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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-

Abbreviations: ACT-B, β-actin; CCNU, lomustine; DMR 1/2, differentially methylated region 1 / 2; FFPE, formalin-fixed paraffin-embedded; HR, hazard ratio; IDH, isocitrate dehydrogenase; MGMT, O-6-methylguanine-DNA methyltransferase; mITT, modified-intent-to-treat; NPV, negative predictive value; OS, overall survival; PFS, progression-free survival; PPV, positive predictive value; PSQ, pyrosequencing; qMSP, quantitative methylation-specific PCR; RPA, recursive partitioning analysis; TMZ, temozolomide.

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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.[1] With a median overall survival of about 17 months in unstratified study populations, IDH-wildtype glioblastoma patients have a dismal prognosis.[2] Standard of care treatment includes surgery followed by radiotherapy and chemotherapy with the DNA alkylating agent temozolomide (TMZ).[3] 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.[4] 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.[5]

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[2,6-9] and also in the CeTeG/NOA-09 trial,[5] the method chosen for *MGMT* promoter methylation testing was a quantitative methylation-specific PCR (qMSP) assay.[10] 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.[10] 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.[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)[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.[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.[1,13,14] 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 analogy to the CeTeG/NOA-09 trial can be based on results from PSQ 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.[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*m*MGMT*/*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.[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_F1 5′-GGATATGTTGGGATAGTT-3′ and MGMT_PSQ_R1 5′-ACCCAA ACACTCACCAAATC-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, Hilden, Germany). As sequencing primer, we used MGMT_PSQ_S1: 5′-GGATAGTTYGYGTTTTTAGAA-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[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.[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.[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,[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 = 129[5]) 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-STP27 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.

$$P(\mathrm{MSP}^- \mid \mathrm{PSQ}^+) = \frac{P(\mathrm{PSQ}^+ \mid \mathrm{MSP}^-) \cdot P(\mathrm{MSP}^-)}{P(\mathrm{PSQ}^+ \mid \mathrm{MSP}^-) \cdot P(\mathrm{MSP}^-) + P(\mathrm{PSQ}^+ \mid \mathrm{MSP}^+) \cdot P(\mathrm{MSP}^+)}$$

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 cut-off 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 cut-off 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%),

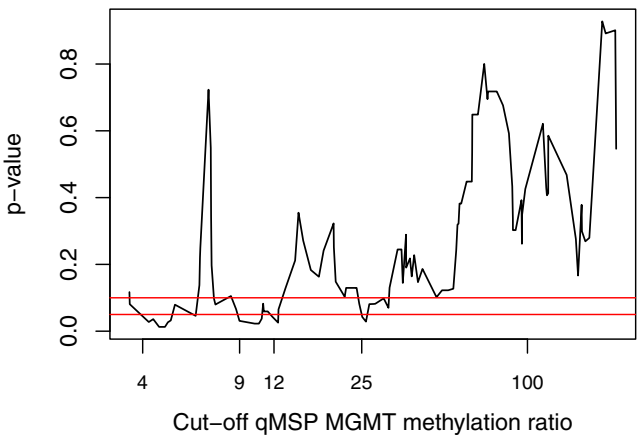

FIGURE 1 Association of the qMSP methylation ratio with overall survival in the CeTeG/NOA-09 population (mITT, n = 129) irrespective of the treatment arm. Depicted are the significance levels (*P* values) for dichotomous survival prediction of different cutoffs for *MGMT* promoter methylation ratio via qMSP for the mITT population CeTeG/NOA-09 trial irrespective of treatment arm considering RPA class and study center. Red lines indicate *P* values 0.1 and 0.05. Significant results were only seen for qMSP methylation ratio cutoffs below 25 [Color figure can be viewed at wileyonlinelibrary.com]

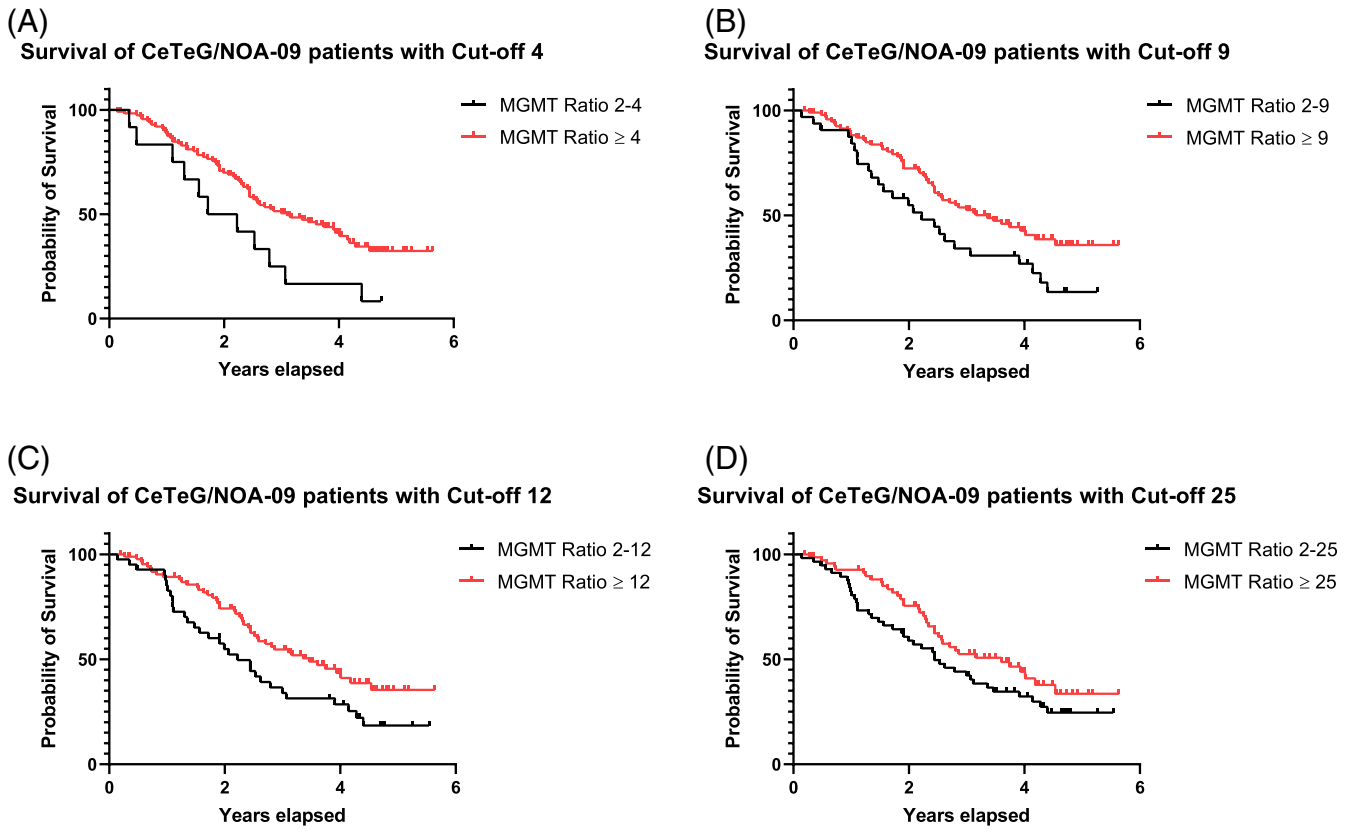

FIGURE 2 A-D, Overall survival of patients with different qMSP-based *MGMT* ratio cutoffs (entire mITT population, no stratification for treatment arm). Patients having tumors with an *MGMT* ratio 2-4, 2-9 and 2-12 had an inferior overall survival compared to patients whose tumors showed an *MGMT* ratio >4/>9 and >12 (log-rank tests: $P = .0251$; $P = .0178$; $P = .0235$). Patients with qMSP ratio of more than 25 showed a nonsignificant trend to superior overall survival compared to patients with qMSP ratios 2-25 (log-rank test: $P = .0961$) [Color figure can be viewed at wileyonlinelibrary.com]

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[5]) for the entire mITT population (A) and separately for the two treatment arms (B).

when regarding the four invalid samples (qMSP) as samples without detection of *MGMT* promoter methylation corresponding to the CeTeG/NOA-09 inclusion criteria requiring unequivocal *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-*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

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/MGMT-STP27 analysis. A concordance of methylation results was found in 57/64 patients (89.1%, Table 3) when MGMT-STP27 was compared to qMSP and invalid qMSP results were classified as unmethylated/no methylation detected corresponding to the CeTeG/NOA-09 inclusion criteria requiring unequivocal *MGMT* promoter methylation with a ratio > 2 (in accordance with the above-mentioned comparison between qMSP and PSQ). When considering only tumors with valid qMSP results, 54/60 (90%) cases were concordant for qMSP and MGMT-STP27. Concordance was found in 59/64 patients (92.2%), when MGMT-STP27 was compared to PSQ. When comparing MGMT-STP27 to the qMSP test used in the trial, the risk for patients not being detected as having an *MGMT* promoter-methylated tumor with MGMT-STP27 inspite of qMSP showing a methylated *MGMT* promoter was 4.5%. The risk of getting the result of a methylated *MGMT* promoter with MGMT-STP27 while qMSP revealed an unmethylated *MGMT* promoter or an invalid result (and thus not matching the criteria for the CeTeG/NOA-09 study population) was 29.9%.

Tumors of 64 patients were tested with all three assays. Full concordance between all three tests was found in 53/60 (88.3%) patients, when only considering tumors with valid qMSP results. When evaluating the inclusion/exclusion of a patient in the CeTeG/NOA-09 trial and thus considering invalid qMSP results as “no methylation detected”, 56/64 (87.5%) cases were concordant. Of note, three of 43 patients (7%) included in the CeTeG/NOA-09 study tested as having *MGMT* promoter-methylated tumors by both qMSP and PSQ scored as unmethylated by MGMT-STP27, and would thus not have been assigned to the study, if only this array-based *MGMT* methylation testing had been used for *MGMT* assessment. The three discordant cases had qMSP ratios of 4.4, 5.6 and 5.1 and comparably low PSQ mean percentages of methylated alleles of 14%, 25% and 9%, respectively. When considering only patients with higher methylation rates (qMSP ratio > 4 and mean PSQ methylated allele frequency > 25% across the seven investigated CpG sites), there was a full concordance of the results obtained with each of the three tests (36/36, 100%).

TABLE 3 Results obtained by quantitative methylation-specific PCR (qMSP), DNA pyrosequencing (PSQ) or MGMT-STP27 for *MGMT* promoter methylation in tumors of 76 patients screened for the CeTeG/NOA-09 trial [Color table can be viewed at wileyonlinelibrary.com]

CpG 74	CpG 75	CpG 76	CpG 77	CpG78	CpG 79	CpG 80	mean CpG 74-80	mean CpG 76-79	qMSP	qMSP res	PSQ res	850 k-STP27
68	60	43	68	42	77	55	59	58	8.1	meth	meth	meth
85	73	85	88	90	95	67	83	90	16.3	meth	meth	meth
66	70	83	62	57	85	66	70	72	192.4	meth	meth	meth
58	52	16	46	56	61	46	48	45	25.9	meth	meth	meth
54	20	10	19	13	53	40	30	24	31.6	meth	meth	meth
62	50	67	65	18	74	45	54	56	29.0	meth	meth	meth
54	48	58	57	58	60	47	55	58	63.0	meth	meth	meth
83	68	83	84	86	91	67	80	86	167.8	meth	meth	meth
9	17	22	66	9	81	58	37	45	36.3	meth	meth	meth
54	48	46	57	12	58	44	46	43	11.8	meth	meth	meth
53	43	29	41	35	64	56	46	42	20.1	meth	meth	meth
33	29	23	20	30	23	35	28	24	14.3	meth	meth	meth
81	71	79	80	14	88	57	67	65	152.7	meth	meth	meth
81	68	79	82	40	89	64	72	73	495.2	meth	meth	meth
87	54	14	41	13	51	42	43	30	90.6	meth	meth	meth
91	81	62	93	21	47	38	62	56	10.2	meth	meth	meth
18	28	18	43	39	51	49	35	38	20.1	meth	meth	meth
83	73	85	83	24	34	29	59	57	119.0	meth	meth	meth
74	63	81	77	79	85	62	74	81	304.0	meth	meth	meth
82	71	80	37	24	86	58	63	57	77.4	meth	meth	meth
85	76	85	82	25	42	48	63	59	24.0	meth	meth	meth
67	65	63	77	39	62	46	60	60	40.0	meth	meth	meth
41	27	22	23	16	67	59	36	32	7.1	meth	meth	meth
28	26	14	20	10	22	45	24	17	2.9	meth	meth	meth
6	7	11	51	6	16	37	19	21	7.1	meth	meth	meth
79	68	77	34	85	84	65	70	70	113.8	meth	meth	meth
43	36	46	47	44	51	37	43	47	8.4	meth	meth	meth
62	76	49	36	31	38	39	47	39	28.0	meth	meth	meth
43	44	48	50	51	53	43	47	51	98.2	meth	meth	not analyzed
17	37	35	9	12	15	30	22	18	11.02	meth	meth	not analyzed
55	73	56	51	48	53	42	54	52	8.7	meth	meth	not analyzed
21	15	19	20	8	55	43	26	26	42.1	meth	meth	meth
30	37	15	32	31	63	47	36	35	11.4	meth	meth	meth

(Continues)

TABLE 3 (Continued)

CpG 74	CpG 75	CpG 76	CpG 77	CpG78	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	30	29	30	26	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	2	3	1	2	2	<0.6	unmeth	unmeth	unmeth
1	1	2	2	1	4	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

TABLE 3 (Continued)

CpG 74	CpG 75	CpG 76	CpG 77	CpG78	CpG 79	CpG 80	mean CpG 74-80	mean CpG 76-79	qMSP	qMSP res	PSQ res	850 k-STP27
3	4	4	2	3	4	2	3	3	<0.6	unmeth	unmeth	not analyzed
43	29	26	15	9	14	36	25	16	<0.6	unmeth	meth	meth
41	40	43	38	44	45	37	41	43	30.7	meth	meth	not analyzed
1	2	3	1	2	2	1	2	2	<0.6	unmeth	unmeth	unmeth
2	2	2	1	2	2	1	2	2	<0.6	unmeth	unmeth	unmeth
2	3	3	2	2	3	2	2	3	<0.6	unmeth	unmeth	not analyzed
3	3	2	1	2	3	1	2	2	<0.6	unmeth	unmeth	unmeth
1	2	1	6	1	2	1	2	3	2.4	meth	unmeth	meth
2	3	2	1	1	3	2	2	2	<0.6	unmeth	unmeth	unmeth
1	2	3	3	1	4	0	2	3	<0.6	unmeth	unmeth	unmeth

Note: For PSQ, the percentages methylated alleles at each of the seven interrogated CpG sites and the mean methylated alleles percentages across all seven CpGs (CpG 74-80) or the CpGs 76-79 are given. For qMSP results, results are classified as meth (qMSP ratio >2), unmeth (unmethylated, ratio < 0.6) or invalid (dark gray). For MGMT-STP27 results are indicated as meth (methylated) or unmeth (unmethylated). Nonhighlighted fields indicate full concordance between all three methods. Yellow color indicates nonconcordant results according to at least one method with inconcordance between qMSP and PSQ. Red color indicates tumors classified as methylated by qMSP and PSQ but unmethylated by MGMT-STP27.

4 | 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

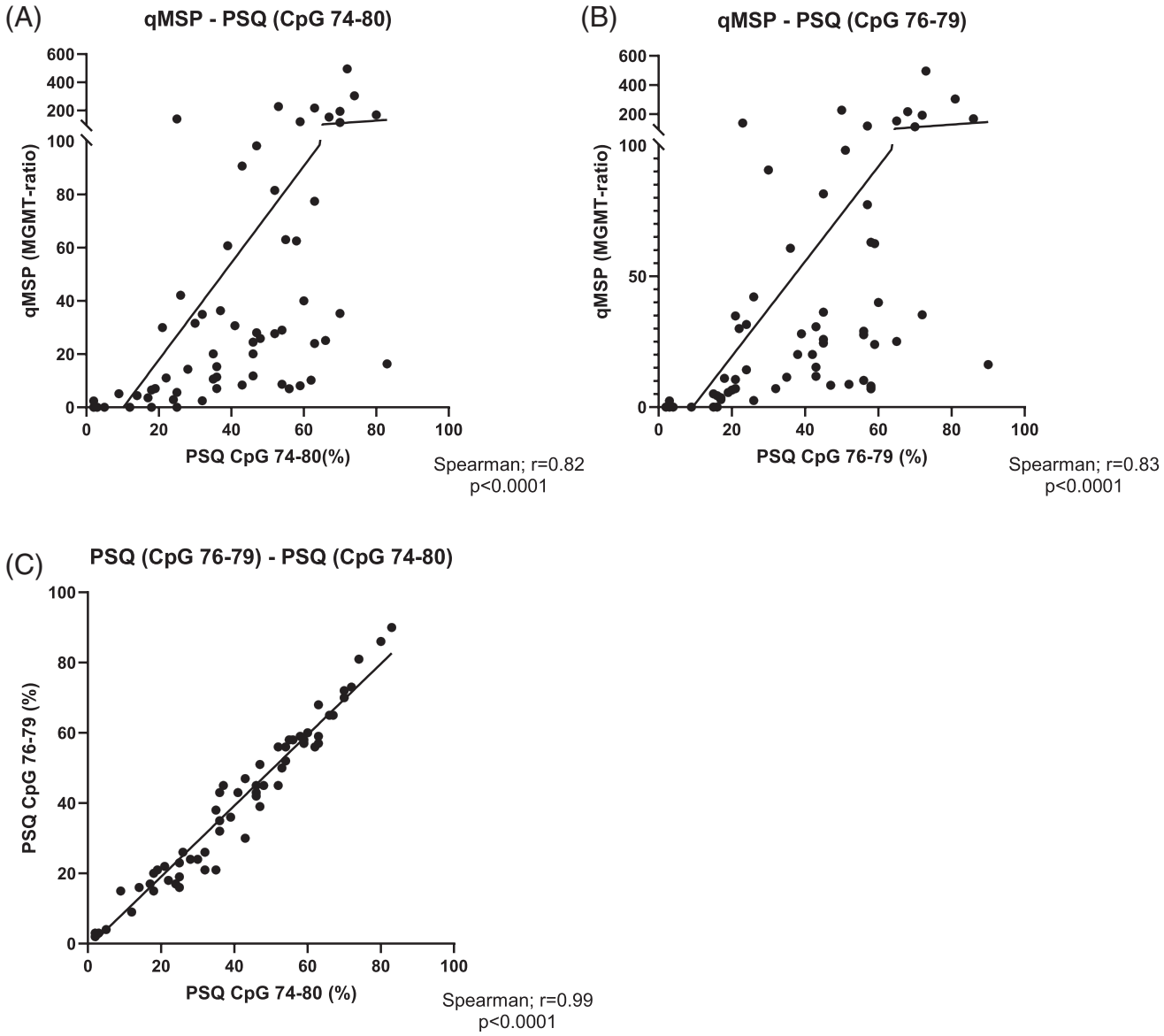

FIGURE 3 Correlation of qMSP and PSQ values of a subset of patients screened for the CeTeG/NOA-09 trial. A,B, Correlation of the *MGMT* promoter methylation ratios determined by qMSP (with valid results) and the *MGMT* promoter-methylated allele percentages determined by PSQ across the investigated CpG 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)

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[19] 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

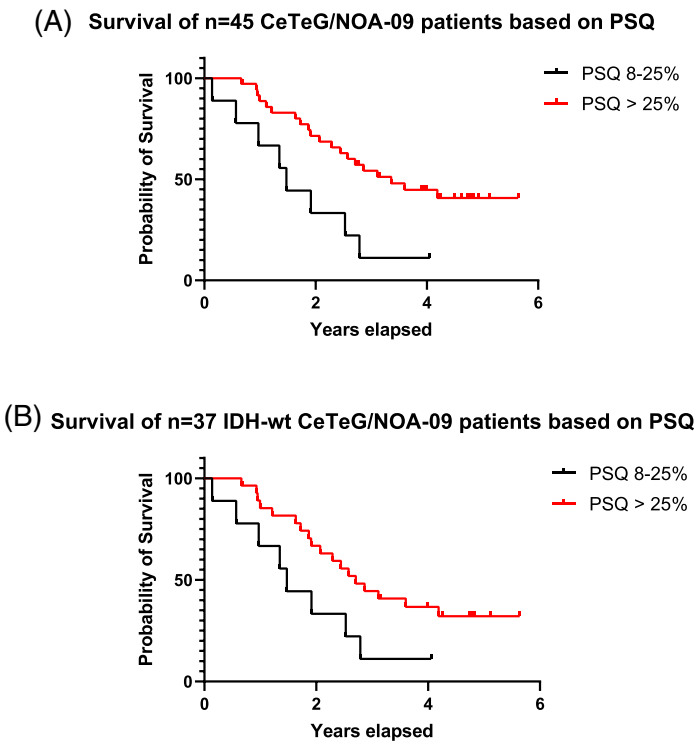

FIGURE 4 Overall survival of a subset of CeTeG/NOA-09 patients irrespective of treatment arm (A, n = 45 patients including IDH-mutant patients; B, n = 36 patients excluding IDH-mutant patients) stratified according to mean values of *MGMT* promoter-methylated alleles of 8%-25% vs >25% across the CpGs 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]

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.[12] 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.[18,21] 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.[18] 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.[16] 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[22]), 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.[22-25] Thus, the cutoff of a mean methylated allele frequency (here: 8%), may differ between different assays. The Infinium EPIC Beadchip (850k) array allows for a robust epigenetic classification of glioblastoma based on DNA methylation and deduced copy number profiles.[26] In addition, the method can determine *MGMT* promoter methylation using the MGMT-STP27 model.[12] Nonetheless, only two CpG sites—with 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

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-STP27, 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-STP27 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-STP27 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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