**Referee: 1 Comments to the author(s)**

The manuscript details determination of the MGMT promoter methylation status in glioma using nanopore sequencing. Multiple datasets are analyzed and compared to the results of the original MGMTp methylation classification by distinct methods commonly used. The overall conclusion is that nanopore sequencing is a good and rapid method to determine the MGMTp methylation status for diagnostic use.

The methodology is well described, and the source code for data analyses is made available. The limitations of the study are discussed.

This is an interesting manuscript, and the analyses are well performed and described.

Specific comments:

1. How do the authors envision the classification for individual patient samples using the nanopore sequencing.

Author response: Every institution has its own standard operating procedures and cut-off points as to how patient samples are classified as *MGMT* methylated or unmethylated. Although we believe the nanopore sequencing adds value, further evaluation with more data on patient survival is necessary to decide the optimal way to classify patients. We have added the following text to Discussion:

*“Here we provide a framework for how nanopore sequencing can be used to evaluate MGMT promoter methylation in the context of established methods (the MGMT-Pyro kit and STP-27 classifier) or based on full sequencing of the MGMT promoter CpG -island (for example by hierarchical clustering of samples). We also provide methylation values for the 98 CpG sites in the MGMT promoter CpG-island in 165 samples. Institutions and research groups are encouraged to download our data and compare our results to their own samples or established methods of MGMT methylation detection.”*

1. The issue of the grey zone samples is not addressed, it would require large datasets to have the statistical power. The grey zone certainly plays a role when decisions of omitting TMZ are addressed e.g. in clinical trials.

Author response: An interesting yet difficult to answer comment. We have added the following text to the Discussion:

*“The definition of the ‘grey-zone’ in the context of MGMT promoter methylation is an active area of research \cite{Pinson2020, Torre2022}. This is complicated by the3 multitude of methods are currently in use for MGMT methylation classification. Hierarchical clustering of our samples based on nanopore sequencing of the MGMT CpG-island (figure 4c) indicates the presence of two distinct groups within methylated samples. However, our data lack the necessary statistical power to evaluate differences in patient survival between these groups. It should be noted that nanopore sequencing data is comparable between platforms and assays, pooling data from future studies will undoubtedly aid in a true clinically significant cut-off for patient classification.”*

Additional comment

The reference list is messed up (likely because the first 2 references have been fused and all references thereafter seem to be wrongly annotated in the text). Hence the rational for parts of the text cannot be followed, as the proper references cannot be linked to get respective background information. The origin and quality of cutoffs chosen in this work from the literature cannot be looked at. Since this is a crucial part for the study, this needs to be corrected for proper evaluation of this work.

Author response: We apologize for this embarrassing mistake, which is a product of converting the manuscript text between different formats. The reference list has been fixed in the reviewed version.

The tables at the end of the document cannot be evaluated due to the unfortunate formatting that cuts the tables into multiple pieces. -

Author response: We apologize again for the unfortunate formatting of the tables. This error is a product of the online submission platform that does not provide the submitter with a final version to review before final submission. We have uploaded a finalized .pdf version of the manuscript that we hope will find its way to the reviewer.

**Referee: 2 Comments to the author(s)**

MGMT promoter methylation status remains the most important predictive biomarker for treatment of IDH-wildtype glioblastoma. The authors performed comparative MGMT promoter methylation analysis using nanopore sequencing, methylation microarray and pyrosequencing. The study provides important performance metrics for nanopore sequencing and is of strong interest to neuropathologists. The study is well written and well illustrated.

Specific aspects:

1. - An important factor to guide implementation of nanopore sequencing for MGMT promoter analysis would be the minimally required read depth for reliable quantitative estimation of promoter methylation. A dedicated analysis to derive a cut-off using e.g. random downsampling would be very valuable. -

Author response: This is a good suggestion. We have now added the following paragraph to the main text and supplementary figure on read depth analysis.

*“The read depth in nanopore sequencing typically falls below standard sequencing methods. Therefore, we investigated how sequence read depth impacts sample classification in nanopore sequencing. To assess the influence of decreasing read depth in the MGMT CpG-island on sample classification, we conducted random downsampling of all samples with over 20 reads. A graphical representation of methylation patterns, ranging from 100 reads to 10, 5, or 2 reads (Supplementary figure 1a), indicates minimal deviation from the smoothed methylation profile until the read depth drops to only two reads. Subsequently, we reclassified the samples using the MGMT-Pyro logistic regression model as described above.*

*Among the 29 samples initially classified as "unmethylated" based on the full sequence depth, 27 (93%) maintained this classification after downsampling to 10 and 5 reads. Similarly, of the 13 samples initially classified as "methylated" based on the full sequence depth, 11 (85%) remained stable through downsampling to 5 reads (Supplementary figure 1b).*

*To assess the impact of read depth on hierarchical clustering based on methylation values of all CpG sites in the MGMT promoter CpG-island, we assigned samples with full or downsampled read depth to the two major clusters (figure 4c) using K-nearest neighbor (KNN) regression. All but two samples (95%) retained their initial clustering from full read depth (> 20 reads) to five reads in this approach (Supplementary figure 1c).”*

1. - For logistic models, it is not clear how training and test cohorts were chosen (retrospective nCATS vs other, Rapid-CNS vs other). Would not random splits of the entire cohort be more appropriate? -

Author response: Good observation, our approach needs more clarity. The objective of the logistic regression models (figure 2c and 3b) was to show that extracting the relevant CpG sites for either the MGMT-pyro kit or STP27 can be used to accurately predict the classification as long as the “known status” was established using the same sites, ie the retrospective nCATs samples for MGMT-pyro evaluation and Rapid-CNS samples for STP-27. However, the regression models perform poorly when used to predict the outcome of different classifiers. The text has been amended for clarity in the following way:

*“The "retrospective nCATs" samples offer direct comparison between nanopore sequencing and the MGMT-pyro kit as the same DNA was analysed via both methods. To compare classification results, a logistic regression model was trained using the average methylation of CpGs 76-79 based on nanopore sequencing and classification based on the MGMT pyro kit as indicator.”*

1. Should maybe non-glioblastoma cases be excluded from regression model fitting/validation because they appear to show different methylation patterns across the entire island (see point below) and predictive value is only relevant for IDH-wildtype glioblastoma?

Author response: - This is a valid point. Ideally, all our samples would have been IDHwt-GBMs. This is unfortunately not the case as only 103 of our 165 samples (62%) are in fact IDHwt-GBMs. We were therefor faced with the choice of excluding a substantial part of our samples because they may not be clinically relevant (as we did in figure 5) even though they have relevant data for the comparisons or including them under the assumption that they provide relevant information for MGMT promoter methylation classification. In this study we chose the latter although arguments can certainly be made for both routes.

1. Hierarchical clustering (Fig 4a) suggests that the majority of IDH-mutant gliomas have a distinct methylation profile compared to MGMT-methylated IDH-wildtype glioblastoma (showing similar methylation approximately along CpG sites 1-14 and 65-98, but distinct methylation along CpG sites 20-54 approximately). This is an interesting finding that might warrant further analysis and discussion (e.g. hierarchical clustering of CpG sites of the CpG island). -

Author response: This is an interesting observation by the reviewer and worth a closer look. To this extent we have examined clustering of IDHmut-glioma samples in more detail, for example by clustering of CpG sites as suggested. It is true that methylation of IDHmut-glioma and IDHwt-GBM samples appears to differ in the central region of the CpG island but there is also in general somewhat lower methylation in the cluster containing most of the IDHmut-glioma samples. We also performed the clustering with only methylated IDH-glioma and IDHwt-GBM samples (see figures below) and observe two main clusters; one, characterized by lower methylation. included 17 of 25 IDHmut-glioma samples (68%) and 16 of 39 IDHwt GBM samples (41%) while the other, characterized by higher overall methylation, contained 8 of 25 IDH-glioma samples (32%) and 23 of 39 IDHwt GBM samples (59%). We also analysed the data via principal component analysis (PCA). As expected, known *MGMT* methylation status was the main separator of samples. No separation of IDHmut-glioma samples and IDHwt-GBM was observed within the first 3 principal components. Finally, we performed supervised clustering via partial least squares discriminatory analysis (PLS-DA) to specifically search for patterns that might separate methylated IDHglioma samples from methylated GBM samples. Some level of separation is apparent but there is still considerable overlap between IDHmut glioma samples and GBM samples. Ultimately, while the observation is interesting, the *MGMT* methylation pattern separating IDHwt GBM and IDHmut glioma samples is interesting but subtle. We believe it does not warrant further discussion in the manuscript at this point. Our primary aim was to show that data generated via nanopore sequencing covering the *MGMT* promoter is a valid way of separating *MGMT* methylated samples from *MGMT* unmethylated samples.

1. - Figure 3: In analogy to Figure 2a, it would be interesting to see the correlation between nanopore methylated allele frequency and microarray beta value for the two CpG sites of the MGMT-STP27 model. -

Author response: We completely agree, a more thorough comparison of nanopore sequencing data and methylation bead array data is warranted. Unfortunately, the array beta values are not available to us for further analysis.

1. - Line 110f: The authors claim that coverage differed between methylated and unmethylated cases in the adaptive sampling cohort. Have MGMT copy number and genome-wide mean depth been accounted for? (There likely is a strong correlation between MGMT/chr10 copy number, read depth and IDH-mutant glioma, which usually are MGMT methylated.) -

Author response: An excellent observation. We assume the reviewer is referring to the gain of chromosome 7 and loss of chromosome 10 typically observed in IDHwt-GBM and if this may influence MGMT promoter read depth in methylated versus unmethylated samples. Of the samples analyzed via adaptive sampling, 49 of 67 were IDHmut GBM samples (40 of which were classified as unmethylated) while 13 of 67 were IDHmut glioma samples (12 of which were methylated). This likely creates a bias where unmethylated samples are far more likely to have loss of heterozygosity on chromosome 10 (and by extension the MGMT gene) while methylated samples are likely to retain both copies and to therefore have higher read depth. We have added this to the results

“This is likely due to the realtively high number of IDHwt GBM samples among the unmethylated samples (40 of 48, 83%) in comparison to the methylated samples (9 of 21, 43%). IDHwt GBM samples commonly exhibit loss of heterozygosity on chromosome 10 and the MGMT gene cite{Reuss2015ATRXGlioblastoma}.”

Minor aspects:

1. - Abstract: “95%” is not an appropriate metric for correlation. Please consider rephrasing as agreement or concordance or provide a correlation coefficient. -

Author response: This has been corrected in the abstract.

1. Figure 3a: the axis range should be 0-100.

Author response: This has been corrected.

1. - STP27 probe and pyrosequencing CpG locations could maybe be indicated in Figures 4a, 4b, 4c and 5a in consistent fashion (annotation track) as in Figure 1a for easier recognition. -

Author response: This has been implemented, the STP-27 and MGMT-Pyro sites have been annotated in figure 4a in the same way as in figure 4b. A column annotation track has also been added to the heatmaps (figures 4c and 5a).

1. - In heatmaps (Fig. 4a and 5a), it might be interesting to indicate MGMT status separately for pyrosequencing vs. STP27 and also include their quantitative readouts.

Author response: A row annotation track has been added to figure 4c that indicates how the samples were originally classified (MGMT-Pyro or STP-27). We do not feel that including this annotation track to figure 5a (heatmap of only GBM samples) adds value. Regarding the quantitative readout of these assays, we agree with the reviewer that this could have been a valuable addition. However, as stated in the comment above, we do not have access to the beta values for the samples classified via the STP-27 classifier, these samples (67 of 165) which would result in multiple missing values in the annotation track.

