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Diagnostic, prognostic and predictive significance of the immunohistochemical loss of H3K27me3 in different CNS and extra-CNS tumors

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Abstract

Epigenetic regulation is essential for normal development, influencing critical biological processes such as cell differentiation, tissue development, and responses to environmental factors. The epigenetic signature in cells is a dynamic process changing over time and adapting to internal and environmental stimuli. Dysregulation of these processes is associated with various diseases, including inflammatory and degenerative conditions, as well as cancers.

Indeed, several epigenetic changes have been implicated in cancer onset, progression, and in modulating the effectiveness of treatments, and the ongoing research in this field holds promise for developing novel therapeutic strategies targeting epigenetic mechanisms.

In recent years, among epigenetic modifications, dysregulation of the methylation status of the lysin at position 28 of Histone 3 (H3K27) has gained significant attention and loss of trimethylation of H3K27 (H3K27me3) has been extensively studied in various tumor types, in some of which it serves as a diagnostic and/or prognostic marker.

In this project we aimed to investigate the diagnostic, prognostic and predictive role of H3K27me3 immunohistochemical loss in different central nervous system (CNS) and extra-CNS tumors.

In diffuse intracranial gliomas, H3K27me3 loss was significantly correlated with the presence of 1p/19q codeletion and ATRX immunohistochemical retention, serving as a diagnostic marker in the differential diagnosis of IDH-mutant gliomas. In these tumors, H3K27me3 loss was also a prognostic marker, its loss being correlated with longer progression-free survival (PFS), independently of *IDH1/2* mutations.

We demonstrated H3 K27me3 loss in rosette forming glioneuronal tumors (RGNTs), without any correlation with clinical outcome.

In intracranial meningiomas and rectal adenocarcinomas, H3 K27me3 loss was significantly associated with shorter PFS after radiotherapy, suggesting that this staining could be used as a predictive biomarker in these tumors.

Therefore, our data demonstrate that H3K27me3 loss has a different and opposite prognostic significance in different tumor types.

Despite the diagnostic, prognostic and predictive value of H3 K27me3 loss in human tumors, a standardized protocol for its assessment and interpretation is urgently needed for its use in routine practice.

Sommario

I meccanismi di regolazione epigenetici sono essenziali per il fisiologico sviluppo degli organismi, influenzando processi biologici critici come la differenziazione cellulare, lo sviluppo dei tessuti e le risposte ai fattori ambientali. La *signature* epigenetica nelle cellule è un processo dinamico, che cambia e si adatta agli stimoli interni e ambientali. Diversi cambiamenti epigenetici sono stati implicati nell'insorgenza e nella progressione del cancro e nella modulazione dell'efficacia dei trattamenti oncologici. Pertanto, strategie mirate che agiscano su questi meccanismi potrebbero essere promettenti come trattamenti innovativi per il cancro.

Negli ultimi anni, tra le alterazioni epigenetiche, le modifiche dello stato di metilazione della lisina in posizione 28 dell'istone 3 (H3K27) sono state al centro di grande interesse clinico, e la perdita di trimetilazione di H3K27 (H3K27me3) è stata ampiamente studiata in vari tumori, con dimostrazione di un suo valore diagnostico e/o prognostico. Dopo la scoperta iniziale di questo marcatore, sono state riportate molte neoplasie umane con alterazioni nei livelli di metilazione di H3K27.

In questo progetto ci siamo prefissi l'obiettivo di studiare il ruolo diagnostico, prognostico e predittivo della perdita immunoistochimica di H3K27me3 in diversi tumori, sia del sistema nervoso centrale (SNC) che extra-SNC.

Nei gliomi intracranici diffusi la perdita di H3K27me3 ha mostrato una correlazione significativa con la codelezione 1p/19q e con la conservazione dell'espressione immunoistochimica di ATRX, suggerendo un suo potenziale utilizzo come marcatore diagnostico per la diagnosi differenziale tra i gliomi diffusi IDH-mutati. Inoltre, in questi tumori la perdita di H3 K27me3 ha mostrato significato prognostico, essendo correlata con una progression-free survival (PFS) più lunga, indipendentemente dallo stato mutazionale di *IDH1/2*.

Il nostro studio ha dimostrato che i tumori glioneuronali formanti rosette si caratterizzano per una perdita, globale o a mosaico, di H3 K27me3, tuttavia in assenza di correlazione con la prognosi.

Nei meningiomi intracranici e negli adenocarcinomi rettali, la perdita immunoistochimica di H3 K27me3 era correlata ad una PFS inferiore dopo trattamento radioterapico, mostrando un potenziale valore predittivo.

Pertanto, i nostri dati, in accordo con quanto precedentemente riportato in letteratura, mostrano un diverso significato biologico della perdita di H3K27me3 in diversi tipi di tumore, tuttavia ai fini di un suo uso nella pratica clinica, è necessario un protocollo standardizzato per questa analisi.

Introduction

Role of epigenetics in normal development and disease

During the latter half of the 20th century, analyses of the correlations between genetic composition and resultant phenotype in eukaryotes led to the realization that classical genetics, defined as the study of genes and alterations directly affecting DNA sequences, could not fully elucidate the multitude of phenotypic variations observed in organisms (Waddington C.H., 2012). Subsequently, epigenetics emerged as "the study of changes in gene function that are mitotically and/or meiotically heritable and that do not entail a change in DNA sequence" (Wu C.T and Morris J.R., 2001). This field has since evolved to encompass the current definition of heritable post-translational modifications targeting histones, DNA, or RNA that reversibly regulate gene expression without altering the DNA sequence (Cheng Y. et al, 2019).

The main epigenetic regulatory events are represented by DNA methylation, microRNA (miRNA) and post-translational histone modifications, mainly represented by methylation and acetylation of specific amino acid residues that eventually act on chromatin remodelling, enhancing or repressing gene expression (Cheng Y. et al, 2019). These mechanisms play a pivotal role in normal development by regulating various biological processes, including cell differentiation, tissue development, and responses to environmental stimuli; indeed, the cellular epigenetic asset changes over time (Murrell A. et al, 2013).
These marks are normally reset in germline cells and can be transmitted from parental cells to the offspring (Murrell A. et al, 2013).
Epigenetic reprogramming though, not only influences and determines the physiological development in eukaryotes, but dysregulation in epigenetic changes has been implicated in the development of numerous inflammatory and degenerative diseases (Murrell A. et al, 2013) and of neoplasms, influencing disease onset, progression, and response to treatments (Cheng Y. et al., 2019).

Epigenetic modifications of Histone proteins

Among epigenetic modifications, particular attention has been paid to mechanisms of chromatin remodelling and heterochromatin formation, in which histone modifications play a crucial role (Bannister A.J., Kouzarides T.,2011).

The fundamental unit of chromatin, the nucleosome, is composed of 147 base pairs of DNA and of a histone octamer, consisting of two copies each of H2A, H2B, H3, and H4 core histone proteins, basic proteins with a highly conserved amino acid sequence (Park J. et al., 2022).

The main post-translational modifications of histones are represented by acetylation and methylation of lysine residues on the N-terminal tail domains, due to their significant impact on transcriptional activity and cellular functions. Histone acetylation involves the addition of acetyl groups ($-COCH_3$) to the lysine residues on histone proteins, mediated by histone acetyltransferases (HATs). This modification is generally associated with transcriptional activation. The introduction of acetyl groups causes the relaxation of the chromatin structure with formation of the euchromatin, which enables the access of transcription factors and RNA polymerase to the DNA, facilitating gene transcription (Kouzarides T., 2007). Histone deacetylation- acethyl group removal- is instead performed by histone deacetylases (HDACs), resulting in a compacted chromatin structure called heterochromatin, less accessible to transcription factors.

Histone methylation implies the addition of methyl groups ($-CH_3$) to specific lysine or arginine residues on histones and is a process mediated by methyltransferases (HMTs). Histone methylation can either activate or repress gene expression depending on the specific residue to which methyl groups are added. For instance, trimethylation of lysine 4 on histone H3 (H3K4me3) is believed to promote transcription, while trimethylation of lysine 27 on histone H3 (H3K27me3) has been mostly linked to transcriptional repression (Kouzarides T., 2007, Berger S.L., 2007).

Methylation marks act as binding sites for proteins that further influence gene expression through chromatin remodelling (Egger et al., 2004).

Histone methylation acts in conjunction with other post-translational modifications that involve the histone proteins, such as phosphorylation, ubiquitination, SUMOylation and ADP-ribosylation (Bannister A.J. and Kouzarides T, 2011). Most modifications are site-

specific and occur only on determined amino acid residues; for example, acetylation occurs exclusively on lysine residues whereas methylation can occur on lysine, arginine and glutamine residues (Zhang Y. et al., 2021).

Histone H3 K27 epigenetic modifications in human cancer

Oncogenesis is regarded as a multifactorial, dynamic process, driven by genetic, epigenetic and environmental factors (Lu Y. et al., 2020).

In recent decades, there has been increasing attention to the oncogenic potential of epigenetic alterations in human cancer, their impact on cancer biology, and the possibility of developing novel cancer treatments targeting these epigenetic modifications. (Lu Y. et al., 2020, Egger G. et al., 2004, Audia J.E. and Campbell R.M., 2016, Zaib S. et al., 2022).

Within the Histone superfamily, H3 histone family is involved in several physiological and pathological processes. In particular, the mono-di-trimethylation of lysin in position 28 (K27) of the N-terminal tail of H3 results in repression of gene transcription (Day C.A. et al 2022).

Methylation of H3 is mainly mediated by Enhancer of Zest Homolog (EZH2), a subunit of the Polycomb Repressive Complex 2 (PRC2) with methyl-transferase activity (Day C.A. et al 2022), whereas its demethylation is driven by demethylases KDM6A (UTX) and KDM6B (JMJD3) (Agger K. Et al 2007).

Alterations involving either EZH2 or the demethylases directly influence H3K27me3 levels. Inactivating mutations in Histone H3K27 demethylase *KDM6A/UTX* were detected in different cancer types, with the highest prevalence in multiple myeloma, myeloid leukaemias, in oesophageal squamous cell carcinomas renal clear cell carcinomas, breast and colorectal cancers and glioblastoma (van Haaften G. et al., 2009). Moreover, the inhibition of UTX enhanced radiosensitivity in breast, lung adenocarcinoma and glioblastoma cell lines (Rath B.H. et al., 2018).

EZH2 overexpression with subsequent global gene silencing, has been implicated in tumor progression and aggressiveness of several tumor entities (Qiu B.Q. et al., 2020;

Gan L. et al., 2018; Pellecchia S. et al., 2020; Krill L. et al., 2019; Kleer C.G. et al., 2003; Weikert S. et al., 2005).

In breast invasive carcinomas, higher levels of EZH2 mRNA were associated with the development of metastases within five years since the initial diagnosis (Kleer C.G. et al., 2003).

In bladder cancer, high EZH2 transcript levels were significantly correlated with higher tumor grade and invasiveness (Weikert S. et al., 2005). In prostate cancer, higher EZH2 mRNA and EZH2 protein levels were associated with metastatization and a poorer prognosis (Varambally S. et al., 2002).

Finally, gain-of-function somatic mutations of *EZH2* have been found in up to one third of diffuse large B cell lymphomas (McCabe MT et al., 2012).

In all cases, increased expression of EZH2 resulted in the increase of H3K27me3 levels in neoplastic cells.

Other tumors, including myeloid neoplasms, T cell lymphomas, or peripheral nerve sheath tumors feature EZH2 inactivation (Ernst T. et al., 2010, Ntziachristos P. et al., 2012, Prieto-Granada C.N. et al., 2016) which result in the loss of H3K27me3 (Schaefer I.M. et al., 2016a).

H3K27me3 loss has been reported in several CNS tumors (Angelico G. et al., 2024, Bender S. et al., 2013, Jain S. U. et al., 2019, Broniscer A. et al, 2017). In addition, several genetic and epigenetic alterations resulting in H3K27me3 loss were firstly identified in tumors of the CNS (Bender S. et al., 2013, Jain S. U. et al., 2019, Khuong- Quang D.A. et al, 2012).

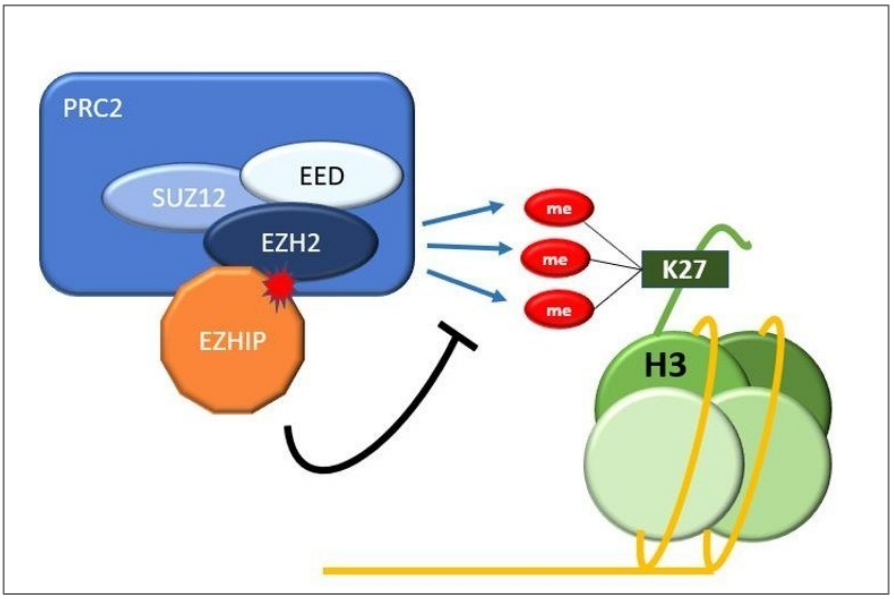
H3K27me3 expression can be determined using immunohistochemistry and monoclonal antibodies targeting the trimethylated form of the lysin residue.

H3K27me3 loss as a diagnostic marker

H3K27me3 immunohistochemical loss is currently used as a diagnostic marker, along with the presence of the K27M mutation in *H3F3A, HIST2H3C* or *HIST3H1B* genes (Lewis P.W. et al, 2013, Bender S. et al., 2013, Castel D. et al., 2015) or EZHIP over-expression, in diffuse midline glioma H3 K27-altered. This subset of pediatric-type diffuse gliomas arises in midline CNS anatomical structures and is characterized by aggressive clinical behaviour and dismal prognosis. The name "H3 K27-altered" refers to the evidence that these tumors are uniformly characterized by the loss of H3 K27me3 resulting from two main mechanisms:

1) a mutation in *H3F3A,* leading to the substitution of the lysin residue in position 28 with a methionine residue (c.83A>T p.K28M, K27M) and determining the binding of PRC2 to the K27M-mutant protein and the inhibition of its methylating activity with consequent global loss of H3K27me3 (Bender S. et al., 2013) (Figure 1); 2) the over- expression of EZHIP, (EZH Inhibitory Protein). This protein shows a small sequence that mimics the K27M mutation on H3 and acts as an allosteric inhibitor on EZH2 active site, preventing methyl group apposition ((Jain S.U. et al. 2019, Hübner J.M. et al., 2019).

The aberrant overexpression of EZHIP is responsible for H3 K27me3 loss in a subgroup of posterior fossa ependymomas, namely the group A (PFA) (Bayliss J. et al., 2016), characterized by higher prevalence in children and a worse prognosis (Pajtler K.W. et al., 2015, Witt H. et al., 2011) (Figure 1).



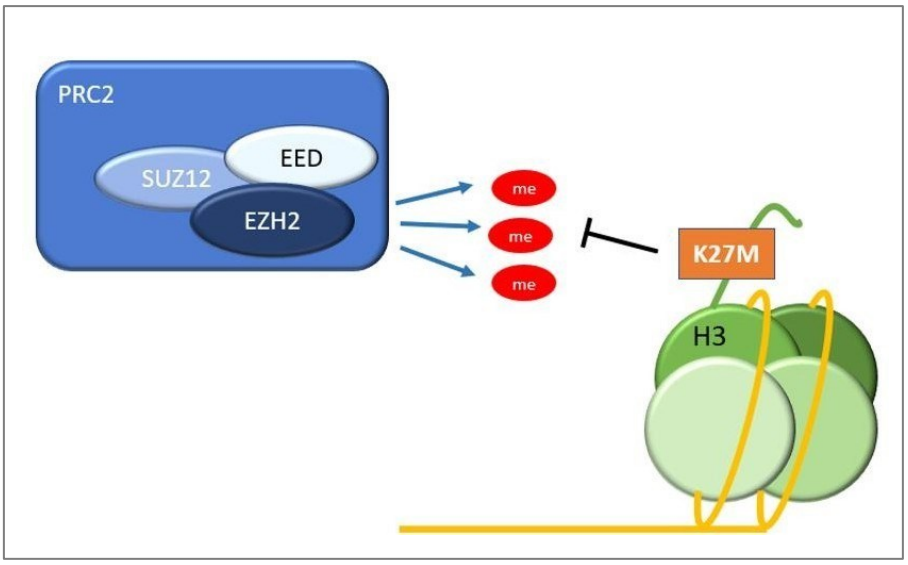


Figure 1. Mechanisms of H3K27 trimethylation loss in diffuse gliomas H3K27-altered. A: EZHIP competes as allosterical inhibitor of PRC2, preventing methilation of H3 K27. B: the mutant H3.3 K27M binds to PRC2 and entraps the complex with global loss of H3K27me3.

H3 K27me3 loss and *H3* K27M mutation were initially thought to be exclusive to diffuse midline gliomas (Wu G. et al., 2012, Khuong-Quang D.A. et al 2012, Lewis P.W. et al, 2013, Bender S. et al., 2013, Castel D. et al., 2015). However, these alterations were also reported in circumscribed gliomas and glioneuronal tumors, such as pilocytic astrocytomas (Orillac C. et al., 2016, Rodriguez F.J. et al., 2018, Pagès M. et al., 2017) ganglioglioma (Orillac C. et al., 2016; Rodriguez F.J. et al., 2018; Pagès M. et al., 2017), and glioneuronal tumors not further specified (Pratt D. et al., 2018). Nonetheless, whether these alterations bear a negative prognostic significance in these tumors remains unclear (Pratt D. et al., 2018).

The evidence that H3 K27me3 loss can be found in more indolent tumors has definitely changed the paradigm that H3K27me3 loss is linked with high biological aggressiveness in CNS tumors (Orillac C. et al., 2016).

Rosette-forming glioneuronal tumor (RGNT) shares the localization in midline CNS structures with diffuse midline gliomas (WHO Classification of Tumours Editorial Board, 2021). However, in contrast to the latter it is characterized by an indolent behavior and is classified as CNS WHO grade 1. Histologically, it is a biphasic tumor composed of rosettes or perivascular pseudorosettes of neurocytic cells and a glial component with pyloid or oligodendroglia-like features. Genetically, the hallmark of RGNTs is the co-occurrence of *FGFR1* mutation and either *PIK3CA* or *PIK3R1* mutations, and in a subset of cases, inactivation of *NF1* (Sievers P. et al., 2019). The differential diagnosis of RGNT *vs* diffuse midline glioma H3 K27-altered may be challenging in small biopsies where only a glial component is apparent. Moreover, whether RGNT may feature H3K27me3 immunohistochemical loss and/or f *H3* K27M is unknown.

H3K27me3 loss has been found also in CNS tumors outside midline anatomical structures, in the cerebral hemispheres (La Rocca G. et al., 2019, Onishi S. et al., 2022, Filipski K. et al., 2019).

Diffuse *IDH*-mutant gliomas include astrocytoma *IDH*-mutant and oligodendroglioma *IDH*-mutant and 1p/19q-codeleted (WHO Classification of Tumours Editorial Board, 2021). The latter is defined by the co-occurrence of an *IDH1* or *IDH2* mutation and the co-deletion of entire chromosomal arms 1p and 19q, caused by a balanced whole-arm

translocation of chromosomes 1 and 19, followed by the loss of one of the resulting chromosomes composed of 1p and 19q. Astrocytoma *IDH*-mutant is characterized by *IDH1/2* mutations, frequent *ATRX* and *TP53* mutations and the absence of 1p/19q codeletion (WHO Classification of Tumours Editorial Board, 2021). Compared to astrocytoma IDH-mutant, oligodendroglioma has a better prognosis and higher response to chemotherapy; therefore, its correct identification is essential for both prognostic and therapeutic purposes (van den Bent M.J. et al., 2013).

Because *ATRX* truncating mutations are mutually exclusive with 1p/19q codeletion, the immunohistochemical loss of ATRX is sufficient for the diagnosis of astrocytoma according to WHO criteria (WHO Classification of Tumours Editorial Board, 2021). Therefore, ATRX immunostaining represents a cost-effective marker to differentiate *IDH*-mutant astrocytomas from oligodendrogliomas (Ikemura M. et al., 2016). However, some *IDH*-mutant astrocytomas retain ATRX immunohistochemical expression (Barresi V. et al, 2020) or this immunostaining may be inconclusive (Filipski K. et al., 2019). In these cases, 1p/19q codeletion testing is fundamental for a correct classification.

Although fluorescent in situ hybridization (FISH) is the most widely used technique for the detection of 1p/19q codeletion, it features a limited specificity owing to its inability to differentiate between complete and partial deletions of 1p and 19q and to confirm 1p/19q co-deletion in case of imbalanced aneuploidy, polyploidy, or polysomy (Woherer A. et al., 2015). Other molecular techniques, such as PCR-based loss of heterozygosity (LOH) analysis, have greater specificity but are more labor-intensive and require a control sample from non-neoplastic tissue (Woherer A. et al., 2015). Therefore, the identification of surrogate markers for 1p/19q co-deletion could aid in the differential diagnosis of *IDH*-mutant diffuse gliomas in clinical practice.

Several studies investigated whether H3K27me3 expression could represent a diagnostic marker in this setting (Feller C. et al., 2020, Filipski K. et al., 2019, Kitahama K. et al., 2021).

Filipski et al. found that H3K27me3 was lost in 25 out of 26 *IDH*-mutant and 1p/19q codeleted oligodendrogliomas, while it was retained in 120 out of 135 astrocytomas (Filipski K. et al., 2019). Another study on 101 gliomas reported a significant

association between H3K27me3 loss and 1p/19q co-deletion, although 12,7% *IDH*-mutant and 1p/19q codeleted oligodendrogliomas retained H3 K27me3 expression (Pekmezci M. et al., 2020). Therefore, additional studies are necessary to assess whether H3K27me3 immunostain can be used as a surrogate for 1p/19q codeletion testing in routine practice.

H3K27me3 loss as a prognostic and predictive factor

In other CNS and extra-CNS tumors, H3K27me3 loss has been investigated as a prognostic and predictive factor.

It has been shown that meningiomas can be prognostically stratified according to their DNA methylation profiles (Olar A. et al., 2017, Sahm F. et al., 2017). In more detail, they can be subdivided into three benign, two intermediate (A and B) and one malignant methylation classes (Sahm F. et al., 2017) (Sahm F. et al., 2017, Sahm F et al., 2024). Notably, the intermediate B and malignant methylation classes were enriched in meningiomas with H3 K27me3 loss, suggesting that this can represent a negative prognostic marker (Katz L.M. et al., 2018). In accordance, H3 K27me3 loss is more frequent in high-grade meningiomas and in recurrent tumors and it is associated with a shorter time to recurrence after surgery (Behling F. et al., 2021, Katz L.M. et al., 2018, Nassiri F. et al., 2021) and/or shorter overall survival (Gauchotte G. et. al, 2020, Jung M. et al., 2021, Tosefsky K. et al, 2024).

However, the lack of uniformity in the immunostaining protocols in the different studies assessing the prognostic significance of H3 K27me3 in meningiomas prevents from drawing definitive conclusions (Sahm et al. 2024).

Whereas many studies were focused on the potential prognostic significance of H3 K27me3 loss in meningiomas, its value in predicting tumor response to adjuvant treatments is understudied, although there is some evidence that H3 K27me3 expression can influence tumor sensitivity to chemo- or radiotherapy. Indeed, high levels of H3K27me3 sensitize colorectal carcinoma cell lines to chemotherapeutic agents (Wang Q. et al., 2020) and in medulloblastoma the loss of H3K27me3 was associated with a radioresistant phenotype, with high relapse rates, and poor overall survival (Gabriel N.

et al., 2022). High levels of H3K27me3 and EZH2 have been correlated with longer overall survival and better prognosis in non-small cell lung cancer (Chen X. et al., 2013). On the contrary, Sun et al. (2020) found that in ovarian carcinoma a combination of EZH2low/H3K27Me3low status predicted a better response to chemotherapy and better progression-free survival (Sun S. et al., 2020). Similarly, a previous study found that in vitro EZH2 was overexpressed in cisplatin-resistant ovarian cancer cells compared to cisplatin-sensitive cells, and that, in vivo, loss of EZH2 enhanced the sensitivity of tumor xenografts to cisplatin (Hu S. et al., 2010).

Therefore, even in tumors outside the CNS, the assessment of H3 K27me3 expression can be used to stratify patients and predict their response to treatments.

Carcinomas located in the rectum represent about one third of all colorectal cancers and are treated according to their clinical Tumor Node Metastasis (cTNM) stage assessed by magnetic resonance imaging (MRI) or endoscopic rectal ultrasound (Glynne-Jones R. et al., 2018). Specifically, to enhance the chances of successful tumor resection and reduce postoperative recurrence risks, neo-adjuvant chemo-radiotherapy (CRT) is restricted to patients with locally advanced clinical stage (cT3/4 or N+) or those with imaging that indicates a threatened circumferential resection margin (Glynne-Jones R. et al., 2018). In patients affected by these tumors, a complete clinical response to neoadjuvant treatments is defined by the absence of any palpable mass during a digital rectal examination, no visible lesions during endoscopy, or no residual tumor detected at the primary site or draining lymph nodes on MRI or endoscopic ultrasound examination (Glynne-Jones R. et al., 2018). On the other hand, the pathological response is evaluated in surgical specimens using the pathological TNM stage (ypTNM) and applying a tumor regression grading (TRG) system, a histological grading system based on the ratio of remaining tumor to stromal fibrosis at the primary tumor site (Chetty R. et al., 2012, Mandard A.M. et al., 1994). For patients achieving a complete clinical response, a "watch-and-wait" strategy may be considered to avoid surgery, though, in many cases clinical and pathological responses do not align, determining the possibility of either over- or underestimate the response at imaging (Glynne-Jones R. Hughes R., 2012). Therefore, identifying additional predictors of pathological tumor response could

help select patients who could safely undergo a "watch-and-wait" approach, specifically finding features that could suggest which tumors respond better to neoadjuvant therapies.

Objectives

Based on this premise, this project aims to:

- Investigate H3K27me3 immunohistochemical expression and H3 mutations, and their eventual prognostic significance in RGNTs;
- Explore the diagnostic and prognostic utility of H3K27me3 immunohistochemical expression in hemispheric diffuse gliomas;
- Evaluate whether H3K27me3 immunohistochemical expression differs according to tumor grade in Oligodendrogliomas, IDH-mutant and 1p/19q- codeleted;
- Analyze the prognostic and predictive role of H3K27me3 expression in primary meningiomas that underwent surgery and subsequent stereotactic radiosurgery for residual or recurrent disease;
- Assess whether H3K27me3 expression can predict response to neo-adjuvant treatments and has prognostic significance in treatment naïve rectal adenocarcinomas.

Materials and Methods

All cases included in the project were retrieved from the files of the Pathology Section of Verona University Hospital.

All samples had been fixed in 10% neutral buffered formalin for 24 hours at room temperature and embedded in paraffin at 55°C.

Rosette forming glioneuronal tumors

Seven RGNTs from 6 female and 1 male patient (18–43 yo, median 29 yo) were included in this study. All tumors were located in midline structures and were described as circumscribed at preoperative imaging.

Extent of surgical resection, follow-up data on tumor recurrence and recurrence free survival, were also available.

Data on tumour localization, extent of surgical resection (gross total *vs* subtotal/partial resection) and development of recurrence were retrieved using clinical records.

Tumor recurrence was defined as the presence of tumour growth at the site of previous surgery, or an increase of tumour residue in case of subtotal or partial surgery, detected at either computed tomography (CT) or magnetic resonance (MR) imaging.

H3K27me3 stain was considered: i) lost when absent in > 95% neoplastic cells with retained expression in the positive internal controls; ii) retained, when present in > 95% of tumor cells; iii) mosaic, when the stain was present in a percentage between 5 and 95% of neoplastic cells.

Immunostains against Histone H3.3 K27M-mutant (polyclonal, Merck KGaA, Darmstadt, Germany; dilution 1:500), and EZHIP/CXorf67 (polyclonal, Merck KGaA, Darmstadt, dilution 1:75) were also performed. Both stainings were considered either as negative (absence of staining in neoplastic and non-neoplastic cells) or positive (presence of staining in neoplastic cells).

H3K27me3 immunonegative cases and/or and/or cases with H3.3 K27M- mutant immunohistochemical expression were further investigated using a next-

generation sequencing (NGS) panel targeting 523 cancer-relevant genes (TruSight Oncology 500, Illumina, San Diego, CA, USA).

DNA was extracted from FFPE tissue sections using Maxwell CSC instrument (Promega, Madison, USA) with Maxwell RSC DNA FFPE kit (Promega, Madison, USA); DNA concentrations were measured on a Qubit 2.0 Fluorometer (Thermo Fisher Scientific, Waltham, USA) using the Qubit dsDNA High Sensitivity. DNA libraries were prepared using the TSO500 Library Preparation Kit (Illumina, San Diego, CA, USA) and sequenced to a mean coverage depth of >500× for up to 500 cancer-related genes. NGS data were analyzed with Illumina TruSight Oncology 500 Local App v2.1 and variant report files were uploaded into the Pierian Clinical Genomics Workspace cloud (Pierian DX software CGW_V6.21.1).

Intracranial diffuse gliomas with oligodendroglial and oligoastrocytic morphology

Cases of diffuse gliomas showing either oligodendroglial or an admixture of oligodendroglial and astrocytic morphology were selected and included in the study. Data on tumour localization, extent of surgical resection (gross total *vs* subtotal/partial resection) and development of recurrence were retrieved using clinical records.

Tumor recurrence was defined as the presence of tumour growth at the site of previous surgery, or an increase of tumour residue in case of subtotal or partial surgery, detected at either computed tomography (CT) or magnetic resonance (MR) imaging.

H3K27me3 immunohistochemical expression was classified according to Peckmezci et al. (Pekmezci M. et al., 2020) as retained when present in ≥ 5% positive neoplastic cells; (ii) lost, when absent in > 95% neoplastic cells with its retention in the internal positive controls; and (iii) non-conclusive when absent in both control and neoplastic cells. Positive internal controls were represented by endothelial cells and non-neoplastic tissue. In a larger cohort composed only of WHO CNS grade 2 and grade 3 *IDH*-mutant and 1p/19q-codeleted oligodendrogliomas, immunohistochemical expression have also been assessed subdividing tumors in 4 groups according to the percentage of H3K27me3-positive neoplastic cells in one hotspot, defined as the tumor area with the highest percentage of positive neoplastic cells at 10X magnification. The cut-off values of each group were respectively: i) <25%; ii) between 25 and 50%; iii) between 50 and 75%; iv) >75% of positive neoplastic cells.

In addition, immunohistochemical stains with antibodies against IDH1-R132H (clone H09, Dianova, GmbH, Germany; dilution 1:200), P53 (clone DO-7, Leica Biosystems, Newcastle, UK; prediluted), ATRX (Polyclonal; Life Science Sigma, St Louis, MO, USA; dilution 1:750) were performed. IDH1-R132H expression was considered either positive (presence of staining in neoplastic elements) or negative (absence of staining); ATRX expression was considered (i) retained, when nuclear staining was observed in both normal and neoplastic cells; (ii) lost, when staining was absent in neoplastic cells and present in normal cells; and (iii) non-conclusive, when staining was absent in both normal and neoplastic cells. P53 expression was considered positive only in case of strong staining in at least 10% of neoplastic cells, otherwise negative (Takami H. et al.,

2015). All IDH1 R132-negative cases were additionally investigated for mutations in *IDH1* and *IDH2* using polymerase chain reaction (PCR) analysis.

Briefly, neoplastic cellularity was enriched to at least 70% by manual microdissection of 10 consecutive 4 μm sections. DNA was purified using the QIAamp DNA FFPE Tissue Kit (Qiagen) and qualified. IDH1 and IDH2 were amplified by PCR, and both strands were sequenced using the ABI PRISM 3500 Genetic Analyzer (Applied Biosystems). PCR conditions were: i) denaturation at 95 °C for 5 min; ii) 40 cycles at 95 °C/30 s, 58 °C/30 s, and 72 °C/30 s; and iii) elongation step at 72 °C/5 min. Primers used were IDH1-F CCATCACTGCAGTTGTAGGTT, IDH1-R GCAAAATCACATTATTGCCAAC, IDH2-F TGCAGTGGGACCACTATTATC, and IDH2-R GTGCCCAGGTCAGTGGAT.

1p/19q-codeletion was assessed using FISH analysis in all cases and, in cases showing imbalance of one of the chromosomal arms, PCR based loss of heterozygosity (LOH) analysis was additionally performed.

Intracranial meningiomas

We selected cases of primary non-syndromic, non-radiation-induced intracranial meningiomas from patients who subsequently underwent gamma knife stereotactic radiosurgery (SRS) after the primary tumor resection. Data on the extent of surgical resection, SRS treatment, and tumor progression after SRS were retrieved.

Tumor progression was defined as a 15% increase in the sum of the products of perpendicular diameters of lesions within the prior 6 months, or the development of a new lesion (Huang R.Y., et al., 2018).

Progression-free survival was defined as the length of survival since SRS treatment to the detection of tumor progression or the last follow-up.

H3K27me3 immunohistochemical expression was classified, as previously described, according to Peckmezci et al. (Pekmezci M. et al., 2020).

Rectal adenocarcinomas

Endoscopic biopsies of treatment naïve rectal adenocarcinomas of patients who underwent neoadjuvant chemo-radiotherapy and subsequent total mesorectal excision were included in the study.

Tumor localization in the upper, mid or lower rectal segment, the cranio-caudal extension of the tumor at diagnosis, tumor progression and progression-free survival were retrieved from clinical records.

Tumor progression was defined as the development of metastatic lesions. Progression free survival was defined as the time in months from the diagnosis to the identification of metastases at imaging. In all cases, a minimum follow-up time of 36 months was available. H3K27me3 immunohistochemical expression was classified, as previously described, according to Peckmezci et al. (Pekmezci M. et al., 2020).

Immunohistochemistry

Immunohistochemistry was performed on 4µm thick sections obtained from each FFPE block. At least one representative slide of the selected case has been stained with H3K27me3 antibody (clone C36B11, Cell Signaling Technology, Danvers, MA, USA), by means of an automated immunostainer (Leica Biosystems, Newcastle, UK).

The chosen antibody is a Rabbit IgG monoclonal antibody that detects endogenous levels of histone 3 (H3) only when tri-methylated on the lysin at position 28 (Lys27) of H3. The antibody does not cross-react with the non-methylated, mono-methylated or di-methylated lysin residue, as well as with mono-methylated, di-methylated or tri-methylated histone H3 at Lys4, Lys9, Lys36 or Histone H4 at Lys20 (https://www.cellsignal.com/products/primary-antibodies/tri-methyl-histone-h3-lys27-c36b11-rabbit-mab/9733?srsltid=AfmBOoqjmIlviZZ5Sw4-Rxnc4BbmNgbaOpSkXNlamGm-SqU-Ubqqh1Rh). In non-neoplastic tissue of female subjects, an eccentric intranuclear dot-like stain can be seen, representing the inactivated X chromosome, called Barr bodies (Schaefer I.M. et al., 2016b). The same nuclear dot can be also identified in neoplastic cells with immunohistochemical loss of H3K27me3 and should not be misinterpreted as H3K27me3 immunohistochemical retention (Filipski K. et al., 2019). For deparaffinization slides were immerged in xylene for 5 minutes and then rehydrated through incubation in ethanol for 5 minutes and then in graded ethanol solutions (100% ethanol for 10 minutes, and 95% ethanol for 10 minutes).

Antigen retrieval was obtained using Heat-Induced Epitope Retrieval (HIER) method: slides were submersed in a microwave in 1X citrate unmasking solution (pH 6.0) until boiling, followed by 10 minutes at a sub-boiling temperature (95°-98°C). After, slides were cooled on bench top for 30 minutes.

To inhibit endogenous peroxidase 3% hydrogen peroxide was used for 10 minutes. A blocking solution (TBST/5% Normal Goat Serum) was used for 1 hour at room temperature to reduce non-specific binding.

After, H3K27me3 antibody was diluted at 1:200 dilution and the sections incubated overnight at 4°C at room temperature and then rinsed slides in phosphate buffered saline (PBS) three times for 5 minutes each, to remove unbound primary antibody.

Diaminobenzidine (DAB) substrate was applied and incubate according to the manufacturer's instructions and ultimately, sections were counterstained with haematoxylin.

All cases included in the studies were revised by an expert pathologist; the histological diagnosis and, when appropriate, tumor grading, were reassessed according to the latest available update of the World Health Organization (WHO) guidelines.

Statistical analyses

The Chi-squared test was used to analyze the statistical correlations between H3K27me3 immuno-expression and other clinical-pathological variables.

The Kaplan–Meier method was used to assess either Recurrence-free survival or progression-free survival.

The Mantel-Cox log-rank test was used to assess the strength of association between progression- or recurrence-free survival and clinical pathological parameters.

A probability (p) value less than 0.05 was considered significant.

All statistical analyses were performed using MedCalc 12.1.4.0 statistical software (MedCalc Software, Mariakerke, Belgium).

Results

Rosette-forming glioneuronal tumors can show H3K27me3 immunohistochemical loss independently of a co-occurring H3K27M mutation

Of the 7 RGNTs analyzed (Table 1, Table 2), 3 displayed complete immunohistochemical loss of H3K27me3 in tumor cells and among these, one showed positive immunostaining for Histone H3.3 K27M-mutant (figure 2) and absence of EZHIP, whereas the other two cases were negative for both H3.3 K27M-mutant and EZHIP (figure 3).

The remaining four cases had heterogeneous H3K27me3 expression (figure 4), with the percentage of neoplastic stained cells ranging between 5 and 95% (mosaic pattern) and none of these was positive for H3.3 K27M-mutant or EZHIP.

The 3 H3K27me3-negative RGNTs showed mutations in *FGFR1* and concurrent mutations in *PIK3CA*. The case showing H3.3 K27M immunohistochemical expression also harbored the K27M mutation in *H3F3A* at NGS analysis. EZHIP immunohistochemical stain was absent in all cases and molecular analysis did not reveal any EZHIP mutations. During a follow-up period ranging from 5 to 134 months, none of the patients presented tumor recurrence nor, in cases of partial resection, an increase in the tumor residue. The RGNT with the K27M mutation in *H3F3A* remained stable in size over a 23-months follow-up time.

Table 1. Clinical and pathological features of the RGNTs analyzed

Case	Sex	Age	Site	Imaging	Resection	Macroscopic description	Recurrence (months)
1	F	38	Mesencephalon	Relatively circumscribed solid	Partial	Soft, well dermarcated, highly vascularized	No (23)
2	F	22	Pineal gland	Relatively circumscribed solid	Partial, endoscopic	Soft, well dermar-cated, highly vascularized	No (27)
3	F	29	Mesencephalon	Relatively circumscribed solid	Partial	Soft, well dermar-cated	No (134)
4	F	33	Pineal gland	Relatively circumscribed cystic-solid	Partial, endoscopic	Soft	No (6)
5	M	43	Mesencephalon	Relatively circumscribed cystic-solid	Partial	Soft, well dermarcated	No (103)
6	F	29	Hypothalamus	Relatively circumscribed solid	Biopsy	Soft	No (64)
7	F	18	Sylvian aqueduct	Relatively circumscribed cystic-solid	Gross total	Soft, well dermarcated	No (11)

Table 2. Mutations found in three RGNTs showing either H3K27me3 global loss and/or positive H3K27M staining. Case 1 showed positive H3.3 K27M IHC and H3F3A (K27M) at NGS analysis

Case	H3K27me3 IHC	H3.3 K27M IHC	EZHIP IHC	Mutations
1	Lost in >95% cells	Positive	Negative	FGFR1 (K638R; Y653C; K656Q); PIK3CA (E110del); H3F3A (K27M)
2	Lost in >95% cells	Negative	Negative	FGFR1 (N546K); PIK3CA (H1047L); NF1 (L2337R; E2339Dfs*; 2340P); Top2A(S1337L); BLM(V4A); ERBB3(G989V); NOTCH4(R1475S); FGF5(D106N); MSH2(N583I)
3	Lost in >95% cells	Negative	Negative	FGFR1 (N546K); PIK3CA(H1047L); NF1(W2317Gfs*2; T2621Lfs*3); PIK3R1 (Y452del; K575del); REKQL4 (L719H); CDKN1B (S7C); FGFR4 (M524I); PAX3 (K183del)

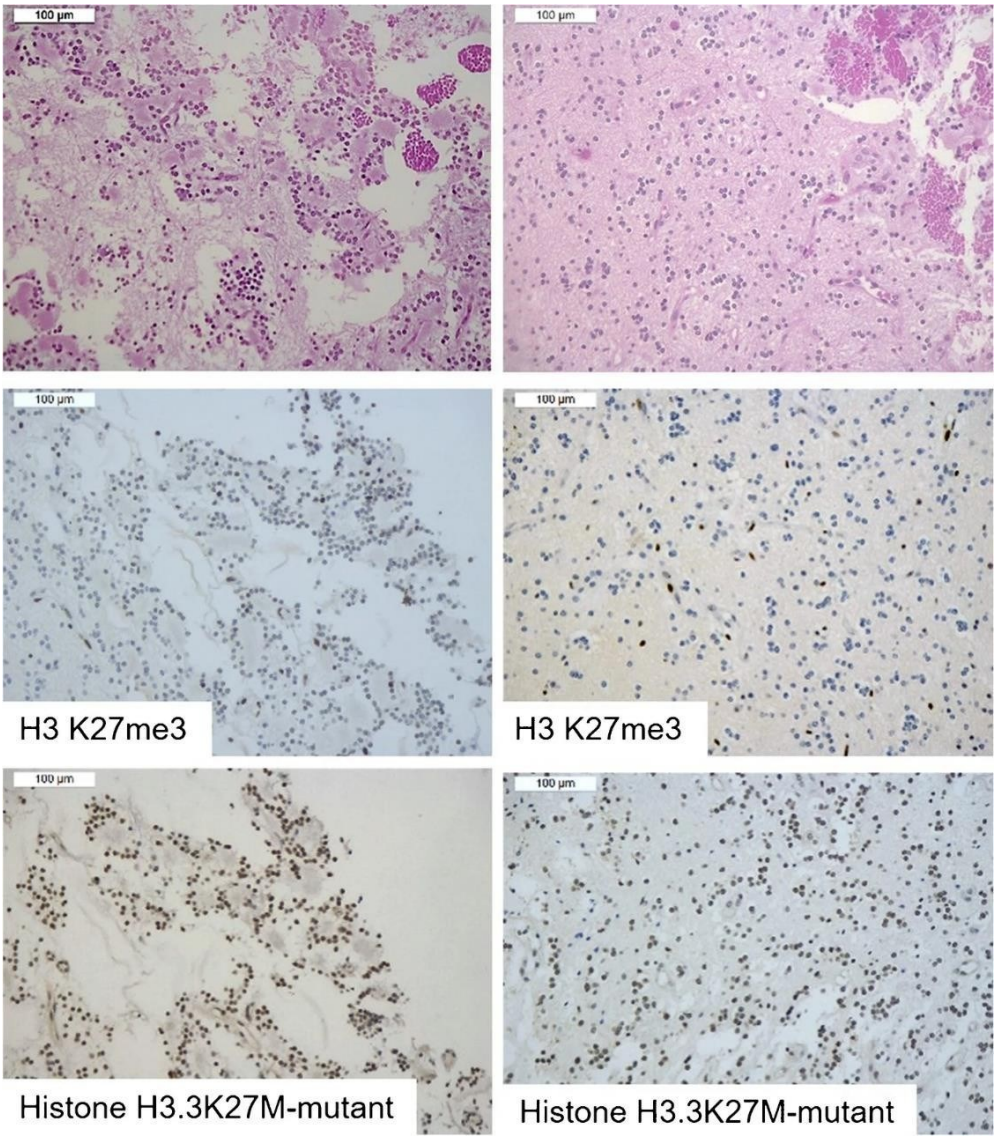


Figure 2. RGNT showing H3K27me3 loss in all neoplastic cells, coupled with Histone H3 K27M-mutant positivity. H3 K27me3 immunohistochemical expression was lost in both the rosette-forming (left) and glial (right) components. The glial component showed Histone H3.3 K27M-mutant immunostaining and were negative for EZHIP. Next-generation sequencing confirmed the presence of *H3.3* K27M mutation

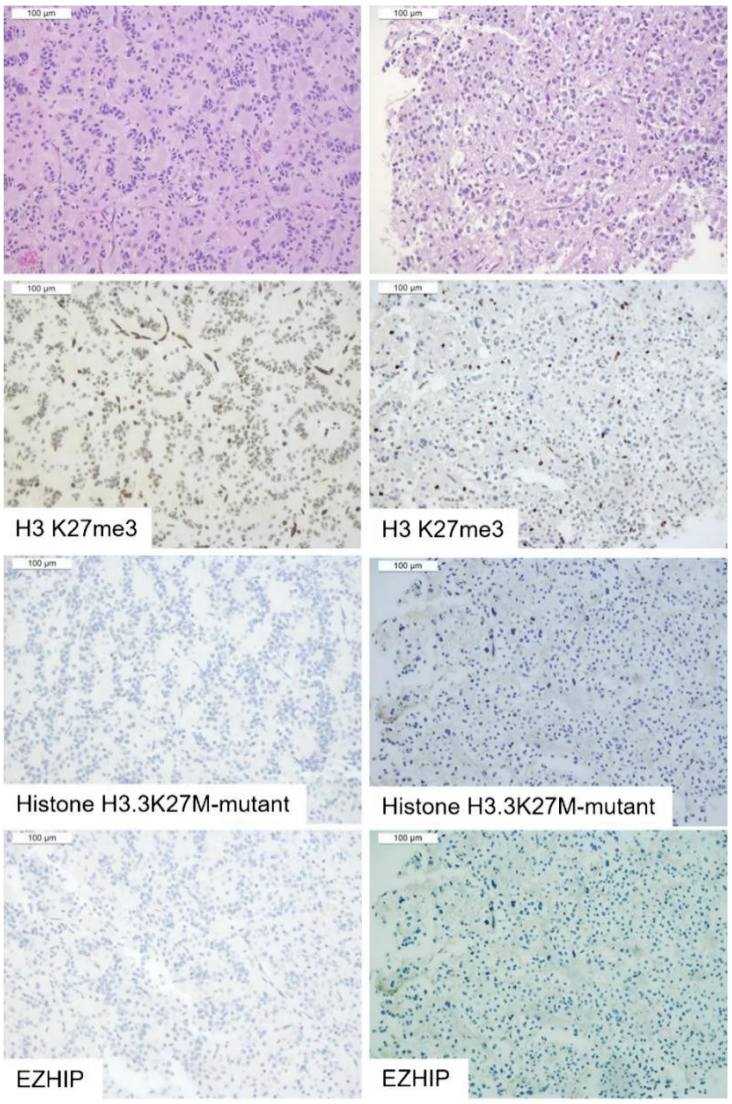


Figure 3: RGNT displaying the immunohistochemical loss of H3K27me3 in all cells and negative Histone H3 K27M-mutant or EZHIP stains. H3 K27me3 immunohistochemical expression was lost in both the rosette-forming (left) and glial (right) components. Both tumor components were negative for either Histone H3.3 K27M-mutant immunostaining or EZHIP.

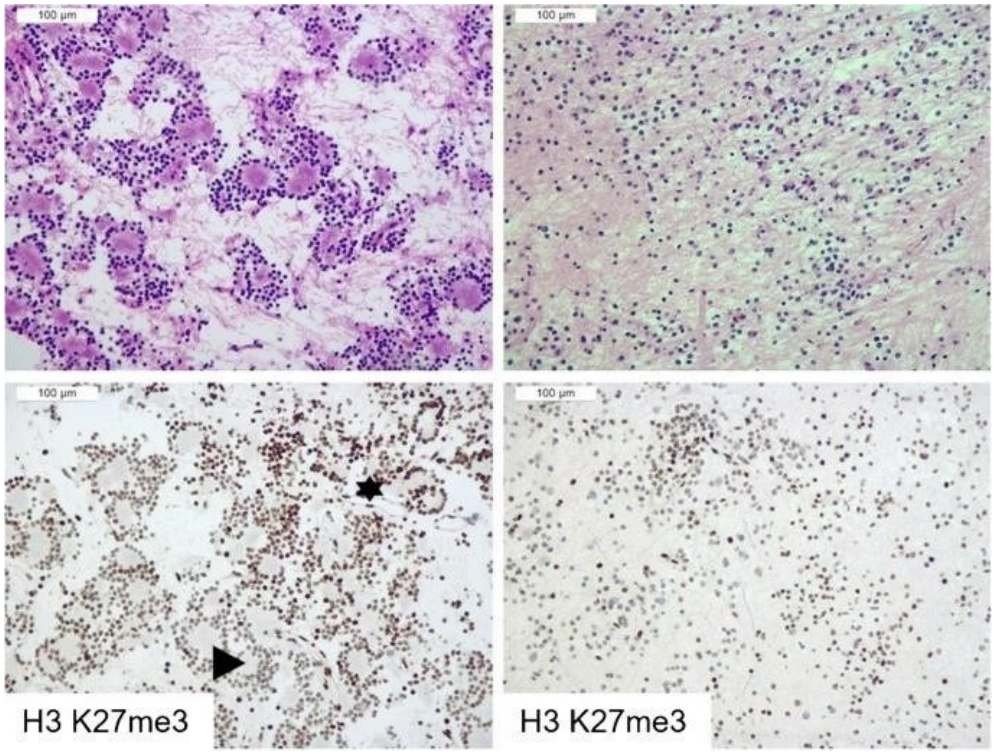


Figure 4: RGNT displaying mosaic immunostaining for H3 K27me3 (case 4) with alternating positive (star) and negative (triangle) areas in both rosette-forming (left) and glial (right) components

Loss of H3K27me3 immunohistochemical expression is significantly correlated to 1p/19q codeletion in IDH-mutant diffuse gliomas and bears prognostic significance

The results of the investigation are summarized in Figure 5.

At morphological evaluation on the Haematoxylin and eosin (H&E) stained glass slide, in 60 tumors the oligodendroglial morphology was predominant. Neoplastic cells showed small, round nuclei with coarsely dispersed chromatin and often a perinuclear halo, an artifact caused by formalin fixation (WHO Classification of Tumours Editorial Board, 2021). Vasculature had the characteristic "chicken wire" appearance and neuronal satellitosis, as well as perivascular and subpial aggregation of neoplastic cells were often present.

In the remaining 7 cases there was an admixture of oligodendroglial and astrocytic features within the tumor, the latter composed of glial elements with irregular and angulated nuclear contours, coarse chromatin and nuclear hyperchromasia (WHO Classification of Tumours Editorial Board, 2021).

After *IDH1/2* mutational analysis and 1p/19q codeletion status assessment, 60 cases were reclassified according to WHO classification in grade 2 (33/60) and grade 3 (27/60) oligodendrogliomas *IDH*-mutant and 1p/19q-codeleted; 3 cases were reclassified as glioblastoma *IDH*-wildtype, and the remaining 6 cases were classified as *IDH*-mutant astrocytomas.

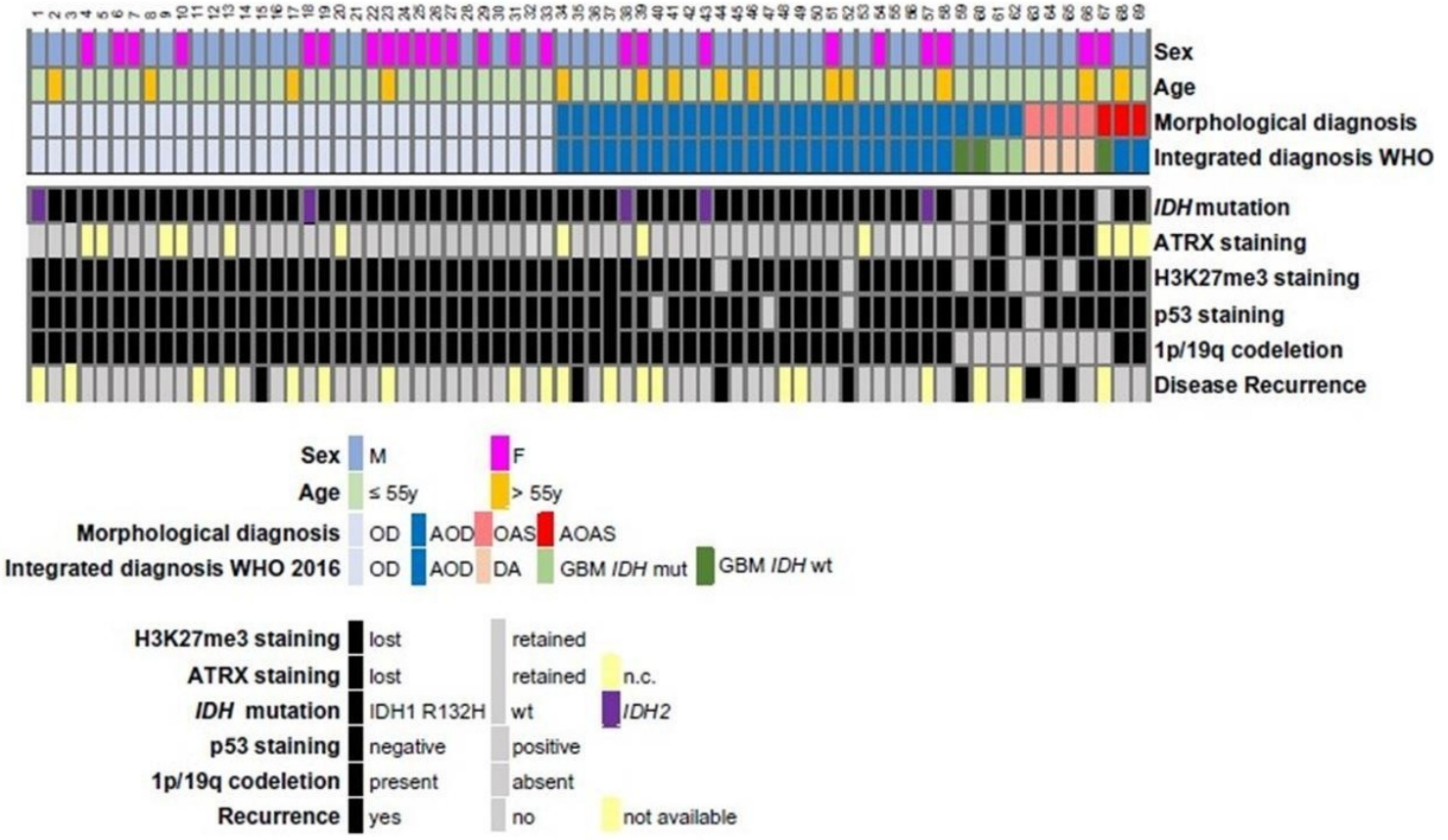


Figure 5. Clinical-pathological, immunohistochemical, and molecular features of 69 diffuse gliomas with oligodendroglial or mixed oligoastrocytic morphology.

M, male; F female; OD, oligodendroglioma; AOD, anaplastic oligodendroglioma; OAS, oligoastrocytoma; AOAS, anaplastic oligoastrocytoma; DA, diffuse astrocytoma; GBM, glioblastoma; Wt, wild type; N.c., non-conclusive

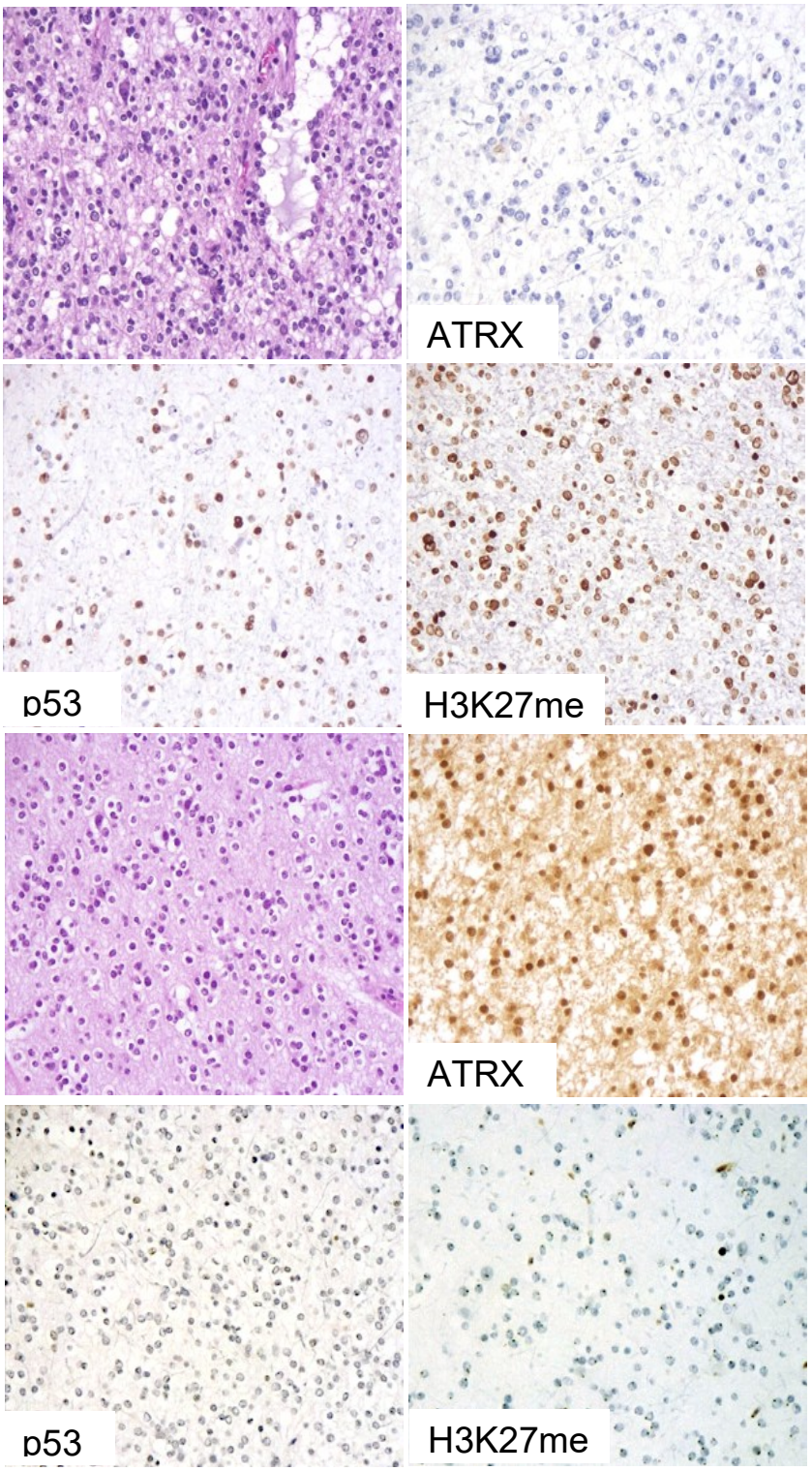
H3K27me3 immunohistochemical loss was found in 58/60 IDH-mutant and 1p/19q codeleted oligodendrogliomas, and in 4/9 astrocytic tumors. The staining was retained in the remaining 2/60 oligodendrogliomas and 5/9 astrocytic tumors (Figure 6).

H3K27me3 loss was significantly associated with the retention of ATRX immuno-expression ($P = 0.025$), presence of 1p/19q codeletion ($P = 0.0001$), and P53 expression in <10% of neoplastic cells ($P = 0.0027$).

In IDH-mutant tumors, an immunohistochemical profile consisting in H3K27me3-, ATRX+ and p53- was diagnostic of oligodendroglioma in 100% cases.

Moreover, the loss of H3K27me3 predicted a longer recurrence free survival at survival analysis ($p = 0.005$) (Figure 7).

Diffuse Astrocytoma IDH-mutant



Oligodendroglioma IDH mutant 1p/19q codeleted

Figure 6. H3K27me3 immunohistochemical expression in one diffuse astrocytoma, IDH-mutant and one oligodendroglioma, IDH-mutant and 1p/19q codeleted form the study

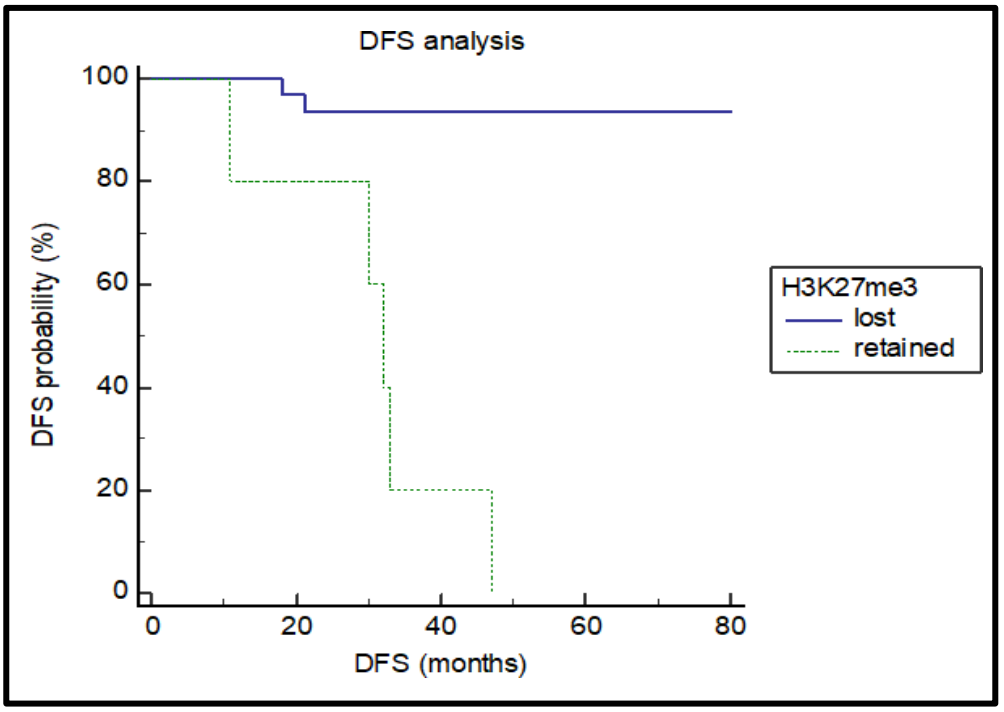


Figure 7. Recurrence-free survival (RFS) analysis of 55 patients with diffuse gliomas, according to H3K27me3 expression. The RFS of patients with a tumour showing H3K27me3 retention was significantly shorter than that of patients harbouring a tumour with H3K27me3 loss ($P < 0.0001$)

H3K27me3 expression is not correlated to high-grade features in Oligodendrogliomas, IDH-mutant and 1p/19q-codeleted

We further explored whether high-grade features correlated with increased H3K27me3 expression in a larger cohort comprising only Oligodendrogliomas, IDH-mutant and 1p/19q codeleted tumors, composed of 41 WHO grade 2 and 36 WHO grade 3 tumors.
Global H3K27me3 loss, intended as < 5% positive neoplastic cells, was seen in 74/77 tumors considering H3K27me3 expression in all neoplastic cells in one glass slide (Pekmezci M. et al., 2020). Instead, using the 4-tiered scoring system (figure 8), grade 2 tumors were classified as having <25% H3K27me3 positive neoplastic cells in 28 cases, 25-50% in 8 cases, 50-75% in 6 cases and >75% in 5 cases; grade 3 tumors showed H3K27me3 retention in <25% neoplastic cells in 17 cases, 25-50% in 10 cases, 50-75% in 5 cases and >75% in 4 cases. No significant correlation between any percentage of H3K27me3 positive cells in the hotspot and tumor grade was found (Table 3).
H3K27me3 retention in any percentage of neoplastic cells did not significantly correlate with tumor progression, ki67 labelling index or the presence of tumor recurrence. Recurrence-free survival data were available in 62 cases and at survival analysis, there weren't significant differences in RFS among groups (figure 9).

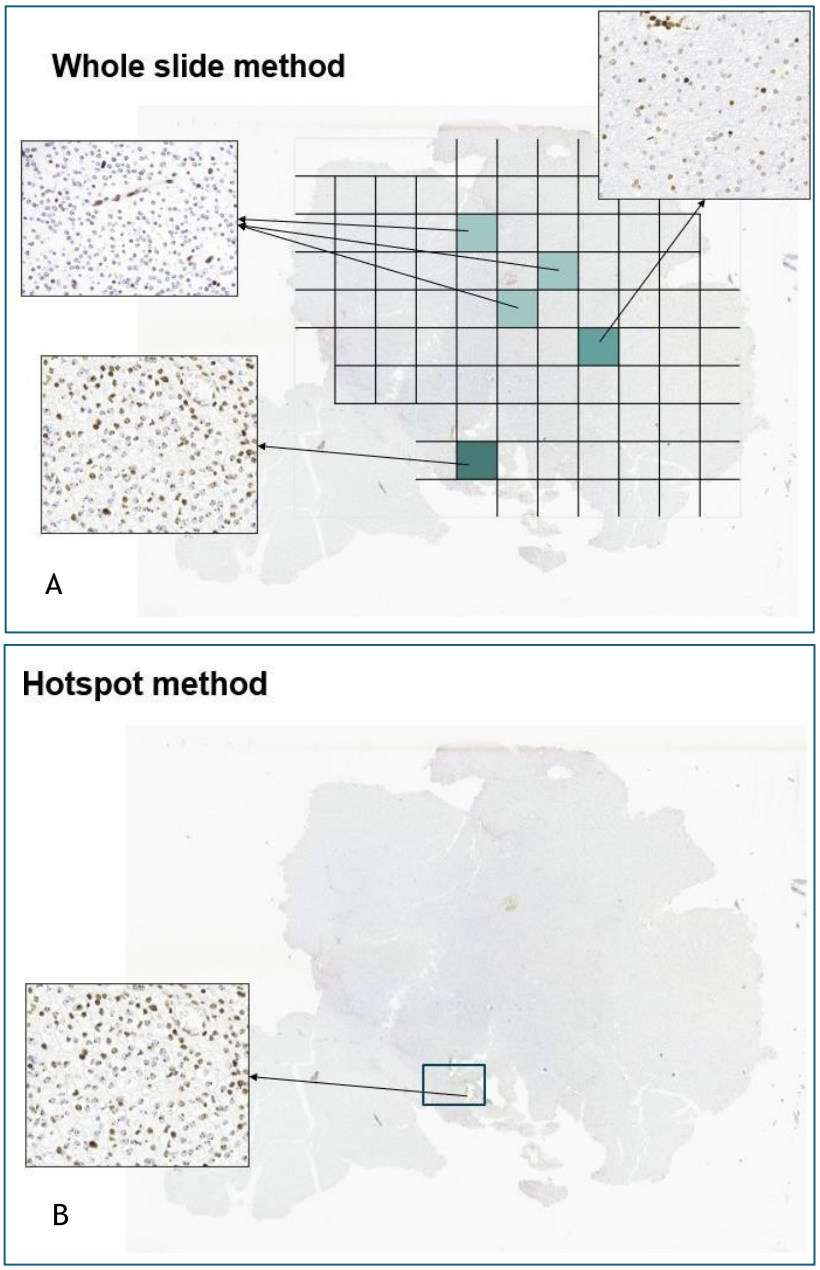


Figure 8. Evaluation methods of H3K27me3 immunohistochemistry in oligodendroglioma, IHD-mutant and 1p/19q-codeleted. Seventy-seven tumors were evaluated considering all tumor cells in 1 tumor slide (whole slide method) and classified according to Pekmezci et al. (A) and then divided in 4 groups according to the percentage of H3K27me3-positive neoplastic cells in one hotspot (<25%, 25-50%,50- 75%,>75%) (B).

Table 3. Seventy-seven oligodendrogliomas divided in 4 groups according to the percentage of neoplastic cells showing H3K27me3 retention in one hotspot at 10X.

H3K27me3+ neoplastic cells (%)	**Oligodendroglioma**	
	Grade 2	Grade 3
<25	22	17
25-49	8	10
50-75	6	5
>75	5	4

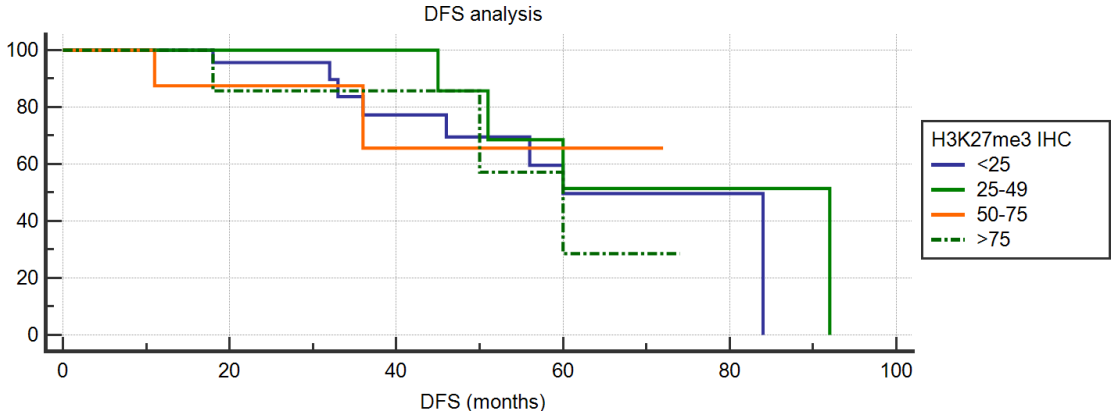


Figure 9. Survival analysis of 62 Oligodendrogliomas, IDH-mutant and 1p/19q codeleted subdivided in groups according to the % of neoplastic cells retaining H3K27me3 expression in 1 hotspot. Disease free survival analysis did not show significant differences among the four groups.

Loss of H3K27me3 is correlated to a shorter progression-free survival after SRS in meningiomas and can be influenced by adjuvant therapies

Among thirty-nine primary intracranial meningioma (6 WHO grade 1 and 33 WHO grade 2) included in the analysis (Figure 10), 7 tumors (6 WHO grade 2 and 1 WHO grade 1 tumors), were H3K27me3 immunonegative. In five cases H3K27me3 staining was non-conclusive. Tumor progression after SRS was detected in a significantly higher proportion among H3K27me3 immunonegative meningiomas (6/7), compared to tumors with retained H3K27me3 stain (9/32) ($p = 0.0143$) (Table 4).

PFS after stereotactic radiosurgery was significantly shorter in cases with H3K27me3 loss compared to those with retained H3K27me3 ($p = 0.0036$) (Table 5). Though, at multivariate analysis, H3K27me3 did not retain its significance as an independent prognostic factor.

Progression-free survival after SRS was significantly shorter in patients with meningiomas with loss of H3K27me3 immunohistochemical expression (figure 12).

In four cases of secondary WHO grade 3 anaplastic meningiomas, the primary untreated tumor and their recurrence before tumor progression to grade 3 were available for immunohistochemical testing. Among the primary four tumors only 1 of 4 showed H3K27me3 loss, while at the second and third surgery respectively in 4/4 and 2/2 recurrent meningiomas H3K27me3 was lost at immunohistochemistry. Indeed, all four secondary WHO CNS grade 3 anaplastic meningiomas had H3K27me3 loss after therapy, though only one, having the worst clinical course, was H3K27me3 negative in the corresponding primary (figure 13).

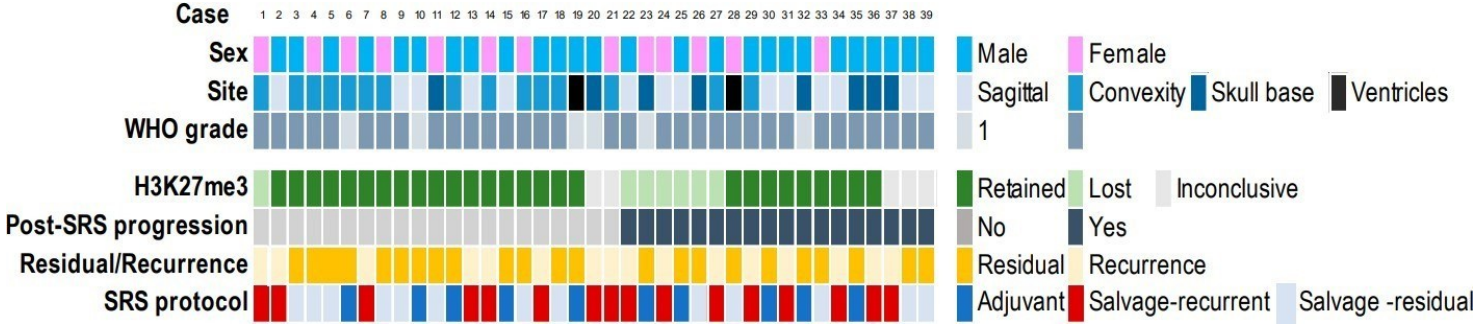


Figure 10. Clinical–pathological features and H3K27me3 immunoexpression of 39 intracranial meningiomas treated with surgery and subsequent stereotactic radiosurgery (SRS).

Table 4. Statistical correlation between H3K27me3 immuno-expression and clinical- pathological features of 34 intra-cranial meningiomas with evaluable H3K27me3 immunostainining

Parameter	H3K27me3 immuno-expression		*P*
	Lost	Retained	
Sex			
Male	3	4	
Female	4	8	0.1811
Age			
<65	3	12	
≥65	4	15	0.9408
Site			
Sagittal	2	12	
Convexity	3	9	
Skull base	2	4	
intraventricular	0	2	0.6599
WHO grade			
1	1	4	
2	6	23	0.9723
Post-SRS tumor progression			
Absent	1	18	
Present	6	9	0.0143
SRS protocol			
Adjuvant	2	8	
Salvage-residual	1	10	
Salvage-recurrent	4	9	0.4241
Treated tumor			
Residue	3	18	
Recurrence	4	9	0.2551

SRS: stereotactic radiosurgery

Table 5. Univariate analyses for progression-free survival after SRS in 39 patients with intracranial meningiomas

Parameter	H.R. (95% C.I.)	*P*
Sex		
Male	1	
Female	0.5 (0.2-1.4)	0.1928
Age		
<65 years	1	
≥65 years	0.9 (0.3- 2.3)	0.8401
Site		
Convexity	1	
Sagittal	7.3 (2.4-21.8)	
Skull base	6.3 (1.9-21)	
Intraventricular	8.4 (0.5-145.2)	0.023
WHO grade		
1	1	
2	1.4 (0.4-5.1)	0,839
H3K27me3		
Retained	1	
Lost	8.9 (2-38.6)	0.036
Treated tumor		
Residue	0.6 (0.2-1.6)	
Recurrence	1	0.2828
SRS protocol		
Adjuvant	1.8 (0.5-5.9)	
Salvage-residual	1	
Salvage-recurrent	2.1 (0.7-6.2)	0.3752

H.R.: hazard ratio. C.I.: confidence interval

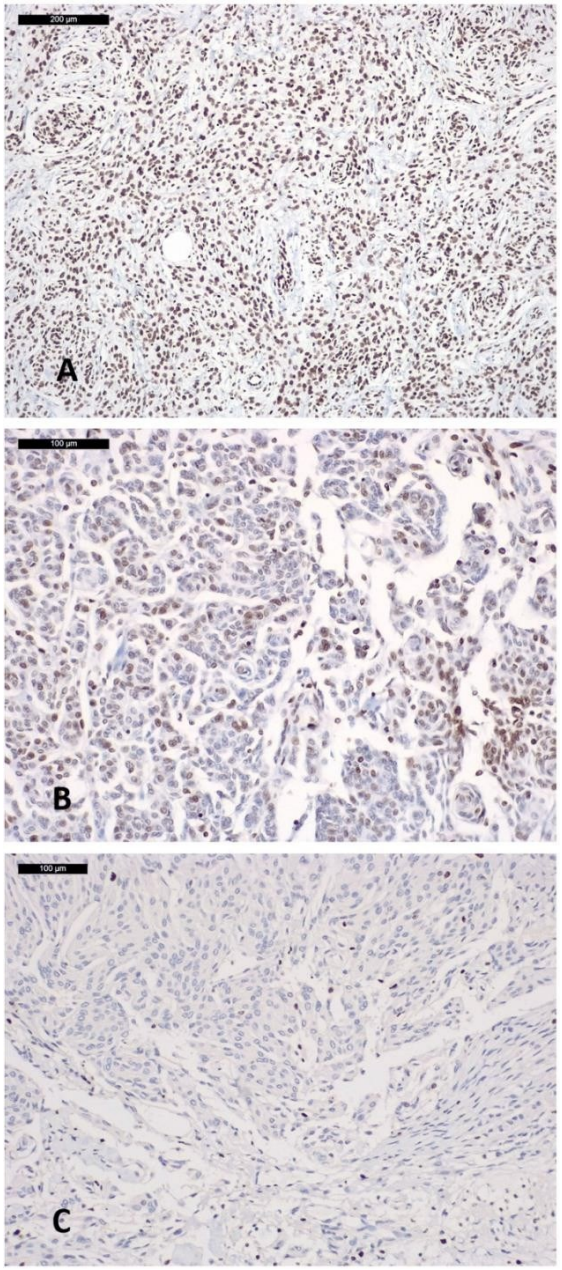


Figure 11. H3K27me3 Immunostaining in meningiomas. (A) meningioma classified H3K27me3 positive, with nuclear staining retained in all the neoplastic cells; (B) meningioma with retained H3K27me3 immunoexpression in 20% neoplastic cells; (C) H3K27me3 loss in >95% of neoplastic cells

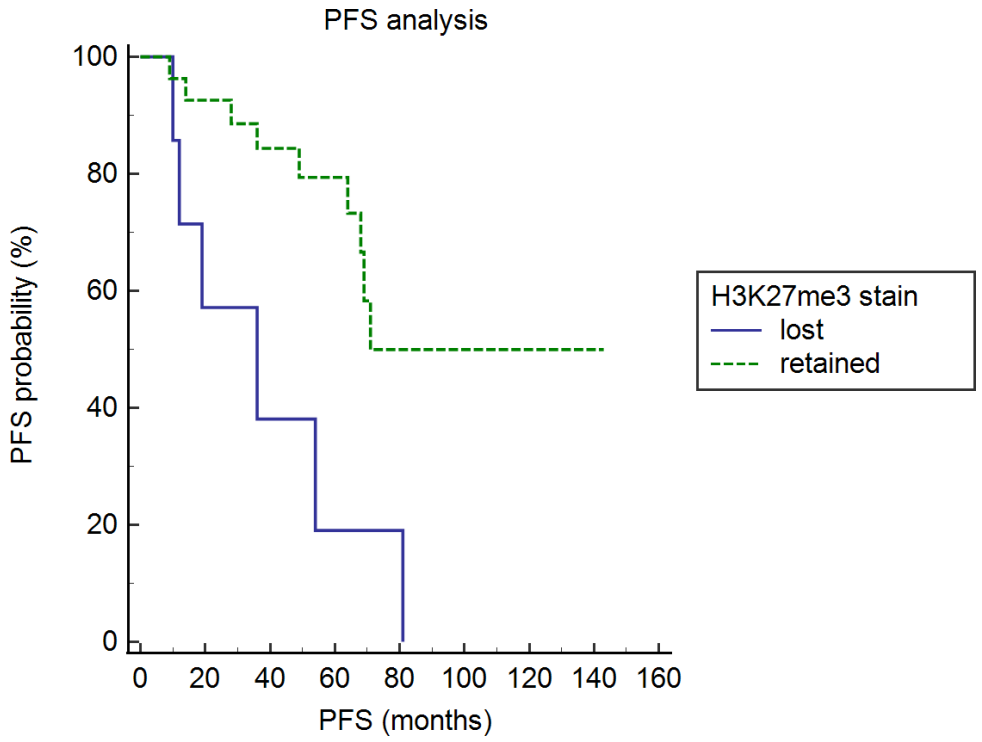


Figure 12. PFS analysis according to H3K27me3 immunoexpression in a cohort of 34 intracranial meningiomas. PFS after SRS is significantly shorter in patients with a meningioma with H3K27me3 loss compared to those harboring a meningioma with H3K27me3 retention (p = 0.0036)

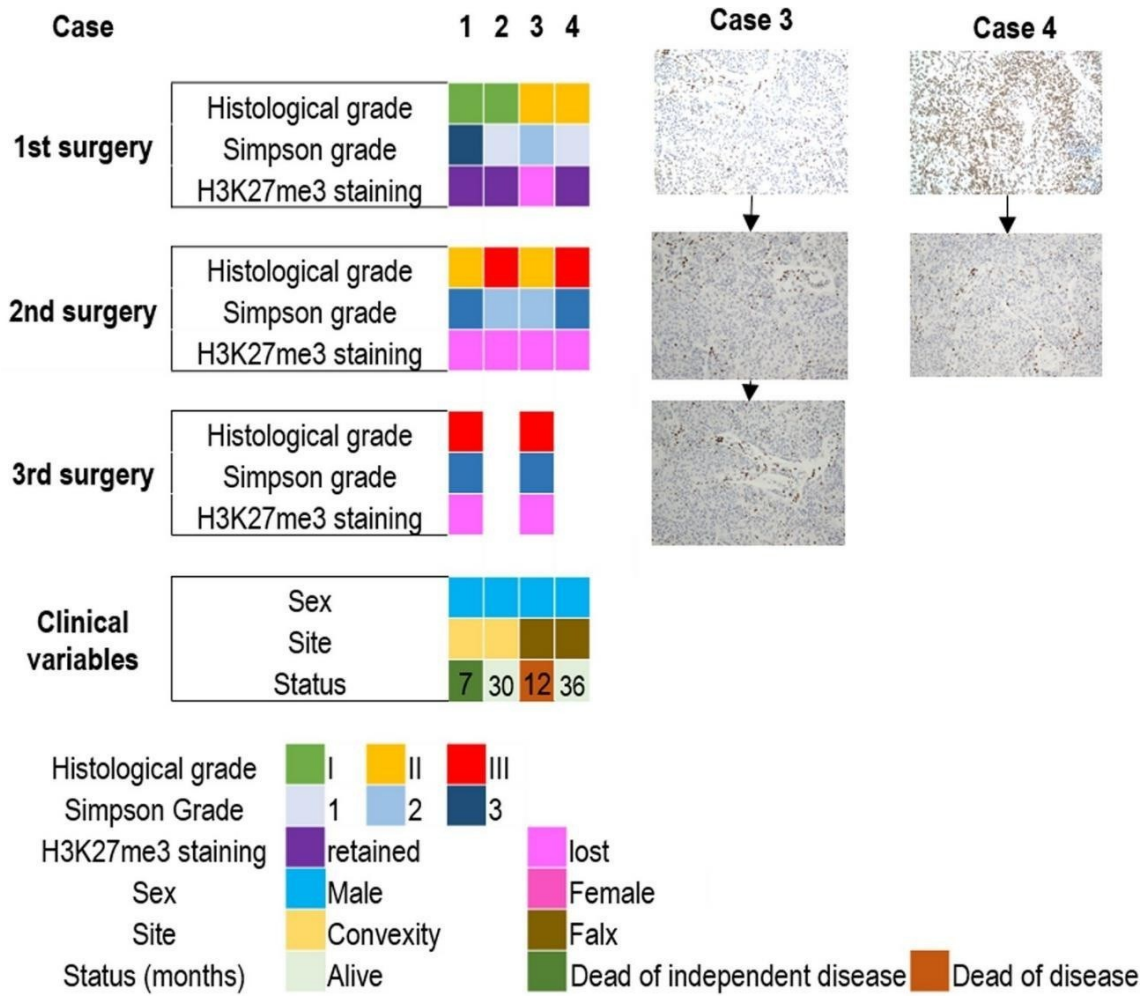


Figure 13. Clinical–pathological features and H3K27me3 immuno-expression in four secondary anaplastic meningiomas and their paired primary tumors. All anaplastic meningiomas showed loss of H3K27me3 immunostaining. In three cases, the corresponding primary tumor showed H3K27me3 retention, which was lost at recurrence; only one primary tumor (case 3), showed H3K27me3 loss. The latter had the worst having the worst prognosis of all cases

Loss of H3K27me3 in treatment-naïve rectal carcinoma is correlated to complete pathological response to neoadjuvant therapies and to higher tumor regression

Clinical pathological features of the 46 biopsies of rectal adenocarcinomas analysed are summarized in Figure 14. H3K27me3 expression (figure 15) was considered lost in 20 cases, retained in 19 cases, and inconclusive, due to lack of positive internal control, in 7 cases.

The immunohistochemical retention of H3K27me3 in the tumor cells of the pre- treatment endoscopic biopsy was significantly associated with a complete pathological response to chemo-radiotherapy (ypTNM 0) (p= 0.0111) and correlated with higher tumor regression histologically assessed in the surgical specimen measured using the 4- tiered (P=0,042) or the 2-tiered (p= 0,0009) Dworak tumor regression grading system ((Dworak O. et al.,1997) (Table 6).

Cases with retained H3K27me3 expression showed less frequently tumor progression, but the correlation did not reach statistical significance.

At univariate analysis for progression-free survival loss of H3K27me3 did not reach statistical significance and at multivariate analysis the only independent factors were the presence of >9 foci of poorly cohesive cells (p = 0.0023) and the involvement of radial margin (p = 0.0001).

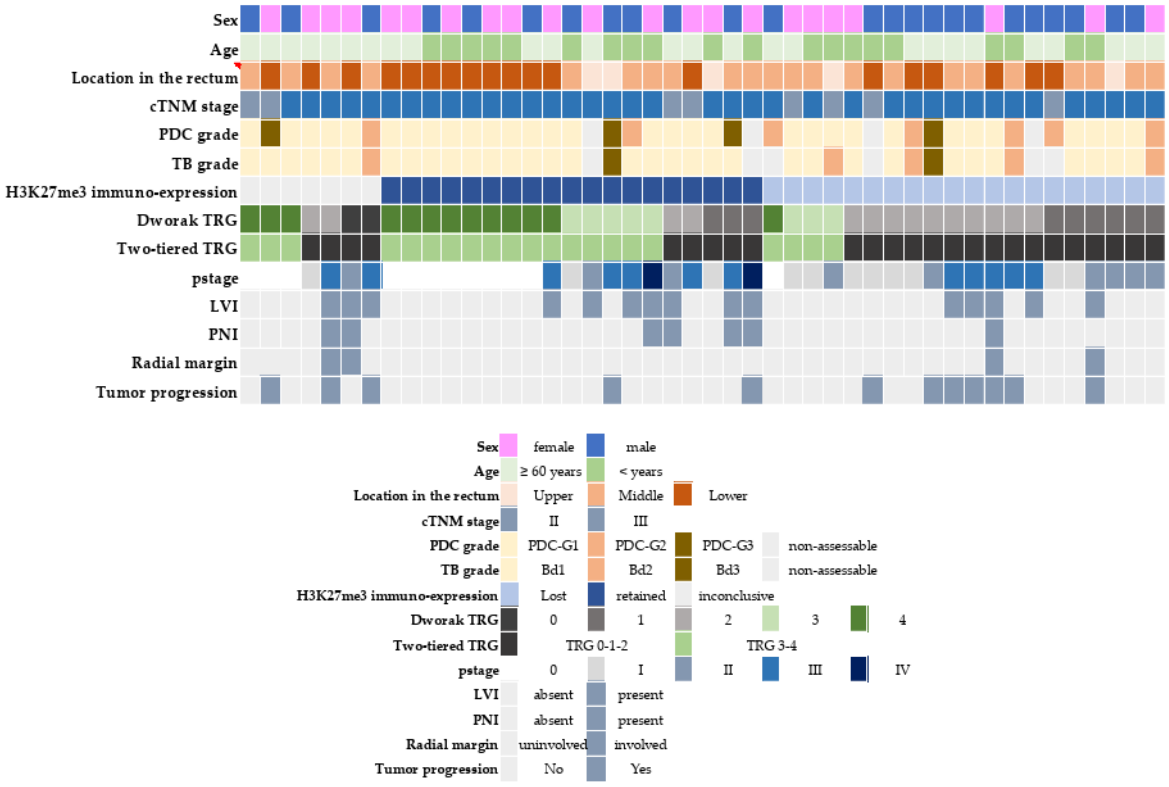


Figure 14. Clinical-pathological features of 46 rectal carcinomas treated with neo-adjuvant chemoradiotherapy and surgery. The loss of H3K27me3 immuno-expression in the pre-treatment endoscopic biopsy was significantly associated with lower Dworak tumor regression grade (TRG).

PDC: poorly differentiated clusters. TB: tumor budding. LVI: lympho-vascular invasion. PNI: peri-neural invasion.

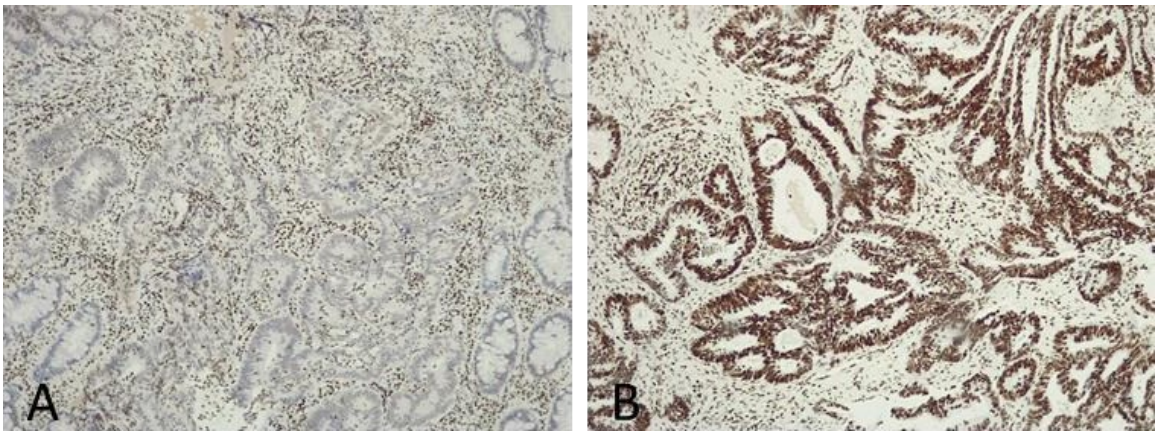


Figure 15. H3K27me3 immuno-expression in the pre-treatment biopsies of two rectal carcinomas. A) Rectal carcinoma with loss of H3 K27me3 immunostaining in the neoplastic cells. Inflammatory cells, which served as internal positive control, showed nuclear immunostaining. B) Rectal carcinoma with retained H3 K27me3 immunostaining.

Table 6. Statistical correlations between H3K27me3 immunoexpression and clinical-pathological features of 39 rectal adenocarcinomas treated with neoadjuvant chemo-radiotherapy and surgery.

Parameter	H3K27me3 immunoexpression		p
	Lost	Retained	
Tumor Localization			
Lower	6	10	
Middle	12	6	
Upper	2	3	0,204
cTNM stage			
II	4	2	
III	16	17	0,418
TB grade			
Bd1	11	16	
Bd2	4	0	
Bd3	1	1	0,086
PDC grade			
PDC G1	12	14	
PDC G2	5	1	
PDC G3	1	2	0,209
yp stage			
0	1	8	
I	8	2	
II	6	2	

Parameter	H3K27me3 immunoexpression		p
	Lost	Retained	
III	5	5	
IV	0	2	0,0111
Lymphovascular invasion			
Absent	15	12	
Present	5	7	0,429
Perineural invasion			
Absent	19	15	
Present	1	4	0,139
Dworak TRG			
0	0	0	
1	6	3	
2	10	2	
3	3	5	
4	1	9	0,0042
Two-tiered Dworak TRG			
TRG 0-1-2	16	5	
TRG 3-4	4	14	0,0009
Tumor progression			
No	13	17	
Yes	7	2	0,0735

Discussion

Currently, H3K27me3 immunohistochemical expression is assessed in diffuse midline gliomas, H3K27me3-altered and for prognostic subgrouping in posterior fossa ependymomas (WHO Classification of Tumors Editorial Board, 2021). The current edition of the WHO classification of brain tumors and the c-IMPACT-NOW consortium do not currently recommend its testing in other CNS tumor types, due to lack of unequivocal evidence of either a diagnostic, prognostic or predictive significance of this mark in other CNS entities (Sahm F. et al. 2024).

Nonetheless, our investigations contributed to enriching the current knowledge on the impact of H3K27 trimethylation status in different tumor entities, providing evidence of a diagnostic and/or prognostic and predictive role of H3K27m3 immunohistochemical expression in IDH-mutant diffuse gliomas, meningiomas, and rectal carcinomas.

We also contributed to increasing the spectrum of midline CNS neoplasms that can harbor the H3 K27M mutation and H3K27me3 loss, as shown in a cohort of rosette- forming glioneuronal tumors.

All the seven RGNTs analyzed showed either partial or global loss of H3K27me3 expression in tumor cells, demonstrating that H3K27me3 and/or *H3F3A* K327M mutation can be found also in these tumors (Marastoni E. et al, 2023), in line with the several reports of these alterations in different low grade tumor types (Orillac C. et al., 2016, Rodriguez F.J. et al., 2019 Pagès M. et al., 2018).

These results underscore that H3K27me3 loss and *H3* K27M mutations are not exclusive to aggressive tumor entities in CNS. They also caution pathologists against diagnosing diffuse midline glioma-H3K27 altered in tumors with *H3* K27M mutation that show non-infiltrative growth, and a non-midline location (Louis D.N. et al., 2018).

This new knowledge is of paramount significance in clinical settings, since extended neurosurgical resection, the standard of care for midline low grade gliomas/glioneuronal tumors, cannot always be performed due to the risks of complications and late effects,

especially in tumors located in midline anatomical structures (Weiß et al., 2023). In such cases, neurosurgery is performed mainly for diagnostic purposes.

In the latter scenario, the presence of H3K27me3 immunohistochemical loss and *H3* K27M-mutant expression evaluated on limited amounts of tumour tissue from biopsy material, could lead to a dangerous misclassification of lower grade entities, generally showing an indolent course, as highly aggressive WHO grade 4 gliomas. Corroborating the c-IMPACT-NOW suggestions (Louis D.N. et al., 2018), in our experience, *H3* K27M mutant and H3K27me3 immunonegative RGNTs did not exhibit a more aggressive biological behavior than *H3.3* wild-type tumors in a follow-up period ranging from 5 to 134 months, with a median follow up of 27 months.

As well, our results on hemispheric diffuse gliomas with oligodendroglial and oligoastrocytic morphology show that H3K27me3 immunohistochemical loss is correlated with better prognosis and confirms the diagnostic utility of this marker in the differential diagnosis of *IDH*-mutant gliomas.

A relationship between H3K27me3 loss and the presence of 1p/19q codeletion in gliomas has already been proposed by several studies (Filipski K. et al., 2019, Feller C et al. 2020, Kitahama K. et al., 2021), despite being questioned by other Authors (Pekmezci M. et al., 2020). While the former found a significant association between these two alterations, the latter found that H3K27me3 staining was retained in 25% of tested oligodendrogliomas and conversely lost in 27% of *IDH*-mutant astrocytomas, concluding that retained expression of the antibody should not be regarded as a marker of astrocytic lineage in *IDH*-mutant tumors, especially if morphological and immunohistochemical features are suggestive of oligodendroglioma (Pekmezci M. et al., 2020).

Our data, in line with those presented by Filipski et al. (Filipski K. et al., 2019), show a significant association between H3K27me3 loss and the presence of 1p/19q codeletion.

Filipski et al., though, found that diffuse gliomas with H3K27me3 loss, retention, or non-conclusive nuclear ATRX and IDH1R132H mutation are 1p/19q-codeleted oligodendrogliomas with a very high predicted probability of 0.9678, whereas

H3K27me3 retention is highly predictive of astrocytic lineage, with a predicted probability of 0.9995.

In our cohort, if we had categorized tumors as astrocytic based on the retention of H3K27me3, without testing for 1p/19q codeletion, two oligodendrogliomas that exhibited both *IDH* mutations and 1p/19q codeletion would have been incorrectly identified as *IDH*-mutant astrocytomas.

To overcome the possibility of misclassification, in *IDH1/2*-mutant gliomas we proposed an immunohistochemistry-based algorithm that relies on the assessment of ATRX, and H3K27me3 immunoexpression that may significantly reduce the need for 1p/19q codeletion assessment by FISH or PCR-based analyses, improving cost efficiency in pathology laboratories (Figure 16).

Finally, since H3K27me3 retention in diffuse gliomas is correlated with poorer outcomes, independent of the presence of *IDH*1/2 mutations we suggest the implementation of this antibody in clinical practice for prognostic purposes in this setting.

Moreover, in our experience with oligodendrogliomas, *IDH*-mutant, and 1p/19q-codeletion, H3K27me3 immunohistochemical expression was not useful in distinguishing between grade 2 and grade 3 tumors. Indeed, 74/77 tumors showed global loss of H3K27me3 expression considering all neoplastic cells in one slide, and despite the consistent intratumoral heterogeneity among tumor areas on the same slide, the percentage of H3K27me3 positive neoplastic cells in one hotspot did not correlate with the grade or other clinical pathological features.

These data are in contrast with those presented at the 20th International Congress of Neuropathology in 2023 by Miller et. al, who found that H3K27me3 partial or complete retained expression is correlated with higher grade in *IDH*-mutant and 1p/19q-codeleted tumors, both inter-and intratumorally (Millner T. et al. 2020). A possible reason for this discrepancy may be the use of different scoring methods to evaluate H3K27me3 expression; indeed, Millner et al. did not disclose the cut-off values used to define

H3K27me3 loss. Further studies are needed to understand the potential of H3K27me3 in identifying tumor progression in oligodendrogliomas.

Despite differences in the sensitivity and specificity of H3K27me3 loss in the differential diagnosis of oligodendrogliomas across studies, H3K27me3 loss represented a mark of favourable prognosis in diffuse gliomas (Ammendola S. et al., 2021). To challenge this hypothesis, a recent study on a total of 20 diffuse gliomas (10 glioblastomas *IDH*-wild type, 5 astrocytomas *IDH*-mutant and 5 oligodendrogliomas IDH-mutant and 1p/19q-codeleted), low levels of H3K27me3 were correlated with higher grade and temozolomide resistance (Zhang X. et al, 2024). This cohort was enriched in glioblastomas *IDH*-wildtype, and the staining was assessed not only by considering the percentage of positive cells (< 1 %, 1 %-25 %, 26 %-50 %, 51 %-75 %,
> 75 %), but also the intensity of the staining, using a three-tiered grading system. This approach to evaluating H3K27me3 immunohistochemical expression had not been performed in other studies. These limitations prevent us from drawing definitive conclusions on the differences between the results obtained by Zhang et al. and ours.

It must be noted that the diffuse gliomas included in our study were categorized according to the diagnostic criteria recommended by the updated 4th edition of the WHO CNS tumor classification of the central nervous system tumors (Louis. D.N. et al., 2016), therefore, astrocytomas *IDH*-mutant have not been tested for *CDKN2/A/B* homozygous deletion and the assigned grade could not represent the real biological aggressiveness of the neoplasm; (WHO Classification of Tumors editorial board, 2021).

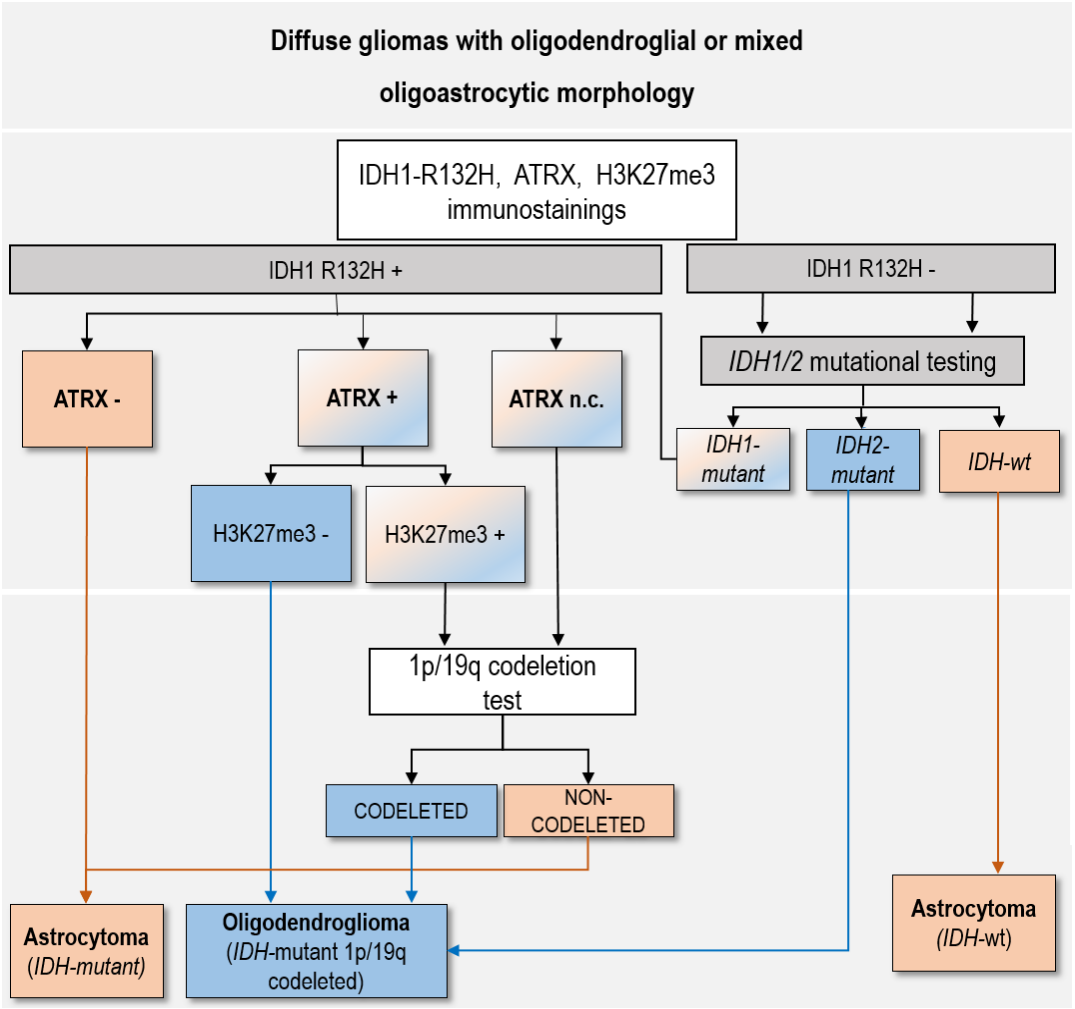


Figure 16. Proposed diagnostic algorithm for diffuse gliomas. Diffuse gliomas are classified starting with the assessment of IDH1 R132H, ATRX, and H3K27me3 immunostainings. Cases with ATRX loss are classified astrocytic, while those positive for IDH1 R132H and with retained ATRX and lost H3K27me3 stain are classified oligodendroglial. Cases with negative IDH1 R132H are tested for other *IDH1/2* mutations: the *IDH*-wt is classified astrocytic, while the *IDH*-mutant with retained ATRX and lost H3K27me3 is classified oligodendroglial. The assessment of 1p/19q codeletion is reserved to *IDH*-mutant tumours with retained ATRX and H3K27me3 immunostainings or non-conclusive ATRX.

In contrast to diffuse gliomas, the loss of H3K27me3 in meningiomas and rectal adenocarcinomas was associated with a poor prognosis (Ammendola S. et al., 2022a; Ammendola S. et al., 2022b, Ammendola S., Barresi V., 2022). In our study, H3K27me3 loss in grade 1 and grade 2 meningiomas significantly correlated with shorter recurrence-free survival, in line with previous findings, (Behling F. et al., 2021, Katz L.M. et al., 2018, Nassiri F. et al., 2021) and with tumor progression after SRS. Indeed, 86% of cases with H3K27me3 loss progressed after SRS compared to 33% of cases with retained H3K27me3.

This previously unreported correlation between H3K27me3 loss and higher risk of recurrence after stereotactic radiosurgery suggests a role of H3K27me3 in predicting resistance to this treatment.

A possible mechanism responsible for resistance to adjuvant treatments in meningioma is represented by the inability to repair double strand breaks due to chromatin condensation, which makes damaged areas inaccessible to enzymatic complexes responsible for DNA repair; conversely, DNA demethylated areas confer easier access to these repair systems, eventually leading to prolonged survival of neoplastic cells (Rath B.H. et al., 2018). Epigenetic modifications induced by radiotherapy and stereotactic radiosurgery, including alterations in H3K27me3 levels in meningiomas, are of paramount importance from a clinical perspective. Notably, previous studies on H3K27me3 expression in meningiomas (Behling F. et al., 2021; Hua L. et al., 2023) examined both primary and secondary meningiomas for H3K27me3 immunohistochemical expression. The group of secondary meningiomas contained a higher proportion of H3K27me3 immunonegative cases, raising the question of whether the loss of H3K27me3 is a poor prognostic indicator in secondary tumors or merely an epiphenomenon influenced by adjuvant therapies, lacking any real prognostic significance.

According to our findings, H3K27me3 loss in untreated primary tumors and in secondary tumors did not show the same prognostic value (Ammendola S., Barresi V., 2021). Our study on four matched primary and secondary meningiomas, the latter resected after radiotherapy treatments, suggest that H3K27me3 loss could be caused by adjuvant chemo-radiotherapies (Ammendola S., Barresi V. 2022) in accordance with

previous studies (Rath B.H. et al., 2018) and underscore that H3K27me3 loss in secondary tumors treated with stereotactic radiosurgery could not confer a worse prognosis if the primary untreated tumor showed H3K27me3 retention.

On the contrary, loss of H3K27me3 in the primary untreated tumor could implicate a more aggressive behavior and worse clinical outcome (Ammendola S. and Barresi V. 2022).

Similar to what was found in meningiomas, in rectal carcinomas, H3K27me3 immunohistochemical loss showed negative prognostic significance and predicted a worse response to neoadjuvant chemoradiotherapy in a cohort of 42 untreated locally advanced adenocarcinomas (Ammendola S. et al., 2022b). In accordance with our results, previous studies have documented significantly lower expression of H3K27me3 in colorectal cancer with advanced pTNM stage (Carvalho S et al. 2018) and a correlation between H3K27me3 loss and shorter disease-free survival (Benard A. et al., 2014). or shorter OS in early-stage colorectal cancer (Benard A. et al. 2013). These data may have a significant clinical impact since immunohistochemical assessment of H3K27me3 could be used to identify patients who respond better to neoadjuvant therapy and be part of the clinical algorithm to select patients who could avoid subsequent surgery.

Indeed, the standard therapeutic approach in patients with locally advanced rectal carcinoma involves neoadjuvant radio-chemotherapy followed by surgical resection in patients with partial or absent clinical response to neoadjuvant treatments or the avoidance of surgery in case of complete clinical response (Glynne-Jones R. et al., 2017). This so-called watch-and-wait approach proposed for the latter patients aims to avoid complications of surgery and improve patients' quality of life (Dossa F. et al., 2017, Glynne-Jones R. et al., 2017).

Despite this, in a small percentage of cases, patients treated with the watch-and-wait approach experience tumor recurrence at follow up (Dossa F. et al., 2017). We found that H3K27me3 expression in pre-treatment biopsies of rectal carcinoma could be assessed, among other clinicopathological features (Ammendola S. et al., 2021; Reggiani Bonetti L. et al., 2017), to predict the complete pathological response to neoadjuvant therapies in these patients.

Retention of H3K27me3 in the untreated tumors was significantly correlated with ypTNM stage 0 using the Dworak tumor regression grading system (Dworak O. et al., 1997). Therefore, as for meningiomas, loss of H3K27me3 correlated with unfavorable clinicopathological features in treatment-naïve tumors.

In conclusion our data suggests different biological significance of H3K27me3 immunohistochemical loss in the tumors analyzed, confirming that both global increases and decreases in H3K27me3 levels can influence the prognosis and drug resistance in a wide range of cancer types, and that any disruption in the regulation of H3K27me3, could contribute to oncogenesis and progression (Bannister A.J., Kouzarides T., 2011). H3K27me3 regulatory mechanisms are indeed crucial for maintaining cellular identity (van Haaften G. et al., 2009). H3K27me3 loss in some CNS tumor entities seems to contribute to the maintenance of glial cell stemness with tumor suppressor gene silencing, whereas an increase in H3K27me3 levels in later phases of tumorigenesis could improve cell migration and neoangiogenesis, contributing to tumor progression (Day C.A. et al., 2022).

Notably, in many neoplasms, the mechanism responsible for H3K27me3 loss is still unknown and presumably prevents a complete understanding of the significance of this alteration from a prognostic and predictive perspective.

For instance, in a small percentage of diffuse midline gliomas, H3K27me3-altered, small in-frame insertions of exon 20 and missense mutations of exon 7 in the *EGFR* gene are the main oncogenic drivers (Mondal G. et al.2020).

Although H3K27me3 loss is usually found in this subset, a direct effect of *EGFR* mutations on H3K27 methylation loss has not been proven and, in the absence of K27M mutation or EZHIP overexpression, trimethylation loss in these tumors is unexplained.

However, beyond its prognostic value, a clinically relevant aspect is represented by the possibility of targeting epigenetic alterations for therapeutic purposes (Cheng Y. et al., 2019). Alongside conventional therapeutic methods, researchers have devoted considerable effort over the last four decades to developing medications that target enzymes involved in the epigenetic control of the genome (Cheng Y. et al., 2019, Mabe N.W. et al., 2024, Tao L. et al., 2024). These treatments are referred to as epigenetic drugs or epi-drugs. Many of them, belonging to three epigenetic inhibitor classes, have

already been approved for clinical use, and many others are currently under evaluation in clinical trials (Mabe N.W. et al., 2024).

The three epigenetic drug classes currently approved are DNA methyltransferase inhibitors (DNMTi), which inhibit DNA methyltransferases, leading to hypomethylation of DNA and reactivation of silenced genes; histone deacetylase inhibitors (HDACi), which determine an increase in acetylation of histones; and Bromodomain Inhibitors, targeting bromodomain proteins that recognize acetylated lysines on histones (Yu X. et al., 2024). Among them, there are several EZH2 inhibitors and UTX inhibitors that directly influence H3K27 methylation status. Therefore, the identification of H3K27me3 loss, independent of tumor type, could be used as a preliminary screening tool for the identification of tumors with druggable epigenetic changes.

In order to implement H3K27me3 immunohistochemical assessment for prognostic and predictive purposes in routine practice, a relevant issue that needs to be addressed regards the variability of protocols for staining and the different scoring methods used across studies. This lack of uniformity inevitably prevents a comparison of the findings among studies and precludes drawing definitive conclusions on this issue.

Indeed, H3K27me3 staining protocols and pathological evaluations have not yet been standardized, leading to conflicting results, even among studies using the same antibody clone. For instance, in the case of IDH-mutant diffuse glioma, Pekmezci (Pekmezci M. et al., 2020), Filipski (Filipski K. et al., 2019) and our group used the same anti- H3K27me3 antibody (clone C36 B11) at different antibody dilution. While Pekmezci worked at a 1:50 dilution, Filipski and our group used a dilution of 1:200, within the range suggested by the antibody producer.

Interestingly, another study in which was used the C36B11 clone found an increase in the specificity of H3K27me3 loss for oligodendrogliomas with a higher dilution of the antibody (Kitahama K. et al., 2021). Likewise, a recent systematic review of the published literature on H3K27me3 immunohistochemical expression in meningiomas highlighted significant differences in the staining protocols (Pietrantoni A., Barresi V., 2024, accepted paper). H3K27me3 immunostaining was performed on whole tissue slides or tissue microarrays (TMAs) using the same antibody clone used in our studies, with a wide range of working dilutions, from 1:50 to 1:700. Moreover, there were also

significant differences in the definition of H3K27me3 loss across studies, with different percentages, ranging from 0 to 50% of positive neoplastic cells, used as cut-off values to define H3K27me3 loss (Pietrantoni A., Barresi V., 2024 accepted paper).

The lack of uniformity in the immunohistochemical assessment of H3K27me3 in meningioma prompted the c-IMPACT-NOW (Consortium to Inform Molecular and Practical Approaches to CNS Tumor Taxonomy—Not Official WHO) working group to state that the available data are still insufficient to recommend its use in clinical practice (Sahm F. et al., 2024).

Despite this, our studies on diffuse gliomas, RGNTs, meningiomas and rectal carcinomas showed that the addition of H3K27me3 staining could play a relevant role in clinical decision-making, where H3K27me3 immunohistochemical loss could be used to better select patients who would benefit from neoadjuvant treatment and conversely predict those who would experience resistance to treatment.

Research in the field of epigenetic modifications in cancer leaves several open questions and it is still unclear which epigenetic changes can be considered solid tumor biomarkers and which are reversible changes induced by neoadjuvant or adjuvant therapies, or stage/grade-dependent modifications. Moreover, precise guidelines on how to detect and evaluate these alterations in clinical settings are needed to reduce inter- observer variability.

Conclusions

Our experience with H3K27me3 immunohistochemical expression in CNS and extra-CNS human solid neoplasms confirms that H3K27me3 is not a "one-size-fits-all" type of alteration.

In IDH1/2-mutant gliomas, we confirmed the significant association between H3K27me3 loss and the presence of 1p/19q codeletion assessed by FISH or PCR-based analyses, providing a reliable tool to aid in the differential diagnosis of IDH-mutant diffuse gliomas of the CNS.

We further showed that in oligodendrogliomas, *IDH*-mutant and 1p/19q-codeleted, H3K27me3 immunohistochemical expression did not distinguish grade 2 from grade 3 tumors, although this assumption should be validated in a larger cohort of cases, starting from a more homogeneous pre-analytical phase, optimizing antibody choice and staining techniques, and subsequently improving the interobserver variability in the pathological assessment.

We demonstrated that global or partial immunohistochemical loss of H3K27me3 and K327M mutation can also be found in rosette-forming glioneuronal tumors. This finding has significant clinical implications because of the risk of misclassification when evaluating limited amounts of tumor tissue in biopsy specimens.

In meningiomas, we found a previously unreported correlation between H3K27me3 loss and a higher risk of recurrence after stereotactic radiosurgery, suggesting a role for H3K27me3 in predicting resistance to this treatment. Our results also suggest that H3K27me3 loss could be induced by chemotherapy and/or radiation treatment, without implying a worse clinical outcome. In contrast, loss of H3K27me3 in the primary untreated tumor could implicate a more aggressive behavior and worse clinical outcome.

Our findings in rectal carcinoma suggest that H3K27me3 immunohistochemical loss predicts the response to neoadjuvant chemo-/radiotherapy and may be used to identify patients who can avoid surgery after neoadjuvant treatment.

Indeed, in agreement with the vast literature on this topic, modifications in H3K27me3 immunohistochemical expression do not bear univocal diagnostic, prognostic, and predictive significance across tumor types. A better understanding of the mechanisms responsible for the suppression of H3K27 methylation and the use of different investigation techniques with better "spatial" resolution of the DNA regions affected by changes in H3K27me3 levels will likely shed light on this intricate subject.

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