Published in final edited form as:

Adv Cancer Res. 2021 ; 151: 231–304. doi:10.1016/bs.acr.2021.02.008.

Genomic and Epigenomic Biomarkers in Colorectal Cancer: From Diagnosis to Therapy

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Abstract

Colorectal cancer (CRC) is the second leading cause of cancer-related deaths in the United States. Despite ongoing efforts aimed at increasing screening for CRC and early detection, and development of more effective therapeutic regimens, the overall morbidity and mortality from this malignancy remains a clinical challenge. Therefore, identifying and developing genomic and epigenomic biomarkers that can improve CRC diagnosis and help predict response to current therapies are of paramount importance for improving survival outcomes in CRC patients, sparing patients from toxicity associated with current regimens, and reducing the economic burden associated with these treatments. Although efforts to develop biomarkers over the past decades have achieved some success, the recent availability of high-throughput analytical tools, together with the use of machine learning algorithms, will likely hasten the development of more robust diagnostic biomarkers and improved guidance for clinical decision-making in the coming years. In this article, we provide a systematic and comprehensive overview on the current status of genomic and epigenomic biomarkers in CRC, and comment on their potential clinical significance in the management of patients with this fatal malignancy, including in the context of precision medicine.

Keywords

biomarkers; colorectal cancer; epigenomics; genomics; liquid biopsy

INTRODUCTION

Colorectal cancer (CRC) is the third leading cause of cancer-related deaths in the western world. Despite ongoing efforts aimed at increasing screening of individuals and improving

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strategies for early detection, ~20% of patients still present with metastatic disease at diagnosis, and ~35% of patients who undergo surgery for primary disease experience relapse (Siegel et al., 2020). Over the past three decades, the median overall survival (OS) of patients with metastatic CRC (mCRC) has gradually increased because of the implementation of combined chemotherapy regimens as well as the introduction of targeted molecular therapies against epidermal growth factor receptor (EGFR), angiogenic factors, and, more recently, *BRAF*. In addition, the discovery that immunotherapy is effective for patients with microsatellite instability-high (MSI-H) or deficient DNA mismatch-repair (dMMR) mCRC has introduced a new therapeutic option for this subset of patients (Ruiz-Bañobre, Kandimalla, & Goel, 2019).

CRC develops through sequential accumulation of genetic and epigenetic alterations in precursor lesions (mainly adenomas and serrated polyps). These precursor lesions progressively develop a more aggressive phenotype, acquiring a range of dysplastic features and eventually progressing to adenocarcinoma, the most malignant stage (Jung, Hernández-Illán, Moreira, Balaguer, & Goel, 2020). Greater understanding of the molecular pathways involved in carcinogenesis of CRC is needed to improve the detection of therapeutically targetable vulnerabilities and to develop more effective early diagnostic strategies in order to detect disease before it progresses to malignancy. To date, three pathways have been well established in CRC carcinogenesis: chromosome instability (CIN), microsatellite instability (MSI), and the serrated pathway. These pathways are characterized by multistep genetic and epigenetic alterations, involving several oncogene and tumor suppressor genes, as described below.

CIN pathway

The CIN phenotype is observed in as many as 80% of sporadic CRCs, and is characterized by chromosome changes such as somatic copy number variations, deletions, insertions, amplifications, or loss of heterozygosity (Grady & Carethers, 2008). Mutation of the *APC* gene is a key event in CRC tumors that exhibit CIN, and is considered the earliest genomic event in CRC carcinogenesis through this pathway (Powell et al., 1992). Loss of APC activity results in translocation of beta-catenin to the nucleus and subsequent activation of the WNT signaling pathway, which leads to constitutive activation of other relevant genes such as *MYC*, *CCND1*, *VEGF*, and *PPARD* (Mann et al., 1999). The WNT signaling pathway is activated in nearly all CRCs that exhibit CIN, and APC mutations have been identified in more than 80% of these cancers (Guinney et al., 2015). Later mutations that are characteristic of CRC arise in a sequential manner after mutation of *APC* as lesions progress toward adenocarcinoma. Activating mutations in *KRAS* often arise following acquisition of mutations in *APC*, and are present in 40–50% of CRCs (Santini et al., 2008). Later in the adenoma–carcinoma sequence, activating mutations in *PIK3CA* and inactivating mutations in *P53* arise in ~15% and 60% of CRCs, respectively (Baker et al., 1990; Liao et al., 2012). Loss of heterozygosity at chromosome 18q, which harbors various tumor suppressor genes from the TGF-beta pathway, such as *SMAD2* and *SMAD4*, is also found in more than 70% of CRCs (Fearon & Vogelstein, 1990).

MSI pathway

MSI is the primary mechanism for the development of colorectal tumors with a hypermutable phenotype. CRC that arises due to MSI develops more rapidly than CRC arising from CIN, having an estimated tumor development time of a few years as compared to approximately two decades for the CIN pathway (Aust, Sommer, & Baretton, 2012). CRCs arising due to MSI are often characterized by the presence of single nucleotide substitutions or insertions and deletions within microsatellite repeats. Microsatellites are short tandem repeat DNA sequences spread throughout the human genome. Because they are formed of highly repetitive sequences, they have a higher propensity for acquiring mutations than do other regions of the genome. The process of repairing errors in microsatellite repeats is tightly governed by an intact MMR system (Ruiz-Bañobre & Goel, 2019). MMR deficiency resulting from somatic or germline DNA alterations in any of the key MMR genes (*MLH1*, *MSH2*, *MSH6*, and *PMS2*), or from deletions in the *EPCAM* gene (which leads to constitutional repression of *MSH2* gene expression through promoter methylation), is the initiating event for MSI-driven carcinogenesis. The loss of *MLH1* expression due to biallelic *MLH1* promoter methylation is the key somatic event responsible for ~75%–80% of sporadic cancers with MSI. Overall, MSI is observed in ~12–15% of sporadic CRCs and in the majority of CRCs from patients with Lynch syndrome and its variants (Muir–Torre or Turcot's syndromes) (Ruiz-Bañobre & Goel, 2019). Moreover, sporadic CRCs with MSI frequently exhibit a CpG island methylator phenotype (CIMP) and harbor *BRAF V600E* mutations, while *APC* and *P53* mutations are found in a smaller proportion of MSI-H CRCs compared with MSS CRCs (Nguyen, Goel, & Chung, 2020; Rajagopalan et al., 2002; TCGA Research Network, 2012).

Serrated pathway

The serrated pathway represents the origin of ~15% of CRCs, and describes the progression of precursor malignant lesions that present as serrated polyps (Toyota et al., 1999). The term "serrated polyp" encompasses a heterogeneous spectrum of lesions that include, in decreasing order of prevalence, benign hyperplastic polyps (the most frequent subtype), sessile serrated adenomas (SSAs), and traditional serrated adenomas (TSAs; the less frequent subtype) (McCarthy, Serra, & Chetty, 2019). SSAs and TSAs are more advanced lesions that have a higher propensity for malignant progression. The earliest driving molecular event in the serrated pathway appears to be *BRAF* V600E mutation. This mutation is present in a large proportion of microvesicular hyperplastic polyps, which subsequently progress to SSA, then to SSA with dysplasia, and finally to CRC (Davies et al., 2002). Following the appearance of the *BRAF V600E* mutation, serrated tumors can follow two different routes and develop either into MSI CRC through acquisition of CIMP and methylation of the MLH1 promoter, or into microsatellite stable (MSS) CRCs (Guinney et al., 2015). In addition, *RNF43* mutation constitutes a subsequent important genomic event for the serrated pathway in both MSI and MSS CRCs (Yan et al., 2017). TSAs are less prevalent than SSAs (TSAs represent 1–7% of all serrated lesions) and therefore are not characterized as well, but they often exhibit three somewhat distinct molecular patterns: *BRAF*-mutated and CIMP-high (CIMP-H), *KRAS*-mutated and CIMP-low, or *BRAF* and *KRAS* wild-type. In addition, TSAs almost never display an MSI phenotype (McCarthy et al., 2019).

Although differences and similarities in the molecular profiles for these three pathways are well defined, a deeper temporospatial understanding of these carcinogenesis pathways will help for more rational biomarker development. Conceptually, events that occur early during carcinogenesis will serve better as potential biomarkers for the early detection of precursor lesions. In contrast, molecular profiles from both early and later lesions will be useful for identifying biomarkers for prognostication, and therapeutic benefit prediction. However, as we will discuss in this chapter, the biomarker field is complex, and it is not simple to identify robust biomarkers, thus reinforcing the importance of comprehensive molecular profiling in a clearly defined clinical context (Figure 1).

DIAGNOSTIC BIOMARKERS

Recently, the incidence of CRC has been rising in adults under the age of 50, and such CRC is referred to as early-onset CRC. This increasing incidence suggests that CRC screening programs for early detection should be expanded to a younger, asymptomatic, average-risk population. Although there is a lack of consensus within the medical community regarding this issue, some medical societies are discussing implementing currently available screening strategies across a wider range of ages, including younger individuals (USPSTF, 2020). Regarding screening strategies, these can be prioritized into several tiers: the first tier could include colonoscopy, fecal immunochemical test (FIT), and fecal occult blood test (FOBT); the second tier computed tomographic colonography, FIT-DNA, and flexible sigmoidoscopy; and the third tier capsule endoscopy. Although colonoscopy is considered the gold standard for CRC screening, it has several limitations, including invasiveness, cost, and low compliance. In contrast, FOBT and FIT, which are the most commonly used non-invasive screening tests in Europe and other Western countries, lack sensitivity and specificity compared to colonoscopy, especially for precursor lesions (Quintero et al., 2012). Given the challenges associated with these commonly used screening approaches, there is a clear need for developing more robust, non-invasive strategies to diagnose premalignant and malignant colorectal lesions.

These strategies are likely to include novel biomarkers for early detection and improved, clinically useful devices and kits that leverage existing and new biomarkers. Some molecular alterations are particularly appealing for potential use as non-invasive biomarkers, especially alterations that can be detected in stool or blood. For example, epigenetic changes, either on their own or, more recently, in combination with DNA mutations and circulating cells, have emerged as front runners for the development of early detection biomarkers in colorectal neoplasia (which includes both precursor lesions and CRC). In this section we provide a summary of genomic and epigenomic diagnostic biomarkers for CRC (Table 1), highlighting well-established assays and describing some of the most promising and novel potential biomarkers in the field.

MicroRNAs (miRNAs)

The potential of miRNAs as biomarker candidates lies in their small size, relatively limited numbers as compared to protein-coding genes/mRNAs, and stability in a variety of biological specimens, such as tissue, blood, and stool (Bovell et al., 2012; Cortez et

al., 2011; Esteller, 2011; J. Lu et al., 2005; Volinia et al., 2006). In addition, there is a wide variety of routine laboratory techniques for identifying and quantifying miRNAs (e.g., microarrays, quantitative reverse transcription PCR) in virtually all specimen types, which would further support application of miRNAs as biomarkers. Over the past decade, the number of studies investigating miRNAs in CRC has increased exponentially, and efforts to evaluate their potential as biomarkers have particularly increased. Despite this enthusiasm, only a few well-designed studies have thus far been conducted that include large patient cohorts, well-defined patient populations, and analysis of independent validation cohorts. None of the biomarkers evaluated in these studies have yet been adopted in clinical practice because of the lack of prospective randomized studies comparing them with current gold standard screening methods. Below, we discuss some of the most promising miRNAs for diagnostic purposes.

miR-92a ––The presence of miR-92a has shown remarkable utility for discriminating patients with CRC from healthy controls when measured in blood or stool samples. In plasma samples, miR-92a showed good performance for detecting CRC, with an area under the receiver operating characteristic curve (AUROC), sensitivity, and specificity of 0.89, 89%, and 70%, respectively (Ng et al., 2009). In the context of adenoma detection, serum miR-92a levels yielded an AUROC of 0.75, and corresponding sensitivity and specificity of 65% and 79%, respectively (Uratani et al., 2016). Recently, Yau et al. (Yau, Tang, Harriss, Dickins, & Polytarchou, 2019) reported the results of a meta-analysis involving 17 studies evaluating the diagnostic potential of several miRNAs, specifically in stool samples. Expression of miR-92a in stool had an AUROC, sensitivity, and specificity of 0.80, 47%, and 91%, respectively, for detecting CRC (Yau et al., 2019), and of 0.64, 43%, and 91%, respectively, for detecting adenoma (Yau et al., 2019). Intriguingly, the diagnostic accuracy of miR-92a for adenoma detection was higher using blood than stool samples (Uratani et al., 2016; Yau et al., 2019).

miR-21 ––The presence of miR-21 has demonstrated high sensitivity and specificity for detecting CRC in both blood and stool samples, although results from stool samples showed greater variability. In a meta-analysis of 11 studies conducted between 2012 and 2014 and involving almost 800 patients, the overall AUROC, sensitivity, and specificity of circulating miR-21 for blood-based early detection of CRC were 0.86, 72%, and 84%, respectively (Peng et al., 2017). For the diagnosis of colorectal adenomas, miR-21 expression in serum showed an AUROC of 0.80 with a sensitivity and specificity of 76.8% and 81.1%, respectively (Toiyama et al., 2013). In a recent meta-analysis, miR-21 expression in stool samples yielded an AUROC, sensitivity, and specificity of 0.84, 60%, and 86%, respectively for CRC (Yau et al., 2019). In the same study, the overall AUROC, sensitivity, and specificity for adenoma detection were 0.77, 60%, and 83%, respectively (Yau et al., 2019).

miRNA panels ––Although miRNA panels have been suggested as a potentially more accurate and robust diagnostic approach than the use of a single miRNA biomarker, these approaches have not yielded consistent findings among studies. For example, in some studies, the analysis of miR-21 and miR-92a in blood samples for the non-invasive diagnosis

of CRC yielded sensitivities and specificities ranging from 90% to 89%, and 90% to 96%, respectively, whereas in another cohort, the combination of these two biomarkers yielded a sensitivity of 68% and a specificity of 91% (Liu et al., 2013). In contrast, in another study, a serum-based four-miRNA panel (miR-21, miR-29a, miR-92a and miR-125b) showed an excellent performance for CRC diagnosis, with an AUROC of 0.95, sensitivity of 85%, and specificity of 99% (Liu et al., 2018). Along similar lines, a two-miRNA panel (miR-223 and miR-92a) exhibited an AUROC of 0.91, sensitivity of 97%, and specificity of 75% for detection of CRC in blood specimens from a cohort of 291 patients with CRC and 452 self-reported healthy controls (Chang et al., 2016). On the other hand, a recent meta-analysis evaluating the potential for CRC diagnosis using a combination panel of miR-21 and miR-92a detected in stool samples yielded an AUROC of 0.84, sensitivity of 0.54, and specificity of 0.88 (Yau et al., 2019). A plasma-based six-miRNA panel (miR-19a, miR-19b, miR-15b, miR-29a, miR-335, and miR-18a), when analyzed in a cohort of 300 individuals, could accurately identify healthy controls from patients with advanced colorectal neoplasms (CRC and advanced adenoma), with an AUROC of 0.92, sensitivity of 85%, and specificity of 90% (Herreros-Villanueva et al., 2019).

Recently, Duran-Sanchon et al. (Duran-Sanchon et al., 2020) proposed a new miRNA-based model to discriminate patients with advanced neoplasms (CRC and advanced adenomas) from those with non-relevant findings at colonoscopy. The authors conducted a study composed of four stages (discovery in tissue samples, technical validation in a subset of stool samples from patients of the discovery phase, clinical validation in an independent set of stool samples, and predictive modeling) and identified a panel of 3 miRNAs (miR-421, miR-130b-3p, and miR-27a-3p) that were significantly up-regulated in fecal samples obtained from participants with advanced neoplasms. Subsequently, they developed a predictive model by combining stool expression levels of miR-421, miR27a-3p, and hemoglobin. This model could identify patients with CRC with an AUROC of 0.93, sensitivity of 97%, and specificity of 43%, as compared with an AUROC of 0.67, sensitivity of 100%, and a specificity of 31% for fecal hemoglobin concentration alone. However, the accuracy of this model for identifying patients with advanced neoplasms was significantly lower when compared with participants who had non-relevant findings at colonoscopy (AUROC of 0.62, sensitivity of 62%, specificity of 58%) or normal colonoscopy findings (AUROC of 0.59, sensitivity of 43%, specificity of 63%). One of the major limitations of this study, together with the small sample size of the validation set (n = 189), was the fact that it included only individuals with a positive result from a FIT, and therefore, as the authors stated, further studies are needed in FIT-naive subjects.

Although saliva is a less explored bodily fluid for CRC diagnosis, it has recently gained attention as a potential biospecimen for non-invasive liquid biopsy because it is easy and safe to collect (Malathi, Mythili, & Vasanthi, 2014). Several studies have reported the existence of various salivary molecular indicators of local and systemic disorders, including cancer (Malathi et al., 2014). In this context, Sazanov et al. recently evaluated the expression levels of miR-21 in plasma and saliva samples from CRC patients (Sazanov, Kiselyova, Zakharenko, Romanov, & Zaraysky, 2017). miR-21 showed a plasma sensitivity and specificity of 65% and 85%, respectively, for discriminating between CRC patients (n = 34) and healthy controls (n = 34), and even greater sensitivity (97%) and specificity (91%)

in saliva. The high sensitivity and specificity values for salivary miR-21 reflect the potential of this saliva-based test for CRC diagnostic purposes. Another study recently identified a set of five miRNAs (miR-186-5p, miR-29a-3p, miR-29c-3p, miR-766-3p, and miR-491-5p) that were significantly upregulated in saliva from CRC patients (Rapado-González et al., 2019). Interestingly, the combined analysis of these five miRNAs yielded an AUROC of 0.754, sensitivity of 72%, and specificity of 67% for differentiating CRC patients (n = 51) from healthy individuals (n = 37). Unfortunately, and in line with Sazanov et al. study (Sazanov et al., 2017), the authors did not explore the accuracy of the panel for identifying adenoma or adenoma or CRC. Furthermore, although both saliva-based studies showed promising results, they must still be evaluated in validation trials in larger retrospective and prospective cohorts.

In general, studies conducted to date on potential miRNA biomarkers share two main characteristics that limit their external validity—relatively small sample size and the lack of detailed clinical-pathological information on the CRC population evaluated in the study. These limitations must be addressed, and the miRNA biomarkers or panels evaluated in randomized prospective studies before they can be translated to the clinic.

Long non-coding RNAs (lncRNAs)

In recent years, lncRNAs have gained increasing attention as potential biomarkers for CRC. In contrast to miRNAs, the precise number of functional lncRNAs remains unclear, because of ongoing discovery of new lncRNAs. Despite promising early results as biomarkers, research on lncRNAs still focuses mostly on discovery of new lncRNAs and their functions. Studies exploring the potential of lncRNAs as biomarkers in large patient cohorts are still elusive. Although tissue-derived lncRNAs have been the primary analyte in the most studies, a few studies have also analyzed blood-derived lncRNAs to determine their potential as non-invasive biomarkers. The lncRNA HOTAIR has been analyzed in both serum and tissues and is upregulated in early stages of CRC (Svoboda et al., 2014; Zhao, Song, Zhang, Kuerban, & Wang, 2015). Along similar lines, upregulation of the lncRNA colon cancer-associated transcript 1 (CCAT1) in tumor tissue and blood also seems to be an early event during colorectal carcinogenesis (Alaiyan et al., 2013; X. He et al., 2014; Nissan et al., 2012; Ozawa et al., 2017). Evaluation of *HOTAIR* and *CCAT1* in a panel combining both markers revealed that upregulated expression of both markers had greater sensitivity and specificity for diagnosing CRC in plasma samples than of either lncRNA separately (Zhao et al., 2015). These two lncRNAs were also recently evaluated in a stool-based lncRNA extended panel (Gharib et al., 2020). The study population consisted of 150 participants, including a training and a validation set, and a group of 30 subjects with colon polyps. Expression levels of lncRNAs were evaluated by quantitative real-time PCR. To design a predictive panel, the investigators selected 10 significantly dysregulated lncRNAs, including *CCAT1*, *CCAT2*, *H19*, *HOTAIR*, *HULC*, *MALAT1*, *PCAT1*, *MEG3*, *PTENP1*, and *TUSC7*. The diagnostic performance of the panel in terms of distinguishing CRC as compared to healthy tissue yielded an AUROC of 0.8554 in the training set and 0.8465 in the validation set. The panel's AUROC for early-stage CRC (I-II TNM stages) was 0.7871 in the training set and 0.8121 in the validation set, and for advanced CRC (III-IV TNM stages) the AUROC was 0.9281 in the training set and 0.9236 in the validation set. The corresponding AUROC values for

CRC vs. colon polyp were 0.9228 (I-IV TNM stages), 0.9042 (I-II TNM stages), and 0.9362 (III-IV TNM stages). Again, in addition to the small sample size of the cohort included in the study, there is a lack of information regarding the clinical-pathological characteristics of the polyps analyzed in the study beyond the sex and age of the corresponding subjects.

Histone modifications

Although some studies have evaluated histone modifications as non-invasive diagnostic biomarkers, the data are still preliminary and further studies are required to determine the feasibility and utility of this approach. Methylation levels of histone 3 at lysine 4 are higher in CRC and in adenomas than in normal colonic mucosa, and acetylation levels of histone 3 at lysine 27 and histone 4 at lysine 12 are markedly greater in CRC compared to normal colonic mucosa (Ashktorab et al., 2009; Karczmarski et al., 2014; Nakazawa et al., 2012). In an attempt to identify non-invasive biomarkers and to take advantage of the stability of nucleosomes within the circulation, some studies have demonstrated the potential of using histone modifications in circulating nucleosomes as CRC diagnostic biomarkers. Chromatin immunoprecipitation studies revealed that reduced levels of trimethylation of histone 3 at lysine 9 and of histone 4 at lysine 20 in circulating nucleosomes were present in patients with CRC as compared with healthy control individuals (Gezer et al., 2013, 2015). Although histone modifications have a central role in cancer pathogenesis, their lack of cancer-specificity and various technical limitations associated with their use as quantitative analytes are, to date, some of the major obstacles to their use as non-invasive biomarkers.

DNA methylation

One of the first epigenetic alterations to be discovered in human cancer was the low level of DNA methylation in tumors as compared to normal tissue (Feinberg & Vogelstein, 1983). The loss of methylation is primarily due to hypomethylation of repetitive DNA sequences (e.g., LINE, SINE and Alu elements) and demethylation of coding regions and introns (Feinberg & Tycko, 2004). During carcinogenesis, the degree of hypomethylation of genomic DNA increases as a lesion progresses from a benign proliferation of cells to an invasive cancer (Fraga et al., 2004). In addition to hypomethylation of genomic DNA, hypermethylation of specific CpG islands in the promoter regions of tumor-suppressor genes is also a major event in the origin of many cancers. All of these changes in DNA methylation modulate gene expression: gene silencing via hypermethylation of CpG islands in promoters, gene activation via hypomethylation of CpG-poor gene promoters, and oncogene overexpression via hypermethylation of gene bodies (Yang et al., 2014).

DNA methylation profiling provides several advantages over somatic mutation analysis for detecting cancer, including greater clinical sensitivity and dynamic range, multiple detectable methylation target regions, greater prevalence in precancerous lesions and early stage cancers, and presence of multiple altered CpG sites within each targeted genomic region (Heyn & Esteller, 2012; Laird, 2003). Importantly, altered methylation is also seen as a field effect in colonic mucosa that predisposes normal tissue to neoplastic transformation (Luo, Yu, & Grady, 2014). In addition, the alterations in CpG methylation are relatively constant in each type of cancer, whereas there are usually no predominant somatic mutations. Despite the relatively high frequency of somatic mutations in cancers,

the patterns of mutations are highly heterogeneous in individual patients, making somatic mutations less than ideal markers for early detection of cancers (Kandoth et al., 2013). Given favorable characteristics of DNA methylation markers and strong supporting evidence of their utility as biomarkers, several assays using DNA methylation biomarkers have been commercialized during the last decade, are used in current clinical practice, or have even entered clinical guidelines.

One of the most widely studied non-invasive DNA methylation biomarkers for CRC diagnosis is methylation of the *SEPT9* gene in plasma. *SEPT9* encodes septin 9, a GTP-binding protein involved in actin dynamics, cytoskeletal remodeling, vesicle trafficking, and exocytosis. Multiple studies have evaluated the diagnostic accuracy of this methylation biomarker in large cohorts of patients with CRC, and in these studies sensitivity and specificity ranged from 48% to 90% and 73% to 97%, respectively (Bergheim et al., 2018; Church et al., 2014; Fu et al., 2018; Song, Jia, Peng, Xiao, & Li, 2017; Song, Peng, et al., 2017; Tänzer et al., 2010; Wu et al., 2016). In 2016, this biomarker (commercialized as the Epi proColon test by Epigenomics) was approved by the US FDA as the first molecular blood-based assay for CRC screening. This test is not recommended for routine CRC screening, and represents an alternative only for individuals who refuse other screening modalities such as FOBT, FIT, flexible sigmoidoscopy, or colonoscopy. In one of the largest studies in which *SEPT9* plasma methylation was analyzed using Epi proColon test 2.0 methodology, this biomarker yielded an overall sensitivity of 73.7% and specificity of 97% in a large cohort (300 patients with CRC and 568 healthy control individuals) (He et al., 2018). However, findings of both this study and a 2017 meta-analysis agree that this test performed statistically significantly better in patients with advanced stage (III-IV) CRC than in those with early-stage (I–II) CRC (He et al., 2018; Song, Jia, et al., 2017). The Epi proColon test requires only a 10 ml blood sample, out of which ~3.5 ml of plasma is used to obtain free circulating DNA. Methylated *SEPT9* DNA within the plasma is amplified via PCR, along with β-actin as an internal control. Because this screening test only requires a blood draw, which can be performed in conjunction with other routine laboratory exams, this test is attractive to patients and has high compliance rates.

In an effort to further improve the diagnostic accuracy of Epi proColon test, which, as noted above, is suboptimal for early-stage CRC, other groups have attempted to combine *SEPT9* plasma methylation levels with additional biomarkers, such as FIT or other plasma-based methylated genes (for example, SHOX2 and ALX4) (Bergheim et al., 2018; Tänzer et al., 2010; Wu et al., 2016). Wu et al. evaluated a combination of *SEPT9* plasma methylation, FIT and serum carcinoembryonic antigen (CEA) levels for CRC screening that included all CRC stages, and achieved an overall sensitivity of 97.2% (Wu et al., 2016). Although the majority of the data for this test were obtained from case–control or cohort studies, *SEPT9* plasma methylation has also been analyzed in asymptomatic, intermediate-risk populations of healthy individuals to further assess its diagnostic potential as an alternative to FIT or colonoscopy (Church et al., 2014). However, when Song et al. (Song, Jia, et al., 2017) compared the data from this study with that from a meta-analysis of FIT in a similar population, they found lower sensitivity (68% vs. 79%) and specificity (80% vs. 94%) for *SEPT9* plasma methylation than for FIT. One of the major limitations of the *SEPT9* plasma methylation biomarker is its poor sensitivity (ranges from 7.9% to 38.7%) for identifying

precursor lesions (adenomas) (Fu et al., 2018; He et al., 2018; Song, Peng, et al., 2017; Wu et al., 2016). *SEPT9* plasma methylation levels exhibited the highest sensitivity (83.3%) in a subgroup of patients with villous adenoma, suggesting this biomarker has potential for identifying advanced adenomas (Song, Peng, et al., 2017). However, the number of patients included in this study was small (n = 18), highlighting the need for the development of more ambitious approaches for detecting precancerous lesions and early-stage CRC (Song, Peng, et al., 2017).

Methylation of the *VIM* gene has also been evaluated as a non-invasive biomarker for CRC diagnosis. *VIM* encodes the intermediate filament protein vimentin, which, together with microtubules and actin microfilaments, constitutes the cytoskeleton. The diagnostic accuracy of VIM methylation for CRC appears be greater for stool samples than for blood specimens. For example, the sensitivity and specificity of *VIM* methylation were 81% and 95%, respectively, in stool samples, with similar values seen across CRC stages (Baek et al., 2009; Carmona et al., 2013; W.-D. Chen et al., 2005; Fu et al., 2018; Itzkowitz et al., 2007; Kisiel et al., 2013; Li et al., 2009; H. Lu et al., 2014). In contrast, in plasma samples, *VIM* methylation had a sensitivity and specificity of 59% and 93%, respectively, with substantially greater sensitivity in advanced disease stages(Li et al., 2009). Given the performance of *VIM* methylation in stool samples, this biomarker has been commercialized as the ColoSure test (LabCorp) (Ned, Melillo, & Marrone, 2011), but has not yet obtained US FDA clearance or approval for use as a CRC screening test.

In contrast to *VIM* methylation, methylation of *secreted frizzled-related protein 2* (*SFRP2*) has shown more promising results for adenomas. *SFRP2* methylation has been studied for detection of precancerous lesions (both adenomas and hyperplastic polyps). The sensitivity of plasma-based *SFRP2* methylation for detecting adenomas ranged from 6.4% to 81.1%, and the specificity from 73% to 100% (Barták et al., 2017; Tang et al., 2011), while the sensitivity and specificity of stool-based *SFRP2* methylation ranged from 27.8% to 76% and from 55% to 100%, respectively (Glöckner et al., 2009; Park et al., 2017; Zhang, Zhu, Wu, Zhang, & Qi, 2014).

As high-throughput tools for molecular development continue to improve, new studies are emerging to evaluate the potential of DNA methylation biomarkers through the use of more comprehensive approaches. In 2020, Luo et al (Luo et al., 2020) identified CRC-specific methylation signatures by comparing CRC tissues to normal blood leukocytes. Using machine learning algorithms, the authors developed a predictive diagnostic model (cd-score) by using cell-free DNA (cfDNA) samples from a cohort of 801 patients with CRC and 1021 normal controls. In the discovery dataset, the cd-score discriminated patients with CRC (n = 528) from normal controls (n = 674) with an AUROC of 0.96, sensitivity of 87.5%, and specificity of 89.9%. This accuracy was confirmed in a validation dataset of 273 CRC patients and 347 normal controls, yielding an AUROC of 0.96, sensitivity of 87.9%, and specificity of 89.6%. With the goal of making screening methods for cancer as simple as possible, the authors prospectively investigated the efficiency of using the methylation status of CpG site cg10673833 for detecting CRC as well as precancerous lesions in high-risk populations. To identify an appropriate population, all enrolled participants (n = 16,890) were invited to take a cancer risk assessment using an established Clinical Cancer Risk

Score System (H. Chen et al., 2019). This identified 1493 participants (ages range: 45–75 years) who were considered to be at high risk for CRC. These high-risk subjects were subsequently scheduled to undergo screening colonoscopy and were recruited into the study to undergo methylation profiling at the time of the screening. The cg10673833 methylation test identified 19 of 21 participants with CRC and 7 of 8 participants with CRC *in situ* (diagnosis as high-grade dysplasia), with an AUROC of 0.90, sensitivity of 89.7%, and specificity of 86.8%. The positive predictive value and negative predictive value were 0.118 (95% CI, 0.101 to 0.138) and 0.998 (95% CI, 0.993 to 0.999), respectively. For advanced precancerous lesions, the sensitivity was 33.3%, much higher than the positivity rate for subjects who did not have CRC or advanced precancerous lesions (12.1%).

More recently in 2020, Liu et al. (Liu et al., 2020) reported the results of a prospective case-controlled substudy from the Circulating Cell-free Genome Atlas (CCGA; NCT02889978) and the STRIVE (NCT03085888) projects. This substudy assessed the ability of targeted methylation analysis of circulating cfDNA to detect and localize multiple cancer types across all stages at high specificity. The CCGA study was designed to determine if genome-wide cfDNA sequencing in combination with machine learning algorithms could detect and localize a large number of cancer types at sufficiently high specificity to be considered for a general population-based cancer screening program. In previous discovery work in a first CCGA substudy, whole-genome bisulfite sequencing to interrogate genome-wide methylation patterns outperformed whole-genome sequencing and targeted sequencing approaches to interrogate copy-number variants and single-nucleotide variants (SNVs)/small insertions and deletions, respectively) (Liu et al., 2018; Oxnard et al., 2019). In addition, targeted sequencing with SNV-based classification was significantly confounded by clonal hematopoiesis of indeterminate potential (Swanton et al., 2018), suggesting that such a test would require concurrent sequencing of white blood cells to return accurate results. The cfDNA methylation-based classifier achieved consistently high overall specificity between the cross-validated training and independent validation sets (>99%) across all cancer types (>50) with a sensitivity of ~55%. Incorporation of clinical baseline demographic information and blood sample quality metrics to the model did not sufficiently improve sensitivity (<10%). Although this study did not evaluate the accuracy specifically across CRC stages, both the training and validation cohorts included patients with CRC (training, n = 122 with CRC; validation, n = 53 with CRC) among all the cancer types included (Liu et al., 2020). Because not all participants with cancer were asymptomatic, additional studies in an asymptomatic screening population are currently ongoing.

Similar efforts to identify methylation-based biomarkers are ongoing that are specifically focused on gastrointestinal (GI) cancers. One of the most promising tests is the EpiPanGI-Dx (Kandimalla et al., 2020), a cfDNA methylation fingerprint for the early detection of several GI cancers, including CRC, esophageal squamous cell and adenocarcinoma (ESCC and EAC), gastric cancer (GC), hepatocellular carcinoma (HCC), and pancreatic ductal adenocarcinoma (PDAC). Using a tissue-based genome-wide DNA methylation analysis of these GI cancers to select the most informative differentially methylated regions (DMRs), Kandimalla et al. (Kandimalla et al., 2020) developed a novel three distinct categories of DMR panels and sequenced 300 plasma specimens from all GI cancers, as well as age-matched healthy controls, with 40X coverage. They found 1) cancer-specific biomarker

panels with AUROC values of 0.98 (CRC), 0.94 (ESCC), 0.90 (EAC), 0.90 (GC), 0.98 (HCC), and 0.85 (PDAC); 2) a pan-GI cancer biomarker panel that detected all GI cancers with an AUROC of 0.90; and 3) a multi-cancer prediction panel, EpiPanGI Dx, with a prediction accuracy around 0.85 for most GI cancers. All three groups of DMR panels, after being trained and tested using the cfDNA cohorts, achieved excellent diagnostic accuracy as indicated by AUROC values ranging from 0.74 to 0.98, even for each of the early-stage GI cancers. This study represents the first specific GI cancer cfDNA methylation test that has potential to be applied in the clinic for the early detection of all GI cancers.

Recently, Chen et al. (Chen et al., 2020) described a new blood-based cancer diagnostic test based on cfDNA methylation analysis, the PanSeer assay. Using publicly available microarray and whole genome bisulfite sequencing data from The TCGA and genomic regions known to be cancer-related in the literature, as well as internal reduced representation bisulfite sequencing data from a variety of cancer tissues, the authors compiled a targeted panel of 595 genomic regions for further interrogation in plasma samples. Because tumor DNA tends to be rare in plasma, especially in patients with early stage cancer, and because conventional methods for constructing sequencing libraries that incorporate bisulfite conversion and double-stranded ligation typically have a high DNA loss rate (Aigrain, Gu, & Quail, 2016), the authors used the Singlera library construction method. This method uses semi-targeted PCR, which requires only a single ligation event and a single PCR primer per amplicon, allowing single-molecule counting at a higher molecular recovery rate than conventional methods (Gansauge & Meyer, 2013; Zheng et al., 2014). Preliminary results of PanSeer on plasma samples from 605 asymptomatic individuals, 191 of whom were later diagnosed with stomach, esophageal, colorectal, lung, or liver cancer within four years of blood draw, demonstrated an accuracy of cancer diagnosis of 95%. Using a similar approach, another plasma-based assay of circulating tumor methylated DNA (ColonES, Singlera Genomics) showed impressive preliminary results, with sensitivities of 91%, 97%, and 94% for detecting adenoma, stage I CRC, and stage II-IV CRC, respectively (Singlera Genomics, 2020). The reported preliminary specificity of the test is 99%. Although additional studies are needed in larger population-based cohorts, DNA methylation genome-wide approaches are leading candidates for biomarker panels and seem to be the future for molecular blood-based diagnostics for CRC and many other tumor types.

Multi-modal approach

Although epigenetic alterations are among the most well-characterized CRC diagnostic biomarkers, considering that CRC evolves through acquisition of many genetic and epigenetic alterations, combinations of multiple types of molecular and cellular biomarkers have also been evaluated to see if they enable more accurate detection of colorectal polyps and CRC. Indeed, several promising combinations have been proposed to improve diagnostic performance. Among the tests that combine different biological approaches, currently, only Cologuard (Exact Sciences), a stool-based multi-target panel, is approved by the US FDA for CRC screening. Cologuard tests three genomic biomarkers (seven point mutations in *KRAS* and methylation status of *NDRG4* and *BMP3*) and includes a quantitative enzyme-linked immunosorbent assay for the presence of hemoglobin. In a study of ~10,000 intermediate-risk individuals, Imperiale et al. (Imperiale et al., 2014) found

that Cologuard had a significantly higher sensitivity for CRC detection compared to FIT (92.3% vs. 73.8%). More importantly, the sensitivity for detecting advanced precancerous lesions (advanced adenomas or sessile serrated lesions ≥1 cm) was almost two-fold higher for Cologuard than for FIT (42.4% vs. 23.8%). However, this increased sensitivity came at the cost of lower specificity (89.8% [Cologuard] vs. 96.4% [FIT]) in patients with negative colonoscopy (Imperiale et al., 2014). Given these promising results, Cologuard has been included in the United States Preventive Services Task Force and National Comprehensive Cancer Network (NCCN) guidelines as a CRC screening option at 3-year intervals, which is on equal standing with the other traditional screening options (colonoscopy, FIT, and FOBT) (USPSTF). Ongoing trials are prospectively evaluating the utility of Cologuard in other scenarios, such as longitudinal testing in an average-risk population at 3-year intervals (NCT02419716), in an average-risk population aged 45 to 49 (NCT03728348), and with FIT to model stool-based molecular surveillance approaches to inform health policy decisions (NCT02715141).

At the 2020 ASCO Virtual Scientific Program, Friedland et al. (Friedland et al., 2020) presented interim results of a study of a new promising diagnostic test, the FirstSight assay. FirstSight is a blood-based assay that evaluates three biomarkers: 1) circulating GI epithelial cells, 2) validated somatic oncogene and tumor suppressor mutations, and 3) methylation of *SEPT9* in cfDNA. Based on results for these three biomarkers, the assay calculates a diagnostic score (CMx Score). FirstSight was evaluated in 354 patients who had no prior history of CRC and were scheduled to undergo a colonoscopy. Eighty-six percent of the patients were asymptomatic and 14% had reported symptoms or a positive-FIT result. Prior to colonoscopy, patient blood samples were analyzed using the FirstSight assay. In this study, FirstSight achieved specificity of 90% and sensitivity of 100% for detecting CRC and sensitivity of 75.5% for detecting advanced adenomas. Overall, the test had a sensitivity of 79.7% for detecting advanced adenomas and CRC. In addition, there was a significant association between CMx score and polyp size (F value = 5.80, p-value = 0.017), but not for DNA mutation (F value = 1.29, p-value = 0.263) or methylation status (F value = 0.34, p-value = 0.560) and polyp size. Similarly, there was a significant association between CMx score and the number of polyps (F value = 23.71, p-value < 0.0001), but again not for DNA mutation (F value = 1.57, p-value = 0.210) or methylation status (F value = 1.34, p-value = 0.248) and the number of polyps. These results suggest that CMx score, which incorporates circulating epithelial cells, provided predictive information for polyp sizes and number above and beyond DNA mutation and methylation status alone.

Analysis of cancer-associated mutations in cfDNA has been also explored in conjunction with plasma protein analysis. In 2018, Cohen et al. (Cohen et al., 2018) reported on a new blood test, called CancerSEEK, for cancer diagnosis. CancerSEEK evaluates plasma levels of eight proteins (cancer antigen 125, CEA, cancer antigen 19-9, proactin, hepatocyte growth factor, osteopontin, myeloperoxidase, and tissue inhibitor of metalloproteinases) together with the presence of mutations (SNVs or INDELs) in 1933 distinct genomic sites. The presence of a mutation in an assayed gene or elevated levels of any of these proteins would classify a patient as positive. In this study, 1005 patients with nonmetastatic, clinically detected cancers of the ovary, liver, stomach, pancreas, esophagus, colorectum, lung, or breast were evaluated using CancerSEEK. The median sensitivity of CancerSEEK

among the eight cancer types evaluated was 70% (78% for stage III, 73% for stage II, and 43% for stage I cancers). The specificity of CancerSEEK was >99% across cancers of all stages. Moreover, the test could localize the anatomic site of origin of the cancer in a median of 83% of the patients. Given that driver gene mutations are not usually tissue-specific, the vast majority of the localization information was derived from protein markers. The accuracy of prediction for primary cancer prediction varied by tumor type, but the test was most accurate for CRC (84%).

Although it is beyond the scope of this article, many other approaches are being evaluated for diagnosing polyps and CRC in various bodily fluids. For example, PolypDx (Deng et al., 2017), is a metabolomic-based urine test for detecting adenomas. Although PolypDx was originally designed on a nuclear magnetic resonance platform, it was subsequently developed as a mass spectrometry-based urine metabolomic test to detect three metabolites: succinic acid, ascorbic acid, and carnitine. Detection of these metabolites used in combination with three clinical features (age, sex, and smoking status) yielded higher sensitivity (43%) and similar specificity (91%) to two different FITs (sensitivities of 18% and 21%, and specificities of 97% and 92%, respectively) for detecting hyperplastic polyps and adenomas (Deng et al., 2017).

Primary tumor of origin

Tissue-based markers are also being developed to guide CRC diagnosis, and there are various molecular platforms that can help define the primary tumor of origin in cases classified as cancer of unknown primary (CUP). Initial work to identify original tumor sites was performed using algorithms based on genome-wide gene expression profiles. This work achieved an accuracy of 88% for primary tumors within 56 categories and of 78% for CUP (Ojala, Kilpinen, & Kallioniemi, 2011). However, despite an accuracy rate of 78.6% for site prediction *in silico*, results suggested that comprehensive genome-wide profiling of gene expression by microarray analysis might not yet be suitable for clinical application in patients with CUP. Currently, two assays, a 92 gene RT- PCR assay and a 64 tissue-specific miRNA assay are commercially available for prediction of tumor sites of origin (Erlander et al., 2011; Meiri et al., 2012). Although comprehensive gene expression profiling can be influenced by irrelevant variants, and dimension reduction is necessary because of the large number of variables, the 92 gene RT-PCR assay might be more informative as a result of the limited number of biologically relevant genes on which it is based (Ma et al., 2006). Other methods that are based on miRNAs, which can regulate the expression of large numbers of protein-coding genes, also have achieved a high accuracy rate (Rosenfeld et al., 2008; Varadhachary et al., 2011). In this regard, recently a miRNA-based tissue signature has been proposed for the specific diagnosis of CRC with mucinous differentiation (Ruiz-Bañobre et al., 2020). Although data are still very preliminary and validation studies are needed, this signature could be especially useful for classifying CRC in those cases where a small tumor biopsy is not able to capture the whole histological nature of the tumor (Moran et al., 2016).

Classifiers of cancer type based on DNA methylation profiles, which can overcome the inherent limitations of working with RNA in formalin-fixed paraffin-embedded (FFPE) tissue samples, are also being developed. One such platform, the EPICUP, showed a

specificity of 99.6%, a sensitivity of 97.7%, a positive predictive value of 88.6%, and a negative predictive value of 99.9% in a validation set of 7691 tumors (Moran et al., 2016). DNA methylation profiling predicted a primary cancer of origin in 188 (87%) of 216 patients with CUP. In addition, patients who received a tumor type-specific therapy consistent with their EPICUP diagnoses showed improved OS compared with those who received empiric therapy. Although this approach shows promise for achieving a real impact in daily clinical practice, the efficacy of any given assay must be demonstrated in randomized clinical trials, a scenario that has been a serious limitation in the majority of previous studies (Hainsworth et al., 2012).

PROGNOSTIC AND PREDICTIVE BIOMARKERS

During recent decades, in parallel with the development of anti-neoplastic strategies, substantial efforts have been devoted to identifying biomarkers that can help in decision-making in the clinic. Although biomarkers likely have complex associations with patient outcomes, robust discrimination between prognostic and predictive biomarkers is challenging in clinical studies that aim to evaluate therapeutic benefits. To demonstrate that a biomarker is predictive of treatment benefit, biomarker status for all patients must be obtained, including patients who were treated with the agent of interest and untreated patients, preferably in the context of a randomized study. A formal statistical test of the treatment-by-biomarker interaction should be significant. To establish whether a marker is purely prognostic, it must be shown that there is significant association between the biomarker and outcome, regardless of treatment, and that treatment effects do not depend on the biomarker. Finally, a biomarker may have both predictive and prognostic implications (Ballman, 2015). This prognostic-predictive complexity is partly driven by the search for more effective therapies for patients who have a poor prognosis if treated with standard therapies (Sveen, Kopetz, & Lothe, 2020). Therefore, genetic alterations that are classically associated with CRC that has a poor prognosis are now targets of some of the most promising targeted-therapies and, consequently, are also considered response prediction biomarkers for these cancers. Because the number of prognostic and/or predictive biomarkers is rising, in this article we have chosen to focus on biomarkers that are already established in clinical practice or those with a very promising future. Our discussion is structured to follow the typical clinical path, from the adjuvant to the palliative setting.

Adjuvant setting

Regarding biomarkers used in the adjuvant setting, only MSI-H/dMMR is thus far established as a useful prognostic indicator. The first reports of a favorable prognostic association of MSI in CRC were published in 1993 (Lothe et al., 1993; Thibodeau, Bren, & Schaid, 1993). This association has since been confirmed for OS and disease-free survival by metanalyses comparing data from 1277 (Popat, 2004) and 12782 (Guastadisegni, Colafranceschi, Ottini, & Dogliotti, 2010) patients with primary MSI-H CRCs to those with MSS CRCs. Retrospective analyses of data from randomized trials showed this association also applies to patients with stage II and III colon cancer patients who have not received adjuvant chemotherapy (Hutchins et al., 2011; Sargent et al., 2010). A corresponding association of MSI with clinicopathological features has also been reported for prognosis;

patients with proximal tumors have a greater favorable prognostic association of MSI than those with distal tumors (Sinicrope et al., 2013). Various studies report stronger effects of MSI in stage II CRC (Klingbiel et al., 2015; Merok et al., 2013), similar prognostic associations across stages II and III CRC (Benatti et al., 2005; Sinicrope et al., 2011), or an even a stronger effect in stage III CRC (Samowitz et al., 2001). However, no statistically significant interactions with clinicopathological features, including tumor stage, were observed in a pooled analysis of data from 7,326 stage II or III colon cancers (Dienstmann et al., 2017), suggesting that the prognostic value of MSI is independent of disease stage.

The prognostic implications of MSI after adjuvant treatment might be confounded by also having a predictive value for 5-FU-based chemotherapy, thus illustrating prognostic–predictive biomarker complexity (Vilar & Gruber, 2010). Loss of MMR function could result in failure to recognize and respond to incorporation of 5-FU into tumor DNA (Jo & Carethers, 2006). However, many retrospective analyses have shown inconsistent results regarding the effects of 5-FU-based regimens in patients with MSI-H tumors (Vilar & Gruber, 2010). Most studies that compared the effects of 5-FU-based chemotherapy to no treatment failed to show significant improvements in OS or disease-free survival (DFS) in patients with MSI-H/dMMR tumors, even though 5-FU-based chemotherapy significantly improved the outcomes of patients with MSS or MMR-proficient colon cancers or CRCs (Benatti et al., 2005; Carethers et al., 2004; Hutchins et al., 2011; Lanza et al., 2006; Sargent et al., 2010). Only two of the seven studies revealed a statistically significant interaction between MSI status and the response to chemotherapy (Jover et al., 2009; Ribic et al., 2003). A meta-analysis including data from 396 patients with MSI-H CRCs revealed a substantial degree of heterogeneity with respect to the effects of 5-FU-based chemotherapy, and a lack of benefit could not be definitively confirmed (Guastadisegni et al., 2010).

MSI status is not predictive of a lack of benefit from the combination chemotherapies typically used in patients with CRC. The addition of oxaliplatin to 5-FU-based adjuvant chemotherapy regimens improves patient survival (André et al., 2004), and this effect is also seen among patients with dMMR stage II or III CRC (Cohen et al., 2020; Flejou et al., 2013; Tougeron et al., 2016). Furthermore, patients with dMMR tumors have better survival outcomes after treatment with FOLFOX compared with those with MMR-proficient tumors (in one study the association was tumor location dependent) (Gavin et al., 2012; Sinicrope et al., 2013, 2014; Zaanan et al., 2011), consistent with the favorable prognostic effect of MSI. Paradoxically, metastatic MSI-H tumors are aggressive and are associated with inferior PFS and OS outcomes relative to metastatic tumors for MSS CRC (Heinemann et al., 2018; Kim et al., 2016; Tran et al., 2011; Venderbosch et al., 2014). Although other reports indicate that no prognostic associations exist and additional data are needed, the survival benefits associated with primary MSI-H CRCs seem to be lost in the metastatic setting (Jin et al., 2018; Margonis et al., 2018). On the other hand, although neoadjuvant therapy is not currently a standard therapeutic strategy in CRC, the combination of nivolumab plus ipilimumab has yielded encouraging results in CRC patients in this setting, especially among those with MSI-H/dMMR tumors. In a recent study reporting the results of a phase II NICHE trial (NCT03026140) (Chalabi et al., 2020), among 35 patients who received both nivolumab and ipilimumab and were evaluable for efficacy (two patients with pMMR

CRCs were deemed ineligible post-surgery), 100% of those with dMMR CRC and 27% with pMMR CRC had pathologically-relevant responses. The majority of responders (19/20 with dMMR CRC and 3/15 with pMMR CRC) had major pathologically relevant responses, including 12 complete responses. Subsequent organoid-based investigations suggested that the lower response rates of patients with pMMR CRCs reflected a lack of highly immunogenic T cell antigens, as opposed to other tumor-intrinsic factors (Chalabi et al., 2020).

In 2012, Ebert et al. (Ebert et al., 2012) proposed the epigenetic biomarker methylation of *TFAP2E* in tumor tissue specimens as a promising negative predictive biomarker for 5-FU-based chemotherapy. The authors suggested the lack of response to 5-FU was probably mediated by DKK4, a downstream effector of *TFAP2E* that is implicated in chemoresistance to 5-FU in CRC cell lines (Xi, Formentini, Nakajima, Kornmann, & Ju, 2008; Xi, Nakajima, Schmitz, Chu, & Ju, 2006). Although the expectations were high, this study presented various important limitations for its clinical usefulness. Primarily, it interrogated a relatively small cohort of patients with CRC (n = 220), which was actually a combined collection of patients with non-metastatic and mCRC from four different prospective trials that were analyzed together as one large cohort. Second, only a very small subset of the entire cohort was analyzed for methylation and expression of *TFAP2E*, as well as expression of the DKK4 protein. Third, although all patients received 5-FU–based chemotherapy, their regimens differed and also included either oxaliplatin, irinotecan and/or cetuximab, or radiotherapy (chemoradiotherapy). Unfortunately, in a subsequent study the negative predictive potential of *TFAP2E* was not confirmed (Murcia et al., 2018). Although methylation of *TFAP2E* intron 3 is tumor-related, it did not correlate with loss of TFAP2E protein expression, and, more importantly, *TFAP2E* methylation did not play any role in predicting response to 5-FU-based chemotherapy in patients with CRC.

In January 2016, Dalerba et al. (Dalerba et al., 2016) reported a novel approach to the problem of identifying patients with CRC who might benefit from adjuvant chemotherapy. They reasoned that the presence of a stem cell-like state could be associated with more aggressive tumors, and performed a bioinformatic search for a gene-expression signature obtained from populations of stem cells and progenitor cells. By mining a large, preexisting database of CRCs, the authors identified a panel of 16 genes for which expression was inversely related to the stem cell-like state. *CDX2* was the most clinically actionable of these genes because it could be detected using immunohistochemical analysis. The investigators performed a series of validation analyses involving multiple independent data sets, which is a necessary approach for data-mining research. The first analysis confirmed an inverse relationship between *CDX2* expression and patient outcomes in which *CDX2*-negative tumors (present in 6.9% of patients in the discovery data set) were associated with significantly lower rates of 5-year DFS compared to *CDX2*-positive tumors (41% vs. 74%). A validation dataset was created by immunohistochemical analysis to confirm CDX2 protein expression. Analysis of the validation dataset, in which 13% of the patients had CDX2-negative tumors, confirmed 5-year survival rates of 48% among patients with CDX2-negative tumors vs. 71% among patients with CDX2-positive tumors. However, this finding was not sufficient to prove that the subgroup of patients with a worse natural history would benefit from adjuvant chemotherapy; instead they might be less responsive

to treatment. To address this, the investigators focused on patients with stage II CRC and confirmed that CDX2-negative cancers were associated with significantly lower rates of survival than were CDX2-positive cancers (48–51% vs. 80–87%). Finally, they used an expanded database to demonstrate that the benefit observed with the administration of chemotherapy in terms of disease-free survival in CDX2-negative cohorts was superior to that observed in CDX2-positive cohorts in both the stage II subgroup and the stage III subgroup. However, despite the rigorous bioinformatics analysis, the number of patients who had stage II CRC and *CDX2*-negative tumors was small, and so this result is not definitive. This retrospective study requires prospective confirmation with uniform interventions. In addition, the immunohistochemical analysis was performed on tissue microarrays, which facilitated rapid throughput but may have underestimated the heterogeneity of CDX2 expression throughout the tumor. Furthermore, these findings raise the question of what mechanism might be at work in silencing *CDX2*; the answer to this question could lead to the discovery of new approaches to treating the fundamental problem. Meanwhile, given these limitations, use of CDX2 has not entered clinical practice to indicate (or not) if adjuvant chemotherapy should be given to stage II CRC patients.

On the other hand, based on the cfDNA methylation analysis, Luo et al. (Luo et al., 2020) recently developed a combined score (cp-score) for the prognostic purposes in CRC. This score also includes clinical characteristics such as age, gender, primary tumor site, and TNM stage. Using machine learning algorithms and proportional hazards regression methods, the authors conducted a variable selection on the training set (n = 528, events = 157) and built a composite score on the validation set (n = 273, events = 77). Uni-Cox and LASSO-Cox methods were implemented to reduce the dimensionality, and a Cox-model was constructed for prognostication using a five-marker panel, which dichotomized the patients into high-risk and low-risk groups. To characterize the discrimination potential of the composite score, TNM stage, CEA levels, primary tumor location, and the combination of all the existing biomarkers, the investigators applied time-dependent receiver operating characteristic curves. Multivariate Cox regression analysis indicated that the cp-score was highly correlated with the risk of death and was an independent prognostic factor in both the training and validation sets. As expected, TNM stage, CEA status, and primary tumor location were also prognostic factors for survival of patients with CRC. An integrative model combining the cp-score and clinical characteristics demonstrated greater prognostic accuracy in both the training (AUROC of 0.82) and validation (AUROC of 0.87) cohorts. Finally, in an attempt to develop an easier-to-use tool, the authors constructed a nomogram with a point scale of the next four variables: cp-score, CEA concentration, TNM stage, and primary tumor location.

The possibility that ctDNA could be a useful prognostic marker for minimal residual disease was also been suggested for a small series of advanced CRC patients who underwent resection of liver metastases (Diehl et al., 2008). This approach has been subsequently demonstrated in localized CRC (Reinert et al., 2019; Jeanne Tie et al., 2020, 2016; Jeanne Tie, Cohen, Wang, Christie, et al., 2019; Jeanne Tie, Cohen, Wang, Li, et al., 2019), and many ongoing clinical trials are evaluating the role of ctDNA status in adjuvant therapy decision-making and the impact on patient survival outcomes (Coakley, Garcia-Murillas, & Turner, 2019).

Finally, although it is not a genomic or epigenomic biomarker itself, Immunoscore, deserves specific attention in this section. Proposed for the first time in 2006 by Jerome Galon (Galon et al., 2006), Immunoscore was recently recognized in the current *Localised Colon Cancer ESMO Clinical Guidelines* as a useful tool for prognostication in the non-metastatic colon cancer (CC) setting. Immunoscore has been validated in a huge prospective cohort of 2,681 stage I-III CC patients, and was a strong predictor for time to recurrence, OS, and DFS, independent of patient age, sex, MSI, and other relevant prognostic factors. Moreover, Immunoscore showed the highest relative contribution to the risk of all clinical parameters, including the UICC TNM staging system (Pagès et al., 2018). Based on this data, the *ESMO Clinical Guidelines* concluded that Immunoscore could help refine the prognosis for colon cancer patients with early stage disease in conjunction with the TNM staging system (level of evidence III – prospective cohort studies, grade of recommendation C – insufficient evidence for efficacy or benefit does not outweigh the risk or the disadvantages, optional). Oppositely, the NCCN Colon Guidelines, to date, do not even mention Immunoscore, probably because of unclear evidence for its prognostic value for predicting the risk of recurrence and death in stage II or III CC separately or for its predictive role in predicting adjuvant chemotherapy benefit. In an attempt to clarify these questions specifically in stage III CC, the International Society for Immunotherapy of Cancer Immunoscore Consortium conducted a retrospective study to evaluate Immunoscore in 763 patients with TNM stage III CC from two retrospective cohorts: cohort 1 (Canada/United States) and cohort 2 (Europe/Asia) (Mlecnik et al., 2020). In this analysis, patients with a high Immunoscore also had the lowest risk of recurrence, and a significantly prolonged TTR, OS, and DFS. The association of Immunoscore with TTR was independent of major prognostic covariates such as sex, T stage, N stage, primary tumor location, and MSI status. Moreover, Immunoscore had the strongest contribution to survival risk for TTR and OS. Importantly, chemotherapy was significantly associated with survival in the high-Immunoscore group for patients with either low-risk or high-risk stage II CC as compared to the low-Immunoscore group (Mlecnik et al., 2020). These studies have paved the path for investigating the prognostic role of Immunoscore in larger prospective studies, and, even more importantly, in randomized clinical trials to evaluate its predictive potential for chemotherapy benefit.

Palliative setting

Although the backbone of treatment in patients with mCRC has historically been chemotherapy, over the last few decades targeted molecular therapies against EGFR and angiogenic factors have been introduced into daily clinical practice. Furthermore, new treatment options have recently been added to the mCRC armamentarium, including BRAF inhibitors (BRAFi) and anti-programmed cell death 1 (PD-1) antibodies. Historically, and in parallel with drug development, multiple research efforts have been undertaken to discover and implement molecular biomarkers to guide therapeutic strategies. This becomes even more important in today's clinical scenario in which multiple therapeutic options are available, and therefore treatment selection aims not only to improve patient survival, but also to spare patients from unnecessary toxicity and reduce the economic burden of expensive treatments. In this section, we summarize the most relevant milestones achieved in the field of biomarkers for various treatments in mCRC (Figure 2) and provide insights into some of the important clinical and methodological aspects.

Conventional Chemotherapy—The backbone of treatment in patients with mCRC has historically been chemotherapy, and several chemotherapeutic agents are now approved in this setting: fluoropyrimidines (fluorouracil [FU] and capecitabine), oxaliplatin, irinotecan, and, since 2015, trifluridine/tipiracil (TAS-102).

***Fluoropyrimidine-based chemotherapy and TAS-102 –:** Several studies have investigated the predictive role of fluoropyrimidine metabolic pathway enzymes in response to FU-based therapies. Various retrospective and prospective studies of the role of thymidylate synthase (TS) in fluoropyrimidine-based therapy (primarily FU plus leucovorin) have yielded discordant results. In this regard, multiple studies have shown that low levels of TS expression in metastatic tumor tissues generally correlate with higher overall response rate (ORR) (Aschele et al., 2002; Aschele, Debernardis, Tunesi, Maley, & Sobrero, 2000; Cascinu et al., 2000; Etienne-Grimaldi et al., 2008). Surprisingly, such a correlation was not evident when TS levels were measured in primary tumor tissues (Aschele et al., 2000; Johnston et al., 2003). Similarly, low levels of TS and dihydropyrimidine dehydrogenase (DPD) in metastatic tumor tissues were associated with a favorable response to FU in patients with mCRC (Salonga et al., 2000); however, a subsequent report in 2006 failed to validate these findings (Smorenburg et al., 2006). Likewise, the role of thymidine phosphorylase as a predictive biomarker was investigated, but the results remain inconclusive (Gustavsson et al., 2009; Lindskog, Derwinger, Gustavsson, Falk, & Wettergren, 2014).

In 2009, a meta-analysis of five studies examining a total of 861 patients with mCRC concluded that, compared with MSS patients, MSI-H patients did not achieve a statistically significant better response rate to FU-based chemotherapy (Des Guetz, Uzzan, Nicolas, Schischmanoff, & Morere, 2009). Similarly, a study of the relationship between *MSH2* gene expression and capecitabine efficacy in patients with mCRC revealed that observed that higher MSH2 expression was associated with a better response (Jensen, Danenberg, Danenberg, & Jakobsen, 2007).

In an attempt to identify noncoding RNA-based predictive biomarkers, a low expression of miR-143 was shown to be associated with improved ORR and progression-free survival (PFS) in patients treated with capecitabine (Simmer et al., 2015). Likewise, low expression of miR-429 correlated with improved 5-year disease-free survival and OS in patients with mCRC treated with FU-based chemotherapy (Dong, Cai, & Li, 2016).

On the basis of the results of the phase III RECOURSE (Study of TAS-102 in Patients With Metastatic Colorectal Cancer Refractory to Standard Chemotherapies) trial, the US FDA approved TAS-102 for patients with mCRC. In 2017, Suenaga et al. (Suenaga et al., 2017) analyzed genomic DNA extracted from 233 samples from three cohorts: an evaluation cohort of 52 patients who received TAS-102, a validation cohort of 129 patients who received TAS-102, and a control cohort of 52 patients who were treated with regorafenib. Single nucleotide polymorphisms of genes involved in homologous recombination repair (*ATM*, *BRCA1*, *BRCA2*, *XRCC3*, *FANCD2*, *H2AX*, and *RAD51*), and cell cycle checkpoints (*ATR*, *CHEK1*, *CHEK2*, *CDKN1A*, *TP53*, *CHE1*, *PIN1*, and *PCNA*) were analyzed by PCR-based direct sequencing. Interestingly, patients who harbored

any G allele at the *ATM rs609429* locus exhibited improved OS rates when compared with patients with a C/C variant (Suenaga et al., 2017).

***Oxaliplatin-based chemotherapy –:** Several key genes involved in the nucleotide excision repair pathway have been explored as potential predictive biomarkers for treatment with oxaliplatin. The most notable studies in this area have been on the *ERCC1* gene, but as reported based on results from the MAVERICC (Marker Evaluation for Avastin Research in CRC) trial (NCT01374425), intratumoral *ERCC1* expression failed to predict response to oxaliplatin treatment (Parikh et al., 2019). The *XRCC1* gene, a base excision repair modulator, has also been evaluated as a possible predictive biomarker for oxaliplatin treatment, and a polymorphism in this gene (*XRCC1-839 Arg/Gln* or *Gln/Gln*) correlated with worse ORR to treatment with FU/oxaliplatin (Stoehlmacher et al., 2001).

Several microRNAs (miRNAs) have also been assessed for predictive response potential to FU, leucovorin, and oxaliplatin (FOLFOX)- or capecitabine plus oxaliplatin (CAPEOX)-based regimens. In this treatment setting, high miR-625-3p and low miR-148a expression were associated with poor response (Rasmussen et al., 2013; Takahashi et al., 2012), whereas high miR- 126 microvessel density was associated with improved PFS (Torben Frostrup Hansen, Nielsen, Jakobsen, & Sorensen, 2013).

***Irinotecan-based chemotherapy –:** The most notable marker studied as a potential predictive biomarker for irinotecan treatment is topoisomerase 1 (TOP1). The first large study in which the predictive power of TOP1 was evaluated used tumor tissue samples from the FOCUS (Fluorouracil, Oxaliplatin, CPT-11: Use and Sequencing; NCT00008060) trial and reported that moderate or high expression of TOP1 was associated with a significant gain in survival after irinotecan-based therapy (Braun et al., 2008). Unfortunately, these findings were not subsequently confirmed in an analysis of samples from 545 patients involved in the CAIRO (Capecitabine, Irinotecan, Oxaliplatin; NCT00312000) study, even though patients received similar treatment regimens and similar analytical approaches were used (Koopman et al., 2009).

A study of the role of genetic polymorphisms in the *TDP1* and *XRCC1* genes in response to irinotecan-based regimens, showed a positive correlation with improved ORR in patients with the *TDP1 IVS12+79G.T* and *XRCC1* GGCC-G/GGCC-G genotypes (Hoskins et al., 2008). Aprataxin, a protein member of the histidine triad superfamily, has also shown potential ability to be used to discriminate responders from nonresponders. In a retrospective cohort of 128 patients treated with irinotecan-based chemotherapy, low expression of aprataxin correlated with improved disease control, PFS, and OS (Dopeso et al., 2010). Although studies of *UGT1A* gene polymorphisms and their predictive value for response to irinotecan treatment have yielded conflicting results (Cecchin et al., 2009; Dias, McKinnon, & Sorich, 2012), higher methylation levels of the *BNIP3* gene in patients treated with irinotecan plus S1 correlated with lower response rates (Hiraki et al., 2010).

Anti-angiogenic Drugs

***Bevacizumab –:** Since the introduction of bevacizumab for the management of patients with mCRC, substantial efforts have been made to discover predictive biomarkers for

this anti-angiogenic drug. To date, the most promising approach evaluated the correlation between VEGF-A levels and clinical benefit from bevacizumab-based chemotherapy. A 2014 study reported higher ORR and improved PFS in patients with a low expression of VEGF-A in primary tumor specimens (Bruhn et al., 2014). Unfortunately, these encouraging results were not confirmed by the MAVERICC trial (Parikh et al., 2019). The possible predictive role of *RAS* was evaluated in a subset of 230 patients with mCRC treated with irinotecan, FU, and leucovorin (IFL) plus placebo, or IFL plus bevacizumab, in a phase III randomized clinical trial. In this study, only the subset of patients who had wild-type *KRAS* showed a significantly higher response rate in the bevacizumab arm (Hurwitz, Yi, Ince, Novotny, & Rosen, 2009).

***Regorafenib –:** While most of the studies have explored biomarkers from a radiomic perspective, others have used tissue- and plasma-based approaches. As a potential tissue-based predictive molecular marker for regorafenib, Wong et al. (Wong et al., 2015) described a correlation between downregulation of p53 and phosphorylated-proline-rich AKT substrate and higher PFS and metabolic response, respectively. The authors also evaluated the association between total plasma circulating cfDNA at baseline and major efficacy outcomes and found a significant inverse correlation with PFS ($p = 0.048$). Moreover, patients with detectable *KRAS* mutation in plasma presented a worse PFS ($p = 0.04$).

Regarding *RAS* gene evaluated in circulating ccfDNA, a small retrospective study involving 21 CRC patients showed a clinically significant association between reduced rates of *RAS* mutation in plasma, as detected by digital droplet PCR, and improved PFS ($p = 0.01$) and OS ($p = 0.06$)(Khan et al., 2017). A prognostic score for regorafenib has also been developed based on combinatorial clinico-molecular models and the REBECCA (Regorafenib in Metastatic Colorectal Cancer: A Cohort Study in the Real-Life Setting) study. This prognostic score included the following parameters that were independently associated with poorer OS: high Eastern Cooperative Oncology Group performance status, a shorter time from initial diagnosis of metastases, an initial regorafenib dose of 160 mg, more than three metastatic sites, presence of liver metastases, and *KRAS* mutations (Adenis et al., 2016).

***Aflibercept and ramucirumab –:** A 2015 study of 87 patients with mCRC who were enrolled on the phase II AFFIRM (Study of Aflibercept and Modified FOLFOX6 as First-Line Treatment in Patients With Metastatic Colorectal Cancer) trial and were treated with aflibercept plus mFOLFOX6 reported that high plasma levels of interleukin-8 at baseline, together with increase in these plasma levels during the course of treatment, correlated with shorter PFS (Lambrechts et al., 2015). In 2017, Tabernero et al. (Tabernero et al., 2018) evaluated the predictive potential of VEGF-D in a translational research study based on the RAISE (Ramucirumab Versus Placebo in Combination With Second-Line FOLFIRI in Patients With Metastatic Colorectal Carcinoma That Progressed During or After First-Line Therapy With Bevacizumab, Oxaliplatin, and a Fluoropyrimidine) trial. Using an adaptive analysis design, the researchers analyzed plasma proteins from two sets of patients, one exploratory ($n = 294$) and one confirmatory ($n = 590$), and found that high VEGF-D basal levels correlated with a better PFS ($p = 0.0013$) and OS ($p = 0.0107$) in patients treated in

the ramucirumab arm. Although VEGF-D is not a genomic or epigenomic biomarker, it is the most compelling study to date in a ramucirumab-treated CRC cohort.

Anti-EGFR Drugs: Cetuximab and Panitumumab—*KRAS* mutations in tumor tissues were the first predictive biomarker approved to guide decision-making and determine eligibility for anti-EGFR therapy in patients with mCRC. One of the first studies of KRAS mutations as predictive biomarkers comprised 30 patients with mCRC treated with cetuximab-based regimens. This study showed a significant correlation between the presence of *KRAS* mutations and the lack of response to anti-EGFR therapy (Lievre et al., 2006), an observation that was subsequently validated in a cohort of 427 patients with mCRC treated with panitumumab (Amado et al., 2008). Similarly, the potential of *NRAS* mutation status as a negative response predictor for panitumumab and cetuximab was later confirmed in several clinical trials and meta-analyses (De Roock, Claes, et al., 2010; De Roock, Jonker, et al., 2010; Douillard et al., 2013; Peeters M, Oliner KS, 2010; Sorich M J, Wiese M D, Rowland A, Kichenadasse G, McKinnon R A, 2015). Interestingly, in addition to the lack of efficacy in patients with *RAS*-mutated mCRC, panitumumab or cetuximab also showed reduced efficacy when given in combination with FOLFOX in a retrospective data analysis from two phase III clinical trials (Bokemeyer et al., 2017; Douillard et al., 2013). Such a negative effect for panitumumab or cetuximab when given in conjunction with FU, leucovorin, and irinotecan (FOLFIRI) has not been confirmed to date (Stintzing et al., 2017). In an attempt to translate these tissue-based predictive biomarkers into circulating tumor DNA (ctDNA)-based liquid biopsy assays, two 2017 studies used an innovative beads, emulsions, amplification, and magnetics (BEAMing) assay and reported promising agreement rates of 89.7% and 93% for the mutational status of RAS between tissue and ctDNA (Grasselli et al., 2017; Vidal et al., 2017).

BRAF mutations have also been evaluated as a predictive biomarker for anti-EGR therapies. The presence of *V600E* mutation within *BRAF* often reflects a poor prognosis in patients with CRC (Yuan et al., 2013). In addition, two meta-analyses reported a lack of benefit in terms of PFS, OS, and ORR when anti-EGFR therapies were combined with standard chemotherapy in a subset of patients harboring BRAF mutations (Pietrantonio et al., 2015; Rowland et al., 2015). Despite these early data suggesting that the presence of the *BRAF V600E* mutation may suggest a lack of response to anti-EGFR-based therapies, there is not yet enough clinical evidence to consider *BRAF* mutational status as a negative predictive biomarker in patients with advanced disease. Moreover, emerging therapeutic strategies that combine anti-EGFR drugs with BRAF inhibitors have recently changed the paradigm of how we must understand these molecular alterations from a therapeutic perspective.

In 2014, Manceau et al. (Manceau et al., 2014) investigated the roles of different miRNAs in response to anti-EGFR therapies. They conducted miRNA profiling in a training set of tissue specimens (33 fresh-frozen [FF] and 35 FFPE samples that were retrospectively collected and 19 prospectively collected FF samples) from 87 patients with mCRC refractory to chemotherapy and treated with anti-EGFR antibodies, and determined that hsa-miR-31-3p expression levels were significantly associated with PFS. They subsequently validated these results in an independent validation cohort consisting of 19 FF and 26 FFPE prospectively collected samples. The percentage of size variation of target lesions by RECIST criteria in

the validation series and risk status were significantly associated with expression levels of hsa-miR-31-3p. In addition, the investigators built and validated nomograms to predict PFS based on hsa-miR-31-3p expression. In the same context, Miller-Phillips et al. (L Miller-Phillips et al., 2018) evaluated the role of miR-21 as a predictive biomarker for anti-EGFR therapies. Using an RT-qPCR assay, they quantified miR-21 expression in FFPE tumor samples from patients enrolled on the FIRE-3 trial (NCT00433927) patients. The median miR-21 expression within the FIRE-3 population was determined and subsequently used to segment the population into low and high miR-21 expression groups. Patients who had wild-type *RAS* wild-type and low miR-21 expression showed significantly improved ORR and OS when cetuximab, but not bevacizumab, was added to FOLFIRI chemotherapy. In contrast, patients who had wild-type RAS but high miR-21 expression showed no significant difference in ORR and OS between the cetuximab and bevacizumab treatment groups. Similarly, patients with mutated *RAS* and either high or low miR-21 expression showed no significant difference in ORR between cetuximab or bevacizumab added to FOLFIRI. Unfortunately, other correlations between groups and PFS or OS were not significant. In a later study to determine the biological changes underlying these differences in efficacy outcomes, Miller-Phillips et al. (Lisa Miller-Phillips et al., 2019) used normalized mRNA microarray data to compare gene expression in the miR-21 low and high groups. This analysis identified 538 genes that were significantly and differentially expressed in patients with wild-type *RAS* after adjustment for multiple testing. Including data from the two groups into single-sample Gene Set Enrichment Analysis showed 23 hallmarks of cancer gene sets significantly differentially enriched, being *KRAS*-signaling higher in the miR-21 high group.

Anti–PD-1 Drugs: Pembrolizumab and Nivolumab—In May and July of 2017, the US FDA approved the anti-PD-1 therapies pembrolizumab and nivolumab for the treatment of patients with MSI-H mCRC for whom the disease has progressed after treatment with fluoropyrimidine, oxaliplatin, and irinotecan. Almost a year later, in July 2018, a nivolumab plus ipilimumab combination regimen was approved, which opened three novel treatment options for patients with MSI-H or dMMR mCRC (patients with MSI-H or dMMR mCRC represent approximately 5% of all patients with mCRC) (Braun et al., 2017). Although patients with MSI-positive mCRC have worse prognosis, it is thought that they derive clinical benefit from anti-PD-1 therapy because of a large proportion of lymphocytic infiltration and the presence of mutation-associated neoantigens (Giannakis et al., 2016) (Le et al., 2017, 2015; Overman et al., 2017, 2018). This exciting discovery has led to universal MSI testing for the management of patients with mCRC.

Not surprisingly, in May 2020 the US FDA approved pembrolizumab as first-line therapy for patients with MSI-H/dMMR mCRC. This approval was based on the results of the KEYNOTE-177 study (NCT02563002), a multicenter, international, open-label, active-controlled, randomized trial that compared first-line therapy with pembrolizumab vs. chemotherapy in 307 patients with MSI-H/dMMR mCRC. This study demonstrated a statistically significant improvement in PFS, with a median PFS of 16.5 months vs. 8.2 months for pembrolizumab compared to chemotherapy standard-of-care. Longer-term analysis is needed to assess the effect on OS. Moreover, in June 2020 the US FDA granted

accelerated approval to pembrolizumab for the treatment of patients with any unresectable or metastatic solid tumor with high mutational burden (as determined by the FDA-approved test, the FoundationOneCDx assay) whose cancer has progressed after previous treatment and has no satisfactory alternative treatment options (Bersanelli, 2020). Several clinical trials evaluating the combination of anti-PD-1 therapy with chemotherapy are ongoing for previously untreated MSI-H/dMMR mCRC patients with the goal of improving on results from previous studies and further extending survival of these patients. Meanwhile, other different immunotherapeutic approaches are being evaluated for treatment of MSS CRC, which is less responsive to immune-checkpoint inhibition than MSI-H mCRC. Although all of these results represent substantial therapeutic advances in the treatment of mCRC, they also emphasize the growing need for more precise predictive biomarkers to support more rational development of immunotherapies. A more comprehensive understanding of the intersection between genomics, epigenomics, and immunology in mCRC seems essential for meeting this need for new strategies.

Recently, Grasso et al. (Grasso et al., 2018) reported the results of a large-scale genomic analysis (TCGA, Nurses' Health Study, and Health Professionals Follow-up Study cohorts) involving 1211 primary CRC tumor specimens. Mutations in genes involved in immune modulatory pathways, as well as in the neoantigen-presentation machinery (mainly *B2M* and *HLA*), significantly correlated with MSI-H. Along with *JAK1/2* and *IFN-gamma receptor 1* mutations, similar alterations have been observed in melanoma, non-small cell lung cancer, and CRC and deemed to be genetic drivers of primary or acquired resistance to immune-checkpoint blockade, reflecting their role as a mechanism of adaptive resistance against T-cell tumor infiltration (Gao et al., 2016; Giannakis et al., 2016; Grasso et al., 2018; Gurjao et al., 2019; Le et al., 2017; Zaretsky et al., 2016). The interaction between somatic alterations and the immune system is complex, as indicated by a recent study in which 11 out of 13 B2M-mutant CRC patients achieved mCRC control with anti-PD-1 or anti-PD-L1 agents, despite the presence of a mutation that, theoretically, conferred primary resistance to ICI (Middha et al., 2019). On the other hand, for both MSS and MSI-H tumors, active WNT/β-catenin signaling was inversely associated with tumor T-cell infiltration, providing evidence of the existence of an anti-immune response mechanism beyond the MSI profile (Grasso et al., 2018).

BRAF Inhibitors: Vemurafenib and Encorafenib—*BRAF* mutations occur in 10–15% of all CRCs and in ~7% of all mCRC (Clarke & Kopetz, 2015; Davies et al., 2002). Although most *BRAF* mutations occur in codon 600 (mainly *BRAF* V600E), which leads to constitutive BRAF kinase activity and sustained MAPK pathway signaling, 2% of mCRCs have atypical *BRAF* mutations that are outside of codon 600, usually in codon 594 (Jones et al., 2017). Surprisingly, although monotherapy with BRAFi has proven effective in the treatment of *BRAF*-mutant melanoma, it was ineffective in *BRAF V600E*-mutant CRCs. Preclinical evidence demonstrated that despite transient inhibition of pERK by BRAFi such as vemurafenib, rapid ERK reactivation occurs through EGFR-mediated activation of RAS and CRAF (Corcoran et al., 2012). Furthermore, the fact that *BRAF V600E*-mutant CRCs express higher levels of pEGFR than do *BRAF*-mutant melanomas, positions them for EGFR-mediated resistance (Corcoran et al., 2012). Collectively, these findings provided

rationale to test dual BRAF and EGFR blockade. Results from preclinical studies and early phase clinical trials, have demonstrated this strategy is feasible and safe, and can potentially improve therapeutic efficacy of BRAFi. Moreover, preclinical studies have suggested that combined inhibition of BRAF and MEK was more effective than dual BRAF and EGFR blockade. This strategy was tested in subsequent phase 1 and phase 2 clinical trials that combined BRAF inhibitors with both anti-EGFR monoclonal antibodies and MEK inhibitors (Corcoran et al., 2018, 2010, 2012). Results of these trials led to US FDA approval (in April 2020) of encorafenib, a BRAF tyrosine kinase inhibitor, used in combination with cetuximab for the treatment of adult patients with *BRAF* V600E-mutated mCRC. The efficacy of this combination of drugs was evaluated in the BEACON CRC study (Kopetz et al., 2019), a phase 3 randomized, active-controlled, open-label, multicenter trial (NCT02928224). In this trial, encorafenib plus cetuximab demonstrated a clinical and statistically significant OS and PFS benefit compared to the control arm of either irinotecan or FOLFIRI plus cetuximab in patients with *BRAF* V600E-mutated mCRC who had progressed on one or two prior regimens. This trial also evaluated the efficacy of triple-therapy with encorafenib, binimetinib (a MEK inhibitor [MEKi]), and cetuximab in a second experimental arm, but although this regimen showed an improved OS and PFS compared to the control arm, it was more toxic than the dual BRAF and EGFR blockade and had similar efficacy.

Another BRAFi, vemurafenib, which has more modest clinical activity, was recently included in the NCCN guidelines as a treatment option for patients with *BRAF* V600E-mutated mCRC when used in combination with cetuximab/panitumumab plus irinotecan (Kopetz et al., 2020; “National Comprehensive Cancer Network. Colon Cancer (Version 4.2020). https://www.nccn.org/professionals/physician_gls/pdf/colon.pdf. Accessed December 6, 2020.,” n.d.). Inclusion in the guidelines was based on results of the randomized phase 2 Southwest Oncology Group (SWOG) 1406 trial, in which the triple-therapy (vemurafenib, cetuximab, and irinotecan) demonstrated improved PFS and ORR as compared with cetuximab plus irinotecan (Kopetz et al., 2020). In addition to the previously described regimens, based on the results of a phase 1 study (Corcoran et al., 2018), the NCCN Panel has recommend the combination of dabrafenib (BRAFi) plus trametinib (MEKi) plus either cetuximab or panitumumab as another treatment option beyond the first line setting for *BRAF* V600E-mutated mCRC (NCCN. Colon Cancer version 4, 2020).

HER-2 Blockade—A large body of evidence, accrued primarily from breast and gastric cancer patients, supports the role of *HER-2* amplification or overexpression as a predictive biomarker for anti-HER-2-based therapies. Therefore, there is renewed interest in evaluating HER-2 as a clinically actionable target in mCRC. Although initial mCRC clinical trials interrogating the anti-HER-2 monoclonal antibody trastuzumab in combination with other chemotherapeutic agents (either FOLFOX or irinotecan) closed early due to lack of patient accrual, mechanistic insights gained from preclinical analyses of *HER-2*-amplified mCRC patient-derived xenografts has led to improved design of new clinical trials (Bertotti et al., 2011; Clark JW, Niedzwiecki D, Hollis D, 2003; Ramanathan et al., 2004). Three phase 2 clinical trials evaluated dual HER-2 blockade in a biomarker-selected subset of heavily pretreated mCRC patients. Study treatment included trastuzumab plus lapatinib (HERACLES trial, NCT03225937), pertuzumab and trastuzumab (MyPathway

trial, NCT02091141), or the antibody-drug conjugate trastuzumab deruxtecan (DESTINY-CRC01, NCT03384940). Results of these studies demonstrated an impressive ORR of ~30–45% (Hainsworth et al., 2018; Sartore-Bianchi et al., 2017; Siena et al., 2020). These data have paved the way for development of ongoing phase 2 clinical trials evaluating the efficacy of new anti-HER-2 agents, such as S1613 (NCT03365882), trastuzumab-emtansine (NCT03418558), or tucatinib (NCT03043313) in this clinical scenario comprising ~5% of RAS wild-type mCRC patients (Valtorta et al., 2015). Furthermore, determining the utility of ctDNA analyses in monitoring therapeutic efficacy and in identifying mechanisms of resistance to dual HER-2 blockade is also an attractive area of study (Siravegna et al., 2018).

Tyrosine Kinase Inhibitors—New drugs that target tyrosine kinase (TK) fusions in genes such as *NRTK1/2/3*, *RET*, *ALK*, and *ROS1* are showing promising preliminary results in phase 1 and 2 clinical trials that include patients with CRC. One agent, LOXO 101 (larotrectinib), is a selective tropomyosin receptor kinase (TRK) inhibitor that demonstrated tumor-agnostic efficacy in patients with *NTRK* fusion-positive malignancies (including four patients with CRC who achieved a partial response) (Drilon et al., 2018). A second agent, entrectinib, an ALK, ROS1, TRKA, TRKB, and TRKC selective inhibitor, demonstrated clinical activity in patients who had fusions in the previously described TK genes (Drilon, Siena, et al., 2017). Patients who responded to entrectinib included two patients whose mCRC harbored *CAD-ALK* or *LMNA-NTRK1* gene fusions (Amatu et al., 2015; Sartore-Bianchi et al., 2016). Anticipating potential resistance mechanisms to larotrectinib based on evidence from other pan-TK inhibitors, Drilon et al. (Drilon, Nagasubramanian, et al., 2017) developed LOXO-195 (selitrectinib), a potent and selective TRK kinase inhibitor designed to have a molecular structure that would overcome typical TRK resistance mutations. LOXO-195 was initially evaluated in a mCRC patient whose cancer had an *LMNA-NTRK1* rearrangement with a G595R larotrectinib-resistance mutation. This patient successfully achieved a durable partial response (Drilon, Nagasubramanian, et al., 2017). Although the prevalence of rearrangements in TK genes in mCRC patients may be as low as 1.5%, the accelerated development of TK inhibitors offers new hope for some heavily pretreated mCRC patients who have no other therapeutic options (Pietrantonio et al., 2017). Given these promising results, the US FDA granted accelerated approval to larotrectinib (November 2018) and entrectinib (August 2019) for patients with *NTRK* gene fusion-positive solid tumors without a known acquired resistance mutation. The Committee for Medicinal Products for Human Use of the European Medicines Agency has also recommended the granting of a conditional marketing authorization for larotrectinib (July 2019) and entrectinib (May 2020) for the same indication.

KRAS Inhibitors—*KRAS* is one of the most commonly altered oncogenes in human cancers, and was long considered an undruggable target because of the small size of abnormal KRAS proteins, the presence of few binding sites, and the rapid, tight binding of active KRAS to GTP. However, recent data have suggested that KRAS may be targetable. For example, preliminary data on the activity of AMG510 (sotorasib), a small covalent inhibitor, have shown that it rapidly and irreversibly occupies *KRAS G12C* and extinguishes its activity through a unique interaction with the P2 pocket (Janes et al., 2018). The *KRAS G12C* mutation occurs in ~4% of CRC (Neumann, Zeindl-Eberhart, Kirchner, &

Jung, 2009). In a recent phase 1 trial, sotorasib showed encouraging anti-tumor activity in heavily pretreated patients who had advanced, *KRAS G12C*-mutated solid tumors (Hong et al., 2020). A total of 129 patients were included in this study, 42 of whom had CRC. Within CRC patients, sotorasib treatment yielded an ORR and disease control rate (DCR) of 7.1% and 73.8%, respectively. The median duration of stable disease was 5.4 months and the median PFS was 4.0 months. Although sotorasib showed promising anticancer activity in patients with heavily pre-treated solid tumors bearing the *KRAS G12C* mutation, inconsistency was seen in tumor response between patients with non-small cell lung cancer and those with CRC, which the authors suggested indicated either that *KRAS G12C* is not the dominant oncogenic driver for CRC or that other pathways, such as the WNT or EGFR pathways, mediate oncogenic signaling beyond *KRAS*. These hypotheses are supported by solid preclinical evidence (Amodio et al., 2020; Lee et al., 2018; Xue et al., 2020), and therefore, clinical trials that combine sotorasib with other agents that block additional pathways have already been initiated (i.e., NCT04185883 and NCT04303780). Although many *KRAS* G12C inhibitors in addition to sotorasib are under development, to date only adagrasib, an irreversible covalent inhibitor, has shown promising antitumor activity in *KRAS G12C*-mutated CRC. Furthermore, inhibitors for mutations other than *KRAS G12C* are being developed. For example, initial preclinical data for MRTX1133, a new, first-in-class *KRAS G12D* inhibitor, have demonstrated significant tumor regression in preclinical animal models (Mirati Therapeutics, 2020). Thus, through development of a range of inhibitors, effective means of targeting KRAS are emerging.

Disease Monitoring by Liquid Biopsies—Liquid biopsies have recently emerged as powerful tools for monitoring disease evolution and therapeutic response through the analysis of cfDNA and RNA biomarkers in bodily fluids. One of the basis for this concept of liquid biopsies came from a study that reported that a gradual decrease in CEA levels during chemotherapy was significantly associated with better survival rates (Al-Sarraf, Baker, Talley, Kithier, & Vaitkevicius, 1979). Such a correlation between CEA flare and improved PFS and OS was confirmed a few years later in a subset of 670 patients with mCRC being treated with first-line chemotherapy (Strimpakos et al., 2010). Although CEA is not a CRC-specific biomarker, CEA monitoring in blood, alone or in addition to CA19-9 monitoring, is still commonly performed in routine clinical practice (Stiksma, Grootendorst, & van der Linden, 2014). In addition to this strategy, analyses of circulating tumor cells and endothelial cells in mCRC have been undertaken by several groups (S. J. Cohen et al., 2008; Ronzoni et al., 2010). In 2015, Hansen et al. (T F Hansen, Carlsen, Heegaard, Sørensen, & Jakobsen, 2015) reported that circulating levels of miRNA-126 in a subset of 68 patients with mCRC were predictive of tumor response to bevacizumab-based chemotherapy. On the other hand, the role of ctDNA in genotyping CRCs and tracking clonal evolution of CRC during and after treatment with anti-EGFR–based regimens has been evaluated (Siravegna et al., 2015). Multiple studies have since reported distinct genetic alterations in ctDNA from patients with primary or acquired resistance to anti-EGFR–based therapies. These alterations were seen in genes such as *KRAS*, *NRAS*, *MET*, *ERBB2*, *FLT3*, *EGFR*, and *MAP2K1* and detected by approaches including droplet digital PCR, BEAMing, and next-generation sequencing methodologies (Siravegna et al., 2015; Van Emburgh et al., 2016). Further demonstrating the potential utility of changes in ctDNA, a massively parallel sequencing-based assay of ctDNA

from a prospective cohort of 53 patients with mCRC showed that early changes in ctDNA during first-line standard chemotherapy can predict subsequent radiologic response (J Tie et al., 2015). Similarly, a 2017 study demonstrated a significant correlation between a decrease in RAS mutant clones in blood after 8 weeks of therapy and improved PFS and OS in a cohort of patients treated with regorafenib (Khan et al., 2017).

Intriguingly, although clonal evolution is dynamic, the emergence of drug-resistant clones in circulation increases during treatment and decreases after drug withdrawal. Understanding of this fact has led to novel treatment strategies that are being evaluated in studies such as the RASINTRO (*RAS* Mutations in ctDNA and Anti-EGFR Reintroduction in mCRC) study (NCT03259009) and the CHRONOS (Rechallenge With Panitumumab Driven by *RAS* Dynamic of Resistance) trial (NCT03227926). Both of these studies are evaluating the predictive impact of ctDNA *RAS* mutations on the efficacy of anti-EGFR monotherapy rechallenge in patients with mCRC with wild-type *RAS* whose disease has progressed after anti-EGFR–free chemotherapy. Another recently described approach involved a five-gene methylation panel for monitoring tumor burden in liquid biopsies using a methyl-BEAMing assay (Barault et al., 2017). Using this method to analyze plasma samples from 182 patients with mCRC treated with chemotherapy and/or targeted therapy, revealed a significant correlation between the dynamics of circulating cfDNA methylation markers and ORR and PFS. Using a similar approach, as part of studies presented in previous sections of this chapter, Luo et al. (Luo et al., 2020) evaluated the utility of ddPCR analysis of cg10673833 methylation for monitoring disease evolution. Dynamic changes in cg10673833 methylation were consistent with treatment response, and these changes were more pronounced than changes in CEA. Patients who had positive responses to treatment had parallel decreases in cg10673833 methylation as compared with untreated patients, and a further reduction in cg10673833 methylation was observed in patients after surgery. In contrast, patients who had progressive or recurrent disease showed increased methylation of cg10673833.

TOXICITY-ASSOCIATED BIOMARKERS

DPYD gene

Severe adverse events and side effects as a result of treatment-related toxicity are a major concern for any cancer therapy. One particular concern in the context of CRC is DPD deficiency, an inherited defect in the enzyme that catabolizes fluoropyrimidines and thus increases the risk of toxicity secondary to fluoropyrimidine-based therapies (Diasio, Beavers, & Carpenter, 1988; Tuchman et al., 1985; Amstutz et al., 2018; Mattison, Soong, & Diasio, 2002). DPD deficiency causes a deficit in the metabolism of thymine and uracil, resulting in excessive amounts of uracil and thymine in the blood, urine, and cerebrospinal fluid (Innocenti et al., 2020). It can be inherited as an autosomal recessive trait, such as homozygosity for deleterious *DPYD* variants. Patients who are homozygous for *DPYD* variants can develop severe DPD deficiency, which can result in neurologic and other severe defects during infancy. However, patients with homozygous variants may also be asymptomatic. The prevalence of DPD deficiency in the population ranges from 2% to 8% depending on ethnicity (Innocenti et al., 2020). The Clinical Pharmacogenetics Implementation Consortium selected 4 *DPYD* variants that

confer DPD deficiency and for which recommendations exist to aid fluoropyrimidine treatment and dosing decisions: *c.1905*1G>A (*2A), c.2846A>T, c.1679T>G,* and *c.1129-5923C>G* (haplotype B3). These patients could be offered alternative regimens or receive dose reductions (Amstutz et al., 2018). Two prospective studies have shown *DPYD* genotyping followed by tailoring of the fluoropyrimidine dose to be feasible in clinical practice and to improve patient safety and cost effectiveness (Deenen et al., 2016; Henricks et al., 2018, 2019). We note that universal *DPYD* genotyping before starting fluoropyrimidine treatment remains controversial. For example the *NCCN Panel* does not currently support this practice, while the *ESMO Clinical Guidelines*, in accordance to the Pharmacovigilance Risk Assessment Committee of the European Medicines Agency statement (March 2020) ("EMA recommendations on DPD testing prior to treatment with fluorouracil, capecitabine, tegafur and flucytosine. https://www.ema.europa.eu/en/news/ema-recommendations-dpd-testing-prior-treatment-fluorouracil-capecitabine-tegafur-flucytosine. Accessed Novem," n.d.), strongly recommend *DPYD* genotyping or DPD phenotyping before initiating fluoropyrimidine-based therapy in the adjuvant setting (Argilés et al., 2020).

UGT1A1

The UDP-glucuronosyltransferase (UGT) enzyme encoded by *UGT1A1* is important in the metabolism of irinotecan, a pro-drug that is widely used in the treatment of mCRC. Irinotecan is converted by carboxylesterase enzymes into the active metabolite SN-38. SN-38 produces severe treatment-related toxicities, including early and late forms of diarrhea, dehydration, and severe neutropenia (Innocenti et al., 2004; "Package Insert. Camptosar® (Irinotecan) Injection, intravenous infusion. 2019. Available at: https://www.accessdata.fda.gov/drugsatfda_docs/label/2019/020571s050lbl.pdf. Accessed November 16, 2020.," n.d.). SN-38 is inactivated and detoxified by the UGT1A1 enzyme. UGT enzymes are responsible for glucuronidation, which transforms lipophilic metabolites into hydrophilic substances that can be easily excreted from the body. Deficiencies in UGT1A1 can be caused by certain genetic polymorphisms and can result in conditions associated with accumulation of unconjugated hyperbilirubinemias, such as types I and II of the Crigler-Najjar and Gilbert syndromes. Moreover, certain genetic polymorphisms *UGT1A1* can result in a decreased glucuronidation of SN-38, resulting in increased drug levels and thus increased risk for toxicity, although severe irinotecan-related toxicity is not experienced by all patients who have these polymorphisms (Innocenti et al., 2004; X. Liu, Cheng, Kuang, Liu, & Xu, 2014; O'Dwyer & Catalano, 2006). *UGT1A* is located on chromosome 2q37, which encodes for multiple genes and pseudogenes. Moreover it exists in many alternatively spliced isoforms (van Es et al., 1993). Variants of *UGT1A1* that decrease UGT1A1 enzyme activity can lead to jaundice. Up to 135 genetic variants of *UGT1A1* have been reported; *UGT1A1*1* is the wild-type allele associated with normal enzyme activity (Strassburg, 2008). The most common variant allele is *UGT1A1*28*, which is also associated with drug toxicity. Current literature suggests that one copy of the *UGTA1A*28* allele results in an approximately 35% decrease in transcriptional activity, and 2 copies *(*28/*28*, homozygous) result in approximately a 70% decrease (Barbarino, Haidar, Klein, & Altman, 2014; Lam, 2019). Approximately 10% of the North American population is homozygous for the *28* allele (*28/*28* genotype, also known as *UGT1A1* 7/7 genotype)

and are at an increased risk of neutropenia following intravenous irinotecan therapy (Hall, Ybazeta, Destro-Bisol, Petzl-Erler, & Di Rienzo, 1999). The rate of severe neutropenia in **28/*28* homozygous patients is as high as 36%, and is strongly associated with a higher hospitalization rate (Etienne-Grimaldi et al., 2015; Shulman et al., 2011). There is less evidence to support a link between *UGT1A1* genotype and irinotecan treatment-related diarrhea, and data is conflicting regarding the influence of *UGT1A* genotype on response to irinotecan therapy (Dias et al., 2012; "Recommendations from the EGAPP Working Group: can UGT1A1 genotyping reduce morbidity and mortality in patients with metastatic colorectal cancer treated with irinotecan?," 2009).

Individuals homozygous for another variant allele, *UGT1A1*6*, which is more prevalent in Asian populations, have reduced UGT1A1 enzyme activity and homozygosity appears to be an important predictor of severe toxicity to irinotecan therapy in this population (Dean, 2012).

Moreover, many other genetic markers, including genetic variations in *ABCC1* and *ABCB2*, SLC transporter genes, *TGFB*, and *NR1I2* may influence the risk of irinotecan toxicity (Dean, 2012). On the other hand, emerging data suggests that other *UGT1* variant alleles may have a protective effect. This could be the case for *UGT1A1 c.-1068A>G*, which seems to have a lower risk of irinotecan-induced neutropenia, probably due to an enhanced capacity for glucuronidation (S. Chen et al., 2015).

FUTURE PERSPECTIVES AND CONCLUSION

Over the past several years, many different approaches have been developed and tested for diagnosing and treating CRC. The rising incidence of early-onset CRC, coupled with the typical screening and diagnosis of the disease in individuals of older ages, represents a tremendous challenge for healthcare systems worldwide (Akimoto et al., 2020). Given current evidence and recent advances, the future of CRC early-diagnosis would appear to be in multimodal liquid-biopsy approaches that include robust DNA methylation analyses. However, much additional study is needed to identify the ideal diagnostic test as well as a better understanding of the molecular characteristics of the various subsets of CRC and premalignant lesions.

Beyond diagnosis, a detailed understanding of CRC from a multi-omic perspective seems necessary to improve clinical outcomes in the adjuvant and palliative settings. Although risk stratification of CRC based on multi-omic molecular analyses has not yet been implements in the clinic, such a strategy together with in-depth knowledge of the interaction among (epi)genetic somatic and germline alterations and the immune system could have an important role in patient selection for adjuvant therapy. Moreover, although current studies have involved only a small number of patients, the growing evidence of the utility of various target genomic alterations such as specific gene mutations and fusions, together with specific genomic phenotypes, are laying the groundwork for developing more precise therapeutic approaches. This is exemplified by, for example, the emerging importance of *NRTK* fusions and *KRAS* mutations as targets for TK and RAS inhibitors, respectively. Moreover, many

alterations that were previously identified in other tumor types such as *HER2* amplification in breast cancer, are also showing promise for CRC.

Finally, an understanding of CRC from a phylogenetic perspective and a better characterization of tumor heterogeneity and evolution should be incorporated into clinical guidelines for future CRC screening and therapeutic strategies. Cancer is a complex disease, and using rational tissue sampling, detailed clinical phenotype annotation, and integrative genomics coupled with comprehensive mathematical models of cancer evolution and biomarker shedding should become the basis for data generation and biomarker development (Avanzini et al., 2020; Wheeler et al., 2020). Implementing all of these approaches will require making use of novel high-throughput molecular analytic techniques for comprehensive molecular profiling as well as rethinking and adapting the design of the next generation of clinical trials. Integrating multi-omic approaches with clinical and epidemiologic data using machine-learning algorithms will hasten biomarker development, and, most importantly, the decision-making process (Ruiz-Bañobre et al., 2019). To make this feasible in daily clinical practice, it will be necessary to implement user-friendly decision support tools that foster interactive treatment planning based on streamlined, expert-driven genomic-data interpretation and reporting (Tamborero et al., 2020).

Gradually, CRC management is moving from a “one-size-fits-all” approach to a more personalized medicine strategy in which increasing numbers of subsets of patients can obtain long-term survival benefits targeting low prevalence driver molecular alterations. With this concept in mind, biomarker research is moving forward, bringing together many types of “omics” data and establishing novel liquid biopsy-based strategies to capture temporo-spatial tumor heterogeneity. The future of CRC diagnosis and treatment is promising as many scientific disciplines come together, generating new knowledge and strategies that will improve patient care and outcomes.

ACKNOWLEDGEMENTS

We thank Raquel Varela-Fernández and Jared Millar for their assistance in medical illustration.

FUNDING INFORMATION

This work was supported by CA72851, CA181572, CA184792 and CA227602 grants from the National Cancer Institute, National Institutes of Health to AG. In addition, JR-B is supported by Río Hortega fellowship from the Institute of Health Carlos III (CM19/00087) and 2020 TTD Research Grant from the Spanish Cooperative Group for the Treatment of Digestive Tumors (TTD).

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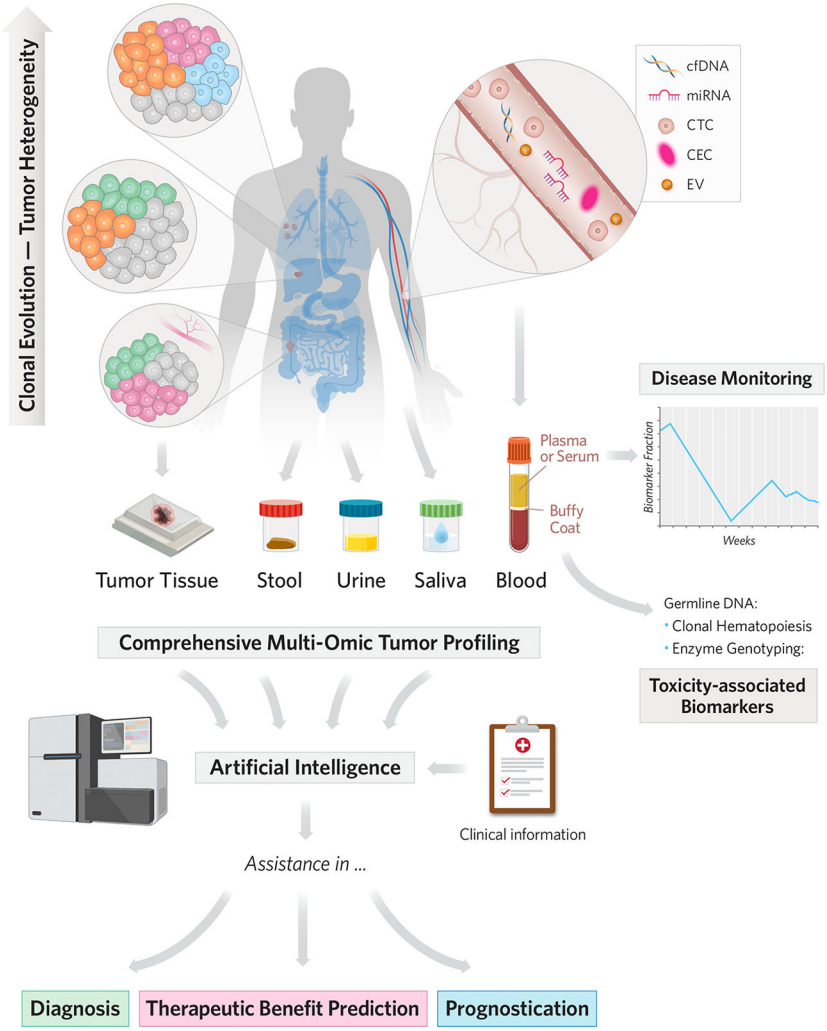

Figure 1. Schematic and general overview of the different biospecimen sources and biomarkers involved in diagnosis, prognosis, and prediction of clinical benefit of therapies for CRC.

Starting from a single ancestral clone, cancer cells undergo different routes of clonal evolution, which are responsible for temporospatial tumor heterogeneity. Although a single tumor tissue biopsy cannot capture all of the tumor variability, it is postulated that different types of liquid biopsies and biospecimens can capture this heterogeneity over time. In addition, there are many sources and types of biomarkers that can be detected in tumor tissue and other biospecimens, with blood, stool, saliva, and urine being the most promising. To date, for CRC early-diagnosis, the most frequently studied biospecimens have been stool and blood and for prognosis and clinical benefit prediction tumor tissue and blood have been most frequently studied. Moreover, because of their capacity to recapitulate tumor heterogeneity, liquid biopsies can facilitate disease monitoring and therapeutic response, especially through serial blood analyses. Identification of genotypic markers encoding drug-metabolizing enzymes is also possible by analyzing germline DNA from peripheral blood mononuclear cells. Last, implementation of novel high-throughput molecular analytic techniques, together with integration of multi-omic approaches from multiple sources

with clinical and epidemiologic data using artificial intelligence, will accelerate biomarker development and assist in the clinical decision-making process.

Abbreviations: CEC, circulating endothelial cells; cfDNA, cell-free DNA; CRC, colorectal cancer; CTC, circulating tumor cell; EV, extracellular vesicle; miRNA, microRNA.

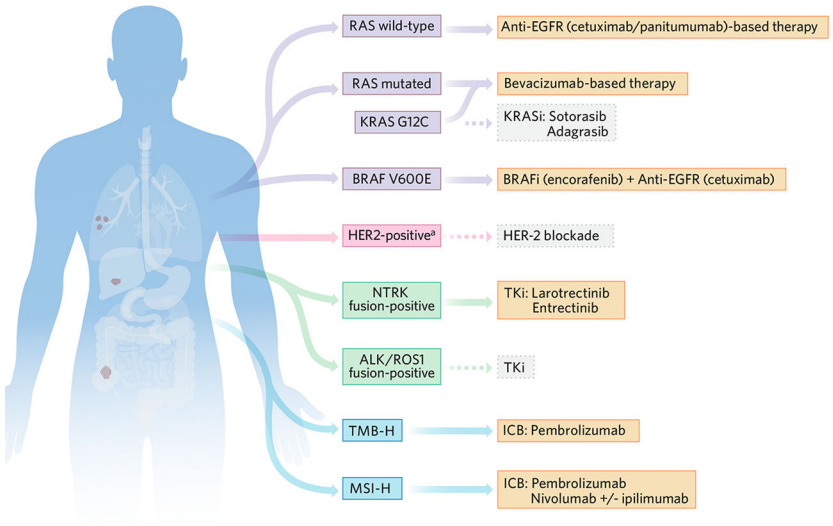

Figure 2. Predictive biomarkers for metastatic CRC treatment.

Summary of currently known molecular tumor alterations that enable improved clinical decision-making regarding use of therapies that are tailored to the metastatic CRC (mCRC) patient. The therapies shown go beyond classical chemotherapeutic agents. Excluding *RAS* mutations for EGFR-targeted therapies, which are considered a negative predictive biomarker, the molecular alterations shown represent positive predictors of benefit with the indicated targeted therapies. Although bevacizumab is considered a useful therapeutic option in combination with chemotherapy in mCRC independent of any particular molecular alteration, currently there is no consistent predictive biomarker to guide bevacizumab use. Although many of these molecular alterations are applicable only to a minority of mCRC patients, collectively these low-prevalence actionable characteristics support a new targeted therapeutic horizon for many patients. Color-coded boxes: yellow, US FDA-approved therapy; grey, not US FDA approved therapy.

[a] *HER2* amplification or overexpression.

Abbreviations: BRAFi, BRAF inhibitors; CRC, colorectal cancer; ICB, immune checkpoint blockade; KRASi, KRAS inhibitors; MSI-H, microsatellite instability high; TKi, tyrosine kinase inhibitors; TMB-H, tumor mutation burden high.

Table 1

Established and potential biomarkers in CRC diagnosis.

References	Biomarker	Specimen	Test	Accuracy	Regulatory status
miRNAs					
Ng et al. (2009) Uratani et al. (2016) Yau, Tang, Harriss, Dickins, and Polytarchou (2019)	miR-92a	Blood	NA	CRC: *AUROC: 0.89 (plasma)* *Sensitivity: 0.89* *Specificity: 0.70* A: *AUROC: 0.75 (serum)* *Sensitivity: 0.65* *Specificity: 0.79* AA: AUROC: *0.84 (serum)*	–
		Stool	NA	CRC: *AUROC: 0.80* *Sensitivity: 0.47* *Specificity: 0.91* A: *AUROC: 0.64* *Sensitivity: 0.43* *Specificity: 0.91*	–
Peng et al. (2017) Toiyama et al. (2013) Yau et al. (2019)	miR-21	Blood	NA	CRC: *AUROC: 0.85 (plasma)/0.87 (serum)* *Sensitivity: 0.69/0.75* *Specificity: 0.86/0.84* A: *AUROC: 0.80 (serum)* *Sensitivity: 0.77* *Specificity: 0.81*	–
		Stool	NA	CRC: *AUROC: 0.84* *Sensitivity: 0.60* *Specificity: 0.86* A: *AUROC: 0.77* *Sensitivity: 0.60* *Specificity: 0.83*	–
Yau et al. (2019)	2-miRNA panel: miR-92 miR-21	Stool	NA	CRC: *AUROC: 0.84* *Sensitivity: 0.54* *Specificity: 0.88*	–
Duran-Sanchon et al. (2020)	GBM model[a]: miR-421 miR-27a-3p	Stool	NA	CRC and AA: *AUROC: 0.74 (training set)/0.63 (test set)* *Sensitivity: 0.74/0.67* *Specificity: 0.63/0.60* CRC: *AUROC: 0.86/0.74*	–

References	Biomarker	Specimen	Test	Accuracy	Regulatory status
				Sensitivity: 0.96/0.96 *Specificity: 0.36/0.33* AA: *AUROC: 0.71/0.64* *Sensitivity: 0.61/0.59* *Specificity: 0.71/0.69*	
Liu, Klein, et al. (2018) and Liu, Liu, et al. (2018)	4-miRNA panel: miR-21 miR-92a miR-29a miR-125b	Blood (serum)	NA	CRC: *AUROC: 0.95* *Sensitivity: 0.85* *Specificity: 0.99*	–
Chang et al. (2016)	2-miRNA panel: miR-223 miR-92a	Blood (plasma)	NA	CRC: *AUROC: 0.91* *Sensitivity: 0.97* *Specificity: 0.75*	–
Herreros-Villanueva et al. (2019)	6-miRNA panel: miR-19a miR-19b miR-15b miR-29a miR-335 miR-18a	Blood (plasma)	NA	CRC and AA: *AUROC: 0.92* *Sensitivity: 0.85* *Specificity: 0.90*	–
Sazanov, Kiselyova, Zakharenko, Romanov, and Zaraysky (2017)	2-miRNA panel: miR-21 miR-92a	Saliva	NA	CRC: *Sensitivity: 0.97* *Specificity: 0.91*	–
Rapado-González et al. (2019)	5-miRNA panel: miR-186-5p miR-29a-3p miR-29c-3p miR-766-3p miR-491-5p	Saliva	NA	CRC: *AUROC: 0.75* *Sensitivity: 0.72* *Specificity: 0.67*	–
DNA methylation					
Church et al. (2014)	SEPT9	Blood (plasma)	Epi proColon	CRC: *Sensitivity: 0.68* *Specificity: 0.79* AN: *Sensitivity: 0.25* *Specificity: 0.79* AA: *Sensitivity: 0.22* *Specificity: 0.79*	Registered trademark. FDA approval: alternative for CRC screening[b]
Baek et al. (2009) Carmona et al. (2013) Chen et al. (2005) Fu et al. (2018) Itzkowitz et al. (2007) Kisiel et al. (2013)	VIM	Blood (plasma)	NA	CRC: *Sensitivity: 0.59* *Specificity: 0.93*	–
		Stool	ColoSure	CRC: *Sensitivity: 0.81* *Specificity: 0.95*	Trademark. Not FDA approval

References	Biomarker	Specimen	Test	Accuracy	Regulatory status
Li et al. (2009) Lu et al. (2014)					
Jung et al. (2020)	SFRP2	Blood (plasma and serum)	NA	CRC: *Sensitivity: 0.64–0.67* *Specificity: 0.97–1.00* A: *Sensitivity: 0.06–0.81* *Specificity: 0.73–1.00*	–
		Stool	NA	CRC: *Sensitivity: 0.56–0.94* *Specificity: 0.77–0.97* A: *Sensitivity: 0.28–0.76* *Specificity: 0.55–1.00*	–
Luo et al. (2020)	cd-score (multi-region panel)	Blood (plasma)	NA	CRC: *AUROC: 0.96* *Sensitivity: 0.88* *Specificity: 0.90*	–
Luo et al. (2020)	cg10673833 (single probe)	Blood (plasma)	NA	CRC: *AUROC: 0.90* *Sensitivity: 0.90* *Specificity: 0.87*	–
Kandimalla et al. (2020)	Multi-region panel	Blood (plasma)	EpiPanGI-Dx	CRC: *AUROC: 0.98*	–
ColonES Product Sheet (2020)	Multi-region panel	Blood (plasma)	ColonEs	CRC and A: *Sensitivity: 0.91 (A)/0.97 (I)/0.94 (II–IV)* *Specificity: 0.99*	–
Multi-modal					
USPSTF. US Preventive Services Task Force et al. (2020)	Multi-modal panel: KRAS mutations (×7) NDRG4 m.s. BMP3 m.s. FIT	Stool	Cologuard	CRC: *Sensitivity: 0.93* *Specificity: 0.85* AN: *Sensitivity: 0.47* *Specificity: 0.89* AA: *Sensitivity: 0.43* *Specificity: 0.89*	Registered trademark. FDA approval: CRC screening[c]
Friedland et al. (2020)	Multi-modal panel: Circulating GI endothelial cells Somatic mutations[d] SEPT9 m.s.	Blood	FirstSight	CRC: *Sensitivity: 1.00* *Specificity: 0.90* AA: *Sensitivity: 0.80*	Trademark
Cohen et al. (2018)	Multi-modal panel[e]: 8 proteins Mutations	Blood (plasma)	CancerSEEK	CRC: *AUROC: 0.85*	–

References	Biomarker	Specimen	Test	Accuracy	Regulatory status
Duran-Sanchon et al. (2020)	GBM model[a]: miR-421 miR-27a-3p FIT	Stool	NA	CRC and AA: *AUROC: 0.72 (training set)/0.70 (test set)* *Sensitivity: 0.74/0.68* *Specificity: 0.63/0.64* CRC: *AUROC: 0.90/0.93* *Sensitivity: 0.96/0.97* *Specificity: 0.36/0.43* AA: *AUROC: 0.70/0.64* *Sensitivity: 0.61/0.49* *Specificity: 0.71/0.71*	–
Metabolomic					
Deng et al. (2017)	Metabolites: Succinic acid Ascorbic acid Carnitine	Urine	PolypDx	HP and A: *Sensitivity: 0.43* *Specificity: 0.91*	Trademark. Available for CLIA-certified laboratories

[a] miRNA-based predictive model included miR-421 and miR-27a-3p, along with age and sex.

[b] Alternative in average-risk individuals who refuse other screening modalities.

[c] CRC screening in average-risk population.

[d] Somatic mutations in oncogenes and tumor suppressor genes.

[e] 8 proteins (cancer antigen 125, carcinoembryonic antigen, cancer antigen 19–9, proactin, hepatocyte growth factor, osteopontin, myeloperoxidase, and tissue inhibitor of metalloproteinases 1) and mutations (SNVs or INDELs) in 1933 distinct genomic positions.

Abbreviations: A, adenoma; AA, advanced adenoma; CRC, colorectal cancer; AN, advanced neoplasia; AUROC, area under the receiver operating characteristic curve; CLIA, Clinical Laboratory Improvement Amendments; FIT, fecal immunochemical test; GBM, gradient boosting machine; GI, gastrointestinal; HP, hyperplastic polyp; m.s., methylation status; NA, not available.