients who received chemotherapy or surgical intervention were excluded.	NA	123	125

AUC	Sensitivity	Specificity	Sensitivity for adenomas	Sensitivity for CRC stage I-II	Significant outcomes and possible limitations	Author / year
0.873	76,60%	95,90%	9,80%	Stage I 64.9% , Stage II 72.7%	• Enhanced technical simplicity, convenience, and lower cost compared with the commercialized kit (Epi proColon 2.0), from which it did not differ in performance. • Sensitivity was further enhanced when the test wascombined with CEA (86.4%) or FIT (94.4%).	Wu et al., 2016
0.766	47%	89%	Stages 0-I-II 32%		• PPV, and NPV were respectively 92% and 22%. A ROC curve (AUC = 0.766) was used to predict CRC based on the sensitivity and specificity of the MS-9 DNA blood test and iFOBT (84% and 55% respectively) with the result that higher rates could be predicted to detect CRC if both tests were positive. • However, the sample size was not large enough due to the high cost of the MS-9 DNA blood test and for the detection of early CRC the sensitivity was much lower than iFOBT.	Chen et al., 2017
NA	82%	81%	NA	Stage I 63.5%, Stage II 87.7%	• The detection rate for each CRC stage was satisfactory with no difference between males and females and it did not discriminate cancer from various locations of colorectal tract. It was found that tests using 1/3 algorithm (in comparison with those using the 2/3 algorithm) exhibited higher detection rate for CRC, adenoma, hyperplastic polyps. • However, also the false positive rate in subjects with NED was higher with 1/3 algorithm compared with the 2/3 one. The false PDR correlated with increase of age.	Song et al, 2017.
0.757	61,80%	89,60%	59.2%	Stage I 60%, Stage II 52.8%	• The AUC value of 0.757 (95% CI: 0.701–0.807) was superior to FOBT (sensitivity: 61.4% specificity: 70.3%; AUC: 0.658), CEA (sensitivity: 35.0% ; specificity: 62.6% AUC: 0.485), and Ca-199 (sensitivity: 17.9% ; specificity: 55.7%; AUC: 0.353). • There were no false positives. • However, the study was unblinded and the authors noted that the small number for the stratifications might have lead to an over-evaluated diagnostic value, in particular for some subgroups of tumor location.	Xie et al., 2018

Table A2. Summary of methylated genes panels feasible for CRC detection.

M-genes panels	Target population	Study design/setting	Inclusion/exclusion criteria	N. of cases	N. of controls	AUC
***SFRP1, SFRP2, SDC2, PRIMA1* gene promoters**	• 166 patients who underwent screening colonoscopy. • 121 plasma and 32 biopsy samples were collected from colorectal adenoma and cancer patients.	• Hungary. • The effect of altered promoter methylation on protein expression was examined by immunohistochemistry. ROC curves were applied to determine the sensitivity and specificity of the four markers. Multiple logistic regression analysis used applying the four markers together.	NA	37	129	0.978
ALX4, BMP3, NPTX2. RARB, SCD2, SEPT9* and *VIM	• 295 subjects from a cohort of CRC patients and a control group of patients suspected for but without CRC. • All the patients received a colonoscopy.	• Denmark. • Cross-sectional case-control study. • Use of methylation specific PCR. Multivariable logistic regression used with stepwise backwards selection, and subsequent leave-pair-out cross validation, to calculate the optimism corrected AUC for all stages. • Between 2003 and 2005.	Blood samples were collected from all patients with diagnosed CRC and available blood samples at the time the study and from patient referred fo colonoscopy with symptoms of, but without CRC, were collected.	193	102	0.86
MGMT, RASSF1A, SEPT9	• 214 CRC patients consecutively diagnosed and treated with surgical resection. • The median age was 60.35. 65.4% were males.	• Portugal. • Promoter methylation levels were assessed using real-time qMSPR. Molecular data were correlated with standard clinico-pathological features. Diagnostic and prognostic performances were evaluated by ROC curve and survival analyses, respectively. • Between 2000 and 2012.	NA	214	50	0.97
IKZF1, BCAT1, IRF4	• 1621 subjects. • Blood was collected before colonoscopy or colonic surgery. • The median age was 63. 56% were males.	• Australia. • Plasma was assayed for methylated BCAT1, IKZF1 and IRF4 using multiplex, methylation-specific real-time PCR.	NA	184	820	NA

Sensitivity	Specificity	Sensitivity and Specificity for adenomas	Sensitivity for CRC stage I-II	Significant outcomes and possible limitations	Author / year
91,50%	97,30%	• 89.2% sensitivity • 86.5% specificity (AUC: 0.937, 95% CI: 0.885 to 0.989).	NA	• All four markers showed increased methylation in adenoma and tumorous samples both in tissue and plasma specimens, as confirmed with diverse techniques including pyrosequencing, MethyLight PCR, and in silico analyses.	Bartak et al., 2017
90,70%	72,50%	NA	• 88,7% sensitivity • 73,5% specificity	• The seven hypermethylated promoter regions along with the covariates sex and age yielded an optimism corrected AUC of 0.86 for all stage CRC and 0.85 for early stage CRC. • However, none of the individual DNA promoter regions provided an overall sensitivity above 30% at a reasonable specificity.	Rasmussen et al., 2017
96.6%	74.0%	NA	100%	• The panel detect cancer both in the colon (proximal and distal) and rectum with a sensitivity of 95.7% for colon and 98.0% for rectum. • Furthermore, the panel was also able to identify tumors at any disease stage with similar efficiency (100, 94.2, 95.9% for stage I/II, stage III and stage IV, respectively). PPV 91.5 (AUC= 0.97)	Freitas et al., 2018
73.9% (95%CI: 67.1-79.1)	90.1% (95%CI: 88.0-92.0)	NA	NA	• Each gene contributed to cancer detection. The OR for presence of cancer associated with detection of three methylated genes compared to a single gene was 42.1 (9.8- 181.5, p<0.0001). • However, there were 81 false-positives (9.9% of 820 non-neoplastic paricipants) of which 67/81 (82.7%) were methylation positive in a single gene only and half (34/67) were due to BCAT1.	Young et al., 2019

Table A3. miRNAs as CRC biomarkers.

miRNA panels	Target population	Study design/ Setting	Inclusion/ Exclusion criteria	N. of cases	N. of controls	AUC
miR-23a-3p, miR-27a-3p, miR-142-5p, miR-376c-3p	• 427 CRC patients who underwent resection or colonoscopy. • All blood serum samples were collected prior to colonoscopy or surgery.	• Czech Republic. • Diagnostic and prognostic potential of identified microRNAs was validated on independent training and validation sets of samples using RT-qPCR. • From 2010 through 2014.	All subjects enrolled in the study were of the same ethnicity (European descent), and colon cancer patients did not receive any neo-adjuvant treatment. Hemolytic serum samples were excluded from the study, as hemolysis may influence the expression of some miRNAs.	427	276	0.922
miR-21	• 65 subjects. • Samples of EDTA stabilized peripheral blood and saliva were collected. • All the patients were tested with tumor markers CEA, CA 19–9, and u-FCC to confirm their diagnosis.	• Russia. • MicroRNA-21 expression was quantified by real-time qRT-PCR in peripheral blood and saliva samples.	All patients had adenocarcinoma located in the distal colon and were under the supervision of the FPSMU Research Institute of Surgery and Emergency Medicine.	31	34	NA
miR-1246, miR-202-3p, miR-21-3p, miR- 1229-3p, miR-532-3p	• 575 patients who underwent colonoscopy to confirm diagnosis.	• China. • A qRT-PCR was performed in three stages and binary logistic regression was used in the validation stage. A ROC curve was drawn to evaluate the diagnostic accuracy. • Between April 2013 and December 2014.	Patients with inflammatory bowel diseases, hereditary adenomatous polyposis, family history of tumors, and other tumors in the preceding or corresponding periods were excluded.	217	190	0.96 (95% CI: 0.937–0.983)
miRNA19a, miRNA19b, miRNA15b, miRNA29a, miRNA335, miRNA18a	• 297 patients who underwent colonoscopy. • The median age was 60.3 for control group, 63.4 for AA, and 72.0 for CRC.	• Spain. • Multicenter case-control study of 297 patients. qRT-PCR was used to quantify a signature of miRNA in plasma samples. Binary classifiers were built for the best predictive model. • Between May 2014 and June 2016.	Patients with any another type of cancer in the previous 5 years, chemotherapy or radiotherapy, previously diagnosed with AA, familial adenomatous polyposis, Lynch syndrome, or inflammatory bowel disease were excluded.	96	100	0.92 (95% CI 0.871–0.962)
hsa-miR-378, hsa-miR-342-3p	• 1684 FIT+ subjects from a CRC screening program.	• Italy. • Three-phase design study comprising cohorts of plasma samples from FIT+ individuals: (1) discovery cohort from 60 subjects who had total colonos- copy collected between Nov. 2008 and Mar. 2012; (2) internal validation cohort prospectively collected from 201 individuals who had total colonoscopy from Feb. 2013 to Jan. 2015 and (3) an external validation cohort from 1,423 subjects prospectively collected from Nov. 2013 to Dec. 2015	Patients with known or suspected inflammatory bowel disease or who already had a colonoscopy/ sigmoidoscopy for any indication, with endoscopic resection of a colonic lesion (including polyps), and patients who had received a diagnosis of cancer during the last 5 years and/or had a suspicion of malignancies other than CRC were excluded.	Look at study design		0.682 (95% CI: 0.580–0.785)

Sensitivity	Specificity	Sensitivity and specificity for adenomas	Sensitivity and specificity for CRC stage I-II	Significant outcomes and possible limitations	Author / year
89%	81%	NA	80% (AUC: 0.877)	• On 168 colon cancer patients the 4-miRNA based panel correctly identified 149 colon cancer patients (89%). • Combining CEA, CA19-9 and the miRNA-based panel the highest sensitivity was reached (96%). • However, the expression of these miRNAs did not allow to distinguish precancerous lesions from cancer and there were no significant changes between the particular TNM stages of colon cancer.	Vychytilova-Faltejskova et al., 2016
65% [C.I 95%: 0.85-1.93; (P > 0.95)]	85% [C.I 95%: 0.85-1.93; (P > 0.95)	NA	NA	• Notably, the sensitivity of miR-21 expression in the saliva was 97%, and specificity 91% [C.I 95%: 0.65 to 2.49, (P > 0.95)]. • However, the sample size was limited.	Sazanov et al., 2017
91,6%,	91,70%	94.4% sensitivity, 84,7% specificity AUC 0.951 (95% CI: 0.922–0.980)	NA	• The panel accurately distinguished CRCs from colorectal adenomas and healthy controls with high sensitivity and specificity.	Guo et al. , 2018
85%	90%	95% sensitivity 90% specificity, AUC 0.91 (95% CI 0.868–0.959) PPV 0.82 and NPV 0.97.	94% sensitivity, 87% specificity	• PPV and NPV were respectively 0.94 0.76 when CRCs and AAs were compared with healthy individuals. The authors assumed a hypothetical screening population in which expected prevalence of CRC and AA were 1:100 and 7:100, respectively: as a result the obtained PPV and NPV were 0.08 and 0.99 respectively for CRC and 0.42 and 0.99 for AA. • However, the authors suggest that further studies in a real CRC screening population are required to validate the model presented in this article.	Herreros-Villanueva et al., 2019
cut off 85% sensitivity	25.71%	NA	NA	• Classifying each subject as positive or negative for the 3 miRNA signatures, the detection rate rose to 95%, with loss of specificity (about 10%). The corresponding NPV was 0.64, indicating that 64% of FIT+ subjects without any endoscopic lesion according to the miRNA test are truly free of the disease. As a result, the authors estimated a 4.4% reduction in colonoscopies after a FIT+ test. • However, the authors suggest that further studies in a real CRC screening population are required to validate the model presented in the article.	Zanutto et al., 2020

Table A4. piRNAs as CRC biomarkers.

piRNA panels	Target population	Study design/ Setting	Inclusion/ Exclusion criteria	N. of cases	N. of controls	AUC	Sensitivity	Specificity
piR-18849, piR-19521 piR-17724	• 83 CRC patients who underwent surgery between January 2016 and January 2018.	• China. • Fresh CRC tissues and matched adjacent non-tumor tissues were collected from the patients. Of the 83 pairs of samples, 3 pairs were used for deep sequencing for small RNAs, and the remaining 80 pairs were used for validation. Differential expression analysis was used to identify upregulated and downregulated piRNAs and qRT-PCR was used to confirme those results.	All patients exhibited no primary tumors in other sites and did not receive chemoradiotherapy or biological therapy prior to surgery.	83	/	NA	NA	NA
piR-54265	• 2429 subject of which 209 healthy controls, 725 CRC+, 1303 patients with other types of digestive cancer and 192 patients with benign colorectal tumors.	• China. • Prospective case-control analysis. • The predictive value of serum piR-54265 was determined by a digital PCR analysis. ROC curve was constructed to quantify the diagnostic performance assessing its sensitivity, specificity and respective AUC. The ORs were computed using multivariate logistic regression models.	NA	725	209	0.896 (95% CI: 0.874-0.914)	85.7% at 1500 copies/µ as a cut-off value.	65.1% at 1500 copies/µL as a cut-off value.
piR-020619, piR-020450	• N. of subjects: look at study design. • Similar distribution of age and gender among control and CRC group. Same ethnicity. All the lesions were diagnosed by histopathology analysis. Serum CEA and CA19-9 levels were measured.	• 4 phases study: piRNA expression profile in sera from 7 CRC patients and 7 controls using small RNA sequencing. Differentially expressed piRNAs were measured in a training cohort of 140 CRC patients and 140 controls using qRT-PCR. The identified piRNAs were evaluated in 2 independent validation cohorts of 180 CRC patients and 180 controls. Finally, the diagnostic value of the identified piRNAs for AAs was assessed, and their expression was measured in 50 lung cancer, 50 breast cancer and 50 gastric cancer patients.	The patients had not received any anticancer treatments before serum collection. All the control individuals were recruited from the physical examination departments of the two hospitals, and were confirmed to have no indication for cancer in the physical xaminations, no history of malignancy or diagnosis of benign colorectal neoplasm.	Look at study design		Validation cohort 1: 0.883 Validation cohort 2: 0.913	• Validation cohort 1: 86.00% (CEA 40.00%, CA19-9 27.00%) • Validation cohort 2: 88.75% (CEA 42.50%, CA19-9 28.75%).	•Validation cohort 1: 92.00% • Validation cohort 2: 93.75%

Sensitivity and specificity for CRC stage I-II	Significant outcomes and possible limitations	Author / year
NA	• The expression levels of piR-18849, piR-19521 and piR-17724 were consistently increased in CRC tissues, compared with adjacent tissues (P<0.05). • The expression of piR-18849 was positively correlated with lymph node metastasis potential and negatively correlated with the degree of tumor differentiation; additionally, CRC with poor differentiation and high lymph node metastasis had significantly increased levels of piR-18849 (P<0.05) correlated with the degree of tumor differentiation (P=0.001). • Limitations: No controls. Clinical setting. No assesment of sensitivity and specificity. Only 3 pairs of samples were used for deep sequencing, which may cause a number of notable piRNAs to be omitted due to the limited samples.	Yin et al., 2019
•83% sensitivity •65,1%specifi--city AUC: 0862, P<0,001 (95%CI, 0,827-0,891)	• The prediagnostic serum piR-54265 levels were significantly associated with future CRC diagnosis, with the ORs of 7.23, 2.80, 2.45, and 1.24 for those whose CRC was diagnosed within 1, 2, 3 and >3 years. • The serum piR-54265 levels in patients declined substantially after surgery but increased significantly again when tumor relapses. However, the authors concluded that the possibility that this trend will also be seen in relapse-free patients cannot be excluded. • Other reported limitations were 1. Because of the low compliance, most of control subjects in the nest case-control analysis from the DFTJ prospective cohort were not diagnosed by colonoscopy; 2. Caution should be taken in preparation of serum/plasma samples - because of the existence of abundant piR-54265 in blood cells - to avoid hemolysis and/or blood cell contamination.	Mai et al., 2020
•76.79% sensitivity •90.94% specificity AUC: 0.839	• When the data from the two cohorts were processed in a mixed-up manner, similar results were obtained. • The expression of these piRNAs was detected in 12 paired serum samples from CRC patients before and one month after surgery: both showed decreased post-operative expression levels. • Stepwise binary logistic regression analysis was performed to evaluate whether the diagnostic usefulness of the piRNA panel was affected by clinical features of CRC cases: the piRNA panel remained a strong predictor of CRC regardless of the subgroupings of the patients in the training and validation cohorts. Moreover the expression levels of piR-020619 and piR-020450 in the sera of lung, breast and gastric cancer patients were similar to those of normal controls, suggesting that the two piRNAs could serve as CRC-specific biomarkers.	Wang et al., 2020

Table A5. t-M2-PK as CRC screening tool.

t-M2-PK test and methodology	Target population	Study design/setting	Inclusion/exclusion criteria	N. of cases	N. of con-trols	AUC	Sensitivity	Specificity
Quantitative ELISA test (ScheBo Biotech AG, Giessen, Germany); cutoff: 4 U/ml.	• 127 subjects who underwent colonoscopy. • Pre-colonoscopy stool samples were stored at -20°C. • The mean age was 63, range 50–75 years. 64 were men and 63 were women.	• Italy. • Clinical setting. • 130 patients were considered eligible for the study during a 12 month period (2015-2016).	• Inclusion criteria: age 50-75, presence of alarm symptoms, anamnestic risk factors for CRC, or family history of CRC. • Exclusion criteria: colon surgery in the previous 3 years, inflammatory bowel disease or other gastrointestinal diseases, coexisting serious illness, medication associated with intestinal inflammation or intestinal infection. Patients with inadequate colon cleansing and incomplete colonoscopy were also excluded.	9	118	NA	63.64% (95% CI: 30.79–89.07%)	74.14% (95%CI: 65.18–81.82%)
Quantitative ELISA test (ScheBo Biotech AG, Giessen, Germany); cutoff: 4 U/ml.	• 60 patients, 3 groups: 20 patients with CRC, 20 patients with CRP (lack criteria for colonic cancer by biopsy), and 20 healty controls. • Pre-colonoscopy stool samples were stored at -20°C. • The studied groups were age- and sex-matched. The mean age was 52.6 ± 9.4.	• Egypt. • Case-control study during June 2016 with follow-up period of 6 months.	Patients who received previous surgical resection of a colonic mass or chemotherapy, patients in whom colonoscopy was not completed due to any cause (bad preparation, intolerability, etc.) and patients with inflammatory bowel disease either ulcerative colitis or Crohn's disease were excluded.	20	20	0.711-0.925	• M2PK > 4: 85.0% (95%CI 68.8–95.7) • M2PK > 25: 75.0% (95%CI: 59.8-75.0)	• M2PK > 4: 40.0% (95%CI 23.8–50.7) • M2PK > 25: 100% (95%CI: 84.4-100.0)
Quantitative ELISA test (ScheBo Biotech AG, Giessen, Germany); cutoff: 4 U/ml.	• 94 patients scheduled for colonoscopy: they all completed faecal t-M2-PK test and FOBT and among them 69 completed colonoscopy, 5 subjects had further colonic examination by CT colonography or barium enema. • The mean age was 56.8 ±15.3 years.	• Malaysia. • Prospective study on patients that underwent colonoscopy between September 2014 and January 2016.	• Patients with past history of colectomy, known to have colorectal cancer and came for surveillance colonoscopy, who received chemotherapy for colorectal cancer, or who had incomplete colonic examination were excluded. • Of the 94 patients 11 subjects had obstructed colonic tumour and were included and the remaining 9 subjects were excluded due to incomplete colonic examination without further imaging	17	58	0.868 (95% CI: 0.794-0.941; p < 0.005).	100%	72.5%

Sensitivity for Aas	Sensitivity for CRC stage I-II	Significant outcomes and possible limitations	Author / year
NA	NA	• NPV 95,56% (95%CI: 89.01–98.78%). FIT+/t-M2-PK+combination showed sensitivity the same as t-M2-PK, specificity 89.66%, PPV 36.84%, and NPV 96.30%. Considering as "positive" patients with at least one positive test it was obtained a very high NPV (98.55%). • Immunohistochemical staining with polyclonal antibodies was used to investigate t-M2-PK expression in tissues surrounding the lesions: there were no false positives and all positive controls showed staining in healthy tissue surrounding the lesion, cytoplasm showed a positive signal in high-risk adenomas and a strong positive signal in CRC surroundings. • The faecal quantification correlates with tissue staining. For this reason the authors hypothesize that it can be used in parallel with histological examination during CRC screening, as a warning tool for a very early CRC diagnosis. • However, compared to t-M2-PK alone, FIT showed higher test accuracy.	Zaccaro et al., 2017
NA	NA	• M2PK with a cutoff > 25 showed high diagnostic accuracy (PPV: 100.0, NPV: 80.0). Fecal M2PK was found to be superior to other tumor markers (CEA, CA19-9, FOBT) and in indicating the progress of colorectal adenomas > 1 cm. • However, M2PK had poor diagnostic performance in differentiating CRP from the control group. Another limitation to the study was that it used a selection of cases for three well-matched groups, and the colonoscopy was performed in well-prepared cases and conditions.	Dabbous et al., 2018
•Sensitivity 20.0%, •Specificity 54.4%	NA	• AUC 0.868 whereas the gFOBT test AUC was 0.765. The sensitivity of M2-PK test in colorectal cancer detection was higher than gFOBT (100.0% vs 64.7%), however the specificity was lower (72.5% vs 88.2%). PPV and NPV of M2-PK test and gFOBT were (47.2% and 100.0%) and (57.9% and 90.9%), respectively. • This study investigated on symptomatic group going for colonoscopy, consequentially the authors suggest that there may be overestimation of sensitivity and underestimation of specificity in hospital settings. Average-risk population was not included in this study.	Che Alhadi et al., 2020

Table A6. Gut microbiota as CRC screening tool.

Gut microbiota markers	Target population	Study design/setting	Inclusion/exclusion criteria	N. of cases	N. of con-trols	AUC
Peptostreptococcus anaerobius (genes 2361423 and 3173495), *Parvimonas micra* (gene 1696299), *Fusobacterium nucleatum* (gene 1704941)	• Cohort C1: 128 individuals, 74 CRC patients (median age 67 years; 26 were females), and 54 controls (median age 62years; 21 were females • Cohort C2: 156 individuals, 47 CRC patients (median age 69 years; 22 were females) and 109 controls (median age 58 years; 69 were females). • Cohort D: 40 individuals, 16 CRC patients with CRC (median age 67.5 years; 6 were females) and 24 controls (median age 65.5 years; 17 were females). Stool samples were collected by individuals at home, followed by immediate freezing at −20°C	• China (Cohort C1 and C2) • Denmark (Cohort D) • Metagenome-wide association analysis was performed on faecal samples from cohort C1, results were validated in cohort D. The biomarkers were further validated in two cohorts from France (F cohort) and Austria (A cohort). Finally, targeted qPCR assays was used to evaluate diagnostic potential of the selected biomarkers in cohort C2.	Discovery cohort C1 only included carcinoma samples, with the exlcusion of all patients with adenoma.	See Target populatio		0.84
clbA1+ bacteria		• Sweden. • Nested case–control study. • qPCR assays targeting the clbA gene and the afaC gene of the afa-1 operon were used to detect pks1 bacteria and Afa-1 adhesin-expressing DAEC, respectively. E. coli Nissle 1917 was used as a positive control for pks. F. nucleatum was assessed by rt-qPCR using the Microbial DNA qPCR Assay (Qiagen) containing a FAM- labeled probe specific for F. nucleatum 16S rRNA gene (Gene- Bank Acc. FJ471654.1). • Between September 2008 and March 2013.	Patients with planned colonoscopy within 1 week, dementia and low-performance status, including mentally or physically disabled were excluded.			NA
Fusobacterium nucleatum	• 238 study subjects selected from a cohort of 1136 patients who went through colonoscopy.			39	66	0.737
Fusobacterium nucleatum and *clbA1+ bacteria*						NA
Fusobacterium nucleatum	• 500 participants were selected from the BliTz study (an ongoing prospective screening study among participants of screening colonoscopy in Germany that was initiated in 2005 with the primary aim of developing novel non-invasive tests for CRC screening). • From all subjects stool sample was collected before colonoscopy.	• Germany. • True screening population. Multiplex PCR was used to perform 16S rRNA gene analysis (sequencing of regions V1–V2). The association of Fusobacterium with presence of CRC was analyzed using logistic regression modeling, and its predictive ability was estimated using the AUC. Between 2005 and 2013.	Inclusion criteria: 50 years or older, adequate bowel cleansing. Exclusion criteria: inflammatory bowel disease or history of CRC.	46	110	NA
Higher total bacterial load.	• 180 samples from FIT+ screenees. • All screenees included underwent complete colonoscopy. • 56% were male with a median age of 64 years	• Netherlands. • Bacterial stability over time and the possibility of bacterial contamination were evaluated using qPCR. FIT+ samples of an average-risk screening cohort were subsequently analysed for universal 16S, and bacteria (E. coli, Fusobacterium nucleatum, Bacteroidetes and Faecalibacterium prausnitzii) by qPCR. • Between February 2013 and August 2014	NA	180 FIT+	/	NA

Sensitivity	Specificity	Sensitivity for Aas	Sensitivity for CRC stage I-II	Significant outcomes and possible limitations	Author / year
NA	NA	NA	These genes were enriched in early-stage (I– II) patient microbiomes.	• In ethnically different cohorts there was a confirmation of the known associations of *F. nucleatum* and *Peptostreptococcus stomatis* with CRC. Other species - including *Parvimonas micra* and *Solobacterium moorei* - were found related to CRC as well. 20 microbial gene markers differentiated CRC and control microbiomes and 4 markers were validated in the D cohort. In the F and A cohorts, these 4 genes distinguished CRC metagenomes from controls with AUC of 0.72 and 0.77, respectively. • qPCR measurements of two of these genes (*butyryl-CoA dehydrogenase* from F. nucleatum, and *RNA polymerase subunit β, rpoB,* from P. micra) identified CRC cases in the independent Chinese cohort with AUC 0.84 and OR of 23. • However, only CRC-enriched genes and species were validated across cohorts therefore the conclusions are limited on species depleted in CRC-associated microbiomes.	Yu et al., 2017
56,40%	81,50%	31%	NA	• *clbA* was more often detected in stool samples of CRC patients compared to patients with dysplasia (p 5 0.004) or controls ($p < 0.001$): with a specificity of 81.5%, the clbA assay detected 56.4% of CRCs and 31.3% of dysplasias.	Eklof et al., 2017
69,20%	76,90%	20,10%	NA	• *F. nucleatum* alone showed to be present at significantly higher levels in stool of patients diagnosed with CRC compared to patients with dysplasia ($p < 0.001$) and controls ($p < 0.001$): with a specificity of 76.9%, it detected 69.2% of CRCs and 20.1% of dysplasias. At the selected cutoff (0.00026), the *F. nucleatum* assay detected CRC with a higher sensitivity (69.2%) than clbA (56.4%) and the immunochemical F-Hb test currently used in the clinic (65.5%).	
63,10%	84,60%	NA	NA	• However, the F-Hb test was more specific (90.2%) than F. nucleatum (76.9%), detecting <10% false positives. Moreover this study was not randomized, as all patients selected for colonoscopy presented with symptoms from the large bowel. Also, there was no validation cohort.	
NA	NA	NA	Dunn's post hoc test showed significant differences between stage I and stage II (unadjusted P-value = 0.012) and stage I and stage III (u. P-value = 0.042).	• *Fusobacterium* was found in stool samples of 27.2% of study participants and it was significantly more common in the CRC group (54.3%) than in all other groups (23.6–25.1%) (Kruskal–Wallis rank sum test, $P < 0.001$). Moreover, within the CRC group, Fusobacterium RA was positively associated with cancer stage (Kruskal–Wallis rank sum test, P-value = 0.049)	Amitay et al. 2017
NA	NA	NA	NA	• As only FIT-positive subjects underwent colonoscopy, it was not possible to evaluate prime indicators of diagnostic performance, including sensitivity, specificity AUC. However, faecal microbiota in FIT were stably measured up to six days for *E. coli* (p 1⁄4 0.53), *F. nucleatum* (p 1⁄4 0.30), Bacteroidetes (p 1⁄4 0.05) and *F. prausnitzii* (p 1⁄4 0.62) and as a result the *total bacterial load* (i.e. 16S) was found to be significantly higher in patients with colorectal cancer and high-grade dysplasia (p 1⁄4 0.006). • For the individual bacteria tested, no association was found with colonic lesions. • Another finding was the observation that the faecal microbial content in FIT samples remains stable for six days.	Grobbee et al., 2020

Table A7. Stool volatile organic compounds (VOCs) based CRC test: chemical analytical and pattern recognition techniques.

	Target population	Study design/ Setting	Inclusion/ Exclusion criteria
Fecal VOCs (by chemical analytical techniques)		**Fecal VOCs (by chemical analytical techniques)**	
No specific VOCs (described as compound A, B, X and Y due to potential future IP).	• 137 patients. • The mean age was 64 (range 22–85), 54% were males.	• UK. • Prospective case–control study. • Gas compounds were collected from the bowel movements of each subject and analyzed with gas chromatography. Logistic regression modelling with 10 fold cross validation was used to test potential biomarkers.	NA
↑ Acetic acid, valeric acid, butyric acid and isovaleric acid, Glu- tamic acid, glycine, aspartic acid, leucine, glyc- erin, proline, serine, valine, phenylala- nine, pheny- lacetic acid, cholesterol derivates ↓ butyrate, oleic acid, trans-oleic acid, linoleic acid, glycerol, monoacyl glycerol, myristic acid, ursodesoxycholic acid and pantothenic acid	• 15 patients with colorectal cancer (nine males and six females) and 12 healthy control individuals. • The average age of the patients was 52.5 (range, 40-60 years).	• China. • Case–control study. • Gas compounds were collected from the bowel movements of each subject and analyzed with gas chromatography. • Between June 2013 and October 2014.	Patients with CRC recurrence post-surgery, chemotherapy, metabolic diseases, who received antibiotics within one month, NSAIDS, statins or probiotics within two months prior, suffered chronic intestinal diseases and had a history of food allergies were excluded.
↑ Oleic acid, Linoleic acid only in male subjects	• 26 newly diagnosed CRC patients and 28 healthy individuals. • CRC was histologically diagnosed in all patients by colonoscopy. Healthy controls were individuals who voluntarily undergo a routine health check with screening colonoscopies.	• Korea. • Prospective case–control study. • Gas compounds were collected from the bowel movements of each subject and analyzed with GC–MS for long chain fatty acids. • Between July 2014 and August 2014	Patients with history of CRC, inherited CRC syndromes such as Lynch syndrome and familial adenomatous polyposis, prior intestinal resection, and concurrent chronic bowel disorders such as inflammatory bowel disease were excluded.
↑ Methyl mercaptan and ↓ Hydrogen	• 56 subjects: 30 patients with CRC who were scheduled for surgery and 26 healthy adult volunteers. • The average age was 68 years.	• USA. • Prospective cross-sectional study. • Gas compounds were collected from the bowel movements of each subject and analyzed with gas chromatography. Multivariate logistic regression analysis was carried out to identify CRC patients: when there were two or more samples, an average value was used for logistic regression analysis. A ROC curve of the discriminant formula (0.137 x MM (mean)-0.582 x H2 (mean) + 0.137) was constructed.	• Inclusion criteria: age 20 years or older, histologically proven adenok, any clinical stage, and elective operation for CRC. • Exclusion criteria: concurrent malignancy in another organ, IBD, infectious enteritis, bowel obstruction or the inability to tolerate surgery under general anesthesia
Propan-2-ol Propan-2-ol and 3-methylbutanoic acid	• 137 subjects. Most participants were recruited from colonoscopy waiting lists. • Mean age 64 years (range 22-85), 54% were male.	• UK. • Gas compounds were collected from the bowel movements of each subject and analyzed with gas chromatography mass spectrometry. Logistic regression modelling and 10-fold cross-validation were used to test potential biomarkers.	Patients who had received antibiotics in the preceding 3-6 months, vegetarians and patients with active colitis were excluded.
Fecal VOCs (by pattern recognition techniques)		**Fecal VOCs (by pattern recognition techniques)**	
• 86 CRC patients who resulted positive to FOBT and participated in the experimental study SCENT A1 before undergoing colonoscopy investigation.		• Italy. • The patented device SCENT A1 [87] was used to analyze the fecal VOC patterns. It consists of a pneumatic system that conveys fecal exhalations to the sensing unit. The sensors, each composed of a film of nanostructured semiconductor material consisting of five MOX sensors, vary their resistance in contact with the gas and can distinguish different mixtures. Peroxidation of tissues and the alteration of the metabolic activity of cells are the main discriminating factors. • Ongoing since May 2016.	NA
• 1039 patients collected a faecal sample prior to colonoscopy and a total of 274 subjects were included. A second sample was collected from 32 polypectomy patients and 32 controls. • Age differed significantly between groups ($P < 0.001$), with the controls displaying the lowest mean age (60 ± 11.8 years) and AA patients the highest (68.8 ± 6.7 years). Gender differed significantly between controls, SA, LA and AA groups ($P < 0.0001$) but not between control and CRC or any of the adenoma groups.		• Netherlands. Prospective multi-centre case–control study. • Gas chromatography–ion mobility spectrometry (GC–IMS; FlavourSpec; G.A.S., Dortmund, Germany) was used to measure the faecal VOC patterns. Random forest, support vector machine, Gaussian process and neural net classification were used to evaluate accuracy. • Between February 2015 and November 2017	NA

Inclusion/ Exclusion criteria	N. of cases	N. of con-trols	AUC	Sensitivity	Specificity	Sensitivity and specificity for adenomas	Sensitivity and specificity for CRC stage I-II
NA	21	60	0.82	87.9%	84.6%	NA	NA
Patients with CRC recurrence post-surgery, chemotherapy, metabolic diseases, who received antibiotics within one month, NSAIDS, statins or probiotics within two months prior, suffered chronic intestinal diseases and had a history of food allergies were excluded.	15	12	NA	NA	NA	NA	NA
Patients with history of CRC, inherited CRC syndromes such as Lynch syndrome and familial adenomatous polyposis, prior intestinal resection, and concurrent chronic bowel disorders such as inflammatory bowel disease were excluded.	26	28	NA	NA	NA	NA	NA
• Inclusion criteria: age 20 years or older, histologically proven adenok, any clinical stage, and elective operation for CRC. • Exclusion criteria: concurrent malignancy in another organ, IBD, infectious enteritis, bowel obstruction or the inability to tolerate surgery under general anesthesia	30	26	0.78	90%	57,70%	NA	NA
Patients who had received antibiotics in the preceding 3-6 months, vegetarians and patients with active colitis were excluded.	21	60	0.76 0.82	83% 87.9%	71% 84.6%	NA	NA
NA	86 FOBT+	/	NA	95%	95%	NA	NA
NA	arget population		NA	27.6 at 40 μg/g, 51.7% at 10 μg/g	94.1% at 40 μg/g, 86.2% at 10 μg/g	• Sensitivity: 17.0% at 40 μg/g; 33.0% at 10 μg/g; • Specificity: 95.1% at 40 μg/g, 88.0% at 10 μg/g	NA

Significant outcomes and possible limitations	Author / year
• Logisitic regression analysis of VOC presence identified a three VOC panel (compounds A, X and Y) AUROC =0.86: a person 6 times more likely to have cancer for each of the VOCs present in their stool (p =< 0.0001). Factor analysis supported these findings.	Bond et al., 2016
• Analysis of fecal metabolites demonstrated that the metabolic profiles of healthy individuals and patients with colorectal cancer were distinct. All p values were < 0.05 to < 0.0001. • However, the sample size of the study was small.	Wang et al., 2017
• The levels of *total ω-6 polyunsaturated fatty acid* , particularly of linoleic acid, were significantly higher in male CRC patients than in healthy men (2.750 ± 2.583 vs. 1.254 ± 0.966 μg/ mg feces, P = 0.040; 2.670 ± 2.507 vs. 1.226 ± 0.940 μg/mg feces, P = 0.034, respectively). In addition, the levels of total *monounsaturated fatty acid*, particularly, of oleic acid were significantly higher in male CRC patients than in healthy men (1.802 ± 1.331 vs. 0.977 ± 0.625 μg/mg feces, P = 0.027; 1.749 ± 1.320 vs. 0.932 ± 0.626 μg/mg feces, P = 0.011, respectively). • However, those differences were not shown in female gender, the sample size was small and there was a significant difference in the mean age between CRC patients and healthy controls: because aging may act as a metabolism modifier, authors stated that the difference in age might have impacted the fecal fatty acid metabolome composition and influenced the results.	Song et al., 2018
• In the gas analysis, *methyl mercaptan* was significantly higher in the CRC group than in the control group. Hydrogen was significantly lower in the CRC group than in the control group and was correlated with tumor depth and advanced disease stage. • Hazard ratio MM 1.224 (95%CI: 0.978-1.532; P value 0.078), Hazard Ration H2 0.557 (95%CI: 0.325-0.952; P value 0.032). Sensitivity, specificity, negative NPV, PPV, and accuracy of the discriminant formula were 90%, 57.7%, 83.3%, 75%, and 75%, respectively. • However, the authors noted that the reason for the differences in gas compounds between CRC patients and healthy controls was unclear: gas composition could have been affected by meals and information regarding the food of CRC patients and controls was not accessible. Also, the sample size was small.	Ishibe et al., 2018
• Logistic regression analysis using the presence/absence of specific VOCs identified a three volatile organic compound panel (*propan-2-ol, hexan-2-one and ethyl 3- methyl- butanoate*) with an AUROC of 0.73, with a person six times more likely to have cancer if all three volatile organic compounds were present (P < 0.0001). • However, the heterogenous nature of the study cohort limits the generalisability of the results to an asymptomatic screening population.	Bond et al., 2019
• All the CRC+HR were correctly identified so as the largest part of HS (97%) and LR (90%). The authors suggest that the addition of a complementary test as SCENT A1 - that relies on a gaseous marker totally different from occult blood - should be fundamental to reduce false positive rate and that the error for low risk adenomas could be solved classifying subjects with low risk adenomas as healthy and re-screening them after two years, which could be a way of reducing colonoscopies to the benefit of the patients' health.	Zonta et al., 2017
•From the results obtained the authors suggest that, since replacing colonoscopy with FIT would reduce colonoscopies by 71%, but would lead to 30–40% missed CRC cases and 40–70% missed AA cases, faecal VOC profiles may be useful for early detection of CRC and adenomas and the timing of polyp surveillance as polypectomy led to a normalization of the VOC profile. • However, the CRC subgroup was relatively small. Gender and age did differ between groups and since age and gender have previously been shown to influence faecal VOC profiles this could have represented a possible bias of this study: nevertheless faecal VOC profiles did return to a 'normal state' after polypectomy, which suggests that these variables had not a great influence on VOC patterns.	Bosch et al., 2020

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