Anti-Tumour Treatment

Biomarker-guided implementation of the old drug temozolomide as a novel treatment option for patients with metastatic colorectal cancer

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**ABSTRACT**

Temozolomide is an oral alkylating agent used for treating several cancers including glioblastoma and melanoma. Promising, albeit limited, activity and efficacy of temozolomide have been reported in pretreated patients with metastatic colorectal cancer bearing MGMT promoter methylation. MGMT silencing and proficiency of the mismatch repair system were considered the major predictive biomarkers of sensitivity to temozolomide. Refinement of established biomarkers and integration with those related to alteration in specific DNA-damage response pathways such as base excision repair are promising strategies for selecting metastatic colorectal patients to this old drug with several potential novel applications. Then, mounting preclinical and clinical observations have linked acquired resistance to temozolomide to emergence of alterations in the mismatch repair system. Whilst accounting for tumor cells capability of escaping apoptosis when exposed to temozolomide, inactivation of key mismatch-repair proteins will ultimately lead to increasing tumor mutational burden. This drug-induced mismatch deficient-like phenotype is being exploited in proof-of-concept trials combining temozolomide and immune checkpoint inhibitors in metastatic colorectal cancer.

**MGMT promoter methylation as predictive biomarker of treatment with alkylating agents in metastatic colorectal cancer**

In patients with metastatic colorectal cancer (mCRC), the occurrence of chemo-refractory disease and the failure of guidelines-recommended standard treatments pose a major challenge, at least when an adequate performance status would allow the potential administration of further treatment lines. In the era of personalized medicine, tumor molecular profiling may lead to the identification of therapeutic targets that may represent predictive biomarkers for pharmacological intervention. The DNA repair gene O6-methylguanine-DNA methyltransferase (MGMT) is responsible for the elimination of alkyl groups from the O6-position of guanine (Fig. 1). MGMT inactivation due to gene promoter hypermethylation may be involved in the early steps of colorectal tumorigenesis accounting for an increase of G-to-A point mutations of cancer-associated genes (e.g. in KRAS and TP53)\(^{[1]}\). MGMT promoter hypermethylation is observed in up to 40% of colorectal cancers and might confer enhanced sensitivity to alkylating agents such as methyltriazen-imidazole-carboximide prodrugs dacarbazine and temozolomide, as previously shown for patients with glioblastoma and advanced melanoma - for whom temozolomide represents a standard option \(^{[2–4]}\). Following the initial case report of two patients with refractory mCRC and tumor loss of MGMT who achieved an exceptional response to single-agent temozolomide \(^{[5]}\), several phase II non-randomized studies were started with the aim of assessing the activity of dacarbazine and temozolomide after failure of the standard treatment options. In the initial trial with dacarbazine, responses according to RECIST criteria were observed only in 2 out of 68 patients, although all responders had tumors bearing MGMT promoter methylation \(^{[6]}\). Therefore, even if the trial failed to meet its primary endpoint of overall response rate (ORR), the biological rationale linking MGMT silencing with clinical benefit was considered strong enough to perform the subsequent temozolomide monotherapy trials in patients with mCRC who were molecularly selected for the presence of MGMT methylation \(^{[7–11]}\). In all these trials, methylation-specific PCR (MSP) was adopted as the assay for qualitatively detecting the presence or absence of methylation.

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MGMT methylation, as previously validated for patients with glioblastoma [12]. The summary of all 6 monocenter trials is shown in Table 1. The mean ORR to single agent temozolomide in MGMT methylated refractory mCRC patients selected by MSP is about 9%. Based on the modest activity of temozolomide in these heavily pretreated patient populations, the median progression-free survival (PFS)

Table 1

<table>
<thead>
<tr>
<th>Schedule</th>
<th>MGMT methylation assessment assay</th>
<th>N</th>
<th>ORR N (%)</th>
<th>DCR N (%)</th>
<th>mPFS (months)</th>
<th>mOS (months)</th>
<th>Non hematological toxicity (% G3-G4)</th>
<th>Hematological toxicity (% G3-G4)</th>
<th>Ref.</th>
</tr>
</thead>
<tbody>
<tr>
<td>Amatu 2013a</td>
<td>DTIC 250 mg/sqm IV d1-d4, q21</td>
<td>MSP</td>
<td>26</td>
<td>2 (8)</td>
<td>11 (44)</td>
<td>-</td>
<td>14</td>
<td>10</td>
<td>[6]</td>
</tr>
<tr>
<td>Hochauer 2013b</td>
<td>TMZ 150 mg/sqm PO 7 d on/7 d off, q28</td>
<td>MSP</td>
<td>37</td>
<td>1 (3)</td>
<td>16 (43)</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>[7]</td>
</tr>
<tr>
<td>Pietrantonio 2014</td>
<td>TMZ 150 mg/sqm PO d1-d5, q28</td>
<td>MSP</td>
<td>32</td>
<td>4 (12)</td>
<td>10 (31)</td>
<td>1.8</td>
<td>8.4</td>
<td>0</td>
<td>[8]</td>
</tr>
<tr>
<td>Pietrantonio 2016</td>
<td>TMZ 75 mg/sqm PO d1-21, q28</td>
<td>MSP; IHC (exploratory)</td>
<td>32</td>
<td>5 (16)</td>
<td>7 (21)</td>
<td>2.3</td>
<td>6.7</td>
<td>9</td>
<td>[9]</td>
</tr>
<tr>
<td>Amatu 2016</td>
<td>TMZ 200 mg/sqm PO d1-5, q28</td>
<td>MSP; MB (exploratory)</td>
<td>29</td>
<td>1 (3)</td>
<td>14 (48)</td>
<td>2.6</td>
<td>6.2</td>
<td>24</td>
<td>[10]</td>
</tr>
<tr>
<td>Calegari 2017</td>
<td>TMZ 200 mg/sqm PO d1-5, q28</td>
<td>MSP</td>
<td>41</td>
<td>4 (10)</td>
<td>13 (31)</td>
<td>1.9</td>
<td>5.1</td>
<td>43</td>
<td>[11]</td>
</tr>
</tbody>
</table>

**Abbreviations.** DTIC: dacarbazine. IHC: immunohistochemistry. IV: intravenous. MB: methyl-BEAMing. mOS: median overall survival. mPFS: median progression-free survival. MSP: methylation-specific PCR. ORR: overall response rate. PO: per-os. TMZ: temozolomide.

*a* Here reported data about patients with MGMT promoter methylated tumors.

*b* Here reported data about patients with metastatic colorectal cancer included in the trial.

Fig. 1. DNA repair pathways involved in processing major temozolomide-induced alkylations. Methyl group in O6 atom of guanine is directly removed by MGMT; in contexts of MGMT deficiency O6 methylguanine is mispaired with thymine and processed by the mismatch repair system. Persistent activation of mismatch repair (MMR) in face of O6-methylguanine and thymine mispairs is implicated in apoptotic cell death. Reliance upon functional MMR for inducing apoptosis after alkylaton-induced DNA damage is explained by different models. In the futile cycle model, an apoptotic response may be triggered by insurmountable DNA damage after continued MMR processing of the O6methylguanine-thymine mispairs. According to the direct signaling model, ATR is activated by the MuTSα complex, thus initiating the G2/M cell-cycle checkpoint pathway. Methyl groups in N3 position of adenine and N7 position of guanine are processed by base excision repair proteins for repair. **Abbreviations.** MTIC: methyl-triazen-imidazole-carboxamide. MMH: monomethylhydrazine. TMZ: temozolomide.
and overall survival (OS) results were overall poor. However, these results clearly suggested that the molecular selection for temozolomide-based treatment based on MSP is necessary but not sufficient and, from a precision medicine point of view, temozolomide cannot represent a new bullet for the treatment of all patients with MGMT methylated mCRC, but only for a relatively small subgroup of them.

Currently, regorafenib and trifluridine/tipiracil represent two approved standard treatments used from the third line setting and beyond in patients with molecularly unselected mCRC. The approval of the two drugs was based on the positive results of several phase III placebo-controlled trials, which led to demonstrate a median OS gain of 1.4 and 1.8 months for regorafenib and trifluridine/tipiracil, respectively, in Western studies [13,14], and 1.8 and 0.7 months, respectively, in Asian ones [15,16]. The OS benefit achieved by these drugs in trial patients was therefore quite limited, albeit statistically significant, whereas their costs and toxicity burden are particularly relevant in the clinical practice, especially if considering the extremely palliative goal of treatments used in the third line setting and beyond. Finally, the ORR according to RECIST was negligible - only about 1% - in trials with both regorafenib and trifluridine/tipiracil, therefore not allowing to potentially use such agents in the clinical practice in order to reduce the tumor burden and potentially improve the health status and quality of life of patients with poor performance status. Even if the use of temozolomide is not supported by level I evidences and phase III trials, such agent may offer several potential advantages compared to the available standard treatment options: 1) significantly lower cost; 2) manageable safety profile; 3) the chance to achieve tumor response; 4) the chance to investigate DNA damage response (DDR)-related biomarkers that may be potentially implemented during the design of clinical trials, whereas no biomarkers have been so far considered as sufficiently promising to potentially benefit from both regorafenib and trifluridine/tipiracil. Besides being a candidate “targeted” chemotherapy agent, temozolomide shares with regorafenib and trifluridine/tipiracil the advantage of oral administration and is safe when used both at the standard schedule (150 mg/sqm days 1–5 q28) and a dose-dense one (75 mg/sqm days 1–21 q28). The only dose-limiting toxicity of temozolomide is myelosuppression, and its safety profile may be favorable when indirectly compared to regorafenib and even the relatively well tolerated agent trifluridine/tipiracil. However, it should be pointed out that several promising strategies are emerging as breakthrough treatments for specific molecular subgroups of patients with mCRC, such as BRAF +/-MEK inhibitors added to anti-EGFR agents for patients with BRAF V600E mutated [17], immunotherapy for MSI-high [18], dual HER2 blockade for HER2-positive [19], entrectinib or larotrectinib for ALK, ROS1 or NTRK1-3 fusion positive [20,21], and RET selective inhibitors such as BLU-667 and LOXO-292 for RET fusion positive disease [22]. However, except for BRAF V600E mutations (observed in about 8% of all patients with mCRC [23]), the individual frequency of all the above-mentioned predictive biomarkers is below 1–4% in the metastatic setting. Therefore, most patients with mCRC are not currently eligible for immunotherapy or novel targeted strategies, highlighting the urgent need of exploring treatment options that may be effective in pretreated patients, particularly those with RAS mutated disease.

Improving the efficacy of temozolomide in patients with metastatic colorectal cancer: combination strategies

An improvement of the efficacy of temozolomide may be expected from its use in earlier treatment lines and its combination with other agents with known activity in patients with mCRC. Considering this rationale, we ran an open-label, phase II non-randomized study on the combination of temozolomide plus irinotecan (TEMIRI), which showed promising and higher than expected activity [24]. Key inclusion criteria were: failure of at least two prior treatment lines including at least one prior irinotecan-based regimen, irinotecan-sensitive disease based on presence of an irinotecan-free interval (time from irinotecan last dose and disease progression) of 3 months or greater, presence of tumor MGMT methylation assessed by MSP and mismatch repair (MMR) proficiency. TEMIRI regimen achieved an ORR of 24%, a median PFS and OS of 4.4 and 13.8 months, respectively, in patients that had been carefully selected by combined clinical and molecular criteria, despite 68% of them had received at least three previous treatment lines. Retained sensitivity to irinotecan might have contributed to the high activity of the combination, as reported also in the setting of anti-EGFR re-treatments [25]. However, given the limited activity of both temozolomide and irinotecan as single-agents in pretreated patients [26], our results suggested that the two drugs may have synergistic rather than additive effects. Such synergistic effect may be partially independent from MGMT and MMR status [27], since O6 methylated guanine is expected to induce a topoisomerase 1 cleavage complex and poison topoisomerase 1 enzyme [28], ultimately leading to DNA replication-mediated double strand breaks [29].

Additionally, the combination of fluoropyrimidines and temozolomide relies on in-vitro evidence of schedule-dependent synergism between 5-fluouracil (5FU) and temozolomide [30]. Depletion of deoxothymidine triphosphate by the capecitabine metabolite fluorodeoxyuridine monophosphate would leave unmatched O6 methylguanine resulting in DNA double-strand breaks in S-phase. Moreover, thymidylate synthase inhibition by capecitabine results in deoxynucleoside triphosphate imbalance and mutagenicity due to 5FU:G mismatched nucleotides; 5FU:G mismatched nucleotides are substrates for MMR network and their processing leads to apoptosis; a similar DNA damage response is evoked upon temozolomide induced O6 methylguanine-thymine mismaps [31]. Based on these preclinical data and reinforcing the use of temozolomide as a combination treatment in earlier lines, we recently reported the results of a multicenter, open-label, randomized phase II study aimed at investigating the efficacy of capecitabine and temozolomide (CAPTEM) regimen versus FOLFIRI after failure of a prior first-line oxaliplatin-based treatment in patients with MGMT methylated and RAS mutated mCRC [32]. This trial enrolled 86 patients that were randomized on 1:1 basis to receive second-line treatment with either intravenous biweekly FOLFIRI regimen (irinotecan, leucovorin and fluorouracil at standard doses) or fully oral four-weekly CAPTEM regimen (temozolomide for 5 days at 75 mg/sqm bid on days 10–14 and capecitabine 750 mg/sqm bid on days 1–14 of a 28-day cycle) until disease progression or unacceptable toxicity. Even if the trial failed to meet its primary endpoint of PFS superiority of CAPTEM over FOLFIRI, activity and efficacy outcomes of the two regimens were super-imposable (11.6% overall response rate and 3.5 months median PFS in both arms, 9.5 versus 10.6 months in CAPTEM and FOLFIRI arm, respectively). Moreover, the fully oral CAPTEM combination was associated with a better safety profile and quality of life, potentially leading to improved treatment compliance. When putting these results in the context of the current treatment landscape, we acknowledge that several effective combinations of chemotherapy plus biological agents are recommended in the second-line. Therefore, since our second-line randomized trial was negative and failed to show superiority of CAPTEM compared to standard FOLFIRI, the most reasonable setting for further investigating the role of temozolomide may be from the third setting and beyond, ideally based on the simultaneous implementation of predictive biomarkers during the trial design.

Refining the selection of patients for temozolomide treatment thanks to microsatellite instability testing and comprehensive MGMT assessment

MMR proteins are involved in processing and repair of single nucleotide misincorporations, as well as small insertion/deletion (indel) loops. MSH2-MSH6 (MutSc) heterodimer recognizes single mismatches and small indels, whereas MSH2-MSH3 (MutSβ) the larger indels. Then MLH1/PM2S (MutLa) complex is recruited thus allowing recruitment
of the exonuclease EXO1, DNA polymerases POLE/POLD and DNA ligases for excision of error-containing strand, DNA re-synthesis and ligation [33]. Reliance upon functional MMR for inducing apoptosis after alkylation-induced DNA damage is explained by different models, including the futile cycle model and a direct signaling model [34,35]. Namely MMR proficiency has been linked to sensitivity to several DNA damaging agents, including temozolomide [36] and MMR deficient cells display intrinsic resistance to several alkylating agents [37]. Therefore, even if MSI-high status is found only in 4% of patients with metastatic disease [38], it is reasonable to exclude such patients from temozolomide-based treatment, even if concomitant MGMT hypermethylation is detected.

In metastatic melanoma the role of MGMT as a biomarker remains still unclear. A small retrospective study has linked MGMT promoter methylation to higher activity of single-agent temozolomide as first-line therapy (ORR 62% vs 15% in unmethylated patients [39]). However, these results were not recalled in similar retrospective studies regarding both standard-schedule dacarbazine or temozolomide [40,41] or dose-dense temozolomide [42]. MGMT promoter methylation assessed by the qualitative MSP is a predictive biomarker in patients with glioblastoma [12,43]. Even if MGMT promoter methylation may also represent a favorable prognostic factor [44], its prognostic role in CRC is controversial and the few available studies reported conflicting results, mainly in early stage disease [45]. Moreover, the prognostic relevance could be masked by its significant association with other negative prognostic biomarkers such as KRAS mutations. The pivotal phase II trial of dacarbazine in pretreated patients clearly suggested the potential clinical usefulness of patients selection based on the presence of MGMT methylation [6], since only patients with MGMT methylation achieved a RECIST response. Nevertheless, the activity of dacarbazine was low even when focusing on this molecular subgroup (2 responders out of 26 patients, accounting for an ORR of 8%). Although subsequent phase II trials of temozolomide restricted the enrollment to patients with MSP-detected MGMT methylation (Table 1), the poor specificity of such assay (highlighted by the dramatically high fraction of non-responders) has been recalled in all the five monotherapy trials, as well as in the combination trials investigating TEMIRI and CAPTEM regimens [24,32]. The low positive predictive values of MSP for the identification of patients with RECIST response are summarized in Supplementary Table S1.

Several issues account for the lack of accuracy in patients’ selection when adopting MSP as the unique assay for assessing MGMT status. Firstly, even if high concordance (90%) has been reported between MGMT promoter methylation in primary and metastatic tissues [6], a lower concordance (27%) has been reported between archival tumor tissue and baseline samples obtained immediately before temozolomide administration [10]. Decline of the quantitative methylation rate from archival and fresh tissues might be related to prior exposure to several chemotherapeutic agents such as 5-FU [46] and has been reported in patients with glioblastoma at the time of disease recurrence [47]. Also, long-term storage of archival tissue might negatively affect the accuracy of the assays used for assessing MGMT status [48]. Second, MSP might not catch real MGMT status as outlined by consistent disagreement of gene promoter methylation and protein expression across several tumors [49]. Several MGMT-centered assays have been investigated with the aim of refining the selection of patients with mCRC for temozolomide-based treatment. Post-hoc analyses of phase II trials have shown that qualitative MSP may be outperformed by digital PCR quantification of MGMT promoter methylation % (i.e. Methyl-Beam [MB] assay, with MGMT quantitative methylation of 63% identified as the optimal cutoff for predicting treatment response) [50–52]. Although increasing specificity, up to 50% of patients with MB-high tumors do not achieve tumor response to temozolomide monotherapy, whereas a small subset of patients with MB-low (less than 10%) still experienced a tumor shrinkage [52]. Interestingly, immunohistochemistry (IHC) was investigated as a fast, low-cost and widespread assay for assessing tumor expression of MGMT protein. In glioblastoma, IHC is not validated due to several limitations linked to operator sensitivity, lack of prognostic relevance, poor concordance with methylation assays and low tumor specificity due to MGMT staining in non-neoplastic cells [53]. In the setting of mCRC, IHC has been retrospectively evaluated as an exploratory biomarker in non-randomized trials with temozolomide monotherapy and temozolomide-based combinations (Supplementary Table S1). Such analyses highlighted a suboptimal concordance between MGMT protein expression (assessed by IHC) and MGMT quantitative methylation (measured by MB) [51], suggesting that different techniques should not be used interchangeably. Despite such limitations, IHC has consistently shown a negative predictive value of 100%, as none of the tumors with retained MGMT staining achieved a RECIST response. To date, the highest accuracy (87%) for predicting RECIST response arose from a comprehensive MGMT assessment thanks to the use of multiple assays (i.e. MB, mass-spectrometry and RNaseq), thus outperforming individual assays such as MB or proteomic analysis by mass-spectrometry [52]. Although the best prediction may be likely dependent on quantitative/semiquantitative methylation assays (MB or pyrosequencing) combined with protein expression with IHC, several techniques are far from clinical-stage spread and their prospective validation is needed.

The potential predictive role of IHC was investigated thanks to the tumor tissue samples centrally collected during the screening procedures of the CAPTEM trial [32]. Notably, MGMT-centered assays had been previously investigated only in patients receiving temozolomide and in absence of a control group, thus not allowing to discriminate between their potential predictive role or just the prognostic one. In the randomized phase II CAPTEM trial a significant difference was reported in disease control rate according to treatment arm between patients with immunohistochemically MGMT positive and negative tumors (interaction test P = 0.028). Similarly, we reported a non-significant difference in outcomes such as ORR, PFS and OS between CAPTEM and FOLFIRI according to MGMT protein expression status, even if the lack of statistically significant interaction test may have been related to the small sample size. Despite identification of IHC as a promising biomarker for clinical benefit to temozolomide-based regimens, ORR to the CAPTEM regimen was still only 16% even in patients with MGMT IHC negative tumors, suggesting that MGMT-centered extensive assessment is still not sufficient to accurately identify the subgroup of patients with the chance of treatment benefit. With this regard, to investigate whether colorectal cancers with MGMT methylation and low-to-absent MGMT expression may represent a distinct molecular subtype with specific features, we performed here an in-silico analysis of The Cancer Genome Atlas [54]. Analysis of DNA-methylation data from TCGA’s colorectal cancer samples (TCGA-COAD and TCGA-READ) showed the presence of MGMT promoter hyper-methylation in 32% of cases (Fig. 2A and Supplementary Material). Interestingly, MGMT itself was the only gene resulting as differently expressed between patients with MGMT hyper-methylated versus unmethylated status, in line with the observation that MGMT methylation may occur early in colorectal tumorigenesis. As expected, MGMT under-expression was observed in patients with promoter hypermethylation (Wilcoxon P < 10^-3, Fig. 2B). Residual expression of MGMT accounted for the majority of cases, with only 27% of cases showing low-to-absent gene expression. Patients with hyper-methylation and low-to-absent gene expression level of MGMT (Meth/Low) showed similar clinical and molecular characteristics than those with residual gene-expression (Meth/Normal) or unaltered methylation status (Unmeth), including sex, age, frequency altered genes and MSI status (Fig. 2C). Interestingly, Meth/Low cases were more prevalent in the right side than Meth/Normal and
Compared to remaining cases, Meth/Low patients' subgroup showed different distribution of consensus molecular subtype (CMS) classes, with a reduction of CMS4 and increase of CMS3 and CMS1 classes. In line with this data, in-silico functional analysis of Meth/low versus Meth/normal patients demonstrates significant depletion of stem-like and epithelial-mesenchymal transition terms (Fig. 2D). Collectively these data show that MGMT promoter methylation assessed by means of qualitative assays (i.e. dichotomized as present or absent) is clearly insufficient for selecting patients with higher chance of response to temozolomide. Supporting this, MGMT assessment by means of

Fig. 2. (A) DNA-methylation level across TCGA CRC samples for CpG sites mapping in the genomic region around MGMT promoter. The horizontal segment refers to the 6 CpG sites considered to define the methylation status of MGMT. (B) Box plots of the distribution of MGMT gene expression level in samples showing hyper-methylated and normal status of MGMT. (C) Bar plots showing the distribution of Hyper/Low, Hyper/Normal, Normal/Normal TCGA CRC patients stratified by (A) Gender, (B) Age, (C) Side, (D) KRAS mutant, (E) BRAF mutant, (F) Microsatellite status status and (G) CMS. (D) Examples from results of GSEA analysis in hyper/low versus hyper/normal CRC TCGA samples, including HALLMARK_EPITHELIAL_MESENCHYMAL_TRANSITION (left, FDR = 0, Enrichment Score = 0.76) and BOQUEST_STEM_CELL_UP (right, FDR = 0, Enrichment Score = 0.80) gene lists.

Unmeth. Compared to remaining cases, Meth/Low patients' subgroup showed different distribution of consensus molecular subtype (CMS) classes, with a reduction of CMS4 and increase of CMS3 and CMS1 classes. In line with this data, in-silico functional analysis of Meth/low versus Meth/normal patients demonstrates significant depletion of
second-level assays (IHC, mass spectrometry, RNAseq and methyl-BeAMing) may refine the predictive accuracy for response. As shown by our in-silico original data reported in this review, low/absent MGMT expression was restricted to a small subset of samples with methylated MGMT promoter. From a practical point of view, the available evidence in colorectal cancer suggests that any residual MGMT expression or a low level of MGMT methylation percentage is associated with lack of activity from temozolomide. However, there is still a long way to optimal molecular selection of patients, that may be also limited by the potential intra- and/or inter-lesion heterogeneity of MGMT status as reported for glioblastoma [55].

Future perspectives on biomarkers: base excision repair status

Temozolomide causes mainly methylation of guanine on the N7 and O6 atoms, and adenine on the N3 atom [56]. While MGMT accounts for direct repair of O6-methyl adducts of guanine, alkylated bases other than O6 methyl-guanine are processed by the base excision repair (BER) network (Fig. 1). BER is a multistep pathway starting from recognition and removal of small DNA lesions by specific DNA glycosylases (alkylpurine DNA-N glycosylase [APNG] is responsible for sensing and removing temozolomide induced alkylated bases). The resulting abasic site is recognized by an apurinic/apyrimidinic endonuclease (APE1) providing excision of damaged nucleotide. DNA single strand break is stabilized by PARP1 and XRCC1 until gap filling synthesis and ligation occur by means of a DNA polymerase (such as POLB) and a DNA ligase [57]. Crucially, cells display higher sensitivity to alkylating agents when BER is dysregulated leaving unrepaired cytotoxic intermediates. This concept is regarded as BER imbalance [58] and might arise from both deficiency of BER key enzymes (e.g. POLB) or overexpression of the BER initiating enzyme APNG coupled with POLB deficiency [59]. Despite BER is responsible for repairing the majority of temozolomide-induced DNA alkylations, few clinical studies have investigated with conflicting results if specific BER alterations might be involved in the susceptibility or resistance to temozolomide. In a small trial of temozolomide followed by fotemustine in patients with metastatic melanoma, lower mRNA levels of APNG, PARP1 and XRCC1 were associated with improved PFS [60]. Then, in a post-hoc analysis of the trial investigating the role temozolomide as concurrent and adjuvant to radiation therapy in patients with newly-diagnosed glioblastoma APNG expression assessed by IHC failed to show any significant association with OS according to treatment regimen (radiotherapy alone or radiotherapy plus temozolomide) in patients with MGMT promoter methylation [61].

Moving from a strong preclinical rationale [62–64], many efforts [65–71] (Table 2) have been made to increase the tumor sensitivity to temozolomide thanks to the pharmacological effect of agents such as PARP1/2 inhibitors or methoxyamine (that inhibits BER by binding to apurinic/apyrimidinic sites and blocking APE1 nuclease activity). Results of phase I/II trials have been overall disappointing. Regarding mCRC, the combination of the PARP inhibitor veliparib with temozolomide was tested by a single-arm phase II trial carried out in MGMT unselected cheemo-refractory patients, including those with MMR deficiency [66]. Even if this trial formally met the primary endpoint of disease-control rate after 2 cycles of temozolomide and veliparib (24%), both median PFS and OS (1.8 and 6.6 months, respectively) were quite disappointing. Moreover, the ORR was only 4% for patients with MMR proficiency treated with 150 mg/sqm of temozolomide and veliparib, and no responses were seen in patients treated with 200 mg/sqm temozolomide plus veliparib. Regarding upstream BER inhibition with the APE1 inhibitor methoxyamine, its combination with temozolomide was investigated by a single-arm phase II trial in patients with chemorefractory mCRC, who were again not selected for MGMT status [71]. The ORR was only 6% (1 out of 16 patients) and occurred in a patient with MGMT promoter methylation.

The reasons for unsuccessful results of the combination trials with temozolomide plus BER pathway inhibitors may be primarily referred to the absence of proper molecular selection. In fact, the combination of PARP inhibitors or methoxyamine with temozolomide should be assessed in the patient subgroup with MGMT hypermethylated and MMR proficient tumors. Moreover, in context of intrinsic BER imbalance, PARP inhibition could paradoxically impair temozolomide-induced cytotoxicity [72]. Consensual upregulation of key genes involved in BER is observed only in a minority of cases of CRC patients [73]. In conclusion, further studies should address clinical-stage assays for evaluating BER status and therefore potentially predicting the expected benefit from the use of BER inhibitors as sensitizers to the DNA damage induced by temozolomide.

The use of temozolomide as a “priming” therapy for sensitizing microsatellite stable colorectal cancer to immune checkpoint inhibitors

Mutational signatures across human cancers might be traced back to enzymatic modifications of DNA, defective DNA repair pathways or mutagen exposure; among the latter, temozolomide is able to induce a specific signature characterized by C > T transitions found in samples of patients with melanoma and glioblastoma previously exposed to this drug [74]. Notably, C > T transitions are frequently found in MSI-high cancers characterized by small insertions and deletions at mono/poly-nucleotide repeats [75]. In glioblastoma, the inactivation of MMR-related genes has been reported at the time disease recurrence after initial temozolomide-based treatment [76–80] (Supplementary Table S2). Overall, the prevalence of glioblastoma relapses with hypermutated phenotype due to MMR inactivation after temozolomide treatment is about 10–20% [81]. As MMR deficient cells are resistant to temozolomide, the emergence of alterations in MMR genes could be regarded as an adaptive mechanism of resistance to temozolomide. Since both temozolomide and carboplatin/cisplatin share dependency on intact MMR for inducing apoptosis [36,82], the emergence of a MMR deficiency-mimic phenotype has been reported in ovarian cancer tumors after exposure to platinum-based chemotherapy [83]. More recently, we were able to confirm the hypermutator effects of temozolomide also in colorectal cancer preclinical models and in patients with mCRC selected for baseline microsatellite stable (MSS) status, low tumor mutational burden (TMB) and MGMT promoter hypermethylation.

From a clinical point of view, patients displaying initial benefit from temozolomide-based treatment were endowed with a deep change of tumor mutational landscape at time of progression. Specifically, 2 out of 5 patients with acquired resistance to temozolomide underwent tumor rebiopsy and the progressing lesions were characterized by the acquisition of high TMB (> 60 mutations/Mb) and pathogenic mutations in key MMR genes (MSH6 p.T1219I and MSH6 p.T1219I/MSH2 p.782L/MSH6 p.G1224E, respectively), while the loss of MGMT expression was retained also after disease progression. Conversely, patients with non-hypermutated progressing lesions (TMB < 10 mutations/Mb) had increased MGMT expression compared to pre-treatment samples, suggesting that the selection and expansion of tumor subclones with MGMT expression may have played a crucial role in determining secondary resistance to temozolomide [84]. Similar findings were reported for another patient treated with the temozolomide plus irinotecan regimen [24]: post-progression tumor tissue displayed high TMB (68 mutations/Mb) in contrast to the pre-treatment sample (4 mutations/Mb). Despite increasing mutational load no somatic mutations in MMR repair were found with MSS status retained, and BRCA2 E2198* emerged likely as a passenger mutation. Acquisition of high tumor mutational burden independently from MMR following temozolomide might be related to alterations in additional DNA repair
pathways responsible for repairing alkylated adducts. BER-initiating enzyme APNG might be involved in temozolomide resistance due to increase in repair of N3 and N7 adducts in clinical setting [61]. Increased expression of APNG has been linked to genomic instability due to increase in spontaneous mutations, especially frameshift ones [85,86]. Overall, these data suggest that acquired resistance to temozolomide might develop independently from the induction of MGMT expression and may include MMR inactivation and/or acquisition of a mutator phenotype (Fig. 3).

In patients with mCRC, clinical benefit from immune checkpoint inhibitors (ICIs) is strictly restricted to MSI-high subgroup [18]. In pretreated patients, both nivolumab +/- ipilimumab [87,88] and pembrolizumab [18,89] led to rapid and durable responses. In first-line setting, an unprecedented ORR of 60% was achieved by the combination of low-dose ipilimumab and nivolumab [90]. Despite such encouraging results, it must be pointed out that MSI-high mCRCs represent only 4% of all cases, and therefore most of remaining patients (i.e. those with MSS status) are currently excluded from immunotherapy approaches. Sensitizing MSS cancers to immunotherapy is a major challenge since several strategies have failed so far, including combination of PD-1/PD-L1 blockade with anti-CTLA-4 monoclonal antibodies [91], MEK inhibitors [92] and post-induction “lightened” combination with 5-FU/LV and the anti-angiogenic agent bevacizumab [93]. However, promising data were reported for the combination of nivolumab and regorafenib thanks to the potential suppression of pro-tumorigenic tumor-associated macrophages induced by regorafenib [94]. We hypothesized that temozolomide could reshape the mutational landscape of MGMT silenced mCRC and could be used as a “priming” treatment able to sensitize MSS cancers to sequential or concomitant ICIs. Three proof-of-concept phase II trials are currently ongoing (Table 3). Different trial design will address unanswered questions regarding the role of temozolomide in potentially inducing the MSI-high like phenotype. Firstly, MSI-high like status is expected only in a patients’ subgroup following temozolomide treatment and, therefore, only those patients who acquire a high TMB might benefit from ICIs. ARETHUSA and NCT03879811 trials will test the feasibility

Table 2
Trials investigating safety and efficacy of base excision repair inhibitors and temozolomide in solid tumors.

| BER target | Phase | Molecular selection | N | Drug | Setting | Schedule | ORR (%) | Exploratory biomarkers | Ref.
|------------|-------|---------------------|---|------|---------|----------|---------|-----------------------|------|
| PARP I – 33 | rucaparib | Solid tumors | | TMZ 100 mg/sqm qd PO | | 9 a | | | [65]
| PARP II – 75 | veliparib | mCRC | | TMZ 150 or 200 mg/sqm qd PO | | 4 a | PTEN, MGMT | [66]
| PARP II – 104 | veliparib | SCLC | | TMZ 200 mg/sqm qd PO | | 39 a | PARP1 IHC, SLFN11 IHC, MGMT (PCR) | CTCs | [67]
| PARP II – 346 | veliparib | Metastatic melanoma | | TMZ 150 to 200 mg/sqm qd PO | | 10.3 a | P16 IHC, ERCC1 IHC | [68]
| APE1 II – 19 | TRC102 | Relapsed GBM | | TMZ 150 mg/sqm qd PO | | 0 a | APNG, MGMT (PCR) | [69]
| APE1 I – 52 | TRC102 | Solid tumors and lymphomas | | TMZ 150 mg/sqm qd PO | | 7.6 a | γH2AX p, NBS1, RAD51 | [70]
| APE1 II – 16 | TRC102 | mCRC | | TMZ 150 mg/sqm qd PO | | 6 a | MGMT (IHC) | [71]


a Recommended dose.

b ORR is referred to patients with mismatch-repair proficient tumors treated with standard-dose temozolomide or high-dose temozolomide. In the cohort of patients with mismatch-repair deficient tumors (N = 5) no objective responses were observed.

c vs 14% in the temozolomide/placebo group.
d Referred to veliparib 20/40/placebo respectively.
e Recommended dose.

Table 3
Trials investigating safety and efficacy of base excision repair inhibitors and temozolomide in solid tumors.

| BER target | Phase | Molecular selection | N | Drug | Setting | Schedule | ORR (%) | Exploratory biomarkers | Ref.
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c (vs 14% in the temozolomide/placebo group)

d Referred to veliparib 20/40/placebo respectively.

e Recommended dose.
of this approach by means of a tumor re-biopsy following disease progression on temozolomide or after a pre-planned number of cycles (1 or 3, respectively). Then, only patients with demonstrated high TMB will be sequentially treated with an anti-PD-1 agent. While these trials are correctly excluding from immunotherapy those patients with retained non-hypermutated status, some warnings exist regarding: 1) rapid performance status deterioration of heavily pretreated patients following several courses of temozolomide; 2) lack of priming phase of naïve T cells at central level by highly immunogenic neoantigens, that may be preferentially stimulated by anti-CTLA-4 compared to anti-PD-1 agents; 3) lack of prevention of the potential occurrence of temozolomide-induced hypermutation that may be again preferentially stimulated by anti-CTLA-4 agents [95]; 4) the induction of mutations and MMR gene alterations in single cells may be cumulative and time-dependent, so that short-term temozolomide treatment may be not sufficient to boost the efficacy of immunotherapy. Moving from these considerations, we designed the MAYA trial (NCT03832621). Patients will be initially treated with temozolomide (priming phase) and, if no progressive disease is observed after 2 temozolomide courses, low dose ipilimumab plus nivolumab will be administered along with temozolomide. Patients progressing at first radiological restaging are considered bona-fide as primary resistant and therefore likely to bearing innate resistance mechanism other than MMR. Those who gained clinical benefit from temozolomide will be treated with ICI combo regardless of TMB status as acquired resistance invariably develops within 6 months after temozolomide as outlined by phase II trials of temozolomide as single agent.

Translational analyses of all the three trials will hopefully help to better understand the clinical relevance, dynamics and potential subclonality of temozolomide-induced hypermutation that might hamper its activity as a priming therapy for immune checkpoint inhibitors. Clonality and antigenicity of neoepitopes rather than just tumor mutational burden are emerging biomarkers for response to immune checkpoint inhibitors [96]. Frameshift neo-epitopes generated in MSI-high tumors are likely to elicit effective anti-tumor immune response in ICI treated tumors [97]. Anyway, high tumor mutational burden might represent a clinical-stage biomarker of response to ICIs irrespective of the underlying DDR-related alteration as shown in both MSI-high [98] or MSS mCRC tumors such as those harboring POLE mutations [99].

Specific strategies and their biological insights could be transferred to other settings such as maintenance treatment following first-line therapy in mCRC or other diseases showing MGMT methylation. A biomarker-guided approach could be explored for tumors with MGMT deficiency and MSS/MMR proficiency status and limited benefit from ICIs, such as triple negative breast cancer. This agnostic approach would provide in many cases two effective treatments sequentially: a tailored chemotherapeutic therapy and immunotherapy at onset of resistance.

Fig. 3. Putative mechanism of temozolomide induced hypermutation in metastatic colorectal cancer. Initially MGMT deficient, microsatellite stable metastatic colorectal cancer is treated with temozolomide (1) with response and lesion shrinkage (2). Acquired resistance to temozolomide might develop due to MGMT re-expression/selection of MGMT expressing subclones (3) of due to inactivation of key genes involved in the mismatch repair and acquisition of a high tumor mutational burden (4). This hypermutant temozolomide-resistant tumor might elicit antitumor response hampered by expression of immune checkpoint proteins as observed in initially MSI-high tumors. Treatment with immune checkpoint inhibitors is expected to boost antitumor immunity (5). Abbreviations. MMR: mismatch repair. MSI-H: microsatellite instable-high. MSS: microsatellite stable. TMZ: temozolomide.
## Table 3

<table>
<thead>
<tr>
<th>Phase</th>
<th>MMR status</th>
<th>MGMT status</th>
<th>RAS/BRAF status</th>
<th>Line of treatment</th>
<th>Design and Schedule</th>
<th>Primary endpoint</th>
<th>Ref.</th>
</tr>
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<tbody>
<tr>
<td>II</td>
<td>MSS (PCR)</td>
<td>MGMT promoter methylation (pyrosequencing)</td>
<td>–</td>
<td>At least 2 prior therapies including oxaliplatin and irinotecan-based regimens</td>
<td>TMZ 150 mg/sqm 1-5 q28 x2 cycles.</td>
<td>8-month PFS “MAYA”</td>
<td>NCT03832621</td>
</tr>
<tr>
<td></td>
<td></td>
<td>MGMT low expression (IHC)</td>
<td></td>
<td></td>
<td>If CR/PR/SD phase 2 follows</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>MSS (IHC)</td>
<td>MGMT promoter methylation (MethylBEAMing)</td>
<td>RAS mutated</td>
<td>–</td>
<td>TMZ 150–200 mg/sqm 1-5 q28</td>
<td>ORR</td>
<td>NCT03519412</td>
</tr>
<tr>
<td></td>
<td></td>
<td>MGMT low expression (IHC)</td>
<td></td>
<td></td>
<td>until PD. Rebiopsy If TMB &gt; 20 mut/ Mb phase 2 follows</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>MSS (IHC or PCR)</td>
<td>MGMT promoter methylation</td>
<td>–</td>
<td>At least 2 prior therapies including oxaliplatin and irinotecan-based regimens</td>
<td>TMZ 150–200 mg/sqm 1-5 q28</td>
<td>ORR</td>
<td>NCT03879811</td>
</tr>
</tbody>
</table>

### Abbreviations.


* Initially 3 patients will be treated with TMZ, after 1 cycle rebiopsy will be performed. If TMB will be found increased in 1/3 patients accrual proceeds with the same schedule. Alternatively, 6 patients will be treated with TMZ and rebiopsied after completing 3 cycles. If TMB will be found increased in 1/6 patients, 12 more patients will be treated at the same schedule.

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### Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

### Appendix A. Supplementary material

Supplementary data to this article can be found online at https://doi.org/10.1016/j.ctrv.2019.101935.

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