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WHO grade has no prognostic value in the pediatric high-grade glioma included in the HERBY trial

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Abstract

Background. The World Health Organization (WHO) adult glioma grading system is questionable in pediatric high-grade gliomas (pHGGs), which are biologically distinct from adult HGGs. We took advantage of the neuropathological review data obtained during one of the largest prospective randomized pHGG trials, namely HERBY (NCT01390948), to address this issue in children with newly diagnosed non-brainstem HGG.

Methods. HGG diagnosis was confirmed by pre-randomization, real-time central pathology review using WHO 2007 criteria, followed by a consensus review blinded to clinical factors and outcomes. We evaluated association between WHO 2007 grade and other clinical/radiological/biological characteristics and the prognostic value of WHO 2007 grade, midline location, and selected biomarkers (Ki-67 index/Olig2/CD34/EGFR/p53/*H3F3A* K27M mutation) on overall survival.

Results. Real-time central neuropathological review was feasible in a multicenter study, with a mean time of 2.4 days, and led to the rejection of HGG diagnosis in 20 of 163 cases (12.3%). The different grading criteria and resulting WHO grade were not significantly associated with overall survival in the entire population ($n = 118$) or in midline and non-midline subgroups. *H3F3A* K27M mutation was significantly associated with poor outcome. No significant prognostic value was observed for grade, even after regrading *H3F3A* K27M-mutated midline glioma as grade IV (WHO 2016). Midline location and a high Ki-67 index (≥20%) were associated with poor outcome ($P = 0.004$ and $P = 0.04$, respectively). A 10% increase in Ki-67 index was associated with a hazard ratio of 1.53 (95% CI: 1.27–1.83; $P < 0.0001$).

Conclusion. Our findings suggest that WHO grade III versus IV has no prognostic value in pediatric HGG.

Key Points

1. WHO grade III versus IV has no prognostic value in pediatric high-grade glioma.
2. High Ki-67 and midline location are the 2 factors associated with poor outcome.

Importance of the Study

The new combined histomolecular WHO 2016 classification makes significant changes in the diagnosis of gliomas especially in pediatrics, but these modifications have not been accompanied by changes in the key elementary grading criteria. We took advantage of the neuropathological review dataset obtained during one of the largest prospective randomized pHGG trials named HERBY, including molecular and radiological central evaluation (phase II, multicenter, comparative study of the addition of an anti-angiogenic agent to radiotherapy and temozolomide in patients between the ages of 3 and 18 years with newly diagnosed non-brainstem HGG to analyze the prognostic value of key grading criteria and interobserver reproducibility. We show that (i) real-time central neuropathological review is feasible in a multicenter study and (ii) the different grading criteria and the resulting WHO grade have no prognostic value in pHGG. Only midline location and a high Ki-67 index were associated with poor outcome.

In the past, pediatric high-grade gliomas (pHGGs) have been considered counterparts of adult malignant diffuse gliomas. As such, they have been diagnosed, subclassified, and graded using the same World Health Organization (WHO) classification criteria and treated similarly. However, in the last 5 years, there has been substantial progress in our understanding of the biology of pHGG. We now know that many of the molecular drivers of pHGG are unique to this age group, including *H3F3A* K27M, H3.3G34R/V, *SETD2*, and *MET* fusions, and can be associated with underlying tumor predisposition syndromes, including constitutional mismatch repair deficiency and Li–Fraumeni syndrome.[1,2] Furthermore, in half of supratentorial tumors, the molecular drivers are not yet identified,[3,4] and some tumors previously considered primitive neuroectodermal tumors can now be diagnosed by genetic classification as HGG.[5]

There have been few trials evaluating pharmacological treatments in addition to radiotherapy for pHGG and most, if not all, evaluated drugs with known efficacy in adult gliomas.[6] The phase II, prospective, randomized controlled HERBY trial (study BO25041; clinicaltrials.gov NCT01390948) showed that the addition of bevacizumab to standard radiotherapy plus temozolomide did not improve event-free survival in pediatric patients with newly diagnosed HGG.[7] The HERBY trial was aligned with large studies testing bevacizumab efficacy in adults with HGG.[8,9] In HERBY, all cases underwent expert neuropathological and radiological panel review, and an extensive molecular assessment was conducted in 80% of patients.[10] We used data from patients screened for this trial to evaluate the usefulness of the WHO glioma grading system.

The WHO grading for adult diffuse glioma has not been extensively validated in defined pediatric cohorts and the results of the few studies (before the era of molecular diagnostics) are contradictory. Gilles et al found no prognostic difference between grade III and IV gliomas,[11] while Finlay et al reported that grade III versus IV had prognostic value in the Children's Cancer Group (CCG)-945 study. Based on this unique historical finding, the randomization within the HERBY trial was stratified according to grade (III vs IV).[12] The high incidence of reclassification from HGG to low-grade glioma (LGG) in the CCG-945 cohort following central review (29.6% of local HGG were reclassified as LGG after randomization) motivated the creation of real-time, pre-randomization, central histological review, which was followed by an independent review by 5 experienced neuropathologists.[13] This independent, comparative histological evaluation in a randomized trial prompted us to work on a potential new grading system for pHGG.

The updated fourth edition (2016) of the WHO classification of central nervous system (CNS) tumors has profoundly modified the classification by: (i) adding well-established molecular parameters, particularly for diffuse gliomas; (ii) acknowledging that "WHO classification has included a grading scheme that essentially constitutes a malignancy scale rather than a strict histological grading system"; and (iii) adopting the principle of grading within a tumor entity for the first time. However, key morphological grading criteria for evaluating adult and pediatric gliomas have not been updated. These elements have been established on cohorts that do not take into account distinctions of molecular subgroups, such as isocitrate dehydrogenase (IDH) mutation in adult gliomas or *H3F3A* K27M mutations in pediatric gliomas. Furthermore, the inter- and intra-observer evaluations of these criteria have shown considerable variability.[14,15] In order to build a more reproducible grading system for pHGG, we evaluated the interobserver agreement of pathologists (local, central, and 5 independent experts) by analyzing grading criteria, histopathological entities, and main biomarkers, while blinded to clinical, radiological, and follow-up data.

Materials and Methods

Study Population

The phase II, open-label, randomized, multicenter HERBY trial has been previously described.[7] Eligible patients aged ≥3 to <18 years with newly diagnosed HGG were randomized to receive standard radiotherapy plus temozolomide with or without bevacizumab. Randomization was stratified by age group (≥3 to <6 vs ≥6 to <13 vs ≥13 to <18 years), WHO 2007 grade (grade III vs grade IV), and extent of resection (total/near-total resection vs others). All procedures performed in studies involving human participants were in accordance with the ethical standards of the institutional and/or national research committee and with the 1964 Helsinki declaration and its later amendments or comparable ethical standards.

Informed consent was obtained from all individual participants included in the study.

All patients screened for the HERBY trial at one of the 51 participating sites in 14 countries between October 2011 and February 2015 were eligible for the current analysis, including patients who were not enrolled in the randomized trial because of exclusion criteria (thereafter called "non-randomized patients"), provided that informed consent was signed.

Three different patient subgroups were considered for the pathology data analyses: (i) All cases confirmed as HGG by central review were included in the interobserver agreement analysis of pathology assessment; (ii) those with a diagnosis of anaplastic astrocytoma, anaplastic oligoastrocytoma, or glioblastoma were included in the grading analysis; (iii) those enrolled in the randomized trial were included in the prognostic factor analysis.

Pathological Evaluation

The first step of the pathology review consisted of real-time, pre-randomization, central review of all cases of HGG by the local pathologist. The central review (performed in Italy [F.G.], in Austria [C.H.], and in France by the lead pathologist, P.V.) was blinded to clinical and radiological information, and confirmed whether the local HGG diagnosis and WHO grading III versus IV was correct (according to WHO 2007 guidelines; stratification factor for randomization) and defined the tumor type (anaplastic oligodendroglioma, oligoastrocytoma, or astrocytoma) within 5 working days.

Six biannual consensus reviews were organized during the recruitment phase of the study. Five experienced neuropathologists (D.F.B., T.P., T.J., F.G., and C.H.) independently re-analyzed all randomized cases and completed an identical evaluation form in addition to the central review. If there was discordance between the different diagnoses (ie, more than 3 of 6 different conclusions) for the WHO grade or tumor type, a multiheaded microscope review was performed to reach a consensus diagnosis. Unlike the central pathologist's conclusion, the nonspecific descriptive term "HGG not otherwise specified" was accepted for the consensus conclusion. These consensus meetings were conducted face to face for randomized patients and using telepathology for non-randomized cases.

All pathologists (local, central, expert consensus panel) used WHO 2007 grading criteria, based on: differentiation (well, anaplasia, poor); cellular density (moderate, increased, high); atypia (occasional, distinct, marked); mitotic activity (absent, single [if biopsy], mitotic rate per 10 high-power fields: <5, 5–10, and >10); necrosis (absent, present); and vascular proliferation (absent, present). Other neuropathological assessments included: Ki-67 index (per 10 high-power fields: <5%, 5–20%, 20–50%, and >50%); P53 nuclear staining (<30%, ≥30% of cells); and epidermal growth factor receptor (EGFR) protein expression according to a modified Hirsh score (varying from 0 to 300: % positive cells × staining intensity, 0–3). Analyses by glial fibrillary acidic protein, oligodendrocyte transcription factor 2 (Olig2), cluster of differentiation (CD)34 (extravascular stellar staining, yes or no), integrase interactor 1, synaptophysin, IDH1-R132H, NF70 (neurofilament staining in tumor cells, yes or no) were also performed depending on material availability and the main differential diagnoses. A minimum of 6 unstained slides were sent, formalin-fixed paraffin-embedded blocks and local immunostains included or not. *H3F3A* K27M immunostatus was evaluated based on the loss of H3K27me3 from June 2013 and completed by *H3F3A* K27M mutation from January 2015.

Computerized Quantification of Ki-67 Index

The Ki-67 index was quantitatively evaluated as the percentage of Ki-67+ nuclei among all detected nuclei using a computerized analysis of whole-slide images, NDPITools,[16] and in-house software (C programs and ImageJ). The tissue area was selected by excluding blurred spaces, tissue-free space, and clusters of red blood cells.[17,18] Reference zones for the blue (hemalun) and brown (Ki-67) stains were selected automatically, and color deconvolution was performed by using the measured optical densities of the reference zones.[18] Objects (Ki-67+ and Ki-67– nuclei) were segmented based on automatically defined thresholds. Objects smaller than 6.13 μm^2 were discarded to avoid false positives due to dust and impurities. The resulting masks of nuclei and unblurred tissue areas, along with digitized images of the samples, were converted to the OpenSeadragon image format using Vips[19] and uploaded to a web server where the pathologist could remotely review the segmentation quality.

Molecular Analyses

Immunohistochemistry assessment (loss of H3K27me3 and *H3F3A* K27M mutation positivity) combined with molecular biology by *H3F3A* Sanger sequencing defined *H3F3A* K27M mutational status. Comprehensive molecular data combined with pathology and radiology data from HERBY have been described previously.[10]

Due to the newly defined entity of diffuse midline glioma (DMG) grade IV, *H3F3A* K27M mutant, introduced in the WHO 2016 classification, the WHO 2007 grade was updated based on the combined presence of *H3F3A* K27M mutation and midline location defined by imaging (ie, midline-located, *H3F3A* K27M-mutated diffuse grade III gliomas regraded as WHO 2016 grade IV). The primary statistical analysis was performed using the WHO 2007 grade classification.

Radiological Data

Baseline imaging was centrally reviewed by an expert radiologist (T.J.) who was blinded to pathological information.[7] For the purpose of the current analysis, 3 categories of radiological data were collected: tumor site (midline or non-midline), radiological enhancement (none, minor, moderate, strong-focal, or strong), and necrosis (no or yes).

Statistical Analysis

Statistical analysis included 4 different parts: (i) descriptive analysis of the central review process and results; (ii) interobserver agreement analysis of pathological

assessment; (iii) association between WHO 2007 pathological grade and other clinical, radiological, and pathological features; and (iv) prognostic factor analysis.

Interobserver Agreement Analysis of Pathology Assessment

Interobserver agreement of pathology assessment was evaluated using kappa, weighted kappa, or Kendall's coefficients, as appropriate (details available in Supplementary Fig. 1).

Factors Associated with WHO 2007 Pathological Grade

We evaluated the correlation between the 6 key grading criteria (differentiation, cellular density, atypia, mitosis, necrosis, and vascular proliferation, as defined by the lead reference pathologist) using Kendall's tau-b coefficients and multiple correspondence analyses. The contribution of these criteria to the grade defined by the expert consensus panel was then assessed. The discriminant value of each criteria, as well as the discriminant value of the different combinations, was evaluated using the area under the curve (AUC) of the corresponding receiver operating characteristics curve.

Finally, we explored the association between grade and the following: (i) clinical characteristics (age, tumor site [midline vs non-midline], extent of resection [total/near-total resection vs incomplete resection]), (ii) radiological evaluation of angiogenesis and necrosis, (iii) biomarker immunophenotype evaluated by the lead pathologist (Ki-67 index, <20% vs ≥20%, Olig2 negative vs positive, CD34 negative vs positive, NF70 negative vs positive, EGFR Hirsch index <150 vs ≥150, P53, <30% vs ≥30%), and (iv) *H3F3A* K27M mutation based on molecular biology when evaluable, otherwise on immunohistochemistry. For descriptive purposes, the association between each factor and grade was measured by the mean of odds ratio and 95% confidence intervals (CIs), which were estimated using a univariate logistic regression model (penalized Firth's approach). This analysis was performed for all cases confirmed as HGG by central review excluding pure oligodendroglioma and patients with HGG not otherwise specified and patients with confirmed HGG but tumoral subtype not defined.

Prognostic Factor Analysis

Overall survival (OS), defined as the time from randomization to death, was used for the prognostic factor analysis. Data were censored at the latest follow-up visit for surviving patients. Cox proportional hazards models were used to evaluate the prognostic value of key clinical and radiological characteristics, grade as defined by the expert consensus panel, each elementary criterion of WHO 2007 grading, other pathological features, and *H3F3A* K27M status. The percentage of Ki-67+ cells was studied as a continuous variable after checking the log-linearity hypothesis. Univariate hazard ratio (HR) and 95% CIs were estimated using the Cox model with the penalized Firth's approach appropriate for small sample size.

The prognostic impact of grade was then evaluated in a multivariable model, adjusted for age, tumor site, extent of resection, and treatment group, as well as biological factors with a *P*-value <0.20 in univariate analysis and <15% of missing data (main multivariable model). The proportional hazards assumption was tested using Schoenfeld residuals and no violation was observed. Heterogeneity of the prognostic value of grade according to tumor site and *H3F3A* K27M mutation status was assessed in multivariable models, including an interaction term.

These analyses included only patients with HGG confirmed by central review who were enrolled in the randomized trial, as follow-up data were not available for the other patients. All tests were performed at a two-sided alpha = 5%; point estimates and 95% CIs were determined. All analyses were performed using SAS v9.4.

Results

Description of the Central Review Process

Of the 174 patients who were screened for the HERBY trial, 163 had a central review; 11 patients were not reviewed due to a screening failure before the histological material shipment (Fig. 1). The real-time central pathology review was deemed feasible (Fig. 2A); results were provided within 3 days for 69% of patients (98/143 patients with information about dates; mean time, 2.4 days; median time, 2 days [interquartile range 1–4 days]), and quality of tumor samples was considered satisfactory in 79% of cases (107/135 informative cases). Only 10% were not acceptable (grading and subtyping not feasible) due to tumoral quantity and/or quality (Fig. 2B).

The pre-randomized central review led to the rejection of HGG diagnosis for 20/163 patients (12.3%): 9 LGGs grades I and II (pilocytic astrocytoma, $n = 4$; astroblastoma, $n = 2$; pleomorphic xanthoastrocytoma, $n = 1$; ganglioglioma, $n = 2$); 2 embryonal tumors (primitive neuroectodermal tumor, $n = 1$; rhabdoid tumor, $n = 1$); and 9 nondiffuse anaplastic gliomas grade III (anaplastic pleomorphic xanthoastrocytoma/anaplastic ganglioglioma). The expert consensus panel confirmed the diagnosis of a diffuse HGG entity by telepathology for 2 of these 20 initially rejected cases (both considered anaplastic gangliogliomas), and rejected the diagnosis of HGG in 3 additional cases after randomization (oligodendroglioma grade III reclassified as oligodendroglioma grade II, $n = 1$; glioblastoma grade IV reclassified as anaplastic ganglioglioma grade III, $n = 2$). The highest discrepancy rate (between local, reference, and expert panel) occurred between malignant diffuse glioma and anaplastic pleomorphic xanthoastrocytoma/anaplastic ganglioglioma (7/9 cases rejected by central pathologists were subsequently confirmed as diffuse HGG by the expert consensus panel). BRAF-V600E immunostaining was not available at the time of enrollment for the HERBY study, therefore could not be used to facilitate the separation between these tumors. The routine establishment of BRAF status will likely improve this distinction in the future.

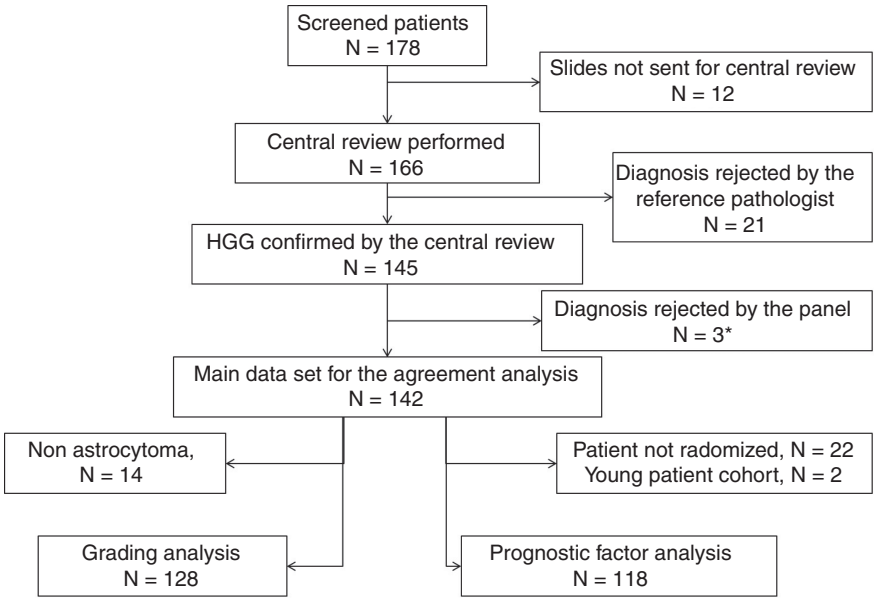

Fig. 1 Inclusion/exclusion of HERBY patients. The three patients for whom the diagnosis was finally rejected by the consensus panel were enrolled in the randomized trial, as the diagnosis of diffuse HGG had been initially confirmed by the reference pathologist. Based on the consensus review, 12 additional patients were excluded for the grading analysis because the diagnosis of astrocytoma was not confirmed: 1 patient with a diagnosis of oligodendroglioma, 7 with a diagnosis of high-grade glioma not otherwise specified, and 4 with HGG confirmed but subtype not defined by the consensus pathologists. Follow-up data were not collected in patients who were finally not enrolled in the randomized controlled trial because they did not meet all eligibility criteria (clinical, radiological) for the trial. Consequently, those patients had to be excluded from the prognostic factor analysis.

A total of 121 patients with HGG were randomized in the HERBY trial: 37 grade III gliomas (anaplastic astrocytoma, $n = 31$; anaplastic oligoastrocytoma, $n = 5$; anaplastic oligodendroglioma, $n = 1$) and 84 grade IV gliomas (glioblastoma, $n = 82$; glioblastoma with oligoid features, $n = 2$).[7]

Interobserver Agreement Analysis of Pathology Assessment

As detailed in Supplementary Fig. 1, interobserver agreement was substantial for grade (kappa = 0.76), vascular proliferation (kappa = 0.67), and necrosis (kappa = 0.82), but moderate for differentiation, cellular density, atypia, and mitosis (kappa ≤0.60). Regarding tumoral types, the distribution of final conclusions was not significantly different between the 6 experts ($P = 0.28$) and the vast majority of cases were classified as pure astrocytic tumors. However, concordance between experts was fair (Kendall's coefficient, 0.29). Agreement regarding Ki-67 index was substantial but not perfect (Kendall's coefficient, 0.71).

Factors Associated with WHO 2007 Grade III versus IV

We observed a significant association between the 6 key criteria for WHO grade, with correlation coefficients varying from 0.34 to 0.63 (Supplementary Table 1 and Supplementary Fig. 2). As expected, the 2 criteria with the highest discriminant ability for differentiating WHO grade III versus IV were necrosis and vascular proliferation (Supplementary Table 2); the AUC was 0.97 when both factors were combined. The discriminant ability was not significantly increased ($P = 0.25$) by adding the 4 other key criteria (AUC = 0.99).

A higher proportion of grade IV gliomas were non-midline tumors (82%) versus midline tumors (66%), but the difference was borderline significant ($P = 0.056$; Table 1). Grade IV glioblastoma was more frequent in cases of total/near-total resection than in cases of incomplete resection (89% vs 61%; $P = 0.0005$). Regarding biological characteristics, glioblastoma was significantly more frequent in cases with a high Ki-67 index versus a low Ki-67 index (81% vs 61%; $P = 0.03$) and in NF70+ versus NF70– cases (94% vs 74%; $P = 0.02$). Among the 86 informative cases, we did not observe a significant association between grade and *H3F3A* K27M mutation ($P = 0.28$). Among the 23 patients classified as grade III and evaluable for the *H3F3A* mutation, 11 (48%) had an *H3F3A* K27M mutation.

WHO 2007 grade was associated with radiological criteria (enhancement or necrosis; $P < 0.0001$). However, the agreement between pathology and radiology was moderate (kappa coefficient, 0.60 [95% CI: 0.41–0.80]). Indeed, among the 99 cases with radiological and pathological assessments, 10 cases classified as grade III had radiological enhancement or necrosis, whereas 3 cases classified as grade IV had neither radiological enhancement nor necrosis on baseline imaging. Further details are shown in Supplementary Tables 3 and 4.

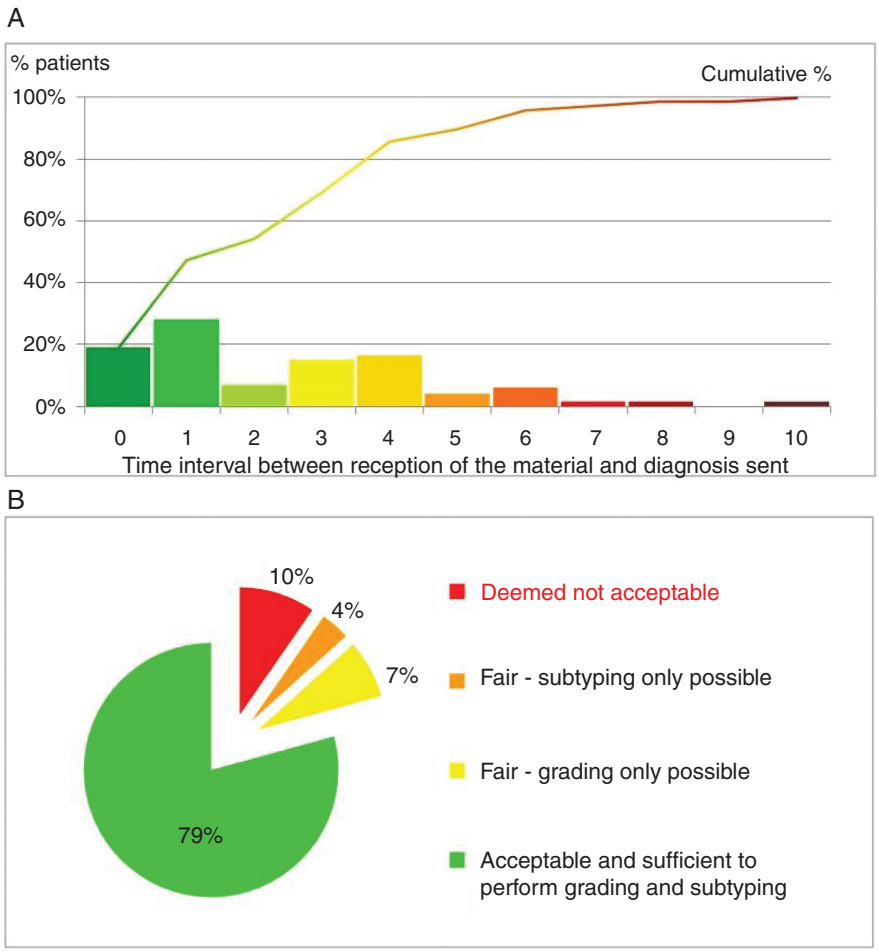

Fig. 2 Feasibility of the real-time central review. (A) Distribution of the time interval, in days, between reception of the material and diagnosis sent back to the center. (B) Quality of tumor samples.

Prognostic Factor Analyses

Among the 118 patients included in the prognostic factor analyses (median follow-up duration, 24.1 mo [range 0.03–46.8]), 86 had an event (tumor progression or recurrence, $n = 78$; death, $n = 6$; second primary non-HGG malignancy, $n = 2$) and 64 died (from disease progression, $n = 63$; due to adverse event, $n = 1$). The median event-free survival and OS were 10.3 months (95% CI: 8.0–12.7) and 18.3 months (95% CI: 15.7–28.1), respectively.

WHO 2007 grade III versus IV was not associated with survival outcome, either in univariate analysis (Fig. 3A and Table 2: $HR_{\text{grade IV vs III}} = 0.75$ [95% CI: 0.44–1.29]; $P = 0.30$) or in the multivariable model including age, tumor site, extent of resection, treatment group, and Ki-67 index (HR = 0.99 [95% CI: 0.53–1.85]; $P = 0.96$). We did not observe any significant heterogeneity regarding prognostic value associated with grade, according to tumor site (midline tumors, $HR_{\text{grade IV vs III}} = 0.90$ [95% CI: 0.43–1.92]; non-midline tumors, $HR_{\text{grade IV vs III}} = 1.12$ [95% CI: 0.38–3.35]; interaction test, $P = 0.74$) or *H3F3A* K27M status (mutation, $HR_{\text{grade IV vs III}} = 0.52$ [95% CI: 0.20–1.35]; no mutation, $HR_{\text{grade IV vs III}} = 1.15$ [95% CI: 0.39–3.41]; interaction test, $P = 0.28$). None of the grade key criteria were significantly associated with OS (Supplementary Table 5). In univariate analysis, *H3F3A* K27M mutation was significantly associated with poor survival outcome (HR = 2.15 [95% CI: 1.19–3.88]; $P = 0.01$; Fig. 3B). However, after reclassification of the 12 patients with *H3F3A* K27M mutation and midline location initially graded III and classified as DMG according to the updated 2016 grading, the updated grading was not significantly associated with OS either ($P = 0.31$).

In contrast, we observed a strong and significant association between OS and tumor site (Fig. 3C; in multivariable analysis, midline vs non-midline location: HR = 2.57 [95% CI: 1.36–4.88]; $P = 0.004$) and Ki-67 index (Fig. 3D; $HR_{\geq 20\% \text{ vs } <20\%} = 2.06$ [95% CI: 1.04–4.10]; $P = 0.04$). As the main analysis included the Ki-67 index evaluated by the lead pathologist, we checked the results when considering the Ki-67 index evaluated by the consensus panel. The HR associated with Ki-67 index in multivariable

Table 1 Univariate association between 2007 WHO grade (IV vs III) and other clinicopathological characteristics (n = 128[a])

Factors	WHO Grade III N = 30 % (n)	WHO Grade IV N = 98 % (n)	Odds Ratio [95% CI][b]	P-value[c]
Age, y				0.15
3–5	16.7 (2)	83.3 (10)	1 (ref)	
6–12	32.8 (20)	67.2 (41)	0.48 [0.09–1.87]	
13–18	15.8 (6)	84.2 (32)	1.19 [0.20–5.58]	
Missing[d]	2	15		
Tumor site				0.056
Non-midline	18.0 (11)	82.0 (50)	1 (ref)	
Midline	34.0 (17)	66.0 (33)	0.44 [0.18–1.02]	
Missing[d]	2	15		
Extent of resection				0.0005
(Near) total resection	10.9 (6)	89.1 (49)	1 (ref)	
Other	39.3 (22)	60.7 (34)	0.20 [0.07–0.51]	
Missing[d]	2	15		
Radiological criteria[e]				<0.0001
No enhancement nor necrosis	82.4 (14)	17.7 (3)	1 (ref)	
Enhancement or necrosis	12.2 (10)	87.8 (72)	28.6 [8.28–inf]	
Missing	6	23		
***H3F3A* K27M mutation**				0.28
No	22.6 (12)	77.4 (41)	1 (ref)	
Yes	33.3 (11)	66.7 (22)	0.59 [0.23–1.54]	
Missing	7	35		
Ki-67 index				0.03
<20%	39.3 (11)	60.7 (17)	1 (ref)	
≥20%	19.1 (17)	80.9 (72)	2.72 [1.09–6.77]	
Missing	2	9		
Olig2				0.93
Negative	20.0 (3)	80.0 (12)	1 (ref)	
Positive	22.6 (19)	77.4 (65)	0.94 [0.22–3.16]	
Missing	8	21		
CD34 (extravascular)				0.59
Negative	22.7 (17)	77.3 (58)	1 (ref)	
Positive	15.8 (3)	84.2 (16)	1.41 [0.43–5.87]	
Missing	10	24		
NF70 in tumor cells				0.02
Negative	25.7 (9)	74.3 (26)	1 (ref)	
Positive	6.4 (3)	93.6 (44)	4.56 [1.31–19.60]	
Missing	18	28		
EGFR Hirsh index				0.77
<150	25.0 (8)	75.0 (24)	1 (ref)	
≥150	28.3 (13)	71.7 (33)	0.86 [0.31–2.33]	
Missing	9	41		
P53 nuclear accumulation				0.84
<30%	24.2 (8)	75.8 (25)	1 (ref)	
≥30%	22.7 (15)	77.3 (51)	1.11 [0.41–2.86]	
Missing	7	22		

[a]Patients with diagnosis of an HGG entity rejected (N = 5) as well as patients with final diagnosis of oligodendroglioma (WHO grade II) (N = 1) and HGG-NOS (N = 9) were excluded, leading to a population of 128 patients. *HGG-NOS* = HGG not otherwise specified.
[b]Odds ratio was estimated from the Firth's penalized method, an approach used for small sample sizes and 95% CIs using the profile likelihood.
[c]Likelihood ratio test.
[d]Missing information for patients not included in the randomized trial.
[e]A case was classified as grade IV in the radiological grading if a radiological enhancement (minor, moderate, strong-focal, or strong) or necrosis was observed on imaging. Association between radiological enhancement and vascular proliferation, as well as association between radiological necrosis and necrosis evaluated on the pathological sample, are detailed in Supplementary Tables 3 and 4.

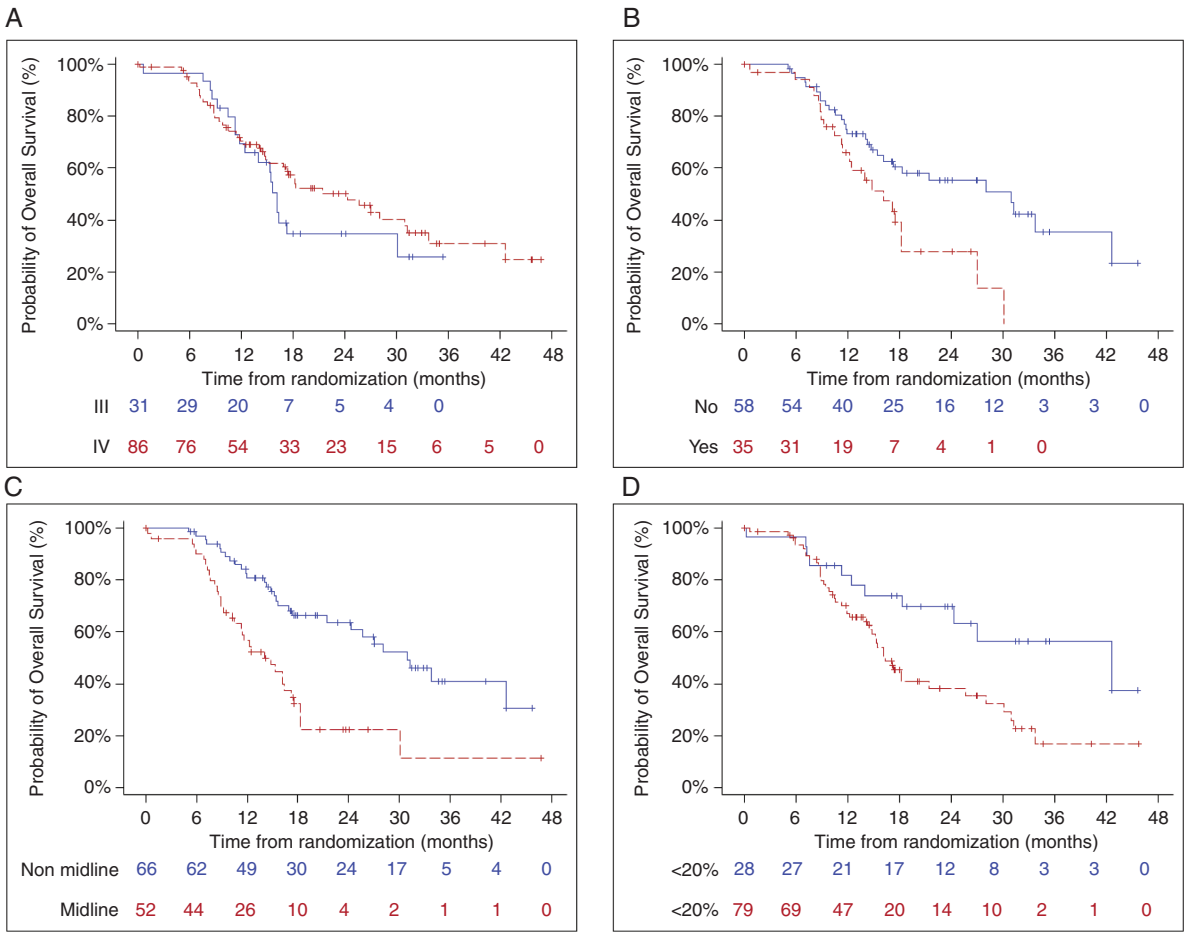

Fig. 3 Kaplan–Meier estimate of overall survival from randomization (A) according to 2007 WHO pathological grade ($N = 117$); (B) according to *H3F3A* K27M mutation status ($N = 93$); (C) according to tumor site (midline vs non-midline, $N = 118$); (D) according to Ki-67 index (>20% vs <20%, $N = 10$).

analysis varied from 2.06 to 2.99 for 5 of the 6 experts with a significant *P*-value, and was equal to 1.54 for the other expert ($P = 0.14$). The prognostic value associated with Ki-67 index did not significantly vary according to tumor site (midline location, $HR_{\geq 20\% \text{ vs } <20\%} = 1.19$ [95% CI: 0.48–2.92]; non-midline location, $HR_{\geq 20\% \text{ vs } <20\%} = 3.60$ [95% CI: 1.22–10.48]; interaction test, $P = 0.13$). After adding *H3F3A* K27M mutation status in the multivariable Cox model, Ki-67 index remained significantly associated with OS ($HR_{\geq 20\% \text{ vs } <20\%} = 3.77$ [95% CI: 1.55–9.16]; $P = 0.003$), whereas tumor site was no longer significantly associated with OS (HR = 1.86 [95% CI: 0.59–5.88]; $P = 0.29$), due to the association between tumor site and *H3F3A* K27M mutation (Supplementary Table 6).

When considering the quantitative measurement of Ki-67 index (percentage of Ki-67+ nuclei), we observed a significant relationship with the risk of death. A 10% increase in Ki-67 positivity was associated with an HR of 1.53 (95% CI: 1.27–1.83; $P < 0.0001$), with no violation of the log-linearity hypothesis ($P = 0.20$ based on spline functions; Supplementary Fig. 3) and no significant interaction with tumor site ($P = 0.95$).

Discussion

The present report assesses the applicability and validity of the WHO grading system for diffuse pHGG using materials and data from the phase II, multicenter, randomized HERBY trial.[7] To our knowledge, this is the first study to demonstrate the feasibility of real-time central histological review in pHGG. Furthermore, re-analysis by the expert consensus panel using telepathology was performed without technical difficulty in a subset of patients who were not diagnosed with HGG upon central review prior to study randomization. Improvements in fast imaging techniques will likely make real-time analysis easier in the future.

In this analysis, the discordance rate between neuropathological diagnoses was 12%, and 5% of cases were reclassified as LGG. This is a substantial improvement on the discordance rate observed in the CCG-945 study from 1985–1990 (30%),[12] and is similar to the more recent ACNS0126 (6.5% of non-HGG tumors at central review) and ACNS0423 (6.1%) trials.[6,20] These diagnostic improvements may be due to the optimization of

Table 2 Cox regression analysis of prognostic factors of overall survival (n = 118)

Factors	Number of Deaths/Patients	Univariate HR [95% CI][a]	*P*-value	Multivariable HR [95% CI][a,c]	*P*-value[b]
Age, y			0.70		0.90
3–5	7/14	1 (ref)		1 (ref)	
6–12	36/64	1.20 [0.54–2.66]		1.13 [0.50–2.56]	
13–18	21/40	0.96 [0.41–2.23]		0.99 [0.41–2.35]	
Tumor site			<0.0001		0.004
Non-midline	29/66	1 (ref)		1 (ref)	
Midline	35/52	2.78 [1.66–4.63]		2.57 [1.36–4.88]	
Extent of resection			0.003		0.10
(Near) total resection	25/59	1 (ref)		1 (ref)	
Other	39/59	2.12 [1.28–3.51]		1.71 [0.91–3.23]	
Radiological criteria (MD: *N* = 14)			0.48		
No enhancement nor necrosis	11/18	1 (ref)		–	
Enhancement or necrosis	50/86	0.79 [0.41–1.51]		–	
WHO 2007 grade (MD: *N* = 1)			0.30		0.96
III	19/31	1 (ref)		1 (ref)	
IV	45/86	0.75 [0.44–1.29]		0.99 [0.53–1.85]	
***H3F3A* K27M mutation (MD: *N* = 25)**			0.01		
No	28/58	1 (ref)		–	
Yes	22/35	2.15 [1.19–3.88]		–	
WHO 2007 grade + *H3F3A* K27M (MD: *N* = 6)			0.31		
III and no DMG [d]	6/14	1 (ref)			
IV or DMG [d]	54/98	1.49 [0.66–3.39]			
Ki-67 index (MD: *N* = 11)[e]			0.01		0.04
<20%	11/28	1 (ref)		1 (ref)	
≥20%	46/79	2.22 [1.14–4.31]		2.06 [1.04–4.10]	
Olig2 (MD: *N* = 25)			0.59		
Negative	10/17	1 (ref)		–	
Positive	36/76	0.83 [0.41–1.65]		–	
CD34 (extravascular) (MD: *N* = 31)			0.32		
Negative	38/69	1 (ref)		–	
Positive	7/18	0.68 [0.31–1.51]		–	
NF70 in tumor cells (MD: *N* = 46)			0.17		
Negative	16/30	1 (ref)		–	
Positive	24/42	1.55 [0.82–2.93]		–	
EGFR Hirsh index (MD: *N* = 46)			0.42		
<150	12/31	1 (ref)		–	
≥150	26/41	1.32 [0.66–2.61]		–	
P53 nuclear accumulation (MD: *N* = 25)			0.04		
<30%	11/28	1 (ref)		–	
≥30%	40/65	1.93 [0.99–3.75]		–	
Treatment arm			0.79		0.87
Without bevacizumab	30/57	1 (ref)		1 (ref)	
With bevacizumab	34/61	1.07 [0.65–1.75]		1.05 [0.60–1.84]	

Abbreviation: MD = missing data.
[a]Hazard ratio was estimated from the Firth's penalized method, an approach used for small sample sizes and 95% CIs using the profile likelihood.
[b]Likelihood ratio test.
[c]The multivariable model includes the following variables: age, tumor site, extent of resection, pathological WHO grade, Ki-67 index, and treatment arm, as defined in the table. *N* = 106 patients (57 deaths). Results of the multivariable analysis, including the same covariates plus *H3F3A* K27M mutation, are detailed in Supplementary Table 6 (*N* = 85, 46 deaths).
[d]In the updated WHO 2016 classification, patients with *H3F3A* K27M mutation and midline location were classified as diffuse midline glioma (DMG), and graded as grade IV. This updated grading concerned 12 patients initially grade III with WHO 2007 and classified as DMG, grade IV.
[e]Results for Ki-67 index presented in the table correspond to the evaluation by the lead reference pathologist. Results from the multivariable analysis were very stable when considering Ki-67 index evaluated by the consensus panel experts, with a significant hazard ratio varying from 2.06 to 2.99 for 5 of the 6 experts (significant *P*-value) and a hazard ratio equal to 1.54 for the other one (*P* = 0.14).

routine immunostaining techniques. In the HERBY study, the most difficult differential diagnoses of pHGG were anaplastic ganglioglioma and anaplastic pleomorphic xanthoastrocytoma (5% of local HGG diagnoses were reclassified as such). However, the differential diagnosis between these tumor entities with frequent BRAF mutations is difficult even among experts, particularly in the absence of clinical and radiological information. Furthermore, the HERBY study was designed before the discovery of histone gene mutations and tumor entities that mimic HGG, such as primitive neuroectodermal tumor variants (eg, CNS NB-FOXR2, HGNET-MN1).[5] The routine use of new immunophenotyping tools with specific antibodies, such as mutated *H3F3A* K27M, H3-G34R, and ATRX, will likely facilitate the differentiation of these entities in the future.[21]

The WHO grading system was primarily established for adult gliomas and has been subsequently used for pediatric gliomas without proper validation in controlled pediatric cohorts. However, pHGG is distinct from adult HGG, with substantial differences in tumor location, contrast enhancement, and driver mutations, and represents a biologically heterogeneous group.[22–29]These differences question the applicability of WHO grading, particularly since the description of diffuse midline glioma with *H3F3A* K27M mutation in the 2016 WHO classification. This is reflected in our findings from the HERBY cohort, which included a high proportion of this new entity (27%).[10] However, the recent separation into different molecular IDH or histone H3 glioma subgroups has not been accompanied by modifications of the different grading criteria within these subgroups. Many studies have focused on these potential adaptations, particularly in adult diffuse gliomas.[30,31] The prognostic value of glioma grading in the WHO 2016 integrated adult subgroups is also unclear, particularly since the differentiation of adult glioma by IDH1/2 gene mutation and 1p/19q chromosomal codeletion status. Some studies highlighted the loss of prognostic significance between WHO grades III and IV for IDH wild type and IDH mutated gliomas without 1p/19q codeletion.[32–34] This suggests that global improvements in prognostic value of the WHO 2016 guidelines is due to better histomolecular characterization, rather than clarifying grading elements.

Grade III versus IV was included as a stratification factor in the HERBY study, due to its prognostic impact, as suggested in the CCG-945 trial.[12] However, we found no prognostic value for grade III versus IV established by the expert consensus panel, or for key grading criteria, particularly endothelial proliferation and necrosis. Furthermore, using the updated WHO 2016 grading guidelines and correcting the grading according to radiological data (ie, grade III reclassified as grade IV if necrosis and/or contrast enhancement present on central radiological review) failed to establish any prognostic value with WHO grading. The conflicting findings between our study and the previous CCG-945 trial may be due to differences in the WHO grading system (2007 vs 1993), central review process, and chemotherapy regimens, as well as exclusion from the HERBY trial of patients with multifocal/metastatic tumors.

Previous research has shown significant interobserver variability in the histological diagnosis of glioma using WHO 2007 criteria.[14,35] We had planned to use the comparative pathologist data from the histological evaluation in the present study to determine the best combination of grading criteria to improve reproducibility. However, the absence of any observable prognostic value in the different grading elements meant our data could not be used to construct a new grading system for pediatric malignant diffuse gliomas. Therefore, we recommend that existing criteria should be used to differentiate malignant diffuse gliomas from nondiffuse anaplastic gliomas and LGGs, but not for determining prognosis.

In the present study, Ki-67 index was the only independent biomarker associated with OS in a multivariable analysis; a quantitative analysis showed that a 10% increase in Ki-67 positivity was associated with an HR of 1.53. Furthermore, there was substantial agreement among the 6 experts for Ki-67 index, according to a semiquantitative score (0–5%, 5–20%, 20–50%, and >50%). The prognostic impact of Ki-67 using a cutoff of 36% has already been demonstrated in the large, randomized CCG-945 trial, both in the overall cohort and in patients with midline tumors.[13,36,37]

We acknowledge some limitations to our study. In particular, even if this represents the largest study so far of pHGG with comprehensive histopathological, molecular, and radiological evaluation, the relatively limited sample size limits the power of the current analyses. In addition, we recognize that, in this rare disease setting, eligibility criteria defined at the design stage of the randomized trial led to a heterogeneous population under the denomination of HGG.

In conclusion, analysis of the histopathological data obtained from the large, prospective, phase II HERBY trial in pediatric patients with newly diagnosed HGG suggests that WHO grade III versus IV has no prognostic value in pHGG. We propose the use of the term "pediatric HGG" rather than anaplastic astrocytoma or glioblastoma and suggest that the Ki-67 index may be more useful than grade for prognostic evaluation in this population. However, this finding should be validated in further controlled studies of homogeneously treated pediatric patients suffering from diffuse HGG.

Supplementary Material

Supplementary data are available at *Neuro-Oncology* online.

KeyWords

grading criteria | high-grade glioma | Ki-67 | pediatric

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