BRAIN TUMORS IN CHILDREN

National population-based studies on classification, diagnostics and long-term follow-up

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To my family
for your endless support and encouragement.

Brain tumors in children

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ABSTRACT

Although the prognosis for pediatric tumors of the central nervous system (CNS) has improved over time it is urgent to reduce mortality and improve long-term quality of life for survivors. With this aim, the first step is to identify the correct diagnosis in order to choose the right therapy and avoid unnecessary treatment. Establishing a correct diagnosis can be challenging and genome-wide DNA methylation profiling has evolved as a valuable tool in the diagnostics of childhood CNS tumors. The aim of this thesis was 1) to provide comprehensive data on children diagnosed with a CNS tumor in Sweden between 1984-2021, and 2) to evaluate the added value of performing DNA methylation profiling in the standard diagnostics of pediatric CNS tumors in Sweden.

In Paper I we found a stable incidence of childhood CNS tumors during the study period of almost 40 years. The distribution of tumor diagnoses was relatively comparable to that reported from other countries. Overall survival for children diagnosed with a CNS tumor has improved over time but for several tumor types the long-term survival rates continued to decrease.

In paper II we determined that integrating DNA methylation profiling into real time diagnostics of pediatric CNS tumors improves diagnostics and provides molecular information that has shown to be essential for choosing the optimal treatment. We demonstrated that methylation profiling has a role in the classification of all types of CNS tumors, also those with a lower tumor cell content.

In paper III we re-classified tumors formerly diagnosed as CNS primitive neuroectodermal tumor (PNET). We confirmed the heterogeneity of these tumors and concluded that DNA methylation has a pivotal role in the diagnostics of rare childhood embryonal tumors. The survival rates for the re-classified tumor types were in line with other studies. All patients with CNS NB-*FOXR2* had received craniospinal irradiation and the prognosis was excellent.

Keywords: Pediatric CNS tumors, population-based, DNA methylation, profiling, CNS-PNET, re-evaluation, epidemiology, incidence rate, long-term outcome, classification

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SAMMANFATTNING PÅ SVENSKA

Av de barn i Sverige som drabbas av cancer får ca 25% en tumör i centrala nervsystemet (CNS), det vill säga i hjärna och ryggmärg. Även om överlevnaden har förbättrats så fortsätter den att minska även långt efter att patienterna fått sin diagnos. Ibland är det utmanande att ställa rätt diagnos då varje enskild CNS tumör i sig är ovanlig och ofta heterogen i sitt utseende. Tumörcellernas DNA ser olika ut. De skiljer sig bland annat åt i sitt metyleringsmönster, d.v.s var metylgrupperna sitter på DNA-strängen. Genom att analysera DNA-metyleringsprofilen i tumörcellerna kan man klassificera hjärntumörer på ett mer specifikt sätt. Målsättningen med denna avhandling var att se om diagnostiken kunde förbättras genom att studera DNA metyleringsmönstret i cancercellerna samt att se hur insjuknandet och överlevnaden av CNS tumörer hos barn har förändrats mellan åren 1984-2021.

I delarbete I kunde vi se att antalet barn som varje år diagnosticerades med en CNS tumör var oförändrat sedan nästan fyrtio år tillbaka. Vi kunde också se att överlevnaden för barn som insjuknat med en hjärntumör har förbättrats men att den tyvärr sjunker även lång tid efter diagnosen, vilket visar hur viktigt det är med livslång uppföljning för denna patientgrupp.

I delarbete II utvärderade vi om diagnostiken av CNS tumörer i Sverige kunde förbättras genom att analysera DNA metylerings profiler på alla nydiagnosticerade CNS tumörer under fyra års tid. Vi konstaterade att metyleringsbaserad tumörklassifikation är viktig att utföra i rutindiagnostiken av CNS tumörer hos barn. Vi såg att i flertalet fall blev diagnosen mer precis då metyleringanalysen bidrog med ytterligare information om vilken undergrupp tumören tillhörde vilket kan ha betydelse för val av behandling och prognos. I 5% av fallen skulle den metyleringsbaserade klassificeringen haft en direkt påverkan på den kliniska handläggningen.

I delarbete III studerade vi de hjärntumörer som tidigare fått diagnosen primitiv neuroektodermal tumör (PNET) i Sverige. Under de senaste åren har man upptäckt att denna tumör utgörs av flera olika tumörtyper. Med hjälp av DNA-metyleringsteknik så reklassificerade vi tumörerna i efterhand. Vi såg att denna tumörgrupp bestod av flera olika tumörtyper. Genom att samla in klinisk information om vilken behandling patienterna fått såg vi att de olika tumörtyperna svarat olika bra på behandling vilket bidrar till att vi idag vet mer om hur vi skall behandla dessa patienter.

LIST OF PAPERS

This thesis is based on the following studies, referred to in the text by their roman numerals.

I. **Schepke E**, Lannering B, Lähteenmäki P, Georgantzi K, Sandström P-E, Nyman P, Eliasson-Hofvander M, Öberg A, Carén H, Sabel M. Incidence and long-term survival in children diagnosed with CNS tumors in Sweden 1984-2021. *Manuscript*.

II. **Schepke E**, Löfgren M, Pietsch T, Olsson Bontell T, Kling T, Wenger A, Ferreyra Vega S, Danielsson A, Dosa S, Holm S, Öberg A, Nyman P, Eliasson-Hofvander M, Sandström P-E, Pfister S M, Lannering B, Sabel M, Carén H. DNA methylation profiling improves routine diagnosis of paediatric central nervous system tumours: A prospective population-based study. *Neuropathol Appl Neurobiol,* 2022, 48(6), e12838. doi.org/10.1111/nan.12838

III. **Schepke E**, Löfgren M, Pietsch T, Kling T, Nordborg C, Olsson Bontell T, Holm S, Öberg A, Nyman P, Eliasson-Hofvander M, Sabel M, Lannering B, Carén H. Supratentorial CNS-PNETs in children; a Swedish population-based study with molecular re-evaluation and long-term follow-up. *Clinical Epigenetics*, 2023, 15(1):40. doi.org/10.1186/s13148-023-01456-2

Additional publications not included in this thesis.

I **Schepke E**, Tisell M, Kennedy C, Puget S, Ferroli P, Chevignard M, Doz F, Pizer B, Rutkowski S, Massimino M, Navajas A, Schwalbe E, Hicks D, Clifford SC, Pietsch T, Lannering B. Effects of the growth pattern of medulloblastoma on short-term neurological impairments after surgery: results from the prospective multicenter HIT-SIOP PNET 4 study. *J Neurosurg Pediatr*, 2020 Jan 17:1-9. doi.org/10.3171/2019.11. Peds 19349

II Von Hoff K, Habereler C, Schmitt-Hoffner, F, **Schepke E,** de Rojas T, Jacobs S, Zapotocky M, Sumerauer D, Perek-Polnik M, Dufour C, van Vuurden D, Salavc I, Gojo J, et al. Therapeutic implications of improved molecular diagnostics for rare CNS-embryonal tumor entities: results of an international, retrospective study. *Neuro Oncol*. 2021, 23(9):1597-1611. doi.org/10.1093/neuonc/noab136

III Wenger A, Ferreyra Vega S, **Schepke E**, Löfgren M, Olsson Bontell T, Tisell M, Nilsson D, Kling T, Carén H. DNA methylation alterations across time and space in paediatric brain tumours. *Acta Neuropathol Commun,* 2022 Jul 16;10(1):105. Doi.org/10.1186/s40478-022-01406-8

IV Wenger A, Larsson S, Danielsson A, Elbæk KJ, Kettunen P, Tisell M, Sabel M, Lannering B, Nordborg C, **Schepke E**, Carén H. Stem cell cultures derived from pediatric brain tumors accurately model the originating tumors. *Oncotarget,* 2017 8(12), 18626-18639. doi.org/10.18632/oncotarget.14826

CONTENT

ABBREVIATIONS

AT/RT	Atypical Teratoid/Rhabdoid Tumors
CNS	Central nervous system
CDKN2A/B	Cyclin-dependent kinase inhibitor 2A/B
CNA	Copy number alterations
CpG	Cytosine Guanine
CSI	Craniospinal irradiation
DIA	Desmoplastic infantile ganglioglioma/ astrocytoma
DNA	Deoxyribonucleic acid
DNET	Dysembryoplastic neuroepithelial tumor
DNMT	DNA methyltransferase
FISH	Fluorescence In situ Hybridization
FFPE	Formalin-fixed paraffin-embedded
H&E	Hematoxylin and Eosin
HDSCR	High-dose chemotherapy with stem cell rescue
ICCC	International Classification of Childhood Cancer
ICD-O	International Classification of Diseases for Oncology
IHC	Immunohistochemistry
MNP	Molecular neuropathology
MRI	Magnetic resonance imaging
OS	Overall survival
PA	Pilocytic astrocytoma
PCR	Polymerase chain reaction
PFS	Progression-free survival
PNET	Primitive neuroectodermal tumor
TET	Ten-eleven translocation
WHO	World Health Organization

01

INTRODUCTION

Every year in Sweden around 330 children are diagnosed with a pediatric neoplasm and about 27% of them have a tumor in the central nervous system (CNS)[1]. The prognosis for CNS tumors has improved over time[2] and the 10-year overall survival (OS) in Sweden is more than 70%, although this is dependent on the histopathological diagnosis, tumor site and treatment. The survival for malignant CNS tumors is still poor. Cancer is the major cause of death in children aged 1-14 years in Sweden, and CNS tumors constitute the largest proportion of neoplasms leading to death in this age group[3]. Childhood cancer survivors suffer from serious side effects and bear an increased risk of developing short-term and long-term sequelae including neurological deficits, endocrinopathy, and cognitive impairments[4-7].

In the last decades it has become evident that cancer is not only a genetic disease, but also an epigenetic disease[8,9]. Childhood neoplasms differ considerably in their molecular alterations compared to adult cancer. The number of mutations is lower but also the molecular driving events are different. The mutations that occur in childhood cancer often involve epigenetic regulation[10]. For the development of childhood tumors, it also seems crucially important in which developmental window the affected cell is in, when the first genetic and epigenetic changes occur[11,12]. In recent years, the rapidly advancing epigenetic research has increased our understanding of how important the epigenetic dysregulation is for the onset of childhood neoplasm. It has also given us new perspectives on how these epigenetic modifications can be used in the diagnostics and prognostics of CNS tumors.

1.1 Epidemiology of CNS tumors in children

Central nervous system tumors are the second most common pediatric neoplasm after leukemia, and the most frequent solid tumors in children. It is a very diverse group of tumors that differ considerably as far as the clinical and biological characteristics are concerned. The etiology of most CNS tumors in children is still unknown. Ionizing radiation and certain genetic syndromes have been described as risk factors for predisposition to the development of a brain tumor[13, 14].

The annual incidence rates for childhood brain tumors vary internationally, with a global age-standardized average incidence rate of 2.8/100 000 children in the age range of newborn to 14 years[15]. However, in Sweden, as well as in the other Nordic countries, the UK, France and the US, the incidence rates for CNS tumors are considerably higher (3.8-5.9/100 000 children) [2, 16-19]. In the mid-1980s, an increase in the incidence of pediatric brain tumors was noted in several countries[18,20,21] probably due to the diagnostic improvements after the introduction of computer tomography and later magnetic resonance imaging (MRI), as well as the introduction of a more accurate registration[21]. This increase does not seem to have continued and recent studies from several countries have reported on a stable incidence of pediatric CNS tumors during the last decades[2,16,18,19,22,23]. However, a trend towards a slightly higher incidence over the last 30 years was noted in a recent publication from Finland (Abuhamed et al., 2022). Furthermore, according to the latest report from the Central Brain Tumor Registry of the United States (CBTRUS), CNS tumors are the most common neoplasm in children aged 0–14 years[17].

The age-distribution of CNS tumors in children is relatively even in the ages 1 - <15 years of age at diagnosis[23]. Overall, brain tumors are more common in males than females even though this varies depending on tumor type. Almost half of all pediatric CNS tumors

are located in the posterior fossa, with the majority sited in the cerebellum (28-31%) or in the brain stem (11-13%). The remaining are situated in the cerebral hemispheres (21-24%), the midbrain (13-27%), or in the spinal cord (3-4%)[24, 25].

Clinical symptoms mainly depend on the localization of the tumor and the age of the patients. The most common initial symptoms are headache and nausea followed by motorical weakness, visual impairment, and seizures[25, 26]. The presenting symptoms are often varying and nonspecific which is a challenge to the clinician to achieve a correct diagnosis[27, 28].

The diagnoses of CNS tumors in children and juveniles differ from adults[17]. Roughly, childhood tumors of the CNS can be divided into tumors of glial (astrocytes, oligodendrocytes, and ependymal cells) and non-glial origin. Glial tumors are most common and include astrocytomas, oligodendrogliomas, ependymomas and glioneuronal tumors (e.g., gangliogliomas). Embryonal tumors, germ cell tumors and craniopharyngiomas for instance, are non-glial tumors (Figure 1)[2,23].

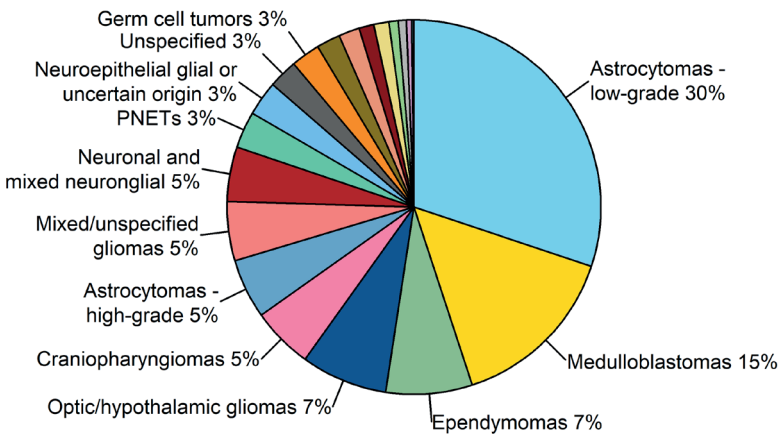

FIGURE 1. Distribution of pediatric CNS tumors in Sweden 1984-2013. Based on data extracted from Gustafsson et al., 2013[23].

1.2 Diagnostic classification of tumors of the CNS

The diagnosis of pediatric CNS tumors is based on the histopathological and molecular criteria from the World Health Organization (WHO)[29]. In 1979, the WHO published the first edition of the histological typing of tumors of the central nervous system[30] and since then the so called "Blue book" has been updated several times[29,31-33]. Previously, all tumors of the CNS were classified solely based on their histopathological features and the histological grading was based on morphological characteristics such as mitotic activity, cytological atypia, etc. The WHO grading system was used to predict the biological behavior of the tumor and thus also a way to predict the clinical course of the patients and ranged from I-II (non-malignant) to III to IV (malignant)[32].

The establishment of a diagnosis based solely on morphology, did not always correlate to the clinical course of the patients[34-36]. Previous studies have also reported on certain inter-observer inconsistency in the diagnostics for some tumor entities[37,38]. A major adjustment in the classification criteria of brain tumors was performed in 2016 in the WHO Classification of CNS tumors[33]. The classification was, for the first time, extended to incorporate molecular markers for some tumor types. By combining molecular information and histological features into an integrated diagnosis, the tumor classification gained enhanced precision, and reduced the heterogeneity for various tumor types[29]. This applied not only to the tumor specific group patterns, but also to the specific response to treatment and clinical outcomes. However, due to the rapidly increased understanding of the molecular mechanisms in CNS tumors in recent years, it has become evident that additional classification updates in-between the standard WHO updates are needed. Therefore, a group of experienced neuropathologists and neuro-oncologists formed the Consortium to Inform Molecular and Practical Approaches to CNS Tumor Taxonomy - Not Official WHO (cIMPACT-NOW) in 2016[39] to provide continuous updates in the molecular classification and to recommend changes to future

CNS tumor classifications. The most recent update of the WHO classification; the 5th edition of the WHO Classification of the Tumors of the Central Nervous system (CNS5) was published in 2021[29] and is based on the recommendations from the cIMPACT-NOW[40-46]. The 5th WHO classification has listed more than 100 different types of tumors in the CNS, with varying biological behavior and different clinical courses. Molecular markers have become increasingly important, both in terms of determining the specific diagnosis and for determining the grading of the tumors (WHO grade 1-4), as well as providing a prognostication for the patient[29]. Several newly recognized tumor types and subtypes have been introduced, and the tumors are grouped into more biologically and molecularly defined entities. This is exemplified in the classification of diffuse gliomas, where the tumors are separated into *adult*-type and *pediatric*-type based on their molecular genetic differences. Moreover, DNA methylation profiling has been recognized in the 2021 WHO CNS5 as an essential diagnostic tool for selected tumor types and a desirable analysis for most of the CNS tumors. Importantly, for some tumor types, the molecular subgrouping has been shown to be superior to the histopathologic grading when it comes to prognosis, e.g., posterior fossa group A ependymoma has a poorer prognosis compared to posterior fossa group B ependymoma. Both of these tumors can be diagnosed through DNA methylation profiling or distinguished by the loss or presence of nuclear H3K27me3 by immunohistochemistry (IHC).

Furthermore, as far as the development of new drugs and research is concerned, molecular classification is essential; as nowadays many patients can be eligible for inclusion in clinical trials of potential targeted therapy which are based on the molecular alteration of the tumor. However, the recent advancement in tumor biology has not yet fully reached the clinical work in regards of therapy adjustment. Still, the main choice for treatment is chemotherapy and sometimes radiotherapy.

1.3 Epigenetics

The term epigenetics, literally "outside or above genetics", refers to the changes in the gene function that do not involve alterations in the underlying DNA sequence[47,48]. All cells in the body contain the same genetic information but are expressed differently in order to form the various existing cell types. This is due to the epigenetic regulation, which alters the expression of genes in different cell types and thus controls gene transcription and cell development[49,50]. These epigenetic changes are transferable during cell division and their regulation of gene expression is crucial for the development of normal human cells[51]. There are three major epigenetic mechanisms; DNA methylation, histone modifications and non-coding RNAs (Figure 2)[52,53]. Histones are proteins that organize the DNA into chromatin and the genome is then further condensed into chromosomes inside the cell nucleus. The histone modifications occur at the histone tails and include for example methylation, phosphorylation and acetylation, which can alter the chromatin structure and affect gene expression[54,55]. Non-coding RNA is RNA that is not translated into a protein. There are long or short non-coding RNAs and they are involved in the regulation of gene expression through posttranscriptional modifications[56,57]. All these epigenetic markers interact with each other and together they regulate the gene expression through the chromatin architecture.

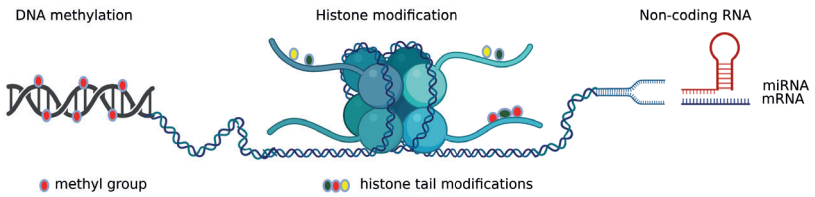

FIGURE 2: The epigenetic machinery. DNA methylation, histone modification and non-coding RNA. Created with Biorender.com

1.3.1 DNA methylation

DNA methylation is a crucial epigenetic mechanism involved in the regulation of gene expression and cell differentiation[58,59]. It is the process where a methyl group (CH_3) is added to the 5th carbon position of the cytosine ring in the DNA sequence. This is catalyzed by DNA methyltransferase enzymes (DNMTs) which transfer a methyl group from S-adenosyl methionine to an unmethylated cytosine[60,61]. The methylation typically occurs at a so called CpG site, where a cytosine is followed by a guanine nucleotide (Figure 3). The DNA methylation process is reversible, either by a passive demethylation or by the active process where the ten-eleven translocation (TET) enzymes remove the methyl group[62,63].

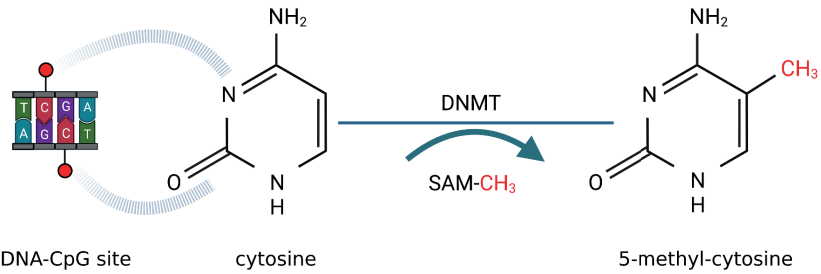

FIGURE 3. The DNA methylation process. A methyl group is transferred from S-adenosyl methionine to a cytosine nucleotide by DNA methyltransferase (DNMT). Created with Biorender.com

There are approximately 28 million CpG sites across the human genome of which the majority are methylated[64]. These CpG sites are not equably distributed, and clusters of CpG sites appear on the DNA strands, so called CpG islands. These islands are usually located in the gene promotor regions and are mainly unmethylated in normal somatic cells[65-67]. The methylation pattern in the promotor region regulates the gene expression; an unmethylated promotor region permits for gene expression, while a hypermethylated promotor silences gene transcription (Figure 4)[68,69].

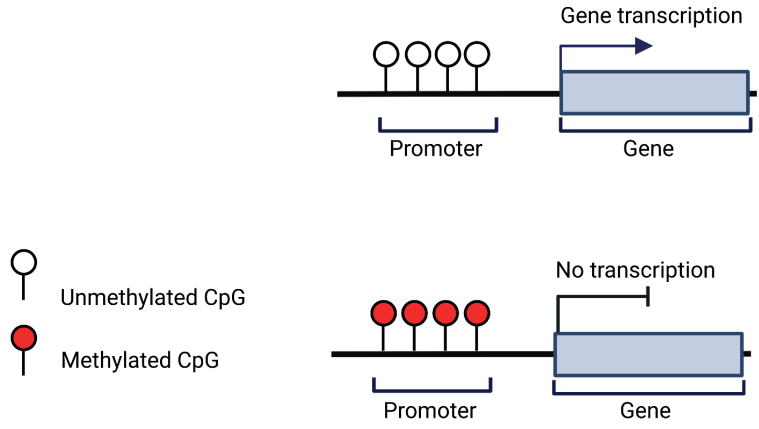

FIGURE 4. DNA methylation and gene expression. Created with Biorender.com

1.3.2 Epigenetics in cancer

The methylation pattern in tumor cells differs from that of normal cells[70]. It has been demonstrated that different cell types, including cancer cells, display a distinct DNA methylation pattern that results in a specific fingerprint which reflects the cell-of-origin[71]. This fact is the background for the diagnostic capacity of DNA methylation.

In cancer tissue, a lower level of DNA methylation is observed genome-wide which leads to an increased frequency of mutations and an instability of chromosomes[51,72]. In tumor suppressor genes, however, there is a hypermethylation of CpG sites in promotor regions which results in inactivation of gene transcription. In oncogenes the hypomethylation of promotor regions leads to an activation of transcription (Figure 5)[71,73].

Pediatric CNS tumors generally have much fewer genetic mutations compared to adult tumors, and the mutations that do occur in pediatric CNS tumors often encode epigenetic regulation[10, 74]. This suggests that epigenetic dysregulation is an important mechanism in tumor development in children, but all aspects are

not yet fully understood[11]. For example, specific histone mutations have been identified as the driving mutations, in pediatric high-grade gliomas. The majority of pediatric high-grade gliomas contains driver mutations in the histone *H3.3*, which cause amino acid substitutions in the histone tail; *K27M* (for diffuse midline gliomas) or *G34R/V* (for diffuse hemispheric gliomas) leading to epigenetic dysregulations.

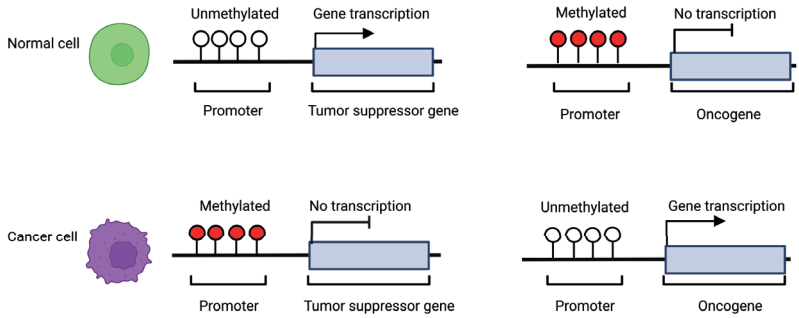

FIGURE 5. Alterations of DNA methylation in cancer.
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1.3.3 DNA methylation for CNS tumor diagnostics and prognostics

As mentioned earlier, CNS tumors in children are rare and heterogenous, which makes diagnostics challenging. A correct diagnosis is highly essential to choose the appropriate therapy. Histopathological evaluation has shown interobserver variability and new diagnostic modalities have been desirable[38]. In DNA methylation, the methyl group is tightly bound to the DNA strand resulting in a stable molecule that is suitable for diagnostic testing. A great advantage of DNA methylation profiling is that it can be performed on paraffin-embedded formalin-fixed (FFPE) material as well as on fresh frozen tissue[75,76]. CNS tumor classification using DNA methylation profiling has been proven to be a trustworthy and robust tool to classify CNS tumors[77-82]. Based on the different methylation signatures from various CNS

tumors, DNA methylation-based classifiers for acknowledged tumor types have been created using a machine learning algorithm. In recent years, several different methylation-based classifiers have been developed and shown to trustworthy classify CNS tumors; MethPed[78], a meningioma classifier[83] and a glioma classifier[84]. This methylation-based tumor classification is not limited to CNS tumors and has also shown to have a potential in the clinical diagnostics for soft tissue and bone sarcomas[85]. The most widely used classifier for CNS tumors is the Molecular neuropathology (MNP classifier) which was published in 2018 by Capper et al. The CNS tumor types and subtypes included in the classifier were based on methylation profiles from 2800 reference cases and the algorithm could classify the tumors into several different classes and subclasses[77,86]. Furthermore, for some tumor types, the methylation-based classification can predict the patients' prognosis more accurately than the histopathological classification. This also applies to medulloblastoma, the most common malignant brain tumor in childhood, where methylation profiling among other molecular techniques has identified four molecular subgroups (wingless (WNT)-activated, Sonic Hedgehog (SHH)-activated, Group 3 and Group 4), which clearly differ in prognosis[35,87,88]. Today, this categorization is the base for the choice of treatment to the patients. The molecular classification of CNS tumors is a dynamic field rapidly evolving, and nowadays the four medulloblastoma subgroups, can be further divided into several subtypes[89]. The clinical implications of this extended taxonomy remain to be elucidated.

Likewise, the ependymomas, which is the third most common tumor type in children, are divided into several subclasses by methylation profiling leading to the revelation of diagnostic and prognostic information beyond histology[90,91].

Another important benefit from methylation-based tumor classification is the maintenance of the methylation signatures between the primary tumor and its relapse which is of great importance in clinical practice[92-94].

Besides the methylation data, the methylation arrays can provide chromosomal copy number data which can identify chromosomal changes, so called copy number alterations (CNA). Some chromosomal gains and losses as well as focal gene deletions or amplifications are more common for certain tumor types and the copy number alterations can assist in diagnostics as well as to identify gene fusions[95].

1.4 Primitive neuroectodermal tumors of the CNS

About 15-20% of all primary brain tumors in children are embryonal tumors, which is a heterogeneous group of malignant CNS tumors (WHO grade 4) that primarily affects young children. Embryonal tumors show a similar morphology characterized by small, round cells with scant cytoplasm and varying degree of differentiation, but the histopathological diagnosis is challenging due to the lack of specific molecular markers. Over the years it has been a discussion how to classify these tumors. For long they were separated into different tumor entities based on the tumor site; a medulloblastoma if located in the posterior fossa, a pineoblastoma if sited in the pineal gland and a supratentorial or primitive neuroectodermal tumor of the CNS (CNS-PNET) if located above the tentorium[96]. Although the tumors had similar morphology, clinical studies showed that survival rates for children with a CNS-PNET were worse compared to children with medulloblastoma treated in the same way[97,98]. In 2002, microarray-based analyses could demonstrate that medulloblastoma, atypical teratoid rhabdoid tumors (AT/RT) and CNS-PNET were molecularly distinct tumor entities[34].

During the last decades, additional molecular markers and DNA methylation profiling have segregated the CNS-PNETs into different tumor entities or new molecular categories[78, 99-101]. In a study by Sturm et al., 323 institutionally diagnosed CNS-PNETs were analyzed using DNA methylation profiling and the majority of tumors clustered to other known tumor entities, including misdiagnosed high-grade gliomas (HGGs), ependymomas (EPNs), AT/RTs, embryonal tumors with multi-layered rosettes (ETMRs) and new previously unknown tumor entities were discovered including CNS neuroblastoma with *FOXR2* activation (CNS-NB-*FOXR2*), CNS high-grade neuroepithelial tumor with *BCOR* alteration (CNS HGNET-*BCOR*), high-grade neuroepithelial tumor with *MN1* alteration (HGNET-*MN1*) and Ewing sarcoma family tumor with *CIC* alteration (EFT-*CIC*) [102]. Therefore, in the WHO classification from 2016 the term CNS-PNET was removed and substituted by the term CNS embryonal tumor

NOS. As a consequence of this reclassification, fewer tumors received the diagnosis of CNS embryonal tumor NOS.

In the WHO CNS5, these newly identified tumor types were incorporated as CNS neuroblastoma, *FOXR2*-activated (CNS NB-*FOXR2*) and CNS tumor with *BCOR* internal tandem duplication (CNS *BCOR* ITD). The tumor type *CIC*-rearranged sarcoma is currently listed under mesenchymal tumors in the CNS[29].

Among the embryonal tumors, medulloblastoma is the most common and constitutes approximately 15% of all CNS tumors in children, whereas CNS-PNET accounted for 3-5% of all pediatric CNS neoplasm[2,24]. The median age at diagnosis for children diagnosed with a CNS-PNET was 3.5 years[103] and approximately 40% of the patients had metastatic disease at diagnosis according to Chang stage[104].

Patients diagnosed with a CNS-PNET were historically treated with the same protocols as high-risk medulloblastoma, including surgical resection of the tumors followed by craniospinal radiation (CSI) for children over the age of 3-5 years (4 years in Sweden) and chemotherapy[105-107]. For younger children (< 3-5 years) radiation-sparing treatment was given, most often according to the German treatment protocols[108,109].

The overall survival for CNS-PNET was poor with 5-year overall survival rates of 43-49%[110-113]. However, after having realized that CNS-PNET consists of different tumor entities, retrospective clinical studies have shown that survival differed between the included tumor types; best OS was seen for CNS NB-*FOXR2* and worst for ETMR[102, 114-117].

Each of these rare embryonal tumors is unusual and specific clinical and treatment data are sparse. Some of the tumor types will be briefly presented beneath. Upon diagnosis, all patients undergo an initial staging procedure, including pre-and postoperative MRI of the brain and spine as well as a postoperative lumbar craniospinal fluid (CSF) cytology evaluation.

1.4.1 Embryonal tumor with multilayered rosettes

Embryonal tumor with multilayered rosettes is a highly malignant tumor primarily occurring in children younger than three years of age[117, 118]. The true incidence of ETMR is currently not known, but it seems like ETMRs constitute a relevant proportion of the former CNS-PNETs[102]. The predominant tumor location is supratentorial (64-70%), followed by the cerebellum and brain stem[119-121]. Disseminated disease at diagnosis has been reported in 24-27%[117-119].

Diagnostic classification of ETMR

The histological patterns in ETMRs are variable, with three main patterns being described, all of which are characterized by the findings of multilayered rosettes[29,122]. Besides the histological findings, the diagnostic criteria for ETMRs are based on molecular alterations such as amplification of the microRNA cluster on chromosome 19q (*C19MC*), which is present in approximately 90% of all ETMRs[101,123] independent of the histological pattern. The expression of the microRNA cluster is often associated with the fusion of the promotor-gene of Tweety family member 1 (*TTYH1*) upstream of the *C19MC* which is thought to be the main driver of the tumor[120,124]. Some of the ETMRs that do not have the microRNA cluster amplification have a biallelic mutation in the *DICER1* gene, which results in an improper processing of the microRNAs[125]. Typically, the biallelic mutations are combinations of a germline mutation and a second somatic mutation and is often part of the cancer predisposition syndrome *DICER1*[125].

Besides the alterations affecting the microRNA pathway, the ETMRs are characterized by a high expression of the RNA-binding protein LIN28A, which can be verified by staining[126]. Although this LIN28A expression is not entirely specific to ETMR (occasionally also seen in AT/RTs, germinomas, high-grade gliomas) it is considered as a characteristic marker for this tumor[127]. In addition, ETMR tumors have been shown to exhibit a distinctive DNA methylation pattern[124,125] and the *C19MC* amplification can, for example, be detected by fluorescence *in*

situ hybridization, single nucleotide polymorphism array or DNA methylation array. A histological image of ETMR and a CNA plot are shown in Figure 6.

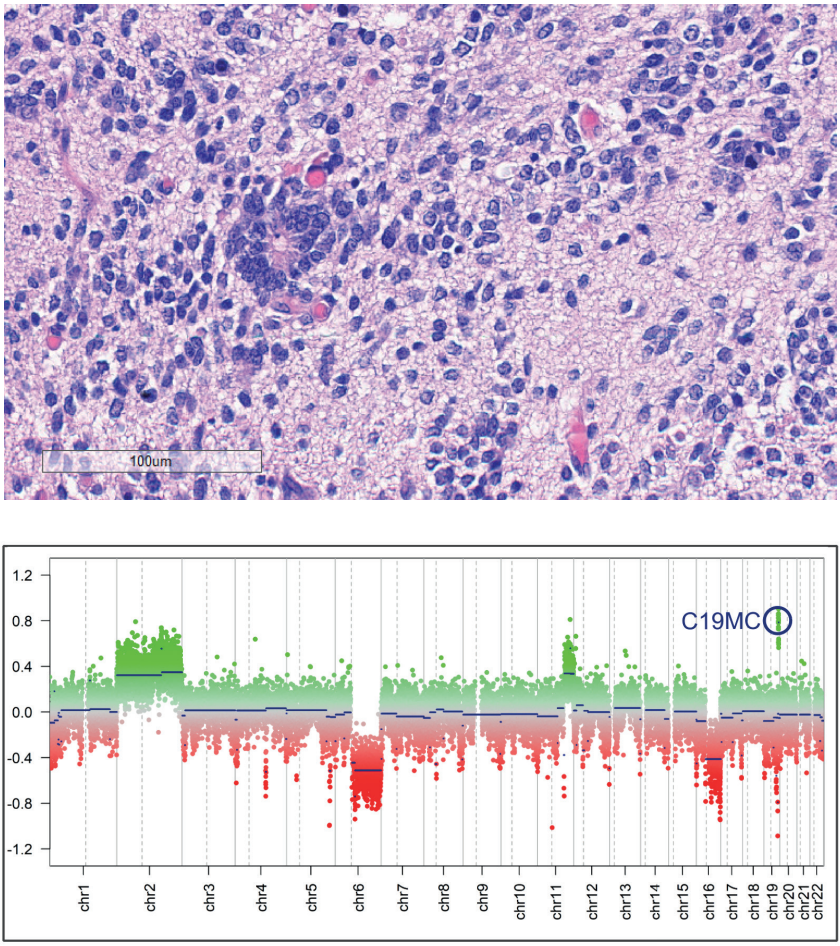

FIGURE 6. Multilayered rosettes typical for embryonal tumors of multilayered rosettes and a CNA plot demonstrating the amplification of the *C19MC* on chromosome 19.

Therapeutic management and clinical outcome

Most of the current knowledge about the clinical features and outcomes of ETMRs relies on retrospective studies on children previously diagnosed with a CNS-PNET. The overall prognosis for children diagnosed with an ETMR tumor is very poor with a reported median survival of 12.3 months and 5-year survival rates of 18-28%[115,117,126] despite intensive treatment. Prognostically important factors associated with a longer survival are localized disease and non-brainstem location[119]. Two recently published studies reported a significant better outcome (2 and 5-year survival rates of 66% and 58%, respectively) for children with non-brainstem, localized ETMR when treatment was combined with complete surgical resection, high-dose chemotherapy with stem cell rescue (HDSCR) and radiation (focal or CSI) [117,119].

1.4.2 CNS neuroblastoma, *FOXR2*-activated

Diagnostic classification of CNS NB-*FOXR2*

In the WHO classification of CNS tumors from 2016, the diagnosis of CNS neuroblastoma was incorporated as a separate tumor entity solely defined by its histological features[33]. CNS neuroblastomas are tumors with neuroectodermal origin, although the exact cell of origin is unknown. Later it became evident that these tumors are characterized by specific chromosomal rearrangements which result in an increased activation of the transcription factor forkhead box R2 (*FOXR2*)[102]. The dysregulation of the *FOXR2* gene is important in the tumorigenesis for different tumors and for inducing CNS embryonal tumors[128,129]. In the WHO CNS5 this tumor is defined as a separate tumor type named CNS neuroblastoma, *FOXR2*-activated[29,45]. In addition to the histopathological findings, the tumors are characterized by a typical methylation profile and can be reliably classified using DNA methylation analysis[116,130]. Nearly all these tumors exhibit typical chromosomal alterations such as gain of 1q, loss of 3p and 16q as well as gain of 17q[114,116,131].

Therapeutic management and clinical outcome

So far, CNS NB-*FOXR2* has only been reported in the supratentorial region and 17-20% of the patients had metastatic lesions at diagnosis[114,132]. The median age at diagnosis was five-eight years[114, 116]. On imaging, the tumors were described as large with mixed solid and cystic lesions and limited perifocal edema. The contrast enhancement was variable[131,132]. An example of a CNS NB-*FOXR2* visualized by MRI is shown in Figure 7.

Most patients have been treated according to various CNS-PNET protocols and retrospective analyses report on favorable survival for patients treated with up-front CSI in combination with maintenance chemotherapy with 5-year survival rates of 82-85%[114-116]. This is also the treatment of choice today. For young children, CSI-sparing regimens including combinations of chemotherapy and HDSCR is recommended.

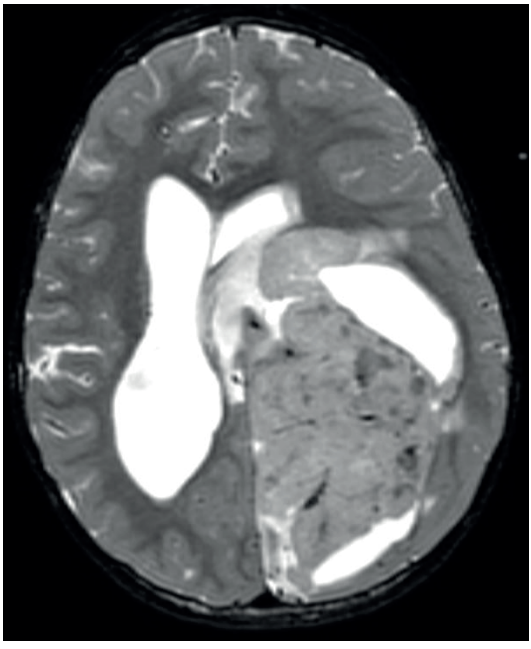

FIGURE 7. Magnetic resonance imaging of a CNS neuroblastoma, *FOXR2*-activated.

1.4.3 CNS tumor with *BCOR* internal tandem duplication (ITD)

Another of these novel rare embryonal tumors is the CNS tumor with *BCOR* ITD which was identified by methylome analysis in 2016 and characterized by an internal tandem duplication in exon 15 of the *BCOR* gene[102]. The gene is located on the X chromosome and is an epigenetic regulator. The *BCOR* ITD is the same genetic alteration described in renal clear cell sarcomas and some soft tissue sarcomas[133,134] and the tumor's relationship to mesenchymal tumors is not yet clear.

The *BCOR* ITD tumors have variable histological features and molecular confirmation of the ITD in exon 15 of the *BCOR* gene by gene expression or by DNA methylation is a required diagnostic criteria according to the WHO CNS5[29].

Clinical data is sparse and based on small series of patients but shows that the tumors predominantly occur in younger children and can appear anywhere along the neuroaxis[135,136]. The prognosis is poor with a 5-year OS of 50-54%[115,136]. However, several long-term survivors have been reported[102,135].

1.5 Epidemiological classification of CNS tumors

As mentioned before, the diagnoses of pediatric CNS neoplasms are based on the diagnostic criteria from the WHO Classification of CNS tumors. The morphological coding system in the WHO Classification relates to the International Classification of Diseases for Oncology (ICD-O) codes[137]. This international standard is based on morphology, localization, and biological behavior of the tumors and is used globally in cancer registries today. As part of the coding system, the tumors are coded depending on their behavior; /0 for benign tumors, /1 for unspecified, borderline, or uncertain behavior, (/2 for in situ lesions) and /3 for malignant tumors. It should be noted that this type of grading is *not* the same as the WHO grading system (1-4)[29].

It is well established that classification of neoplasm in children is based on morphology and not, as in adults, on the primary site of the cancer. The third edition of International Classification of Childhood Cancer (ICCC-3) was published in 2005 and is based on the ICD-O-3 (third edition) codes[138]. This provides a standardized classification of cancer, which is essential for comparing incidence and survival worldwide and over time. ICCC-3 classifies all childhood cancers into 12 main groups, in which CNS constitute group III. Tumors of the CNS are divided into six subgroups; a – ependymomas and choroid plexus tumors, b - astrocytomas, c – embryonal tumors, d - other gliomas, e - other specified tumors and f - unspecified tumor diagnoses and these subgroups are then further subdivided (Table 1).

TABLE 1 Diagnostic groups by International Classification of Childhood Cancer, Third Edition

Diagnostic groups by International Classification of Childhood Cancer, Third Edition	
Main group	III. CNS and miscellaneous intracranial and intraspinal neoplasms
Subgroup	IIIa. Ependymomas and choroid plexus tumor
	IIIa1. Ependymomas
	IIIa2. Choroid Plexus tumor
Subgroup	IIIb. Astrocytomas
Subgroup	IIIc. Embryonal tumors
	IIIc1. Medulloblastomas
	IIIc2. PNET
	IIIc3. Medulloepithelioma
	IIIc4. Atypical teratoid/rhabdoid tumor
Subgroup	IIId. Other gliomas
	IIId1. Oligodendrogliomas
	IIId2. Mixed and unspecified gliomas
	IIId3. Neuroepithelial glial tumors of uncertain origin
Subgroup	IIIe. Other specified intracranial and intraspinal neoplasms
	IIIe1. Pituitary adenomas and carcinomas
	IIIe2. Tumors of the sellar region (craniopharyngiomas)
	IIIe3. Pineal parenchymal tumors
	IIIe4. Neuronal and mixed neuronal-glial tumors
	IIIe5. Meningiomas
Subgroup	IIIf. Unspecified intracranial and intraspinal neoplasms
Main group	X. Germ cell tumors
Subgroup	Xa. Intracranial and intraspinal germinomas

All CNS tumors can unfortunately, independent of tumor type, cause severe damage to the affected patient due to the anatomic conditions and a morphological diagnosis is not always possible. Therefore, *all* CNS tumors are, irrespective whether classified as malignant (behavior code 3) or non-malignant, included within the ICCC-3 classification system, which is different from other non-malignant neoplasms.

Globally, different cancer registries have varying registry policies, e.g., some registries include only tumor types with a malignant behavior code which challenges tumor registration and statistical comparisons across international registries[139,140]. For example, in the US, registration of non-malignant tumors was previously not mandatory and therefore limited. After 2004, it was mandated by law to include non-malignant tumors. In addition, the ICD-O behavior code for the most common tumor in children, Pilocytic astrocytoma (PA), changed from /3 (malignant) in the ICD-O 2nd edition to /1 (non-malignant) in the 3rd, which also affects the accuracy of cancer registries for CNS tumors. Examples of other tumors with a non-malignant behavior code are craniopharyngiomas, dysembryoplastic neuroepithelial tumor (DNET) and desmoplastic infantile astrocytoma (DIA) which all are tumors that typically appear in childhood.

With the rapidly evolving molecular classification new tumor types are identified. It is not always obvious into which group these new tumors should be sorted in the current ICCC-3 classification.

1.5.1 Childhood CNS tumor registration in Sweden

Since 1958, all Swedish inhabitants diagnosed with cancer in Sweden have been registered in the Swedish Cancer Registry. This registration is mandatory, and the aim is to monitor the incidence and time trend of different cancers. To create a more complete registry for children with more detailed information, the Swedish *Childhood* Cancer Registry (SCCR) was started. Since 1984, all children living in Sweden diagnosed with a neoplasm before the age of 18 have been registered by clinicians in this nationwide clinical database. The SCCR contains individual-based data on diagnosis, treatment and long-term follow-up. The registration also facilitates the evaluation of treatment outcomes. All six pediatric oncology centers in Sweden administer the registration of the patients belonging to their population area. The unique Swedish personal identity number ensures that no double registration can occur.

All CNS tumors have been registered in the SCCR regardless of their grade of malignancy. The variables included in the SCCR are gender, date of birth, date of diagnosis, histopathological diagnosis or clinical diagnosis in the absence of morphological diagnosis. The WHO tumor grade, tumor location as well as treatment details and follow up are registered. This systematic registration now allows for a very long-term follow-up.

1.6 Treatment and long-term follow up

Currently, the treatment for CNS tumors includes surgery and sometimes chemotherapy and/or radiotherapy. In the last decade, targeted therapy has emerged as a choice of treatment as molecular alterations in pediatric CNS tumors have been deciphered.

The overall survival for children with a brain tumor has improved over the last years and the 5-year OS is now over 70% (73-75%)[16, 19] The prognosis for malignant tumors still remains poor[2,19] and brain tumors are the leading cause of death from cancer in children in higher income countries[17]. For survivors, cure often has a high price, with an increased risk of developing short-term and long-term sequelae including neurological deficits, endocrinopathy and cognitive impairments.[4,5,141-143] as well as the risk of premature death later than five years after diagnosis[144,145]. The excess late mortality risk seems to persist even 25 years after diagnosis[146] and depends on non-neoplastic causes as well as on recurrence of the primary tumor or secondary neoplasms[147,148].

In the last years it has become clear that long-term follow-up is very important for these patients and that national guidelines are essential to achieve a structured and standardized follow-up[149,150].

02

AIM

The overall aim of this PhD thesis was to study the role of DNA methylation profiling in the diagnostics of children diagnosed with a CNS tumor in Sweden. Furthermore, a secondary aim was to study epidemiological data for CNS tumors with respect to tumor classification, incidence, and long-term outcome.

Paper I:

- To study descriptive epidemiological data on the incidence, the distribution of tumor diagnoses, and long-term follow-up for children diagnosed with a tumor in the CNS in Sweden 1984-2021.

Paper II:

- To evaluate whether the systematical implementation of DNA methylation profiling in real time diagnostics strengthens the routine diagnostics for all children diagnosed with a CNS tumor during a four-year period (2017-2020).

Paper III:

- To study the re-classification of historical CNS-PNETs and long-term outcome for the different rare embryonal tumor types in a national population-based setting.

03

MATERIALS AND METHODS

Patient cohorts (paper I-III)

In **paper I**, children younger than 18 years of age at diagnosis were included if they had received the diagnosis of a primary CNS tumor in Sweden between January 1st, 1984, and December 31st, 2021, and were registered in the Swedish Childhood Cancer Registry. Clinical information was collected from the registry or from the patients' medical records. All patients were followed until death or until December 31st, 2021. The study was approved by the ethics committee in Sweden (Dnr 2019-06586, 2021-06259-02).

In **paper II**, all children (<18 years at diagnosis) diagnosed with a CNS tumor in Sweden between January 1st, 2017, and December 31st, 2020, were qualified for inclusion in the study if sufficient FFPE material was available for DNA methylation array and informed consent could be obtained. All patients were diagnosed at one of the six pediatric neuro-oncology centers in Sweden. The study was approved by the regional ethics committee in Gothenburg, Sweden (Dnr 604-12, T1162-16).

In **paper III**, all children (<18 years of age at diagnosis) diagnosed with a CNS-PNET in Sweden between January 1st, 1984, and December 31st, 2015 that were registered in SCCR were eligible. Clinical data were collected from the registry or from the medical charts. The study was approved by the regional ethics committee in Gothenburg, Sweden (Dnr 604-12, T581-15, T821-17).

Epidemiological tumor classification (paper I)

The tumor diagnoses were coded based on the ICD-O codes. Missing or unclear diagnoses were checked with the patients' charts and in the absence of a morphological diagnosis a clinical diagnosis was obtained in most cases.

The tumors were classified according to the ICCC-3 classification system but with the incorporation of additional subgroups to the classification system to accomplish a more relevant clinical approach.

Immunohistochemistry (paper II-III)

All immunohistochemistry and additional molecular analyses were performed according to WHO 2016 (in paper II)[33] and WHO 2021 (in paper III)[29].

Estimation of tumor cell content (paper II)

The percentage of tumor cell content in each sample was estimated by two neuropathologists, using hematoxylin and eosin-stained slides, under the light microscope. The percentage ranged from 0% (no neoplastic cells) to 100% (only neoplastic cells). A high tumor cell content was defined as ≥ 70%. All tumor samples collected in the study were analyzed independently of the tumor cell content.

DNA methylation profiling (paper II-III)

DNA methylation analysis

To analyze the methylation pattern in samples, bisulfite modification was used which converts unmethylated cytosine (C) to uracil but leaves methylated cytosine unchanged[151]. The bisulfite-treated DNA was then amplified by polymerase chain reaction, where unmethylated C is amplified as thymine (T) and methylated C as C. All tumor samples in paper II and III were retrieved from FFPE tumor blocks from six different pathology departments in Sweden. DNA was extracted from the tumor samples and approximately 500ng from each sample was bisulfite-modified. The bisulfite-modified DNA from the FFPE samples was thereafter repaired/restored before the methylation profiling.

Analysis of genome-wide DNA methylation patterns was performed using Infinium Human Methylation Bead-Chip array (Illumina) and the methylation values of 450 000 or 850 000 CpG sites of the genome were measured with the 450K array or the EPIC array. The arrays measure the methylation pattern using probes that produce fluorescent signals for each CpG site, which represent methylated (red) and unmethylated (green) status. Depending on the methylation status of the analyzed DNA, different signals are produced (Figure 8).

The raw methylation data was further analyzed using established biostatistics software in R[152] where the relationship between methylated and unmethylated sites was calculated and presented as a β value. The β value ranges between 0-1 where β = 0 refers to completely unmethylated CpG sites and β = 1 to completely methylated CpG sites.

FIGURE 8. The process of DNA methylation analysis. Created using Biorender.com

DNA methylation-based classification

The methylation-based tumor classification was performed using the MNP classifier, which is open and available on the website (www.molcularneuropathology.org). The brain tumor classifier version 11b4 comprises 82 CNS tumor methylation classes and nine control tissue classes[77]. Since its publication, the MNP classifier has been updated several times. The latest brain tumor classifier version 12.5 (unpublished) includes 184 molecular tumor classes, subclasses and control tissue classes[153]. The classifier version 12.5 classifies the tumor samples into four hierarchical levels; a superfamily, a family, a class and a subclass.

The methylation raw data from the tumor samples was uploaded and the results from the brain tumor classifier were represented as a calibrated score (CS). This score ranges from 0-1 and reflects the probability that the analyzed tumor belongs to a given tumor class or subclass included in the classifier. In version 11b4, the recommended threshold is set to ≥ 0.9 for a valid classification to a known tumor class and to ≥ 0.5 to a subclass[77]. In version 12.5, the threshold is set to ≥ 0.9 on all four hierarchical levels[153]. In paper II, we used the MNP classifier 11b4 but used the alternate calibrated score of ≥ 0.84 for a valid classification which is suggested for clinical samples by the classifier's developers[130]. In paper III, we used version 12.5 with the cutoff of ≥ 0.9 for a valid classification.

Additionally, in paper II, all non-WNT/non-SHH medulloblastomas were additionally classified in the separate medulloblastoma classifier: medulloblastoma classifier group 3/4 version 1.0, which classified these medulloblastomas into the subtypes I-VIII.

Chromosomal copy number alterations (paper II-III)

DNA methylation data can be used to generate information on genome-wide copy number alterations, CNAs[95]. Certain CNAs are more prevalent in certain tumors and this information is therefore important and helpful in the diagnostics[130]. In paper II and III we analyzed the CNA profiles inferred from the methylation data to determine chromosomal rearrangements in the tumor samples.

Statistical methods (paper I-III)

The age-standardized incidence rate (ASR) per 100 000 person-years was adjusted using the weight of the world standard population. The population size was retrieved from Statistics Sweden which covers the complete childhood population living in Sweden. Exact estimations of person-years at risk were gained by annual age-stratified data from all of Sweden. The average child population (age 0 -15 years) was 1.5 million.

The probability of OS after diagnosis was estimated using the Kaplan-Meier method and the differences in outcome between diagnostic groups were tested using the log-rank method. P values <0.05 were considered significant. Death was defined as an event. Dates of death were retrieved from the SCCR through the Swedish population registry.

04

RESULTS AND DISCUSSION

4.1 Paper I: Incidence and long-term survival in children diagnosed with CNS tumors in children 1984-2021

The incidence and mortality rates of pediatric CNS tumors vary considerably between countries[15]. Different national cancer registries have different registration practices regarding CNS tumors, for example they differ whether all non-malignant CNS tumors are registered or not. Globally, CNS tumors are classified according to the ICCC-3 classification system to facilitate comparison of incidence, tumor distribution and survival across countries. Registration in the Swedish Childhood Cancer Registry began in 1984 and both benign and malignant CNS tumors have been registered since then. In paper I, we reviewed the SCCR and evaluated epidemiological data on incidence, short- and long-term outcome for children diagnosed with a tumor in the CNS in Sweden 1984-2021.

A total of 3361 individuals (under 18 years of age at diagnosis) diagnosed with a primary CNS tumor were identified in the SCCR. Children with non-classical CNS tumors were excluded. For a better comparison to other studies, we divided the study-population into two groups; children aged <15 years at diagnosis and older children, aged 15 - <18 years at diagnosis (Figure 9).

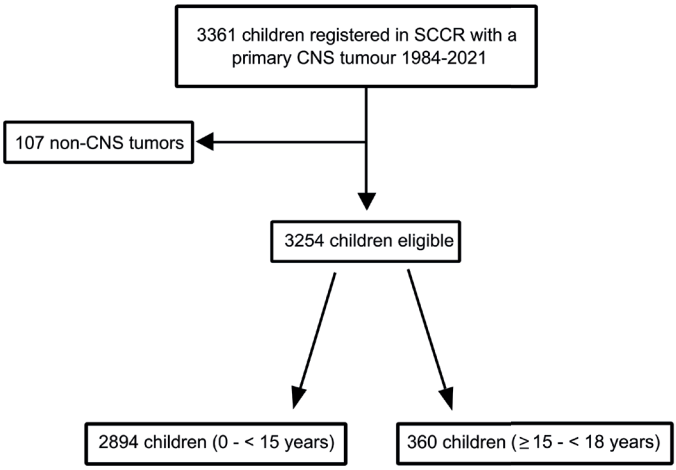

FIGURE 9. Cohort description, paper I as described in methods. SCCR, Swedish Childhood Cancer Registration.

The majority of the patients were males (54%), and the location of the tumors was supratentorial in 50%, infratentorial in 43% and spinal in 4% (Figure 10).

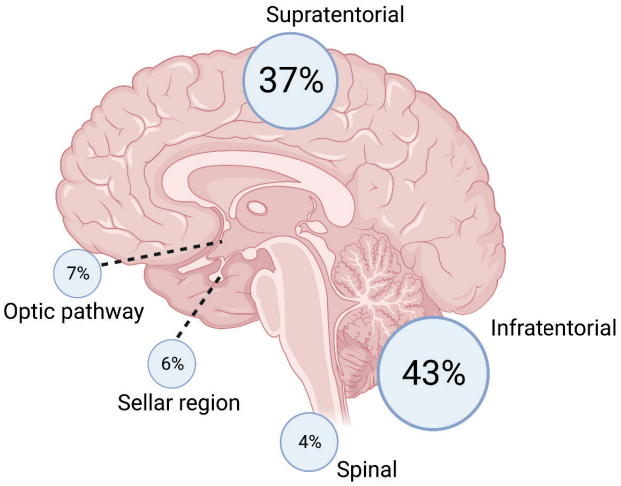

FIGURE 10. Localization of CNS tumors in children <18 years of age at diagnosis. Created with Biorender.com

A. Children aged <15 years of age at diagnosis

We found a relatively high annual ASR compared to what has been reported from many other countries[16,18,19]. The incidence rate remained stable over the entire study period. Since 1984, Sweden has carefully and systematically registered CNS tumors independently of tumor type and the coverage rate for CNS tumors is believed to be high. The Swedish national identification number ensures that no double registration have occurred. Almost 83% of the tumors had a registered morphological diagnosis.

The astrocytomas were the largest diagnostic tumor group, comprising 49% of the CNS tumors in this age group. This number is higher compared to a previous Swedish epidemiological study[2] as well as the number that is reported in other studies[16,18,19,154]. This can be partly explained by the carefully performed registry review that allocated the Diffuse intrinsic pontine gliomas (DIPGs) to the astrocytoma group. The distribution of other tumor groups was similar to findings in other studies[16,18,19].

The 5-year OS for the CNS tumors in children in this age group was 78% which declined to 72% at 20 years of follow-up, similar to survival rates reported in Denmark[16] and France[19]. Long-term survivors of childhood brain tumors suffer not only from increased late-appearing morbidity[155-157] but they also have a high risk of late mortality[158,159]. We evaluated the long-term outcomes for the different tumor groups and tumor diagnoses and concluded that survival rates for some tumor types continued to decrease for a long time after diagnosis. This was not only seen in patients with malignant tumors, like medulloblastomas and ependymomas but also in craniopharyngiomas, optic pathway gliomas and other non-malignant tumors. The survival rates for some of the tumor groups are shown in Figure 11. These findings, indeed, emphasize the need for a life-long follow-up independently of tumor type or the treatment modalities received.

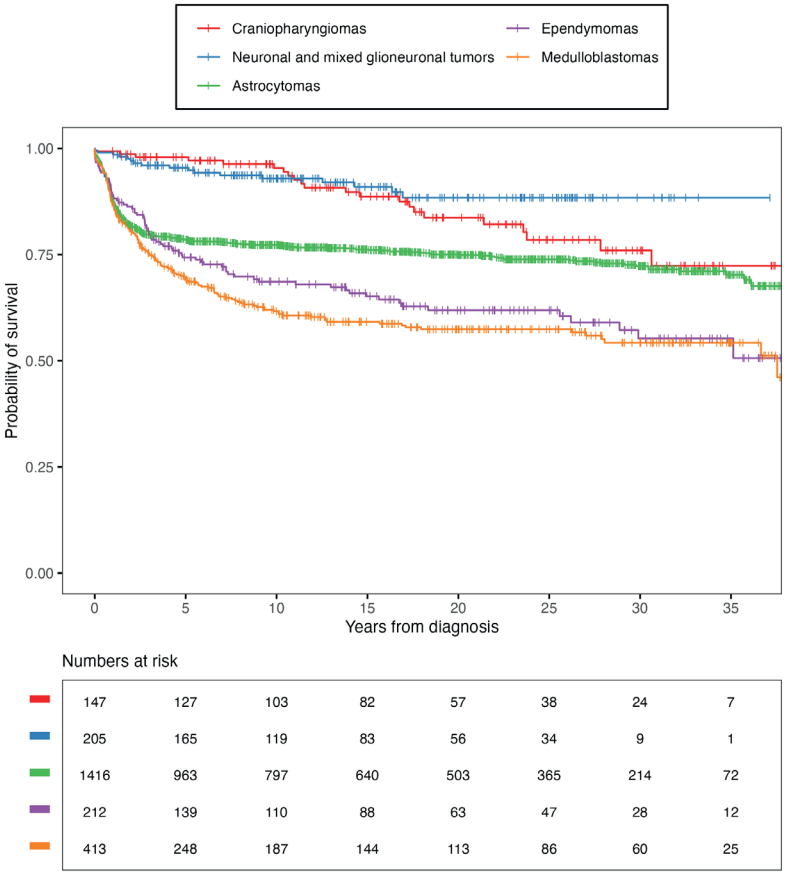

FIGURE 11. Kaplan-Meier plots of survival by some diagnostic groups of CNS tumors in Sweden in children 0-<15 years of age at diagnosis.

The classification system ICCC-3 was established in the 1990s as a tool to compare incidence and survival of childhood cancer on an international level. From a clinical perspective, there are several shortcomings in this general classification system. All astrocytomas are not part of the same condition and should not be combined as one diagnostic group as the different tumor types differ in survival and prevalence. This became evident in our study. We therefore further subdivided the ICCC-3 tumor groups into additional tumor subgroups or tumor diagnoses to make

the classification system more clinically relevant and to receive information on long-term outcome for specific tumor diagnoses and tumor types. Also, as new tumor diagnoses have emerged, the classification system has not yet been adapted to the reclassified tumors. In the future, the newly identified and reclassified tumor types will be relocated to different ICCC-3 subgroups, which will affect the distribution of the tumors and requires updates of the classification system on a regular basis.

B. Children aged 15-<18 years of age at diagnosis

The age group 15-<18 years is not treated by pediatric oncologists all over the world. Therefore this age group is excluded in many epidemiological studies. However, it is important to have profound knowledge about the epidemiological perspective and clinical course for CNS tumors in this age group as well. We therefore also analyzed the epidemiological findings in this age group. The spectrum of CNS tumor types and tumor sites differed compared to that in younger children. A higher proportion of the tumors were supratentorial and spinal. Pilocytic astrocytomas were most common, followed by neuronal and mixed glioneuronal tumors and germ cell tumors. The proportion of pituitary tumors and craniopharyngiomas were lower compared to findings in other countries[17,160] which may be an underrepresentation in our material. The long-term overall survival rates for this patient cohort were superior to that of younger children but also varied between the tumor types.

4.2 Paper II: DNA methylation profiling improves routine diagnosis of paediatric central nervous system tumours: A prospective population-based study

Classification of pediatric CNS tumors is challenging as the tumors are heterogenic and rare. DNA methylation profiling has emerged as an important technique in the diagnostics of CNS tumors[161-164] and has been more frequently requested and used in the clinical diagnostics in recent years. In paper II[165], we investigated whether methylation analysis could improve routine diagnostics if used up-front by performing DNA methylation profiling on all childhood CNS tumors diagnosed in Sweden during a period of four years in a prospective setting. A total of 250 tumor samples from pediatric patients were collected from pathology departments in all of Sweden. We analyzed the tumor samples with Illumina 450K or EPIC array. The methylation-based tumor classification was compared to the histopathological reports and in case of incongruent results they were re-evaluated by an experienced neuropathologist who was masked for the original histopathological diagnoses as well as the methylation-based classifications (Figure 12). During the study-period the 2016 WHO classification was applied.

In total, 372 pediatric patients (<18 years of age) were diagnosed with a CNS tumor in Sweden during the study-period and 250 samples were included in the study. The histopathological diagnoses of the included tumor samples were representative of the general distribution of CNS tumors in Sweden (Figure 13)[2].

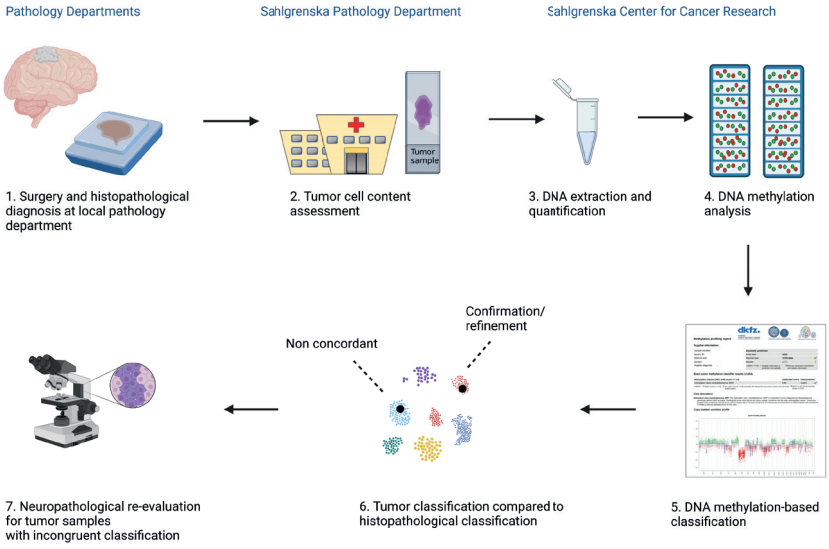

FIGURE 12. National methylation study set up. Created with Biorender.com

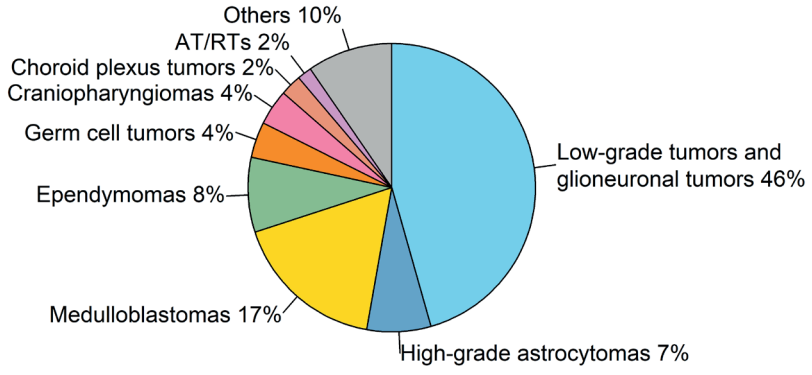

FIGURE 13: Cohort description and histopathological diagnoses of the included patients. Based on data extracted from Paper II, Schepke et al., 2023[165] (Neuropathology and applied neurobiology).

The ten patients with germ cell tumors were excluded as this diagnosis was not included in the classifier version 11b4 and the study cohort thus constituted of 240 tumor samples. Classification of the tumors by methylation profiling was accomplished in 78% (187/240 tumors) (Figure 14). The histopathological tumor diagnoses were confirmed in 69% and refined in 25%. In 6% (14/240) the predicted tumor diagnoses from the methylation-based classification were incongruent to the histopathological diagnoses. On re-evaluation by the reference neuropathologist, the diagnoses for these 14 tumor samples were *all* in favor of the methylation-based classification. The implications of the changed diagnoses would have had a direct impact on management of the patients in 11 of these 14 cases, corresponding to 5% of the total cohort. Similar numbers have been reported in other publications[161,166].

During the study-period, the MNP brain tumor methylation classifier version 12.5 became available for use[153]. We re-analyzed all the 240 tumor samples and found that several more samples reached a high CS independently of tumor cell content and new tumor classes were identified (Figure 14).

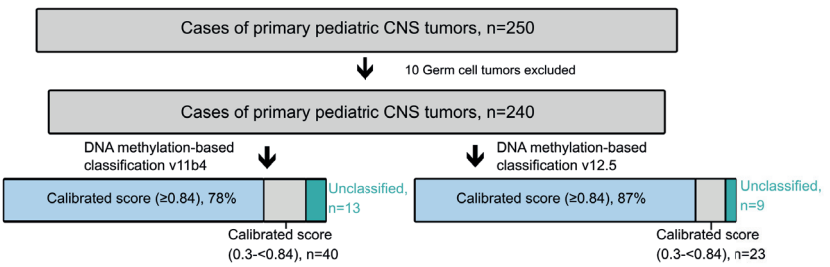

FIGURE 14. Result of DNA methylation classification of pediatric CNS tumors. Image adapted from Paper II, Schepke et al., 2022[165] (Neuropathology and applied neurobiology).

Our study demonstrated many benefits of DNA methylation profiling; the methylation-based classification provided an additional layer in the molecular diagnostics and had an added value in real-time diagnostics. Also, the CNAs generated from the methylation data provided information on molecular alterations that was important in the diagnostics. None of the FFPE tumor samples failed technically, which demonstrates the stability of the method.

However, there are also limitations in the algorithm-based classification[167,168]. For example, when the classifier confidently classifies the tumor but in a potential misleading way or when the algorithm cannot find a class prediction with a high calibrated score (CS ≥ 0.9), the interpretation may be problematical. When the calibrated score is low there is a higher rate of misleading diagnoses[161]. Several factors can lead to a lower CS; low tumor cell content, poor DNA quality, DNA heterogeneity or that the actual tumor diagnosis is not included in the classifier[169,170]. The proportion of neoplastic cells in a tumor sample is important as the methylation array detects signals generated from the included CpG sites from all the analyzed cells. The majority of the tumor samples in our cohort that were classified as "normal brain control tissue" classes or received a CS < 0.84 had a low tumor cell content which probably explains the low scores. As with all diagnostic testing it is crucial to interpret the result from the methylation profiling in the clinical, radiological, and morphological context. A limitation of the study was that neither micro- nor macrodissection of the tumor samples were performed, due to logistical reasons in the set-up of the study.

The proportion of tumor cell content is important in the interpretation of the results. A tumor cell content of >70% in the samples was recommended for the MNP classifier[130] but even though more than 30% of the tumor samples in our cohort had a tumor cell content <70% the majority was confidently classified, and we concluded that one should not refrain from performing the profiling solely based on the tumor cell content in the sample (Figure 15).

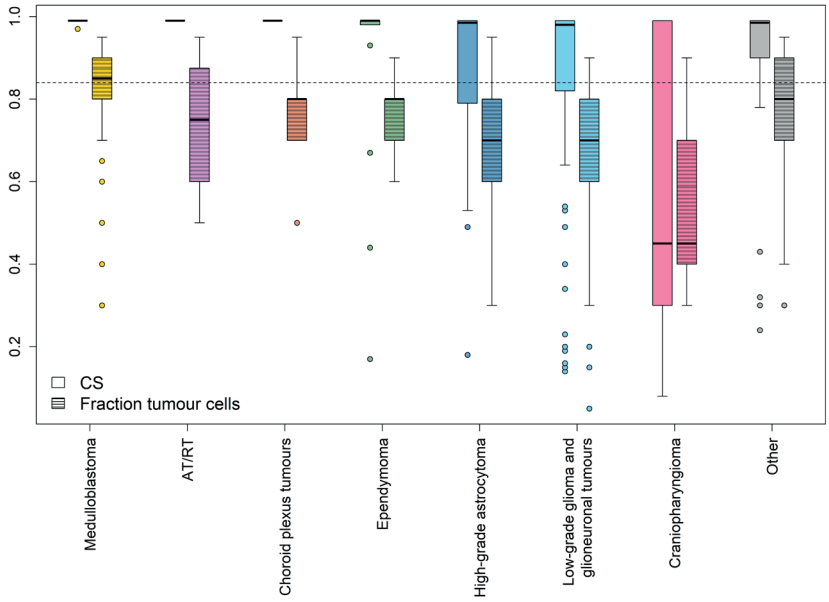

FIGURE 15. Tumor cell content and calibrated score in relation to different tumor types. Image taken from Paper II, Schepke et al., 2022[165] (Neuropathology and applied neurobiology).

This was a population-based study with the intention to evaluate the added value of performing DNA methylation profiling on all tumor diagnoses. One could argue that the exclusion of germ cell tumors was unjustified as these tumors potentially could be classified as a different tumor type. But as the germ cell tumors were not included in version 11b4 there was no precondition given to include them. The samples were re-analyzed in version 12.5 and 9/10 samples classified as different germ cell tumors.

4.3 Paper III: Supratentorial CNS-PNETs in children; a Swedish population-based study with molecular re-evaluation and long-term follow-up

CNS-PNET was no longer recognized as a single entity in the 2016 WHO classification of CNS tumors[33]. By using molecular analyses, many tumors previously known as CNS-PNETs, are now reclassified into different tumors with specific genetic characteristics[102]. They differ in their clinical course and outcome[114,115]. These rare CNS embryonal tumors are now incorporated into the latest WHO classification of CNS tumors (2021)[29]. In paper III, we collected all the tumors from patients diagnosed with a CNS-PNET that we could identify in the SCCR, n=71 and reclassified them by histopathology and DNA methylation profiling and collected clinical data (Figure 16).

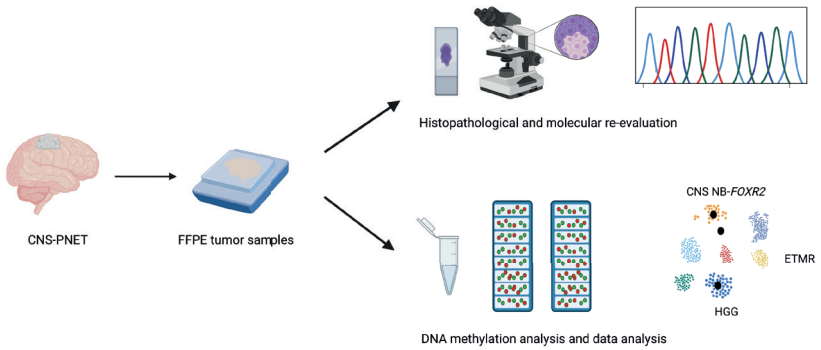

FIGURE 16. Study set up for re-evaluation of CNS-PNETs. Created with Biorender.com

Our study demonstrated the heterogeneity within this tumor cohort. We found that most of the re-evaluated CNS-PNETs were different types of HGGs, AT/RTs, CNS NB-*FOXR2s* and a minor part were ETMRs and other rare CNS embryonal tumors. The distribution of tumor types was in line with previous publications[102, 114,171]. Our results showed that DNA methylation confirmed the new morphological diagnoses and could further subdivide the tumors into more specific subgroups (Figure 17). DNA methylation also identified a newly suggested CNS embryonal tumor with *PLAG*-family amplification which is not yet incorporated in the 2021 WHO classification of CNS tumors.

The reviewing neuropathologist in our study is one of the most experienced in this field, being a member of the WHO expert committee. The use of DNA methylation profiling for these rare tumors is therefore probably even more helpful in the everyday diagnostics. Some of the tumor samples were more than 30 years old and still the methylation analysis was technically successful which shows that it works well also on old tumor material.

The 13 tumors that could not be confidently classified (CS <0.9) by methylation profiling were further analyzed by visualizing the tumor classification in a t-SNE plot. The majority of the tumors clustered to the different HGGs in the reference cohort, which was in agreement with the diagnoses given by the reference neuropathologist.

Clinical data was collected for all the 71 CNS-PNET patients. The OS rates for the whole cohort were poor with a 5-year OS of 45%±12%. As expected, the survival rates varied extensively between the different re-evaluated tumor types and best survival rates were seen for CNS NB-*FOXR2* and lowest for HGG, AT/RTs and ETMRs. Other publications have shown similar results[114,115]. All children in our cohort diagnosed with a CNS NB-*FOXR2* had received craniospinal irradiation and all are long-term survivors, and it seems that this is important for survival for this diagnosis[114].

All these tumors mentioned are rare. A correct diagnosis is the foundation for deciding on further treatment and our study confirms the additional layer in diagnostics provided by DNA methylation analysis. At present the variety in treatment modalities is limited and lags behind the discovery of new tumor types. With the development of targeted therapies there is hope that this will change in the future. Retrospective studies help in investigating the prevalence of these tumors and describe the treatment given which hopefully also can help in evaluating the most appropriate treatment.

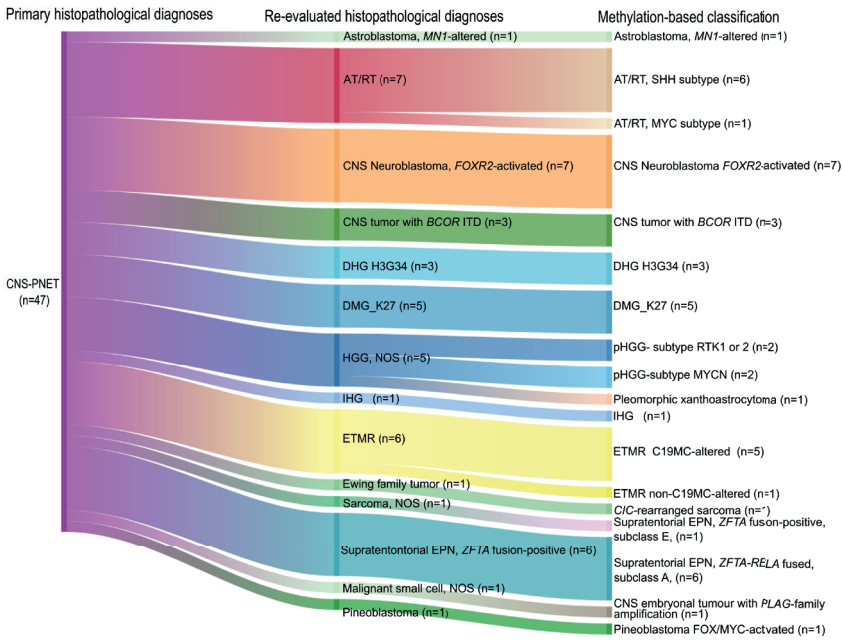

FIGURE 17. Sankey diagram over the 47 CNS-PNETs that were classified by DNA methylation. Adapted from Paper III, Schepke et al[172] (BioMed Central, Clinical Epigenetics).

05

CONCLUSION

In our national-based studies, we (1) collected comprehensive data on all children diagnosed with a CNS tumor in Sweden during a time period of almost 40 years, (2) investigated whether DNA methylation profiling could improve routine diagnostics on pediatric CNS tumors if used up-front and (3) re-classified the former CNS tumors diagnosed as CNS-PNET through DNA methylation and histopathology.

Paper I:

- The incidence rate of pediatric CNS tumors was relatively high but remained stable over time.
- The spectrum of CNS tumors was similar to reports from other countries.
- The overall survival for patients diagnosed with a CNS tumor has improved over time but for several tumor types it continued to decline long time after the diagnosis which demonstrate the necessity of lifelong follow-up.

Paper II:

- DNA methylation profiling has an important role in real-time diagnostics for pediatric CNS tumors.
- Methylation-based tumor classification improved the diagnostics and facilitated identification of rare tumor types.
- The diagnostic guidance from the methylation-based tumor classification allowed for a change in the management of the patients.
- Methylation profiling has a role in the classification of CNS tumors with a lower tumor cell content.

Paper III:

- CNS-PNETs were re-classified into several different tumor types and the distribution of different tumors were similar to reports from other countries.
- DNA methylation is important in the diagnostics of rare childhood embryonal tumors.
- The survival rates for the re-classified tumor types were in line with other studies. All patients with CNS NB-*FOXR2* had received craniospinal irradiation and the prognosis was excellent.

06

FUTURE PERSPECTIVES

The advancement of molecular analyzes in the diagnostics of pediatric CNS tumors is tremendous. The rapidity in which these are developed and their importance in the clinical diagnostics have become evident during the time that this thesis has been in progress. In 2017, when we started the "national methylation study", DNA methylation profiling was not performed at an early step in the diagnostic workflow of pediatric CNS tumors but was rather considered for difficult cases. Today it is no longer a discussion in pediatric neuro-oncology whether methylation profiling has an added value in the daily clinical work or not. We know it has. We must now rather discuss how to implement this analysis into standard diagnostics in a structured manner and with as short turnaround times as possible[173]. Nowadays, in the national project Genomics Medicine Sweden, all pediatric CNS tumors diagnosed in Sweden are subjected to methylation profiling. It is of great importance to interpret the methylation-based classification with consideration of clinical information, radiological imaging, histopathology and molecular findings. The final diagnosis must be made in the context of all available diagnostic modalities and preferably discussed on national or international multidisciplinary tumor boards.

The methylation-based tumor classification will be further refined and for sure many more algorithm-based tumor subclasses will be discovered. Naturally, the clinical value for each individual patient concerning prognostication and clinical implications by identifying many more methylation subclasses needs to be discussed. But as new possible targeted drugs are becoming available there is hope for better treatment and for improvement of the prognosis for a child diagnosed with a CNS tumor. The prerequisite for this is, as always, a thorough and accurate diagnosis.

The methylation-based classification will undoubtedly in the nearby future include other tumor diagnoses as well. For example, there is a methylation-based classifier for sarcomas accessible and this classifier seems to have the potential to become a useful diagnostic tool for sarcomas[85]. A methylation-based classifier for carcinomas of unknown primary is being developed which hopefully will optimize the clinical management for these patients[174].

The more we will evaluate the benefits and limitations of the methylation technique, the more the method will develop. For example, an ultrafast methylation-based nanopore technique appears to enable intraoperative tumor classification of CNS tumors which can facilitate surgical decisions and speed up the postoperative diagnostic workflow[175,176]. The usefulness of this method remains to be elucidated.

One of the most important cornerstones of pediatric oncology is the international collaboration which has enabled the improved survival for children diagnosed with neoplasms worldwide. International collaboration is of high importance as rare new tumor types have been and will be discovered. In order to optimize efficient workflows for diagnostics and treatment, improve cancer registrations and develop joint guidelines for follow-up of long-term survivors of childhood cancer, collaboration across borders is required.

We also need to remember that the vast majority of the children in the world live in countries where the resources are limited[177]. Most of the pediatric oncology patients in these countries are currently not yet being helped by these techniques. Through continued and persistent global collaborations by inventing and exploring universal accessible techniques to lower costs, sharing of clinical expertise, increasing the availability of effective treatment more children can benefit from improved diagnostics[178-180]. By joining all available efforts, we hopefully can reach the global survival target recently agreed upon by the World Health Organization: 60% survival for all children with cancer by 2030[181].

07

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