A phase 2 study of temozolomide in pretreated metastatic colorectal cancer with *MGMT* promoter methylation

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Background: Presently, few options are available for refractory colorectal cancer (CRC). O6-methyl-guanine-DNA-methyltransferase (*MGMT*) promoter methylation is a frequent and early event in CRC tumourigenesis. This epigenetic silencing is a predictor of response to the alkylating drug temozolomide in glioblastoma. Preclinical evidences and some case reports showed temozolomide activity in CRC with *MGMT* silencing, but the available data from clinical trials are inconsistent.

Methods: This was a multicentre, phase 2 trial, planned according to a two-stage Simon's optimal design to investigate activity and safety of temozolomide in refractory CRC harbouring *MGMT* promoter methylation. The primary end point was overall response rate (ORR). Patients who failed two or more prior treatments received temozolomide at a dose of 150–200 mg m^{-2} per day on days 1–5 every 28 days.

Results: From July 2012 to June 2016, 225 patients were screened, 80 showed *MGMT* promoter methylation and 41 were enrolled. Overall response rate was 10% and disease control rate was 32%. Median progression-free survival and overall survival were 1.9 and 5.1 months, respectively.

Conclusions: Temozolomide showed a modest activity in this heavily pretreated population and the study did not meet its primary end point. The role of temozolomide in CRC remains still controversial and further research is warranted.

Worldwide, colorectal cancer (CRC) is the third most commonly diagnosed malignancy and one of the leading causes of cancer-related deaths, with an estimated 1.4 million cases and 693 900 deaths occurring in 2012 (Torre *et al*, 2015). Around 25% of patients present with metastatic disease at diagnosis and at least 50% of those with early disease eventually develop metastases (Van Cutsem *et al*, 2010). Historically, for patients with unresectable metastatic CRC (mCRC) the only available systemic treatment was chemotherapy, including fluoropyrimidines, oxaliplatin and irinotecan, with a median overall survival (mOS) of ~20 months (Tournigand *et al*, 2004). Over the past decade, the introduction of anti-EGFR antibodies (cetuximab and panitumumab) for patients with *RAS* wild-type (WT) tumours (Douillard *et al*, 2014; Van Cutsem *et al*, 2015) and antiangiogenic agents (bevacizumab, aflibercept and ramucirumab) has significantly improved the outcome (Slatz *et al*, 2008; Van Cutsem *et al*, 2012; Tabernero *et al*, 2015), extending mOS to almost 30 months (Cremolini *et al*, 2015). More recently, the multikinase inhibitor regorafenib

(Grothey *et al*, 2013) and the novel oral fluoropyrimidine TAS-102 (Mayer *et al*, 2015) have demonstrated efficacy in refractory patients.

Despite such advances, prognosis of patients failing standard therapies remains poor. However, many refractory patients still maintain a good performance status (PS) and are potentially suitable for further active treatment. Therefore, the development of novel approaches for pretreated mCRC patients represents an unmet clinical need.

Temozolomide (TMZ) is an oral alkylating agent with a broad spectrum of antitumour activity. It is indicated for the treatment of patients with glioblastoma (Stupp *et al*, 2005, 2009), and widely used in patients with refractory metastatic melanoma (Middleton *et al*, 2000; Patel *et al*, 2011) and advanced pancreatic neuroendocrine tumours (Chan *et al*, 2012). Its antitumour activity relies on methylation of DNA at the O6 position of guanine, which leads to DNA double-strand breaks and subsequent DNA replication inhibition and apoptosis (Newlands *et al*, 1997). The predominant mechanism of resistance to TMZ is the expression of O6-methyl-guanine-DNA-methyltransferase (MGMT), a DNA repair enzyme that removes the O6-methyl-guanine DNA adducts in a one-step suicide reaction (Pegg, 1990; van Nifterik *et al*, 2010). O6-methyl-guanine-DNA-methyltransferase levels are regulated through an epigenetic mechanism (Heigi *et al*, 2005). Indeed, methylation of the CpG dinucleotides in the promoter region leads to a transcriptional silencing, thus causing a failure to repair the TMZ-induced DNA damage (Esteller and Herman, 2004). Both *MGMT* methylation and the absence of MGMT protein have been associated with *in vitro* sensitivity to TMZ in different human tumour cell lines. Moreover, *MGMT* methylation has been identified as a prognostic factor and a predictor of response to TMZ in glioblastoma (Hegi *et al*, 2005; Dunn *et al*, 2009).

O6-methyl-guanine-DNA-methyltransferase methylation represents a quite frequent as well as relevant event in colorectal cancerogenesis. About 27–40% of mCRC present a low expression of MGMT protein due to gene promoter methylation (Esteller and Herman, 2004), with higher prevalence among CRC with *KRAS* mutation (Esteller *et al*, 2000). In fact, in a series of 244 CRC, *MGMT* methylation was found in 71% of tumours with *KRAS* G > A mutations, in 32% of those with non-G > A *KRAS* mutations and in 35% of WT *KRAS* tumours. Temozolomide showed *in vitro* activity against a large variety of human tumour cell lines, including colorectal cancer (Raymond *et al*, 1997). In 2011, Shacham-Shmueli *et al* (2011) reported two cases of impressive clinical response to TMZ in patients with *MGMT* loss of expression. This evidence provided the rationale for investigating TMZ in mCRC. Four mono-institutional, phase 2 trials have been published so far with different results (Hochhauser *et al*, 2013; Pietrantonio *et al*, 2014, 2016; Amatu *et al*, 2016). Here, we report the results of a multicentre phase 2 trial assessing activity and safety of TMZ in refractory patients with mCRC.

PATIENTS AND METHODS

Study population. Patients with histologically confirmed mCRC harbouring *MGMT* promoter methylation were eligible for inclusion if they met all of the following criteria: age $\geqslant 18$ years; Eastern Cooperative Oncology Group (ECOG) PS $\leqslant 2$; life expectancy of at least 3 months; at least one measurable lesion as defined by Response Evaluation Criteria in Solid Tumours (RECIST) v.1.1; failure of at least two previous chemotherapy regimens (including fluoropyrimidines, irinotecan, oxaliplatin, bevacizumab and, if *RAS* WT, an anti-EGFR antibody); radiological progressive disease (PD) during or within 6 months from the end of the previous treatment or unacceptable toxicity to the

previous treatment; adequate hepatic, renal and bone marrow function (defined as white blood cell count $\geqslant 3 \times 10^9/l$ with absolute neutrophil count (ANC) $\geqslant 1.5 \times 10^9/l$, platelet count $\geqslant 100 \times 10^9/l$ and haemoglobin $> 9 \, g \, dl^{-1}$; total serum bilirubin < 1.5 times upper limit of normal (ULN), unless documented Gilbert's syndrome; ALT $\leqslant 2.5 \times$ ULN or $\leqslant 5 \times$ ULN in patients with liver metastases; serum creatinine $\leqslant 1.5 \times$ ULN or calculated creatinine clearance $\geqslant 50 \, ml \, min^{-1}$ according to Crockcroft and Gault; INR and aPTT $\leqslant 1.5 \times$ ULN, unless on therapeutic anticoagulation). Patients were ineligible if they met any of the following exclusion criteria: history of any other severe or uncontrolled disease; pregnancy or lactation; existing acute effects of prior treatment; history of any other malignancy within the previous 5 years, except for patients appropriately treated for skin carcinoma (other than melanoma) or *in situ* cervical carcinoma, or those treated with curative intent for any other cancer with no evidence of disease for at least 5 years. The presence of *MGMT* promoter methylation was assessed by methylation-specific polymerase chain reaction (MS-PCR), the evaluation was performed centrally at the Institute of Pathological Anatomy of Catholic University of the Sacred Heart. *RAS* and *BRAF* mutational genetic analyses and mismatch repair system (MMR) status evaluation by immunohistochemistry (IHC) were also performed. The trial was approved by the Ethics Committee of Catholic University of the Sacred Heart Rome and by the Institutional Review Board of each participating centre. All patients provided written informed consent before study entry and any protocol-specific procedure.

Study design, treatment and evaluations. This was a multicentre, single-arm, phase 2 trial (EudraCT number: 2012-002766-13), evaluating activity and safety of TMZ in patients with *MGMT*-methylated mCRC who have failed all standard therapeutic options.

Enrolled patients received TMZ (TEVA) orally under fasting conditions once a day for five consecutive days every 28 days. At each cycle, treatment was administered if ANC and platelet count were $\geqslant 1.5 \times 10^9/l$ and $\geqslant 100 \times 10^9/l$, respectively. Temozolomide was administered at a dose of $150 \, mg \, m^{-2}$ per day for the first cycle (level 0). If no grade 3–4 neutropenia, thrombocytopenia and non-haematological adverse events (AEs) (except from nausea and vomiting) occurred, the dose was increased to $200 \, mg \, m^{-2}$ per day starting from the next cycle (level 1). In case of grade 3 or 4 toxicity with the exception of nausea and vomiting, two dose reductions were allowed up to $100 \, mg \, m^{-2}$ per day (level -1). If further toxicity occurred, patients were discontinued from treatment. Treatment was continued until occurrence of PD, unacceptable toxicity, death or withdrawal of consent.

Pre-treatment evaluation were performed within 28 days before starting treatment and consisted of: collection of tumour tissue samples (blocks or slides from primary tumours or metastases) and assessment of *MGMT* methylation status by MS-PCR, *RAS* and *BRAF* mutational status and MMR expression status, medical history, ECOG PS, physical examination, routine haematology, biochemistry and urine analyses, electrocardiogram and contrast-enhanced computed tomography (CT) scans or magnetic resonance imaging of the chest and abdomen. On day 1 of each cycle, treatment compliance, concomitant medications, symptoms, physical examination, vital signs, PS and all AEs, graded according to the National Cancer Institute Common Toxicity Criteria for Adverse Events (NCI-CTCAE) v.4.03, were recorded. Complete blood count and serum chemistry were obtained every month before each cycle. Radiographic assessment of tumour response was carried out every 8 weeks according to RECIST v.1.1 (Eisenhauer *et al*, 2009) until PD or consent withdrawal for any reason.

Study end points and statistical analysis. The primary end point was overall response rate (ORR), defined as the proportion of

patients that achieved a confirmed complete response (CR) or a partial response (PR) as best response. Secondary end points were: disease control rate (DCR), defined as the proportion of patients that achieved CR, PR or stable disease (SD), duration of response (DoR), defined as the time from first documented response to the date of PD or death due to any cause, whichever occurred first; progression-free survival (PFS), defined as the time from the start of the treatment to the date of the first documented PD or death due to any cause, whichever occurred first; overall survival (OS), defined as the time from the start of the treatment to the date of death due to any cause, or censored at the date of last follow-up for alive patients; safety profile. Preplanned exploratory analyses evaluated the correlation between ORR and *RAS* and *BRAF* mutational status. All assessments were conducted on the intent-to-treat population (ITT). The Kaplan–Meier method was used to estimate DoR, OS and PFS. Median values are presented with 95% confidence interval (CI). The correlation between ORR and mutational status was assessed by two-tailed Fisher's exact test. Adverse events are presented according to NCI-CTCAE v.4.03.

This study was planned according to a two-stage Simon's optimal design (Simon, 1989; Jung *et al*, 2008). The null hypothesis that the true response rate is $\leq 5\%$ (p_0) was tested against a one-sided alternative that the response rate is $\geq 20\%$ (p_1). According to this design, in the first stage, 21 patients (n_1) were accrued: if 1 or fewer responses (r_1) were observed in this cohort of patients, the study would be stopped early for futility; otherwise, 20 additional patients would be accrued for a total of 41 patients (n). The null hypothesis would be rejected if more than four responses (r_2) were observed in 41 patients (n). Thus, if four or fewer responses would be observed by the end of stage 2, then no further investigation would be warranted. This design yields a type I error rate of 5% and power of 90% when the true response rate is 20%.

Data were analysed using IBM SPSS Statistics version 22 software (IBM, Armonk, NY, USA).

Assessment of MGMT gene promoter methylation with MS-PCR. After DNA modification with Methylamp DNA Modification Kit (Epigentek, Farmingdale, NY, USA), MS-PCR for *MGMT* were determined as described previously (Martini *et al*, 2008). Briefly, bisulphite-modified DNA (100–200 ng) was amplified in a mixture containing $1 \times$ PCR buffer (20 mM Tris (pH 8.3), 50 mM KCl, 1.5 mM MgCl$_2$), deoxynucleotide triphosphates (0.2 mM each), primers (20 pM each) and 0.75 U GoTaq Hot Start polymerase (Promega, Madison, WI, USA) in a final volume of 25 μl. Polymerase chain reaction conditions were: an initial denaturation of 95 °C for 8 min, followed by 35 cycles of 95 °C for 60 s, 60 °C for 60 s and 72 °C for 60 s. Polymerase chain reaction products were electrophoresed in a 2.5% agarose gel, stained with ethidium bromide and visualised under ultraviolet illumination. Methylation-specific-PCR analysis was performed in duplicate for all samples. Normal lymphocyte DNA supermethylated with SssI methyltransferase (New England Biolabs, Beverly, MA, USA) and treated with bisulphite was used as the unmethylated and methylated control, water as a negative control and untreated DNA as an internal PCR control. We also carried out MS-PCR on granulocyte DNA obtained from 10 healthy individuals, as the control group.

Assessment of RAS and BRAF mutational status. DNA was extracted from three 10 μm slides from paraffin-embedded tissues using QIAamp DNA Mini Kit (Qiagen, Milan, Italy), following the manufacturer's protocol. *KRAS* and *NRAS* gene (2, 3 and 4 exons) and *BRAF* gene (11 and 15 exons) were amplified using the same primers and PCR conditions described previously (Basso *et al*, 2013). Briefly, DNA (100–200 ng) was amplified in a mixture containing $1 \times$ PCR buffer (20 mM Tris, pH 8.3, 50 mM KCl, 1.5 mM MgCl$_2$), deoxyribonucleotide triphosphates (200 mM each), primers (20 pM each) and 0.5 U GoTaq (Promega, Milan, Italy) in

a final volume of 25 μl. After visualisation onto agarose gel, stained with ethidium bromide and visualised under UV light, PCR products were treated with ExoSAP-IT (GE Healthcare, Milan, Italy) following the manufacturer's protocol, amplified with the BigDye Terminator Cycle-Sequencing Kit (version 3.1; Applied Biosystems, Milan, Italy) using forward and reverse primers, and sequenced with an ABI PRISM 3100-Avant Genetic Analyser (Applied Biosystems). Water was used as a negative control. The sensitivity of this method is 15% in our laboratory.

Assessment of MMR expression status. Mismatch repair system analysis was performed on formalin-fixed paraffin-embedded (FFPE) tissue with antibodies against proteins MLH1, MSH2, MSH6 and PMS2. Briefly, FFPE sections (4 μm thick) were mounted on positive charged glass slides. For antigen retrieval, deparaffinised and rehydrated sections were treated with citric acid buffer (pH 6.0), two cycles of 3 min each at 500 W, followed by inhibition of endogenous peroxidase with 3% H$_2$O$_2$ for 5 min. Then, the sections were incubated for 1 h at room temperature with mouse monoclonal anti-MLH-1, anti-MSH2 and anti-MSH6 (clone M1; clone G219-1129; clone 44; Roche, Monza, Italy) and mouse monoclonal anti-human PMS2 (clone 2G5; Novus Biologicals, Milan, Italy). The primary antibodies were visualised using the avidin–biotin–peroxidase complex. 3,3′-Diaminobenzidine was used as the enzyme substrate to observe the specific antibody localisation and Mayer haematoxylin was used as a nuclear counterstain. Samples were stained more than once, and the results were highly reproducible. Staining patterns of MMR proteins were evaluated using normal epithelial, stromal or inflammatory cells, or centres of lymphoid follicles as internal controls. Stained slides were scored as positive when showing nuclear staining in at least some tumour cells ($> 0\%$).

RESULTS

Patients characteristics. Between July 2012 and June 2016, a total of 225 patients were screened. Archival tissue samples collected from each patient were assessed for *MGMT* promoter methylation: 80 samples (35%) resulted positive, whereas 134 were negative and 11 did not provide adequate quality/quantity DNA to perform the evaluation. Among the 80 patients with *MGMT* promoter methylation, 41 met all the inclusion criteria, were enrolled in the study and received study treatment (ITT population) (Figure 1). The remaining *MGMT* methylated patients resulted ineligible due to: hepatic impairment (51%), worsening of clinical condition with increase of the ECOG PS > 3 and reduction of life expectancy to < 3 months (41%) and absence of adequate bone marrow function due to persistence of AEs of previous treatments (8%).

Baseline patient and disease characteristics are summarised in Table 1. Twenty-four patients (58.5%) were female and 17 (41.5%) male. Median age was 66 years (range 47–81). Eastern Cooperative Oncology Group PS was 0 for 12 (29%) patients, 1 for 23 (56%) patients and 2 for 6 (15%) patients. Primary tumour location was rectum in 6 (15%), left colon in 27 (67.5%) and right colon in 7 (17.5%) patients. The median number of metastatic sites was 3. Six (15%) patients had only one site involved and 14 (34%) had more than three metastatic sites. The median number of previous treatment lines was 3. Ten (24%) patients received more than three lines and 3 (7%) were previously treated with regorafenib. Twenty-three (63%) tumours were *RAS* mutant (MT), 2 (5%) were *BRAF* MT and 13 (32%) were *RAS/BRAF* WT. Only one tumour (2.5%) presented a deficient MMR, because of the lack of MLH1 expression.

Outcome. A total of 109 cycles were administered and patients received a median of three cycles (range, 1–8). Temozolomide
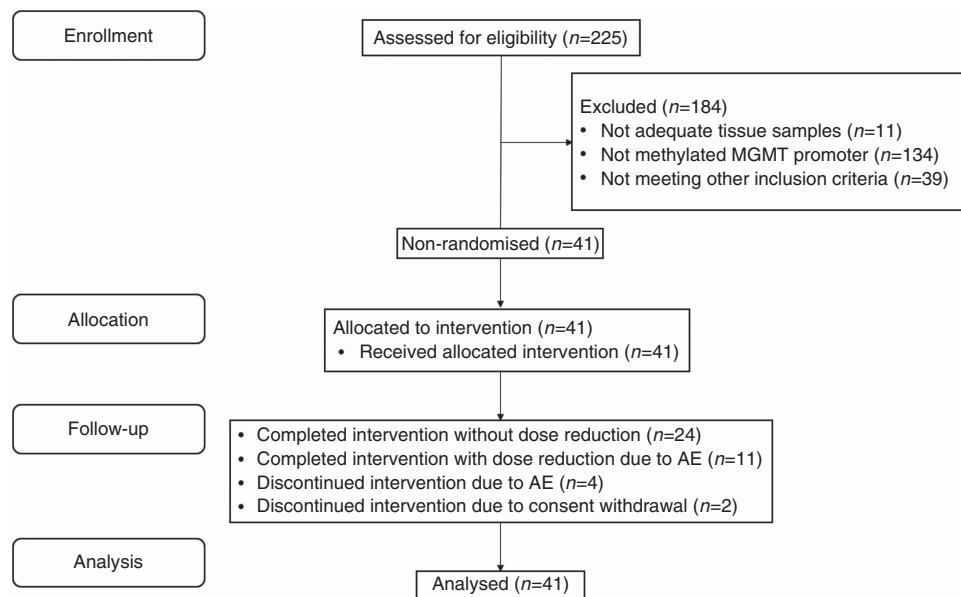
Figure 1. Study flowchart.

activity was evaluated in the ITT population and is summarised in Table 2. Overall response rate was 10% with no CR and 4 (10%) PR. Stable disease was achieved in 9 (22%) patients, accounting for a DCR of 32%. Twenty-eight (68%) patients experienced PD as best response (Figure 2). Thus, the primary end point of the study was not met, not reaching the prespecified level for promising activity. The DoR was 3.05 months (range, 2–6).

At a median follow-up time of 9 months, all patients experienced PD and 36 (88%) patients died due to CRC. Kaplan–Meier curves for PFS and OS are reported in Figure 3a and b respectively. The median PFS was 1.9 months (95% CI: 1.6–2.35) (Figure 3a) and the median OS (Figure 3b) was 5.1 months (95% CI: 3.9–6.2). The median PFS was significantly higher for patients achieving PR or SD as compared with patients who experienced PD (4.7 *vs* 1.7 months; $P < 0.001$). No significant difference was observed between patients achieving PR or SD and those with PD in terms of median OS (9.8 *vs* 6.4 months; $P = 0.245$).

All patients who had a PR were *RAS* MT (three *KRAS* MT and one *NRAS* MT). Of the nine patients who presented SD as best response, six had a *RAS* MT tumour (five *KRAS* and one *NRAS*) and three a *RAS/BRAF* WT tumour. Among the 28 patients who experienced PD, 16 tumours were *RAS* MT (15 *KRAS* and 1 *NRAS*), 2 *BRAF* MT and 10 *RAS/BRAF* WT. Therefore, with regard to the exploratory analysis, no significant correlation between *RAS/BRAF* mutational status and RR (χ^2 test $P = 0.49$) or survival (PFS $P = 0.967$; OS $P = 0.912$) was observed.

The only patient with MMR-deficient tumour achieved PD as best response; the PFS and OS reached by this patient were 1.7 and 29.7 months, respectively.

Overall, 20 patients (49%) received poststudy therapy. Chemotherapy rechallenge was administered in 10 (24%) patients, regorafenib in 6 (14.6%), capecitabine monotherapy in 4 (10%), mitomycin C in 2 (5%) and hepatic intra-arterial injection of irinotecan-eluting bead (DEBIRI) plus systemic capecitabine in 2 (5%).

Safety. All patients received at least one cycle of treatment and formed the safety population. Overall, 109 cycles were administered. Treatment-related AEs are summarised in Table 3. Any-grade AEs were reported in 31 patients (75.6%). The most frequent haematological AE was platelet count decreased, reported in 16 patients (39%); 13 cases of anaemia (31.7%) and 3 cases

(7.3%) of ANC decreased occurred. Two patients received blood transfusion, whereas colony-stimulating factors were never required. Regarding non-haematological AEs, nausea was observed in 23 (56.1%) patients, vomiting in 15 (36.5%), constipation in 10 (24.4%), liver enzyme increased in 22 (53.6%) and fatigue in 4 (9.7%). Most AEs were grade 1 or 2 (84%). Grade 4 AEs occurred in 6 (14.6%) patients. Overall, dose reduction was required in 11 (27%). All patients discontinued study treatment; the primary reason for discontinuation was PD (35 patients; 85%), whereas 4 (10%) patients discontinued due to AE and 2 (5%) patients withdrawn the consent. No serious AEs nor toxic deaths were recorded.

DISCUSSION

Nowadays, research is actively committed in the identification of treatment options for refractory mCRC after failure of standard therapy. The unresolved issue is the absence of effective drugs that could be offered to patients progressing after all approved treatment but still in adequate clinical conditions to face further therapy. Given the known role of *MGMT* promoter methylation status as predictive biomarker of response to TMZ in glioblastoma (Esteller and Herman, 2004; Heigi *et al*, 2005; van Nifterik *et al*, 2010), the high frequency of this epigenetic alteration in CRC cancerogenesis (Dunn *et al*, 2009) and the preliminary evidence (both from *in vitro* studies and clinical reports) of TMZ activity in *MGMT*-methylated mCRC (Raymond *et al*, 1997; Shacham-Shmueli *et al*, 2011, Inno *et al*, 2014), we hypothesised that TMZ might represent a treatment option in pretreated mCRC selected according to *MGMT* promoter methylation status. Thus, we conducted this phase 2 study to assess the activity and safety of TMZ in refractory mCRC harbouring *MGMT* promoter methylation.

In the present study, TMZ showed some activity, with an ORR of 10%. However, the primary end point of the study was not met since the prespecified threshold for activity was not reached. In fact, we observed 4 PR among 41 patients, while at least five responses should have been observed according to the study protocol for considering TMZ worthy of further evaluation in this setting.

Table 1. Patient's and tumour's characteristics

	No.	%
Patients	41	
Median age (years)	66	
Gender		
Male	17	41.5
Female	24	58.5
PS (ECOG)		
0	12	29
1	23	56
2	6	15
Primary tumour location		
Right colon	7	17.5
Left colon	27	67.5
Rectum	6	15
Number of metastatic sites		
1	6	15
2	9	22
3	12	29
>3	14	34
Peritoneal carcinomatosis		
Yes	15	37
No	26	63
Liver metastases		
Yes	30	73
No	11	27
Prior adjuvant chemotherapy		
Yes	10	24
No	31	76
Number of treatment lines for advanced disease		
2	15	37
3	16	39
>3	10	24
***RAS-BRAF* status**		
KRAS-mutated	23	56
NRAS-mutated	3	7
BRAF-mutated	2	5
All genes wild type	13	32
MMR status		
MMR-deficient	1	2.5
MMR-proficient	40	97.5

Abbreviations: ECOG = Eastern Cooperative Oncology Group; MMR = mismatch repair system; PS, performance status.

Table 2. Efficacy results according to RECIST 1.1

Response end point	N (%)
ORR	4 (10)
CR	0
PR	4 (10)
SD	9 (22)
PD	28 (68)
DCR	13 (32)
Survival end point	**Months (95% CI)**
DoR	3.05 (2–6)
PFS	1.9 (1.6–2.35)
OS	5.1 (3.9–6.2)

Abbreviations: CI = confidence interval; CR = complete response; DCR = disease control rate; DoR = duration of response; ORR = overall response rate; OS = overall survival; PFS = progression-free survival; PD = progressive disease; PR = partial response; RECIST = Response Evaluation Criteria in Solid Tumours; SD = stable disease.

well tolerated with an acceptable toxicity profile, consisting mostly of grade 1 or 2 AEs (84%).

Taken together, these data suggest that TMZ has activity against *MGMT*-methylated mCRC, but probably further investigation of other possible predictive biomarkers may contribute to select truly responsive patients leading to better results. A proposed biomarker is the mutational status of *RAS/BRAF* genes. In fact, in their study with standard schedule of TMZ, Pietrantonio *et al* (2014) observed objective responses only in *RAS/BRAF* WT patients. It was therefore postulated that *RAS* mutations could enhance *MGMT* transduction through hyperactivation of the MAPK pathway, leading to a MGMT-mediated resistance to TMZ. On the contrary, when TMZ was given with a dose-dense schedule (Pietrantonio *et al*, 2016), the authors observed responses exclusively in *RAS*- or *BRAF*-mutated patients, speculating that a dose-dense schedule could enhance sensitivity to TMZ in *RAS*-mutated mCRC as a result of MGMT depletion due to cumulative dose and protracted drug exposure. In our study, we did not observe a significant difference in terms of TMZ activity among *RAS* MT and WT patients. Moreover, all patients who experienced an objective response had *RAS* MT tumours (three *KRAS* and one *NRAS*). This observation is in contrast with the hypothesis that *RAS* mutations are responsible for resistance to TMZ. Therefore, data on the role of *RAS/BRAF* mutational status as a biomarker of TMZ activity, as well as data on a possible correlation of the schedule with TMZ activity according to *RAS/BRAF* mutational status, are not conclusive. Another suggested biomarker is the MMR pathway status, on whose integrity could rely the mechanism of cell death induced by TMZ (Inno *et al*, 2014). The study by Hochhauser *et al* (2013) suggested that a proficient MMR seems to be required to achieve the therapeutic effects of TMZ. Indeed, all patients achieving a PR as best response were MMR-proficient and most patients with an SD or PD were MMR-deficient. However, given the low number of PR no inference could be drawn by the authors. Moreover, the absence of MMR-deficient tumours in the studies by Pietrantonio *et al* (2014, 2016) and the low frequency in our cohort do not allow to identify a correlation between MMR status and response to TMZ.

Preclinical models suggest other possible mechanisms of resistance to TMZ, including the base excision repair enzyme alkylpurine-DNA *N*-glycosylase involved in repairing N3-methyladenine and N7-methylguanine (Agnihotri *et al*, 2012), but its role in the clinical setting is not yet defined.

A possible limitation of our study is represented by the assessment of *MGMT* methylation on archival biopsies. Amatu *et al* (2016) reported a significant decline in *MGMT* methylation from archival samples compared with tumour or liquid biopsies taken at the time of therapy start. In our study, the median time
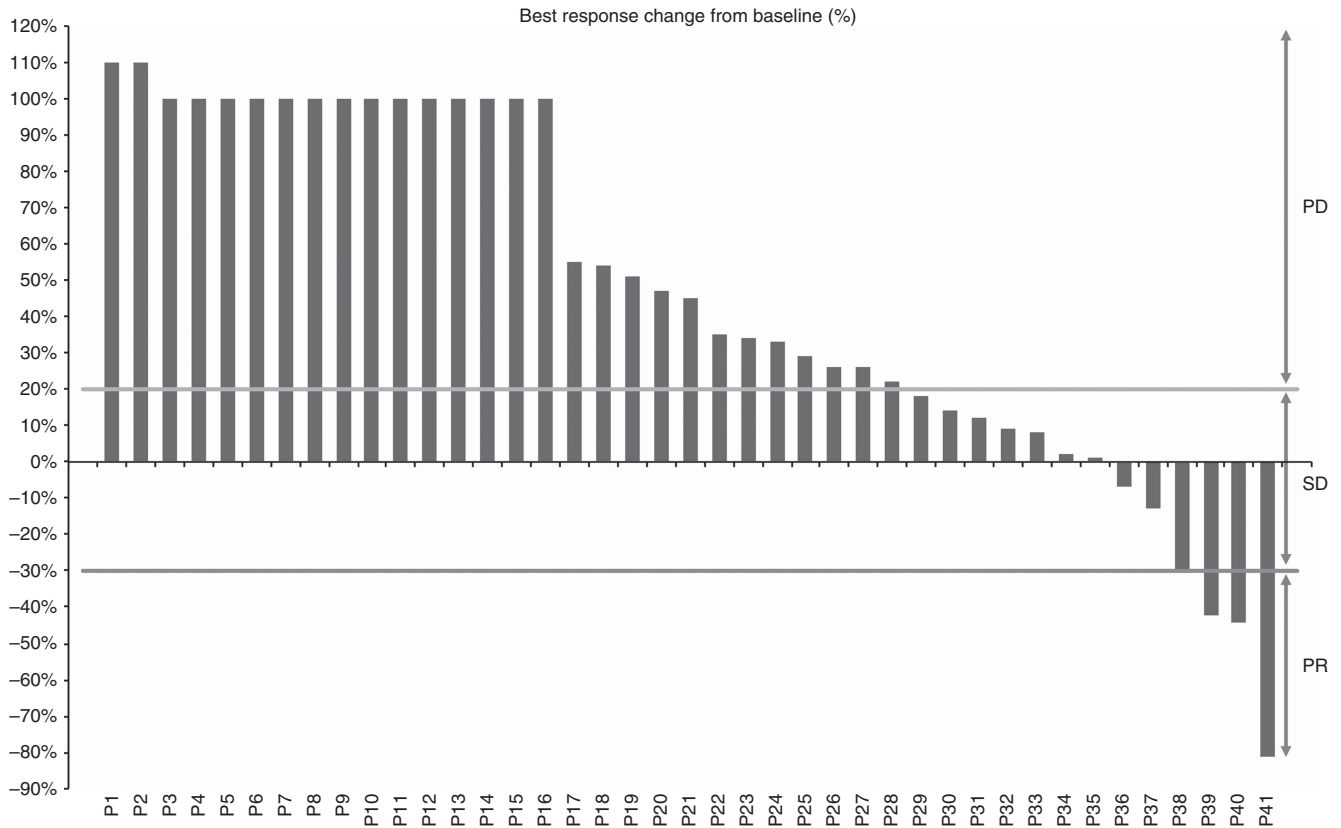
While the trial was still ongoing, four similar phase 2 studies on patients with *MGMT*-methylated refractory mCRC were published, with conflicting results (Hochhauser *et al*, 2013; Pietrantonio *et al*, 2014, 2016; Amatu *et al*, 2016). Hochhauser *et al* (2013) reported a disappointing RR of 3% among 31 patients treated with a 7-day-on/7-day-off schedule for each 28-day cycle. Similarly, Amatu *et al* (2016) failed to demonstrate a meaningful activity of TMZ, reporting a PFS rate at 12 weeks of 10.3% for 29 patients treated with a standard schedule of TMZ (200 mg m^{-2} on days 1–5 of 28-day cycles). In contrast, Pietrantonio *et al* (2014, 2016) observed a promising activity of TMZ in this setting, reporting a RR of 12% among 32 patients receiving the standard schedule (150 mg m^{-2} on days 1–5 of 28-day cycles) and of 16% among 32 patients treated with a dose-dense schedule (75 mg m^{-2} on days 1–21 of 28-day cycles).

Interestingly, the observed RR in the present study (10%) and median survival times (median PFS and OS of 1.9 and 5.1 months, respectively) are consistent with those reported by Pietrantonio *et al* (2014, 2016), and somewhat comparable to those achieved by regorafenib (Grothey *et al*, 2013) or TAS-102 (Mayer *et al*, 2015) in randomised phase 3 trials. Furthermore, in our study TMZ was

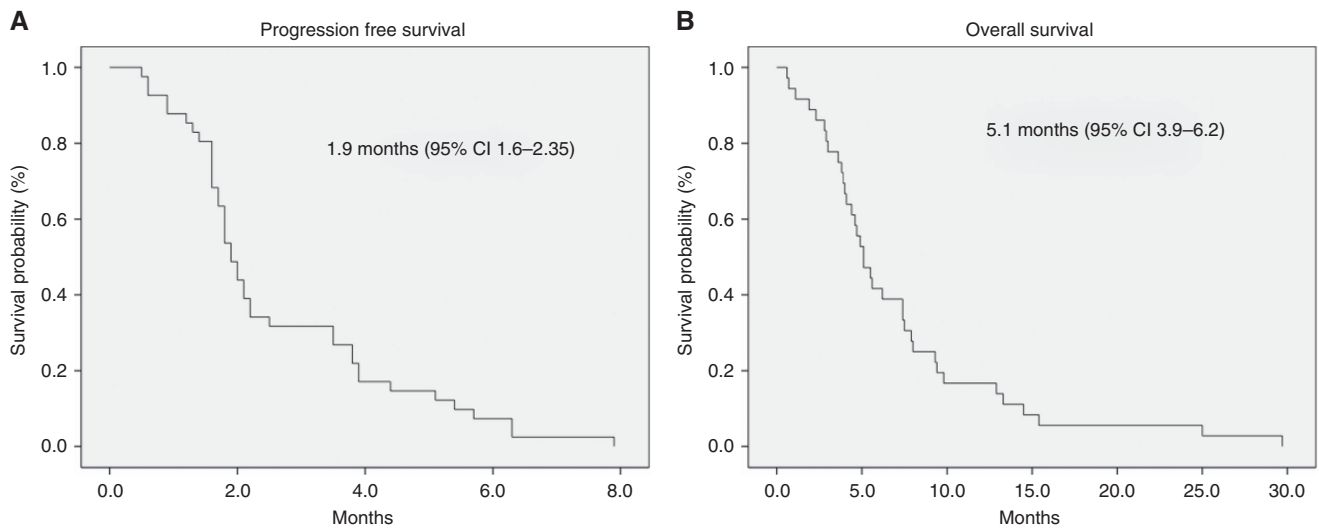Figure 2. Waterfall plot: responses to TMZ.

Figure 3. (**A**) Kaplan–Meier curve for PFS in the ITT population and (**B**) Kaplan–Meier curve for OS in the ITT population.

from histological diagnosis on primary tumour or metastases and the start of TMZ treatment was 3 years (range 1–7, 4 years). Thus, given the dynamic epigenetic nature of this biomarker, during the long interval between tumour biopsy and start of TMZ and under the selective pressure of previous lines of therapy, *MGMT* methylation might have been lost in an unpredictable percentage of patients, leading to a lower than expected RR in our study. On the other hand, a long-term storage of paraffin-embedded samples could cause a reduction in *MGMT* methylation status, lowering the sensitivity of MS-PCR (Kalmár *et al*, 2015).

Another limit might be related to the assay used to perform molecular selection of patients in this setting. Sartore-Bianchi *et al*

(2017) recently conducted a pooled retrospective analysis on MS-PCR (MSP)-positive tumour samples from 105 patients treated within four phase 2 studies on alkylating agents (TMZ or dacarbazine) (Amatu *et al*, 2013, 2016; Pietrantonio *et al*, 2014, 2016). This analysis assessed MGMT expression by IHC and digital PCR methylation assay (methyl-BEAMing, MB) and evaluated the association with clinical outcome, identifying a subgroup of patients with a combined IHC low score/MB high level displaying a significant reduction in the hazards of progression and death. The authors showed that selection for MGMT deficiency by MSP might not be sufficient to achieve the best efficacy of alkylating treatment, whereas a combination of IHC and MB testing could

Table 3. Treatment-related AEs according to NCI-CTCAE v.4.03

Adverse event	Patients, n (%)				
	All grades	G1	G2	G3	G4
Blood and lymphatic system disorders					
Anaemia	13 (31.7)	6 (14.6)	5 (12.2)	2 (4.9)	
Gastrointestinal disorders					
Constipation	10 (24.4)	5 (12.2)	1 (2.4)	1 (2.4)	3 (7.3)
Diarrhoea	2 (4.9)	1 (2.4)	1 (2.4)		
Mucositis	3 (7.3)	3 (7.3)			
Nausea	23 (56.1)	9 (22)	13 (31.7)	1 (2.4)	
Vomiting	15 (36.5)	6 (14.6)	8 (12.5)	1 (2.4)	
General disorders					
Fatigue	4 (9.7)		4 (9.7)		
Investigations					
Bilirubin increased	4 (9.7)	2 (4.9)	1 (2.4)	1(2.4)	
AST increased	2 (4.9)	2 (4.9)			
ALT increased	3 (7.3)	3 (7.3)			
ALP increased	6 (14.6)	4 (9.7)	2 (4.9)		
GGT increased	7 (17)	4 (9.7)	1 (2.4)	1 (2.4)	1 (2.4)
Neutropenia	3 (7.3)	1 (2.4)			2 (4.9)
Thrombocytopenia	16 (39)	7 (17)	4 (9.7)	3 (7.3)	2 (4.9)
Metabolism and nutrition disorders					
Anorexia	2 (4.9)		2 (4.9)		

Abbreviations: ALP = alkaline phosphatase; ALT = alanine transaminase; AST = aspartate aminotransferase; GGT = γ-glutamyl transferase; NCI-CTCAE = National Cancer Institute Common Toxicity Criteria for Adverse Event.

enhance patient selection in this setting. Based on these results, future prospective trials could require an optimised molecular selection with combined techniques.

In conclusion, in the present study, TMZ achieved an RR of 10% in heavily pretreated patients with *MGMT*-methylated mCRC, not reaching the prespecified threshold of activity. However, taking into account the evidence from other similar studies and also the possible unreliability of the assessment of *MGMT* methylation in archival samples, a role of TMZ in mCRC cannot be definitively excluded. Future research should elucidate the appropriate timing of *MGMT* methylation assessment as well as the mechanisms of primary resistance to TMZ, to enhance TMZ activity by identifying responsive patients. Therefore, when planning future trials with TMZ in mCRC, a liquid or tissue biopsy at the moment of starting therapy and a wide panel of potential biomarkers should be considered. Moreover, as *MGMT* methylation is an early event of CRC tumourigenesis that could be lost over time, the activity of TMZ may be higher in earlier lines of treatment than in subsequent lines. In this regard, a randomised phase 2 trial comparing 1:1 TMZ plus capecitabine (CAPTEM) *vs* FOLFIRI in patients with MGMT-methylated, RAS-mutated mCRC after failure of prior first-line oxaliplatin-based treatment is currently ongoing (Berenato *et al*, 2016).

CONFLICT OF INTEREST

The authors declare no conflict of interest.

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