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Biomarker-guided therapy for colorectal cancer: strength in complexity

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Abstract

The number of molecularly stratified treatment options available to patients with colorectal cancer (CRC) is increasing, with a parallel increase in the use of biomarkers to guide prognostication and treatment decision making. This increase in both the number of biomarkers and their use has resulted in an increasingly complex situation, which is evident both from the extensive interactions between biomarkers and from their sometimes complex associations with patient prognosis and treatment benefit. Current and emerging biomarkers also reflect the genomic complexity of CRC, and include a wide range of aberrations such as point mutations, amplifications, fusions and hypermutator phenotypes, in addition to global gene expression subtypes. In this Review, we provide an overview of current and emerging clinically relevant biomarkers and their role in the management of patients with CRC, illustrating the intricacies of biomarker interactions and the growing treatment opportunities created by the availability of comprehensive molecular profiling.

Introduction

Clinical outcomes and treatment responses of patients with colorectal cancer (CRC) vary greatly[1], and the use of stratified treatment options remains limited in standard practice[2,3]. In the primary setting, patients undergo surgery with a curative intent, as well as perioperative chemoradiation and/or adjuvant chemotherapy predominantly determined by cancer stage and tumour location[4]. However, considerable potential exists to improve the risk:benefit ratio of adjuvant chemotherapy regimens by adopting more personalized approaches. This potential is illustrated by the noninferiority of 3 months of adjuvant chemotherapy versus the

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Authorship contributions

All authors made substantial contributions to all aspects of the preparation of this manuscript.

Competing interests

A.S. and R.A.L. are co-inventors of a pending patent application (Attorney Docket Number: INVEN-35063/US-1/PRO) regarding the use of HSP90 inhibitors in relation to the consensus molecular subtypes of colorectal cancer. S.K. is a co-inventor of a pending patent application regarding a clinical classifier for the consensus molecular subtypes of colorectal cancer.

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standard-of-care treatment duration of 6 months in patients with low-risk stage III colon cancer[5]. In those with metastatic disease, the treatment repertoire has been extended to include biologically targeted agents including monoclonal antibodies targeting EGFR, such as cetuximab or panitumumab, and anti-angiogenic agents targeting VEGF signalling, such as bevacizumab or ramucirumab,[6] as well as the broad-spectrum kinase inhibitor regorafenib[7]. As a result of improved treatment options, the overall survival (OS) of patients with metastatic CRC has increased from approximately 1 year in the era of 5-fluorouracil (5-FU) therapy alone, to approximately 3 years with currently available therapies[8]. No clinically validated predictive biomarkers of response to the anti-angiogenic agents are currently available, although VEGF-D expression[9] and *KDR* mutations[10] are potential candidates. By contrast, activating mutations in *KRAS*/*NRAS* are contraindications for the use of EGFR targeted therapies, and this approach remains the gold standard for the stratified treatment of patients with CRC[11]. Addition of cetuximab to 5-FU, leucovorin and irinotecan (FOLFIRI) has been shown to increase the median progression-free survival (PFS) of patients with previously untreated *RAS* wild type metastatic disease by ~3 months[12]. However, most patients either have a poor initial response to treatment, or develop secondary resistance to all standard therapies[13]. Mechanisms of resistance to targeted agents commonly include secondary, reactivating mutations in the affected signalling pathways, as demonstrated by the emergence of resistant subclones harbouring mutations in the MAPK signalling pathway during EGFR inhibition[14,15].

Several advances from the past few years indicate the potential to improve the effectiveness of treatments through patient stratification based on tumour biology. Arguably, the most prominent examples are provided by the successes with anti-programmed cell death protein-1 (PD-1) and anti-cytotoxic T lymphocyte associated protein 4 (CTLA4) antibodies in metastatic cancers with a microsatellite instability (MSI) or hypermutator phenotype[16-19], and the use of targeted combination therapies in patients with CRCs harbouring $BRAF^{V600}$ mutations[20,21]. Furthermore, moving from the single-marker and single-agent approach to a more integrated perspective[3], the gene expression-based consensus molecular subtypes (CMS) have provided a new and biologically rational stratification framework[22]. This framework defines four groups of CRCs based on intrinsic biological characteristics: CMS1-MSI/immune; CMS2-epithelial/canonical; CMS3-epithelial/metabolic; and CMS4-mesenchymal/stromal. Finally, the search for effective therapies has been greatly facilitated by the availability of ex vivo drug screening of patient-derived preclinical models in culture using tumour organoid models[23].

Biomarker-guided treatment of patients with CRC is currently based on the presence or absence of individual markers that are associated with either prognosis or expected benefit from a specific therapy. However, as the treatment options are expanding, an increasing level of complexity is becoming apparent. Firstly, biomarkers often interact with each other, thus reinforcing the importance of comprehensive molecular profiling (FIG. 1). For example, the experience with therapies targeting alterations in the MAPK signalling pathway illustrates the potential to improve initial response rates and/or to identify effective drug combinations based on features associated with primary resistance. In addition, monitoring the dynamics of resistance-bearing subclones provides the potential for rechallenge with anti-EGFR antibodies in later lines of treatment[15,24]. Nonetheless, the majority of patients with CRC

currently have no targeted therapy options available. Even low-prevalence markers can substantially increase the use of molecularly-guided treatments, as demonstrated by the introduction of anti-PD-1 antibodies for patients with MSI-high (MSI-H)/mismatch repair deficient (dMMR) cancers[25]. However, other subgroups provide examples of cancers with more than one molecular target, including MSI-H and *RAS* wild type cancers that also harbour either $BRAF^{V600E}$ mutations or kinase fusions. The presence of several targetable alterations might extend the range of treatment options when faced with resistance, but also emphasizes the need for appropriate treatment sequencing. Secondly, biomarkers might have complex associations with patient outcomes. Discriminating between prognostic and predictive biomarker values[26] is sometimes challenging in clinical studies evaluating therapeutic benefit. This prognostic–predictive complexity is partly driven by the search for more effective therapies for patients who have a poor prognosis with standard treatments.

In this Review, we provide an overview of current and emerging biomarkers with therapeutic implications in the treatment and management of patients with CRC. We illustrate biomarker complexity by highlighting interactions between different biomarkers and discuss both potential prognostic and predictive associations (TABLE 1).

Biomarker complexity and MSI

Adjuvant chemotherapy in primary cancers

According to current guidelines, patients with stage II colon cancer with MSI-H and/or dMMR should not be offered adjuvant 5-FU-based chemotherapy[27-29]. This recommendation is based on a low risk of recurrence and a lack of treatment benefit. The first reports of a favorable prognostic association of MSI in CRC were published in 1993[30,31], preceding clinical implementation by approximately two decades. This association has now been confirmed for both OS and disease-free survival (DFS) in meta-analyses comparing data from over 1,200 patients with primary MSI-H CRCs to those with MSS CRCs[32,33]. This association also applies specifically to patients with stage II and III colon cancers who have not received adjuvant chemotherapy, as shown by retrospective analyses of data from randomized trials[34,35]. The biological basis of this prognostic association is likely the high tumour mutational burden (TMB) owing to MMR deficiency, and in particular the higher number of frameshift mutations in repetitive sequences[36-40]. This results in the generation of neoantigens capable of activating cytotoxic T cells, and intra-epithelial infiltration of activated lymphocytes causes a strong antitumour immune response[41-45].

Approximately 15% of primary CRCs are MSI-H[46]. However, the prevalence differs according to clinicopathological factors and MSI is more common in older female patients (>70 years of age) with right-sided and poorly differentiated stage II colon cancers[35,47-53]. A corresponding association with clinicopathological features has also been reported for prognosis, with patients with proximal tumours having a greater relative benefit from MSI than those with distal tumours[54]. Various studies report stronger effects of MSI in stage II[55,56], similar prognostic associations across stages II and III[57,58], or even a stronger effect in stage III[49] among patients with CRC. However, no statistically significant interactions with clinicopathological features, including tumour stage, were observed in a pooled

analysis of data from >7,000 stage II or III colon cancers[52], suggesting that the prognostic value of MSI is independent of disease stage.

The prognostic implications of MSI after adjuvant treatment might be confounded by a predictive value for 5-FU-based chemotherapy, thus illustrating prognostic–predictive biomarker complexity[51]. A loss of MMR function could result in a failure to recognize and respond to the incorporation of 5-FU into tumour DNA[59]. However, results have been inconsistent in the many retrospective analyses of the effects of 5-FU-based regimens in patients with MSI-H tumours[51]. Most studies comparing the effects of 5-FU-based chemotherapy versus no treatment have reported no significant improvements in OS or DFS in patients with MSI-H/dMMR tumours (TABLE 2), despite 5-FU-based chemotherapy significantly improving the outcomes of patients with MSS/MMR proficient colon cancer or CRC[34,35,58,60,61]. However, only two of the seven studies revealed a statistically significant interaction between MSI status and the response to chemotherapy[62,63]. A meta-analysis including data from almost 400 patients with MSI-H CRCs revealed a substantial degree of heterogeneity with respect to the effects of 5-FU-based chemotherapy, and a lack of benefit could not be definitively confirmed[33].

MSI-status is not predictive of a lack of benefit from the combination chemotherapies commonly used in patients with CRC. The addition of oxaliplatin to 5-FU-based adjuvant chemotherapy regimens (FOLFOX/FLOX or CAPOX) has been shown to improve patient survival[64], and this is also seen separately for patients with dMMR stage II or III colon cancers[65,66]. Furthermore, patients with dMMR tumors have improved survival outcomes compared to the MMR proficient subgroup after treatment with FOLFOX[67,68] (dependent on tumour location in one study[54,69]), and this is consistent with the favorable prognostic effect of MSI. Irinotecan is also part of the chemotherapy regimens received by patients with metastatic CRC, although this agent is not used in the adjuvant setting for those with primary CRC[70-72]. Data from studies investigating the predictive value of MSI status are again conflicting, indicating both a survival benefit[73] and a lack of benefit[56] from the addition of irinotecan to 5-FU. Preclinical investigations of the effects of these agents are challenging, partly owing to differences in the in vitro and in vivo drug metabolism. These differences have been illustrated by discordant levels of 5-FU sensitivity in matched patient-derived organoid and xenograft (PDX) models[74]. In conclusion, the complex prognostic–predictive association between MSI and benefit from adjuvant chemotherapy in patients with primary CRC is not fully understood. Nonetheless, adjuvant chemotherapy is not recommended for patients with low-risk MSI-H/dMMR stage II CRCs owing to the generally good prognosis of such patients and a lack of treatment benefit[27]. In those with stage III disease, the risk of recurrence is higher, therefore, patients should receive standard chemotherapy irrespective of MSI status.

Heterogeneity and immunity—MSI-H CRCs have a distinct clinicopathological, biological and molecular profile[51]; nonetheless, these tumours are still heterogeneous. The hypermutated phenotype seen in MSI-H tumors (>10 mutations per megabase (mut/Mb)[75]) might augment this heterogeneity, and MSI-H CRCs are more heterogeneous in terms of point mutations and indels compared with MSS cancers, according to both intratumoral analyses[76] and comparisons of biopsy material from metastatic cancers with matched

circulating tumour DNA[77]. MMR deficiency in sporadic MSI-H tumours is primarily caused by promoter hypermethylation of *MLH1*, but MSI also occurs in a hereditary setting, resulting from germline mutations in the MMR genes and causing hereditary nonpolyposis CRC (HNPCC) syndrome[78]. Sporadic MSI can be further distinguished from HNPCC by the presence of the CpG island methylator phenotype (CIMP) and frequent *BRAF* mutations[79]. The implications of this distinction for patient outcomes and possible treatment benefit are uncertain[57,58,66,80-83], and this information is not included in adjuvant chemotherapy guidelines[84]. Furthermore, loss of expression of the transcription factor CDX2 is common in MSI-H tumours and, in contrast to MSI, is a proposed biomarker of benefit from adjuvant chemotherapy[85]. Clinical data for this association are currently limited to retrospective analyses, but preclinical data indicate higher sensitivity of CRC cell lines harbouring a loss of CDX2 expression to several chemotherapies[86], and further clinical investigation is warranted to determine the precise interpretation of the co-occurrence of MSI and loss of CDX2 expression in this setting. Sporadic MSI-H tumours also interact with other prominent CRC biomarkers and therapeutic targets, such as oncogenic kinase fusions, which are mutually exclusive to *BRAF* mutations. Kinase fusions are generally rare in patients with CRC (<1-2%), although they have been reported in 55% of *BRAF* and *KRAS* wildtype MSI-H tumours harbouring *MLH1* methylation[83,87,88].

Most, but not all, MSI-H tumours are of the immunogenic gene expression-based consensus molecular subtype CMS1-immune[22]. The level of immune cell infiltration in MSI tumours seems to differ according to these gene expression subtypes and to be particularly high in CMS1. This distinction is supported by a potentially favorable prognosis with MSI-H CMS1 tumours compared with MSI-H CMS2–4[89]. Variations in the extent of tumour immunity among patients with MSI-H CRCs have also been clearly demonstrated by the immunoscore, a standardized immunohistochemistry-based scoring system that summarizes the density of tumour infiltrating T cells ($CD3^+$ and $CD8^+$) in the tumour centre and at the invasive margins. This approach provides a level of prognostic discriminatory power superior to that of MSI in primary CRC[90]. In 2018, an international multicentre consortium demonstrated the additive value of the immunoscore beyond that of clinicopathological prognostic factors and MSI status among 2,681 patients with stage I–III colon cancers[45]. This prompted the development of an attempt to integrate immunoscore with TNM staging to better guide adjuvant treatment decision-making. This approach seems particularly appealing considering the power of immunoscore in determining the prognosis of patients with stage II MSS CRCs who had not received adjuvant chemotherapy[91].

Immunotherapy in metastatic cancers

Owing to associations with a more favourable prognosis, MSI is less common among patients with metastatic disease compared to those with primary CRCs and is typically reported in ≤5% of patients with metastatic CRC in clinical trials[92-94]. T cell infiltration is also a positive prognostic factor in metastatic disease, even after conventional chemotherapy and/or surgical resection[95-98]. However, heterogeneity in the density of immune-cell infiltration has been reported in comparisons of biopsy material from matched primary tumour and liver metastases[99,100], as well as between liver metastases from individual patients[96]. Paradoxically, metastatic MSI-H tumours are aggressive and have been found to

be associated with inferior PFS and OS outcomes relative to those of patients with metastatic MSS CRC in several studies[92,101-103]. While other reports indicate no prognostic associations and additional data are needed, the survival benefits associated with primary MSI-H CRCs seem to be lost in the metastatic setting[104-106].

Several mechanisms have been proposed in an attempt to explain the paradoxical effects of MSI status on patient outcomes, including variations in patterns of metastatic spread between MSI-H and MSS cancers, with lower rates of liver metastases and higher rates of peritoneal metastases in MSI-H[80,94,101,107]. The paradoxical effect might also be driven by enrichment with *BRAF* mutations in MSI-H[92,101,108]. Curative surgical removal of metastases (metastasectomy) can improve the OS of patients with MSI-H CRCs[109], but these patients might be less likely to undergo surgery, partly owing to a lack of benefit from conversion chemotherapy[102]. Whether MSI-H confers chemoresistance in patients with metastatic disease remains unclear[11], although 'tumour-sidedness' has been identified as a prognostic factor in clinical trial cohorts receiving chemotherapy and/or targeted agents[110-113]: those with cancers originating from the right side of the colon have inferior outcomes. The prevalence of MSI-H, like multiple other molecular features, differs in a gradient-like fashion along the anatomical sections of the colorectum[114], and although the independent prognostic contribution of each clinicopathological and biological factor is not clear, collectively, these data support inferior outcomes for patients with MSI-H metastatic disease. Metastasizing MSI-H cancer cells might also be particularly effective at immunoediting and subsequently to evade immune surveillance[115]. However, a dependency on immunoediting for continued growth could also render tumours more sensitive to treatments directed towards the immune microenvironment.

The first tissue-agnostic approval of a cancer therapy based on the presence of a molecular marker was granted to the anti-PD-1 antibody pembrolizumab in patients with advanced-stage MSI-H/dMMR solid tumours[116]. The introduction of this biomarker-driven approach was responsible for a jump in the use of molecularly-guided cancer therapies in 2017 (0.33% increase in number of patients eligible for genomically informed therapies)[25]. Three drugs are currently approved for patients with metastatic and chemotherapy-refractory MSI-H/dMMR CRCs based on data from phase II clinical trials[16,18,117]; pembrolizumab, another anti-PD-1 antibody, nivolumab, as well as the anti-CTLA4 antibody ipilimumab. Results from the totally 125 patients with MSI-H/dMMR treatment-refractory metastatic CRCs who received anti-PD-1 antibodies alone indicate an overall objective response rate (ORR) and disease control rate (DCR) of 39% and 75%, respectively (TABLE 3). Promisingly, the majority of responses were durable. The median PFS[16,117], or median duration of response[18], was not yet reached after a median follow-up of >36 weeks. Two randomized phase III trials evaluating the efficacy of immune-checkpoint inhibitors (ICIs) as a first-line treatment in patients with metastatic MSI-H/dMMR CRCs are currently ongoing. The KEYNOTE-177 study[118] is designed to compare the efficacy of single-agent pembrolizumab with that of investigator's choice of chemotherapy[119]. Similarly, COMMIT[120] is designed to compare the efficacy of the anti-PD-L1 antibody atezolizumab, either as a single-agent or in combination with a standard treatment regimen (FOLFOX and a VEGF targeted therapy), to standard treatment alone. Additionally, the possibility of treatment benefit in the adjuvant setting is being evaluated in a randomized phase III trial

comparing the efficacy of adjuvant FOLFOX with and without atezolizumab in patients with dMMR stage III colon cancers[121].

Data published in 2018 show an apparent synergistic effect of ICIs when used in combination, including anti-PD-1/PD-L1 antibodies with anti-CTLA4 antibodies. Among 119 patients with chemotherapy-refractory MSI-H/dMMR metastatic CRCs receiving nivolumab and ipilimumab, 55% and 80% had objective responses and disease control, respectively (TABLE 3). Again, the responses were durable, lasting ≥6 months in 83% of patients[122], and were found to be independent of poor prognostic factors such as the presence of *BRAF* and *KRAS* mutations. Initial results from 45 patients treated with this combination in the first-line revealed objective responses and disease control in 60% and 84%, respectively[123]. Promising preliminary data with the same combination have been presented also in the neoadjuvant setting in patients with early stage colon cancers, showing pathological responses in all seven patients with MMR deficiencies, including four complete responses[124]. A similar combination of durvalumab and tremelimumab is currently under investigation, irrespective of MSI-status[125,126]. Based on the successes and the level of interest with which ICIs are currently being investigated, the indications for use of these agents are likely to soon extend beyond patients with treatment refractory metastatic cancers with MSI-H/dMMR. However, primary resistance remains common and further research into the determinants of response and resistance in patients with MSI-H cancers is needed in order to improve patient selection for treatment.

Optimization of the use of immunotherapy.—The sensitivity of MSI-H tumours to ICIs is attributed to the hypermutated phenotype[16]. Mechanisms of response mirror those that explain the favourable prognosis of patients with primary MSI-H CRCs, involving the generation of cancer-specific neoantigens and subsequent activation of cytotoxic T cells[76,127,128]. The immunosuppression induced by cancers with a high TMB as a protective trait is, to a large extent, mediated by upregulated expression of PD-L1, as well as immunomodulatory receptors on T cells including CTLA4 and PD-1[129,130]. ICIs target this ligand–receptor interaction and reactivate T cell responses to tumour-associated antigens[131]. Consequently, treatment responses are expected also in cancers with a hypermutated phenotype that is unrelated to MSI. A prominent example is provided by MSS tumours with defective replication repair caused by mutations in the proofreading domain of the DNA polymerase POLE. Mutations in this enzyme often lead to an ‘ultra-hypermutator’ phenotype with a TMB >100 mut/Mb, exceeding that of MSI-H tumours (>10 mut/Mb)[75], and with a similar extent of cytotoxic T cell infiltration[132]. Responses to pembrolizumab have been documented in two case reports describing patients with *POLE*-mutated MSS CRCs[19,133] (TABLE 3), and additional clinical data are awaited from an ongoing study investigating the efficacy of the anti-PD-L1 antibody avelumab in patients with MSI-H or *POLE*-mutated CRC[134]. However, patients with non-metastatic *POLE*-mutated tumours also have a favourable prognosis compared to those with MSS CRCs[132], and the prevalence of *POLE* mutations is <1% in patients with advanced-stage CRCs[135]. This observation suggests that this biomarker has only a modest level of potential to extend the indications for use of ICIs.

A high TMB (>10-20 nonsynonymous mut/Mb) is emerging as a separate positive predictive biomarker of benefit from ICIs in several cancer types[136,137]. The majority of hypermutated CRCs are identified by the presence of either MSI or *POLE* mutations, although an increased TMB has been found in 3% of MSS tumours, of which only 21% could be attributed to *POLE*[133]. A report showing a complete and durable response to nivolumab in one patient with *POLE* wild-type MSS cancer with an elevated TMB suggests the potential to refine the criteria for hypermutated CRCs in relation to treatment[133]. The criteria for hypermutation will need to be carefully adjusted based on additional clinical data, and the most appropriate TMB threshold is likely to vary across different cancer types, as well as according to the gene panel used for sequencing[136]. The presence of a pre-existing $CD8^+$ cytotoxic T cell antitumour immune response might be a prerequisite for response to anti-PD-1 antibodies[138] and tumour immunoreactivity provides another possible strategy for the refinement of patient eligibility criteria. Immunoscore enables the identification of cancers with an in situ adaptive immune response and is a potential biomarker. Up to 12% of patients with primary MSS CRCs that later recur might be classified as immunoscore high (>70% density of $CD3^+$ and $CD8^+$cells)[139], although whether this is reflected in responsiveness to immune-checkpoint inhibition in metastatic lesions has yet to be shown.

Investigators attempting to optimize the clinical efficacy of ICIs in patients with CRC should have two immediate objectives (FIG. 2): identification of the mechanisms of innate resistance in patients with hypermutated and/or immunogenic cancers and; identification of the most effective combination strategies, in particular for patients with tumours of an immunologically ‘cold’, non-hypermutated phenotype[140]. Whether MSI is the result of either sporadic or hereditary (epi-)genetic alterations does not seem to affect the responses of patients to treatment[117,122]. However, a range of mechanisms of resistance have been proposed in patients with various cancer types, and several of these are likely to apply to patients with CRC. These can be broadly classified into one of four categories, including defects in the antigen presentation machinery (for example loss of expression of TAP, B2M and HLA molecules), insensitivity to cytotoxic T cells (for example owing to loss of IFNγ signalling), activation of inhibitory immune checkpoints (such as CTLA-4, PD-1 and others, including lymphocyte activation gene 3 protein (LAG-3) and V-type immunoglobulin domain-containing suppressor of T-cell activation (VISTA)), and infiltration of immunosuppressive cells (such as regulatory T cells and tumour-associated macrophages) into the tumour[140].

The IFNγ signalling pathway has a key role in responsiveness to ICIs, and the loss of IFNγ signalling is one of few mechanisms of resistance that have been recognized to exist in patients with CRC. This mechanism arises from homozygous or hemizygous loss-of-function mutations in *JAK1*, eventually leading to a loss of PD-L1 expression[141] (FIG. 2). Mutations in *JAK1* occur in 20% of patients with primary MSI-H CRCs, and are associated with transcriptional profiles that are predictive of resistance to ICIs[142]. However, these alterations are also associated with a more favorable prognosis in the primary cancers, and are therefore likely to be less common among patients with metastatic disease[89]. Furthermore, anti-PD-1 antibodies have been shown to be effective in tumours harbouring loss-of-function mutations in one allele of *JAK1*, thus supporting the need for a ‘second hit’ and complete loss of JAK1 function to confer resistance. Preclinical data indicate that IFNγ

secretion might be increased by treatment with an anti-LAG-3 antibody, and that this causes proliferation of isolated T cells, similarly to treatment with anti-PD-1 antibodies[143]. Evidence from patients with melanoma indicates that mutations in other genes involved in IFNγ signaling might also affect responsiveness to ICIs[144]. Another example is provided by mutations in *STK11*, which are associated with T cell depletion and primary resistance to anti-PD-1 antibodies in patients with *KRAS*-mutant lung cancer[145]. In summary, no robust biomarkers that might enable treatment stratification within the subgroup of patients with MSI-H CRCs are currently available. Moving forward, patient monitoring with repeated longitudinal sampling and analysis of immune signatures during treatment will likely be a successful strategy for identifying additional mechanisms of resistance as they occur[146].

Targeting poor prognostic *BRAF* mutations

Guidelines for molecular testing in patients with metastatic CRC include assessment of *BRAF* mutation status for prognostic stratification[29]. The prognostic value of $BRAF^{V600E}$ is a prominent example of complexity caused by biomarker interactions[147]. The mutations are strongly enriched in, and occur in up to 60% of sporadic primary MSI-H CRCs, but in no more than 5–10% of MSS CRCs[148-153]. Consequently, the majority of epidemiological and clinicopathological associations are similar to those of the MSI subtype[154], and in primary CRC, the poor prognosis associated with this alteration might be limited to those with MSS disease[150]. Retrospective analyses of clinical trial data show that the $BRAF^{V600E}$ confers inferior OS[68,155,156] and DFS outcomes[54,82,156] in patients with stage II and III MSS colon cancers, and the MSS-dependent poor prognostic value of $BRAF^{V600E}$ has also been shown in population-based series of CRCs[157,158]. The MSI–BRAF interaction is supported also at the level of gene expression, and $BRAF^{V600E}$ has a greater impact on mutation-associated gene expression patterns in MSS compared with MSI-H CRCs[158]. This effect might be related to the different tumorigenic roles of this mutation in the two subtypes. ‘Classical’ CRC tumorigenesis is initiated by activation of WNT signalling, eventually causing the development of MSS tumours that are chromosomally unstable[159,160]. In this model, $BRAF^{V600E}$ mutations are mutually exclusive to the more frequent *KRAS* mutations, which contribute to adenoma development but are not required for adenoma initiation[161]. However, in CIMP and MSI-H tumours arising from serrated polyps, $BRAF^{V600E}$ mutations are tumour-initiating events[79,162,163]. A role in the formation, rather than the development of MSI-H tumours provides a potential rationale for the weaker prognostic associations of $BRAF^{V600E}$ in this tumour subtype. Nonetheless, the size of the prognostic effect in MSS cancers has led to calls for stratification according to *BRAF* and MSI status in future trials involving adjuvant treatments[29,156].

The association between $BRAF^{V600E}$ and survival in primary CRC might primarily reflect reduced OS after disease relapse, rather than an increased risk of relapse[68,149], indicating a prognostic effect also in metastatic cancers. Furthermore, the dependence on MSI status seems to change in the metastatic setting, and here $BRAF^{V600E}$ also has prognostic relevance in patients with tumours of the MSI-H subtype[109]. This difference might reflect the more aggressive biology of MSI in metastatic disease. The median OS of patients with metastatic CRCs harbouring $BRAF^{V600E}$ is commonly reported to be <10 months[164], but might reach 13 months depending on treatment[165]. The mutation affects both PFS and

OS[109,154,164-168] and survival after metastasectomy[166,169-171]. Consequently, the current level of benefit from standard therapies is inadequate[29,172], and oxaliplatin-based and irinotecan-based therapies both seem to confer similar outcomes[173,174]. Improved survival and response rates might be obtained by the use of combination therapies in the first-line setting, including triplet or doublet chemotherapy (FOLFOXIRI or FOLFIRI) in combinations with VEGF targeted therapies (bevacizumab)[165,175], although small cohort sizes preclude the statistical significance of this observation. Early indications suggested that *BRAF*^{V600E} was predictive of a lack of response to EGFR targeted therapies (cetuximab or panitumumab) in the *RAS* wild type population[176,177]. This observation is consistent with the biological rationale that BRAF is a principal effector of KRAS signalling. However, as summarized in a review published in 2017[172], several retrospective analyses of *BRAF*^{V600E} in data from randomized trials exploring the efficacy of EGFR targeted therapies have been conducted[94,178-185], and only one[186] was able to confirm this association. Data from meta-analyses have either indicated a negative predictive value[187], or concluded that insufficient evidence exists to support such an association[188]. The presence of *BRAF*^{V600E} is not a contraindication for EGFR targeted therapy, according to guidelines published in 2017[29].

Considerable effort has been applied to the development of effective treatments for patients with *BRAF*^{V600E}-mutant CRCs and the strongest clinical benefits have been achieved with agents targeting the mutated protein itself. Monotherapy with the BRAF inhibitor vemurafenib has not mirrored the encouraging initial response rates obtained in patients with melanoma[189,190], although several targetable mechanisms of resistance have been identified, which has paved the way for more effective combination therapies. Re-activation of EGFR signalling is perhaps the most prominent mechanism of resistance[191-193], and clinical studies evaluating the efficacy of different combinations of BRAF and EGFR targeted therapies have revealed disease regression in 52%[190] and 67%[194] of patients who have previously progressed on other treatments. However, formal criteria for a partial response were not met in all patients, and response rates according to RECIST have been lower and reported in the range of 4–22%, potentially related to selection of resistance[190,194-197]. Nevertheless, BRAF and EGFR targeted agents in combination with chemotherapy (vemurafenib, irinotecan and cetuximab)[20] are now included in treatment guidelines for patients with treatment-refractory *BRAF*^{V600E}-mutant metastatic CRC[84]. This recommendation is based on initial data from a randomized phase II trial that revealed superior PFS and an ORR of 16% among patients in the triple therapy arm, compared to 4% in those who did not receive vemurafenib[21]. Final results from this study are awaited.

Activation of the PI3K/AKT signalling pathway is another potential mechanism of resistance to BRAF inhibition[193]. A triplet including BRAF, EGFR and PI3K targeted agents (encorafinib, cetuximab and alpelisib) has been shown to prolong PFS in patients with treatment-refractory disease, relative to encorafinib plus cetuximab alone in an interim analysis of a randomized phase II trial[197]. However, the difference in survival was not statistically significant, and the triplet also increased the risk of adverse events (grade 3–4 adverse events occurred in 79% versus 58% of patients).

MEK is a downstream effector of BRAF, and responses to the combination of BRAF and MEK inhibition (with dabrafenib plus trametinib) have been reported in 12% of a cohort of

43 patients[198]. These outcomes do not parallel those of patients with melanoma[199], although in a phase I study involving a triplet including an EGFR targeted agent (dabrafenib, panitumumab and trametinib), responses were achieved in 21% of patients, compared to 10% with dabrafenib plus panitumumab alone[200,201]. Results are awaited from the ongoing randomized phase III BEACON CRC study, in which the efficacy of encorafinib plus cetuximab, with or without the MEK inhibitor binimetinib will be evaluated in comparison with a control arm, in which patients are receiving chemotherapy plus cetuximab[202]. Early results from the safety lead-in phase of this trial show an ORR of 48% with good tolerability among the 29 patients receiving the triplet combination[203]. If similar outcomes are observed in the randomized phase, this combination will be confirmed as having unprecedented potential to improve the outcomes of patients with this aggressive CRC subtype. However, any improvements in PFS are likely to be limited to only a few months[200,203], and an improved understanding of the biology of *BRAF*^{V600E}-mutant CRC could enable the development of even more effective strategies.

Identifying additional targets

Tumours of the immunogenic CMS1-MSI/immune subtype are strongly enriched with *BRAF*^{V600E} mutations[22]. This high prevalence is related not only to the co-occurrence of these mutations with MSI, but also to enrichment at a similar magnitude observed specifically among CMS1 MSS cancers, with a mutation frequency of 34%[158]. This observation suggests that inflammation and immunogenicity are defining characteristics of *BRAF* mutated CRCs. Interestingly, the combination of nivolumab plus ipilimumab resulted in an ORR of 55% in patients with *BRAF*-mutant MSI-H CRCs[122]. This response rate is the same as that of the overall MSI-H cohort, and is a very promising result for patients with *BRAF*^{V600E}-mutant disease. If these data are confirmed in ongoing studies, the co-occurrence of MSI and *BRAF*^{V600E} could become defined as a ‘double-target’ subgroup with a need for therapy prioritization between ICIs and BRAF targeted agents. In this setting, the longer duration of response achieved with ICIs compared to that obtained with BRAF inhibition will be an important consideration. Furthermore, improved characterization of the immune context of *BRAF*^{V600E} metastatic CRCs is an important strategy for the potential to design combinations therapies including both ICIs and BRAF targeted agents.

Gene expression profiling has enabled the *BRAF*^{V600E}-mutant phenotype to be extended to a subpopulation of *BRAF* wild type CRCs with a similarly poor prognosis[204]. Preclinical data suggest that the subgroup of tumours with this ‘*BRAF*-like’ gene expression pattern might be particularly vulnerable to silencing of the microtubule regulator *RANBP2* and repurposing of the tubulin-binding agent vinorelbine[205]. However, in a phase II trial, in which patients with *BRAF*^{V600E} metastatic CRC received vinorelbine, no clinical activity was reported and this treatment strategy has not been translated into clinical use[206]. Gene expression subtyping offers another potential method of stratifying patients with *BRAF*^{V600E}-mutant disease who might benefit from combined BRAF plus MEK inhibition. *BRAF*^{V600E} CRCs can be divided into two distinct gene expression subtypes: one subtype confers a poor prognosis and is characterized by activation of KRAS/AKT signalling and epithelial-to-mesenchymal transition (EMT), but with potentially greater sensitivity to BRAF plus MEK inhibition than the second subtype, which is characterized by cell-cycle

dysregulation[207]. Clinical testing is required to evaluate these putative associations, which are currently derived from CRC cell lines.

Tumour heterogeneity is likely to have a role in acquired resistance to BRAF-targeted therapies. This association has been demonstrated in a case report describing a patient with expansion of a *MET*-amplified subclone during combination therapy with panitumumab and vemurafenib[208]. This case report revealed a new potential treatment approach following a successful switch to combination therapy with the ALK and MET inhibitor crizotinib plus vemurafenib, although the patient later also progressed on this drug combination owing to *MET* hyperamplification[209]. Acquired resistance to such regimens of two or three targeted therapies often leads to reactivation of the MAPK signalling pathway. Combined inhibition of EGFR, BRAF and ERK signalling therefore provides a new strategy that might circumvent the development of acquired resistance[210]. These studies clearly demonstrate that genomic monitoring of patients' disease during treatment is a powerful approach that is likely to improve disease management, based on the early detection of treatment-resistant subclones.

Rare *BRAF* mutations

Approximately 2% of metastatic CRCs have *BRAF* mutations located outside of the hotspot in codon 600, most frequently in codon 594[211,212]. In contrast to $BRAF^{V600E}$, some of these mutations might lead to inactivation of the kinase[213], co-occur with *RAS* mutations, and are not enriched in MSI-H CRCs. Consequently, rare *BRAF* alterations have distinct clinical associations, including a propensity for left-sided or rectal primary locations and fewer peritoneal metastases, relative to $BRAF^{V600E}$-mutant CRCs[211,214]. These tumours also confer a favorable prognostic association, at least in comparison with $BRAF^{V600E}$-mutant CRCs (median OS approximately 60 months among patients with non-V600E *BRAF*-mutant metastatic CRCs)[211,214]. This association has also been observed in patients undergoing surgery for CRC liver metastases[171]. Insufficient data are available to determine whether non-V600E mutations confer an absence of poor prognostic associations, or also a better prognosis than wild-type *BRAF*. Differences in prognosis might be related to the specific codon affected[215]. Nonetheless, patients with these cancers might not need the same aggressive treatments as those with $BRAF^{V600E}$-mutant disease, although clinical trials are required in order to determine the optimal treatment approach, and again this might depend on the specific mutated codon[212]. Patients with cancers harbouring certain non-V600E mutations in *BRAF* might benefit from EGFR inhibition, although currently available data are inconclusive[216-218]. Responses to approved BRAF inhibitors are unlikely, considering that these inhibitors bind to and inhibit monomeric BRAF, which is seen only with V600E mutations[212].

Complexity among emerging biomarkers

HER2 overexpression

HER2 overexpression can be found in approximately 20% of breast cancers. This biomarker is associated with a poor prognosis following treatment with standard therapies, but improved outcomes with HER2-targeted agents[219]. In CRC, HER2 overexpression has a

limited prevalence, occurring in approximately 2% of patients. This effect is caused by *ERBB2* amplification in >90% of the patients[220]. The prognostic value of HER2 overexpression in patients with CRC remains uncertain[220,221], although HER2 might have a dual predictive value relating to response to targeted therapies.

HER2 can activate the MAPK signalling pathway and preclinical data suggest that *ERBB2* amplification is involved in both primary and acquired resistance to EGFR inhibition[222,223]. The prevalence of HER2 overexpression in this therapeutically relevant setting, among patients with *RAS* or *BRAF* wild type metastatic CRCs, is increased to approximately 5%[220,224,225]. However, clinical data are limited to retrospective analyses of a small number of patients. Data are available on the effects of *ERBB2* amplifications among patients with *KRAS* wild type solid tumours treated with anti-EGFR monoclonal antibodies alone or in combination with chemotherapy[223,226]; from patients with *RAS* and/or *BRAF* wild type cancers receiving anti-EGFR antibodies after failure of first-line chemotherapy[227,228]; and from 15 patients with HER2 overexpressing cancers refractory to previous anti-EGFR therapies[225]. These data consistently confirmed a negative predictive value of HER2 overexpression, with a similar magnitude of effect on PFS after EGFR blockade to that of *RAS* mutations. However, prospective studies are needed in order to confirm any negative associations between HER2 overexpression and responses to anti-EGFR therapy.

The suggested dual predictive role of HER2 overexpression stems from the potential to directly target this protein. The first evidence of clinical responses to HER2 targeted therapies in patients with metastatic, HER2-overexpressing CRCs was obtained more than a decade ago, in combination with oxaliplatin-based[229] or irinotecan-based chemotherapies[230]. However, both of these phase II trials were prematurely terminated owing to limited accrual of patients with the relevant biomarkers. Later preclinical studies suggested that dual inhibition improves the efficacy of HER2-targeted therapies. Long-lasting tumour regressions were achieved in cetuximab-resistant PDX models of liver metastases with wild type *RAS*, *BRAF* and *PIK3CA* harbouring *ERBB2* amplifications following combined treatment with the dual HER2/EGFR inhibitor lapatinib and antibodies targeting either HER2 (pertuzumab) or EGFR (cetuximab), but not with monotherapies[222]. Encouraged by these preclinical data, the phase II HERACLES trial was conducted to evaluate the efficacy of dual HER2 inhibition with lapatinib plus trastuzumab in chemotherapy and anti-EGFR antibody refractory *KRAS* wild type, HER2-positive metastatic CRCs[225]. Objective responses were seen in 30% of the 27 eligible patients, with disease control in 59%. Furthermore, a combination of the two anti-HER2 antibodies, trastuzumab and pertuzumab, enabled objective responses in 38% of the 37 patients with HER2-positive metastatic CRCs enrolled in the MyPathway phase II basket study[231]. Confirmatory case-reports describing durable responses to trastuzumab monotherapy[232] and in combination with chemotherapy, lapatinib or pertuzumab have also been published[233,234]. Therapies targeting HER2 in patients with HER2-positive metastatic CRC are currently considered investigational, although enrollment of such patients in clinical trials is encouraged[84] and offers a therapeutic option for those with resistance to EGFR targeted therapies. The optimal timing of HER2 inhibition in relation to use of standard-of-care chemotherapies also needs to be addressed in future studies, in addition to the efficacy of

this approach as an alternative to EGFR-targeted therapies in *RAS* wild type and HER2-overexpressing cancers.

Initial clinical investigations demonstrated that primary resistance to dual HER2 inhibition is a frequent occurrence in patients with CRC, and the development of resistance during treatment is almost inevitable. An initial report suggested that *ERBB2*, *RAS* and *PIK3CA* mutations are involved in the development of resistance[235], although further genomic analyses of biopsy samples from relevant clinical cohorts is currently awaited. An antibody–drug conjugate (ADC) that combines trastuzumab with the cytotoxic agent emtansine has shown efficacy in patients with trastuzumab-resistant CRCs[236,237]. This agent is currently being evaluated in the HERACLES B and RESCUE trials, the latter including patients who progressed in the initial HERACLES study[238]. Preliminary data from an ongoing phase I trial[239] combining another ADC, trastuzumab deruxtecan, which combines trastuzumab with a DNA topoisomerase I inhibitor show an ORR of 25% and a DCR of 83%, respectively, among 12 patients with HER2-expressing, *KRAS* wild type advanced-stage CRCs[240]. Translational studies designed to identify synergistic combination partners will be crucial to further improvements in the level of clinical benefit derived from HER2 inhibition, although such approaches are challenged by the limited prevalence of this biomarker.

In addition to amplifications, *ERBB2* is also targeted by point mutations or indels in an additional 2% of metastatic CRCs[233]. These aberrations might be associated with an inferior prognosis[241], and preclinical data indicate that activating mutations and amplifications have similar therapeutic implications. Resistance to cetuximab has been demonstrated in *KRAS* wild type cell lines and PDX models of CRCs harbouring activating *ERBB2* mutations[242,243]. Monotherapy with HER2 inhibitors has no clinical efficacy in patients with these mutations[244], although preclinical data suggest that dual inhibition of HER2 signalling might be more effective[243]. Accordingly, point mutations and indels add to the complexity of *ERBB2*/HER2 as a biomarker in CRC.

Oncogenic addiction to kinase fusions

In November 2018 the tropomyosin kinase (TRK) receptor inhibitor larotrectinib was granted accelerated FDA approval for patients with metastatic solid tumours harbouring *NTRK1*/*2*/*3* (*NTRK*) fusions[245]. Of note, this was the second tissue-agnostic FDA approval of a cancer therapy. Gene fusions resulting in increased kinase activity and subsequent oncogene addiction are, arguably, among the most obvious targetable vulnerabilities. These kinase fusions are likely to be intimately associated with disease progression, although the limited prevalence of these alterations in CRCs creates a challenge for those attempting to analyze possible clinical associations. Kinase fusions have been reported in <1-2% of patients with CRC and primarily involve *RET*, *NTRK*, *ALK* or *ROS1* (in addition to *BRAF* and *FGFR*s)[88,246,247]. Nonetheless, in an analysis of 27 metastatic CRCs harbouring *ALK*, *NTRK*, or *ROS1* rearrangements identified from molecular screening programs, rearrangements were found to be most prevalent among patients with right-sided MSI-H *RAS* wild type tumours[248]. In comparison with a set of 319 fusion-negative cancers collected through similar means, the presence of these rearrangements also conferred an inferior prognosis including shorter median OS, independent of both MSI status and primary

tumour location. Similar results were found by a study with a similar design in a cohort of 24 patients with metastatic CRCs harbouring *RET* fusions: shorter median OS was observed independent of MSI status and primary tumour location[249].

Associations between kinase fusions and inferior outcomes might be augmented by poor responses to standard therapies. Preclinical analyses have shown that excessively high expression of *ALK*, *NTRK* and *RET* is associated with primary resistance to EGFR targeted therapy in *RAS* and *BRAF* wild type CRC cell lines[250]. Later studies have confirmed disease progression on such treatments in fusion-positive cancers[248,249,251]. Preclinical analyses have also suggested that selective tyrosine-kinase inhibitors (TKIs) might be effective[250,252], and clinical efficacy has been confirmed in a few studies. These observations include disease regression in three of four patients with *NTRK* fusion-positive colon cancers who received larotrectinib as part of a basket trial[253]; responses to the ALK/ROS1/pan-TRK inhibitor entrectinib in two patients with treatment-refractory cancers harbouring either an *ALK*[254] or an *NTRK1* rearrangement[255], as well as in two of three patients with *NTRK*-fusion positive CRCs from a pooled analysis of data from three tissue-agnostic phase I/II trials[256]; an exceptional 9-month response to the ALK inhibitor ceritinib in a patient with *ALK* fusion-positive CRC[257]; and a complete response to the broad-spectrum kinase inhibitor RXDX-105 in a patient with *RET*-rearranged CRC[249]. Additional clinical data are needed to support these anecdotal reports. In order to better identify eligible patients, understanding the likelihood of the co-occurrence of kinase fusions with other biomarkers might reveal a patient population with a frequency of such fusions that is appropriate for molecular screening[258]. For example, *RET* rearrangements can be found in two thirds of patients with right-sided MSI-H tumours that lack *RAS* or *BRAF* mutations[249]. Enrichment for *NTRK* fusions in MSI tumours has also been documented in clinically sequenced MSI-H metastatic CRC samples in a single-institution study, albeit with a prevalence of only 8% and 1% in MSI-H and MSS CRCs, respectively[259].

Owing to associations with MSI, most patients with CRCs harbouring kinase fusions are also eligible for ICIs. This association provides an appealing possibility for combination therapies in patients with resistance to single-agent ICIs. Data supporting the use of TKIs in this setting are currently unavailable, although the combination of lorlatinib plus avelumab has shown clinical efficacy in patients with *ALK*-mutated non-small-cell lung cancers with no previous exposure to ICIs[260]. Of note, a durable response to single-agent nivolumab has been reported in one patient with MSI-H metastatic CRC harbouring an *ALK* rearrangement[248]. This observation suggests that this rearrangement is not predictive of innate resistance to ICIs. Again, the duration of response among patients with CRC who respond to ICIs is longer than that of patients who respond to TKIs: this will be an important consideration for therapy prioritization.

The R-spondins RSPO2/3 represent another class of potentially targetable oncogenic rearrangements in patients with CRC. These proteins are secreted proteins that regulate WNT signalling, and upon translocation to high-affinity promoter regions, can cause aberrant activation of WNT signalling in CRCs[261]. Virtually all CRCs of both the MSI and MSS subtypes have activating mutations in the WNT pathway, most commonly loss-of-function mutations in *APC* (in approximately 50% and 80% of MSI and MSS CRCs

respectively)[262]. However, *RSPO2/3* fusions are mutually exclusive to *APC* mutations, supporting an independent role of each alteration in the promotion of WNT signalling. These fusions are, in contrast to kinase fusions, primarily found in MSS CRCs and were initially reported to occur in 10% of MSS CRCs[261]. However, this initial report might be an overestimate and later studies suggest mutation frequencies of 0.35%[88] and 4%[263]. RNF43 is another component in the same WNT regulatory complex[264] and mutant forms of this tumour suppressor protein are also mutually exclusive to mutations in *APC*. Truncating alterations in *RNF43* are most frequent in MSI-H CRCs and might occur in up to 80% of these tumours[89,265]. CRCs harbouring *RSPO* fusions or *RNF43* mutations are, unlike tumours with many other types of WNT pathway alterations, dependent on secreted WNT ligands. This dependency offers an opportunity for targeted interventions, which is particularly relevant in light of the safety concerns associated with other therapeutic attempts to target this pathway[266] and given that WNT signalling is essential for the homeostasis of nonmalignant adult tissues[267,268]. Indeed, preclinical data from an *RNF43*-mutated organoid model of CRC indicated sensitivity to PORCN inhibition, which blocks both the secretion and activity of WNTs[23]. Inhibition of PORCN signalling has been used to arrest tumour growth with on-target effects also reported in *PTPRK–RSPO3*[269] or *RSPO2*-fusion-positive[270] PDX models, and the same effect was obtained with an anti-RSPO3 antibody in a *PTPRK–RSPO3* fusion positive PDX model[271]. A response to PORCN inhibition has been reported in one patient with an *RNF43*-mutated cancer who was treated in an ongoing phase I trial[272]; however, virtually no clinical data are currently available on the efficacy of *RNF43* or *RSPO*-guided therapies in patients. Nonetheless, combination strategies based on translational and/or preclinical data are currently being investigated in early phase trials. These include the combination of agents that inhibit PORCN, BRAF and EGFR in patients with $BRAF^{V600E}$-mutant metastatic CRCs harbouring either an *RSPO* fusion or an *RNF43* mutation[273]. The combination of the porcupine inhibitor WNT974 with ICIs is also currently under investigation[274], based on the association between activated WNT signalling and T cell exclusion observed in patients with CRC[275].

Biomarkers of inferior responses

Several biomarkers are associated with a poor outcome in patients with CRC receiving standard-of-care therapies. In addition to the low-prevalence markers described previously, these include more common biomarkers that can be used to pinpoint particularly important target populations for the development of novel targeted therapies.

RAS mutations

Genetic testing for *RAS* mutations is recommended in clinical guidelines for the management of patients with metastatic CRC as a negative predictor of benefit from EGFR targeted therapies[29,84]. Associations between mutations in *RAS* and survival outcomes have also been intensively investigated in other therapeutic settings, although the prognostic value is less clear.

Hotspot mutations in *KRAS* or *NRAS* are mutually exclusive to mutations in *BRAF* and other recurrent mutations in the MAPK signalling pathway. *KRAS* mutations are the most

frequent and occur in approximately 35% of stage I–IV primary CRCs (SUPPLEMENTARY TABLE 1), most commonly in right-sided tumours. Similar to *BRAF*, the prevalence of *KRAS* mutations decreases in a stepwise manner along the right-to-left axis of the large bowel. However, in contrast to $BRAF^{V600E}$, *KRAS* mutations are twice as frequent in MSS compared with MSI-H colon cancers[52,276]. Furthermore, the potential size of the prognostic effect of these mutations is substantially smaller than that of $BRAF^{V600E}$ and remains debatable. However, the majority of studies of the prognostic implications of *KRAS* alterations revealed a negative effect on patient survival[35,158,277-290] (SUPPLEMENTARY TABLE 1), including studies in which use of EGFR targeted therapies[276,291] might have influenced the results.

Retrospective analyses of data from several randomized trials investigating the efficacy of adjuvant chemotherapies revealed no prognostic associations of *KRAS* mutations in patients with stage II or III CRCs[68,292-294]. However, a negative prognostic association was confirmed in an analysis of data from a pooled cohort of 7,326 patients with stage II and III colon cancers, which included some patients from the clinical trial cohorts[52]. Furthermore, the prognostic value of *KRAS* mutations in patients with primary CRCs might be limited to specific subgroups, including MSS cancers[52,158,276,285,290], cancers with a distal primary tumour location[295,296], or even to MSS cancers of the ‘epithelial-like’ CMS2/3 gene expression subtypes[158]. Accordingly, *KRAS* mutations have been suggested as a biomarker for the prognostic stratification of primary CRCs in trials in the adjuvant setting, in combination with both *BRAF* and MSI status[29]. Furthermore, *KRAS* mutations might be associated with inferior survival after relapse from stage III CRCs, although the result from this analysis was not statistically significant[291]. This association suggests that *KRAS* alterations have a stronger prognostic effect in patients with metastatic disease, and indeed, the majority of large-cohort studies have identified statistically significant negative prognostic associations (SUPPLEMENTARY TABLE 1)[174,218,259,297-307], which possibly relates to the greater prevalence of MSS disease in this setting. Conflicting data on this association do exist[308-312], although the prognostic effect of *KRAS* alterations is supported by a multivariable analysis of pooled data from 26 randomized trials, including 22,674 patients with metastatic CRC (HR for OS 1.35, 95% CI 1.30– 1.39; $P<0.001$)[313].

NRAS mutations are less frequent and occur in only 3–4% of metastatic CRCs[298,304,314]. These mutations are, therefore, commonly grouped with *KRAS* alterations, with which they share some clinicopathological associations[297,299,300,305,314]. However, an independent association with a poor prognosis has also been demonstrated[305], along with indications that mutant *NRAS* confers a somewhat worse prognosis than mutant *KRAS*[298,314].

Importantly, *RAS* mutations seem to have prognostic value in patients who do not undergo metastasectomy, in patients undergoing partial liver resection[300], and in patients with disease recurrence after partial liver resection[297]. An association with an inferior prognosis following hepatectomy has also been confirmed in a meta-analysis of data from 1,833 patients (HR for OS, 1.67, 95% CI 1.34–2.09; $P<0.001$)[315]. It has been suggested that surgical treatment might not be beneficial in some patients with *RAS*-mutated liver metastases [300]; however, current guidelines for determining resectability do not consider the use of genetic testing for *RAS* or *BRAF* mutations[84]. Adding *RAS* mutations to clinical risk

scores might help improve the risk:benefit ratio of therapeutic interventions in patients with resectable liver metastases, but prospective validation of such a personalized approach is needed[316]. In summary, although a substantial amount of evidence (SUPPLEMENTARY TABLE 1) supports a role for *KRAS* mutations in the prognostic stratification of patients with CRC, no appropriate clinical setting has been defined and the modest prognostic effect size limits clinical relevance.

The strongest associations between *RAS* mutations and outcome in patients with CRC relate to responsiveness to anti-EGFR therapies. Current expert consensus guidelines recommend extended pretreatment genetic testing for *RAS* alterations in patients with metastatic CRC, including *KRAS* and *NRAS* codons 12 and 13 (exon 2), 59 and 61 (exon 3), and 117 and 146 (exon 4)[29]. *RAS* mutations cause the aberrant activation of MAPK signalling downstream of EGFR, resulting in a poor response to EGFR inhibition[218,317], and the recommendations for stratified treatment are based on large volumes of data from almost 75,000 patients. However, fewer than half of all patients with *RAS* wild type metastatic CRCs respond to anti-EGFR therapies[218] and responsiveness might depend on the primary tumour location: patients with right-sided primary tumours derive no benefit from EGFR inhibition[318]. In future, the predictive biomarker panel for the detection of primary resistance might be further expanded to include *ERBB2*, *KRAS* and *MET* amplifications as well as mutations in the ectodomain of *EGFR*, *MAP2K1*, *ERBB2*, *PIK3CA* and possibly *BRAF*[3]. Indeed, a MAPK gene expression signature that enables tumours to be classified as either ‘wild-type like’ or ‘activated oncogenic’ has been shown to better predict treatment responsiveness than *KRAS* mutation status alone[319]. This example highlights the potential to improve the response rates of patients receiving targeted therapies using more comprehensive molecular screening approaches. Nevertheless, secondary resistance invariably develops, even among patients with responsive ‘multi-wild-type’ cancers, and the mechanisms largely converge on the same aberrations as in primary resistance. Again, tumour heterogeneity has a detrimental effect, and analyses of liquid biopsy samples have repeatedly demonstrated treatment-induced expansion of resistant subclones harbouring aberrations in the MAPK signalling pathway[14,15,320-324]. Importantly, rechallenge with anti-EGFR antibodies after therapy withdrawal might be possible owing to a decline in levels of the treatment-resistant subclone[15,24]. This phenomenon highlights the importance of molecular monitoring of patients during targeted therapy, both to identify mechanisms of resistance and to develop management strategies.

More than 40% of patients with metastatic CRC have *RAS*-mutant disease (SUPPLEMENTARY TABLE 1). This classification thus defines a large and heterogeneous patient population that routinely receives 5-FU-based chemotherapy with or without bevacizumab. A limited number of alternative options are available when treatment resistance develops, and RAS has for a long time been deemed undruggable[325]. Renewed optimism exists[326] for example based on the in vitro effects of direct RAS inhibition by a selective KRAS-G12C inhibitor that locks the mutant protein in an inactive state[327]. However, KRAS-G12C constitutes only approximately 10% of *KRAS* mutations in CRC[328] and data on clinical efficacy in patients are awaited. KRAS-targeted cellular immunotherapies might offer a new option, and tumor regressions have been demonstrated after infusion of autologous cytotoxic T cells with specific reactivity towards a KRAS-G12D

neoantigen in a case report describing a patient with metastatic CRC[329]. Considering the genomic heterogeneity of *RAS*-mutant CRCs, different strategies are likely to be needed for different subsets of *RAS*-mutated cancers[326]. To this end, an international collaborative effort to subtype *RAS*-mutant metastatic CRCs according to their unique signalling dependencies is currently ongoing, and will hopefully provide a basis to design matched therapeutic interventions (https://www.colossusproject.eu/researchers/).

Combination strategies in CMS4 CRCs

The CMS4 gene expression subtype is found in approximately 25% of primary CRCs[22], and the mesenchymal/stromal characteristics associated with this subtype have repeatedly been shown to confer a poor patient prognosis[330-340]. The poor prognostic value of CMS4 is independent of cancer stage and has been validated in an independent series of patients with stage I–IV CRCs[341], as well as in a pooled analysis of tumours that partly overlaps with the original analysis cohort[339]. Adaptation of the CMS classification to the analysis of formalin-fixed paraffin-embedded tumour samples has further enabled the validation of the poor prognostic associations of CMS4 in almost 1,800 stage III colon cancer specimens from a randomized trial cohort[342], and in a smaller multicentre series of patients with stage II cancers[343]. Bioinformatic modelling of intratumour heterogeneity in the former study suggests that both ‘pure’ CMS4 tumours and tumours in which CMS4 signals are heterogeneous and mixed with signals from any of the other subtypes confer a poor prognosis[344].

The CMS classification of metastatic CRCs is complicated by several factors, including an expected enrichment with the poor-prognostic CMS4 group, dependence on the sample source used for gene expression profiling, and the effects of specific treatments prior to sample collection[345]. Analyses have largely been limited to profiling of the primary tumours, owing to effects of the tumour microenvironment on gene expression and the subsequent challenge of translating the classification to samples of metastatic tumours obtained from other organs. Accordingly, the extent of subtype heterogeneity in metastatic CRC is largely unknown, although a concordance in subtypes of 60% between primary tumours and their metastases have been reported (47% for the CMS4 subtype)[346]. The potential for CMS subtype-switching effects, as observed after neoadjuvant chemotherapy, increases the prevalence of a CMS4-like subtype in patients with pretreated metastatic tumours[347]. Furthermore, intratumour CMS heterogeneity in primary CRCs[348], which is at least partly related to EMT in regions of tumour budding[349], also suggests an increased prevalence of the CMS4 subtype in patients with metastatic disease. The poor prognostic value of CMS4 has been indicated relative to CMS2/3 in the metastatic setting, by a retrospective analysis of trial cohorts[350]. However, there is accumulating evidence that the CMS1 subtype is associated with inferior survival compared with CMS4 in patients with metastatic CRC[346,351]. This is likely related to the enrichment with MSI-H tumours in the CMS1 subtype. The treatments received by the patients might influence the prognostic analyses, and data on therapeutic outcomes associated with CMS4 are conflicting. CMS4-like subtypes have been suggested to be associated with limited benefit from standard-of-care therapies, including both 5-FU[337] and oxaliplatin[352] in the adjuvant setting in patients with stage II/III CRCs, as well as from EGFR targeted therapies in those with *KRAS* wild-

type metastatic CRCs[335,350]. Associations with responsiveness to irinotecan-based chemotherapy[334,353] and the broad-spectrum TKI regorafenib[354] have also been reported in this disease subtype. However, chemotherapy resistance has been corroborated in preclinical models[341,355] and overcoming treatment resistance in the CMS4 subtype is currently an important area of research. The potential to overcome resistance has been demonstrated using HSP90 inhibitors, which have synergistic antitumour effects when combined with 5-FU in PDX models of CMS4 CRC[341].

Therapies designed to alter the tumour microenvironment of CMS4 CRCs are another area of considerable research interest. CMS4 cancers typically have robust activation of TGFβ-signalling, and inhibition of TGFβ signalling has been shown to inhibit crosstalk between cancer cells and cancer-associated fibroblasts, thus reducing the metastatic capacity of preclinical models[338]. CRCs of a mesenchymal phenotype are also immunosuppressive[3,356], making the CMS4 group an attractive candidate for ‘immune-conversion’ strategies designed to render immunologically ‘cold’ tumours vulnerable to ICIs (FIG. 2). Several chemotherapies and molecularly targeted agents have been shown to temporarily promote antitumour immunity via various mechanisms[140]. Oxaliplatin has the potential to elicit immunogenic cell death[357], and improvements in PFS observed with perioperative FOLFOX chemotherapy[358] might be partly attributable to activation of a localized immune response[97]. The combination of FOLFOX with an anti-PD-1 antibody has a strong synergistic effect in mouse models of CRC[359]. However, these preclinical data have not been confirmed in patients, and a preliminary report indicates that the addition of atezolizumab to FOLFOX plus bevacizumab induction therapy does not improve the outcomes of patients with *BRAF* wild type metastatic CRCs[360]. Inhibition of TGFβ signalling also causes a cytotoxic T cell response and resensitizes PDX models of metastatic CRC to ICIs. This effect suggests that TGFβ-mediated activation of the tumour-associated stroma might be an important mechanism of immune evasion[361]. M7824, a bifunctional molecule simultaneously targeting PD-L1 and TGFβ confers long-term antitumour immunity and suppression of tumour growth[362]. Clinical benefit from this agent has been confirmed in one patient with MSS metastatic CRC of the CMS4 subtype in a phase I trial[363]. MEK inhibition also promotes the recruitment of cytotoxic T cells and synergizes with anti-PD-L1 antibodies in a mouse model of colon cancer[364]. Objective responses to this combination were observed in 10% of patients with metastatic MSS CRCs in a phase I trial[365]; however, the subsequent phase III study failed to demonstrate improvements in OS compared with regorafenib[366]. Finally, CMS4 tumours are characterized by a high proportion of myeloid-derived suppressor cells (MDSCs) in the tumour microenvironment[367]. MDSCs are known to prevent the activation of T cells during immune-checkpoint inhibition and might serve as negative predictors of treatment response in melanoma[368,369]. These cells can be targeted using epigenetic-modulating agents, which have synergistic effects when combined with ICIs in mouse models of moderately immunogenic colon cancers[370].

In summary, the clinical translation of the CMS classification is currently premature, both regarding associations with therapeutic outcomes and the standardization of appropriate assays. However, the CMS classification does provide an improved biological taxonomy of CRCs and a new framework for patient stratification in biologically guided clinical trials.

Conclusions

Biomarker-guided treatment options for patients with primary CRCs remain limited. Upstaging of patients with high-risk primary CRCs to enable them to receive experimental therapies currently reserved for those with metastatic disease might offer the potential for cure, although this is currently not a well-explored approach. In this respect, data from the phase III trial in which patients with dMMR stage III colon cancers are receiving ICIs in the adjuvant setting are eagerly awaited[121]. In the metastatic setting, the accumulation of experimental data is broadening the applicability of established biomarkers. Associations with a poor prognosis in the context of standard-of-care therapies, albeit with the potential for improved outcomes by targeting the marker itself seem to be a common theme. Most established biomarkers have a low prevalence, although the number of biomarkers is increasing and CRC might, in this respect, eventually be considered an umbrella diagnosis encompassing numerous rare disease subtypes (FIG. 3). Beneath this umbrella, a growing level of biomarker complexity is emerging, primarily caused by interactions between different biomarkers. These interactions reinforce the importance of expanded genetic testing to enable improved treatment-related decision making. A rapid increase in the level of biomarker complexity is also expected to emerge from resources such as large-scale preclinical drug screens and comprehensive molecular profiles generated in translational studies. In this setting, artificial intelligence offers a new and intriguing opportunity to develop improved molecular prediction algorithms from the wealth of available data, and to develop synergistic drug combinations[371].

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Key points

- The expanded use of biomarkers to guide the treatment of patients with colorectal cancer (CRC) has revealed a level of complexity arising from interactions between different biomarkers.
- An improved understanding of the causes of primary resistance might improve response rates among patients receiving targeted therapies and enable more-effective drug combinations, exemplified by mutations in the MAPK signalling pathway for EGFR and/or BRAF targeted therapies.
- Immune-checkpoint inhibition (ICI) has provided the largest contribution to the increased use of molecularly guided therapies, and biomarkers that complement patient stratification by MSI status are likely to provide further benefit.
- Biomarkers that indicate a poor prognosis have motivated the search for more effective therapies for specific molecular subgroups; these biomarkers typically have a limited prevalence, but their accumulation could expand the eligibility for, and benefit from, targeted treatment.
- Some CRCs harbour more than one molecular target and treatment sequencing both in relation to standard and targeted therapies is a growing challenge.

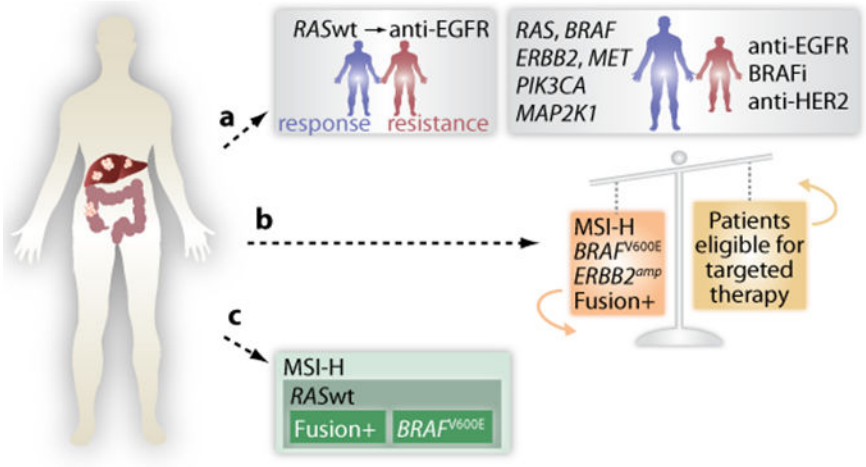

Figure 1. Clinical implications of biomarker interactions in CRC.

Interactions between established and emerging clinical biomarkers suggest that more comprehensive molecular profiling would improve patient outcomes **a**. Detection of, and stratification based on genetic and/or clinical features associated with primary resistance, such as alterations in the MAPK signalling pathway, might improve responsiveness to targeted therapies and/or enable the identification of more effective drug combinations. **b**. The accumulation of low-prevalence 'actionable' alterations has the potential to increase the total use of biomarker-guided therapies **c**. The co-occurrence of more than one 'actionable' alteration might enable new treatment options when resistance develops, although the most appropriate treatment sequence and/or drug combinations need to be determined. BRAFi, BRAF inhibitor; *ERBB2*^{amp}, amplification of *ERBB2*/HER2; Fusion+, positive for kinase gene fusions; MSI-H, microsatellite instability-high; *RAS*wt, *RAS* wild-type.

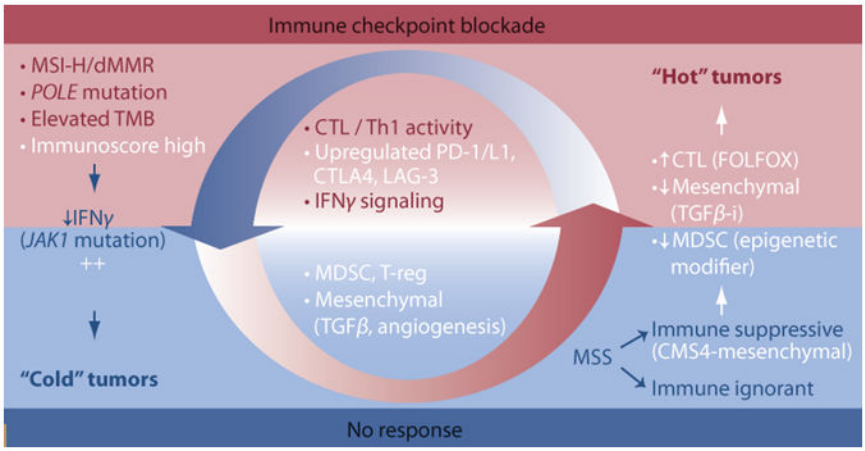

Figure 2. Optimization of immunotherapy in CRC.

Many of the genetic and/or clinical features that determine responsiveness to immune-checkpoint inhibitors (ICIs) (inside circle) are associated with genotypes and phenotypes (outside circle) that can be modulated. In patients with CRC, clinical (red and blue text) or pre-clinical (white text) data are available on a few biomarkers and/or mechanisms that might enable the modification of treatment responses. The best-described mechanisms of resistance in patients with hypermutated and/or immunogenic cancers include loss of IFNγ response owing to *JAK1* mutations. The potential to promote immune-cell infiltration is strongest in tumours with an immunosuppressive phenotype, although limited clinical data are available on this possibility in patients with CRC. Experimental data suggest that chemotherapies, inhibition of TGFβ, as well as epigenetic modifiers that target MDSCs might all promote immune-cell infiltration. The expansion of this simplified model is an important task for the optimization of ICIs in the coming years. CMS, consensus molecular subtypes; CTL, CD8+ cytotoxic lymphocyte; IFNγ, interferon-gamma; MDSC, myeloid-derived suppressor cells; MSI-H, microsatellite instability-high; MSS, microsatellite stable; TGFβ-i, TGFβ inhibition; Th1, T helper 1 cell; T-reg, regulatory T cell; white arrow up/down; upregulation or increased levels/down-regulation or decreased levels.

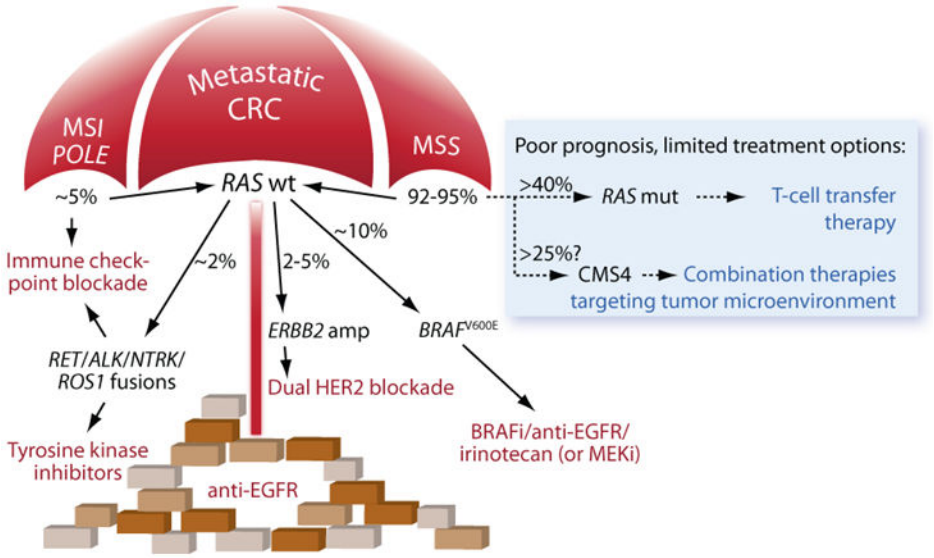

Figure 3. Treatment options and biomarker interactions in metastatic CRCs.

EGFR targeted therapies, guided by *RAS* mutation status remain the foundation of biomarker stratified medicine in patients with metastatic CRC, although treatment options are expanding, guided by several low-prevalence biomarkers and biomarker combinations. CRCs of the CMS4 subtype and/or those harbouring *RAS* mutations are important target populations for the development of new treatment strategies. BRAFi, BRAF inhibition; CMS, consensus molecular subtypes; MEKi, MEK inhibition; MSI, microsatellite instability; MSS, microsatellite stability; *RAS*, *KRAS*/*NRAS*.

Table1.

Biomarkers with therapeutic implications in patients with CRC

Biomarker	Cancer stage	Prevalence in relevant cancer stage	Biomarker complexity		
			Patient prognosis	Treatment benefit	Interactions with therapeutic implications
MSI-H/dMMR	Stage II	>15%[46]	[a]Favorable OS in meta-analysis of stage II or III (HR 0.67, 95% CI 0.58-0.78[32]) and DFS in retrospective analysis of patients with untreated stage II or III colon cancers in randomized trials (HR 0.51, P = 0.009)[34].	[a]Lack of benefit from 5-FU-based chemotherapy (no effect of treatment on survival in retrospective analyses of patients with stage II or III CRCs in randomized trials; Table 2).	Enriched for immunoscore-high tumours, and immunoscore may have superior prognostic value[45,90].
	Metastatic	3-5%[92,93]	[b]Inferior OS (HR 1.35, 95% CI 1.13-1.61) and PFS (HR 1.33, 95% CI 1.12-1.57) in retrospective analysis of pooled data from patients treated with standard therapies in randomized trials[92].	[a]Benefit from ICIs (ORR 39% and DCR 75%; summarized from 125 patients treated with single-agent ICIs, mostly in prospective single-arm trials[16,18,117]; Table 3).	Enriched for the drug targets *BRAF*^{V600E}and kinase fusions[83,148]. Hypermutated phenotype (including *POLE* mutations) may be a better predictive marker for ICIs (Table 3).
BRAF^{V600E}	Metastatic	~10%[211]	[a]Median OS < ~1 year for patients treated with standard therapies[164,165]. Inferior OS also after metastasectomy (HR for OS > 2.7, P < 0.01)[170,171]. Retrospective analyses..	[a]Benefit from targeted combination therapies in prospective randomized trials with vemurafenib plus irinotecan plus cetuximab (ORR 16%)[21], and with encorafenib plus binimetinib plus cetuximab (ORR 48%)[203].	Prognostic value limited to MSS cancers in the primary setting[150], possibly independent of MSI status in metastatic disease. Response to ICIs if MSI-H (ORR 55% with nivolumab plus ipilimumab[122]).
HER2 over-expression/ *ERBB2* amplification	Metastatic	~2%[220]	-	[b]No efficacy of anti-EGFR antibodies in retrospective analyses of *RAS* and *BRAF* wild-type cancers (HR for PFS ≥2.8, P < 0.001 in patients with amplification versus no amplification)[228]).	Predictive value in *RAS* wild-type cancers.
				[b]Benefit from dual HER2 inhibition in prospective trials with lapatinib plus trastuzumab (ORR 30% and DCR 59%)[225], and with trastuzumab plus pertuzumab (ORR 38%)[231].	
Kinase fusions (*ALK*/*NTRK*s/ *RET*/*ROS1*)	Metastatic	<2%[88]	[b]Poor prognosis on standard therapies in retrospective analyses of selected patient series (HR for OS ≥ 2.17, P < 0.001)[248,249].	[b]Benefit from tyrosine kinase inhibitors (responses have been reported in a few patients treated as part of prospective basket/umbrella trials[249,253-257]).	Strongly enriched in sporadic MSI-H and *RAS* wild-type cancers. Response to ICI has been reported in one patient with MSI-H cancer[248].
RAS mutations	Metastatic	~40% (Supplementary Table 1)	[b]Inferior OS in pooled analysis of randomized trials of standard therapies (HR 1.35, P <0.001[313]), also after	[a]No benefit from anti-EGFR antibodies in prospective randomized trials (HR for OS 0.72, P < 0.01 and HR for PFS 0.60, P <0.001 in retrospective	Mutually exclusive with mutations in other components of the MAPK signaling pathway which may

Biomarker	Cancer stage	Prevalence in relevant cancer stage	Biomarker complexity		
			Patient prognosis	Treatment benefit	Interactions with therapeutic implications
			metastasectomy (HR 1.67, P <0.001; meta-analysis[315]) (Supplementary Table 1).	analyses of pooled data for patients with *RAS* wild-type versus *RAS*-mutant cancers treated with anti-EGFR antibodies[29])	confer resistance to anti-EGFR antibodies in RAS wild-type cancers[29].
CMS4-mesenchymal/ stromal gene expression subtype	Primary and metastatic	Primary: ~25%[22]; advanced stage: >25%[345]	[b]Inferior OS (HR >1.5, P ≤0.021) in retrospective analyses of patients with primary CRC[22,341,342]. CMS1 might confer inferior OS compared with CMS4 in metastatic cancers (HR 2.9, P = 0.017)[346].	[b]Poor benefit from standard therapies in retrospective analyses of 5-FU[337] and oxaliplatin[352] in primary cancers, and anti-EGFR antibodies in *KRAS* wild-type metastatic cancers[335,350].	Mostly MSS[22].

[a]Biomarker recommended for clinical testing in this setting.

[b]Biomarker currently not recommended for clinical testing in this setting (owing to conflicting data and/or small patient numbers).95% CI, 95% confidence interval; DCR, disease control rate; DFS, disease-free survival; ICI, immune checkpoint inhibitors; ORR, objective response rate; OS, overall survival; PFS, progression-free survival.

Table 2.

Retrospective analyses of survival outcomes of patients with MSI-H/dMMR stage II or III CRCs receiving 5-FU-based chemotherapy

Study	Study design	Stage	Treatment	MSI-H/dMMR cancers (*n*)	Treatment benefit in MSI-H/dMMR	Interaction (MSI/dMMR-status and treatment)
Ribic *et al.*[63]	Pooled analysis of 5 randomized phase III trials	II and III[a]	Randomization to 5-FU + LV/LEV versus no chemotherapy	95	Worse OS (non-significant) with treatment versus no treatment in MSI-H (HR = 2.14, P = 0.11)	Significant (P = 0.01)
Carethers *et al.*[60]	Consecutive single-centre series	II and III	No randomization: 5-FU or no chemotherapy	36	No effect of treatment on OS in MSI-H (P = 0.52)	NA (although treatment was associated with improved OS in non-MSI-H [P = 0.048])
Benatti *et al.*[58]	Patients from 3 centres	II and III	No randomization: 5-FU-based or no chemotherapy	206	No effect of treatment on DSS in MSI-H (HR = 0.55, 95% CI 0.20-1.69)	NA (although treatment was associated with improved DSS in stage III MSS [P = 0.02], but not in stage II MSS [(P = 0.32])
Lanza *et al.*[61]	Consecutive single-centre series	III	No randomization: 5-FU + FA or no chemotherapy	41	No effect of treatment on DSS in dMMR (P = 0.91)	NA (although treatment was non-significantly associated with improved DSS in non-dMMR [P = 0.08])
Jover *et al.*[62]	Patients from 10 centres	II and III	No randomization: 5-FU or no chemotherapy	60	No effect of treatment on OS (69.2% and 73.5% in treated and untreated, P = 0.8) or DFS (57.7% and 67.6% in treated and untreated, P = 0.6) in dMMR	Significant (HR = 2 for OS and DFS, P = 0.0001)
Sargent *et al.*[34]	Pooled analysis of 5 randomized trials	II and III[a]	Randomization to 5-FU + LV/LEV versus no chemotherapy	70[b]	No effect of treatment on DFS in dMRR (multivariable HR = 1.39, P = 0.56)	Non-significant (P = 0.18; although treatment was associated with improved DFS in MMR proficient [HR = 0.67, P = 0.02])
Hutchins *et al.*[35]	Randomized phase III trial	II and III (mainly II)	No randomization: 5-FU + FA or no chemotherapy	218	No effect of treatment on risk of recurrence in dMMR (OR = 0.8, 95% CI 0.29-2.22)	NA (although treatment was associated with lower risk of recurrence in MMR proficient [OR = 0.59, 95% CI 0.46-0.78])

[a]Colon cancer only

[b]Only patients independent from Ribic et al., N Eng J Med 2003 are included. 5-FU, 5-fluorouracil; 95% CI, 95% confidence interval; DFS, disease-free survival; DSS, disease-specific survival; FA, folinic acid; HR, hazard ratio; LEV, levamisole; LV, leucovorin; NA, not analyzed; OR, odds ratio; OS, overall survival.

Table 3.

Data from clinical studies of immune-checkpoint inhibition in hypermutated metastatic CRCs

Study	Study design	Agent	Hypermutated metastatic CRCs (*n*)	ORR[a]	DCR[b]
Monotherapies					
Lipson *et al.* (2013)[372]	Single-arm phase I trial in patients with treatment-refractory solid tumours	Nivolumab	1 MSI-H	Durable complete response	NA
Le *et al.* (2015)[16]	Single-arm phase II trial in patients with treatment-refractory metastatic MSI-H CRCs, MSS CRCs and MSI-H other	Pembrolizumab	10 MSI-H (HNPCC and sporadic)	40%	90 %
Le *et al.* (2017)[117]	Single-arm phase II of treatment-refractory metastatic MSI-H/dMMR cancers from 12 cancer types	Pembrolizumab	40 MSI-H/dMMR (HNPCC and sporadic)	52%	82 %
Overman *et al.* (2017)[18]	Reporting from monotherapy arm of a phase II study of treatment-refractory metastatic MSI-H/dMMR CRCs[c]	Nivolumab	74 MSI-H/dMMR (ongoing)	31%	69 %
Gong *et al.* (2017)[19]	Case report of treatment-refractory MSS metastatic CRC	Pembrolizumab	1 *POLE*-mutated	Clinical response	NA
Fabrizio *et al.* (2018)[133]	Case report of treatment-refractory MSS metastatic CRC	Pembrolizumab	1 *POLE*-mutated	Radiographic response	NA
Combination therapies					
Overman *et al.* (2018)[122]	Reporting from combination therapy arm of a phase II study of treatment-refractory metastatic MSI-H/dMMR CRCs[c]	Nivolumab plus ipilimumab	119 MSI-H/dMMR (ongoing)	55%	80 %
Lenz *et al.* (2018)[123]	First-line treatment[c]	Nivolumab plus ipilimumab	45 MSI-H/dMMR (ongoing)	60%	84%
Hochster *et al.* (2017)[373]	Reporting from one treatment arm of a phase Ib study	Atezolizumab plus bevacizumab	10 MSI-H	30%	90 %

CRC, colorectal cancer; DCR, disease control rate; HNPCC, hereditary nonpolyposis colorectal cancer; MSI, microsatellite instability; MSS, microsatellite stable; NA, not applicable; ORR, objective radiographic response rate

[a]Complete or partial response according to RECIST1.1.

[b]Complete/partial response or stable disease according to RECIST1.1.

[c]Same clinical study.