

Review

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# Pharmacogenomics in colorectal cancer: current role in clinical practice and future perspectives

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## Abstract

The treatment scenario of colorectal cancer (CRC) has been evolving in recent years with the introduction of novel targeted agents and new therapeutic strategies for the metastatic disease. An extensive effort has been directed to the identification of predictive biomarkers to aid patients selection and guide therapeutic choices. Pharmacogenomics represents an irreplaceable tool to individualize patients treatment based on germline and tumor acquired somatic genetic variations able to predict drugs response and risk of toxicities. The growing knowledge of CRC molecular characteristics and complex genomic makeup has played a crucial role in identifying predictive pharmacogenomic biomarkers, while supporting the rationale for the development of new drugs and treatment combinations. Clinical validation of promising biomarkers, however, is often an issue. More recently, a deeper understanding of resistance mechanisms and tumor escape dynamics under treatment pressure and the availability of novel technologies are opening new perspectives in this field. This review aims to present an overview of current pharmacogenomic biomarkers and future perspectives of pharmacogenomics in CRC, in an evolving scenario moving from a single drug-gene interactions approach to a more comprehensive genome-wide approach, comprising genomics and epigenetics.

**Keywords:** Colorectal cancer, pharmacogenomics, RAS, BRAF, microsatellite instability, dihydropyrimidine dehydrogenase, UDP-Glucuronosyltransferase A1, epidermal growth factor receptor, vascular endothelial growth factor, DNA methylation



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## INTRODUCTION

Colorectal cancer (CRC) is one of the leading causes of cancer-related deaths in the western world and ranks third among the most frequent malignancies in both men and women<sup>[1]</sup>. Although still unsatisfactory, the median overall survival (OS) of patients with metastatic CRC (mCRC) has notably increased in the past 20 years, reaching around 30 months in recent phase III clinical trials<sup>[2,3]</sup>, thanks to the introduction of innovative medical and surgical treatment strategies. The availability of new drugs and treatment combinations, both in terms of cytotoxic chemotherapy regimens and new targeted therapies, has been crucial in order to reach this result. However, patients' outcome and response to treatment can be highly heterogeneous, thus an extensive effort has been directed towards the identification of reliable predictive biomarkers to aid clinical management of patients and identify subgroups more likely to benefit from different treatment strategies.

Pharmacogenomics represents an irreplaceable tool in order to tailor patients treatment to an individualized approach based on germline and somatic acquired genetic variations able to predict drugs response and risk of toxicities<sup>[4]</sup>. Moving from early studies exploring the genetic bases of individual predisposition to severe toxicities from chemotherapy agents [i.e. 5-fluorouracil (5-FU) or irinotecan] in mCRC patients, the introduction of targeted agents such as anti-epidermal growth factor receptor (EGFR) drugs, has prompted the discovery of predictive molecular biomarkers (i.e. RAS mutational status) which are now tested as part of routine clinical practice<sup>[5]</sup>. Over time, additional mechanisms of primary and secondary resistance to targeted agents have emerged as promising novel predictive biomarkers and potentially actionable target of treatment, although validation is still an issue in most cases, and many steps forward have been made in the biological understanding and molecular characterization of CRC<sup>[6]</sup>. Finally, new perspectives have been recently opened following innovative results of immunotherapy treatment, and the development of new analytical techniques which allow dynamic tumor profiling and a sensitive detection of coexisting alterations underlying tumor heterogeneity, such as liquid biopsy<sup>[7]</sup>.

In this review, we present an overview of current pharmacogenomic biomarkers validated in clinical practice and future perspectives of pharmacogenomics in CRC [Tables 1 and 2], in an evolving scenario moving from a single drug-gene interactions approach to a more comprehensive genome-wide approach, comprising genomics and epigenetics.

## CURRENT PHARMACOGENOMIC BIOMARKERS IN CLINICAL PRACTICE

### RAS

EGFR signaling pathway plays a crucial role in the regulation of cellular responses to growth signals and its constitutive activation is one of the main actor promoting CRC growth and proliferation through the KRAS/RAF/MAPK and the PI3K/AKT/mTOR axes<sup>[8]</sup>. EGFR inhibitors are nowadays well-established therapeutic agents incorporated into standard care for mCRC<sup>[9,10]</sup>. To date, two anti-EGFR monoclonal antibodies have been approved by the Food and Drug Administration (FDA) and European Medicines Agency (EMA) for the treatment of mCRC: Cetuximab (Erbix<sup>®</sup>, Merck KGaA/Lilly USA) and Panitumumab (Vectibix<sup>®</sup>, Amgen Inc). At the time when the efficacy of these drugs was first proven in advanced lines of treatment<sup>[11,12]</sup>, no predictive biomarker was available, although a subgroup effect on the activity of these agents was evident. KRAS is a small GTPase member of the RAS protein family<sup>[13]</sup>, and somatic gene mutations can lead to its constitutive activation resulting in independent cell proliferation and survival<sup>[14]</sup>. KRAS mutations, more frequently involving exon 2<sup>[15]</sup>, can be found in approximately 40% to 50% of mCRCs. The identification of KRAS exon 2 (codons 12 and 13) mutations as a negative predictive marker of response to anti-EGFRs represented the turning point on biomarker selection for anti-EGFR treatment.

First evidence of the negative predictive role of KRAS exon 2 mutation came from retrospective series<sup>[16]</sup> and was then confirmed through *post-hoc* analyses of randomized phase III trials<sup>[11,17-20]</sup>. Moving from these data, in 2008 FDA and EMA restricted the use of anti-EGFR drugs to patients with KRAS exon 2 wild-

**Table 1. Summary of main presented biomarkers**

Biomarker	Type of alteration	Frequency in CRC	Approved for clinical practice	Predictive value	Ref.
KRAS	Exon 2 (codons 12 and 13), exon 3 (codons 59 and 61) and exon 4 (codons 117 and 146) mutations	40%-50% mCRC	Y	Resistance to anti-EGFRs	[5]
NRAS	Exon 2 (codons 12 and 13), exon 3 (codons 59 and 61) and exon 4 (codons 117 and 146) mutations	3%-5% mCRC	Y	Resistance to anti-EGFRs	[5]
BRAF	V600E mutations	8%-10%	Y (prognostic value, Lynch Sdr screening in MSI-H)	Resistance to anti-EGFRs (accumulating evidence)	[5]
MSI	MMR-D (MSI-H)	20% stage I-II, 12% stage III, 4%-5% stage IV	Y (Lynch Sdr screening, prognostic value in early stage CRC)	Response to immune-checkpoint inhibitors (mCRC) Lack of efficacy of 5-FU adjuvant therapy in stage II (low evidence)	[5,81,100,101]
DPYD	DPYD*2A (IVS14+1G>A)	1%-2% heterozygous (caucasian population)	Y	5-FU severe toxicity	[9,120]
UGT1A1	UGT1A1*28	45% heterozygous 10% homozygous (caucasian population)	Y	Irinotecan severe toxicity	[9,10]
HER2	HER2 amplification	5% RAS WT mCRC	N	Resistance to anti-EGFRs Response to anti-HER2 treatment	[133-135]
PI3K	Exon 9 and 20 hotspot mutations	10%-18%	N	Resistance to anti-EGFRs	[5]
CIMP	Aberrant DNA hypermethylation at select CpG islands	10%-15%	N	Response to 5-FU adjuvant therapy Potential resistance to anti-EGFRs Potential sensitivity to demethylating agents	[161]
MGMT	MGMT promoter hypermethylation	40% mCRC	N	Response to alkylating agents	[172]

Y: yes; N: no; CRC: colorectal cancer; mCRC: metastatic CRC; EGFR: epidermal growth factor receptor; 5-FU: 5-fluorouracil; MSI-H: high microsatellite instability

type (WT) tumors. However, in the same year, the possible existence of additional predictive biomarkers of resistance to anti-EGFR treatment was highlighted by an independent meta-analysis<sup>[21]</sup> showing a low sensitivity for *KRAS* exon 2 mutations in predicting acquired resistance to anti-EGFRs. Shortly after, rare *RAS* activating mutations in exon 3 (codons 59 and 61) and exon 4 (codons 117 and 146) of *KRAS* and exons 2, 3, and 4 of *NRAS* (codons 117 and 146), were reported as novel negative predictive markers<sup>[22,23]</sup>. Outcome data from the extended *RAS* analyses in the large randomized phase III PRIME trial, comparing FOLFOX with or without panitumumab as first-line treatment in mCRC patients, provided definitive evidence in this regard. In this study, patients with any *RAS* mutation in their tumors showed a worse outcome when treated with panitumumab [hazard ratio (HR) for progression free survival (PFS) = 1.31 ( $P = 0.008$ ,  $P$  for interaction < 0.002); HR for OS = 1.21 ( $P = 0.04$ ,  $P$  for interaction = 0.001)]<sup>[24]</sup>. Following this evidence, results of all recent randomized trials with anti-EGFR-based therapies were retrospectively re-evaluated according to the extended *RAS* mutational status<sup>[25-27]</sup> and several meta-analyses were performed. Data were consistent across different chemotherapy backbones, anti-EGFR agents and lines of therapy, showing no improvement in outcome results, both in term of PFS and OS, with the addition of anti-EGFRs in tumors harboring any *RAS* mutation ( $P > 0.05$ )<sup>[28]</sup>. Notably, in the selected extended *RAS* WT population efficacy results from the addition of anti-EGFR treatment were highly improved<sup>[29]</sup>. Based on these results, the use of anti-EGFRs has been currently restricted to *RAS* WT (exons 2, 3, and 4 of each *KRAS* and *NRAS*) tumors<sup>[30]</sup>, and regulatory authorities recommend that every patient being considered for anti-EGFR therapy must receive *RAS* mutational testing including *KRAS* and *NRAS* codons 12, 13 of exon 2; 59, 61 of exon 3; and 117 and 146 of exon 4, performed only in highly qualified and certified laboratories<sup>[5]</sup>.

**Table 2. Promising future pharmacogenomics biomarkers**

Biomarker	Description	Potential predictive value	Ref.
CMS1	Microsatellite instability immune (14%): - high TML -MSI -CIMP+ -BRAF mutation -strong immune activation -right sided	Response to anti-VEGF	[181-186]
CMS2	Canonical (37%): -epithelial signature -WNT- $\beta$ -catenin and MYC activation -CIN -left sided	Response to anti-EGFRs Response to anti-HER2 Chemo-sensitivity	[181-186]
CMS3	Metabolic (13%): -metabolic dysregulation	-	[181-186]
CMS4	Mesenchymal (23%): -TGF- $\beta$ activation -stromal invasion -angiogenesis	Resistance to anti-EGFRs Lack of benefit from 5-FU and oxaliplatin	[181-186]
Liquid biopsy	Mutational analysis of circulating tumor DNA	Identification of predictive mutations for targeted treatments at baseline Dynamic monitoring Early detection of secondary resistance	[187-191]
MiRNA	Micro RNA: noncoding single-stranded RNA molecules, < 200 nucleotides, with post-transcriptional regulatory functions	Response/resistance to chemotherapy and targeted agents	[195]

TML: tumor mutational load; EGFR: epidermal growth factor receptor; 5-FU: 5-fluorouracil; MSI: microsatellite instability; TGF: transforming growth factor; VEGF: vascular endothelial growth factor

More recently, KRAS mutations have been shown to be associated with suppressed Th1/cytotoxic immunity in CRC, irrespective of mismatch repair (MMR) status, tumor location, neoantigen load and transcriptional subtype, with a differential effect modulated by the underlying tumor consensus molecular subtypes (CMS, discussed more extensively in section 4)<sup>[31]</sup>. These findings may have a role in explaining the heterogeneity of treatment response and outcomes in RAS mutated tumors and provide a rationale for novel treatment strategies in these patients.

## BRAF

The serine/threonine protein kinase BRAF is another player in the EGFR-mediated signaling pathway which is well-known to be implicated as an oncogenic driver in CRC. In normal cells, MEK, ERK and RAF are part of a tyrosine kinase signaling cascade activated by RAS, which affects cell proliferation, growth and differentiation, and regulates key cellular function such as apoptosis, cell migration and survival<sup>[32]</sup>. Mutations in *BRAF* can be found in approximately 8%-10% of CRCs<sup>[33]</sup>, the majority of which (about 80%) involve the substitution of glutamic acid for valine at residue 600 within the protein kinase domain (V600E). BRAF constitutive activation resulting from V600E mutation promotes signaling transduction through the MEK-ERK-MAP kinase pathway even in absence of RAS-mediated signals. RAS and BRAF V600E mutations, as they work through the same pathway, are considered mutually exclusive, and their concomitant detection is extremely rare (< 0.001%)<sup>[34]</sup>.

The negative prognostic value of *BRAF* V600E mutation in mCRC has been extensively described in several univariate and multivariate models. Life expectancy for this subgroup of patients is poor when compared to *BRAF* WT ones. When retrospectively evaluated, in fact, metastatic *BRAF*-mutated patients were showed to have a median OS ranging from 10 to 19 months across multiple series, even when treated with association therapies<sup>[35-38]</sup>. Additionally, *BRAF* V600E-mutated tumors share distinct clinicopathological features: they are more frequent in women, elderly, and are often right-sided; they more often present a mucinous histology, poor differentiation and high microsatellite instability (MSI-H); more often are diagnosed as advanced disease with preferential spread to lymph nodes and peritoneum<sup>[39-41]</sup>. When oligo-metastatic liver

disease is radically resected, *BRAF*-mutated tumors tends to relapse early with extra-hepatic lesions<sup>[42,43]</sup>. A specific carcinogenesis pathway<sup>[44]</sup> and a distinct gene signature<sup>[45]</sup> have also been associated with *BRAF* V600E mutation. More recently, gene expression analyses allowed to identify two different *BRAF* V600E subtypes in a large cohort of *BRAF* V600E mutated patients unselected for tumor stage: the BM1 subtype characterized by *KRAS*/*AKT* activation, *mTOR*/*4EBP* deregulation and EMT, and the BM2 subtype characterized by cell cycle and checkpoint pathway deregulation<sup>[46]</sup>. In contrast with *BRAF* V600E mutation, metastatic tumors harboring rare mutations of *BRAF* codons 594 and 596 (less than 1% of CRCs) have been shown to have different prognosis and clinical outcome. These rare mutations are associated with a non-mucinous histology, a rectal primary tumor location, microsatellite stability, and lack of peritoneal disease. Moreover, no negative prognostic impact was observed although in a small series of patients (median OS 62.0 vs. 12.6 months; HR, 0.36; 95% CI, 0.20-0.64;  $P = 0.002$  for *BRAF* 594 or 596 mutant vs. *BRAF* V600E)<sup>[47]</sup>. Similar results on the impact and characteristics of *BRAF* nonV600E mutations were confirmed in a recent retrospective evaluation of a large cohort of patients<sup>[48]</sup>.

Although still debated, growing evidence is accumulating on the role of *BRAF* mutations as a negative predictive marker for anti-EGFR agents activity. Retrospective series showed that the response rate to anti-EGFR treatment with or without chemotherapy was significantly lower in *BRAF*-mutated vs. WT patients<sup>[22,23,49]</sup>. On the other hand, *BRAF* V600E mutation failed to demonstrate its predictive value in several sub-group analyses of phase III trials, possibly because of the small number of *BRAF*-mutated patients and lack of statistical power<sup>[24,50]</sup>. More recently, two meta-analyses showed a lack of improvement in PFS and OS in patients with *BRAF*-mutated mCRCs when treated with either cetuximab- or panitumumab-containing regimens compared to chemotherapy alone<sup>[51,52]</sup>. Additionally, a retrospective evaluation of the randomized phase III FIRE-3 trial, comparing FOLFIRI plus cetuximab or bevacizumab as first-line treatment in *KRAS* exon 2 WT mCRC patients, confirmed poorer survival outcomes for *BRAF*-mutated tumors irrespective of cetuximab and bevacizumab administration<sup>[53]</sup>. Based on these data, it appears that anti-EGFRs do not demonstrate a clear outcome benefit in *BRAF*-mutated tumors, and their use should be restricted to patients with no alternative therapeutic options. Notably, however, in FIRE-3 cetuximab arm a small subgroup of *BRAF*-mutated tumors achieving an early tumor shrinkage  $\geq 20\%$  (9/17) showed significantly longer median PFS (9.0 vs. 1.9 months, log-rank test  $P = 0.002$ ; HR = 0.14) and OS (29.8 vs. 5.9 months, log-rank test  $P = 0.047$ ; HR = 0.3) than those not achieving it<sup>[53]</sup>. Despite the limitations due to the retrospective nature of this evaluation and the small patients numbers, these results highlight a significant heterogeneity among *BRAF*-mutated mCRCs warranting further investigation.

While FOLFOXIRI plus bevacizumab represents the most promising treatment option in the first-line setting for clinically selected *BRAF*-mutated patients<sup>[2,54]</sup>, outcomes are still unsatisfactory. An extensive effort has been made in the last few years aiming to develop possible effective anti-*BRAF* strategies for mCRC patients. In contrast to melanoma, the use of *BRAF* inhibitors, such as vemurafenib and dabrafenib, as single-agents did not show significant activity in *BRAF*-mutated mCRC<sup>[55]</sup>. Dual blockade of *BRAF* and alternative survival pathways, such as MEK and EGFR, have been tested as well in clinical trials without convincing results<sup>[56-58]</sup>. Promising results are coming instead from a triple inhibition strategy combining *BRAF*-inhibitors, MEK-inhibitors and EGFR-inhibitors<sup>[59,60]</sup>. An additional strategy under study to increase the activity of dual targeted *BRAF* inhibition is its association with standard cytotoxic chemotherapy, such as the combination of vemurafenib with cetuximab plus irinotecan which have been explored in the SWOG 1406 trial with encouraging results<sup>[61]</sup>. Moreover, several other promising strategies designed to overcome resistance pathways to *BRAF*-inhibitors are currently under investigation<sup>[62,63]</sup>. Final results from ongoing trials are warranted to improve targeted treatment options for *BRAF*-mutated patients.

### Microsatellite Instability

MMR is a highly conserved DNA repair mechanism that ensures genomic integrity by correcting mispaired or unpaired bases which have escaped the proofreading activity of DNA polymerases during DNA replication

and recombination, as well as repairing some forms of DNA damage. The loss of MMR proteins activity leads to an accumulation of DNA replication errors, a phenomenon known as MSI, characterized by high frequency of frameshift mutations in microsatellite DNA which translates into a high somatic mutational burden in MMR-deficient (MMR-D) cells (mutator phenotype)<sup>[64]</sup>.

The prevalence of MSI in CRC depends on the stage of the disease. Approximately 20% of CRCs in stage I-II, 12% in stage III and 4%-5% in stage IV, are deficient in one or more DNA MMR proteins, with one-quarter of these resulting from Lynch syndrome (LS), an autosomal dominant condition characterized by germline mutations in genes coding for MMR proteins (i.e. *MLH1*, *MSH2*, *MSH6*, *PMS2* or *EPCAM*)<sup>[65]</sup>. The vast majority (circa 80%-90%) of sporadic MSI cases are due to hypermethylation of the *MLH1* gene promoter<sup>[66,67]</sup>, associated with a high CpG island methylation phenotype (CIMP+) and about 30% harbor a *BRAF* V600E mutation<sup>[6,68]</sup>. The remaining cases of sporadic MSI can be explained mainly by the presence of multiple somatic mutations in the MMR genes without an identifiable germline MMR mutation ("double somatic" MSI cases)<sup>[69]</sup>, found to be associated with a higher frequency of somatic mutations in *PIK3CA*<sup>[70]</sup>. According to the recent CMS classification MSI is associated with CMS1<sup>[6,71]</sup>. MSI detection is currently based on two different approaches: immunohistochemical staining (IHC) for *MLH1*, *MSH2*, *MSH6*, and *PMS2* on tumor samples to identify the loss of protein expression which characterizes MMR deficiency as a surrogate for MSI<sup>[72]</sup>; DNA MSI testing through a polymerase chain reaction (PCR)-based approach evaluating specific panels of microsatellite markers<sup>[73]</sup>. If either MSI or MMR deficiency is detected, further evaluation is recommended to rule out LS, rather than sporadic MSI. Of note, recently new computational approaches based on the evaluation of next generation sequencing (NGS) data have been proposed as a tool for MSI assessment<sup>[74-77]</sup>, as well as the evaluation of mutational burden on circulating cell-free tumor-DNA testing as a surrogate marker of mismatch repair deficiency or microsatellite instability in patients with CRC<sup>[78]</sup>.

MSI-H CRCs are characterized by distinct clinical and pathological features such as right-sided colon location, early-stage at diagnosis, prominent lymphocytic infiltrate, poor differentiation and mucinous histology<sup>[79]</sup>. When diagnosed in the metastatic setting, MSI-H mCRCs arise more frequently in women and in elderly; presenting often with synchronous metastases involving peritoneum, lymph nodes and lung rather than liver. Notably, distinct patterns characterize inherited and sporadic MSI-H mCRCs<sup>[80]</sup>. In addition to LS screening, in patients with early-stage (especially stage II) CRCs, MMR status provides important prognostic and predictive information, with MMR deficiency being associated with both a good prognosis and apparently a lack of efficacy from fluorouracil treatment, although data regarding whether or not MSI status predicts response to adjuvant chemotherapy in this setting has been controversial<sup>[81-85]</sup>. The most solid data derive from the analyses of the ACCENT database investigating the impact of MSI in stage II and III CRCs treated with surgery *vs.* surgery followed by 5-FU-based adjuvant therapy across 17 different trials. Stage II and III patients with MSI tumors showed better outcome with surgery alone compared to those with microsatellite stable (MSS) tumors. Conversely, stage III patients showed a significant survival benefit from the addition of 5-FU adjuvant therapy after surgery both in case of MSS and MSI tumors<sup>[84]</sup>. To date, adjuvant chemotherapy is not recommended for patients with low risk stage II MSI-H tumors due to their excellent prognosis, while stage III patients should receive adjuvant treatment irrespective of MSI status. Of note, MSI etiology (germline *vs.* sporadic) seems to affect the predicted benefit from 5-FU, as Sinicrope *et al.*<sup>[86]</sup> showed, in a retrospective evaluation of stage II and III CRC patients who received either adjuvant 5-FU or placebo, that individuals with MSI-H CRCs due to germline mutations (i.e. LS) had an improved disease free survival (DFS) with 5-FU compared to those with sporadic MSI-H tumors. The role of MSI as a predictive marker with modern combination regimens, such as FOLFOX and FOLFIRI, has less evidence<sup>[87-89]</sup>, and although an MSI-H status was retrospectively shown to predict improved DFS with adjuvant irinotecan and 5-FU (IFL regimen) in the CALGB (Alliance) 89803 trial, these results were inconsistently demonstrated in other exploratory analyses<sup>[90,91]</sup>. In the metastatic setting, recent data suggest a greater activity of irinotecan in MSI-H mCRC and better outcomes in favor of bevacizumab treatment compared to anti-EGFRs<sup>[92]</sup>. Indeed, vascular endothelial growth factor (VEGF) is known to play a crucial

role in tumor microenvironment immuno-modulation and anti-angiogenic treatment has been proposed as an effective modality to potentiate immunotherapy<sup>[93]</sup>. No definitive evidence is available on the prognostic role of MSI-H in mCRC; recent data suggest no statistically significant difference in OS between MSI-H and MSS mCRCs, although a trend toward a worse OS has been reported for MSI-H<sup>[94]</sup>. Some studies suggest the correlation with *BRAF* mutational status as a potential confounding factor affecting the estimation of MSI-H impact on survival in mCRC<sup>[95]</sup>. However, the prognostic role of *BRAF* in these tumors is still object of debate and in a recent analysis *BRAF* V600E mutation was not associated with a worse survival in MSI-H CRC<sup>[80]</sup>. Additionally, a possible negative prognostic effect of immune checkpoint expression in MSI-H CRCs have been recently reported, which seems to be able to counterbalance the positive effect of tumor-infiltrating cytotoxic T-cell lymphocytes in these tumors<sup>[96]</sup>.

MSI assessment has lately gained a prominent role in the metastatic setting due to the recent groundbreaking success of immunotherapy with checkpoint inhibitors in MMR-D mCRCs which has opened a new era in the treatment of MSI-H tumors. In the phase II KEYNOTE 016 trial, pembrolizumab demonstrate its activity in 28 MSI-H mCRC patients with refractory disease, significantly improving response rate (RR), disease control rate (DCR), median PFS and OS compared to MSS patients (RR: 50% vs. 0% and DCR 89% vs. 16%, respectively; HR for PFS = 0.135,  $P < 0.001$ , HR for OS = 0.247,  $P = 0.001$ )<sup>[97,98]</sup>. The combination of ipilimumab (an anti-CTLA4) and nivolumab (an anti-PD1), under investigation in the phase II CHEKMATE142 trial, showed as well significant results with a recently reported RR of 31.1% (95% CI, 20.8-42.9) in patients receiving nivolumab ( $n = 74$ ) and 55% (95% CI, 45.2-63.8) in those receiving ipilimumab plus nivolumab ( $n = 119$ ), and remarkable 12 months PFS rate and 12 months survival rate (50% and 73% respectively, for nivolumab monotherapy; 71% and 85% respectively, for nivolumab plus ipilimumab)<sup>[99,100]</sup>. Responses were irrespective of tumor *RAS* and *BRAF* mutational status, immune cell PD-L1 expression or clinical history of LS. Notably, both pembrolizumab and ipilimumab/nivolumab showed a trend towards a plateau in the tail of patients' survival curves, suggesting the possibility of long term responders similar to the previous experience with immunotherapy in melanoma. Following these striking results, FDA approval was granted for the use of checkpoint inhibitors pembrolizumab (Keytruda®, Merck & Co., Inc.)<sup>[101]</sup> and nivolumab (Opdivo®, Bristol-Myers Squibb)<sup>[100]</sup> in the treatment of MSI-H or MMR-D mCRC.

Despite the clinical success of anti-CTLA4 and PD-L1/PD-1 inhibitors, however, only a subset of selected patients exhibits durable responses, suggesting that a broader view of cancer immunity is required. A complex set of dynamic tumor, host and environmental factors modulate the strength and timing of immune anticancer response, and several key immunoregulatory pathways have been identified and involved in the definition of an immune signature to predict responses to immunotherapy<sup>[102-105]</sup>. Alongside the ongoing extensive effort to identify additional predictive biomarkers<sup>[106,107]</sup>, understanding the mechanisms limiting immunotherapy efficacy, both in terms of innate and acquired resistance, represents a challenge which needs to be addressed in order to improve treatment outcomes and develop new actionable strategies<sup>[108-110]</sup>.

### Dihydropyrimidine dehydrogenase

Fluoropyrimidine analog 5-FU and its pro-drug capecitabine represent the backbone of chemotherapy treatment for colorectal cancer<sup>[10]</sup>. The mechanism of action of these drugs is based on thymidylate synthase (TYMS) inhibition through the formation of a ternary complex between the active metabolite 5-fluoro-2-deoxyuridine-5-monophosphate (5-FdUMP), TYMS and 5,10-methylenetetrahydrofolate, leading to the suppression of DNA synthesis<sup>[111]</sup>. The rate-limiting enzyme for 5-FU catabolism is the enzyme dihydropyrimidine dehydrogenase (DPD), responsible for the inactivation of more than 80% of the administered dose of 5-FU<sup>[112]</sup>.

Up to one-third of patients treated with these agents experience severe (and in 0.5%-1% of cases lethal) toxicities including myelosuppression, mucositis and diarrhea<sup>[113]</sup>. Functional DPD gene (*DPYD*) variants

leading to a decreased enzymatic activity have been found to correlate with the risk of 5-FU and capecitabine severe toxicities in several pharmacogenetic studies. Over 30 single nucleotide polymorphisms (SNPs) in the *DPYD* gene have been studied over the last 20 years, although many of these variants did not appear to have any functional effect. Among the most well-known, the c.2846 A>T and c.1679 T>G variants, alongside the G>A mutation (DPYD\*2A) of the invariant splice site in exon 14 (IVS14+1G>A), coding for a truncated protein with no enzymatic activity, have been consistently associated with decreased DPD activity and a 4-fold increase of risk of developing 5-FU related toxicities<sup>[114]</sup>. DPYD\*2A is the most frequent SNPs in the Caucasian population, nevertheless its incidence is low (about 1%-2% for the heterozygote genotype) and shows substantial ethnic variations. Homozygous for DPYD\*2A have been associated with cases of lethal toxicities in patients treated with fluoropyrimidine-based chemotherapy<sup>[115,116]</sup>. More recently a large meta-analysis from Meulendijks *et al.*<sup>[117]</sup> confirmed the predictive role for drug-related toxicities for four *DPYD* variants: DPYD\*2A, c.2846A>T, c.1679 T>G and c.1236G>A/haplotype B3. Data from retrospective pharmacogenetic analyses from the Italian adjuvant TOSCA trial confirm the role of DPYD\*2A as a risk factor for fluoropyrimidine-related toxicities<sup>[118]</sup>. Additionally, a prospective study enrolling 2,038 patients candidate to receive a fluoropyrimidine-based chemotherapy demonstrated the feasibility and cost-effectiveness of upfront DPYD\*2A genotyping before treatment start. DPYD\*2A variant allele carriers were treated with a reduced dose-intensity leading to a significant reduction of the risk of grade  $\geq 3$  toxicity (28% vs. 73% in historical controls,  $P < 0.001$ ) and a reduction of drug-induced death from 10% to 0%<sup>[119]</sup>. The low frequencies of the aforementioned risk alleles, however, cannot fully explain the estimated risk of DPD-linked fluoropyrimidine-related adverse events, underlining the complex multi-level modulation of DPD activity, involving both transcriptional and post-transcriptional mediators, and the need to investigate additional *DPYD* risk variants. Nevertheless, available data support the role of *DPYD* testing as a pre-treatment screening in patients undergoing 5-FU and capecitabine treatment in order to improve the safety of fluoropyrimidine-based therapies and potentially allow genotype-guided dose adaptations, as recently recommended by the clinical pharmacogenetics implementation consortium<sup>[120]</sup>.

Evidence on the role of DPD deficiency as a toxicity biomarker led the FDA to include a warning annotation on the label of fluorouracil for patients with low or absent DPD activity, recommending to withheld or permanently discontinue fluorouracil in patients with evidence of acute early-onset or unusually severe toxicity, which may indicate near complete or total absence of DPD activity. On the other hand, latest published ESMO clinical practice guidelines on metastatic colorectal cancer management suggest for the first-time pre-treatment DPYD testing as an option<sup>[9]</sup>. This indication, however, is focused on those patients who experience severe 5-FU toxicity before 5-FU re-introduction and routine testing is not recommended, despite the authors stating that patients with known partial DPD deficiency benefit from dose adaptation of 5-FU/capecitabine therapy to avoid severe toxicity, while in patients with complete DPD deficiency fluoropyrimidines should be avoided and an alternative treatment offered. The lack of recommended standardized assessment techniques represents an additional issue to the introduction of routine DPD testing.

The predictive role of genetic variants in other key genes involved in the folate pathway, such as TYMS and 5,10-methylenetetrahydrofolate reductase, has not been validated and their use in clinical practice is not recommended.

### UDP-Glucuronosyltransferase A1

Irinotecan, a topoisomerase I inhibitor, is another key drug in the chemotherapy treatment of mCRC, which can be used as a monotherapy or in combination with 5-FU and/or other agents in different treatment lines<sup>[9,10]</sup>. This agent is administered as a pro-drug which is metabolized to its active form, SN-38, via carboxylation. SN-38 catabolism and excretion are subsequently dependent on conversion to its inactive form, SN-38G, operated by hepatic UDP-Glucuronosyltransferases (UGT) such as UGT1A1<sup>[121]</sup>. Additionally, the pharmacokinetics of irinotecan involves several other enzymes, such as CYP3A4, which control its



metabolism modulating the available dose of the active drug. A genetic variation in these enzymes can affect tolerability and toxicity profile in patients.

Up to 36% of patients treated with irinotecan-containing regimens experience severe and potentially life-threatening adverse events, such as neutropenia and diarrhea<sup>[122]</sup>. Variations in the UGT1A1 activity have been shown to be associated with irinotecan-induced toxicities. The most common gene variants are the UGT1A1 \*1 and \*28 alleles, representing 98%-99% of all variants in the Caucasian population. The \*28 variant, responsible for Gilbert syndrome, is characterized by the presence of an extra TA repeat in the promoter of the *UGT1A1* gene which is associated with a remarkably reduced enzymatic activity and correlates with higher incidence of drug-related adverse events due to a slower catabolism of SN-38G<sup>[123]</sup>. In USA, about 45% of the population is heterozygous for the \*28 allele (\*1/\*28) while around 10% carries a homozygous genotype for this variant. The frequency increases in the African population and is lower in South-East Asian and Pacific populations. The role of UGT1A1 genotyping has been evaluated in several clinical trials, and two large meta-analyses including nearly 2000 patients confirmed that carriers of the UGT1A1 \*28/\*28 genotype were at a higher risk for neutropenia compared to WT \*1 patients even at a low irinotecan dosage (80-145 mg/m<sup>2</sup>)<sup>[124]</sup>, while carriers of the \*28 allele were at risk of severe diarrhea at doses above 125 mg/m<sup>2</sup><sup>[125]</sup>. Consistently, genotyping analyses of patients treated with 5-FU and irinotecan within the randomized phase III Nordic IV trial<sup>[126]</sup> and the randomized phase III TRIBE trial<sup>[127]</sup>, confirmed the association between the UGT1A1\*28/\*28 genotype and higher risk of neutropenia. Subsequent meta-analyses most recently supported once again the role of UGT1A1\*28 as predictive of irinotecan-related severe toxicities, as well as the role of additional variants such as UGT1A1\*6, a missense variant frequent in the Asian population<sup>[128,129]</sup>. Finally, a recent dose-finding and pharmacokinetic study suggests that irinotecan treatment dose should be individualized based on UGT1A1 genotype. Results from this study, in fact, show that the maximum tolerated dose of irinotecan, administered as an intravenous infusion every 3 weeks, was 850, 700, and 400 mg in patients bearing the \*1/\*1, \*1/\*28, and \*28/\*28 genotypes, respectively<sup>[130]</sup>.

Based on available data the latest ESMO guidelines suggest UGT genotyping as an option in patients with a suspicion of UGT1A1 deficiency and when the administration of a dose of irinotecan >180 mg/m<sup>2</sup> is planned<sup>[9]</sup>. On the other hand, the National Comprehensive Cancer Network guidelines version 2.2017 states that irinotecan should be used with caution and at a decreased dose in patients with Gilbert syndrome or elevated serum bilirubin, but routine genotyping of UGT SNPs is not recommended<sup>[10]</sup>. It has to be noted, however, that FDA has modified irinotecan label to include a toxicity warning for the UGT1A1\*28 polymorphism, suggesting an initial dose reduction when treating patients carrying the UGT1A1\*28 homozygous allele.

## EMERGING BIOMARKERS OF SPECIAL INTEREST

### HER2

Although tumor *RAS* WT status is, as previously described, a crucial prerequisite for anti-EGFRs activity in mCRC, several patients with *RAS* and *BRAF* WT tumors still do not benefit from anti-EGFR treatment. Based on preclinical data and retrospective evaluations, additional mechanisms of primary resistance to anti-EGFR agents have been identified over time in *RAS* WT mCRC, including human epidermal growth factor receptor 2 (*HER2/neu*) amplification. *HER2* is a member of the EGRF family which regulates key cellular processes such as proliferation and apoptosis through the activation of the *RAS/RAF/ERK* and the *PI3K/PTEN/AKT* signalling pathways. *HER2* role as a driver oncogene in CRC and as potential biomarker for targeted treatment in the metastatic setting has recently been the object of great interest.

First data were reported in 2011 when *HER2* amplification (which can be found in approximately 5% of *RAS* WT mCRCs), was detected in a subset of *KRAS/NRAS/BRAF/PIK3CA* WT cetuximab-resistant patient-derived xenografts. Following this first evidence, a proof-of-concept study in the subgroup of *HER2*-amplified

xeno-patients demonstrated a significant tumor regression after combined treatment with HER2 and EGFR blockade<sup>[131]</sup>. These results were subsequently challenged in an Italian phase II clinical trial, the HERACLES study. More than 1000 mCRC cases were analysed in order to identify strict criteria for the definition of *HER2* amplification<sup>[132]</sup> in the dedicated HERACLES diagnostic. Afterwards, the activity of an HER2 double blockade with trastuzumab and lapatinib was evaluated in chemorefractory mCRC patients with *HER2*-positive tumors. Initial results of the study have been published, showing a 30% objective response rate (95% CI, 14-50), with one patient achieving a complete response, and a 44% stable disease rate (95% CI, 25-63)<sup>[133]</sup>. Of note, none of the 15 patients (56%) evaluable for response to anti-EGFRs achieved an objective response to previous treatment with either cetuximab or panitumumab, supporting the role of *HER2* amplification as a mechanism of primary resistance to anti-EGFR targeted agents. Moving from such promising results, a second cohort of the study has enrolled patients to treatment with a combination of trastuzumab-emtansine (TDM1) and pertuzumab, and patients experiencing disease progression after treatment with trastuzumab and lapatinib are receiving TDM1 monotherapy within the HERACLES Rescue trial. New results from these studies are highly anticipated.

Confirmatory results on HER2 as a possible target in mCRC came also from the phase II MyPathway trial, and retrospective series confirmed data on *HER2* as a possible predictive biomarker of resistance to anti-EGFRs<sup>[134]</sup>. Additionally, *HER2* amplification detected on tissue or on circulating tumor DNA (ctDNA) was identified as a possible mechanism of acquired resistance in *HER2* negative, *RAS/BRAF* WT, patients progressed during anti-EGFR treatment<sup>[135]</sup>. Of note, a randomized phase II trial, the S1613 study, has been recently opened to explore the efficacy of trastuzumab and pertuzumab compared to cetuximab and irinotecan in pre-treated anti-EGFR naïve mCRC patients carrying a tumor with *HER2/neu* amplification<sup>[136]</sup>.

Supported by a strong preclinical rationale and confirmatory clinical data *HER2* testing might be soon implemented in clinical practice for patients with mCRC candidate to receive anti-EGFR and/or anti-HER2 treatments.

#### **Anti-EGFR agents: other biomarkers of primary and acquired resistance**

Alongside *HER2* amplification, several other mechanisms of primary resistance to anti-EGFR targeted treatment have been identified so far, including phosphatidylinositol-3-kinase catalytic subunit alpha (*PIK3CA*) mutations (exon 9 and 20 hotspot mutations), *MET* amplification, *FGFR1* and *PDGFRA* mutations, loss of *PTEN* function and low *EGFR* copy number<sup>[137]</sup>. However, the routine use of these biomarkers in clinical practice cannot be recommended at present, and further prospective validation of their predictive role is warranted. Nevertheless, different combined strategies and novel targeted agents aimed to overcome primary resistance to anti-EGFRs are currently under investigation, such as the combination of anti-EGFR agents with mammalian target of rapamycin (mTOR) inhibitors<sup>[138]</sup>. Recently, a panel of genomic alterations (the PRESSING panel) comprising activating mutations of the MAPKs or PI3K/AKT axis, *HER2* amplification or mutations, *MET* amplification and *NTRK/ROS1/ALK/RET* rearrangements, have been tested in an interesting retrospective case-control study aiming to dissect primary resistance to anti-EGFR treatment, demonstrating the negative predictive impact of these mutations in *RAS/BRAF* WT mCRCs treated with anti-EGFRs<sup>[139]</sup>. The study included 47 cases (patients resistant to anti-EGFR-containing regimens) and 47 controls (patients who responded to single agent anti-EGFRs or to a combination of irinotecan with anti-EGFRs if previously clearly irinotecan refractory). Aforementioned genomic alterations were reported in 20 (42.6%) cases and 1 (2.1%) control ( $P < 0.001$ ), meeting the primary endpoint of the study. Additionally, primary tumor right-sidedness was found to be associated with resistance to anti-EGFRs, confirming recent literature evidence, and the combined evaluation of PRESSING panel and primary tumor location demonstrated the best predictive accuracy. These results open promising perspectives on the clinical application of a more comprehensive molecular characterization of *RAS/BRAF* WT mCRCs to further improve and refine patients selection.

Secondary resistance to anti-EGFRs is often dependent on clonal selection induced by targeted treatment pressure. Emerging mutations in the RAS/RAF/MAPK signaling pathway can be detected after disease progression in tumor biopsies from previously *KRAS* wild-type tumors and multiple mutations can coexist at the same time in the same sample<sup>[140]</sup>. This seems to be the result of the amplification of pre-existing minor sub-clones, suggested by a significant overlap in the genetic events associated with primary and acquired resistance<sup>[141]</sup>. Moving from these data, several trials are currently exploring different approaches to multiple targeted inhibition based on the emergence of selected resistance drivers, such as the combination of anti-EGFRs with MEK or MET inhibitors. Mutations in the ectodomain of EGFR represent an additional mechanism of resistance limited to the acquired setting<sup>[142,143]</sup>. Notably, a subset of mutations including *EGFR* S492R as well as other acquired mutations recently identified (S464L, G465R and I491M) appears to confer resistance to cetuximab but not panitumumab. The binding epitopes of cetuximab and panitumumab on EGFR, in fact, overlap but are not identical<sup>[144,145]</sup>. Retrospective analyses from the ASPeCCT trial, comparing panitumumab to cetuximab in chemorefractory mCRC patients, revealed that EGFR S492R mutations occurred in 1% vs. 16% of patients treated with panitumumab and cetuximab, respectively<sup>[146]</sup>. The possible rationale for using panitumumab after the detection of these mutations as a mechanism of resistance to cetuximab still need further validation. Other strategies to overcome acquired resistance to anti-EGFRs include treatment with novel antibodies targeting different epitopes of the EGFR ectodomain, which can increase receptor internalization and degradation such as MM-151<sup>[147]</sup> and Sym004<sup>[148]</sup>.

### VEGF pathway

Angiogenesis plays a key role in CRC development and progression, and VEGF is a key regulator in both physiological and pathological angiogenesis. Therapeutic agents targeting VEGF/VEGFR signaling (i.e. bevacizumab, aflibercept, ramucirumab and regorafenib) proved to be effective across different treatment lines in mCRC and contributed greatly to improve patients' survival in recent years<sup>[9,10]</sup>. However, despite extensive efforts to identify predictive biomarkers for antiangiogenic therapies in the last decade, no predictive marker is available in clinical practice yet<sup>[149]</sup>. The complexity of the angiogenesis signaling network and the overlap between various angiogenic factors, in fact, represent a challenge to pharmacogenomic biomarkers discovery.

In 2012, Bates *et al.*<sup>[150]</sup> retrospectively analyzed CRC tumor samples from the phase III bevacizumab E3200 trial to explore the predictive value on treatment outcomes of VEGF165b, a VEGF splice isoform. Despite not reaching a statistical significance, patients with a lower level of VEGF165b appeared to benefit more from bevacizumab treatment. Focusing on a different candidate marker, recently published data demonstrated that patients treated with first-line bevacizumab-containing regimens had a significantly longer PFS when affected by *Homeobox B9* (*HOXB9*)-negative tumors compared with those with *HOXB9*-positive tumors (18.0 vs. 10.4 months,  $P = 0.048$ ). *HOXB9* is known as a highly conserved homeobox transcription factor gene which drives neoplastic transformation and tumor progression exerting an anti-apoptotic effect and promoting tumor cell invasion. The authors demonstrated, both with preclinical and clinical data, that transcription factor *HOXB9* mediates resistance of CRC to bevacizumab modulating a complex network of alternative pro-angiogenic and pro-inflammatory secreted factors<sup>[151]</sup>. A prospective validation of these promising results is highly anticipated. In another interesting analysis, NOTCH1 expression has been proposed as a detrimental prognostic factor in mCRC patients treated with chemotherapy plus bevacizumab<sup>[152]</sup>. Of note, a phase Ib trial is ongoing exploring safety and preliminary efficacy of a bispecific antibody targeting VEGF and the NOTCH ligand DLL4 (OMP-305B83) in combination with FOLFIRI as second-line treatment in mCRC<sup>[153]</sup>. Finally, a novel emerging player in the angiogenesis regulatory pathways is the protein apelin (APLN). APLN signaling takes part in multiple physiological functions including angiogenesis, and interacts at different levels with key mechanisms regulating cell growth, survival and apoptosis. Recent preclinical data based on the analysis of tumor-derived endothelial cells from patients receiving bevacizumab showed that APLN mRNA levels are significantly associated with treatment response. In fact, APLN levels were high

in non-responders and low in patients who benefitted from bevacizumab ( $P = 0.0001$ )<sup>[154]</sup>. All these potential biomarkers, however, still need validation.

As novel anti-angiogenic agents have entered clinical practice in recent years, the interest was directed to identify specific biomarkers for each compound. A retrospective analysis of ctDNA from liquid biopsies collected from about 350 patients treated with regorafenib in the CORRECT trial was performed to investigate the impact of *KRAS*, *PIK3CA* and *BRAF* mutations on regorafenib efficacy. Results were consistent with previous data and confirmed that the benefit from regorafenib on survival and treatment outcomes was irrespective of *KRAS* and *PIK3CA* mutational status<sup>[155]</sup>. The analysis according to *BRAF* mutational status, on the other hand, was not feasible due to the small number of *BRAF*-mutated patients. Data on *RAS*, *BRAF* and sidedness as biomarkers in patients treated with aflibercept in the VELOUR trial have been recently presented as well. No significant interactions according to *RAS* and *BRAF* status were found in this analysis, although a trend for better outcomes was observed for *BRAF*-mutated tumors treated with aflibercept in comparison with the control arm (mOS 10.3 vs. 5.5 months, respectively, HR 0.42; 95% CI, 0.16-1.09;  $P = 0.08$ )<sup>[156]</sup>. Similar results were observed in patients treated with ramucirumab within the RAISE trial. In fact, the ramucirumab favorable treatment effect was similar between *RAS*-mutated and all *RAS/RAF* WT tumors; however, the benefit was more notable in *BRAF*-mutated tumors both for OS (HR 0.54; 95% CI 0.25-1.13) and PFS (HR 0.55; 95% CI 0.28-1.08)<sup>[157]</sup>. Additionally, Taberero *et al.*<sup>[158]</sup> assessed the correlations of a series of baseline marker levels (including VEGFR-2 immunohistochemistry in tumor tissue) with clinical outcomes in the RAISE patients population. Only VEGF-D circulating serum levels were found to be statistically significant with higher levels of this soluble factor ( $\geq 115$  pg/mL) associated with improved ramucirumab efficacy in comparison with placebo<sup>[158]</sup>.

Several SNPs in different genes involved in VEGF signaling pathway have been investigated over time. Results from a large meta-analysis including 158 SNPs and 1348 patients enrolled in five phase III randomized trials suggested an association between VEGFA rs699946 and VEGFR-2 rs11133360 polymorphisms and improved PFS in bevacizumab-treated patients<sup>[159]</sup>. Unfortunately, additional promising retrospective findings on different candidate SNPs of VEGF/VEGFR pathway genes were not prospectively validated in a dedicated study<sup>[160]</sup>.

### DNA methylation

Over the last decade, evidence on the role of the epigenome in CRC has been largely explored and it is now recognized that among thousands of epigenetic alterations which can be present in each tumor, a small subgroup may be considered a driver event in CRC development<sup>[161]</sup>. Different epigenetic mechanisms, in fact, can play a key role in carcinogenesis, such as DNA methylation, nucleosome positioning, histone modifications and non-coding RNAs expression<sup>[162]</sup>. Technological advances have considerably increased our ability to detect a wide number of epigenetic alterations which can eventually have a role as clinical biomarkers for early detection, prognostic stratification and treatment efficacy prediction in CRC patients. Of note, recently the availability of more refined genome-wide mapping technologies, highlighted that the function of DNA methylation can vary depending on its context, underlining a deep complexity that warrants further evaluations<sup>[163]</sup>.

Aberrant DNA methylation is the most extensively studied epigenetic mechanism in CRC. Global DNA hypomethylation is currently considered a common feature of CRC; on the other hand, however, evidence on the role of CpG islands DNA hypermethylation in promoting CRC by silencing the expression of tumor suppressor genes led to the identification of the CpG Island Methylator Phenotype (CIMP), consisting in a subset of CRCs characterized by distinct epidemiological, histological and molecular features and prognosis<sup>[164]</sup>. CIMP+ tumors are associated with female gender and older age, show more frequently a right-sided colon location, a high incidence of *BRAF* V600E mutation and MSI-H status as a consequence of *MLH1*

epigenetic silencing through promoter DNA hypermethylation, diploid copy number and absence of TP53<sup>[165]</sup>. CIMP status has been proposed as a promising prognostic marker for CRCs, however, several studies reported contradictory results, possibly due to the overlap between the CIMP+ phenotype and the MSI-H phenotype, associated in 30%-50% of cases with *BRAF* mutation<sup>[166]</sup>. The lack of global consensus in defining CIMP+ tumors, together with these controversial results, has hindered the uptake of CIMP as a relevant biomarker in clinical practice and further studies are warranted to explore its predictive and prognostic value<sup>[167]</sup>.

Long interspersed nucleotide element-1 (LINE-1) methylation measured by pyrosequencing has been shown to correlate with global DNA methylation levels<sup>[168]</sup>. LINE-1 is a retrotransposon related to key CRC features involved in the carcinogenesis process: LINE1 hypomethylation is associated with 18q loss of heterozygosity (LOH); whereas an inverse correlation has been demonstrated between LINE-1 hypomethylation, CIMP-H and MSI-H status. LINE-1 methylation levels have been reported to impact CRC prognosis with hypomethylation conferring poor prognosis in terms of overall mortality (OM) and colorectal cancer-specific mortality<sup>[169]</sup>. Additionally, LINE-1 hypomethylation in MSS/CIMP+ stage II and III CRC has been showed to predict benefit from adjuvant chemotherapy with oral fluoropyrimidines<sup>[170]</sup>. These data suggest that DNA demethylation may play, as well, a crucial role in CRC development, prognosis and response to treatment. Although promising, however, these findings need further validation.

The DNA repair gene O6-methylguanine-DNA methyltransferase (*MGMT*) has recently gained attention and has been object of several studies. This gene encodes a DNA repair protein which removes alkylating groups from O6-guanine and is involved in protecting cells against damages from alkylating agents. *MGMT* has been shown to undergo epigenetic silencing by promoter hypermethylation in more than 40% of mCRCs<sup>[171]</sup>. The loss of *MGMT* gene expression impairs the ability of DNA repair mechanisms to remove alkyl groups, potentially enhancing the cytotoxic effects of alkylating drugs, such as dacarbazine and temozolomide. On these bases, several phase II clinical trials<sup>[172]</sup> evaluating the efficacy of alkylating agents in mCRC have been conducted with promising results. In these studies, *MGMT* methylation has been used as a predictive biomarker for patients' selection, supporting a possible role for this novel marker in clinical practice.

In an era in which immuno-oncology is revolutionizing cancer treatment strategies, novel possible relevant implications of aberrant DNA methylation come from its tight connection with the immune cells system. To date, immune-checkpoint inhibitors (ICI) have shown striking results in selected cancer types, although only a minority of patients are sensitive to these drugs. *De novo* DNA methylation has been recently reported to have a central role in maintaining a T cell exhaustion status that contributes to resistance to ICI treatment<sup>[173]</sup>. On the other hand, previous studies demonstrated that DNA demethylating drugs can enhance CTLA-4 blockade-mediated T cell responses<sup>[174]</sup>. Moreover, treatment of epithelial cancer cell lines (including CRC cell lines) with demethylating agents, i.e. 5-azacitidine, has been reported to promote a significant enrichment of immunomodulatory pathways<sup>[175]</sup>. As a possible explanation, cryptic transcription of thousands of treatment-induced non-annotated transcriptional start sites (TINATs) may contribute to cancer immunogenicity through the translation of novel potential antigenic proteins, as recently shown by Brocks and colleagues in their work exploring DNA methyltransferases inhibitors (DNMTi) treatment consequences on epigenetic and genome-wide transcription<sup>[176]</sup>. Overall, this growing evidence supports a strong immunomodulatory effect of DNA demethylating agents in cancer cells, and the rationale to combine these drugs with immunotherapy in cancer patients. Based on these premises, a deeper understanding of the interplay between epigenetic modifications, cancer cells and immune cells could reveal novel potential strategies to enhance ICI treatment efficacy and overcome primary and acquired resistance mechanisms to immunotherapy.

Finally, aberrant DNA methylation may exert a direct effect modulating well-established molecular pathways in CRC. Notably, *EGFR* promoter DNA methylation has been reported to occur in 58% of primary colon

tumors and to be strongly correlated with shorter patients' PFS and OS (PFS 2.4 vs. 7.4 months,  $P < 0.0001$ ; OS 6.1 vs. 17.8 months,  $P < 0.0001$ )<sup>[177]</sup>. On the other hand, Khambata-Ford *et al.*<sup>[178]</sup> discovered that patients with overexpression of epiregulin (EREG) and amphiregulin (AREG), two EGFR ligands, are more likely to achieve disease control when treated with cetuximab and show a significantly longer PFS. These data have been confirmed by Jacobs *et al.*<sup>[179]</sup> showing a significant association between cetuximab response and AREG/EREG expression. In a recent work, EREG and AREG expression has been found to have a strong inverse correlation with methylation and to be inversely associated with right-sided tumor location, CIMP-H status and BRAF mutation<sup>[180]</sup>. Additionally, the authors reported that treatment with hypomethylating agents (i.e. azacitidine) increased EREG expression, and that a CIMP-H status was associated with shorter PFS outcomes, also in *BRAF/NRAS* WT patients. Based on these data, promoter DNA methylation may be the main regulatory mechanism of AREG/EREG expression, which may explain, at least in part, the association between right-sided tumor location, CIMP-status and anti-EGFR treatment response in mCRC. DNA methylation may, then, partially account for primary anti-EGFRs resistance, supporting the rationale to explore the possible synergistic treatment effect of demethylating agents in combination with anti-EGFR drugs.

Despite promising evidence, the complexity and heterogeneity of epigenetic alterations in CRC still represent a considerable challenge, which needs to be further addressed in order to identify reliable biomarkers and translate current knowledge into actionable therapeutic strategies.

## FUTURE PERSPECTIVES

### CRC consensus molecular subtypes

In recent years, great advances have been made in understanding the complexity of tumor biology and genetic landscape underlying tumor development and response to treatment. In 2015 an international consortium developed the Consensus Molecular Subtypes, which classifies CRC into four distinct biological groups, based on gene expression signatures and correlated with distinct genetic, epigenomic, transcriptomic, microenvironmental, prognostic and clinical features<sup>[181]</sup>. CMS1 (microsatellite instability immune, 14%) tumors are associated with high tumor mutational load (TML), microsatellite instability, hypermethylation status (CIMP+), *BRAF* mutation, and strong immune activation. The CMS2 (canonical, 37%) subtype is characterized by an epithelial signature, marked WNT- $\beta$ -catenin pathway and MYC signaling activation. CMS3 (metabolic, 13%) tumors feature metabolic dysregulation; and CMS4 (mesenchymal, 23%) a prominent transforming growth factor (TGF)- $\beta$  activation, stromal invasion and angiogenesis. Samples with mixed features (13%) are considered to represent a transition phenotype or intratumoral heterogeneity. CMS subgroups show a strong prognostic value independent of tumor stage, with CMS4 associated with worse survival. Moreover, retrospective analyses of clinical trials have suggested a potential predictive value for CMS subtypes, including a better outcome following bevacizumab treatment for CMS1<sup>[182]</sup>, and a lack of benefit from oxaliplatin<sup>[183]</sup> and anti-EGFRs (irrespective of *RAS* mutational status)<sup>[184]</sup> for the mesenchymal-like phenotype. Although not yet implemented in clinical practice, this classification system has the potential to better inform clinicians of prognosis and therapeutic response, and to guide novel therapeutic strategies with subtype-based targeted interventions<sup>[6]</sup>. In fact, data have been published from very recent preclinical studies exploring models of CMS in large panels of CRC cell lines, primary cultures and patient-derived xenografts (PDX), with the aim of developing "adapted" classifiers optimized for pre-clinical research and investigate specific drug sensitivity of individual CMS<sup>[185,186]</sup>. Results from these studies show interesting initial findings highlighting subtype-dependent response profiles, with a different sensitivity to chemotherapy (either 5-FU or oxaliplatin)-induced apoptosis between CMS2 and CMS4, which relates to the *in vivo* efficacy of chemotherapy in PDX models where a delay in outgrowth of CMS2, but not CMS4 xenografts, was observed. Additionally, a strong response to anti-EGFRs and HER2 inhibitors was observed in the CMS2 subtype. Indeed, a deeper understanding of the unique drug-sensitivity profile of each CMS subtype and the possibility of performing high-throughput *in vitro* and *in vivo* drug screening using PDX technology have the potential to greatly advance precision medicine in CRC.

### Liquid biopsy

Another field of major interest is the rapid development of liquid biopsies technology and the analysis of ctDNA as a more comprehensive and less invasive approach to pharmacogenomic profiling in CRC patients<sup>[187,188]</sup>. Allowing large-scale genomic profiling and being able to capture the molecular heterogeneity of different tumor sub-clones coexisting in the same patients, these techniques are expected to play a pivotal role in improving patients stratification and selection for targeted treatments. Moreover, the possibility to perform serial testing over time represents a valid opportunity to guide treatment strategies through an early detection of the emergence of treatment resistance and a dynamic tumor molecular profiling<sup>[189]</sup>. Indeed, data from repeated ctDNA analyses have been able to show the emergence of *RAS* and/or *BRAF* mutations during treatment with anti-EGFRs in *KRAS* WT patients, closely dependent on treatment exposure, with a dynamic increase during EGFR blockade followed by a rapid decline after treatment withdrawal<sup>[190]</sup>. Recently, a large study on genomic profiling through liquid biopsy analyzing next generation sequencing data from cell-free DNA of 1397 CRC patients, confirmed the reliability of this methodology in detecting genomic alterations when compared with corresponding tissue-based sequencing. Additionally, results of this study highlighted the possibility of detecting the development of multiple distinct concomitant mechanisms of resistance after targeted treatment with anti-EGFRs in the same subject, proving that ctDNA sequencing can generate a valuable insight into tumor heterogeneity and therapeutic resistance<sup>[191]</sup>. Although still needing extensive investigations and prospective validation, liquid biopsy approaches to profile tumor dynamics and response to treatment and to guide rechallenge strategies based on detection of circulating genomic alterations are currently under investigation in several clinical trials.

### MiRNAs

Finally, noncoding RNAs represent an evolving field in cancer diagnosis and prognosis, and several studies have suggested their possible role as treatment target in different diseases<sup>[192,193]</sup>. miRNAs are noncoding single-stranded RNA molecules, less than 200 nucleotides in length, with a post-transcriptional regulatory function involved in the modulation of a broad range of biological processes comprising cellular signaling, metabolism, proliferation and differentiation<sup>[194]</sup>. The role of several miRNAs has been implied in CRC evolution and progression, moreover different miRNAs have been identified as predictive of treatment response to standard chemotherapy (i.e. miR-429 and miR-148a with 5-FU) and targeted agents (i.e. miR-7 and miR-375 with anti-EGFRs)<sup>[195]</sup>. Although promising these findings still need validation; nevertheless, the possible clinical application of miRNAs as biomarkers or as a potential target of treatment in CRC deserves further investigation. Of note, new strategies are currently under study to develop miRNA based inference methods to extensively infer drug-disease causal relationship (miRDDCR) to assist in experimental design for drug discovery and disease treatment<sup>[196]</sup>.

### CONCLUSION

In the era of precision medicine, optimizing therapeutics and drugs combination for a narrow subset of patients based on patients' and tumors genetic makeup is of paramount importance in order to improve outcomes and minimize unrequired toxicities. The field of pharmacogenomics is constantly growing, and with the availability of new technologies it has been moving beyond candidate gene approaches and genome-wide association studies towards a comprehensive evaluation of genomic and epigenomic markers to drive treatment choices and optimize targeted therapies. Several biomarkers have entered clinical practice so far, and many more are currently being tested in clinical trials. Biomarker discovery and validation however still encounter many issues, due often to the small subsets of patients bearing selected alterations, the retrospective nature of most studies and the difficulty in proving the cost-effectiveness of a specific novel marker. Implementing biomarker-driven clinical trials and prospective pharmacogenomic profiling in clinical research, possibly integrating companion diagnostic tests since the early stages of novel drug development, is thus a priority for future research. Finally, dynamic profiling of tumor genomics under treatment pressure will play a critical role in uncovering acquired mechanism of resistance and directing personalized treatment strategies.

## DECLARATIONS

### Authors' contributions

Manuscript drafting: Battaglin F, Puccini A

Directly provided contributions, read and approved the final manuscript: all authors

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### Conflicts of interest

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### Patient consent

Not applicable.

### Ethics approval

Not applicable.

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## REFERENCES

- 1 Siegel RL, Miller KD, Jemal A. Cancer statistics, 2018. *CA Cancer J Clin* 2018;68:7-30.
- 2 Cremolini C, Loupakis F, Antoniotti C, Lupi C, Sensi E, Lonardi S, Mezi S, Tomasello G, Ronzoni M, Zaniboni A, Tonini G, Carlomagno C, Allegrini G, Chiara S, D'Amico M, Granetto C, Cazzaniga M, Boni L, Fontanini G, Falcone A. FOLFOXIRI plus bevacizumab versus FOLFIRI plus bevacizumab as first-line treatment of patients with metastatic colorectal cancer: updated overall survival and molecular subgroup analyses of the open-label, phase 3 TRIBE study. *Lancet Oncol* 2015;16:1306-15.
- 3 Stintzing S, Modest DP, Rossius L, Lerch MM, von Weikersthal LF, Decker T, Kiani A, Vehling-Kaiser U, Al-Batran SE, Heintges T, Lerchenmüller C, Kahl C, Seipelt G, Kullmann F, Stauch M, Scheithauer W, Held S, Giessen-Jung C, Moehler M, Jagenburg A, Kirchner T, Jung A, Heinemann V; FIRE-3 investigators. FOLFIRI plus cetuximab versus FOLFIRI plus bevacizumab for metastatic colorectal cancer (FIRE-3): a post-hoc analysis of tumour dynamics in the final RAS wild-type subgroup of this randomised open-label phase 3 trial. *Lancet Oncol* 2016;17:1426-34.
- 4 McDermott U, Downing JR, Stratton MR. Genomics and the continuum of cancer care. *N Engl J Med* 2011;364:340-50.
- 5 Sepulveda AR, Hamilton SR, Allegra CJ, Grody W, Cushman-Vokoun AM, Funkhouser WK, Kopetz SE, Lieu C, Lindor NM, Minsky BD, Monzon FA, Sargent DJ, Singh VM, Willis J, Clark J, Colasacco C, Rumble RB, Temple-Smolkin R, Ventura CB, Nowak JA. Molecular biomarkers for the evaluation of colorectal cancer: Guideline from the American Society for Clinical Pathology, College of American Pathologists, Association for Molecular Pathology, and American Society of Clinical Oncology. *J Mol Diagn* 2017;19:187-225.
- 6 Dienstmann R, Vermeulen L, Guinney J, Kopetz S, Tejpar S, Tabernero J. Consensus molecular subtypes and the evolution of precision medicine in colorectal cancer. *Nat Rev Cancer* 2017;17:79-92.
- 7 Wan JCM, Massie C, Mouliere F, Brenton JD, Caldas C, Pacey S, Baird R, Rosenfeld N. Liquid biopsies come of age: towards implementation of circulating tumour DNA. *Nat Rev Cancer* 2017;17:223-38.
- 8 Hynes NE, Lane HA. ERBB receptors and cancer: the complexity of targeted inhibitors. *Nat Rev Cancer* 2005;5:341-54.
- 9 Van Cutsem E, Cervantes A, Adam R, Sobrero A, Van Krieken JH, Aderka D, Aranda Aguilar E, Bardelli A, Benson A, Bodoky G, Ciardiello F, D'Hoore A, Diaz-Rubio E, Douillard JY, Ducreux M, Falcone A, Grothey A, Gruenberger T, Haustermans K, Heinemann V, Hoff P, Köhne CH, Labianca R, Laurent-Puig P, Ma B, Maughan T, Muro K, Normanno N, Österlund P, Oyen WJ, Papamichael D, Pentheroudakis G, Pfeiffer P, Price TJ, Punt C, Ricke J, Roth A, Salazar R, Scheithauer W, Schmoll HJ, Tabernero J, Taïeb J, Tejpar S, Wasan H, Yoshino T, Zaanen A, Arnold D. ESMO consensus guidelines for the management of patients with metastatic colorectal



- cancer. *Ann Oncol* 2016;27:1386-422.
- 10 National Comprehensive Cancer Network. Clinical Practice Guidelines in Oncology. Colon Cancer. Version 2. 2017. Available from: [https://www.nccn.org/professionals/physician\\_gls/pdf/colon.pdf](https://www.nccn.org/professionals/physician_gls/pdf/colon.pdf) [Last accessed on 16 Dec 2017]
  - 11 Jonker DJ, O'Callaghan CJ, Karapetis CS, Zalberg JR, Tu D, Au HJ, Berry SR, Krahn M, Price T, Simes RJ, Tebbutt NC, van Hazel G, Wierzbicki R, Langer C, Moore MJ. Cetuximab for the treatment of colorectal cancer. *N Engl J Med* 2007;357:2040-8.
  - 12 Van Cutsem E, Peeters M, Siena S, Humblet Y, Hendlisz A, Neyns B, Canon JL, Van Laethem JL, Maurel J, Richardson G, Wolf M, Amado RG. Open-label phase III trial of panitumumab plus best supportive care compared with best supportive care alone in patients with chemotherapy-refractory metastatic colorectal cancer. *J Clin Oncol* 2007;25:1658-64.
  - 13 Malumbres M, Barbacid M. RAS oncogenes: the first 30 years. *Nat Rev Cancer* 2003;3:459-65.
  - 14 Schubert S, Shannon K, Bollag G. Hyperactive Ras in developmental disorders and cancer. *Nat Rev Cancer* 2007;7:295-308.
  - 15 Peeters M, Kafatos G, Taylor A, Gastanaga VM, Oliner KS, Hechmati G, Terwey JH, van Krieken JH. Prevalence of RAS mutations and individual variation patterns among patients with metastatic colorectal cancer: a pooled analysis of randomised controlled trials. *Eur J Cancer* 2015;51:1704-13.
  - 16 Lievre A, Bachet JB, Le Corre D, Boige V, Landi B, Emile JF, Côté JF, Tomic G, Penna C, Ducreux M, Rougier P, Penault-Llorca F, Laurent-Puig P. KRAS mutation status is predictive of response to cetuximab therapy in colorectal cancer. *Cancer Res* 2006;66:3992-5.
  - 17 Karapetis CS, Khambata-Ford S, Jonker DJ, O'Callaghan CJ, Tu D, Tebbutt NC, Simes RJ, Chalchal H, Shapiro JD, Robitaille S, Price TJ, Shepherd L, Au HJ, Langer C, Moore MJ, Zalberg JR. K-ras mutations and benefit from cetuximab in advanced colorectal cancer. *N Engl J Med* 2008;359:1757-65.
  - 18 Amado RG, Wolf M, Peeters M, Van Cutsem E, Siena S, Freeman DJ, Juan T, Sikorski R, Suggs S, Radinsky R, Patterson SD, Chang DD. Wild-type KRAS is required for panitumumab efficacy in patients with metastatic colorectal cancer. *J Clin Oncol* 2008;26:1626-34.
  - 19 Van Cutsem E, Köhne CH, Láng I, Folprecht G, Nowacki MP, Cascinu S, Shchepotin I, Maurel J, Cunningham D, Tejpar S, Schlichting M, Zube A, Celik I, Rougier P, Ciardiello F. Cetuximab plus irinotecan, fluorouracil, and leucovorin as first-line treatment for metastatic colorectal cancer: updated analysis of overall survival according to tumor KRAS and BRAF mutation status. *J Clin Oncol* 2011;29:2011-9.
  - 20 Douillard JY, Siena S, Cassidy J, Tabernero J, Burkes R, Barugel M, Humblet Y, Bodoky G, Cunningham D, Jassem J, Rivera F, Kocáková I, Ruff P, Błasińska-Morawiec M, Šmakal M, Canon JL, Rother M, Oliner KS, Wolf M, Gansert J. Randomized, phase III trial of panitumumab with infusional fluorouracil, leucovorin, and oxaliplatin (FOLFOX4) versus FOLFOX4 alone as first-line treatment in patients with previously untreated metastatic colorectal cancer: the PRIME study. *J Clin Oncol* 2010;28:4697-705.
  - 21 Linardou H, Dahabreh IJ, Kanaklopiti D, Siannis F, Bafaloukos D, Kosmidis P, Papadimitriou CA, Murray S. Assessment of somatic k-RAS mutations as a mechanism associated with resistance to EGFR-targeted agents: a systematic review and meta-analysis of studies in advanced non-small-cell lung cancer and metastatic colorectal cancer. *Lancet Oncol* 2008;9:962-72.
  - 22 Loupakis F, Ruzzo A, Cremolini C, Vincenzi B, Salvatore L, Santini D, Masi G, Stasi I, Canestrari E, Rulli E, Floriani I, Bencardino K, Galluccio N, Catalano V, Tonini G, Magnani M, Fontanini G, Basolo F, Falcone A, Graziano F. KRAS codon 61, 146 and BRAF mutations predict resistance to cetuximab plus irinotecan in KRAS codon 12 and 13 wild-type metastatic colorectal cancer. *Br J Cancer* 2009;101:715-21.
  - 23 De Roock W, Claes B, Bernasconi D, De Schutter J, Biesmans B, Fountzilias G, Kalogeras KT, Kotoula V, Papamichael D, Laurent-Puig P, Penault-Llorca F, Rougier P, Vincenzi B, Santini D, Tonini G, Cappuzzo F, Frattini M, Molinari F, Saletti P, De Dosso S, Martini M, Bardelli A, Siena S, Sartore-Bianchi A, Tabernero J, Macarulla T, Di Fiore F, Gangloff AO, Ciardiello F, Pfeiffer P, Qvortrup C, Hansen TP, Van Cutsem E, Piessevaux H, Lambrechts D, Delorenzi M, Tejpar S. Effects of KRAS, BRAF, NRAS, and PIK3CA mutations on the efficacy of cetuximab plus chemotherapy in chemotherapy-refractory metastatic colorectal cancer: a retrospective consortium analysis. *Lancet Oncol* 2010;11:753-62.
  - 24 Douillard JY, Oliner KS, Siena S, Tabernero J, Burkes R, Barugel M, Humblet Y, Bodoky G, Cunningham D, Jassem J, Rivera F, Kocáková I, Ruff P, Błasińska-Morawiec M, Šmakal M, Canon JL, Rother M, Williams R, Rong A, Wizek J, Sidhu R, Patterson SD. Panitumumab-FOLFOX4 treatment and RAS mutations in colorectal cancer. *N Engl J Med* 2013;369:1023-34.
  - 25 Bokemeyer C, Köhne CH, Ciardiello F, Lenz HJ, Heinemann V, Klinkhardt U, Beier F, Duecker K, van Krieken JH, Tejpar S. FOLFOX4 plus cetuximab treatment and RAS mutations in colorectal cancer. *Eur J Cancer* 2015;51:1243-52.
  - 26 Van Cutsem E, Lenz HJ, Köhne CH, Heinemann V, Tejpar S, Melezinek I, Beier F, Stroh C, Rougier P, van Krieken JH, Ciardiello F. Fluorouracil, leucovorin, and irinotecan plus cetuximab treatment and RAS mutations in colorectal cancer. *J Clin Oncol* 2015;33:692-700.
  - 27 Peeters M, Oliner KS, Price TJ, Cervantes A, Sobrero AF, Ducreux M, Hotko Y, André T, Chan E, Lordick F, Punt CJ, Strickland AH, Wilson G, Ciuleanu TE, Roman L, Van Cutsem E, He P, Yu H, Koukakis R, Terwey JH, Jung AS, Sidhu R, Patterson SD. Analysis of KRAS/NRAS mutations in a phase III study of panitumumab with FOLFIRI compared with FOLFIRI alone as second-line treatment for metastatic colorectal cancer. *Clin Cancer Res* 2015;21:5469-79.
  - 28 Sorich MJ, Wiese MD, Rowland A, Kichenadasse G, McKinnon RA, Karapetis CS. Extended RAS mutations and anti-EGFR monoclonal antibody survival benefit in metastatic colorectal cancer: a meta-analysis of randomized, controlled trials. *Ann Oncol* 2015;26:13-21.
  - 29 Schwartzberg LS, Rivera F, Karthaus M, Fasola G, Canon JL, Hecht JR, Yu H, Oliner KS, Go WY. PEAK: a randomized, multicenter phase II study of panitumumab plus modified fluorouracil, leucovorin, and oxaliplatin (mFOLFOX6) or bevacizumab plus mFOLFOX6 in patients with previously untreated, unresectable, wild-type KRAS exon 2 metastatic colorectal cancer. *J Clin Oncol* 2014;32:2240-7.

- 30 Allegra CJ, Rumble RB, Hamilton SR, Mangu PB, Roach N, Hantel A, Schilsky RL. Extended RAS gene mutation testing in metastatic colorectal carcinoma to predict response to anti-epidermal growth factor receptor monoclonal antibody therapy: American Society of Clinical Oncology Provisional Clinical Opinion Update 2015. *J Clin Oncol* 2016;34:179-85.
- 31 Lal N, White BS, Goussous G, Pickles O, Mason MJ, Beggs AD, Taniere P, Willcox BE, Guinney J, Middleton GW. KRAS mutation and consensus molecular subtypes 2 and 3 are independently associated with reduced immune infiltration and reactivity in colorectal cancer. *Clin Cancer Res* 2018;24:224-33.
- 32 Matallanas D, Birtwistle M, Romano D, Zebisch A, Rauch J, von Kriegsheim A, Kolch W. Raf family kinases: old dogs have learned new tricks. *Genes Cancer* 2011;2:232-60.
- 33 McCubrey JA, Steelman LS, Chappell WH, Abrams SL, Wong EW, Chang F, Lehmann B, Terrian DM, Milella M, Tafuri A, Stivala F, Libra M, Basecke J, Evangelisti C, Martelli AM, Franklin RA. Roles of the Raf/MEK/ERK pathway in cell growth, malignant transformation and drug resistance. *Biochim Biophys Acta* 2007;1773:1263-84.
- 34 Rajagopalan H, Bardelli A, Lengauer C, Kinzler KW, Vogelstein B, Velculescu VE. Tumorigenesis: RAF/RAS oncogenes and mismatch-repair status. *Nature* 2002;418:934.
- 35 Souglakos J, Phillips J, Wang R, Marwah S, Silver M, Tzardi M, Silver J, Ogino S, Hooshmand S, Kwak E, Freed E, Meyerhardt JA, Saridaki Z, Georgoulas V, Finkelstein D, Fuchs CS, Kulke MH, Shivdasani RA. Prognostic and predictive value of common mutations for treatment response and survival in patients with metastatic colorectal cancer. *Br J Cancer* 2009;101:465-72.
- 36 Richman SD, Seymour MT, Chambers P, Elliott F, Daly CL, Meade AM, Taylor G, Barrett JH, Quirke P. KRAS and BRAF mutations in advanced colorectal cancer are associated with poor prognosis but do not preclude benefit from oxaliplatin or irinotecan: results from the MRC FOCUS trial. *J Clin Oncol* 2009;27:5931-7.
- 37 Yokota T, Ura T, Shibata N, Takahari D, Shitara K, Nomura M, Kondo C, Mizota A, Utsunomiya S, Muro K, Yatabe Y. BRAF mutation is a powerful prognostic factor in advanced and recurrent colorectal cancer. *Br J Cancer* 2011;104:856-62.
- 38 Saridaki Z, Papadatos-Pastos D, Tzardi M, Mavroudis D, Bairaktari E, Arvanity H, Stathopoulos E, Georgoulas V, Souglakos J. BRAF mutations, microsatellite instability status and cyclin D1 expression predict metastatic colorectal patients' outcome. *Br J Cancer* 2010;102:1762-8.
- 39 Tran B, Kopetz S, Tie J, Gibbs P, Jiang ZQ, Lieu CH, Agarwal A, Maru DM, Sieber O, Desai J. Impact of BRAF mutation and microsatellite instability on the pattern of metastatic spread and prognosis in metastatic colorectal cancer. *Cancer* 2011;117:4623-32.
- 40 Tie J, Gibbs P, Lipton L, Christie M, Jorissen RN, Burgess AW, Croxford M, Jones I, Langland R, Kosmider S, McKay D, Bollag G, Nolop K, Sieber OM, Desai J. Optimizing targeted therapeutic development: analysis of a colorectal cancer patient population with the BRAF(V600E) mutation. *Int J Cancer* 2011;128:2075-84.
- 41 Loupakis F, Moretto R, Aprile G, Muntoni M, Cremolini C, Iacono D, Casagrande M, Ferrari L, Salvatore L, Schirripa M, Rossini D, De Maglio G, Fasola G, Calvetti L, Pilotto S, Carbognin L, Fontanini G, Tortora G, Falcone A, Sperduti I, Bria E. Clinico-pathological nomogram for predicting BRAF mutational status of metastatic colorectal cancer. *Br J Cancer* 2016;114:30-6.
- 42 Yaeger R, Cercek A, Chou JF, Sylvester BE, Kemeny NE, Hechtman JF, Ladanyi M, Rosen N, Weiser MR, Capanu M, Solit DB, D'Angelica MI, Vakiani E, Saltz LB. BRAF mutation predicts for poor outcomes after metastasectomy in patients with metastatic colorectal cancer. *Cancer* 2014;120:2316-24.
- 43 Schirripa M, Bergamo F, Cremolini C, Casagrande M, Lonardi S, Aprile G, Yang D, Marmorino F, Pasquini G, Sensi E, Lupi C, De Maglio G, Borrelli N, Pizzolitto S, Fasola G, Bertorelle R, Ruge M, Fontanini G, Zagonel V, Loupakis F, Falcone A. BRAF and RAS mutations as prognostic factors in metastatic colorectal cancer patients undergoing liver resection. *Br J Cancer* 2015;112:1921-8.
- 44 Weisenberger DJ, Siegmund KD, Campan M, Young J, Long TI, Faasse MA, Kang GH, Widschwendter M, Weener D, Buchanan D, Koh H, Simms L, Barker M, Leggett B, Levine J, Kim M, French AJ, Thibodeau SN, Jass J, Haile R, Laird PW. CpG island methylator phenotype underlies sporadic microsatellite instability and is tightly associated with BRAF mutation in colorectal cancer. *Nat Genet* 2006;38:787-93.
- 45 Popovici V, Budinska E, Tejpar S, Weinrich S, Estrella H, Hodgson G, Van Cutsem E, Xie T, Bosman FT, Roth AD, Delorenzi M. Identification of a poor-prognosis BRAF-mutant-like population of patients with colon cancer. *J Clin Oncol* 2012;30:1288-95.
- 46 Barras D, Missiaglia E, Wirapati P, Sieber OM, Jorissen RN, Love C, Molloy PL, Jones IT, McLaughlin S, Gibbs P, Guinney J, Simon IM, Roth AD, Bosman FT, Tejpar S, Delorenzi M. BRAF V600E mutant colorectal cancer subtypes based on gene expression. *Clin Cancer Res* 2017;23:104-15.
- 47 Cremolini C, Di Bartolomeo M, Amatu A, Antoniotti C, Moretto R, Berenato R, Perrone F, Tamborini E, Aprile G, Lonardi S, Sartore-Bianchi A, Fontanini G, Milione M, Lauricella C, Siena S, Falcone A, de Braud F, Loupakis F, Pietrantonio F. BRAF codons 594 and 596 mutations identify a new molecular subtype of metastatic colorectal cancer at favorable prognosis. *Ann Oncol* 2015;26:2092-7.
- 48 Jones JC, Renfro LA, Al-Shamsi HO, Schrock AB, Rankin A, Zhang BY, Kasi PM, Voss JS, Leal AD, Sun J, Ross J, Ali SM, Hubbard JM, Kipp BR, McWilliams RR, Kopetz S, Wolff RA, Grothey A. Non-V600BRAF mutations define a clinically distinct molecular subtype of metastatic colorectal cancer. *J Clin Oncol* 2017;35:2624-30.
- 49 Di Nicolantonio F, Martini M, Molinari F, Sartore-Bianchi A, Arena S, Saletti P, De Dosso S, Mazzucchelli L, Frattini M, Siena S, Bardelli A. Wild-type BRAF is required for response to panitumumab or cetuximab in metastatic colorectal cancer. *J Clin Oncol* 2008;26:5705-12.
- 50 Bokemeyer C, Van Cutsem E, Rougier P, Ciardiello F, Heeger S, Schlichting M, Celik I, Köhne CH. Addition of cetuximab to chemotherapy as first-line treatment for KRAS wild-type metastatic colorectal cancer: pooled analysis of the CRYSTAL and OPUS randomised clinical trials. *Eur J Cancer* 2012;48:1466-75.
- 51 Pietrantonio F, Petrelli F, Coinu A, Di Bartolomeo M, Borgonovo K, Maggi C, Cabiddu M, Iacovelli R, Bossi I, Lonati V, Ghilardi

- M, de Braud F, Barni S. Predictive role of BRAF mutations in patients with advanced colorectal cancer receiving cetuximab and panitumumab: a meta-analysis. *Eur J Cancer* 2015;51:587-94.
- 52 Therkildsen C, Bergmann TK, Henrichsen-Schnack T, Ladelund S, Nilbert M. The predictive value of KRAS, NRAS, BRAF, PIK3CA and PTEN for anti-EGFR treatment in metastatic colorectal cancer: a systematic review and meta-analysis. *Acta Oncol* 2014;53:852-64.
- 53 Stintzing S, Miller-Phillips L, Modest DP, Fischer von Weikersthal L, Decker T, Kiani A, Vehling-Kaiser U, Al-Batran SE, Heintges T, Kahl C, Seipelt G, Kullmann F, Stauch M, Scheithauer W, Held S, Moehler M, Jagenburg A, Kirchner T, Jung A, Heinemann V; FIRE-3 Investigators. Impact of BRAF and RAS mutations on first-line efficacy of FOLFIRI plus cetuximab versus FOLFIRI plus bevacizumab: analysis of the FIRE-3 (AIO KRK-0306) study. *Eur J Cancer* 2017;79:50-60.
- 54 Loupakis F, Cremolini C, Salvatore L, Masi G, Sensi E, Schirripa M, Michelucci A, Pfanner E, Brunetti I, Lupi C, Antoniotti C, Bergamo F, Lonardi S, Zagonel V, Simi P, Fontanini G, Falcone A. FOLFOXIRI plus bevacizumab as first-line treatment in BRAF mutant metastatic colorectal cancer. *Eur J Cancer* 2014;50:57-63.
- 55 Kopetz S, Desai J, Chan E, Hecht JR, O'Dwyer PJ, Maru D, Morris V, Janku F, Dasari A, Chung W, Issa JP, Gibbs P, James B, Powis G, Nolop KB, Bhattacharya S, Saltz L. Phase II pilot study of vemurafenib in patients with metastatic BRAF-mutated colorectal cancer. *J Clin Oncol* 2015;33:4032-8.
- 56 Corcoran RB, Atreya CE, Falchook GS, Kwak EL, Ryan DP, Bendell JC, Hamid O, Messersmith WA, Daud A, Kurzrock R, Pierobon M, Sun P, Cunningham E, Little S, Orford K, Motwani M, Bai Y, Patel K, Venook AP, Kopetz S. Combined BRAF and MEK inhibition with dabrafenib and trametinib in BRAF V600-mutant colorectal cancer. *J Clin Oncol* 2015;33:4023-31.
- 57 Yaeger R, Cercek A, O'Reilly EM, Reidy DL, Kemeny N, Wolinsky T, Capanu M, Gollub MJ, Rosen N, Berger MF, Lacouture ME, Vakiani E, Saltz LB. Pilot trial of combined BRAF and EGFR inhibition in BRAF-mutant metastatic colorectal cancer patients. *Clin Cancer Res* 2015;21:1313-20.
- 58 Taberero J, Guren TK, Yaeger RD, Spreafico A, Faris JE, Yoshino T, Yamada Y, Kim TW, Bendell JC, Schuler MH, Lenz HJ, Eskens F, Desai J, Hochster HS, Avsar E, Demuth T, Sandor V, Elez E, Schellens JHM. Phase 2 results: encorafenib (ENCO) and cetuximab (CETUX) with or without alpelisib (ALP) in patients with advanced BRAF-mutant colorectal cancer (BRAFM CRC). *J Clin Oncol* 2016;34:abstr 3544.
- 59 Corcoran RB, Yoshino T, Bendell JC, Atreya CE, Schellens JHM, Ducreux MP, McRee A, Siena S, Middleton G, Gordon M, Humblet Y, Muro K, Elez E, Yaeger R, Sidhu R, Squires M, Jaeger S, Rangwala F, Van Cutsem E. Efficacy and circulating tumor DNA (ctDNA) analysis of the BRAF inhibitor dabrafenib (D), MEK inhibitor trametinib (T), and anti-EGFR antibody panitumumab (P) in patients (pts) with BRAF V600E-mutated (BRAFM) metastatic colorectal cancer (mCRC). *Ann Oncol* 2016;27:149-206.
- 60 Van Cutsem E, Cuyle PJ, Yaeger R, Huijberts S, Schellens JHM, Elez E, Taberero J, Fakih M, Montagut C, Peeters M, Desai J, Yoshino T, Ciardiello F, Wasan H, Kopetz S, Maharry K, Christy-Bittel J, Gollerkeri A, Grothey A. BEACON CRC study safety lead-in (SLI) in patients with BRAFV600E metastatic colorectal cancer (mCRC): efficacy and tumor markers. *J Clin Oncol* 2018;36:abstr 627.
- 61 Kopetz S, McDonough SL, Lenz HJ, Magliocco AM, Atreya CE, Diaz LA, Allegra CJ, Raghav KPS, Morris VK, Wang SE, Lieu CH, Guthrie KA, Hochster HS. Randomized trial of irinotecan and cetuximab with or without vemurafenib in BRAF-mutant metastatic colorectal cancer (SWOG 1406). *J Clin Oncol* 2017;35:abstract 3505.
- 62 Morris EJ, Jha S, Restaino CR, Dayananth P, Zhu H, Cooper A, Carr D, Deng Y, Jin W, Black S, Long B, Liu J, Dinunzio E, Windsor W, Zhang R, Zhao S, Angagaw MH, Pinheiro EM, Desai J, Xiao L, Shipps G, Hruza A, Wang J, Kelly J, Paliwal S, Gao X, Babu BS, Zhu L, Daublain P, Zhang L, Lutterbach BA, Pelletier MR, Philippar U, Siliphaivanh P, Witter D, Kirschmeier P, Bishop WR, Hicklin D, Gilliland DG, Jayaraman L, Zawel L, Fawell S, Samatar AA. Discovery of a novel ERK inhibitor with activity in models of acquired resistance to BRAF and MEK inhibitors. *Cancer Discov* 2013;3:742-50.
- 63 Ahronian LG, Sennott EM, Van Allen EM, Wagle N, Kwak EL, Faris JE, Godfrey JT, Nishimura K, Lynch KD, Mermel CH, Lockerman EL, Kalsy A, Gurski JM Jr, Bahl S, Anderka K, Green LM, Lennon NJ, Huynh TG, Mino-Kenudson M, Getz G, Dias-Santagata D, Iafrate AJ, Engelman JA, Garraway LA, Corcoran RB. Clinical acquired resistance to RAF inhibitor combinations in BRAF-mutant colorectal cancer through MAPK pathway alterations. *Cancer Discov* 2015;5:358-67.
- 64 Jiricny J. The multifaceted mismatch-repair system. *Nat Rev Mol Cell Biol* 2006;7:335-46.
- 65 Hampel H, Frankel WL, Martin E, Arnold M, Khanduja K, Kuebler P, Nakagawa H, Sotamaa K, Prior TW, Westman J, Panescu J, Fix D, Lockman J, Comeras I, de la Chapelle A. Screening for the Lynch syndrome (hereditary nonpolyposis colorectal cancer). *N Engl J Med* 2005;352:1851-60.
- 66 Boland CR, Goel A. Microsatellite instability in colorectal cancer. *Gastroenterology* 2010;138:2073-87.
- 67 Lynch HT, de la Chapelle A. Hereditary colorectal cancer. *N Engl J Med* 2003;348:919-32.
- 68 Lynch HT, Lynch JF, Lynch PM. Toward a consensus in molecular diagnosis of hereditary nonpolyposis colorectal cancer (Lynch syndrome). *J Natl Cancer Inst* 2007;99:261-3.
- 69 Haraldsdottir S, Hampel H, Wu C, Weng DY, Shields PG, Frankel WL, Pan X, de la Chapelle A, Goldberg RM, Bekaii-Saab T. Patients with colorectal cancer associated with Lynch syndrome and MLH1 promoter hypermethylation have similar prognoses. *Genet Med* 2016;18:863-8.
- 70 Cohen SA, Turner EH, Beightol MB, Jacobson A, Gooley TA, Salipante SJ, Haraldsdottir S, Smith C, Scroggins S, Tait JF, Grady WM, Lin EH, Cohn DE, Goodfellow PJ, Arnold MW, de la Chapelle A, Pearlman R, Hampel H, Pritchard CC. Frequent PIK3CA mutations in colorectal and endometrial tumors with 2 or more somatic mutations in mismatch repair genes. *Gastroenterology* 2016;151:440-7.e1.
- 71 Becht E, de Reynies A, Giraldo NA, Pilati C, Buttard B, Lacroix L, Selves J, Sautès-Fridman C, Laurent-Puig P, Fridman WH. Immune and stromal classification of colorectal cancer is associated with molecular subtypes and relevant for precision immunotherapy. *Clin Cancer Res* 2016;22:4057-66.

- 72 Senter L. Genetic testing by cancer site: colon (nonpolyposis syndromes). *Cancer J* 2012;18:334-7.
- 73 Umar A, Boland CR, Terdiman JP, Syngal S, de la Chapelle A, Rüschoff J, Fishel R, Lindor NM, Burgart LJ, Hamelin R, Hamilton SR, Hiatt RA, Jass J, Lindblom A, Lynch HT, Peltomaki P, Ramsey SD, Rodriguez-Bigas MA, Vasen HF, Hawk ET, Barrett JC, Freedman AN, Srivastava S. Revised Bethesda guidelines for hereditary nonpolyposis colorectal cancer (Lynch syndrome) and microsatellite instability. *J Natl Cancer Inst* 2004;96:261-8.
- 74 Salipante SJ, Scroggins SM, Hampel HL, Turner EH, Pritchard CC. Microsatellite instability detection by next generation sequencing. *Clin Chem* 2014;60:1192-9.
- 75 Niu B, Ye K, Zhang Q, Lu C, Xie M, McLellan MD, Wendl MC, Ding L. MSIsensor: microsatellite instability detection using paired tumor-normal sequence data. *Bioinformatics* 2014;30:1015-6.
- 76 Kautto EA, Bonneville R, Miya J, Yu L, Krook MA, Reeser JW, Roychowdhury S. Performance evaluation for rapid detection of pancreatic microsatellite instability with MANTIS. *Oncotarget* 2017;8:7452-63.
- 77 Hause RJ, Pritchard CC, Shendure J, Salipante SJ. Classification and characterization of microsatellite instability across 18 cancer types. *Nat Med* 2016;22:1342-50.
- 78 Kasi PM. Mutational burden on circulating cell-free tumor-DNA testing as a surrogate marker of mismatch repair deficiency or microsatellite instability in patients with colorectal cancers. *J Gastrointest Oncol* 2017;8:747-8.
- 79 Raut CP, Pawlik TM, Rodriguez-Bigas MA. Clinicopathologic features in colorectal cancer patients with microsatellite instability. *Mutat Res* 2004;568:275-82.
- 80 Cohen R, Buhard O, Cervera P, Hain E, Dumont S, Bardier A, Bachel JB, Gornet JM, Lopez-Trabada D, Dumont S, Kaci R, Bertheau P, Renaud F, Bibeau F, Parc Y, Vernerey D, Duval A, Svrcek M, André T. Clinical and molecular characterisation of hereditary and sporadic metastatic colorectal cancers harbouring microsatellite instability/DNA mismatch repair deficiency. *Eur J Cancer* 2017;86:266-74.
- 81 Sargent DJ, Marsoni S, Monges G, Thibodeau SN, Labianca R, Hamilton SR, French AJ, Kabat B, Foster NR, Torri V, Ribic C, Grothey A, Moore M, Zaniboni A, Seitz JF, Sinicrope F, Gallinger S. Defective mismatch repair as a predictive marker for lack of efficacy of fluorouracil-based adjuvant therapy in colon cancer. *J Clin Oncol* 2010;28:3219-26.
- 82 Hutchins G, Southward K, Handley K, Magill L, Beaumont C, Stahlschmidt J, Richman S, Chambers P, Seymour M, Kerr D, Gray R, Quirke P. Value of mismatch repair, KRAS, and BRAF mutations in predicting recurrence and benefits from chemotherapy in colorectal cancer. *J Clin Oncol* 2011;29:1261-70.
- 83 Des Guetz G, Schischmanoff O, Nicolas P, Perret GY, Morere JF, Uzzan B. Does microsatellite instability predict the efficacy of adjuvant chemotherapy in colorectal cancer? A systematic review with meta-analysis. *Eur J Cancer* 2009;45:1890-6.
- 84 Sargent DJ, Shi Q, Yothers G, Tejpar S, Bertagnolli MM, Thibodeau SN, Andre T, Labianca R, Gallinger S, Hamilton SR, Monges G, Pogue-Geile KL, Paik S, Klingbiel D, Roth A, Pavay ES, Kim GP, Sinicrope FA; ACCENT Collaborative Group. Prognostic impact of deficient mismatch repair (dMMR) in 7,803 stage II/III colon cancer (CC) patients (pts): a pooled individual pt data analysis of 17 adjuvant trials in the ACCENT database. *J Clin Oncol* 2014;32:abstr3507.
- 85 Dienstmann R, Mason MJ, Sinicrope FA, Phipps AI, Tejpar S, Nesbakken A, Danielsen SA, Sveen A, Buchanan DD, Clendenning M, Rosty C, Bot B, Alberts SR, Milburn Jessup J, Lothe RA, Delorenzi M, Newcomb PA, Sargent D, Guinney J. Prediction of overall survival in stage II and III colon cancer beyond TNM system: a retrospective, pooled biomarker study. *Ann Oncol* 2017;28:1023-31.
- 86 Sinicrope FA, Foster NR, Thibodeau SN, Marsoni S, Monges G, Labianca R, Kim GP, Yothers G, Allegra C, Moore MJ, Gallinger S, Sargent DJ. DNA mismatch repair status and colon cancer recurrence and survival in clinical trials of 5-fluorouracil-based adjuvant therapy. *J Natl Cancer Inst* 2011;103:863-75.
- 87 Des Guetz G, Uzzan B, Nicolas P, Schischmanoff O, Perret GY, Morere JF. Microsatellite instability does not predict the efficacy of chemotherapy in metastatic colorectal cancer. A systematic review and meta-analysis. *Anticancer Res* 2009;29:1615-20.
- 88 Vilar E, Gruber SB. Microsatellite instability in colorectal cancer—the stable evidence. *Nat Rev Clin Oncol* 2010;7:153-62.
- 89 Sinicrope FA, Mahoney MR, Smyrk TC, Thibodeau SN, Warren RS, Bertagnolli MM, Nelson GD, Goldberg RM, Sargent DJ, Alberts SR. Prognostic impact of deficient DNA mismatch repair in patients with stage III colon cancer from a randomized trial of FOLFOX-based adjuvant chemotherapy. *J Clin Oncol* 2013;31:3664-72.
- 90 Bertagnolli MM, Niedzwiecki D, Compton CC, Hahn HP, Hall M, Damas B, Jewell SD, Mayer RJ, Goldberg RM, Saltz LB, Warren RS, Redston M. Microsatellite instability predicts improved response to adjuvant therapy with irinotecan, fluorouracil, and leucovorin in stage III colon cancer: Cancer and Leukemia Group B Protocol 89803. *J Clin Oncol* 2009;27:1814-21.
- 91 Fallik D, Borriani F, Boige V, Viguier J, Jacob S, Miquel C, Sabourin JC, Ducreux M, Praz F. Microsatellite instability is a predictive factor of the tumor response to irinotecan in patients with advanced colorectal cancer. *Cancer Res* 2003;63:5738-44.
- 92 Tougeron D, Sueur B, Sefrioui D, Gentilhomme L, Lecomte T, Aparicio T, Guetz GDES, Artru P, De La Fouchardiere C, Moulin V, Hautefeuille V, Coriat R, Toucheffeu Y, Lecaille C, Gael G, Ferru A, Tourani JM, Emambux S, Taieb J, Zaanani A. A large multicenter study evaluating prognosis and chemosensitivity of metastatic colorectal cancers with microsatellite instability. *J Clin Oncol* 2017;35:abstr3536.
- 93 Huang Y, Goel S, Duda DG, Fukumura D, Jain RK. Vascular normalization as an emerging strategy to enhance cancer immunotherapy. *Cancer Res* 2013;73:2943-8.
- 94 Jin Z, Sanhueza CT, Johnson B, Smyrk TC, Larson DW, Nagorney DM, Hubbard JM, Grothey A. Outcome of mismatch repair deficient metastatic colorectal cancer (CRC): The Mayo Clinic Experience. *J Clin Oncol* 2017;35:abstr15030.
- 95 Goldstein J, Tran B, Ensor J, Gibbs P, Wong HL, Wong SF, Vilar E, Tie J, Broadus R, Kopetz S, Desai J, Overman MJ. Multicenter retrospective analysis of metastatic colorectal cancer (CRC) with high-level microsatellite instability (MSI-H). *Ann Oncol*

- 2014;25:1032-8.
- 96 Marisa L, Svrcek M, Collura A, Becht E, Cervera P, Wanherdrick K, Buhard O, Goloudina A, Jonchère V, Selves J, Milano G, Guenot D, Cohen R, Colas C, Laurent-Puig P, Olschwang S, Lefèvre JH, Parc Y, Boige V, Lepage C, André T, Fléjou JF, Dérangère V, Ghiringhelli F, de Reynies A, Duval A. The balance between cytotoxic T-cell lymphocytes and immune checkpoint expression in the prognosis of colon tumors. *J Natl Cancer Inst* 2018;110:djx136.
- 97 Le DT, Uram JN, Wang H, Bartlett BR, Kemberling H, Eyring AD, Skora AD, Lubner BS, Azad NS, Laheru D, Biedrzycki B, Donehower RC, Zaheer A, Fisher GA, Crocenzi TS, Lee JJ, Duffy SM, Goldberg RM, de la Chapelle A, Koshiji M, Bhaijee F, Huebner T, Hruban RH, Wood LD, Cuka N, Pardoll DM, Papadopoulos N, Kinzler KW, Zhou S, Cornish TC, Taube JM, Anders RA, Eshleman JR, Vogelstein B, Diaz LA Jr. PD-1 blockade in tumors with mismatch-repair deficiency. *N Engl J Med* 2015;372:2509-20.
- 98 Le DT, Uram JN, Wang H, Bartlett B, Kemberling H, Eyring A, Azad NS, Laheru D, Donehower RC, Crocenzi TS, Goldberg RM, Fisher GA, Lee JJ, Greten TF, Koshiji M, Kang SP, Anders RA, Eshleman JR, Vogelstein B, Diaz LA. Programmed death-1 blockade in mismatch repair deficient colorectal cancer. *J Clin Oncol* 2016;34:abstr 103.
- 99 Overman MJ, Lonardi S, Wong KYM, Lenz HJ, Gelsomino F, Aglietta M, Morse MA, Van Cutsem E, McDermott R, Hill A, Sawyer MB, Hendlitz A, Neyns B, Svrcek M, Moss RA, Ledezne JM, Cao ZA, Kamble S, Kopetz S, André T. Durable clinical benefit with nivolumab plus ipilimumab in DNA mismatch repair-deficient/microsatellite instability-high metastatic colorectal cancer. *J Clin Oncol* 2018; doi: 10.1200/JCO.2017.76.9901.
- 100 Overman MJ, McDermott R, Leach JL, Lonardi S, Lenz HJ, Morse MA, Desai J, Hill A, Axelson M, Moss RA, Goldberg MV, Cao ZA, Ledezne JM, Maglinte GA, Kopetz S, André T. Nivolumab in patients with metastatic DNA mismatch repair-deficient or microsatellite instability-high colorectal cancer (CheckMate 142): an open-label, multicentre, phase 2 study. *Lancet Oncol* 2017;18:1182-91.
- 101 Le DT, Durham JN, Smith KN, Wang H, Bartlett BR, Aulakh LK, Lu S, Kemberling H, Wilt C, Lubner BS, Wong F, Azad NS, Rucki AA, Laheru D, Donehower R, Zaheer A, Fisher GA, Crocenzi TS, Lee JJ, Greten TF, Duffy AG, Ciombor KK, Eyring AD, Lam BH, Joe A, Kang SP, Holdhoff M, Danilova L, Cope L, Meyer C, Zhou S, Goldberg RM, Armstrong DK, Bever KM, Fader AN, Taube J, Housseau F, Spetzler D, Xiao N, Pardoll DM, Papadopoulos N, Kinzler KW, Eshleman JR, Vogelstein B, Anders RA, Diaz LA Jr. Mismatch repair deficiency predicts response of solid tumors to PD-1 blockade. *Science* 2017;357:409-13.
- 102 Chen DS, Mellman I. Elements of cancer immunity and the cancer-immune set point. *Nature* 2017;541:321-30.
- 103 Fridman WH, Zitvogel L, Sautes-Fridman C, Kroemer G. The immune contexture in cancer prognosis and treatment. *Nat Rev Clin Oncol* 2017;14:717-34.
- 104 Roelands J, Kuppen PJK, Vermeulen L, Maccalli C, Decock J, Wang E, Marincola FM, Bedognetti D, Hendrickx W. Immunogenomic classification of colorectal cancer and therapeutic implications. *Int J Mol Sci* 2017;18:E2229.
- 105 Patel SJ, Sanjana NE, Kishton RJ, Eidizadeh A, Vodnala SK, Cam M, Gartner JJ, Jia L, Steinberg SM, Yamamoto TN, Merchant AS, Mehta GU, Chichura A, Shalem O, Tran E, Eil R, Sukumar M, Gujjarro EP, Day CP, Robbins P, Feldman S, Merlino G, Zhang F, Restifo NP. Identification of essential genes for cancer immunotherapy. *Nature* 2017;548:537-42.
- 106 Lesterhuis WJ, Bosco A, Millward MJ, Small M, Nowak AK, Lake RA. Dynamic versus static biomarkers in cancer immune checkpoint blockade: unravelling complexity. *Nat Rev Drug Discov* 2017;16:264-72.
- 107 Nishino M, Ramaiya NH, Hatabu H, Hodi FS. Monitoring immune-checkpoint blockade: response evaluation and biomarker development. *Nat Rev Clin Oncol* 2017;14:655-68.
- 108 Sharma P, Hu-Lieskovan S, Wargo JA, Ribas A. Primary, adaptive, and acquired resistance to cancer immunotherapy. *Cell* 2017;168:707-23.
- 109 Gotwals P, Cameron S, Cipolletta D, Cremasco V, Crystal A, Hewes B, Mueller B, Quarantino S, Sabatos-Peyton C, Petruzzelli L, Engelman JA, Dranoff G. Prospects for combining targeted and conventional cancer therapy with immunotherapy. *Nat Rev Cancer* 2017;17:286-301.
- 110 Syn NL, Teng MWL, Mok TSK, Soo RA. De-novo and acquired resistance to immune checkpoint targeting. *Lancet Oncol* 2017;18:e731-41.
- 111 Longley DB, Harkin DP, Johnston PG. 5-fluorouracil: mechanisms of action and clinical strategies. *Nat Rev Cancer* 2003;3:330-8.
- 112 Heggie GD, Sommadossi JP, Cross DS, Huster WJ, Diasio RB. Clinical pharmacokinetics of 5-fluorouracil and its metabolites in plasma, urine, and bile. *Cancer Res* 1987;47:2203-6.
- 113 Saif MW, Syrigos K, Mehra R, Mattison LK, Diasio RB. Dihydropyrimidine dehydrogenase deficiency (Dpd) in GI malignancies: experience of 4-years. *Pak J Med Sci* 2007;23:832-9.
- 114 Morel A, Boisdron-Celle M, Fey L, Soulie P, Craipeau MC, Traore S, Gamelin E. Clinical relevance of different dihydropyrimidine dehydrogenase gene single nucleotide polymorphisms on 5-fluorouracil tolerance. *Mol Cancer Ther* 2006;5:2895-904.
- 115 van Kuilenburg AB, Muller EW, Haasjes J, Meisma R, Zoetekouw L, Waterham HR, Baas F, Richel DJ, van Gennip AH. Lethal outcome of a patient with a complete dihydropyrimidine dehydrogenase (DPD) deficiency after administration of 5-fluorouracil: frequency of the common IVS14+1G>A mutation causing DPD deficiency. *Clin Cancer Res* 2001;7:1149-53.
- 116 Raida M, Schwabe W, Häusler P, Van Kuilenburg AB, Van Gennip AH, Behnke D, Höffken K. Prevalence of a common point mutation in the dihydropyrimidine dehydrogenase (DPD) gene within the 5'-splice donor site of intron 14 in patients with severe 5-fluorouracil (5-FU)- related toxicity compared with controls. *Clin Cancer Res* 2001;7:2832-9.
- 117 Meulendijks D, Henricks LM, Sonke GS, Deenen MJ, Froehlich TK, Amstutz U, Largiadèr CR, Jennings BA, Marinaki AM, Sanderson JD, Kleibl Z, Kleiblova P, Schwab M, Zanger UM, Palles C, Tomlinson I, Gross E, van Kuilenburg AB, Punt CJ, Koopman M, Beijnen JH, Cats A, Schellens JH. Clinical relevance of DPYD variants c.1679T>G, c.1236G>A/HapB3, and c.1601G>A as predictors of severe fluoropyrimidine-associated toxicity: a systematic review and meta-analysis of individual patient data. *Lancet Oncol* 2015;16:1639-50.

- 118 Ruzzo A, Graziano F, Galli F, Galli F, Rulli E, Lonardi S, Ronzoni M, Massidda B, Zagonel V, Pella N, Muciarini C, Labianca R, Ionta MT, Bagaloni I, Veltri E, Sozzi P, Barni S, Ricci V, Foltran L, Nicolini M, Biondi E, Bramati A, Turci D, Lazzarelli S, Verusio C, Bergamo F, Sobrero A, Frontini L, Menghi M, Magnani M. Dihydropyrimidine dehydrogenase pharmacogenetics for predicting fluoropyrimidine-related toxicity in the randomised, phase III adjuvant TOSCA trial in high-risk colon cancer patients. *Br J Cancer* 2017;117:1269-77.
- 119 Deenen MJ, Meulendijks D, Cats A, Sechterberger MK, Severens JL, Boot H, Smits PH, Rosing H, Mandigers CM, Soesan M, Beijnen JH, Schellens JH. Upfront genotyping of DPYD\*2A to individualize fluoropyrimidine therapy: a safety and cost analysis. *J Clin Oncol* 2016;34:227-34.
- 120 Amstutz U, Henricks LM, Offer SM, Barbarino J, Schellens JHM, Swen JJ, Klein TE, McLeod HL, Caudle KE, Diasio RB, Schwab M. Clinical Pharmacogenetics Implementation Consortium (CPIC) Guideline for dihydropyrimidine dehydrogenase genotype and fluoropyrimidine dosing: 2017 update. *Clin Pharmacol Ther* 2018;103:210-6.
- 121 Mathijssen RH, van Alphen RJ, Verweij J, Loos WJ, Nooter K, Stoter G, Sparreboom A. Clinical pharmacokinetics and metabolism of irinotecan (CPT-11). *Clin Cancer Res* 2001;7:2182-94.
- 122 Douillard JY, Cunningham D, Roth AD, Navarro M, James RD, Karasek P, Jandik P, Iveson T, Carmichael J, Alakl M, Gruia G, Awad L, Rougier P. Irinotecan combined with fluorouracil compared with fluorouracil alone as first-line treatment for metastatic colorectal cancer: a multicentre randomised trial. *Lancet* 2000;355:1041-7.
- 123 Mathijssen RH, Marsh S, Karlsson MO, Xie R, Baker SD, Verweij J, Sparreboom A, McLeod HL. Irinotecan pathway genotype analysis to predict pharmacokinetics. *Clin Cancer Res* 2003;9:3246-53.
- 124 Hu ZY, Yu Q, Pei Q, Guo C. Dose-dependent association between UGT1A1\*28 genotype and irinotecan-induced neutropenia: low doses also increase risk. *Clin Cancer Res* 2010;16:3832-42.
- 125 Hu ZY, Yu Q, Zhao YS. Dose-dependent association between UGT1A1\*28 polymorphism and irinotecan-induced diarrhoea: a meta-analysis. *Eur J Cancer* 2010;46:1856-65.
- 126 Glimelius B, Garmo H, Berglund A, Fredriksson LA, Berglund M, Kohnke H, Byström P, Sørbye H, Wadelius M. Prediction of irinotecan and 5-fluorouracil toxicity and response in patients with advanced colorectal cancer. *Pharmacogenomics J* 2011;11:61-71.
- 127 Del Re M, Cremolini C, Loupakis F, Marmorino F, Citi V, Palombi M, Bergamo F, Schirripa M, Rossini D, Cortesi E, Tomasello G, Spadi R, Buonadonna A, Amoroso D, Vitello S, Di Donato S, Granetto C, D'Amico M, Danesi R, Falcone A. DPYD c.1905+1G>A and c.2846A>T and UGT1A1\*28 allelic variants as predictors of toxicity: pharmacogenetic translational analysis from the Phase III TRIBE study in metastatic colorectal cancer. *J Clin Oncol* 2015;33:abstr 3532.
- 128 Liu X, Cheng D, Kuang Q, Liu G, Xu W. Association of UGT1A1\*28 polymorphisms with irinotecan-induced toxicities in colorectal cancer: a meta-analysis in Caucasians. *Pharmacogenomics J* 2014;14:120-9.
- 129 Campbell JM, Stephenson MD, Bateman E, Peters MD, Keefe DM, Bowen JM. Irinotecan-induced toxicity pharmacogenetics: an umbrella review of systematic reviews and meta-analyses. *Pharmacogenomics J* 2017;17:21-8.
- 130 Innocenti F, Schilsky RL, Ramirez J, Janisch L, Undevia S, House LK, Das S, Wu K, Turcich M, Marsh R, Karrison T, Maitland ML, Salgia R, Ratain MJ. Dose-finding and pharmacokinetic study to optimize the dosing of irinotecan according to the UGT1A1 genotype of patients with cancer. *J Clin Oncol* 2014;32:2328-34.
- 131 Bertotti A, Migliardi G, Galimi F, Sassi F, Torti D, Isella C, Corà D, Di Nicolantonio F, Buscarino M, Petti C, Ribero D, Russolillo N, Muratore A, Massucco P, Pisacane A, Molinaro L, Valtorta E, Sartore-Bianchi A, Risio M, Capussotti L, Gambacorta M, Siena S, Medico E, Sapino A, Marsoni S, Comoglio PM, Bardelli A, Trusolino L. A molecularly annotated platform of patient-derived xenografts ("xenopatients") identifies HER2 as an effective therapeutic target in cetuximab-resistant colorectal cancer. *Cancer Discov* 2011;1:508-23.
- 132 Valtorta E, Martino C, Sartore-Bianchi A, Pennault-Llorca F, Viale G, Risio M, Rugge M, Grigioni W, Bencardino K, Lonardi S, Zagonel V, Leone F, Noe J, Ciardiello F, Pinto C, Labianca R, Mosconi S, Graiff C, Aprile G, Frau B, Garufi C, Loupakis F, Racca P, Tonini G, Lauricella C, Veronese S, Truini M, Siena S, Marsoni S, Gambacorta M. Assessment of a HER2 scoring system for colorectal cancer: results from a validation study. *Mod Pathol* 2015;28:1481-91.
- 133 Sartore-Bianchi A, Trusolino L, Martino C, Bencardino K, Lonardi S, Bergamo F, Zagonel V, Leone F, Depetris I, Martinelli E, Troiani T, Ciardiello F, Racca P, Bertotti A, Siravegna G, Torri V, Amatu A, Ghezzi S, Marrapese G, Palmeri L, Valtorta E, Cassingena A, Lauricella C, Vanzulli A, Regge D, Veronese S, Comoglio PM, Bardelli A, Marsoni S, Siena S. Dual-targeted therapy with trastuzumab and lapatinib in treatment-refractory, KRAS codon 12/13 wild-type, HER2-positive metastatic colorectal cancer (HERACLES): a proof-of-concept, multicentre, open-label, phase 2 trial. *Lancet Oncol* 2016;17:738-46.
- 134 Raghav KPS, Overman MJ, Yu R, Meric-Bernstam F, Menter D, Kee BK, Muranyi A, Singh S, Routbort M, Chen K, Shaw KR, Shanmugam K, Maru DM, Fakih M, Kopetz S. HER2 amplification as a negative predictive biomarker for anti-epidermal growth factor receptor antibody therapy in metastatic colorectal cancer. *J Clin Oncol* 2016;34:abstr 3517.
- 135 Pietrantonio F, Vernieri C, Siravegna G, Mennitto G, Berenato R, Perrone F, Gloghini A, Tamborini E, Lonardi S, Morano F, Piccioni B, Busico A, Volpi CC, Martinetti A, Battaglin F, Bossi I, Pellegrinelli A, Milione M, Cremolini C, Di Bartolomeo M, Bardelli A, de Braud F. Heterogeneity of acquired resistance to anti-EGFR monoclonal antibodies in patients with metastatic colorectal cancer. *Clin Cancer Res* 2017;23:2414-22.
- 136 US National Library of Medicine. S1613, Trastuzumab and Pertuzumab or Cetuximab and Irinotecan Hydrochloride in Treating Patients With Locally Advanced or Metastatic HER2/Neu Amplified Colorectal Cancer That Cannot Be Removed by Surgery. Identifier: NCT NCT03365882. Available from: <https://clinicaltrials.gov/ct2/show/NCT03365882> [Last accessed on 17 Jan 2018]
- 137 Bertotti A, Papp E, Jones S, Adleff V, Anagnostou V, Lupo B, Sausen M, Phallen J, Hruban CA, Tokheim C, Niknafs N, Nesselbush M, Lytle K, Sassi F, Cottino F, Migliardi G, Zanella ER, Ribero D, Russolillo N, Mellano A, Muratore A, Paraluppi G, Salizzoni M,

- Marsoni S, Kragh M, Lantto J, Cassingena A, Li QK, Karchin R, Scharpf R, Sartore-Bianchi A, Siena S, Diaz LA Jr, Trusolino L, Velculescu VE. The genomic landscape of response to EGFR blockade in colorectal cancer. *Nature* 2015;526:263-7.
- 138 Lee MS, Kopetz S. Current and future approaches to target the epidermal growth factor receptor and its downstream signaling in metastatic colorectal cancer. *Clin Colorectal Cancer* 2015;14:203-18.
- 139 Cremolini C, Morano F, Moretto R, Berenato R, Tamborini E, Perrone F, Rossini D, Gloghini A, Busico A, Zucchelli G, Baratelli C, Tamburini E, Capone I, Volpi C, Milione M, Di Maio M, Fontanini G, De Braud FG, Falcone A, Pietrantonio F. Dissecting primary resistance to anti-EGFRs in RAS and BRAF wt metastatic colorectal cancer (mCRC): a case-control study. *J Clin Oncol* 2017;35:abstr 11508.
- 140 Russo M, Siravegna G, Blazskowsky LS, Corti G, Crisafulli G, Ahronian LG, Mussolin B, Kwak EL, Buscarino M, Lazzari L, Valtorta E, Truini M, Jessop NA, Robinson HE, Hong TS, Mino-Kenudson M, Di Nicolantonio F, Thabet A, Sartore-Bianchi A, Siena S, Iafrate AJ, Bardelli A, Corcoran RB. Tumor heterogeneity and lesion-specific response to targeted therapy in colorectal cancer. *Cancer Discov* 2016;6:147-53.
- 141 Misale S, Di Nicolantonio F, Sartore-Bianchi A, Siena S, Bardelli A. Resistance to anti-EGFR therapy in colorectal cancer: from heterogeneity to convergent evolution. *Cancer Discov* 2014;4:1269-80.
- 142 Montagut C, Dalmases A, Bellosillo B, Crespo M, Pairet S, Iglesias M, Salido M, Gallen M, Marsters S, Tsai SP, Minoche A, Seshagiri S, Serrano S, Himmelbauer H, Bellmunt J, Rovira A, Settleman J, Bosch F, Albanell J. Identification of a mutation in the extracellular domain of the epidermal growth factor receptor conferring cetuximab resistance in colorectal cancer. *Nat Med* 2012;18:221-3.
- 143 Esposito C, Rachiglio AM, La Porta ML, Sacco A, Roma C, Iannaccone A, Tatangelo F, Forgione L, Pasquale R, Barbaro A, Botti G, Ciardiello F, Normanno N. The S492R EGFR ectodomain mutation is never detected in KRAS wild-type colorectal carcinoma before exposure to EGFR monoclonal antibodies. *Cancer Biol Ther* 2013;14:1143-6.
- 144 Voigt M, Braig F, Göthel M, Schulte A, Lamszus K, Bokemeyer C, Binder M. Functional dissection of the epidermal growth factor receptor epitopes targeted by panitumumab and cetuximab. *Neoplasia* 2012;14:1023-31.
- 145 Arena S, Bellosillo B, Siravegna G, Martínez A, Cañadas I, Lazzari L, Ferruz N, Russo M, Misale S, González I, Iglesias M, Gavilan E, Corti G, Hobor S, Crisafulli G, Salido M, Sánchez J, Dalmases A, Bellmunt J, De Fabritiis G, Rovira A, Di Nicolantonio F, Albanell J, Bardelli A, Montagut C. Emergence of multiple EGFR extracellular mutations during cetuximab treatment in colorectal cancer. *Clin Cancer Res* 2015;21:2157-66.
- 146 Price TJ, Newhall K, Peeters M, Kim TW, Li J, Cascinu S, Ruff P, Suresh AV, Thomas A, Tjulandin S, Boedigheimer M, Zhang K, Sidhu R, Murugappan S. Prevalence and outcomes of patients (pts) with EGFR S492R ectodomain mutations in ASPECCT: panitumumab (pmab) vs. cetuximab (cmab) in pts with chemorefractory wild-type KRAS exon 2 metastatic colorectal cancer (mCRC). *J Clin Oncol* 2015;33:abstr740.
- 147 Arena S, Siravegna G, Mussolin B, Kearns JD, Wolf BB, Misale S, Lazzari L, Bertotti A, Trusolino L, Adjei AA, Montagut C, Di Nicolantonio F, Nering R, Bardelli A. MM-151 overcomes acquired resistance to cetuximab and panitumumab in colorectal cancers harboring EGFR extracellular domain mutations. *Sci Transl Med* 2016;8:324ra314.
- 148 Dienstmann R, Patnaik A, Garcia-Carbonero R, Cervantes A, Benavent M, Roselló S, Tops BB, van der Post RS, Argilés G, Skartved NJ, Hansen UH, Hald R, Pedersen MW, Kragh M, Horak ID, Braun S, Van Cutsem E, Tolcher AW, Taberero J. Safety and activity of the first-in-class Sym004 Anti-EGFR antibody mixture in patients with refractory colorectal cancer. *Cancer Discov* 2015;5:598-609.
- 149 Cidon EU, Alonso P, Masters B. Markers of response to antiangiogenic therapies in colorectal cancer: where are we now and what should be next? *Clin Med Insights Oncol* 2016;10:41-55.
- 150 Bates DO, Catalano PJ, Symonds KE, Varey AH, Ramani P, O'Dwyer PJ, Giantonio BJ, Meropol NJ, Benson AB, Harper SJ. Association between VEGF splice isoforms and progression-free survival in metastatic colorectal cancer patients treated with bevacizumab. *Clin Cancer Res* 2012;18:6384-91.
- 151 Carbone C, Piro G, Simionato F, Ligorio F, Cremolini C, Loupakis F, Ali G, Rossini D, Merz V, Santoro R, Zecchetto C, Zanotto M, Di Nicolantonio F, Bardelli A, Fontanini G, Tortora G, Melisi D. Homeobox B9 mediates resistance to anti-VEGF therapy in colorectal cancer patients. *Clin Cancer Res* 2017;23:4312-22.
- 152 Paiva TF Jr, de Jesus VH, Marques RA, da Costa AA, de Macedo MP, Peresi PM, Damascena A, Rossi BM, Begnami MD, de Lima VC. Angiogenesis-related protein expression in bevacizumab-treated metastatic colorectal cancer: NOTCH1 detrimental to overall survival. *BMC Cancer* 2015;15:643.
- 153 US National Library of Medicine. A Study of OMP-305B83 in Subjects With Metastatic Colorectal Cancer. Identifier: NCT03035253. Available from: <https://clinicaltrials.gov/ct2/show/NCT03035253?term=bispecific+oncomed&rank=1> [Last accessed on 17 Jan 2018]
- 154 Zuurbier L, Rahman A, Cordes M, Scheick J, Wong TJ, Rustenburg F, Joseph JC, Dynoodt P, Casey R, Drillenburger P, Gerhards M, Barat A, Klingner R, Fender B, O'Connor DP, Betge J, Ebert MP, Gaiser T, Prehn JHM, Griffioen AW, van Grieken NCT, Ylstra B, Byrne AT, van der Flier LG, Gallagher WM, Postel R. Apelin: a putative novel predictive biomarker for bevacizumab response in colorectal cancer. *Oncotarget* 2017;8:42949-61.
- 155 Taberero J, Lenz HJ, Siena S, Sobrero A, Falcone A, Ychou M, Humblet Y, Bouché O, Mineur L, Barone C, Adenis A, Yoshino T, Goldberg RM, Sargent DJ, Wagner A, Laurent D, Teufel M, Jeffers M, Grothey A, Van Cutsem E. Analysis of circulating DNA and protein biomarkers to predict the clinical activity of regorafenib and assess prognosis in patients with metastatic colorectal cancer: a retrospective, exploratory analysis of the CORRECT trial. *Lancet Oncol* 2015;16:937-48.
- 156 Wirapati P, Pomella V, Vandenbosch B, Kerr P, Maiello E, Jeffery GM, Curca ROD, Karthaus M, Bridgewater JA, Mihailov AC, Kiss I, Merino S, McKendrick JJ, Saridaki Z, Sagaert XJA, Tejpar S. Velour trial biomarkers update: impact of RAS, BRAF, and sidedness on

- affibercept activity. *J Clin Oncol* 2017;35:abstr3538.
- 157 Yoshino T, Obermannova R, Bodoky G, Prausová J, Garcia-Carbonero R, Ciuleanu TE, Alfonso PG, Portnoy DC, Cohn AL, Van Cutsem E, Yamazaki K, Clingan P, Muro K, Kim TW, Wijayawardana SR, Hozak R, Nasroulah F, Taberero J. Are BRAF mutated metastatic colorectal cancer (mCRC) tumors more responsive to VEGFR-2 blockage? Analysis of patient outcomes by RAS/RAF mutation status in the RAISE study - a global, randomized, double-blind, phase III study. *J Clin Oncol* 2018;36:abstr 622.
- 158 Taberero J, Hozak RR, Yoshino T, Cohn AL, Obermannova R, Bodoky G, Garcia-Carbonero R, Ciuleanu TE, Portnoy DC, Prausová J, Muro K, Siegel RW, Konrad RJ, Ouyang H, Melemed SA, Ferry D, Nasroulah F, Van Cutsem E. Analysis of angiogenesis biomarkers for ramucirumab (RAM) efficacy in patients with metastatic colorectal cancer from RAISE, a global, randomized, double-blind, phase 3 study. *Ann Oncol* 2017; doi: 10.1093/annonc/mdx767.
- 159 Lambrechts D, Delmar P, Miles DW, Leigh N, Saltz L, Escudier B, Van Cutsem E, Scherer SJ, Carmeliet P, de Haas S. 1414 POSTER single nucleotide polymorphism analysis and outcome in advanced-stage cancer patients treated with bevacizumab. *Eur J Cancer* 2011;47:S173.
- 160 Loupakis F, Cremolini C, Yang D, Salvatore L, Zhang W, Wakatsuki T, Bohanes P, Schirripa M, Benhaim L, Lonardi S, Antoniotti C, Aprile G, Graziano F, Ruzzo A, Lucchesi S, Ronzoni M, De Vita F, Tonini G, Falcone A, Lenz HJ. Prospective validation of candidate SNPs of VEGF/VEGFR pathway in metastatic colorectal cancer patients treated with first-line FOLFIRI plus bevacizumab. *PLoS One* 2013;8:e66774.
- 161 Puccini A, Berger MD, Naseem M, Tokunaga R, Battaglin F, Cao S, Hanna DL, McSkane M, Soni S, Zhang W, Lenz HJ. Colorectal cancer: epigenetic alterations and their clinical implications. *Biochim Biophys Acta* 2017;1868:439-48.
- 162 Sharma S, Kelly TK, Jones PA. Epigenetics in cancer. *Carcinogenesis* 2010;31:27-36.
- 163 Jones PA. Functions of DNA methylation: islands, start sites, gene bodies and beyond. *Nat Rev Genet* 2012;13:484-92.
- 164 Ang PW, Loh M, Liem N, Lim PL, Grieu F, Vaithilingam A, Platell C, Yong WP, Iacopetta B, Soong R. Comprehensive profiling of DNA methylation in colorectal cancer reveals subgroups with distinct clinicopathological and molecular features. *BMC Cancer* 2010;10:227.
- 165 Weisenberger DJ, Levine AJ, Long TI, Buchanan DD, Walters R, Clendenning M, Rosty C, Joshi AD, Stern MC, LeMarchand L, Lindor NM, Daftary D, Gallinger S, Selander T, Bapat B, Newcomb PA, Campbell PT, Casey G, Ahnen DJ, Baron JA, Haile RW, Hopper JL, Young JP, Laird PW, Siegmund KD; Colon Cancer Family Registry. Association of the colorectal CpG island methylator phenotype with molecular features, risk factors, and family history. *Cancer Epidemiol Biomarkers Prev* 2015;24:512-9.
- 166 Puccini A, Berger MD, Zhang W, Lenz HJ. What we know about stage II and III colon cancer: it's still not enough. *Target Oncol* 2017;12:265-75.
- 167 Min BH, Bae JM, Lee EJ, Yu HS, Kim YH, Chang DK, Kim HC, Park CK, Lee SH, Kim KM, Kang GH. The CpG island methylator phenotype may confer a survival benefit in patients with stage II or III colorectal carcinomas receiving fluoropyrimidine-based adjuvant chemotherapy. *BMC Cancer* 2011;11:344.
- 168 Ogino S, Kawasaki T, Nosho K, Ohnishi M, Suemoto Y, Kirkner GJ, Fuchs CS. LINE-1 hypomethylation is inversely associated with microsatellite instability and CpG island methylator phenotype in colorectal cancer. *Int J Cancer* 2008;122:2767-73.
- 169 Ogino S, Nosho K, Kirkner GJ, Kawasaki T, Chan AT, Schernhammer ES, Giovannucci EL, Fuchs CS. A cohort study of tumoral LINE-1 hypomethylation and prognosis in colon cancer. *J Natl Cancer Inst* 2008;100:1734-8.
- 170 Kawakami K, Matsunoki A, Kaneko M, Saito K, Watanabe G, Minamoto T. Long interspersed nuclear element-1 hypomethylation is a potential biomarker for the prediction of response to oral fluoropyrimidines in microsatellite stable and CpG island methylator phenotype-negative colorectal cancer. *Cancer Sci* 2011;102:166-74.
- 171 Shen L, Kondo Y, Rosner GL, Xiao L, Hernandez NS, Vilaythong J, Houlihan PS, Krouse RS, Prasad AR, Einspahr JG, Buckmeier J, Alberts DS, Hamilton SR, Issa JP. MGMT promoter methylation and field defect in sporadic colorectal cancer. *J Natl Cancer Inst* 2005;97:1330-8.
- 172 Cremolini C, Pietrantonio F. How the lab is changing our view of colorectal cancer. *Tumori* 2016;102:541-7.
- 173 Ghoneim HE, Fan Y, Moustaki A, Abdelsamed HA, Dash P, Dogra P, Carter R, Awad W, Neale G, Thomas PG, Youngblood B. De novo epigenetic programs inhibit PD-1 blockade-mediated T cell rejuvenation. *Cell* 2017;170:142-57.e119.
- 174 Chiappinelli KB, Strissel PL, Desrichard A, Li H, Henke C, Akman B, Hein A, Rote NS, Cope LM, Snyder A, Makarov V, Budhu S, Slamon DJ, Wolchok JD, Pardoll DM, Beckmann MW, Zahnow CA, Merghoub T, Chan TA, Baylin SB, Strick R. Inhibiting DNA methylation causes an interferon response in cancer via dsRNA including endogenous retroviruses. *Cell* 2016;164:1073.
- 175 Li H, Chiappinelli KB, Guzzetta AA, Easwaran H, Yen RW, Vatapalli R, Topper MJ, Luo J, Connolly RM, Azad NS, Stearns V, Pardoll DM, Davidson N, Jones PA, Slamon DJ, Baylin SB, Zahnow CA, Ahuja N. Immune regulation by low doses of the DNA methyltransferase inhibitor 5-azacitidine in common human epithelial cancers. *Oncotarget* 2014;5:587-98.
- 176 Brocks D, Schmidt CR, Daskalakis M, Jang HS, Shah NM, Li D, Li J, Zhang B, Hou Y, Laudato S, Lipka DB, Schott J, Bierhoff H, Assenov Y, Helf M, Ressenrova A, Islam MS, Lindroth AM, Haas S, Essers M, Imbusch CD, Brors B, Oehme I, Witt O, Lübbert M, Mallm JP, Rippe K, Will R, Weichenhan D, Stoecklin G, Gerhäuser C, Oakes CC, Wang T, Plass C. DNMT and HDAC inhibitors induce cryptic transcription start sites encoded in long terminal repeats. *Nat Genet* 2017;49:1052-60.
- 177 Scartozzi M, Bearzi I, Mandolesi A, Giampieri R, Faloppi L, Galizia E, Loupakis F, Zaniboni A, Zorzi F, Biscotti T, Labianca R, Falcone A, Cascinu S. Epidermal growth factor receptor (EGFR) gene promoter methylation and cetuximab treatment in colorectal cancer patients. *Br J Cancer* 2011;104:1786-90.
- 178 Khambata-Ford S, Garrett CR, Meropol NJ, Basik M, Harbison CT, Wu S, Wong TW, Huang X, Takimoto CH, Godwin AK, Tan BR, Krishnamurthi SS, Burris HA 3rd, Poplin EA, Hidalgo M, Baselga J, Clark EA, Mauro DJ. Expression of epi-regulin and amphiregulin



- and K-ras mutation status predict disease control in metastatic colorectal cancer patients treated with cetuximab. *J Clin Oncol* 2007;25:3230-7.
- 179 Jacobs B, De Roock W, Piessevaux H, Van Oirbeek R, Biesmans B, De Schutter J, Fieuws S, Vandesompele J, Peeters M, Van Laethem JL, Humblet Y, Pénault-Llorca F, De Hertogh G, Laurent-Puig P, Van Cutsem E, Tejpar S. Amphiregulin and epiregulin mRNA expression in primary tumors predicts outcome in metastatic colorectal cancer treated with cetuximab. *J Clin Oncol* 2009;27:5068-74.
- 180 Lee MS, McGuffey EJ, Morris JS, Manyam G, Baladandayuthapani V, Wei W, Morris VK, Overman MJ, Maru DM, Jiang ZQ, Hamilton SR, Kopetz S. Association of CpG island methylator phenotype and EREG/AREG methylation and expression in colorectal cancer. *Br J Cancer* 2016;114:1352-61.
- 181 Guinney J, Dienstmann R, Wang X, de Reyniès A, Schlicker A, Soneson C, Marisa L, Roepman P, Nyamundanda G, Angelino P, Bot BM, Morris JS, Simon IM, Gerster S, Fessler E, De Sousa E Melo F, Missiaglia E, Ramay H, Barras D, Homicsko K, Maru D, Manyam GC, Broom B, Boige V, Perez-Villamil B, Laderas T, Salazar R, Gray JW, Hanahan D, Taberero J, Bernards R, Friend SH, Laurent-Puig P, Medema JP, Sadanandam A, Wessels L, Delorenzi M, Kopetz S, Vermeulen L, Tejpar S. The consensus molecular subtypes of colorectal cancer. *Nat Med* 2015;21:1350-6.
- 182 Lenz HJ, Ou FS, Venook AP, Hochster HS, Niedzwiecki D, Goldberg RM, Mayer RJ, Bertagnolli MM, Blanke CD, Zemla T, Qu X, Innocenti F, Kabbarah O. Impact of consensus molecular subtyping (CMS) on overall survival (OS) and progression free survival (PFS) in patients (pts) with metastatic colorectal cancer (mCRC): analysis of CALGB/SWOG 80405 (Alliance). *J Clin Oncol* 2017;35:abstr3511.
- 183 Song N, Pogue-Geile KL, Gavin PG, Yothers G, Kim SR, Johnson NL, Lipchik C, Allegra CJ, Petrelli NJ, O'Connell MJ, Wolmark N, Paik S. Clinical outcome from oxaliplatin treatment in stage II/III colon cancer according to intrinsic subtypes: secondary analysis of NSABP C-07/NRG oncology randomized clinical trial. *JAMA Oncol* 2016;2:1162-9.
- 184 Trinh A, Trumpi K, De Sousa E Melo F, Wang X, de Jong JH, Fessler E, Kuppen PJ, Reimers MS, Swets M, Koopman M, Nagtegaal ID, Jansen M, Hooijer GK, Offerhaus GJ, Kranenburg O, Punt CJ, Medema JP, Markowitz F, Vermeulen L. Practical and robust identification of molecular subtypes in colorectal cancer by immunohistochemistry. *Clin Cancer Res* 2017;23:387-98.
- 185 Sveen A, Bruun J, Eide PW, Eilertsen IA, Ramirez L, Murumägi A, Arjama M, Danielsen SA, Kryeziu K, Elez E, Taberero J, Guinney J, Palmer HG, Nesbakken A, Kallioniemi O, Dienstmann R, Lothe RA. Colorectal cancer consensus molecular subtypes translated to preclinical models uncover potentially targetable cancer-cell dependencies. *Clin Cancer Res* 2018;24:794-806.
- 186 Linnekamp JF, Hooff SRV, Prasetyanti PR, Kandimalla R, Buikhuisen JY, Fessler E, Ramesh P, Lee KAST, Bochove GGW, de Jong JH, Cameron K, Leersum RV, Rodermond HM, Franitza M, Nürnberg P, Mangiapane LR, Wang X, Clevers H, Vermeulen L, Stassi G, Medema JP. Consensus molecular subtypes of colorectal cancer are recapitulated in in vitro and in vivo models. *Cell Death Differ* 2018; doi: 10.1038/s41418-017-0011-5.
- 187 Bettegowda C, Sausen M, Leary RJ, Kinde I, Wang Y, Agrawal N, Bartlett BR, Wang H, Luber B, Alani RM, Antonarakis ES, Azad NS, Bardelli A, Brem H, Cameron JL, Lee CC, Fecher LA, Gallia GL, Gibbs P, Le D, Giuntoli RL, Goggins M, Hogarty MD, Holdhoff M, Hong SM, Jiao Y, Juhl HH, Kim JJ, Siravegna G, Laheru DA, Lauricella C, Lim M, Lipson EJ, Marie SK, Netto GJ, Oliner KS, Olivi A, Olsson L, Riggins GJ, Sartore-Bianchi A, Schmidt K, Shih IM, Oba-Shinjo SM, Siena S, Theodorescu D, Tie J, Harkins TT, Veronese S, Wang TL, Weingart JD, Wolfgang CL, Wood LD, Xing D, Hruban RH, Wu J, Allen PJ, Schmidt CM, Choti MA, Velculescu VE, Kinzler KW, Vogelstein B, Papadopoulos N, Diaz LA Jr. Detection of circulating tumor DNA in early- and late-stage human malignancies. *Sci Transl Med* 2014;6:224ra24.
- 188 Diaz LA, Jr., Williams RT, Wu J, Kinde I, Hecht JR, Berlin J, Allen B, Bozic I, Reiter JG, Nowak MA, Kinzler KW, Oliner KS, Vogelstein B. The molecular evolution of acquired resistance to targeted EGFR blockade in colorectal cancers. *Nature* 2012;486:537-40.
- 189 Siravegna G, Mussolin B, Buscarino M, Corti G, Cassingena A, Crisafulli G, Ponzetti A, Cremolini C, Amatu A, Lauricella C, Lamba S, Hobor S, Avallone A, Valtorta E, Rospo G, Medico E, Motta V, Antoniotti C, Tatangelo F, Bellosillo B, Veronese S, Budillon A, Montagut C, Racca P, Marsoni S, Falcone A, Corcoran RB, Di Nicolantonio F, Loupakis F, Siena S, Sartore-Bianchi A, Bardelli A. Clonal evolution and resistance to EGFR blockade in the blood of colorectal cancer patients. *Nat Med* 2015;21:795-801.
- 190 Morelli MP, Overman MJ, Dasari A, Kazmi SM, Mazard T, Vilar E, Morris VK, Lee MS, Herron D, Eng C, Morris J, Kee BK, Janku F, Deaton FL, Garrett C, Maru D, Diehl F, Angenendt P, Kopetz S. Characterizing the patterns of clonal selection in circulating tumor DNA from patients with colorectal cancer refractory to anti-EGFR treatment. *Ann Oncol* 2015;26:731-6.
- 191 Strickler JH, Loree JM, Ahronian LG, Parikh AR, Niedzwiecki D, Pereira AAL, McKinney M, Korn WM, Atreya CE, Banks KC, Nagy RJ, Meric-Bernstam F, Lanman RB, Talasaz A, Tsigelny IF, Corcoran RB, Kopetz S. Genomic landscape of cell-free DNA in patients with colorectal cancer. *Cancer Discov* 2018;8:164-73.
- 192 Zhang S, Chen L, Jung EJ, Calin GA. Targeting microRNAs with small molecules: from dream to reality. *Clin Pharmacol Ther* 2010;87:754-8.
- 193 Shomron N. MicroRNAs and pharmacogenomics. *Pharmacogenomics* 2010;11:629-32.
- 194 Pichler M, Calin GA. MicroRNAs in cancer: from developmental genes in worms to their clinical application in patients. *Br J Cancer* 2015;113:569-73.
- 195 Goblirsch M, Richtig G, Slaby O, Berindan-Neagoe I, Gerger A, Pichler M. MicroRNAs as a tool to aid stratification of colorectal cancer patients and to guide therapy. *Pharmacogenomics* 2017;18:1027-38.
- 196 Chen H, Zhang Z, Peng W. miRDDCR: a miRNA-based method to comprehensively infer drug-disease causal relationships. *Sci Rep* 2017;7:15921.