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# The impact of methylome analysis on the diagnosis and treatment of CNS tumours in children and adolescents: A population-based study in Greece

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ABSTRACT

*Background:* The recently published WHO classification of central nervous system (CNS) tumours recognizes DNA methylation profiling as a desirable and, for some diagnoses, essential diagnostic tool adjunctive to conventional histopathology. DNA methylation profiling is not routinely available in many countries, including Greece.
*Methods:* In this collaborative study, we report the DNA methylation results in a series of children and adolescents with CNS tumours in Greece (2018–2023). In total, 130 tumour samples were analyzed using the latest applicable version of the Heidelberg brain tumour classifier.
*Results:* Upon initial analysis, 80 % (104/130) achieved calibrated scores (Cs) $\geq$ 0.9 and matched an established methylation class family/subclass. Among them, methylation results confirmed (90/104, 86.5 %), refined (50/104, 48 %) or changed (10/104, 9.6 %) the histological diagnosis. Only four results were regarded as non-contributing (4/104, 3.9 %). Twenty-six tumour samples received Cs $<$ 0.9. Despite low scores, methylation results supported the initial diagnosis with lower confidence in 38.5 % (10/26) and established the diagnosis in two tumours with non-conclusive histopathology. Additional t-distributed stochastic neighbour embedding (t-SNE) analysis allowed the possible classification of twelve tumours. Nine more samples reached high Cs using the newer brain tumour classifiers, since available. Samples co-tested in Greece demonstrated excellent test reproducibility, supporting the analysis' local implementation. Methylome profiling impacted the clinical management of 40 % of patients, modifying stratification, prognosis, or treatment approach.
*Conclusions:* This study supports the need to integrate methylome analysis into routine diagnostics in our country and highlights the importance of collaboration between European pediatric oncology centres.

## 1. Introduction

Tumours of the central nervous system (CNS) comprise a heterogeneous group of neoplasms with distinct clinical and biological characteristics ranging from benign to highly aggressive. They remain the leading cause of cancer-related death in patients under 19 years and a major cause of morbidity in cancer-survivors [1]. One of the reasons for this unsatisfactory outcome is the lack of accuracy in classifying these tumours into distinct types and subtypes, leading to less precise prognosis, stratification, and treatment approaches.

Tumour classification was based on morphological and immunohistochemical features for nearly a century. Recent advances in genome and epigenome research refined this process by i) subdividing heterogeneous tumour types, such as medulloblastomas (MB), ependymomas (EPN), atypical teratoid/rhabdoid tumours (AT/RT), and pineoblastomas into molecular subtypes [2–5], ii) re-classifying tumours with questionable diagnoses, like in primitive neuroectodermal tumours of CNS (CNS-PNETs) and tumours at the grey zone between pediatric high-grade (HGG) and low-grade (LGG) gliomas, into more specific types [6,7], and iii) introducing new tumour types with characteristic molecular findings, e.g. diffuse midline glioma H3K27-altered, diffuse astrocytoma, *MYB*- or *MYBL1*-altered, CNS tumour with *BCOR* internal tandem duplication or astroblastoma, *MN1*-altered [8,9].

The recently published 5th Edition of the WHO classification of CNS tumours (2021), CNS5, encompasses more than 150 tumour types that cannot be easily distinguished morphologically. CNS5 recognizes DNA methylation profiling as a desirable or rather essential diagnostic tool adjunctive to conventional histopathology for many tumour types [8–11]. However, this brings further challenges to routine diagnostic practice in many countries, including Greece, as DNA methylation profiling is not yet reimbursed by the national health system and, thus, not routinely available.

To enable access to novel molecular diagnostics, including DNA methylation profiling, for pediatric oncology patients across Greece, a collaborative program was established between the Pediatric Oncology/Hematology Unit of First Department of Pediatrics, National and Kapodistrian University of Athens at "Aghia Sophia" Children's Hospital in Athens (POHemU) and the German Cancer Research Center in Heidelberg (DKFZ).

In this study, we report the results of DNA methylation profiling in 125 pediatric and adolescent patients. Our goals are to assess the impact of DNA methylation profiling on clinical practice using real-world data and evaluate the technique's reproducibility in our lab. Our findings support the integration of methylome analysis in routine diagnostics for pediatric CNS tumours in Greece and highlight the importance of collaboration between pediatric oncology centers around Europe.

## 2. Materials and methods

### 2.1. Patients and samples

We prospectively collected patients diagnosed between 2018 and 2023 with (a) histologically confirmed CNS tumours upon initial diagnosis, relapse, or progression of disease, (b) primary diagnosis until the age of 18, and (c) available tumour tissue and blood samples. Not all patients treated in Greece at that period were included in this study. Priority was initially given to patients with challenging diagnoses, relapsed or primary high-grade tumours and tumours for which molecular classification was important, but gradually patients with all types of tumours could be included.

Clinical data, histopathological reports, DNA methylation and molecular results were captured for each patient. Central reference neuropathological evaluation was performed for most samples at the Pathology Department of Aghia Sophia Children's Hospital in Athens. For each patient, tumour tissue (FFPE and/or fresh frozen) or nucleic acid extracted from fresh-frozen tissue areas with more than 70 % tumour cell content and blood samples were forwarded to the Department of Neuropathology of the Heidelberg University Hospital. Depending on the patient's diagnosis and the availability of the material, DNA methylation profiling and molecular analyses were performed in the context of the Pediatric Precision Oncology INFORM Registry [12], Molecular Neuropathology 2.0 [7], Pediatric Targeted Therapy 2.0 [13], LOGGIC core [14] or EURHAB [15] studies.

The study was conducted according to the Declaration of Helsinki guidelines and approved by institutional review boards. Parents or legal representatives of all patients provided written informed consent before enrollment.

### 2.2. DNA methylation analysis

All samples were analyzed using the Illumina Methylation EPIC platform and classified by the DNA methylation-based CNS tumour classifier, available through https://www.molecularneuropathology.org/. Consistent with previous studies, the threshold calibrated class prediction score (Cs) was set at $\geq$ 0.9 [16].

Tumours were classified using the latest applicable brain tumour classifier version available at the time of request - that is, versions 11b4–11b6 (n = 85) and 12.3–12.8 (n = 45). Samples with low scores,

initially analyzed with versions 11b4–11b6, were retrospectively reclassified with the updated brain tumour classifier versions (versions 12.3–12.8), which include more brain tumour classes and subclasses, such as germinomas, since these versions became available.

Apart from Cs, the results of methylation analysis included chromosomal copy number variation (CNV) plots generated from raw methylation data. Additional experimental analysis based on the visualization of DNA methylation patterns by *t*-distributed stochastic neighbour embedding (*t*-SNE), and subsequent class assignment by visual inspection was performed in tumours with low scores [7,16,17] and was taken into account clinically only when the result was compatible with histology or molecular findings.

To assess technique's reproducibility in our lab, we performed DNA methylation profiling using the Infinium Methylation EPIC v2.0 Array Bead Chip (Illumina). Raw data were analyzed using the openly available DNA methylation-based brain tumour classifier, V12.8. Inter-laboratory blind test validation was performed by comparing the methylation-based tumour classification and scores from seven tumours co-tested at our lab and DKFZ.

### 2.3. Evaluation of methylation results

To evaluate the impact of DNA methylation results, we compared the methylation-based classification with initial histopathology report, radiological findings, and any additional diagnostic examinations triggered by histopathological or methylation results. For tumours with:

i) High Cs $\geq$ 0.9, the impact was further categorized as (I) confirmation of diagnosis; (II) confirmation and refinement of diagnosis; (III) alteration of diagnosis; IV) non-contributing or misleading.
ii) Low Cs < 0.9, the impact was further categorized as (I) confirmation of diagnosis with lower confidence; (II) introduction of new entities; (III) unclassifiable.

DNA methylation results were considered clinically important when they affected the patients' clinical management by changing the stratification, prognosis or treatment approach (e.g., change of treatment protocol, delivery of radiotherapy, chemotherapy or targeted therapy).

## 3. Results

### 3.1. Cohort characteristics

Over a 6-year period (2018–2023), 125 patients (65 males) were included in the study, with mean age at diagnosis 8.9 years (range 0.3–17.9 years, median age 10 years). In total, 130 samples were analyzed either at primary diagnosis (77 %; 100/130) or relapse/progression (23 %; 30/130). Tumour samples of five patients were analyzed both at primary diagnosis and relapse. The majority (79 %; 103/130) came from patients diagnosed and/or treated in POHemU; the rest (21 %; 27/130) were referred from other departments/hospitals across Greece.

Tumours from different CNS locations were represented with the following distribution: posterior fossa (40 %; 52/130), hemispheres (33.8 %; 44/130), spine (6.9 %; 9/130), brainstem (6.2 %; 8/130) followed by more rare locations. Based on histopathology, LGGs (28.5 %; 37/130), MBs (24.6 %; 32/130) and HGGs (22.3 %; 29/130, including six patients diagnosed with Diffuse Intrinsic Pontine Glioma), accounted for most cases (Table 1, Suppl. Table 1).

### 3.2. DNA methylation results upon initial request

Upon initial request, using the latest applicable version of the brain tumour classifier, 104/130 (80 %) of the profiled samples reached Cs $\geq$0.9 (Fig. 1). Among them, the methylation class family/subclass matched the histological diagnosis in 90/104 (86.5 %), whereas in 50/104 (48 %), the additional information gained by DNA methylation profiling not only confirmed the neuropathological diagnosis but also refined it (Fig. 2, Suppl. Table 1, Suppl. Table 2). In 10/104 (9.6 %) the predicted methylation class was discordant from the initial neuropathological assessment and the diagnosis was established in favour of the DNA methylation result after interdisciplinary tumour board discussion (Table 2). In 4/104 (3.9 %), the result was deemed rather non-contributing than misleading to the final diagnosis when taken in the context of radiological and pathological data (Table 3).

Twenty-six tumour samples (20 %, 26/130) obtained Cs < 0.9

**Table 1**
Characteristics of the studied patients (n = 125) and the analyzed samples (n = 130), including sex, age upon diagnosis, timepoint of analysis, type of samples analyzed, location of the tumour, initial histological diagnosis, year of analysis and source of samples. Tumour samples of five patients were analyzed both at primary diagnosis and relapse. *Three tumours had no conclusive diagnosis, although examined by at least two experienced pathologists including a reference neuropathologist. [Diffuse Intrinsic Pontine Gliomas (DIPG)].

| Characteristic | No. | % |
|---|---|---|
| Sex | | |
| Male | 65 | 52 % |
| Female | 60 | 48 % |
| Age upon diagnosis | | |
| Range | 0.3–18 years | |
| Mean | 8.9 years | |
| Median | 10 years | |
| Analysis performed at | | |
| Primary diagnosis | 100 | 77 % |
| Relapse | 30 | 23 % |
| (Both Primary and Relapse) | (5) | |
| Samples | | |
| Formalin-fixed Paraffin-Embedded Blocks | 90 | 69 % |
| DNA extracted from Fresh Frozen Tissue | 40 | 31 % |
| Location | | |
| Hemispheres | 44 | 33.9 % |
| Optic Chiasm | 3 | 2.3 % |
| Sellar/Suprasellar | 5 | 3.8 % |
| Thalamus/Hypothalamus | 5 | 3.8 % |
| Pineal Region | 3 | 2.3 % |
| Brainstem | 8 | 6.2 % |
| Posterior fossa | 52 | 40.0 % |
| Spine | 9 | 6.9 % |
| Meninges | 1 | 0.8 % |
| Histological Diagnosis | | |
| Low-Grade Gliomas | 37 | 28.5 % |
| High-Grade Gliomas, incl. DIPG | 29 | 22.3 % |
| Ependymomas | 9 | 6.9 % |
| Medulloblastomas | 32 | 24.6 % |
| Atypical Teratoid/Rhabdoid Tumours | 6 | 4.6 % |
| Other Embryonal Tumours | 9 | 6.9 % |
| Meningiomas | 2 | 1.5 % |
| Germ cell tumours | 2 | 1.5 % |
| Rosai-Dorfman Syndrome | 1 | 0.8 % |
| Not Definite Diagnosis* | 3 | 2.4 % |
| Year of analysis | | |
| 2018 | 14 | 10.8 % |
| 2019 | 20 | 15.4 % |
| 2020 | 14 | 10.8 % |
| 2021 | 28 | 21.5 % |
| 2022 | 21 | 16.1 % |
| 2023 | 33 | 25.4 % |
| Samples' source | | |
| Pediatric Hematology and Oncology Unit (POHemU), University of Athens, "Aghia Sophia" Children's Hospital | 103 | 79.2 % |
| "Aglaia & Panagioti Kyriakou" Hospital of Athens-Dept of Hematology and Oncology | 7 | 5.4 % |
| Crete, University Hospital | 5 | 3.9 % |
| MITERA, Children's Hospital, Athens | 5 | 3.9 % |
| Hippokration Hospital, Thessaloniki | 4 | 3.0 % |
| "Aghia Sophia" Children's Hospital of Athens - Dept of Hematology and Oncology | 3 | 2.3 % |
| AHEPA Hospital, Thessaloniki | 3 | 2.3 % |

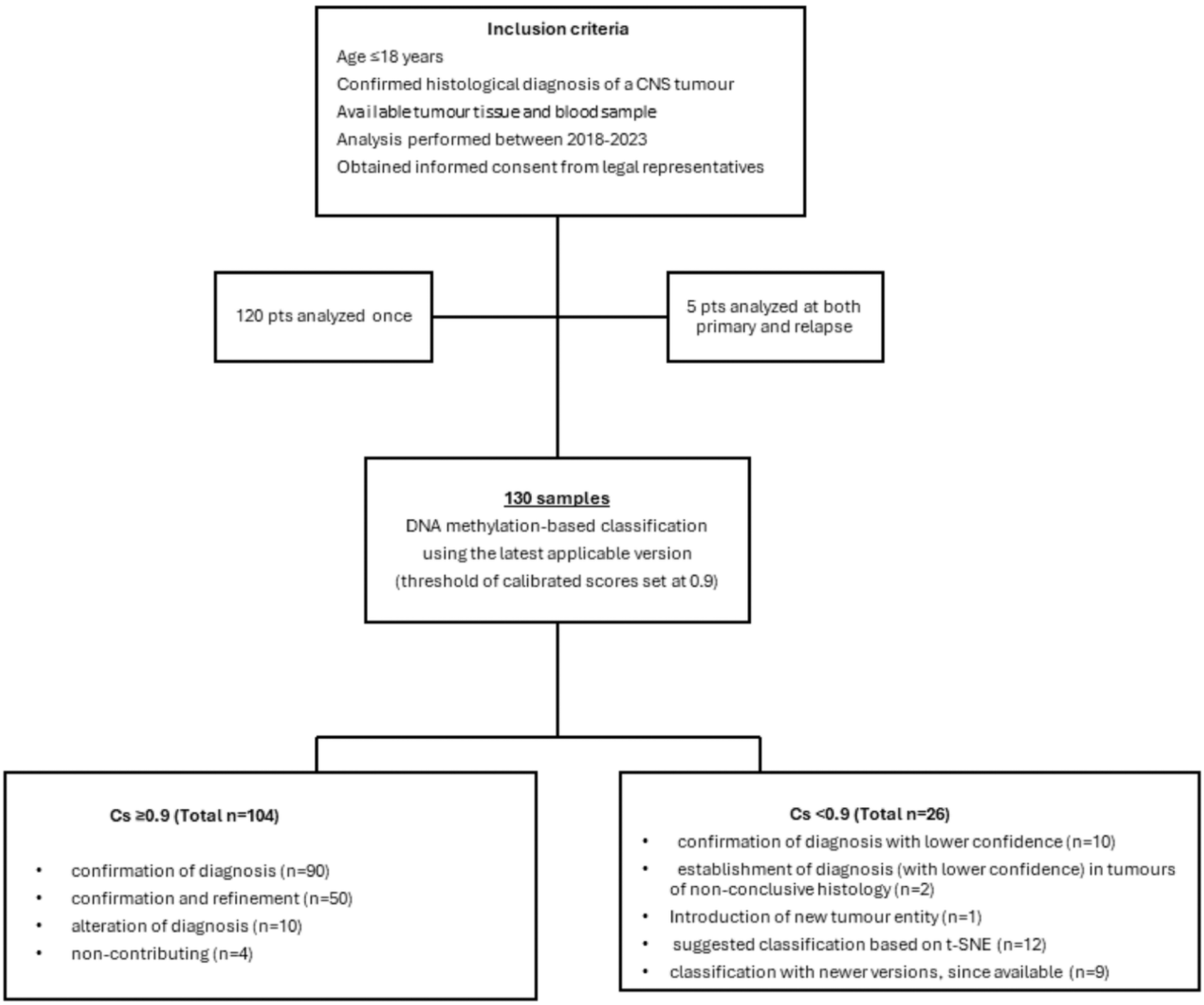


**Fig. 1.** Schematic description of the analyzed cohort of patients and the results of DNA methylation classification of central nervous system (CNS) tumours. Among 130 analyzed tumour samples, 104 tumours (80 %) obtained a high calibrated score (Cs ≥ 0.9) and 26 tumours (20 %) a low calibrated score (Cs <0.9). The diagnostic impact of methylation profiling on the initial histopathological diagnosis was categorized into (I) confirmation of the diagnosis (n = 90); (II) confirmation and refinement of diagnosis (n = 50); (III) alteration of diagnosis (n = 10); (IV) non-contributing (n = 4). In tumours with Cs < 0.9, the diagnosis was supported with lower confidence in accordance with histology (n = 10) or established with lower confidence in tumours with non-conclusive diagnosis (n = 2), by experimental t-distributed stochastic neighbour embedding (t-SNE) analysis (n = 12) or by the latest version of brain tumour classifier, since available (n = 9). One case was indicative of a new tumour entity.

(Suppl. Table 1, Suppl. Table 3). In 10/26 (38.5 %), the diagnosis with the highest Cs was in accordance with histopathology (confirmation of initial diagnosis with lower confidence). In two tumours with non-conclusive histological diagnosis, examined by two experienced pathologists, including a reference neuropathologist (7.7 %, 2/26), the methylation result was compatible with one of the proposed differential diagnoses (establishment of diagnosis with lower confidence); GR_010 (ependymoma or HGG) achieved Cs 0.84 for glioblastoma IDH-wildtype, subclass RTK III; and GR_020 with known Tuberous Sclerosis (Subependymal Giant cell Astrocytoma or HGG) received Cs 0.73 for glioblastoma, IDH-wildtype, subclass MYCN. Only nine tumours (6.9 %; 9/130) obtained no score and were characterized as unclassifiable (Suppl. Table 1). Additional experimental *t*-SNE analysis allowed possible classification of twelve tumours with low Cs (HGG, n = 6; EPN, n = 2; IHG, n = 1; anaplastic astrocytoma, n = 1; meningioma, n = 1), while one tumour was suggestive of a novel molecular class (Neuroepithelial tumour with PATZ1 fusion, NET-PATZ1). Nine of these (HGG, n = 6; EPN, n = 2; NET-PATZ1, n = 1) were subsequently confirmed using the newest classifier V12.8, achieving Cs ≥ 0.9 (Fig. 1, Suppl. Table 3).

### 3.3. Reclassification using newer classifier versions

Using retrospectively the recently released newer classifier versions (V12.3, V12.5 and V12.8) from DKFZ, nine more samples reached Cs ≥0.9 (Suppl. Table 3). In four cases, there was an underlying cancer predisposition syndrome (Mismatch Repair Syndrome, n = 2; Tuberous sclerosis, n = 1; Ataxia Telangiectasia, n = 1). Two samples obtained high Cs for methylation classes not included in previous versions; GR_063 (embryonal tumour NOS with a *ZFTA::NCOA2* fusion) achieved high score for Supratentorial EPN, ZFTA fusion-positive; and GR_096 (pleomorphic xanthoastrocytoma or EPN, with a *MN1::PATZ1* fusion) obtained high score for NET-PATZ1, a new tumour entity recently described [18]. Summing up all cases with high Cs, 113/130 (87 %) matched recognized methylation classes using the initial or newer classifier versions.

### 3.4. Identification of newly recognized diagnoses of CNS5

In 24 samples (18.5 %; 24/130), we identified newly recognized

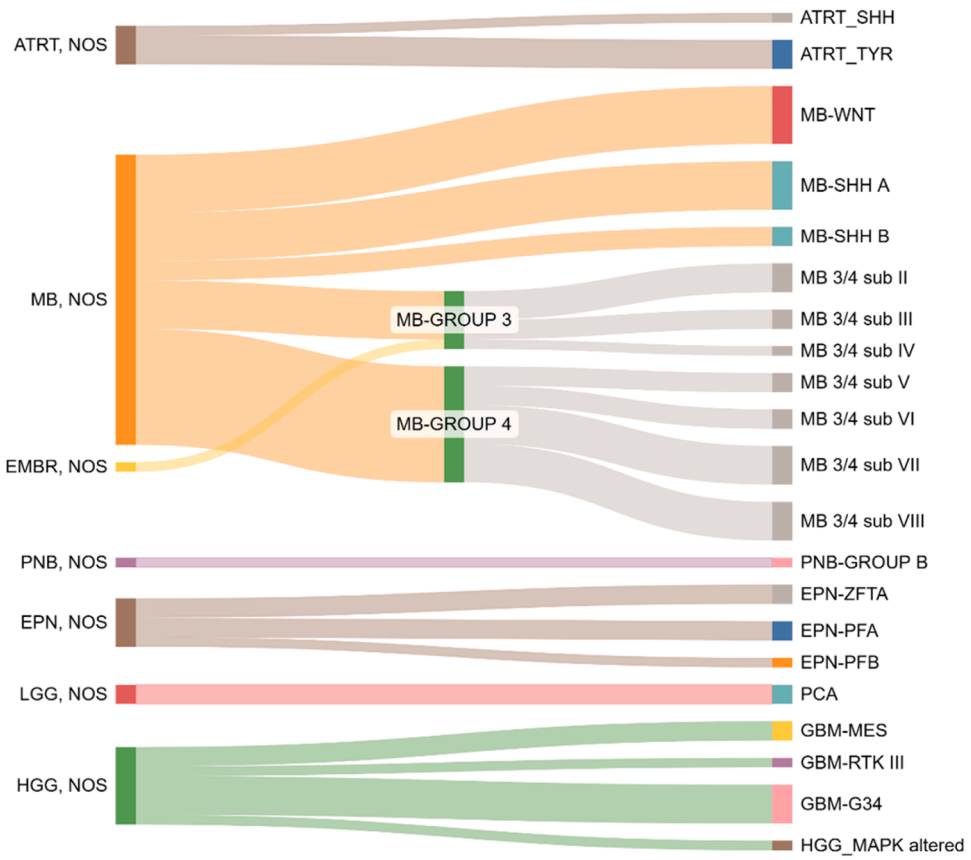


**Fig. 2.** Refinement of diagnosis by methylation profiling in 50 tumours with varying initial histological diagnoses according to WHO [left] and corresponding methylation classes [right]. Atypical teratoid/rhabdoid tumours (AT/RTs n = 4), AT/RT, subclass SHH (n = 1), ATRT, subclass TYR (n = 3). Medulloblastoma (MB, n = 30); MB-WNT-activated (n = 6), MB-SHH-activated (n = 7), child and adolescent group (MB-SHH-A, n = 5); MB-SHH-activated, infant group (MB-SHH-B, n = 2); MB-Group-3 (n = 5); MB-group-4 (n = 12); Embryonal CNS tumour, Not otherwise specified (EMBR, NOS, n = 1); Pineoblastoma (PNB n = 1), Pineoblastoma group B (n = 1); Ependymoma (EPN, n = 4), ZFTA-fusion positive ependymoma (n = 2); Posterior fossa ependymoma subgroup A (PFA, n = 2); Posterior fossa ependymoma subgroup B (PFB, n = 1) Low-Grade Gliomas Not otherwise specified (LGG, NOS n = 2), Pilocytic astrocytoma (PCA n = 2), High-Grade gliomas Not otherwise specified (HGG, NOS n = 6), methylation class glioblastoma, IDH wildtype, Mesenchymal type (GBM-MES, n = 2), RTK III (n = 1) H3.3 G34 mutant (GBM_G34 n = 4), Methylation family high-grade astrocytoma with piloid features, MAPK pathway altered (HGG_MAPK altered n = 1). Further refinement of 18 tumours diagnosed as non-WNT/non-SHH medulloblastoma (MB-Group-3 and MB-Group-4) into further subtypes I-VIII (Sub I, n = 0; Sub II, n = 3; Sub III, n = 2; Sub IV, n = 1; Sub V, n = 2; Sub VI, n = 2; Sub VII, n = 4; Sub VIII, n = 4), using the medulloblastoma classifier or the brain tumour classifier V12.8.

diagnoses based on their methylome profile, demonstrating that DNA methylation analysis is essential for the classification of these tumour types (Diffuse hemispheric glioma, H3.G34-mutant (n = 5); CNS high-grade astrocytoma with piloid features, MAPK pathway altered (n = 1) [19]; Diffuse pediatric-type HGG, H3-wildtype and IDH-wildtype (n = 7) [20]; Infant type hemispheric glioma (IHG, n = 1) [21], Diffuse Leptomeningeal Glioneuronal Tumour (DLGNT, n = 3) [22]; EPN- PFA (n = 3) and EPN-PFB (n = 1) [23]; CNS tumour with *BCOR* internal tandem duplication (n = 2) [24], and LGG with *MYB* alteration (n = 1) [25]) (Fig. 3, Suppl. Table 1).

### 3.5. Clinical impact

The methylome profiling impacted the clinical management of 52 (40 %; 52/130) patients (Suppl. Table 4). For patients diagnosed with MB, the methylation profiling confirmed and refined the diagnosis by providing molecular subclassification (Suppl. Table 2), based on which the attending physicians subsequently modified the risk stratification and the treatment approach [2,26]. For example, low-risk patients with MB-WNT received less intensified treatment. For MB-SHH-activated, further analysis with sequencing triggered by the methylation results confirmed mutated TP53 in two patients (one constitutional; one somatic), changing the prognosis dramatically; both patients showed progression during treatment and succumbed to their disease [27]. All MB, non-WNT/non-SHH, were subclassified into further subtypes I–VIII (Suppl. Table 2); this provided more information about the clinical behaviour of each tumour [28,29]. Patients with EPN were subclassified into supratentorial EPN with ZFTA fusion (ZFTA is also used for the previously known EPN-RELA fusion-positive), EPN-PFA and EPN-PFB; subgroups with distinct features, outcome and relapse patterns [3,30,31].

For 14 patients (Suppl. Table 4), the methylation results altered the diagnosis even with lower confidence or established a final diagnosis when that was not definite by histology, giving more information regarding the prognosis and the treatment approach. For instance, GR_014 (histologically diagnosed as ependymoma) was predicted to be an IHG with an *ALK::MSI2* fusion and started treatment with ALK-inhibitor (Alectinib) [21], achieving complete remission. GR_016 (histologically diagnosed as oligoastrocytoma) obtained high score for DLGNT; additional investigation revealed a *TNS3::NTRK2* fusion and targeted treatment with NTRK-Inhibitor (Larotrectinib) was initiated [32], showing partial remission for over 18 months.

### 3.6. Tumour samples checked at initial and recurrent disease

For five patients both materials from primary and recurrent disease were analyzed, and suggested methylation classes remained the same in both analyses (Table 4).

**Table 2**
List of tumours for which the initial histological diagnosis was changed or established in favor of the DNA methylation result, after interdisciplinary tumour board discussion.

| ID | Clinical information | Histological diagnosis, based on WHO classification | Methylation class upon initial request (plus, molecular findings) | Calibrated score | Revised diagnosis | Clinical impact of methylation result |
|---|---|---|---|---|---|---|
| GR_012 | 13 years old, male (primary) | Anaplastic astrocytoma, NOS, grade 3 | glioblastoma, IDH wildtype, subclass RTK III, grade 4 | 0.98 | Glioblastoma, grade 4 | Change of prognostication (upgrade) |
| GR_014 | 1 year old, female (relapse/ progression) | Ependymoma anaplastic, grade 2/3 | Infantile hemispheric glioma - *ALK* fusion detected | 1.00 | Infantile hemispheric glioma | New tumour type with ambiguous prognostication Targeted treatment |
| GR_017 | 11 years old, male (relapse) | Anaplastic Oligoastrocytoma, NOS, grade 3 | diffuse leptomeningeal glioneuronal tumour | 0.95 | diffuse leptomeningeal glioneuronal tumour | New tumour type with ambiguous prognostication |
| GR_053 | 1 year old, male (primary) | Medulloblastoma, NOS, grade 4 | embryonal tumour with multilayered rosettes, grade 4 -amplification of C19MC | 0.98 | embryonal tumour with multilayered rosettes, grade 4 | New tumour type with ambiguous prognostication |
| GR_059 | 9 years old, female (primary) | Pineoblastoma, grade 4 | CNS high grade neuroepithelial tumour with *BCOR* alteration - *BCOR* duplication confirmed with DNA sequencing | 0.98 | CNS high grade neuroepithelial tumour with *BCOR* alteration | New tumour type with ambiguous prognostication |
| GR_074 | 14 years old, female (primary) | Anaplastic astrocytoma, NOS, grade 3 | glioblastoma, IDH wildtype, subclass *MYCN*, grade 4 | 0.99 | Glioblastoma, grade 4 | Change of prognostication (upgrade) |
| GR_075 | 15 years old, male (relapse) | Anaplastic astrocytoma, NOS, grade 3 | glioblastoma, IDH wildtype, subclass RTK II, grade 4 | 1.00 | Glioblastoma, grade 4 | Change of prognostication (upgrade) |
| GR_101 | 2 years old, female (primary) | High grade glioma, NOS, Grade 4 | CNS high grade neuroepithelial tumour *BCOR* alteration (internal tandem duplication) -*BCOR* duplication confirmed with DNA sequencing | 1.00 | CNS high grade neuroepithelial tumour with *BCOR* alteration | New tumour type with ambiguous prognostication, change of treatment |
| GR_104 | 14 years old, female (relapse) | Anaplastic astrocytoma, NOS, grade 3 | glioblastoma, IDH wildtype, H3.3 G34 mutant, grade 4 | 0.94 | Glioblastoma, grade 4 | Change of prognostication (upgrade) |
| GR_119 | 9 years old, male (relapse) | Anaplastic astrocytoma, NOS, grade 3 | glioblastoma, IDH wildtype, subclass RTK III, grade 4 | 0.90 | Glioblastoma, grade 4 | Change of prognostication (upgrade) |

**Table 3**
Summary of tumours which received a high methylation score, but the result was deemed as non-contributing to the final diagnosis.

| ID | Clinical information | Histological diagnosis, based on WHO classification | Methylation class upon initial request | Discussion at the interdisciplinary tumour board | Revised diagnosis |
|---|---|---|---|---|---|
| GR_036 | 10 years old, female (primary) | Pilocytic Astrocytoma (PCA) | control class of non-neoplastic tissue (V12.8) | -Radiology: temporal lobe tumour more compatible with a low-grade glioma (LGG)<br>-Pathology: histological findings compatible with PCA<br>-Molecular results: detection of *BRAF V600E*-mutation | PCA, *BRAF V600E* mutation |
| GR_072 | 8 years old, male (relapse) | Atypical Teratoid/Rhabdoid Tumour (AT/RT) | control class of non-neoplastic tissue (V11b6) | -Radiology: parietal lobe tumour, more compatible with AT/RT relapse<br>-Pathology: histological findings compatible with AT/RT<br>-Molecular results: already known SMARCB1 germline mutation - rhabdoid tumour predisposition syndrome<br>-Comments: *difficulty of methylation classification in cases of underlying cancer predisposition syndromes* | AT/RT |
| GR_093 | 10 years old, male (relapse) | Medulloblastoma NOS | Pineoblastoma (V12.5) | - Radiology: no involvement of the pineal region<br>-Pathology: immunophenotypic features of medulloblastoma<br>-Molecular results: second higher calibrated score for medulloblastoma, group-3, detection of *SMARCA4* mutation, which also points towards MB-group-3<br>-Comments: *In our experience, several examples of small round blue cell tumours arising in the cerebellum would fit well with a histologic diagnosis of medulloblastoma yet appear by methylation to show a more pineal origin, and, therefore, may indicate a so far elusive background possibly relating to a distinct cell of origin of a rare embryonal tumour variant with epigenetic features of pineoblastoma* | MB-Group-3 |
| GR_130 | 10 years old, male (relapse) | yolk sac tumour | control class of non-neoplastic tissue (V11b6) | -Radiology: cerebellar tumour<br>-Pathology: histological findings compatible with yolk sac tumour, with elevated tumour markers (AFP) | Yolk Sac Tumour |

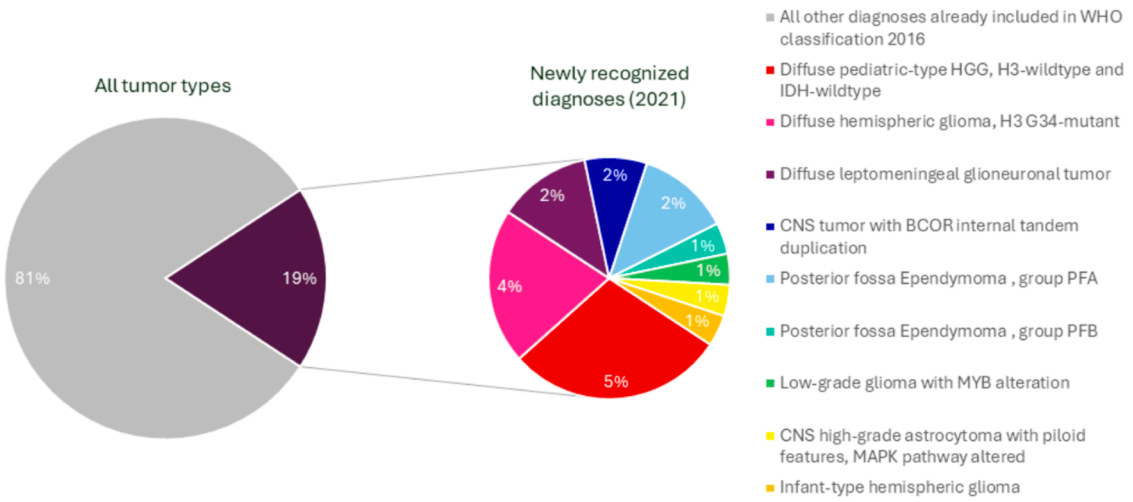


**Fig. 3.** Pie of pie chart presenting the percentage of newly recognized tumour diagnoses, introduced in WHO CNS5, based on their methylome profile, in our cohort. We have identified the following newly recognized tumour diagnoses in 18.5 % of all samples: Diffuse hemispheric glioma, H3 G34-mutant (n = 5); CNS high-grade astrocytoma with piloid features, MAPK pathway altered (n = 1) [19]; Diffuse pediatric-type HGG, H3-wildtype and IDH-wildtype (n = 7) [20]; IHG (n = 1) [21]; DLGNT (n = 3) [22]; EPN-PFA (n = 3) and EPN-PFB (n = 1) [23]; CNS tumour with *BCOR* internal tandem duplication (n = 2) [24]; and LGG with MYB alteration (n = 1).

**Table 4**
Results from 5 patients, whose samples have been analyzed in both primary diagnosis and recurrence of their disease. Cases with low scores have been reanalyzed with the newer classifier versions.

| Case no | ID | Status of disease | Histological diagnosis, according to WHO classification | Methylation class upon initial request | Classifier version | Score |
|---|---|---|---|---|---|---|
| 1 | GR_010 | Primary | Non conclusive: Ependymoma, grade 2/3 vs High-Grade Glioma, NOS | methylation class family Glioblastoma, IDH wildtype, subclass RTK III | Brain classifier V11b4 | 0.84 |
| | | | | *Reanalysis: Diffuse pediatric-type high grade glioma, H3 wildtype and IDH wild type, Subtype A&B (novel), highest score for subtype B* | Brain classifier V12.5 | 0.99 |
| | GR_011 | Recurrence | Glioblastoma, NOS | methylation class family Glioblastoma, IDH wildtype, subclass RTK III | Brain classifier V11b4 | 0.62 |
| | | | | *Reanalysis: Diffuse pediatric-type high grade glioma, H3 wildtype and IDH wild type, Subtype A&B (novel), highest score for subtype B* | Brain classifier V12.5 | 0.99 |
| 2 | GR_016 | Primary (retrospective analysis) | Anaplastic oligoastrocytoma, grade 3 | methylation class glioneuronal tumour, not otherwise specified, subtype A | Brain classifier V12.5 | 0.89 |
| | GR_017 | Recurrence | Anaplastic oligoastrocytoma, grade 3 | methylation class diffuse leptomeningeal glioneuronal tumour. | Brain classifier V11b4 | 0.95 |
| 3 | GR_037 | Primary | Embryonal brain tumour, more compatible with an embryonal tumour with multilayered rosettes NOS | No match | Brain classifier V11b4 | No |
| | GR_038 | Recurrence | Embryonal brain tumour, more compatible with an embryonal tumour with multilayered rosettes NOS | No match | Brain classifier V11b4 | No |
| 4 | GR_114 | Primary | Medulloblastoma, NOS | methylation class family Medulloblastoma, SHH | Brain classifier V11b4 | 0.98 |
| | GR_115 | Recurrence | Medulloblastoma, NOS | methylation class family Medulloblastoma, SHH | Brain classifier V11b4 | 0.98 |
| 5 | GR_117 | Primary | Glioblastoma, NOS | methylation class family Glioblastoma, IDH wildtype, mesenchymal subtype | Brain classifier V12.5 | 0.93 |
| | GR_118 | Recurrence | Glioblastoma, NOS | methylation class family Glioblastoma, IDH wildtype, mesenchymal subtype | Brain classifier V12.5 | 0.97 |

### *3.7. Reproducibility of the method*

DNA methylation results obtained from the blind testing of seven samples that were processed at our laboratory in Greece in parallel to DKFZ experimental pipeline, were fully concordant with DNA methylation results from DKFZ (Table 5).

## 4. Discussion

Methylome profiling is a recently introduced method which provides powerful information for the classification and diagnosis of CNS tumours. It has been shown to be highly robust, reliable, and reproducible for analyzing FFPE or fresh-frozen tumour samples, including samples of small size or poor-quality [16,33,34].

In this study, we investigated the integration of DNA methylation analysis with standard histopathological diagnostics in a cohort of 125 pediatric and adolescent patients with primary or recurrent CNS tumours in Greece. Upon initial request, 80 % of the profiled tumours obtained high Cs (≥ 0.9) and matched an established methylation class family/subclass. Previous studies report classification rates of 49–88 %,

**Table 5**
Results from seven samples that were processed at our laboratory in Greece in parallel to DKFZ experimental pipeline, fully concordant for the diagnosis based on the brain tumour classifier V12.8.

| Histology | Classifier result (txt_TUMOUR_450K_lang) | DKFZ score | Athens score |
|---|---|---|---|
| Pilocytic Astrocytoma | MC Pilocytic astrocytoma, infratentorial (PA_INF) | 0.99999804 | 0.99999 |
| Dysembryoplastic neuroepithelial tumour | MC Dysembryoplastic neuroepithelial tumour (DNET) | 0.98038565 | 0.98565 |
| Germinoma | MC Germinoma, subtype KIT mutant (novel) (GCT_GERM_KIT) | 0.99458 | 0.99318 |
| Pilocytic astrocytoma | MC Pilocytic astrocytoma, infratentorial (PA_INF) | 0.98289 | 0.98965 |
| Anaplastic Ependymoma | MC Posterior fossa group B (PFB) ependymoma, subclass 4 (novel) (EPN_PFB_4) | 0.99999 | 0.99999 |
| Medulloblastoma, NOS | MC Medulloblastoma, non-WNT/non-SHH, Group 4 subtype, subclass VI (MB_G34_VI) | 0.97159 | 0.98853 |
| Myxopapillary Ependymoma | MC Myxopapillary ependymoma (EPN_MPE) | 0.99998 | 0.99998 |

depending mainly on the inclusion criteria for analyzed patients, as cohorts focusing on difficult-to-diagnose tumours achieve lower scores [35–37] compared to population-based studies [38,39], including our study.

The methylation profile confirmed the initial histopathology in 86.5 % of tumours with high Cs, whereas in 48 %, it also refined the initial diagnosis by providing molecular subgrouping data unavailable with standard diagnostics. In ten tumours with high Cs, the diagnosis was established in favour of methylation results after interdisciplinary tumour board discussion, showing that integrating methylation analysis in routine diagnostics improves diagnostic accuracy by giving class prediction and providing guidance for additional testing, e.g. *ALK* fusions in IHG [21]. In four tumours with high Cs, the results were characterized as non-contributing, demonstrating that it is crucial to interpret methylation results in the context of histopathological, radiological, and clinical findings.

Upon initial request, 20 % of all tumours achieved low Cs, possibly due to low tumour cell content, low amount, or poor quality of extracted DNA [40]. Although we tried to analyze samples with more than 70 % tumour cells, the estimation of tumour cell percentage by pathologists is not always accurate [41]. In some patients, there was an association with hereditary tumour syndromes [17], or the tumour type was not included in the initial classifier cohort, e.g., the NET-PATZ1 tumour, which did not match with any methylation class initially but was classified with V12.5 as a novel tumour type [18]. Despite the low scores, in many cases we could still yield informative results, as we confirmed the initial diagnosis with less confidence or identified focal copy-number changes suggesting fusion events that we could subsequently confirm using targeted methods. Additional *t*-SNE analysis allowed possible class assignment by visual inspection and suggested novel molecular classes not represented in the original reference cohort. However, t-SNE is an experimental and subjective method and should be used cautiously for clinical decision-making.

Using newer classifier versions, more samples matched an established methylation class, demonstrating that the classification ability is expected to improve further through the increase of collected data. In 19 % of all samples, we identified newly recognized tumour diagnoses accepted into the CNS5 [9]. Although many of these new molecular entities could be possibly identified with conventional methods such as DNA/RNA sequencing, DNA methylation-based profiling is more efficient, by limiting costs, and saving time and tumour tissue [42]. Proper financial cost-effectiveness analyses need to be performed to support these initial observations.

Methylome profiling impacted the clinical management of almost 40 % of our patients. The molecular subgrouping of MB and EPN provided a framework to define low and high-risk patients more accurately, taking into consideration that these stratifications continue to evolve and become more refined with the accumulation of new data [2,3,26,28,30,43]. Precise classification better defined prognosis and allowed treating physicians to adjust oncological interventions by implementing targeted treatment, intensifying therapy or avoiding unnecessary chemo-and/or-radiotherapy and their associated side effects.

When materials from both initial and recurrent disease were analyzed, the suggested methylation class remained the same in both analyses, indicating that methylation profiling is robust regardless of the administered chemo- and/or radiotherapy, probably because it reflects the tumour cell of origin [16,38]. Finally, in accordance with previous published studies with large cohorts of patients [7,39], our local methylation profiling results support that the method is reproducible and efficient to set up locally.

## 5. Conclusions

Methylation-based classification is a well-established and effective diagnostic tool that has become essential in managing patients with CNS tumours. Our study supports the need to integrate methylome analysis into routine diagnostics in our country and highlights the importance of collaboration between European pediatric oncology centres to provide optimal care to young patients.

## CRediT authorship contribution statement

**Roser Pons:** Writing – review & editing, Investigation. **Christina Kanaka-Gantenbein:** Writing – review & editing, Investigation. **Dominik Sturm:** Writing – review & editing, Investigation, Formal analysis. **Maria Filippidou:** Writing – review & editing, Writing – original draft, Project administration, Methodology, Formal analysis, Data curation, Conceptualization. **Steffen Hirsch:** Writing – review & editing, Investigation. **Stavros Glentis:** Writing – review & editing, Project administration, Investigation, Formal analysis, Data curation. **Nicola Dikow:** Writing – review & editing, Investigation. **Ilona Binenbaum:** Writing – review & editing, Project administration, Investigation, Formal analysis, Data curation. **Nikolaos Katzilakis:** Writing – review & editing, Investigation. **Vita Ridola:** Writing – review & editing, Investigation. **Evgenia Papakonstantinou:** Writing – review & editing, Investigation. **Vassilios Papadakis:** Writing – review & editing, Investigation. **Emmanouel Hatzipantelis:** Writing – review & editing, Investigation. **Eleftheria Kokkinou:** Writing – review & editing, Investigation. **David T. W. Jones:** Writing – review & editing, Investigation, Formal analysis, Data curation. **Andreas Von Deimling:** Writing – review & editing, Investigation, Formal analysis. **Felix Sahm:** Writing – review & editing, Investigation, Formal analysis, Data curation. **Mirjam Blattner-Johnson:** Writing – review & editing, Investigation, Formal analysis, Data curation. **Kalliopi Stefanaki:** Writing – review & editing, Validation, Investigation, Formal analysis, Data curation. **Kathrin Schramm:** Writing – review & editing, Investigation, Formal analysis, Data curation. **Stefan M. Pfister:** Writing – review & editing, Writing – original draft, Supervision, Methodology, Conceptualization. **Clio Trougkou:** Writing – review & editing, Investigation, Formal analysis, Data curation. **Antonis Kattamis:** Writing – review & editing, Writing – original draft, Supervision, Methodology, Conceptualization. **Dimitrios Doganis:** Writing – review & editing, Investigation. **Kristian W. Pajtler:** Writing – review & editing, Investigation, Formal analysis. **Martin Sill:** Writing – review & editing, Formal analysis, Data curation. **Cornelis M. van Tilburg:** Writing – review & editing, Investigation. **Kleoniki Roka:** Writing – review & editing, Investigation, Data curation. **Michael C. Frühwald:** Writing – review & editing, Investigation, Formal analysis. **Antonia Vlachou:** Writing – review & editing, Investigation, Data

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## Declaration of Competing Interest

The authors declare the following financial interests/personal relationships which may be considered as potential competing interests: Andreas Von Deimling, Martin Sill, Felix Sahm, Stefan M Pfister and David T. W. Jones are co-founders and shareholders of Heidelberg Epignostix GmbH.

All remaining authors have declared no conflicts of interest.

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## Appendix A. Supporting information

Supplementary data associated with this article can be found in the online version at doi:10.1016/j.ejcped.2024.100198.

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