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DNA methylation-array interlaboratory comparison trial demonstrates highly reproducible paediatric CNS tumour classification across 13 international centres

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Abstract

Aims: DNA methylation profiling, recently endorsed by the World Health Organisation (WHO) as a pivotal diagnostic tool for brain tumours, most commonly relies on bead arrays. Despite its widespread use, limited data exist on the technical reproducibility and potential cross-institutional differences. The LOGGIC Core BioClinical Data Bank registry conducted a prospective laboratory comparison trial with 12 international laboratories to enhance diagnostic accuracy for paediatric low-grade gliomas, focusing on technical aspects of DNA methylation data generation and profile interpretation under clinical real-time conditions.

Methods: Four representative low-grade gliomas of distinct histologies were centrally selected, and DNA extraction was performed. Participating laboratories received a DNA aliquot and performed the DNA methylation-based classification and result interpretation without knowledge of tumour histology. Additionally, participants were required to interpret the copy number profile derived from DNA methylation data and conduct DNA sequencing of the *BRAF* hotspot p.V600 due to its relevance for low-grade gliomas. Results had to be returned within 30 days.

Results: High technical reproducibility was observed, with a median pairwise correlation of 0.99 (range 0.94–0.99) between coordinating laboratory and participants. DNA methylation-based tumour classification and copy number profile interpretation were consistent across all centres, and *BRAF* mutation status was accurately reported for all cases. Eleven out of 12 centres successfully reported their analysis within the 30-day timeframe.

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Conclusion: Our study demonstrates remarkable concordance in DNA methylation profiling and profile interpretation across 12 international centres. These findings underscore the potential contribution of DNA methylation analysis to the harmonisation of brain tumour diagnostics.

KEYWORDS
CNS tumour, DNA methylation, interlaboratory comparison trial

INTRODUCTION

Diagnostic pathology plays a crucial role in cancer treatment as it helps guide therapeutic decisions and identifies additional treatment options. Inaccurate or wrong diagnoses significantly hinder optimal patient care and can lead to either under- or overtreatment. Therefore, ensuring the harmonisation of pathological diagnoses across laboratories is of utmost importance. Several factors may influence such diagnostic harmonisation, with interobserver variability (subjectivity in interpreting results) and the technical reproducibility of procedures across laboratories likely being the most relevant.

In particular, the long-recognised challenge of interobserver variability among pathologists on histological criteria for diagnostic precision [1, 2] appears even more pressing in the context of paediatric cases [3, 4]. In recent years, DNA methylation-based tumour classification has emerged in neuro-oncology and other cancer fields [5–8]. This approach typically employs machine-learning classifiers to categorise genome-wide methylation patterns of diagnostic samples against a central reference cohort. This ensures that all diagnostic samples are consistently compared to the same reference, aiming to mitigate the impact of interobserver variability (although the interpretation of classifier score values can still involve a degree of subjectivity). The feasibility of implementing this approach into pathological laboratories has been explored by several diagnostic centres [2, 9–11]. Consequently, the current World Health Organisation (WHO) Classification of Tumours of the Central Nervous System recognises the significance of DNA methylation profiling. The majority of WHO-recognised tumour types have been shown to exhibit specific DNA methylation profiles (e.g., *MYB*- or *MYBL1*-altered diffuse astrocytoma, high-grade astrocytoma with piloid features or diffuse leptomeningeal glioneuronal tumour) [12–15].

Far less research has been conducted on the technical reproducibility of DNA methylation profiling across laboratories. In the initial publication of the most commonly used brain tumour classifier, a selection of samples was analysed in two research laboratories with broad agreement [2]. However, a study specifically investigating the multicentre reproducibility and technical stability of the method has yet to be undertaken.

One of the objectives of the LOGGIC Core BioClinical Data Bank (LOGGIC Core) registry is to enhance diagnostic accuracy for paediatric low-grade gliomas (pLGG) and thus harmonise molecular inclusion criteria for clinical trials in pLGG [16]. With this objective, we organised an international laboratory comparison trial involving one lead laboratory and 12 participating diagnostic laboratories, utilising centrally extracted DNA from four different low-grade gliomas. The participating centres were tasked with conducting DNA methylation profiling and *BRAF* mutation analysis and reporting on the molecular results, within a 30-day timeframe in a blinded manner. Additionally, the centres were also instructed to report copy number changes of diagnostic significance, as the information in the form of copy number profiles can help identify further molecular traits of a tumour, such as amplifications or homozygous deletions (e.g. homozygous *CDKN2A/B* deletions), specific chromosomal patterns (e.g., 1p/19q co-deletions, chromosome +7/−10), focal duplications (e.g., as frequently observed in *BRAF* fusions) or others [17, 18]. Many of these changes are of high diagnostic as well as prognostic and therapeutic relevance for brain tumours.

Key Points

- Prospective laboratory comparison trial shows remarkable concordance in DNA methylation profiling and profile interpretation across 12 international centres.
- DNA methylation is a highly technical reproducible method. which it could help with the harmonisation of brain tumour diagnostics.

MATERIAL AND METHODS

Sample selection

Four tumour samples with prototypical molecular alterations and sufficient material representing relevant tumour types for differential diagnosis of pLGG were identified in the archives of the Institute of Neuropathology at Charité—Universitätsmedizin Berlin (coordinating centre): one pilocytic astrocytoma (PA) with *KIAA1549::BRAF* fusion, one pleomorphic xanthoastrocytoma (PXA) with *CDKN2A/B* homozygous deletion and BRAF p.V600E mutation, one IDH-mutant and 1p/19q co-deleted oligodendroglioma (O-IDH) and one *MYB*/*MYBL1*-altered diffuse astrocytoma. All four tumours were resected at the Charité - Universitätsmedizin Berlin. The selected cases included three female and one male patient. DNA methylation profiling using the 'Brain Tumour Classifier' v11.b4 (www.molecular-neuropathology.org) at the coordinating centre in Berlin demonstrated

that all cases had high calibrated class scores (all 0.99) for their respective tumour class [2].

Participating centres

The participating laboratories were from the following centres: Hospital Sant Joan de Déu (Barcelona, Spain), Universitätsspital Basel (Basel, Switzerland), Rigshospitalet (Copenhagen, Denmark), Beaumont Hospital (Dublin, Ireland), UZ Leuven (Leuven, Belgium), Sapienza Università di Roma (Rome, Italy), Children's Cancer Institute (Sydney, Australia), Princess Maxima Center (Utrecht, Netherlands), Medical University of Vienna (Vienna, Austria), University Hospital in Motol (Prague, Czech Republic), Great Ormond Street Hospital (London, UK) and Oslo University Hospital (Oslo, Norway). At the time of planning of the laboratory comparison trial, all participants had already established DNA methylation analysis in their diagnostic process and wanted to qualify as a molecular reference centre in their respective countries within the LOGGIC Core. LOGGIC Core was registered with the German Clinical Trial Register, number DRKS00019035. For further details on LOGGIC Core, please refer to Hardin et al. [16]. To be included in the laboratory comparison trial, each centre had to fulfil a list of minimum requirements (Data S1). These referred primarily to their capacity to generate DNA methylation data and testing for *BRAF* mutation within 30 calendar days as well as having the local expertise for histopathological diagnostics, performing standard quality control, interpreting the classification score of the generated data and identifying copy number alterations.

Study design and data analysis, sample preparation

The Institute of Neuropathology at Charité-Universitätsmedizin Berlin, which also serves as the German National Reference Center for pLGG, acted as the coordinating centre (Figure 1). For each of the four cases, multiple DNA extractions from formalin-fixed, paraffin-embedded (FFPE) tissue were performed according to the manufacturer's instructions (Maxwell RSC FFPE Plus DNA Purification Kit, Custom, AX4920, Promega, USA) and pooled. For each case, all participating centres received an aliquot containing 1 µg DNA in a 30 µL end volume.

The study included the prior submission of a predefined structured questionnaire by the participating centres (see Data S1). DNA was sent out sequentially to the 12 centres between 07.2020 and 12.2023. The participating centres did not receive further information such as patient age, tumour location or coordinating centre diagnosis. The following information was requested from the participating centres as part of the final report: (1) The exact scores of local DNA methylation-based classification (classifier output), (2) the local interpretation of the classifier output, (3) the interpretation of the locally generated copy number profiles for all alterations that were locally regarded as diagnostically relevant [18] and (4) the result of *BRAF* mutation analysis. The reports had to be returned within 30 calendar days from receiving the DNA aliquot.

All participating sites performed array-based DNA methylation analysis and *BRAF* mutation analysis based on established local protocols. For the DNA methylation analysis, all centres used the Infinium MethylationEPIC v1.0 BeadChip Array Platform from Illumina. All centres performed DNA methylation data analysis using the machine-

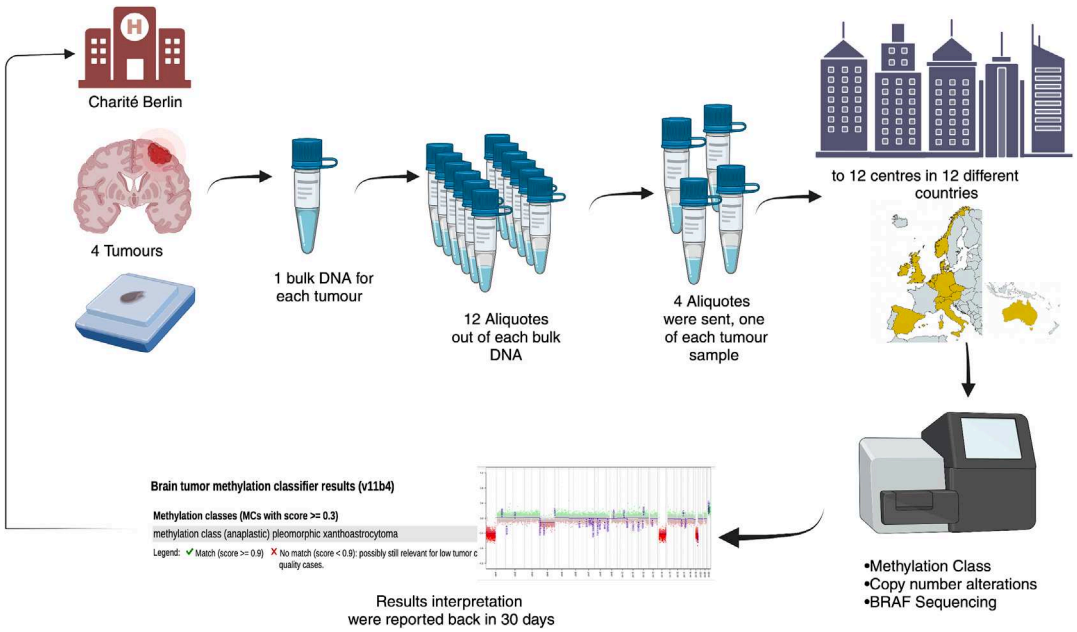

FIGURE 1 Illustration outlining the design of the interlaboratory comparison trial. A bulk DNA sample was extracted for each of the four samples, and each centre received an aliquot for analysis. The findings regarding tumour classification, pertinent copy number alterations and BRAF status were required to be submitted to the coordinating centre within 30 calendar days.

learning algorithm described by Capper et al. [2]. After analysis and interpretation, the participants submitted their reports and raw data to the coordinating centre for central evaluation. The coordinating centre calculated mean detection *p*-values for all raw data sets. Data analysis at the coordinating centre was performed using RStudio Server version 1.4.1717 using the R/Bioconductor package minfi (version 1.38.0). Copy number profiles were derived from raw DNA methylation data using the R/Bioconductor package conumee (version 1.26.0) [17].

RESULTS

Case selection and internal analysis

In this prospective, multinational laboratory comparison trial four representative low-grade gliomas were selected. The cases exhibited a variety of molecular features, enabling an assessment of the participating centres' capabilities in performing DNA methylation analysis, copy number interpretation and *BRAF* mutation analysis. To determine the most suitable region for DNA extraction, we re-evaluated the histology of all four cases (Figure 2). Before dispatching the samples, a secondary methylation analysis was conducted on the pooled DNA. Additionally, a *t*-distributed stochastic neighbour embedding (t-SNE) was generated to confirm whether the samples were classified as anticipated.

The copy number analysis (Figure 2) unveiled specific findings for each case: a *BRAF* duplication in the case of PA, a homozygous deletion of *CDKN2A/B* for PXA, a typical 1p/19q co-deletion for the oligodendroglioma and a partial loss of chromosome 6—without involvement of the *MYB* gene—for the *MYB*-associated diffuse glioma.

Analysis of DNA methylation by participating centres

The results of the DNA methylation analysis for all participating centres are summarised in Table S1. Despite the participants being

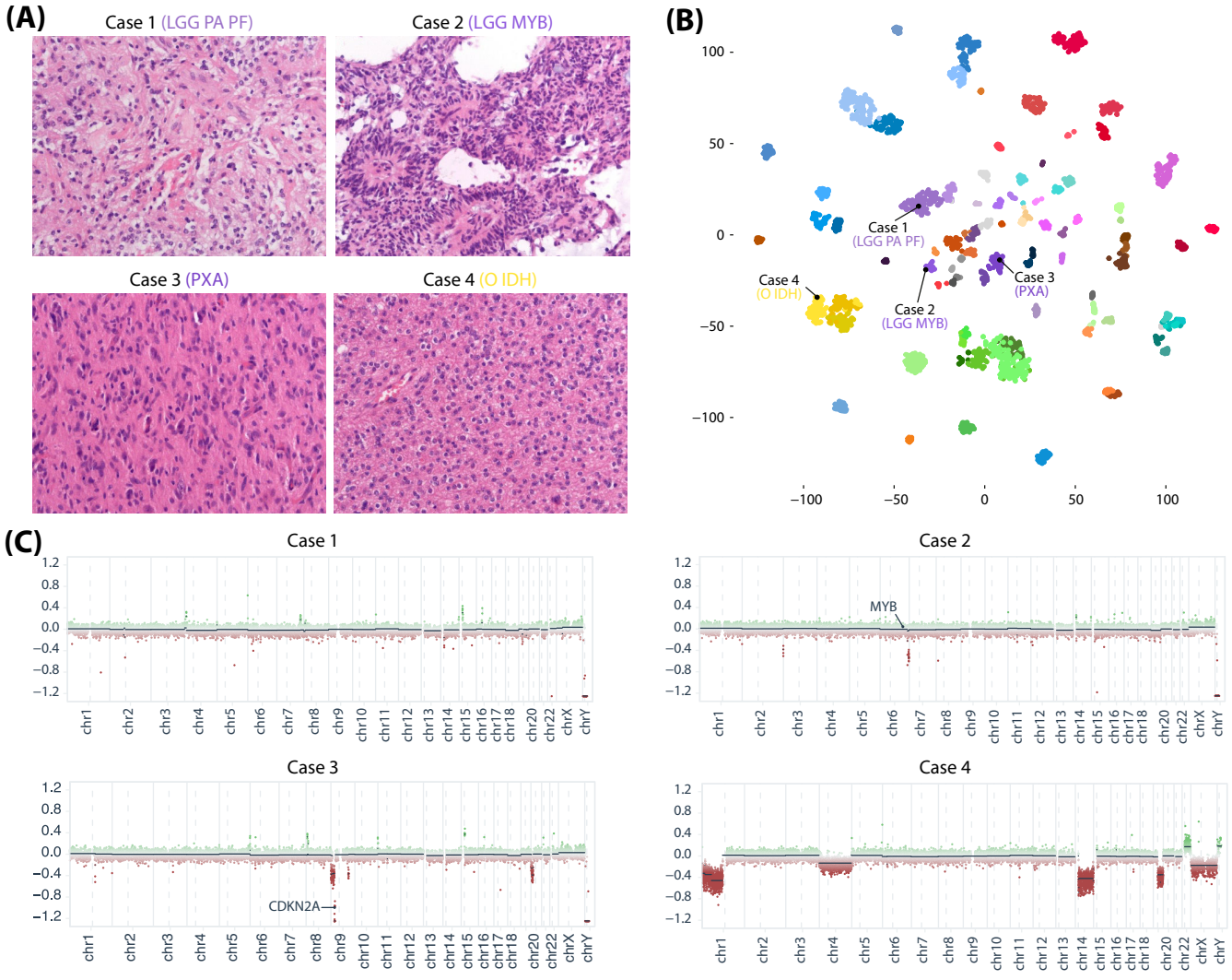

FIGURE 2 (A) Representative haematoxylin and eosin stained images illustrating the histopathology of the four test cases. (B) t-Distributed stochastic neighbour embedding (t-SNE) representation of the four test cases mapped against the brain tumour classifier cohort [2]. (C) Copy number profiles calculated from the DNA methylation data, with relevant molecular features marked (Case 1: BRAF duplication; Case 2: partial loss of chromosome 6 near MYB locus; Case 3: homozygous deletion of CDKN2A/B; Case 4: 1p/19q co-deletion).

distributed across 12 different countries and having access to various laboratory pipelines and local expertise, all centres accurately identified the methylation class for each of the four tumours. The time required for data generation varied from 11 to 30 days, except for one centre that faced delivery difficulties due to the COVID-19 pandemic and could not obtain the Illumina arrays on time. We acknowledged this as an external factor beyond their control.

The calibrated score was 0.99 across all centres and samples in 45 out of 48 measurements (94%) with the remaining three measurements having a score of 0.94, 0.95 and 0.96, respectively. These were not related to one centre or a specific sample. The highest score was identical across all centres, indicating a unanimous classification of all tumours with a calibrated score well above the required cut-off of $> = 0.9$ [19].

The mean detection *p*-value, serving as a quality control measure for the signal reliability of the samples, was consistently <0.01 across all cases and centres. This suggests a consistently high technical quality of the generated DNA methylation data throughout all participating centres.

Copy number profiling and *BRAF* sequencing by participating centres

All centres generated highly comparable copy number profiles. For PA, all centres (12/12) identified a *BRAF* duplication in copy number analysis and no *BRAF* mutation (refer to Table 1 and Table S1). In the case of PXA, all centres (12/12) correctly identified a *CDKN2A/B* deletion in the copy number profile and a *BRAF* p.V600E mutation. The molecular profile of the oligodendroglioma case was also accurately interpreted by all centres (12/12), identifying a chr 1p/19q co-deletion and a *BRAF* wild-type sequence. Lastly, for the *MYB*-associated diffuse glioma, the coordinating centre did not identify copy number changes of diagnostic significance but noted the presence of a partial loss of chromosome 6q. This was also reported by nine centres (9/12), whereas three centres detected no alterations (3/12), and all centres reported a *BRAF* wild-type sequence (12/12).

Correlation analysis of coordinating centre and participating centre results

We further conducted a pairwise correlation analysis across all CpG DNA methylation sites on the DNA methylation array between the coordinating centre and the participating centres (Figure 3A). The median pairwise correlation was nearly perfect (0.99; range 0.94–0.99), indicating an exceptionally high level of technical reproducibility across all centres. In an additional clustering analysis of the CpG sites utilised by the Brain Tumour Classifier v11.b4 for all samples from all centres (Figure 3B), we observed that the same specimens consistently fell into a shared cluster, regardless of the laboratory where the analysis was conducted.

DISCUSSION

In this study, we present the results of a prospective, international interlaboratory comparison trial involving 12 diagnostic centres and a coordinating centre, assessing the technical stability and reproducibility of diagnostic DNA methylation profiling under clinical real-time conditions. Our findings indicate that, when provided with identical

TABLE 1 Interpretation of copy number profiles and *BRAF* mutation analysis.

	Analysis	Coordinating centre	Participating centres (N)	Centres with correct results
Case 1 (PA)	Copy number analysis	Diagnostically relevant: BRAF duplication	12	12
	BRAF sequencing	wt	12	12
Case 2 (MYB-associated diffuse glioma)	Copy number analysis	Diagnostically relevant: none. Observation of partial loss of chromosome 6	12	12 (9 additionally reported partial loss chromosome 6)
	BRAF sequencing	wt	12	12
Case 3 (PXA)	Copy number analysis	Diagnostically relevant: CDKN2A/B homozygous deletion	12	12
	BRAF sequencing	BRAF p.V600E	12	12
Case 4 (O-IDH)	Copy number analysis	Diagnostically relevant: 1p/19q co-deletion	12	12
	BRAF sequencing	wt	12	12

Abbreviations: O-IDH, oligodendroglioma; PA, pilocytic astrocytoma; PXA, pleomorphic xanthoastrocytoma.

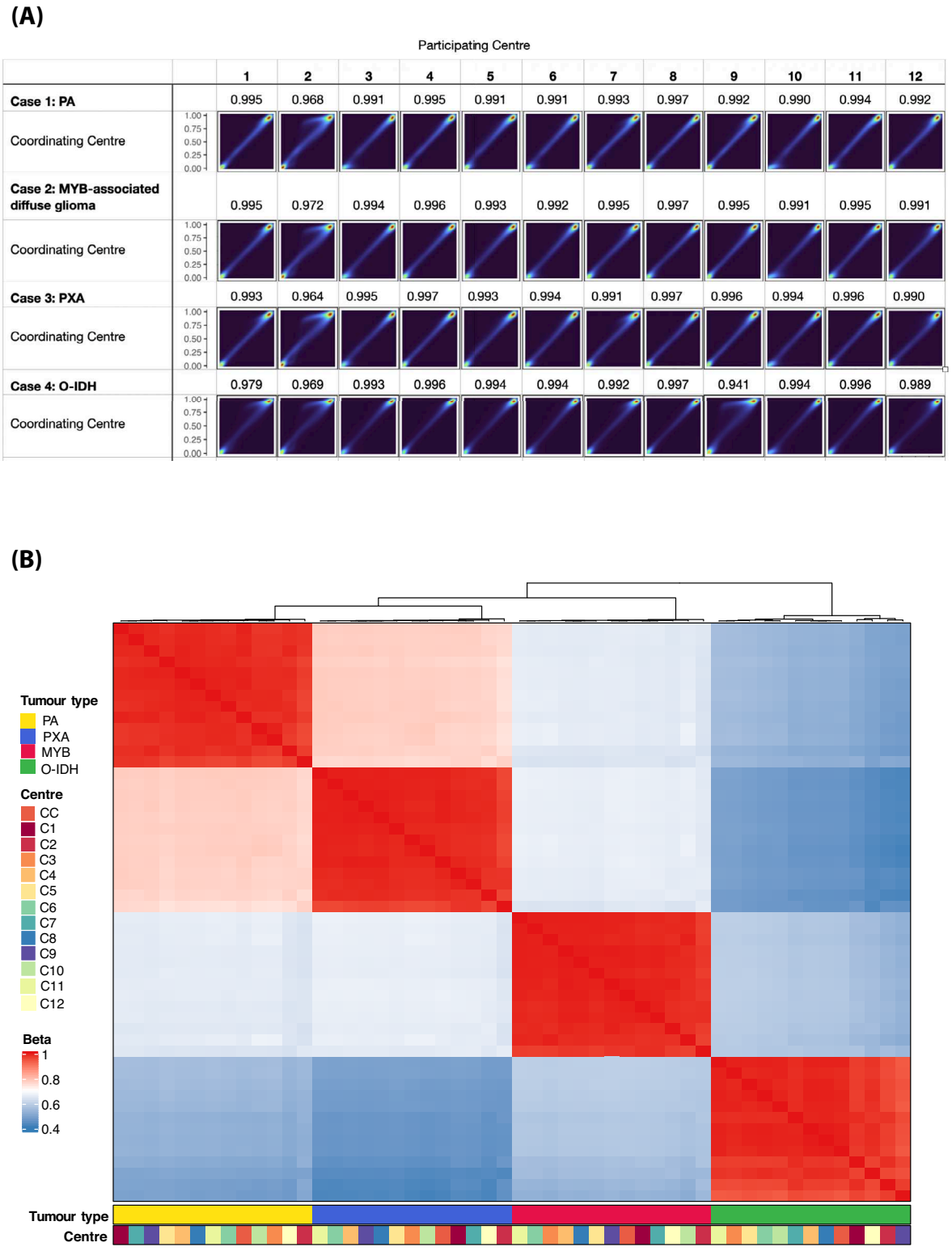

FIGURE 3 (A) The pairwise correlation coefficients between the coordinating centre and the participating centres for all four samples indicate a high level of concordance. The heatmap (B) illustrates the results of a correlation analysis across all centres using the CpG sites selected by the random forest algorithm in the Heidelberg brain tumour classifier v11.b4. This highlights that tumours are consistently grouped together, regardless of the centre where the data was generated (C, centre; CC, coordinating centre).

DNA samples, different testing centres consistently produce highly homogeneous results both in terms of generated data and their interpretation. Given that the application in routine diagnostics necessitates a rapid and reproducible sample classification, a 30-day timeframe for analysis was a stipulated requirement. Eleven centres successfully reported results within this timeframe, with one

centre missing the deadline due to the COVID-19 pandemic. All reported mean detection *p*-values were <0.01, indicating a high quality of the generated data, and all centres accurately classified the tumour methylation class using the brain tumour classifier with high calibrated scores. This underscores the robustness of DNA methylation profiling as a diagnostic tool, supporting international diagnostic harmonisation [2]. Since the commencement of this laboratory comparison trial, an updated version of the DNA methylation array ("EPIC v2") has been released. Additionally, an updated classification algorithm, which includes a larger number of classes and adaptations for compatibility with EPIC v2 array data, has been developed (unpublished data). Despite these updates, the technical pipeline for generating array data and the core concept of the classifier remain unchanged. Consequently, the results of this laboratory comparison trial are likely applicable to the newer array version and can provide valuable guidance for the design of future laboratory comparison trials.

In this study, our goal was to assess the technical stability of DNA methylation data generation and its interpretation. The selection of the tumour area for DNA extraction is another factor that is likely to influence reliable classification, given that DNA methylation profiling relies on a high tumour cell content [2]. In this initial study, we deliberately omitted this crucial aspect of the diagnostic process. Including it at this stage would have made it challenging to distinguish between technical factors in the data generation pipeline and issues related to tumour area selection in cases of discrepancies or low scores. Now that our data have demonstrated remarkable interlaboratory technical stability, it appears feasible to build on our observations and to incorporate the DNA extraction process, as well as a larger sample cohort, in future comparison trials for DNA methylation profiling.

One significant challenge hindering the harmonisation of copy number evaluation is the absence of defined cut-off values for gains and losses of chromosomal aberrations. Conversely, defining cut-off values is exceedingly difficult, given that the intensity scores of the copy number profile depend on the tumour cell content, which may vary dramatically between samples of the same tumour type. Consequently, the interpretation of copy number alterations relies on personal experience and, thus, is subjective. Drawing lessons from histopathology, we understand that this subjectivity may reduce the reproducibility of the diagnostic method. In our evaluation of copy number assessment, instead of specifying certain copy number alterations, we formulated the requirement that 'diagnostically relevant copy number alterations should be reported'. With this somewhat vague directive, all centres correctly identified the sought-after chromosome 1p/19q co-deletion, *BRAF* duplication and homozygous *CDKN2A/B* deletion. However, the situation was less clear for the *MYB*-associated diffuse glioma, which exhibited a focal deletion on chromosome 6q close to the *MYB* locus. This may not be 'diagnostically relevant' in the traditional sense, and accordingly, this alteration was only reported by some participants. It is conceivable that the continued development of bioinformatic algorithms will contribute to the task of harmonising the assessment of copy number alterations [19].

This study was conducted in the framework of the LOGGIC CORE BioClinical Data Bank registry, which was set up in 2019 as a multinational registry for prospective collection of clinical and molecular diagnostics data [16]. In addition, interventional clinical trials conducted by the LOGGIC Consortium require patients to be enrolled in LOGGIC Core to ensure quality controlled and harmonised molecular data from all trial subjects for patient stratification and correlative biology studies. Thus, the data presented here are highly supportive of using DNA methylation data as part of the inclusion criteria in a multinational clinical trial setting.

In conclusion, our study demonstrates that DNA methylation is a highly stable and reproducible method, both technically and in terms of data interpretation. These findings emphasise the potential substantial impact of objective DNA methylation-based classification values as one element supporting the standardisation of CNS tumour diagnostics, indicating that future endeavours should concentrate on enhancing the accessibility of this diagnostic method.

AUTHOR CONTRIBUTIONS

David Capper, Olaf Witt and David T. W. Jones designed the study. Mihaela Chirica wrote the initial manuscript. David Capper, Daniel Teichmann, Mihaela Chirica, Simone Schmid, Arend Koch, Eilís Perez, Michèle Simon and Pablo Hernáiz Driever selected the study cases and performed the internal analysis. David Capper, Mihaela Chirica, Philipp Jurmeister and Eilís Perez analysed the data. Mihaela Chirica and Carina Bodden coordinated contact with participating centres. Cornelis M. van Tilburg, Emily C. Hardin, Cinzia Lavarino, Jürgen Hench, David Scheie, Jane Cryan, David Scheie, Ales Vicha, Francesca R. Buttarelli, An Michiels, Christine Haberler, Paulette Barahona, Bastiaan B. J. Tops, Tom Jacques and Tore Stokland generated and interpreted participating centre data. David Capper, Mihaela Chirica and Philipp Jurmeister performed central data analysis and interpretation. All authors reviewed and approved the final manuscript.

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CONFLICT OF INTEREST

The Editors of Neuropathology and Applied Neurobiology are committed to peer-review integrity and upholding the highest standards of review. As such, this article was peer-reviewed by independent, anonymous expert referees, and the authors (TSJ) had no role in either the editorial decision or the handling of the paper. D. Capper and D.T.W. Jones are co-founders and shareholders of Heidelberg Epignostix GmbH. C. van Tilburg: Advisory boards Alexion, Bayer and Novartis. OW: Advisory Boards of Novartis, Bayer, Roche, Astra Zeneca, BMS, Janssen as well as research support from BioMed Valley Discoveries, Day One Biopharmaceuticals. All other authors declare no conflicts of interest.

PEER REVIEW

The peer review history for this article is available at https://www.webofscience.com/api/gateway/wos/peer-review/10.1111/nan.13010.

DATA AVAILABILITY STATEMENT

The data that support the findings of this study are available from the corresponding author upon reasonable request.

ETHICS STATEMENT

The usage of the samples for the interlaboratory comparison trial was approved by the local ethics committee (EA2/171/21).

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SUPPORTING INFORMATION

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

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