



## Anti-Tumour Treatment

## Immunotherapy of colorectal cancer: Challenges for therapeutic efficacy

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## ABSTRACT

A better knowledge of the complex interactions between cancer cells and the immune system has led to novel immunotherapy approaches. Treatment with selective anti-PD1, anti-PD-L1 and/or anti-CTLA-4 monoclonal antibodies (mAbs) has been a revolution in the therapeutic scenario of several cancer types, with the highest clinical efficacy in melanoma and in lung cancer. Colorectal cancer is one of the tumours in which immunotherapy has been shown less effective. Whereas in deficient mismatch repair (MMR) or in highly microsatellite instable (MSI-H) metastatic colorectal cancer there is clear clinical evidence for a therapeutic role of immune checkpoint inhibitors, the vast majority of patients with proficient MMR or with microsatellite stable (MSS) tumours do not benefit from immunotherapy. Defining the molecular mechanisms for immunogenicity in metastatic colorectal cancer is needed in order to develop predictive biomarkers and effective therapeutic combination strategies. A major challenge will be to identify, among the heterogeneous spectrum of this disease, those patients with specific tumour and tumour infiltrating stroma molecular and functional characteristics, that could be effectively treated with immunotherapy. In this review, we discuss the role of immune response in the context of metastatic colorectal cancer. We summarize the available clinical data with the use of anti PD-1/PD-L1 mAbs as single agents or in combination with anti CTLA-4 mAbs in MSI-H patients. Finally, we address the challenges and the potential strategies for rendering the more frequent microsatellite stable (MSS) tumours “immune-competent” and, therefore, amenable for effective immunotherapy interventions.

## Introduction

Colorectal cancer (CRC) is the third most commonly diagnosed cancer and the second type in terms of mortality. Over 1.8 millions of CRC cases and 881.000 CRC-related deaths were estimated to occur in 2018 in the World [1]. Despite significant improvements in CRC treatment, the prognosis of patients with metastatic CRC (mCRC) remains poor, with a median overall survival (OS) of approximately 30 months [2]. For this reason, novel and more effective therapeutic strategies are necessary for metastatic disease.

In the last few years, a better knowledge of the complex interactions between cancer cells and the immune system has led to novel immunotherapy approaches. The development of immune checkpoint inhibitors, such as anti-CTLA-4 monoclonal antibodies (mAbs) (ipilimumab, tremelimumab), anti-PD-1 mAbs (nivolumab, pembrolizumab) and anti-PD-L1 mAbs (atezolizumab, durvalumab, avelumab), has dramatically changed the therapeutic scenario for several types of cancer, including melanoma, lung, head and neck, kidney, bladder, Merkel cell carcinoma and mCRC with high microsatellite instability

(MSI-H) [3–21]. However, the use of immune checkpoint inhibitors has demonstrated little or no clinical activity in the majority of patients with mCRC [22].

In this review, we discuss the role of immune response in the context of mCRC. We summarize the available clinical data with the use of anti PD-1/PD-L1 mAbs as single agents or in combination with anti CTLA-4 mAb in MSI-H mCRC patients. Finally, we address the challenges and the potential strategies for rendering the more frequent microsatellite stable (MSS) mCRC tumours “immune-competent” and, therefore, amenable for effective immunotherapy interventions.

## Evidence for immune competence in colorectal cancers with microsatellite instability

Microsatellites are short segments of DNA that consist of multiple repetitions of one to ten nucleotide base pairs. During DNA synthesis by DNA polymerase, these sequences are frequently subjected to mutations, like base insertions or deletions, thus leading to frameshift mutations. The mismatch-repair (MMR) system has a major function in

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recognizing and correcting these errors in the microsatellite region, thus preventing genomic alterations [23].

The presence of microsatellite instability, determined by the deficiency of one MMR protein, has been found in approximately 15–20% CRC, with some differences according to tumour stage [24,25]. In fact, the prevalence of deficient mismatch repair (dMMR) is higher in Stage II (20%) and Stage III (12%) as compared to Stage IV (4%) CRC [25–27]. The majority of MSI-H CRC are sporadic tumours and are associated to the inactivation by methylation of MLH1 [28]. However, approximately 3% of dMMR CRC are related to the Lynch Syndrome (LS), which is associated with germ line mutations in the genes involved in MMR (MLH1, MSH2, MSH6 and PMS2) [29]. Interestingly, patients with MSI-H CRC share a characteristic clinical-pathological profile such as the more frequent association with female sex, older age, BRAF activating mutations, peritoneal disease, the presence of mucin-rich and signet ring cancer cells, right sided primary tumours, early stage and poorly differentiated tumours, with an abundance of tumour infiltrating lymphocytes (TILs) and a Crohn-like lymphoid reaction [30,31].

Diagnosis of MSI status can be assessed by various methodologies, like real time polymerase chain reaction (PCR), immunohistochemistry (IHC) and next generation sequencing (NGS), with a good level of concordance [32–35]. dMMR CRC is characterized by a high tumour mutational burden (TMB), as a consequence of a large number of tumour DNA damages, including deletions, insertions and frameshift mutations during cancer cell replication.

Measurement of TMB in the primary tumour and/or in blood samples from melanoma or lung cancer patients treated with immune checkpoint inhibitors has been suggested as a potential biomarker of therapeutic efficacy [36,37]. However, these results are largely from retrospective analyses and are obtained with different NGS-based techniques. In fact, it is still under investigation, in these tumour types, which is the most appropriate cut-off for defining high TMB for therapeutic use. In this respect, the evaluation of TMB has been recently reported in a retrospective, subgroup and hypothesis-generating analysis of tumour samples from CRC patients that received as first line treatment for metastatic disease a 5-FU-based chemotherapy doublet plus bevacizumab or cetuximab [38]. In the 35 patients with MSI-H tumours, the median TMB was 52 mutations/Mb, whereas in the 475 patients with MSS tumours, the median TMB was 6 mutations/Mb. Authors defined high TMB cases those with 8 or more mutations/Mb. According to this selected cut-off, better OS could be observed in mCRC patients with high TMB, regardless of the first line treatment [38]. These data need to be validated in a prospective fashion in appropriately designed studies in order to define the TMB cut-off for clinical use and if high TMB could be confirmed as a positive prognostic marker in mCRC.

The presence of a high number of tumour-associated neoantigens could favour the identification of cancer cells by the host immune system [39–41]. In this respect, MSI-H CRC are correlated with an increased infiltration of TILs, such as CD8<sup>+</sup> cytotoxic lymphocytes, T<sub>H</sub>1 activated cells that produces IFN $\gamma$ , and CD45 RO<sup>+</sup> T memory cells, which are also correlated with a better survival as compared to MSS CRC [42–44]. Moreover, the results of the international validation of the consensus immunoscore for the classification of colon cancer have demonstrated that a high immunoscore, based on CD3<sup>+</sup> and CD8<sup>+</sup> T cell densities within the tumour, as measured by IHC and quantified by digital pathology, is an independent positive prognostic biomarker for the risk of recurrence in Stage I–III CRC [45]. High immunoscores were observed in 45% of cases with MSI-H tumours (138/304) and only in 21% of cases with MSS tumours (273/1275) [45].

Further, a tissue microarray analysis of a series of CRC samples detected high tumour PD-L1 expression in 5% of cases (19/394) and high tumour infiltrating lymphocytes PD-1 expression in 19% of cases (76/392) [46]. dMMR tumours had significantly higher levels of PD-1 and PD-L1 as compared to proficient MMR (pMMR) tumours (18% vs 2% and 50% vs 13%, respectively;  $P < 0.001$  for both). In multivariate

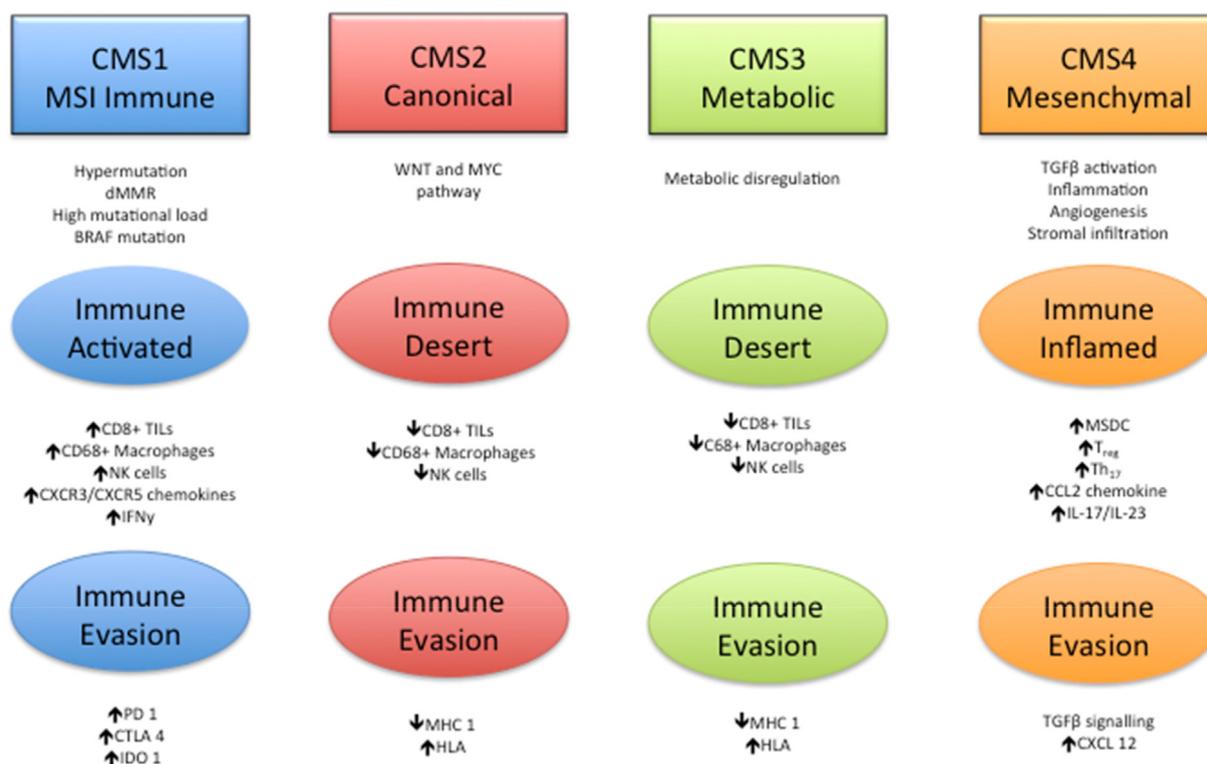
analysis, both PD-1 and PD-L1 high expression were independent factors associated with better recurrence-free survival, but only in dMMR cases [46].

### Molecular classification of colorectal cancer: implications for immune competence

Colorectal cancer is a genetically heterogeneous disease in which several and different molecular pathways could be involved in tumour initiation, growth and progression. According to a comprehensive re-evaluation and comparison of CRC molecular gene expression profiling that has been obtained by the use of different platforms, a Consensus Molecular Subtype (CMS) classification has been developed, that is based on both tumour as well as infiltrating stroma gene expression. According to the CMS classification, four major groups can be identified. CMS1 (MSI Immune, approximately 14% of cases) are hypermutated tumours, generally with MSI-H features, can harbour BRAF mutations and show a robust immune infiltration. CMS2 (Canonical, approximately 37% of cases) are tumours, which are characterized by the activation of the *Wnt* and *Myc* pathways. CMS3 (Metabolic, approximately 13% of the cases) tumours have frequently KRAS mutations and display a deregulation in cancer cell metabolic pathways. CMS4 (Mesenchymal, approximately 23% of the cases) tumours are characterized by transforming growth factor beta (TGF $\beta$ ) pathway activation, enhanced angiogenesis, stromal activation and inflammatory infiltrate [47].

Interestingly, the four CRC CMSs are characterized by relevant differences in tumour microenvironment (TME). CMS1 and CMS4 are “hot” tumours with an intense immune infiltration, whereas CMS2 and CMS3 are “cold” tumours and lack immune activation. Therefore, the CMS classification represents a useful model to define potential therapeutic options for activating host immune responses, taking in account that different strategies are needed for “hot” and for “cold” CRCs. In this respect, CMS1 CRC has a diffuse immune infiltrate with CD8<sup>+</sup> TILs and CD68<sup>+</sup> macrophages. However, the concomitant up-regulation of immune checkpoint molecules (CTLA-4, PD-1, PD-L1) may represent a major mechanism of immune evasion in these tumours [48]. Thus, the use of immune checkpoint inhibitors could reverse the immune blockade and could activate an effective antitumor immune response in this CRC subtype. On the other hand, CMS4 tumours are characterized by a different immune infiltration pattern, in which T regulatory cells (T<sub>reg</sub>), myeloid-derived suppressor cells (MDSCs), monocyte-derived cells and T helper 17 (T<sub>H</sub>17) cells play a major role [49]. This ‘inflamed’ subtype, that is generally found in the microenvironment of immune-tolerant malignancies, is characterized by marked up-regulation of immunosuppressive factors, such as TGF $\beta$  and CXCL12, and high expression of genes encoding for chemokines that attract myeloid cells, including C-C motif chemokine ligand 2 (CCL2), interleukin 23 (IL-23) and IL-17 [50]. Therefore, the potential immune response in CMS4 tumours is blocked by the activation in the stroma of several mechanisms that favour an inflammatory environment and that, therefore, suppress the immune response against cancer cells. In this respect, TGF $\beta$  and other functional activators of epithelial to mesenchymal transition (EMT) and of angiogenesis may be major players for tumour immune evasion. It has been recently demonstrated by using a mouse genetic model of colon cancer development, that cancer progression and metastatic spreading is due to the key role of TGF $\beta$  activation in the tumour stroma that specifically inhibits the immune response [51]. In this respect, a therapeutic strategy of combining selective TGF $\beta$  inhibitors with immune checkpoint inhibitors is able to re-activate a robust immune response in this mouse colon cancer model [51].

In contrast to CMS1 and CMS4 tumours, in which high levels of immune infiltrates are observed, although with different functional characteristics, CMS2 and CMS3 tumours lack immune activation and, therefore, could be defined as “immune desert” cancers. Different mechanisms may be responsible for this phenomenon, including



**Fig. 1.** Consensus molecular classification according to the immune phenotype. CMS1 and CMS4 show activation of the immune system, with different orientation. On the other hand CMS2 and CMS3 are poorly immunogenic and display a little activation of the immune response. dMMR: deficient mismatch repair; Transforming growth factor beta: TGFβ; TILs: tumour infiltrating lymphocytes; NK: natural killer; CXCR3/CCR5: Chemokine (C-X-C motif) receptor 3/C-C chemokine receptor type 5; IFNγ: interferon gamma; MSDCs: myeloid suppressor derived cells; CCL2: chemokine (C-C motif) ligand 2; IL-17/IL-23: interleukin 17/interleukin 23; PD1: programmed death protein 1, CTLA4: cytotoxic T-lymphocyte-associated protein 4, IDO1: Indoleamine-pyrrole 2,3-dioxygenase; MHC I: major histocompatibility complex 1; HLA: human leukocyte antigens; CXCL12: Chemokine (C-X-C-motif) ligand 12.

specific oncogenic-driven cancer cell pathways, lack of major histocompatibility complex (MHC) class 1 molecules, up-regulation of non-classical human leukocyte antigens (HLA), that all may concur to cancer immune evasion [52]. For these reasons, novel therapeutic approaches are clearly needed to render immunogenic these tumours and possibly offer effective immunotherapies to CRC patients whose tumours are CMS2 or CMS3 (Fig. 1).

#### Clinical efficacy of immune checkpoint inhibitors in mCRC patients with microsatellite unstable tumours

Several trials have evaluated the clinical activity of immune checkpoint inhibitors in dMMR mCRC. In the phase II clinical trial KEYNOTE 016, 41 patients with pMMR and dMMR chemorefractory mCRC or with dMMR non-CRCs were treated with the anti-PD1 mAb pembrolizumab at the dose of 10 mg/kg every 3 weeks [22]. In the cohort of patients with dMMR mCRC (10/41), the overall response rate (ORR) was 40% (4/10), with durable clinical responses, whereas ORR was 0% in pMMR mCRC (18/41). Moreover, a high number of somatic mutations were associated with longer progression free survival (PFS). Pembrolizumab therapy was well tolerated: Grade 3–4 treatment related adverse events (TRAEs) occurred in 17% of patients. Updated results of the KEYNOTE 016 trial which included data on 86 patients with dMMR cancers, reported 52% ORR with 12% complete responses (CR) in the cohort of CRC patients. The 2-year progression free survival rate was 59% and the 2-year overall survival rate was 72% [15].

In the CheckMate 142, a nonrandomised phase II clinical trial, a first cohort of 74 patients with chemorefractory dMMR mCRC were treated with the anti PD-1 mAb nivolumab as single agent at the dose of 3 mg/kg, every 2 weeks [10]. The ORR was 31.1%. Disease control longer than 12 weeks was achieved in 69% of patients, with median time to

response of 2.8 months. Twelve months PFS was 50.4%, while 12 months OS was 73.4%. Grade 3–4 TRAEs occurred in 20% of patients. These adverse events were mainly asthenia, diarrhoea and pruritus. In a different cohort, 119 patients with pre-treated dMMR mCRC received as induction treatment the combination of nivolumab, 3 mg/kg, plus the anti-CTLA-4 mAb ipilimumab, 1 mg/kg, every 3 weeks, followed by nivolumab, 3 mg/kg, every 2 weeks as maintenance [11]. The combination of anti PD-1 and anti-CTLA-4 immunotherapy displayed an ORR of 55% and a disease control rate at 12 weeks of 80%. Twelve month PFS rate was 71%, while 12 month OS was 85%. Grade 3–4 TRAEs were reported in 32% of patients.

Recently, the promising preliminary results of the combination with nivolumab, 3 mg/kg, every 2 weeks plus low-dose ipilimumab, 1 mg/kg, every 6 weeks in an untreated patient population with MSI-H mCRC have been presented [53]. This study has enrolled 45 patients. The ORR was 60% (27/45), with 7% CRs (3/45). The 12 months PFS and OS rates were 77% and 83%, respectively. Grade 3–4 TRAEs occurred in 16% of patients, with only 7% of patients that had to discontinue therapy for an adverse event. Based on these results, the USA Food and Drug Administration (FDA) has approved pembrolizumab, nivolumab, and nivolumab plus ipilimumab, for the treatment of MSI-H mCRC patients that have progressed after therapy with fluoropyrimidines plus irinotecan or oxaliplatin.

Several phase III clinical trials are currently on-going to evaluate the efficacy of anti-PD1, anti-PD L1 and CTLA-4 mAbs in dMMR mCRC (Table 1). Keynote 177 (NCT02563002) is a phase III randomized trial, which is comparing pembrolizumab versus a standard of care chemotherapy as first line treatment in dMMR mCRC. Further, the COMMIT trial (NCT02997228) is exploring the clinical efficacy of treatment with the anti-PD L1 mAb atezolizumab as single agent or in combination with FOLFOX plus bevacizumab, as compared to FOLFOX

**Table 1**  
Ongoing phase II/III clinical trials evaluating the use of immune-checkpoint inhibitors in microsatellite instable colorectal cancer.

Study name	Agent	Target	Study population	Primary endpoint	Phase	Recruitment status
ATOMIC NCT02912559	FOLFOX/Atezolizumab Vs FOLFOX	PD-L1	Stage III MSH-I CRC	DFS	III	Active, recruiting
POLEM trial (NCT02912559)	Avelumab Vs Observation After adjuvant chemotherapy	PD-L1	Stage III MSH-I, POLE mutant CRC	DFS	III	Active, recruiting
KEYNOTE-177 (NCT02563002)	Pembrolizumab vs Standard therapy	PD-1	First line MSH-I mCRC	PFS OS	III	Active, recruitment completed
COMMIT Trial (NCT02997228)	Atezolizumab Vs FOLFOX/Atezolizumab/Bevacizumab Vs FOLFOX/Bevacizumab	PD-L1	First line MSH-I mCRC	PFS	III	Active, recruiting
NCT02227667	Durvalumab	PD-L1	Pretreated mCRC	ORR	II	Active

MSH-I: High microsatellite instability; mCRC: Metastatic colorectal-cancer; DFS: Disease free survival; PFS: Progression free survival; OS: Overall survival; ORR: Overall response rate.

plus bevacizumab as first line therapy of dMMR mCRC patients. Two trials are currently assessing the use of immune therapy in the adjuvant setting. The ATOMIC trial (NCT02912559) is investigating the combination of FOLFOX plus atezolizumab vs FOLFOX as adjuvant treatment in Stage III MSH-I CRC; while the POLEM trial (NCT02912559) is addressing the role of avelumab as maintenance treatment after 5-FU based adjuvant chemotherapy for Stage III MSI-H or POLE mutant CRC.

#### Are POLE and POLD1 mutations predictive for immune competence of colorectal cancer?

DNA polymerase epsilon (POLE) and DNA polymerase delta (POLD1) are two key enzymes involved in the process of DNA synthesis and repair, which guarantee the correct replication of the genome during cell cycle [54]. Interestingly, exonuclease domain mutations in POLE or in POLD1 genes determine a specific alteration of the enzyme proof-reading activity, with a deficit in DNA repair; thus, leading to the accumulation of a very high number of mutations, up to ten times higher than MSH-I CRC. This translates in an increased risk of developing several types of cancer, including endometrial, colorectal, gastric, pancreatic, breast and brain tumours [55–57].

POLE mutations have been found in approximately 1–2% in the overall population of pMMR CRCs. However, the frequency of this mutation arises to 5–7% in tumours of patients aged less than 50 years. This rare type of “ultra-mutated” tumour shares similar clinical-pathological features with dMMR CRC, such as high levels of TILs, up-regulation of immune checkpoint molecules, increase in cytotoxic T cell markers and in effector cytokines, suggesting a potentially enhanced immunogenicity. Moreover, Stage II-III CRC patients harbouring POLE mutations showed a reduced risk of recurrence after surgery as compared to pMMR POLE wild type CRC patients [57]. A few clinical data are currently available regarding the potential activity of immune checkpoint inhibitors in mCRC patients whose tumours carry POLE/POLD1 mutations. Of note, durable clinical responses were reported with the use of the anti PD-1 mAb pembrolizumab in a patient with POLE mutated endometrial cancer and in a patient with POLE mutated pMMR CRC [58,59]. Probably in the near future, based on these promising findings, POLE mutations will be tested alongside MMR in order to better define CRC patients that could benefit from immunotherapy.

#### Is it possible to render MSS tumours immune-competent and amenable for immunotherapy?

The use of immune-checkpoint inhibitors in patients with dMMR mCRC determines significant and durable clinical responses, even in

heavily pre-treated patient populations. Nevertheless, this relevant clinical benefit is limited to only a small group of tumours, which represents approximately 4% of mCRC [60,61]. In fact, therapy with anti-PD1 mAbs has no efficacy in pMMR mCRC patients. Novel therapeutic strategies to render these tumours immune-competent are urgently needed.

It has been shown that chemotherapy, molecular targeted therapies and radiotherapy could cause immunogenic cell death (ICD) in cancer cells. This may be due to the release of damage associated molecular patterns (DAMPs), which in turn are recognised by host dendritic cells (DC), that are able to present these antigens to CD8<sup>+</sup> cytotoxic lymphocytes and, therefore, to activate these cells against cancer cells [62–65]. These observations have led to the hypothesis that combining treatment with immune checkpoint inhibitors and other anticancer therapies could potentially overcome the primary resistance to immunotherapy of MSS CRC.

#### The potential role of radiotherapy

The “abscopal effect” is a rare phenomenon described for the first time in 1953. It consists of tumour regression in a site distant from the field of irradiation [66]. This may be due to the reactivation of host immune response against cancer cells. In fact, radiotherapy (RT) can increase the expression of MHC class I on cell membrane, and, thus, can improve antigen presentation by DC with a strong immune activation and subsequent ICD [67,68]. Currently, a few data are available for understanding the role of the combination of immune checkpoint inhibitors and RT for the treatment of CRC. One study has evaluated the combination of AMP224, a PD-1 inhibitor, with stereotactic body radiation therapy (SBRT) directed to liver metastases in patients with mCRC. The treatment was safe and feasible. However, no responses were observed [69]. Another trial investigated the clinical activity of pembrolizumab in combination with RT (cohort 1) or with local ablation (cohort 2) in a pre-treated population of mCRC patients. In the first cohort, one major response (ORR 9%, 1/11) was observed in a metastatic site distant from the irradiation field [70]. Currently, several clinical trials are on-going (Table 2).

#### The role of chemotherapy and of molecular targeted agents

In mCRC the combination of chemotherapy (5-fluorouracil, irinotecan, oxaliplatin) with molecular targeted agents that inhibit angiogenesis (bevacizumab, aflibercept, ramucirumab) or epidermal growth factor receptor (EGFR), such as cetuximab and panitumumab (in RAS wild type tumours) represents currently the standard of care for

**Table 2**  
Principal clinical trial investigating the combination of immune checkpoint inhibitors and radiotherapy.

Study name	Agent	Target	Study population	Primary endpoint	Phase	Recruitment status
NCT03102047	Chemoradiation/Durvalumab	PD-L1	Stages II-IV MSS CRC	Median Neoadjuvant Rectal Score	II	Active, recruiting
NCT03104439	Nivolumab/Ipilimumab/Radiation	PD-L1 CTLA-4	MSS/MSH-I mCRC	Disease control rate	II	Active, recruiting
NCT03007407	Durvalumab/Tremelimumab/Radiotherapy	PD-L1 CTLA-4	MSS mCRC	ORR	II	Active, recruiting
NCT02888743	Durvalumab/Tremelimumab ± Radiotherapy	PD-L1 CTLA-4	Metastatic to liver MSS CRC	ORR	II	Active, recruiting

MSS: Microsatellite stability; MSH-I: High microsatellite instability; ORR: Overall response rate.

the first two lines of treatment [71–75].

Novel experimental evidence suggests that the anti-tumour activity of chemotherapy is not only due to the direct cytotoxic effects on cancer cells but also potentially to the stimulation of the host immune response [76]. In fact, conventional chemotherapy can augment the immunogenicity of cancer cells by inducing immunogenic cell death and by blocking mechanisms of immune-tolerance [77]. In particular, myeloid-derived suppressor cells (MDSCs) can promote immune escape by inhibiting the activation and proliferation of CD8<sup>+</sup> lymphocytes. In preclinical models, 5-fluorouracil treatment determines the apoptosis of MDSCs, favouring the production of IFN $\gamma$  by CD8<sup>+</sup> cells and, therefore, increasing T-cell mediated anti-tumour response [78]. Oxaliplatin can also induce ICD by leading to the release of high mobility box binding protein-1 (HMGB-1) from cancer cells, which is recognised by the toll like receptor 4 (TLR4) and activates T CD8<sup>+</sup> cells [77,79].

Vascular endothelial growth factor (VEGF) plays a central role in physiologic angiogenesis and tissue and wound repair processes and is often up-regulated in numerous malignancies in which significantly contributes to tumour-induced neoangiogenesis [80]. Further, VEGF may be involved in regulating immune functions in the TME by several mechanisms, such as by decreasing T-cell migration from lymph nodes to tumour sites; by up-regulating suppressive immune checkpoint molecules (PD-1, PD-L1, CTLA-4, LAG 3); by increasing MDSCs; by inhibiting DC maturation; by down-regulation of MHC I and, consequently, by reducing the activation of CD8<sup>+</sup> cells [81–89]. In this respect, a study has recently evaluated the clinical activity of the combination of the anti-PD-L1 mAb atezolizumab with bevacizumab (cohort A) or the same combination plus modified FOLFOX6 chemotherapy (cohort B) [90]. In cohort A, 14 chemo-refractory mCRC patients, who had received at least two previous lines of chemotherapy, were treated with atezolizumab; 20 mg/kg, plus bevacizumab, 15 mg/kg, every 3 weeks. One patient achieved a partial response (ORR, 1/14, 7%) and 9 patients had stable disease as best clinical response. In cohort B, 23 mCRC patients received as first line of treatment atezolizumab 800 mg, bevacizumab 10 mg/kg and modified FOLFOX6 every 2 weeks. The ORR was 52% (12/23) with a median PFS of 14.1 months and a median duration of response of 11.4 months [91]. The treatment had an acceptable safety profile. Grade 3–4 TRAEs were observed in 67% patients. These preliminary results that suggest a potential synergistic activity of atezolizumab with chemotherapy plus bevacizumab need to be confirmed in appropriately sized, larger, randomized clinical trials. On the other hand, MODUL is a multicentre, randomized clinical trial, in which the best strategy of maintenance treatment in mCRC following a first line induction therapy with FOLFOX plus bevacizumab is being investigated with different therapeutic options. Recently, the results of the cohort of patients, that have been treated the addition of atezolizumab to standard treatment with fluoropyrimidine and bevacizumab after chemotherapy plus bevacizumab have been presented [92]. Unfortunately, no clinical advantage of this triplet maintenance treatment as compared to fluoropyrimidine plus bevacizumab was reported.

Cetuximab is a IgG 1 chimeric mouse-human mAb that inhibits the EGFR. Preclinical data have demonstrated that *in vitro* cetuximab could activate the opsonisation and phagocytosis of human colon cancer cell

by DC, and, therefore, may favour the activation of T-cell mediated immune response [89]. Furthermore, cetuximab stimulates NK-mediated cell antibody-dependent cellular cytotoxicity (ADCC) [93]. In a recent phase II clinical trial (AVETUX), 43 mCRC patients with all RAS and BRAF wild type tumours were treated with the anti-PD-L1 mAb avelumab plus cetuximab and FOLFOX6, as first line of therapy. An interim analysis of the AVETUX study on 20 patients has reported a promising ORR of 75% (15/20) with DCR of 95% (19/20), and an acceptable tolerability profile [94]. Furthermore, rechallenge with anti-EGFR mAb treatment in mCRC patients, that have previously obtained a clinical response to anti-EGFR therapy in previous lines of treatment, is a potentially effective strategy, as recently suggested by small size, proof of concept studies [95,96]. In this respect, our research group is currently conducting a large, single arm, multicentre phase II clinical study, which evaluates the combined treatment with cetuximab plus avelumab in pretreated mCRC patients, that obtained a partial or a complete response with an anti-EGFR mAb-based chemotherapy in the first line of treatment. Overall survival (OS) is the primary endpoint of this study (CAVE Colon study, Eudract Number 2017-004392-32).

Table 3 summarizes the clinical trials that are currently on going to evaluate the role of combination strategies with chemotherapy, molecular targeted agents and immune checkpoint inhibitors in mCRC.

#### *The role of MEK inhibition in combination with immune checkpoint inhibitors*

MEK is a key signalling molecule in the MAPK signalling pathway. In preclinical models, it has been suggested that MEK inhibition can lead to up-regulation of MHC I and can increase the infiltration of CD8<sup>+</sup> lymphocytes into tumours. Moreover, in an *in vivo* mouse model the combination of PD-1 and MEK blockade has determined a more effective inhibition of tumour growth as compared to single agent treatments, indicating a potential synergistic effect [97,98].

In 2016, Bendell presented the initial results of a phase Ib clinical trial, that was evaluating the clinical activity of the combination of atezolizumab, 800 mg every 2 weeks, plus cobimetinib, 60 mg orally, once a day (21 day on/7 day off) in chemo-refractory mCRC patients [99]. Twenty-three patients were treated. The ORR was 17% (4/23). Interestingly, 3 patients with a major response had an MSS tumour, while in the fourth patient with major response the tumour microsatellite status was unknown. Updated results from an expansion cohort of 84 heavily pre-treated patients reported an ORR of 8% (7/84), with durable responses (median duration of response 14.8 months) and median OS of 10 months [100]. On the basis of these promising results, the IMblaze370 (COTEZO) phase III randomized clinical trial was initiated. The study enrolled 363 chemo-refractory mCRC patients, that were randomized (2:1:1) to receive atezolizumab, 1200 mg plus cobimetinib 60 mg (21 day on/7day off schedule); atezolizumab monotherapy at the dose of 840 mg every 2 weeks or regorafenib at standard dose as control, standard arm of treatment. Unfortunately, the IMblaze370 trial did not met its primary endpoint. The median OS was 8.9 month with atezolizumab plus cobimetinib vs. 8.5 month with regorafenib monotherapy [hazard ratio (HR) 1.00, 95% CI: 0.73, 1.38

**Table 3**

Selection of ongoing clinical trial investigating the combination of immunotherapy with chemotherapy and target agents.

Study name	Agent	Target	Study population	Primary endpoint	Phase	Recruitment status
CheckMate9X8 (NCT03414983)	FOLFOX/Bevacizumab/Nivolumab Vs FOLFOX/Bevacizumab	PD-1	First line CRC (MMR not spciefied)	PFS	II/III	Active, recruiting
MEDITREME (NCT03202758)	FOLFOX/Durvalumab/Tremelimumab	PD-1 CTLA- 4	First line CRC (MMR not spciefied)	Safety	Ib/II	Active,recruiting
EleveTiON: CRC 101 (NCT03176264)	FOLFOX/Bevacizumab/PDR001	PD1	First line MSS CRC	DLT ORR	I	Terminated
AVETUX (NCT03174405)	FOLFOX/Cetuximab/Avelumab	PD-L1	First line CRC (MMR not spciefied)	PFS	II	Active, recruitment completed
BACCI (NCT02873195)	Capecitabine Bevacizumab/Atezolizumab vs Capecitabine/Bevacizumab	PD-L1	Pretreated metastatic CRC (MMR not spciefied)	PFS	II	Active, recruitment completed
NCT02860546	TAS-102/Nivolumab	PD-1	Pretreated metastatic MSS CRC	irORR	II	Completed
NCT03396926	Capecitabine/Bevacizumab/Pembrolizumab	PD-1	Pretreated metastatic MSS CRC	ORR	II	Active, recruiting
NCT02848443	TAS 102/Oxaliplatin/Nivolumab ± Bevacizumab	PD-1	Pretreated metastatic MSS CRC	Safety	I	Active, recruiting
CAVE Colon (Eudract 2017-004392-32)	Cetuximab/Avelumab	PD-L1	Pretreated metastatic CRC (MMR not spciefied)	OS	II	Active, recruiting

CRC: Colorectal-cancer; MMR: mismatch repair; PFS: Progression free survival; MSS: Microsatellite stability; DLT: Dose limiting toxicities; ORR: Overall response rate; PFS: Progression free survival; irORR: Immune related overall response rate; OS: Overall survival.

( $p = 0.987$ ]). Median OS was 7.1 month with atezolizumab monotherapy [(HR vs. regorafenib, 1.19 (95% CI: 0.83, 1.71)]. Moreover, there were no differences in terms of PFS and ORR across the treatment arms [101].

#### The role of combined PD-1 and CEA CD3 TCB blockade

Carcinoembryonic antigen (CEA) is a member of the immunoglobulin supergene family. It is localized on the cell membrane of enterocytes in the colonic mucosa. CEA is over-expressed in the majority of mCRC with levels of expression which could be up to 60 times higher than in normal cells [102,103].

CEA CD3 TCB (RG7802, RO6958688) is a T-cell bispecific antibody (CEA-TCB) that binds simultaneously CEA on tumour cells and CD3 on T cells. Thanks to its 2:1 ratio design, with two domains that bind CEA on tumour cells and one domain which recognizes CD3 on T cells, CEA-TCB can induce T cell migration, activation and proliferation in tumour sites with a potential significant anti-cancer activity [104]. Taberero has recently presented the first results of a phase I clinical trial evaluating CEA-TCB as monotherapy or in combination with atezolizumab in patients with CEA positive metastatic tumours [105]. In the monotherapy cohort, 31 patients with chemo-refractory mCRC were treated with CEA-TCB at doses of 60 mg or higher. Disease control rate was 45%, with two patients having a partial response (PR, 2/31, 6%) and with 12 patients reporting as best response a stable disease (SD, 12/31, 39%). In the combination arm, 11 patients were treated at doses of CEA-TCB between 80 and 160 mg that were shown to have a clinical activity. Interestingly, in this heavily pre-treated mCRC population, two patients had a partial response (PR, 2/11, 18%) and 7 a stable disease (SD, 7/11, 64%). CEA-TCB treatment displayed a complex safety profile. The most common grade 3 TRAEs, at doses of 40 mg or higher, were diarrhoea, infusion related reactions, pyrexia, with five patients in the single agent treatment arm that experienced a dose limiting toxicity (DLT) and two patients that experienced a DLT in the combination arm. Thus, the safety profile represents a major issue for the future development of this drug.

#### The role of cancer vaccines

The activation of a selective host immune response against cancer cell antigens could be an effective strategy to boost specific

immunotherapy. Several studies have been done to develop cancer vaccines for human CRC. However, little or no clinical efficacy has been observed so far [106].

DCs are able to process tumour-associated antigens (TAA), present them on the cell surface, causing the activation of T-cell and leading a cancer specific immune response. Dendritic cell vaccines can be obtained by isolating DC (autologous DC, ADC) from the host, pulsing them *ex vivo* with TAA, tumour lysate and then after activation, re-infusing them into the patient. The results of a phase II clinical trial, which was aimed to compare autologous tumour lysate DC plus best supportive care versus best supportive care, in pre-treated mCRC patients, were recently published [107]. Despite the fact that ADCs generated a tumour specific immune response, there was no clear benefit in terms of PFS or OS for the experimental treatment as compared with the control arm.

Peptide vaccines are based on combination of one or multiple antigenic epitopes derived from tumour-associated antigens with a vaccine adjuvant. These peptides are recognized and processed by DCs, stimulating a T-cell specific activation against cancer cells. A phase II study evaluated the combination of 5 peptides (RNF43, TOMM34, KOC1, VEGFR1 and VEGFR2) with an oxaliplatin-based chemotherapy, as first line treatment for patients with mCRC, but failed to demonstrate any clinical benefit [108].

OncoVAX is an active specific immunotherapy (ASI) that consists in the administration to patients of autologous irradiated cancer cells, which could combine with the Bacillus Calmette-Guerin (BCG), as immune-adjuvant. In a phase III study, 254 patients with stage II or III resected colon cancer were randomized to receive ASI or not as adjuvant treatment [109]. ASI consisted in 3 weekly vaccinations, starting 28 days after surgery, with a fourth vaccination at 6 months with irradiated tumour cells. Interestingly, for patients with stage II disease, it was observed a 61% reduction of the risk of the recurrence with ASI as compared to surgery alone with a trend toward improvement in OS. Based on these results, a confirmatory, randomized phase III trial is now recruiting patients with stage II colorectal cancer to receive OncoVAX or observation after surgery (ACTIVE trial, NCT02448173).

Oncolytic viruses are genetically modified viruses that could be able to selectively recognize and kill cancer cells; thus, avoiding damages to the host normal tissues. In a preclinical model, AD881, an oncolytic adenovirus, was able *in vitro* to induce ICD in CT26 cancer cells [110]. Similarly, G207 (a multi-mutated herpes simplex virus type I) had a

**Table 4**  
Selection of ongoing trial investigating the combination of immunotherapeutic agents.

Study name	Agent	Target	Study population	Primary endpoint	Phase	Recruitment status
NCT02448173	Surgery/OncoVAX Vs Surgery	OncoVAX	Stage II CRC (MRR non specified)	DFS	III	Active, recruiting
NCT02981524	GVAX colon vaccine/Pembrolizumab	PD-1	Pretreated metastatic MSS CRC	ORR	II	Active, non recruiting
NCT02959437	Azacitidine/Epacadost/Pembrolizumab	PD-1 IDO	Pretreated metastatic MSS CRC	Safety ORR	I/II	Active non recruiting
NCT02559024	MEDI6469	OX-40	Liver metastasis of CRC (MRR non specified)	Safety Immune score	I	Active, non recruiting
NCT03241173	INCAGN01949, INCAGN01949/Ipilimumab, INCAGN01949/ Ipilimumab,	OX-40	Advanced solid tumour	Safety ORR	I/II	Active, non recruiting
NCT02777710	PEXIDARTINIB/Durvalumab	CSF-1R PD-1	Pretreated metastatic CRC (MRR non specified)	Safety ORR	I	Active, non recruiting
NCT02503774	Oleclumab ± Durvalumab	CD73 PD-L1	Advanced solid tumour	Safety	I	Active, recruiting
NCT03207867	PDR001/NIR178	A2AR PD-1	Advanced solid tumour (including MSS CRC)	ORR	II	Active, recruiting
NCT03549000	NZV930, NZV930/PDR001, NZV930/NIR178, NZV930/NIR178/PDR001	A2AR CD73 PD-1	Advanced solid tumour	ORR	I	Active, recruiting

CRC: Colorectal-cancer; MMR: mismatch repair; DFS: Disease free survival; MSS: Microsatellite stability; ORR: Overall response rate.

significant cytotoxic activity in a panel of five human colon cancer cells lines [111]. Despite these compelling preclinical results, to date limited evidence is available in patients with mCRC. A phase I/II clinical trial evaluated the safety and the anti-tumour activity of NV1020, a genetically engineered oncolytic herpes simplex virus, in pre-treated chemo-refractory mCRC patients with liver metastasis [112]. Among the twenty-two patients that received the optimal dose treatment, disease control rate was 68% (1 partial response and 14 stable disease as best responses); median time to progression was 6.4 months and median OS was 11.8 months.

#### The role of IDO inhibitors

Indolamine 2,3-dioxygenase (IDO) is an enzyme that catalyses the conversion of tryptophan in kynurenine. Tryptophan is an essential amino acid for T-lymphocytes functions. Its depletion induces apoptosis and prevents T cell activation. On the other hand, kynurenine can promote T<sub>reg</sub> activation and has an immunosuppressive effect [113–115].

It was found that high IDO expression was present in nearly 40% of CRC, it correlates with poor prognosis and with liver metastasis [116]. In a mouse model of colon cancer, IDO inhibitions determined a modification in tumour microenvironment by reducing T<sub>reg</sub> and by increasing the levels of pro-inflammatory cytokines. However, IDO inhibition alone was not able to induce tumour regression, indicating that a combination treatment with immune checkpoint inhibitors could be a better strategy. A few data are available on the use of IDO inhibitors in a clinical setting in mCRC. A phase I study has assessed the tolerability of the IDO inhibitor epacadost in 52 patients with pre-treated solid tumours, including 29 patients with mCRC [117]. Further, a phase I/II clinical trial evaluating the combination of epacadostat with pembrolizumab and the epigenetic agent azacitidine in patients with advanced non small cell lung cancer and MSS mCRC is currently on-going (NCT02959437).

#### The role of OX40 agonists

OX40 (CD134) is a member of the tumour necrosis factor family of receptors, which is expressed by activated T cell, and acts as a co-stimulatory molecule. Its ligand OX40L is exposed on the cell membrane of antigen-presenting cells, such as Langerhans cells, mast cells, NK

cells, epithelial cells, endothelial cells and smooth muscle cells. Interactions between OX40 and OX40L stimulate proliferation, activation and cytokine productions of CD4+ and CD8+ cells, the expansion of antigen-specific memory T cells and suppress the differentiation of T<sub>reg</sub> cells [118]. Interestingly, it has been reported that high levels of expression of OX40 in TILs were found in 50% of tumours samples and were correlated with a increased overall survival [119].

An *in vivo* study using the Colon 26 cancer model showed that treatment with an OX40-agonist induced depletion of T<sub>reg</sub> and induced tumour-regression [120]. Several phase I/II trials are investigating the safety and activity of OX40 agonists as monotherapy or in combination with immune checkpoint inhibitors in different tumours including mCRC (see Table 4).

#### The role of macrophage colony-stimulating factor 1 receptor (CSF1R) inhibition

Myeloid-derived cell infiltration in TME could represent a mechanism of tolerance and immune escape. In fact, MSDs can suppress the maturation of DCs, inhibit the activation and proliferation of T-cells and contribute to invasiveness and metastasis [121].

CSF1R is a receptor that is expressed on monocytoïd cells. its activation by CSF favours the differentiation in MSDs. In preclinical models of colon cancer, CSF1R inhibition suppressed MSDs and delayed tumour growth [122]. Thus, a phase I study with the combination of a CSF1R inhibitor, pexidartinib, with an anti-PD-L1 antibody, durvalumab, in patients including colorectal and pancreatic cancer, is now under evaluation (NCT02777710).

#### The role of CD73 inhibition

High levels of adenosine in TME elicit the migration of cancer cells, stimulate angiogenesis, activate stromal remodelling and determine an immunosuppressive microenvironment. In fact, adenosine binds the adenosine A2A receptor (A2AR) and inhibits proliferation and activation of cytotoxic lymphocytes. Moreover, adenosine promotes the infiltration of immunosuppressive cells, such as T<sub>reg</sub> and MDSCs [123]. The enzyme CD73 is expressed on cancer and immune cells and catalyse the conversion of adenosine monophosphate to adenosine. Recently, it has been shown in a mouse model that dual inhibitions of A2AR and CD73 improve anti-cancer immune response [124]. To date, three

clinical trial evaluating the feasibility and activity of immune checkpoint inhibitors with anti-CD73 antibodies (NCT02503774), A2AR antagonists (NCT03207867) and combinations of both (NCT03549000) are on-going.

## Conclusions

Immunotherapy of cancer has become a novel effective therapeutic strategy since the role of immune checkpoint inhibitors has been elucidated in activating host immune response. Treatment with selective anti-PD1, anti-PD-L1 and/or anti-CTLA4 mAbs has been a revolution in the therapeutic scenario of several cancer types, with the highest clinical efficacy so far in melanoma and in lung cancer. However, even for these cancers, it is evident that immunotherapy is not effective in all patients and that biomarker selection is needed for the optimization of treatments.

Unfortunately, colorectal cancer is currently one of the tumour types in which immunotherapy has been shown less effective. Whereas in dMMR or MSI-H mCRC there is clear clinical evidence for a therapeutic role of immune checkpoint inhibitors, the majority of mCRC patients with pMMR or MSS tumours do not benefit from immunotherapy. Up to date, although promising preclinical findings and preliminary clinical results, we do not yet have clear evidence for immunotherapy efficacy in these patients.

A better knowledge of the molecular mechanisms that define the immune competence of a mCRC is needed in order to develop predictive biomarkers and effective therapeutic combination strategies such as with chemotherapies or with other molecular targeted agents or with co-stimulatory molecules. A major challenge will be in the next future to identify, among the heterogeneous spectrum of mCRC, those patients with specific tumour and tumour infiltrating stroma molecular and functional characteristics, that could be effectively treated with immunotherapy.

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## Conflict of interests

FC has participated to Advisory Boards for Merck KgA, Bayer, Amgen, Roche, Servier, Pfizer, and he has received institutional research grants from Merck KgA, Bayer, Amgen, Roche, Ipsen. The other authors have no interests to disclose.

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