Colorectal cancer biomarkers and their impact on the clinical practice

Biomarcadores de câncer colorretal e seu impacto na prática clínica

James Crespo[1,2], Ana Paula Victorino[1,2], Kelly Araujo[1,2], Luiz Henrique Araujo[1,2], Fernando Meton de Alencar Camara Vieira[1,2]

ABSTRACT

Colorectal cancer (CRC) holds third place in the global ranking of malignancies worldwide. Patients with CRC commonly show distinct outcomes and treatment responses due to their biological features and tumoral biomarkers. This review explores the repertoire of molecular biomarkers in CRC, comprised of chromosomal aberrations and genomic instability and genetic mutations. We also underline the stratification of CRC patients into four clinically defined subsets: CMS1 (MSI, immune); CMS2 (canonical); CMS3 (metabolic); and CMS4 (mesenchymal), as well as novel techniques to be applied very soon in the field, such as cell-free DNA, tumor mutational burden, and microbiome profiling.

Keywords: Biomarcadores; Genômica; Prognóstico; Agentes antineoplásicos; Neoplasias colorretais.

RESUMO

O câncer colorretal (CCR) ocupa o terceiro lugar no ranking mundial de doenças malignas. Pacientes com CCR geralmente apresentam resultados e respostas ao tratamento distintos devido às suas características biológicas e biomarcadores tumorais. Esta revisão explora o repertório de biomarcadores moleculares no CCR, composto por aberrações cromossômicas e instabilidade genômica e mutações genéticas. Também destacamos a estratificação dos pacientes com CCR em quatro subconjuntos clinicamente definidos: CMS1 (MSI, imune); CMS2 (canônico); CMS3 (metabólico); e CMS4 (mesenquimal), bem como novas técnicas a serem aplicadas muito em breve na área, como DNA livre de células, carga mutacional tumoral e perfil do microbioma.

Descritores: Biomarcadores; Genômica; Prognóstico; Agentes antineoplásicos; Neoplasias colorretais.

1. Americas Centro de Oncologia Integrado, Medical Oncology - Rio de Janeiro - RJ - Brazil.
2. Instituto COI, Medical Oncology - Rio de Janeiro - RJ - Brazil.

INTRODUCTION

According to the World Health Organization, there are about 1.9 million colorectal cancer (CRC) cases worldwide, being the third place in the global ranking of malignancies.[1] CRC has favorable prognosis when detected in early stages with relative survival of 65% in five years (91% for localized and 14% metastatic tumors, approximately).[2] These tumors can develop in different anatomical sites (ascending colon, transverse colon, descending colon, sigmoid colon, and rectum) and show distinct clinical and biological features.[3-6] Nonetheless, metastatic CRC (mCRC) is understood as a unique disease regardless of its origin.

There are many critical clinical aspects to define prognosis and treatment in CRC. Tumor location can be correlated to outcome,[7-9] although the underlying reasons are still under discussions, like its embryonic origin and microbiome divergence.[10-12] Also, tumors with abundant extracellular mucin content drive patients into worse outcome. About 5-15% of CRC are classified as mucinous colorectal adenocarcinoma and are associated with poor survival,[13] increased chance of metastasis,[14] and less chance of response to chemotherapy.[15,16]. When mucin is located intracellularly, pushing the nucleus aside, CRC is understood as signet ring cell carcinoma (SRCC) – a rare subset representing 1% of all cases and related to worse prognosis when compared to other histological subtypes.[17,18] In addition, the evaluation of non-invasive glycoprotein tumor markers in peripheral blood, such as CEA, CA19-9, and CA125, has been largely used for outcome assessment and patient monitoring.[19,20]

Overall survival (OS) of patients with CRC has been increasing over the last years, given remarkable advances with novel therapies.[21] Most cases require surgery to remove the primary tumor and, if necessary, the metastasis, which might be associated with radiotherapy and chemotherapy as neoadjuvant or adjuvant treatment.[22,23] However, several targeted therapies have been approved by Food and Drug Administration (FDA) since the last decade, including small molecule inhibitors of EGFR pathway-related key proteins (EGFR, HER-2, and BRAF), angiogenesis inhibitors (anti-VEGF/VEGFR), and more recently, immune checkpoint inhibitors (anti-PD-1).[24,25] These novel drugs were successfully associated with long-lasting response, prolonged survival, and reduced toxicity compared to conventional therapeutic options. Nevertheless, only a subset of patients benefits from these new drugs.

Herein, we discuss relevant molecular biomarkers in CRC that either are being routinely evaluated in the clinic or will be incorporated in short-term, mainly in metastatic disease. Our main focus is reviewing those clinical biomarkers with emphasis on molecular alterations, their hallmarks, and how they drive us to perform patients' prognosis stratification and identification of drug-responders.

Chromosomal aberrations and genomic instability

Genome instability is one of the hallmarks of cancer commonly observed in patients with CRC, especially those with worse outcomes. The gain of whole chromosomes and structural aberrations involving one or different regions of the genome has been used over the years to explain the CRC evolution from carcinoma to metastatic CRC.[26] At the molecular level, the genomic instability assessment seems to be a valuable tool to interrogate patients who might benefit from specific therapies.

Chromosomal instability (CIN): over 80% of all CRC cases display CIN, the most common type of genomic aberration in this tumor.[27] CIN is mainly defined by the presence of aneuploidy or polyploidy, which is typically assessed by flow cytometry. The mechanisms underlying this genomic instability in CRC progression remain unclear; however, CIN is frequently associated with worse prognosis, therapy resistance, and poor survival.[28,29]

Microsatellite instability (MSI): it can be determined using molecular biology approaches based on amplification (e.g.: PCR) or immunohistochemistry. The immunohistochemistry method uses antibodies against mismatch repair (MMR) proteins (MLH1, MSH2, MSH6, and PMS2). If all proteins are present, the tumor is considered MMR proficient. Otherwise, the tumor is MMR deficient (dMMR), which is correlated with the presence of microsatellite instability.[30] CRC is divided into MSI-high (MSI-H) or MSI-low (MSI-L), depending on the number of unstable markers identified by PCR. Tumors not classified as MSI-H or MSI-L are called stable microsatellites (MSS).[31] Somatic defects in *MMR* genes have been reported in approximately 19% of CRC, while 52% showed hypermethylation in *MLH1* gene promoter, which is associated with gene inactivation.[32,33] Therefore, if dMMR is detected by immunohistochemistry, it should prompt the evaluation of genes involved in the Lynch syndrome. Mutations in MMR genes are observed in patients with Lynch syndrome, also known as hereditary nonpolyposis colorectal cancer (HNPCC) syndrome, the most common cause of hereditary CRC.[34]

In general, MSI-H patients have no benefit from 5-FU treatment after surgery. Instead, these patients demonstrate lower survival than those who undergo surgery alone.[35] Likewise, results from a retrospective study of adjuvant treatment showed that tumors with dMMR displayed poor results with adjuvant therapy with 5-FU in stage II, but not in stage III.[36] Results obtained from analysis of ACCENT database, with compilation of 12 randomized clinical trials, suggest that adding oxaliplatin to adjuvant fluoropyrimidine improves OS in patients with MSI stage III, and then it could be the standard-of-care adjuvant treatment for patients with CRC MSI/dMMR stage III.[37] PETACC-3 study pointed out that tumors characterized by MSI-H are more frequently in stage II than III (22% versus 12%, respectively) and only 3% of patients with stage IV. These results suggest

that tumors with MSI-H/dMMR still have little chance of metastasis. Favorable outcome was observed in those MSI-H.[38-40] In summary, MSI predicts good outcome in CRC, but it has no benefit to the treatment with fluoropyrimidine (5-FU).[41] Therefore, the MSI evaluation using immunohistochemistry or molecular biology techniques-based is important to select patients at stages III and IV to adjuvant chemotherapy.[42]

CRC is commonly affected by tumor-infiltrating lymphocytes (TILs).[43] Although the expression of immune checkpoints (PD-1, CTLA-4, and their ligands) is variable across the samples, higher expressions of PD-1 and PD-L1 correlates with better prognosis in CRC patients.[44] Furthermore, MSI-H/dMMR patients display a high-density of T helper 1 (TH1) CD4+ cells, important to promote anti-tumor response, associated with upregulation of immune checkpoint.[45] This fact could, at least in part, explain the low response ratio observed in the first clinical trials dedicated to investigating immune checkpoint inhibitors in unselected CRC patients. In 2015, it was shown the first evidence that only CRC MSI- H/dMMR patients would benefit from anti-PD-1 inhibitor (pembrolizumab) due to the incredible progression-free (PFS) and long-term OS reached upon treatment.[46] Subsequent studies reinforced these findings exploring other anti-PD-1 inhibitors solely or associated with CTLA-4 inhibitors,[47] accelerating their approval by the FDA in 2017. Finally, the phase III trial, KEYNOTE-177, showed an improvement in progression-free survival (16.5 months vs. 8.2 months; hazard ratio=0.60; 95%CI: 0.45-0.80; p=0.0002) for patients with MSI- H/dMMR who received pembrolizumab versus chemotherapy in the first line mCRC setting.[48]

Loss of heterozygosity on chromosome 18q (18qLOH): despite CIN and MSI, CRC might also harbor punctual chromosomal abnormalities. Loss of heterozygosity (LOH) on chromosome 18q (18qLOH) is the most frequent cytogenetic alteration in CRC, corresponding to 70% of cases, approximately.[49] Patients with 18qLOH have worse prognosis likely due to its association with CIN;[50] however, it is still unclear whether 18qLOH represents an independent prognostic biomarker.

Fusion genes: fusion genes arising from chromosomal rearrangements contribute to the hallmarks of CRC, even being infrequent. Several fusion genes were reported, mostly involving actionable genes, such as *NTRK, ALK, BRAF, RET*, and *FGFR*. However, there is no still evidence of benefits for those patients upon treatment with tyrosine kinase inhibitors (TKI), but patients harboring *NTRK*- treated with entrectinib and larotrectinib.[51,52] *NTRK*- fusions are associated with MSI-H, *RAS* and *BRAF* wild type (WT), dismal prognosis, and seems to explain part of the cases with primary resistance to EGFR therapy.[53,54] Other fusion genes do not show impact on outcome or even correlation with disease subtype.

Genetic mutations and aberrant expression

The presence of mutations in genes implicated to cell signaling pathways that control proliferation, dif-ferentiation, apoptosis, angiogenesis, and invasion is as crucial as genomic instability for the pathogenesis of CRC. The most common pathways dysregulated in CRC are WNT-β-catenin, β growth factor (TGFβ), epidermal growth factor receptor via mitogen-activated protein kinases (EGFR-MAPK), and phosphatidylinositol 3-kinase (PI3K) signaling pathway (Figure 1).[55,56]
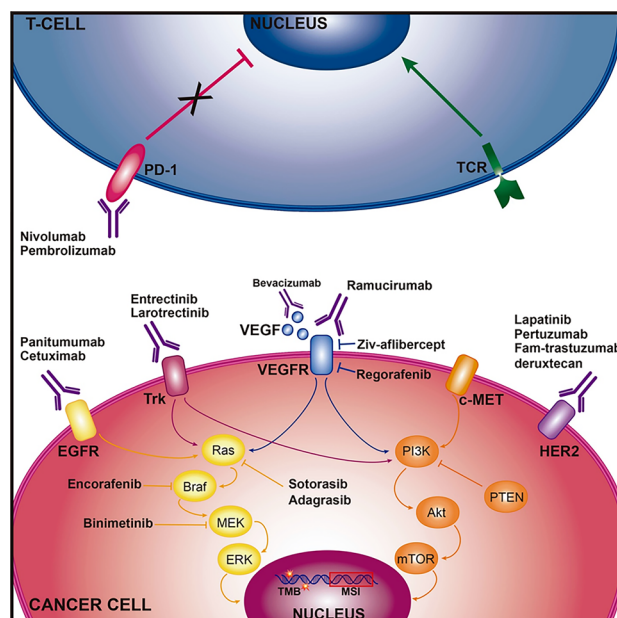

Figure 1. Biomarkers, signaling pathways and drugs in CRC. Genetic alterations frequently observed in CRC affect mainly signaling pathway receptors and their downstream partners. The target therapies already in clinical use can act on these biomarkers or immune cells, blocking checkpoint inhibitors to suppress T-cell response.

PI3KCA and PTEN: mutations in *PI3KCA* are observed in about 40% of CRC cases. In *PTEN*, a tumor suppressor gene that negatively regulates PI3K signaling, the mutations are present in about 30% of MSI and 9% of CIN tumors.[56] The PI3K pathway is modulated by EGFR and through KRAS activation, but there is no evidence about the role of *PIK3CA* or *PTEN* mutations as predictive markers of anti-EGFR therapy.[57,58] Mutations in those genes seem not to have an impact as prognostic biomarkers in CRC.[59,60]

KRAS and NRAS: KRAS mutations in codons 12 and 13 (exon 2) induce activation of MAPK/ERK cell signaling pathway regardless of the binding of growth factor to the cell surface receptor (e.g.: EGFR).[61,62] These somatic variants can predict the lack of drug- response to cetuximab and panitumumab, both anti-EGFR therapy, and are present in about 40% of individuals with CRC.[63,64] In Brazil, a cohort study analyzed over eight thousand patients with mCRC with average age of 59 years-old.[65] In the study, authors performed *KRAS* genetic sequencing of codons 12 and 13 and revealed 31.9% of mutated cases. The PRIME study reported that 17% of 641 CRC patients did not present *KRAS* mutation in exon 2, but instead in exon 3 and 4, or in *NRAS* (exon 2-4). The authors concluded that patients with any *KRAS* or *NRAS* mutation who received anti-EGFR therapy did not have better rates

of PFS and OS compared to those who received only chemotherapy.[64] In addition, the presence of *KRAS* mutations do not correlate with age.[66] Otherwise, the FIRE-3 trial included patients with *KRAS* (exon 2) codon 12/13 wild-type mCRC and displayed longer overall survival in patients who received FOLFIRI plus cetuximab compared to FOLFIRI plus bevacizumab. [67] Currently, all CRC patients with stage IV are strongly suggested to be evaluated for -*RAS* and *BRAF* mutations to be treated with an anti-EGFR drug, as long as the tumor is not located in the right colon, given those patients do not benefit from that therapy at first-line regardless -*RAS* status. Recently, sotorasib and adagrasib showed efficacy in heavily pre-treated advanced solid tumors harboring the KRAS G12C mutation. Of the 42 patients with mCRC treated with sotorasib, 73.8% (31 patients) had disease control and 7.1% (3 patients) had an objective response; the median progression-free survival was 4.0 months.[68] Likewise, adagrasib showed acceptable safety profile and promising clinical activity in a small cohort of pre-treated mCRC patients with KRAS G12C mutation.[69] Interesting, CRC patients that developed lung metastasis harbor *KRAS* mutations more frequently suggesting the screening for those alterations might be a relevant to lead patients to surgical resection.[70]

BRAF: approximately 5-9% of the cases are characterized by a specific point mutation in *BRAF* (V600E), mutually exclusive with *KRAS* exon 2 variants.[71] It is clear that *BRAF* V600E mutation is a strong prognostic factor. However, about 1/5 of CRC patients with BRAF mutations harbor another different type than not V600E, in general, associated with younger men individuals with low-grade tumor. Also, the non-V600E-BRAF were related to longer survival in those cases. [72] Despite the strong correlation between the presence of *BRAF* mutations and MSI, these alterations might also affect MSS patients and correlate with lower survival, whereas no impact is observed in MSI cases.[73] The PETACC-3 study demonstrated that mutations in this gene were strong predictors of poor OS in patients with MSI-L/MSS in stage II or III, being these data confirmed by CRYSTAL and AGITG MAX trials.[74-76] The evaluation of CRC refractory to chemotherapy suggested that *BRAF* mutations display significantly lower response rate to cetuximab than WT tumors (8.3% vs. 38.0%).[77] Additionally, patients with *BRAF* V600E-mutated mCRC who had disease progression after one or two previous regimens had a longer OS with the combination of encorafenib, binimetinib, and cetuximab or encorafenib and cetuximab compared to the control group (cetuximab plus irinotecan or cetuximab plus FOLFIRI).[78]

Vascular endothelial growth factor (VEGF): although VEGF and its receptor can be targeted by monoclonal antibodies (MoAb), their role as a predictive biomarker is not established. Bevacizumab (MoAb targeting VEGF-A) has shown improvements in PFS and OS in the first- and second-line treatment of mCRC when combined to fluoropyrimidine-based chemotherapy backbone.[79-81] Moreover, ramucirumab (MoAB that

binds to VEGFR-2) and aflibercept (recombinant fusion protein that binds VEGF-A, VEGF-B and placental growth factor) has shown improvements in PFS and OS when added to fluoropyrimidine-based chemotherapy backbone in the second-line treatment of mCRC.[82,83]

SMAD4: in 2012, Isaksson-Mettävainio et al.[84] showed that high expression of *SMAD4* was significantly correlated with a favorable prognosis in CRC MSI-H. Previous studies have revealed that loss of *SMAD4* expression was associated with advanced stage, metastatic potential, adverse prognosis, and 18qLOH, regardless of MSI status.[85] However, the underlying mechanism and prognostic value of variations in *SMAD4* expression in CRC are not completely elucidated.

HER-2: *ERBB2* oncogene amplification or HER2 protein overexpression accounts for 3-5% of CRC, but the frequency of alterations in the gene might increase after treatment with to anti- EGFR.[86] Therefore, screening for such alterations through more sensitive and less invasive approaches after EFGR-therapy resistance might be a trend. Although alterations in *MET* e *HER-2* seem to confer resistance to this type of therapy, some patients with HER2^{amp} can benefit from HER-2 inhibitor.[87-90] The phase II HERACLES trial showed that the combination of trastuzumab and lapatinib in 27 eligible patients with HER-2-overexpressing mCRC and *KRAS* exon 2 WT resulted in 30% objective response and 44% stable disease.[87] Furthermore, MyPathway study showed 32% objective response with trastuzumab plus pertuzumab in HER-2 overexpressed or amplified mCRC.[91] Lately, the phase II DESTINY-CRC01 trial showed that the use of Fam-trastuzumab deruxtecan resulted in 45% objective response rate in pre- treated HER-2-overexpressing mCRC patients with a median PFS of 6.9 months and duration of response not reached.[92]

POLE and POLD1: polymerase proofreading-associated polyposis is a dominant-inheritance and high-penetrance hereditary syndrome, caused by variants in the exonuclease domain (EDMs) in *POLE* and *POLD1* genes, and it is correlated with a predisposition to attenuated colorectal polyposis and early-onset CRC. *POLE* mutations have been reported in approximately 2% of patients and were related to better outcomes in stage II-III CRC.[93] Domingo et al. (2016)[94] also reported upregulation of immune checkpoints in patients with *POLE* EDMs. These findings further suggested that CRC patients with these mutations might respond to immune checkpoint inhibitors when clinically indicated their administration.[95]

PD-L1: most of these genetic variants cannot predict response to immunotherapy, which in many cases can be associated with PD-L1 expression. PD-1 is a cell surface receptor expressed by activated T-cells, β lymphocytes, natural killer cells and myeloid-derived suppressor cells.[96] PD-L1 is a cell surface receptor expressed in tumor cells. PD-1 and PD-L1 inhibitors can revert the immunotolerance state caused by the tumor. A recent

study suggested that PD-L1 expression has a broader role as a potential biomarker for predicting response to immune treatment.[97] PD-L1 expression was significantly correlated with lymphatic metastasis, tumor diameter and differentiation, vascular invasion, and could act as an independent poor prognostic factor in CRC.[98] Nonetheless, the correlation between PD-L1 expression and clinicopathological features and prognosis of CRC is still controversial.

Molecular subtypes of CRC – International CRC Subtyping Consortium Classification

Like other solid tumors, it is unfeasible to define CRC stratification at the molecular level only by one mutation or just a few events. The classification of patients into robust clinically defined subsets requires the combination of multiple biomarkers using different approaches, such as mutational evaluation, gene expression, and protein analysis, through a comprehensive assessment (Table 1).

It has been shown over the years the impact of epigenetics impairment on leukemia and solid tumors.[99] The disbalance in gene expression of key regulators implicated in proliferation, apoptosis and other cancer hallmarks can be explained beyond chromosomal damage, copy number alterations (CNA) and point mutations, as discussed so far. Epigenome mainly affects gene expression regulation and can act on different levels – DNA methylation, histone modifications and non-coding RNAs.

The International Colorectal Cancer Subtyping Consortium classified CRC patients into four different groups based on the tumor gene expression profile: CMS1 (MSI, immune), CMS2 (canonical), CMS3 (metabolic), and CMS4 (mesenchymal).[100] All groups show distinct outcome and seem to exhibit a specific mutational pattern.

CMS1 group represents about 14% of all CRC cases; it has low prevalence of somatic CNA and is generally hypermethylated. These tumors show abundant immune infiltrate and are associated with MSI. Interestingly, CMS1 shows overactivation of JAK/STAT pathway, which seems to be related to the upregulation of proteins involved in immune response and enrichment of cases with *BRAF* V600 mutation. Besides the molecular landscape, CMS phenotypes can display a correlation with tumor location and histopathological features. For instance, most of the CMS1 tumors are observed in women with right-sided lesions and high histopathological grade. Compared to the other groups, CMS1 patients hold favorable outcome, which could be explained by the high diffuse immune infiltrate, mainly compounded by cytotoxic cells.[101]

The canonical (CMS2) and mesenchymal (CMS4) phenotypes are characterized by CNI, which was initially measured through high levels of CNA. On the other hand, the distribution of nonsynonymous somatic mutation events is not high in those cases. CNI, commonly observed in CMS2 tumors, is the main reason to explain why over 1/3 of all CRC patients are classified into this subset, thus being named canonical subtype. *APC* mutations/loss are enriched in CMS2 tumors, as well as somatic variants in *KRAS,* and activation of Wnt/c-Myc, which is consistent with an

Table 1. Surrogate biomarkers in CRC and their clinical features.

Biomarker	Alteration	Clinical Implication	References
CIN	Aneuploidy and polyploidy	Poor prognosis	(28)
MSI / dMMR	Amplification / deficient MMR proteins	Good prognosis; increased RFS	(37-39)
18qLOH	Loss of heterozygosity on chromosome 18q	Response to immunotherapy; increased PFS	(46,47)
		Poor prognosis	(49)
NTRK	Fusion	Poor prognosis	(50-53)
KRAS	Mutation	Response to TKI	(62)
		Resistance to anti-EGFR therapy	
BRAF	Mutation	Response to TKI	(64,65)
		Poor prognosis	(67,69)
		Resistance to anti-EGFR therapy	(70)
		Response to TKI	(71)
MET	Overexpression	Resistance to anti-EGFR therapy	(81)
HER2	Amplification or overexpression	Resistance to anti-EGFR therapy	(83)
		Response to anti-HER2 therapy	(80,82,84,85)
ctDNA	Mutation	Predictive of recurrence	(107)
TMB	Mutation (/per Mb)	Response to immunotherapy	(113)

Abbreviations: CIN: Chromosomal instability; MSI: Microsatellite instability; dMMR: Deficient mismatch repair proteins; RFS: Relapse-free survival; PFS: Progression-free survival; EGFR: Epidermal growth factor receptor; TKI: Tyrosine kinase inhibitor; cfDNA: Circulating tumor DNA; TMB: Tumor mutational burden; Mb: Megabase.

upregulation of its downstream targets. Conversely observed in CMS1, CMS2 tumors are mainly left-sided and display superior survival rates after relapse. Regarding the morphological appearance, CMS2 and CMS3 are more epithelial-like (tubular adenoma), whereas CMS1 and CMS4 are mesenchymal.[102]

CMS3 and CMS4 represent 13% and 23% of CRC cases, respectively, having CMS4 the worst prognosis across all molecular subtypes.[100] Such as CMS2, CMS3 display features of epithelial-like characteristics, as well as metabolic dysregulation, with mixed MSI and ubiquitous MAPK pathway alterations, marked by mutations in *KRAS* gene. As previously discussed, CRC is characterized by genome instability commonly arising from dMMR and global genome hypermethylation, resulting in CpG island methylator phenotype (CIMP).[103] Despite its strong association with MSI, presence of mucinous features, poor tumor differentiation, and mutations in *BRAF*, CIMP is enriched in CMS1 and underrepresented in CMS3. On the other hand, CMS4 group characterized by CIN showed upregulation of genes implicated to angiogenesis, matrix remodeling, and epithelial-mesenchymal transition pathways, which in part respond why most mCRC cases are found in this molecular subtype. Notwithstanding the poor prognosis, only CMS4 patients seem to benefit from cetuximab, although with reduced sensitivity to chemotherapy.[104,105]

NOVEL APPROACHES

Circulating tumor DNA: for many years, clinicians and researchers pursued reliable strategies to access tumoral genetic and epigenetic features using non-invasive techniques. Regardless of underlying conditions, individuals can carry DNA in the blood, generally fragments not larger than 200kb.[106] This circulating cell-free DNA (cfDNA) seems to be more abundant in cancer patients (circulating cell-free tumor DNA - ctDNA) than in healthy individuals or subjects with non-malignant conditions. These molecules have been studied as surrogate markers in diagnosis establishment, prognosis assessment, predict treatment response, but mainly to monitor minimum residual disease (MRD).[106-109] A strong correlation between ctDNA and anatomical site of CRC was reported. In summary, patients with colon tumors tend to exhibit higher amounts of ctDNA in plasma than individuals with rectum tumors,[110] although this is not a consensus. Most of the studies investigating ctDNA in CRC context screened mutations previously identified in the tumor site to determine whether the findings obtained from liquid biopsy are reliable and can translate faithfully the genetic landscape of the tumor. Mutations in *BRAF* and *KRAS* genes had >95% of concordance between tumor and ctDNA analysis, especially in patients with advanced disease.[111,112] In addition, those variants were successfully tracked in plasma following tumor evolution, endorsing that this is a promising approach to be used instead of tumor-section investigation.[113] The identification of fusion genes and other actionable genes were reported also.[114] There is a dramatic drop in the levels of plasmatic ctDNA in patients subjected to surgery or other therapies, but it is still possible to detect it over time. In 2019, Reinert et al.[115] proposed that longitudinal investigation of ctDNA presence in samples from postoperative patients would be able to predict relapse up to 16 months earlier. Liquid biopsy is being used also to track the clonal dynamics of malignant cells after anti-EGFR therapy in CRC.[116] Mutations in *RAS* and *EGFR* showed relative allele frequency decays after therapy with a cumulative half-life of 4.4 months. Interesting, ctDNA monitoring was useful to guide the timing of re-challenge therapies for those patients, guiding them to higher responses. The Colon and Rectal-Anal Task Forces of the United States National Cancer Institute has been discussing how ctDNA might improve the clinical management of CRC by the detection of MRD and monitoring responses to therapy.[117]

Tumor mutational burden (TMB): the comprehensive tumoral investigation to discovery new biomarkers is going beyond the mutational landscape and gene expression signatures. Most exploratory studies with satisfactory response upon immunotherapeutic agents were performed on solid tumors, such as melanoma, non-small-cell lung carcinoma, advanced renal cell carcinoma, and CRC.[118] Levels of PD-1 and CTLA-4 expression, immune landscape (tumor-infiltrating immune cells profiling), and TMB have been mostly used to explain clinical response and predict which are the cases that will benefit from these immune checkpoint inhibitors. TMB is defined as the number of somatic variants found across the DNA of cancer cells and based on that, it is classified into hypermutated (TMB high) or non-hypermutated (TMB low). TMB and *POLE* mutations have been associated with clinical outcomes becoming promising biomarkers for both predictive and prognostic value in diverse cancers, including CRC. Studies have shown that besides patients with MSI-H tumors, CRC patients with MSS tumors and high TMB may respond to immunotherapy.[119,120] In MSI-H mCRC TMB is likely an independent biomarker and recent studies associated TMB with more favorable prognosis in CRC patients treated with chemotherapy and targeted therapy.[121] Also, there is no universal definition of high TMB considering that cut-points associated with improved survival between cancer types is varied.[122] The data from KEYNOTE-158, a phase II clinical trial study in multiple cancer types investigating the efficacy and safety of pembrolizumab, demonstrated that high TMB was associated with higher ORR (28.3% vs. 6.5%) in patients with select advanced solid tumors treated with pembrolizumab monotherapy, which accelerated the pembrolizumab's agnostic approval by FDA in patients with high TMB.[123] However, there was no CRC patients included in this trial.

Microbiome: the whole community of microorganisms living within a particular individual is defined as

microbiome. These microbes habit and interact actively with different body tissues, such as skin, gut, and stomach, and drive a remarkable role in immune cells ontogeny. There is no direct evidence that the commensal microbiome plays a determinant role in cancer pathogenesis, even though it has been shown its cooperation on tumor initiation and progression.[124] Intriguingly, patients with metastatic melanoma responsive to PD-1/PD-L1 axis inhibition seem to have differences in the gut microbiota composition compared to unresponsive cases.[125,126] In CRC, *Fusobacterium nucleatum* modulates the expression of genes implicated in immune cells recruitment leading to poor T-cell infiltration, which could explain at some point the differences observed in the profile of immunotherapy response.[127,128] In general, cancerous and normal tissue's overall microbiome is very similar. In contrast, CRC patients display more *Lactobacillales* and reduced *Faecalibacterium* than healthy individuals.[129] These differences are also observed in the CRC virome (whole viral composition), which could explain in part the bacterial composition of these tissues, given that viruses such as *Inovirus* and *Tunalikevirus* are enriched in CRC patients and can infect gram- negative bacteria.[11] Most of the studies in microbiome field only observed a correlation between microbiological composition in a given tumor. Although it is not totally understood how the microbiome influences cancer pathogenesis and progression, in vivo experiments suggest those bacterias promote inflammation and metabolic changes leading normal cells toward malignant phenotype or even cancer progression.[130]

More recently, studies have identified the relationship between certain strains of *Escherichia coli* with a pathogenic island, named *pks*+ *Escherichia coli,* which is attributed to polyketide synthetases (*pks*) that produces the genotoxin colibactin. This colibactin-producing bacteria promotes DNA damage, across interstrand crosslinks and double-strand breaks in cultured cells, with pro-tumorigenic effect. Using organoid technology, Pleguezuelos-Manzano et al. (2020)[131] identified two unique mutational signatures (single base substitution named SBS-*pks* and small insertion and deletion, the ID-*pks*) caused by exposure to *pks*+ *E. coli.* Those signatures were correlated with colorectal metastases and primary tumors through two WGS datasets. The first analysis of data was from a Dutch collection of 3,668 solid cancer metastases (496 from CRC) revealed that 7.5% of CRC samples were enriched for SBS-*pks* and 8.8% for ID-*pks*, while the second analysis of the Genomics England 100,000 Genomes Project with 2,208 CRC tumours, confirmed the fingerprint of SBS and ID-*pks* in 5% and 4.4% of patients, respectively. Furthermore, the researchers also analyzed seven cohorts of patients with colorectal cancer and their driver mutations, defining *pks* signatures as oncogenic mutations, once they were observed in 2.4% of 4,712 CRC drivers mutations, and associated with mutations in APC gene, which harbored the highest number of mutations containing these signatures (5.3%).[131] In 2019, Lee-Six et al.[132] identified two mutational signatures occurring in healthy colonic crypts named as SBS-A and ID-A in 29 of 42 individuals, and data analysis suggest that these signatures were acquired in children before 10 years of age. These signatures were confirmed by Pleguezuelos-Manzano et al. (2020)[131] with the same aetiology of SBS-*pks* and ID-*pks.* Although more investigation is needed, researchers highlights about the colibactin mutagenic effect in vitro, the mutational signatures associated with a risk of developing CRC and promotes discussion about re-evaluation of probiotics that contain genotoxic strains of *E. coli*.[131]

CONCLUSION

Over the last decades, the molecular mechanisms of CRC carcinogenesis have been unraveled. The identification of signaling pathways committed to CRC, as well as their genetic vulnerabilities, allowed the establishment of new biomarkers, druggable targets, and therapeutic agents. These new findings resulted from the development of genomic high-throughput approaches, such as next-generation sequencing and array-based techniques. New biomarkers in CRC range from chromosomal changes to specific variations in the DNA sequence, and when grouped, allow the establishment of clinically homogeneous groups with a well-defined prognosis and treatment.

Most of these markers are already being used in clinical practice, and many others will be inserted into the routine as soon as possible. In precision medicine, the biggest challenge will be the development of algorithms that combine these new findings to efficiently predict each patient's response profile to the respective drugs.

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AUTHOR CONTRIBUTIONS

Conceptualization: J.C, A.P.V, L.H.A and F.M.A.C.V. Writing-original draft preparation: J.C, A.P.V and F.M.A.C.V.

Writing-review and editing: J.C, A.P.V, K.B.A, L.H.A and F.M.A.C.V.

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CONFLICT OF INTEREST STATEMENT

The authors declare no conflicts of interest.

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