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# Current and future biomarkers in colorectal cancer

**George Zarkavelis[a,b], Stergios Boussios[a,b], Alexandra Papadaki[a,b], Konstantinos H. Katsanos[c], Dimitrios K. Christodoulou[c], George Pentheroudakis[a,b]**

Medical School, University of Ioannina; Society for Study of the Clonal Heterogeneity of Neoplasia, Ioannina, Greece

**Abstract**

Colorectal cancer (CRC), one of the leading causes of death among cancer patients, is a heterogeneous disease and is characterized by diversions in multiple molecular pathways throughout its evolutionary process. To date, specific mutations in RAS and RAF genes are tested in everyday clinical practice along with mismatch repair gene deficiency, serving either as prognostic or predictive biomarkers, providing information for patient risk stratification and the choice of appropriate therapy. However, ongoing studies are focusing on the potential role of recently discovered genetic and epigenetic alterations in the management of CRC patients and their potential prognostic or predictive value. To overcome the problem of tumor heterogeneity as well as the practical obstacles of access to tumor tissue, and to achieve real-time monitoring of disease and therapy efficacy, liquid biopsies constitute a novel technology worth exploring. CRC screening and management is entering a new era where molecular testing will be applied to genomic material extracted from easily accessible bodily fluids.

**Keywords** Colorectal cancer, biomarkers, prognostic and predictive markers, liquid biopsies

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## Introduction

Colorectal cancer (CRC) is one of the leading causes of death among cancer patients worldwide. Risk factors associated with the incidence of CRC include older age, male sex, lifestyle, inflammatory bowel disease and a previous personal history of CRC. A positive family history is also strongly correlated with an increased relative risk of CRC diagnosis during lifetime. However, CRC is an indolent disease in its early stages and usually becomes symptomatic when it progresses to more advanced stages. Many efforts have been made to establish appropriate screening methods, but, to date, these remain invasive, resulting in lower participation rates among the healthy population [1].

Recent advances in our knowledge of the molecular basis and cellular mechanisms of CRC have led to the adoption of specific molecular tests in every day clinical practice. Based on the test's results the patient's risk is stratified and therapy is determined. Molecular biomarkers that serve as prognostic factors are already in use and specific genomic mutations serving as predictive biomarkers are examined in formalin-fixed tumor tissues. However, ongoing research for the identification of noninvasive biomarkers may lead to a new era in diagnosis, risk prediction and choice of treatment [2].

## Right- vs. left-sided CRC biology

There is no uniform and consistent definition of the dividing point between right- and left-sided CRC. The most common distinction defines cancers proximal to the splenic flexure as right-sided and cancers at or distal to the splenic flexure as left-sided [3]. This cutoff point is often used because approximately two thirds of the transverse colon arises embryologically from the midgut, and only the distal one third arises from the hindgut (Table 1). Vascular supply has also been proposed as a defining characteristic of embryologic origin: the superior and inferior mesenteric arteries supply the midgut and hindgut, respectively.

There are also differences in gene expression profiles between normal right and left colon epithelium. Normal

[a]Department of Medical Oncology, Medical School, University of Ioannina (George Zarkavelis, Stergios Boussios, Alexandra Papadaki, George Pentheroudakis); [b]Society for Study of the Clonal Heterogeneity of Neoplasia, Ioannina, Greece (George Zarkavelis, Stergios Boussios, Alexandra Papadaki, George Pentheroudakis); [c]Department of Gastroenterology, University Hospital of Ioannina, Faculty of Medicine, School of Health Sciences, University of Ioannina, Ioannina (Konstantinos H. Katsanos, Dimitrios K. Christodoulou), Greece

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Correspondence to: Dr Stergios Boussios MD, PhD, Medical Oncologist, Department of Medical Oncology, Medical School, University of Ioannina, Greece, Stavros Niarchou Avenue, 45110, Ioannina, Greece, e-mail: stergiosboussios@gmail.com

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right colon has a higher expression of cytochrome P450 family genes than does the left colon [4]. Likewise, there are significant differences in the patterns of gene methylation between the right and left colon [5]. Notably, the prevalence of promoter methylation of the mismatch repair (MMR) gene *hMLH1* and the O-6-methylguanine-DNA methyltransferase (*MGMT*) is significantly greater in normal right colon mucosa, especially in older women, suggesting epigenetic aberrations in preneoplastic right colon mucosa that may be reflected in subsequent right-sided adenocarcinoma biology [6].

There are different rates of mutation in key oncogenes and tumor suppressors between right- and left-sided CRC [7]. Mutations in *BRAF* V600E are significantly more common in right-sided CRC [8] (Table 1). Conversely, mutations in *APC* and *TP53* are enriched in left-sided CRC [7]. Recently, different patterns of mutations in *APC*, *TP53*, and *KRAS* were identified as conferring differential prognoses in CRC [9]. Besides point mutations, potentially targetable amplifications of receptor tyrosine kinases, such as *ERBB2* and epidermal growth factor receptor (*EGFR*), are also more common in left-sided CRC [4].

The *EGFR* ligands epiregulin (*EREG*) and amphiregulin (*AREG*) are differentially expressed between right- and left-sided CRCs [10]. EREG and AREG expressions are significantly higher in left-sided CRC [4,11], and are inversely correlated with promoter methylation [11].

There is differential prognosis by stage between patients with right- and left-sided CRC (Table 1). Retrospective studies suggest that right-sided tumors have a slightly better prognosis in stage II CRC, but a slightly worse prognosis in stage III disease, which is probably associated with the higher prevalence of good-prognosis microsatellite unstable (MSI-High) tumors in right-sided stage II cancers [3] (Table 1). Furthermore, analyses of prospective clinical trials of patients with stage III CRC who received adjuvant chemotherapy also demonstrated inferior progression-free survival (PFS) in those with right-sided CRC (hazard ratio [HR], 0.70; 95% confidence interval [CI] 0.61-0.81) [12]. Patients who have metastatic CRC with a right-sided primary also have an inferior prognosis compared to those with a left-sided primary [13]. This was highlighted by a pooled analysis of 3 studies of 2027 evaluable patients treated with first-line chemotherapy, in which those with left-sided CRC had significantly better PFS and overall survival (OS) compared to those with right-sided CRC, even after adjustment for *BRAF* mutation and mucinous histology [14].

The primary CRC site is prognostic but not predictive of outcome with therapy based on the anti-angiogenic monoclonal antibody bevacizumab. An analysis of 2 prospective randomized controlled trials of chemotherapy, with or without bevacizumab, revealed that side is not a predictive biomarker for or against the benefit of bevacizumab. The statistical interaction test between side and bevacizumab use was nonsignificant [14].

However, the primary CRC site is both prognostic and predictive of benefit with anti-EGFR therapy among patients with *KRAS* wild-type, refractory, metastatic CRC. In the CO.17 randomized trial, among those with *KRAS* codon 12/13 wild-type disease, there was a significant improvement in PFS with cetuximab in patients with a left-sided primary (HR 0.28; 95%CI 0.18-0.45), whereas there was no difference in PFS among those with a right-sided primary (HR 0.73; 95%CI 0.42-1.27; interaction P=0.002) [15]. Additional retrospective studies also showed that patients with left-sided CRC had better PFS with anti-EGFR therapy compared with those with right-sided CRC, even among patients with *KRAS*/*BRAF* wild-type [4] and extended *RAS* and *BRAF* wild-type mutations [16] (Table 1).

## *POLE* and *POLD1* germline mutations in familial CRC and polyposis

Germline mutations in DNA polymerase 1 (*POLE*) and d (*POLD1*) have recently been identified in families with multiple colorectal adenomas and CRC. All reported cases carried *POLE* c.1270C>G (p.Leu424Val) or *POLD1* c.1433G>A (p.Ser478Asn) mutations. Because of the scarcity of cases reported so far, an accurate clinical phenotype has not been defined. The two pathogenic mutations show dominant inheritance and confer high risk to multiple colorectal adenomas, large adenomas, early-onset CRC or multiple CRC. *POLD1* p.S478N also confers an increased risk to endometrial cancer in female carriers [17-21].

Phenotypic data from the 69 carriers (29 families) of *POLE*/*POLD1* exonuclease pathogenic mutations reported to date [17-21], point to an associated phenotype characterized by attenuated or oligo-adenomatous colorectal polyposis (>80% of *POLE* and >60% of *POLD1* mutation carriers were diagnosed with ≥2 adenomas), CRC (60-64% of carriers), and probably brain tumors (5.8%). Gastroduodenal (mostly duodenal) adenomas were detected in 57.1% of carriers who underwent gastroduodenoscopy [20]. Moreover, the *POLD1* phenotypic spectrum includes endometrial (57.1% of female carriers) and breast (14.3% of female carriers) tumors. All 21

**Table 1** Molecular features of CRC by site

| | CIMP High | MSI High | MLH1 Methylation | *BRAF* mutation | CIN | CMS | Prognosis | Outcome with cetuximab | Embryogenesis |
|---|---|---|---|---|---|---|---|---|---|
| Right-sided CRC | High prevalence | High prevalence | High prevalence | High prevalence | Low prevalence | 1, 3 | Poorer | Inferior | Midgut |
| Left-sided CRC | Low prevalence | Low prevalence | Low prevalence | Low prevalence | High prevalence | 2, 4 | Better | Superior | Hindgut |

*CIMP, CpG island methylator phenotype; MSI, microsatellite instability; CIN, chromosomal instability; CMS, consensus molecular subtypes; CRC, colorectal cancer*

*POLE*/*POLD1* mutation carriers without cancer underwent resection of colorectal adenomas, indicating complete or very high expressivity of the associated phenotype. A better characterization of the syndrome is currently required to establish specific criteria for *POLE* and *POLD1* exonuclease mutation screening and to help define the clinical management of mutation carriers.

### *RAS* mutational status

*KRAS* proto-oncogene encodes a GTPase protein (*KRAS*) that has a substantial role in many molecular pathways. Approximately one third of CRC have point mutations in exon 2 (codons 12 and 13) or exon 3 of *KRAS*. Those mutations cause permanent activation of the *RAS* (*RAS/RAF/MAPK*) pathway. In addition, 15% of CRC carry mutations in exons 2, 3 and 4 of the *NRAS* gene [22]. These mutations can predict resistance to anti-EGFR therapy consisting of cetuximab or panitumumab monoclonal antibodies [23,24]. The clinical benefit of anti-EGFR antibody therapy is only observed in *RAS* wild-type tumors. Detection of *KRAS* mutations has become of high utility as a negative predictive factor, when deciding about the use of anti-EGFR therapy. In a retrospective series, the clinical characteristics of *KRAS*- and *NRAS*-mutated tumors were very similar, with the exceptions of an extremely low incidence of mucinous histology in *NRAS*-mutated tumors (4% vs. 26%, P=0.012) and a slightly lower prevalence of lung metastases (30% vs. 36%, P=0.012) [25]. In terms of clinical outcome, worse median OS was observed in *NRAS*- and *KRAS*-mutated patients (25.6 months and 30.2 months, respectively) compared with all wild (42.7 months). Among 47 *NRAS*-mutated patients, 19 (40%) received an anti-EGFR in advanced lines. Eight of them were evaluated for response to anti-EGFR therapy. Five, 2 and 1 patients received cetuximab plus irinotecan, cetuximab monotherapy and panitumumab monotherapy, respectively. At the first reassessment, 7 patients experienced disease progression and only 1 achieved initial disease stabilization, though it progressed after 8 weeks. Median PFS and OS were 2.4 and 8.5 months, respectively [25]. In patients with *NRAS* mutations, expanded RAS mutational status (both tumor *KRAS* and *NRAS*) should be evaluated in all candidates for anti-EGFR therapy [26]. There is a dire need for the identification and validation of positive predictive factors that are able to select, rather than exclude, tumors amenable to therapeutic EGFR axis modulation.

In another retrospective study, 84 patients with *NRAS*-mutant metastatic CRC were analyzed in terms of clinical characteristics, outcomes, and response to therapy [27]. OS was significantly shorter for *NRAS* exon 3 mutant metastatic CRC patients compared to *RAS* wild-type metastatic CRC patients (HR 2.85; 95%CI 1.87-4.36, P<0.01) and to *NRAS* exon 2 mutant metastatic CRC patients (HR 2.0; 95%CI 1.04-4.0, P=0.039). *NRAS* mutation represents a clinically and molecularly distinct subgroup of metastatic CRC, with increased left-sided colon primary. An increased proportion of *RAS*-mutant tumors, both *KRAS* and *NRAS*, was detected among African-American patients compared to Caucasians that was more pronounced for *NRAS* [27]. Approximately 68% of metastatic CRC in African Americans had a *RAS* mutation, possibly contributing to the poor outcomes among African Americans.

### *BRAF* mutations

In the *RAS* signaling pathway (*RAS/RAF/MAPK/RTK*) the direct downstream target of KRAS is *BRAF*, which encodes serine threonine kinase proteins [28,29]. CRC that carry *BRAF* mutations have been associated with poor prognosis. *BRAF* mutations are more frequent in right colon tumors, poorly differentiated, with a mucinous histology and infiltrating lymphocytes that are usually MSI-High [30]. Approximately 8% of CRC carry the distinct *BRAF* V600E mutation. This point mutation is mutually exclusive with *KRAS* mutations and is an adverse prognostic marker in advanced disease [31,32]. Contrary to the high response rates in melanoma, *BRAF* V600E positive colorectal adenocarcinomas are resistant to *BRAF* inhibition (i.e., vemurafenib), because of feedback activation of the EGFR/PI3K/AKT pathway [33-35]. Thus, resistance to *BRAF* inhibitors in this subset of CRC cases has prompted the investigation of combined *EGFR-BRAF-MEK* inhibition, with or without chemotherapy [36] (Table 2). Minimal activity has recently been reported with the combination of *BRAF* plus *MEK* inhibitors in patients with *BRAF* V600E-mutant metastatic colon cancer, in contrast to promising response rates of vertical *BRAF*/*MEK* inhibition combined with anti-EGFR monoclonal antibodies in patients with refractory CRC [37].

In a retrospective study, Jones *et al* performed a detailed analysis of several large next-generation sequencing databases to determine the range of non-V600 mutations present in metastatic CRC [38]. They found that 2.2% of patients harbored a different *BRAF* point mutation in their cancers, many of them within 10 bases of the 600 location. Obviously, in a common disease like metastatic CRC the absolute number of patients affected is significant. Clinical characteristics and outcomes for these patients were carefully collected, and the investigators demonstrated that patients with non-V600 *BRAF*-mutant metastatic CRC were significantly younger (58 vs. 68 years, respectively), more often male (65% vs. 46%, respectively), and less likely to have high-grade tumors (13% vs. 64%, respectively) or right-sided primary tumors (36% vs. 81%, respectively) compared to those with V600E *BRAF*-mutant metastatic CRC. Additionally, patients with non-V600 BRAF mutations were more likely to have concomitant *RAS* mutations than patients with V600E *BRAF* mutations (26% vs. 2%; P<0.001), but less likely to have MSI (6% vs. 30%; P<0.001). Median OS was significantly longer in patients with non-V600 *BRAF*-mutant metastatic CRC as compared to those with either V600E *BRAF*-mutant or wild-type *BRAF* metastatic CRC (60.7 vs. 11.4 vs. 43.0 months, respectively; P<0.001). In multivariable analysis, non-V600 *BRAF* mutation was independently associated with improved OS (HR 0.18; 95% CI 0.10-0.32; P<0.001). It seems that non-V600 *BRAF* mutations

**Table 2** Activity of BRAF inhibitors and combinations of targeted therapies in *BRAF* V600E-mutated colorectal cancer

| Author/Reference | Year of publication | Treatment | Phase | Number of patients | OR (%) | SD (%) | PFS (months) |
|---|---|---|---|---|---|---|---|
| Yaeger *et al* [36] | 2015 | Vemurafenib + panitumumab | 1/2 | 15 | 13 | 53 | 3.2 |
| Corcoran *et al* [37] | 2015 | Dabrafenib + trametinib | 1/2 | 43 | 7 | 56 | 3.5 |
| Gomez-Roca *et al* [84] | 2014 | Encorafenib | 1 | 18 | 0 | 67 | 4 |
| Kopetz *et al* [85] | 2015 | Vemurafenib | 2 | 21 | 5 | 33 | 2.1 |
| Hyman *et al* [86] | 2015 | Vemurafenib | 2 | 10 | 0 | 50 | 4.5 |
| | | Vemurafenib + cetuximab | 1/2 | 27 | 23 | 62 | 3.7 |
| Hong *et al* [87] | 2016 | Vemurafenib + cetuximab + CPT11 | 1b | 19 | 35 | - | 7.7 |
| Corcoran *et al* [88] | 2016 | Dabrafenib + panitumumab | 1/2 | 20 | 10 | 80 | 3.5 |
| | | Dabrafenib + trametinib + panitumumab | 1/2 | 91 | 21 | 59 | 4.2 |
| Tabernero *et al* [89] | 2016 | Encorafenib + cetuximab + alpelisib | 1b/2 | 52 | - | - | 5.4 |
| | | Encorafenib + cetuximab | 1b/2 | 50 | - | - | 4.2 |

*OR, objective response; SD, stable disease; PFS, progression-free survival*

define a clinically distinct molecular subtype of metastatic CRC with an excellent prognosis, and that these findings have immediate clinical implications.

Several *post hoc* analysis of phase 3 randomized trials evaluated the predictive impact of the *BRAF* V600E mutation on the efficacy of anti-EGFR therapies. The results of these retrospective analyses did not reach statistical significance and were insufficiently powered to conclude whether or not the *BRAF* V600E mutation is a biomarker of primary resistance to anti-EGFR agents in CRC [39-43]. Pietrantino *et al* conducted a meta-analysis of randomized trials to evaluate whether cetuximab or panitumumab in monotherapy or in combination with chemotherapy improved survival in patients with *BRAF* V600E-mutated CRC [44]. In this meta-analysis, the addition of anti-EGFR agents to standard treatment in the *RAS* wild-type/*BRAF* V600E-mutated subgroup did not significantly improve PFS (HR 0.88; 95%CI 0.67-1.14; P=0.33) and OS (HR 0.91; 95%CI 0.62-1.34; P=0.63). However, another meta-analysis by Rowland *et al* evaluated whether the efficacy of anti-EGFR therapies differed according to the *BRAF* V600E mutational status by performing interaction tests [45]. Trials comparing anti-EGFR monoclonal antibodies to bevacizumab were excluded, taking into consideration that they were not comparable to those evaluating standard treatment with or without anti-EGFR agent. The reported HRs for PFS and OS which benefit from anti-EGFR therapies were 0.86 (95%CI 0.61-1.21) and 0.97 (95%CI 0.67-1.41) for *RAS* wild-type/*BRAF* V600E-mutated tumors and 0.81 (95%CI 0.70-0.95) and 0.62 (95%CI 0.50-0.77) for *RAS* wild-type/*BRAF* wild-type tumors. Tests of interaction of PFS and OS HRs between the two populations were not statistically significant (P=0.43 and P=0.07, respectively), suggested that the observed differences in survival benefit with anti-EGFR agents according to *BRAF* mutational status may be due to chance alone. Conversely, there may be insufficient evidence to justify the exclusion of anti-EGFR therapies for patients with *RAS* wild-type/*BRAF* V600E-mutated metastatic CRC.

## DNA MMR genes/MSI

Microsatellites are repeating sequences of DNA in coding and non-coding areas, with a length of 1-6 base pairs; they are also known as simple sequence repeats [46]. MSI is the result of the MMR gene's inability to fix DNA errors occurring during replication. Somatic mutations in those repeating sequences, consisting mainly of insertions or deletions, lead to unstable genomic loci. The MMR genes are inactivated as a result of sporadic MLH1 promoter hypermethylation or germline mutations in *MLH1*, *MSH2*, *MSH6* and *PMS2* genes [47]. The germline genetic abnormality of MSI is the molecular basis of Lynch syndrome, also known as hereditary non-polyposis colon cancer (HNPCC) [48]. Localized CRC that are MMR-deficient (MSI-H) have a more favorable prognosis compared to MMR proficient (MSI-L) tumors. Stage II colon cancer patients who are MSI-H do not benefit from adjuvant 5-fluorouracil therapy and have a very good prognosis. Among patients with stage III tumors who participated in adjuvant chemotherapy trials, those whose tumors were MMR-deficient experienced better clinical outcomes compared to those with MMR proficient tumors [12]. However, the association of MMR-deficient status with prognosis is less robust in stage III than in stage II disease, and limited data are available in patients treated with the adjuvant FOLFOX (folinic acid, 5-fluorouracil, and oxaliplatin) regimen in contrast to fluorouracil alone. *BRAF* V600E mutations are associated with poorer outcomes in a metastatic setting [12,32] and are significantly enriched in sporadic colon cancers with MMR-deficient or MSI tumors [7,49]. In this regard, *BRAF* should be analyzed in conjunction with MMR for prognostic stratification of CRC.

The analysis of Sinicrope *et al* revealed a significant association of MMR-deficient CRC with better survival after recurrence, for cancers of the proximal rather than distal colon [12]. Likewise, analysis of *KRAS* mutations by primary tumor site suggested a significantly shorter survival after recurrence for patients with distal but not proximal cancers.

Conversely, among patients with *KRAS* wild-type tumors, those with distal cancers achieved better survival than patients with proximal cancers. However, patients with stage III *KRAS* wild-type tumors, treated with FOLFOX with or without cetuximab, had similar survival, irrespective of the tumor site. In the metastatic setting, it seems that the treatment benefit of cetuximab is more pronounced for cancers of the distal colon than for proximal tumors [50]. Patients whose tumors harbored *BRAF* V600E mutations had significantly poorer survival, and this was more prominent in distal tumors.

It is becoming increasingly common to test all newly diagnosed CRC cases for MSI, as it serves as a prognostic marker (stage II CRC), as a screening marker for Lynch syndrome, or as a future predictive biomarker (stage IV CRC). Le *et al* reported a phase II study in which patients with metastatic MMR-deficient (MSI-H) colon cancer were treated with the anti PDL1 antibody pembrolizumab, resulting in a 62% objective response rate compared to MSI-L tumors [51]. This was probably due to the high index of infiltrating lymphocytes and the increased expression of neoantigens in MSI-H tumors as a result of their high genomic instability [52].

## CpG island methylator phenotype (CIMP) – DNA hypermethylation status

The main characteristic of CIMP+ phenotype is the high frequency of aberrantly methylated CpG islands (i.e., cytosine residues preceding guanines). It is more often observed in older patients, females and high-grade proximal tumors, which are usually *BRAF*-mutated and MMR-deficient (MSI-H) [49,53-56]. In addition, MLH1 promoter hypermethylation leading to MMR deficiency through gene silencing has been found in a subset of CIMP+ tumors [53]. CIMP status seems to be an emerging biomarker in CRC, because of its distinct mutations [57]. Furthermore, a plethora of hypermethylated genes, such as *SLC5A8*, *ITGA4*, *SFRP2*, *CDKN2A*, *HLTF,* and *MGMT*, seem to play crucial role in colon carcinogenesis [58]. Specific families of GTP-binding proteins, the septins, also participate in cell division. From the 13 known septins (*SEPT1–SEPT13*), *SEPT9* gene silencing due to hypermethylation leads to a compromise of cell cycle functions and promotes carcinogenesis, making it a plausible future biomarker [59].

## EGFR/HER family

EGFR is targetable in *KRAS* wild-type colorectal tumors by using the anti-EGFR antibodies cetuximab and panitumumab. However, after a few months of antibody administration, resistance to therapy emerges. *HER2* has recently been evaluated as a possible resistance pathway to anti-EGFR antibody therapy in *KRAS* wild-type tumors [60]. In 5% of *KRAS* non-mutated cancers, *HER2* is amplified and the administration of combined trastuzumab and lapatinib has been tested in a phase II study. The dual *HER2* blockade resulted in a 35% overall response rate and a median time to progression of about 5.5 months in heavily pretreated patients harboring HER2-amplified CRC. This is a hopeful indication that anti-HER2 therapy may be effective in this subset of metastatic CRC and *HER2* expression may serve as a predictive biomarker [61].

*EREG* and *AREG*, which are EGFR ligands, have recently been investigated as possible biomarkers in the therapy of *KRAS* wild-type colorectal tumors receiving anti-EGFR antibody therapy. Studies of the mRNA expression of *EREG* and *AREG* have found controversial results regarding the response to anti-EGFR therapy while evaluating their role as predictive biomarkers [10,62,63]. A recent meta-analysis of studies investigating the impact of *EREG* and *AREG* mRNA levels in primary colon tumors came to the conclusion that they both serve as independent favorable prognostic and predictive biomarkers. High *EREG* and *AREG* expression was associated with longer PFS in patients receiving anti-EGFR therapy, indicating the significance of *EREG* and *AREG* expression as biomarkers in CRC. More recently, evaluation of *EREG* and *AREG* levels and their association with PFS and OS was conducted in the FIRE1 clinical trial. Statistical analysis came to the conclusion that high *EREG* mRNA levels may serve as a positive prognostic marker regarding both PFS and OS, whereas high AREG levels did not affect patients' outcome [64,65].

## Chromosomal instability

Changes in the structure or number of chromosomes results in tumor karyotype alterations. Mutations that take place in oncogenes or tumor suppressor genes, along with defective telomeres, are the main causes of instability. Loss of heterozygosity, aneuploidy or amplifications are found in about 65% of CRC. Aneuploidy, which is an alteration in chromosomal number, is the result of defective mitotic checkpoint leading to abnormal segregation. Shorter telomeres, resulting from excessive telomere breakage, cause chromosomal instability, thus leading to carcinogenesis through transition from adenoma to carcinoma. Loss of heterozygosity, meaning loss of one parental allele, has been linked to CRC. Specifically, loss of heterozygosity in chromosome 18 has been linked to a poorer prognosis among CRC patients, especially those at stage II or III, who show lower survival rates compared to those who retain parental alleles in chromosome 18. Chromosomal instability and MMR status are the main pathways that give rise to CRC [66-68].

## TP53-APC/β-catenin

*TP53* protein, encoded by the *TP53* tumor suppressor gene located on the short arm of chromosome 17, has a regulatory role in cell growth arrest, DNA repair and apoptosis but also in oxidative stress, DNA damage and cell aberrant proliferation,

thus maintaining cell cycle homeostasis. *TP53* mutations result in dysfunctional *TP53* protein, which has a critical role in tumor carcinogenesis. Mutated *TP53* can be spotted in both malignant cells and in adenomas, and is expressed in about 60% of CRC. Point mutation in codon 72 - resulting in the substitution of proline to arginine - leads to dysfunction of the cell cycle "gatekeeper" that promotes the malignant process [69-70].

Germline *APC* mutations are the hallmark of familial adenomatous polyposis. Somatic mutations occurring in the *APC* gene activate the *Wnt* pathway in the early stages of colon carcinogenesis. The *APC* tumor-suppressor protein, encoded by the APC gene, has a crucial role in cellular processes while interacting and inactivating *glycogen synthase-kinase-3β* and *β-catenin*. Apart from somatic mutations that occur in the *APC* gene, promoter hypermethylation has also been recognized as a distinct cause of *APC* silencing [71-72].

## MicroRNAs

Small RNAs, called microRNAs, play a key role in tumor suppression or growth. MicroRNAs are highly stable structures with a hairpin-loop shape and small size. They can be extracted not only from fixed tissues, but also from body fluids, especially peripheral blood. They are found in exosomes, distinct microvesicles secreted by tumor cells, achieving a high level of stability and avoiding degradation of the genomic material [69,73]. These RNA sequences can lead to silencing of targeted genes and interfere with the invasion and progression of tumors by epithelial mesenchymal transition to metastatic sites [74]. In CRC where *BRAF* mutations are present, the expression of *miR-31* is noted, which could potentially be used as a diagnostic biomarker [75]. Furthermore, in *KRAS* wild-type tumors that respond to anti-EGFR antibody therapy, *miR-99a* and *miR-125b* may have a predictive role, whereas the expression of *miR-181a* correlates with a poor prognosis in this subset of patients [76]. Finally, poor responders to radiation therapy in rectal cancer cases have been found to express *miR-622* making it a plausible predictive marker [77]. Overall, expression of 500 different microRNAs has been noted in CRC.

## Blood and stool biomarkers

Circulating tumor cells (CTC) originating from either the primary or metastatic sites, are detected in the sera of colon cancer patients. Their detection is associated with active disease, tumor progression and metastatic potential, making them a strong prognostic biomarker, but also providing the advantage of avoiding a new biopsy [78]. Following the isolation of CTC, genomic analyses can provide information regarding the biology of the tumor and its evolution, even when the patient is on therapy, achieving real-time monitoring of the disease and therapy effectiveness. However, given the small number of circulating tumor cells, circulating free DNA may constitute a more practical noninvasive biomarker [79].

Cell-free DNA (cfDNA) refers to DNA fragments originating from tumor cells that are detected in patients' sera or plasma. It can be extracted from peripheral blood and then examined for mutations and genomic abnormalities, providing real-time information about tumor progression [79]. cfDNA is more accurate than CTC regarding tumor burden and can be used as both a diagnostic and prognostic biomarker. It also has predictive potential in the assessment of antineoplastic therapy through molecular analysis and mutation identification. *TP53* and *KRAS* mutations, MSI or loss of heterozygosity, along with DNA hypermethylation can be detected using cfDNA. cfDNA assays constitute a powerful tool for study of the molecular heterogeneity as well as the clonal divergence of a malignancy. Not surprisingly, the ability of circulating mRNA to resist degradation, mainly due to its presence in exosomes, has prompted investigation regarding the isolation of cell-free mRNA and the consequent use of reverse transcription polymerase chain reaction techniques in order to isolate a more accurate tumor signature using peripheral blood [80-81].

## Stool-based tests

To date, fecal immunohistochemical tests and fecal occult blood tests are of utility for the detection of CRC, but show little specificity and sensitivity. Thus, detection of mutated DNA in stool may be a promising technique for the detection of CRC. The technical restriction of stool DNA detection is the fact that only 0.01% of total stool DNA derives from the patient, whereas the remainder comes from intestinal bacteria and microbial flora [82]. Specific panels that are able to detect mutated human DNA in stool could help in the diagnosis of CRC and contribute to the avoidance of invasive techniques for the diagnosis and molecular profiling of colon cancers [2,82].

## Molecular classification of CRC

With the advent of molecular profiling and gene expression signatures, four distinct consensus molecular subtypes of CRC have been proposed. Current TNM staging remains the cornerstone for subsequent therapeutic decisions; however, there is a plethora of new molecular-based information for a CRC patient, indicating the implementation of personalized medicine according to the tumor's genetic signature. *CMS1* MSI-immune CRCs are hypermutated, *CIMP* (+), frequently *BRAF*-mutated with MMR deficiency, and may respond to immunotherapy because of higher levels of tumor-infiltrating lymphocytes and tumor neoantigen load. These tumors have a dismal prognosis at their recurrence or in the metastatic setting. *CMS2 Wnt*-canonical tumors are characterized by Wnt and MYC activation with high somatic copy number alterations. *CMS3*-metabolic tumors have a high prevalence of *KRAS* mutations, discrete metabolic dysregulation, low CIMP and mixed MSI status. Finally, *CMS4*-mesenchymal tumors are the ones with high somatic copy number alterations, intense

stromal infiltration and active angiogenesis, having the worse relapse-free and OS [83]. The molecular classification of CRC further reinforces the need for the establishment and use of molecular biomarkers.

## Concluding remarks

CRC development is highly heterogeneous, with distinct molecular alterations taking place throughout the natural course of the disease. In everyday clinical practice, *KRAS* and *NRAS* mutations serve as predictive biomarkers for the selection of patients eligible for anti-EGFR therapy, with a benefit recorded only in *RAS* wild-type tumors. Mutations in *BRAF* have an adverse prognostic value and are associated with worse patient outcomes; they may also have a negative predictive value for the benefit from anti-EGFR therapy. According to the latest CRC guidelines, every newly diagnosed patient with stage II CRC must have the tumor checked for MMR status: in those who are MMR-deficient adjuvant 5-fluorouracil therapy is of no benefit, while the patient's prognosis is better. As our knowledge regarding the molecular landscape of colorectal carcinogenesis advances, new molecular biomarkers with prognostic and predictive information are being discovered. Liquid biopsies are a promising tool for real-time evaluation of the tumor clonal evolution, response to therapy, presence of minimal residual disease and acquired resistance. Such noninvasive biomarkers may lead to real-time tumor molecular classification and personalized treatment of CRC patients.

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