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TARGETED ONCOLOGY - Review

Immune checkpoint inhibition in colorectal cancer: microsatellite instability and beyond

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1 **ABSTRACT**

2 Immune checkpoints inhibitors (ICI) have been a breakthrough, with unique response and
3 survival patterns compared with chemotherapy, for patients with advanced Mismatch Repair-
4 deficient/Microsatellite instable (dMMR/MSI) colorectal cancer but have shown
5 disappointing results in Mismatch Repair-proficient/Microsatellite stable (pMMR/MSS)
6 colorectal cancer. As up to 50% of patients harboring dMMR/MSI advanced cancers will
7 ultimately progress after PD-1 blockade, biomarkers are needed to predict response/resistance
8 to immunotherapy and select patients for immunomodulating combinations. Patients with
9 pMMR/MSS colorectal cancer present with distinct immune profiles compared to
10 dMMR/MSI tumors, giving evidence of different immune escape mechanisms which could be
11 overcome through individualized immunotherapeutic strategies.

12 In this review we discuss the latest developments in the field of immunotherapy for
13 dMMR/MSI and pMMR/MSS colorectal cancers and unresolved questions and considerations
14 concerning use of ICI therapies in this population. Future immunomodulation strategies based
15 on biomarker selection (tumor mutational burden, Immunoscore®, mutational profile) are
16 exposed.

1 **KEYPOINTS**

2 Despite dramatic responses to immune checkpoint inhibitors in patients with MSI
3 (microsatellite instability) colorectal cancers (CRC), nearly half of the patients will ultimately
4 progress and no biomarker is currently validated to correctly predict the resistance or benefit
5 derived from immunotherapy.

6 *POLE* exonucleasic domain mutations are a major alternative biomarker to select the best
7 candidates for immunotherapy in MSS (microsatellite stable) CRC.

8 The incorporation of immuno-genomicanalyses into future studies that assess
9 immunomodulation/targeted therapeutic combinations would improve the identification of
10 subsets of patients who would benefit from these treatments as well as provide support to
11 move the field towards individualized immunotherapeutic strategies in CRC.

1 **ABBREVIATIONS**

- 2 Best supportive care: BSC
- 3 Beta-2-microglobulin: B2M
- 4 Blood Tumor mutational burden (bTMB)
- 5 Colorectal cancer: CRC
- 6 Disease-free Survival: DFS
- 7 Disease response rate: DCR
- 8 Food and Drug Administration: FDA
- 9 Hazard Ratio: HR
- 10 Immune checkpoint inhibitor: ICI
- 11 Immune Response Evaluation Criteria in Solid Tumors criteria: iRECIST
- 12 Immune-related Response Evaluation Criteria in Solid Tumors criteria: irRECIST
- 13 Insertion/deletion mutations: InDels
- 14 Interferon gamma: IFN- γ
- 15 Major histocompatibility complex: MHC
- 16 Metastatic colorectal cancer: mCRC
- 17 Microsatellite instability: MSI
- 18 Microsatellite stable: MSS
- 19 Mismatch repair: MMR
- 20 Mismatch-repair deficiency: dMMR

- 1 Mismatch repair-proficient: pMMR
- 2 Mutations per megabase: mt/mb
- 3 Objective response rate: ORR
- 4 Overall survival: OS
- 5 Polymerase proofreading-associated polyposis: PPAP
- 6 Progression-free survival: PFS
- 7 Response Evaluation Criteria in Solid Tumors criteria: RECIST
- 8 Tumor mutational burden: TMB
- 9 Vascular Endothelial Growth Factor: VEGF

1. Introduction

Immune checkpoint inhibitors (ICI) represent one of the major therapeutic breakthroughs in the history of medical oncology. In this context, metastatic colorectal cancer (mCRC) represent an intriguing entity, with a minority (4 to 5%) of tumors being highly sensitive to these compounds (*i.e.* CRC harboring microsatellite instability (MSI) and/or mismatch-repair deficiency (dMMR) [1]) but a vast majority are cold tumors refractory to immunotherapeutic strategies [2–4]. It has been known for years that MSI/dMMR tumors are highly infiltrated by immune cells [5]. These tumors are characterized by a high tumor mutational burden (TMB) with highly immunogenic neoantigens arising from frameshift mutations [6]. Moreover, they are associated with an upregulation of checkpoint inhibitors that exhaust intratumoral cytotoxic T lymphocytes and consequently protect MSI cancer cells from their hostile immune microenvironment [7,8].

Following the durable complete response of one patient with an MSI/dMMR mCRC treated with anti-PD1 ICI in a phase I study that included various types of unselected solid tumors, research and development of ICI for CRC focused on MSI/dMMR mCRC [9]. The impressive results of several phase II studies demonstrated that immune checkpoint inhibition is a breakthrough therapeutic strategy for dMMR/MSI mCRC [1,10,11]. Pembrolizumab or nivolumab alone or in combination with ipilimumab have been therefore approved by the Food and Drug Administration (FDA) for the treatment of chemoresistant MSI/dMMR mCRC patients. More, the FDA granted accelerated approval to pembrolizumab for advanced MSI/dMMR tumors progressing following prior treatment, in a tissue/site-agnostic fashion [12]. The efficacy of ICI in MSI/dMMR mCRC is now well-established, and trials are currently ongoing to assess ICI efficacy in first line, adjuvant or even neoadjuvant setting.

However, questions remain concerning the role of ICI for the treatment of microsatellite stable (MSS) and mismatch repair-proficient (pMMR) CRC. The combination of ICI with

1 other anticancer drugs is currently being evaluated in MSS/pMMR mCRC. However, the
2 disappointing results of the phase III IMblaze 370 trial (atezolizumab with or without
3 cobimetinib *versus* regorafenib) raises concerns regarding the testing of therapeutic ICI
4 strategies without a guiding biomarker in these “cold” tumors [13]. It has been shown that
5 TMB could be a biomarker for ICI efficacy in various histologies and some MSS/pMMR
6 CRCs show a high TMB, notably due to mutations within genes coding for DNA polymerase
7 [14,15]. Furthermore, the consensus molecular subtype (CMS) classification, which allows for
8 better comprehension of CRC molecular heterogeneity, might be a tool worth considering for
9 the development of new immunotherapeutic strategies in MSS/pMMR CRC [16].

10 In this review, we highlight how MSI/dMMR has emerged as a powerful biomarker for ICI
11 efficacy in mCRC. Then we will focus on the currently explored ways to expand the use of
12 ICI in CRC beyond MSI/dMMR and also in adjuvant and neoadjuvant settings.

2. Immune checkpoint blockade in colorectal cancer: from disappointment to microsatellite instability

The evaluation of immune checkpoint inhibitors (ICI) in CRC has been initially disappointing. The results of phase I basket trials including molecularly unselected mCRC patients did not show evidence of clinical activity. Notably, no objective responses were observed amongst the 3, 19 and 18 mCRC patients treated with pembrolizumab, nivolumab (anti PD-1 monoclonal antibodies), or BMS-936559 (anti-PD-L1 monoclonal antibody), respectively [2–4].

2.1. Clinical activity of ICI in patients with CRC MSI/dMMR

The breakthrough of immunotherapy in CRC came from one out of the 14 mCRC patients included in the basket phase I trial CA209-001 (NCT00441337), who experienced a durable complete response with nivolumab. This patient's tumor presented an MSI phenotype [9].

The proof-of-concept phase II study (MK-3475-016, NCT01876511) was designed to assess the efficacy of pembrolizumab in three cohorts of chemoresistant metastatic cancer patients: MSI/dMMR mCRC, MSI/dMMR non-CRC and MSS/pMMR CRC [1]. Preliminary results published in the New England Journal of Medicine in 2015 showed a 40%, 57% and 0% objective response rates (ORR), respectively, in the three pembrolizumab-treated cohorts (N = 11; N = 9; N = 21). This was the first step to recognize MSI/dMMR as a major predictive tissue-agnostic biomarker for the efficacy of ICI [12]. The updated data after 28 MSI/dMMR mCRC patients treated with pembrolizumab confirmed these encouraging results, with 11% of complete responses, 46% of partial responses and only 4% of progressive diseases [17].

Results of immune checkpoint blockade strategy in MSI/dMMR mCRC patients are summarized in Table 1. In the Keynote-164 study, pembrolizumab was evaluated in two distinct cohorts: (i) cohort A, after 2 or more prior lines of therapy including

1 fluoropyrimidine, oxaliplatin and irinotecan, and; (ii) cohort B, after 1 or more prior line of
2 therapy. In these 2 cohorts, pembrolizumab was associated with disease control rate (DCR) of
3 51% and 44%, and 1-year progression-free survival (PFS) rate of 34% and 41%, respectively
4 [18–20].

5 Nivolumab has been also evaluated in the context of MSI/dMMR mCRC. In the CheckMate-
6 142 phase II trial, nivolumab alone (nivolumab 3 mg/kg every 2 weeks) and its combination
7 with ipilimumab (nivolumab 3 mg/kg every 3 weeks + ipilimumab 1 mg/kg every 3 weeks for
8 4 cycles then nivolumab 3 mg/kg every 2 weeks) until disease progression exhibited a 34%
9 and 58% ORR, respectively, with 36% and 12% RECIST1.1 progressive disease at first
10 evaluation, respectively. The 2-year PFS and OS (overall survival) rates were 60% and 74%
11 with the combination of nivolumab and ipilimumab [21,22]. In both cohorts, patients were
12 resistant or intolerant to at least 1 prior line of chemotherapy before inclusion: 54% and 40%
13 of patients, respectively, received 3 lines of systemic treatment or more before ICI. In a third
14 cohort of the CheckMate-142 trial, 45 patients with no prior treatment for MSI/dMMR mCRC
15 were treated with nivolumab 3 mg/kg every 2 weeks plus low-dose ipilimumab every 6 weeks
16 until disease progression. ORR was 77% and only 1 progressive disease was observed; 1-year
17 PFS and OS rates were 77% and 83%, respectively [23].

18 Durvalumab, an anti-PD-L1 monoclonal antibody, has also been tested in the context of
19 MSI/dMMR mCRC. Amongst 36 MSI/dMMR mCRC patients treated with durvalumab 10
20 mg/kg IV every 2 weeks (NCT01693562 and NCT02227667.), ORR was 22%, DCR for more
21 than 24 weeks was 47% and the 2-year OS rate 54% [24]. Interestingly, patients were treated
22 for a maximum of 12 months whereas in other trials ICI treatment was maintained for 2 years
23 or until disease progression.

24 2.2. ICI compared to conventional chemotherapy

1 Based on the positive results from these clinical trials [1,10–12], pembrolizumab as well as
2 nivolumab alone or in combination with ipilimumab have been granted accelerated approval
3 by the FDA for chemoresistant MSI/dMMR mCRC patients. However, the European
4 Medicine Agency has not yet approved ICI for these subsets of patients.

5 Several randomized trials are currently evaluating the efficacy of ICI as compared to standard-
6 of-care chemotherapy \pm targeted therapy in first or second-line metastatic setting (Table 2).
7 Notably, the KEYNOTE-177 and COMMIT phase III studies might lead to the approval of
8 pembrolizumab and atezolizumab, respectively, in frontline MSI/dMMR mCRC if they meet
9 their primary endpoint. The ongoing randomized phase II trial PRODIGE 54 – SAMCO(
10 NCT03186326) evaluates the efficacy of avelumab in second-line *versus* standard of care
11 treatment (chemotherapy \pm targeted therapy). In the randomized phase III CA209-8HW study
12 (NCT04008030), patients are randomized to receive nivolumab alone, nivolumab plus
13 ipilimumab or investigator's choice chemotherapy \pm targeted therapy. The main objective is to
14 compare the clinical benefit, as measured by PFS, ORR and OS, achieved by nivolumab in
15 combination with ipilimumab or by nivolumab monotherapy in patients with MSI/dMMR
16 mCRC.

17 Importantly, 2 phase III trials are currently assessing the efficacy of ICI in adjuvant setting.
18 The Alliance A021502 trial (NCT02912559) is evaluating mFOLFOX6 \pm atezolizumab in
19 patients with stage III colon cancer. In the experimental arm, patients receive 6 months of
20 adjuvant mFOLFOX6 concurrently with atezolizumab, followed by 6 additional months of
21 atezolizumab monotherapy. The POLEM trial (EudraCT 2017000370_10) is testing the
22 benefit of adding ICIs after the completion of capecitabine-based adjuvant chemotherapy (3
23 months of capecitabine plus oxaliplatin or 6 months of capecitabine monotherapy) in
24 MSI/dMMR or *POLE*-mutated stage III CRC. At the end of adjuvant chemotherapy, patients

who are randomized to the investigational arm will receive additional 24 weeks of treatment with avelumab. The primary endpoint is disease-free survival in both trials.

2.3. Biomarkers within the MSI/dMMR population

Potential biomarkers of ICI efficacy amongst MSI/dMMR population are summarized in table 3.

Despite high rates of response and durable clinical benefit with ICI, 10 to 50% of MSI/dMMR mCRC tumors exhibit primary resistance to immunotherapy. However, a significant amount of these refractory tumors are mistakenly diagnosed as MSI/dMMR. In a cohort study of 38 mCRC patients included in ICI clinical trials based on a positive MSI and/or dMMR status as determined by local laboratories, misdiagnosis of MSI or dMMR status was responsible for 3 of 5 cases of primary resistance to ICI [25]. Thus, the first concern when observing immediate tumor progression under ICI is the possibility of a misdiagnosed MSI phenotype that requires confirmatory analysis by immunohistochemistry or molecular assay, the alternate hypothesis being tumor pseudo-progression [25,26].

Preliminary results on potential biomarkers for the efficacy of ICI amongst MSI/dMMR mCRC did not find significant predictive impact neither in *RAS/RAF* mutational status, tumor PD-L1 expression, the inherited (*i.e.* Lynch syndrome) nor sporadic origin of MMR deficiency nor the tumor mutational load [1,10,11,27]. Concerning the mechanism underlying MMR deficiency, it is worthy to note that the results remain questionable since the definition of Lynch syndrome-related cancers and sporadic cases is partly equivocal. In the analysis of ICI clinical trials, MMR deficiency origin was determined by investigators based on past medical history collected from clinical records without a systematic molecular approach that might skew the results [1,11,28]. Moreover, it has been recently proven that Lynch-like tumors (MSI tumors without *MLH1* promoter hypermethylation, *BRAF* mutation, or germline

MMR gene mutation) arise from biallelic somatic MMR gene inactivation in approximately 50% of patients [29]. Therefore, further studies are warranted to confirm these findings.

Several potential mechanisms of resistance are under evaluation, such as notably deleterious mutations of *JAK* (found in approximately 10 to 20% of MSI/dMMR tumors), loss of major histocompatibility complex (MHC) molecules or *B2M* (beta-2-microglobulin) loss-of-function mutations (20-30% of MSI CRC) [30–33]. The latter was not found to be associated with ICI resistance in a cohort of 13 *B2M*-mutated MSI/dMMR mCRC with ORR of 85% (11/13). Remarkably, the authors showed that MHC class I expression is variable in *B2M*-mutated MSI CRC [34] but not associated with response to ICI. To note, there is currently no available data on the impact of gut microbiota in MSI/dMMR cancer patients treated with ICI [35,36]. Histopathological characteristics such as the amount of extracellular mucin and PD-L1 expression at the invasive front might be associated with resistance to ICI [37].

The impact of tumor mutational load remains controversial within the MSI/dMMR population. Important recently published works detected a positive correlation between tumor mutational load and the efficacy of ICI amongst MSI/dMMR population [38,39]. However, the sample sizes remain small, with potential tumors misdiagnosed as MSI/dMMR amongst ICI-resistant cases [25]. Moreover, the threshold of tumor mutational load to be considered as predictive biomarker may be different from other tumor types. Larger translational studies are therefore warranted to establish the role of TMB in MSI/dMMR tumors. Finally, intra- and inter-tumoral heterogeneity might negatively influence ICI efficacy on sporadic MSI/dMMR tumors especially in cases of mixed dMMR/pMMR tumors [40,41].

The quality of the neoantigens seems capital as a potential new biomarker, as demonstrated in MSI/dMMR tumors treated by ICI [12]. Even in the context of high TMB and predicted neoantigens, very few effective immune responses directed against tumor associated neoantigens are detected, suggesting that immune response to ICI may rely only on a few

highly immunogenic antigens. These antigens are derived mostly from insertion/deletion mutations (InDels), a hallmark of dMMR tumors [38,42]. Importantly, this phenomenon may be difficult to overcome in MSS/pMMR tumors where InDels are rarely detected .

2.4. Controversies in the clinical management of MSI/dMMR mCRC patients treated with ICI

Pseudo-progression is a noted phenomenon of ICI therapy. Pseudo-progression is an initial radiographic tumor enlargement (consistent with progression per Response Evaluation Criteria in Solid Tumors criteria (RECIST)) followed by measurable tumor regression, sometimes months to years after therapy initiation. The frequency of pseudo-progression in MSI/dMMR population is currently unknown but it may be common in this highly sensitive population. Amongst patients from the CheckMate-142 trial with progression of disease as best response (n=14) and who continued treatment beyond progression (N = 11), those with a reduction (N = 3) or stabilization of target lesions (N = 3) were more likely to survive more than 12 months [21]. Distinguishing pseudo-progression from true disease progression is tricky, and irRECIST (Immune-related RECIST) or iRECIST (immune RECIST) criteria can be helpful [43]. Clinical parameters such as the improvement of performance status, improvement of symptoms or decrease of serum tumor marker levels may help in the clinician's decision to continue the ICI and wait for later radiologic evaluations. To note, the KEYNOTE-016 trial used irRECIST to assess response, meanwhile others such as CheckMate-142 and KEYNOTE-164 used RECIST v.1.1. This difference in radiologic evaluation criteria might explain the difference in ORR across studies [10,11,17,18,20].

Concerning secondary resections of primary tumor or metastasis after ICI treatment, the data are scarce. Some case reports suggested that pre-operative immunotherapy followed by resection of primary tumor and or metastatic sites is associated with high rates of pathological

1 complete response, despite the presence of residual lesions on radiological exams [44]. This
2 brings to light some issues in the therapeutic management of ICI-treated patients with
3 MSI/dMMR cancer: (i) what is the best therapeutic strategy for long responder patients: ICI
4 continuation, surgical removal of residual lesions or ICI discontinuation without surgery?, and
5 ; (ii) Would those patients experiencing disease progression confined to a single site benefit
6 from surgery? These issues are currently emerging since the number of ICI-treated
7 MSI/dMMR mCRC patients is growing exponentially.

8 Another emerging crucial concern is the optimal duration of treatment for patients who
9 respond to ICI. The strategy varies across trials, with a fixed duration of ICI (usually limited
10 to 1 or 2 years of treatment) or the continuation of treatment until progression or unacceptable
11 toxicity, which is a burden for patients and healthcare systems. The question remains
12 unanswered and clinical trials are needed to find the optimal treatment duration.

13

3. Immunotherapy in colorectal cancer beyond MSI/dMMR

Ongoing clinical trials evaluating therapeutic strategies with ICI for MSS/pMMR CRC patients are displayed in table 4.

3.1. Hypermutability amongst microsatellite stable colorectal cancer: DNA polymerase mutations

The biological substratum of ICI efficacy on MSI/dMMR tumors relies on immunogenic frameshift mutations and high TMB. Several studies have shown that MSI/dMMR CRC could be detected through TMB evaluation with similar accuracy as polymerase chain reaction or immunohistochemistry [45,46]. Moreover, there is a high-TMB population that do not exhibit MSI/dMMR but displays an ultramutated phenotype with significantly higher TMB than MSI/dMMR CRC: all these tumors harbor mutations in DNA polymerase genes (Figure 1) [15,45,47].

In the TCGA cohort (N = 276), 16% of CRC samples (N=44) exhibited hypermutation (defined as a TMB greater than 12 mutations per 10^6 bases). Among these, all MSS tumors (N=7) harbored exonucleasic proofreading domain *POLE*-mutations (DNA epsilon polymerase) and presented the highest TMB. Similarly, in a study by Stadler and colleagues, 31 out of 224 CRC samples were hypermutated, of which 3 MSS *POLE*-mutated tumors presented a TMB 3-fold higher than MSI/dMMR cases [45]. In a study by Vanderwalde and colleagues, 93 out of 1395 CRC (6.7%) samples were hypermutated (TMB high if ≥ 17 mutations per megabase (mt/mb)) and 80 (5.7%) were MSI (and only 4 MSI tumors had low TMB) [46].

DNA polymerase enzymes are involved in DNA synthesis and repair during S phase of cell cycle. Delta and epsilon polymerases (*POLD* and *POLE*) are the main DNA polymerases in humans, with a proofreading exonuclease domain activity allowing error correction during

1 replication. Deleterious mutations in the exonuclease domain of *POLD* and *POLE* genes are
2 responsible for a dramatic genetic instability associated with an ultramutated tumor
3 phenotype. Germline mutations of *POLD* and *POLE* genes are associated with an autosomic
4 dominant familial cancer syndrome favoring notably endometrial and colorectal cancers
5 occurrence (PPAP: polymerase proofreading-associated polyposis). Somatic *POLE* gene
6 mutations may also occur [48,49]. Interestingly, *POLE* mutations have been described in
7 MSI/dMMR cancers, but arise distantly to the exonuclease domain and are therefore
8 considered as passenger mutations due to the high genetic instability of MSI/dMMR tumors.
9 This hypothesis is supported by the higher TMB of *POLE*-mutated MSS CRC compared to
10 *POLE*-mutated MSI CRC tumors [42,50].

11 Exonucleasic domain *POLE*-mutated CRCs typically arise from left colon and rectum in
12 younger male patients. Not all *POLE* mutations seem to be driving hypermutagenesis and the
13 high mutation load phenotype seems restricted to specific hotspots. These mutations are
14 observed in 1% of stage II and III tumors and are associated with favorable prognosis. This
15 population is very rare in the metastatic setting, accounting for less than 1% of cases [47,51],
16 and its prognosis and response to conventional treatments are still poorly understood.
17 Considering ICI in this population, the literature is scarce but nevertheless promising with
18 case reports suggesting major clinical activity for *POLE*-mutated CRC patients [52–54].
19 Several ongoing clinical trials are dedicated to this population (NCT03827044,
20 NCT02715284, NCT03012581).

21 3.2. Tumor Mutational Burden and efficacy of ICI in CRC

22 TMB is an exonic genomic measure of non-synonymous mutations in tumor cells. Using
23 algorithms comparing DNA sequences from control tissues to tumor samples, TMB helps to
24 predict the number of somatic mutations within tumors. Therefore, it is an indirect measure of
25 tumoral predicted neoantigens [55]. The usefulness of TMB is based on the concept that when

1 it is high, the likelihood of observing an effector T cell response directed against a specific
2 tumor antigen is increased. This concept seems to be verified in MSI/dMMR tumors, as both
3 are hypermutated and rich in effector immune cells. A recent study evaluated the predictive
4 value of TMB in MSI/dMMR tumors treated by anti PD-1 [41]. Authors showed that patients
5 with high TMB (> 37 mt/Mb) tumors displayed an ORR of 100% ($N = 13$) and improved PFS
6 compared with patients with low TMB tumors ($N = 9$) [39]. While these results are promising,
7 confirmation in prospective larger cohorts is needed before being translated to clinical
8 practice because, as previously mentioned, the impact of the mutation load varies from one
9 study to another.

10 MSS/pMMR CRC tumors may also display a high TMB but with a low frequency, including
11 exonucleasic domain *POLE*-mutated tumors and other CRCs in which the mechanism of
12 hypermutation remains unclear. A recent 2:1 randomized phase II clinical trial, CCTG CO.26,
13 assessed the combination of tremelimumab, an anti-CTLA-4 and durvalumab, *versus* Best
14 Supportive Care (BSC) in 180 MSS chemoresistant mCRC patients [56]. The trial reached its
15 primary endpoint, with a median OS of 6.6 months for the ICI combination *versus* 4.1 months
16 for BSC (HR 0.72 $P = 0.07$ for a preplanned threshold <0.1 for statistical significance) but
17 without any improvement in the median PFS which were 1.8 and 1.9 months, respectively.
18 DCR was 22.7% for the experimental arm *versus* 6.6% for BSC. TMB was assessed on cell
19 free DNA (cfDNA) using a 500-gene next generation sequencing panel [57], and, excluding 2
20 MSI/dMMR patients, the mean TMB was 20.4 ± 16.3 mts/Mb (range: 0.96 - 114.0).
21 Interestingly, 21% of tumors had a high TMB (defined as ≥ 28 mt/Mb), which was associated
22 with better OS compared to patients with low or intermediate TMB tumors (5.5 month *versus*
23 3 months respectively, HR 0.34, 90% CI 0.18-0.63). Nevertheless, the pre-specified threshold
24 of 20 mt/Mb was not predictive of benefit either for DCR, PFS or OS.

1 Genotyping of circulating tumor DNA (ctDNA) allows for a non-invasive quantification and
2 characterization of the tumoral molecular profile and can indicate tumor heterogeneity and
3 changes in TMB in real time. High blood TMB (bTMB) has been recently associated with an
4 increase of response and survival in other tumor types treated with immunotherapy becoming
5 a promising biomarker for ICI [58–60]. However, bTMB shows higher number of mutations
6 per megabase compared to classic TMB assessed in tissue resulting in variable concordance
7 data between tissue and liquid samples. Moreover, in CRC it remains unclear why a benefit is
8 observed in OS but not in other outcomes. Further prospective studies are needed for
9 implementation of bTMB into clinical practice.

10 The theoretical presence of tumoral neoantigens may not be able to independently predict
11 their immunogenicity. A high number of neoantigens and/or high quality neoantigens are
12 necessary to induce a durable antitumor immune response. A recent study underlined that
13 non-hypermutated MSS CRC have a very low predicted number of neoantigens (45 ± 22)
14 compared to MSI (635 ± 308), or hypermutated MSS CRC (1651 ± 1475) [61]. Notably, the
15 number of predicted neoantigens decreases from early to advanced stage in the context of an
16 active immunoediting, resulting in the elimination of the immunoreactive subclones and
17 selection of immune privileged CRC subclones [62]. Specific translational immunological
18 studies should be performed at latter stage to better understand the immune escape
19 mechanisms in mCRC.

20 3.3. Immunoscore® and efficacy of ICI in CRC

21 The immunoscore® is a measure of the tumor-associated inflammatory stroma, assessing pan-
22 / cytotoxic lymphocyte densities ($CD3^+$ and $CD8^+$, respectively) inside the tumor and at the
23 invasion margin [63]. The immunoscore® is a validated independent prognostic factor in
24 early stage CRC to predict disease-free survival. Patients with a low immunoscore®, *i.e.* low
25 infiltration of $CD3^+$ and $CD8^+$ cells, have higher risk of recurrence thereby justifying its use

as a stratification factor in adjuvant clinical trials [64]. In this publication, when stratified into two immunoscore® categories (0-1, and 2-4), 49 MSI tumors (16%) had a low immunoscore® (0-1) and 255 (84%) had intermediate and high immunoscore® (2-4). Patients with intermediate and high immunoscore® had prolonged DFS and OS, irrespective of their microsatellite status (unadjusted HR for high versus low immunoscore® in MSI tumors 0.56, 95%CI 0.34-0.90; P = 0.0150). DFS of patients with immunoscore® high was similar for MSS and MSI tumors (around 80% at 3 years) and DFS of patients with immunoscore® low was similar for MSS and MSI tumor (around 60%).

The usefulness of immunoscore® in advanced disease seems more complex, as it relies on the assessment of the immune contexture of the invasion margin. Indeed, discordances between the primary tumor and the metastases has been observed and only the immunoscore® of metastases remained an independent prognostic factor in multivariate analyses [65]. In other tumors, the lymphocytic infiltration or intratumoral PD-L1 expression is not always associated with a benefit from immunotherapy, which is better predicted by transcriptional signatures where interferon gamma (IFN-γ) response genes are included [66]. The immunoscore® correlation with IFN-γ response remains to be established and, if confirmed, would allow the prospective assessment of ICI according to immunoscore® both in dMMR and pMMR tumors.

3.4. Molecular alterations and immune escape mechanisms in CRC

The international consensus molecular classification of CRC has been proposed, with 4 subtypes described so far: CMS1 (immune), CMS2 (canonical), CMS3 (metabolic), and CMS4 (mesenchymal) [16]. Interestingly, the CMS are associated with specific immune and stromal profile [67,68]. The CMS was developed using a set of localized tumors and its value in the metastatic setting remains controversial: (i) spatial CMS heterogeneity has been described, as some tumors have a homogeneous CMS and others have a heterogeneous CMS,

1 which complicates the allocation of tumors to a specific group and modulate the prognosis
2 [69]; (ii) CMS modifications has been observed within metastatic tumor under active
3 antitumoral treatment such as anti-EGFR agents [70]. Consequently, CMS clinical
4 applicability in the metastatic setting remains limited and even reinforced by the complexity
5 of bioinformatical analyses. Nevertheless, CMS helped to identify at least three main immune
6 escape mechanisms in CRC that are related to distinct molecular pathway alterations.
7 Importantly, there is also an increasing body of evidence showing that the genomic landscape
8 of CRC correlates with specific immune profiles and immune escape mechanisms, which are
9 consistent with the one described in the CMS. Figure 2 proposes an immune classification of
10 CRC based on molecular pathway alterations and the potential treatment strategies to
11 immunomodulate each tumor subtype.

12 Firstly, tumors with DNA repair impairment, such as MMR deficiency or mutations within
13 the polymerases exonucleasic domain, are prone to accumulate immunogenic mutations.
14 These tumors are immune inflamed and characterized by high infiltration in CD8 cells, a high
15 number of neoantigens, and increased expression of CMH-I and immune checkpoints such as
16 PD-1, PD-L1, CTLA-4, resulting in an immune profile that is highly favorable for ICI
17 activity.

18 Secondly, CRC with TGF- β pathway dependency presents a strong angiogenesis activation
19 and an immune inflamed profile. The role of TGF- β in colorectal carcinogenesis is complex
20 as it may activate multiple transduction pathways and has a dual anti- and pro-tumoral role
21 from early stage to late stage cancer [71]. Multiple alterations in genes implicated in TGF- β
22 transduction pathways have been described in CRC, such as in the *SMAD* family genes,
23 *BMPRIA* or *TGFb* receptors itself. The impact of these alterations on immune cell recruitment
24 in human CRC remains to be elucidated, but multiple preclinical models have shown that
25 impairment of these genes results in a chronic inflammation favoring colorectal cancer

1 progression [71]. Even if immune inflamed, the immune contexture of TGF- β dependent
2 tumors is different from DNA repair impaired tumors and is characterized by an imbalance
3 between the pro- and anti-tumoral immune cells with higher Treg, M0 and M2 macrophages
4 and lower number of CD8⁺ cells. Enrichment in immunosuppressive and complement factors
5 such as high expression of chemokines attracting myeloid cells is also observed, reinforcing
6 the pro-tumoral immune response. Recent preclinical studies suggest that targeting the TGF- β
7 pathway in CRC favors the recruitment of T cells and enhances the Th1 response leading to
8 an increased sensitivity to ICI [72]. Another seductive target is angiogenesis, as Vascular
9 Endothelial Growth Factor (VEGF) pathway inhibitors have shown encouraging results in
10 other tumor types in combination with ICI [73,74]. Indeed, in CRC, anti-VEGF agents were
11 shown to increase CD8⁺ cells infiltration and decrease Treg in resected metastases, giving a
12 rationale for combination strategies with ICI [75].

13 Finally, tumors with WNT/ β -catenin pathway activation derived from *APC* or *CTNNB1*
14 mutations have been shown to be poorly infiltrated by immune cells through the lack of
15 immune cells trafficking [76] and characterized by: (i) few nonsynonymous mutations; (ii)
16 low intratumoral immune infiltrate with a global decrease in lymphocytes, CD8⁺ cells, Th1,
17 activated NK, and PD-L1⁺ mono/macrophages, and; (iii) low CMH-1 and B2M expression,
18 suggesting poor antigen presentation. Preclinical models of CRC suggested that inhibition of
19 WNT/ β -catenin pathway could increase the immunological “hotness” of these tumors and
20 generate sensitivity to ICI [77].

21 Compared to CMS assessment, immuno-genomic profiling could be more easily transposable
22 in clinical practice as it relies on DNA sequencing and/or pathological assessment. This
23 immuno-genomic profiling should probably be repeated overtime during the course of the
24 metastatic disease and after exposure to treatments due to constant immunoediting, which
25 actively modulates the immuno-genomic phenotype [62].

1 Setting aside the issue of the CMS classification, a recent early phase I trial assessing the
2 combination of nivolumab and regorafenib (anti-VEGF Receptor inhibitor) in MSS mCRC
3 showed encouraging results with an ORR of 33% and a median PFS of 6.3 months warranting
4 further development [78], suggesting that angiogenesis inhibitors and ICI are synergistic in a
5 subset of CRC, possibly the TGFβ/angiogenesis subgroup. Nevertheless, the use of other
6 kinase inhibitors, such as MEK inhibitors, in combination with anti PD-L1 failed to show an
7 improvement compared to regorafenib monotherapy in MSS mCRC tumors, despite showing
8 promising results in phase Ib trials [13].

9

4. Conclusion and perspectives

ICI has been a major oncologic breakthrough in dMMR/MSI CRC but showed very limited results in low-TMB pMMR/MSS patients, with disappointing results in several clinical trials. Despite dramatic responses to pembrolizumab or nivolumab in patients with dMMR/MSI tumors, nearly half of the patients would finally progress, and no biomarker is currently validated to correctly predict the resistance or the benefit derived from ICI, precluding further clinical development. Potential biomarkers are controversial because they are assessed retrospectively or in *post hoc* translational studies of small cohorts. Two examples are (i) *B2M* mutations thought to be predictive of resistance in early studies and not confirmed in latter studies, and (ii) the discrepancies between mutation load and TMB correlations with clinical outcome, potentially explained by technical limitations and non-consensual bioinformatical analyses. Combination of anti-PD-1/PD-L1 and anti CTLA-4 agents seems to increase the benefit with an acceptable safety profile in selected patients with MSI/dMMR mCRC. Currently, there are still many unresolved questions concerning the clinical management of patients with ICI-treated MSI/dMMR mCRC such as duration of therapy, surgical removal of post-immunotherapy residual lesions, the best predictive factors of efficacy, or the choice of the best treatment scheme approach.

POLE/D exonucleasic domain mutations are an important alternative biomarker to select best candidates for ICI in MSS/pMMR mCRC. *POLE*-mutated tumors seem rare and confirming ICI activity in this subset may be challenging prospectively as the pathogenicity of these mutations remains unclear outside of the described hotspots. TMB is a seductive biomarker to offer ICI to patients with MSI/pMMR CRC with high mutation load. First results seem to indicate a limited benefit on OS but not on other outcomes. However, TMB assessment remains expensive, without a clear threshold and technical/bioinformatical pitfalls, and its predictive value remains to be established both for MSS/pMMR and MSI/dMMR tumors. The

1 Immunoscore® and IFN- γ response signature are also emerging biomarkers but
2 preclinical/translational studies are still necessary to confirm their value in CRC, especially in
3 pMMR patients before designing prospective clinical trials. Finally, immuno-genomic
4 classification may be useful, especially to separate MSS/pMMR CRC in clinical trials in
5 accordance with immune escape mechanisms. Incorporating immune-genomic profiling in
6 future studies assessing immunomodulation/targeted therapeutic combinations would be of
7 added value to identify subsets of patients benefiting from these treatments and move to
8 individualized immunotherapeutic strategies in CRC.

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2

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4

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1 **Table 1: Immune checkpoint inhibitors in MSI/dMMR metastatic colorectal cancer**

	Prior systemic treatment	N	CR (%)	PR (%)	SD (%)	PD (%)	NE (%)	1-year PFS rate (%)	1-year OS rate (%)	2-year PFS rate (%)	2-year OS rate (%)	Median follow-up (months)
Keynote-016 [17]												
Pembrolizumab	≥ 1	28	11	46	32	4	7	-	-	-	-	8.7
Keynote-164 [18–20]												
Pembrolizumab, cohort A	≥ 2	61	0	28	23	46	3	34	-	-	-	13.2
Pembrolizumab, cohort B	≥ 1	63	3	29	25	40	3	41	-	-	-	12.6
CheckMate-142 [21–23]												
Nivolumab	≥ 1	74	9	24	31	31	5	44	-	-	-	21
Nivolumab + Ipilimumab	≥ 1	119	6	52	28	12	3	71	85	60	74	25.4
Nivolumab + Ipilimumab	0	45	7	53	24	2	0	77	83	-	-	13.8

CD-ON-MEDI4736-

1108 [24]

Durvalumab	≥ 1	36	-	22	38	54	29
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NCT02227667 [24]

Durvalumab	≥ 1	11	-	27	36		30
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1 CR: complete response; PR: partial response; SD: stable disease; NE: not evaluable; PFS: progression-free survival; OS: overall survival

1 **Table 2: Randomized trials with immune checkpoint inhibitors for MSI colorectal cancer**

Study name	NCT number	Study type	Setting	Disease	Intervention	Status
KEYNOTE-177	NCT02563002	Phase III	First line	Metastatic colorectal cancer	Pembrolizumab (anti-PD1) Standard-of-care chemotherapy (mFOLFOX6 or FOLFIRI alone or in combination with bevacizumab or cetuximab)	Closed to recruitment
COMMIT	NCT02997228	Phase III	First line	Metastatic colorectal cancer	Atezolizumab (anti-PD-L1) Atezolizumab + mFOLFOX6 + bevacizumab mFOLFOX6 + bevacizumab	Recruiting
CA209-8HW	NCT04008030	Phase III		Metastatic colorectal cancer	Nivolumab Nivolumab and ipilimumab	Recruiting

					Investigator's Choice chemotherapy ± targeted therapy	
PRODIGE 54 - SAMCO	NCT03186326	Randomized phase II	Second line	Metastatic colorectal cancer	Avelumab (anti-PD-L1)	Recruiting
					Standard-of-care chemotherapy (mFOLFOX6 or FOLFIRI alone or in combination with bevacizumab or cetuximab)	
Alliance A021502	NCT02912559	Phase III	Adjuvant	Stage III colon cancer	Atezolizumab (anti-PD-L1) + mFOLFOX6	Recruiting
					mFOLFOX6	
POLEM	NCT03827044	Phase III	Adjuvant	Stage III colon cancer (MSI or POLE-mutated)	Avelumab (anti-PD-L1) + CAPOX 3 months or capecitabine 6 months	Recruiting
					CAPOX 3 months or capecitabine 6 months	

Table 3: Emerging biomarkers predicting the activity / resistance to immune checkpoint blockade in patients with MSI/dMMR and MSS/pMMR metastatic colorectal cancer

Population	Biomarker	Outcomes	References
dMMR	High frameshift mutation load	Associated with Response and PFS	[38]
	High TMB	Associated with response and OS	[39]
	MSIsensor score	Associated with response and OS	[38]
	<i>RAS</i> and <i>BRAF</i> mutations	Not associated with resistance or benefit	[1,10,11]
	Germline MMR mutations	Not associated with resistance or benefit	[1,10,11]
	JAK mutations	Associated with resistance?	[30]
	HLA class I and <i>B2M</i> mutations	Controversial, not associated with outcome	[34]
	Immunoscore®, immune profile	Higher CD8 infiltration associated with better response	
pMMR	High blood TMB	Associated with better survival	[56]
	PD-L1	No benefit associated with PD-L1 expression by tumor cells	[27]
	Immunoscore®	No data	
	CMS classification	No data	

B2M: beta-2-microglobulin; CMS: consensus molecular subtype; dMMR: mismatch repair-deficient; OS: overall survival; PFS: progression-free survival; pMMR: mismatch repair-proficient; TMB: tumor mutation burden

1 **Table 4: Ongoing immunotherapy clinical trials for metastatic colorectal cancer beyond MSI**

Clinical trials identifiers	Study phase	Intervention	Target
Combination of ICIs			
NCT03867799	II	Nivolumab + relatlimab	PD1, LAG3
NCT03642067	II	Nivolumab + relatlimab	PD1, LAG3
ICIs and chemotherapy [79]			
NCT03202758	I-II	Durvalumab + tremelimumab + FOLFOX	PDL1, CTLA4
NCT03626922	I	Pembrolizumab + oxaliplatin + pemetrexed	PD1
ICIs and antiangiogenics / vascular disrupting agents [80]			
NCT03647839	II	Nivolumab + BNC105 or nivolumab + BBI608	PD1, STAT3, disrupting agent
NCT04126733	II	Nivolumab + regorafenib	PD1
NCT03396926	II	Pembrolizumab + capecitabine + bevacizumab	PD1
NCT04110093	I-II	Nivolumab + regorafenib	PD1
NCT03946917	I-II	JS001 + regorafenib	PD1
NCT02298959	I	Pembrolizumab + ziv-aflibercept	PD1
ICIs and anti-EGFR agents and/or tyrosine kinase inhibitors [81,82]			
NCT03608046	II	Avelumab + cetuximab + irinotecan	PDL1, EGFR

NCT03442569	II	Nivolumab + ipilimumab + panitumumab	PD1, CTLA4, EGFR
NCT03428126	II	Duvalumab + trametinib	PDL1, MEK
NCT04044430	I-II	Nivolumab + encorafenib + binimetinib	PD1, BRAF, MEK
NCT04017650	I-II	Nivolumab + encorafenib + cetuximab	PD1, BRAF, EGFR
NCT03711058	I-II	Nivolumab + copanlisib	PD1, PI3K

ICIs and radiotherapy [83]

NCT03101475	II	Radiofrequency ablation or stereotactic body radiotherapy + durvalumab + tremelimumab	PDL1, CTLA4
NCT03927898	II	Stereotactic body radiotherapy + toripalimab	PD1
NCT04030260	II	Radiotherapy + regorafenib + nivolumab +/- irinotecan	PD1
NCT04108481	I-II	Yttrium-90 radioembolization + durvalumab	PDL1

ICIs and DNA methylation agents [84]

NCT03832621	II	Nivolumab + ipilimumab + temozolomide	PD1, CTLA4, MGMT
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Immunotherapies beyond ICIs [85]

NCT03950154	III	CAPOX + bevacizumab +/- PD-1 monoclonal antibody- activated autologous peripheral blood T-lymphocyte (PD1-T)	T cells
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NCT04119830	II	Rintatolimod and pembrolizumab	PD1, immunomodulation
NCT03222089	II	FOLFOXIRI + interleukin-2 + GM-CSF	IL2
NCT03631407	II	Vicriviroc + pembrolizumab	CCR5 receptor, PD1
NCT03206073	I-II	Pexa-Vec Oncolytic Virus + durvalumab + tremelimumab	Antitumor virus, PDL1, CTL4
NCT04068610	I-II	FOLFOX + bevacizumab + durvalumab + oleclumab	PDL1, CD73
NCT02834052	I-II	Poly-ICLC + pembrolizumab	PD1, immunomodulation
NCT03555149	I-II	Atezolizumab + Imprime PGG + bevacizumab, or atezolizumab + isatuximab, or atezolizumab + selicrelumab + bevacizumab, or atezolizumab + idasanutlin	Immunomodulation, CD-38, CD-40, MDM2
NCT04062721	I-II	Radiofrequency ablation + chemotherapy + in situ hydrogel with TLR agonist and GM-CSF	TLR
NCT03507699	I	Liver radiation therapy + nivolumab + ipilimumab + intra- tumoral CMP-001	PD1, PDL1, TLR9
NCT03720678	I	FOLFOX + AB928	Adenosine receptor
NCT03692429	I	FOLFOX + Allogeneic NKG2D-based CYAD-101 Chimeric Antigen Receptor T-cells	NKG2D

NCT03256344	I	Talimogene Laherparepvec + atezolizumab	Antitumor vaccine, PDL1
NCT03866239	I	Obitunuzumab, followed by cibisatamab + atezolizumab + tocilizumab	CD20, PDL1, CEA-positive tumor cells and CD3+ T-cells, IL6-R

1 **FIGURE CAPTIONS**

2 **Figure 1: Association of *Polymerase E* mutations with MSI status and hypermutability in**
3 **colorectal cancer (data from the The Cancer Genome Atlas)**

4 **Figure 2: Immune classification of colorectal cancer subtypes based on molecular**
5 **pathways** **alteration**

Figure 1: Association of *Polymerase E* mutations with MSI status and hypermutability in colorectal cancer (data from the The Cancer Genome Atlas)





