MGMT promoter methylation analysis for allocating combined CCNU/TMZ chemotherapy: Lessons learned from the CeTeG/NOA-09 trial

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Abstract
The CeTeG/NOA-09 trial showed a survival benefit for combined CCNU/TMZ therapy in MGMT-promoter-methylated glioblastoma patients (quantitative methylation-
specific PCR [qMSP] ratio > 2). Here, we report on the prognostic value of the MGMT promoter methylation ratio determined by qMSP and evaluate the concordance of MGMT methylation results obtained by qMSP, pyrosequencing (PSQ) or DNA methylation arrays (MGMT-STP27). A potential association of qMSP ratio with survival was analyzed in the CeTeG/NOA-09 trial population (n = 129; log-rank tests, Cox regression analyses). The concordance of MGMT methylation assays (qMSP, PSQ and MGMT-STP27) was evaluated in 76 screened patients. Patients with tumors of qMSP ratio > 4 showed superior survival compared to those with ratios 2-4 (P = .0251, log-rank test). In multivariate analysis, the qMSP ratio was not prognostic across the study cohort (hazard ratio [HR] = 0.88; 95% CI: 0.72-1.08). With different cutoffs for qMSP ratio (4, 9, 12 or 25), the CCNU/TMZ benefit tended to be larger in subgroups with lower ratios (eg, for cutoff 9: HR 0.32 for lower subgroup, 0.73 for higher subgroup). The concordance rates with qMSP were 94.4% (PSQ) and 90.2% (MGMT-STP27). Discordant results were restricted to tumors with qMSP ratios ≤ 4 and PSQ mean methylation rate ≤ 25%. Despite a shorter survival in MGMT-promoter-methylated patients with lower methylation according to qMSP, these patients had a benefit from combined CCNU/TMZ therapy, which even tended to be stronger than in patients with higher methylation rates. With acceptable concordance rates, decisions on CCNU/TMZ therapy may also be based on PSQ or MGMT-STP27.

KEYWORDS
CCNU/TMZ, glioblastoma, MGMT promoter methylation

1 INTRODUCTION

Glioblastomas lacking isocitrate dehydrogenase (IDH) 1 or 2 mutations (IDH-wildtype glioblastomas) are the most common malignant primary brain tumors in adults. With a median overall survival of about 17 months in unstratified study populations, IDH-wildtype glioblastoma patients have a dismal prognosis. Standard of care treatment includes surgery followed by radiotherapy and chemotherapy with the DNA alkylating agent temozolomide (TMZ). Promoter methylation of the O-6-methylguanine-DNA methyltransferase (MGMT) gene is so far the most important prognostic and predictive biomarker in IDH-wildtype glioblastoma patients. Patients whose tumors exhibit MGMT promoter methylation have a superior overall survival when treated with alkylating chemotherapy due to impaired DNA repair mechanisms. For this subset of glioblastoma patients, the randomized phase III multicenter CeTeG/NOA-09 trial recently showed a survival benefit when they were treated with a combination of lomustine (CCNU) and TMZ (CCNU/TMZ) instead of TMZ monotherapy.

Due to the encouraging results of the CeTeG/NOA-09 trial, some centers have started using combined CCNU/TMZ therapy off-study for patients with MGMT promoter-methylated glioblastoma. However, no current standard exists for MGMT promoter methylation testing in clinical routine and results from different tests may not be completely concordant. In several prospective phase II/III glioma trials and also in the CeTeG/NOA-09 trial, the method chosen for MGMT promoter methylation testing was a quantitative methylation-specific PCR (qMSP) assay. This assay yields a methylation ratio between MGMT and β-actin (ACT-B) at a logarithmic scale after sodium bisulfite conversion of tumor DNA and amplification of methylated sequences from the MGMT-associated 5′ CpG island. Using the qMSP assay, two major issues remain for routine clinical use in decision making for or against a combined CCNU/TMZ therapy: (i) The cutoff for MGMT promoter methylation is set at an MGMT methylation ratio of 2, yet

What's new?
In patients with IDH-wildtype glioblastoma, methylation of the MGMT promoter allows for improved survival after chemotherapy, due to reduced ability to repair DNA damage. Here, the authors set out to evaluate the use of different tests for promoter methylation, with an eye toward their usefulness at allocation of chemotherapy, and on their prognostic applicability. They show that three different methods of testing methylation—quantitative methylation-specific PCR, pyrosequencing, and DNA methylation arrays—agree more than 90% of the time. Patients with lower MGMT promoter methylation had shorter survival times, but still benefited from CCNU/TMZ therapy.
uncertainty is reported for ratios closely above the cutoff of 2.\textsuperscript{10} The question remains, whether patients harboring methylated tumors with methylation ratios in the lower range (a) have shorter survival than patients with higher methylation rates and (b) have a benefit from the more aggressive combination treatment with CCNU/TMZ as compared to TMZ monotherapy. (ii) Nowadays, qMSP is not commonly used in clinical routine, while pyrosequencing (PSQ)\textsuperscript{11} of sodium bisulfite-modified DNA seems to be the preferred method for MGMT promoter methylation testing in many European centers. Another assay that is increasingly being used to assess the MGMT promoter methylation status is based on DNA methylation profiling using the Infinium Methylation Epic 850k array (Illumina, San Diego, California) and the MGMT-STP27 algorithm.\textsuperscript{12} In this algorithm, methylation of two CpG islands in the differentially methylated region 1 and 2 (DMR1/2) of the MGMT 5’ CpG island respectively is determined. Several studies have analyzed different MGMT promoter methylation assays regarding their reliability, feasibility in different tumor samples and prediction of survival.\textsuperscript{13-14} However, there are only limited results concerning the concordance of these tests and no study has directly compared PSQ or MGMT-STP27 to the qMSP used in most clinical trials including CeTeG/NOA-09 trial. Thus, it remains unclear to date whether a decision for combined CCNU/TMZ treatment in clinical trials including CeTeG/NOA-09 trial can be based on results from qMSP or MGMT-STP27.

Here, we performed a post hoc analysis using data from the CeTeG/NOA-09 trial evaluating the prognostic and predictive value of low-level MGMT promoter methylation ratios as determined by qMSP. In addition, we investigated the concordance of the results obtained for MGMT promoter methylation by qMSP, PSQ or MGMT-STP27.

## 2 Materials and Methods

### 2.1 Quantitative methylation-specific PCR

qMSP-based MGMT promoter methylation testing was prospectively conducted within the CeTeG/NOA-09 trial in central laboratories of MDxHealth (Herstal, Belgium) as reported.\textsuperscript{10} In short, DNA was extracted from representative Formalin-fixed Paraffine-embedded (FFPE) sections and sodium bisulfite conversion was performed, followed by real-time MSP using primers for the methylated sequence. The results were normalized to ACTB as reference gene. For calculation of the MGMT methylation ratio, log2 (1000*mMGMT/ACTB) was used. In a dichotomized manner, a log2 value of above 2 was considered as MGMT promoter-methylated and below 2 as MGMT promoter-unmethylated. In case of a copy number below 20 for β-actin/ACTB, the result was considered as invalid.

### 2.2 DNA pyrosequencing

PSQ was performed as reported using modified oligonucleotide primer sequences.\textsuperscript{15} Briefly, DNA was extracted from representative FFPE tissue samples. Extracted tumor DNA was treated with sodium bisulfite using the MethylEdge Bisulfite Conversion System (Promega GmbH, Mannheim, Germany). MGMT promoter-methylated and promoter-unmethylated DNA control samples as well as a no template DNA control were run with each experiment. The following oligonucleotide primers were used for amplifying a 99-bp genomic fragment from the MGMT-associated 5’-CpG island: MGMT_PSQ_R1 5’-GGATATGTGGATAGTT-3’ and MGMT_PSQ_R2 5’-ACCCAAACACTACCAAATC-3’. The 5’-end of the reverse primer was conjugated with biotin. An initial incubation of 15 minutes at 95°C for activation of the Hot-Star Taq-polymerase and 45 cycles at 95°C for 30 seconds, 52°C for 30 seconds and 72°C for 30 seconds were performed, followed by a terminal elongation step at 72°C for 5 minutes. Pyrosequencing was carried out on a PyroMark Q24 system (Qiagen GmbH, Hilden, Germany). As sequencing primer, we used MGMT_PSQ_S1: 5’-GGATATGTGGATAGTTGGTTTTTAGAA-3’. For each tumor, the percentage of methylated alleles was determined at each of the CpG sites analyzed within the MGMT-associated 5’-CpG island and the mean percentage of methylated alleles was calculated across all investigated CpG sites. In total, seven CpG sites were sequenced that corresponded to CpGs 74-80 according to Malley et al\textsuperscript{16} and are located in MGMT exon 1 between nucleotides chr. 10:131 265 507 and 131 265 539 according to the UCSC GRCh37/hg19 MGMT reference sequence NM_002412. The seven CpG sites investigated here cover the four CpG sites (CpG 76-79) interrogated by a commercial PSQ assay from Qiagen (Hilden, Germany). For categorization of tumors as either MGMT promoter-methylated or unmethylated, we used a cutoff of <8% for the mean percentage of methylated alleles across CpGs 74-80 for MGMT promoter-unmethylated tumors, as reported in previous comparative analyses between PSQ and non-quantitative PCR.\textsuperscript{15}

### 2.3 850k methylation bead chip hybridization and calculation of MGMT promoter methylation status by the MGMT-STP27 model

DNA extracted from FFPE tissue samples was quantified and processed as previously described.\textsuperscript{17} After hybridization of 500 ng bisulfite-converted DNA to 850k Illumina bead chips, MGMT promoter methylation status was calculated by the MGMT-STP27 model according to Bady et al\textsuperscript{18} with a cutoff level of 0.3582. The model is based on the methylation of two CpG sites, one in the DMR1 and one in the DMR2 (CpG84) region of the MGMT promoter.

### 2.4 Statistical analysis

For survival analysis, all patients of the modified-intent-to-treat (mITT) population as the target population for the primary analysis of the CeTeG/NOA-09 trial (n = 1295) were considered. For multivariate analysis, Recursive Partitioning Analysis (RPA) class, study center and IDH mutation status were considered as covariates. To further
explore the correlation between qMSP and overall survival, we analyzed significance levels for dichotomized MGMT promoter methylation via qMSP in a Cox-regression model with RPA class and study centers as co-variates using varying cutoffs. Tests were conducted using the SAS software, R version 3.5.2 (SPSS Inc, Chicago, Illinois). Survival curves were generated using Kaplan-Meier plots and a log-rank test (GraphPad 8.0 Software, La Jolla/San Diego, California).

For calculation of the PSQ and MGMT-STRP27 probability of missing patients who would have been included in the CeTeG/NOA-09 trial according to qMSP and, on the other hand, the probability of treating patients with experimental CeTeG/NOA-09 protocol who would have been excluded from the CeTeG/NOA-09 trial based on qMSP, the Bayes’ theorem was used. As an example, we show the formula for calculation of the risk considering a sample as methylated by PSQ, but nonmethylated by qMSP.

\[
P(\text{PSQ}^+|\text{MSP}^-) = \frac{P(\text{PSQ}^+|\text{MSP}^+) \cdot P(\text{MSP}^-)}{P(\text{PSQ}^+|\text{MSP}^+) \cdot P(\text{MSP}^-) + P(\text{PSQ}^+|\text{MSP}^+) \cdot P(\text{MSP}^+)}
\]

Correlation of qMSP and PSQ was performed using nonparametric Spearman’s rank correlation coefficient (GraphPad 8.0 Software).

3 | RESULTS

3.1 | qMSP-based MGMT promoter methylation ratio: analysis of its prognostic potential

In the CeTeG/NOA-09 trial, tumors of patients of the mITT population (n = 129) exhibited a median MGMT promoter methylation ratio of 31.6 (range: 2.5-526.9) as determined by qMSP. To unravel a general prognostic relevance of qMSP ratio, we serially varied the cutoff point for qMSP (dichotomized variable) for the Cox analysis of OS throughout the range of qMSP ratios in the entire mITT population irrespective of treatment arm (Figure 1). Patients whose tumors had a methylation ratio greater than 4, 9 or 12 showed a significantly superior overall survival, when compared to patients whose tumors had lower MGMT methylation levels (ratios 2-4, 2-9 or 2-12, Figure 2A-D). We saw a nonsignificant trend toward inferior survival for patients with methylation levels 2 to 25 compared to patients with ratios >25 (Figure 2D). Beyond a qMSP ratio cut-off value of 25, no association of higher ratios with OS was found in this dichotomized analysis (Figure 1). Similar survival correlations were found for qMSP ratio cutoff values of 9 and 12, when considering progression-free survival (PFS) with patients whose tumors had lower methylation values exhibiting a higher PFS as compared to those with lower ratios (Figure S1B,C). A nonsignificant PFS curve separation was observed for qMSP cutoff of 4 (Figure S1A), while no correlation with PFS was found for a cut-off of 25 (Figure S1D). No overt imbalances of relevant prognostic and clinical factors (treatment arm, RPA class, sex) were seen in subgroups separated by different cutoffs (Table S1). A multivariate Cox regression analysis of the CeTeG/NOA-09 mITT population (n = 129 patients) with MGMT promoter methylation ratio as a continuous variable, treatment arm, RPA class and clinical center as covariates (in analogy to the confirmatory survival analysis of the trial)5 did not show a significant correlation of MGMT promoter methylation ratio with overall survival (Table 1) and confirmed the effect of the treatment arm as previously reported.19 Moreover, we did not observe a correlation of MGMT promoter methylation ratio with number of chemotherapy courses (CCNU/TMZ for the experimental and TMZ for the standard arm, Figure S2).

We also studied the treatment arm effect of CCNU/TMZ vs TMZ monotherapy separately in different subgroups defined by the qMSP cutoff ratios 4, 9, 12 and 25 (chosen based on the results of the dichotomized analyses of the mITT population, Figure 1). As shown in Table 2, experimental treatment with CCNU/TMZ showed hazard ratios substantially below 1 in each subgroup, yet significance was mostly not reached in any of the subgroups potentially due to small patient numbers. Interestingly, there was a tendency to lower HRs and thus a more pronounced effect of CCNU/TMZ therapy in subgroups with lower qMSP ratios (2-4; 2-9; 2-12; 2-25, Table 2).

3.2 | Concordance between PSQ and qMSP results

We compared the results obtained for the MGMT promoter methylation status by PSQ and qMSP in a subset of patients screened for the CeTeG/NOA-09 trial (n = 76; Table 3): Patient populations studied are outlined in Figure S3). qMSP showed evidence for MGMT promoter methylation in 56/76 patients (73.7%), while PSQ showed methylated results for 58/76 patients (76.3%) using a mean methylation rate of 8% across the seven investigated CpGs as cutoff. A concordance between the two methods was observed in 72/76 cases (94.7%),
when regarding the four invalid samples (qMSP) as samples without detection of \textit{MGMT} promoter methylation corresponding to the CeTeG/NOA-09 inclusion criteria requiring unequivocal \textit{MGMT} promoter methylation with a ratio > 2. When considering only the 72 patients with valid qMSP results, concordance was observed in 68/72 (94.4%) cases. When correlating the values of qMSP-\textit{MGMT} ratio (unmethylated values calculated as zero) and mean percentages of methylated alleles according to PSQ, we saw a high Spearman-correlation of 0.82 (Figure 3A). Similar results were obtained when restricting the PSQ evaluation to the four CpG sites covered in the Qiagen assay and comparing those with qMSP (Figure 3B), while both evaluations of the PSQ data showed an excellent Spearman-correlation of 0.99 (Figure 3C).

Out of the four tumors showing different results in PSQ vs qMSP, three were classified as unmethylated by qMSP, while being found methylated by PSQ (mean methylation percentages across the seven CpGs of 12%, 18% and 25%). One tumor was found to be methylated by qMSP (ratio 2.4) but unmethylated by PSQ (mean methylation percentage across the seven CpGs of 2%; Table 3).

The proportion of MGMT-methylated tumors as measured by qMSP (56/76; 73.7%) in the subgroup of patients analyzed was not representative of the entire CeTeG/NOA-09 screening population.

\textbf{TABLE 1}  Cox regression analyses investigating the potential influence of the qMSP-based MGMT promoter methylation ratio on overall survival

| Parameter | Patient population | n patients | Hazard ratio | 95% CI   |
|-----------|--------------------|------------|--------------|----------|
| (A)       |                    |            |              |          |
| qMSP MGMT ratio | mITT               | 129        | 0.88         | 0.715-1.084 |
| CCNU/TMZ treatment | mITT               | 129        | 0.579        | 0.333-1.006 |
| (B)       |                    |            |              |          |
| qMSP MGMT ratio | TMZ arm            | 63         | 0.792        | 0.584-1.072 |
| qMSP MGMT ratio | CCNU/TMZ arm       | 66         | 0.882        | 0.591-1.318 |

Note: Multivariate analysis with strata MGMT ratio determined by qMSP and treatment arm (+ RPA class and study center in analogy to the primary analysis of the trial\textsuperscript{5}) for the entire mITT population (A) and separately for the two treatment arms (B).
TABLE 2  Cox regression analyses investigating the effect of treatment arm in different subgroups based on different qMSP MGMT promoter methylation ratio cutoffs

| Parameter                  | Subgroup     | n patients | Hazard ratio | 95% CI      |
|----------------------------|--------------|------------|--------------|-------------|
| CCNU/TMZ treatment         | qMSP 2-4     | 12         | 0.294        | 0.057-1.509 |
| CCNU/TMZ treatment         | qMSP >4      | 117        | 0.661        | 0.368-1.186 |
| CCNU/TMZ treatment         | qMSP 2-9     | 32         | 0.317        | 0.114-0.881 |
| CCNU/TMZ treatment         | qMSP >9      | 97         | 0.731        | 0.379-1.413 |
| CCNU/TMZ treatment         | qMSP 2-12    | 41         | 0.404        | 0.16-1.019  |
| CCNU/TMZ treatment         | qMSP >12     | 88         | 0.712        | 0.35-1.447  |
| CCNU/TMZ treatment         | qMSP 2-25    | 57         | 0.476        | 0.221-1.024 |
| CCNU/TMZ treatment         | qMSP >25     | 72         | 0.731        | 0.329-1.628 |

Note: Multivariate analysis with strata treatment arm considering RPA class and study center in analogy to the primary analysis of the trial (5) in different subgroups defined by different cutoffs of qMSP MGMT ratio.

(35.8%).5 Assuming an overall methylation rate of 35.8% and using the Bayes theorem, the risk of labeling a tumor as MGMT promoter-methylated using PSQ although qMSP did not detect MGMT promoter methylation (either unmethylated MGMT promoter or invalid result) is therefore 21.5% in an unselected population. The probability of patients being tested by PSQ as having an MGMT-unmethylated tumor, while qMSP detected a methylated MGMT promoter is only 1.2%. Accordingly, the positive predictive value (PPV) of PSQ in relation to qMSP as a reference was 78.5% and the negative predictive value (NPV) was 98.8%.

Similar to the results obtained for the dichotomized OS analyses based on low qMSP cut-offs (Figure 2), dichotomized OS analyses irrespective of treatment arm based on mean PSQ methylation rates showed significantly longer OS in the patients with higher mean PSQ rates (cut-off 25%; Figure 4): The median OS in the group of nine patients (6 TMZ, 3 CCNU/TMZ) with mean PSQ methylation rate of 8-25% was 17.6 months while median OS was 40.2 months in the group of 36 patients (16 TMZ, 20 CCNU/TMZ) with a mean PSQ methylated allele frequency of more than 25% (P = .009, log-rank test; Figure 4A). In an analysis restricted to IDH-wild-type tumors (n = 37, 28 with PSQ methylated allele frequency above 25% and nine with PSQ methylated allele frequency of 8%-25%), median survival was 17.6 months in patients with mean methylation percentages between 8% and 25% and 32.4 months with mean methylation percentages above 25% (P = .04, log-rank test). An additional analysis for the effect of the treatment arm separately for the low and the high PSQ methylation rate subgroup (in analogy to Table 2) was not possible due to the small case numbers in the low PSQ methylation rate subset (n = 9).

3.3 Concordance rates of MGMT-STP27 with qMSP and between all three methods

We compared the results of MGMT-STP27 and qMSP in the same subset of patients screened for the CeTeG/NOA-09 trial, but had to restrict our analyses to 64/76 patients from whom sufficient tumor DNA was available to perform an EPIC methylation beadchip/
| CpG 74 | CpG 75 | CpG 76 | CpG 77 | CpG 78 | CpG 79 | CpG 80 | mean CpG 74-80 | mean CpG 76-79 | qMSP 850 k-STP27 |
|--------|--------|--------|--------|--------|--------|--------|---------------|---------------|------------------|
| 68     | 60     | 43     | 68     | 42     | 77     | 55     | 59            | 58            | 8.1 methmethmeth |
| 85     | 73     | 85     | 88     | 90     | 95     | 67     | 83            | 90            | 16.3 methmethmeth |
| 66     | 70     | 83     | 62     | 57     | 85     | 66     | 70            | 72            | 192.4 methmethmeth |
| 58     | 52     | 16     | 46     | 56     | 61     | 46     | 48            | 45            | 25.9 methmethmeth |
| 54     | 20     | 10     | 19     | 13     | 53     | 40     | 30            | 24            | 31.6 methmethmeth |
| 62     | 50     | 67     | 65     | 18     | 74     | 45     | 54            | 56            | 29.0 methmethmeth |
| 54     | 48     | 58     | 57     | 58     | 60     | 47     | 55            | 58            | 63.0 methmethmeth |
| 83     | 68     | 83     | 84     | 86     | 91     | 67     | 80            | 86            | 167.8 methmethmeth |
| 9      | 17     | 22     | 66     | 9      | 81     | 58     | 37            | 45            | 36.3 methmethmeth |
| 54     | 48     | 46     | 57     | 12     | 58     | 44     | 46            | 43            | 11.8 methmethmeth |
| 53     | 43     | 29     | 41     | 35     | 64     | 56     | 46            | 42            | 20.1 methmethmeth |
| 33     | 29     | 23     | 20     | 30     | 23     | 35     | 28            | 24            | 14.3 methmethmeth |
| 81     | 71     | 79     | 80     | 14     | 88     | 57     | 67            | 65            | 152.7 methmethmeth |
| 81     | 68     | 79     | 82     | 40     | 89     | 64     | 72            | 73            | 495.2 methmethmeth |
| 87     | 54     | 14     | 41     | 13     | 51     | 42     | 43            | 30            | 90.6 methmethmeth |
| 91     | 81     | 62     | 93     | 21     | 47     | 38     | 62            | 56            | 10.2 methmethmeth |
| 18     | 28     | 18     | 43     | 39     | 51     | 49     | 35            | 38            | 20.1 methmethmeth |
| 83     | 73     | 85     | 83     | 24     | 34     | 29     | 59            | 57            | 119.0 methmethmeth |
| 74     | 63     | 81     | 77     | 79     | 85     | 62     | 74            | 81            | 304.0 methmethmeth |
| 82     | 71     | 80     | 37     | 24     | 86     | 58     | 63            | 57            | 77.4 methmethmeth |
| 85     | 76     | 85     | 82     | 25     | 42     | 48     | 63            | 59            | 24.0 methmethmeth |
| 67     | 65     | 63     | 77     | 39     | 62     | 46     | 60            | 60            | 40.0 methmethmeth |
| 41     | 27     | 22     | 23     | 16     | 67     | 59     | 36            | 32            | 7.1 methmethmeth |
| 28     | 26     | 14     | 20     | 10     | 22     | 45     | 24            | 17            | 2.9 methmethmeth |
| 6      | 7      | 11     | 51     | 6      | 16     | 37     | 19            | 21            | 7.1 methmethmeth |
| 79     | 68     | 77     | 34     | 85     | 84     | 65     | 70            | 70            | 113.8 methmethmeth |
| 43     | 36     | 46     | 47     | 44     | 51     | 37     | 43            | 47            | 8.4 methmethmeth |
| 62     | 76     | 49     | 36     | 31     | 38     | 39     | 47            | 39            | 28.0 methmethmeth |
| 43     | 44     | 48     | 50     | 51     | 53     | 43     | 47            | 51            | 98.2 methmethmeth |
| 17     | 37     | 35     | 9      | 12     | 15     | 30     | 22            | 18            | 11.02 methmethmeth |
| 55     | 73     | 56     | 51     | 48     | 53     | 42     | 54            | 52            | 8.7 methmethmeth |
| 15     | 19     | 20     | 8      | 55     | 43     | 26     | 26            | 26            | 42.1 methmethmeth |
| 30     | 37     | 15     | 32     | 31     | 63     | 47     | 36            | 35            | 11.4 methmethmeth |

(Continues)
| CpG 74 | CpG 75 | CpG 76 | CpG 77 | CpG 78 | CpG 79 | CpG 80 | mean CpG 74-80 | mean CpG 76-79 | qMSP | qMSP res | PSQ res | 850 k-STP27 |
|-------|-------|-------|-------|-------|-------|-------|--------------|-------------|------|--------|--------|-------------|
| 43    | 47    | 32    | 68    | 80    | 42    | 49    | 52           | 56          | 27.7 | meth   | meth   | not analyzed |
| 26    | 52    | 15    | 52    | 13    | 22    | 42    | 32           | 26          | 2.5  | meth   | meth   | meth         |
| 64    | 73    | 74    | 61    | 71    | 83    | 62    | 70           | 72          | 35.3 | meth   | meth   | not analyzed |
| 60    | 58    | 62    | 61    | 59    | 53    | 50    | 58           | 59          | 62.5 | meth   | meth   | not analyzed |
| 9     | 9     | 19    | 18    | 5     | 20    | 15    | 14           | 16          | 4.4  | meth   | meth   | unmeth       |
| 46    | 61    | 12    | 11    | 12    | 50    | 50    | 35           | 21          | 10.6 | meth   | meth   | meth         |
| 50    | 48    | 29    | 21    | 5     | 30    | 39    | 32           | 21          | 34.9 | meth   | meth   | meth         |
| 64    | 59    | 55    | 39    | 46    | 60    | 47    | 53           | 50          | 227.6 | meth   | meth   | meth         |
| 63    | 59    | 66    | 65    | 66    | 76    | 44    | 63           | 68          | 217.0 | meth   | meth   | meth         |
| 30    | 30    | 1     | 39    | 8     | 18    | 28    | 25           | 23          | 138.8 | meth   | meth   | meth         |
| 12    | 14    | 15    | 22    | 6     | 44    | 35    | 21           | 22          | 30.0  | meth   | meth   | meth         |
| 50    | 49    | 48    | 51    | 52    | 30    | 39    | 46           | 45          | 24.5  | meth   | meth   | meth         |
| 38    | 37    | 10    | 39    | 8     | 18    | 28    | 25           | 19          | 5.6   | meth   | meth   | unmeth        |
| 5     | 20    | 19    | 20    | 4     | 25    | 24    | 17           | 17          | 3.6   | meth   | meth   | not analyzed |
| 66    | 66    | 57    | 65    | 21    | 36    | 51    | 52           | 45          | 81.5  | meth   | meth   | not analyzed |
| 67    | 72    | 32    | 73    | 72    | 84    | 63    | 66           | 65          | 25.1  | meth   | meth   | meth         |
| 36    | 20    | 54    | 32    | 56    | 28    | 23    | 36           | 43          | 15.3  | meth   | meth   | not analyzed |
| 10    | 16    | 12    | 11    | 8     | 48    | 22    | 18           | 20          | 6.5   | meth   | meth   | meth         |
| 1     | 2     | 2     | 19    | 36    | 4     | 2     | 9            | 15          | 5.1   | meth   | meth   | unmeth        |
| 1     | 2     | 2     | 1     | 1     | 5     | 1     | 2            | 2           | –     | invalid | unmeth | unmeth       |
| 56    | 56    | 58    | 57    | 54    | 64    | 49    | 56           | 58          | 7.0   | meth   | meth   | meth         |
| 1     | 3     | 3     | 1     | 3     | 3     | 1     | 2            | 3           | <0.6  | unmeth | unmeth | unmeth       |
| 1     | 2     | 2     | 1     | 3     | 3     | 1     | 2            | 2           | <0.6  | unmeth | unmeth | unmeth       |
| 2     | 12    | 4     | 3     | 3     | 4     | 5     | 5            | 4           | –     | invalid | unmeth | meth         |
| 18    | 26    | 12    | 10    | 4     | 8     | 5     | 12           | 9           | <0.6  | unmeth | meth   | meth         |
| 1     | 2     | 2     | 1     | 1     | 2     | 1     | 2            | 2           | <0.6  | unmeth | unmeth | unmeth       |
| 1     | 1     | 2     | 2     | 1     | 1     | 1     | 2            | 2           | –     | invalid | unmeth | unmeth       |
| 1     | 2     | 3     | 1     | 2     | 4     | 1     | 2            | 3           | <0.6  | unmeth | unmeth | unmeth       |
| 13    | 29    | 13    | 17    | 13    | 18    | 22    | 18           | 15          | <0.6  | unmeth | meth   | meth         |
| 2     | 2     | 2     | 1     | 2     | 3     | 1     | 2            | 2           | –     | invalid | unmeth | unmeth       |
| 2     | 3     | 3     | 2     | 2     | 4     | 2     | 3            | 3           | <0.6  | unmeth | unmeth | unmeth       |
| 3     | 3     | 3     | 3     | 3     | 4     | 2     | 3            | 3           | <0.6  | unmeth | unmeth | unmeth       |
| 47    | 46    | 29    | 29    | 47    | 37    | 39    | 39           | 36          | 60.7  | meth   | meth   | meth         |
DISCUSSION

The present post hoc analyses of the CeTeG/NOA-09 trial were intended to shed light on the use of different tests for MGMT promoter methylation especially in terms of allocating chemotherapy with CCNU and TMZ and on their prognostic potential in the context of this trial: (a) Using the qMSP test (ie, the test used in CeTeG/NOA-09 and most other previous phase III trials), we found that, irrespective of treatment arm, MGMT-promoter-methylated patients with low levels of MGMT promoter methylation (ie, MGMT methylation ratios 2-4, 2-9, 2-12 or 2-25) tended to have a shorter survival compared to patients with MGMT promoter methylation above these cutoffs. This observation could not be made for a qMSP MGMT ratio cutoff higher than 25. Similarly, lower methylation levels obtained by PSQ analysis (mean value of methylated alleles ≤ 25% across CpG islands 74-80) defined a subgroup of patients with inferior overall survival. (b) We observed a relatively high but not complete concordance between results obtained for MGMT promoter methylation testing by qMSP, PSQ and MGMT-STP27. When considering patients with higher methylation levels according to qMSP and PSQ (ie, qMSP ratio greater than 4 and mean PSQ methylated allele frequency of more than 25%), the concordance between the three methods was complete.

In our dichotomized survival analyses (Figures 2 and 4), we found that patients whose tumors exhibited MGMT promoter methylation below certain cutoffs (qMSP ratios up to 25; PSQ mean methylation 25%) had a worse prognosis as compared to patients with tumors above these cutoffs. This was not further supported by the Cox regression analysis with qMSP as a continuous variable, where we found that the qMSP-based MGMT promoter methylation ratio in general was not an independent prognostic factor (Table 1). The latter is in line with recently published data showing that MGMT promoter methylation ratios beyond a newly defined optimized cutoff ratio of 1.27 did not convey an extra survival benefit in a large cohort of 4041 glioblastoma patients from different trials.20 In the discussion of the divergence of results between dichotomized Kaplan-Meier and multivariable Cox regression analyses, the following points should be considered: (a) the Cox regression analysis is dominated by the majority of patients with high MGMT ratios (median ratio 31.6), where no influence of MGMT ratio is observed, so that differences in the lower range may not sufficiently influence the analysis as a whole. (b) The cutoff point analysis (Figure 1) is purely exploratory and thus not corrected for multiple testing. Overall, the dichotomized analysis provides an interesting hypothesis for further analyses in a prospective independent cohort.

Interestingly, the beneficial effect of combination treatment with CCNU/TMZ appeared to be more pronounced in the subgroups with lower methylation ratios (lowest HR of 0.317 [95% CI: 0.114-0.881] for subgroup of patients with qMSP ratios of 2-9). A potential explanation for this could be that combination treatment might prolong survival in patients, who have per se inferior prognosis due to lower MGMT promoter methylation ratios. In these patients, the effects that CCNU exerts beyond TMZ (eg, interstrand crosslinks) could play a particularly important role. These results have to be considered with
great caution due to the post hoc nature of the analysis, the risk of bias through multiple testing and the low patient numbers involved.

We found a high concordance rate between results obtained by qMSP and PSQ in the 76 patients tested with both methods. Based on our data comparing PSQ and qMSP, it appears to be acceptable to use PSQ results of $MGMT$ promoter methylation for making decisions to apply combined alkylating chemotherapy. Doing so, there is a minimal risk of missing $MGMT$ promoter-methylated patients (risk of missing a patient with PSQ who would have been included in the CeTeG/NOA-09 study according to qMSP: 1.2%). Nevertheless, one has to accept that 21.5% of patients who would be tested as having $MGMT$ promoter-methylated tumors by PSQ with 8% mean methylation rate as cutoff, yet would be tested as unmethylated or invalid in the qMSP test and thus excluded from the CeTeG/NOA-09 trial. Using off-study CCNU/TMZ treatment and PSQ for allocating CCNU/TMZ, one would thus treat approximately one out of five patients with a more intense regimen, although this patient would not match the inclusion criteria of the CeTeG/NOA-09 trial. This appears to be acceptable, as combined CCNU/TMZ therapy was well tolerated with an only slightly increased rate of adverse events and since it cannot be excluded that such a patient could have a benefit from combined chemotherapy. One could even provocatively postulate that based on the

**FIGURE 3** Correlation of qMSP and PSQ values of a subset of patients screened for the CeTeG/NOA-09 trial. A,B, Correlation of the $MGMT$ promoter methylation ratios determined by qMSP (with valid results) and the $MGMT$ promoter-methylated allele percentages determined by PSQ across the investigated CpG sites 74-80 or across CpG sites 76-79 ($n = 72$). Data are based on 76 patients screened within the CeTeG/NOA-09 trial and having valid qMSP results (72/76, 94.7%). For qMSP, linear values of the methylation ratio were used, while unmethylated values were set at 0. The results of the two methods showed a high correlation as calculated using Spearman’s rank correlation coefficient. C, Comparison of the two different PSQ evaluations showed an excellent correlation ($n = 76$).
nonconcordant results, one out of five patients would miss an effective treatment when the qMSP test is used as the decision tool. The above-mentioned discussion should be an explicit part of the informed consent process for each individual patient when using PSQ as the molecular test for decision making. Similar conclusions can be drawn about the use of the EPIC Infinium methylation array and the MGMT-STP27 algorithm. The concordance with the qMSP assay used in CeTeG/NOA-09 and particularly the positive predictive value in relation to qMSP as a reference was lower (71.1%) than found for PSQ (78.5%), but still remains in an acceptable range considering the same arguments as made above for PSQ. This comparison is highly relevant since some centers determine MGMT promoter methylation using the MGMT-STP27 model, which however has so far not been used in the screening phase of published clinical trials and only few studies have compared results obtained by this algorithm with those obtained by other methods like MSP or PSQ. One way to get a higher concordance of MGMT-STP27 and qMSP could be the adjustment of the cutoff for MGMT-STP27 test from 0.3582 to 0.405 when considering CCNU/TMZ treatment. This cutoff has previously been discussed, yet was dismissed due to a slight increase of specificity at cost of low sensitivity. Interestingly, patients with higher MGMT promoter methylation levels according to both qMSP and PSQ (ie, qMSP ratio > 4 and PSQ mean methylation percentage > 25%), show a full concordance of MGMT promoter methylation status for all three methods. With the above-mentioned limitations, PSQ and MGMT-STP27 can thus also be used for clinical decision making, as long as confirmatory long-term survival data for patients treated with CCNU/TMZ therapy off-study are missing.

In a more general perspective, the discussion of concordance and cutoff issues between different MGMT tests is just at the beginning: qMSP is the only test validated in large phase III clinical trials so far, yet it relies only on the methylation status of the CpG sites where the forward and reverse primers bind. This may lead to results not reflecting the methylation status across the entire 5' CpG island of MGMT or at least the differentially methylated regions therein that have been associated with regulation of transcriptional activity. Of note, qMSP is the only method with a comparatively high rate of invalid results (in our cohort: 4/76, 5.3%). Pyrosequencing protocols usually encompass several CpG sites within a differentially methylated region (in our case CpG sites 74-80, in Felsberg et al 74-78), and thus may produce more reliable and quantitative information on the MGMT promoter methylation status, but PSQ tests applied in different centers may interrogate different CpG sites and use distinct evaluation methods. Nonetheless, only two CpG sites—wit one (No 84) located in the DMR2 region of the MGMT promoter—are evaluated with this method. Furthermore, the array-based method needs a much (approximately 10-fold) higher DNA input compared to the other assays, which may represent a limiting factor in small biopsies. In our cohort, we saw a concordance for all three methods of 88.3%. This comparison is limited by the fact that the qMSP and the PSQ assays used for FFPE-derived DNA in our study do not cover CpG 84. Overall, our study has several limitations: The post hoc nature of the analysis, the low patient number involved and the different subgroups underline that our results should be interpreted with caution. To ultimately answer the question of the optimal

![FIGURE 4 Overall survival of a subset of CeTeG/NOA-09 patients irrespective of treatment arm (A, n = 45 patients including IDH-mutant patients; B, n = 36 patients excluding IDH-mutant patients) stratified according to mean values of MGMT promoter-methylated alleles of 8%-25% vs >25% across the CpGs 74-80 assessed by pyrosequencing (A, log-rank test: P = .0085; B, log-rank test: P = .0403) [Color figure can be viewed at wileyonlinelibrary.com]
method for MGMT promoter methylation testing and define generally accepted cutoffs, clinical trials with prospective, systematic testing of tumor samples with more than one method are warranted.

In conclusion, MGMT-promoter-methylated glioblastoma patients whose tumors show a low methylation ratio as defined by qMSP (2-25%) or a low MGMT promoter methylation as defined by PSQ (8%-25%) tend to have an inferior overall survival, but seem to benefit from CCNU/TMZ treatment nonetheless. Based on concordance rates found for qMSP with PSQ and MGMT-SP27, we conclude that a decision for CCNU/TMZ treatment in patients with MGMT-promoter-methylated glioblastoma could be based on MGMT promoter methylation testing via PSQ or MGMT-SP27 results. Further studies with systematic assessment of different MGMT promoter methylation tests and their cutoffs especially in the context of treatment with CCNU/TMZ are needed. Meanwhile, we would consider PSQ and, despite a slightly lower concordance rate with qMSP, MGMT-SP27 to be suitable when offering treatment with CCNU/TMZ to MGMT methylated glioblastoma patients.

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CONFLICT OF INTEREST
Ulrich Herrlinger reports speaker honoraria from Medac, Bayer and Novartis and advisory board honoraria from Bayer, Janssen, Noxxon and Karyopharm. Joachim-Peter Steinbach reports honoraria for lectures, travel or advisory board participation from Med-Update, Abbvie, Bristol-Myers Squibb, Medac, Roche, Novocure and UCB. Uwe Schlege reports honoraria for lectures from Medac, GSK and Novartis. Niklas Schäfer reports personal fees and other support from Roche and received honoraria for advisory board from Bayer. Martin Glas reports grants, personal fees and other support from Novocure and Medac, and personal fees from Merck. All other authors declare no competing interests.

DATA AVAILABILITY STATEMENT
The data that support the findings of our study are available on request from the corresponding author. The data are not publicly available due to privacy or ethical restrictions.

ETHICS STATEMENT
Our study was approved by the Ethical Committee of the University of Bonn (AZ: 093/10) and written informed consent was obtained by every patient. The registration number of the CeTeG/NOA-09 trial is NCT01149109.

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SUPPORTING INFORMATION
Additional supporting information may be found online in the Supporting Information section at the end of this article.

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