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Review

Paediatric-type diffuse high-grade gliomas in the 5th CNS WHO Classification

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Summary

As a relevant element of novelty, the fifth CNS WHO Classification highlights the distinctive pathobiology underlying gliomas arising primarily in children by recognizing for the first time the families of paediatric-type diffuse gliomas, both high-grade and low-grade. This review will focus on the family of paediatric-type diffuse high-grade gliomas, which includes four tumour types: 1) Diffuse midline glioma H3 K27-altered; 2) Diffuse hemispheric glioma H3 G34-mutant; 3) Diffuse paediatric-type high-grade glioma H3-wildtype and IDH-wildtype; and 4) Infant-type hemispheric glioma. The essential and desirable diagnostic criteria as well as the entities entering in the differential will be discussed for each tumour type. A special focus will be given on the issues encountered in the daily practice, especially regarding the diagnosis of the diffuse paediatric-type high-grade glioma H3-wildtype and IDH-wildtype. The advantages and the limits of the multiple molecular tests which may be utilised to define the entities of this tumour family will be evaluated in each diagnostic context.

Key words: paediatric high-grade glioma, diffuse midline glioma K27M-altered, DMG with *EZHIP* overexpression, diffuse hemispheric glioma H3 G34-mutant, infant-type hemispheric glioma, infant-type hemispheric glioma with atypical location, RTK fusions, *ZCCHC8-ROS1, MEF2D-NTRK1, ETV6-NTRK3*, diffuse paediatric-type high grade glioma H3-wildtype and IDH-wildtype, pHGG MYCN, pHGG RTK1, pHGG RTK2, *MYC* amplification, *MYCN* amplification, *PDGFRA* amplification, radiation-induced gliomas

Introduction

One of the main changes in the fifth CNS WHO Classification is the introduction of the term 'paediatric-type' reflecting the different pathobiology underlying gliomas arising primarily in children compared to adults (adult-type) [1,2]. Paediatric-type diffuse high-grade gliomas are less common than their low-grade counterpart and different types are defined depending on typical location, age and molecular alterations. They comprise four main entities: 1) Diffuse midline glioma H3 K27-altered; 2) Diffuse hemispheric glioma H3 G34-mutant; 3) Diffuse paediatric-type high-grade glioma H3-wildtype and IDH-wildtype; and 4) Infant-type hemispheric glioma. The first type, which was recognised by the prior WHO edition, has been revised and renamed, whereas the remaining three types are

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novel. The current WHO classification provides essential and desirable diagnostic criteria for each type (Tab. I). Before discussing the single entities, there are some general concepts which are worth highlighting. This tumour family is defined as 'paediatric-type diffuse high-grade gliomas' and this designation overall reflects their characteristics. However, firstly, although they are qualified as 'diffuse', a circumscribed pattern of growth is typically found in the infant type [3,4] and may be also encountered in the H3-wildtype and IDH-wildtype type. Secondly, with respect to the definition of 'high-grade', although most cases display high-grade features, a low-grade morphology may occur in the H3K27-altered type which sometimes appears remarkably hypocellular without evidence of mitotic activity [5] or in the infant type which may even show low-grade glioneural features with a ganglionic differentiation [3,4]. Notably, regardless of the morphological features, the paediatric-type diffuse high-grade gliomas are biologically aggressive tumours with a dismal prognosis and are designated as grade 4, except for the infant-type to which a formal grade has not been assigned due to limited experience. Finally, although classified as 'gliomas', they sometimes show morphological and phenotypic features typical of the embryonal tumours, especially if belonging to the H3 G34-mutant type or to the H3-wildtype and IDH-wildtype type [6,7].

Diffuse midline glioma H3 K27-altered

Diffuse midline glioma (DMG) is an aggressive astrocytic tumour occurring in the brainstem or the midline structures of the brain, recognised as a CNS WHO grade 4 malignancy regardless of its histological features. In children it is located mostly in the pons (diffuse intrinsic pontine glioma - DIPG), representing 15% of all paediatric brain tumours [8], or it can be bithalamic. In adolescents and adults, it is usually monothalamic or spinal representing about 5% and 40% of brain tumours and spinal astrocytomas in children [9,10], respectively. It is characterised by the loss of H3K27me3 and is molecularly defined into three subtypes: 1) DMG, with H3K27M/I mutation, encompassing H3.3-mutant and H3.1 or H3.2-mutant cases; 2) DMG, H3-wildtype with overexpression of EZHIP; and 3) DMG, with *EGFR* alteration, including either mutation, e.g. *EGFRvIII*, or amplification.

The DMG H3.3 K27-mutant occurs in children with a median age of 7-8 years and is localized through the midline or within the pons [11]. H3.1 or H3.2 K27–mutant DMG occurs in the pons in younger patients with a median age of 5 years [12]. The H3-wildtype DMG with EZHIP overexpression is rarer and has the same location and age distribution of the H3.3-mutant subgroup. *EGFR*-mutant DGM involves the thalami bilaterally

Table I. Diagnostic criteria according to the 5th CNS WHO Classification

	Essential	Desirable
K27 M-altered	• Diffuse glioma with loss of K27me3 by immunohistochemistry, midline location **AND** • K27M mutation for H3 K27–mutant subtype, **OR** • Pathogenic mutation or amplification of EGFR for the EGFR-mutant subtype, **OR** • Overexpression of EZHIP for the H3-wildtype with EZHIP overexpression subtype, **OR** • Methylation profile of one of the subtypes of diffuse midline glioma	• Molecular results discriminating the H3.1 or H3.2 subtype from the H3.3 subtype
H3 G34-mutant	• Cellular, infiltrative glioma with mitotic activity **AND** • H3.3 G34R or G34V mutation **AND** • Hemispheric location **AND** • Methylation profile of diffuse hemispheric glioma, H3 G34–mutant (for unresolved lesions)	• OLIG2 immunonegativity • Loss of ATRX expression • Diffuse p53 immunopositivity
pHGG H3/IDH WT	• A diffuse glioma in a child or young adult with high mitotic activity **AND** • Lack of IDH1/IDH2 mutations **AND** • Lack of H3 mutations **AND** • Methylation profile: pHGG RTK1, RTK2 or MYCN **OR** • PDGFRA alteration, EGFR alteration, or MYCN amplification	• Microvascular proliferation • Necrosis, typically palisading • K27me3 retained
Infant-type hemispheric glioma	• Cellular astrocytoma **AND** • Presentation in early childhood **AND** • Hemispheric location **AND** • Receptor tyrosine kinase alterations (e.g. fusion in NTRK family gene or ROS1, MET1, or ALK) **OR** • Methylation profile: infant-type hemispheric glioma	

and affects children with a median age of 7-8 years [13]. Interestingly, DMG H3K27-mutant can be separated into distinct sub-groups based on the specific type of histone H3 variant mutated and their respective gene expression and DNA methylation profile [14]. The oncogenic driver is the mutation of the *H3-3A* (also known as *H3F3A)*, *HIST1H3B*, *HIST1H3C* or *HIST2H3C* genes, leading to an amino acid substitution of lysine (K) to methionine (M) or, rarely, isoleucine (I) [15-17]; it affects the enzymatic activity of EZH2, a subunit of the Polycomb Repressive Complex2 (PRC2) involved in gene silencing, causing an extensive loss of H3K27me3 [18].

DMG with *EZHIP* overexpression is associated with the loss of H3K27me3 via inhibition of PRC2 activity, a mechanism found also in posterior fossa A (PFA) ependymoma [19].

The pathogenic alterations in *EGFR*-mutant DGM are small in-frame insertions or missense mutations in the *EGFR* gene and/or *EGFR* amplification [20].

H3K27M may co-occur with BRAF V600E mutation and more rarely with IDH1 mutation [15,21] whereas the co-occurrence with H3.3 G34R/V mutation is extremely rare [22].

On MRI it appears as a solid mass mostly centred in the pons or within the thalamus, with a diffuse infiltrative growth. An exophytic component can be observed. When localised in the pons, referred to as DIPG, the tumour exhibits high T2/FLAIR signal intensity and variable contrast enhancement, which is most frequently absent (Fig. 1A-B) [23] and often shows an extension to the midbrain, cerebellar peduncle and cerebellar hemispheres. Due to these unique imaging features, MRI is still standardly used for diagnosis. When located within the thalamus, DMG can extend to the cerebral peduncle, brainstem or basal ganglia and periventricular white matter [13].

Macroscopically, a distortion of the involved structures is present; discolorations may represent haemorrhagic or necrotic areas. Histologically, there are no relevant differences among the different subtypes. The majority of DMGs look like grade 3 or 4 astrocytomas with a morphologic spectrum varying from cells with hyperchromatic oval to elongated nuclei with scant cytoplasm, to cells with epithelioid features, or, rarely, giant cells [24]. Mitotic figures are frequently observed and microvascular proliferation and/or necrosis may be present (Fig. 2A). Cases of grade 2 histology have also been described [5]. The immunophenotype is consistent with the glial nature of this tumour. Thus, neoplastic cells are reactive for OLIG2 and S100 and variably for GFAP. Of note, antibodies for H3K27M (mutated protein), H3K27me3 (trimethylated protein) and EZHIP represent a valuable molecular surrogate to identify DMG subtypes. The nuclear positivity for H3K27M combined with the nuclear loss of H3K27me3

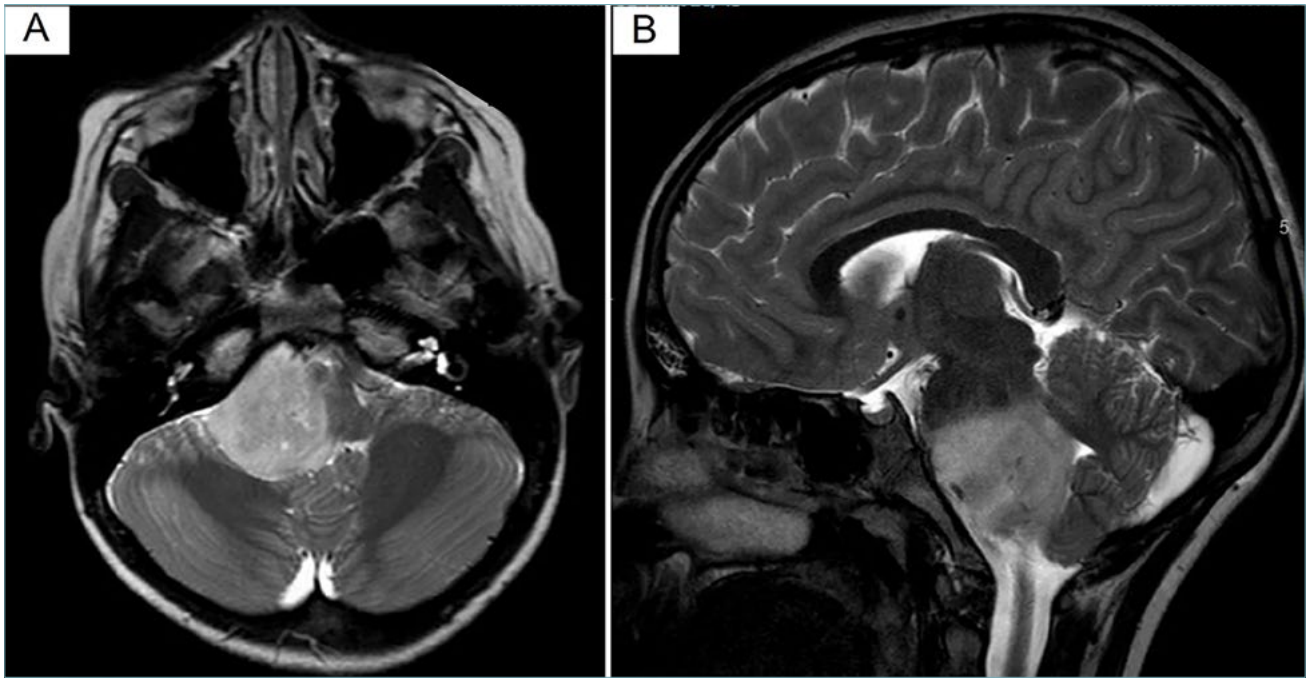

Figure 1. Axial (A) and sagittal (B) T2 MRI of DMG-H3 K27- altered. A large solid mass involving the right cerebellar peduncle and the right pons with an exophytic component.

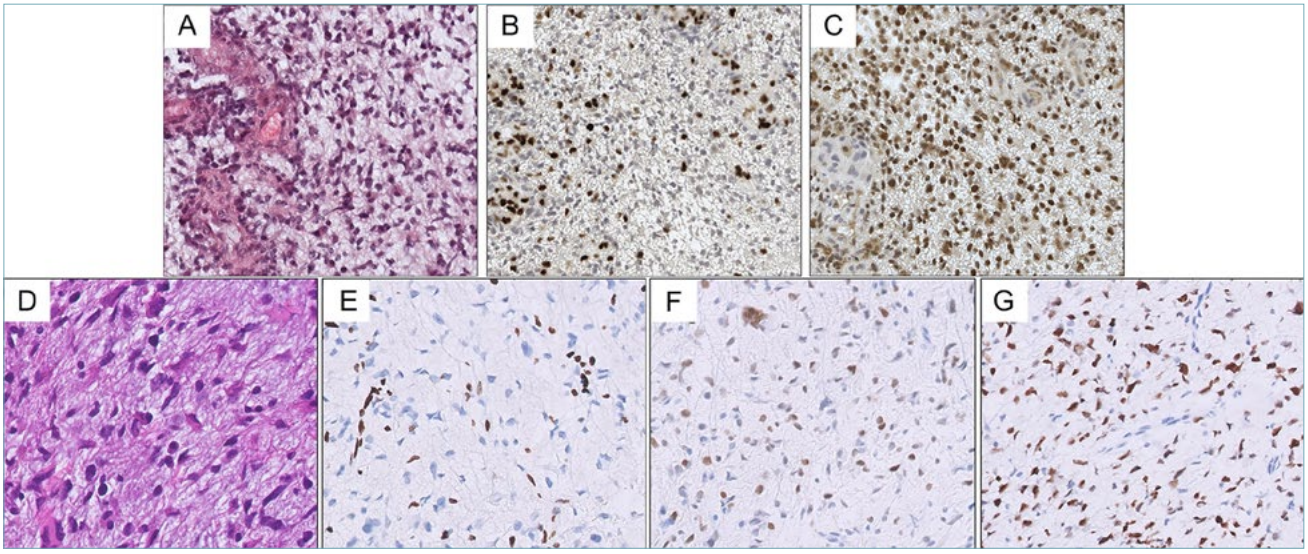

Figure 2. DMG-H3 K27- altered. (A-C): DMG H3 K27–mutant subtype. (A): The tumour is highly cellular with hyperchromatic nuclei and fibrillary eosinophilic cytoplasm. There is evidence of one mitosis and endothelial vascular proliferation. (B): Nuclear loss of H3K27me3 immunostain. C: Nuclear positivity for H3K27M antibody. (D-G): DMG subtype with EZHIP overexpression. D: neoplastic cells exhibit pleomorphic nuclei, some multinucleated cells and a big amount of eosinophilic cytoplasm with fibrillary processes. E. Nuclear loss of H3K27me3 in neoplastic cells. (F): Nuclear positivity for EZHIP antibody. (G): Diffuse nuclear positivity for p53 immunostain consistent with *TP53* mutation.

antibody identifies H3 K27-altered DMG (Fig. 2B-C) [25]. On the other hand, the loss of H3K27me3 associated with a negative staining for H3K27M should prompt the evaluation of the expression of EZHIP (CXorf67); in this context, the immunopositivity for EZHIP antibody recognises DMG, H3-wildtype with overexpression of EZHIP (Fig. 2D-E) [11]. A subset of tumours, in addition to H3K27me3 loss, can also show p53 expression (Fig. 2G) and/or nuclear loss of ATRX immunostain resulting from *TP53* and *ATRX* mutations espectively [11].

The differential diagnosis includes circumscribed glial/glioneural tumours, i.e. pilocytic astrocytoma and ganglioglioma, and the distinction from DMG is particularly challenging for those cases carrying H3K27M mutation, relying only on a morphological ground [26]. Diffuse gliomas are also in the differential. The possibility of a infratentorial IDH-mutant astrocytoma, although rare in children, has to be considered. Notably, in this context, the immunohistochemistry for IDH1 Arg132His mutant and ATRX is of limited use, as the infratentorial IDH-mutant astrocytomas frequently carry alternative nonArg132His *IDH1* mutations, and usually lack *ATRX* mutations [21]. Hence, sequencing of *IDH1* and *IDH2* hot spot region is warranted [21]. Moreover, in paediatric IDH-mutant astrocytoma a possible association with a mismatch repair deficiency syndrome has to be evaluated [27]. Cases of high-grade glioma, H3-wildtype and IDH-wildtype with *MYCN* amplification arising in the brainstem have also been described and should be considered in the differential [7]. Besides glioneural and glial tumours, even embryonal tumours such as atypical teratoid/rhabdoid tumour and embryonal tumour with multilayered rosettes enter in the differential diagnosis, which may be addressed by immunohistochemistry.

The prognosis of DMG is extremely poor with a 2-year survival of < 10% and a median survival of approximately 10-12 months in H3- and *EGFR*-mutant tumours [28]. DMG with *EZHIP*-overexpression has a slightly higher survival [11]. This dismal prognosis is partially due to its critical location, which involves vital nervous system structures making its resection extremely difficult, and to its tendency to diffusely involve adjacent structures. There is an urgent need to develop personalised treatments [29], although the intra-tumour heterogeneity characterising DMG represents an additional obstacle in the development of effective therapeutic approaches for this tumour type [30]. A clinical trial with GD2-CAR T cell therapy is on going and seems to be promising [31]. For essential and desirable diagnostic criteria, see Table I.

Diffuse hemispheric glioma, H3 G34-mutant

Diffuse hemispheric glioma, H3 G34-mutant is a CNS WHO grade 4 astrocytoma, diffusely infiltrative, arising in the cerebral hemispheres, and is now considered a distinct tumour type. Although it is classified as a glioma, it seems neuronal in origin based on its transcriptomic and epigenomic profile [32]. It affects children and young adults with a median age of 15 years, accounting for 16% of cortical tumours, mainly located in the temporal and parietal lobe [22].

The driver alteration is a point mutation at codon 35 of the histone H3.3 gene *H3-3A*, corresponding to glycin 34 of the mature H3.3 protein [16,33]. This leads to the glycine to arginine (G34R) or less frequently to valine (G34V) amino acid substitution [34] and to a consequent inhibition of SETD2 methyltransferase [35] and KDM2A lysine demethylase activity [36]. Concurrent *TP53* and *ATRX* mutations are present in ~90% of cases; *MGMT* promoter is frequently methylated [34]. Between 50% and 70% of diffuse hemispheric glioma H3 G34-mutant harbour *PDGFRA* mutations [32,37].

On MRI it shows aspects similar to other high-grade gliomas including contrast enhancement, necrosis, haemorrhage and oedema. A characteristic feature is the high frequency of contact with leptomeninges and ependymal regions [38].

The macroscopic appearance of this tumour reflects its malignancy, exhibiting soft consistence and necrotic areas. Histologically it displays a classic glioblastoma appearance, i.e. diffuse growth pattern, astrocytic cytology, high cellularity, high mitotic activity, microvascular proliferation and necrosis. A subset of tumours exhibits an embryonal (PNET-like) appearance, composed of cells with hyperchromatic nuclei and scant cytoplasm, and structures resembling Homer–Wright rosettes (Fig. 3A) [6,34].

The glioblastoma-like pattern shows a strong expression of GFAP; the PNET-like variant is diffusely positive for synaptophysin and exhibits a focal expression of GFAP. Both variants are negative for OLIG2 (Fig 2. 3B). The nuclear expression of p53 immunostain in a substantial fraction of neoplastic cells and the nuclear loss of ATRX protein reflects the underlying *p53* and *ATRX* gene mutation, respectively (Fig. 3C-D). Two antibodies to detect G34R/V-mutated cases are now available for immunohistochemistry (Fig. 3E) but false negative immunoreactivity in H3 G34-mutant cases has been described [39]. The absence of OLIG2 and ATRX expression together with the presence of a diffuse p53 expression is the typical phenotypic constellation of this entity and strongly suggests the diagnosis in the appropriate clinico-pathological context. However, it is not highly specific as it may be encountered in rare cases of diffuse paediatric-type

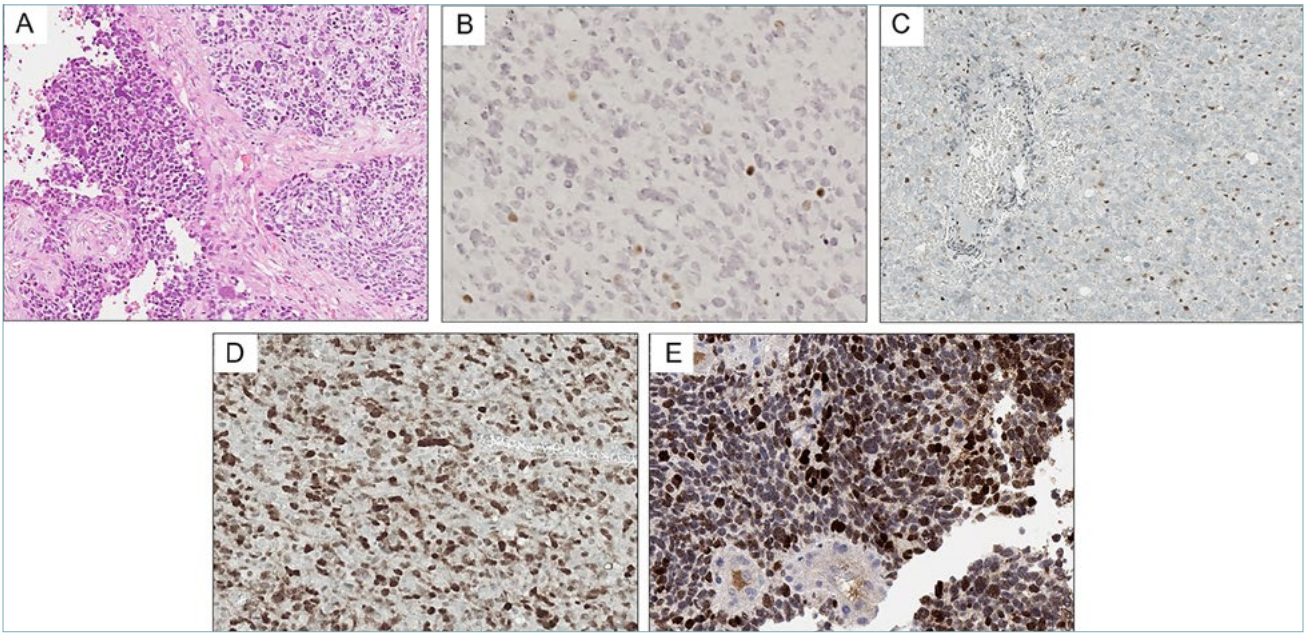

Figure 3. Diffuse hemispheric glioma, H3 G34-mutant. (A): A glioblastoma-like (right side) and PNET-like (left side) component is evident within the same tumour (H&E, 10X magnification). (B-C): The neoplastic cells show absence of nuclear expression of OLIG2 (B) and ATRX (C). (D): Diffuse nuclear positivity for p53 and H3G34R antibodies.

high-grade glioma, H3-wildtype and IDH-wildtype (personal observation). Hence, molecular analyses are needed to address the differential.

The median survival of patients with G34-mutant tumours is 22 months. The presence of MGMT promoter methylation is related to a better prognosis, while the presence of amplification of oncogenes (e.g. *EGFR, CDK4, MDM2*) is associated with a worse outcome [34]. The identification of *PDGFRA* mutations in more than 50% of the cases may offer new therapeutic opportunities for these patients [32,37]. For essential and desirable diagnostic criteria, see Table I.

Diffuse paediatric-type high-grade glioma, H3-wildtype and IDH-wildtype

Diffuse paediatric-type high-grade glioma H3-wildtype and IDH-wildtype (pHGG H3/IDH WT) is a heterogeneous entity currently defined by the combination of a high-grade morphology, either glial or primitive/undifferentiated, and the following molecular features: i) the absence of IDH1/2 mutations, ii) the absence of oncohistone alterations, e.g. H3.3/H3.1/H3.2 pK27M, iii) the alignment of the tumour with one of three paediatric high grade glioma (pHGG) methylation groups, i.e. pHGG RTK1, pHGG RTK2, pHGG MYCN [40] or, alternatively, the identification of genetic alterations which are considered characteristic of these groups, affecting *PDGFRA, EGFR* or *MYCN*, respectively (Tab. I). The documentation of the absence of IDH and H3 abnormalities even when occurring in an appropriate clinico-pathological context is not *per se* sufficient to make a diagnosis of pHGG H3/IDH WT. Hence, differently from what its name could erroneously suggest, the diagnosis of pHGG H3/IDH WT is not a diagnosis of exclusion. Rather, based on the WHO definition, it is essential to demonstrate the alignment of the tumour methylation profile with the pHGG RTK1, pHGG RTK2, pHGG MYCN or the occurrence of *PDGFRA, EGFR* or *MYCN* alterations. It is important to consider that a fraction of pHGG MYCN lacking *MYCN* amplification on the short arm of chromosome 2 show *MYC* amplification on the long arm of chromosome 8, which is still compatible with the diagnosis [40].

However, there are several critical issues regarding this entity and its diagnostic criteria which still need to be addressed:

1 A substantial proportion of cases falling in these methylation clusters lack the expected alterations in *PDGFRA, EGFR or MYCN*/*MYC* [40-42]. Hence, caution should be exercised when ruling out the diagnosis of pHGG H3/IDH WT based on the absence of *PDGFRA*/*EGFR*/*MYCN*/*MYC* alterations.

2 *PDGFRA, EGFR* or *MYCN*/*MYC* alterations are not highly specific for the three RTK1, RTK2 and MYCN methylation groups. For instance, *MYCN* amplification may be found also in a low proportion of pHGG RTK1 and RTK2 [40] or even in adult-type glioblastoma IDH WT with a primitive neuronal component [43]. Conversely, co-amplification of nearby *ID2* gene on the short arm of chromosome 2, which is frequently observed in pHGG MYCN, seems more specific than *MYCN* amplification alone, making the FISH test for *ID2* a valuable diagnostic tool [40,41,44]. Similar to *MYCN* amplification, *PDGFRA* amplification is frequently encountered in other gliomas, e.g. diffuse midline gliomas H3K27-altered [40]. Overall, these data caution against embracing the diagnosis of pHGG H3/IDH WT upon the identification of *PDGFRA, EGFR* or *MYCN*/*MYC* alterations [41,42], indicating that methylation analysis is the diagnostic gold standard for this entity (Fig. 4A-B) [41]. Gene expression profiling may potentially represent a valuable option, but this technique has not found wide application in routine diagnostics, its implementation remaining mostly limited to the research field.

3 Another issue regarding this entity as currently defined is related to the substantial amount of cases encountered in our daily diagnostic practice which are wildtype for IDH and H3, negative for receptor tyrosine kinase (RTK) fusions and do not classify as pHGG RTK1, pHGG RTK2, pHGG MYCN. These tumours are designated as 'NEC' according to the WHO classification after thorough molecular characterisation [2]. Additional studies with accurate molecular characterization and clinico-pathological correlations are needed to further dissect the heterogeneous group of paediatric HGG with no evidence of IDH and H3 alterations.

There are a few studies investigating the clinico-pathological correlates of pHGG H3/IDH WT and further investigations are needed. pHGG H3/IDH WT are estimated to represent a large proportion (about 40%) of paediatric high-grade gliomas with pHGG MYCN and pHGG RTK2 representing the largest and the smallest subgroups respectively. As for the aetiology, it is of note that the pHGG RTK1 subgroup includes most of the radiation-induced gliomas developing in patients previously treated for medulloblastomas or acute lymphoblastic leukaemia as well as most of the gliomas arising in syndromic contexts (e.g. Li Fraumeni, constitutional mismatch repair deficiency and Lynch syndrome) [45-47]. With the exception of a higher degree of genomic instability associated with radiation-induced DNA damage, no relevant biological differences have emerged between sporadic and radiation-induced

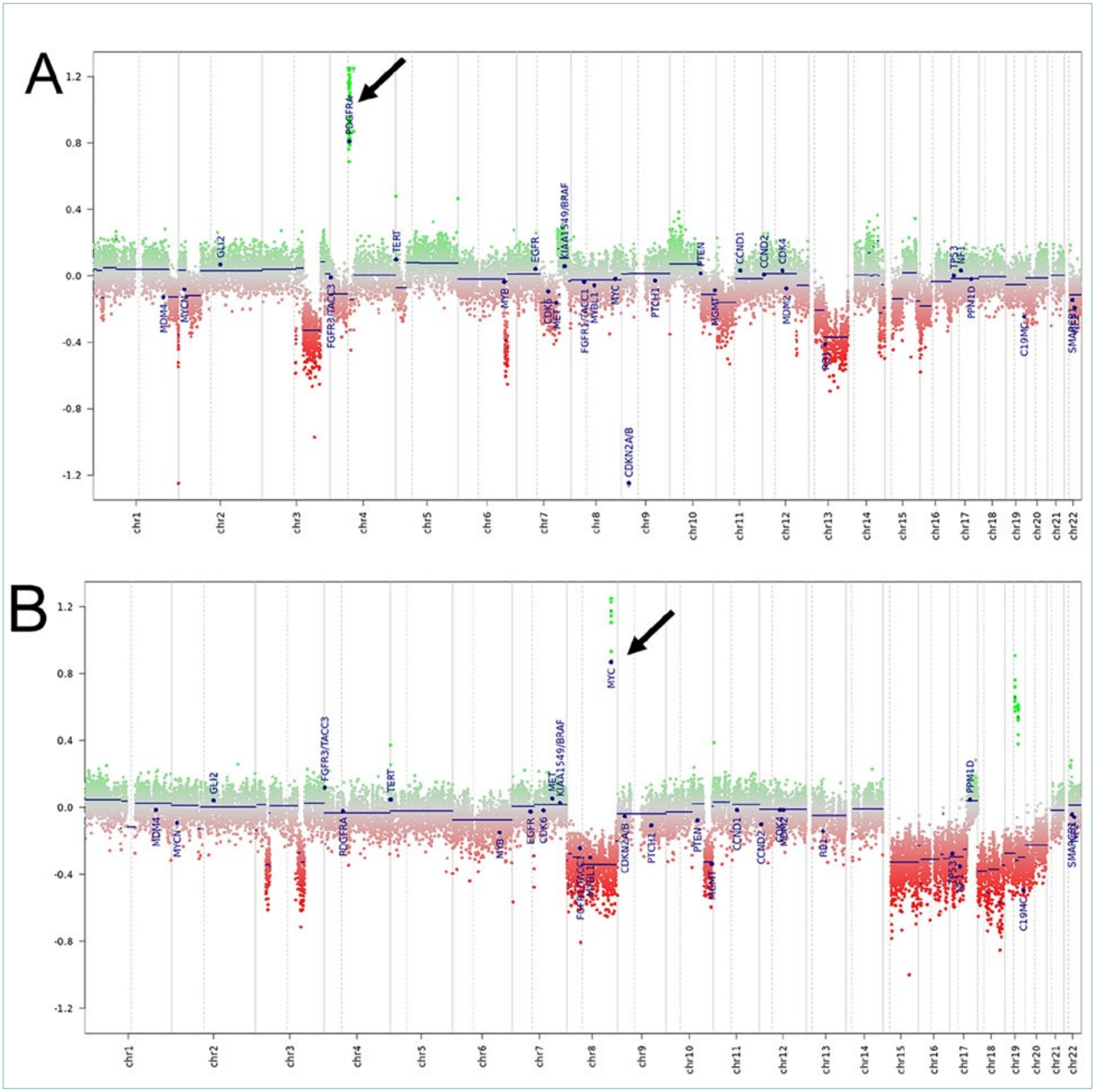

Figure 4. Diffuse paediatric-type high-grade glioma, H3-wildtype and IDH-wildtype. A: CNV obtained from DNA methylation analysis of a pHGG RTK1 showing losses of multiple chromosomal segments and the amplification of PDGFRA at 4q12. B: CNV obtained from DNA methylation analysis of a pHGG MYCN showing losses of multiple chromosomal segments and amplification of MYC at 8q24. A subset of pHGG MYCN are driven by amplification of MYC [43].

pHGG RTK1 [46,47]. As for the location, the vast majority of pHGG H3/IDH WT occur in the supratentorial anatomic compartment. A minority of pHGG MYCN (about 15%) arise in the brainstem [7,48,49]. pHGG RTK1 may also occur in infratentorial/brainstem structures, for instance in patients treated for medulloblastoma who received the highest radiotherapy dose in the posterior fossa [45-47,50]. As for the outcome, the overall prognosis of pHGG H3/IDH WT is poor (WHO grade 4). pHGG MYCN are associated with the lowest survival rates [40] and, within this subgroup, the pontine tumours behave more aggressively than the supraten-

torial counterpart, likely due to tumour location, median overall survival being 16.5 and 1.5 months for supratentorial and pontine HGG-MYCN respectively [41]. Within the pHGG RTK1 subgroup, survivals of the radiation-induced pHGG RTK1 are remarkably shorter than the sporadic counterpart, and the reasons for this difference are currently debated [46,47]. On the one hand, a more aggressive underlying biology linked to a high intrinsic genomic instability may account for the worse prognosis in these cases. On the other hand, clinical reasons, e.g. low number of therapeutic options due to prior treatments, may impact on the survival rates of these patients [47]. pHGG RTK2 have been shown to be the least aggressive [40]. The occurrence of *TP53, ATRX* and mismatch repair genes mutations in the context of pHGG H3/IDH WT have been associated with adverse outcomes [42].

Microscopically, pHGG H3/IDH WT may feature classical diffuse astrocytic morphology, with brisk mitotic activity, foci of necrosis and microvascular proliferation, showing a conventional phenotype with the expression of glial markers, i.e. GFAP and OLIG2 (Fig. 5A-C). Some cases, especially belonging to pHGG MYCN or more rarely to pHGG RTK1, may display a primitive/embryonal morphology and pushing borders with only minimal infiltration of the surrounding parenchyma (Fig. 5D-E). In such cases, the expression of the glial markers may be focal or even absent, whereas neuronal markers may be diffusely expressed, e.g. synaptophysin (Fig. 5F). A biphasic pattern may be also present, and embryonal nodules may be found in a background of an infiltrating high grade astrocytoma [7]. A remarkable nuclear pleomorphism may be seen in pHGG H3/IDH WT arising in syndromic contexts, i.e. Li-Fraumeni syndrome [51]. As for the immunophenotype, p53 is commonly found overexpressed in pHGG H3/IDH WT (Fig. 5G) [40]. ATRX is lost in a minority of cases (Fig. 5H) [40] which, in combination with p53 overexpression, may suggest the possibility of an astrocytoma IDH-mutant. H3K27me3 expression may be remarkably decreased or lost (personal observation).

The differential diagnosis depends on the age of the patient, location and morphological features of the tumour and requires molecular investigations. Diffuse hemispheric gliomas H3 G34-mutant and adult-type glioblastoma IDH WT may be taken into consideration when dealing with adolescents/young adults, whereas infant-type hemispheric gliomas enter in the differential for cases arising in the early childhood. Although the pHGG RTK2 share some molecular features with

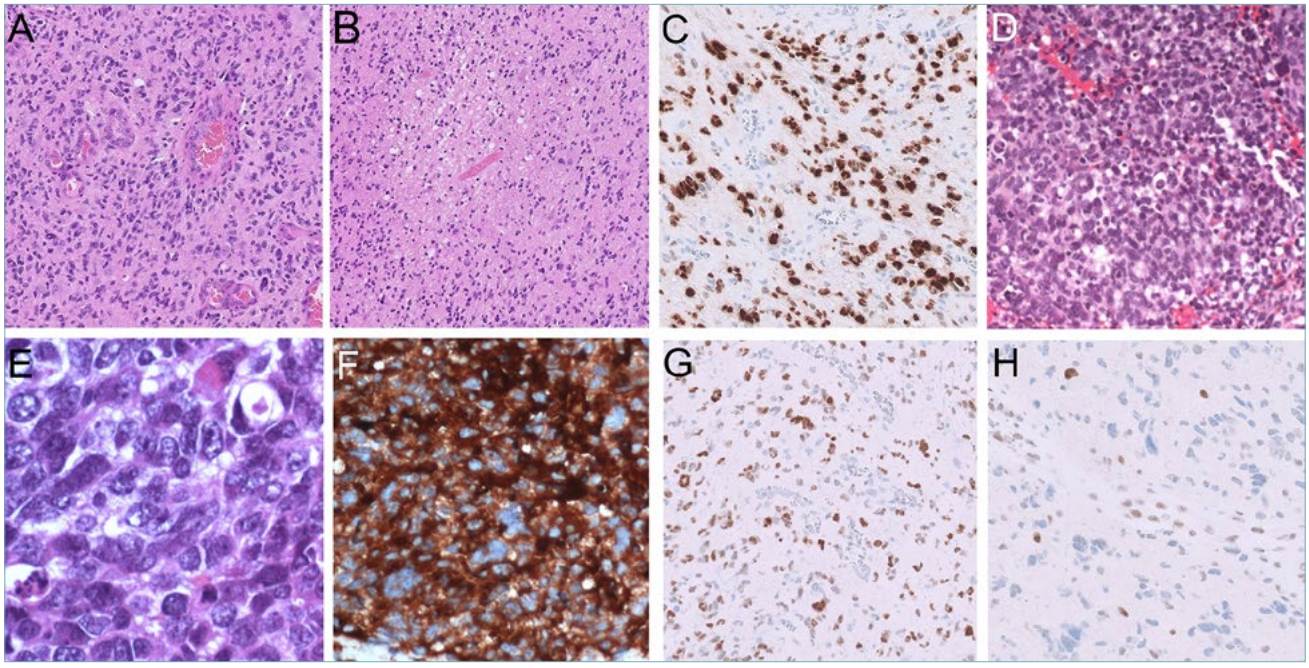

Figure 5. Diffuse paediatric-type high-grade glioma, H3-wildtype and IDH-wildtype. A-C: A case of pHGG RTK1 featuring a diffuse astrocytic morphology, microvascular proliferation (A) and necrosis (B) showing the expression of glial markers, e.g. OLIG2 (C). D-F: A case of pHGG RTK1 displaying an embryonal morphology (D-E) with a diffuse and strong expression of synaptophysin (F). Both these cases were radiotherapy-induced pHGG RK1 arising in the posterior fossa of adolescents with a previous history of medulloblastoma. G-H: A case of pHGG RTK1 showing overexpression of p53 (G) and loss of ATRX (H).

adult-type gliomas, i.e. TERT promoter mutations or CDKN2A/B deletion, concomitant +7/-10 chromosomal pattern is not considered a feature of pHGG H3/IDH WT. For cases showing a primitive morphology, embryonal tumours are considered. In this regard, the distinction of a cerebellar radiation-induced pHGG RTK1 from a relapse of medulloblastoma may be particularly challenging. For pHGG MYCN arising in the brainstem, the implementation of appropriate immunostains, i.e. LIN28, H3K27M, H3K27me3, EZHIP, as well as FISH for *MYCN*/*ID2* amplification may help in the differential with ETMR and midline glioma H3 K27-altered [5,7]. For essential and desirable diagnostic criteria, see Table I.

Infant-type hemispheric glioma

The essential diagnostic criteria for infant-type hemispheric glioma are a combination of clinico-pathological and molecular characteristics. Cellular astrocytic morphology, presentation in early childhood (usually before age 5) and hemispheric location are fundamental to the diagnosis. Infant-type hemispheric glioma is typically driven by a fusion involving a receptor tyrosine kinase (RTK) gene, i.e. *NTRK 1/2/3, ALK, ROS1* and *MET*, as 3' partner. Either the presence of the fusion or the methylation profile of infant-type hemispheric glioma is also required for the diagnosis (Tab. I). It is worth highlighting that the identification of an RTK fusion, for instance *NTRK*, within the context of a high-grade glioma, does not equate to a diagnosis of infant-type hemispheric glioma, as RTK rearrangements are also found occasionally in adult-type glioblastoma IDH-wildtype [52,53] where they likely represent additional molecular events resulting from clonal evolution.

Clinical presentation is usually acute typically occurring in the first year of life with non-specific symptoms ranging from seizures to lethargy or irritability; congenital cases are also reported, with increased head circumference and bulging fontanelles being common clinical signs [54].

Radiology usually shows a large intra-axial tumour with a solid and a cystic component, often with a marked mass effect (Fig. 6A-B). Intratumoural bleeding is observed in some cases with spontaneous intracranial haemorrhage representing the main radiological differential diagnosis (Fig. 6L). Location is typically hemispheric. However, spinal examples are also reported (Fig. 6P-Q) [55] raising the question of whether the inclusion of tumour location within the WHO definition of this entity may be too restrictive.

From a histological viewpoint, a sharp demarcation between the tumour and the adjacent cerebral parenchyma is frequently identified with absent or minimal neoplastic infiltration of the surrounding tissue (Fig. 6 C). Infant-type hemispheric glioma shows a typically uniform astrocytic cytomorphology, consisting of monotonous sheets of gemistocytes/minigemistocytes or fascicles of spindle cells (Fig. 6D, M, R) [3,4]. The majority of cases show a high-grade morphology with brisk mitoses, palisading necrosis and microvascular proliferation (Fig. 6E-F, N-O, S-T). A low-grade component, sometimes with ganglionic differentiation, may co-exist in some cases (Fig. 6G) [3,4]. Notably, less than 20% of infant-type gliomas show low grade features throughout [3]. Thus, the absence of high-grade features is still compatible with the diagnosis of infant-type hemispheric glioma. Focal ependymal differentiation may also be present [4,56]. As for the immunophenotype, infant-type hemispheric gliomas express glial markers, i.e. OLIG2 and GFAP (Fig. 6H-I); EMA expression with a dot-like pattern may be seen; synaptophysin and H3K27M are negative; H3K27me3, INI-1 and BRG1 nuclear expression is retained. The diagnosis relies on the demonstration of the appropriate methylation class or the translocation of the RTK genes (Fig. 6J, K, U). It is worth highlighting that, in this context, the utility of the gene fusion identification is superior to the methylation profile assessment as it not only has a diagnostic value but also relevant therapeutic implications.

The differential diagnosis includes other entities occurring during infancy such as desmoplastic infantile ganglioglioma/astrocytoma (DIG/DIA), supratentorial ependymoma, astroblastoma MN1-altered, CNS tumour with BCOR internal tandem duplication (ITD) and, for cases with a low-grade morphology, ganglioglioma [4,57]. DIG/DIA and infant-type hemispheric glioma share some radiological and pathological characteristics, e. g. large size, presence of a prominent cystic component, superficial location with meningeal involvement, well-defined borders, possible ganglionic differentiation and, in some cases, even a fascicular growth pattern and a spindle cell cytology. Compared with infant-type hemispheric glioma which mostly shows high-grade features, DIA/DIG are grade 1 tumours consisting of desmoplastic areas with a bland morphology (Fig. 7A) [58]. However, DIA/DIG may display areas with an embryonal/primitive morphology and brisk mitoses which may wrongly suggest the possibility of a malignant tumour (Fig. 7B) [58]. Notably, the key feature of the molecular landscape of DIA/DIG is the activation of the MAPK pathway, through either *BRAF* or *RAF1* alterations, and their identification in the appropriate clinico-pathological context may support the diagnosis in difficult cases [59,60]. Supratentorial

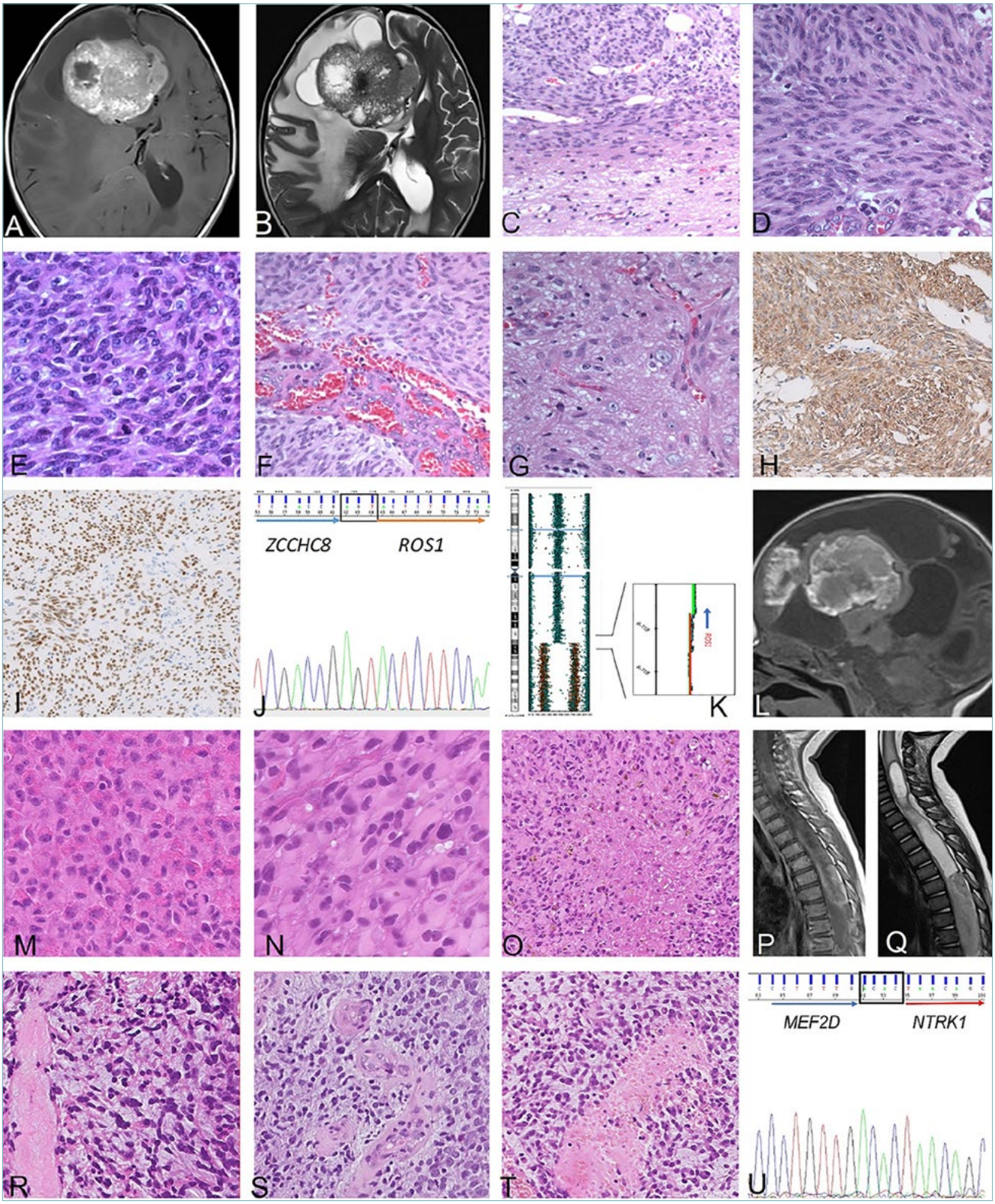

Figure 6. Infant-type hemispheric glioma: illustrative cases. (A-J): A case of a 3-year-old girl with a right baso-frontal tumour showing multiple foci of cystic change (A, axial T1 image; B, T2 image). Microscopically, the case displayed well-defined borders (C) and consisted of monotonous fascicles of spindle cells (D), featuring brisk mitotic activity (E) and microvascular proliferation (F); an area of low-grade morphology with ganglionic differentiation was also present (G). The tumour showed a diffuse expression of GFAP (H) and OLIG2 (I). NGS analysis with a targeted panel (Ampliseq Childhood Cancer panel, Il-

Continues ►

► *Follows*

lumina) revealed a ZCCHC8-ROS1 fusion transcript, which was confirmed with RT-PCR and Sanger sequencing (breakpoint including ZCCHC8 exon 1/2 and ROS1 exon 36 fusion) (J). CGH/SNParray (Illumina CytoSNP-850K v1.2) showed multiple chromosomal imbalances, including a deletion at the region 6q22.1q27 (53MB), interrupting ROS1 coding sequence, in line with the identification of ZCCHC8-ROS1 fusion (K). (L-O): A congenital case of infant-type hemispheric glioma. Sagittal T1 images showed a large haemorrhagic bilobed mass occupying the right hemisphere. The central hemorrhagic area was surrounded by a rim of neoplastic tissue of variable thickness (up to 15 mm) (L). The tumour consisted of monotonous sheets of gemistocytes (M), showing brisk mitoses (N) and palisading necrosis (O). This case carried a ETV6-NTRK3 fusion and was included in a previously published series (Clarke et al. 2021). (P-U): A case of a 3-year-old girl with a spinal lesion extending between C3 and T12 showing a solid hyperintense caudal component and a cystic cranial component (P, sagittal T1 image; Q, sagittal T2 image). Microscopically the tumour consisted of small somewhat spindle cells with atypical hyperchromatic nuclei, surrounded by a myxoid background (R), featuring mitoses, microvascular proliferation (S) and necrosis (T). A NGS panel (Archer Fusion Plex) revealed a MEF2D-NTRK1 transcript which was confirmed via RT-PCR and Sanger sequencing (breakpoint including MEF2D exon 6 and NTRK1 exon 10) (U).

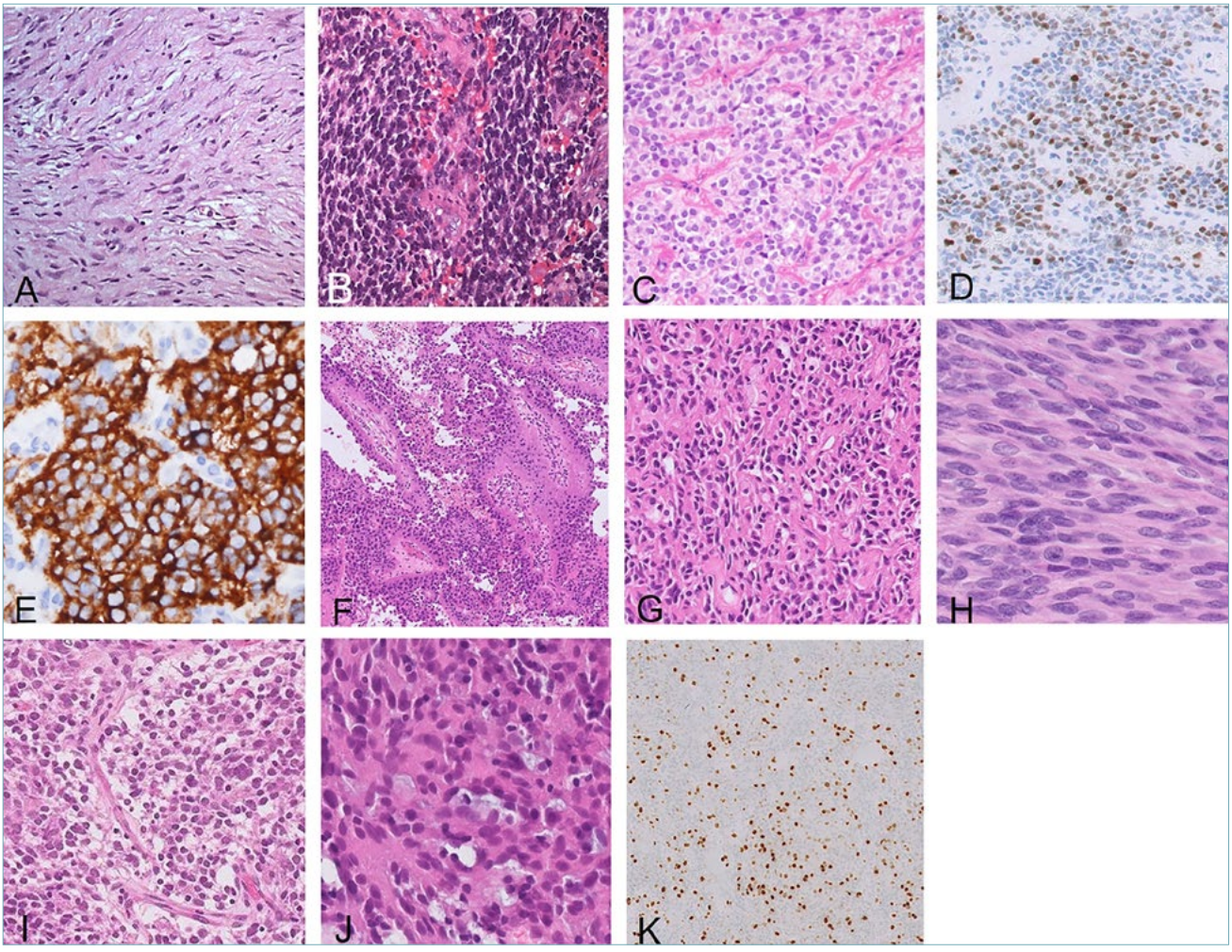

Figure 7. Entities entering in the differential diagnosis of infant-type hemispheric glioma. (A-B): A case of desmoplastic infantile ganglioglioma/astrocytoma characterized by astrocytic areas with marked desmoplasia (A) and by areas with a primitive morphology (B). This case carried a BRAF V600D mutation. (C-E): A case of supratentorial ependymoma ZFTA-fusion positive showing a clear cell morphology with branching capillaries (C), with expression of OLIG2 in multiple areas (D) and a diffuse L1CAM (E). (F-G): A case of astroblastoma MN1-altered featuring the typical astroblastic pseudorosettes (F) and stromal sclerosis (G). This tumour carried the typical *MN1-BEND2* translocation. H: A case of astroblastoma MN1-altered consisting of fascicles of monomorphous spindle cells and carrying *MN1-CXXC5* fusion. (I-K): A case of CNS tumour with BCOR ITD characterized by the co-existence of myxoid areas with delicate curvilinear capillaries (I) and microcystic areas (J); the tumour showed focal OLIG 2 expression (K).

ependymomas, e.g. ZFTA fusion-positive ependymomas, may represent a diagnostic pitfall as infant-type hemispheric glioma may feature ependymal differentiation and express EMA with a dot-like pattern of staining (Fig. 7C) [3,4,61]. Ependymomas generally lack OLIG2 expression. Thus, the absence of OLIG2 is currently used as a relevant clue to ependymoma over astrocytoma. However, this rule does not always apply to ZFTA fusion-positive ependymomas, where OLIG2 may be expressed in multiple areas, thereby limiting the utility of OLIG2 immunostain in this differential (Fig. 7D). Helpful features favouring ZFTA fusion-positive ependymomas are a remarkable network of capillaries, an oligo-like or clear cell morphology and the expression of L1CAM and p65/RELA markers (Fig. 7E). Astroblastoma MN1-altered, in its classical form, shows characteristic features, i.e. astroblastic pseudorosettes and prominent vascular and stromal hyalinization (Fig. 7F-G) [62]. However, the differential diagnosis with infant-type hemispheric glioma may be challenging when dealing with non-classical examples, where astroblastic pseudorosettes are hard to find and the tumour consists of uniform fascicles of spindle cells (fig. 7H), as the diagnosis relies *de facto* on the identification of *MN1* fusion, i.e. *MN1-BEND2* or *MN1-CXXC5,* and/or on the methylation profile [63]. CNS tumour with BCOR ITD may also enter in the differential [63]. Although in typical cases the combination of a remarkably myxoid background and a well-developed network of delicate arcuate and branching capillaries may suggest the diagnosis (Fig. 7I), cases with a glioma-like fibrillary background have also been described (Fig. 7J). The utility of the immunostains is limited as CNS tumours with BCOR ITD may express glial markers, e.g. OLIG2 (Fig. 7K). Furthermore, even a diffuse and strong nuclear expression of BCOR, although sensitive, is not specific for CNS tumours with BCOR ITD and has been described in other glial tumours [64,65], highlighting the need to resort to molecular analysis to address the differential.

As to the prognosis, a formal grade has not been assigned to infant-type hemispheric glioma as follow-up data are still limited. However, the outcome of infant-type hemispheric glioma, even in cases with high-grade features, seems favourable compared with other gliomas with similar morphology but different underlying biology. It has been suggested that the type of RTK gene involved in the fusion may influence tumour biological behaviour with ALK possibly being associated with a low-grade histology and high survival rates [3]. Whether the type of gene fusion yields a different outcome needs to be further explored. However, what is unquestioned is the importance of identifying the 3' gene partner of the fusion to predict tumour response to targeted therapy and choose the most appropriate molecular agent, e.g. Larotrectinib for NTRK rearrangement or, for instance, Alectinib for ALK rearrangement [54]. In this setting, the implementation of high-throughput sequencing technologies, e.g. RNA-based NGS panels or whole transcriptome sequencing, is of paramount importance, whereas methylation analysis is of limited use.

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Conflict of interest

The authors declare no conflict of interest.

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Ethical consideration

This study was approved by the ethics committee (202105_INNOV_Onetti).

Authors' contributions

Study conception: SR, FG, RA, FG; acquisition of data: SR, FG, BC, SB, SM, VA, FRB, FD-C, EM, MA, IG, GSC, AC, CP, AM, AC; analysis and interpretation of data: SR, FG, SB, EM, VA, AC, GSC. Drafting of manuscript: SR, FG. Critical revision: RA, FG, MV, EV, AM. All authors approved the final version of the manuscript to be published.

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