DNA methylation profiling of CNS tumors; implications for clinical diagnostics

Sandra Ferreyra Vega

Department of Clinical Neuroscience
Institute of Neuroscience and Physiology
at Sahlgrenska Academy
University of Gothenburg

Gothenburg, Sweden 2022

UNIVERSITY OF
GOTHENBURG

Cover illustration: “DNA methylation rainbow”
by Sandra Ferreyra Vega

DNA methylation profiling of CNS tumors; implications for clinical diagnostics

sandra.ferreyra.vega@gu.se

ISBN 978-91-8009-899-1 (PRINT)
ISBN 978-91-8009-900-4 (PDF)

Printed in Borås, Sweden 2022
Printed by Stema Specialtryck AB

Dedicado a mis amados padres y hermanos.

“This is the way”
-The Mandalorian

DNA methylation profiling of CNS tumors; implications for clinical diagnostics

Sandra Ferreyra Vega
Department of Clinical Neuroscience,
Institute of Neuroscience and Physiology,
Sahlgrenska Academy, University of Gothenburg, Sweden

Abstract

Diffuse gliomas and meningiomas are the most common primary tumors of the central nervous system (CNS) in adults and these tumors cause significant morbidity and mortality worldwide. Deregulation of the epigenetic mechanisms, e.g. in the form of aberrant changes of DNA methylation patterns, are important for the formation and development of many diseases including cancer. Genome-wide DNA methylation profiling is an emerging molecular technique that offers a new way for characterization of CNS tumors with potential use in routine clinical diagnostics. In this thesis, we used DNA methylation profiling for evaluation of patient diagnosis and prognosis and further provide new insights into intratumor heterogeneity, highlighting the potential implications that this could bring into the clinical setting.

In Paper I, we assessed the value of using DNA methylation profiling as a diagnostic tool for molecular classification of diffuse lower-grade gliomas. We demonstrated that methylation profiling provided accurate diagnostic and prognostic information and enabled a reliable molecular classification of the tumors according to the World Health Organization classification criteria.

In Paper II, we studied DNA methylation profiles across distinct regions of glioblastomas and found methylation subclass differences within the tumors as well as variable methylation status of the clinical prognostic and predictive biomarker *MGMT*.

In Paper III, we further explored DNA methylation and chromosomal copy number variability within adult-type diffuse gliomas and meningiomas and shed light on the effect of varying tumor cell

content on methylation analyses. Diffuse gliomas and high-grade meningiomas were characterized by spatial methylation and chromosomal heterogeneity after accounting for tumor purity. In addition, we found heterogeneity of the clinical biomarker *CDKN2A/B* homozygous deletion in *IDH*-mutant gliomas.

In Paper IV, we investigated DNA methylation changes during progression of *IDH*-mutant gliomas. The tumors accumulated methylation alterations over time, but methylation patterns were mostly maintained upon recurrence.

In conclusion, we demonstrated the potential of using DNA methylation profiling for improved CNS tumor diagnostics and prognostics. We further provided a better understanding of the methylation and chromosomal heterogeneity in diffuse gliomas and meningiomas, which could affect the clinical diagnosis and treatment management of these patients.

Keywords: Central nervous system tumors, DNA methylation, epigenetics, molecular biomarkers, classification, World Health Organization, adult-type diffuse gliomas, meningiomas, methylation EPIC array, clinical diagnostics, prognostics

ISBN 978-91-8009-899-1 (PRINT)
ISBN 978-91-8009-900-4 (PDF)
http://hdl.handle.net/2077/72551

SAMMANFATTNING PÅ SVENSKA

Epigenetiska mekanismer styr hur gener uttrycks i cellerna och avvikelser i den epigenetiska kontrollen kan kopplas till uppkomsten av cancer och dess tillväxt. DNA metylering är en epigenetisk mekanism där metylgrupper (CH_3) adderas till DNA strängar på cytosinnukleotider. Kartläggning av tumörcellernas DNA metyleringsprofiler har visat sig vara mycket användbart för att diagnosticera olika typer av tumörer i centrala nervsystemet (CNS). Diffusa gliom och meningiom är de vanligaste primära CNS tumörerna hos vuxna och i denna avhandling studerar vi DNA metyleringsprofiler med fokus på den kliniska diagnostiken av dessa tumörer.

I delarbete I utvärderade vi DNA metyleringsprofiler för molekylär klassificering av diffusa gliom av lägre grad. Vi visade att metyleringsprofilering gav korrekt diagnostisk och prognostisk information och möjliggjorde en tillförlitlig molekylär klassificering av tumörerna enligt WHO:s klassificeringskriterier.

I delarbete II och III studerade vi DNA metyleringsmönster och kromosomkopienummer och avvikelser från tumörprover tagna från olika områden inom enskilda CNS tumörer. Metyleringsmönster och kromosomvariationer skilde sig inom tumörerna, vilket påverkade metyleringsbaserad diagnostisk subgruppering och biomarkörer som används idag inom klinisk diagnostik.

I delarbete IV studerade vi förändringar i DNA metyleringsmönstret under progression av *IDH*-muterad gliom. Vi visade att dessa förändringar kan ackumuleras och dessa profiler förblev, i stora drag, oförändrade vid återfall.

Sammanfattningsvis har vi i denna avhandling visat hur vi kan förfina och förbättra diagnostiken av CNS tumörer med hjälp av DNA metyleringsprofiler. Dessutom har vi identifierat hur metyleringsmönstret skiljer sig inom CNS tumörer, vilket potentiellt kan påverka den kliniska diagnostiken av dessa tumörer, behandlingen av patienterna och framtida utveckling av kliniska biomarkörer.

RESUMEN EN ESPAÑOL

Las neoplasias del sistema nervioso central (SNC) son tumores que se originan en el cerebro o en la médula espinal. Según la Organización Mundial de la Salud (OMS), existen hasta más de 100 tipos de tumores del SNC en los cuales los gliomas difusos y meningiomas destacan por su mayor incidencia en la población adulta. Según la Institución Nacional de Enfermedades Neoplásicas del Perú, se registraron 392 nuevos casos de tumores en el SNC en el año 2018. A pesar de los grandes avances en el tratamiento de estos tumores, los gliomas difusos y meningiomas se siguen caracterizando por su gran morbilidad y mortalidad a nivel mundial.

La clasificación de los tumores del SNC se basa en el criterio de la OMS, la cual integra información histopatológica y molecular para establecer el diagnóstico clínico final. La metilación del ADN es un mecanismo epigenético que se encarga de activar o desactivar genes a través de grupos metilos (un átomo de carbón unido a tres átomos de hidrogeno, CH_3) que se adhieren a la molécula del ADN. Una metilación aberrante del ADN puede promover la carcinogénesis.

El objetivo de esta tesis fue caracterizar los perfiles de metilación del ADN y variación de números de copia cromosómicas en los gliomas difusos y meningiomas con la finalidad de entender y mejorar el diagnóstico y pronostico clínico de estos tumores.

En el estudio I, evaluamos el uso de los perfiles de metilación en la clasificación molecular de los gliomas de bajo grado acatando el criterio de clasificación propuesto por la OMS. Nuestros resultados demuestran se puede llegar a un diagnóstico y pronostico clínico eficaz y confiable examinando los patrones de metilación en estos tumores.

En el estudio II, estudiamos los perfiles de metilación en diferentes zonas del glioblastoma de grado 4 y encontramos que la subclasificación dentro de este tumor varia así también como el estado de metilación del marcador clínico *MGMT*.

En el estudio III, exploramos más a fondo la metilación del ADN y la variabilidad del número de copias cromosómicas en los gliomas

difusos y meningiomas. Además, nos enfocamos en examinar la influencia de la pureza tumoral en los análisis de metilación. Los gliomas difusos y los meningiomas de alto grado se caracterizaron por metilación heterogénea y variación cromosómica después de tener en cuenta la pureza del tumor.

En el estudio IV, finalmente estudiamos la asociación de los cambios en el perfil de metilación del ADN con la recurrencia de gliomas con mutación de *IDH*. Nuestros resultados demuestran que estos cambios se acumulan y reflejan la progresión del tumor hasta la recurrencia.

En conclusión, nosotros hemos determinado los perfiles de metilación y variación de número de copias cromosómicas en gliomas difusos y meningiomas con la finalidad de evaluar las implicancias en el diagnóstico clínico. En esta tesis hemos demostrado que se puede mejorar el diagnóstico y pronóstico de tumores del SNC utilizando los perfiles de metilación del ADN. Además, hemos identificado que el patrón de metilación varía dentro de los tumores, lo que podría afectar el diagnóstico clínico de estos tumores, el manejo del tratamiento de los pacientes y el desarrollo de biomarcadores clínicos.

LIST OF PAPERS

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

I. **Ferreyra Vega S**, Olsson Bontell T, Corell A, Smits A, Jakola AS, Carén H. DNA methylation profiling for molecular classification of adult diffuse lower-grade gliomas. *Clinical Epigenetics*, 2021, 13(1):102. doi: 10.1186/s13148-021-01085-7

II. Wenger A, **Ferreyra Vega S**, Kling T, Olsson Bontell T, Jakola AS, Carén H. Intratumor DNA methylation heterogeneity in glioblastoma: implications for DNA methylation-based classification. *Neuro-Oncology*, 2019, 21(5):616-27. doi: 10.1093/neuonc/noz011

III. **Ferreyra Vega S**, Wenger A, Kling T, Olsson Bontell T, Jakola AS, Carén H. Spatial heterogeneity in DNA methylation and chromosomal alterations in diffuse gliomas and meningiomas. *Modern Pathology*, 2022, 1-11. doi: 10.1038/s41379-022-01113-8

IV. **Ferreyra Vega S**, Olsson Bontell T, Kling T, Jakola AS, Carén H. Longitudinal DNA methylation analysis of adult-type *IDH*-mutant gliomas. *Manuscript*.

Additional relevant publications not included in this thesis.

I. Corell A, **Ferreyra Vega S**, Hoefling N, Carstam L, Smits A, Olsson Bontell T, Björkman-Burtscher IM, Carén H, Jakola AS. The clinical significance of the T2-FLAIR mismatch sign in grade II and III gliomas: a population-based study. *BMC Cancer*. 2020, 20(1):450. doi: 10.1186/s12885-020-06951-w

II. Carstam L, Corell A, Smits A, Dénes A, Barchéus H, Modin K, Sjögren H, **Ferreyra Vega S**, Olsson Bontell T, Carén H, Jakola AS. WHO grade loses its prognostic value in molecularly defined diffuse lower-grade gliomas. *Frontiers in Oncology*. 2022, 11:803975. doi: 10.3389/fonc.2021.803975

III. Corell A, Gomez Vecchio T, **Ferreyra Vega S**, Dénes A, Neimantaite A, Hagerius A, Barchéus H, Solheim O, Lindskog Bergström C, Olsson Bontell T, Carén H, Jakola AS, Smits A. Stemness and clinical features in relation to the subventricular zone in diffuse lower-grade glioma; an exploratory study. *Neuro-Oncology Advances*. 2022, 4(1). doi: 10.1093/noajnl/vdac074

IV. 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 Neuropathologica Communications*. 2022, 10(1):1-:14. doi: 10.1186/s40478-022-01406-8

V. 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, Lannering B, Sabel M, Carén H. DNA methylation profiling improves routine diagnosis of paediatric CNS tumours: a prospective population-based study. *Neuropathology and Applied Neurobiology*. 2022, i159-i160. doi: 10.1111/nan.12838

CONTENT

ABBREVIATIONS

1p/19q	Chromosomal arms 1p and 19q
ATRX	Alpha-thalassemia/mental retardation, X-linked
BSM	Bisulfite modification
CDKN2A/B	Cyclin-dependent kinase inhibitor 2A/B
CNA	Copy number alteration
cIMPACT	The Consortium to Inform Molecular and Practical Approaches to CNS Tumor Taxonomy
CNS	Central nervous system
CpG	Cytosine guanine
CSC	Cancer stem cell
dLGG	Diffuse lower-grade glioma (CNS WHO grade 2 & 3)
DMP	Differentially methylated positions
DNA	Deoxyribonucleic acid
DNMT	DNA methyltransferase
EANO	European Association of Neuro-Oncology
EGFR	Epidermal growth factor receptor
FFPE	Formalin-fixed paraffin-embedded
FISH	Fluorescence *In situ* Hybridization
G-CIMP	Glioma CpG island methylator phenotype
H&E	Hematoxylin and Eosin
IDH	Isocitrate dehydrogenase
IHC	Immunohistochemistry
MDM2	Murine double minute clone 2
MGMT	O6-methylguanine-DNA methyltransferase
MLPA	Multiplex ligation-probe amplification
MNP	Molecular neuropathology
PCR	Polymerase chain reaction
RTK	Receptor tyrosine kinase

TCGA	The Cancer Genome Atlas
TERT	Telomerase reverse transcriptase
TET	Ten-eleven translocation
TMZ	Temozolomide
WHO	World Health Organization

INTRODUCTION

The human body is made up of trillions of cells that constitute our different organs and tissues. Normal cells obey signals of cell growth and replication by which our bodies are built and maintained. Cancer cells, in contrast, function without restrains. The cells grow uncontrollably and may eventually give rise to an abnormal mass of tissue referred to as a tumor, or neoplasm[1].

Cancer is the second leading cause of death and cancer burden is estimated to rise globally[2]. In early 2000, Hanahan and Weinberg first proposed the six hallmarks of cancer - six traits of the cells that provided a solid foundation for our understanding of cancer initiation and development: "*Self-sufficiency in growth signals, insensitivity to anti-growth signals, tissue invasion and metastasis, limitless replicative potential, sustained angiogenesis and evading apoptosis"*[3]. Cancer has long been considered as a genetic disease, depending on somatic mutations that are passed through cell division in order to form and drive the disease. But years of advanced cancer research has revealed that a single genetic alteration is not always enough to cause cancer. Cancer is much driven by the epigenetic machinery, a group of mechanisms involved in the control of gene expression[4,5]. By 2021, the "*nonmutational epigenetic reprogramming"* was incorporated in the core hallmarks of cancer, further emphasizing the importance studying epigenetics for cancer research.

The rapidly expanding research on the human epigenome has improved our understanding of the epigenetic modifications occurring in cancer and has opened up new perspectives on how we see the disease in a diagnostic and therapeutic point of view.

Epidemiology of tumors of the central nervous system

Cancer may arise anywhere in the human body, even in the most complex and fascinating organ: the brain.

Brain cancer affects children and adults and has a considerable impact on the patient's quality of life. Approximately 300,000 individuals were diagnosed with a tumor of the central nervous system (CNS) in 2020 worldwide[6]. In children, CNS tumors are rare but due to the rarity of cancer in children still constitute the second most prevalent cause of cancer-related death following leukemia[7]. In Sweden, the incidence of primary CNS tumors is about 26 per 100,000 people and in 2020, 1 103 adult patients were newly diagnosed with a CNS tumor[8].

The World Health Organization (WHO) has listed over 100 types of tumors of the CNS, each being of distinct biological behavior and exhibit a variable prognosis and clinical course. CNS tumors are graded based on their stages of malignancy, which is mainly evaluated by histomorphological assessment of the tumor under a light microscope. For selected tumors only, molecular grading markers have been introduced in the current 2021 WHO classification criteria of CNS tumors[2]. The highest percentage of primary malignant CNS tumors develops in the frontal lobe in the cerebral cortex while the most frequent benign tumors occur in the meninges[7].

There are few established risk factors for developing CNS tumors such as increasing age, male sex (except in e.g. meningioma), Caucasian ethnicity, ionizing radiation and a few hereditary genetic syndromes[9-12]. Some hereditary genetic syndromes predisposing to gliomas and/or meningiomas includes disorders caused by germline mutations such as Li-Fraumeni syndrome[13,14], Neurofibromatosis type 1 and 2 (NF1/2)[15,16] and Turcot's syndrome[17,18]. Exposure to ionizing radiation has been linked to increased risk to develop meningioma[9,12]. Intracranial volume was strongly associated with the risk of developing high-grade gliomas[19], whereas atopy/allergy has been associated with reduced glioma risk[20-22].

Diagnostic classification of tumors of the central nervous system

The WHO has played a key role in the taxonomy of tumors of the CNS since 1979[23]. In the first international publication of the "blue book", CNS tumors were categorized based on histomorphological phenotypes, and histology became the cornerstone technique for tumor typing. However, the establishment of a diagnosis based on histology alone carried high interobserver discordance among neuropathologists[24-27]. A major restructuring of the CNS tumor taxonomy came with the 2016 WHO classification criteria, an update of the 4th edition from 2007[28,29]. For the first time, molecular information was incorporated to define many CNS tumors. The use of an integrated approach, involving phenotypic and genotypic parameters, has led to an improvement in the diagnostic and prognostic accuracy of CNS neoplasms. The traditional histological WHO grading scheme, however, remained unchanged. To keep in line with recent molecular advances of tumor diagnostics, and to enable a rapid update of future WHO classifications, the "*Consortium to Inform Molecular and Practical Approaches to CNS Tumor Taxonomy"* (cIMPACT-NOW) was established in 2016[30]. The fifth edition of the WHO classification of CNS tumors is the latest version published in 2021 and follows the recommendations of the cIMPACT-NOW[31-37] to simplify and precise the nomenclature of the tumors. Among the new updates from the 2021 WHO criteria is the use of DNA methylation profiling, as an essential diagnostic approach for selected tumor types or as desirable for the majority of CNS tumors. Moreover, the histological WHO grading scheme of CNS tumors has been adapted to prognostic molecular markers within specific tumor types, e.g. homozygous deletion of the cell cycle regulator *cyclin-dependent kinase inhibitor 2A and 2B (CDKN2A/B),* defines a CNS WHO grade 4 in *IDH*-mutant astrocytomas and a CNS WHO grade 3 in meningiomas[2].

Gliomas

Gliomas represent a heterogeneous group of neoplasms of presumed neuroepithelial origin, arising from glial stem cells or precursor cells in the cerebral hemispheres or spinal cord[26,27]. Gliomas fall into the

"*gliomas, glioneuronal tumors and neuronal tumors"* category of the 2021 WHO classification criteria of CNS tumors[2], under which four families of gliomas are defined: *Circumscribed gliomas, pediatric-type high-grade diffuse gliomas, pediatric-type low-grade diffuse gliomas and adult-type diffuse gliomas*. Circumscribed gliomas (e.g. pilocytic astrocytomas CNS WHO grade 1 and pleomorphic xanthoastrocytomas CNS WHO grade 2 and 3) are astrocytic gliomas with well-defined tumor margins. Pilocytic astrocytomas are the most common CNS tumors in children and young adults, accounting for 15.2% of cases[7]. The diffuse gliomas in turn are characterized by invasive growth into the surrounding brain without a distinct border. The pediatric-type diffuse gliomas are biologically and prognostically different from gliomas developing in adults. For instance, pediatric diffuse gliomas harbor less chromosomal aberrations and have a higher prevalence of histone gene mutations compared to adult-type diffuse gliomas. In addition, *IDH* mutations are rarely present in pediatric gliomas and also malignant transformation is less common in these tumors[2,38-40]. Although rare, adult-type diffuse gliomas may occur in children just as the pediatric-type could also manifest in adults, especially in young adults. This thesis is focused on the adult-type diffuse gliomas and these are described further below.

Adult-type diffuse gliomas

Adult-type diffuse gliomas have a peak incidence of age between 35-44 years and an increased dominance in the male population[7]. The clinical course and outcome are highly variable, depending on the tumor type and grade of malignancy. Diffuse lower-grade gliomas (dLGGs) is a term introduced by The Cancer Genome Atlas (TCGA) group to define gliomas of CNS WHO grade 2 and 3[41]. dLGGs have a continuous tumor expansion where the tumors initially grow continuously and slow, and faster as the tumor undergoes malignant transformation into higher grades[42,43]. In turn, the CNS WHO grade 4 gliomas, astrocytomas, *IDH*-mutant and glioblastoma, *IDH*-wildtype, are more aggressive than dLGGs, accounting for 14.3% of all primary CNS tumors[7]. Astrocytomas, *IDH*-mutant CNS WHO grade 4 are more commonly diagnosed in younger patients (median age 44 years) and have better prognosis compared to

glioblastoma patients who are typically older (65+ years) and have a poor prognosis (median survival rate of <2 years)[7]. An example of a diffuse glioma on magnetic resonance imaging (MRI) is provided in Figure 1.

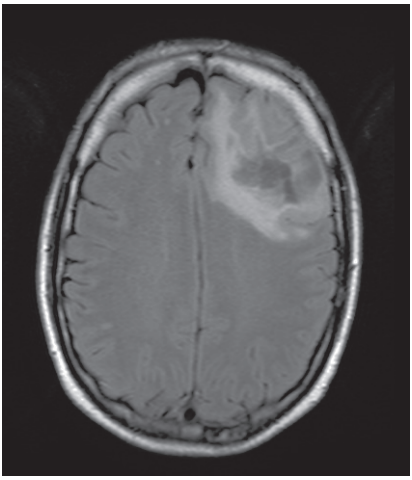

Figure 1. Diffuse glioma. Magnetic resonance imaging (MRI), FLAIR sequence of a diffusely infiltrating glioma located in the left frontal lobe of the brain.

Diagnostic classification of diffuse gliomas

The classification of diffuse gliomas has undergone major modifications since the 2007 WHO classification criteria of CNS tumors, where the histological glioma entities astrocytoma, oligodendroglioma and oligoastrocytoma (a mixture of astrocytic and oligodendroglial phenotypes) were established on presumed cell of origin and graded based on malignancy into grade II-IV. The integration of molecular biomarkers in the revised 2016 WHO classification refined the 2007 WHO diagnoses into distinct molecular groups that better predict clinical outcome[28,29]. As such, mutational status of the isocitrate dehydrogenase genes 1 and 2 (*IDH1/IDH2*) and complete codeletion of chromosome arms 1p and 19q (1p/19q-codeleted) became shared genetic determinants for stratification of diffuse gliomas. The preceding diagnosis of oligoastrocytoma was excluded from the revised 2016 WHO classification as these tumors could be categorized as astrocytomas or oligodendrogliomas by molecular information. The current 2021 WHO

classification criteria have further simplified and refined the nomenclature of adult-type diffuse gliomas into solely 3 tumor types:

- Astrocytoma, *IDH*-mutant (CNS WHO grade 2, 3, and 4)
- Oligodendroglioma, *IDH*-mutant and 1p/19q-codeleted (CNS WHO grade 2 and 3)
- Glioblastoma, *IDH*-wildtype (CNS WHO grade 4)

The diagnosis of astrocytoma, *IDH*-mutant requires histological features of diffuse astrocytoma (e.g. hyperchromatic cell nuclei with irregular contour and scanty cytoplasm[44]) and the presence of *IDH1 or IDH2* mutations, retained 1p/19q chromosomal arms and loss of nuclear expression/mutation of alpha-thalassemia/mental retardation, X-linked (*ATRX*). According to the 2021 WHO criteria, astrocytomas, *IDH*-mutant can be graded as CNS WHO grade 2, grade 3 or grade 4. The WHO grading is no longer restricted to histological criteria since the presence of *CDKN2A/B* homozygous deletion in the tumor designates a CNS WHO grade 4. An astrocytoma, *IDH*-mutant with e.g. hypercellularity, nuclear atypia, microvascular proliferation and/or necrosis also designates a CNS WHO grade 4 even in the absence of *CDKN2A/B* homozygous deletions[2].

In turn, the essential diagnostic criterion of oligodendrogliomas is based on *IDH1 or IDH2* mutations and codeletions of chromosomal arms 1p and 19q. Retained *ATRX* expression and promotor mutation of the telomerase reverse transcriptase (*TERT*) gene can also lead to the diagnosis of oligodendroglioma[2]. In comparison to astrocytomas, *IDH*-mutant, oligodendrogliomas are graded mainly based on histology into CNS WHO grade 2 or 3. Homozygous deletion of *CDKN2A/B* has been observed in <10% of grade 3 oligodendrogliomas cases whereas the grade 2 oligodendrogliomas lacked this alteration. Therefore, *CDKN2A/B* homozygous deletion may serve as a grading marker of oligodendrogliomas[45]. Histomorphological features of oligodendroglioma include round to oval nuclei with uniform shape, perinuclear cytoplasmic clearing resembling a “fried-egg” pattern and branching or network of small blood vessels with a “chicken wire” appearance[44].

The minimal criteria for the diagnosis of glioblastoma are an *IDH*-wildtype gene and at least amplification of the epidermal growth factor receptor (*EGFR)* and/or combined chromosomal 7 gain and chromosomal 10 loss and/or *TERT* promotor mutation and this tumor is classified as CNS WHO grade 4[2]. Although glioblastoma, *IDH*-wildtype can share histological criteria of *IDH*-mutant astrocytomas CNS WHO grade 4 (e.g. features of atypia, hypercellularity and necrosis) they are separated in the 2021 WHO classification.

Molecular markers and chromosomal alterations

Isocitrate dehydrogenase gene mutations

IDH enzymes play a role in many cellular processes including glucose-, lipid- and glutamine metabolism. These enzymes catalyze the conversion of isocitrate to alpha-ketoglutarate, an essential molecule in the citric acid cycle (Kreb's cycle)[46]. Somatic mutations in *IDH1/IDH2* are considered as early events during glioma initiation[47,48]. An amino acid substitution in *IDH1/IDH2* results in the production of the oncometabolite D2-hydroxyglutarate (2HG), which drives glioma formation through e.g. inhibition of glioma stem cell differentiation and upregulation of vascular endothelial growth factors[49]. IDH1 (R132H) is the most frequent gene variant in glioma, which involves the missense mutation of arginine to histidine (CGT → CAT). Other less common variants include mutation of arginine to cysteine (R132C), leucine (R132L), serine (R132S) and glycine (R132G). *IDH2* mutations have been identified at codon R172 (R172K, R172M and R172G)[47]. In the clinic, molecular examination of IDH mutations is primarily carried out by immunohistochemical labelling of IDH1 (R132H), and occasionally (e.g. if further examination is required), high-throughput sequencing analyzes are used, such as Next generation sequencing and Sanger sequencing. Patients with gliomas harboring *IDH* mutations have better clinical outcomes compared to patients with *IDH* wildtype gliomas with molecular features of glioblastoma[47].

Codeletion of 1p and 19q chromosomal arms

Combined deletion of 1p and 19q is considered to be a result of whole-arm translocation between chromosomes 1 and 19. Inactivation of tumor suppressor genes located on 1p and 19q, through deletion of these chromosomal regions, is likely to drive the pathogenesis of oligodendrogliomas[50,51]. 1p/19q status is commonly examined in the clinical setting by Fluorescence *In situ* Hybridization (FISH) and polymerase chain reaction (PCR)-based methods, e.g Multiplex ligation-dependent probe amplification (MLPA®[52])[53-56].

Homozygous deletion of cyclin-dependent kinase inhibitor 2A/B

The cell cycle is regulated by several types of cycling dependent kinases such as the *CDKN2A* and *CDKN2B* genes residing on chromosome band 9p21. *CDKN2A/B* induces cell cycle arrest of neoplastic cells via tumor suppressor proteins and therefore, alteration of the *CDKN2A/B* inhibitory activity is thought to contribute to unrestricted tumor cell growth[57]. *CDKN2A/B* homozygous deletion is associated with poor prognosis in distinct cancer types including diffuse gliomas and meningiomas[58,59].

Amplification of epidermal growth factor receptor

EGFR is located on chromosome 7 and it belongs to the family of tyrosine kinase growth factor receptors[60,61]. EGFR is normally activated through ligand binding, leading to a cascade of downstream signals that promote cell growth and proliferation[61,62]. In glioblastoma, *IDH*-wildtype, activation of *EGFR* can occur through e.g. high-level amplification of *EGFR*, which enhances the EGFR signaling pathway, and thereby an unrestricted tumor cell growth is sustained. *EGFR* amplification has been found in approximately 40% of glioblastomas, *IDH*-wildtype[63].

Mutation of telomerase reverse transcriptase promotor

TERT is located on chromosome band 5p15 and encodes the core catalytic subunit of telomerase that is responsible for maintaining the length of telomeric DNA[64,65]. In most somatic cells, telomerase activity is limited due to downregulation of TERT, causing shortening of

telomeres with every cell cycle and restricting the number of times a cell can divide[66-68]. Somatic point mutations in *TERT* promotor leads to overexpression of TERT, causing reactivation of telomerase activity and elongation of telomeres, which confers cells the ability to divide infinitely. *TERT* promotor mutation is frequently found in glioblastomas, *IDH*-wildtype[63,69,70] and in nearly all oligodendrogliomas, *IDH*-mutant and 1p/19q-codeleted[71].

Gain of chromosome 7 and loss of chromosome 10

The most frequent chromosomal CNAs observed in glioblastomas, *IDH*-wildtype involves the gain of several copies of the entire chromosome 7 and loss of one copy of chromosome 10[63], and these chromosomal alterations have been identified as early events in glioblastoma formation[72]. Chromosome 7 gain and chromosome 10 loss have been found in approximately ~80%[72] and 60-80%[73,74] of glioblastomas, respectively. It has been shown that genes located in chromosome 7 that are involved in the pathogenesis of glioblastoma (e.g. *PDGFRA*) are important drivers of chromosome 7 gain. Loss of function of tumor suppressor genes located on chromosome 10 (e.g. *PTEN* on 10q23.31 *MMAC1* on 10q23–24 and *DMBT1* on 10q25.3-q26.1) through chromosome 10 deletion indicates their involvement in the pathogenesis of glioblastoma[74-77].

Therapeutic management

Upon initial diagnosis, the treatment of diffuse gliomas is usually based on the patient's performance status, age, tumor burden and location. Moreover, the oncological therapy can further be guided by tumor tissue diagnosis and molecular markers[78]. In the vast majority of cases, surgery is feasible and it is considered the first in-line therapeutic approach. Jakola et al. 2017 demonstrated that early maximal safe surgical resection sustained survival benefit compared to biopsy and watchful waiting[79]. After surgery, a watch-and-wait strategy follows unless further treatment beyond surgery is necessary, which in that instance includes radiotherapy with adjuvant chemotherapy (e.g. fractionated radiotherapy with PCV [procarbazine, lomustine and vincristine]).

Postoperatively patients are routinely followed with MRI examination at regular intervals according to guidelines[78,80,81]. There is no standardized treatment for patients that have a recurrence of the tumor, in that instance, the chosen treatment approach is based on the patient's condition and may also be influenced by the initial treatment provided. Common oncological treatment options include temozolomide (TMZ) and a single treatment with lomoustine (as an alternative to PCV), and occasionally re-irradiation may be considered. Prior to a second-line oncological treatment the suitability for a second surgical intervention should always be considered.

The standard treatment of care for patients with CNS WHO grade 4 astrocytomas *IDH*-mutant and glioblastomas, *IDH*-wildtype, is maximal safe tumor resection followed by adjuvant radiation therapy and chemotherapy with the alkylating agent TMZ[78,81]. Fluorescence-guided surgery using 5-aminolevulinic acid (5-ALA, Gliolan®) is often performed for visualization of malignant tissue during surgery[81,82]. The O6-methylguanine-DNA methyltransferase (*MGMT*) promotor methylation status helps guiding the therapeutic management in these patients[83,84]. Patients with CNS WHO grade 4 gliomas may also be eligible for treatment with tumor treating fields (TTFields or Optune), a device that delivers electric fields to the brain to disrupt neoplastic cell division[81,85,86]. Treatment with TTFields in combination with TMZ chemotherapy, has shown to prolong the median progression-free and median overall survival to 7 and 20 months, respectively[85,86].

Meningiomas

Meningiomas are intracranial neoplasms that are believed to originate from the arachnoid cap cells within the meninges, a membrane of tissue that surrounds the cerebral hemispheres and spinal cord[87]. In comparison to diffuse gliomas, meningiomas are extra-axial tumors that have a circumscribed growth pattern and rarely infiltrates surrounding regions of the brain. According to "*The Central Brain Tumor Registry of the United States*" (CBTRUS), meningioma has the highest incident rate among intracranial tumors, accounting for 39% of all CNS tumors and with an annual incidence of 9.12 cases per 100,000 individuals[7]. The incidence of meningioma increases with age, and these are more common in female compared to males[7]. Meningiomas are typically slow growing tumors with benign behavior. Yet, ~20% of meningiomas have a more aggressive clinical course, with malignant progression into higher grades and/or focal recurrence[28]. An example of a meningioma on MRI is provided in Figure 2.

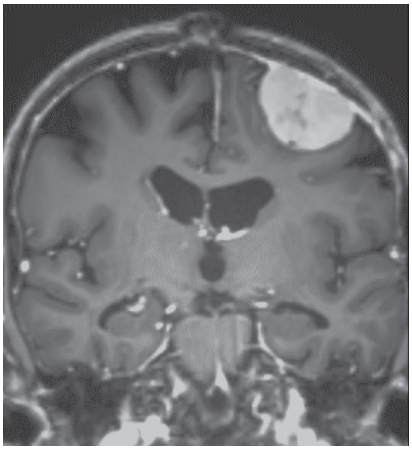

Figure 2. Meningioma. Magnetic resonance imaging (MRI), T1-weighted sequence with contrast of a meningioma located at left the cerebral convexity.

Diagnostic classification of meningiomas

The 2021 WHO criteria recognize meningioma as a single tumor type with 15 distinct histological/molecular variants categorized by their grade of malignancy as *benign* (CNS WHO grade 1), *atypical* (CNS WHO grade 2) and *anaplastic* (CNS WHO grade 3)[88-90]. The majority of

meningiomas (~80%) are benign tumors characterized by a low risk of recurrence and indolent growth pattern. WHO has listed 9 benign subtypes of meningiomas where meningothelial and fibrous meningiomas are the most common subtypes[7]. Atypical meningiomas constitute ~17% of meningiomas under which three variants (atypical, choroid and clear-cell) are present. These tumors are characterized by increased mitotic activity (mitotic index ≥4), small cells with high nuclear-to-cytoplasmic ratio, prominent nucleoli, sheeting-like growth, spontaneous necrosis and brain invasion[89,90]. Also, the tumors have a higher risk of recurrence compared to benign meningiomas. Anaplastic meningiomas are rare, constituting about 1.7% of meningiomas and these are considered malignant. CNS WHO grade 3 comprises three meningioma variants (anaplastic, papillary and rhabdoid), which are characterized by a mitotic index ≥20 and frank anaplasia[89,90]. According to the 2021 WHO classification criteria, the presence of *CDKN2A/B* homozygous deletion or *TERT* promotor mutation in meningioma is sufficient to make the diagnosis of anaplastic meningioma, CNS WHO grade 3 despite the absence of anaplastic phenotypes on histology[2,45]. In addition, DNA methylation profiling can be employed for molecular stratification of meningiomas into prognostically relevant subtypes as methylation profiling has proven to better correlate with the patient's clinical outcome compared to WHO criteria[2,91,92].

Molecular markers and chromosomal alterations

Frequent gene mutations and chromosomal alterations have been identified in meningiomas, where loss of heterozygosity (LOH) of chromosome 22q and NF2 mutations are the most common alterations occurring in any grade of meningioma[88]. Other major genetic mutations are present in *AKT1*, *TRAF7*, *SMO* and *KLF4*, *SMARCE1*, *BAPI1* and *PBRM1*. *AKT1*, *TRAF7* and *SMO* are associated with CNS WHO grade 1 meningiomas. *SMARCE1* mutations are typically found in clear-cell meningiomas (CNS WHO grade 2) while *BAPI1*, *PBRM1* are commonly encountered in rhabdoid and papillary meningiomas (CNS WHO grade 3), respectively[93]. *TERT* promotor mutations and *CDKN2A/B* homozygous deletions are associated with high risk of recurrence and poor clinical outcome[94-96].

Murine double minute clone 2 (*MDM2*) amplification is a rare genomic event in atypical meningiomas, with only two cases reported in the literature[97,98]. *MDM2* is a proto-oncogene located on chromosome arm 12q that acts as a negative regulator of the *TP53* tumor suppressor gene. Overexpression of *MDM2* by gene amplification has been reported in other human malignancies such as lung and colon cancer[99] where it was found to induce genomic instability by inhibition of the DNA double-strand break repair mechanism.

Therapeutic management

Therapeutic management of meningiomas depends on the patients' performance status, symptoms, tumor size and location, and when known histopathology also the malignancy of the tumor. The European Association of Neuro-Oncology (EANO) guidelines for treatment of meningioma recommends a radiological diagnosis by MRI[93]; histological diagnosis is not mandatory if radiology strongly suggest meningioma. The management of asymptomatic meningiomas consists of an observational approach with periodic MRI scans[93]. Surgical resection is the primary treatment approach in most patients with meningioma. Surgery is deemed necessary in patients with e.g. symptomatic meningiomas and/or patients who developed a significant tumor growth. The aim of a surgical intervention is to achieve a gross total resection. The extent of resection is a prognostic factor in meningiomas as a gross total tumor resection is often curative. Fractionated radiotherapy can be offered to patients with inoperable meningiomas or after partial removal of the tumor tissue. One dose irradiation (i.e. radiosurgery) may be considered in patients with small-sized meningiomas without mass effect and/or with tumor remnants. The efficacy of pharmacotherapeutic agents for the treatment of meningioma is still unclear, although the use of anti-angiogenic compounds including bevacizumab, sunitinib and vatalanib have shown promising results for the treatment of atypical and anaplastic meningiomas[100-102]. According to EANO recommendations, pharmacotherapy should be considered for patients with progressive atypical meningiomas[93].

Tumor heterogeneity

Tumors are heterogeneous, consisting of several populations of normal cells and neoplastic cells with distinct differentiation status and genomic alterations. These differences in cellular traits can be observed between patients with tumors of the same type of disease (*intertumor heterogeneity*), or within single tumors (*intratumor heterogeneity*)[103,104], Figure 3.

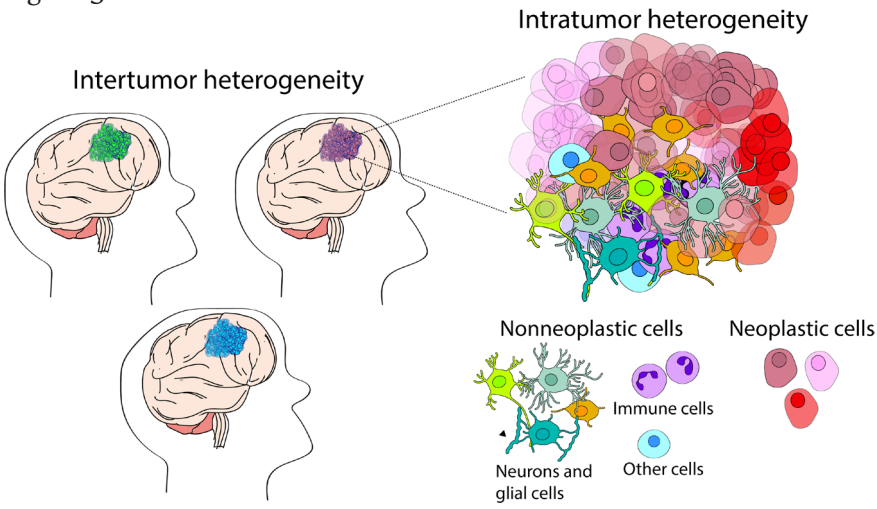

Figure 3. Tumor heterogeneity. Heterogeneity can be observed between patients with the same type of tumor (intertumor heterogeneity), or within single tumors (intratumor heterogeneity).

Intratumor heterogeneity can develop across time (*temporal heterogeneity*), as cell populations can evolve gradually over time, or it can manifest in space (*spatial heterogeneity*) as population of cells can arise across different regions of the tumors[103,104]. Regardless of how heterogeneity within tumors develops, such heterogeneity complicates disease classification, prognosis and treatment. Studies assessing the epigenetic heterogeneity within CNS tumors are limited in the literature, thus in Paper II and III, we investigated the role of intratumor heterogeneity in clinical diagnostics and prognostics.

Two central models have been proposed to explicate the origin and maintenance of tumor heterogeneity: "*the stochastic model*" and "*the cancer stem cell (CSC) model*". The stochastic model, also known as the

clonal evolution model, states that any nonneoplastic cell accumulates genetic/epigenetic alterations over time. These alterations may confer the cells with a selective growth advantage (e.g. invasiveness potential and treatment resistance), and consequently, the most dominant subpopulation of cells drives the tumor grow further[105]. Unlike the stochastic model, the CSC model postulates that tumor growth is initiated and sustained by a subpopulation of cells in a hierarchical manner[106]. These cells, commonly referred to as CSCs or tumor initiating cells, have the ability of indefinite self-renewal and differentiation and can generate heterogeneous tumors consisting of populations of cells at various degrees of differentiation[107].

Epigenetics

In 1942, Conrad Waddington conceived the term "*epigenetics*" as to explain the interaction of genes with their environment that lead to the development of phenotypes[108]. A redefined and modern definition of epigenetics concerns the study of heritable changes in genetic functions that do not entail any change in the underlying DNA sequence. A way of seeing epigenetics is to think of the cells in the human body; the cells have the same genetic code, yet we have a diversity of cell types with different gene expression profiles and functions. This is possible through the epigenetic machinery, which plays a pivotal role in controlling a wide variety of cell regulatory mechanisms as well as processes of cellular differentiation[109]. The three major epigenetic mechanisms consist of DNA methylation, histone modifications and non-coding RNA, Figure 4. Histones are proteins that packages DNA into chromosomes within the nucleus. Post-translational modifications on histones, by for instance acetylation, methylation, phosphorylation and ubiquitination, can occur at the N-terminal of core histone tails and these can lead to the activation/repression of gene transcription[110,111]. Post-translational modifications can also occur at the RNA level, specifically at short non-coding RNAs, which are segments of RNA that do not encode a protein[112].

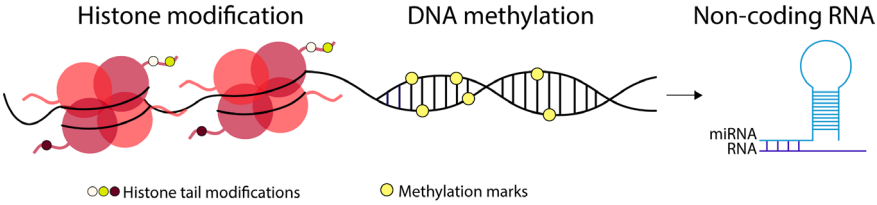

Figure 4. The epigenetic machinery. Histone modification, DNA methylation and short non-coding RNAs such as miRNAs are the most studied epigenetic mechanisms.

DNA methylation

DNA methylation has a central role in diverse biological processes including embryonic development, aging and cellular differentiation[113]. DNA methylation is a process controlled and maintained by DNA methyltransferases (DNMTs) and ten-eleven translocation (TET) enzymes. DNMTs catalyze the transfer of methyl groups (-CH3) from S-adenosyl methionine (SAM) to the 5th carbon of cytosine (C) rings in the

DNA strand (Figure 5), whereas TETs play a role in demethylation processes. In the mammalian genome, the methylation process typically occurs at C nucleotides that precede guanines (G) nucleotides, in a so called *CpG site*. High frequency of CpG sites are found in genomic regions termed *CpG islands*, usually enriched in gene promoter regions.

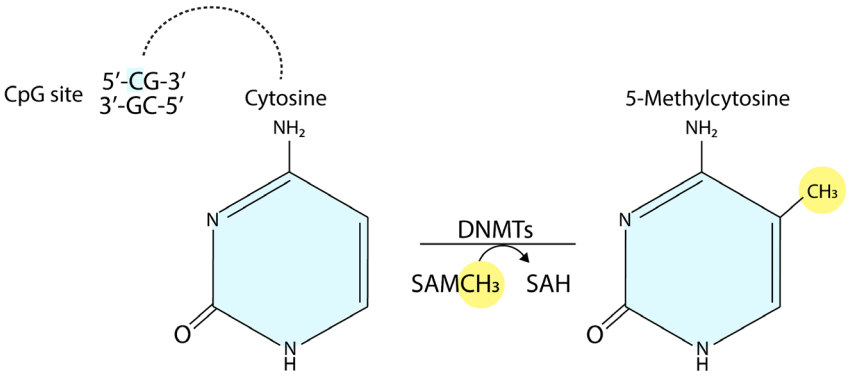

Figure 5. DNA methylation. A methyl group (CH_3) is transferred from S-adenosyl methionine (SAM) to a cytosine based followed by a guanine base (CpG site) by DNA methyltransferases (DNMTs), producing S-adenosyl homocysteine (SAH) as a byproduct.

Increased levels of methylation at a specific DNA locus is defined as hypermethylation and it is associated with gene silencing. In turn, decreased levels of methylation is referred to hypomethylation, which allows gene expression. The ~28 million CpG sites present in the human genome are mainly methylated while CpG islands are mainly unmethylated[114]. In the human brain, 5.7% of CpG islands are methylated whereas 6.4%, 8.2% and 8.3% methylation is found in blood, muscle tissue and spleen respectively[115].

DNA methylation in cancer

Proper methylation of the DNA is essential to ensure normal cellular function as aberrant alterations of the methylome can lead to various diseases including cancer[116,117]. Compared to nonneoplastic tissue, the tumor presents a distinct methylome, characterized by genome-wide hypomethylation accompanied by focal hypermethylation in specific promoter regions at CpG islands. Hypermethylation contributes to the

transcriptional repression of tumor suppressor genes[118] whereas hypomethylation is associated with e.g. chromosomal instability through demethylation of repetitive genomic sequences and also the activation of oncogenes, Figure 6.

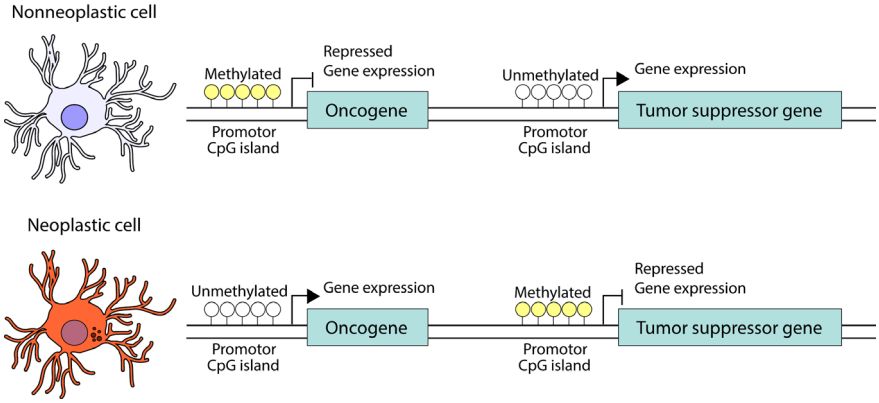

Figure 6. DNA methylation in cancer. DNA methylation can regulate the expression of oncogenes and tumor suppressor genes through methylation/unmethylation of gene promotor regions.

DNA methylation for CNS tumor diagnostics and prognostics

Methylation of *MGMT* promotor for prediction of therapy response and prognostication of survival

MGMT encodes a DNA repair protein that counteracts the damage inflicted by alkylating agents such as the chemotherapeutic drug TMZ. Epigenetic inactivation of *MGMT* through promotor methylation has been associated with decreased expression of MGMT and inhibition of the DNA repair mechanism, thereby enhancing the sensitivity to TMZ[83]. Patients with glioblastomas harboring a methylated *MGMT* promotor have shown survival benefits when treated with TMZ[84,119,120]. Therefore, the methylation status of the *MGMT* promotor has become an independent predictor of response to TMZ and it is used as a clinical biomarker for treatment allocation in the elderly patients[81]. Yet, there is a lack of consensus regarding the molecular technique and threshold used to define the methylation status of the *MGMT* promotor[121]. In a

recent international survey conducted by Malmström et al. 2020, methylation-specific PCR and pyrosequencing were the most common methods for *MGMT* analysis[121]. The prognostic and predictive impact of *MGMT* promotor methylation has been investigated in *IDH*-mutant gliomas, although the value of *MGMT* in these patients is still quite debatable. For instance, a study indicated that *MGMT* promotor methylation can be employed as a prognostic biomarker in patients with *IDH*-mutant gliomas of CNS WHO grade 2 treated with combined TMZ and radiotherapy[122]. However, other studies reported the lack of prognostic and predictive value of *MGMT* promotor in *IDH*-mutant gliomas of lower grades[123,124].

Glioma CpG island methylator phenotype (G-CIMP) for determination of *IDH* mutation status

Another DNA methylation marker highlighting the importance of epigenetic biomarkers in clinical oncology is the extensive hypermethylation of CpG islands, also known as the *CpG island methylator phenotype* (CIMP), which was first described in colorectal cancer by Toyota et al. in 1999[125]. In 2010, the glioma CIMP phenotype (G-CIMP) was identified in a proportion of glioblastomas[126], and it has since then also been examined across all diffuse glioma types and grades[127-130]. *IDH* mutations can impair the function of DNA demethylation enzymes through e.g. inhibition of TET, resulting in the CIMP phenotype[130]. Closely all *IDH*-mutant gliomas possess a CIMP phenotype (G-CIMP+) and carry a favorable prognosis compared to gliomas without this phenotype (G-CIMP-), and thus the analysis of G-CIMP can be useful to identify patients with *IDH* mutations. Further segregation of G-CIMP+ tumors based on the extent of DNA methylation (G-CIMP-high and G-CIMP-low) revealed differences in overall survival where a G-CIMP-low signature was associated with the poorest prognosis among the G-CIMP tumor patients[127]. In addition, a loss of DNA methylation in G-CIMP-high cases was linked to *IDH*-mutant glioma recurrence with poor clinical outcome compared to *IDH*-mutant gliomas that retained the G-CIMP-high at recurrence[128], highlighting the importance of further studying the molecular features and clinical implications of G-CIMP during tumor progression.

DNA methylation profiling for molecular diagnosis

Histomorphological examination of tumor tissues has long been the mainstay approach for cancer diagnostics, but it has been shown over time to suffer from high interobserver variation between pathologists[24,25]. New innovative tools have therefore been developed for improved diagnostics in neuro-oncology. One of the most promising approaches is DNA methylation array profiling, which enables the classification of tumors into molecular subgroups with different prognoses and outcomes[91,127,129,131,132].

The classification of a tumor sample with respect to its DNA methylation profile is commonly done using a *methylation-based classifier*, which is a machine-learning algorithm that provides a standardized and rapid classification of a tumor sample based on the tumor's methylation values at specific CpG sites[127,131,133]. To date, there are several methylation classifiers with potential use in pediatric tumor diagnostics, e.g. Methped[131], glioma diagnostics[127], meningioma diagnostics[91,92,134] as well as many other CNS tumor diagnostics[133]. One of the most widely used classifier for CNS tumors is the Molecular neuropathology (MNP) classifier published in 2018 by the German Cancer Research Center (DKFZ) with the University Hospital Heidelberg and in collaboration with many international research institutes. With presumed adoption of methylation-based diagnostic classification in clinical neuro-oncology, we (Paper I) and others have examined the robustness of methylation profiling for molecular stratification of pediatric and adult CNS tumors[135-141].

Other proposed biomarkers with potential value in the clinical setting

Recent advances have shown that epigenetic alterations at specific CpG sites occur in an age-related manner meaning that individual CpG sites becomes hyper- or hypomethylated with age[142-146]. This have led to the development of multiple models for prediction of chronological age based on DNA methylation, so called epigenetic clocks. Models of epigenetic age have demonstrated to accurately predict chronological age in a broad range of cell types and tissues[113,142,147]. Since the risk on

getting cancer increases as we age, studies on the age-related epigenetic alterations in cancer have been of interest. In cancer, epigenetic age signatures are altered and an accelerated methylation age (DNA methylation age higher than the chronological age) has been observed in diffuse gliomas[143,148], where an accelerated methylation age in glioblastoma was associated with better overall survival and molecular subtypes[143]. Epigenetic clocks provide thus another way on how DNA methylation can inform cancer research. The robustness of methylation age as a prognostic biomarker for CNS tumors necessitates further validation as well as to clarify the potential clinical utility of methylation age/epigenetic clocks for disease prognosis and tumor progression.

CSCs have profound implications for cancer therapy as these can contribute to tumor aggressiveness and resistance to target radio- and chemotherapy[149]. Therefore, identification of CSCs in a rapid and automated way may facilitate the development of more effective treatment strategies to restrict the role of CSCs to tumor growth and progression. In 2018, Malta et al. proposed the stemness index algorithm to measure the degree of oncogenic dedifferentiation, providing a potential prognostic and predictive biomarker for patient outcome. Stemness signatures have been defined reflecting gene expression (mRNAsi) and DNA methylation-based signatures (mDNAsi). The lowest stemness value was found in normal cells and it increased in tumors, with the highest index typically encountered in metastatic tumors[150]. In diffuse gliomas, mDNAsi was strongly correlated with the tumor's histological grade of malignancy and prognostic molecular subtypes; *IDH*-mutant gliomas showed lower stemness indices compared to *IDH*-wildtype gliomas, and the stemness index in G-CIMP-low tumors was comparable with glioblastoma *IDH*-wildtype[150,151]. The glioma stemness index has also been studied in relation to the subventricular zone (SVZ), a possible niche for CSCs, where no correlation was found between the stemness index and the proximity of the tumor to the SVZ[152]. The prognostic/predictive role of the stemness index with mDNAsi has not been studied in meningioma.

AIMS

The overall aim of this thesis was to characterize and analyze DNA methylation and chromosomal copy number profiles of diffuse gliomas and meningiomas, with focus on the clinical implications for CNS tumor diagnostics.

Specific aims

Paper I:

- To investigate the robustness of DNA methylation profiling for molecular stratification of adult diffuse lower-grade gliomas.
- To explore whether DNA methylation profiling refines the clinical diagnosis and prognosis of the patients with morphological diffuse lower-grade gliomas.

Paper II:

- To profile the intratumor DNA methylation-based heterogeneity in glioblastoma.
- To investigate the effects of DNA methylation-based heterogeneity on clinical diagnostic properties.

Paper III:

- To study tumor heterogeneity in adult-type diffuse gliomas and meningiomas with focus on DNA methylation, chromosomal copy number alterations and molecular markers.
- To examine the extent of tumor cell content as a source of tumor heterogeneity.

Paper IV:

- To investigate DNA methylation changes associated with progression of *IDH*-mutant gliomas.

MATERIALS AND METHODS

Patient material

The studies included in this thesis were approved by the Regional Ethics Committee in the Västra Götaland region in Sweden (Dnr 604-12, Dnr 1067-16 and T688-18).

Archived FFPE tumor tissue specimens were provided by the Pathology department at the Sahlgrenska University Hospital. Paper I included FFPE tumor samples from adult patients diagnosed with primary diffuse lower-grade glioma (2007 WHO) whereas Paper IV included a group of the primary tumors presented in Paper I in addition to their matched tumor recurrences.

In Papers II and III, spatially-separated tumor biopsies were collected intraoperative and tumor touch imprints were prepared on super-frost glass slides before snap-freezing the samples in liquid nitrogen. On some occasions, tumor samples were freshly-frozen at -80°C and collected after surgery. All tumor tissue samples were provided by the Department of Neurosurgery at the Sahlgrenska University Hospital after informed and signed consent from the patients. The studies included adult patients with a clinical diagnosis of diffuse glioma or meningioma (2016 WHO).

Histology

Fresh-frozen tumor samples (Paper II and III) were fixated in 4% paraformaldehyde over night at 4°C prior dehydration using increasing concentrations of ethanol baths. The tumors were embedded in paraffin wax and stored at room temperature until sectioning. 5um tumor sections were cut using a microtome and the tissue slices were mounted onto super-frost glass slides. Tumor imprints and fresh—frozen FFPE tumor biopsy sections were stained with Mayer's hematoxylin and eosin (H&E) for estimation of tumor cell content.

Immunohistochemistry

Immunohistochemistry is a widely used technique for localization of antigens in tissue specimens. Based on antigen-antibody interaction, the antigen of interest can be detected[153]. In Paper III, immunohistochemistry was performed to evaluate the expression of MDM2 and mutation of IDH1 (R132H) in meningiomas and diffuse gliomas, respectively. FFPE tissue sections were deparaffinized and rehydrated with decreased concentrations of ethanol baths prior heat-induce antigen retrieval and blocking of endogeneous peroxidase. The samples were incubated with a horseradish peroxidase (HRP) secondary antibody and visualized with DAB.

Sanger sequencing

In Paper I and II, the mutational status of *IDH1, IDH2* and/or *BRAF* were examined by Sanger sequencing to validate methylation-based predictions.

DNA methylation profiling

Bisulfite modification

Bisulfite modification is a well-established method for detection of genomic DNA methylation[154]. Bisulfite treatment consists of the deamination of unmethylated cytosines to uracils, while methylated cytosines remain unaltered by bisulfite. The bisulfite-converted DNA is then amplified by PCR where uracils are amplified as thymines - and considered *unmethylated* - while methylated cytosines are maintained as cytosines and considered *methylated*, Figure 7. In this thesis, genomic DNA from fresh-frozen tumors biopsies (Paper II and III) and FFPE tumors tissue samples (Paper I and IV) was isolated, and ~500 ng – 1000 ng DNA was bisulfite converted according to the manufacturer instructions. In Paper I and IV, we restored degraded FFPE DNA prior methylation array profiling.

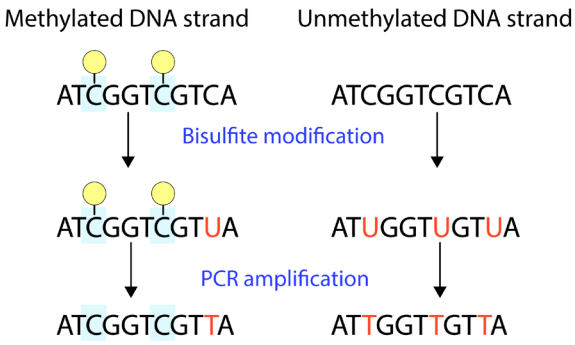

Figure 7. Bisulfite modification of DNA followed by amplification with polymerase chain reaction (PCR). Bisulfite converts unmethylated cytosines into uracils, while methylated cytosines are retained as cytosines. During PCR reaction, cytosines are amplified as cytosines and uracils as thymines.

DNA methylation array

Analysis of genome-wide DNA methylation patterns was carried out using the Infinium Methylation EPIC beadchip microarray, which enables a quantitative measurement of methylation levels of more than 850 000 CpG sites based on genotyping of bisulfite-converted DNA. The Infinium beadchip assay employs a two color-based probe design (red and green signals) to interrogate the methylation levels of CpG sites[155].

The DNA methylation level at a specific CpG loci, referred as beta value, was calculated as:

$$\beta = \frac{\max(y_{methylated})}{\max(y_{unmethylated}) + \max(y_{methylated}) + \alpha}$$

Where y is the intensities of methylated and unmethylated probes respectively. The beta value ranges from 0 (referring to a completely unmethylated CpG site) to 1 (referring to a completely methylated CpG site). α is a constant to avoid a small denominator.

The raw methylation data from the EPIC array is then retrieved in the form of *intensity data files* (.idat files), which are compilations of intensity signals for each methylated and unmethylated probe on the methylation array. The statistical software R with R studio[156] was used to read, normalize, and process methylation array data with the

packages minfi[157] and ChAMP[158,159]. We filtered out probes hybridizing to non-target genomic regions, probes hybridizing to single nucleotide polymorphism sites and targeting sex chromosomes prior methylation analyzes conducted in this thesis.

DNA methylation-based classification

Classification of CNS tumors was performed using the MNP classifier, which is freely available online at www.molecularneuropathology.org. In Paper I and II we applied the MNP classifier version 11b4, which comprises 82 CNS tumor methylation classes and nine nonneoplastic tissue classes (Control classes)[133]. The latest and current version 12.5, comprises many more tumor classes and hierarchical levels for classification[160] and this was employed in Paper III and IV. The MNP classifier was used as a research tool only and examined as recommended by the developers[132,133,160].

To classify the tumor samples, .idat files for each sample were uploaded into the MNP classifier's online platform. The output of the MNP classifier was a list of predicted methylation classes and subclasses (version 11b4) or superfamily and family members (version 12.5) with respective probability estimates (calibrated scores) that together added up to 1. In version 11b4, a threshold value of 0.90 and 0.50 had to be reached for a valid classification and subclassification, respectively[133]. Nevertheless, in Paper I we applied the alternative threshold of 0.84 for a successful classification as it was suggested for clinical samples by the classifier's developers[132]. In Paper III, we applied version 12.5 with the cutoff of 0.90 for a valid prediction in the class hierarchy as indicated[160].

Methylation classifications predicted for each tumor sample were inspected in terms of the tumor's histopathological diagnosis, neoplastic cell content, chromosomal copy number alterations (CNAs) and other available molecular information to verify whether a valid classification was generated by the classifier. If a tumor sample in our cohort received a classification score ≤0.30, it was considered as an *unclassified* sample.

Chromosomal copy number analysis

Multiple CNAs have been described in the formation and progression of many CNS tumors, including diffuse gliomas[129] and meningiomas[161], and thus copy number analysis has become increasingly important for CNS tumor diagnostics and prognostics. High-resolution CNA profiles can be retrieved from the methylation array data, and these profiles can be used as an independent layer of diagnostic/prognostic information. In Papers I-IV, we inferred CNA profiles from the methylation EPIC array with the R package conumee[162], and we used these profiles to determine chromosomal rearrangements in the genomes of the tumor samples such as 1p/19q codeletions (Figure 8), *CDKN2A/B* homozygous deletions, *MGMT* homozygous deletion, *EGFR* amplification, *MDM2* amplification, chromosome 7 gain and chromosome 10 loss.

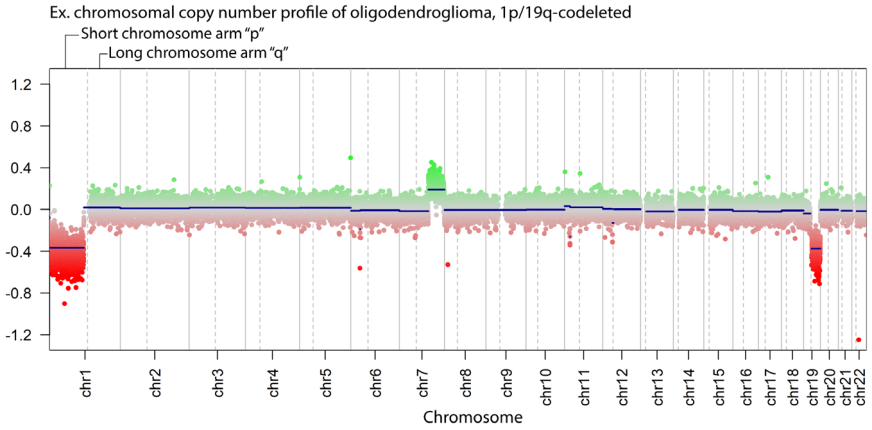

Figure 8. Example of copy number alteration (CNA) profile of an oligodendroglioma with deletions of the short chromosmal arm 1p and the long arm 19q. Copy number losses/deletions are vizualized in red whereas gains/amplifications are shown in green. The CNA profile was retrieved from the methylation array data with the conumee[162] R-package.

Estimation of tumor cell content

Tumor purity plays an important role in the readouts of the DNA methylation array data. Tumor tissue samples obtained in the clinical setting present a varying degree of cancer cells and nonneoplastic tissue components, and thus the bulk tumor array data is confounded by

normal cell admixture[163,164]. Therefore, it is important to estimate tumor cell content for reliable interpretation of the tumor's true methylation signal.

The proportion of tumor cells relative to other nonneoplastic tissue elements was estimated by a specialist in clinical neuropathology by visual morphological inspection of H&E stained samples under a light microscope. Tumor purity scores ranged from 0%-~100%, where 0% represented the lowest tumor purity (referred as suspected nonneoplastic tissue) and 100% the highest tumor purity. The tumor purity scores were based on the varying degree of e.g. immune cells, nerve cells and vascular proliferation (including white blood cells and endothelial cells) that were present in the evaluated tumor sample. In Paper II, tumor biopsies with a tumor purity of 70% or higher were included for further analysis.

Tumor purity was also estimated from bulk tissue DNA methylation data. In Paper I, III and IV, we used the R package InfiniumPurify[165,166] with the reference cohort for *lower-grade gliomas* or *glioblastoma multiforme* from TCGA. In Paper III we additionally calculated tumor purity with PAMES[163] and the RFpurify[167] algorithms RF_ABSOLUTE and RF_ESTIMATE. In Paper III we demonstrated the reliability of histopathological evaluation of tumor purity in comparison to methylation-based predictions of neoplastic cell content.

Statistical methods

The Wilcoxon two-sided t-test was used to evaluate statistical significance between groups. We used the Pearson correlation to evaluate the linear association between two numerical variables. A p value < 0.05 was considered statistically significant unless otherwise specified. In paper I, we conducted a survival analysis using the Kaplan-Meier method in IBM SPSS® Statistics software version 25 to examine the prognostic value of methylation subclasses compared to WHO CNS criteria.

RESULTS AND DISCUSSION

Paper I: DNA methylation profiling for molecular classification of adult diffuse lower-grade gliomas

The revised 2016 WHO classification of CNS tumors integrated molecular biomarkers to improve the diagnosis and prognosis of the patients[28]. DNA methylation profiling is an emerging technique for molecular classification of CNS tumors, and it is moving towards implementation in the clinical setting. In Paper I[135], we therefore investigated DNA methylation profiling as a tool for molecular diagnosis of adult-type dLGGs (2007 WHO grade II and III) according to the 2016 WHO classification system, which was the diagnostic criteria in current use at the time we worked with this study. We further investigated whether methylation analysis could add improved molecular characterization of the tumors as well as to capture prognostic differences beyond the conventional histomorphological WHO grading in addition to clinical biomarkers (i.e *IDH* mutation and 1p/19q codeletion status). A total of 166 FFPE tumor samples from patients diagnosed with dLGG (2007 WHO grade II and III) were retrospectively collected from the pathology department at the Sahlgrenska University Hospital. We processed the tumors with the Illumina methylation EPIC beadChip array to generate genome-wide DNA methylation profiles and CNA profiles of the tumors, Figure 9.

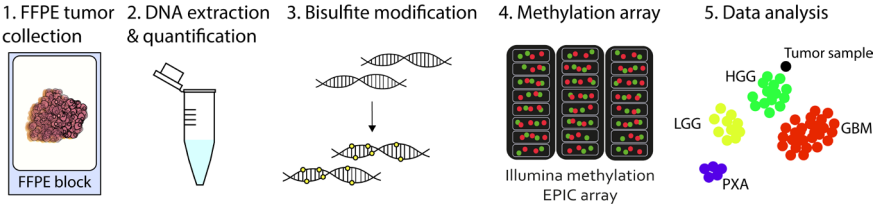

Figure 9. Workflow of DNA methylation profiling: from collection of archived formalin-fixed paraffin-embedded (FFPE) diffuse lower-grade glioma samples to data analysis.

Our results demonstrated that methylation profiling provided accurate detection of the clinical markers *IDH* mutation and 1p/19q codeletion that are required in the WHO diagnosis of diffuse gliomas. We characterized the G-CIMP phenotype from the methylation profiles of

the classified gliomas and showed that all *IDH*-mutant gliomas exhibited the G-CIMP pattern (G-CIMP+) whereas the *IDH*-wildtype gliomas did not (G-CIMP-). To validate this outcome, we analyzed *IDH1* and *IDH2* mutations with Sanger sequencing where methylation profiling yielded a sensitivity and specificity of 100% compared to Sanger sequencing. Other studies reported *IDH*-mutant gliomas carrying a G-CIMP- phenotype and/or *IDH*-wildtype gliomas exhibiting a G-CIMP+, however these events were observed in a vast minority of the cases analyzed[126,127,130,168]. This means that characterization of the G-CIMP phenotype could be used to identify *IDH* mutations in gliomas.

We next used CNA profiles inferred from the methylation array data to examine the status of 1p and 19q chromosomal arms. Combined 1p and 19q whole-arm loses were detected with high accuracy from CNA profiles compared to FISH and MLPA, and further resolved the cases with inconclusive cytopathology results, Figure 10. The 2021 WHO diagnosis of oligodendroglioma requires determination of 1p/19q but there is no consensus regarding the optimal approach for 1p/19q testing. FISH has long been the preferred technique for routine clinical testing of 1p/19q codeletion as it discriminates between nonneoplastic and neoplastic cells[53], but it cannot differentiate between partial deletions and chromosomal whole-arm losses[54], which is a limitation since only whole-arm loses of 1p and 19q are of diagnostic and prognostic relevance. MLPA could be used instead of FISH but this technique is often time consuming and the data can be difficult to analyze[169]. Furthermore, MLPA cannot distinguish CNAs among e.g. polyploidy and diploidy[170], which could lead to misdiagnosis. Based on this and other studies[132,133,137,139], CNA profiles from the methylation array data, allow for reliable determination of 1p/19q (and also other chromosomal CNAs) since it provides genome-wide overview of the structural alterations in the tumors. CNA can visually and/or quantitatively be interpreted, although estimation of tumor cell content is necessary for proper CNA analysis.

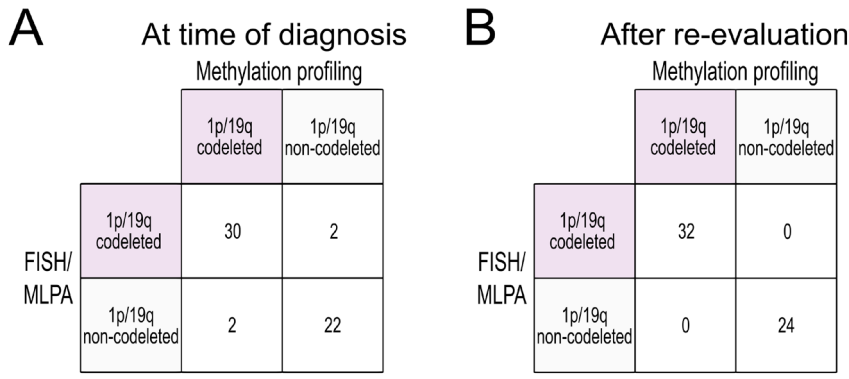

Figure 10. Copy number alteration (CNA) profiles, retrieved from the DNA methylation array, provided accurate status of 1p/19q, validated by Fluorescent *In situ* Hybridization (FISH) and Multiplex ligation-probe amplification (MLPA). We compared 1p/19q status from CNA profiles with FISH/MLPA data generated A) at time of diagnosis and B) after reevaluation of the discrepant cases. Image taken from Paper I, Ferreyra Vega et al., 2021[135] (BioMed Central, Clinical Epigenetics).

We evaluated the potential of methylation profiling as tool for the diagnosis and prognosis of dLGG. Classification of the tumors by methylation profiling was achieved in 76% (126/166 tumors) using the MNP classifier version 11b4 (classification score >0.84 and subclassification score >0.50). The DNA methylation classes identified in our study cohort were strongly associated with the *IDH* mutation and 1p/19q codeletion status, Figure 11A. Moreover, methylation profiling could further refine the 2016 WHO diagnosis of astrocytoma, *IDH*-wildtype as it could clearly separate the indolent angiocentric/lower-grade gliomas from the more aggressive glioblastomas, *IDH*-wildtype, which were commonly misclassified based on WHO histologic criteria. *IDH*-wildtype diffuse astrocytomas of lower histological grade (grade 2 and 3) harboring *EGFR* amplification, *TERT* promotor mutation and/or chromosome 7 gain and chromosome 10 loss are now recognized as CNS WHO grade 4 glioblastomas by the 2021 WHO criteria. In turn, the lower-grade *IDH*-wildtype astrocytomas that does not harbor molecular features of glioblastoma could be considered as pediatric-type diffuse gliomas but additional examination of other molecular biomarkers is necessary, which means extra workload and probably a delayed establishment of the diagnosis. The use of methylation profiling in standard diagnostics therefore holds great promise for the classification of diffuse gliomas since it can resolve diagnostic discrepancies where

other conventional techniques does not yield informative results and thereby improve the diagnostic accuracy and treatment decision making.

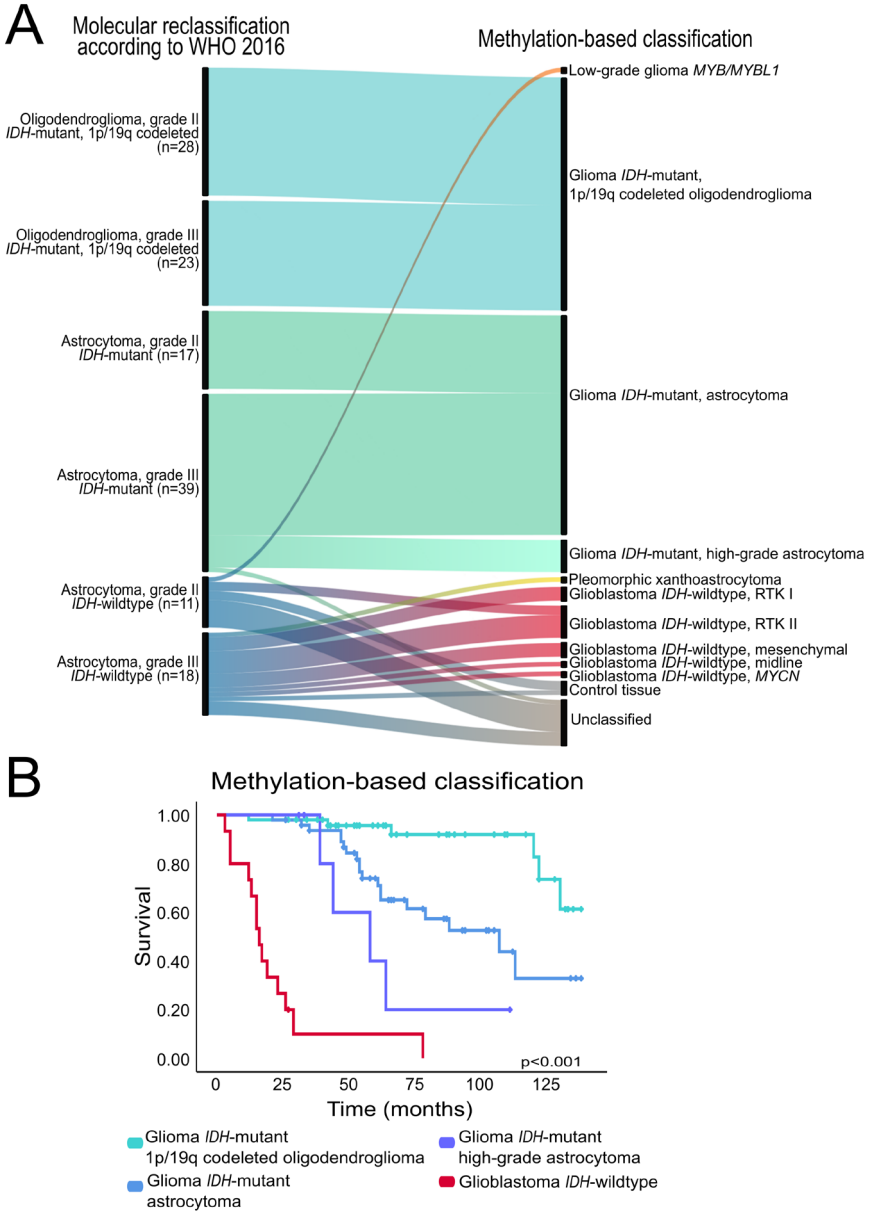

Figure 11. A) Sankey diagram of molecular reclassification according to 2016 WHO (left) and DNA methylation-based classification with the Molecular neuropathology classifier (right). B) Methylation-based classification for prediction of overall survival. Image adapted from Paper I, Ferreyra Vega et al., 2021[135] (BioMed Central, Clinical Epigenetics).

Methylation profiling was also able to predict similar survival outcomes of the patients compared to the 2016 WHO CNS classification system; patients with tumors classified as 1p/19q-codeleted oligodendrogliomas by methylation profiling had better prognosis than the patients with astrocytoma subclasses, Figure 11B. The patients with glioblastoma subclasses showed the worst survival. This indicates the value of methylation profiling to obtain prognostic information of the patients, which could help in evaluating their clinical course and guide the treatment decision-making.

The Illumina methylation EPIC array has the capacity to cover up to 8 samples in a single array[155]. The turnover time, from tumor collection to methylation profiling, has been estimated to be ~5-30 days[133,136,137,139] across different diagnostic laboratory facilities. The turnover time is commonly dictated by e.g. the availability of laboratory staff that can run the methylation array, acquisition of the required equipment, availability of reagents and supplies, the number and type of tumor tissue specimens (fresh tissue vs FFPE) selected for methylation testing and monetary resources. In our experience, the turnaround time is of a maximum of 2 weeks (including weekend days) as the samples are sent to an external laboratory facility to process the methylation array, which demands additional logistic time. Analysis of *IDH* mutation, 1p/19q codeletion and *MGMT* promotor methylation by IHC, FISH and pyrosequencing, respectively requires indeed less time (1-2 days) compared to DNA methylation array[54,169]. Nevertheless, methylation array enables the analysis of these and also other 2021 WHO required biomarkers (e.g. *CDKN2A/B* homozygous deletion) from one single array, which facilitates and reduces the number of tests of individual biomarkers in the routine clinical diagnostics. In addition, a great variety of R-packages are freely available for research use that allow a standardized and automated processing of the array data.

As any other molecular technique used in the clinical setting, tumor cell content should be addressed for proper analysis of the methylation array data as the presence of high degree of nonneoplastic cell content may mask the cancer cells' methylation signal. In this study, we did not assess tumor purity before running the samples on the array since we aimed to evaluated methylation classification performance regardless of tumor cell content. Nevertheless, among the benefits of using methylation profiling for diagnostic classification is that tumor purity can be assessed *in silico* using methylation-based algorithms such as InfiniumPurify[165] and PAMES[163] with high accuracy compared to histology/IHC[171,172].

Among other benefits of using methylation array in the routine clinical diagnostic, in comparison to the conventional molecular techniques, is that several tumor tissue specimens (fresh, fresh-frozen and FFPE) are suitable for the analysis[173]. Formalin-fixation can cause chemical modification and fragmentation of DNA, yielding degraded DNA and thus it may be unsuitable DNA for methylation array analysis. This can, however, be overcome by restoring the degraded DNA using "restoration kits" provided by the Illumina developers. Previous studies have demonstrated that the age of the FFPE tumor, i.e. the length of time between the tumor was FFPE and methylation analysis does not influence the methylation array performance[139,173-175], allowing for a robust analysis of archival FFPE samples, which are often used in population-based studies. Furthermore, about 250ng DNA is needed as input material[132], which is beneficial when processing small sample volumes such as stereotactic biopsies.

Paper II: Intratumor DNA methylation heterogeneity in glioblastoma: implications for DNA methylation-based classification

Glioblastoma is the most aggressive brain tumor in adults distinguished by extensive heterogeneity at the genetic and transcriptional level[176-178], but methylation variability in this disease is less explored. In Paper II[179], we profiled the intratumor DNA methylation heterogeneity in glioblastoma *IDH*-wildtype/*IDH*-mutant (2016 WHO) and examined how methylation heterogeneity within the tumor affects clinical diagnostics. Twelve adult patients with primary glioblastoma *IDH*-wildtype/*IDH*-mutant (2016 WHO) were included in the study. For comparison we also included intratumor biopsies from three patients with grade I meningiomas. Since grade I meningiomas are of benign nature[28], we expected them to be intratumor homogeneous. The glioblastoma and meningioma patients were operated between 2016 and 2018 at the Sahlgrenska University Hospital. Three to four spatially separated biopsies were collected and subsequently processed with the Infinium MethylationEPIC beadchip array for genome-wide characterization of DNA methylation patterns, Figure 12.

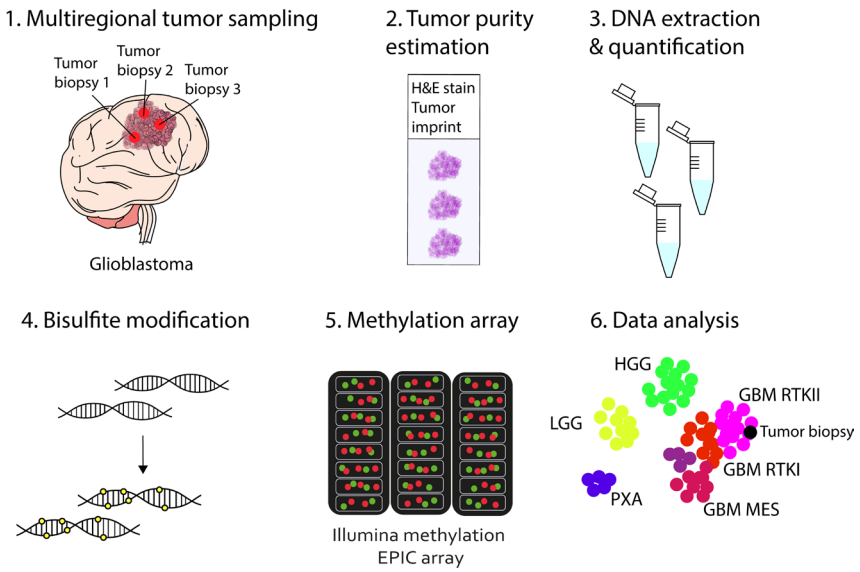

Figure 12. Workflow of DNA methylation profiling: from collection of spatially-separated glioblastoma biopsies during surgery to data analysis.

Tumor cell content was examined by a specialist in clinical neuropathology based on H&E-stained tumor imprints; tumors with ≥70% tumor cell content were analyzed further in this study.

Methylation-based classification with the MNP classifier (version 11b4) successfully classified all samples as glioblastoma (classification score >0.90). However, we observed subclass heterogeneity within five out of 12 cases as the tumors presented combinations of the mesenchymal, RTKI and RTKII subclasses (subclassification scores >0.50), Figure 13. The subclass heterogeneity found was not caused by differing neoplastic cell content within the tumors. An explanation of the subclass heterogeneity could be the presence of subclonal cell populations coexisting within the tumor. Although, subclass heterogeneity could also be an indication that these methylation subtypes require more refinement. A later publication by Verbug et al. in 2021 further supported intratumor methylation subclass heterogeneity in glioblastoma and to a similar extent[171].

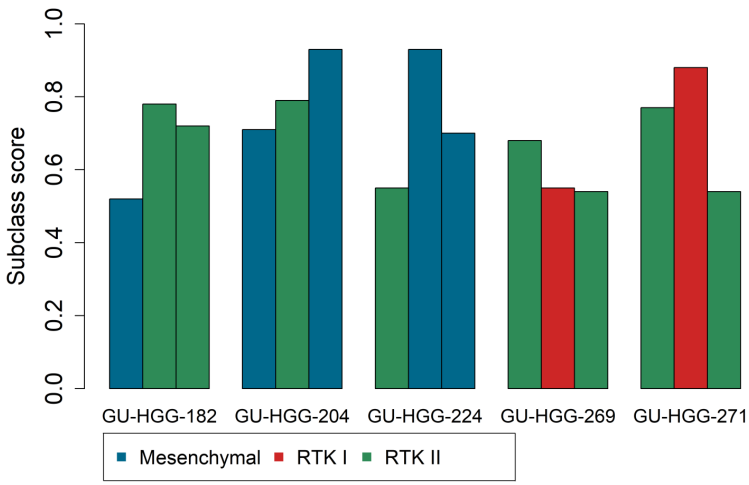

Figure 13. Distinct methylation-based subclasses were identified within 5 out of 12 glioblastoma patients using the Molecular neuropathology classifier version 11b4. Image adapted from Paper II, Wenger et al., 2019[179] (Society for Neuro-Oncology, Neuro-Oncology).

The methylation-based subclasses of glioblastoma, *IDH*-wildtype are currently not of clinical relevance. Therefore, in the current clinical setting, the presence of methylation subclass heterogeneity within

glioblastoma would not alter the diagnosis nor guide the treatment of the patients. The clinical relevance of glioblastoma methylation subclasses remains to be fully elucidated if these are to be introduced in e.g. future updates of WHO criteria, EANO/national treatment guidelines and/or in the design of clinical trials.

Clinical determination of intratumor heterogeneity would ideally require multiregional tumor sampling with at least three tumor tissue biopsies sampled to resolve which subclone is the most malignant in the tumor. Although multisampling strategy might not be suitable for patients with small tumor volumes or with inoperable tumors such as tumors injuring eloquent areas of the brain. The presence of distinct methylation subclasses within the glioblastoma, *IDH*-wildtype would probably demand a multimodal therapeutic strategy adjusted to the subclones present in the tumor. As we observed in Paper II, the mesenchymal, RTKI and/or RTKII methylation subclasses coexisted within glioblastoma *IDH*-wildtype. A combination therapy with drug agents targeting the mesenchymal (e.g. NF1 inhibitors) and RTK I/II (e.g. PDGFRA/EGFR inhibitors) subclasses could potentially be beneficial to treat such heterogeneous tumor. Nevertheless, as tumor subclones may evolve over time and potentially become resistant to the initial treatment, perhaps an intermittent therapeutic approach providing distinct subclass-specific drugs at different time periods could also be an alternative strategy[103,180].

We further observed that the methylation levels at specific CpG sites largely varied intratumorally in glioblastoma in comparison to the grade I meningioma; ~17 000 differentially methylated CpG sites were found within glioblastoma whereas meningioma had an average of 100 differentially methylated sites. Such CpG-site methylation variability affected the clinical biomarker *MGMT* promotor methylation. Our results suggest that the *MGMT* promotor methylation is prone to spatial sampling bias as we found that it varied within one out of 12 glioblastoma cases. This could have important implications in the clinical management of glioblastoma patients since the patients could be treated with TMZ or abstained from TMZ treatment based on the area of the tumor wherefrom *MGMT* was analyzed.

Paper III: Spatial heterogeneity in DNA methylation and chromosomal alterations in diffuse gliomas and meningiomas

In light of our results in Paper II, in Paper III[172] we continued to investigate DNA methylation and CNAs within *IDH*-mutant gliomas and high-grade meningiomas and further examined the intratumor heterogeneity in glioblastomas, *IDH*-wildtype. Our focus was towards elucidating how intratumor methylation heterogeneity could impact diagnostic classification, the analysis of CNAs and methylation markers. Furthermore, we studied the degree of neoplastic cell content as potential factor contributing to tumor heterogeneity. Between three and five spatially-separated tissue biopsies were sampled per tumor at the neurosurgery department at the Sahlgrenska University Hospital, Figure 14. Each of the biopsies were processed with the Infinium MethylationEPIC beadchip array. In addition, we included methylation array data from tumor samples analyzed in Paper II. We examined neoplastic cell content by histology and based on methylation array data.

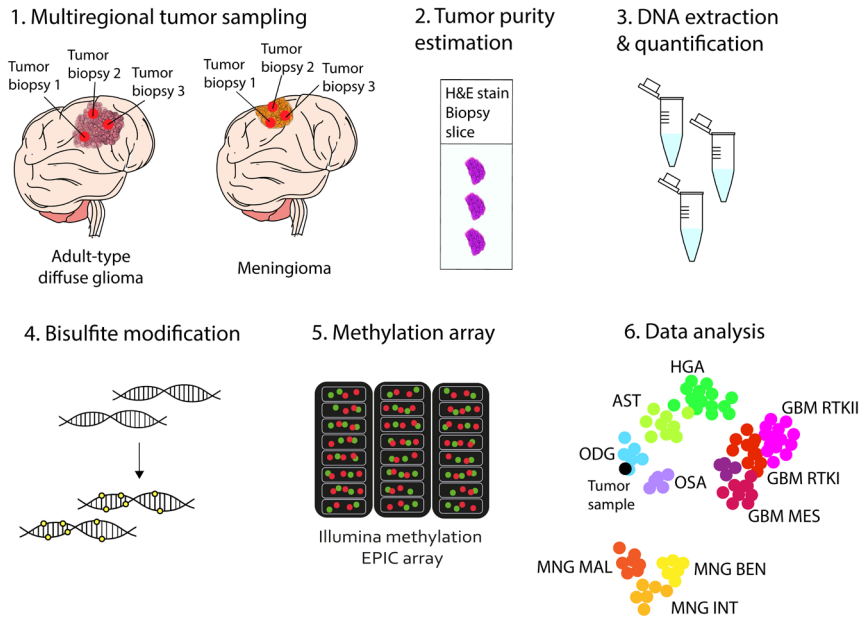

Figure 14. Workflow of DNA methylation profiling: from collection of spatially-separated diffuse glioma and meningioma biopsies during surgery to data analysis.

Our results demonstrated that distinct methylation subclasses coexist within meningioma, and further corroborated subclass heterogeneity in glioblastoma, *IDH*-wildtype after accounting for neoplastic cell content. The average difference of tumor purity within tumors with subclass heterogeneity ranged from 3% to 6%, suggesting that the varying degree of tumor cell content was not a factor leading to heterogeneity in these tumors, Figure 15.

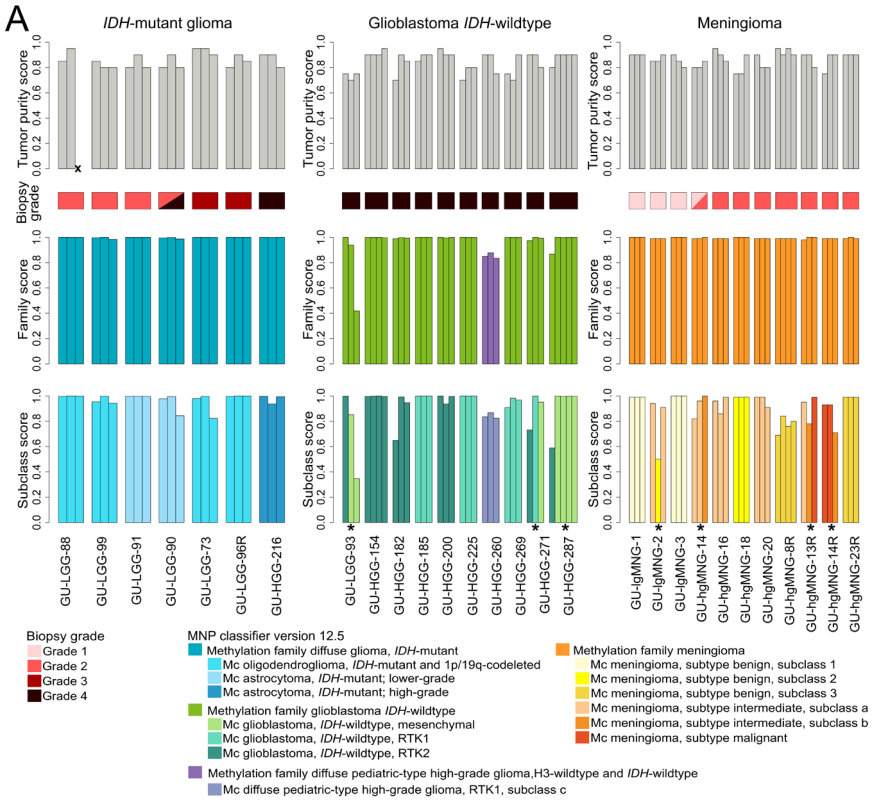

Figure 15. Distinct DNA methylation subclasses were identified in glioblastoma *IDH*-wildtype and meningioma after accounting for tumor purity. *IDH*-mutant gliomas showed, however, homogeneous subclassification within the tumors. Image adapted from Paper III, Ferreyra Vega et al., 2022[172] (Springer Nature, Modern Pathology).

The *IDH*-mutant gliomas (n=7) showed homogeneous methylation-based subclassification, yet a recent study reported subclass heterogeneity in one out of 15 diffuse *IDH*-mutant gliomas analyzed[171]. This indicates that *IDH*-mutant gliomas are less heterogeneous than

glioblastomas, *IDH*-wildtype in terms of methylation-based subclassification. The methylation subclass homogeneity or low subclass heterogeneity in *IDH*-mutant gliomas is promising for a diagnostic point of view since it implies less risk of sampling bias. Further studies with larger cohorts are thus needed to corroborate the methylation subclass homogeneity and/or extent of subclass heterogeneity in *IDH*-mutant gliomas and how it may evolve during tumor progression over time.

The status *CDKN2A/B* homozygous deletion varied within one out of two *IDH*-mutant astrocytomas CNS WHO grade 4 in our cohort. Homozygous deletion of *CDKN2A/B* is currently being analyzed in the clinic as a grading and prognostic factor in *IDH*-mutant astrocytomas as recommended by 2021 WHO criteria. If *CDKN2A/B* homozygous deletion is detected in the tumor, the tumor will be graded as grade 4 regardless of the histopathological grade 2 or 3 lesions present in the tumor. Intratumor variability of *CDKN2A/B* homozygous deletion could thus have great implications in the clinic. Variable status of *CDKN2A/B* deletion increases the risk of incorrect assessment of the tumor malignancy due to sampling bias, which in turn could affect the therapeutic management of the patients (e.g withhold treatment if grade 2 or 3 or provide more aggressive treatment if grade 4).

We also detected heterogeneous amplification of the proto-oncogene *MDM2* in one meningioma patient, but interestingly we found homogeneity of *MDM2* amplification in the paired recurrent tumor. Amplification of *MDM2* has been associated with malignant meningiomas[97,98], which could explain the tumor evolution in this case; the meningioma subclones with *MDM2* amplifications might have outcompeted the other less aggressive tumor subclones over time, leading to the recurrence of the tumor. *MDM2* amplification is not listed as a biomarker for meningiomas in the 2021 WHO criteria and the clinical relevance of this biomarker remains to be addressed. Examination of *MDM2* amplification on a single meningioma biopsy should be avoided as it may not be representative of other regions of the tumor. Therefore, future clinical trial designs with e.g. *MDM2* inhibitors would have to considered intratumor *MDM2* heterogeneity when establishing the inclusion criteria of the patients.

It is plausible that other biomarkers are also prone to sampling bias due to spatial heterogeneity[177], but due to the small cohort of patients/number of biopsies analyzed in our study, we might be missing to detect heterogeneity of other biomarkers. Genetic alterations occurring at a very early stage in tumorigenesis, e.g. *IDH* mutations in gliomas[47,48], could perhaps be exempted to sampling bias due to intratumor heterogeneity, whereas later putative driver aberrations giving rise to distinct tumor subclones could be more susceptible to sampling bias.

Variability of molecular biomarkers in CNS tumors imposes the adoption of new analysis strategies to overcome the risk of sampling bias. Biomarker assessment on multiregional tumor biopsies could be a way to surpass this issue, although the analysis of several tumor biopsies in parallel will of course lead to an increased workload in the daily routine clinical practice and could be a cost-issue in some health centers. The sampling of liquid biopsies (i.e. sampling of bodily fluids such as blood[181] and cerebrospinal fluid[182,183]) offers a promising alternative to tissue sampling for screening of molecular markers as these are minimally invasive and can be obtained at low-cost. Liquid biopsies allow for detection for tumor-specific DNA somatic alterations[184] and it could potentially also be used for detection of cancer-specific DNA methylation markers[185-187].

Paper IV: Longitudinal DNA methylation analysis of adult-type *IDH*-mutant gliomas

IDH-mutant gliomas are characterized by global hypermethylation at CpG islands (G-CIMP) and have better prognosis than *IDH*-wildtype gliomas. Yet, patients with tumors that loses such hypermethylation phenotype during progression have been shown to have an accelerated disease and exhibit a worse prognosis compared to patients with tumors that retained G-CIMP upon recurrence. In Paper IV, we therefore evaluated how DNA methylation changes are associated with tumor progression in gliomas with mutations in *IDH*. We analyzed DNA methylation array data from primary and paired recurrences, Figure 16. DNA methylation patterns were examined in terms of methylation-based classification, CNAs, differential methylation status and methylation-based biomarkers.

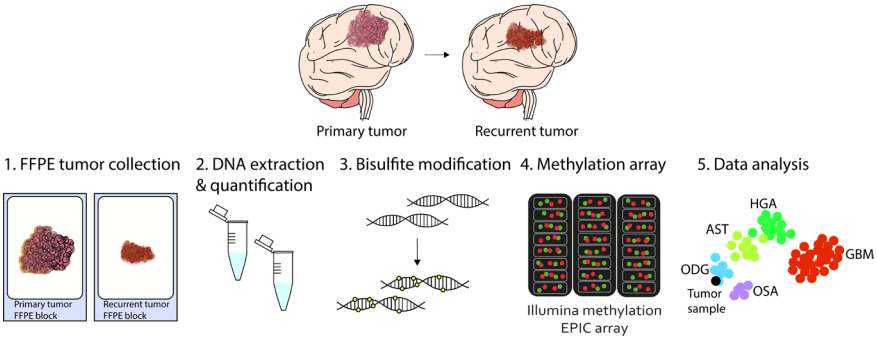

Figure 16. Workflow of DNA methylation profiling: from collection of temporal formalin-fixed paraffin-embedded (FFPE) diffuse *IDH*-mutant gliomas to data analysis.

Our results demonstrated that DNA methylation patterns are mostly maintained during *IDH*-mutant glioma progression, but specific alterations do occur. In most of the cases, the methylation profiles of the tumors were maintained upon recurrence as the primary and recurrent tumors clustered by patient identity as shown by hierarchical clustering of the methylation array data. Yet for some *IDH*-mutant astrocytoma cases, we observed a methylation subclass shift from lower-grade astrocytomas to high-grade astrocytomas. This could indicate that DNA methylation changes did occur in these tumors and these were associated with the tumor's malignant transformation into higher

grades. Nevertheless, these changes in DNA methylation were possibly not enough to induce global DNA methylation changes in the tumors. In some other few cases, we detected high methylation dissimilarities as the paired tumors did not group together by clustering analysis. We also noticed a subclass switch in some of these cases. The DNA methylation changes that occurred in these tumors could be explained by methylation alterations in the cells. Alternatively, the observed methylation dissimilarities between the primary and paired recurrent tumors could also indicate the presence of tumor subclones with different methylation profiles that emerged during tumor progression.

Oligodendroglioma with sarcomatous histological features (oligosarcoma) was first described in 1972 by Rubinstein[188]. Since then, several studies have subsequently reported oligosarcomas arising as primary tumors or from oligodendrogliomas[189-194]. In our study, we identified a primary oligodendroglioma with oligosarcoma recurrences based on DNA methylation profiling. The observed DNA methylation dissimilarities between the primary and recurrent tumors was probably because oligosarcomas have a unique DNA methylation pattern that can be distinguished from the profiles of oligodendrogliomas[195]. Oligosarcoma is not listed as a tumor type in the 2021 WHO CNS criteria but rather sarcomatous areas in oligodendrogliomas are described as rare features in grade 3 oligodendrogliomas. Genome-wide DNA methylation profiling could therefore facilitate the current designation of CNS WHO grade 3 oligodendrogliomas. Although it is debatable whether oligodendrogliomas with sarcomatous features are more appropriately diagnosed as oligosarcomas, *IDH*-mutant, and oligosarcoma should probably be recognized as a separate diffuse glioma subtype in a future update of WHO criteria[195].

One of the most frequent genetic alterations we observed in recurrent *IDH*-mutant astrocytomas was homozygous deletion of *CDKN2A/B*, which is not unexpected as this is an event associated with the malignant transformation of the tumor[2,59]. Notably, few grade 4 astrocytomas cases having homozygous deletions of *CDKN2A/B* matched to the lower-grade astrocytoma *IDH*-mutant methylation subclass according to the classifier tool. One can speculate that these tumors form a subgroup of *IDH*-mutant astrocytomas with a less aggressive clinical course

compared to high-grade astrocytomas as we showed in Paper I that the high-grade astrocytomas had overall shorter survival than the lower-grade astrocytomas irrespective of *CDKN2A/B* status[135]. The lower-grade astrocytomas with *CDKN2A/B* deletions may be indicative of an event preceding higher-grade astrocytoma. Studies with large cohorts are therefore warranted to elucidate the prognostic significance of the *IDH*-mutant astrocytoma methylation subgroups compared to *CDKN2A/B* homozygous deletion and/or histological grade 4 features.

CONCLUSIONS

We generated and analyzed DNA methylation and chromosomal copy number profiles from adult-type diffuse gliomas and meningiomas for a better understanding of the dynamics of DNA methylation in primary and recurrent tumors. Our focus was to study the implications of this approach in clinical diagnostics and prognostics.

Paper I:

- We demonstrated that the diagnostic molecular biomarkers, *IDH* mutation and 1p/19q codeletion, can be accurately identified using DNA methylation and chromosomal copy number profiles inferred from the methylation arrays.
- Methylation profiling can be employed for molecular classification of diffuse lower-grade gliomas.

Paper II:

- We found that methylation-based variability exists intratumorally in glioblastoma.
- Hence, intratumor heterogeneity in glioblastoma should be considered for methylation-based biomarkers and allocation of patients into clinically relevant subtypes.

Paper III:

- We demonstrated that methylation-based heterogeneity exists within *IDH*-mutant gliomas and high-grade meningiomas and further corroborated methylation heterogeneity within glioblastoma *IDH*-wildtype.
- Tumor purity was not, in the majority of the cases, a confounding factor for the observed heterogeneity within the tumors.

Paper IV:

- We concluded that methylation patterns are mostly maintained during tumor progression of *IDH*-mutant gliomas.
- In some cases, the molecular diagnosis of CNS WHO grade 4 astrocytomas with *CDKN2A/B* homozygous deletions was discrepant with methylation-based classification. The clinical significance of this remains to be elucidated.

FUTURE PERSPECTIVES

Molecular biomarkers are increasingly being used in the daily medical practice for the diagnosis and prognosis of CNS tumors, to tailor the treatment strategy of the patients as well as to determine the eligibility criteria in clinical trials. To obtain the minimal diagnostic/prognostic molecular information requires that several distinct analytical methods have to be run in parallel. Conventional molecular techniques including FISH, MLPA, IHC and Sanger sequencing render a relatively fast analysis time and are cost-effective, but these have their own limitations[54,169,196]. Furthermore, when a small amount of tumor tissue is available for molecular testing (e.g. tissue sampling from stereotactic biopsy) or when there is not enough time to carry out the distinct tests, these "issues" can limit the number of tests that can be performed to cover all the molecular biomarkers required and thereby influence the choice of method for analysis[54,197,198], possibly leading to an incorrect diagnosis[199]. Implementation of modern high-throughput technologies that enables an accurate, automated, reproducible and fast identification of the genetic landscape of the tumors in a single assay represents an important step towards improved diagnostic precision in neuro-oncology and possibly also better individualized treatments when tailored treatments become available.

Implementation of genome-wide DNA methylation and chromosomal copy number alteration profiling in the routine clinical diagnostics

Over the past few years, a growing number of studies has supported the robustness and reliability of genome-wide DNA methylation and chromosomal copy number profiling in clinical neuro-oncological practice[91,133,136-139]. For instance, methylation-based classification of meningioma has proven to be superior to WHO criteria for risk stratification of the patients[91,92,200], and novel and clinically relevant pediatric subgroups have been identified for medulloblastomas and ependymomas[201-203]. In addition, methylation profiling has helped in the identification of rare cases and resolved challenging cases or cases where conventional techniques ended in inconclusive results[135,137,141]. In

Sweden, genome-wide DNA methylation profiling is used to aid in the routine clinical diagnostics for children with CNS tumors and challenging cases in adults, and it is plausible to think that methylation profiling will progressively be integrated (if not already implemented) in standard diagnostics in many other medical centers as well. Although the overall diagnostic capabilities of DNA methylation profiling are promising for establishing a diagnosis, there is still limited knowledge on the usefulness of some of the methylation-based subclasses (e.g. glioblastoma subclasses) in relation to overall survival and also the potential benefits of implementing subclass-target therapies.

Implementation of methylation profiling in the clinical setting will require the introduction of a clinical workflow and the establishment of guidelines of *standard operating procedures* to facilitate and standardize the translation of DNA methylation array data into clinical information. It can be debated whether a single prediction cutoff value should be strictly adopted for all CNS tumors using the methylation classifier or whether it should be handled on a case-by-case basis. For instance, we observed in Paper IV that recurrent gliomas commonly get lower predictions scores (<0.90) compared to the primary tumors and still obtained a robust prediction. The same reasoning will probably concern setting a rigid "optimal" score of tumor cell content for methylation analysis. Indeed, high degree of nonneoplastic cell content in the tumor may mask the tumors' "true" methylation signals and thus tumor purity must be considered for proper interpretation of the array data. Nevertheless, we and others have observed that tumors with low tumor purity (<70%) can still get valid classifications and high prediction scores, and therefore those tumors should not be refrained from methylation analysis barely based on low tumor purity.

Intratumor heterogeneity

Intratumor heterogeneity poses significant challenges for personalized medicine. Characterization of the multiple layers of tumor heterogeneity (genetic, epigenetic, transcriptomic, etcetera) is therefore a key step towards refined diagnosis and improved cancer treatment. Tumor tissue biopsy is a widely used medical procedure to establish a diagnosis.

Tumor tissue biopsy sampling is often performed on a single location of the tumor, therefore failing to capture the entire genomic landscape of the tumor and increasing the risk for sampling bias due to intratumor heterogeneity.

Towards the aim to investigate the intratumor DNA methylation and chromosomal copy number heterogeneity in adult-type diffuse gliomas and meningiomas, we applied a multisampling approach to collect tumor tissue biopsies from distinct regions of the tumors. This multisampling strategy facilitated the identification of DNA methylation and chromosomal copy number heterogeneity within the tumors. Introduction of a multiple sampling strategy during surgery is thus an optimal approach to determine the extent of heterogeneity within tumors, unless of course the tumor volume is too small or this strategy cannot be adapted to the patient. Further, neurosurgery is associated with risks, and complications during surgery can occur[204]. Also, not all patients are eligible for surgery, precluding the analysis of tumor heterogeneity. Liquid biopsy using ultrasensitive sequencing is an emerging area that aims to examine the tumor's (epi)genomic landscape in a rapid and non-/minimal invasive way[205,206]. Furthermore, a repeated liquid biopsy sampling over time could facilitate monitoring tumor evolution throughout the course of the disease.

Routine clinical determination of intratumor heterogeneity should be a prerequisite for establishing a correct diagnosis and it should be discussed in e.g. a multidisciplinary medical board to choose the optimal treatment for the patients. A significant challenge will be to find and design effective therapeutic strategies to tackle spatial heterogeneity and prevent the transformation of too heterogeneous tumors at recurrence that may be even more difficult to treat due to e.g. acquired therapeutic resistance. A combination of multiple drugs with differential effects on each tumor subclone will presumably be beneficial to manage heterogeneity, but of course, a tradeoff between efficacy and toxicity must be considered in the design of these therapies. An alternating therapeutic approach targeting one subpopulation at a time could be advantageous to prevent drug sensitivity over time[103,180].

ACKNOWLEDGEMENTS

I would like to express my deepest gratitude to all people who have made this thesis possible, including the patients with CNS tumors that have donated material to our studies.

I want to thank my supervisors who have supported me and helped me grow professionally during these years. My main supervisor **Asgeir Store Jakola**, thank you so much for welcoming me into the clinical world and letting me be "the bridge" between the lab and the hospital (as you sometimes refer to me as). I am grateful for your supervision, for constantly bringing exciting ideas to this thesis and for our insightful scientific discussions. Your enthusiasm, competence and dedication for your work is truly admirable!

Helena Carén, my co-supervisor, the boss, epigenetic master and source of inspiration. I said it many times now but I always mean it: Thank you so much! For always being there for me through the highs and lows, for supporting my career and life goals and for the never-ending patience with my experiments, manuscript writing and conference preparations. What is the worst that can happen? - Nothing because I know you always have my back!

Anja Smits, my co-supervisor, thank you so much for your guidance, for the many brain-storming ideas and valuable advice on my PhD studies.

Thomas Olsson Bontell, thank you for introducing me to the fantastic world of histopathology of CNS tumors, for always been so helpful and taken the time to go through my numerous H&E slices and for kindly letting me borrow your precious WHO CNS book. **Alba Corell**, you are always so generous and cheerful! Muchas gracias for valuable scientific discussions, for helping me out with my last-minute MRI images, for always being my party-supporter and for your friendship! Both of you, thank you so much for your incredible work in the different studies of this thesis.

I also would like to thank everyone at the **Institute of Neuroscience and Physiology** for making my PhD education smoother and the **Clinical Brain Tumor Research group**, specially to **Louise C**, **Anna D** and **Tomás GV** for great scientific discussions and research collaborations.

To the hospital staff at the **Neurokirurgen-Operation5** at the Sahlgrenska University Hospital. I am very grateful for your patience and assistance every time I had to collect samples from surgeries. You are all so kind!

Big thanks to my present colleagues at the **Helena Carén Research group**. **Teresia K**, thank you for saving me too many times with R-codes and for making statistics a bit more comprehensible. I enjoyed our conversations about research and life! **Ida K**, I am so glad that you started your PhD journey in our group. Thanks for your constant help and company in the lab, I particularly enjoyed our talks about dogs. I hope we can go to more conferences together. **Maja L**, thank you for your constant support and encouragement over the years. When are we going to beat our EPIC array record? **Elizabeth S**, my Sankey diagram partner, I am grateful for our scientific discussions and for cheering me on when I needed it most. **Katja W**, your optimism is contagious! It was an honor to hand you the Carén lab half-time trophy. **Medha S**, **Shiva R**, and **Stina L**, thank you so much for helping out with the lab work every day!

…and former colleagues **Susanna L**, **Ágota T** and **Anna D**, you are always so kind! Thank you for sharing your expertise on how to work in the Big lab and the times we spend together. When are you coming back to the lab by the way? **Angelo S**, I admire your enthusiasm for science! Thanks for introducing me to the Helena Carén Research group and for your wonderful friendship! **Anna W**, my Chalmers Phadder, my desk-partner, reliable colleague and friend. It feels weird to have you in the section of "former colleagues", I truly miss you in the lab. Thank you for your pep-talks, for celebrating with me when I got my first-author publication, for helping me out when I was "confused in my own confusions", for research collaborations, for movies and tv-series reviews and foremost for making sure that I do not miss any deadline of my dissertation plan (I am REALLY grateful for that).

To the present and former members at the **Sahlgrenska Center for Cancer Research**, specially to **Junchi H**, **Elin S**, **Anna L**, **Andreas S**, **Delila T**, **Shawn L**, **Amanda OW**, **Sara S, Peter M, Dorota R, Agnieszka P and Gautam K**, thanks for your company during the countless coffee breaks, fikas and lunches, for our short life-updates in the elevator (or

while waiting for it), after works, experiment tips and tricks, career advice, laughs and more! **Karin L** and **Therese C,** your patience and guidance regarding my (immuno)histological stainings meant a lot to me. Mis compatriotas **Anuntxi M** y **Monica R**, gracias por hacerme sentir como en Perú en el SCCR.

To my friends who have been standing by my side since the day I met them. My childhood friends from Peru, **Muriel**, **Fiorella**, **Marita**, **Natalia** y **Diego**. Gracias por su bella amistad, consejos, chongos, viajes, por extrañarme y haberme llevado de fiesta de vez en cuando. 我爱你. **Caroline**, **Karin**, **Josefin**, **Olivia** and **Sara**, you are all wonderful friends. Thank you for always having my back, cheering me on and making sure I do not go crazy with this thesis.

To my swedish family "**Los Wessberg & Wennström**", thank you for your support and taking care of me. Our family gatherings mean a lot to me.

A mi familia, especialmente a mis padres **Lily** y **Juan Carlos** y a mis hermanos **Karen**, **Carla** y **Juan Carlos**. Gracias por su amor y apoyo incondicional y por celebrar cada logro de mi carrera a pesar de la distancia. ¡Por fin lo logré! Los amo. **Jeancarlo**, muchas gracias por ser siempre tan atento y generoso para conmigo. **Peluchin**, **Lulu**, **Princesa** y **Lola**, gracias por siempre sacarme una sonrisa. **Bo**, tack så mycket för att du har stöttat mig genom alla år!

My beloved **Jonas**, words cannot express how grateful I am for your patience, care, love and support during my PhD studies and always! I do not know how I would have finished this thesis on time without your hejarramsor. Jag älskar dig.

I also would like to thank the grants that supported the work in this thesis: The Sahlgrenska University Hospital Foundation, the Assar Gabrielssons Foundation and the Wilhelm and Martina Lundgren Research Foundation.

REFERENCES

1 Bruce Alberts AJ, Julian Lewis, Martin Raff, Keith Roberts, Peter Walter. *Molecular biology of the cell, 5th edition*. (Garland Science: New York, NY, USA, 2008).

2 Louis D, Perry A, Wesseling P, Brat D, Cree I, Figarella-Branger D *et al.* The 2021 WHO Classification of Tumors of the Central Nervous System: a summary. *Neuro Oncol.* **23**, 1231-1251 (2021).

3 Hanahan D & Weinberg RA. The hallmarks of cancer. *Cell* **100**, 57-70 (2000).

4 Hanahan D. Hallmarks of Cancer: New Dimensions. *Cancer Discov.* **12**, 31-46 (2022).

5 Hanahan D & Weinberg RA. Hallmarks of cancer: the next generation. *Cell* **144**, 646-674 (2011).

6 World Health Organization. *Cancer today: Estimated number of new cases in 2020, brain, central nervous system, both sexes, all ages*, https://gco.iarc.fr/today/ (2020). Accessed May 01 2022.

7 Ostrom QT, Cioffi G, Waite K, Kruchko C & Barnholtz-Sloan JS. CBTRUS Statistical Report: Primary Brain and Other Central Nervous System Tumors Diagnosed in the United States in 2014-2018. *Neuro Oncol.* **23**, iii1-iii105 (2021).

8 Socialstyrelsen. *Statistikdatabas för cancer*, https://socialstyrelsen.se/statistik-och-data/register/cancerregistret/ (2019). Accessed May 01 2022.

9 Wolf A, Naylor K, Tam M, Habibi A, Novotny J, Liscak R *et al.* Risk of radiation-associated intracranial malignancy after stereotactic radiosurgery: a retrospective, multicentre, cohort study. *Lancet Oncol.* **20**, 159-164 (2019).

10 Abi Jaoude S, Peyre M, Degos V, Goutagny S, Parfait B & Kalamarides M. Validation of a scoring system to evaluate the risk of rapid growth of intracranial meningiomas in neurofibromatosis type 2 patients. *J. Neurosurg.* **134**, 1377-1385 (2020).

11 Bondy ML, Scheurer ME, Malmer B, Barnholtz-Sloan JS, Davis FG, Il'yasova D *et al.* Brain tumor epidemiology: consensus from the Brain Tumor Epidemiology Consortium. *Cancer* **113**, 1953-1968 (2008).

12 Longstreth WT, Jr., Dennis LK, McGuire VM, Drangsholt MT & Koepsell TD. Epidemiology of intracranial meningioma. *Cancer* **72**, 639-648 (1993).
13 Malkin D. p53 and the Li-Fraumeni syndrome. *Biochim Biophys Acta* **1198**, 197-213 (1994).
14 Olivier M, Goldgar DE, Sodha N, Ohgaki H, Kleihues P, Hainaut P *et al.* Li-Fraumeni and related syndromes: correlation between tumor type, family structure, and TP53 genotype. *Cancer Res.* **63**, 6643-6650 (2003).
15 Evans DG, Sainio M & Baser ME. Neurofibromatosis type 2. *J. Med. Genet.* **37**, 897-904 (2000).
16 Gutmann DH, James CD, Poyhonen M, Louis DN, Ferner R, Guha A *et al.* Molecular analysis of astrocytomas presenting after age 10 in individuals with NF1. *Neurology* **61**, 1397-1400 (2003).
17 Dipro S, Al-Otaibi F, Alzahrani A, Ulhaq A & Al Shail E. Turcot syndrome: a synchronous clinical presentation of glioblastoma multiforme and adenocarcinoma of the colon. *Case Rep Oncol Med* **2012**, 720273 (2012).
18 Itoh H, Hirata K & Ohsato K. Turcot's syndrome and familial adenomatous polyposis associated with brain tumor: review of related literature. *Int. J. Colorectal Dis.* **8**, 87-94 (1993).
19 Fyllingen EH, Hansen TI, Jakola AS, Haberg AK, Salvesen O & Solheim O. Does risk of brain cancer increase with intracranial volume? A population-based case control study. *Neuro Oncol.* **20**, 1225-1230 (2018).
20 Wiemels JL, Wiencke JK, Sison JD, Miike R, McMillan A & Wrensch M. History of allergies among adults with glioma and controls. *Int. J. Cancer* **98**, 609-615 (2002).
21 Linos E, Raine T, Alonso A & Michaud D. Atopy and risk of brain tumors: a meta-analysis. *J. Natl. Cancer Inst.* **99**, 1544-1550 (2007).
22 Disney-Hogg L, Cornish AJ, Sud A, Law PJ, Kinnersley B, Jacobs DI *et al.* Impact of atopy on risk of glioma: a Mendelian randomisation study. *BMC Med.* **16**, 42 (2018).
23 Kleihues P, Burger PC & Scheithauer BW. The new WHO classification of brain tumours. *Brain Pathol.* **3**, 255-268 (1993).

24 Bruner JM, Inouye L, Fuller GN & Langford LA. Diagnostic discrepancies and their clinical impact in a neuropathology referral practice. *Cancer* **79**, 796-803 (1997).

25 van den Bent MJ. Interobserver variation of the histopathological diagnosis in clinical trials on glioma: a clinician's perspective. *Acta Neuropathol.* **120**, 297-304 (2010).

26 Claes A, Idema AJ & Wesseling P. Diffuse glioma growth: a guerilla war. *Acta Neuropathol.* **114**, 443-458 (2007).

27 Glioma through the looking GLASS: molecular evolution of diffuse gliomas and the Glioma Longitudinal Analysis Consortium. *Neuro Oncol.* **20**, 873-884 (2018).

28 Louis DN, Perry A, Reifenberger G, von Deimling A, Figarella-Branger D, Cavenee WK *et al.* The 2016 World Health Organization Classification of Tumors of the Central Nervous System: a summary. *Acta Neuropathol.* **131**, 803-820 (2016).

29 Louis DN, Ohgaki H, Wiestler OD, Cavenee WK, Burger PC, Jouvet A *et al.* The 2007 WHO classification of tumours of the central nervous system. *Acta Neuropathol.* **114**, 97-109 (2007).

30 Louis DN, Aldape K, Brat DJ, Capper D, Ellison DW, Hawkins C *et al.* cIMPACT-NOW (the consortium to inform molecular and practical approaches to CNS tumor taxonomy): a new initiative in advancing nervous system tumor classification. *Brain Pathol.* **27**, 851-852 (2017).

31 Louis DN, Wesseling P, Paulus W, Giannini C, Batchelor TT, Cairncross JG *et al.* cIMPACT-NOW update 1: Not Otherwise Specified (NOS) and Not Elsewhere Classified (NEC). *Acta Neuropathol.* **135**, 481-484 (2018).

32 Louis DN, Giannini C, Capper D, Paulus W, Figarella-Branger D, Lopes MB *et al.* cIMPACT-NOW update 2: diagnostic clarifications for diffuse midline glioma, H3 K27M-mutant and diffuse astrocytoma/anaplastic astrocytoma, IDH-mutant. *Acta Neuropathol.* **135**, 639-642 (2018).

33 Brat DJ, Aldape K, Colman H, Holland EC, Louis DN, Jenkins RB *et al.* cIMPACT-NOW update 3: recommended diagnostic criteria for "Diffuse astrocytic glioma, IDH-wildtype, with molecular features of glioblastoma, WHO grade IV". *Acta Neuropathol.* **136**, 805-810 (2018).

34 Ellison DW, Hawkins C, Jones DTW, Onar-Thomas A, Pfister SM, Reifenberger G *et al.* cIMPACT-NOW update 4: diffuse gliomas characterized by MYB, MYBL1, or FGFR1 alterations or BRAF(V600E) mutation. *Acta Neuropathol.* **137**, 683-687 (2019).

35 Brat DJ, Aldape K, Colman H, Figrarella-Branger D, Fuller GN, Giannini C *et al.* cIMPACT-NOW update 5: recommended grading criteria and terminologies for IDH-mutant astrocytomas. *Acta Neuropathol.* **139**, 603-608 (2020).

36 Louis DN, Wesseling P, Aldape K, Brat DJ, Capper D, Cree IA *et al.* cIMPACT-NOW update 6: new entity and diagnostic principle recommendations of the cIMPACT-Utrecht meeting on future CNS tumor classification and grading. *Brain Pathol.* **30**, 844-856 (2020).

37 Ellison DW, Aldape KD, Capper D, Fouladi M, Gilbert MR, Gilbertson RJ *et al.* cIMPACT-NOW update 7: advancing the molecular classification of ependymal tumors. *Brain Pathol.* **30**, 863-866 (2020).

38 Ryall S, Tabori U & Hawkins C. Pediatric low-grade glioma in the era of molecular diagnostics. *Acta Neuropathol Commun* **8**, 30 (2020).

39 Pollack IF, Hamilton RL, Sobol RW, Nikiforova MN, Lyons-Weiler MA, LaFramboise WA *et al.* IDH1 mutations are common in malignant gliomas arising in adolescents: a report from the Children's Oncology Group. *Childs Nerv. Syst.* **27**, 87-94 (2011).

40 Zhang J, Wu G, Miller CP, Tatevossian RG, Dalton JD, Tang B *et al.* Whole-genome sequencing identifies genetic alterations in pediatric low-grade gliomas. *Nat. Genet.* **45**, 602-612 (2013).

41 Cancer Genome Atlas Research Network. Comprehensive, integrative genomic analysis of diffuse lower-grade gliomas. *New England Journal of Medicine* **372**, 2481-2498 (2015).

42 Mandonnet E, Delattre JY, Tanguy ML, Swanson KR, Carpentier AF, Duffau H *et al.* Continuous growth of mean tumor diameter in a subset of grade II gliomas. *Ann. Neurol.* **53**, 524-528 (2003).

43 Jooma R, Waqas M & Khan I. Diffuse Low-Grade Glioma - Changing Concepts in Diagnosis and Management: A Review. *Asian. J. Neurosurg.* **14**, 356-363 (2019).

44 Wood MD, Halfpenny AM & Moore SR. Applications of molecular neuro-oncology - a review of diffuse glioma integrated diagnosis and emerging molecular entities. *Diagn. Pathol.* **14**, 29 (2019).

45 WHO Classification of Tumours Editorial Board. *World Health Organization Classification of Tumours of the Central Nervous System*. 5th edn, Vol. 6 (Lyon, 2021).

46 Han S, Liu Y, Cai SJ, Qian M, Ding J, Larion M *et al.* IDH mutation in glioma: molecular mechanisms and potential therapeutic targets. *Br. J. Cancer* **122**, 1580-1589 (2020).

47 Yan H, Parsons D, Jin G, McLendon R, Rasheed B, Yuan W *et al.* IDH1 and IDH2 mutations in gliomas. *New England Journal of Medicine* **360**, 765-773 (2009).

48 Watanabe T, Nobusawa S, Kleihues P & Ohgaki H. IDH1 mutations are early events in the development of astrocytomas and oligodendrogliomas. *Am. J. Pathol.* **174**, 1149-1153 (2009).

49 Huang J, Yu J, Tu L, Huang N, Li H & Luo Y. Isocitrate Dehydrogenase Mutations in Glioma: From Basic Discovery to Therapeutics Development. *Front. Oncol.* **9**, 506 (2019).

50 Reifenberger J, Reifenberger G, Liu L, James CD, Wechsler W & Collins VP. Molecular genetic analysis of oligodendroglial tumors shows preferential allelic deletions on 19q and 1p. *Am. J. Pathol.* **145**, 1175-1190 (1994).

51 Bello MJ, Leone PE, Vaquero J, de Campos JM, Kusak ME, Sarasa JL *et al.* Allelic loss at 1p and 19q frequently occurs in association and may represent early oncogenic events in oligodendroglial tumors. *Int. J. Cancer* **64**, 207-210 (1995).

52 Jeuken J, Cornelissen S, Boots-Sprenger S, Gijsen S & Wesseling P. Multiplex ligation-dependent probe amplification: a diagnostic tool for simultaneous identification of different genetic markers in glial tumors. *J. Mol. Diagn.* **8**, 433-443 (2006).

53 Brandner S. Molecular Diagnostics of Adult Gliomas in Neuropathological Practice. *Acta Med Acad* **50**, 29-46 (2021).

54 Priesterbach-Ackley LP, Wesseling P, Snijders TJ, de Vos F & de Leng WWJ. Molecular tools for the pathologic diagnosis of central nervous system tumors. *Neurooncol Pract* **6**, 4-16 (2019).

55 Woehrer A & Hainfellner JA. Molecular diagnostics: techniques and recommendations for 1p/19q assessment. *CNS Oncol* **4**, 295-306 (2015).

56 Brandner S, McAleenan A, Jones HE, Kernohan A, Robinson T, Schmidt L *et al.* Diagnostic accuracy of 1p/19q codeletion tests in oligodendroglioma: A comprehensive meta-analysis based on a Cochrane systematic review. *Neuropathol Appl Neurobiol* **48**, e12790 (2022).

57 Lu VM, O'Connor KP, Shah AH, Eichberg DG, Luther EM, Komotar RJ *et al.* The prognostic significance of CDKN2A homozygous deletion in IDH-mutant lower-grade glioma and glioblastoma: a systematic review of the contemporary literature. *J. Neurooncol.* **148**, 221-229 (2020).

58 Appay R, Dehais C, Maurage CA, Alentorn A, Carpentier C, Colin C *et al.* CDKN2A homozygous deletion is a strong adverse prognosis factor in diffuse malignant IDH-mutant gliomas. *Neuro Oncol.* **21**, 1519-1528 (2019).

59 Shirahata M, Ono T, Stichel D, Schrimpf D, Reuss DE, Sahm F *et al.* Novel, improved grading system(s) for IDH-mutant astrocytic gliomas. *Acta Neuropathol.* **136**, 153-166 (2018).

60 Cohen S, Carpenter G & King L, Jr. Epidermal growth factor-receptor-protein kinase interactions. Co-purification of receptor and epidermal growth factor-enhanced phosphorylation activity. *J. Biol. Chem.* **255**, 4834-4842 (1980).

61 Mitsudomi T & Yatabe Y. Epidermal growth factor receptor in relation to tumor development: EGFR gene and cancer. *FEBS J.* **277**, 301-308 (2010).

62 Wieduwilt MJ & Moasser MM. The epidermal growth factor receptor family: biology driving targeted therapeutics. *Cell Mol Life Sci* **65**, 1566-1584 (2008).

63 Stichel D, Ebrahimi A, Reuss D, Schrimpf D, Ono T, Shirahata M *et al.* Distribution of EGFR amplification, combined chromosome 7 gain and chromosome 10 loss, and TERT promoter mutation in brain tumors and their potential for the reclassification of IDHwt astrocytoma to glioblastoma. *Acta Neuropathol.* **136**, 793-803 (2018).

64 Bryce LA, Morrison N, Hoare SF, Muir S & Keith WN. Mapping of the gene for the human telomerase reverse transcriptase,

hTERT, to chromosome 5p15.33 by fluorescence in situ hybridization. *Neoplasia* **2**, 197-201 (2000).

65 Liu T, Yuan X & Xu D. Cancer-Specific Telomerase Reverse Transcriptase (TERT) Promoter Mutations: Biological and Clinical Implications. *Genes (Basel)* **7** (2016).

66 Blackburn EH. Structure and function of telomeres. *Nature* **350**, 569-573 (1991).

67 Greider CW. Telomerase is processive. *Mol Cell Biol* **11**, 4572-4580 (1991).

68 Chiba K, Johnson JZ, Vogan JM, Wagner T, Boyle JM & Hockemeyer D. Cancer-associated TERT promoter mutations abrogate telomerase silencing. *Elife* **4** (2015).

69 Brennan CW, Verhaak RG, McKenna A, Campos B, Noushmehr H, Salama SR *et al.* The somatic genomic landscape of glioblastoma. *Cell* **155**, 462-477 (2013).

70 Killela PJ, Reitman ZJ, Jiao Y, Bettegowda C, Agrawal N, Diaz LA, Jr. *et al.* TERT promoter mutations occur frequently in gliomas and a subset of tumors derived from cells with low rates of self-renewal. *Proc. Natl. Acad. Sci. U. S. A.* **110**, 6021-6026 (2013).

71 Arita H, Narita Y, Fukushima S, Tateishi K, Matsushita Y, Yoshida A *et al.* Upregulating mutations in the TERT promoter commonly occur in adult malignant gliomas and are strongly associated with total 1p19q loss. *Acta Neuropathol.* **126**, 267-276 (2013).

72 Ozawa T, Riester M, Cheng YK, Huse JT, Squatrito M, Helmy K *et al.* Most human non-GCIMP glioblastoma subtypes evolve from a common proneural-like precursor glioma. *Cancer Cell* **26**, 288-300 (2014).

73 Inda MM, Fan X, Munoz J, Perot C, Fauvet D, Danglot G *et al.* Chromosomal abnormalities in human glioblastomas: gain in chromosome 7p correlating with loss in chromosome 10q. *Mol. Carcinog.* **36**, 6-14 (2003).

74 Mohapatra G, Bollen AW, Kim DH, Lamborn K, Moore DH, Prados MD *et al.* Genetic analysis of glioblastoma multiforme provides evidence for subgroups within the grade. *Genes Chromosomes Cancer* **21**, 195-206 (1998).

75 Li J, Yen C, Liaw D, Podsypanina K, Bose S, Wang SI *et al.* PTEN, a putative protein tyrosine phosphatase gene mutated in human

brain, breast, and prostate cancer. *Science* **275**, 1943-1947 (1997).

76 Steck PA, Pershouse MA, Jasser SA, Yung WK, Lin H, Ligon AH *et al.* Identification of a candidate tumour suppressor gene, MMAC1, at chromosome 10q23.3 that is mutated in multiple advanced cancers. *Nat. Genet.* **15**, 356-362 (1997).

77 Mollenhauer J, Wiemann S, Scheurlen W, Korn B, Hayashi Y, Wilgenbus KK *et al.* DMBT1, a new member of the SRCR superfamily, on chromosome 10q25.3-26.1 is deleted in malignant brain tumours. *Nat. Genet.* **17**, 32-39 (1997).

78 Weller M, van den Bent M, Preusser M, Le Rhun E, Tonn J, Minniti G *et al.* EANO guidelines on the diagnosis and treatment of diffuse gliomas of adulthood. *Nature Reviews Clinical Oncology* **18**, 170-186 (2021).

79 Jakola AS, Skjulsvik AJ, Myrmel KS, Sjavik K, Unsgard G, Torp SH *et al.* Surgical resection versus watchful waiting in low-grade gliomas. *Ann. Oncol.* **28**, 1942-1948 (2017).

80 Munkvold BKR, Solheim O, Bartek J, Jr., Corell A, de Dios E, Gulati S *et al.* Variations in the management of diffuse low-grade gliomas-A Scandinavian multicenter study. *Neurooncol Pract* **8**, 706-717 (2021).

81 Region Cancercentrum i Samverkan. *Nationellt vårdprogram tumörer i hjärna och ryggmärg*, https://kunskapsbanken.cancercentrum.se/diagnoser/hjarna/vardprogram/ (2020). Accessed May 02 2022.

82 Stummer W, Pichlmeier U, Meinel T, Wiestler OD, Zanella F, Reulen HJ *et al.* Fluorescence-guided surgery with 5-aminolevulinic acid for resection of malignant glioma: a randomised controlled multicentre phase III trial. *Lancet Oncol.* **7**, 392-401 (2006).

83 Hegi ME, Diserens A-C, Gorlia T, Hamou M-F, de Tribolet N, Weller M *et al.* MGMT gene silencing and benefit from temozolomide in glioblastoma. *New England Journal of Medicine* **352**, 997 (2005).

84 Malmstrom A, Gronberg B, Marosi C, Stupp R, Frappaz D, Schultz H *et al.* Temozolomide versus standard 6-week radiotherapy versus hypofractionated radiotherapy in patients

older than 60 years with glioblastoma: the Nordic randomised, phase 3 trial. *Lancet Oncol.* **13**, 916-926 (2012).

85 Stupp R, Taillibert S, Kanner AA, Kesari S, Steinberg DM, Toms SA *et al.* Maintenance Therapy With Tumor-Treating Fields Plus Temozolomide vs Temozolomide Alone for Glioblastoma: A Randomized Clinical Trial. *JAMA* **314**, 2535-2543 (2015).

86 Stupp R, Taillibert S, Kanner A, Read W, Steinberg D, Lhermitte B *et al.* Effect of Tumor-Treating Fields Plus Maintenance Temozolomide vs Maintenance Temozolomide Alone on Survival in Patients With Glioblastoma: A Randomized Clinical Trial. *JAMA* **318**, 2306-2316 (2017).

87 Bondy M & Ligon BL. Epidemiology and etiology of intracranial meningiomas: a review. *J. Neurooncol.* **29**, 197-205 (1996).

88 Shibuya M. Pathology and Molecular Genetics of Meningioma: Recent Advances. *Neurol. Med. Chir. (Tokyo)* **55 Suppl 1**, 14-27 (2015).

89 Mawrin C & Perry A. Pathological classification and molecular genetics of meningiomas. *J. Neurooncol.* **99**, 379-391 (2010).

90 Riemenschneider MJ, Perry A & Reifenberger G. Histological classification and molecular genetics of meningiomas. *Lancet Neurol.* **5**, 1045-1054 (2006).

91 Sahm F, Schrimpf D, Stichel D, Jones D, Hielscher T, Schefzyk S *et al.* DNA methylation-based classification and grading system for meningioma: a multicentre, retrospective analysis. *Lancet Oncol.* **18**, 682-694 (2017).

92 Nassiri F, Liu J, Patil V, Mamatjan Y, Wang J, Hugh-White R *et al.* A clinically applicable integrative molecular classification of meningiomas. *Nature* **597**, 119-125 (2021).

93 Goldbrunner R, Stavrinou P, Jenkinson M, Sahm F, Mawrin C, Weber D *et al.* EANO guideline on the diagnosis and management of meningiomas. *Neuro Oncol.* **23**, 1821–1834 (2021).

94 Goutagny S, Nault JC, Mallet M, Henin D, Rossi JZ & Kalamarides M. High incidence of activating TERT promoter mutations in meningiomas undergoing malignant progression. *Brain Pathol.* **24**, 184-189 (2014).

95 Mirian C, Duun-Henriksen AK, Juratli T, Sahm F, Spiegl-Kreinecker S, Peyre M *et al.* Poor prognosis associated with

TERT gene alterations in meningioma is independent of the WHO classification: an individual patient data meta-analysis. *J Neurol Neurosurg Psychiatry* **91**, 378-387 (2020).

96 Sahm F, Schrimpf D, Olar A, Koelsche C, Reuss D, Bissel J *et al.* TERT Promoter Mutations and Risk of Recurrence in Meningioma. *J. Natl. Cancer Inst.* **108** (2016).

97 Wylleman R, Debiec-Rychter M & Sciot R. A rare case of atypical/anaplastic meningioma with MDM2 amplification. *Rare Tumors* **10**, 2036361318779511 (2018).

98 Weber R, Bostrom J, Wolter M, Baudis M, Collins V, Reifenberger G *et al.* Analysis of genomic alterations in benign, atypical, and anaplastic meningiomas: toward a genetic model of meningioma progression. *Proc. Natl. Acad. Sci. U. S. A.* **94**, 14719-14724 (1997).

99 Momand J, Jung D, Wilczynski S & Niland J. The MDM2 gene amplification database. *Nucleic Acids Res.* **26**, 3453-3459 (1998).

100 Kaley TJ, Wen P, Schiff D, Ligon K, Haidar S, Karimi S *et al.* Phase II trial of sunitinib for recurrent and progressive atypical and anaplastic meningioma. *Neuro Oncol.* **17**, 116-121 (2015).

101 Lou E, Sumrall AL, Turner S, Peters KB, Desjardins A, Vredenburgh JJ *et al.* Bevacizumab therapy for adults with recurrent/progressive meningioma: a retrospective series. *J. Neurooncol.* **109**, 63-70 (2012).

102 Nayak L, Iwamoto FM, Rudnick JD, Norden AD, Lee EQ, Drappatz J *et al.* Atypical and anaplastic meningiomas treated with bevacizumab. *J. Neurooncol.* **109**, 187-193 (2012).

103 Dagogo-Jack I & Shaw A. Tumour heterogeneity and resistance to cancer therapies. *Nature Reviews Clinical Oncology* **15**, 81-94 (2018).

104 Heppner GH & Miller BE. Tumor heterogeneity: biological implications and therapeutic consequences. *Cancer Metastasis Rev.* **2**, 5-23 (1983).

105 Nowell PC. The clonal evolution of tumor cell populations. *Science* **194**, 23-28 (1976).

106 Bonnet D & Dick JE. Human acute myeloid leukemia is organized as a hierarchy that originates from a primitive hematopoietic cell. *Nat. Med.* **3**, 730-737 (1997).

107 Clevers H. The cancer stem cell: premises, promises and challenges. *Nat. Med.* **17**, 313-319 (2011).
108 Waddington CH. The epigenotype. 1942. *Int. J. Epidemiol.* **41**, 10-13 (2012).
109 Goldberg AD, Allis CD & Bernstein E. Epigenetics: a landscape takes shape. *Cell* **128**, 635-638 (2007).
110 Bernstein BE, Meissner A & Lander ES. The mammalian epigenome. *Cell* **128**, 669-681 (2007).
111 Jenuwein T & Allis CD. Translating the histone code. *Science* **293**, 1074-1080 (2001).
112 Holley CL & Topkara VK. An introduction to small non-coding RNAs: miRNA and snoRNA. *Cardiovasc. Drugs Ther.* **25**, 151-159 (2011).
113 Fernandez AF, Assenov Y, Martin-Subero JI, Balint B, Siebert R, Taniguchi H *et al.* A DNA methylation fingerprint of 1628 human samples. *Genome Res.* **22**, 407-419 (2012).
114 Luu PL, Ong PT, Dinh TP & Clark SJ. Benchmark study comparing liftover tools for genome conversion of epigenome sequencing data. *NAR Genom Bioinform* **2**, lqaa054 (2020).
115 Illingworth R, Kerr A, Desousa D, Jorgensen H, Ellis P, Stalker J *et al.* A novel CpG island set identifies tissue-specific methylation at developmental gene loci. *PLoS Biol.* **6**, e22 (2008).
116 Jones P, Issa J & Baylin S. Targeting the cancer epigenome for therapy. *Nature Reviews Genetics* **17**, 630-641 (2016).
117 Baylin SB & Jones PA. Epigenetic Determinants of Cancer. *Cold Spring Harb. Perspect. Biol.* **8** (2016).
118 Jones PA & Laird PW. Cancer epigenetics comes of age. *Nat. Genet.* **21**, 163-167 (1999).
119 Wick A, Kessler T, Platten M, Meisner C, Bamberg M, Herrlinger U *et al.* Superiority of temozolomide over radiotherapy for elderly patients with RTK II methylation class, MGMT promoter methylated malignant astrocytoma. *Neuro Oncol.* **22**, 1162-1172 (2020).
120 Wick W, Platten M, Meisner C, Felsberg J, Tabatabai G, Simon M *et al.* Temozolomide chemotherapy alone versus radiotherapy alone for malignant astrocytoma in the elderly: the NOA-08 randomised, phase 3 trial. *Lancet Oncol.* **13**, 707-715 (2012).

121 Malmstrom A, Lysiak M, Kristensen BW, Hovey E, Henriksson R & Soderkvist P. Do we really know who has an MGMT methylated glioma? Results of an international survey regarding use of MGMT analyses for glioma. *Neurooncol Pract* **7**, 68-76 (2020).

122 Bell EH, Zhang P, Fisher BJ, Macdonald DR, McElroy JP, Lesser GJ *et al.* Association of MGMT Promoter Methylation Status With Survival Outcomes in Patients With High-Risk Glioma Treated With Radiotherapy and Temozolomide: An Analysis From the NRG Oncology/RTOG 0424 Trial. *JAMA Oncol* **4**, 1405-1409 (2018).

123 Lam K, Eldred BSC, Kevan B, Pianka S, Eldred BA, Zapanta Rinonos S *et al.* Prognostic value of O (6)-methylguanine-DNA methyltransferase methylation in isocitrate dehydrogenase mutant gliomas. *Neurooncol Adv* **4**, vdac030 (2022).

124 Tesileanu CMS, Gorlia T, Golfinopoulos V, French PJ & van den Bent MJ. MGMT promoter methylation determined by the MGMT-STP27 algorithm is not predictive for outcome to temozolomide in IDH-mutant anaplastic astrocytomas. *Neuro Oncol.* **24**, 665-667 (2022).

125 Toyota M, Ahuja N, Ohe-Toyota M, Herman JG, Baylin SB & Issa JP. CpG island methylator phenotype in colorectal cancer. *Proc. Natl. Acad. Sci. U. S. A.* **96**, 8681-8686 (1999).

126 Noushmehr H, Weisenberger DJ, Diefes K, Phillips HS, Pujara K, Berman BP *et al.* Identification of a CpG island methylator phenotype that defines a distinct subgroup of glioma. *Cancer Cell* **17**, 510-522 (2010).

127 Ceccarelli M, Barthel F, Malta T, Sabedot T, Salama S, Murray B *et al.* Molecular Profiling Reveals Biologically Discrete Subsets and Pathways of Progression in Diffuse Glioma. *Cell* **164**, 550-563 (2016).

128 de Souza C, Sabedot T, Malta T, Stetson L, Morozova O, Sokolov A *et al.* A Distinct DNA Methylation Shift in a Subset of Glioma CpG Island Methylator Phenotypes during Tumor Recurrence. *Cell Rep.* **23**, 637-651 (2018).

129 Sturm D, Witt H, Hovestadt V, Khuong-Quang D, Jones D, Konermann C *et al.* Hotspot mutations in H3F3A and IDH1 define

distinct epigenetic and biological subgroups of glioblastoma. *Cancer Cell* **22**, 425-437 (2012).

130 Turcan S, Rohle D, Goenka A, Walsh LA, Fang F, Yilmaz E *et al.* IDH1 mutation is sufficient to establish the glioma hypermethylator phenotype. *Nature* **483**, 479-483 (2012).

131 Danielsson A, Nemes S, Tisell M, Lannering B, Nordborg C, Sabel M *et al.* MethPed: a DNA methylation classifier tool for the identification of pediatric brain tumor subtypes. *Clin. Epigenetics* **7**, 62 (2015).

132 Capper D, Stichel D, Sahm F, Jones D, Schrimpf D, Sill M *et al.* Practical implementation of DNA methylation and copy-number-based CNS tumor diagnostics: the Heidelberg experience. *Acta Neuropathol.* **136**, 181-210 (2018).

133 Capper D, Jones D, Sill M, Hovestadt V, Schrimpf D, Sturm D *et al.* DNA methylation-based classification of central nervous system tumours. *Nature* **555**, 469-474 (2018).

134 Olar A, Wani K, Wilson C, Zadeh G, DeMonte F, Jones D *et al.* Global epigenetic profiling identifies methylation subgroups associated with recurrence-free survival in meningioma. *Acta Neuropathol.* **133**, 431-444 (2017).

135 Ferreyra Vega S, Olsson Bontell T, Corell A, Smits A, Jakola A & Caren H. DNA methylation profiling for molecular classification of adult diffuse lower-grade gliomas. *Clin. Epigenetics* **13**, 102 (2021).

136 Jaunmuktane Z, Capper D, Jones DTW, Schrimpf D, Sill M, Dutt M *et al.* Methylation array profiling of adult brain tumours: diagnostic outcomes in a large, single centre. *Acta Neuropathologica Communications* **7** (2019).

137 Karimi S, Zuccato J, Mamatjan Y, Mansouri S, Suppiah S, Nassiri F *et al.* The central nervous system tumor methylation classifier changes neurooncology practice for challenging brain tumor diagnoses and directly impacts patient care. *Clin. Epigenetics* **11** (2019).

138 Pickles JC, Fairchild AR, Stone TJ, Brownlee L, Merve A, Yasin SA *et al.* DNA methylation-based profiling for paediatric CNS tumour diagnosis and treatment: a population-based study. *The Lancet Child & Adolescent Health* **4**, 121-130 (2020).

139 Priesterbach-Ackley LP, Boldt HB, Petersen JK, Bervoets N, Scheie D, Ulhøi BP *et al.* Brain tumour diagnostics using a DNA methylation-based classifier as a diagnostic support tool. *Neuropathology and Applied Neurobiology*, urn:issn:0305-1846 (2020).

140 Sturm D, Orr BA, Toprak UH, Hovestadt V, Jones DTW, Capper D *et al.* New Brain Tumor Entities Emerge from Molecular Classification of CNS-PNETs. *Cell* **164**, 1060-1072 (2016).

141 Schepke E, Lofgren M, Pietsch T, Olsson Bontell T, Kling T, Wenger A *et al.* DNA methylation profiling improves routine diagnosis of paediatric central nervous system tumours: A prospective population-based study. *Neuropathol Appl Neurobiol* 10.1111/nan.12838, e12838 (2022).

142 Horvath S. DNA methylation age of human tissues and cell types. *Genome Biol.* **14**, R115 (2013).

143 Lin Q & Wagner W. Epigenetic Aging Signatures Are Coherently Modified in Cancer. *PLoS Genet.* **11**, e1005334 (2015).

144 Fraga MF, Agrelo R & Esteller M. Cross-talk between aging and cancer: the epigenetic language. *Ann. N. Y. Acad. Sci.* **1100**, 60-74 (2007).

145 Fraga MF & Esteller M. Epigenetics and aging: the targets and the marks. *Trends Genet.* **23**, 413-418 (2007).

146 Issa JP. CpG-island methylation in aging and cancer. *Curr Top Microbiol Immunol* **249**, 101-118 (2000).

147 Field AE, Robertson NA, Wang T, Havas A, Ideker T & Adams PD. DNA Methylation Clocks in Aging: Categories, Causes, and Consequences. *Mol. Cell* **71**, 882-895 (2018).

148 Liao P, Ostrom QT, Stetson L & Barnholtz-Sloan JS. Models of Epigenetic Age Capture Patterns of DNA Methylation in Glioma Associated with Molecular Subtype, Survival, and Recurrence. *Neuro Oncol.* 10.1093/neuonc/noy003 (2018).

149 Reya T, Morrison SJ, Clarke MF & Weissman IL. Stem cells, cancer, and cancer stem cells. *Nature* **414**, 105-111 (2001).

150 Malta T, Sokolov A, Gentles A, Burzykowski T, Poisson L, Weinstein J *et al.* Machine Learning Identifies Stemness Features Associated with Oncogenic Dedifferentiation. *Cell* **173**, 338-354 e315 (2018).

151 Wang X, Zhang H, Ye J, Gao M, Jiang Q, Zhao T *et al.* Genome Instability-Associated Long Non-Coding RNAs Reveal Biomarkers for Glioma Immunotherapy and Prognosis. *Front Genet* **13**, 850888 (2022).

152 Corell A, Gomez Vecchio T, Ferreyra Vega S, Denes A, Neimantaite A, Hagerius A *et al.* Stemness and clinical features in relation to the subventricular zone in diffuse lower-grade glioma: an exploratory study. *Neurooncol Adv* **4**, vdac074 (2022).

153 Coons A, Creech H & Jones R. Immunological properties of an antibody containing a fluorescent group. *Proceedings of the society for experimental biology and medicine* **47**, 200-202 (1941).

154 Clark SJ, Statham A, Stirzaker C, Molloy PL & Frommer M. DNA methylation: bisulphite modification and analysis. *Nat. Protoc.* **1**, 2353-2364 (2006).

155 Illumina Infinium. *Data sheet: Infinium MethylationEPIC BeadChip*, https://science-docs.illumina.com/documents/Microarray/infinium-methylation-epic-data-sheet-1070-2015-008/Content/Source/Microarray/Infinium/MethylationEPIC/infinium-methylation-epic-data-sheet.html (2019).

156 R Core Team. *R: A language and environment for statistical computing. R Foundation for Statistical Computing, Vienna, Austria*, https://www.R-project.org/ (2021).

157 Aryee MJ, Jaffe AE, Corrada-Bravo H, Ladd-Acosta C, Feinberg AP, Hansen KD *et al.* Minfi: a flexible and comprehensive Bioconductor package for the analysis of Infinium DNA methylation microarrays. *Bioinformatics* **30**, 1363-1369 (2014).

158 Morris TJ, Butcher LM, Feber A, Teschendorff AE, Chakravarthy AR, Wojdacz TK *et al.* ChAMP: 450k Chip Analysis Methylation Pipeline. *Bioinformatics* **30**, 428-430 (2014).

159 Tian Y, Morris TJ, Webster AP, Yang Z, Beck S, Feber A *et al.* ChAMP: updated methylation analysis pipeline for Illumina BeadChips. *Bioinformatics* **33**, 3982-3984 (2017).

160 Molecular neuropathology. *Brain classifier 12.5*, https://www.molecularneuropathology.org/mnp (2022).

161 Pfisterer W, Hank N, Preul M, Hendricks W, Pueschel J, Coons S *et al.* Diagnostic and prognostic significance of genetic regional heterogeneity in meningiomas. *Neuro Oncol.* **6**, 290-299 (2004).
162 Hovestadt V & Zapatka M. conumee: Enhanced copy-number variation analysis using Illumina 450k methylation arrays. *R package version 0.99* **4** (2015).
163 Aran D, Sirota M & Butte A. Systematic pan-cancer analysis of tumour purity. *Nature Communications* **6**, 8971 (2015).
164 Jaffe A & Irizarry R. Accounting for cellular heterogeneity is critical in epigenome-wide association studies. *Genome Biol.* **15**, R31 (2014).
165 Qin Y, Feng H, Chen M, Wu H & Zheng X. InfiniumPurify: An R package for estimating and accounting for tumor purity in cancer methylation research. *Genes & diseases* **5**, 43-45 (2018).
166 Zheng X, Zhang N, Wu HJ & Wu H. Estimating and accounting for tumor purity in the analysis of DNA methylation data from cancer studies. *Genome Biol.* **18**, 17 (2017).
167 Johann P, Jager N, Pfister S & Sill M. RF_Purify: a novel tool for comprehensive analysis of tumor-purity in methylation array data based on random forest regression. *BMC Bioinformatics* **20**, 428 (2019).
168 Wiestler B, Capper D, Sill M, Jones DT, Hovestadt V, Sturm D *et al.* Integrated DNA methylation and copy-number profiling identify three clinically and biologically relevant groups of anaplastic glioma. *Acta Neuropathol.* **128**, 561-571 (2014).
169 Scheie D, Kufaishi HHA, Broholm H, Lund EL, de Stricker K, Melchior LC *et al.* Biomarkers in tumors of the central nervous system - a review. *APMIS* **127**, 265-287 (2019).
170 Homig-Holzel C & Savola S. Multiplex ligation-dependent probe amplification (MLPA) in tumor diagnostics and prognostics. *Diagn. Mol. Pathol.* **21**, 189-206 (2012).
171 Verburg N, Barthel F, Anderson K, Johnson K, Koopman T, Yaqub M *et al.* Spatial concordance of DNA methylation classification in diffuse glioma. *Neuro Oncol.* **23**, 2054-2065 (2021).
172 Ferreyra Vega S, Wenger A, Kling T, Olsson Bontell T, Jakola AS & Caren H. Spatial heterogeneity in DNA methylation and

chromosomal alterations in diffuse gliomas and meningiomas. *Mod. Pathol.* 10.1038/s41379-022-01113-8 (2022).

173 Kling T, Wenger A, Beck S & Caren H. Validation of the MethylationEPIC BeadChip for fresh-frozen and formalin-fixed paraffin-embedded tumours. *Clin. Epigenetics* **9**, 33 (2017).

174 de Ruijter TC, de Hoon JP, Slaats J, de Vries B, Janssen MJ, van Wezel T *et al.* Formalin-fixed, paraffin-embedded (FFPE) tissue epigenomics using Infinium HumanMethylation450 BeadChip assays. *Lab. Invest.* **95**, 833-842 (2015).

175 Thirlwell C, Eymard M, Feber A, Teschendorff A, Pearce K, Lechner M *et al.* Genome-wide DNA methylation analysis of archival formalin-fixed paraffin-embedded tissue using the Illumina Infinium HumanMethylation27 BeadChip. *Methods* **52**, 248-254 (2010).

176 Patel A, Tirosh I, Trombetta J, Shalek A, Gillespie S, Wakimoto H *et al.* Single-cell RNA-seq highlights intratumoral heterogeneity in primary glioblastoma. *Science* **344**, 1396-1401 (2014).

177 Sottoriva A, Spiteri I, Piccirillo S, Touloumis A, Collins V, Marioni J *et al.* Intratumor heterogeneity in human glioblastoma reflects cancer evolutionary dynamics. *Proc. Natl. Acad. Sci. U. S. A.* **110**, 4009-4014 (2013).

178 Verhaak R, Hoadley K, Purdom E, Wang V, Qi Y, Wilkerson M *et al.* Integrated genomic analysis identifies clinically relevant subtypes of glioblastoma characterized by abnormalities in PDGFRA, IDH1, EGFR, and NF1. *Cancer Cell* **17**, 98-110 (2010).

179 Wenger A, Ferreyra Vega S, Kling T, Olsson Bontell T, Jakola AS & Carén H. Intratumor DNA methylation heterogeneity in glioblastoma: implications for DNA methylation-based classification. *Neuro Oncol.* **21**, 616-627 (2019).

180 Marusyk A, Janiszewska M & Polyak K. Intratumor Heterogeneity: The Rosetta Stone of Therapy Resistance. *Cancer Cell* **37**, 471-484 (2020).

181 Cohen JD, Li L, Wang Y, Thoburn C, Afsari B, Danilova L *et al.* Detection and localization of surgically resectable cancers with a multi-analyte blood test. *Science* **359**, 926-930 (2018).

182 Wang Y, Springer S, Zhang M, McMahon KW, Kinde I, Dobbyn L *et al.* Detection of tumor-derived DNA in cerebrospinal fluid of

patients with primary tumors of the brain and spinal cord. *Proc. Natl. Acad. Sci. U. S. A.* **112**, 9704-9709 (2015).

183 Pentsova EI, Shah RH, Tang J, Boire A, You D, Briggs S *et al.* Evaluating Cancer of the Central Nervous System Through Next-Generation Sequencing of Cerebrospinal Fluid. *J. Clin. Oncol.* **34**, 2404-2415 (2016).

184 Leon SA, Shapiro B, Sklaroff DM & Yaros MJ. Free DNA in the serum of cancer patients and the effect of therapy. *Cancer Res.* **37**, 646-650 (1977).

185 Shen SY, Burgener JM, Bratman SV & De Carvalho DD. Preparation of cfMeDIP-seq libraries for methylome profiling of plasma cell-free DNA. *Nat. Protoc.* **14**, 2749-2780 (2019).

186 Taiwo O, Wilson GA, Morris T, Seisenberger S, Reik W, Pearce D *et al.* Methylome analysis using MeDIP-seq with low DNA concentrations. *Nat. Protoc.* **7**, 617-636 (2012).

187 Moss J, Magenheim J, Neiman D, Zemmour H, Loyfer N, Korach A *et al.* Comprehensive human cell-type methylation atlas reveals origins of circulating cell-free DNA in health and disease. *Nat Commun* **9**, 5068 (2018).

188 Rubinstein LJ. Tumors of the central nervous system. In: *Armed Forces Institute of Pathology* (Washington, DC, 1972).

189 Rodriguez FJ, Scheithauer BW, Jenkins R, Burger PC, Rudzinskiy P, Vlodavsky E *et al.* Gliosarcoma arising in oligodendroglial tumors ("oligosarcoma"): a clinicopathologic study. *Am. J. Surg. Pathol.* **31**, 351-362 (2007).

190 Hiniker A, Hagenkord JM, Powers MP, Aghi MK, Prados MD & Perry A. Gliosarcoma arising from an oligodendroglioma (oligosarcoma). *Clin. Neuropathol.* **32**, 165-170 (2013).

191 Tanaka S, Hitotsumatsu T, Sugita Y, Ishido K, Ito O, Hatae R *et al.* Gliosarcoma arising from oligodendroglioma (Oligosarcoma): A case report with genetic analyses. *Pathol. Int.* **68**, 567-573 (2018).

192 Shoji T, Saito R, Kanamori M, Sonoda Y, Watanabe M & Tominaga T. Sarcoma-like tumor originating from oligodendroglioma. *Brain Tumor Pathol.* **33**, 255-260 (2016).

193 Vajtai I, Vassella E, Hewer E, Kappeler A & Reinert MM. Sarcomatous evolution of oligodendroglioma ("oligosarcoma"): confirmatory report of an uncommon pattern of malignant

progression in oligodendroglial tumors. *Pathol. Res. Pract.* **208**, 750-755 (2012).

194 Yasuda T, Nitta M, Komori T, Kobayashi T, Masui K, Maruyama T *et al.* Gliosarcoma arising from oligodendroglioma, IDH mutant and 1p/19q codeleted. *Neuropathology* **38**, 41-46 (2018).

195 Suwala AK, Felix M, Friedel D, Stichel D, Schrimpf D, Hinz F *et al.* Oligosarcomas, IDH-mutant are distinct and aggressive. *Acta Neuropathol.* **143**, 263-281 (2022).

196 Feldman AZ, Jennings LJ, Wadhwani NR, Brat DJ & Horbinski CM. The Essentials of Molecular Testing in CNS Tumors: What to Order and How to Integrate Results. *Curr. Neurol. Neurosci. Rep.* **20**, 23 (2020).

197 Cree IA, Deans Z, Ligtenberg MJ, Normanno N, Edsjo A, Rouleau E *et al.* Guidance for laboratories performing molecular pathology for cancer patients. *J. Clin. Pathol.* **67**, 923-931 (2014).

198 Jaeckle KA, Decker PA, Ballman KV, Flynn PJ, Giannini C, Scheithauer BW *et al.* Transformation of low grade glioma and correlation with outcome: an NCCTG database analysis. *J. Neurooncol.* **104**, 253-259 (2011).

199 Jackson R, Fuller G, Abi-Said D, Lang F, Gokaslan Z, Shi W *et al.* Limitations of stereotactic biopsy in the initial management of gliomas. *Neuro Oncol.* **3**, 193-200 (2001).

200 Maas S, Stichel D, Hielscher T, Sievers P, Berghoff A, Schrimpf D *et al.* Integrated Molecular-Morphologic Meningioma Classification: A Multicenter Retrospective Analysis, Retrospectively and Prospectively Validated. *J. Clin. Oncol.* **39**, 3839-3852 (2021).

201 Sharma T, Schwalbe EC, Williamson D, Sill M, Hovestadt V, Mynarek M *et al.* Second-generation molecular subgrouping of medulloblastoma: an international meta-analysis of Group 3 and Group 4 subtypes. *Acta Neuropathol.* **138**, 309-326 (2019).

202 Kool M, Korshunov A, Remke M, Jones DT, Schlanstein M, Northcott PA *et al.* Molecular subgroups of medulloblastoma: an international meta-analysis of transcriptome, genetic aberrations, and clinical data of WNT, SHH, Group 3, and Group 4 medulloblastomas. *Acta Neuropathol.* **123**, 473-484 (2012).

203 Pajtler Kristian w, Witt H, Sill M, Jones David tW, Hovestadt V, Kratochwil F *et al.* Molecular Classification of Ependymal

Tumors across All CNS Compartments, Histopathological Grades, and Age Groups. *Cancer Cell* **27**, 728-743 (2015).

204 Landriel Ibanez FA, Hem S, Ajler P, Vecchi E, Ciraolo C, Baccanelli M *et al.* A new classification of complications in neurosurgery. *World Neurosurg* **75**, 709-715; discussion 604-711 (2011).

205 Weaver KD, Grossman SA & Herman JG. Methylated tumor-specific DNA as a plasma biomarker in patients with glioma. *Cancer Invest.* **24**, 35-40 (2006).

206 Lavon I, Refael M, Zelikovitch B, Shalom E & Siegal T. Serum DNA can define tumor-specific genetic and epigenetic markers in gliomas of various grades. *Neuro Oncol.* **12**, 173-180 (2010).