cIMPACT-NOW Update 9: Recommendations on Utilization of Genome-Wide DNA Methylation Profiling for Central Nervous System Tumor Diagnostics

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Abstract

Genome-wide DNA methylation signatures correlate with and distinguish central nervous system (CNS) tumor types. Since the publication of the initial CNS tumor DNA methylation classifier in 2018, this platform has been increasingly used as a diagnostic tool for CNS tumors, with multiple studies showing the value and utility of DNA methylation – based classification of CNS tumors. A Consortium to Inform Molecular and Practical Approaches to CNS Tumor Taxonomy (cIMPACT-NOW) Working Group was therefore convened to describe the current state of the field and to provide advice based on lessons learned to date. Here, we provide recommendations for the use of DNA methylation-based classification in CNS tumor diagnostics, emphasizing attributes and limitations of the modality. We emphasize that methylation classifier is one diagnostic tool to be used alongside previously established diagnostic tools in a fully integrated fashion. In addition, we provide examples of the inclusion of DNA methylation data within the layered diagnostic reporting format endorsed by the World Health Organization (WHO) and the International Collaboration on Cancer Reporting. We emphasize the need for backwards compatibility of future platforms to enable accumulated data to be compatible with new versions of the array. Finally, we outline the specific connections between methylation classes and CNS WHO tumor types to aid in the interpretation of classifier results. It is hoped that this update will assist the neuro-oncology community in the interpretation of DNA methylation classifier results to facilitate the accurate diagnosis of CNS tumors and thereby help guide patient management.

Keywords: DNA methylation profiling, CNS tumors, Molecular classification, Neuropathology

Key points

- DNA methylation profiling is a valuable tool for the diagnosis of CNS tumors
- Results should be interpreted within the entire context of the case
- Inclusion of methylation results into a layered diagnostic format is recommended

Introduction

DNA methylation profiling has emerged as a practical platform for the classification of central nervous system (CNS) tumors. Since the initial description of its potential across CNS tumor types and development of a classifier by the Heidelberg group in 2018 [1], use of this diagnostic strategy has expanded to additional centers. Notably, The World Health Organization (WHO) 2021 Classification of Tumours of the Central Nervous System (WHO CNS 'Blue Book') [2] highlights the utility of DNA methylation profiling by indicating this modality as either an "essential" or "desirable" diagnostic criterion in a large proportion of tumor types. Key attributes of DNA methylation profiling include the generation of objective, reproducible and transferable data metrics that contribute to more precise and accurate tumor classification, prognostic information (in some tumor types), and the potential to discover new tumor types/subtypes.

With the increasing use of DNA methylation-based classification in clinical diagnostics, and in line with previous updates,[3-10] the Consortium to Inform Molecular and Practical Approaches to CNS Tumor Taxonomy (cIMPACT-NOW) assembled a group of experienced neuropathologists and clinical neuro-oncologists to provide recommendations regarding the use of genome-wide DNA methylation profiling in CNS tumor diagnostics. These recommendations, based on the literature and collective experience, relate to methylation classifier results in daily practice, observed attributes and limitations of the classifier, and selection of cases for methylation profiling.

General principles and practice of DNA methylation profiling for CNS tumor diagnostics

DNA methylation profiling is based on the empirical observation that patterns of DNA methylation across the genome is correlated with tumor type in a reproducible manner. Epigenetic changes occurring because of specific genomic alterations can also influence the DNA methylation profile of a neoplasm. By interrogating that pattern on a given sample, and by then comparing the findings to a reference set of defined methylation classes, the closest 'match' can be determined, along with a confidence score. Therefore, the reference set is a defining feature of a classifier, since it determines what methylation-defined groups can or cannot be classified. The selection of cases and groups to be included in the reference set is of primary importance for the characteristics of a classifier. A major step forward was the publication of the initial CNS tumor classifier in 2018, spearheaded by a team from Heidelberg, Germany, where the first reference set was defined for 82 tumor methylation classes and 9 non-neoplastic control tissue classes [1]. The reference set data for this classifier was made publicly available. Since that time subsequent classifiers have been developed by the Heidelberg team, the most recent of which (Version 12.8) has 184 classes. The publication of this new version in a scientific journal is currently in preparation. A complementary classifier (NCI/Bethesda classifier) is in clinical use in some institutions. A manuscript describing the NCI/Bethesda classifier and its classes is in preparation (Aldape et al., in preparation). It is noted that while some classes represent well-established CNS WHO tumor types, other classes are less well-defined, often delineated based on the identification of a new group emerging from an ever-growing dataset. In that sense, it is understood that each classifier is a 'data freeze' at a specific time point, of an essentially dynamic process in the development and annotation of methylation-defined groups as they emerge.

In terms of a DNA methylation profiling platform, the most used platform, and with which the initial Heidelberg classifier was developed, is based on high-throughput methylation arrays. The initial classifier was based on the Infinium HumanMethylation450 BeadChip or '450k array', so named because it had approximately 450,000 interrogated CpG sites on the array. Subsequent iterations of this array (version 1 and version 2 of the 'EPIC' array) have increased the total number of CpG sites but decreased the number of common interrogatable probes (present on all 3 versions of the array: 485512 in 450K, 452822 in 450K/EPICv1, and 394380 in 450K/EPICv1/EPICv2). Consequently, this has necessitated to re-train and re-validate the classifiers for every microarray release in a backwards compatible manner so that previously established reference case cohorts could still be used. The rationale for this approach is that it allows the large number of samples profiled with older array versions to contribute to the reference set and classifier. A relevant and practical consideration is that a comprehensive reference set requires the inclusion of multiple samples per class, which can be a challenge with respect to the accrual of rare tumor types and methylation classes. Of note, additional analytical issues for the classifier arise when a new array version drops off chemically modified probes from a previous version of the array or changes the probe chemistry for a particular genetic location.

Following the determination of the reference set, classifiers are then developed using machine learning. The original Heidelberg classifier used a Random Forest algorithm [1]. Alternative machine-learning algorithms and classifier approaches have also been proposed [11,12]. Classifier performance may differ depending on the specific machine learning algorithm, but these differences are likely modest compared to the quality and diversity of the reference set in determining the overall classifier performance. With respect to application of the classifier to tumor diagnostic practice, the experience from multiple centers indicates improved diagnostic accuracy following the use of DNA methylation–based classification [13-16], an opportunity that may be further enhanced using multi-omic profiling [17]. Beyond its application to routine diagnostics, methylation profiling also has the potential to discover new tumor types that were

not (yet) discernable using more conventional pathological approaches. As an example, *High-grade astrocytoma with piloid features* (HGAP, recognized in the 2021 WHO classification) was discovered in this way [18]. The use of methylation profiling has further led to an appreciation of clinically relevant subtypes of recognized histologic entities (for example medulloblastoma and ependymoma) where methylation classes can define them [19,20]. Further attributes of DNA methylation profiling include the ability to determine the promoter methylation status of specific genes (for example methylguanine methyltransferase [*MGMT]* [21]), as well as in the determination of genome-wide DNA copy number profiles, particularly helpful for the diagnosis of tumor types characterized by stereotypical DNA copy number aberrations (e.g., *Oligodendroglioma, IDH-mutant and 1p/19q-codeleted*). Finally, results of methylation profiling can be an important component of teaching/case review for trainees, in a manner similar to the inclusion and integration of other types of molecular data for diagnostics. By establishing additional evidence to support a specific diagnosis, cases confirmed by methylation profiling used for training purposes can be chosen that are relatively impervious to differences of opinion; this can be especially important for uncommon and rare tumor types. It may also serve as a diagnostic aid in regions of the world with a scarcity of well-trained neuropathologists. To this end, a consortium is being launched to help systematically build capacity in low- and middle-income countries. That said, it is expected that the availability of methylation profiling will likely be a challenge, for a variety of reasons, at many centers in the foreseeable future.

Methylation classes and CNS WHO-defined tumor types

Relevant to reporting of methylation profiling results is knowledge of the relationships of methylation classes, as defined in the Heidelberg classifier, to defined CNS WHO tumor types. While methylation classes generally track with WHO-defined CNS tumor types, they do not always do so in a one-to-one

correlation. As an example (mentioned above), two methylation classes in the Heidelberg classifier correspond to *Astrocytoma, IDH mutant*, and five methylation subtypes correspond to *Supratentorial ependymoma, ZFTA fusion-positive*. In other examples, the classifier name may not always fully represent the corresponding matching tumor types. For example, the methylation class Subependymoma, posterior fossa includes unequivocal posterior fossa subependymomas, but also tumors with unequivocal ependymoma histopathology, a situation that is currently being addressed by an independent cIMPACT-NOW Working Group. The "Melanocytoma" methylation class includes *Melanocytoma*, but also primary melanocytic tumors of higher grade. A practical consequence to this may occur when a recipient of a pathology report sees a match to the 'melanocytoma' family when the tumor is actually a melanoma, which can be initially confusing to those less experienced with CNS methylation profiling results. Some CNS WHO-defined tumor types do not have identified methylation classes, including *Diffuse low-grade glioma, MAPK pathway-altered*, and *Embryonal tumors NOS/NEC*, both of which are a 'basket' of tumors with diverse biological features. Additional examples include *Multinodular and vacuolating neuronal tumor* and *Dysplastic cerebellar gangliocytoma (Lhermitte-Duclos disease)*, which may lack distinctive methylation signatures relative to control brain tissues. Such tumors may not yet have sufficient methylation profiling data available to be included in a classifier, but it is not clear yet whether such tumors correspond to defined methylation classes. *Chondrosarcoma* and *Mesenchymal chondrosarcoma* are WHO CNS tumor types that are not represented in the Heidelberg CNS classifier but that are represented in a separate sarcoma classifier [22]. Finally, many of the tumors described in the "Genetic tumour syndromes involving the CNS" of the 2021 CNS WHO and other tumors resulting from cancer predisposition syndromes are not represented well in the classifier and specific examples of such tumors may have distinct methylation signatures when compared to their sporadic counterparts [23].

Conversely, a substantial number of methylation classes exist that do not correspond to a current CNS WHO tumor type, many of which have been recently described in the literature by virtue of distinctive methylation profiling signatures as well as additional defining features. Examples include "Adult-type diffuse high grade glioma, IDH-wildtype, subtype F", where tumors matching to this class were given a proposed name of "Gliomatosis Cerebri-like Glioma, IDH-wildtype" based on clinical presentation (gliomatosis based on imaging studies), frequent *TERT* and *PIK3R1* mutations and an intermediate clinical course [24]. Another example is "Glioneuronal tumor with ATRX alteration, kinase fusion and anaplastic features", defined by both frequent ATRX loss as well as recurrent fusions, most often involving members of the NTRK family [25]. A third instance is "High-grade glioma with pleomorphic and pseudopapillary features" (HPAP), a glioma in adults with frequent *TP53* mutations and highly recurrent monosomy 13 [26], a class which is present in the NCI/Bethesda classifier (https://methylscape.ccr.cancer.gov/) but not in the Heidelberg classifier. Additional examples of recently described methylation classes of CNS tumors that are not represented by a CNS WHO tumor type exist [27-40] and development of criteria for evaluation of such novel methylation classes for inclusion in future CNS WHO classifications is the subject of a separate cIMPACT-NOW Working Group. Finally, several methylation classes exist in the Heidelberg CNS tumor classifier that are in alternative WHO classifications outside the CNS, including olfactory neuroblastoma, retinoblastomas, intraocular medulloepithelioma, and several classes representing neuroblastoma. Overall, such complexities in the relationships of methylation classes to WHO-defined tumor types can be gained with experience and interdisciplinary discussion. That said, efforts to clarify these relationships more precisely to ensure that class names are, when possible, aligned with corresponding WHO tumor type designations, are recommended. A notable issue with the Heidelberg classifier is that the CNS WHO correlate is sometimes at the level of the methylation Superfamily (e.g., *Meningioma*), sometimes at the level of the methylation Family (e.g., *Glioblastoma, IDH-wildtype*), and sometimes at the level of the methylation

Class (e.g., *Posterior fossa ependymoma group A*). A future goal may be to harmonize the terminology used for the different methylation levels with that of the CNS WHO tumor taxonomy and nomenclature to maximize clarity and facilitate integration of methylation results with CNS WHO designations. As a resource designed to clarify relationships of CNS WHO tumor types with currently defined methylation classes (and vice versa), we display this information in tabular form. Table 1 connects methylation classes in the Heidelberg CNS classifier that correspond to current CNS WHO tumor types. Table 2 shows CNS tumor methylation classes that do not correspond to a current CNS WHO tumor type. CNS WHO tumor types that are not represented in methylation classifiers are shown in Table 3.

Methylation classifier results should be integrated with appropriate clinical, radiological, histological, and genomic information.

For each tumor tested, the classifier typically assigns the neoplasm to a methylation class in the reference set that shows the most epigenetic similarity to the tumor sample being evaluated, as well as a calibrated score ranging from 0 (low-confidence) to 1 (high-confidence) [1], providing the level of confidence for the tumor being placed in that class. The tumor is generally considered a 'match' if the calibrated score is above a pre-determined threshold value. As with all diagnostic testing modalities, machine learning-based (supervised and unsupervised) classifier predictions from methylation profiling data in clinical diagnostics require critical review and integration with clinical, radiological, histological, and genomic data. A methylation profile does not replace a pathologist's opinion; rather it is intended to add additional important data that helps shape their opinion. While specific confidence score cutoffs can be used as a threshold for designating the obtained result as 'match' or 'no-match' in clinical reporting, the scores that result from the classifier are a continuum that can be used to determine contribution to a definitive diagnosis. In this respect, subthreshold matches can nevertheless be contributory to a

diagnosis, especially when ancillary data are supportive. An example is a low-confidence match to *Glioblastoma IDH-wildtype* in a low tumor purity sample from an older adult, in which ancillary data show a stereotypical +7/-10 chromosomal copy number signature as well as a *TERT* promoter mutation; in this situation, the methylation result can be seen as contributory, even in the face of a lower confidence score. Clustering and/or dimensionality reduction using t-distributed stochastic neighbor embedding (t-SNE) or uniform manifold approximation and projection for dimensionality reduction (UMAP) can also often be contributory [41], especially for tumors whose confidence scores do not reach the clinical threshold. A platform (UMAP or t-SNE) using large and well-annotated reference sets that allows embedding and visualization of the methylome profile of a tumor sample within a group can provide additional support for that class designation, even in the setting of a lower confidence score (e.g., methylscape.ccr.cancer.gov, epidip.org). Class designation using such a visualization tool is not easily standardized and great caution is required when the appropriate diagnostic class is not included in the reference set, as diagnostic cases for which the appropriate class is lacking will often fall into one of the other available cases in the UMAP or t-SNE.

Methylation class and tumor grading

The relationship of methylation class with WHO-defined tumor types that occur in multiple grades is complex. In some cases, a single methylation class accounts for multiple WHO grades. Examples include *Pleomorphic xanthoastrocytoma* and *Solitary fibrous tumor* (SFT), for which the current iteration of the Heidelberg classifier has a methylation class designation that correlates to the tumor type, but a relationship of methylation profile and tumor grade is not defined within each tumor type. Nonetheless, it remains possible that further work has the potential to elucidate prognostic methylation subgroups,

as has been shown for SFT [42]. In other instances, some relationships between methylation classes and tumor grades do exist; examples include *IDH-mutant astrocytoma* and *Meningioma*. Classes corresponding to *IDH-mutant astrocytoma* include "Astrocytoma, IDH-mutant; high grade" and "Astrocytoma, IDH-mutant; lower grade". While methylation class for IDH-mutant astrocytomas is not a grading tool *per se*, WHO grade 2 tumors tend to match to the lower-grade class, while WHO grades 3 and 4 tumors more frequently match to the high-grade class, although these correlations are not absolute. A similar situation exists with meningiomas, which have Benign, Intermediate and Malignant classes (three classes exist for Benign and two for Intermediate). In such cases, the meningioma methylation class does not determine the WHO grade but may provide additional prognostic information helpful to estimate recurrence risk [43]. A concurrent cIMPACT-NOW Working Group recently published recommendations on how molecular (including methylome profiling-based) risk parameters can be used for improved grading of meningiomas [10]. Overall, the specific relationships of tumor grade with methylation class appear to be tumor-type specific and elucidation of these relationships specifically in future classifier versions is recommended to maximize clarity.

Selection of cases for clinical diagnostic DNA methylation profiling

A variety of approaches can be taken for case selection for diagnostic DNA methylation profiling. DNA methylation can be considered as a useful first-line molecular triage for to decide what further molecular testing is needed or when the first line of immunohistochemical stains is non-informative, particularly for samples with limited tissue [13]. Experience indicates that while difficult-to-classify cases may have the highest benefit from this profiling, occasional cases can lead to unexpected findings on methylation profiling that prompt a revision of diagnostic possibilities.

- One study of a series of consultation cases sent for DNA methylation profiling found that nearly 10% of cases (15/160) with a pre-methylation diagnosis of *Glioblastoma, IDH wildtype* were given an alternative integrated diagnosis after DNA methylation-based classification [44].
- As an additional example type, it has been suggested that some methylation classes suggest a broader biology compared to a histopathology-based definition. Tumors matching to the *Pleomorphic xanthoastrocytoma* (PXA) methylation class include both histologically defined PXAs as well as tumors which do not fit this histological definition. Conversely, some histologically defined PXAs matched to classes suggesting alternative tumor types, including *Glioblastoma, IDH-wildtype* and *Ganglioglioma* [45].
- Tumors with histopathological characteristics of ependymoma, while largely matching to an ependymoma class, also can on occasion suggest alternative diagnoses, based on their methylation class results [46].
- An additional example type includes tumors that match to a methylation class that is unexpected compared to the anatomic location of the tumor. Tumors whose methylation class suggests a diagnosis of *Papillary tumor of the pineal region* have been described outside of the pineal region [47]. Occasional cases of *Supratentorial ependymoma, ZFTA fusion-positive* are encountered in the posterior fossa [48], a point to be addressed separately by another cIMPACT-NOW Working Group. Tumors matching to a medulloblastoma class have been reported in the pineal region[49].
- DNA methylation-based classification can be specifically useful when the differential diagnosis includes a tumor type that can only be diagnosed by methylation profile, such as *Diffuse glioneuronal tumour with oligodendroglioma-like features and nuclear clusters* and *High-grade astrocytoma with piloid features.*

- Tumor type definition of medulloblastomas and ependymomas (for example *Posterior fossa group A (PFA) ependymoma* and *Posterior fossa group B (PFB) ependymoma*) is efficiently performed based on methylation signatures.

Based on these considerations, cases that appear unusual, show unexpected results on ancillary studies, or which cannot be precisely classified with 'conventional' pathology diagnostics warrant the use of methylation profiling where possible. While an argument can be made for routine methylation profiling of all CNS tumors, this may not be practical in the current environment for many institutions, and decisions in routine practice need to be made in the context of test availability, resource-based considerations and local expertise.

The classification of pediatric tumors, in general, appears to benefit from methylation profiling. For instance, one study selected tumors that were deemed diagnostically uncertain after two rounds of review by neuropathology experts. In this study, DNA methylation was considered contributory to a final diagnosis in 71% of these cases [50]. A population-based study in the UK assessed the impact of DNA methylation profiling on 306 pediatric brain tumors, finding that the classifier contributed to the diagnosis in 35% of cases [51]. An additional feature of this study was an examination of misleading results, in which three (1%) cases were determined to be initially misleading, but these tumors could be readily resolved with re-review of associated clinical and ancillary data [51]. Another study focused on tumors in adult patients, which included both diagnostically difficult cases, as well as tumors for which methylation-based subtyping was required (e.g., medulloblastoma and ependymoma). In this series, only cases above the pre-specified 0.84 confidence score threshold were considered in the analysis. Among these cases, the diagnosis was changed in 45 (25%), refined in 86 (48%) and confirmed in 44 cases (25%) [52]. It is worth noting that these series were analyzed by highly experienced

neuropathologists. The expectation would be that the impact might be even higher in regions with pathologists less experienced in CNS tumor diagnostics. Additional scenarios that are observed with some frequency are histologically low-grade diffuse gliomas in older adults, where methylation profiling of such cases can at times reveal features of *Glioblastoma, IDH-wildtype*, both by virtue of a methylation match to this family as well as the presence of characteristic DNA copy number changes (e.g. +7/-10 changes and/or *EGFR* amplification). Conversely, occasional IDH-wildtype high-grade diffuse gliomas are encountered where *Glioblastoma, IDH-wildtype* is considered in the differential diagnosis, but where such accompanying genomic changes are not observed. Such cases can at times be resolved into alternative diagnoses (for example, *Diffuse paediatric-type high-grade glioma, H3-wildtype and IDH-wildtype* or *Diffuse hemispheric glioma, H3 G34-mutant*).

Special cases exist in which the diagnosis may not be in question, but the methylation profiling data may provide additional information As mentioned above, another use of methylation profiling is in the determination of prognostic subclass in meningiomas [43]. Yet another additional use of the methylation array is in the determination of copy number status such as the status of 1p/19q in glial neoplasms for the diagnosis of *Oligodendroglioma, IDH-mutant and 1p/19q-codeleted* [53]. Additional examples exist, including the identification of *MYCN* amplification in subsets of pediatric-type high-grade gliomas and ependymomas [54-56]. This technique can also be used as a single tool to obtain synchronously different molecular data as copy number variations (CNVs--deletions, amplifications), although it is noted that the resolution of CNVs from methylation data is not as high as it is from dedicated copy number platforms. Determination of *MGMT* promoter methylation status in *Glioblastoma, IDH-wildtype* [21], is important because the benefit from temozolomide is likely to be lower if their tumors can be shown to be unambiguously unmethylated at the *MGMT* promoter [57]. *MGMT* promoter methylation status is also readily obtained from the methylation array data [21,58,59], which is particularly useful when tumoral material is limited.

While both methylation profiling and NGS provide valuable information, a blanket recommendation cannot be made in the choice of one over the other when tumor tissue is limiting, and decisions are best made on a case-by-case basis. DNA methylation profiling generally provides a wider range of possibilities for challenging cases, but NGS can be helpful to rule in or rule out specific diagnostically meaningful alterations. Given challenges of DNA methylation profiling in a setting of low tumor purity, NGS may be more generally informative in such cases. In addition, precise delineation of low grade glial/glioneuronal tumors can be a challenge for methylation-based classification, a consideration which may influence the choice of molecular platforms to provide helpful diagnostic information. While not all cases can be resolved, collectively, the evidence from multiple studies and institutions demonstrates the value and contribution of DNA methylation-based classification in many, but not all, CNS neoplasms encountered in daily practice, especially for cases that either require subtyping or cases in which the diagnosis cannot be readily resolved using other diagnostic modalities.

An additional consideration arises when the tumor with a high confidence score matches to a methylation class of a tumor type whose definition requires the determination of a specific genomic alteration. A relevant example is a finding of a Supratentorial ependymoma, ZFTA fusion-positive class on methylation. While the WHO CNS5 criteria require determination of the defining ZFTA fusion to make the diagnosis, there may be cases where testing for the defining fusion is not available or practical. In this situation, a final diagnosis of Supratentorial ependymoma, ZFTA fusion-positive is considered reasonable. Addition of a comment is advisable, indicating the particular circumstances of the case and notation that a determination of the defining genomic alteration is not possible, but that a diagnosis can nonetheless be rendered.

Use of additional data layers for diagnostics and molecular classification

Current methylation classifiers are based only on the methylation data *per se*, even though the methylation array data enable alternate outputs, including DNA copy number determination. Although the DNA copy number profile can be helpful diagnostically, copy number data are not formally included in currently available CNS tumor classifiers. DNA copy number profiles derived from DNA methylation profiling data can be used to orthogonally support a suspected diagnosis, which can be helpful in cases of low-confidence-score matches. High-level amplifications for specific genes/small genomic regions can also be helpful for individual cases (for example, *EGFR* amplifications in cases of *Glioblastoma, IDH-wildtype* and *C19MC* amplification in *Embryonal tumor with multilayered rosettes, C19MC-altered*). An additional use of the copy number data is the finding of breakpoints within specific chromosomal regions that suggest the presence of diagnostically helpful fusions, such as such as *KIAA1549*::*BRAF* [60]. Some limitations of DNA copy number data derived from array-based methylation profiling include the inability to detect copy number-neutral loss of heterozygosity, as well as its inability to determine ploidy levels. Still, the determination of copy number from DNA methylation data is of value in many cases, and improvements to the originally described method ('conumee') are in development [61]. Multi-omic approaches, such as leveraging methylation data with next-generation sequencing in formal classification algorithms, have been suggested to improve the ability to classify CNS tumors [17], but specific recommendations cannot be made at this time.

DNA methylation detection platforms

Currently, the methylation array is the most widely used platform for CNS tumors, and methylation array-derived data serve as the basis for all commonly used classifier reference sets. More recently alternative platforms have emerged, including the use of Nanopore technology [41,62-64] for the detection of diagnostic DNA methylation profiles, which has the potential advantage of faster turnaround time.

Next-generation sequencing (for example, following bisulfite or enzymatic conversion) may also be used in the future for DNA methylation profiling [41,63] emphasizing that technological advances may allow for alternative platforms to be used. Existing experience, including current classifier reference sets, can potentially be used for training these newer approaches, saving time and resources when new platforms are being developed and evaluated.

DNA methylation profiling results and diagnostic pathology reports

Reporting methylome results in pathology reports has recently been addressed [64], and key elements can be emphasized here. Briefly, a diagnostic pathology report that includes methylome profiling analysis should contain information on the specific classifier version(s) used, the classifier match, estimated tumor cell content, calibrated confidence score and range or threshold for positive, indeterminate and negative results. Where applicable, reporting on both the diagnostic category (for example the confidence score of a match to the methylation family "Glioblastoma, IDH-wildtype"), as well as any additional subclass determination (for example, the confidence score, in this hypothetical case, of a match to the "Glioblastoma RTK2 subclass") should be included.

A 'layered' reporting format has been suggested for CNS tumor diagnostic reports that incorporate both histologic and molecular information [65,66]. Such a format promotes the communication of relevant data and emphasizes the integration of relevant information. Because methylation profiling provides information distinct from other data sources, the inclusion of methylation classifier results (class match and confidence score) is recommended in the Molecular line of the layered report format. Suggested formats are shown with examples of a case with a high-confidence match to family and class (Figure 1); a case that showed no match on methylation-based classification but sufficient alternative/orthogonal data were available to render a definitive diagnosis (Figure 2); a case that showed a high-confidence

match to a methylation family, but not to a specific methylation class, in which a descriptive (not elsewhere classified (NEC)) diagnosis is warranted (Figure 3); and a case showing a high-confidence match to a class that does not correspond to a current CNS WHO tumor type (Figure 4). Finally, an example of a case that leverages the recent cIMPACT-NOW recommendation on meningioma [10] is shown (Figure 5). For further clarifications within a pathology report and/or the accompanying genome-wide methylation report, additional information is suggested, including the specifics of the methylation classifier used, and the matches and confidence scores within the hierarchy of the methylation classifier (e.g., Superfamily, Family, Class and Subclass, example shown in Figure 6A). Additional technical results that are recommended for inclusion include the Sample type (e.g., FFPE/frozen), Tumor cell fraction/tumor purity, Amount of DNA input, Quality of bisulfite conversion, Reference set used, Classifier/Algorithm/version used (example shown in Figure 6B).

Given the importance of integration of the methylation classifier result within the context of the entire clinical and histomolecular aspects of the case, interpretation of methylation profiling data is optimal when a neuropathologist experienced in molecular diagnostics is directly involved. One salient reason for this is that misleading methylation classifier results with a high calibrated score can occasionally be obtained, highlighting the need for an interdisciplinary tumor board interpretation by content experts who are aware of specific attributes and limitations of the classifier under use. As discussed above, knowledge of such examples from a specific classifier would clearly be of benefit in coming to an accurate diagnosis in specific cases.

Biologic and clinical significance of methylation subclasses

For several CNS tumor types, multiple methylation subclasses exist. For example, five classes exist for *Medulloblastoma, SHH-activated*, encompassing both *TP53*-wildtype and *TP53*-mutant CNS WHO types. Medulloblastoma groups 3 and 4 comprise a total of eight subclasses, and enrichment for specific genomic aberrations are described in these subclasses. As examples, of the groups 3 and 4 medulloblastoma, methylation subclass 1 generally shows a balanced CNV profile but is enriched for *OTX2* amplifications, while subclass 2 is enriched for *MYC* amplification and subclass 5 is enriched for both *MYC* and *MYCN* amplifications [67]. In SHH medulloblastomas, subclass 1 is highly enriched for *TP53* mutations [68]. As such, methylation subclasses can inform as to likelihood of corresponding genomic aberrations in medulloblastoma. Most *Glioblastoma, IDH-wildtype* fall into one of three subclasses, mesenchymal, RTK1 and RTK2. Mesenchymal glioblastomas have been shown to have increased myeloid cell compartment, including macrophages [69], and there have been suggestions of survival differences among these methylation-defined subclasses [70]. The RTK2 subclass of *Glioblastoma IDH-wildtype* is described as showing a high frequency of *EGFR* amplification in the Heidelberg classifier (https://www.molecularneuropathology.org/mnp/classifiers/14). While direct clinical translation of these glioblastoma methylation subclasses has yet to be demonstrated, they provide insights into biology that may suggest directions for future therapeutic approaches. As a practical matter, methylation profiling of *Glioblastoma, IDH-wildtype* can on occasion reveal a high-confidence match to the Glioblastoma IDH-wildtype methylation family, but the scores can split among the glioblastoma methylation subclasses. In this context, a low score for a specific glioblastoma class would not affect the integrated diagnosis. A hypothetical example of such a case, in the layered diagnostic format, is shown in Figure 7. Most cases of the *Diffuse pediatric-type high-grade glioma, H3 wildtype and IDH-wildtype* fall into three broad methylation-defined categories: Diffuse paediatric-type high-grade glioma, MYCN subtype; Diffuse paediatric-type high-grade glioma, RTK1 subtype; or Diffuse paediatric-type high-grade

glioma, RTK2 subtype[71]. The RTK1 subtype is then divided into subgroups A,B and C, and the RTK2 subtype is divided into A and B subtypes. As with the glioblastoma classes, enrichments of specific biologic events correlate with these classes. For example, the *Diffuse paediatric-type high-grade glioma, MYCN subtype* is associated with *MYCN* amplification [71], and the *Diffuse paediatric-type high-grade glioma, RTK1 subtype* is associated with both prior cranial radiation as well as patients with constitutional mismatch repair deficiency syndrome or Lynch syndrome (https://www.molecularneuropathology.org/mnp/classifiers/14). It has been noted that patients whose tumors that fall into these methylation classes are not restricted to children and adolescents [72], and can (not infrequently) occur in adults, when it is important to distinguish them from *Glioblastoma IDH-wildtype* based on genomic aberrations and biological characteristics. Currently the significance of the clinical distinction between *Diffuse pediatric-type high-grade glioma, H3 wildtype and IDH-wildtype* versus *Glioblastoma IDH-wildtype* in adult patients is not clear, although biologic differences are identified with respect to epigenomic and genomic characteristics. Overall, methylation profiling is useful to elucidate the diagnosis of *Diffuse pediatric-type high grade glioma, H3-wildtype and IDH-wildtype* in specific cases, but future work is required to establish the clinical relevance, if any, of the subclasses in this methylation family.

DNA methylation-based classifiers for tumors outside the CNS

The utility of CNS DNA methylation classification has prompted exploration of this platform for additional tumor types and organ systems. A comprehensive review of these is beyond the scope of the current work, but awareness of their existence is important. Classifiers for sarcomas [22,73] and sinonasal tumors [74] have been reported. Additional classifiers have been proposed [41,75] and are in various stages of development. Their existence may be useful in the diagnostic process of CNS neoplasms as such lesions

are occasionally encountered in neuropathology practice. For example, the class "*Malignant peripheral nerve sheath tumor*" is included in both the CNS and sarcoma classifiers, and similarly "*Olfactory neuroblastoma*" is included in both the CNS and sinonasal classifiers. In these instances, dual classifiers can potentially be leveraged in a complementary fashion to evaluate such diagnoses from methylation data. Since some classes overlap among different classifiers, the same data (methylation IDAT files) can be used for multiple applications, adding efficiencies and lowering costs. Additionally, secondary involvement of a tumor to the CNS (direct extension, metastasis) can potentially be interrogated productively using extra-CNS classifiers that may be developed in the future.

Methylation classifier limitations

Optimal interpretation of methylation classifier results for tumor diagnostics is ideally reviewed by individuals with specific expertise in CNS tumor diagnostics, as it requires content expertise as well as awareness of specific classifier characteristics. CNS tumors are highly diverse, and a precise diagnosis can require knowledge of the relevant literature and clinical, histopathologic, genomic, and epigenomics features of the tumor type at hand. This point is even more relevant with uncommon CNS tumors. Here we highlight some examples of limitations of methylation-based classification that have been commonly encountered in current practice:

- *A low content of neoplastic cells in a sample can mask the neoplastic signal in the methylation profile*. The methylation profile of a tumor sample is a composite of the cell types included, which inevitably includes non-neoplastic cells. Samples with relatively high content of non-neoplastic cells (e.g., preexistent glial cells, neurons, inflammatory/reactive cells) can lead to either low confidence matches to tumor types or matches to control/reactive CNS tissue. In this context, one must review samples for appropriate neoplastic content in selecting tissues for methylation profiling, and interpretation of methylation profiling results must occur with awareness of overall neoplastic

content in the specimen. Concurrent evaluation of the amplitudes of copy-number-alterations, if applicable, and/or sequence variant allele frequency may aid in the interpretation of tumor cell content. The Heidelberg classifier has some inbuilt quality measures in this regard, but some cases can still remain ambiguous. It is possible that the addition of deconvolution algorithms will help to resolve such cases. On a practical level, we note that some cases exist that match to the "Control tissue, reactive tumour microenvironment" or to the "Inflammatory microenvironment" classes which can be demonstrated by other means to represent neoplasms of low tumor purity. As such, cases that match to these classes are often not contributory to diagnosis. Of note, class descriptions are available for each class in the Heidelberg classifier (https://www.molecularneuropathology.org/mnp/classifiers) which can clarify the characteristics and common features of each class. For the "Control tissue, reactive tumour microenvironment" and the "Inflammatory microenvironment" classes, the descriptions of each of these 2 classes clarify this point, which highlights the need to provide such descriptions in the pathology report (as noted below) for clarity of interpretation.

- *A substantial proportion of cases that undergo DNA methylation profiling do not result in a high confidence match*. Most studies report that a subset of cases do not match with a confidence score at a pre-specified threshold range [1,44,50,76]. Reasons for 'no-match' cases vary, ranging from issues with pre-analytical variables, assay quality metrics and low tumor purity. An additional important consideration in no-match cases is whether the tumor type in question is represented in the classifier reference set since the classifier cannot determine the tumor type otherwise. Experience indicates that CNS tumors arising in the setting of predisposition syndromes (e.g., Li-Fraumeni syndrome, Neurofibromatosis type 1) more often do not match to specific classes with high confidence scores [23,51]. Still the fact that the classifier does not force each sample into a class is beneficial, since a low classifier score immediately raises a concern regarding the precise diagnosis.

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- *Glioblastoma, matching to Ganglioglioma*. Experience of some of the authors of this work indicates that occasional cases of *Glioblastoma IDH-wildtype*, especially in the setting of low tumor purity, can match (often, but not always, with low confidence score) to *Ganglioglioma* in the version 12 iterations of the Heidelberg classifier. Such cases can usually be resolved using, for example, orthogonal data as outlined above and require awareness to ensure diagnostic accuracy.
- *SMARCB1- and SMARCA4-deficient tumors (of various types) matching to Atypical teratoid/rhabdoid tumor (ATRT)*. The methylation profile of a tumor cell is a composite of cell-of-origin as well as epigenetic changes occurring as a result to specific genomic alterations [77]. Loss of *SMARCB1* (or *SMARCA4*) gene function appears to result in a profound and stereotypic epigenetic change such that non-ATRT tumor types usually match to an ATRT class (often MYC subtype) in the CNS tumor classifier. Anecdotal cases of *SMARCB1*-altered tumor types originating outside the CNS (for example, metastatic malignant rhabdoid tumors of the kidney) that match to AT/RT are identified. Caution is therefore warranted in the setting of matches or high scores for ATRT depending on the histopathological setting.
- *Classifier matches to low-grade glial/glioneuronal tumors can be ambiguous*. The methylation profiles of several WHO-defined low grade glial/glioneuronal tumors appear to be quite similar to each other, making precise distinction difficult. An example is the difficulty in distinguishing *Ganglioglioma* from *Polymorphous low-grade neuroepithelial tumor of the young* in the methylation classifier. An additional ambiguity is the finding of frequent matches to *Dysembryoplastic neuroepithelial tumor* (DNT) in cases that lack typical histologic features of this diagnosis. Whether and how this finding contributes to our understanding of DNT awaits further study. Detailed studies on these tumors indicated that diagnostic criteria of low-grade glial/glioneuronal tumors does not adequately reflect their tumor biology, and in particular the

histopathologic characteristics of this group of tumors is only partially reflective of their underlying molecular profiles [78,79]. As a solution, it has been suggested that integration of methylation and radiological data can improve diagnostic precision for these tumors [79].

- *Reproducible misclassification of Intracranial mesenchymal tumor, FET::CREB fusion-positive as meningioma.* The commonly used Heidelberg version 12 classifier does not include *Intracranial mesenchymal tumor, FET::CREB Fusion-Positive* (ICMT-FET-CREB) as a defined class, and experience indicates that most of these tumors match on the version 12 classifier to meningioma, sometimes with high confidence scores. Future classifier iterations that include ICMT-FET-CREB as a distinct class with well-curated reference cases may serve to resolve this issue, but in the interim genomic testing for a fusion involving genes in the FET and CREB families will provide a definitive diagnosis.

Overall, therefore, while DNA methylation-based classification has many attributes and can be instrumental in specific cases to reach a definitive diagnosis, awareness of its limitations is important in assessing its performance in individual cases. It is also prudent to comment on logistical challenges, which include the time and costs required to perform the assay, as well regulatory issues that may be operative in relation to the launch of a clinically approved test. On that point, progress is evident, and as one example, in the United States a Current Procedural Terminology (CPT) code has been made available for the Epignostix CNS Tumor Methylation Classifier from Heidelberg Epignostix GmbH, which is an important step towards broader adoption of such a test in the United States. A CPT code designation for a clinical test in the United States is a step that can lead to health insurance reimbursement and potential economic viability for institutions conducting the test.

Classifier updates and use

While technical aspects of test validation are outside the scope of this manuscript, a brief comment is in order. Minimum criteria have been proposed, [80] and include determination of assay sensitivity and specificity, establishment of minimum sample criteria, as well as implementation of quality control metrics. Challenges to clinical testing include tumor purity estimation, availability of appropriate reference set data, availability of and access to specialized bioinformatics resources and changes that occur with new versions of the array.

The initial Heidelberg classifier described 91 classes (82 of these concerning neoplasms and nine concerning non-neoplastic/reactive CNS tissue) in a 'version 11' classifier [1]. Subsequently, several iterations of a 'version 12' classifier were developed, with an increased number of classes. Such classifiers are freely available for use (https://www.molecularneuropathology.org/mnp/) and their existence and availability have enabled adoption of methylation profiling at a number of centers. Concurrently, several centers have developed in-house classifiers, which are undergoing evaluation for diagnostics, and such classifiers may also be subject to broader use (for example https://methylscape.ccr.cancer.gov, epidip.org[41]). Differences in existing classifiers include alternative machine learning algorithms, differences in specific samples included in reference sets, as well as differences in specific class types and designations and number of cases that make up the reference sets. However, the original Heidelberg classifiers are currently the most widely known and used in the community. It is expected that the choice of classifiers will depend on specific needs of individual institutions as well as specific applications. As the use of methylation profiling broadens and new classes are proposed, it is expected that classifiers will continue to evolve, with the refinement of classes and designations. The field will thus benefit from consensus and harmonization of agreed-upon methylation

classes and class designations. Alongside the need for harmonization is the knowledge that no single classifier is likely to be perfect, and each will have its strengths and limitations. In this context, a case can be made for the use of multiple, complementary classifiers based, for example, on nonoverlapping reference sets and complementary machine learning algorithms. These points are raised for discussion and specific recommendations on the use of specific classifiers, either singularly or as an ensemble, cannot be made at this time. Nonetheless, it is important to use different pieces of information (e.g. histology, methylation class, NGS, etc.) to support a diagnosis. In this context, a case where multiple classifiers give the same answer could be considered 'higher' confidence. Relevant to this point is the potential for retroactive identification of classifier matches as new classes are defined. In other words, a specific case, while not yielding a match to a prior classifier version, might represent a newly identified class in a future classifier version. In the same spirit, DNA methylation profiling would be suitable for retrospective or prospective clinical trials to ensure reproducibility of diagnosis, but also to find prognostic or predictive markers.

The utility and accuracy of DNA methylation-based classification as a diagnostic tool are highly dependent on a comprehensive and well-annotated reference set that serves as the basis for the classifier. There is a need for the inclusion of all recognized classes in a classifier, based on the principle that classes can be detected only if they are included in the reference set and are included in the classifier. This highlights the benefit of comprehensive 'data warehouses' that collate very large numbers of samples, including collections of rare classes that cannot practically be obtained in a single institution. Given these considerations, evaluation of classifiers, and criteria for their use should be prioritized based on the quality of the reference set. The Heidelberg classifiers, in particular, have leveraged this principle to positive effect, by virtue of the inclusion of data obtained from multiple institutions worldwide. In this context, data-sharing initiatives that promote the collection and annotation of large datasets are likely to result in further improvements in methylation-based

classification. Regulatory policies of specific laboratories or countries may require the availability of both a specific algorithm and the primary reference set data, a limitation that may exist in some situations [81], which further illustrates the advantages of data sharing to promote the availability and expansion of this important diagnostic modality.

Conclusions

DNA methylation profiling represents an important and useful tool in the armamentarium of CNS tumor diagnostics. Appropriate application of this platform requires knowledge of its strengths and limitations, as well as the need to integrate the profiling results within the overall context of a specific case. While recommendations of the use of specific classifiers or inclusion of specific classes are not within the scope of this cIMPACT Working Group, it is evident that harmonization and sharing of classifier reference sets, along with a collaborative process that allows for consensus recommendations and periodic updates is warranted to maximize the utility of this tool to promote best practices for CNS tumor diagnostics.

Summary of recommendations

1. DNA methylation profiling of CNS tumors should be considered whenever it may help resolve a differential diagnosis. Prioritization is recommended for cases where the diagnosis is otherwise challenging, where there is discrepancy among the findings of a case, where further molecular subtyping is needed to come to a diagnosis, or where DNA methylation-based classification is the only method to define a tumor type.

2. DNA methylation profiling is included in essential or desirable criteria for the diagnosis of many CNS WHO tumor types, and therefore awareness of both attributes and limitations of this platform as a diagnostic tool is important for pathologists and clinicians involved in the care of patients with CNS tumors.
3. As with any diagnostic modality (e.g., histologic interpretation, IHC, genomic findings) DNA Methylation classifier results should be interpreted in the context of all appropriate information available for the case. A DNA methylation classifier result that is unexpected or that does not fit within the context of these additional data requires expert, sometimes multidisciplinary review to determine an appropriate diagnosis.
4. DNA methylation classifiers may exhibit limitations in the resolution of specific methylation classes, as well as reveal findings that are unexpected compared to prior knowledge. Experience with the classifier and awareness of these limitations and attributes are therefore critical for appropriate use of DNA methylation data in clinical diagnostics. Review of DNA methylation classifier results by a (neuro)pathologist who can interpret and integrate within the context of the case as a whole is therefore recommended as good clinical practice.
5. Current classifiers utilize all 3 BeadChip versions of the array, and the recent versions developed were not fully compatible with the original ('450k') version. As new versions of the methylation array are formulated, ensuring that these new versions are fully backward-compatible is highly recommended, to provide maximal probe overlap across versions as well as to eliminate the need to re-develop a classifier with each array version.
6. Inclusion of DNA methylation results into a layered diagnostic format is recommended, with the inclusion of specifics as to the classifier used as well as the family and class match, including the confidence scores(s). The inclusion of additional data on quality control and the full output of the classifier results in the pathology report is also recommended.

7. As more cases are profiled by DNA methylation, classifiers will be improved by the inclusion of additional samples of rare tumor types into the reference set. This will also provide the opportunity for the discovery of new methylation classes. Data sharing to promote the development of comprehensive datasets will thus be of benefit to the field.

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Figure Legends:

Figure 1. Layered Diagnosis example: High-confidence match to family and class.

Figure 2. Layered Diagnosis example: No match on methylation-based classification; with sufficient orthogonal data for definitive diagnosis.

Figure 3. Layered Diagnosis example: Match to a methylation family, no match to a methylation class; descriptive diagnosis warranted.

Figure 4. Layered Diagnosis example: High-confidence match to a class that does not correspond to a current WHO tumor type.

Figure 5. Layered Diagnosis example: High-confidence match to meningioma with reference to recent cIMPACT-NOW Working Group recommendations.

Figure 6. Additional reporting recommendations: A. Classifier detailed results; B. Methylation testing parameters.

Figure 7. Layered Diagnosis example: High-confidence match to the Glioblastoma IDH-wildtype methylation family, but low-confidence matches to glioblastoma classes.

Table Legends:

Table 1: WHO CNS5 tumor types that correspond to methylation classes*, with their relationships identified.

Table 2: Methylation classes that do not correspond to a WHO CNS5 tumor type.

Table 3: WHO CNS5 tumor types that do not directly correspond to methylation classes in the CNS classifier.

Table 1

Site	CNS WHO Category	CNS WHO Family	CNS WHO Tumor Type	Methylation Class(es)
Gliomas, glioneuronal tumours, and neuronal tumours	Gliomas, glioneuronal tumours, and neuronal tumours	Adult-type diffuse gliomas	Astrocytoma, IDH-mutant	Astrocytoma, IDH-mutant; high grade and lower grade
			Oligodendroglioma, IDH-mutant and 1p/19q-codeleted	Oligodendroglioma, IDH-mutant and 1p/19q-codeleted
			Glioblastoma, IDH-wildtype	Glioblastoma, IDH-wildtype, subtype posterior fossa Glioblastoma, IDH-wildtype, mesenchymal (incl. subtype B), RTK1, RTK2 and posterior fossa subtypes Glioblastoma, IDH-wildtype with primitive neuronal component
		Paediatric-type diffuse low-grade gliomas	Diffuse astrocytoma, MYB or MYBL1-altered	Diffuse astrocytoma, MYB or MYBL1-altered, subtypes B, C and D
			Angiocentric glioma	Angiocentric glioma, MYB/MYBL1-altered
			Polymorphous low-grade neuroepithelial tumour of the young	Polymorphous low-grade neuroepithelial tumor of the young
		Paediatric-type diffuse high-grade gliomas	Diffuse midline glioma, H3 K27-altered	Diffuse midline glioma, H3 K27-altered, subtype EGFR-altered Diffuse midline glioma, H3 K27-altered, subtype H3 K27-mutant or EZHIP expressing
			Diffuse hemispheric glioma, H3 G34-mutant	Diffuse hemispheric glioma, H3 G34-mutant
			Diffuse pediatric-type high grade glioma, H3 wildtype and IDH-wildtype	Diffuse pediatric-type high grade glioma, H3 wildtype and IDH-wild type, Subtypes A and B Diffuse pediatric-type high grade glioma, MYCN subtype Diffuse pediatric-type high grade glioma, RTK1 and RTK2 subtypes
			Infant-type hemispheric glioma	Infant-type hemispheric glioma
		Circumscribed astrocytic gliomas	Pilocytic astrocytoma	Pilocytic astrocytoma (all classes)
			High-grade astrocytoma with piloid features	High-grade astrocytoma with piloid features
			Pleomorphic xanthoastrocytoma	Pleomorphic xanthoastrocytoma
			Subependymal giant cell astrocytoma	Subependymal giant cell astrocytoma, TSC1/TSC2-altered
			Chordoid glioma	Chordoid glioma, PRKCA-mutant
			Astroblastoma, MN1-altered	Astroblastoma, MN1-altered, MN1:BEND2-fused Neuroepithelial tumor, MN1:CXXC5-fused
		Glioneuronal and neuronal tumours	Ganglioglioma/Gangliocytoma	Ganglioglioma
			Desmoplastic infantile astrocytoma and ganglioglioma	Desmoplastic infantile ganglioglioma / desmoplastic infantile astrocytoma
			Dysembryoplastic neuroepithelial tumour	Dysembryoplastic neuroepithelial tumor
			Diffuse glioneuronal tumour with oligodendroglioma-like features and nuclear clusters	Diffuse glioneuronal tumor with oligodendroglioma-like features and nuclear clusters (DGONC)
			Papillary glioneuronal tumour	Papillary glioneuronal tumor; PRKCA-fused
			Rosette forming glioneuronal tumour	Rosette forming glioneuronal tumor
			Myxoid glioneuronal tumour	Myxoid glioneuronal tumor, PDGFRA-mutant
			Diffuse leptomeningeal glioneuronal tumour	Diffuse leptomeningeal glioneuronal tumor, subtypes 1 and 2
			Central neurocytoma	Central neurocytoma
			Extraventricular neurocytoma	Extraventricular neurocytoma
			Cerebellar liponeurocytoma	Cerebellar liponeurocytoma
		Ependymal tumours	Supratentorial ependymoma, ZFTA fusion-positive	Supratentorial ependymoma, ZFTA fusion-positive (all subclasses)
			Supratentorial ependymoma, YAP1 fusion-positive	Supratentorial ependymoma, YAP1 fusion-positive
			Posterior fossa group A (PFA) ependymoma	Posterior fossa group A (PFA) ependymoma (all subclasses)
			Posterior fossa group B (PFB) ependymoma	Posterior fossa group B (PFB) ependymoma (all subclasses)
			Spinal ependymoma	Spinal ependymoma
			Spinal ependymoma, MYCN-amplified	Spinal ependymoma, MYCN-amplified
			Myxopapillary ependymoma	Myxopapillary ependymoma
			Subependymoma	Subependymoma (all classes)**
Choroid plexus tumours	Choroid plexus tumours	Choroid plexus tumours	Choroid plexus tumours	Choroid plexus papilloma and carcinoma (all classes)
Embryonal tumours	Medulloblastoma	Medulloblastomas, molecularly defined	Medulloblastoma, WNT-activated	Medulloblastoma, WNT-activated
			Medulloblastoma, SHH-activated and TP53-wildtype, Medulloblastoma, SHH-activated and TP53-mutant	Medulloblastoma, SHH-activated (subclasses 1- 4 & subclass IDH-mutant)
			Medulloblastoma, non-WNT/non-SHH	Medulloblastoma, non-WNT/non-SHH, Groups 3 and 4 (subclasses I - VIII)
		Medulloblastoma, histologically defined	Medulloblastoma, histologically defined	Medulloblastoma (all classes)
	Other CNS embryonal tumours	Other CNS embryonal tumours	Atypical teratoid/rhabdoid tumour	Atypical teratoid/rhabdoid tumor (MYC, SHH and TYR subtypes)
			CNS neuroblastoma, FOXR2-activated	CNS neuroblastoma, FOXR2-activated
			CNS tumor with BCOR internal tandem duplication	CNS tumor with BCOR internal tandem duplication
			Cribriform neuroepithelial tumour	Cribriform neuroepithelial tumor, SMARCB1-altered
			Embryonal tumour with multilayered rosettes	Embryonal tumor with multilayered rosettes, C19MC-altered Embryonal tumor with multilayered rosettes, non-C19MC-altered
Pineal tumours	Pineal tumours	Pineal tumours	Pineocytoma	Pineocytoma
			Pineal parenchymal tumour of intermediate differentiation	Pineal parenchymal tumor of intermediate differentiation, KBTBD4-altered, subtypes A and B
			Pineoblastoma	Pineoblastoma, (all classes)
			Papillary tumour of the pineal region	Papillary tumor of the pineal region, subtypes A and B
			Desmoplastic myxoid tumour of the pineal region, SMARCB1-mutant	Desmoplastic myxoid tumor of the pineal region, SMARCB1-mutant***
Cranial and paraspinal nerve tumours	Cranial and paraspinal nerve tumours	Cranial and paraspinal nerve tumours	Schwannoma	Schwannoma
			Neurofibroma	Neurofibroma/Plexiform neurofibroma
			Malignant melanotic nerve sheath tumour	Malignant melanotic nerve sheath tumor
			Malignant peripheral nerve sheath tumour	Atypical malignant peripheral nerve sheath tumor Malignant peripheral nerve sheath tumor
			Cauda equina neuroendocrine tumour [previously paraganglioma]	Cauda equina neuroendocrine tumor [paraganglioma], subtype non-CIMP
Meningioma	Meningioma	Meningioma	Meningioma	Meningioma, (Benign, Intermediate and Malignant classes)
			Clear cell meningioma	Meningioma, clear cell subtype, SMARCE1-altered
Mesenchymal, non-meningothelial tumours involving the CNS	Soft tissue tumours	Fibroblastic and myofibroblastic tumours	Solitary fibrous tumour	Solitary fibrous tumor
		Vascular tumours	Haemangioblastoma	Hemangioblastoma
		Skeletal muscle tumours	Rhabdomyosarcoma	Rhabdomyosarcoma (all classes)
		Tumours of uncertain differentiation	Intracranial mesenchymal tumour, FET::CREB fusion-positive	Intracranial mesenchymal tumor, FET::CREB fusion-positive***
			CIC-rearranged sarcoma	CIC-rearranged sarcoma
			Primary intracranial sarcoma, DICER1-mutant	Primary intracranial sarcoma, DICER1-mutant
			Ewing sarcoma	Ewing sarcoma
	Chondro-osseous tumours	Chondrogenic tumours	Mesenchymal chondrosarcoma	Mesenchymal chondrosarcoma***
			Chondrosarcoma	Chondrosarcoma***
	Notochordal tumours	Notochordal tumours	Chordoma	Chordoma
Melanocytic tumours	Melanocytic tumours	Circumscribed meningeal melanocytic neoplasms	Primary CNS circumscribed melanocytic tumour	Melanocytoma****
Haematolymphoid tumours involving the CNS	Lymphomas	CNS lymphomas	Diffuse large B-cell lymphoma of the CNS	Diffuse large B-cell lymphoma of the CNS
	Histiocytic tumours	Histiocytic tumours	Langerhans cell histiocytosis	Langerhans cell histiocytosis
Germ cell tumours	Germ cell tumours	Germ cell tumours	Germinoma	Germinoma, subtypes KIT mutant and KIT wildtype
			Mature teratoma	Teratoma
			Yolk sac tumour	Yolk sac tumor
Tumours of the sellar region	Tumours of the sellar region	Tumours of the sellar region	Adamantinomatous Craniopharyngioma	Adamantinomatous Craniopharyngioma
			Papillary Craniopharyngioma	Papillary craniopharyngioma
			Pituicytoma, granular cell tumour of the sellar region, and spindle cell oncocytoma	Pituicytoma, granular cell tumor of the sellar region, and spindle-cell oncocytoma
			Pituitary adenoma / pituitary neuroendocrine tumour	Pituitary adenoma (all classes)
			Pituitary blastoma	Pituitary blastoma***
Metastases to the CNS	Metastases to the CNS	Metastases to the brain and spinal cord parenchyma/Metastases to the meninges	Metastatic melanoma	Metastatic melanoma

* For this comparison, the methylation classes listed in the Heidelberg version 12.8 (https://www.molecularneuropathology.org/mnp/classifiers/14) are used

** The subependymoma methylation classes can include, in addition to CNS WHO grade 1 subependymoma, ependymal-like tumors that are not yet represented in the current CNS WHO classification

*** Desmoplastic myxoid tumor of the pineal region, SMARCB1-mutant, Intracranial mesenchymal tumor, FET::CREB fusion-positive, Mesenchymal chondrosarcoma, Chondrosarcoma, and Pituitary blastoma are classes in the NCI/Bethesda classifier but are not present in the current Heidelberg CNS tumor classifier

**** The melanocytoma methylation class includes Circumscribed meningeal melanocytic neoplasms of all grades, including meningeal melanoma

Table 2

Methylation class
Adult-type diffuse high grade glioma, IDH-wildtype, subtypes B, E, and F
CNS embryonal tumour with BRD4::LEUTX fusion
CNS Embryonal tumour with PLAG-family amplification
CNS Schwannoma, VGLL-fused
CNS tumour with BCOR/BCORL1 fusion
Dural angioleiomyoma*
Glioneuronal tumor with ATRX alteration, kinase fusion and anaplastic features
Glioneuronal tumour, subtype A
High-grade glioma with pleomorphic and pseudopapillary features (HPAP)*
Intraocular medulloepithelioma
Neuroblastoma, ALT/TERT TMM positive
Neuroblastoma, MYCN type
Neuroblastoma, TMM negative
Neuroepithelial tumour with PATZ1 fusion
Neuroepithelial tumour, PLAGL1-fused
Olfactory neuroblastoma, IDH-wildtype
Oligosarcoma, IDH-mutant**
Plasmacytoma of the CNS
Retinoblastoma
Retinoblastoma, subtype MYCN-altered
Sinonasal undifferentiated carcinoma, IDH2-mutant
*Dural angioleiomyoma and High-grade glioma with pleomorphic and pseudopapillary features (HPAP) are classes in the NCI/Bethesda classifier but are not present in the current Heidelberg classifier
**Oligosarcoma, IDH mutant includes some neoplasms that do not harbor IDH mutations and/or 1p/19q-codeletion (see PMID 36520193 and PMID 34967922)

Table 3

CNS WHO Tumor type
Diffuse low-grade glioma, MAPK pathway-altered
Multinodular and vacuolating neuronal tumour
Dysplastic cerebellar gangliocytoma (Lhermitte-Duclos disease)
CNS embryonal tumour NEC/NOS
Perineurioma
Hybrid nerve sheath tumours
Haemangiomas and vascular malformations
Rare Haematolymphoid tumours involving the CNS*
*Immunodeficiency-associated CNS lymphomas, Lymphomatoid granulomatosis, Intravascular large B-cell lymphoma, MALT lymphoma of the dura, Other low-grade B-cell lymphomas of the CNS, Anaplastic large cell lymphoma (ALK+/ALK−), T-cell and NK/T-cell lymphomas, Erdheim-Chester disease, Rosai-Dorfman disease, Juvenile xanthogranuloma, Histiocytic sarcoma

Figure 1

Layered Diagnosis example: High-confidence match to family and class			
Integrated diagnosis	Oligodendroglioma, IDH-mutant and 1p/19q-codeleted, CNS WHO grade 2		
Histopathological classification	Oligodendroglioma, high-grade features absent		
Relevant molecular information	Mutation and/ or Fusion (NGS)	*TERT* promoter mutation; *IDH1* R132S mutation detected (NGS)	
	CNV (from methylation array)	1p/19q full-arm codeletion	
	DNA Methylation-based classification (Heidelberg version 12.8)	Family: Diffuse glioma, IDH-mutant	Family score: 0.99
		Class: Oligodendroglioma, IDH-mutant and 1p/19q-codeleted	Class score: 0.98
CNS-WHO grade	2		

Figure 2

Layered Diagnosis example: No match on methylation-based classification; with sufficient orthogonal data for definitive diagnosis			
Integrated diagnosis	Glioblastoma, IDH-wildtype, CNS WHO grade 4. See Note		
Histopathological classification	High grade diffuse glioma		
Relevant molecular information	Mutation and/ or Fusion (NGS)	*TERT* promoter mutation	
	CNV (from methylation array)	+7/-10 chromosomal copy number changes	
	DNA Methylation-based classification (Heidelberg version 12.8)	Family: No high-confidence match	
		Class: No high-confidence match	
CNS-WHO grade	4		

Note: DNA methylation-based classification suggested the "Glioblastoma, IDH-wildtype" family, but the confidence score in this case (0.75) was below the 0.90 pre-defined threshold for a high-confidence match. In the context of the histologic findings of a high-grade diffuse glioma, together with the *TERT* promoter mutation and +7/-10 chromosomal copy number changes, a diagnosis of Glioblastoma, IDH-wildtype, CNS-WHO grade 4 is appropriate in this case.

Figure 3

Layered Diagnosis example: Match to a methylation family, no match to a methylation class; descriptive diagnosis warranted			
Integrated diagnosis	Low-grade glial/glioneuronal tumor, not elsewhere classified (NEC). See Note		
Histopathological classification	Low-grade glial/glioneuronal tumor		
Relevant molecular information	Mutation and/ or Fusion (NGS)	*FGFR1* activating mutation	
	CNV (from methylation array)	No chromosomal copy number changes	
	DNA Methylation-based classification (Heidelberg version 12.8)	Family: Low-grade glioneuronal tumor	Family score: 0.95
		Class: No high-confidence match	
CNS-WHO grade	N/A		

Note: DNA methylation-based classification indicated a high-confidence match to the "Low-grade glioneuronal tumor" family, but did not reach a high-confidence match to a specific class. While Dysembryoplastic neuroepithelial tumor was suggested (confidence score =0.70), the match was not definitive and therefore a descriptive diagnosis of Low-grade glial/glioneuronal tumor is warranted.

Figure 4

Layered Diagnosis example: High-confidence match to a class that does not correspond to a current WHO tumor type			
Integrated diagnosis	Glioneuronal neoplasm, *NTRK2* fusion-positive, not elsewhere classified. See Note.		
Histopathological classification	Glial/glioneuronal tumor		
Relevant molecular information	Mutation and/ or Fusion (NGS)	*DIAPH2*::*NTRK2* fusion	
	CNV (from methylation array)	No chromosomal copy number changes	
	DNA Methylation-based classification (Heidelberg version 12.8)	Family: Diffuse glioneuronal tumor, non-defined type	Family score: 0.95
		Class: Glioneuronal tumor kinase-fused (GNT_KinF_A)	Class score: 0.98
CNS-WHO grade	N/A		
Note: DNA methylation-based classification indicated a high-confidence match to the "Glioneuronal tumour, subtype A class", which does not correspond to a currently recognized WHO tumor type. Tumors in this class are known to exhibit a rage of fusion types, including fusions involving NTRK family genes, as was observed in this case. A descriptive diagnosis, indicating the match to this methylation class and supportive NGS findings, is therefore warranted.			

Figure 5

Layered Diagnosis example: High-confidence match to meningioma with reference to recent cIMPACT-NOW working group recommendations			
Integrated diagnosis	Meningioma (meningothelial subtype), CNS WHO grade 1, with concerning genomic features. See Note.		
Histopathological classification	Meningioma, meningothelial phenotype, histologically grade 1		
Relevant molecular information	Mutation and/ or Fusion (NGS)	N/A	
	CNV (from methylation array)	Complete loss of chr. 1p and of chr. 22q, no homozygous *CDKN2A/B* loss	
	DNA Methylation-based classification (Heidelberg version 12.8)	Family: Meningioma	Family score: 0.95
		Class: Meningioma, benign	Class score: 0.98
CNS-WHO grade	See note		
Note: While this tumor according to the WHO CNS5 is graded as CNS WHO grade 1, given the presence of combined chromosomes 1p loss and 22q loss, it is likely that it may follow a clinical course similar to meningioma CNS WHO grade 2, as presented in cIMPACT-NOW Update 8 (PMID: 39212325).			

Figure 6

A

Heidelberg v12b8 classifier detailed results		
Level	**Designation**	**Confidence score**
Methylation Superfamily	Ependymal tumors	0.99
Methylation Family	Posterior fossa ependymoma group A	0.95
Methylation Class	Posterior fossa ependymoma group A1	0.72
Methylation Subclass	Posterior fossa group A (PFA) ependymoma, subclass 1c	0.61
Methylation Class description	The Methylation family "Posterior fossa group A (PFA) ependymoma" comprises the methylation classes posterior fossa ependymoma group A1 and A2 with their provisional molecular subclasses 1a-f and 2a-c, respectively. They represent the molecularly rendered tumor type of PFA ependymomas, histologically typically demonstrating pseudorosettes or ependymal rosettes and comprising uniform small cells with round nuclei embedded in a fibrillary matrix. They typically show a loss of nuclear H3 p.K28me3 (K27me3) expression in tumor cells.	

B

Methylation testing parameters	
Metric	**Value**
Sample type	Formalin-fixed paraffin-embedded (FFPE) tissue
Tumor purity	70%
Amount of DNA input	250 ng
Testing platform	Infinium MethylationEPIC v2.0
Technical metrics	Passed Quality Control metrics
Reference set/Classifier	Heidelberg version 12.8 reference set and classifier
Algorithm used	Random Forest

Figure 7

Layered Diagnosis example: High-confidence match to the Glioblastoma IDH wildtype methylation family, but low-confidence matches to glioblastoma classes			
Integrated diagnosis	Glioblastoma, IDH-wildtype, CNS WHO grade 4. See Note		
Histopathological classification	High grade diffuse glioma		
Relevant molecular information	Mutation and/ or Fusion (NGS)	*TERT* promoter mutation	
	CNV (from methylation array)	+7/-10 chromosomal copy number changes	
	DNA Methylation-based classification (Heidelberg version 12.8)	Family: Glioblastoma IDH-wildtype	Family score: 0.95
		Class: Glioblastoma, IDH-wildtype, RTK2 subtype	Class score: 0.55
		Class: Glioblastoma, IDH-wildtype, mesenchymal subtype	Class score: 0.40
CNS-WHO grade	4		
Note: DNA methylation-based classification matched to the "Glioblastoma, IDH-wildtype" family, with a high confidence score, warranting a definitive integrated diagnosis to this CNS WHO tumor type.			