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Invited Review: DNA methylation-based classification of paediatric brain tumours

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DNA methylation-based machine learning algorithms represent powerful diagnostic tools that are currently emerging for several fields of tumour classification. For various reasons, paediatric brain tumours have been the main driving forces behind this rapid development and brain tumour classification tools are likely further advanced than in any other field of cancer diagnostics. In this review, we will discuss the main characteristics that were important for this rapid advance, namely the high clinical need for improvement of paediatric brain tumour diagnostics, the robustness of methylated DNA and the consequential possibility to generate high-quality molecular data from archival formalin-fixed paraffin-embedded pathology specimens, the implementation of a single array platform by most laboratories allowing data exchange and data pooling to an unprecedented extent, as well as the high suitability of the data format for machine learning. We will further discuss the four most central output qualities of DNA methylation profiling in a diagnostic setting (tumour classification, tumour sub-classification, copy number analysis and guidance for additional molecular testing) individually for the most frequent types of paediatric brain tumours. Lastly, we will discuss DNA methylation profiling as a tool for the detection of new paediatric brain tumour classes and will give an overview of the rapidly growing family of new tumours identified with the aid of this technique.

Keywords: epigenetics, methylation, molecular pathology, neuropathology, paediatric brain tumour, tumour classification

Introduction

Differentiation of cancers into biologically meaningful classes is the basis for optimal cancer treatment and patient care. For over a century, histopathology has been the central platform for cancer diagnostics. More recently, classification schemes have been continuously refined by the incorporation of molecular information. The pace of refinement was particularly fast for the classification of CNS tumours which resulted in the need for an update of the 4th edition of the WHO classification in 2016 [1,2]. For several brain tumour entities this update incorporated specific, characteristic molecular alterations. The most fundamental changes however were seen in a select few entities where molecular features were incorporated into the entity definition, most notably affecting adult diffuse gliomas with the two now established categories of *IDH* mutant and *IDH* wild-type gliomas.

In summer 2015 at the time of preparation of the update of the WHO CNS tumour classification [2],

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one previously less central type of molecular information, namely DNA methylation, had just started to demonstrate its profound classificatory power [3–7] but it was not yet quite ripe for the central stage. This review will recapitulate the rapid emergence of DNA methylation-based profiling for the classification of paediatric brain tumours, will report on the current possibilities for diagnostic implementation and will briefly introduce suggested new tumour classes that were identified with the help of DNA methylation profiling.

DNA methylation

There are several layers of epigenetic control which converge in a cell-type-specific manner to modulate gene expression, encompassing histone variants, nucleosome remodelling, DNA methylation and non-coding RNAs. The interplay of these mechanisms orchestrates chromatin structure and accessibility dynamics [8]. While there is some overlap, the information encrypted in each of these layers also adds a unique component to the epigenetic code. Even though it is highly likely that each of these layers can play a fundamental role in health and disease, DNA methylation has acquired a prominent role in the currently ongoing reshaping of tumour classification, due to its accessibility, measurability and consequential clinical utility.

DNA methylation occurs almost exclusively at the carbon-5 position of specific cytosines (then called 5-methylcytosine) in sequences of a cytosine followed by a guanine separated by a phosphate group (a 'CpG' site). At a given time point, around 75% of CpG sites in the human genome are expected to be methylated [9]. Likely due to the propensity of 5-methylcytosine to deaminate to thymine spontaneously, large parts of the genome are CpG deficient or at least these sites occur at a clearly lower frequency (reportedly ~0.8%) compared to the expected 4% [10,11]. In contrast, small areas have resisted this evolutionary depletion and have been coined CpG Islands. The working definition of a CpG island has not changed much since 1987 and is, in brief, a DNA region of at least 200 base pairs length with a combined G and C content of at least 50% and a frequency of CpG sites nearer to what would be expected (observed/expected ratio > 0.6) [12,13]. About half of all mammalian gene promoters are associated with at least one CpG island [14] and these regions play an important role in transcriptional control by DNA methylation [9,15–17].

The mechanism of the repression of gene expression by DNA methylation at gene promoters and enhancer regions is well described [9,16,18]. Essentially, the positioning of the methyl group in the major DNA-helix groove may attract specific proteins (methyl-CpG binding domain proteins) for repressor complex recruitment or may directly prevent transcription factor binding. In addition, DNA methylation may be present at active genes and increased gene body methylation has been found to be positively correlated with gene expression depending on the context and genomic location [19–21].

The gene expression regulation actioned by DNA methylation has a variety of established functions that have been reviewed extensively elsewhere [9,16,18,22] ranging from genomic stability maintenance via repetitive element silencing, to developmental roles such as spatiotemporal and tissue-specific gene expression, cell differentiation orchestration, genomic imprinting and X chromosome inactivation in females. In humans, DNA methylation patterns are 'reprogrammed' through a process of extensive demethylation by active (TET1, TET2 and TET3 protein associated) and passive (replication associated) mechanisms in the zygote [23] followed by de novo re-methylation associated with the upregulation of DNA-methyltransferases DNMT3A and DNMT3B during early embryonic development [9]. Changes are then introduced in a cell lineage specific context in coordination with differentiation [24] which are subsequently upheld throughout cell divisions via DNMT1 enzyme recruitment to hemi-methylated sites by the protein UHRF1 [9,23,25].

DNA methylation in cancer

The development and maintenance of the cancer methylome are not entirely understood. While age related and environmentally induced DNA methylation changes combined with increased replication and clonal selection may account for some degree of the altered DNA methylome [24,26], the extent of the role of the cell of tumour origin signature still requires clarification [19]. In non-cancerous tissues, a general stability of DNA methylation patterns across cell division has been reported resulting in universal conservation of the cellular methylome in human tissues [26,27]. For cancer and in particular for brain tumours, the persistence

of the DNA methylation profile needs further verification. For ependymomas it was noted that on a large scale DNA methylation patterns are retained over many years and treatments [4]. Still, such analyses may well overlook more subtle or focal changes of the methylome and at least for adult glioblastoma some degree of temporal evolution has been reported [28]. Nonetheless there seems to be a relative persistence of the tumoral methylation pattern which provides the foundation for the utility of DNA methylation-based analyses for cell lineage tracing [27] and by extension, the identification of the origin of metastases of unknown primaries [29,30] and the rapidly developing field of DNA methylation-based tumour classification [29,31–36]. A graphical summary of the evolution of the field within the context of brain tumours is displayed in Figure 1. DNA methylation is further gaining importance for tumour entity definition and is increasingly being used for the allocation of rare cancers into either known tumour classes [37–40] or the identification of new entities [4,31,41–49], a collection of which are summarized in Figure 2.

The development of DNA methylation-based brain tumour classifiers

Several practical properties and biological traits have made DNA methylation extremely suitable for the development of laboratory tests for brain tumour classification and for machine-learning-based tumour classifiers.

Methylated DNA is highly robust

In DNA methylation, the methyl (CH_3) group connects to the cytosine via a strong carbon-carbon covalent bond which, in combination with the high stability of the DNA molecule itself, results in an extremely robust material that seems almost ideal for diagnostic testing. This property confers one of the fundamental advantages of DNA methylation profiling over other profiling methods (like RNA expression profiling) which is its applicability to formalin-fixed and paraffin-embedded material [6]. This trait allows easy integration of DNA methylation analysis into the widely established tissue and material flows used worldwide that mostly rely on formalin fixation and paraffin embedding. In our personal experience the very high robustness of methylated DNA may even allow analysis of several days old non-fixed tumour material stored at room temperature. Even though we would definitely not recommend this as routine practice, this may in some cases help to at least retrieve some information from material where standard fixation was accidentally not performed. A further example of the ensuing versatility comes from the retrospective analysis of the tumour material of the COG ACNS0332 trial on CNS primitive neuroectodermal tumours (PNETs) [50]. DNA methylation profiling was initially not planned and for many of the cases only archived haematoxylin and eosin or immunohistochemically stained slides were available. Because of the robustness of methylated DNA the coverslips of the histological slides could be removed and the material could still be used for DNA methylation profiling for the majority of samples.

Besides this broad clinical applicability, the robustness of this method further allows the retrospective testing of routinely archived tumour specimens from pathology departments. This has suddenly allowed the generation of high-quality molecular data of rare tumour specimens with no available frozen tissue and thus the generation of extensive reference cohorts that also include rare differential diagnoses in substantial numbers. The reference cohort of the German Cancer Research Center (DKFZ) CNS tumour classifier incorporates 2801 samples from 81 tumour classes [31]. Some of these classes are exceedingly rare tumour types and could only be brought together by the cooperation of over 50 different institutions worldwide. The robustness of methylated DNA allowed going back for many years with the (likely) oldest sample in the reference cohort dating back as far as 1984 [31].

Single technique allows easy pooling of data

A further important factor for the rapid development in this field may be the limited number of available techniques to test genome-wide DNA methylation. This may seem counterintuitive at first as one would generally expect concurring techniques to speed up developments. While this may indeed be true for the future evolution of this field where we may see a further diversification of methods towards such techniques as reduced representation bisulphite sequencing [28] or nanopore sequencing [51] for the initiation of methylation diagnostics it may have been a substantial advantage that the majority of published data were generated

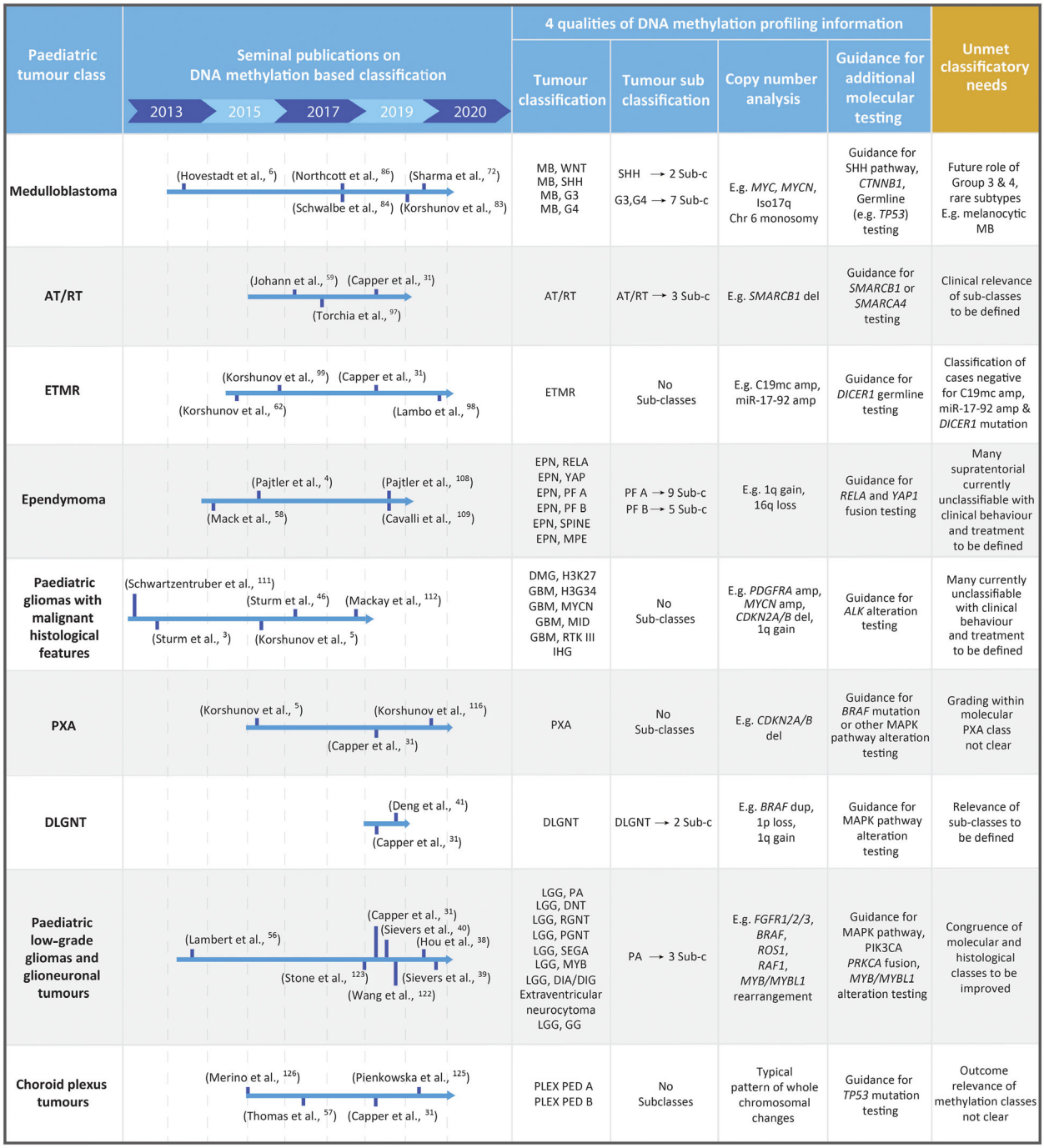

Figure 1. Graphical summary of the four qualities of information derivable from DNA methylation profiling in the context of the established paediatric tumour entities discussed in the text, along with a timeline of evolution in the field detailing seminal papers with their references. The final column lists some as of yet unmet needs regarding DNA methylation profiling as a diagnostic tool for each tumour type. Sub-c, sub-class; del, deletion; dup, duplication; amp, amplification; MB, medulloblastoma; AT/RT, atypical teratoid rhabdoid tumour; ETMR, embryonal tumour with multi-layered rosettes; PXA, pleomorphic xanthoastrocytoma; DLGNT, diffuse leptomeningeal glioneuronal tumour ETANTR, embryonal tumour with abundant neuropil and true rosettes; PF, posterior fossa; RTK, receptor tyrosine kinase; PXA, pleomorphic xanthoastrocytoma; EPN, ependymoma; LGG, low-grade glioma; PA, pilocytic astrocytoma; DNT, dysembryoplastic neuroepithelial tumour; RGNT, rosette-forming glioneuronal tumour; DMG, diffuse midline glioma; DLGNT, diffuse leptomeningeal glioneural tumour; PGNT, papillary glioneural tumour; SEGA, subependymal giant cell astrocytoma; DIA/DIG, desmoplastic infantile astrocytoma/ganglioglioma; GG, ganglioglioma; GBM, glioblastoma; IHG, infantile hemispheric glioma. [Colour figure can be viewed at wileyonlinelibrary.com]

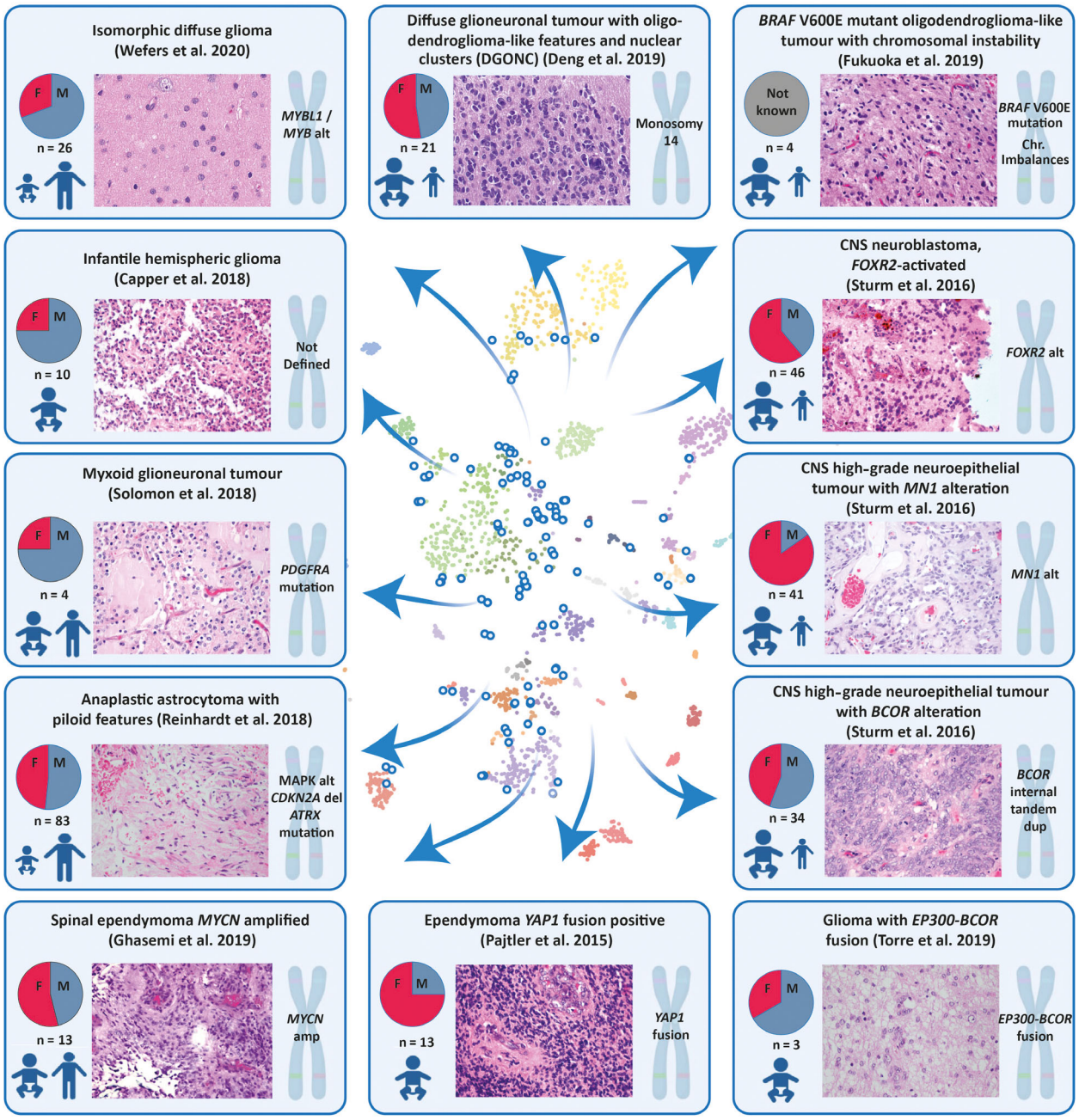

Figure 2. New tumour classes emerging from DNA methylation profiling. The centre shows a section of a t-distributed stochastic neighbour embedding of the reference cohort and parts of the validation cohort of the Capper et al. data set [31]. Classifiable cases are in the same colour as in the initial publication, blue circles represent the non-classifiable cases of the validation cohort. The non-classifiable cases are highly enriched for cases constituting new tumour classes. The outer rim represents examples of recently described putative new tumour classes, several of which were detected by in depth analysis of non-classifiable samples. For others a distinct DNA methylation profile was seen as additional argument that the cases represent distinct new entities. For each suggested class the left gives the sex distribution, the number of cases in the study and the age distribution (child and adult in relative size to each other), the middle represents an HE image (original magnification 400×, some cases with additional electronic zoom), the right represents typical additional genetic alterations if known. alt, alteration; Chr, chromosome; dup, duplication; amp, amplification; del, deletion. [Colour figure can be viewed at wileyonlinelibrary.com]

with a single technique. The Illumina 450K array [52] and EPIC array [53], which while admittedly do not boast the same coverage as whole-genome sequencing-based methods, provide a relatively cost effective, time efficient, highly robust and scalable option for high-throughput DNA methylation profiling that requires only 500 ng of starting DNA. The data of both array types can be adjusted to allow combination in the same data sets (e.g. by 'combineArrays' function of minfi Bioconductor package) [54]. Furthermore, the methylation array data can be pooled across different studies. This was done for the DKFZ CNS tumour classifier where alongside newly tested specimens data from 18 previously published brain tumour series were incorporated [3,4,19,46,55–68]. Extensive testing demonstrated surprisingly few batch effect problems in this combined data set [31]. Along these lines, over 50,000 tumours have now been pooled into a single database at the German Cancer Research Center and unsupervised analyses on this data are extremely powerful for the identification of rare but biologically distinct new tumour classes [as an example see 'Spinal Ependymoma, *MYCN*-amplified' below [47] and Figure 2].

A perfect coupling of DNA methylation and machine learning

Pooling of data and unsupervised analysis allows the identification of previously unrecognisable methylation patterns that are key to the detection of new tumour classes (see below and Figure 2), but such massive DNA methylation data sets have an even more game-changing effect for diagnostic cancer medicine when coupled with machine learning algorithms [69]. This step transforms the data sets into powerful diagnostic tools that allow the identification of all distinct methylation classes that are a part of the reference cohort by a single test. Such diagnostic algorithms are frequently referred to as 'Classifiers' [31]. Such Classifiers will attempt to classify a new ('diagnostic') sample into one of its previously defined methylation profile classes. The decision for a class is usually done by a majority vote. Optionally, cut-offs for classification may be included [see extensive discussion in Ref. [70]]. Classifiers evolve by increasing the number of samples in the reference cohort and the number of distinct methylation classes. Some classifiers attempt to classify a sample into broad diagnostic categories/entities reminiscent of current traditional classifications [31] whereas other classifiers are specialized on the sub-classification of specific entities [71,72]. The relatively small, easily exchangeable and highly standardized methylation array data seems like the perfect fuel for the newly ignited fire of machine-learning-based cancer classification. It is possible that the near future will see the development of far more powerful bioinformatic tools for class prediction, outcome prediction, data deconvolution and others.

A strong clinical need for improved paediatric brain tumour classification

Without doubt, paediatric brain tumours were the drivers for the development of DNA methylation-based tumour classification. For several reasons the need for new diagnostic tools was especially urgent in this field and has pushed the frontline of development. Paediatric brain tumours are altogether rather rare and medical expert training is therefore limited. Interlaboratory comparison showed low consensus for some entities [73]. As a consequence of their low frequency, paediatric brain tumours are often categorized according to their histological similarities to the more frequent adult counterparts, irrespective of possible biological differences. This is particularly problematic for malignancy assessment of paediatric glial tumours and can result in an over estimation of malignancy for some patients as exemplified by a subset of paediatric glioblastoma [5] and anaplastic pilocytic astrocytoma [74]. On top of this, the consequences of a misdiagnosis are particularly grave in the paediatric context, for example the application of an unnecessary brain irradiation due to an overestimation of the malignancy of a glioma may lead to lifelong cognitive, endocrine and cerebral vascular dysfunction as well as the risk of radiation induced secondary brain tumours [75,76].

The combination of these factors has led to the surprisingly rapid emergence of DNA methylation profiling algorithms for paediatric brain tumours and is likely to strongly impact the next years of development in this field. The following section will shortly describe the type of information relevant for the classification of paediatric brain tumours that can be extracted from the data.

What is in the data?

For diagnostic paediatric brain tumour analysis, four main output qualities can be derived from methylation

profiling. These have varying importance from class to class.

- *Tumour classification*: First and likely most central is the possibility to use DNA methylation data for tumour classification into specific DNA methylation classes. This is usually done by machine learning algorithms that draw on the DNA methylation levels of several thousand defined CpG sites. Potentially less reproducible methods like clustering or t-distributed stochastic neighbour embedding are also implemented to allocate samples according to reference groups but the interpretation of the results still has to be defined and may be quite unclear with these methods. In the brain tumour field, so called Random Forest machine learning algorithms are widely applied [31] but other types of machine learning algorithms also hold promise [69]and for other classificatory questions the inferiority of Random Forest to other machine or deep learning techniques has been suggested [33].
- *Tumour sub-classification*: A second level of information that is closely related to the above is the possibility to sub-classify certain classes into subclasses. This also relies on DNA methylation levels at specific CpG sites. Whether this is really a data quality of its own or if this is rather a consequence of an uneven 'focused' development in specific tumour classes and a lagging behind of the less flexible broader classification tools can be debated. But for the time being it is the reality that for certain methylation classes, additional sub-classification can be done that may reveal additional important diagnostic information.
- *Copy number analysis*: The third layer is copy number information calculated from the DNA methylation array data. For this the probe intensities are used to calculate low-density copy number variation plots [54]. Even though they are clearly less dense than other arrays used for brain tumour classification [77] and there may be a danger of missing subclonal events [78], the data offers broad diagnostic applications recently reviewed elsewhere [70].
- *Guidance for additional molecular testing*: The fourth level of information is slightly more abstract and is derived from the mostly unexplained but very tight association of DNA methylation classes with certain mutation events or specific gene fusions. This tight association has substantial potential to guide additional molecular testing, in particular in the rapidly expanding field of gene fusions. For example methylation profiling can readily identify cases that have a high likelihood of harbouring a *MYBL1* or *MN1* fusion, events that are otherwise exceedingly rare in brain tumours. Several of these frequent molecular alterations are summarized in the methylation class description texts that are automatically passed on when the Web-based Capper *et al.*, classification tool is used [31] or can be found online here: https://www.molecularneuropathology.org/mnp/classifier/2. To what extent methylation profiling may even replace more direct sequencing-based methods for identifying such genetic events remains to be demonstrated but its promise for identifying gold standard lines of testing to be pursued are apparent.

Unmet classificatory needs

It must be made clear that many aspects of this new technique are still under development and several diagnostic and classificatory needs are currently unmet. It is possible that some of these may resolve by further refinement of the technique, others may require the implementation of different techniques. We have previously in detail commented on how we would recommend handling cases with problematic material (e.g. too little extracted DNA, low tumour content) or cases that cannot be classified [70]. Concerning paediatric CNS tumours, there are certain entity specific unmet classificatory needs that are listed in Figure 1. One further unresolved issue of relevance for several entities is surely how to best combine DNA methylation-based classification with conventional histological WHO grading. Among paediatric tumours this particularly affects pleomorphic xanthoastrocytomas, ependymomas, low- and high-grade gliomas and choroid plexus tumours. The example of *IDH* mutation in adult diffuse gliomas has taught us that histologically defined grading criteria may have less meaning in molecularly defined series and may have to be re-established in 'molecularly clean series' [79]. Thus, it will likely also be required to re-establish histological grading criteria for tumour classes defined by DNA methylation, because these new classes only partly overlap with the historical classes. It may also turn out, that the newly defined molecular tumour entities show a clinically more homogenous behaviour

and that additional histological grading is not further required or will be increasingly replaced by molecular grading criteria.

In the following we will briefly summarize and discuss the current state of DNA methylation profiling for established paediatric brain tumour classes in respect to the above four qualities. Figure 1 gives an overview of the discussed established classes and illustrates seminal publications on DNA methylation profiling for these.

Methylation profiling of established paediatric brain tumour classes

Medulloblastoma

All four qualities of DNA methylation profiling play an important role for DNA methylation-based classification of medulloblastoma (MB). Molecular *tumour classification* for MB is far advanced with the four molecular groups of MB WNT, MB SHH (*TP53* mutant or wild-type), MB Group 3 and MB Group 4 (provisional, collectively non-WNT/non-SHH) readily recognized by the WHO classification of CNS tumours [1,80]. While these clinically relevant molecular groups were initially identified by gene expression studies [81,82] it has been shown that DNA methylation-based profiling may in fact be the method of choice for reliable detection of these groups [6,83]. There have been multiple studies aimed at further DNA methylation or gene expression based MB *sub-classification* using diverse bioinformatic approaches [84–86]. For the clinically and biologically heterogeneous Group 3 and Group 4 MB several cohorts were recently combined and expanded by Sharma *et al.* in an attempt to reconcile the varying proposed systems for sub-classification. This led to the recognition of eight distinct molecular subtypes largely based on DNA methylation data that showed significant differences in clinical presentation, cytogenetics, age of incidence, and/or survival outcomes [72]. Findings such as these support the genetic and epigenetic overlap between consensus Group 3 and Group 4 tumours previously shown by DNA methylation [84] and more recently by single-cell transcriptomics [87]. Two DNA methylation subgroups of SHH MB have also been described correlating with patient age group and underlying molecular SHH pathway alteration [88]. *Copy number analysis* from DNA methylation arrays offers the identification of various relevant alterations for MB. Among these amplifications (e.g. *MYCN*, *MYC*, *GLI2*), isochromosome 17q, deletion of chromosome 6 and whole-chromosomal aberration signatures may be the most relevant [77]. It must be taken into consideration that as with every tumour bulk analysis, subclonal events (such as some amplifications) may be missed [78]. DNA methylation profiling further offers certain *guidance for additional molecular testing.* This may be particularly relevant for the selection of cases to perform a directed SHH pathway mutation analysis [88] or *CTNNB1* testing [83]. Further guidance may be for directed germline testing as germline variants in *PTCH1*, *SUFU*, *GPR161* and *TP53* are mostly restricted to SHH MBs, whereas *APC* germline alterations may be specific to WNT tumours and *BRCA2* and *PALB2* were identified across SHH, Group3 and Group 4 tumours [89,90]. Recently, highly recurrent hotspot mutations of U1 spliceosomal small nuclear RNAs have been identified in around 50% of SHH MBs [91]. Intriguingly, the mutations are almost exclusively found in the methylation class of adolescent and adult tumours and are virtually absent in infant cases, thus DNA methylation profiling may be used to identify cases to further test for this alteration. This unusual non-protein-coding mutation results in disrupted RNA splicing and may represent a future target for therapy [91].

Atypical teratoid rhabdoid tumour

Atypical teratoid rhabdoid tumours (AT/RT) have a DNA methylation profile sufficiently distinct for robust *tumour classification* [31]. Most AT/RTs can also be reliably diagnosed by demonstration of immunohistochemical *INI1* loss, so the diagnostic benefit of DNA methylation profiling may be restricted to cases with unclear *INI1* immunohistochemistry, the rare cases with *SMARCA4* alterations, categorization of other embryonal tumours that occasionally may present with a *SMARCB1* alteration [92], or other highly unusual presentations like AT/RTs developing from lower grade precursor lesions [93,94]. Even for cases with a combined methylation class of AT/RT and *SMARCB1* alteration, caution is required if histology and/or age differ from the expected, because other rare entities may mimic this constellation [95,96]. In our opinion, it is further important not to automatically equate a *SMARCB1* alteration with the diagnosis of AT/RT for cases with lower grade precursors, even if a tumour

may have developed prominent rhabdoid histology (so called 'secondary AT/RT'). This issue has recently been nicely discussed by Nobusawa *et al.* [94] although methylation profiling of their cases was unfortunately not available and further research on this rare constellation seems required. Analysis of large cohorts have led to the *sub-classification* of AT/RT into three distinct molecular subgroups (ATRT-TYR, ATRT-SHH and ATRT-MYC) [59,97]. The subgroups share the presence of the prototypical alterations of *SMARCB1* or *SMARCA4* but show distinct types of *SMARCB1* deletion as well as distinct localisations, ages of diagnosis and epigenetic and transcriptomic profiles [59]. The clinical utility of the sub-classification into the three subclasses is currently not clear. For AT/RT *copy number analysis* may be used to demonstrate the inactivation of *SMARCB1* and may be helpful to separate the different types of *SMARCB1* deletion if considered of relevance. The methylation class AT/RT does not otherwise indicate *additional molecular testing*.

Embryonal tumour with multi-layered rosettes

The embryonal rosette-forming neuroepithelial brain tumours have historically comprised the histological variants embryonal tumour with abundant neuropil and true rosettes, ependymoblastoma and medulloepithelioma. However, owing to their molecular similarity, these tumours are collectively diagnosed as embryonal tumour with multi-layered rosettes (ETMR) according to the WHO 2016 classification [2,62]. Currently, the diagnosis of ETMR is tightly associated with the presence of a *C19MC* microRNA cluster amplification on chromosome 19q. Recent data demonstrate that this amplification is present in 90% of ETMR, with the majority of remaining tumours showing disruption of the microRNA machinery by other mechanisms, mostly compound heterozygous mutations of the microRNA-processing gene *DICER1*, amplification of a microarray cluster on chromosome 13 (miR-17-92) or structural alterations of the *C19MC* locus not associated with an amplification [98]. Regardless of *C19MC* amplification status, ETMRs form a single methylation class that is sufficiently distinct for robust *tumour classification* [31,98]. A further DNA methylation-based *subclassification* of ETMR is currently not possible [98]. However, it has been recognized that the histologically similar intraocular medulloepithelioma represents a distinct entity with a distinct DNA methylation profile [99]. *Copy number analysis* will reveal a *C19MC* microRNA cluster amplification in around 90% of cases and may identify cases with the rare mutually exclusive amplification of the miR-17-92 cluster [98]. *C19MC* amplifications are frequently associated with *TTYH1* fusions [100] but other fusion partners have also been identified [98]. The DNA methylation profile offers some *guidance for additional molecular testing* for cases without *C19MC* or miR-17-92 cluster amplification. For these cases a high rate of germline *DICER1* alterations can be expected and genetic counselling for *DICER1* syndrome should be considered [98].

Ependymoma

Six molecular ependymoma classes are by now well established, and have a DNA methylation profile sufficiently distinct for robust *tumour classification* [31]. It was previously shown that classification using this DNA methylation-based subgrouping was more informative for risk stratification than histopathological grading [4]. The earliest identified groups were the posterior fossa (PF)A and PFB molecular groups [58,101,102]. In 2015, DNA methylation profiling of a series of 500 ependymal tumours across all histological grades and locations revealed six molecular ependymoma classes and three molecular classes of subependymoma. The ependymal classes constituted the known PFA and PFB groups as well as the previously described supratentorial *RELA*-fusion-positive ependymoma [103], and three new molecular classes consisting of supratentorial *YAP1*-fusion-positive ependymomas, a benign spinal ependymoma class and a class closely related to the histological group of myxopapillary ependymoma [4]. Several series of histologically diagnosed ependymomas that were additionally classified by DNA methylation profiling have demonstrated a high rate of histological misdiagnoses, in particular within histologically diagnosed supratentorial ependymomas or ependymomas of the non-classical histological subtypes [46,104–107]. In contrast, a missed diagnosis of an ependymoma seems to be a rare event [31]. Therefore, for ependymoma the most central role of DNA methylation profiling may be the validation of the histological diagnosis, in particular for supratentorial cases. A second observation from histology validation studies is that a substantial number of

cases cannot be molecularly classified yet [104–107]. This may indicate further rare molecular classes of ependymal tumours not yet established as methylation classes of their own and the requirement for further improvement of DNA methylation-based classification. For PFA and PFB ependymomas large cohort studies have recently proposed further *sub-classification* [108,109]. For PFA ependymoma, nine subclasses have been identified that differ with respect to age at diagnosis, gender ratio, outcome, and/or frequencies of genetic alterations [108]. For PFB, five subclasses have been proposed that also harbour distinct demographics, copy number alterations and gene expression profiles [109]. For the other ependymoma classes no subclasses have yet been identified. For diagnostic *copy number analysis*, the most relevant feature among PF ependymomas to date is likely the presence of chromosome 1q gain as a strong negative prognostic factor [110]. Recent data indicate that chromosome 1q gain may only be of prognostic relevance for PFA ependymomas and is enriched in the DNA methylation subclass PFA-1c that is the subclass with the worst outcome [108,109]. In addition, the six main ependymoma methylation classes have characteristic chromosomal patterns [4,70]. DNA methylation profiling offers some *guidance for additional molecular testing* for ependymomas. In particular the tight association of *RELA* and *YAP1* fusion proteins with certain molecular classes strongly indicates which tumours to test for these gene fusions and in which tumours testing can be omitted [104,106,107]. Among ependymomas, recurrent mutations in *EZHIP*, *HIST1H3C*, *HIST1H2B* and *H3F3A* seem to be specific for PFA ependymomas and thus for other ependymoma classes testing may likely be less relevant [108].

Paediatric gliomas with malignant histological features

Among paediatric gliomas with malignant histological features (also referred to as high-grade gliomas), seven main molecular groups have been repeatedly identified that have a DNA methylation profile sufficiently distinct for robust *tumour classification* [3,31,46,111–113]. These include four methylation classes that are mostly composed of histologically diagnosed glioblastomas and are enriched for additional features that are also reflected in the current nomenclature: glioblastoma midline (these cases frequently have a midline tumour location), glioblastoma MYCN (*MYCN* amplifications are present in 20–30% of cases of this class), glioblastoma receptor tyrosine kinase (RTK) III (frequent RTK activations e.g. *EGFR* amplification are seen in these cases, other RTK classes exist but are rarer in the paediatric population) and glioblastoma G34 (the vast majority of cases display *H3F3A* codon 34 mutations). A further class is defined by histone mutations resulting in pK27M (or pK28M depending on nomenclature used) alterations of the *H3F3A* gene or more rarely other histone genes. A further group are the 'adult type' *IDH* mutant gliomas that may also occasionally be observed in the paediatric population [112]. Lastly, gliomas with malignant histological features includes a methylation class where further characterisation is pending and that has been provisionally named 'infantile hemispheric glioma' [31]. A relation of this methylation class to the recently described infantile hemispheric gliomas driven by alterations of either *ALK*, *ROS1*, *NTRK1/2/3* or *MET* [114] seems likely but has to be further substantiated. A central role of DNA methylation profiling for gliomas with malignant histological features is the validation of diagnosis and in particular the exclusion of a lower grade lesion in 'disguise'. Several studies have demonstrated that tumours with a more benign clinical course and molecular profiles of more indolent entities such as pilocytic astrocytomas or pleomorphic xanthoastrocytomas may frequently be identified among series of histological glioblastomas [5,31,112]. A recent study further demonstrated that paediatric cases diagnosed as anaplastic pilocytic astrocytoma are an extremely heterogeneous group [74]. The majority of samples represent molecular pilocytic astrocytomas and only rarely molecular glioblastomas or anaplastic astrocytomas with piloid features [42,74]. Thus, the most central role of DNA methylation-based classification for paediatric gliomas with malignant histological features is the exclusion of a less malignant neoplasm with misleading histological features. A 'missed diagnosis' of a paediatric glioblastoma may also occasionally occur, in particular from cases diagnosed as CNS embryonal tumour, not otherwise specified or anaplastic astrocytoma, *IDH* wild-type [31] or in the setting of treatment induced glioblastomas that may in fact be underestimated [115]. *Tumour sub-classification* of the above seven classes is currently not established. *Copy number analysis* can be used to identify several features that may be of diagnostic relevance among the above classes [70]. Of higher relevance may

be the detection of amplifications observed in glioblastoma G34 tumours that are associated with a worse outcome [61] or analysis of the 1p/19q co-deletion status for paediatric *IDH* mutant gliomas that would then be classified as oligodendroglial tumours [2]. The identification of one of the above methylation classes may further give *guidance for additional molecular testing*, in particular the strong association of the histone and *IDH* mutation with the respective methylation classes gives a strong indication of which tumours to test for these alterations. To what extent the methylation class infantile hemispheric glioma may prompt the testing for alterations of *ALK*, *ROS1*, *NTRK1/2/3* or *MET* needs to be further determined.

Pleomorphic xanthoastrocytoma

To date, a single DNA methylation pattern sufficiently distinct for robust *tumour classification* has been identified for pleomorphic xanthoastrocytoma (PXA) [5,31,116]. Currently, WHO grade II and III PXA share this DNA methylation class that was therefore named (anaplastic) pleomorphic xanthoastrocytoma [31]. DNA methylation indicates that PXA may be considered as something like the chameleon of brain tumours, as tumours with this distinct molecular profile have been identified in substantial numbers among histological series of paediatric glioblastomas [5,31] epithelioid glioblastomas [116,117] and astroblastomas [118–120], and at lower frequency among embryonal tumours [46], gangliogliomas [31] and AT/RTs [31]. Thus, in contrast to several other discussed tumours (e.g. see ependymomas) PXA may have a higher frequency than indicated by histology and methylation profiling may greatly aid in identifying PXA with unusual morphology. Further studies are needed to identify if the DNA methylation class covers the full spectrum of PXA, because some tumours with histological features of PXA currently cannot be classified by methylation profiling. A further *sub-classification* has not yet been established. *Copy number analysis* frequently indicates the prototypical homozygous *CDKN2A/B* deletions and additional chromosomal changes [70] but none of these changes seem to be highly class specific. The methylation class (anaplastic) pleomorphic xanthoastrocytoma gives clear *guidance for additional molecular testing* as high rates of the therapeutic target *BRAF* V600E are typical for this entity. Cases without *BRAF* mutations likely harbour other mitogen-activated protein kinase (MAPK) pathway activating alterations. High rates of *BRAF* V600E are found in both cases with typical histology [121] or the above mentioned 'chameleon cases'.

Diffuse leptomeningeal glioneuronal tumours

Diffuse leptomeningeal glioneuronal tumours (DLGNT) is a relatively newly defined pathological entity that has been assigned the status of a provisional entity in the current WHO classification [2]. DLGNT was shown to have a DNA methylation profile sufficiently distinct for robust *tumour classification* [31]. Analysis of a large data set of brain tumour DNA methylation profiles indicated that the morphological and clinical spectrum of tumours of this molecular class may be broader than previously anticipated [41] and DNA methylation profiling may play an important role in the identification of cases with such atypical presentation. The same study also identified two molecular *tumour sub-classes* that differ in their methylation profiles as well as chromosome 1q status and outcome [41]. *Copy number analysis* can be used to identify chromosome 1p loss that is present in virtually all cases. *BRAF* fusion is also a frequent event and may be evident from the copy number profile [70]. The molecular DLGNT class strongly implies *additional molecular testing* for MAPK pathway alterations as up to 80% of cases show *BRAF* fusion or other alterations [70].

Paediatric low-grade gliomas and glioneuronal tumours

Among paediatric low-grade gliomas and glioneuronal tumours, at least nine main DNA methylation classes have so far been established and seem sufficiently distinct for *tumour classification* [31,38,39,56,122–124]. The molecular classes are clearly related to the established histological tumour classes although there is typically some degree of reallocation when molecular classes are overlaid on histological series [31,38–40,123,124]. Figure 1 lists the main molecular classes. An important role of methylation profiling lies in the classification of low-grade gliomas that present with unusual or malignant histological features (see also paragraph on Paediatric gliomas with malignant histological features above). It has been observed that methylation profiling may be less straight forward for tumours from the spectrum of low-

grade gliomas [78], possibly owing to the frequently low tumour cell content or the missing representation of several methylation classes in commonly used classifiers [31]. *Tumour sub-classification* has been established for pilocytic astrocytoma where three different location-dependent subclasses are defined [31,56]. The possibility to perform *copy number analysis* of these tumours is a great asset for routine diagnostics [70]. Some but not all gene fusions of, for example *BRAF*, *FGFR1*, *RAF1*, *MYB*, *ROS1*, etc. may be identified on the copy number profile if tumour content is sufficiently high. By this and by the molecular class this analysis gives a strong *guidance for additional molecular testing* in many cases of alterations of the MAPK pathway with different mutational patterns assigned to the different molecular classes.

Choroid plexus tumours

Three DNA methylation classes have so far been established for choroid plexus tumours and seem sufficiently distinct for *tumour classification* [31,57,125,126]. One of these classes is largely restricted to adults and fourth ventricle location [57]. The other classes may occur in children and have differences in prognosis and association with *TP53* mutation status [57,125]. The congruence of these molecular classes to the established histological classes is not absolute and needs further clarification. DNA methylation profiling may be of help to clarify if certain tumours represent choroid plexus tumours *per se* [70] but the advantages of prognostication beyond histological assessment require further clarification. Additional *tumour sub-classification* has not been established. *Copy number analysis* can be used to identify the characteristic whole chromosomal patterns that are associated with the molecular classes [70]. The high enrichment of *TP53* mutation in one of the molecular classes may give *guidance for additional molecular testing*.

New or emerging paediatric tumour classes identified by DNA methylation profiling

In parallel to the above described development of classification tools for established tumour classes, DNA methylation profiling has demonstrated great power for the identification of new tumour classes. This was most impressively shown for tumours previously designated as PNETs and has revealed the existence of three new brain tumour classes and one tumour now allocated to sarcomas [46]. Large cohorts of brain tumours have since been aggregated and have allowed the identification of additional putative tumour classes several of which are described below and are depicted in Figure 2. Whether these will eventually be accepted as defined tumour classes remains to be seen. What is very likely though is that the next years will see a DNA methylation-based identification of many more such examples in neuropathology and general pathology.

Myxoid glioneuronal tumour

Myxoid glioneuronal tumour is a low-grade glioneuronal neoplasm of the septum pellucidum, the lateral ventricle, the corpus callosum or the periventricular white matter with DNT-like or RGNT-like histological features that is defined by recurrent *PDGFRA* p.K385 mutations [44,45]. DNA methylation patterns seem to be close to DNT [45].

Ependymoma *YAP1*-fusion-positive

This ependymal tumour class was identified by DNA methylation profiling of a series of 500 ependymal tumours across all histological grades and locations and is characterized by *YAP1* fusions most frequently with the *MAMLD1* gene [4]. Patients are typically young children and outcome may be better than what would be expected from these often histologically malignant appearing tumours [4].

Infantile hemispheric glioma

These rare supratentorial tumours commonly display high-grade glioma features, but morphology varies greatly in this group. All cases so far observed have occurred in infants. Additional molecular features of this class are not currently known [31]. A possible relation of this methylation class to infantile hemispheric gliomas with *ALK*, *ROS1*, *NTRK1/2/3*, or *MET* alterations requires further investigation [114].

BRAF V600E mutant oligodendroglioma-like tumours with chromosomal instability

These tumours share a histology reminiscent of oligodendroglioma but were all *BRAF* V600E mutated and

had a high number of chromosomal gains and losses but no chromosome 1p/19q codeletion or *CDKN2A/B* deletion [49]. The tumours have a distinct DNA methylation profile also when compared to other tumours with frequent *BRAF* mutations like pleomorphic xanthoastrocytomas or gangliogliomas [49].

Diffuse glioneuronal tumour with oligodendroglioma-like features and nuclear clusters

Histologically these tumours somewhat resemble oligodendroglioma or other clear-cell tumours but may display an unusual nuclear clustering and mostly occur in the paediatric population. They do not show the common genetic alterations associated with other paediatric glioneuronal tumours or oligodendrogliomas. An unusual finding is the high rate (30 of 31 cases) of chromosome 14 monosomy, an alteration that does not appear to be typical in other so far defined entities [127].

Isomorphic diffuse glioma

While the majority of these rare cases are operated on in adulthood, most patients have a history of longstanding epilepsy since childhood. Histologically these tumours have a low cell density and are composed of indistinct glial appearing cells. Signs of histological malignancy are not observed and these lesions may rather contrarily be mistakenly classified as normal or reactive brain tissue. The majority of cases display chromosomal alterations in either *MYBL1* or *MYB* and the tumours have a distinct DNA methylation profile [43].

Anaplastic astrocytoma with piloid features

This rare tumour generally affects mid-aged adults with only very few documented paediatric examples to date [42,74]. The tumours show some reminiscence of classical pilocytic astrocytoma (frequent MAPK pathway activation, predominantly located in the cerebellum, some tumours have histological resemblance to pilocytic astrocytoma) but also clear differences (MAPK pathway alteration is much less frequently *BRAF* fusion, rather *NF1* and *FGFR1* alteration; additionally frequent alterations of *CDKN2A* and *ATRX*, frequent *MGMT* promoter hypermethylation, often signs of histologic malignancy). Outcome data are scarce and currently of low quality, but indicate a clinical behaviour in the range of a WHO III tumour [42]. For the paediatric population these tumours are of interest for their possible relationship to classical pilocytic astrocytoma. Indeed, for some cases a low-grade precursor many years in the past has been documented indicating that, against common conception, pilocytic astrocytoma may in very rare cases show a malignant progression.

Spinal ependymoma, *MYCN*-amplified

MYCN amplification has previously been demonstrated in small series of spinal ependymomas of adolescent and young adult women and was reported to be likely associated with an unfavourable prognosis [128,129]. Recently, an additional 13 cases of these intriguing *MYCN* amplified tumours were identified by their highly specific DNA methylation pattern within a massive data set of over 50 000 tumoral DNA methylation profiles [47]. The sex distribution of this series was balanced, the age range was 12–56 years (median 32). Again these tumours showed aggressive behaviour with a significantly worse median survival than the previously described spinal ependymoma molecular subgroups [4].

CNS neuroblastoma with *FOXR2* activation

Tumours with this methylation pattern typically present with an embryonal histology with a small-cell phenotype and mostly occur in children (6 years median, 2–16 years range) [46]. The majority of cases designated CNS NB-*FOXR2* display increased expression of the *Forkhead Box R2* (*FOXR2)* gene likely owing to various recurrent genetic alterations that resulted in inter- and intrachromosomal rearrangement. Of note, the only case in this subgroup that did not display overexpression of *FOXR2* had a focal amplification of the *MYC* oncogene [46]. Additionally, characteristic copy number alterations seen in this subgroup include gains of chromosome 1q and losses of chromosome 16q.

CNS high-grade neuroepithelial tumours with *BCOR* alteration

Tumours with this methylation pattern frequently have an embryonal histology and may exhibit perivascular pseudorosettes. Other examples may appear more

primitive glial or even ependymal. [31,46,70]. The tumours typically affect children (5 years median, range 1–26 years). Gene expression of *BCOR* is found at higher levels, usually owing to internal tandem duplications in *BCOR* or less frequently, *BCOR* mutations. These tumours generally have a relatively flat copy number profile, with the exception of gains of chromosome 1q in ~20% of cases [31,46].

CNS high-grade neuroepithelial tumours with *MN1* alteration

Tumours with this methylation pattern likely represent the core class of histologically diagnosed astroblastoma [118–120] but tumours with histological features of embryonal tumours or ependymal tumours may also fall into this group [46]. These tumours are characterized by *MN1* transcription regulator-associated chromosomal rearrangements leading to increased expression of the fusion partners [46]. The most frequent copy number alterations seen in this subgroup are losses of chromosome X (~60% of cases). Interestingly, these cases occur significantly more frequently in females [31,46].

Paediatric gliomas with *EP-300-BCOR* fusions

This likely very rare tumour class has so far only been suggested on the basis of three paediatric cases [48]. The cases showed a distinct methylation profile even from CNS high-grade neuroepithelial tumours with *BCOR* alterations and all cases demonstrated fusions of the epigenetic regulator *EP300* to *BCOR* [48].

Future outlook and conclusion

At present, DNA methylation profiling offers relevant diagnostic information for the vast majority of paediatric brain tumours. A rational implementation of DNA methylation profiling into pathological routine, especially for cases with ambiguous histology or non-informative or contradictory molecular information will improve diagnostic precision and will have a substantial impact on patient management [78,130]. For the currently rapidly expanding number of brain tumour classes and sub-classes it may represent a unifying diagnostic tool that can be applied across many types of cancer and will frequently give sound diagnostic suggestions and guidance for additional molecular testing. The easy exchangeability of data could foster the development of worldwide cancer registries that would enable the identification of exceedingly rare tumour types and the consecutive enrolment into specific trials, but the same easy exchangeability may also raise new questions related to data protection and patient privacy that will also have to be addressed. While accessibility is still a limiting factor for the applicability of the technique, this will likely improve once medical systems recognize the benefits provided by DNA methylation profiling and have adapted to this need. Considering the swiftness with which the field is currently expanding and the exponential evolution of technology and data systems, it seems likely that DNA methylation profiling will play a prominent role in the reshaping of cancer diagnostics for the years to come.

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Conflict of interest

DC has a patent pending: DNA methylation-based method for classifying tumour species of the brain (EP3067432A1). DC has patents relating to IDH1 R132H antibody and BRAF V600E antibody.

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