Tumor MGMT promoter hypermethylation changes over time limit temozolomide efficacy in a phase II trial for metastatic colorectal cancer

Amatu, A; Barault, L; Moutinho, C; Cassingena, A; Bencardino, K; Ghezzi, S; Palmeri, L; Bonazzina, E; Tosi, F; Ricotta, R; Cipani, T; Crivori, P; Gatto, R; Chirico, G; Marrapese, G; Truini, M; Bardelli, A; Esteller, M; Di Nicolantonio, F; Sartore-Bianchi, A; Siena, S. Tumor MGMT promoter hypermethylation changes over time limit temozolomide efficacy in a phase II trial for metastatic colorectal cancer. ANNALS OF ONCOLOGY. 27 (6) pp: 1062-1067.
DOI: 10.1093/annonc/mdw071

Tumor *MGMT* promoter hypermethylation changes over time limit temozolomide efficacy in a phase II trial for metastatic colorectal cancer

Alessio Amatu[1], Ludovic Barault[2], Càtia Moutinho[3], Andrea Cassingena[1], Katia Bencardino[1], Silvia Ghezzi[1], Laura Palmeri[1], Erica Bonazzina[1], Federica Tosi[1], Riccardo Ricotta[1], Tiziana Cipani[1], Patrizia Crivori[4], Rosalinda Gatto[4], Giuseppe Chirico[1], Giovanna Marrapese[1], Mauro Truini[1], Alberto Bardelli[2,5], Manel Esteller[3], Federica Di Nicolantonio[2,5], Andrea Sartore-Bianchi[1§] and Salvatore Siena[1, 6§]

[1]Niguarda Cancer Center, Ospedale Niguarda Ca' Granda, Milan, Italy;
[2]Candiolo Cancer Institute-FPO, IRCCS; SP 142 km 3.95, 10060 Candiolo (TO), Italy;
[3]Cancer Epigenetics and Biology Program (PEBC), Bellvitge Biomedical Research Institute (IDIBELL);
[4]CLIOSS s.r.l. , Viale Pasteur 10, 20014 Nerviano (MI) Italy
[5]Department of Oncology, University of Torino, SP 142 km 3.95, 10060 Candiolo (TO), Italy;
[6]Università degli Studi di Milano, Milan. Italy
§ Equally contributed as senior authors

Running title: temozolomide in MGMT deficient metastatic colorectal cancer

Keywords: colorectal cancer, temozolomide, MGMT, methylbeaming, liquid biopsy

Conflict of interest disclosure: The authors have no potential conflicts of interest to disclose.

Acknowledgments: Investigators at Niguarda Cancer Centers are supported by the following grants: Terapia Molecolare dei Tumori (A.S-B, S. S.) from Fondazione Oncologia Niguarda Onlus; Associazione Italiana per la Ricerca sul Cancro (AIRC) 2010 Special Program Molecular Clinical Oncology 5x1000, project 9970 (A.B and S.S.); European Union Seventh Framework Programme, grant 259015 COLTHERES (S.S, M.E. and A.B.) and 602901 MErCuRIC (A.B. and F.D.N.); study conducted within Rete Oncologica Lombarda (ROL); partially funded by grant Regione Lombardia “Fondazione Regionale per

la Ricerca Biomedica", DGR VIII/010761 on Dec 11 2009 e DGR IX/1485 on March 30 2011. The study was partly supported by 5 per mille 2009 MIUR—from Fondazione Piemontese per la Ricerca sul Cancro—ONLUS grant "Farmacogenomica" (F.D.N.); Associazione Italiana per la Ricerca sul Cancro IG 12812 (A.B.); Associazione Italiana per la Ricerca sul Cancro MFAG 11349 (F.D.N.); Fondazione Piemontese per la Ricerca sul Cancro-ONLUS 5 per mille 2010 and 2011 Ministero della Salute (F.D.N. and A.B.). Ludovic Barault was the recipient of a post-doctoral fellowship from Fondazione Umberto Veronesi in 2013 and 2015.

Corresponding Author:

Andrea Sartore-Bianchi, MD
Clinical Molecular Oncology
Niguarda Cancer Center
Ospedale Niguarda Ca' Granda,
Piazza Ospedale Maggiore, 3
20162 Milano
Phone +39 02 6444 3708 – Fax +39 02 6444 2957
e-mail: andrea.sartorebianchi@ospedaleniguarda.it

KEY MESSAGE

This study indicates that temozolomide has limited activity in mCRC selected by MGMT hypermethylation, and show for the first time that tumor MGMT methylation can change from diagnosis, decaying after several lines of treatments. This result points out the need to test baseline tumor biopsy or plasma in order to refine target selection in trials with alkylating agents performed in this setting.

Abstract

Background: Objective response to dacarbazine, the intravenous form of temozolomide (TMZ), in metastatic colorectal cancer (mCRC) is confined to tumors harboring O(6)-methylguanine-DNA-methyltransferase (MGMT) promoter hypermethylation. We conducted a phase II study of TMZ enriched by MGMT hypermethylation in archival tissue (AT), exploring dynamic of this biomarker in baseline tumor (BT) biopsy and plasma (liquid biopsy).

Patients and methods: We screened 150 mCRC patients for MGMT hypermethylation with methylation-specific PCR on AT from FFPE specimens. Eligible patients (n=29) underwent BT biopsy and then received TMZ 200 mg/m2 days 1-5 q28 until progression. A Fleming single-stage design was used to determine whether PFS rate at 12 week would be ≥35% (H0≤15%, type I error = 0.059 (1-sided), power = 0.849). Exploratory analyses included comparison between MGMT hypermethylation in AT and BT, and MGMT methylation testing by Methyl-BEAMing in solid (AT, BT) and LB with regard to tumor response.

Results: PFS rate at 12 weeks was 10.3% (90%CI: 2.9-24.6). Objective response rate was 3.4% (90% CI: 0.2 – 15.3), disease control rate 48.3% (90%CI: 32.0 – 64.8), median OS 6.2 months (95%CI: 3.8 – 7.6), and median PFS 2.6 months (95%CI: 1.4 – 2.7). We observed absence of MGMT hypermethylation in BT in 62.7% of tumors.

Conclusion: Treatment of mCRC with TMZ driven by MGMT promoter hypermethylation in AT samples did not provide meaningful PFS rate at 12 weeks. This biomarker changed from AT to BT, indicating that testing baseline tumor biopsy or plasma is needed for refined target selection.

Introduction

Colorectal cancer (CRC) is the third most common type of cancer in men and the second in women worldwide [1]. CRC is also the fourth leading cause of cancer deaths worldwide, accounting for 9.7% of global new cancers, with 1.4 million cases and 694,000 deaths [2]. Approximately 25% of patients have metastatic disease at diagnosis, and almost 50% of resected patients with early stage disease will eventually develop metastases, accounting for the relevant mortality rates [2]. In the last twenty years, research efforts in mCRC have led to the approval of several targeted agents in addition to standard chemotherapy, including bevacizumab [3], cetuximab [4], panitumumab [5], aflibercept [6], and regorafenib [7]. Apart from the anti-EGFR monoclonal antibodies cetuximab and panitumumab, for which *RAS* gene mutations have been shown to play a negative predictive role [8], in CRC there is a lack of clinically validated biomarkers effectively directing therapy.

MGMT is a repair protein which removes alkylating groups from the O^6-guanine in DNA. MGMT protects normal and tumor cells from this type of DNA damage, moving the alkylating group to a cysteine residual within its own protein [9, 10]. Approximately 40% of mCRC show silencing of the *MGMT* gene leading this to absence of the corresponding protein [11]. Due to this deficiency, the tumor cell is not able to effectively repair O^6-methylguanine adducts, thus determining a higher frequency of G:C > A:T transitions and potentially enhancing the cytotoxic effect of alkylating agents such as temozolomide (TMZ) or dacarbazine [10, 12].

We previously reported that objective response to dacarbazine, the intravenous form of TMZ, is confined to tumors harboring *MGMT* promoter hypermethylation [13]. Initial reports indicated that mCRC selected by MGMT deficiency achieved remarkable responses to TMZ [14]. Subsequent phase II studies enriched for *MGMT* methylation showed objective responses up to 12% [15, 16].

We designed the present study in order to evaluate the anti-tumor activity of TMZ in mCRC after failure of standard therapies selecting patients based on *MGMT* promoter hypermethylation assessed in individual archival tumor (AT) samples. In addition, we explored baseline tumor biopsy (BT) and blood (liquid biopsy) as biomarkers of the actual epigenetic status of the tumor before and during therapy.

Materials and methods

Study design - The study was designed as a single institution, open label, single arm phase II trial (TEMECT – TEMozolomide Evaluation in ColorecTal cancer, EUDRACT number 2012-003338-17). The aim of the study was to evaluate the efficacy of TMZ treatment in a molecularly selected population of mCRC patients by assessing its ability to achieve a clinically meaningful prolongation of progression-free survival (PFS) as compared to the expected outcome in this setting [7]. Patients were treated with TMZ until progression or unacceptable toxicity. Primary endpoint was PFS rate at 12 weeks, i.e. the proportion of patients known to be alive and progression free at 12 weeks or later since TMZ treatment start. Secondary endpoints included Objective Response Rate (ORR=CR+PR) according to RECIST 1.1 [17], Disease Control Rate (Confirmed ORR + SD rate), Overall Survival (OS), and overall safety profile evaluated by the National Cancer Institute Common Terminology Criteria for Adverse Events (NCI-CTCAE) Version 3.0. Computed tomography (CT) or magnetic resonance imaging (MRI) scan for tumor evaluation were carried out every 6±1 weeks until tumor progression. Pre-planned exploratory analyses included quantitative MGMT methylation assessed by new generation PCR (Methyl-BEAMing) and its relationship with primary clinical efficay, both in tumor tissue from AT samples as well as blood and BT collected at baseline within 28 days prior to initiation of study treatment. *KRAS* mutations in exons 2, 3 and 4 have been also evaluated in AT specimens and tested for association with clinical outcome. As for the

exploratory endpoints, tumor shrinkage/increase was also computed as absolute difference (mm) between sum of target lesions at baseline and at best response. Patients provided written informed consent and the study followed the Declaration of Helsinki and Good Clinical Practice, being approved by Ethic Committee of Ospedale Niguarda Ca' Granda (Milan, Italy).

Patient population - All patients met the following inclusion criteria: age ≥ 18 years, histological confirmed diagnosis of metastatic CRC, measurable disease (by RECIST criteria v1.1), and progressed on standard treatment with fluoropyrimidines, oxaliplatin, irinotecan, and cetuximab or panitumumab (if tumor *KRAS* wild-type). Study protocol allowed but not required prior treatment with bevacizumab or regorafenib. Previous treatment with dacarbazine was an exclusion criterion. An adequate bone marrow, liver and renal function was required.

Analysis of MGMT promoter hypermethylation – All patients eligible authorized molecular screening for MGMT promoter hypermethylation on AT tissue formalin-fixed paraffin-embedded (FFPE). FFPE samples were sent to central laboratory (Bellvitge Biomedical Research Institute- IDIBELL; Barcelona, Spain) for evaluation of MGMT promoter methylation status. Genomic DNA was extracted following manufacturer's instructions (E.Z.N.A. FFPE DNA Kit; Omega Bio-Tek). DNA was then subjected to sodium bisulfite treatment using EZ DNA methylation kit (Zymo Research, Orange, CA). MGMT promoter methylation status was analyzed by methyl specific polymerase chain reaction (MSP). It was performed in a 15 µl volume containing 1µl of the sodium bisulfite modified DNA. The characteristics of the MSP reactions and the primer sequence have been described previously [9]. SW48 cell line was used as a positive control for hypermethylated alleles of MGMT, and DNA from normal lymphocytes used as a negative control. The

above analysis was performed also in the BT collected before treatment in double blind fashion at the end of patients' accrual.

MGMT analysis by Methyl-BEAMing in tumor tissue and in plasma (liquid biopsy) – DNA from the AT tissues were retrospectively assessed by Methyl-BEAMing (MB) using the same DNA extracted and sodium bisulfite converted at central laboratory. DNA from BT was freshly extracted and bisulfite converted in Candiolo Cancer Institute-FPO, IRCCS, Candiolo. Biopsy tissue DNAs were extracted from two 10 μm FFPE tissues slides using the QIAamp DNA FFPE Tissue Kit (Qiagen) following manufacturer's protocol. Twenty microliters of DNA were bisulfite converted using the EZ DNA Methylation-Gold Kit (Zymo Research); elution was performed in twice 20 μl of M-Elution Buffer. *MGMT* analysis by MB was carried out as previously described, with normalization according to tumor content [18]. Ninety-nine samples were available for MGMT analysis in plasma (29 baseline, 70 follow-up time points). Cell free circulating DNA (cfDNA) was extracted at the University of Torino from 1ml of plasma using the QIAamp Circulating Nucleic Acid Kit (Qiagen) following manufacturer's protocol. Bisulfite conversion of 20μl of each cfDNA was performed using the EZ DNA Methylation-Gold Kit (Zymo research) according to manufacturer's protocol. Elution was performed using twice 10 μl of M-Elution Buffer. Methyl-BEAMing was then carried out as previously described [18].

Treatment – TMZ (Temozolomide TEVA, Teva Italia, Assago, Milan, Italy) was administered orally at a starting dose of 150 mg/m^2 day 1-5 for each 28-day cycle. Patients without hematological toxicities >1 CTCAE were allowed to escalate to 200 mg/m^2 day 1-5 for subsequent cycles, otherwise treatment continued at the initial dose level.

Statistical analysis – A Fleming single-stage procedure was applied for sample size

calculation [19]. The success rate was defined as the proportion of patients in a progression-free status at ≥ 12 weeks since treatment start out of the total number of treated patients. According to a recently published phase III trial in a similar patient population [7], the PFS rate at 12 weeks of untreated patients was estimated to be approximately 15%. Therefore, a rate of 35% of patients alive and progression-free at 12 week (H_1) was identified as a meaningful improvement for the proposed TMZ single agent treatment against the null hypothesis of a PFS rate at 12 weeks ≤ 15% (H_0). Assuming a type I error $\alpha = 0.059$ (1-sided) with power $1-\beta = 0.849$, 29 patients were required. At least 8 or more patients alive and free from progression at 12 weeks were needed to reject the null hypothesis. Point and 90% confidence interval (CI) estimates were calculated for the PFS rate at 12 weeks, objective tumor response and disease control rates. All other confidence intervals were calculated at 95% unless otherwise stated. Kaplan-Meier method was used to analyze time-to-event endpoints.

Results

Patient's characteristics - From December 2012 to May 2014, we screened 150 patients for *MGMT* promoter hypermethylation by MSP on AT FFPE tissue samples from primary tumor or metastases. Twenty-nine patients were enrolled (**Figure 1**, CONSORT diagram). AT consisted of primary tumors in 21/29 (72.4%) and metastases in 8/29 (27.6%) (6 lung, 2 liver metastases); median time from histological diagnosis on primary or metastases and enrollment was 3.2 (range 1.1– 8.8) and 1.9 years (range 0.3 – 6.7), respectively. Ninety-three percent of patients had received prior bevacizumab, 28% regorafenib (**Table 1**) and 69% more than 4 lines of treatment.

MGMT molecular evaluation in archival tissue (AT) and in pre-TMZ treatment tumor biopsies (BT) – At pre-screening we detected 95/149 (63.8%) unmethylated and 54/149

(36.2%) methylated CRCs, among the latter 29/54 (53.7%) were enrolled. All patients enrolled in the study underwent BT of a metastatic site prior to the start of treatment with TMZ (median time 2 days, range 0-18 days). Twenty-two of 29 matched pairs of AT and BT were evaluable by the MSP assay (**Figure 1**). Concordance in *MGMT* promoter methylation status was found in 6 of 22 evaluable (27.3%) sample pairs. There was no difference in concordance rates between the matched pairs of metastases/metastases (0/16) or primary/metastases (6/16) (Fisher's exact test p= 0.1328). Further molecular characterization was performed on AT and included status of *KRAS* and *BRAF* (**Table 1**).

Treatment Efficacy and Toxicity – The primary endpoint of the study was not met, since PFS rate at 12 weeks was 10.3% (CI 2.9 – 24.6%). Median follow-up was 9.8 months (CI 9.46 – not reached). Median PFS was 2.6 months (CI 1.4 – 2.7) (**Figure 2, A**). We observed one partial response (PR, 3.4%, CI 0.2–15.3%) and 13 SD, accounting for a Disease Control Rate (DCR: CRs/PRs and SDs) of 48% (CI 32.0 – 64.8 %) (**Figure 2, B**). The OS was 6.2 months (CI 3.8 – 7.6). The median number of treatment cycles was 2 (range 1-5), with a median dose intensity of 208.3 (range 102.6-242.4) mg/m^2/week. Treatment modifications, i.e. dose delays or reductions, occurred in 11 (37.9%) patients (6 patients [20.7%] required ≥1 dose reduction; 11 [37.9%] required ≥1 dose delay). Hematological adverse events were the most common reason for dose modification (7 patients, 24%). Common adverse events (occurring in > 10% of patients) are reported in **Table 2.**

Exploratory biomarkers analysis – Since despite *MGMT* silenced status selection by the qualitative MSP assay (yielding either positive or negative), TMZ showed poor efficacy in this setting, a quantitative measurement of MGMT, i.e. the PCR-based method MethylBEAMing, was then applied in individual AT and BT tissue samples as well as in

plasma (liquid biopsy) in order to evaluate whether the percentage of tumor MGMT promoter hypermethylation in individual patients could explain the lack of efficacy.

Methylation status of *MGMT* by methylBEAMing was retrospectively assessed in 28 AT and 25 BT. Median methylation value by this methodology was 26.17% (range 1.9-100) and 10.9% (range 0-100) respectively and this difference was statistically significant (Wilcoxon rank sum test, p=0.01). We did not found a linear correlation between tumor shrinkage and the percentage of *MGMT* promoter hypermethylation by methylBEAMing in AT (p=0.6) (**Figure 3A**). However, a significant correlation was retrieved when the same analysis was performed in BT (p=0.03) (**Figure 3B**). In particular, all patients displaying tumor increase segregated below a cutoff value of 17.4% of MGMT methylation in BT.

In plasma, we found that median MGMT methylation by methylBEAMing at baseline was 14.6% (range 0.0-81.0%). This value is significantly different as compared to the same method applied to AT (p=0.025 by Wilcoxon rank sum test), but not to BT (p=0.627). Interestingly, 7 patients displayed no methylation at all in plasma (0%), mirroring the results by MSP in baseline biopsy. After 1 cycle of TMZ treatment, we found a correlation between methylation variation in plasma and tumor shrinkage (p=0.008) (**Figure 4**). Finally, we found no association between *KRAS* mutations assessed in AT and DCR (p=0.48).

Discussion

Three phase II clinical trials have previously assessed the clinical efficacy of alkylating agents in mCRC. All of them have assessed the role of MGMT promoter hypermethylation as a predictive biomarker of response by the analysis of archival tumor tissues [13, 15, 16]. Despite some evidence of improved disease control rate in patients with MGMT hypermethylated tumors, the role of this biomarker in mCRC remains unclear. In the present study, we report that, in a cohort of 29 patients, all selected for *MGMT*

promoter hypermethylation by MSP in individual archival tumor, TMZ treatment did not overcome the threshold of a meaningful PFS rate at 12 weeks of 35%, with ORR of 3.4% and DCR of 48.3%. The innovative study design included MGMT assessment in archival tumor specimens, in biopsy of tumor at baseline and in plasma (liquid biopsy), leading to the following novel findings about the dynamic of this epigenetic biomarker.

First, we observed a previously unreported change in *MGMT* status occurring over time, i.e., MGMT methylation declined significantly from archival tumor samples compared to a biopsy taken at the actual moment of starting treatment with TMZ. It has been previously reported that in glioblastoma *MGMT* promoter methylation status can change after chemotherapy [20], but very limited data are available for CRC [12, 21, 22]. It should be acknowledged that in our study the time between sample collection and analysis of *MGMT* methylation in archival tumor *vs.* baseline tumor biopsy was remarkably different (median 2.8 years *vs.* 2 days), therefore the observed reduction in *MGMT* methylation of the tumors may be due also to long term storage [23]. Further, we cannot exclude that sampling procedures along with tumor heterogeneity could have affected the performance of *MGMT* testing on tissue, thus reducing its sensitivity. Indeed, large-scale analyses addressing the issue of epigenomics heterogeneity in colorectal cancer are missing and future studies might shed light on the extent or implications of this phenomenon. As a consequence, regardless of the possible cause or explanation, we highlight that molecular screening of MGMT hypermethylation by MSP on archival tumor does not properly select a mCRC population for a biomarker-enriched design. Accordingly, we found that only the percentage of *MGMT* methylation in baseline tumor biopsy, but not that in archival tumor, was correlated with tumor shrinkage after TMZ treatment. However, the observed correlation was weak (R=0.24; **Figure 3B**), possibly because in an advanced setting the heavy pretreatment (in present study 5 median previous lines) gives rise to multiple resistance mechanisms to DNA damaging agents, limiting sensitization by MGMT loss.

Moreover, the only patient who achieved partial response did not display *MGMT* methylation in baseline tumor biopsy, dictating caution in the interpretation of data. Altogether, these findings suggest that MGMT as a biomarker should be evaluated at the time of treatment, and not relying on previous older specimens as it was done in most of published trials for mCRC [13, 15]. Only Hochhauser et al. [16] made a patients selection by using also blood-based MSP assessment at the study entry, but data of matched pairs of tissue/plasma were not provided.

Second, we analyzed *MGMT* methylation status in plasma to test whether liquid biopsy, being performed on a fresh blood sample collected at the time of enrolment, could overcome the previously described spatial and time-dependent variations of the biomarker. This was performed by the quantitative assay methylBEAMing in order to study fluctuations during treatment. Given its blood-based nature, there might be a role for this test in dynamically assessing epigenetic status of *MGMT* by providing updated results without the need of repeated tumor biopsies. Hochhauser *et al* [16] previously reported an assessment of *MGMT* by MSP from blood in a phase II trial with miscellaneous tumor, including aereodigestive, colorectal and head-and-neck cancers. They show that 61% of patients with positive *MGMT* methylation by MSP in tissue did not confirm this finding in blood, even though distinct data for CRC histology were not provided. In our study, we found that plasma MGMT methylation before treatment was significantly different from that determined, with the same method, in the archival tumor, but not from that assessed on baseline tumor biopsy, confirming also in plasma the loss of MGMT methylation after time. Interestingly, 7 patients displayed no methylation at all (0%) in plasma baseline, which could be explained by absence of DNA from tumor origin. However, five of the matched biopsies also displayed very low methylation fraction (<1%) suggesting a loss of the methylated status in the tumor. Among the 22 remaining matched pairs of baseline tumor biopsies/plasma samples (n=22), we found high sensitivity (100%) and poor specificity

(37.5%) for liquid biopsy to detect methylation, thus suggesting its potential use as a pre-screening procedure before tumor biopsy, i.e. excluding those patients with negative results. We did not find an association between pre-treatment values of *MGMT* methylation in plasma and tumor response. However, we report a trend between the variation of *MGMT* methylation in plasma after 1 cycle of TMZ therapy and tumor response, indicating that liquid biopsy provides a surrogate marker of response rather than a predictive marker. Results of a concordance study between tissue and liquid biopsy are clearly warranted prior to further test it as a biomarker in this setting.

In conclusion, TMZ treatment driven by selection according to *MGMT* promoter hypermethylation in archival tumor samples does not provide meaningful PFS. A possible explanation, implied by this study, resides in the novel finding that *MGMT* hypermethylation in archival tissue is not maintained in paired tumor biopsies assessed at the moment of treatment. The latter, as well as liquid biopsies, might better capture the dynamic epigenetic changes of the tumor. All in all, MGMT as a biomarker for therapeutic intervention in metastatic colorectal cancer remains not clinically applicable as in other malignancies such as gliobastoma [18]. Methodological improvements in assessing MGMT in tissue or blood at the actual moment of treatment might overcome some limitations, but clearly further research is needed in order to identify potential mechanisms of tumor sensitivity acting synergistically with MGMT deficiency.

Table 1: Patient's demographic and characteristics.

Clinical characteristics	All patients (n=29) (%)
Age	
Median age (range)	60 (38-77)
≥ 65 years	7 (24%)
Gender – n (%)	
Female	9 (31%)
Male	20 (69%)
Performance status ECOG	
0	22 (76%)
1	7 (24%)
Number of previous treatments	
Median (range)	5 (3-9)
Previous treatment with bevacizumab	27 (93%)
Previous treatment with regorafenib	8 (28%)
Molecular Status (n=evaluable patients)	
KRAS mut (n=29)	18 (62%)
BRAF mut* (n=22)	1* (5%)

* BRAF p.S602S, c.1806T>A

Table 2: Adverse events occurring with most frequency (>10% of patients).

Clinical Adverse events	Any Grade % (n)	Grade 3-4 % (n)
Nausea	44,8 (13)	3,4 (1)
Vomiting	44,8 (13)	
Fatigue	37,9 (11)	3,4 (1)
Constipation	34,5 (10)	
Abdominal pain upper	17,2 (5)	
Back pain	17,2 (5)	10,3 (3)
Decreased appetite	17,2 (5)	
Pyrexia	17,2 (5)	
Asthenia	13,8 (4)	6,9 (2)
Anemia	10,3 (3)	
Cough	10,3 (3)	
Diarrhoea	10,3 (3)	
Laboratory abnormalities		
Platelet count decreased	34,5 (10)	27,6 (8)
Neutrophil count decreased	17,2 (5)	10,3 (3)
Thrombocytopenia	10,3 (3)	3,4 (1)

Figure Legends

Figure 1: Consort diagram showing patients' screening and enrollment.

Figure 2: A. Kaplan-Meyer analysis of PFS for ITT population. **B**. Waterfall plot showing changes in sum of target lesions at best response in evaluable patients. Dotted line shows +20% increases and -30% of tumor shrinkage. Different patterns in bars show the MGMT status in baseline tumor biopsy prior to starting TMZ treatment.

Figure 3: Scatterplot showing relationship between variations in sum of target lesions (mm) and MGMT promoter hypermethylation values (%) in archival tumor (**A**) or baseline tumor biopsy by MethylBEAMing prior to starting TMZ treatment (**B**). Dotted line shows a threshold of 17.4% of MGMT in baseline tumor biopsy segregating patients with an increased sum of target lesion as best response. PD, progression; SD, stable disease; PR, partial response, according RECIST criteria 1.1

Figure 4: Scatterplot showing relationship between variations in sum of target lesions (mm) and variation of MGMT promoter hypermethylation between values at baseline and before 2^{nd} cycle (%) in liquid biopsies. Empty circles represent pairs with absent MGMT methylation in both baseline and 2^{nd} cycle liquid biopsy. PD, progression; SD, stable disease; PR, partial response, according RECIST criteria 1.1

References

1. Chawla N, Butler E, Lund J et al. Patterns of colorectal cancer care in Europe, Australia, and New Zealand. Journal of the National Cancer Institute. Monographs 2013; 2013(46):36–61.
2. Ferlay J, Soerjomataram I, Dikshit R et al. Cancer incidence and mortality worldwide: Sources, methods and major patterns in GLOBOCAN 2012. International journal of cancer. Journal international du cancer 2014. doi:10.1002/ijc.29210.
3. Hurwitz H, Fehrenbacher L, Novotny W et al. Bevacizumab plus irinotecan, fluorouracil, and leucovorin for metastatic colorectal cancer. The New England journal of medicine 2004; 350(23):2335–42.
4. Cunningham D, Humblet Y, Siena S et al. Cetuximab monotherapy and cetuximab plus irinotecan in irinotecan-refractory metastatic colorectal cancer. The New England journal of medicine 2004; 351(4):337–45.
5. Amado R, Wolf M, Peeters M et al. Wild-type KRAS is required for panitumumab efficacy in patients with metastatic colorectal cancer. Journal of clinical oncology : official journal of the American Society of Clinical Oncology 2008; 26(10):1626–34.
6. Cutsem E, Tabernero J, Lakomy R et al. Addition of Aflibercept to Fluorouracil, Leucovorin, and Irinotecan Improves Survival in a Phase III Randomized Trial in Patients With Metastatic Colorectal Cancer Previously Treated With an Oxaliplatin-Based Regimen. Journal of Clinical Oncology 2012; 30(28):3499–506.
7. Grothey A, Cutsem E, Sobrero A et al. Regorafenib monotherapy for previously treated metastatic colorectal cancer (CORRECT): an international, multicentre, randomised, placebo-controlled, phase 3 trial. Lancet 2013; 381(9863):303–12.
8. Douillard J-Y, Oliner K, Siena S et al. Panitumumab-FOLFOX4 treatment and RAS mutations in colorectal cancer. The New England journal of medicine 2013; 369(11):1023–34.
9. Esteller, Garcia-Foncillas, Andion et al. Inactivation of the DNA-repair gene MGMT and the clinical response of gliomas to alkylating agents. The New England journal of medicine 2000; 343(19):1350–4.
10. Esteller M, Herman J. Generating mutations but providing chemosensitivity: the role of O6-methylguanine DNA methyltransferase in human cancer. Oncogene 2004; 23(1):1–8.
11. Esteller, Toyota, Sanchez-Cespedes et al. Inactivation of the DNA repair gene O6-methylguanine-DNA methyltransferase by promoter hypermethylation is associated with G to A mutations in K-ras in colorectal tumorigenesis. Cancer research 2000; 60(9):2368–71.
12. Ju H, An B, Okamoto Y et al. Distinct Profiles of Epigenetic Evolution between Colorectal Cancers with and without Metastasis. The American Journal of Pathology 2011. doi:10.1016/j.ajpath.2010.12.045.
13. Amatu A, Sartore-Bianchi A, Moutinho C et al. Promoter CpG island hypermethylation of the DNA repair enzyme MGMT predicts clinical response to dacarbazine in a phase II study for metastatic colorectal cancer. Clinical cancer research : an official journal of the American Association for Cancer Research 2013; 19(8):2265–72.
14. Hoff D, Stephenson J, Rosen P et al. Pilot Study Using Molecular Profiling of Patients' Tumors to Find Potential Targets and Select Treatments for Their Refractory Cancers. Journal of Clinical Oncology 2010; 28(33):4877–83.
15. Pietrantonio, Perrone, de Braud et al. Activity of temozolomide in patients with advanced chemorefractory colorectal cancer and MGMT promoter methylation. Annals of oncology : official journal of the European Society for Medical Oncology / ESMO 2014; 25(2):404–8.

16. Hochhauser D, Glynne-Jones R, Potter V et al. A phase II study of temozolomide in patients with advanced aerodigestive tract and colorectal cancers and methylation of the O6-methylguanine-DNA methyltransferase promoter. Molecular cancer therapeutics 2013; 12(5):809–18.
17. Eisenhauer, Therasse, Bogaerts et al. New response evaluation criteria in solid tumours: revised RECIST guideline (version 1.1). European journal of cancer (Oxford, England : 1990) 2009; 45(2):228–47.
18. Barault, Amatu, Bleeker et al. Digital PCR quantification of MGMT methylation refines prediction of clinical benefit from alkylating agents in glioblastoma and metastatic colorectal cancer. Annals of oncology : official journal of the European Society for Medical Oncology / ESMO 2015. doi:10.1093/annonc/mdv272.
19. Fleming. One-sample multiple testing procedure for phase II clinical trials. Biometrics 1982; 38(1):143–51.
20. Brandes A, Franceschi E, Tosoni A et al. O(6)-methylguanine DNA-methyltransferase methylation status can change between first surgery for newly diagnosed glioblastoma and second surgery for recurrence: clinical implications. Neuro-oncology 2010; 12(3):283–8.
21. Maglio G, Casagrande M, Guardascione M et al. MGMT promoter methylation status in brain metastases from colorectal cancer and corresponding primary tumors. Future Oncology 2015; 11(8):1201–1209.
22. Konishi K, Watanabe Y, Shen L et al. DNA methylation profiles of primary colorectal carcinoma and matched liver metastasis. PloS one 2011; 6(11):e27889.
23. Kalmár A, Péterfia B, Hollósi P et al. Bisulfite-Based DNA Methylation Analysis from Recent and Archived Formalin-Fixed, Paraffin Embedded Colorectal Tissue Samples. Pathology & Oncology Research 2015. doi:10.1007/s12253-015-9945-4.

Figure 1

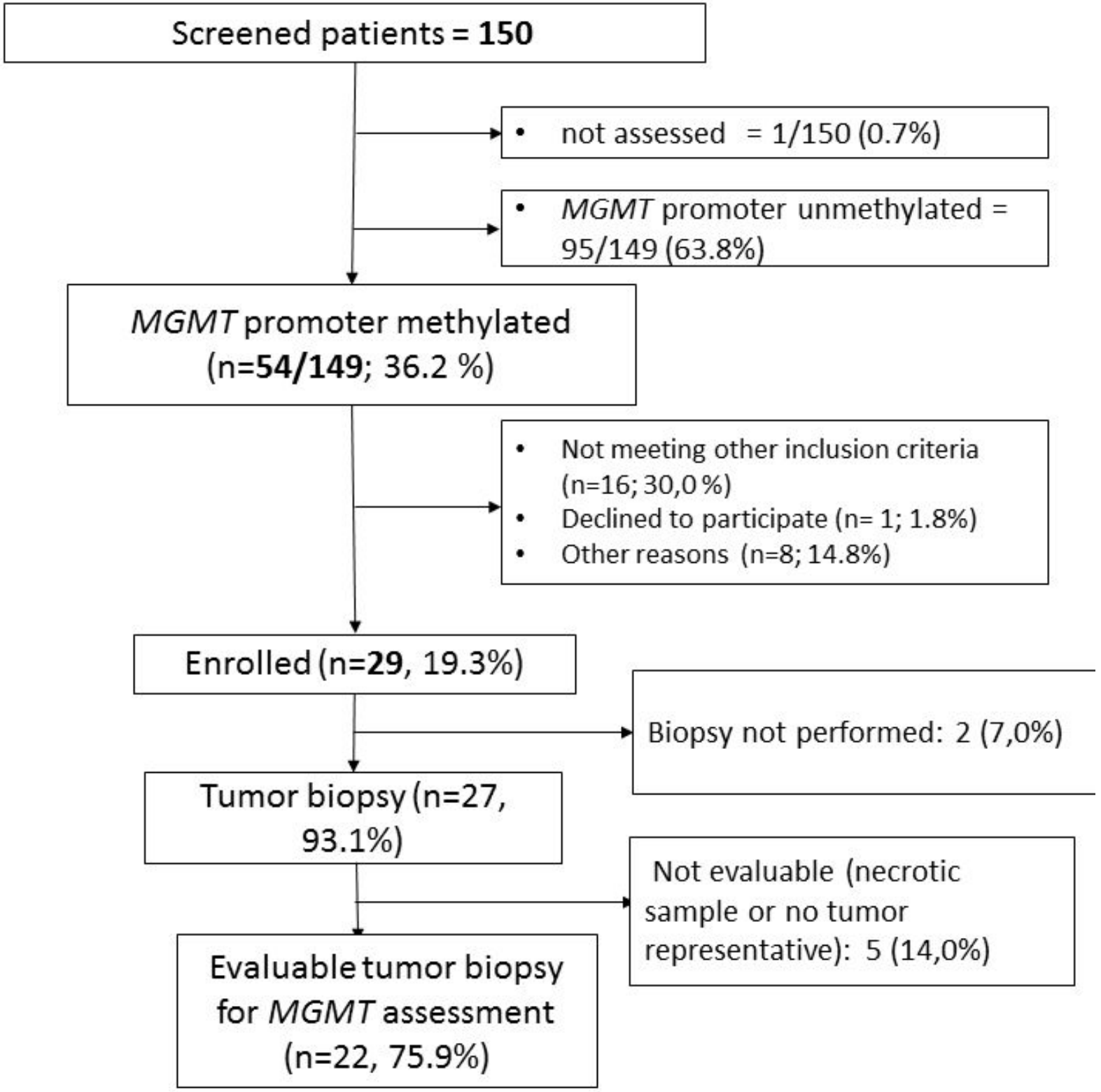
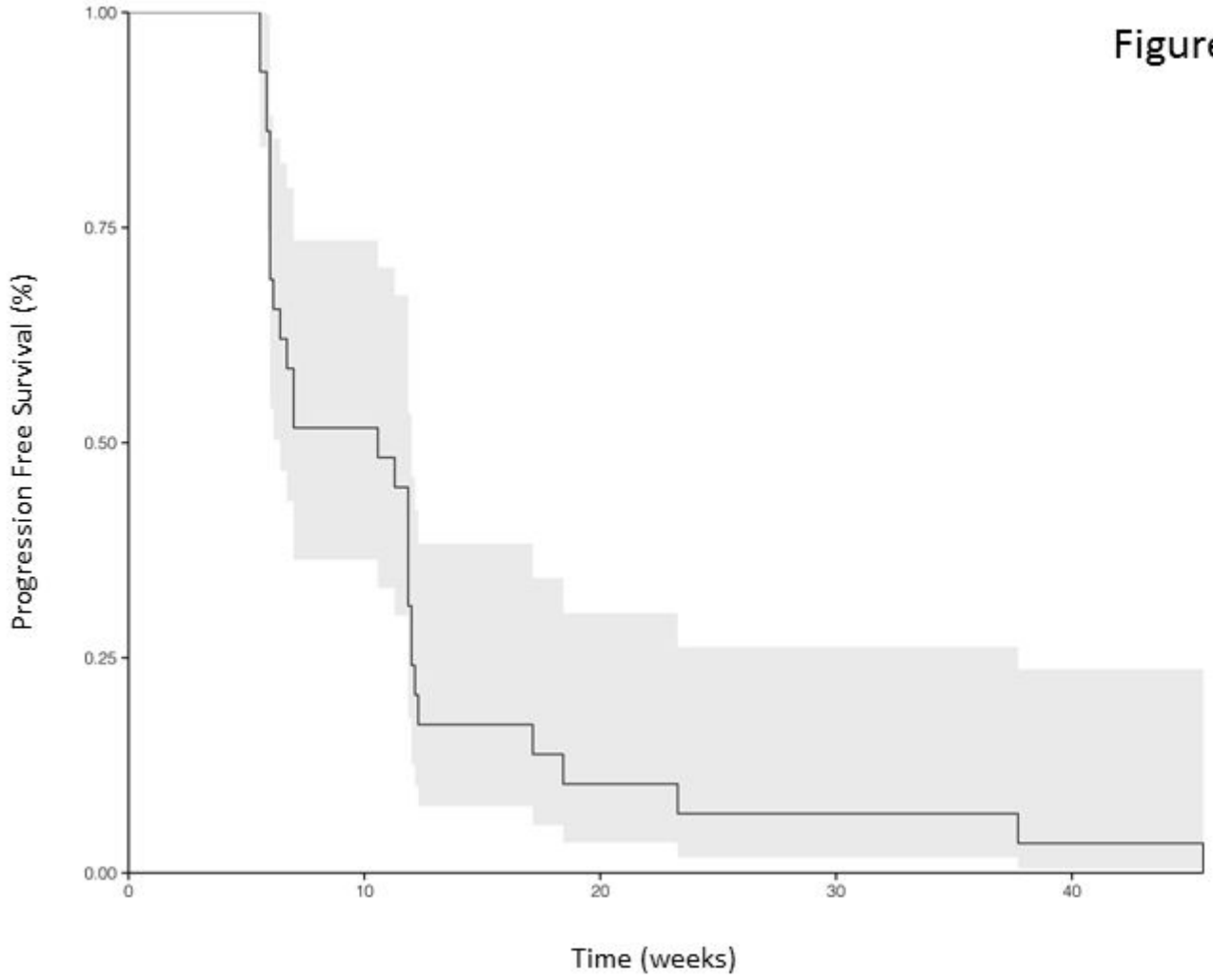

Screened patients = **150**
• not assessed = 1/150 (0.7%)
• *MGMT* promoter unmethylated = 95/149 (63.8%)
MGMT promoter methylated (n=**54/149**; 36.2 %)
• Not meeting other inclusion criteria (n=16; 30,0 %)
• Declined to participate (n= 1; 1.8%)
• Other reasons (n=8; 14.8%)
Enrolled (n=**29**, 19.3%)
Biopsy not performed: 2 (7,0%)
Tumor biopsy (n=27, 93.1%)
Not evaluable (necrotic sample or no tumor representative): 5 (14,0%)
Evaluable tumor biopsy for *MGMT* assessment (n=22, 75.9%)

Figure 2

A


1.00
0.75
0.50
0.25
0.00
Progression Free Survival (%)
0
10
20
30
40
Time (weeks)

B

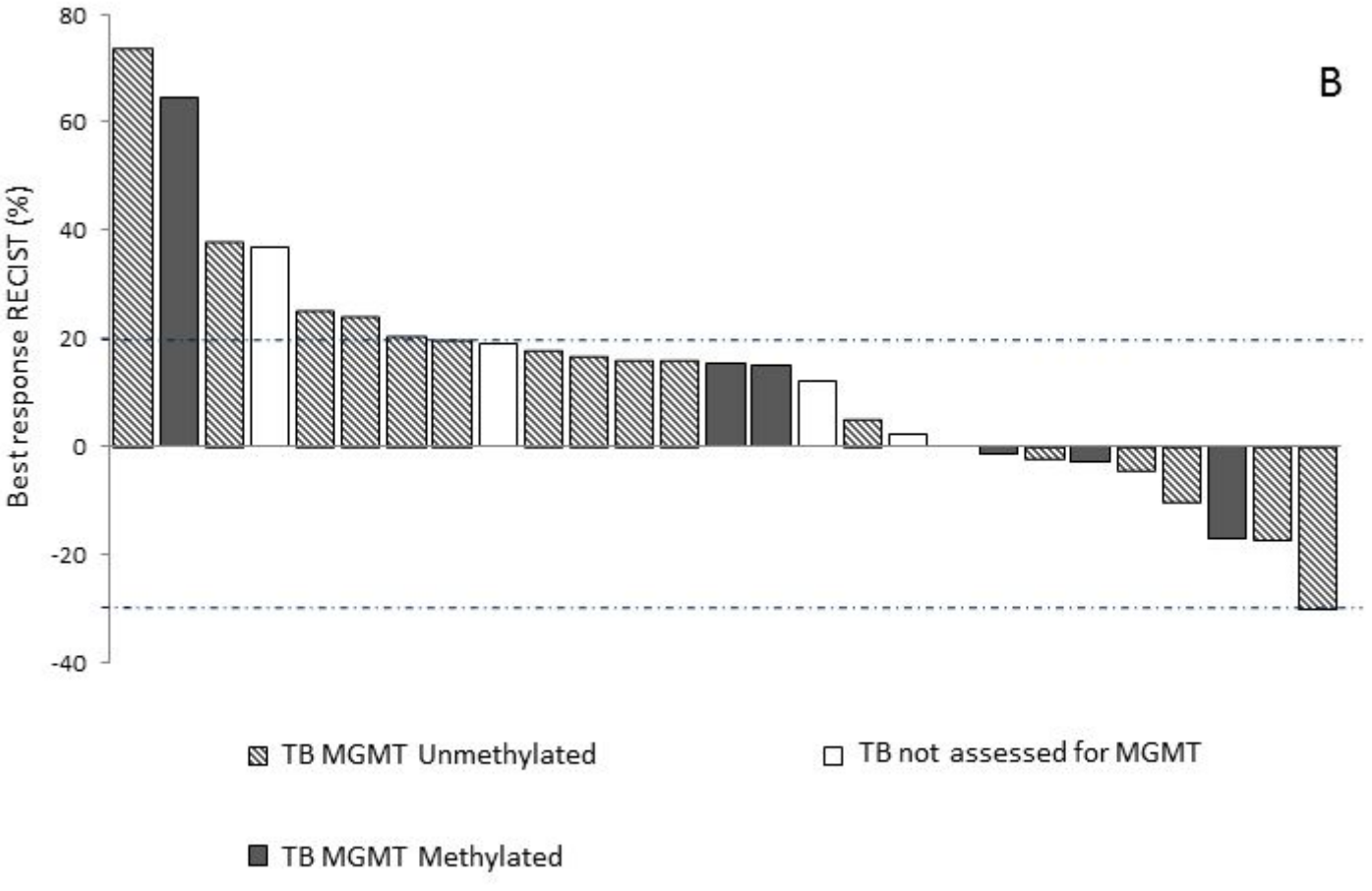
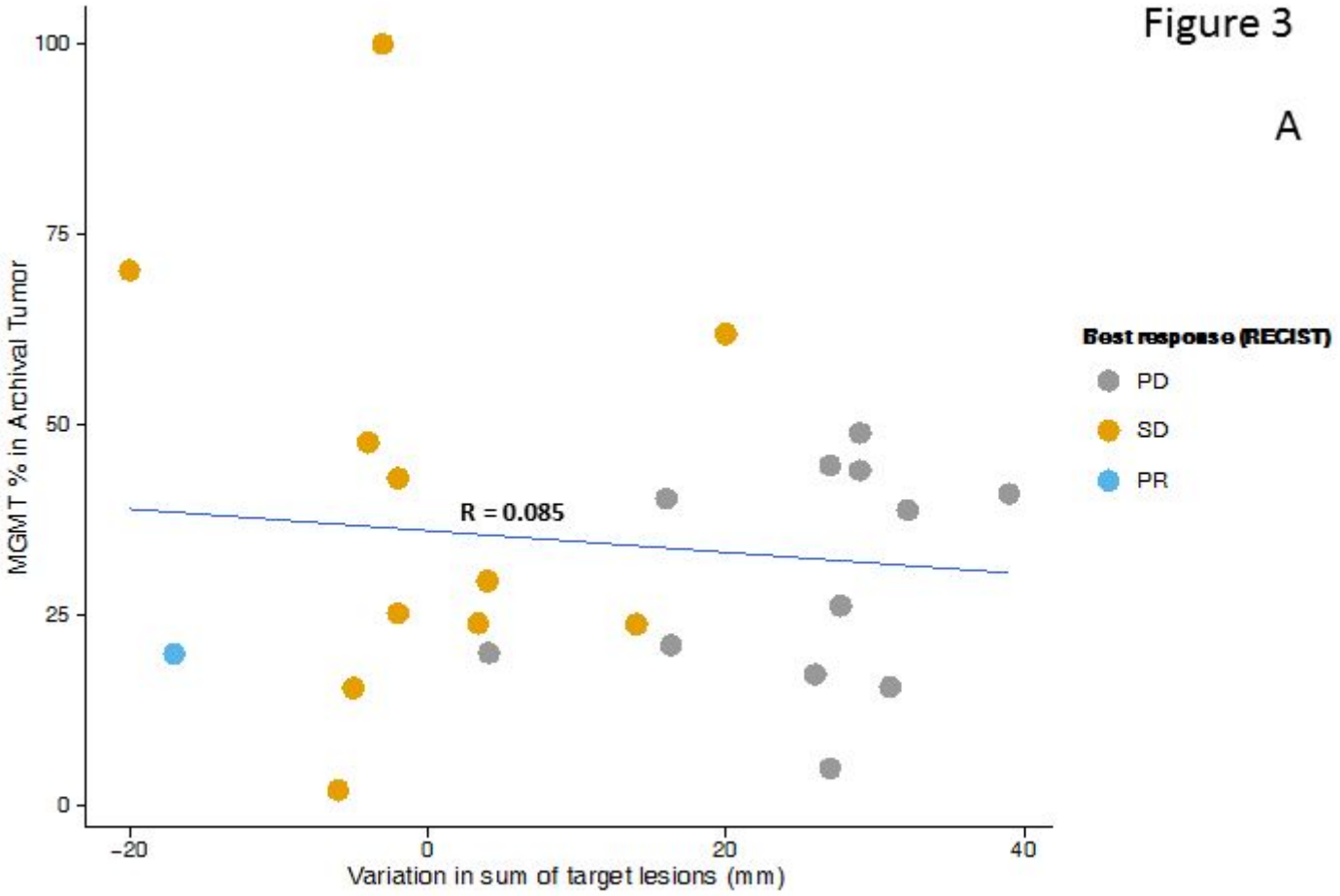

80
60
40
20
0
-20
-40
Best response RECIST (%)
TB MGMT Unmethylated
TB not assessed for MGMT
TB MGMT Methylated

Figure 3

A


MGMT % in Archival Tumor
100
75
50
25
0
R = 0.085
−20
0
20
40
Variation in sum of target lesions (mm)
Best response (RECIST)
PD
SD
PR

B

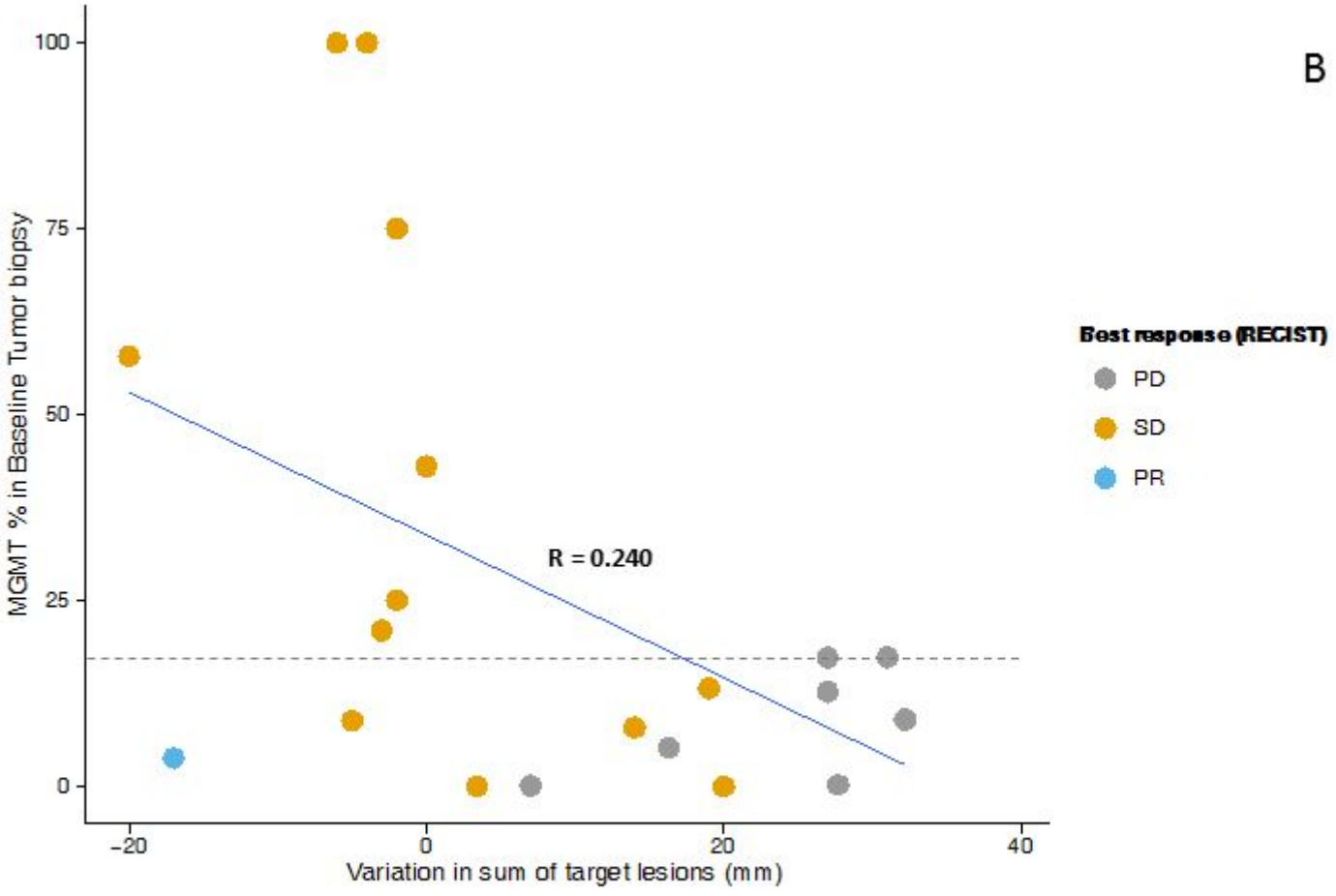
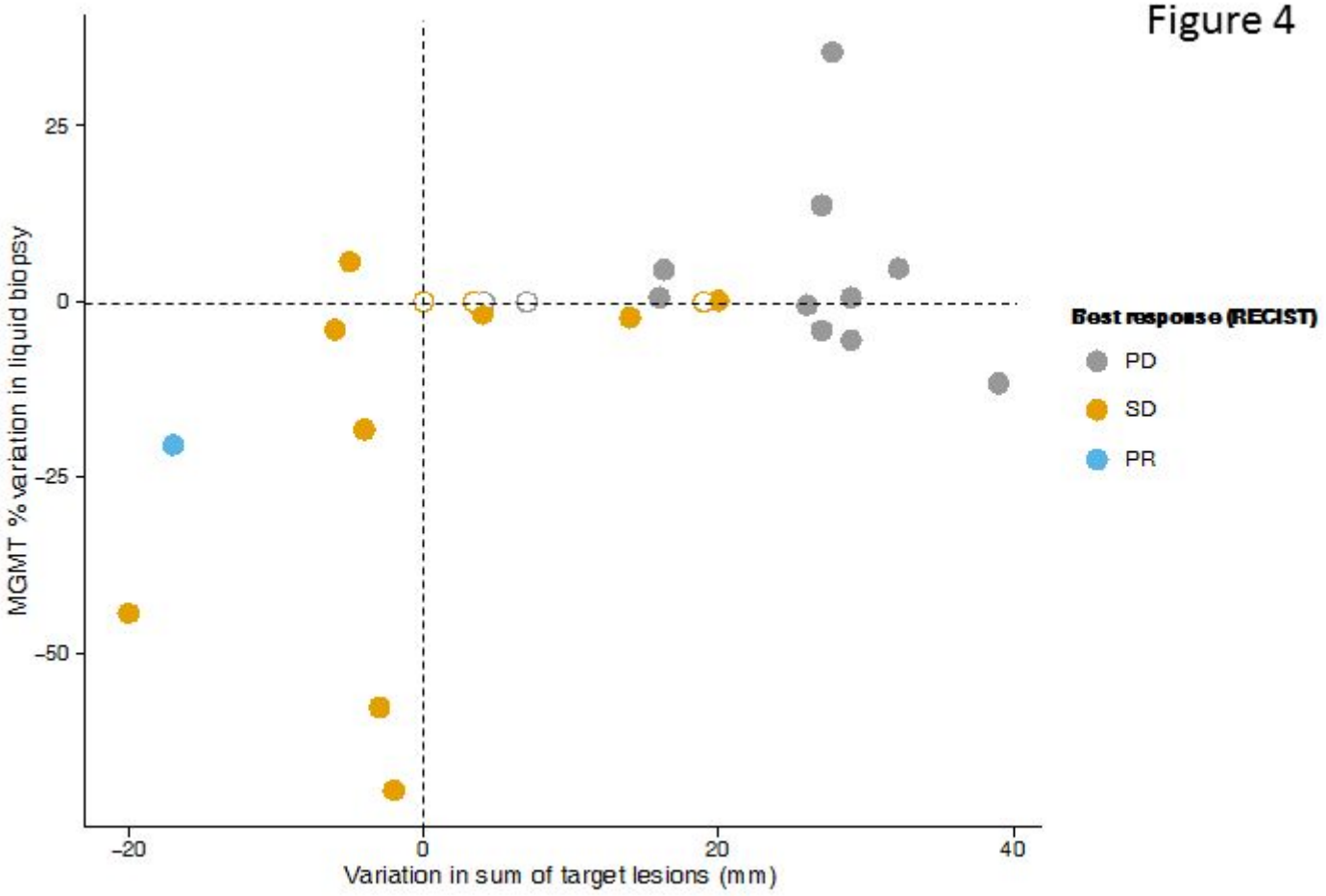

MGMT % in Baseline Tumor biopsy
100
75
50
25
0
R = 0.240
−20
0
20
40
Variation in sum of target lesions (mm)
Best response (RECIST)
PD
SD
PR

Figure 4


MGMT % variation in liquid biopsy
25
0
−25
−50
−20
0
20
40
Variation in sum of target lesions (mm)
Best response (RECIST)
PD
SD
PR