Gastroenterology Report, 8(1), 2020, 11–24

doi: 10.1093/gastro/goz061
Advance Access Publication Date: 25 November 2019
Review

REVIEW

Immunotherapy with immune checkpoint inhibitors in colorectal cancer: what is the future beyond deficient mismatch-repair tumours?

Nicolas Huyghe[1], Paméla Baldin[2] and Marc Van den Eynde[1,3,*]

[1]Institut de Recherche Clinique et Expérimentale (Pole MIRO), UCLouvain, Brussels, Belgium; [2]Department of Pathology, Cliniques Universitaires St-Luc, Institut Roi Albert II, Brussels, Belgium; [3]Department of Medical Oncology, Cliniques Universitaires St-Luc, Institut Roi Albert II, Brussels, Belgium

*Corresponding author. Service d'Oncologie Médicale, Institut Roi Albert II, Cliniques Universitaires Saint-Luc and Institut de Recherche Clinique et Expérimentale, Université catholique de Louvain (UCLouvain), Avenue Hippocrate 10, 1200 Brussels, Belgium. Tel: +32-27641041; Fax: +32-27645428; Email: marc.vandeneynde@uclouvain.be

Abstract

Following initial success in melanoma and lung tumours, immune checkpoint inhibitors (ICIs) are now well recognized as a major immunotherapy treatment modality for multiple types of solid cancers. In colorectal cancer (CRC), the small subset that is mismatch-repair-deficient and microsatellite-instability-high (dMMR/MSI-H) derive benefit from immunotherapy; however, the vast majority of patients with proficient MMR (pMMR) or with microsatellite stable (MSS) CRC do not. Immunoscore and the consensus molecular subtype classifications are promising biomarkers in predicting therapeutic efficacy in selected CRC. In pMRR/MSS CRC, biomarkers are also needed to understand the molecular mechanisms governing immune reactivity and to predict their relationship to treatment. The continuous development of such biomarkers would offer new perspectives and more personalized treatments by targeting oncological options, including ICIs, which modify the tumour-immune microenvironment. In this review, we focus on CRC and discuss the current status of ICIs, the role of biomarkers to predict response to immunotherapy, and the approaches being explored to render pMMR/MSS CRC more immunogenic through the use of combined therapies.

Key words: colorectal cancer; immunotherapy; immune checkpoint inhibitors; immune response; immunoscore

Introduction

Colorectal cancer (CRC) is the second most common cancer in women and the third most common in men. Despite advances in the diagnosis and management of this disease, CRC remains the fifth cause of cancer-related death in women and the fourth cause in men [1]. Moreover, the global CRC burden is expected to increase by 60% by 2030 [2].

The immune system distinguishes self from non-self through the binding of T-cell receptors (TCR) on T-cells to complexes of peptides with major histocompatibility complex (MHC) class I molecules presented on the surface of all cells, including tumour cells [3, 4]. Recognition of peptide–MHC class I complexes by the TCR alone is insufficient for T-cell activation. TCR–MHC signalling pathways are modulated by co-stimulatory

Submitted: 7 August 2019; **Revised:** 25 September 2019; **Accepted:** 22 October 2019

or co-inhibitory signals, which tumour cells exploit to escape destruction [5, 6]. Immune checkpoint inhibitors (ICIs) are a type of immunotherapy often made from antibodies. ICIs target co-inhibitory receptors, such as cytotoxic T-lymphocyte-associated antigen 4 (CTLA-4) and programmed cell death protein 1 (PD-1) on T-cells and other immune-cell subpopulations, or their ligands, such as programmed cell death protein 1 ligand 1 (PD-L1) on tumour cells and various immune cells. Over the past decade, ICIs have revolutionized the field of oncology through demonstrated clinical efficacy in several cancers, including melanoma and non-small-cell lung cancer. To date, other inhibitory receptors such as lymphocyte-activation gene 3 (LAG-3), T-cell immunoglobulin (Ig) mucin 3 (TIM-3), T-cell immunoreceptor with Ig and ITIM domains (TIGIT), and activating receptors such as the tumour necrosis factor receptor (TNFR) superfamily, member 4 (OX40), the glucocorticoid-induced TNFR-related protein, the inductible T-cell costimulator, and CD40 have been identified and are currently evaluated as targets of monoclonal antibodies in different clinical trials [7].

In CRC, it has been shown that only patients with the subset of mismatch-repair-deficient or microsatellite instability-high (dMMR/MSI-H) tumours are likely to respond to treatment with ICIs [8–10]. This subset is characterized by an increased number of tumour mutations [11] due to inactivation of one of the four mismatch-repair (MMR) genes: *MSH2*, *MLH1*, *MSH6*, and *PMS2* [12–15]. In 12% of CRC cases, epigenetic changes cause sporadic dMMR/MSI-H, in particular methylation of the *MLH1* promoter. While, in 3% of CRC cases, dMMR/MSI-H is due to germ-line MMR mutation (Lynch syndrome) [16]. In 2017, the Food and Drug Administration (FDA) approved the anti-PD-1 inhibitors pembrolizumab (Keytruda®, Merck) and nivolumab (Opdivo®, Bristol-Myers Squibb) for the treatment of patients with dMMR/MSI-H CRC, but the European Medicines Agency is still waiting for the results of phase III randomized–controlled studies.

Unlike dMMR/MSI-H CRC patients, ICIs alone provide limited to no clinical benefit in CRC patients with proficient MMR or microsatellite stable (pMMR/MSS) tumours [8]. For these patients, ICIs are being actively explored in combination with treatments that aim to increase the intra-tumoural immune response and render the tumour 'immune-reactive'. In this review, we discuss the current use of ICIs in CRC, the role of biomarkers to predict CRC response to immunotherapy, and approaches currently under investigation to render pMMR/MSS CRC more immunogenic through the use of combined therapies.

Immunotherapy in CRC: current status

Ipilimumab (Yervoy®, Bristol-Myers Squibb) is a monoclonal antibody that targets the CTLA-4 protein receptor to activate the immune system [17–21]. Its rapid success, and that of monoclonal antibodies against PD-1 and its ligand PD-L1 [22–25], led to the active investigation of ICIs in all cancer types. In the initial trials, which included patients with unselected metastatic CRC (mCRC), only three out of >100 patients with treatment-refractory mCRC experienced a partial or complete response following anti-CTLA-4 or anti-PD-1/PD-L1 treatment [23, 26–28]. Retrospectively, it was found that all responders harboured dMMR/MSI-H tumours. Most of these tumours foster an immunogenic microenvironment characterized by a high overall mutation burden (>12 mutations per 10^6 DNA bases), associated tumour neoantigens and T helper 1 (Th1) cytotoxic immune response with upregulation of PD-1/PD-L1-positive cells [29–33]. Based on the observed impressive tumour response, enthusiasm for immunotherapy in CRC grew and several studies investigated the therapeutic potential of PD-1 inhibitors.

Le and colleagues reported the results of a phase II proof-of-concept study (KEYNOTE-016) of dMMR/MSI-H tumours treated with pembrolizumab (10 mg/kg every 2 weeks) [8]. In this trial, which included 41 patients with dMMR/MSI-H and pMMR/MSS chemorefractory mCRC and dMMR/MSI-high non-CRC patients, the overall response rate (ORR) was 40% (4 of 10 patients). Clinically durable responses were observed in patients with dMMR/MSI-H mCRC, whereas no response (ORR = 0%) was observed in those with pMMR/MSS mCRC (0/18). Treatment was well tolerated overall, but 17 of 41 patients experienced a grade 3–4 treatment-related adverse event (TRAE). The updated results of this trial, which included 86 dMMR/MSI-H cancers, confirmed an ORR of 53%, with 21% complete responses. In CRC, objective responses were observed in 52% of patients [34]. The 2-year overall survival (OS) rate was 64% for these highly pretreated cancers [34].

CheckMate-142, a multicohort non-randomized phase II study, evaluated the efficacy and safety of nivolumab (3 mg/kg every 2 weeks) in combination with ipilimumab (1 mg/kg every 3 or 6 weeks), or nivolumab as a single agent in previously treated or treatment-naïve dMMR/MSI-H mCRC [9, 10, 35]. The results of this study confirmed the impressive treatment benefit of these drugs in this setting. In chemorefractory mCRC patients, the ORR for nivolumab monotherapy ($n = 74$) was 31% and for the nivolumab/ipilimumab combination ($n = 119$) it was 55%; 1-year OS rates were 73.4% and 85%, respectively [9, 10]. In treatment-naïve patients, the corresponding ORR and 1-year OS rates for first-line patients treated with the combination regimen ($n = 45$) were 60% and 83%, respectively [10]. With >2 years of follow-up at the time of reporting, the median OS of all cohorts had not been reached. Grade 3–4 TRAEs were higher in the combination regimen (32%) except for the cohort that received combined therapy with low-dose ipilimumab (1 mg/kg every 6 weeks). In these patients, grade 3–4 TRAEs were 16%, suggesting that the low-dose combination is the best treatment regimen in this setting. Efficacy of nivolumab and ipilimumab combination was also recently reported in the preoperative setting. In this ongoing study, authors preliminarily reported that a short preoperative treatment (6 weeks) with ipilimumab (1 mg/kg, day 1) plus nivolumab (3 mg/kg, day 1, 15) in non-metastatic colon cancer was safe and led to major pathological response (≤2% of residual vital tumour cells) in all dMMR/MSI-H tumours (seven of seven patients) [36]. No sign of pathological response (85%–100% of residual vital tumour cells) was observed in the eight patients with pMMR/MSS CRC tumours.

Several randomized phase III trials are currently ongoing in dMMR/MSI-H mCRC to evaluate the efficacy of anti-PD-1, anti-PD-L1, and anti-CTLA-4 either combined with or compared to chemotherapy and with or without targeted therapy [37]. However, dMMR/MSI-H status, well recognized for its favourable prognosis in localized CRC [38], results in a lower proportion of patients (3%–4%) with metastatic dMMR/MSI-H CRC [39] who are available to benefit from such trials.

Unlike patients with dMMR/MSI-H mCRC, immunotherapy alone has not demonstrated a clinical benefit in pMMR/MSS mCRC and this subset constitutes most tumours. In the pivotal KEYNOTE-016 study with pembrolizumab, no responses were observed in these patients [8], consistently with the lack of efficacy of immunotherapy in early studies with non-selected patients, most of whom had pMMR/MSS mCRC. In the CheckMate-142 study, limited responses were seen in pMMR/MSS tumours. The lack of CRC immunoreactivity and recruitment of immune cells seems to be the fundamental obstacle to efficacy. Combination treatment with PD-1 inhibitors and

modulators of other immune checkpoint molecules, such as CTLA-4, might be beneficial in a subset of patients with pMMR/MSS tumours, as reported in a recent study [40]. Nevertheless, alternative approaches to modulate the tumour-immune microenvironment are currently being explored for the many CRC patients who harbour the pMMR-MSS subtype.

Biomarkers for response to immunotherapy beyond dMMR/MSI status

The presence of dMMR/MSI-H in solid tumours, including CRC, is now a clear potential biomarker for response to immunotherapy [7]. However, given the complexity of the antitumour immune response and the intra-tumoural and inter-metastasis heterogeneity, dMMR status alone is presumably not enough to accurately identify responders to ICIs [41]. Identification of more precise and reliable predictive biomarkers continues to be an unmet clinical need. A summary of promising biomarkers discussed hereafter is shown in Figure 1.

PD-1/PD-L1 expression

PD-1 is expressed by activated T-cells, B-cells, and natural killer (NK) cells and can bind to its ligand, PD-L1, as expressed on tumour cells [42]. This expression is, in part, induced by interferon-gamma (IFN-γ) that is produced by activated lymphocytes. PD-L1 expression, measured by immunohistochemical staining, has been extensively evaluated as a predictive biomarker of response to ICIs. Interestingly, in some tumour types, such as non-small-cell lung carcinoma (NSCLC), gastric cancer, and oesophageal tumours, PD-L1 expression might be useful as a predictive marker of response to anti-PD-1 therapy [43–46]. However, several issues prevent PD-L1 expression from being a clinically useful biomarker. The use of various PD-L1-detection antibodies, the lack of standardization of PD-L1-expression evaluation, and the percentage of positive cells cut-off (ranging from >1% to >50% of tumour cells stained) limit its statistical significance. Moreover, PD-L1 expression can be induced by IFN-γ or constitutive oncogene activation [47]. PD-L1 expression is a dynamic process that changes according to the tumour microenvironment and disease stage, and it could well be influenced by treatment [48]. PD-L1 expression in tumours is not uniform, and the sampling time and location may affect the results of PD-L1 staining [49]. Finally, patients who respond to ICIs, but do not express PD-L1, limit its clinical impact.

In CRC, PD-L1 expression was poorly correlated with dMMR/MSI-H status [50] and not found to be associated with response

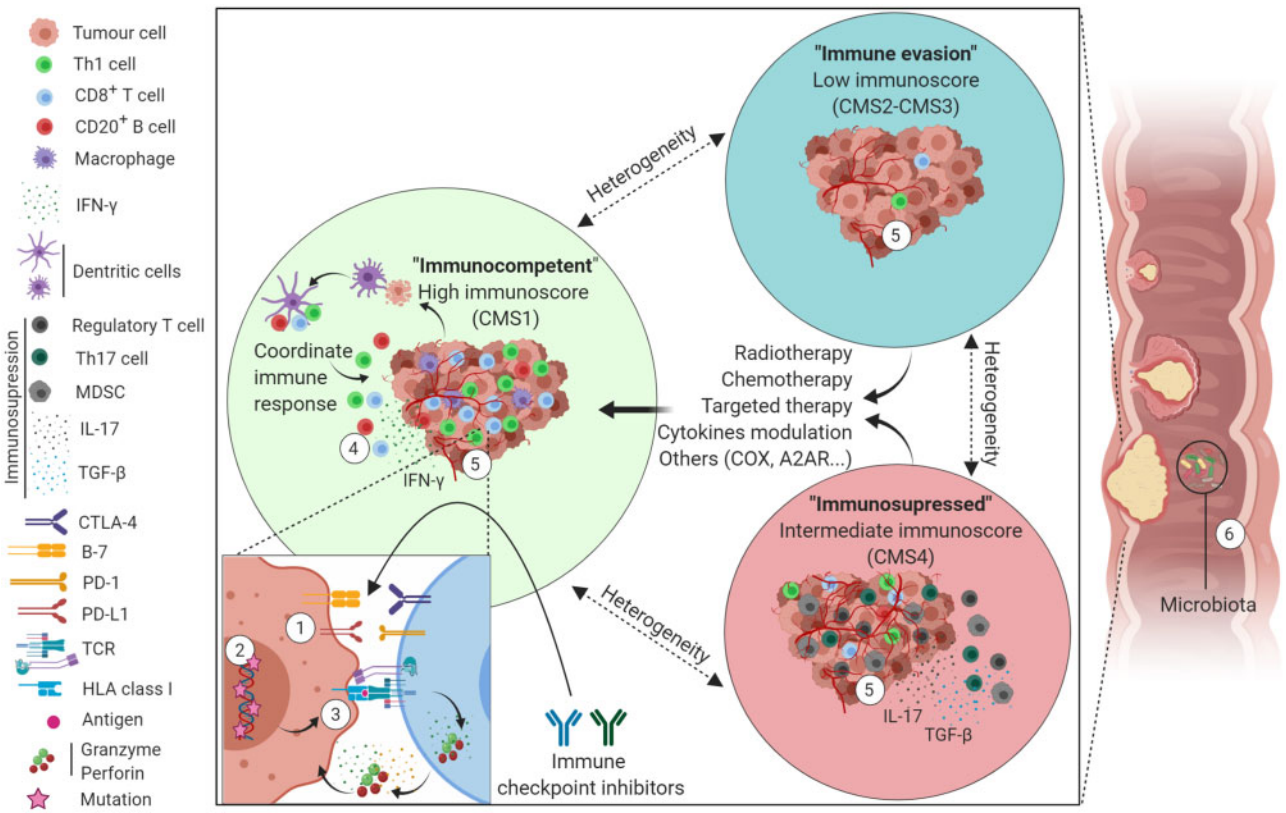

Figure 1. Schematic representation of colorectal-cancer-immune subgroups, linked biomarkers, and potential treatment strategies. 'Immunocompetent' tumour (green circle) is characterized by a coordinated immune response with high T-cells (CD3, CD8, and Th1), macrophage infiltration, and upregulation of immune checkpoint molecules (CTLA-4, PD-1, and PD-L1). 'Immune evasion' group (blue circle) is characterized by poor immune cell infiltration. 'Immunosuppressed' group (red circle) is characterized by high immune cell infiltration as well as a high infiltration of suppressor cells with suppressive cytokine release. The 'immune evasion' and the 'immunosuppressed' groups could be treated with radiotherapy, chemotherapy, targeted therapy, cytokine modulation, or other approaches such as COX inhibition or A2AR inhibition, to render the tumours more immunogenic so that they benefit from ICIs. Possible biomarkers of response to ICIs are marked from 1 to 6. (1) PD-1/PD-L1 expression. (2) Tumour-mutation burden. (3) Mutation-associated neoantigens presentation on HLA class I. (4) High cytotoxic immune cells infiltration (immunoscore). (5) Gene-expression signature (including CMS classification). (6) Gut microbiome. A2AR, adenosine A2A receptor; COX, cyclooxygenase-2; CTLA-4, cytotoxic T-lymphocyte-associated protein 4; HLA, human leucocyte antigens; IFN-γ, interferon-gamma; IL-17, interleukin 17; MDSC, myeloid-derived suppressor-derived cells; PD-1, programmed death protein 1; PD-L1, programmed death-ligand 1; TCR, T-cell receptor; TGF-β, transforming growth factor-beta; Th17, T helper 17; CMS, consensus molecular subtype.

or survival in the registration studies [35], thus limiting its impact in this particular type of solid tumour. A recent meta-analysis revealed that PD-L1 expression can serve as a significant biomarker for negative prognosis that is not related to clinicopathological characteristics [51].

Endogenous antitumour T-cell immunity is largely restricted to PD-1-high cytotoxic lymphocytes. Infiltration of NSCLC with such PD-1 high $CD8^+$ T-cells has recently been associated with clinical response to anti-PD-1 [52, 53]. Interestingly, Llosa and colleagues similarly reported that pMMR/MSS tumours infiltrated by PD-1-high $CD8^+$ cytotoxic lymphocytes (without suppressor T helper 17 [Th17] cells) and expressing a high level of PD-L1 have an immune microenvironment resembling that of the dMMR/MSI-H and were associated with pembrolizumab benefit [54]. As previously reported in digestive cancers and melanoma [52], these PD-1-high $CD8^+$ cytotoxic lymphocytes were characterized by an exhausted/memory transcriptome, suggesting the presence of an antitumour T-cell repertoire [54].

Neoantigens and mutational burden

Due to the hypermethylation of *MLH1* [55, 56] or mutations in MMR genes [57], dMMR/MSI-H tumours harbour a high frequency of insertions/deletions (indels) in microsatellite sequences [58] and a high tumour mutational burden (TMB) [59] that result in a high mutation-associated neoantigen (MANA) load [29–31]. These neoantigens can be processed and presented by dendritic cells leading to the priming of a coordinated adaptive anticancer immune response [32], which explains the higher density of tumour-infiltrating lymphocytes (TILs) and activated Th1 cells, as well as increased type I interferon production, observed in these tumours. This tumour-immune surveillance leads to the immunoediting concept. Three essential phases have been proposed: elimination, equilibrium, and escape [60]. During the elimination, innate and adaptive coordinate immune responses act together for the successful eradication of tumour cells. During the equilibrium phase, the continuous immune-selection pressure also induces a Darwinian selection process in which new tumour variants emerge, carrying various genetic and epigenetic changes in tumour behaviour such as the upregulation of checkpoint receptors including CTLA-4, PD-1, PD-L1, indolamine 2, 3-dioxygenase 1 (IDO1), and LAG-3 [33], or the downregulation of antigen-presenting molecules. During the escape phase, various tumour-derived soluble factors contribute to the induction of a local immunosuppressive environment favouring immune escape, progression, and metastasis [61, 62].

In CRC, 12.8% of tumours simultaneously present TMB-high, dMMR-MSI-H, and PD-L1 expression [50]. A high percentage of concordance (44.2%) of TMB-high and dMMR-MSI-H was observed [50] and was predictive of response to ICIs [40, 63]. However, PD-L1 expression demonstrates great variability and can be expressed even in dMMR-MSI-H-negative and/or TMB-low cases. Interestingly, it is possible to have a TMB-high in the absence of dMMR-MSI-H, whereas dMMR-MSI-H with TMB-low is rare [50]. There is the need to standardize PD-1/PD-L1 expression and define cut-offs for TMB interpretation. The development of a liquid-biopsy method to identify tumours with dMMR-MSI-H or TMB-high is a non-invasive technique demonstrating its efficacy for the determination of response to ICIs [64].

Additionally, tumours harbouring mutations in the region encoding for the *POLE* exonuclease domain also presented high TMB and MANA load [65, 66]. *POLE* mutations are present in 1%–2% of all CRC tumours. Similarly to dMMR/MSI-H CRC, patients with *POLE*-mutated CRC have a good prognosis, resulting in a lower proportion of mCRC than in stage I–III CRC [67]. They also present a higher level of $CD8^+$ lymphocyte infiltration, expression of cytotoxic T-cell markers, and effector cytokines [68]. Given the similarly enhanced immunogenicity of *POLE*-mutated CRCs to dMMR/MSI-H CRCs, the therapeutic potential of immune checkpoint blockade in the subset of POLE-mutated CRCs is of particular interest [69]. Further investigation is currently underway in clinical trials. Therefore, a high TMB and MANA load, associated with a high T-lymphocyte infiltration, and T-cell diversity [70–72] have emerged as biomarkers of response to ICIs in several tumour types [73, 74].

Immune infiltration (immunoscore)

Interestingly, a high TMB might not always be necessary to drive an immune response. Evaluation of the presence of tumour-infiltrating $CD3^+$ and $CD8^+$ lymphocytes, through assignment of an immunoscore based on the density and the location of subsets of T-cells, was prognostic of clinical outcome in patients with stage I–III CRC and performed better than MSI and MMR status [75–77]. The immunoscore is a simple scoring system based on the numeration of two lymphocyte populations (CD3/CD45RO, CD3/CD8, or CD8/CD45RO), both in the core of the tumour and in the invasive margin of tumours. The immunoscore provides a score ranging from Immunoscore 0 (I0) when low densities of both cell types are found in both regions to Immunoscore 4 (I4) when high densities are found in both regions [78].

Recently, the prognostic impact of the immunoscore was validated in a study with samples from 2,681 stage I–III colon cancer patients from 14 centres in 13 countries [77]. Interestingly, the authors showed that patients with a high immunoscore had similar tumour relapse and survival, and that this was independent of their dMMR/MSI-H status. Therefore, it is possible to conclude that the favourable prognosis observed in dMMR/MSI-H-localized CRC is essentially related to high immune infiltration. Furthermore, the prognostic value of the immunoscore was validated in a meta-analysis of eight studies published between 2011 and 2018 [79], supporting the idea that TILs play an important role in disease control across all disease stages by preventing the dissemination of metastasis to lymph nodes and organs.

In mCRC characterized by multiple tumour lesions among different organs, the picture is more complex. We previously reported a comprehensive analysis of patients having undergone complete resection of all metastases and revealed the heterogeneity of the metastatic disease and its clinical impact [80–83]. Complex tumour-immune interrelations shape the metastatic landscape, not only in terms of lesion size, number, or mutational pattern, but also in terms of immune-cell infiltration. We observed a heterogeneous immune infiltrate, immunoscore, and mutational diversity within the multiple resected synchronous and metachronous metastases of patients. Adaptive immune cells and immunoscore quantified in the least-infiltrated metastasis per patient were the most associated with patient long-term survival. Within any specific patient, a high immune infiltrate/high immunoscore correlated with fewer metastases and improved long-term survival [80, 81]. In patients with unresectable mCRC, where adequate immunoscore assessment is impossible, we reported that a single biopsy of a metastasis was able to accurately identify low-infiltrated metastases, but that the overall intra-metastatic

immune infiltrate might be better estimated with multiple biopsies or the sampling of larger tumour areas [81]. Importantly, on biopsies, the performance of the immunoscore was superior to that of PD-L1 in estimating the reality of concordance across the whole metastatic slide [80, 81]. This illustrated the fact that PD-L1 stainings were more heterogeneous in a given metastasis.

High immunoscores reported in pMMR/MSS CRCs raise the question of whether immunophenotyping might predict which patients will benefit from immunotherapy. Just recently, some authors reported that higher $CD3^+$ and $CD8^+$ T-cell densities (but not PD-L1 expression on cancer cells) were associated with a higher ORR and duration of disease control in a small subgroup of patients with dMMR/MSI-H mCRC treated with pembrolizumab [84]. This suggests that a lower immunoscore is associated with immunotherapy resistance in dMMR/MSI-H tumours, which is consistent with the reported finding that lower immunoscores are associated with worse survival in patients with dMMR/MSI-H stage III colon cancers receiving cytotoxic chemotherapy [85]. Hence, combining the immunogenic features of the tumour microenvironment with TMB may be more precise in predicting immunotherapy response than either feature alone. Further validation of immunophenotyping as a predictive biomarker of immunotherapy response, specifically in metastatic disease, is needed for broad clinical utility and is currently being explored in our trials (NCT03608046 and NCT03127007).

Gene-expression signature

A comprehensive re-evaluation and comparison of CRC molecular gene-expression profiles has enabled the CRC Subtyping Consortium (CRCSC) to identify four robust consensus molecular subtype (CMS) classifications. The first type, CMS1—immune, is mainly composed of dMMR/MSI-H tumours and is characterized by a high TMB, high immune infiltration and activation, and *BRAF* mutations. The second type, CMS2—canonical, is characterized by *WNT* and *MYC* activation. The third, CMS3—metabolic, is characterized by cancer-cell metabolic deregulation and *KRAS* mutation. The fourth type, CMS4—mesenchymal, is characterized by stromal infiltration, transforming growth factor-beta (TGF-β) activation, and angiogenesis [65]. Interestingly, the immune microenvironment of each CMS type is different. Stage-independent prognostic values and significant associations with clinical, biological, and treatment features have been demonstrated and recently validated in phase III clinical studies [86–88].

CMS1 and CMS4 are both 'hot' tumours; they are considered to be immune-reactive and highly infiltrated by immune cells as opposed to CMS2 and CMS3, which are 'cold' tumours. Despite CMS1 and CMS4 being immune-reactive, they each present distinct immune features and escape mechanisms. CMS1 present $CD8^+$ T-cells and $CD68^+$ macrophage infiltration as well as frequent upregulation of immune checkpoint molecules (CTLA-4, PD-1, and PD-L1) responsible for the main immune escape mechanism in these tumours [89]. CMS4 present a different pattern of immune infiltration, which is mainly suppressive throughout the infiltration of myeloid-derived suppressor cells (MDSCs), T-regulatory cells (Tregs), monocyte-derived cells, and Th17 cells. In these tumours, immunosuppressive factors such as TGF-β and CXCL12 are upregulated along with chemokines, such as interleukin 23 (IL-23) and interleukin 17 (IL-17) [90]. CMS1 and CMS4 tumours are therefore likely to respond well to immune therapies, but they should each be treated distinctly. Patients harbouring CMS1 tumours could theoretically benefit from ICIs alone, whereas those with the CMS4 subtype would be best suited to strategies combining TGF-β inhibitors, Tregs, MDSCs inhibition, and ICIs. The CMS classification is a promising new biomarker of response to immune therapies; however, as is the case with other biomarkers, care should be taken when selecting biopsies and resection specimens for CMS classification. It should be borne in mind that CMS classification was mainly derived from samples of primary non-metastatic CRC (92% of the samples); it was not totally reproducible on metastatic samples (e.g. liver metastases) [91] or validated in the metastatic setting [86]. Additionally, spatial- and temporal-tumour heterogeneity, predominant in the metastatic setting, can misevaluate CMS status. Thus, the source of the sample and prior treatments before collection must be carefully considered.

A recent study described an innate immune response in some CRC as being due to the upregulation of PD-L1 and IDO1 linked to DNA damage. In order to identify this subtype of tumours with defective DNA-damage response (DDR), the authors developed a 44-gene signature assay and reported that 80% of dMMR/MSI-H and 25% of pMMR/MSS tumours presented the signature. The DDR assay could therefore enable the identification of not only dMMR/MSI-H tumours, but also rare cases of pMMR/MSS tumours likely to respond to ICIs [92].

Microbiota

The gut microbiome also influences the outcome of cancer therapy by modulating the host inflammatory response [93, 94]. An intact microbiome is required for successful tumour control in response to genotoxic (e.g. oxaliplatin used in CRC) and immunomodulatory therapies (e.g. cyclophosphamide). Recent studies have reported the important role of the gut microbiome with ICI treatment [95–97]. It was found that primary resistance to ICIs (in melanoma and lung cancers) could be attributed to an abnormal gut microbiome due to the use of antibiotics. One study raises the hypothesis that transplanting faecal material from responding patients to non-responders could lead to improved tumour control. In this study, it was shown that, when germ-free mice were transplanted with faecal material from melanoma patients, they experienced improved tumour control, augmented T-cell responses, and greater efficacy of anti-PD-LI therapy [98]. Metagenomics of patient stool samples at diagnosis have also revealed correlations between clinical responses to ICIs and the relative abundance of *Akkermansia muciniphila* [95]. This influence of the microbiome in the outcome of cancer treatment and the function of anticancer immunity poses new questions from a preclinical and clinical standpoint in the CRC field. Despite some evidence of an association between the gut microbiome and CRC, its role in the treatment of advanced and metastatic CRC remains largely unexplored.

Immunotherapy for pMMR/MSS CRC: strategies and development

The recent success of anti-PD-1 and anti-CTLA-4 treatments in dMMR/MSI-H patients and preclinical data showing that dMMR/MSI-H tumours are not the only subgroup of CRC that could, theoretically, benefit from ICIs have led to the design of new clinical studies. These trials aim to select the subset of CRC patients most likely to respond to ICIs or to design novel therapeutic strategies to render these tumours 'immune-competent' (Figure 1). Some oncological treatments may also be able to cause immunogenic cell death (ICD)—a form of cell apoptosis

that can induce an antitumour immune response and potentially overcome the primary resistance of pMMR/MSS CRC to ICIs. The main treatment strategies currently being investigated in clinical trials are summarized in this section and in Table 1.

Radiation therapy

Preclinical and early clinical studies have suggested that radiation therapy (RT) or chemo-radiation therapy (CRT) may be a clever way to expose neoantigens. By damaging DNA, radiotherapy induces tumour-cell death. This releases neoantigens creating immune-mediated antitumour responses [99] and ICD [100, 101]. In some patients, this 'neoantigens release' can act as an *in situ* radiation-induced vaccine [102, 103]. This immune effect is applicable not only to the irradiated tumour site, but also to distant sites through the 'abscopal effect' [104], which theoretically could be enhanced with ICIs. It has been shown, for example, that anti-CTLA-4 and/or anti-PD-L1 in combination with RT can work synergistically to significantly improve treatment response (including metastatic lesions outside the radiation field) and patient outcomes in metastatic melanoma and NSCLC [104, 105].

The data to understand the role of RT and ICIs in CRC are, however, limited. In mCRC, stereotactic body radiation therapy (SBRT) directed to a site of liver metastasis in combination with a PD-1 inhibitor did not illicit any response [106]. In another trial in mCRC evaluating pembrolizumab with palliative RT or with local ablation, one major response (1/11 patients, ORR = 9%) in a metastatic site distant from the irradiated field was observed in the RT cohort [107]. Recently, dual blockade of CTLA-4 (ipilimumab) and PD-1 (nivolumab) with RT (8 Gy in three fractions to a single metastatic lesion) demonstrated feasibility and promising activity in a phase II study that included 40 patients with chemorefractory mCRC [108]. Among the 27 patients treated with protocol-defined RT (33% of patients never received RT due to progression or grade 3–4 TRAE), the ORR and disease-control rate (DCR) were 15% and 37%, respectively. The durability of disease control was impressive, with a median of >15 months. Modification of the dosing schedule to reduce the dropout rate should be considered for future development. The outcome of correlative studies (biopsies, whole-exome sequencing) are eagerly awaited.

Several clinical trials evaluating the efficacy of conventional or stereotactic RT in combination with ICIs are ongoing (Table 1). Our research group is currently conducting a multicentre randomized phase II trial (R-Immune trial, NCT03127007) evaluating the benefit of atezolizumab (anti-PD-L1) with preoperative CRT (45–50 Gy over 5 weeks in combination with 5-fluorouracil) for locally advanced rectal cancer. The trial design delays atezolizumab administration until 2 weeks after CRT initiation in order to explore the role of CRT alone on the tumour-immune microenvironment. The trial also incorporates multiple tumour, blood, and stool collections to investigate the role of several biomarkers (as previously described) in multiple correlative sub-studies.

Chemotherapy and targeted therapies

The first two lines of treatment in mCRC currently involve a combination of targeted therapies that inhibit the epidermal growth factor receptor (EGFR; cetuximab and panitumumab), or angiogenesis (bevacizumab, aflibercept, or ramucirumab), together with chemotherapy (5-fluorouracil, irinotecan, oxaliplatin) [109–113]. Recent evidence suggests that these chemotherapy regimens can induce ICD by releasing damage-associated molecular patterns (DAMPs) [114, 115] and activating necrotic or apoptotic pathways [116]. The translocation of calreticulin is then recognized as a signal by dendritic cells (DCs) to mediate the phagocytosis of dying tumour cells. Agents such as 5-fluorouracil can induce the apoptosis of MDSCs, therefore inhibiting their immunosuppressive function and increasing $CD8^+$ T-cell function [117]. The analysis of the immune microenvironment of resected CRC liver metastases revealed that patients treated with preoperative chemotherapy had a significantly higher density of cytotoxic and memory T-cells compared with metastases of untreated patients [80, 81, 118]. Moreover, CRC liver metastases, which had achieved pathological and radiological responses, were associated with a significantly higher immunoscore, reflecting an increased adaptive immune response [81].

Vascular endothelial growth factor (VEGF) is a signal protein that stimulates the formation of blood vessels. It is often upregulated in cancer and contributes to tumour angiogenesis [119]. It also plays a role in the immune microenvironment by upregulating immune checkpoint molecules (PD-1, PD-L1, CTLA-4, LAG-3) and downregulating antigen-presentation molecules. Additionally, VEGF inhibits DC maturation and increases the function of suppressor cells [120–128]. Studies combining anti-angiogenic agents with ICIs, with or without chemotherapy (FOLFOX), initially suggested potentially synergistic activity [129, 130]; however, the randomized phase III MODUL trial failed to confirm the preliminary findings. In this trial, maintenance treatment with combined atezolizumab/bevacizumab/fluoropyrimidine after first-line induction with FOLFOX/bevacizumab did not demonstrate any clinical benefit in progression-free survival (PFS) or OS compared to bevacizumab and fluoropyrimidine alone.

Preclinical data have shown that cetuximab (a chimeric immunoglobulin G1 [IgG1] monoclonal antibody directed to the EGFR) combined with FOLFIRI (a combination chemotherapy regimen that does not induce ICD) induces ICD in a mouse model and CRC cell lines [131] and may favour the activation of T-cell-mediated immune response [128]. Cetuximab can also stimulate NK-mediated cell-antibody-dependent cellular cytotoxicity [132]. We previously reported that patients treated preoperatively with chemotherapy and cetuximab had a higher infiltration of global ($CD3^+$), cytotoxic ($CD8^+$), and memory ($CD45RO^+$) T-cells into the core of their resected CRC liver metastases and higher immune-related gene expression compared to other treatments [80, 81]. This modification of the tumour-immune microenvironment has also been reported by another group [133] and observed in the small subgroup of patients with *RAS*-mutated mCRC [80]. This suggests that the immunological effect of cetuximab, unlike its cytotoxic activity, is independent of *RAS*-mutation status [80]. This promising treatment approach is currently being evaluated in a phase II trial combining avelumab (anti-PD-L1) with FOLFOX and cetuximab in first-line *RAS* and *BRAF* wild-type (wt) mCRC (AVETUX trial). The preliminary results from 20 patients have been reported demonstrating an ORR of 75% and a DCR of 95% [134]. Another multicentre phase II trial is currently investigating the question of cetuximab in combination with avelumab as a rechallenge in mCRC patients who have already experienced a partial or complete response with an anti-EGFR plus chemotherapy in first-line treatment (CAVE Colon study). In the same way, our group is currently investigating the efficacy of avelumab combined with cetuximab and irinotecan (AVETUXIRI trial, NCT03608046) in mCRC patients refractory to chemotherapy (cohort A: *RAS*-mutated)

Table 1. Selection of ongoing trials investigating different treatment strategies with ICIs for pMMR-MSS CRC

Combined therapy	Target	Clinical compound	ICI compound	Trial type	Trial identifier
Radiotherapy	Stereotactic body radiation		Toripalimab	Phase IImCRC with oligometastasis	NCT03927898
	Liver radiation therapy		Nivolumab, Ipilimumab, and CMP-001	Phase ImCRC	NCT03507699
	Radiation therapy		Nivolumab + Ipilimumab	Phase IIpMMR-MSS and dMMR-MSI CRC	NCT03104439
	Radio-chemotherapy	5-FU	Atezolizumab	Phase I/IILocalized rectal cancer	NCT03127007
	Radiation therapy		Durvalumab ± Tremelimumab	Phase IImCRC	NCT02888743
	Radiation therapyOr ablation		Durvalumab ± Tremelimumab	Phase IImCRC	NCT03122509
	Radiation therapy		Durvalumab ± Tremelimumab	Phase IImCRC pMMR-MSS	NCT03007407
	Radio-chemotherapy	Capecitabine	Nivolumab	Phase I/IIRectal cancer	NCT02948348
	Radio-chemotherapy	Standard Radio-chemotherapy	Durvalumab	Phase IIRectal cancer pMMR-MSS	NCT03102047
Targeted therapies	VEGFR and KIT	Cediranib	Durvalumab	Phase I/IIRefractory CRC	NCT02484404
	EGFR	Panitumumab	Nivolumab ± Ipilimumab	Phase IIRAS-wild-type CRC	NCT03442569
	VEGFR, PDGFR, FGFR	Nintedanib	Pembrolizumab	Phase I/IImCRC	NCT02856425
	EGFR	Cetuximab	Pembrolizumab	Phase Ib/IIPretreated mCRC	NCT02713373
	EGFR	Cetuximab + Irinotecan	Avelumab	Phase IImCRC pMMR-MSS	NCT03608046
	EGFR	Cetuximab + FOLFOX	Avelumab	Phase IIUntreated mCRC	NCT03174405
	EGFR	Cetuximab	Avelumab	Phase IIPretreated RAS-wild-type mCRC	CAVE Colon
	VEGFA	Bevacizumab + Capecitabine	Atezolizumab	Randomized phase IIRefractory CRC	NCT02873195
	VEGFA	Bevacizumab + FOLFOX	PDR001	Phase IFirst-line mCRC	NCT03176264
	VEGFA	Bevacizumab + FOLFOX	Nivolumab	Phase II/IIFirst-line CRC	NCT03414983
	VEGFA	Bevacizumab + Capecitabine	Pembrolizumab	Phase IIPretreated mCRC	NCT03396926
	Multikinase	Regorafenib	PDR001	Phase IbPretreated mCRC	NCT03081494
	MEK	Combimetinib and Regorafenib	Atezolizumab	Phase IIImCRC	NCT02788279
	MEK	Cobimetinib	Atezolizumab	Phase IIFirst-line mCRC	NCT02291289
	MEK	Binimetinib	Nivolumab ± Ipilimumab	Phase I/IIPretreated mCRC	NCT03271047
	MEK	Binimetinib+ FOLFOX or FOLFIRI	Pembrolizumab	Phase IbmCRC	NCT03374254
	MEK	Trametinib	Nivolumab ± Ipilimumab	Phase I/IIPretreated mCRC	NCT03377361
	MEK	Trametinib	Durvalumab	Phase IImCRC pMMR-MSS	NCT03428126
	MEK and VEGFA	Combimetiniband Bevacizumab	Atezolizumab	Phase ImCRC	NCT02876224
	MEK, CD38, LAG-3	Cobimetinib, Daratumumab, anti-LAG-3 antibody	Nivolumab ± Ipilimumab	Phase IIRefractory CRC	NCT02060188
	PI3K	Copanlisib	Nivolumab	Phase I/IIUnresectable or mCRC pMMR-MSS	NCT03711058
	MNK	eFT508	Avelumab	Phase IIRelapsed or refractory pMMR-MSS	NCT03258398
Cytokines	IL-15 superagonist	ALT-803	Pembrolizumab, or Nivolumab, or Atezolizumab, or Avelumab	Phase IIAdvanced cancer including CRC	NCT03228667
	CXCL12	Olaptesed pegol	Pembrolizumab	Phase I/IImCRC	NCT03168139
	Cytokines release	Poly-ICLC	Pembrolizumab	Phase I/IImCRC	NCT02834052
	GM-CSF	talimogene laherparepvec	Atezolizumab	Phase ImCRC	NCT03256344

(continued)

Table 1. (continued)

Combined therapy	Target	Clinical compound	ICI compound	Trial type	Trial identifier
	CSF-1R	Pexidartinib	Durvalumab	Phase ICRC	NCT02777710
Others	COX-2	Celecoxib	Nivolumab ± Ipilimumab	Phase IIStage I–III CRC	NCT03026140
	IDO1	Epacadostat	Nivolumab	Phase I/IISolid tumours including CRC	NCT02327078
	IDO1 and DNMT	Epacadostat and Azacitidine	Pembrolizumab	Phase I/IIRefractory CRC and NSCLC	NCT02959437
	DNMT	Azacitidine	Durvalumab	Phase IImCRC	NCT02811497
	DNMT and HDAC	Azacitidine and romidepsin	Pembrolizumab	Phase IPretreated mCRC	NCT02512172
	Thymidine phosphorylase	TAS-102	Nivolumab	Phase IIRefractory CRC	NCT02860546
	Thymidine phosphorylaseVEGFA	TAS-102Bevacizumab and Capecitabine	Nivolumab	Phase IIPretreated mCRC	NCT02848443
	Glucose metabolism	Metformin	Nivolumab	Phase IIRefractory pMMR-MMS CRC	NCT03800602
	Adenosine receptor	AZD4635	Durvalumab	Phase ISolid malignancies including CRC	NCT02740985
	Adenosine receptor	NIR178	PDR001	Phase IIAdvanced solid tumours including CRC	NCT03207867
	Adenosine receptorCD73	NIR178NZV930	PDR001	Phase IAdvanced solid tumours including CRC	NCT03549000
	EGFR-CAR T-cells expressing anti-PD-1 and anti-CTLA-4 antibodies			Phase I/IIEGFR positive advanced malignant solid tumours	NCT03182816
	EGFR-CAR T-cells expressing anti-PD-1 antibodies			Phase I/IIEGFR positive advanced malignant solid tumours	NCT02873390
	MUC1-CAR T-cells expressing anti-PD-1 and anti-CTLA-4 antibodies			Phase I/IIMUC1 positive advanced malignant solid tumours	NCT03179007

Clinical trial details can be accessed at ClinicalTrials.gov.
5-FU, 5-fluorouracil; Avelumab, anti-PD-L1; Atezolizumab, anti-PD-L1; CMP-001, anti-TLR9; CAR, chimeric antigen receptor; CRC, colorectal cancer; CSF-1R, colony stimulating factor 1 receptor; COX-2, cyclooxygenase-2; CXCL12, C-X-C motif chemokine 12; DNMT, DNA methyltransferase; Durvalumab, anti-PD-L1; dMMR/MSI, mismatch-repair-deficient and microsatellite instable; EGFR, epidermal growth factor receptor; FGFR, fibroblast growth factor receptor; FOLFIRI, 5-fluorouracil, leucovorin, and irinotecan; FOLFOX, 5-fluorouracil, leucovorin, and oxaliplatin; GM-CSF, granulocyte-macrophage colony stimulating factor; HDAC, histone deacetylase; IDO1, indolamine 2.3-dioxygenase 1; IL-15, interleukin 15; Ipilimumab, anti-CTLA-4; KIT, tyrosine kinase Kit; LAG-3, lymphocyte-activation gene 3; mCRC, metastatic colorectal cancer; MEK, mitogen-activated protein kinase; MNK, mitogen-activated protein kinase interacting protein kinase; MUC1, mucin-1; Nivolumab, anti-PD-1; NSCLC, non-small-cell lung cancer; PDGFR, platelet-derived growth factor receptor; PDR001, anti-PD-1; Pembrolizumab, anti-PD-1; pMMR/MSS, mismatch-repair-proficient and microsatellite stable; Toripalimab, anti-PD-1; Tremelimumab, anti-CTLA-4; VEGFA, vascular endothelial growth factor A; VEGFR, vascular endothelial growth factor receptor.

and anti-EGFR treatment (cohort B: *RAS* wt). We hope to confirm that the immunological effect of cetuximab can provoke a tumour response when combined with avelumab irrespective of *RAS* mutation. We also plan to prospectively investigate the previously described efficacy biomarkers. For this reason, multiple tumour biopsies and blood collection are planned in the correlative sub-study.

Preclinical studies have reported that MEK inhibitors may be associated with a tumour immunological effect [135, 136]. MEK inhibition upregulates MHC class I expression [137], which promotes antigen presentation on the surface of tumour cells for recognition by $CD8^+$ T-lymphocytes, which then recognize and kill tumour cells. In mouse models, the combination of PD-1 and MEK inhibitors has synergistic tumour-growth inhibition compared to single-agent treatment [135, 136]. This efficacy was clinically observed in a phase Ib study combining atezolizumab (anti-PD-L1) and cobimetinib (anti-MEK) in 23 chemorefractory mCRC patients with an ORR of 17% (4 of 23 patients) [129]. The IMblaze370 (Cotezo) randomized phase III trial failed, however, to confirm this efficacy. Chemorefractory mCRC patients randomized in the combined experimental arm (cobimetinib/atezolizumab) did not experience any increased tumour response or survival benefit (PFS, OS) compared to patients treated with atezolizumab alone or regorafenib [138].

The PI3K-AKT-mTOR pathway is implicated in cell survival, migration, and proliferation, and is often dysregulated in cancer [139]. Interestingly, recent observation suggests that inhibition of this pathway could have not only an effect on tumour cells, but also an effect on the immune microenvironment by preventing the activation of the immunosuppressive pathway [140]. The strategy of combining a PI3K inhibitor and an ICI may lead to an increased ICI response in tumours with an immunosuppressive environment, such as pMMR/MSS CRC. Ongoing trials evaluating chemo and targeted therapies are summarized in Table 1.

Cytokines

Cytokines and chemokines are immune-system molecular messengers. Therefore, targeting these molecules in

combination with ICIs is another approach currently under investigation [141].

Interleukin 15 (IL-15) is a glucoprotein that belongs to the 4-alpha-helix bundle family of cytokines. In the immune system, IL-15 is mainly expressed by monocytes, macrophages, and DCs, and has been characterized as a T-cell growth factor [142]. Upon binding to its receptor, highly expressed on $CD8^+$ and NK cells, IL-15 promotes the proliferation and function of these cells. The efficacy of IL-15 administration is limited by its short half-life *in vivo* [143], but a chimeric fusion protein (ALT-803) that increases the *in vivo* half-life has been developed. This treatment is currently being tested in combination with different ICIs in patients with advanced cancer, including mCRC patients (NCT03228667).

CXCL12, a chemokine mainly expressed by cancer-associated fibroblasts, has been reported to mediate immune exclusion in mouse models [144]. Therefore, the idea of blocking CXCL12 and the binding to its receptors, CXCR4 and CXCR7, is an interesting novel approach to increase the efficacy of ICIs in immune-excluded tumours with a low immunoscore. This approach is being investigated in a study combining olaptesed (NOX-A12)—a heptamer that binds CXCL12 with high affinity—with pembrolizumab (NCT03168139).

Another way to potentially increase efficacy is to stimulate cytokine release. A phase I/II trial is evaluating how the combination of pembrolizumab and poly-ICLC—a molecule that stimulates cytokine release by inducing IFN-γ production—generates an inflammatory response (NCT02834052).

Even if its function in cancer is controversial [145], human cytokine granulocyte-macrophage colony stimulating factor (GM-CSF) is known to regulate cell differentiation [146] and local recruitment of dendritic cells [147]. This may enhance tumour-associated antigen presentation to T-cells [148] and activate other effectors of the immune response, including macrophages and NK cells [147, 149]. A trial is evaluating a virus, talimogene laherparepvec, that encodes the immunostimulating factor GM-CSF. This virus infects and replicates in tumour cells, leading to cell lysis and GM-CSF release. Combining the talimogene laherparepvec virus with atezolizumab is therefore a promising approach to increase the antitumour immune response in mCRC (NCT03256344).

Others

Other trials underway aim to combine ICIs with molecules that target metabolic pathways. Two trials are currently testing nivolumab with or without ipilimumab combined with celecoxib, a COX-2 inhibitor (NCT03926338 and NCT03026140). The expression of COX-2 may induce expression of IDO1 [150]. IDO1 is an intracellular enzyme that catalyses tryptophan along the kynurenine pathway [151] and induces depletion of tryptophan, leading to an immunosuppressive environment [152, 153]. Higher IDO1 expression in CRC tumours correlates with progressive disease and impaired clinical outcome [154]. For this reason, inhibitors of IDO1, such as epacadostat, are currently being tested in combination with ICIs (NCT02327078 and NCT02959437).

One trial is assessing the combination of metformin with nivolumab (NCT03800602) based on its preliminary reported efficacy in metastatic melanoma [155].

The activation of the A2a and A2b adenosine receptors on immune cells inhibits their proliferation and activation resulting in strong immunosuppression and T-cell anergy [156, 157]. Adenosine has been found to be one of the mechanisms used by Tregs to maintain immunotolerance [158]. Inhibition of the adenosine A2a receptor may potentiate ICIs, such as durvalumab (anti-PD-L1), and could prove to be a worthwhile treatment approach. This strategy is currently being evaluated in a safety trial in patients with advanced solid malignancies, including CRC (NCT02740985).

Cibisatamab (CEA CD3 TCB; RG7802, RO6958688) is a T-cell bispecific antibody (TCB) that simultaneously binds to carcinoembryonic antigen (CEA) on tumour cells and CD3 on T-cells, thus cross-linking cancer cells and T-cells. This leads to T-cell engagement and activation independently of pre-existing immunity, T-cell infiltration, and tumour inflammation. In ongoing studies, encouraging clinical activity has been reported in patients with metastatic pMMR/MSS CRC treated with CEA-TCB monotherapy, and its activity was enhanced when combined with atezolizumab [159]. In the combination therapy group, the ORR was 18% ($n = 2$) and the observed DCR was 82%. Toxic effects were manageable. CEA-TCB is the first T-cell bispecific antibody to show efficacy in solid tumours and specifically in pMMR-MSS CRC.

Finally, adoptive cell-based immunotherapy with genetically modified T-cells represents a promising emerging modality for CRC treatment. Adoptive cell therapy is based on collection of T-cells from patients, *in vitro* expansion, and transfusion of T-cells into patients. These T-cells can be engineered to express chimeric antigens receptors (CARs) or selected for their ability to bind tumour antigens. Moreover, CAR T-cells can be engineered to not only recognize tumour antigens, but also to produce cytokines or ICIs. However, despite the fact that CAR T-cells are successfully used for treating haematological cancers such as B-cell malignancies, the efficacy and applicability of cell-based immunotherapy remain to be proved in CRC and other solid tumours [7, 160]. Nevertheless, EGFR-CAR-T or Mucin-1 (MUC1)-CAR T-cells expressing anti-PD-1 or anti-PD-1 and anti-CTLA-4 are currently clinically tested on solid tumours expressing these antigens (EGFR and MUC1).

Conclusion

It is undeniable that considerable advances have been made with the recent FDA approval of ICIs for the treatment of dMMR/MSI-H mCRC. Unfortunately, dMMR/MSI-H CRC represent only a small subgroup of all CRC and most pMMR/MSS mCRC does not benefit from ICIs alone.

Beyond the dMMR/MSI-H tumour status, the continuous development of new biomarkers, such as immunoscore and CMS classification, has led to a better understanding of the molecular mechanisms that define the immune reactivity of CRC and their relationship with oncological treatments. This provides new perspectives, enables a more personalized approach towards patient management, and should continue to be investigated in the translational aspect of clinical trials. That said, many of these biomarkers are governed by a heterogeneous expression pattern in time and space. Therefore, the source of the tumour sample, and any treatments administered prior to sample collection, must be carefully considered.

The key remaining challenge is to identify, among the heterogeneous spectrum of mCRC, which patients are most likely to benefit from ICIs alone or in combination with other oncological treatments, because of their specific genomic and immune tumour characteristics. This question is currently under investigation in ongoing clinical trials.

Funding

N.H. is a research fellow supported by a grant from the Belgian National Fund for Scientific Research [Télévie/FNRS 7460918F].

Acknowledgements

The authors wish to thank Aileen Eiszele for English-language and writing assistance. The Figure 1 has been created with Biorender.com.

Conflicts of interest

The authors have no conflicts of interest to declare.

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