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MGMT testing—the challenges for biomarker-based glioma treatment

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Abstract: Many patients with malignant gliomas do not respond to alkylating agent chemotherapy. Alkylator resistance of glioma cells is mainly mediated by the DNA repair enzyme O(6)-methylguanine-DNA methyltransferase (MGMT). Epigenetic silencing of the MGMT gene by promoter methylation in glioma cells compromises this DNA repair mechanism and increases chemosensitivity. MGMT promoter methylation is, therefore, a strong prognostic biomarker in paediatric and adult patients with glioblastoma treated with temozolomide. Notably, elderly patients (>65-70 years) with glioblastoma whose tumours lack MGMT promoter methylation derive minimal benefit from such chemotherapy. Thus, MGMT promoter methylation status has become a frequently requested laboratory test in neuro-oncology. This Review presents current data on the prognostic and predictive relevance of MGMT testing, discusses clinical trials that have used MGMT status to select participants, evaluates known issues concerning the molecular testing procedure, and addresses the necessity for molecular-context-dependent interpretation of MGMT test results. Whether MGMT promoter methylation testing should be offered to all individuals with glioblastoma, or only to elderly patients and those in clinical trials, is also discussed. Justifications for withholding alkylating agent chemotherapy in patients with MGMT-unmethylated glioblastomas outside clinical trials, and the potential role for MGMT testing in other gliomas, are also discussed.

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MGMT testing in neurooncology - A paradigm for prospects and challenges of biomarker-based treatment decisions

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Running head: *MGMT* testing for treatment decisions in neurooncology

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Abstract

Many patients with malignant gliomas do not respond to alkylating agent chemotherapy with temozolomide or nitrosoureas. Alkylator resistance of glioma cells is mainly mediated by the DNA-repair protein O^6-methylguanine-DNA-methyltransferase (MGMT). Epigenetic silencing of the *MGMT* gene by promoter methylation in glioma cells compromises this DNA-repair mechanism and increases chemosensitivity. *MGMT* promoter methylation is a strong prognostic factor in pediatric and adult glioblastoma patients treated with temozolomide. In particular elderly glioblastoma patients whose tumors lack *MGMT* promoter methylation appear to benefit minimal from chemotherapy. Thus, the *MGMT* promoter methylation status has become a frequently requested biomarker test in neurooncology. Here, we discuss the current data on the prognostic and predictive relevance of *MGMT* testing, review the trials on targeted agents with *MGMT* status-restricted participation, evaluate methodological and quality control issues concerning the molecular testing procedure, and address the necessity for molecular context-dependent interpretation of *MGMT* testing results. A particular focus is placed on the following questions: (i) Should all glioblastoma patients be tested for *MGMT* promoter methylation or should testing be restricted to elderly patients and patients in clinical trials with *MGMT*-restricted entry? (ii) Is it justified withholding alkylating agent chemotherapy in patients with *MGMT*-unmethylated glioblastomas outside clinical trials? (iii) What is the role for *MGMT* testing in other gliomas? The answers to these issues depend not only on data from controlled clinical trials, but also on the availability of alternative treatment options to alkylating agents treatment, as well as on the access to molecular testing and its sensitivity and specificity.

Keywords

bevacizumab, cilengitide, enzastaurin, glioma, IDH1, MGMT, promoter methylation, temozolomide, temsirolimus,

Key points

- Epigenetic inactivation of *MGMT* may facilitate the occurrence of point mutations in *TP53* and other genes during tumorigenesis and progression
- *MGMT* promoter methylation is not a prognostic biomarker in *IDH wildtype* gliomas, but predictive for the use of alkylating chemotherapies in glioblastoma
- Treatment decisions in elderly patients with glioblastoma should consider *MGMT* status
- *MGMT* testing for entry of patients into clinical glioblastoma trials is feasible
- It is justified to withhold TMZ from patients with newly diagnosed glioblastoma without *MGMT* promoter methylation in the context of clinical trials
- MGMT-mediated resistance is not overcome by alternative schedules of alkylating chemotherapies, but may be circumvented by the use of alternative treatments
- Quality-ensured MGMT testing should be implemented as a molecular diagnostic method in the next WHO classification of brain tumors

Introduction

Glioblastoma, the most common primary central nervous system tumor, accounts for 12% to 15% of all intracranial tumors and 50% to 60% of gliomas [1]. Overall, patients diagnosed with glioblastoma die within a few months if untreated. Standard treatment consisting of surgery followed by combined radiochemotherapy with temozolomide (TMZ) increases median survival to 12-15 months, although the disease progresses within 6-9 months and the 2-year survival rate is less than 25% [2]. The EORTC 26981/22981 NCIC CE.3 trial demonstrated that survival benefit derived from radiochemotherapy with TMZ increases significantly with tumors harboring a methylated *MGMT* promoter [3]. This foremost predictive value of *MGMT* promoter methylation has since been confirmed [4]. In the absence of non-chemotherapy arms, older studies cannot separate prognostic from predictive properties of *MGMT* promoter methylation, and even the most recent RTOG-0525 trial only confirms a prognostic impact, as there was no TMZ-free treatment arm in this trial [5-9] (Table 1).

Although there may be small numbers of glioblastoma patients who benefit from combined radiochemotherapy although their tumors lack *MGMT* promoter methylation, there is limited if any benefit for the whole subgroup of patients with *MGMT*-unmethylated glioblastomas. This observation calls for different therapeutic approaches in glioblastoma patients depending on the *MGMT* status. The primary question to address in patients with *MGMT*-unmethylated glioblastomas is the identification of novel concepts (drugs) that provide a larger survival benefit than radiotherapy alone or in combination with TMZ. These concepts have been addressed in several phase II-III clinical trials offering the opportunity to test novel targeted compounds in their interaction with radiotherapy (without TMZ) only, and thereby to avoid additive toxicity with the alkylating chemotherapy. A separation of patients with *MGMT*-methylated and -unmethylated glioblastomas into separate trials introduced major challenges for the trial logistics and necessitates the resolution of key questions regarding the sensitivity and specificity of the *MGMT* testing methods. In addition, the challenge for these trials also extends to the definition of the scientifically sound comparator

(standard therapy) arm. Results from studies exploring safety and efficacy of vascular endothelial growth factor (VEGF) inhibitors, [10-13], the integrin inhibitor cilengitide [14,15], the protein kinase C (PKC) inhibitor enzastaurin [16], the inhibitor of the mammalian target of rapamycin, temsirolimus [17] have been eagerly awaited, and will be discussed.

Lastly, recent trials have suggested two subpopulations of glioma patients, in whom *MGMT* testing may be introduced into the daily clinical practice; these are elderly patients with glioblastoma and patients with anaplastic gliomas of World Health Organization (WHO) grade III that lack mutations in the *isocitrate dehydrogenase type 1 (IDH1)* gene. In contrast, the practice to determine *MGMT* status to substantiate the diagnosis of a pseudoprogression [18], is not supported by current analyses that do not find a differential likelihood for pseudoprogression dependent on *MGMT* status [19].

Search Strategy and Selection Criteria

Publications in the PubMed database published between January 1995 and November 2013 were identified using the search terms “glioblastoma”, “glioma”, “MGMT”, “integrin”, “mTOR”, and “PKC”. Only papers published in English were reviewed. Relevant clinical trials were identified by searching http://www.clinicaltrials.gov/ using the search terms “glioblastoma” and “glioma”.

Glioblastoma therapy

The lack of alternatives to the standard of care [20], the observation that at least some patients with *MGMT* promoter-unmethylated tumors may benefit from TMZ treatment, as well as the limitations of the *MGMT* tests especially in day-to-day management, has prevented *MGMT* biomarker assessment to be generally introduced into clinical decision-making. The EORTC 26981/22981 NCIC CE.3 trial compared radiotherapy alone with radiochemotherapy with TMZ [20]. In this trial, chiefly patients with glioblastoma showing a methylated *MGMT* promoter by methylation-specific PCR (MSP) analysis benefited from TMZ [3].

Based on the hypothesis that intensifying TMZ therapy would sensitize *MGMT*-unmethylated tumors to alkylating DNA damage and enhance the effect of TMZ in *MGMT*-methylated tumors [21], the Radiation Therapy Oncology Group trial 0525 (RTOG0525) compared a 21/28 days dose-intensified TMZ maintenance therapy after regular radiochemotherapy with the standard of care. The trial randomized 833 patients, from whom at least one large biopsy specimen was available for molecular analysis. Median OS for the trial cohort was 17.7 months and progression-free survival (PFS) was 8.2 months. *MGMT* promoter methylation was prognostic with a median OS in the patients with *MGMT* promoter-methylated tumors of 23.2 months versus 16 months in patients whose tumors lacked *MGMT* promoter methylation. However, there was no difference between the standard-dosed TMZ and the dose-intensified regimen [9] (Table 1).

In 2012, trials of the Neurooncology Working Group (NOA)-08 of the German Cancer Society and the Nordic group provided strong evidence for a predictive role of *MGMT* status assessment in selecting the appropriate treatment of elderly glioblastoma patients who are not commonly treated with combined modality treatment (RT/TMZ→TMZ) [22,23] (Table 2).

Because no standard treatment option exists following recurrence, the use of lomustine or rechallenge with TMZ is common practice in countries without regular access to bevacizumab, which is approved in the US and many countries outside the European Union for treatment of recurrent glioblastoma [10,24]. Except from uncontrolled series [26-30], *MGMT* data for lomustine or TMZ from recent trials at progression are missing, though testing could be done on the tissue from original diagnosis and would not necessitate a new biopsy or resection [25].

Regulation of expression and function of MGMT

The *MGMT* gene is located on chromosome band 10q26 and consists of five exons. A 5'-CpG island (CGI) of 762 base pairs (bp), including 98 CpG dinucleotides, encompasses large parts of the promoter region and the first exon. A few studies conducted correlative analyses between methylation at individual CpG sites in the *MGMT*-associated CGI and

gene expression [31-34]. However, it is still not completely clear yet how many and which particular CpG sites in the *MGMT* CGI have to be methylated in order to (i) cause transcriptional silencing in primary tumor cells and (ii) to demonstrate the best association with treatment outcome and patient survival. In fact, each diagnostic methylation-specific assay – whether qualitative, semi-quantitative or quantitative – interrogates distinct sets of CpG sites within the *MGMT* CGI [35,36] (Fig. 1).

In addition to promoter methylation MGMT expression is regulated also by histone modifications, aberrant expression or function of transcriptional activators or repressors, as well as posttranscriptional regulation by various microRNA (miRNA) species. Increased methylation of histone H3 lysine 9 (H3K9) and concomitant binding of MeCP2 to the *MGMT* promoter region was found to be associated with promoter methylation and transcriptional down-regulation, while histone H3 and H4 acetylation and methylation of H3 lysine 4 were detected in MGMT-expressing cells [32, 37]. Increased acetylation of H3K9 and decreased dimethylation of this residue have been linked to *MGMT* upregulation and acquired TMZ resistance in glioblastoma cell lines. In line with these findings, treatment with histone deacetylase inhibitors potentiated the evolution of acquired TMZ resistance [38].

Various transcription factors have been reported to enhance activity of the *MGMT* promoter, including specificity protein (SP)1 [39], nuclear factor (NF)kappaB [40], cAMP response element-binding protein (CBP)/p300 co-activator complex [41] and activator protein (AP)1 [42], while p53 has been implicated as a repressor of *MGMT* transcription [43]. Moreover, there is first evidence that hypoxia may up-regulate *MGMT* expression *via* hypoxia-inducible factor (HIF) 1α signaling [44], which may contribute to TMZ resistance of glioma stem cells located e.g. in perinecrotic hypoxic niches. These cells in turn may be sensitized by treatment with bone morphogenetic protein 2 (BMP2), which can down-regulate HIF-1α-mediated MGMT induction [45]. Induction of resistance may also be conferred by the hypoxia- and steroid-inducible N-myc downstream-regulated gene (NDRG1), which binds to and thus probably sustains action of MGMT [46].

Recent studies addressed a further level of MGMT regulation through direct binding of specific miRNAs to the 3'-UTR of *MGMT* transcripts, which may lead to decreased mRNA stability and/or reduced protein translation [47]. Distinct miRNAs that have been implicated as direct regulators of *MGMT* expression include miR-181b [48,49], miR-181d [49], mir221/222 [50], as well as miR-767-3p and miR-648 [47]. Thus, also aberrant expression of miRNAs may contribute to the variable MGMT expression values observed in *MGMT*-unmethylated tumors (Fig. 2). However, the relative importance of these different regulatory systems for response to TMZ is not known.

MGMT is an evolutionary highly conserved and ubiquitously expressed suicide DNA repair protein. It counteracts the lethal effects of alkylating agents by removing alkyl adducts from the O^6-position of guanine [51]. This persistent O^6-methylguanine adduct induced by methylating agents, such as TMZ and nitrosourea derivatives, causes base mispairing which is recognized by mismatch repair (MMR) during DNA replication and according to the futile repair hypothesis ultimately, induces cell cycle arrest and cell death [52-55]. The methylation damage induced by these agents can be reverted by MGMT. This DNA repair activity provides resistance against cytotoxic effects of DNA alkylating drugs, further demonstrated by small molecule inhibitors of MGMT that revert the effect [56] (Fig. 3). However, for cytotoxic activity of alkylated O^6-guanine an intact MMR machinery is required (see below).

Although many studies have shown that a deficiency of MGMT can increase the sensitivity of high-grade glioma to alkylating agents [5,55-57], tumors with low levels of MGMT may still exhibit resistance to these drugs, suggesting that other mechanisms are also involved in the resistance of some tumors to chemotherapy.

The first striking observations on a potential value of MGMT protein expression as a biomarker determined by immunofluorescence microscopy were made in malignant glioma patients who appeared to derive much more benefit from carmustine (BCNU) treatment when the MGMT protein expression levels in their tumors were low [51,58]. A decreased expression of MGMT protein is mainly attributed to epigenetic silencing mediated by *MGMT* gene promoter methylation. This is assessable by a methylation-specific PCR reaction but

also other tests, which are discussed below (Fig. 1). First correlations of *MGMT* promoter methylation with survival in uncontrolled series were demonstrated for glioma patients treated with nitrosoureas [5] or TMZ [59].

Reasons and consequences of *MGMT* promoter methylation in gliomas

The questions why *MGMT* is epigenetically silenced in subsets of gliomas and how this alteration may provide a tumor-promoting effect have not yet been resolved completely. In *IDH1/2* mutant gliomas, *MGMT* is part of a set of genes that become methylated due to global changes in DNA methylation as a consequence of increased levels of 2-hydroxyglutarate (2HG), which is aberrantly produced by mutant IDH1 or IDH2 proteins [60]. 2HG in turn inhibits various α-ketoglutarate-dependent enzymes, including *ten-eleven translocation* (TET) hydroxylases and histone demethylases [61]. Inhibition of these enzymes leads to aberrant DNA and histone methylation, which eventually causes a CpG island methylator phenotype in *IDH1/2* mutant gliomas (gCIMP) that leads to epigenetic silencing of many different genes including *MGMT* [62,63]. The reasons for *MGMT* promoter methylation in *IDH1/2* wildtype gliomas, including approximately 40% of primary glioblastomas, and the underlying molecular mechanisms are less clear. Functionally, *MGMT* promoter methylation and transcriptional silencing may not only lower TMZ resistance but also has been reported to increase genetic instability that may promote tumorigenesis, in particular by facilitating the appearance of G:C to A:T transition mutations [64], In fact, MGMT-deficient mice are more sensitive to cancer development upon challenge with DNA-alkylating cancerogenic substances [65]. In addition, associations of *MGMT* promoter methylation with higher frequencies of G:C to A:T transition mutations in tumor suppressor genes, including *KRAS* and *TP53*, have been reported in various cancers including gliomas [64,66,67]. These findings are corroborated in the TCGA data set of glioblastomas that also reveals a higher frequency of *TP53* and *PTEN* point mutations in *MGMT* promoter-methylated versus unmethylated glioblastomas (TCGA, accessed Nov 11, 2013). Moreover, TMZ treatment of *MGMT*-inactivated glioblastomas frequently induces a

hypermutator phenotype in glioblastomas, which may lead to secondary resistance by causing mutations in DNA mismatch repair genes [68]. Thus epigenetic inactivation of *MGMT* may facilitate point mutations in *TP53* and other genes during tumorigenesis and progression and TMZ treatment facilitates the emergence of secondary alkylant resistance by inactivating mutations of MMR genes. In line with this hypothesis, the *MGMT* promoter methylation status is usually homogeneous within malignant gliomas as demonstrated by the analysis of serial stereotactic biopsies [69], arguing for MGMT inactivation as an early event in tumor development in both *IDH1/2* mutant and wildtype gliomas. While acquired (somatic) mutations in *MGMT* that reduce enzymatic activity have been reported in certain epithelial cancers [70,71], *MGMT* mutations only occur in 0.4% of malignant gliomas and (TCGA data set, accessed Nov 11, 2013).

Towards standardized testing for diagnostic assessment of the *MGMT* status

A standardized MGMT test both for clinical trials and daily decision-making should be sensitive and specific, reproducible and applicable to formalin-fixed and paraffin-embedded (FFPE) tissues. The most commonly used DNA-based diagnostic method for promoter methylation analysis is methylation-specific polymerase chain reaction (MSP) [72]. MSP also can be reliably performed on small stereotactic biopsy specimens, e.g. in case of non-resectable gliomas [73]. Moreover, MSP of serial stereotactic biopsy specimens revealed homogeneous distribution of *MGMT* promoter methylation in glioblastoma [74]. Alternatives to MSP are summarized in **Figure 1**. Additional methods are based on *MGMT* mRNA expression analysis by quantitative reverse transcription polymerase chain reaction (qRT-PCR) [77], MGMT protein detection by immunohistochemistry or Western blot analysis [79,80], and assessment of MGMT enzymatic activity [81]. In general, assays based on expression analyses and determination of enzymatic activity are more prone to contamination of tissue samples by non-neoplastic, MGMT-positive cells giving rise to false-positive results.

In prospective clinical trials, either simple MSP or qMSP assays have been most commonly used (Table 3, Fig. 1,4,6). In the NOA-08 trial, good concordance was found between results obtained by qMSP [75] or conventional MSP [72]. However, sensitivity and specificity to detect hypermethylation with these assays critically depends on the employed PCR protocols. The definition and prospective validation of reliable cut-off values in quantitative assays like qMSP, DNA PSQ require substantial resources and different methods may yield slightly different results. Usually, cut-off values for the distinction of methylated *versus* unmethylated cases are set at the nadir of the distribution of a large number of testing results, which reflects a technical cut-off. The distributions may overlap, and the behavior close to the cut-off is not so clear given the heterogeneous methylation patterns across the *MGMT* 5'-CpG island in malignant gliomas. Variable contamination of tumor tissue with non-neoplastic cells, may complicate the distinction of methylated versus non-methylated cases. This has led to the introduction of a "grey-zone" of weakly or partly methylated tumors that cannot be unequivocally assigned to either the methylated or unmethylated category. While this concept may be acceptable for clinical trials with *MGMT* methylation status-dependent entry criteria (which simply exclude patients with "grey-zone" testing results), it is hardly acceptable in, where treatment decisions have to be made for each individual patient. One possible approach for routine clinical practice would be to evaluate those cases that demonstrate an equivocal, “borderline” or “grey-zone” result in one test, e.g. MSP, to an independent analysis with a second method, e.g. PSQ (or *vice versa*) (Fig. 6). If the independent method shows a clear result, the particular tumor can be accordingly assigned to either *MGMT*-methylated or not methylated, respectively. Although this approach may not provide an unequivocal result in all cases, it may at least decrease the fraction of patients without a definite testing result.

The most commonly used techniques for *MGMT* promoter methylation testing and their individual advantages and limitations have been thoroughly reviewed before [35]. Although MSP has evolved as the ‘most commonly applied standard’ due to its simplicity and cost effectiveness, it has the disadvantage that it cannot detect heterogeneous patterns of

methylation [82], especially when performed on low quality DNA extracted from FFPE tissue [78,83] (Fig. 4). PSQ overcomes this problem as it provides quantitative information on the extent of methylation at each individual CpG site that is sequenced, however, it loses allele-specific information [65] (Fig. 5). At present only one qMSP assay has been prospectively validated in a phase III trial for glioblastoma [9]. Irrespective of the method applied, care should be taken to standardize diagnostic procedures and establish internal quality control measures according to the guidelines. Moreover, it would be highly recommendable that each laboratory evaluates its diagnostic performance by regular participation in external quality assessments, which are urgently needed to ensure reproducibility and interlaboratory comparability of testing results across different places (see below).

The current WHO classification of 2007 does not consider *MGMT* testing in the classification of glioblastomas and anaplastic gliomas. There is an ongoing debate about whether or not *MGMT* testing should be incorporated into the next revision of the WHO classification for reasons of clinical relevance. If so, a prerequisite would be that the respective molecular analysis can be performed worldwide, i.e., that it is affordable, easy to perform and does not require special equipment or consumables aside from what is present in most medical laboratories anyway. This would be an argument for the use of MSP but certainly does not exclude other approaches. In addition to inclusion of molecular testing in the WHO classification, national and international treatment guidelines for patients with malignant gliomas need to be amended to include predictive molecular testing for treatment stratification. This in turn would be important to establish appropriate reimbursement policies for clinically relevant, new molecular tests, which in most countries are currently not considered in the health care and insurance systems.

Importance of internal and external quality control measures for *MGMT* testing

With *MGMT* promoter methylation testing entering clinical routine, internal and external quality control measures need to be reinforced, as both false positive and false negative

results may be harmful for patients. In addition to the consideration of general guidelines for good laboratory practice, internal quality control measures for diagnostic *MGMT* testing should include histological quality checks of each tissue specimen to be tested, regular evaluations for reproducibility of testing results, implementation of measures for avoidance and detection of contamination, as well as guidelines for troubleshooting and data interpretation. Standardized operating procedures should be established for the entire testing procedures. Each tissue sample, which should get the neurosurgical labeling “not derived from the necrotic areas” needs to be histologically checked for sufficient tumor cell content (macro-dissection) of ≥80% in order to avoid false negative results due to low tumor cell content. Repeated testing of the same specimens is recommendable to ensure test reproducibility. To avoid cross-contamination, the implementation of separate laboratory areas for pre-PCR and post-PCR procedures is mandatory. Appropriate positive (e.g. *in vitro* methylated DNA or DNA from a *MGMT*-methylated tumor or cell line) and negative controls (*MGMT*-unmethylated DNA and a no-template control sample) should be run with each test. A modification of the commonly used method for prevention of cross-contamination, i.e., substitution of dTTP by dUTP in the PCR reaction and treatment of each template DNA by uracil-DNA glycosidase before PCR amplification has ben reported for the application to bisulfite-modified DNA [83]. Interpretation of the results should be performed by experienced staff being aware of potential methodological problems and appropriately trained in trouble-shooting. The use of a second independent testing method for cases with borderline results is recommended (see above). In addition to these local issues, external quality assessment (EQA) measures are becoming of increasing importance. A European Consensus Conference has recently developed guidelines for implementation of appropriate EQA schemes concerning predictive molecular tests in oncology, such as *KRAS* mutation in colon cancer, *EGFR* mutation and *ALK* rearrangement in non-small-cell lung cancer, and *BRAF* mutation in melanoma [86]. Such EQA measures involve the implementation of centrally organized proficiency testing programs in the form of standardized interlaboratory comparisons (round robin trials) at the regional, national and international levels. Such EQA

measures have recently been established in different countries for several molecular markers in tumor entities outside the nervous system. The results clearly indicate a paramount importance of EQA measures to foster harmonization of molecular diagnostic testing across different laboratories [86-88]. In neurooncology, a first interlaboratory comparison of *IDH1* mutation detection in gliomas revealed that testing for *IDH1-R132H* mutation by immunohistochemistry was consistent across the participating institutions, while *IDH1* sequencing yielded inconsistent results in 2 of 6 participating laboratories [89]. A recent pilot study for the determination of *MGMT* status performed under the auspices of the German Society of Neuropathology and Neuroanatomy (DGNN) involved 23 centers in Germany, Austria and the Netherlands. Preliminary results of this trial have only been published as abstract [90] and indicate that the overall concordance rate in cases that either were strongly methylated or completely lacked methylation was good, with few outliers, while results in tumors with partial or borderline methylation were highly variable across different laboratories. These data lend further support for the necessity of EQA measures to assure the quality in diagnostic *MGMT* testing, which will be indispensable in the future. Participation in such studies should be strongly encouraged. This will provide reassurance for laboratories showing good performance while laboratories failing to attain sufficient testing results may be guided to improve their procedures or, alternatively, may withdraw this test from their catalogue.

Targeted therapies in patients with *MGMT* promoter-unmethylated glioblastomas

Due to its high prognostic and predictive relevance, assessment of the *MGMT* status has become state-of-the-art in current and planned clinical trials in glioma as a prognosticator and to stratify patients [9] or even patient selection into trials accordingly [14-17]. In addition, it is nowadays frequently requested in routine diagnostics as a prognostic tool. This said, this molecular biomarker, which exists for more than 10 years now, only now has a guideline-suggested use in clinical neurooncology [91]. Arguments that prevented a regular clinical use for patient stratification on the one hand include the technical challenges, including the

grey- zone problem, and, most importantly, the lack of meaningful and strategically developed therapeutic alternatives to the current standard of care in glioblastoma. In fact, it is considered not very appealing to offer a patient whose tumor lacks *MGMT* promoter methylation mere radiotherapy. From the individual patient's view, even a faint chance of success despite molecular evidence may be worth the try, particularly given the generally favorable safety and tolerability profile of TMZ and the higher probability to be a long-term survivor [92]. From the academic and caregivers perspective, however, it will be difficult to generate progress if each patient is offered the same treatment despite better knowledge. In this area of conflicts, several trial concepts have been developed, with some trial concept for patients with *MGMT*-unmethylated glioblastomas aiming at the replacement of TMZ in the experimental arm for newly diagnosed patients. The biggest challenges in this respect include the molecular testing procedure, a non-opportunistic but hypothesis-driven choice for the experimental arm, and the definition of the standard treatment. Here, not radiotherapy alone but radiochemotherapy with TMZ has been chosen. The reasons being the fear of facing false-negative test results or not treating outliers that may still benefit from TMZ. So far, this concept was feasible only in Europe, but not in the US.

Optimizing primary treatment in patients with MGMT-unmethylated tumors – alternatives to TMZ

The first trial restricting patient entry according to the *MGMT* promoter methylation status was the S039 trial of the PKC-beta inhibitor enzastaurin added to radiotherapy in patients with newly diagnosed MGMT-unmethylated glioblastoma. This single-arm phase II trial set the primary efficacy endpoint (PFS at 6 months, PFS6) to 55% to demonstrate superiority over a PFS6 of 40% achieved with radiochemotherapy in patients with *MGMT*-unmethylated glioblastoma in the EORTC 26981/22981 NCIC CE.3 trial. Despite interesting data and the demonstration that multicenter clinical trials with a molecularly defined entry criterion are feasible, this study failed to meet the preset efficacy endpoint and suffered from the missing control arm [15]. This trial also suggested that omission of TMZ in the experimental arm of

clinical trials in this patient population is not unethical because of potential “undertreatment” of these patients since efficacy data in the TMZ-free arm are comparable to the data with standard of care. The GLARIUS trial aimed to replace TMZ in the primary treatment with bevacizumab and irinotecan (BEV/IRI) in glioblastoma patients lacking *MGMT* promoter methylation. This randomized multicenter phase II study demonstrated an increase of PFS6 to 71% in the BEV/IRI arm compared with 26% in the TMZ arm. Despite the challenge of radiographic definition of progression, this is the first trial thus far reporting efficacy of an alternative chemotherapy added to radiotherapy in the treatment of patients with newly diagnosed glioblastoma without hypermethylation of the *MGMT* promoter [17]. The EORTC 26082 trial has chosen the mTOR inhibitor temsirolimus as a targeted therapy to replace TMZ in patients with *MGMT*-unmethylated newly diagnosed glioblastoma. Data from this European randomized controlled phase II study, which completed accrual in 2012, will become available in 2014 [16]. The CORE study is a formal companion to the CENTRIC trial, in which the integrin inhibitor cilengitide was added to (not replacing) radiochemotherapy with TMZ in *MGMT*- unmethylated glioblastoma patients [14]. In this study, radiochemotherapy with TMZ was compared with radiochemotherapy with TMZ plus cilengitide at two different dosing schedules in a randomized phase II three-arm design. Although negative in this trial, the RT-TMZ plus cilengitide twice-weekly arm showed an intriguing OS of 16.3 months when compared with 13.4 months in the control arm and 16.6 months in the experimental arm of the GLARIUS trial [17] (Table 3). Clearly, the poor treatment results in this molecularly defined group should stimulate more activities in this prognostically unfavorable patient population. The main advantages are the analysis of the interaction of the experimental compound with radiotherapy alone and hence potential detection of radiosensitization, the limitation of side effects without the TMZ regimen, and the enrichment of the patient population studied. The disadvantages and challenges include the heterogeneity of tests used and the more opportunistic than rational drug development strategy, which is not focusing on concepts that may be working for patients with *MGMT*-unmethylated glioblastomas but simply avoid substrates of MGMT.

We expect that there will be more concepts in the future that may benefit from the absence of TMZ but rather combine more than one *MGMT*-independent regimen or use more than one molecular biomarker.

Optimizing primary treatment in patients with MGMT-methylated glioblastomas – companions for TMZ

There is only one concluded trial that specifically included newly diagnosed glioblastoma patients with *MGMT*-methylated tumors, i.e. the CENTRIC trial. In this multicenter phase III study newly diagnosed glioblastoma patients were randomized after central *MGMT* testing to receive standard radiochemotherapy with TMZ or radiochemotherapy with TMZ plus cilengitide (twice per week). The primary endpoint was not met and there was no difference between the treatment arms in any of the parameters analyzed. A median overall survival of 26 months in both treatment arms illustrates the challenge of optimizing an effective primary treatment in this favorable patient population [13].

Changing practice: *MGMT* promoter methylation testing in elderly patients with glioblastoma

Since the initial data from the EORTC 26981/22981 NCIC CE.3 trial [3], there is an ongoing debate on a mere prognostic versus predictive and prognostic role for *MGMT* promoter methylation in glioblastoma. Since RTOG0525 did not contain a TMZ-free control group, it was designed only to confirm a prognostic role for *MGMT* promoter methylation, which was convincingly shown (Table 1). An important role for *MGMT* testing in elderly patients with glioblastoma was already suggested by the non-randomized ANOCEF trial [93] and a prospective cohort study of the German Glioma Network [94]. Recently, biomarker subgroup data from two randomized trials, both using either radiotherapy or TMZ chemotherapy but not the combination of both as the primary treatment, defined a predictive role of *MGMT* promoter methylation for response to TMZ chemotherapy. These trials suggest the practice-changing concept of *MGMT* promoter methylation testing in elderly patients with

glioblastoma. In the NOA-08 trial, PFS was longer in patients with *MGMT* promoter-methylated tumors who received a dose-intensified TMZ schedule than in those who underwent radiotherapy, whereas the opposite was true for patients with *MGMT*-unmethylated tumors. Data for overall survival showed the same trend, but were not significant [22]. The Nordic trial showed a prolonged overall survival in *MGMT*-methylated glioblastoma patients who received TMZ compared to those who underwent one of either (30 x 2 Gy or 10 x 3.4 Gy) radiotherapy regimens (9.7 *versus* 8.2 months), but similar overall survival outcomes for patients whose tumors lacked *MGMT* promoter methylation (6.8 *versus* 7.0 months) [23]. It is tempting to speculate that the higher number of crossovers to TMZ (58.5% at recurrence after RT; to RT 46.1% at recurrence after TMZ) in the NOA-08 trial diluted the overall survival differences. There is increasing evidence to suggest fundamental molecular differences between malignant gliomas of different age groups. *TP53* mutation, EGFR amplification, *EGFRvIII* mutation, *PTEN* deletion and *IDH1* mutation are differentially distributed among in glioblastomas of young adults (19-40 years of age) *versus* patients older than 40 years [94]. Glioblastoma of older patients mostly were classified into mesenchymal, receptor tyrosine kinase 1 (RTK 1), “PDGFRA” or RTK 2 “classic” subtypes [101]. In this cohort, but also in the GGN cohort [94] and the NOA-08 trial [22], *IDH* mutations are very rare in malignant glioma patients above the age of 60. The lack of *IDH* mutations, which are sufficient to generate the prognostically favorable glioma CpG island hypermethylator phenotype (G-CIMP) [63] might contribute to the worse prognosis of elderly patients. In contrast, the distribution of *MGMT* promoter methylation in *IDH1/2* wildtype primary glioblastomas does not appear to vary with age [96]. Therefore, it may be that the absence of yet unknown positive prognostic factors allows a clear singling out of predictive properties of MGMT in this population.

Although *MGMT* promoter methylation testing and also a widely accepted definition of an “elderly patients” implies challenges [35,84], current evidence calls for including *MGMT* promoter methylation status determination in the routine diagnostic procedures for elderly patients with malignant astrocytoma.

***Interdependence of MGMT promoter methylation and IDH1/2 mutation* suggests a role for *MGMT* testing outside glioblastoma**

A support for a contextual role for *MGMT* promoter methylation comes from a retrospective analyses of anaplastic gliomas. In the NOA-04 and in the EORTC 26951 cohort [97,98], *MGMT* promoter methylation was prognostic and not predictive for better outcome independent of treatment with alkylating chemotherapy or radiotherapy. Among the potential explanations for these differences was a confounding influence of *IDH1/2* mutations, which are associated with a CpG island methylator phenotype in glioma (gCIMP) [99]. The most obvious difference known between glioblastoma and WHO grade II and III gliomas is the frequency of *IDH1/2* mutations of 50-70% in diffuse and anaplastic gliomas of WHO grades II and III as well as secondary glioblastomas, of 5-10% in primary glioblastomas of younger patients, and almost zero in elderly patients with glioblastoma [100, 101].

A retrospective subgroup analysis from the NOA-04 trial suggested a simple interaction model to explain the prognostic *versus* predictive relevance of the *MGMT* status in WHO grade III versus WHO grade IV gliomas. *MGMT* promoter methylation is prognostic for patients with *IDH1/2* mutant gliomas, and thus in the majority of WHO grade III gliomas. In contrast, in patients with *IDH1/2* wildtype tumors, hypermethylation of the *MGMT* promoter is predictive for benefit from alkylating chemotherapy. This model explains a mainly prognostic role in younger patients with anaplastic gliomas and *IDH1/2*-mutant glioblastoma, and a predictive role in elderly patients with *IDH1/2*-wildtype glioblastoma [102]. The EORTC 26951 also provides evidence for this interaction, but case numbers available to tackle that question were not sufficient. In addition, this trial also showed that *MGMT* methylation, here determined using 450 k data and the MGMT-STP27 model [78], which associated methylation at two particular CpG (Fig. 1), is of utmost relevance for the prediction of the effect of the procarbazine/lomustine/vincristine (PCV), also mainly alkylating and methylating chemotherapy [103] (Fig. 7).

Discussion

In clinical neurooncology, molecular biomarkers are currently in the transition phase from primarily research-oriented investigations towards routine application. As outlined in this review article, diagnostic testing for *MGMT* promoter methylation has entered the guidelines as a predictive biomarker for elderly patients with glioblastoma based on practice-changing academic trials [22,23,91]. In addition, *MGMT* methylation testing may help in treatment decisions in patients with *IDH1/2* wildtype anaplastic gliomas [102]. In fact, at many institutions the scientific evidence for *MGMT* being a useful predictive biomarker on the one hand and day-to-day decisions in the clinical management of glioma patients on the other hand are not well connected yet. This is in part due to limitations in the testing procedure and the lack of attractive therapeutic alternatives to the standard of care, but also the grim prognosis of the disease, which makes therapeutic approaches with reduced treatment intensity less attractive. Until recently, it has been a general consensus that treatment decisions outside clinical trials should not be based on the *MGMT* status. This concept is shifting, as many centers would nowadays recognize testing as being relevant in particular in the elderly patient population with glioblastoma to determine who should be treated with alkylating chemotherapy (with or without radiotherapy, a question that needs to be addressed in the future), and who should receive radiotherapy only. However, as novel molecular techniques evolve the question of defining clinically relevant molecular subgroups of patients in different age groups needs to be addressed. Recent integrative high-throughput analyses at the genetic, epigenetic and expression levels already have demonstrated their value in subclassifying different kinds of brain tumors, including glioblastomas with certain prognostic relevance but as yet unknown predictive implications [101,104]. However, as next-generation sequencing techniques and microarray-based approaches are becoming more widely available, easier to standardize and less subject to bias, single marker assessments, as exemplified by the current status of *MGMT* testing, may be replaced by more comprehensive assessments of multiple genetic and epigenetic markers, e.g. with 450 k methylation arrays, which may require dedicated expertise in

bioinformatics and –statistics. Nevertheless, the requirements for routine testing will remain similar to those outlined for MGMT, ranging from availability and reliability of the testing procedures, applicability to routinely processed and even tiny tissue specimens, establishment of internal and external quality control measures to cost-effectiveness and speed of testing.

Extending the role of MGMT testing in elderly patients with glioblastoma, all future clinical trials involving alkylating chemotherapy should assess the *MGMT* status and include this as a stratification parameter. In clinical trials, the concomitant assessment of *MGMT* promoter methylation status by different methods, e.g. MSP and PSQ, as well as critical development of biomarkers [105, 106] is encouraged. The relevance of parallel analyses of MGMT mRNA and protein expression is still under debate. In most EU trial centers, it is considered justified to withhold TMZ from patients with newly diagnosed glioblastoma without *MGMT* promoter methylation in the context of clinical trials. This approach has been used in phase II studies led by the EORTC, industry and other study groups in Europe and may have generated clinically relevant data for patients with MGMT unmethylated glioblastoma from just one [17] out of only three evaluated trials [14-17].

The present data from the analyses of patients with anaplastic gliomas [102] will stimulate the discussion on the standard-of-care arm, RT, in the *IDH1* wildtype, *MGMT* promoter methylated patients of the CATNON trial. Also the TMZ alone arm in the reopened CODEL trial for patients with unmethylated tumors is at stake, despite the low frequency of 1p/19q-codeleted/*MGMT*-unmethylated tumors. Data from these trials need to further validate the role of *MGMT* as a predictive biomarker in the group of patients with *IDH1/2* wildtype anaplastic gliomas. It may confirm that alkylating chemotherapy produces no benefit in patients with *MGMT*-unmethylated, *IDH1/2* wildtype tumors, but will provoke the question whether TMZ alone, with deferred RT, may be a sufficient treatment in patients with *MGMT* promoter-methylated and *IDH1/2* wildtype tumors.

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Figure legends

Fig. 1. Assays to determine MGMT promoter methylation.

Particularly difficult is the definition and validation of the technically and clinically relevant cut-off for *MGMT* promoter methylation. The majority of samples will be classified similarly by most tests although different CpGs are interrogated. Misclassification is mainly due to incomplete methylation of the CpG interrogated by a given test or larger amounts of non-neoplastic tissue impacting the signal in the quantitative tests. Numbering of the CpGs, 1-98, in the CpG island encompassing the translation start site of the *MGMT* genes is defined by the following coordinates in University of California at Santa Cruz (UCSC) Genome Browser GRCh37/hg19, Chr10:131264949-131265710. See visualization of CpG locations in Refs 75 and 82.

Fig. 2. Regulation of MGMT. Expression and activity of MGMT are regulated *via* epigenetic mechanisms, but also transcription factors (+/- at the promoter), protein-protein interaction and miRNA.

Abbreviations: activating protein (AP)1, cAMP response element-binding protein (CREB)-binding protein (CBP)/p300 co-activator, hypoxia inducible factor (HIF)1α, N-myc downstream-regulated gene (NDRG)1, nuclear factor (NF)kB, specificity protein (SP)1.

Fig. 3. DNA repair mechanisms

MGMT and other DNA repair mechanisms deal with DNA damage produced by the methylating therapeutic drug, temozolomide, in human cells. Temozolomide (TMZ) and related drugs cause potentially cytotoxic DNA lesions such as O^6-methylguanine (O^6-meG, *red circle*) and N^7-methylguanine (N7-meG, *yellow circle*). *A,* MGMT (O^6-meG DNA methyltransferase) removes the O^6-alkylguanine DNA adduct through covalent transfer of the alkyl group to the conserved active-site cysteine and restores the guanine to normal. After receiving a methyl-group from O^6-meG, MGMT is inactivated, and subject to ubiquitin-

mediated degradation. A similar suicidal enzyme reaction occurs when MGMT transfers and accepts an alkyl-group from O^6-benzylguanine (O^6-BG) or O^6-(4-bromothenyl)guanine (PaTrin-2), two therapeutic strategies. *B,* if an O^6-meG DNA adduct escapes MGMT repair, it would form a base pair with thymine (*blue circle*) during DNA replication. The mismatched base pair of the persistent O^6-meG with thymine is recognized by the mismatch repair pathway, resulting in futile cycles of repair leading to cell death. *C,* N7-meG DNA adducts (>70% of total DNA adducts formed by temozolomide) are efficiently repaired by the base excision repair (BER) pathway, and normally they contribute little to the cytotoxicity of temozolomide. Methoxyamine binds to AP sites produced by methylpurine glycosylase (MPG), the first step in BER processing. Methoxyamine-bound AP sites are refractory to AP endonuclease (APE, *green circle*) cleavage, resulting in the blockage of the BER pathway. This leads to strand breaks, disrupted replication, and increased cytotoxicity of temozolomide.

(from Clinical Cancer Research 2006, 12(2):328-31 Lili Liu and Stanton L. Gerson, Targeted Modulation of MGMT: Clinical Implications [49])

Fig. 4. Methylation-specific PCR. This most commonly applied method of methylation analysis uses bisulphite-treated DNA. Methylation-specific primer pairs are designed by including sequences complementing only unconverted 5-methylcytosines, or, in contrast, unmethylation-specific, complementing thymines converted from unmethylated cytosines. Methylation is determined by the ability of the specific primer to achieve amplification.

Fig. 5. Pyrosequencing. PSQ for MGMT promoter methylation determines the bisulphite-converted sequence of specific CpG sites in the region. The ratio of C-to-T at individual sites is determined quantitatively based on the amount of C and T incorporation during the sequence extension.

In detail, sequencing of a single strand of DNA is done by synthesizing the complementary strand one base pair at a time and detecting which base was added at each step. The

single-stranded template is immobilized and solutions of A, C, G, and T nucleotides are sequentially added. Further, the enzymes DNA polymerase, ATP sulfurylase, luciferase and apyrase as well as the substrate adenosine 5'-phosphosulfate (APS) and luciferin are added (a).

The addition of one of the four deoxynucleoside triphosphates initiates the second step (b). DNA polymerase incorporates the complementary dNTPs onto the template. This incorporation releases pyrophosphate (PPi) stoichiometrically (c).

ATP sulfurylase quantitatively converts PPi to ATP in the presence of adenosine 5´ phosphosulfate. This ATP acts as fuel to the luciferase-mediated conversion of luciferin to oxyluciferin that generates visible light in amounts that are proportional to the amount of ATP. The light produced in the luciferase-catalyzed reaction is detected by a camera and analyzed in a program. Light is produced only when the nucleotide solution complements the first unpaired base of the template (d). The sequence of solutions, which produce chemiluminescent signals allows the determination of the sequence of the template. Unincorporated nucleotides and ATP are degraded by the apyrase before the reaction restarts with the next nucleotide (e) [72].

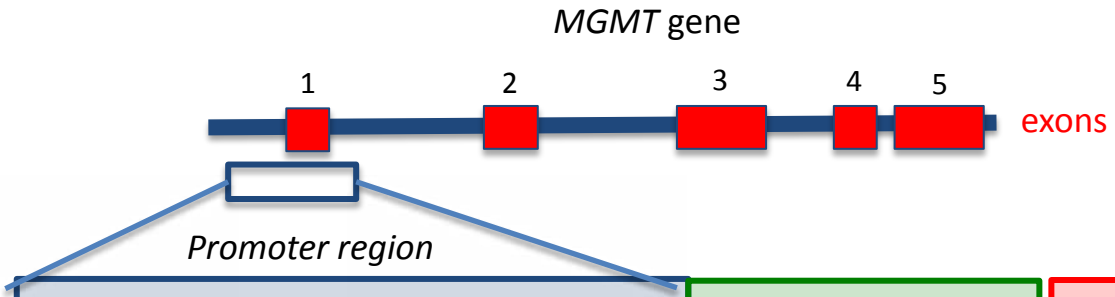
Fig. 6. Pragmatic approach to MGMT testing

Necessary steps to reach an efficient and cost-effective diagnosis of the *MGMT* status.

Fig. 7. Biomarker-driven algorithm for glioma treatment

Algorithm for the use of molecular markers in gliomas, overcoming separation according WHO grades, with resulting treatment recommendations.

Figure 1



Options for MGMT status determination	Advantage	Disadvantage
non-quantitative methylation-specific polymerase chain reaction (MSP) [5,113]: forward M primer, CpG76-80; forward U primer, CpG75-80; reverse MU primers, CpG84-87	•PCR of bisulfite converted DNA, visualization, agarose gel	•undefined limit of detection and cut-off
quantitative MSP (qMSP) [75]: forward M primer, CpG75-80; reverse M primer, CpG 88-90; normalized to unmethylated *ACTB*	•quantitative •technical cut-off prospectively validated in GBM [9] •high throughput	•normalizing to different gene on different chromosome
methylation-specific multiplex ligation-dependent probe amplification (MS-MLPA) [98]: CpG 9,23,81	•no bisulfite conversion •high-throughput	•limited to restriction sites •cut-off definition •Kit changed probes!
combined bisulfite restriction analysis (COBRA) [72]: CpG72,73,76,79,80	•specificity	•limited to restriction sites •cut-off definition
pyrosequencing [77,84]: five adjacent CpGs interrogated by PSQ (CpG74-78)	•quantitative	•expensive •cut-off definition
HM27k & HM450k BeadChip : MGMT-STP27 [78] – logistic regression model (CpG31, cg12434587; CpG84, cg12981137)	•quantitative •validated across data-sets and tumor grades •high throughput •methylome (27K / 450K CpGs)	•not readily available •minimum sample number 24

Figure 2

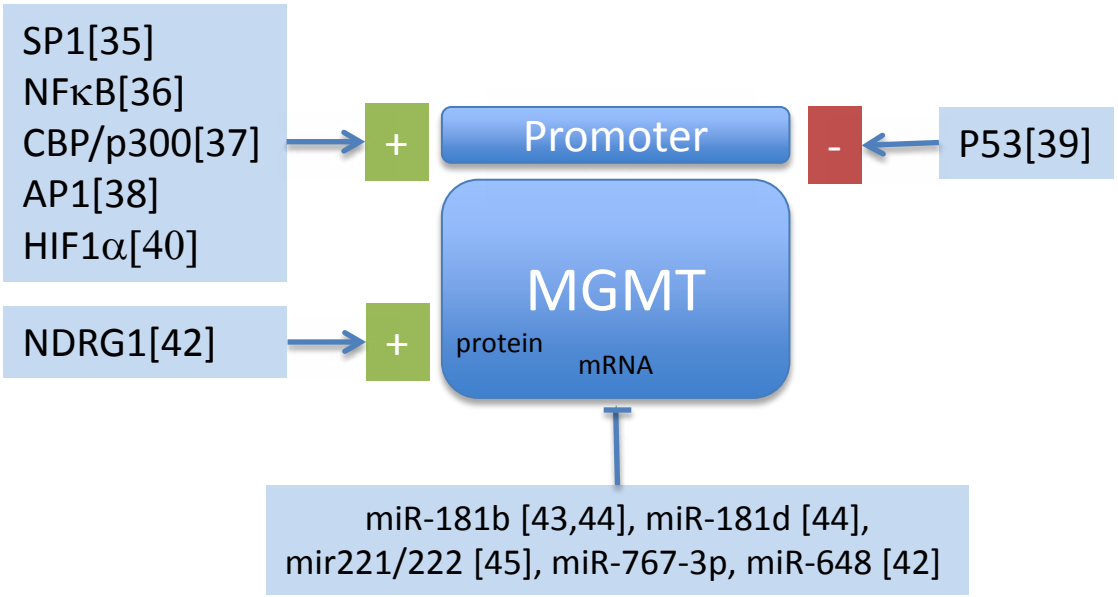

SP1[35]
NFκB[36]
CBP/p300[37]
AP1[38]
HIF1α[40]
+
Promoter
-
P53[39]
NDRG1[42]
+
MGMT
protein
mRNA
miR-181b [43,44], miR-181d [44],
mir221/222 [45], miR-767-3p, miR-648 [42]

Figure 3

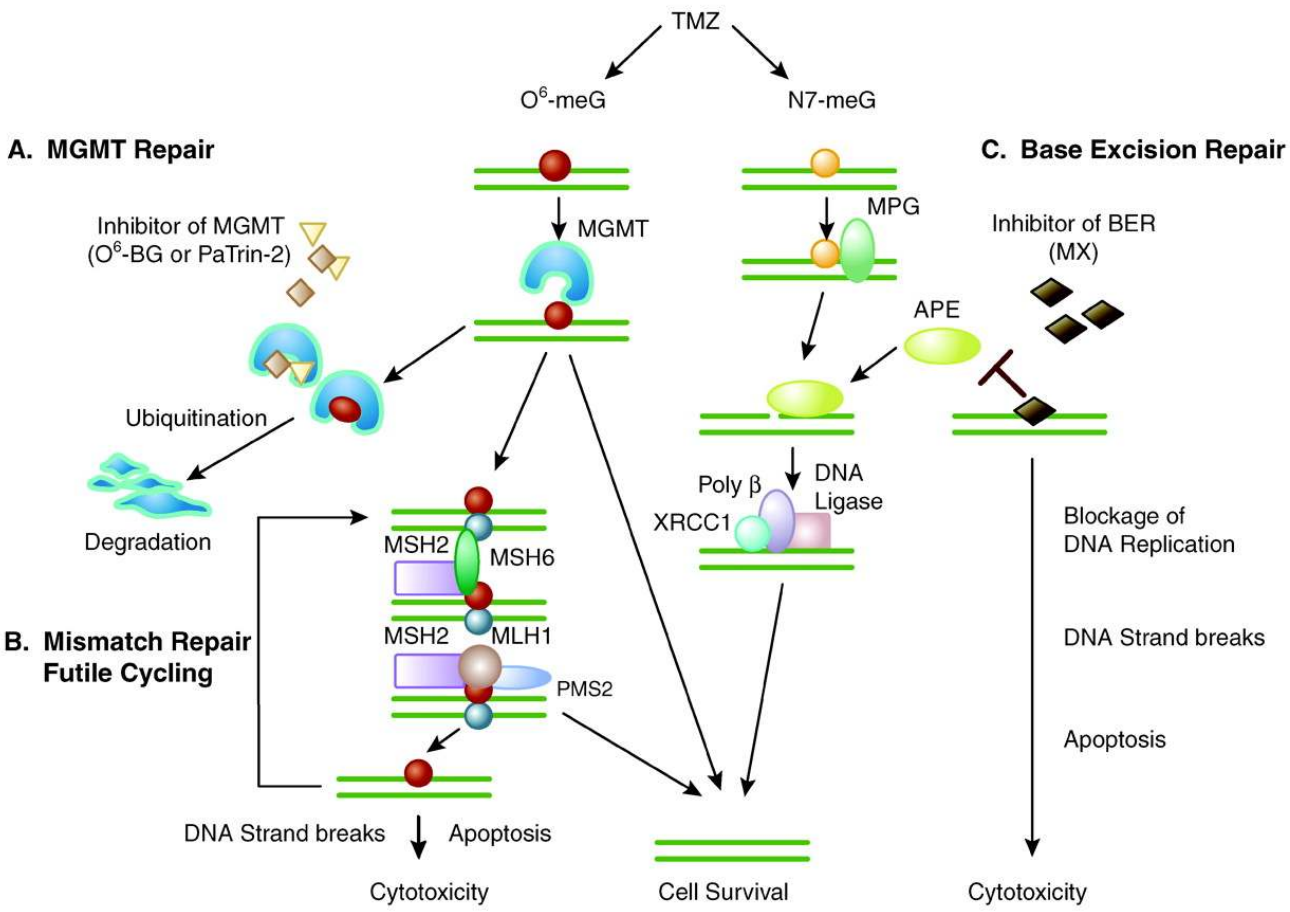
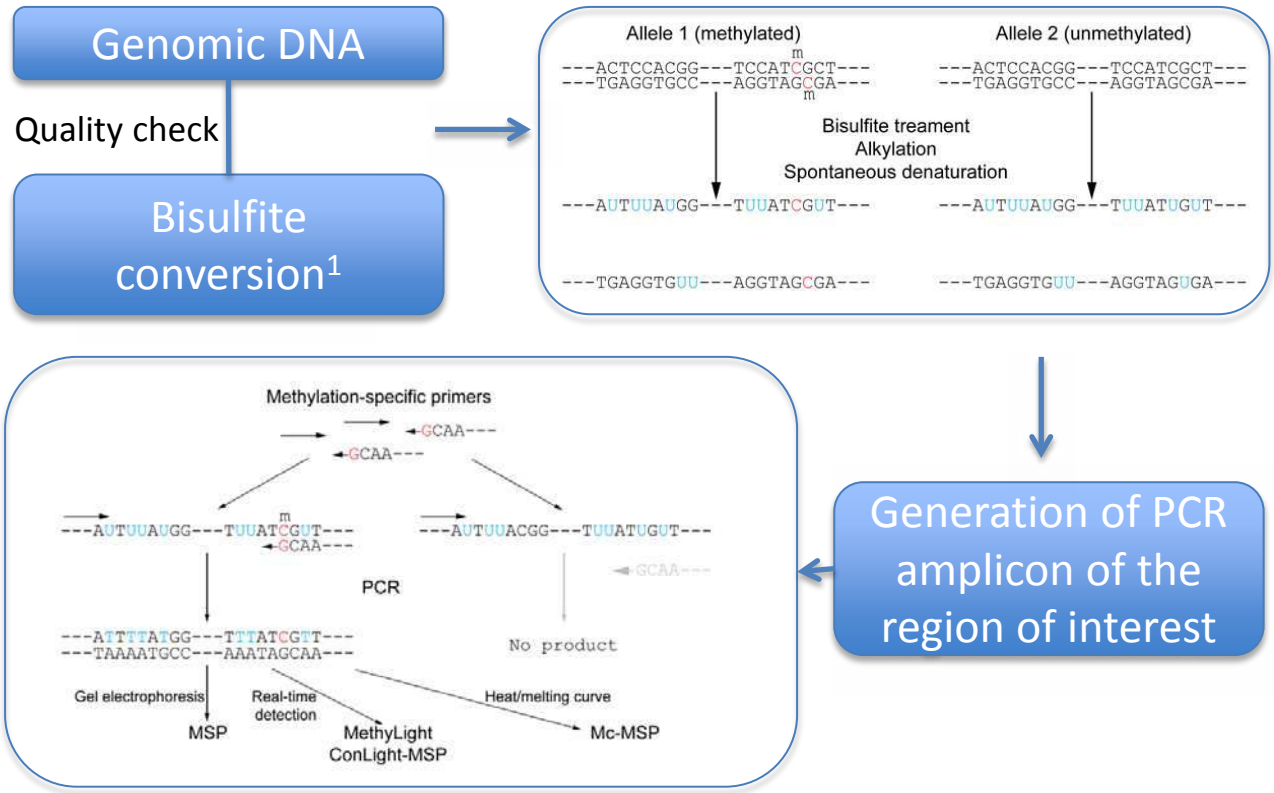
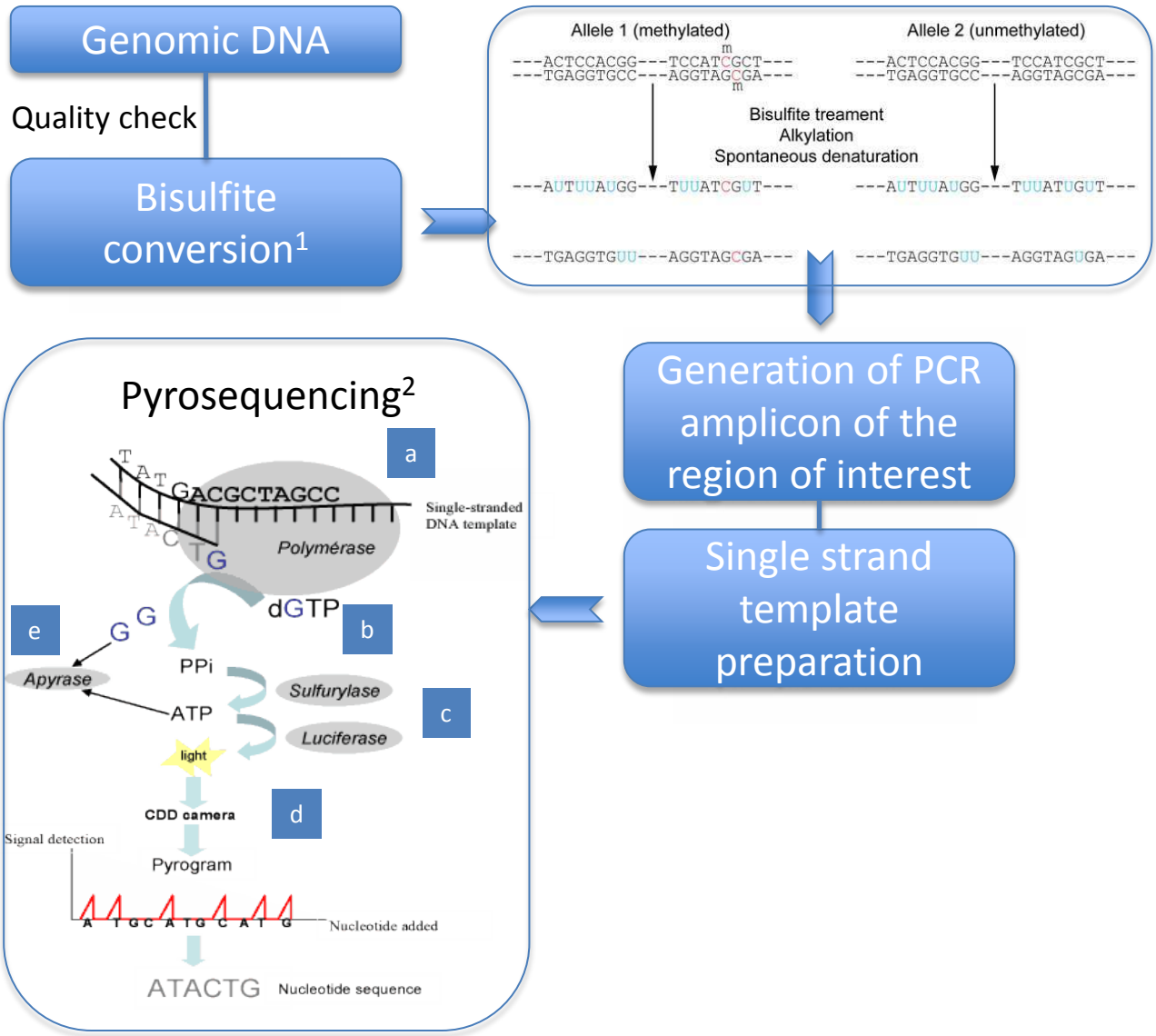

TMZ
O6-meG
N7-meG
A. MGMT Repair
C. Base Excision Repair
Inhibitor of MGMT
(O6-BG or PaTrin-2)
MGMT
MPG
Inhibitor of BER
(MX)
APE
Ubiquitination
Degradation
Poly β
DNA
Ligase
XRCC1
MSH2
MSH6
MSH2
MLH1
PMS2
B. Mismatch Repair
Futile Cycling
Blockage of
DNA Replication
DNA Strand breaks
Apoptosis
DNA Strand breaks
Apoptosis
Cytotoxicity
Cell Survival
Cytotoxicity

Figure 4



[1]Bisulfite conversion: Treatment of DNA with bisulfite converts cytosine residues into uracil, but leaves 5-methylcytosine residues unaltered.

Figure 5



[1]Bisulfite conversion: Treatment of DNA with bisulfite converts cytosine residues into uracil, but leaves 5-methylcytosine residues unaltered.

Figure 6

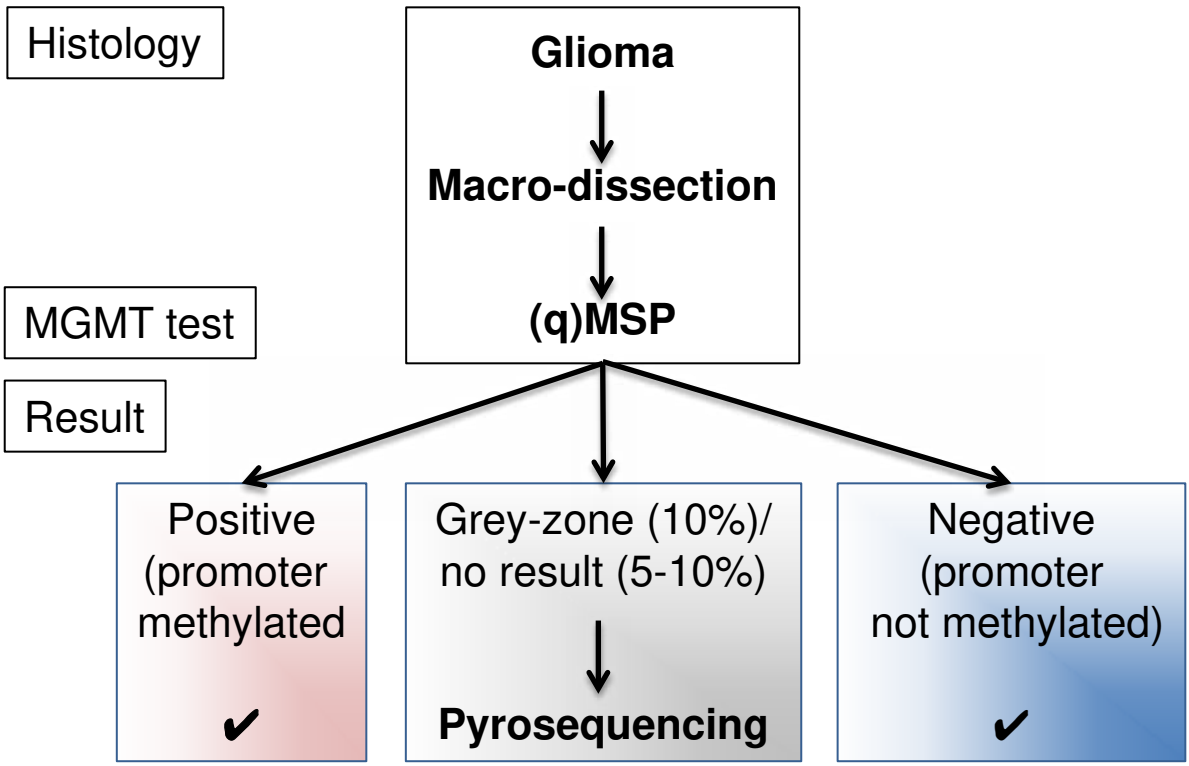

Histology
Glioma
Macro-dissection
(q)MSP
MGMT test
Result
Positive (promoter methylated
✔
Grey-zone (10%)/ no result (5-10%)
Pyrosequencing
Negative (promoter not methylated)
✔

Figure 7

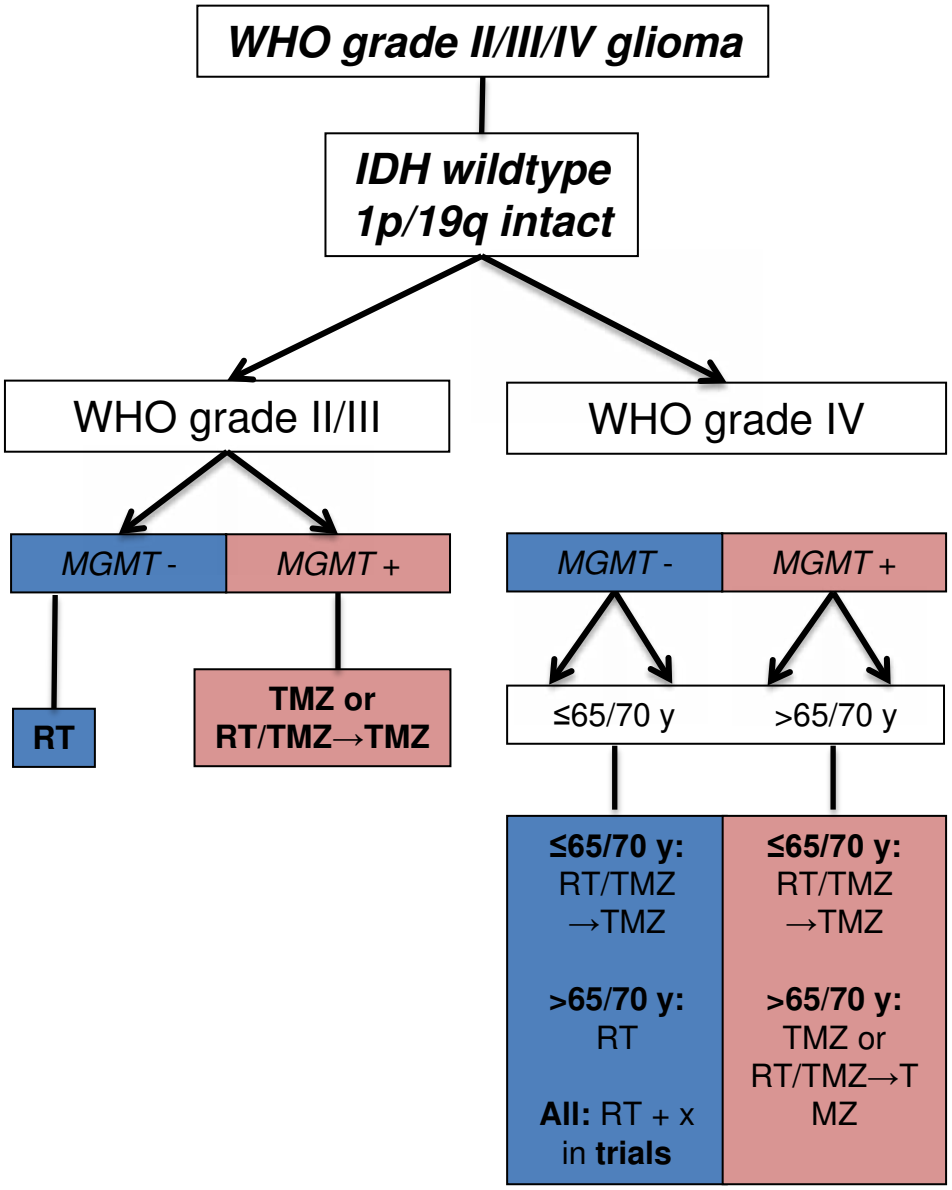

WHO grade II/III/IV glioma
IDH wildtype
1p/19q intact
WHO grade II/III
WHO grade IV
MGMT -
MGMT +
MGMT -
MGMT +
RT
TMZ or
RT/TMZ→TMZ
≤65/70 y
>65/70 y
≤65/70 y:
RT/TMZ
→TMZ
>65/70 y:
RT
All: RT + x
in trials
≤65/70 y:
RT/TMZ
→TMZ
>65/70 y:
TMZ or
RT/TMZ→T
MZ

Table 1 Evidence for the clinical impact of MGMT in glioblastoma

			PFS [months]		OS [months]				
Treatment regimen	**Age [years]**	**Patient population**	**MGMT meth.**	**MGMT unmeth.**	**MGMT meth.**	**MGMT unmeth.**	**MGMT determination**	**MGMT meth./unmeth.**	**Reference**
WBRT + Cisplatin + BCNU*	38-70	Newly diagnosed anaplastic astrocytoma/ glioblastoma (n=49)	21	8	>30	21	MSP	40%/60%	[5]
RT/TMZ -> TMZ	≥18	Newly diagnosed glioblastoma with KPS >70 (n=38)	nr	nr	62% at 18 months	8% at 18 months	MSP	68%/32%	[59]
EORTC 26981 RT (30 x 2 Gy) vs. RT/TMZ -> TMZ	18-70	Newly diagnosed glioblastoma with KPS >70 (n=573)	5.9 10.3	4.4 5.3	15.3 21.7	11.8 12.7	MSP	45%/55%	[3]
UKT-03 RT/TMZ -> TMZ/lomustine	18-70	Newly diagnosed glioblastoma with KPS >70 (n=31)	19	6	34.3	12.5	MSP	42%/58%	[111,112]
GGN* with RT (n=72) with RT/TMZ -> TMZ(n=229)	≥18	Newly diagnosed glioblastoma	 7.1 11.4 HR (MGMT-status): 0.51 [0.38-0.68]	 7.1 6.9	 9.9 24.1 HR (MGMT-status): 0.39 [0.28-0.54]	 8.8 12.9	MSP	44%/56%	[4]

			HR (treatment): 0.51 [0.38-0.68]		HR (treatment): 0.51 [0.38-0.68]				
RTOG 0525 RT/TMZ -> TMZ (5/28 days) vs. RT/TMZ -> TMZ (21/28 days)	>18	Newly diagnosed glioblastoma with KPS >60 and resection (n=833)	8.8 11.7	7.1 8.2	23.5 21.9	16.6 15.4	RT-MSP		[9]

Abbreviations: European Organization for Research and Treatment of Cancer (EORTC), methylation-specific polymerase chain reaction (MSP); O^6-methylguanine DNA methyltransferase (MGMT), not reported (nr), real-time quantitative MSP (RT-MSP), Radiation Therapy Oncology Group (RTOG), temozolomide (TMZ), radiotherapy (RT), progression-free survival (PFS), overall survival (OS), whole brain radiotherapy (WBRT)
*Indicate cohorts as opposed to clinical trials with a planned intervention.

Table 2 Trials for elderly patients with glioblastoma

			PFS [months]		OS [months]				
Treatment regimen	**Age [years]**	**Trial population**	**MGMT meth.**	**MGMT unmeth.**	**MGMT meth.**	**MGMT unmeth.**	**MGMT determination**	**MGMT meth./unmeth.**	**Reference**
Controlled trials									
NOA-08 TMZ 100 mg/m^2 (7/14) until progression *vs.* RT (30 x 2 Gy)	>65 (n=373)	Newly diagnosed anaplastic astrocytoma and glioblastoma with KPS $\geq$ 60	8.4 4.6	3.3 4.6	n.r. 9.6	7.0 10.4	MSP and RT-MSP	35%/65%	[22]
NORDIC Elderly trial TMZ 200 mg/m^2 5/28, 6 cycles *vs.* RT (30 x 2 Gy) *vs.* hypofractionated RT (10 x 3.4 Gy)	>60 (n=291)	Newly diagnosed glioblastoma with KPS >70	n.d.	n.d.	9.7 8.2 (both RT)	6.8 7.0 (both RT)	RT-MSP	45%/55%	[23]
Uncontrolled trials [selection]									
All Alkylating chemotherapy RT RT+ alkylating	≥18 (n=301)	Newly diagnosed glioblastoma	5.2 7.7 4.5 7.3	4.7 3.2 5.2 7.2	8.4 8.1 7.8 13.1	6.4 3.7 8.8 10.4	MSP	58%/42%	[4]

chemotherapy									
RT/TMZ -> TMZ	≥65 (n=29)	Newly diagnosed glioblastoma	4.5	5.5	7.4	7.3	MSP	55%/45%	[107]
RT/TMZ -> TMZ (n=40) RT (n=14) unkown (n=10)	≥70 (n=64)	Newly diagnosed glioblastoma	10.8	5.7	16.1	8.6	MSP	58%/42%	[108]
RT/TMZ -> TMZ	≥65 (n=37)	Newly diagnosed glioblastoma	22.9	9.5	n.r.	13.7	MSP	41%/59%	[109]
RT/TMZ -> TMZ	≥70 (n=83)	Newly diagnosed glioblastoma	10.5	5.5	15.3	10.2		51%/49%	[110]

Abbreviations: methylation-specific polymerase chain reaction (MSP); real-time quantitative MSP (RT-MSP)

Table 3 Trials for patients with glioblastoma restricted to MGMT promoter status

Treatment regimen	**MGMT promoter**	**Trial population**	**PFS [months]**	**OS [months]**	**Endpoint**	**Outcome**	**Reference**
S039 RT/Enzastaurin	unmeth.	Newly diagnosed glioblastoma with KPS ≥ 70	6.6	15.0	PFS-6 (53.6%)	negative	[16]
Glarius RT/TMZ *vs.* RT/Bevacizumab+Irinotecan	unmeth.	Newly diagnosed glioblastoma with KPS ≥70	6 9.7	14.8* 16.6*	PFS-6 (26.2%) (71.1%)	positive	[13]
EORTC 26082 RT/TMZ vs. RT/Temsirolimus	unmeth.	Newly diagnosed glioblastoma with KPS ≥70	ongoing	ongoing	OS12	ongoing	[17]
CORE RT/TMZ vs. RT/TMZ + Cilengitide (x2/week) vs. RT/TMZ + Cilengitide (x5/week)	unmeth.	Newly diagnosed glioblastoma with KPS	4.1 5.6 5.9	13.4 16.3 14.5	OS	negative	[15]
CENTRIC RT/TMZ vs. RT/TMZ + Cilengitide	meth.	Newly diagnosed glioblastoma with KPS	10.5 13.5	26.3 26.3	OS	negative	[14]

*50% events (immature data)

Abbreviations: Karnofsky Performance Status (KPS), radiotherapy (RT), O6-methylguanine DNA-methyltransferase (MGMT), temozolomide (TMZ), overall survival (OS)