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ERK1/2 Phosphorylation Predicts Survival in Recurrent Glioblastoma Following Intracerebral and Adjuvant PD-1/CTLA-4 Immunotherapy: A REMARK-guided Analysis

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ABSTRACT

Purpose: Evidence suggests that MAPK pathway activation, as measured by ERK1/2 phosphorylation (p-ERK), predicts overall survival (OS) in patients with recurrent glioblastoma receiving anti-PD-1 therapy. We aimed to validate these findings in independent cohorts.

Experimental Design: In a 24-patient clinical trial on recurrent glioblastoma and high-grade gliomas, we examined the link between p-ERK levels and OS. Patients received intravenous nivolumab, followed by maximal safe resection and an intracerebral injection of either ipilimumab alone or combined with nivolumab. Biweekly adjuvant nivolumab was then administered up to five times (NCT03233152). Using REporting recommendations for tumor MARKER prognostic studies (REMARK) criteria, we conducted independent analyses for p-ERK quantification and statistical evaluations. Additional comparative analysis included prior cohorts, totaling 65 patients. Cox proportional hazards models and meta-analysis were employed to assess p-ERK as a predictive biomarker after immunotherapy.

Results: Lower median p-ERK+ cell density was observed compared with prior studies, likely due to variable tissue processing across cohorts. Nonetheless, high p-ERK was associated with prolonged OS, particularly in isocitrate dehydrogenase wild-type glioblastomas ($P = 0.036$). Median OS for high and low p-ERK patients were 55.6 and 30 weeks, respectively. Multivariable analysis reinforced p-ERK's significance in survival prediction ($P = 0.011$). Upon p-ERK normalization across cohorts ($n = 65$), meta-analysis supported the survival benefit of elevated tumor p-ERK levels ($P = 0.0424$).

Conclusions: This study strengthens the role of p-ERK as a predictive biomarker for OS in patients with glioblastoma on immune checkpoint blockade. Future research should focus on further validation in prospective trials and the standardization of preanalytical variables influencing p-ERK quantification.

Introduction

Glioblastoma is a devastating disease with limited treatment options, particularly at time of recurrence. At diagnosis, established treatments include radiation, chemotherapy, and tumor-treating fields (1). However, at recurrence, no therapy has been shown to prolong overall survival (OS) in unselected patients. Furthermore, the 5-year survival rate for patients with glioblastoma is less than 10% underscoring the critical need for innovative and effective therapies (1).

One promising area of research in cancer therapy is immune checkpoint inhibition, which involves the use of therapeutic mAbs targeting the programmed cell death protein 1 (PD-1), programmed death-ligand 1(PD-L1), or CTL-associated antigen 4 (CTLA-4). However, immune checkpoint inhibition has not shown efficacy in unselected patients with glioblastoma (2–4). The highly immunosuppressive tumor microenvironment that characterizes glioblastoma constitutes a significant barrier to effective immune checkpoint blockade (5). Yet, several cases of clinical benefit and response to this form of immunotherapy where PD-1/PD-L1 blockade has been evaluated in patients with glioblastoma have been reported (6–8).

Recent analyses identified molecular features associated with the response to PD-1 blockade. Specifically, activating mutations in *BRAF* and *PTPN11*, which drive ERK signaling of the MAPK pathway, were enriched in recurrent glioblastomas from patients that responded to anti-PD-1 therapy (8). Considering these results, we evaluated the predictive value of ERK1/2 phosphorylation (p-ERK), as an indicator of MAPK pathway activation, in glioblastomas from patients treated with adjuvant PD-1 blockade (7). In two independent cohorts of patients with recurrent glioblastoma, we determined that in pretreatment tumor samples (obtained within weeks prior to initiating immunotherapy), p-ERK demonstrated being predictive of OS

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Translational Relevance

This study highlights the translational potential of ERK1/2 phosphorylation (p-ERK) as a predictive biomarker for the efficacy of immune checkpoint inhibitors in patients with recurrent glioblastoma. The immunosuppressive tumor microenvironment of glioblastoma has posed challenges to the effectiveness of immune checkpoint inhibitors. However, the presented data, validated across three cohorts, offer a pathway toward a more nuanced, personalized therapeutic strategy, aiming to pinpoint those patients most poised for a positive response to immune checkpoint inhibition. Before p-ERK can be considered for patient selection in clinical settings, it is important to establish standardized protocols for evaluating p-ERK to improve accuracy and reliability. Alternatively, the use of cost-effective and readily available tests to measure MAPK pathway activation could be integrated into personalized treatment strategies for patients with glioblastoma undergoing immune checkpoint blockade therapy.

following adjuvant anti-PD-1 therapy (7). Although this study provided supportive evidence on a means of identifying patients with glioblastoma that are likely to respond to PD-1 blockade, these studies were not performed in a blinded fashion. Further rigorous validation in additional independent cohorts of patients with glioblastoma was deemed necessary before this biomarker could be considered for patient selection in the clinical setting.

Considering the evidence of tolerability and early indications of activity of intratumoral administration of ipilimumab in extracranial metastases of solid tumors (9, 10), a phase I clinical trial was conducted to explore the potential of the combination of ipilimumab plus nivolumab in patients with recurrent glioblastoma (11). This trial investigated a novel approach by performing an intracerebral administration of ipilimumab with or without nivolumab in combination with intravenous nivolumab in patients with resectable high-grade gliomas and glioblastoma at recurrence.

In this study, we aimed to investigate the predictive value of p-ERK in a cohort of patients with high-grade gliomas and glioblastoma treated with intracerebral and intravenous immune checkpoint blockade within this trial. We analyzed p-ERK in tumor samples from patients treated with intracerebral administration of either nivolumab only or nivolumab plus ipilimumab followed by postoperative immunotherapy. To add rigor to our analysis, we investigated this biomarker in line with the REporting recommendations for tumor MARKER prognostic studies (REMARK) criteria (12). Utilizing a systematic approach involving multiple teams, one team was dedicated to p-ERK quantification, another consolidated relevant clinical, demographic, and outcome data, while a third specialized statistics team managed predetermined analyses. Building on this primary analysis, we embarked on a validation across three distinct cohorts of patients with glioblastoma. Such validation was essential to not only fortify our initial findings but also ensure their generalizability. The combined results from this study support the use of p- ERK as a predictor of OS in patients with glioblastoma treated with immune checkpoint blockade.

Materials and Methods

Study design and analysis by REMARK criteria

This was an analysis of a clinical trial in which p-ERK was evaluated as a biomarker for response to immune checkpoint blockade (anti-PD-1 and anti-CTLA-4 therapies) in patients with recurrent glioblastoma. All patients included in the analysis received treatment with either ipilimumab (10 mg) or ipilimumab (5 mg) plus nivolumab (10 mg) followed by postsurgical intravenous nivolumab (10 mg; NCT03233152; ref. 11). Within the treated patients, there were two cohorts: cohort 1 (intracerebral injection of nivolumab only) and cohort 2 (intracerebral injection of nivolumab plus ipilimumab). The study was conducted in accordance with institutional ethical regulations and the Declaration of Helsinki principles. All patients provided written informed consent. Clinicopathologic data are provided in Supplementary Table S1.

We collected the tumor samples from 27 patients enrolled in the phase I trial cohort that was previously published for which we had tumor samples available for analysis. We employed the same methodology described previously for the staining and quantification of p-ERK cell density in tumor samples from these patients (7). A neuropathologist evaluated whether the samples contained a sufficient amount of tumor tissue and delineated these regions for further quantification of p-ERK cell density. Out of 27 tumor samples, we analyzed 24 samples from patients whose tumors were of good quality and acceptable for IHC analysis (**Fig. 1A**). To strengthen the rigor of the analysis performed in this cohort, we adhered to the REMARK criteria for our workflow (12). In this way, we specified the conditions to be analyzed *a priori* which included the association of p-ERK cell density with OS evaluated by log-rank test and Cox proportional hazards model in the complete patient cohort [recurrent isocitrate dehydrogenase (*IDH*)-mutant high-grade glioma and recurrent glioblastoma] and only in patients with wild-type *IDH* glioblastoma (**Fig. 1B**). We specified that the staining for p-ERK and the quantification of p-ERK cell density to be done in a blinded fashion with regard to outcomes and other clinicopathological characteristics.

After the initial analysis, to enhance the statistical power and robustness, we integrated data from two other independent cohorts with the current one. This pooling resulted in an amalgamated dataset from three different cohorts.

IHC staining

Tumor samples from patients with glioblastoma were preserved in paraffin blocks. IHC and hematoxylin and eosin staining was performed using standard immunoperoxidase staining of formalin-fixed paraffin-embedded tissue section of 5 μm from glioblastomas obtained during surgery. Paraffin sections were deparaffinized with xylene in the stainer followed by heat-mediated antigen retrieval with sodium citrate buffer. Next, slides were stained against mouse anti-phospho-p44/42 (p-ERK1/2; Cell Signaling Technology, 1:500 dilution). Sections were counterstained with hematoxylin, dehydrated, and mounted with coverslips. Staining was performed in the DAKO Autostainer Link48 slide stainer (Agilent Technologies). Slides were scanned with the Hamamatsu K.K. Nanozoomer 2.0 HT and visualized with NDP.view2 Viewing software for tumor delineation.

Quantification of p-ERK cell density in tumor samples

Following the scanning of stained slides, a neuropathologist outlined the tumoral regions on each sample in a blinded fashion regarding treatments, survival outcomes, and other clinical characteristics. In delineated regions, HistoQuest v.6.0 software (TissueGnostics) was employed for the quantification of p-ERK cell density in the delineated tumor regions (Supplementary Fig. S1). In brief, we employed a segmentation method in which the nucleus was identified and separated from the cytoplasm using a ring mask with an interior radius of

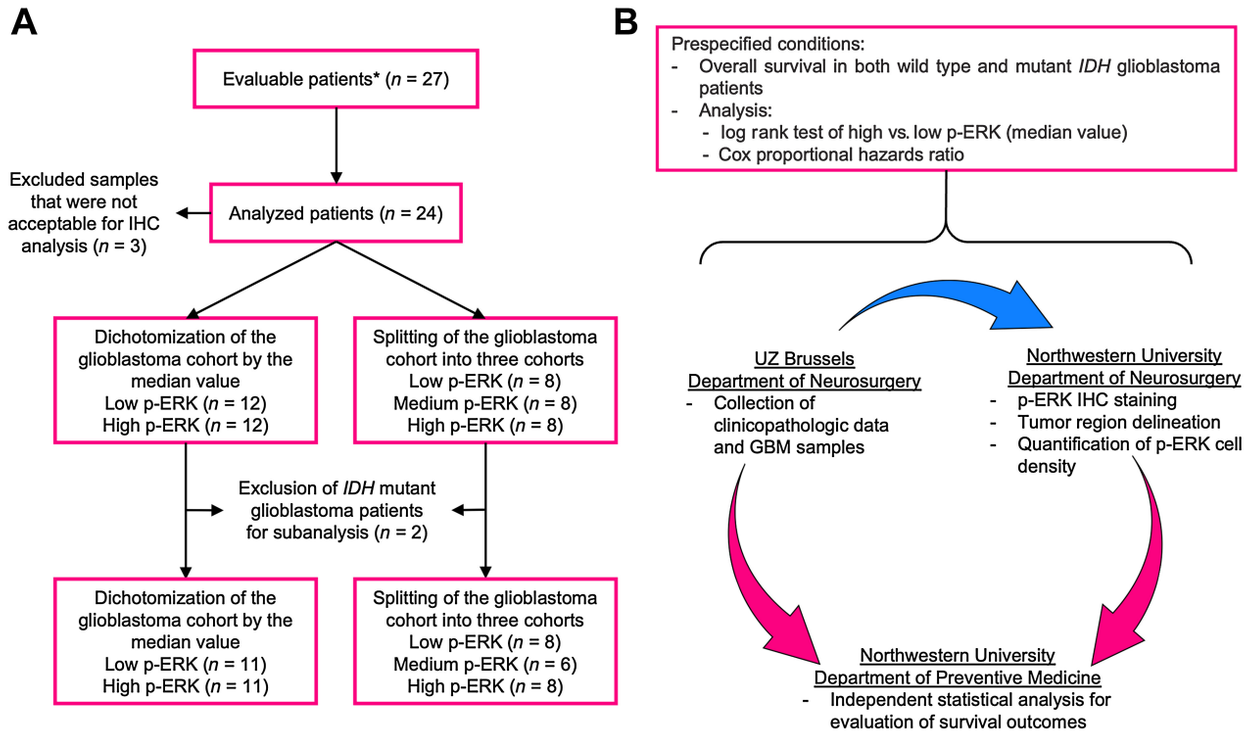

Figure 1.

Analysis workflow and application of the REMARK criteria. **A,** Diagram showing the selection of glioblastoma patient samples and formation of groups based on p-ERK cell density. **B,** Survival analysis workflow using the REMARK criteria for both complete patient cohort and patients with wild-type *IDH* glioblastoma. The blue arrow represents the transfer of tumor samples and the pink arrows represent the transfer of data. *Patients from the phase I clinical trial with tissue available for analysis.

−0.45 μm and an exterior radius of 0.91 μm with a cytoplasm cell mask. With this, the cytoplasm was defined for quantification. For nuclei detection, the software parameters were adjusted to detect hematoxylin [red, green, blue (RGB): 30, 45, 84] and 3,3′-Diaminobenzidine (DAB) chromogen (RGB: 94, 48, 14) in combination with hematoxylin. A mean intensity threshold of 150 was set up to define p-ERK$^+$ cells. For slides where there were multiple regions for a given tumor, these regions were quantified, and the resulting values were averaged to generate a single value for one tumor sample. Similar to our previous study (7), p-ERK cell density was defined as the number of p-ERK$^+$ cells in a given area (mm^2).

For the quantification of p-ERK staining intensity in endothelial cells, these cells were subjected to segmentation by creating regions of interest (ROI). Next, we employed HistoQuest v.6.0 software (TissueGnostics) to quantify the mean intensity of the sum of brown vessels where the ROI was created.

Survival and statistical analysis

Following the REMARK criteria for biomarker validation, we investigated p-ERK as a predictive of OS in 24 evaluable tumor samples of patients with recurrent glioblastoma from a phase I clinical trial. Prespecified conditions to analyze were established *a priori*, which included the association of p-ERK cell density with OS evaluated by log-rank test and Cox proportional hazards model in the complete patient cohort (patients with wild-type and *IDH*-mutant) and only in the patients with wild-type *IDH* glioblastoma. OS was defined as the time from informed consent to death or last seen alive. Age was also determined at the time when informed consent was obtained. OS was censored for patients who were alive at the time of the cut-off date. We ensured that the staining and quantification of p-ERK were performed in a blinded fashion concerning outcomes and other clinicopathologic characteristics. The p-ERK quantification results, survival outcomes, and clinicopathologic characteristics were sent to an independent statistician for survival analysis. The dichotomization of the cohort into high and low p-ERK patients with glioblastoma was determined using the median of all values in the cohort derived from software-based quantification of p-ERK cell density. Patients were also divided into three groups (high, intermediate, and low) based on p-ERK cell density. We employed a two-sided log-rank test for survival analysis between high and low p-ERK groups. For exploratory purposes, patients were divided into three groups ($n = 8$ per group) based on p-ERK cell density.

To support our primary findings regarding p-ERK's predictive capacity for survival after immunotherapy in patients with glioblastoma, we pooled data from two previously reported cohorts (7, 13), and the current cohort (11), which expanded the sample size to 65 patients with glioblastoma. Focusing on these cohorts, we scaled p-ERK values by the median within each cohort and applied a Cox proportional hazards model. The initial approach utilized a Cox univariable model for p-ERK followed by a multivariable Cox model to adjust for potential confounders. The model considered levels of the biomarker, age, steroid use, and differences in cohorts. The pooling of data from multiple cohorts enhances the statistical power of this analysis. For a comprehensive validation, we obtained the HRs for

p-ERK from the multivariable Cox model and undertook a meta-analysis using a random-effects model integrating data from the three distinct cohorts, inclusive of the current study cohort. R v.4.0.2 and GraphPad Prism v. 9.5.1 were used for statistical analyses.

Data availability

The data generated in this study are available upon request from the corresponding author.

Results

Patient baseline characteristics

We sought to evaluate p-ERK as a predictive biomarker in a cohort of patients with recurrent high-grade glioma and glioblastoma treated with immune checkpoint blockade where tumor samples and clinical outcomes were collected prospectively (11). This cohort included 2 patients with *IDH*-mutant high-grade recurrent gliomas and 25 patients recurrent with wild-type *IDH* glioblastoma. As part of the phase I clinical trial, patients received a fixed dose of nivolumab (10 mg) through intravenous administration and underwent a maximal safe resection within 24 hours. Following this, patients received an injection of either ipilimumab (10 mg) in cohort 1 or a combination of ipilimumab (5 mg) and nivolumab (10 mg) in cohort 2, directly into the brain tissue surrounding the brain cavity. Nivolumab was administered intravenously again every 2 weeks after surgery.

To identify potentially clinically significant p-ERK cut-off points, we dichotomized p-ERK at the median value of 540.5 cells/mm^2, categorizing glioblastoma samples into high p-ERK (>540.5 cells/mm^2) and low p-ERK (≤540.5 cells/mm^2). For a more nuanced analysis, we further divided samples into low (≤55), medium (55–895), and high (>895) p-ERK groups. The baseline and clinical characteristics were similar between groups (**Table 1**).

Out of 27 evaluable subjects, 25 had died at the time of analysis from tumoral progression. The median OS for all evaluable patients was 38.3 weeks [95% two-sided confidence interval (CI): 30–75.7] with 25th and 75th quantiles corresponding to 20.1 and 87 weeks, respectively (Supplementary Fig. S2). In the analysis cohort that included 24 evaluable subjects, we determined that there were 22 deaths. The median OS for the analysis cohort ($n = 24$) was 36.1 weeks (95% two-sided CI: 30–87) with 25th and 75th quantiles corresponding to 19.3 and 97.3 weeks, respectively (**Fig. 2**).

Table 1. Demographics and clinical data of patients with glioblastoma.

Patient characteristics	High p-ERK ($n = 12$)	Low p-ERK ($n = 12$)	Total number of patients ($n = 24$)
Cohort (%)			
Cohort 1 (nivo)	2 (16.67)	1 (8.33)	3 (12.5)
Cohort 2 (nivo + ipi)	10 (83.33)	11 (91.67)	21 (87.5)
Age			
Mean ± s.d.	59.2 (10.5)	51.8 (10.1)	55.5 (10.8)
Median (range)	62.2 (41.5–72.4)	53.4 (37.7–70.3)	55.1 (37.7–72.4)
ECOG PS (%)			
0	3 (25)	7 (58.33)	10 (41.67)
1	7 (58.33)	5 (41.67)	12 (50)
2	2 (16.67)	0	2 (8.33)
IDH status, *n* (%)			
Wild type	11 (91.67)	11 (91.67)	22 (91.67)
Mutant	1 (8.33)	1 (8.33)	2 (8.33)
Use of corticosteroids (%)			
No	10 (83.33)	6 (50)	16 (66.67)
Yes	2 (16.67)	6 (50)	8 (33.33)

Note: High p-ERK: ≥ 540.5 cells/mm^2, Low p-ERK: ≤ 540.5 cells/mm^2.
Abbreviations: ipi, ipilimumab; nivo, nivolumab; s.d, standard deviation.

Comparison of p-ERK values across glioblastoma cohorts

Considering that preanalytical variables can influence the integrity of phosphorylated proteins in glioblastoma (7), we performed a comparison of the distribution of p-ERK cell density in tumor regions obtained from the current clinical trial cohort, and previous cohorts of patients with glioblastoma from different institutions (7). We determined that the cohort of patients from both Northwestern University and Columbia University (NU/CU) had a median value of 3,207 cells/mm^2 (25%–75%: 1,786–4,148 cells/mm^2), the cohort from University of California, Los Angeles (UCLA) had a median value of 3,616 cells/mm^2 (25%–75%: 1,410–5,053 cells/mm^2), and the current cohort had a median value of 540.5 cells/mm^2 (25%–75%: 39.16–1,287 cells/mm^2; **Fig. 3A**). Whereas the NU/CU cohort and the UCLA cohort did not show differences in the cell density of p-ERK$^+$ cells in tumors ($P = 0.8131$, one-way ANOVA), tumors from the UZ Brussels patient cohort had lower values of p-ERK$^+$ cells compared with the NU/CU cohort ($P < 0.0001$, one-way ANOVA) and the UCLA cohort ($P < 0.0001$, one-way ANOVA).

Determination of p-ERK epitope integrity across cohorts through staining intensity on endothelial cells

Given that a high percentage of endothelial cells express p-ERK (7) which is predominantly expressed during neovasculogenesis (14), we evaluated the intensity of p-ERK staining in endothelial cells in glioblastomas from previous and current cohorts and compared them against endothelial cells from tumor samples fixed at different ischemic timepoints (0, 0.5, 1, 2, and 3 hours; ref. 7). We noticed that the p-ERK intensity in endothelial cells from glioblastomas from the UZ Brussels cohort most closely resembled the endothelium staining intensity of the samples that were fixed after 3 hours of ischemic time ($P < 0.8165$, one-way ANOVA; **Fig. 3B**). These results suggest some degradation of the p-ERK phosphoepitope across samples in the UZ Brussels cohort and highlight the need for standardized protocols and methods to evaluate p-ERK and its association with OS following immune checkpoint blockade in patients with glioblastoma.

Association of p-ERK with survival in patients with *IDH* wild-type glioblastoma treated with immune checkpoint blockade

Whereas we found differences in p-ERK values and intensities in glioblastomas from patients enrolled in the phase I clinical trial, we adhered to our initial prespecified conditions for OS analysis and used the p-ERK median value to partition groups (high and low p-ERK groups). Thus, through independent statistical analysis of survival outcomes, we evaluated whether p-ERK was associated with increased OS in the complete cohort (patients with *IDH* wild-type glioblastoma and *IDH*-mutant high-grade glioma, $n = 24$) and in *IDH* wild-type glioblastoma only patients ($n = 22$). By analyzing the complete analysis cohort ($n = 24$), we found that patients with high p-ERK tumors (>540.5 cells/mm^2) treated with PD-1 blockade had longer median OS (47.6 weeks) compared with those with low-p-ERK tumors (≤540.5 cells/mm^2) although not statistically significant ($P = 0.29$, log-rank test; **Fig. 4A**). However, in the subset of patients with *IDH* wild-type glioblastoma, we found that patients

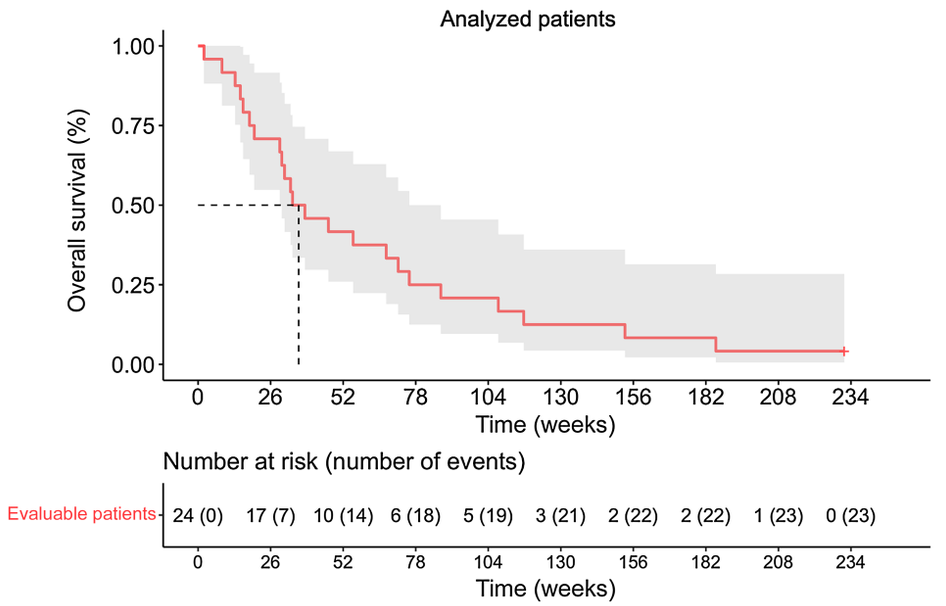

Figure 2.
Survival outcomes of patients with high-grade glioma. Kaplan–Meier curve showing the OS of analyzed patients with glioblastoma.

with high-p-ERK tumors (>540.5 cells/mm^2) treated with PD-1 blockade exhibited prolonged survival [median OS: 55.6 weeks (95% CI: 31–Not Applicable (NA)] compared with those with low-p-ERK tumors [median OS: 30 weeks (95% CI: 18.34, NA)] ($P = 0.036$, log-rank test; **Fig. 4B**). In addition, we also applied the median value (3,207 cells/mm^2) obtained from the p-ERK quantification of a previous analysis we performed to dichotomize the current glioblastoma patient cohort (7). This dichotomization identified one patient with glioblastoma with a tumor classified as high p-ERK that lived 185.57 weeks after therapy (Supplementary Fig. S3).

For exploratory purposes, patients with *IDH* wild-type glioblastoma were divided into three groups (high, intermediate, and low) based on p-ERK cell density. We observed an incremental improvement in OS with high p-ERK (>895, $n = 8$) patients with glioblastoma exhibiting a median OS of 81.6 weeks (95% CI: 33.86–NA), intermediate p-ERK (55–895, $n = 6$) median OS of 39.9 weeks (95% CI: 33.14–NA), and low p-ERK (≤55, $n = 8$) group with a median OS of 19.3 weeks (95% CI: 16.14–NA) ($P = 0.038$, log-rank test; **Fig. 4C**).

These preliminary results show the potential of p-ERK as a predictive biomarker for patients with glioblastoma treated with immune checkpoint inhibitors demonstrated in an additional independent cohort of patients.

Validation of p-ERK as a predictive biomarker across three independent cohorts

To strengthen the major finding that p-ERK predicts response to immune checkpoint blockade in patients with recurrent glioma, we explored p-ERK as a predictive biomarker by pooling data from two previously published cohorts (7, 13) as well as the current cohort (11), yielding a cohort of 65 patients with *IDH* wild-type glioblastoma. Then, we conducted a multivariate Cox proportional hazards model incorporating the p-ERK levels and factors that influence survival such as age, *IDH* mutation, and steroid usage. Considering the differences in p-ERK values between cohorts, we introduced "cohort" (UZ Brussels, NU/CU, and UCLA) as a stratification factor to separate baseline hazard functions for each cohort while estimating a common HR for the biomarker. The results of this analysis indeed reveal that p-ERK remains a significant predictor of survival, even after adjusting for the aforementioned covariates ($P = 0.011$; **Fig. 5A**). The adjusted HR for p-ERK is 0.711, reinforcing its potential utility as a biomarker.

To further substantiate our findings, a complementary analytical strategy was employed. After consolidating data across the three cohorts and deriving the multivariable Cox model outputs, we obtained the cohort-specific adjusted HRs. These were used to perform a meta-analysis employing a random-effects model to determine whether overall p-ERK is associated with a decreased risk of death across cohorts. After adjusting for differences among the three cohorts within a random-effects model, we observed the average HR effect across the studies for p-ERK is 0.19 (95% CI: 0.04–0.94; $P = 0.0424$; **Fig. 5B**). This value suggests that as p-ERK increases, the risk of death decreases in patients treated with immune checkpoint blockade.

Leveraging our preliminary insights and the link of p-ERK with OS in patients with *IDH* wild-type glioblastoma from the phase I clinical trial (**Fig. 4B**), we analyzed the data from patients with *IDH* wild-type glioblastoma from the three cohorts, yielding a comprehensive sample of 56 patients. To account for the intrinsic variations in p-ERK values across three cohorts and other pivotal factors that influence OS, we performed a multivariable Cox model. We incorporated biomarker levels, cohort differences, age, and steroid consumption. This model pointed toward a statistically significant reduction in the risk of death in patients with glioblastoma treated with immune checkpoint blockade. Specifically, for every unit increase in p-ERK (scaled by the median), there was a decrease in death risk by 32.5% (adjusted HR = 0.675; 95% CI: 0.5162–0.8827; $P = 0.004085$; **Fig. 5C**). Notably, both age (adjusted HR = 1.048; 95% CI: 1.019–1.077; $P = 0.000886$) and steroid use (adjusted HR = 1.846; 95% CI: 1.033–3.297; $P = 0.038273$) were significant survival predictors associated with an increased death risk. The concordance statistic of our model, standing at 0.707, showcases the reliable ability of these covariates in predicting survival outcomes.

Through analyses spanning multiple independent cohorts and employing different statistical survival methodologies, our results support the predictive properties of p-ERK levels in patients with recurrent glioblastoma. Specifically, elevated p-ERK expression is

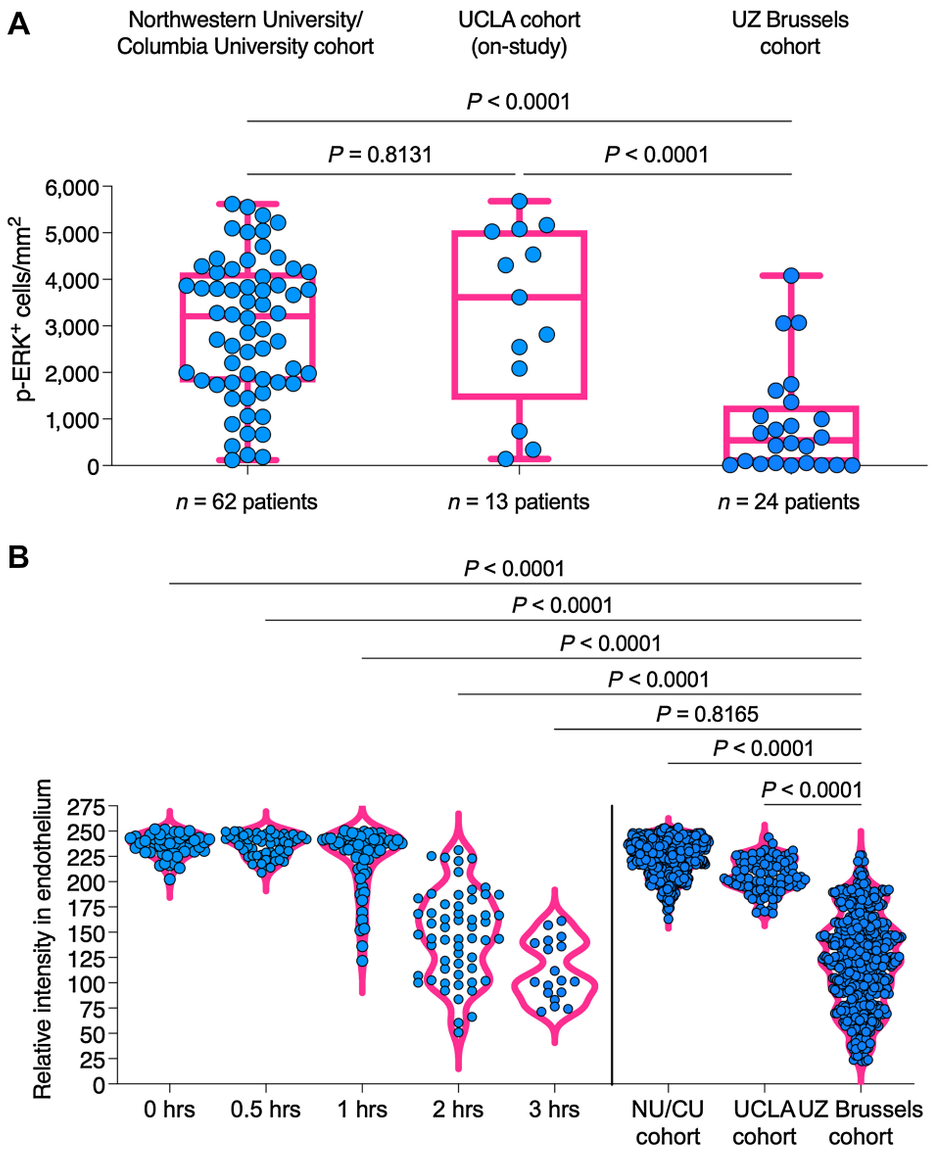

Figure 3.
Comparison of p-ERK values across independent glioblastoma cohorts. **A,** Distribution of p-ERK cell density values for three different glioblastoma patient cohorts [NU/CU cohort from Arrieta and colleagues (7), UCLA cohort from Cloughesy and colleagues (13), and UZ Brussels]. **B,** Intensity of p-ERK staining in endothelial cells from three glioblastomas at different periods of ischemic time and glioblastomas across the three cohorts. Each dot represents p-ERK intensity on individual endothelial cells within the same samples. *P* values by two-sided Kruskal–Wallis test with *post hoc* Dunn multiple comparison test.

associated with a reduced risk of mortality, in patients undergoing immune checkpoint blockade therapy.

Discussion

The quest to identify patients with glioblastoma who are likely to respond to immunotherapy is of paramount importance, particularly in light of the negative outcomes of clinical trials evaluating immune checkpoint blockade in unselected patient populations (2–4). Several clinical trials assessing the use of nivolumab, an anti-PD-1 therapy, in patients with glioblastoma have failed to demonstrate any survival advantage. The CheckMate 143 clinical trial showed that the median OS of nivolumab-treated patients was similar to that of bevacizumab-treated control patients (2). Likewise, the CheckMate 498 clinical study failed to meet its primary endpoint of OS improvement in patients with newly diagnosed glioblastoma with unmethylated *MGMT* (3). Nivolumab in combination with Temozolomide (TMZ) and radiotherapy was also found not to be superior to TMZ, radiotherapy, and placebo in patients with newly diagnosed glioblastoma with methylated *MGMT* promoter (4).

Despite these discouraging outcomes, several studies and case reports have shown that certain subsets of patients with glioblastoma exhibit prolonged OS and durable radiographic responses following immune checkpoint blockade (5, 6, 8, 13, 15–17). Clinical evidence has suggested that tumor phenotype and the composition of the tumor microenvironment might influence responses to immunotherapy in glioblastoma (5, 7, 18). This highlights the need to identify patients who are likely to respond to immunotherapy and which variables may impact treatment efficacy.

In this study, we sought to evaluate the potential of p-ERK as a predictive biomarker for OS in patients with recurrent glioblastoma treated with immune checkpoint blockade. Our analysis, based on a cohort of 24 patients with recurrent glioblastoma enrolled in a phase I clinical trial, provides additional evidence that p-ERK could serve as a valuable biomarker for the outcome of patients with glioblastoma receiving immune checkpoint inhibitors. This finding has important

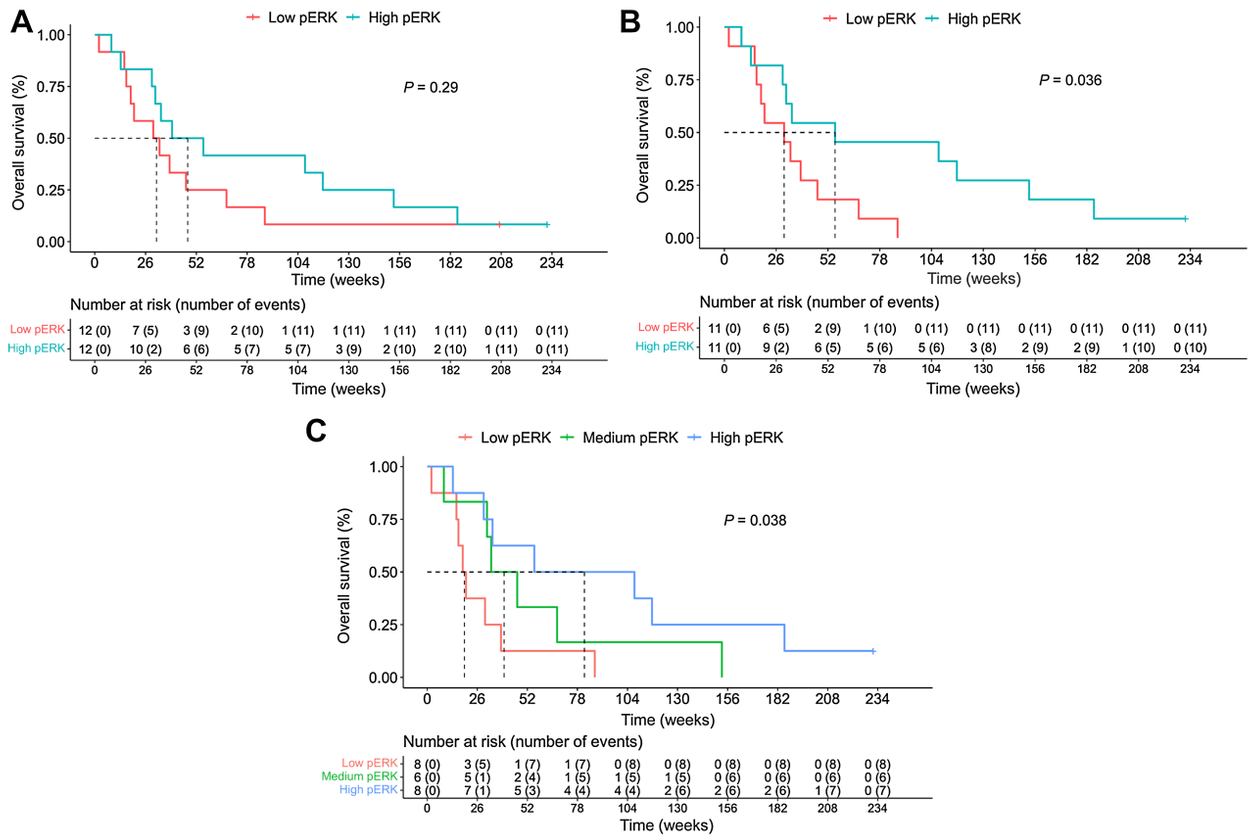

Figure 4.

Association of p-ERK with survival in patients with glioblastoma treated with immune checkpoint blockade. **A,** OS comparison between high and low p-ERK groups in the complete glioblastoma patient cohort. **B,** OS comparison between high and low p-ERK groups in patients with wild-type *IDH* glioblastoma. **C,** OS comparison among high, medium, and low p-ERK groups in the exploratory analysis.

implications for the clinical management of glioblastoma, as it may help identify patients who are more likely to benefit from immunotherapy and optimize treatment strategies.

Our results add to the growing body of literature supporting that MAPK pathway activation in gliomas is associated with favorable outcomes following immunotherapy (7, 8). We recently showed that activation of the MAPK/ERK pathway, indicated by phosphorylation of ERK1/2, was associated with OS following adjuvant anti-PD-1 immunotherapy in two independent recurrent glioblastoma patient cohorts (7). This observation suggests that the activation of this pathway may be a critical determinant of response to PD-1 inhibitors in patients with recurrent glioblastoma, including those who do not harbor *BRAF/PTPN11* mutations.

Whereas the MAPK pathway signaling is associated with response to immunotherapy in gliomas, no causal relationship has been established to date. Interesting observations suggest that gliomas with activation of the MAPK pathway have a distinct tumor immune microenvironment. We observed that glioblastoma tumors with elevated p-ERK were associated with a distinct microglial phenotype including elevated expression of MHC-class II molecules (7). Moreover, we also showed that when gliomagenesis occurs in the absence of CD8 T cells in murine models, the resultant gliomas exhibit elevated p-ERK and increased abundance of $Iba1^+$ myeloid cells (19). Additional recent studies have provided further insight into the relationship between MAPK activation and a distinct microenvironment in gliomas (20, 21).

The use of the REMARK criteria was important to ensure the rigor of our analysis. By specifying the conditions to be analyzed *a priori* and employing a blinded approach to p-ERK quantification, we minimized potential sources of bias in our study. Moreover, our use of an independent statistician for survival analysis further bolstered the reliability of our results.

An important aspect of this study was the comparison of p-ERK values in independent glioblastoma cohorts from different institutions. We found significant differences in $p\text{-}ERK^+$ cell density and staining intensity, highlighting the need to develop and establish standardized protocols and methods for evaluating p-ERK and its association with OS following immune checkpoint blockade in patients with glioblastoma.

Our study has several strengths, including the prospective collection of tumor samples and clinical data, as well as the adherence to the REMARK criteria for survival analysis. Nevertheless, our study also has limitations. The relatively small sample size and the heterogeneity of p-ERK values across cohorts necessitate further investigation in a larger, more diverse patient population. An important limitation of p-ERK to identify patients with glioma who might be susceptible to immunotherapy is the relatively unstable integrity of the phosphoepitope, which is subject to degradation with increasing ischemia

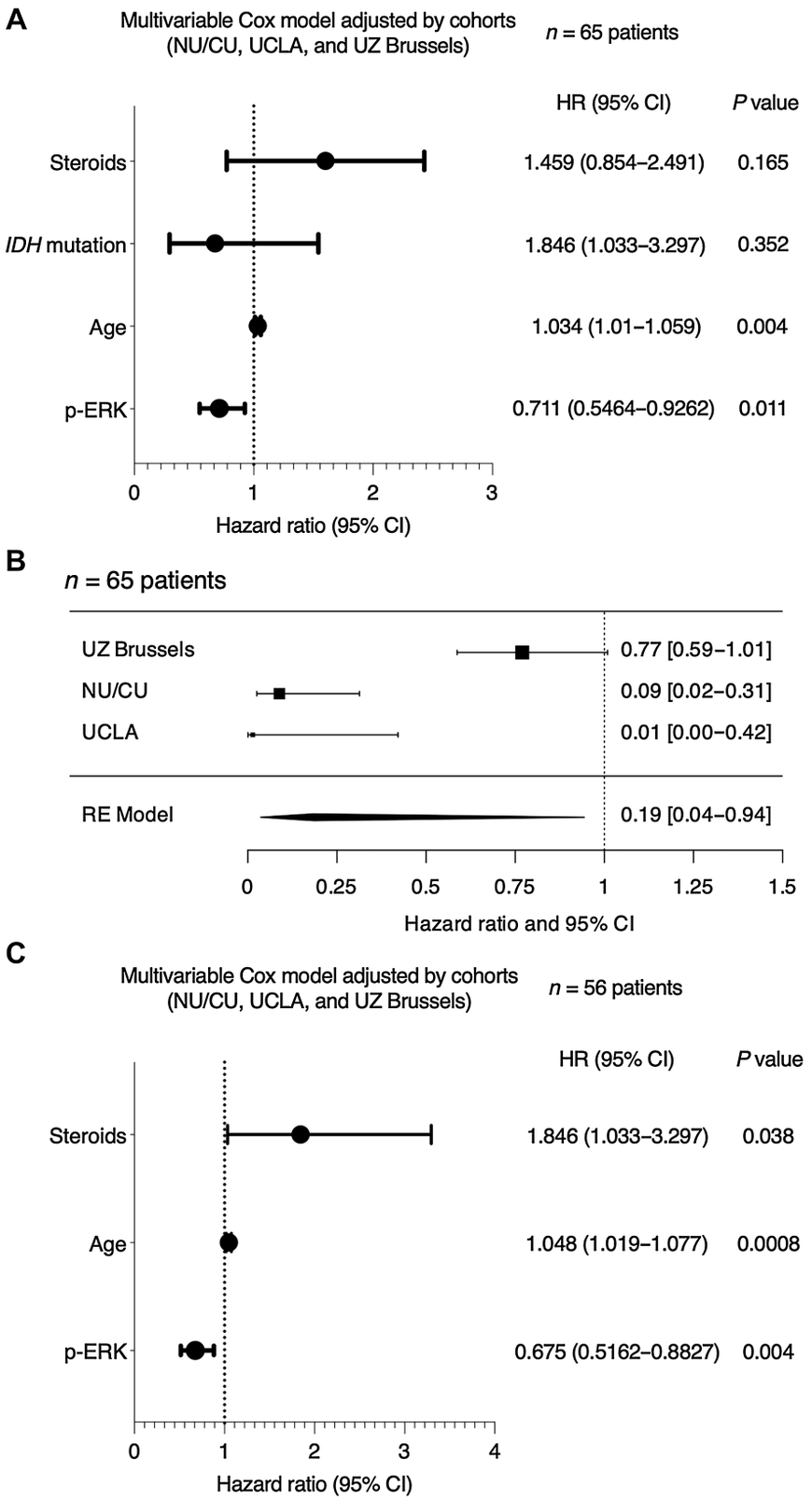

Figure 5.

Multivariable analysis of p-ERK and covariates influencing survival trends in patients with recurrent glioma receiving immune checkpoint blockade. **A,** Forest plots showing the association of steroids, *IDH* mutation, age, and p-ERK after adjusting the multivariable Cox model with cohorts considered as a covariate (N = 65 patients with recurrent glioma). **B,** Forest plot showing the results from the meta-analysis employing a random-effects model in three different cohorts (UZ Brussels, NU/CU, and UCLA; N = 65 patients with recurrent glioma). **C,** Forest plots showing the association of steroids, age, and p-ERK after adjusting the multivariable Cox model with cohorts considered as a covariate (N = 56 patients with recurrent glioma). Results are presented as HR (95% CI) in **A**, **B**, and **C**. *P* value by two-sided Wald test in **A** and **C**.

time. p-ERK determination in tumor-related endothelial cells can help identify samples with suboptimal quality. Yet our analysis suggests that even in the context of some degradation of p-ERK, this biomarker might segregate patients with favorable outcomes following immunotherapy. We have shown that p-ERK integrity remains reasonable when tumor samples are fixed within 30 minutes to 1 hour after excision. Ideally, timely processing of specimens would overcome this limitation of p-ERK as a biomarker. Ultimately, controlling p-ERK phospho-epitope integrity will be important for implementing this biomarker for patient care in the future.

To supplement our initial findings from a limited sample size and to further bolster the argument for p-ERK as a biomarker, we consolidated data from three independent cohorts and undertook comprehensive statistical analyses. Utilizing a meta-analysis approach and incorporating the Cox proportional hazards model, our findings revealed that elevated p-ERK levels corresponded with a marked reduction in mortality risk.

While clinical trials have offered mixed outcomes, our study delineates that a subset of patients, identifiable through molecular markers like p-ERK, might achieve significant therapeutic advantages. Our investigation underscored the potential of p-ERK as a promising biomarker for discerning patients with glioblastoma who may benefit from immune checkpoint blockade. However, variations across different cohorts and the inherent challenges with p-ERK stability emphasize the necessity for methodologic standardization, rigorous validation, and continual reassessment. As we move forward, integrating such biomarkers into clinical practice requires combining research with practical clinical considerations.

Authors' Disclosures

V.A. Arrieta reports a patent for METHODS OF TREATING MALIGNANT GLIOBLASTOMA pending. K.B. Burdett reports grants from NIH during the conduct of the study; in addition, K.B. Burdett has a patent for Disc-ID-23-05-10-002 issued to 2023-102. J.K. Schwarze reports personal fees from Novartis and nonfinancial support from Amgen and MSD outside the submitted work. M. McCord reports grants from NCI during the conduct of the study. H. Zhang reports grants from NIH during the conduct of the study. R. Stupp reports personal fees from Alpheus Medical, Northwest Biotherapeutics, Zai Lab, Celularity, GT Medical Technologies, AstraZeneca, Boston Scientific, and Novartis, grants and personal fees from Carthera, and grants from BMS outside the submitted work; in addition, R. Stupp has a patent for IP pending and issued. A.M. Sonabend reports non-financial support from BMS, non-financial support and other support from Agenus and Carthera, and personal fees from Carthera and EnClear therapeutics outside the submitted work; in addition, A.M. Sonabend has a patent for Patents filed by Northwestern University issued. No disclosures were reported by the other authors.

Authors' Contributions

V.A. Arrieta: Conceptualization, data curation, formal analysis, investigation, visualization, methodology, writing–original draft, writing–review and editing. **J. Duerinck:** Supervision, validation, investigation, methodology, writing–review and editing. **K.B. Burdett:** Data curation, formal analysis, investigation, visualization, methodology, writing–review and editing. **K.J. Habashy:** Formal analysis, investigation, writing–review and editing. **W. Geens:** Data curation, methodology, writing–review and editing. **A. Gould:** Data curation, investigation. **J.K. Schwarze:** Data curation. **C. Dmello:** Investigation. **K.-S. Kim:** Investigation. **R. Saganty:** Investigation. **L. Chen:** Investigation. **A. Moscona:** Writing–review and editing. **M. McCord:** Methodology, writing–review and editing. **C. Lee-Chang:** Formal analysis, investigation. **C.M. Horbinski:** Validation, investigation, methodology. **H. Zhang:** Data curation, formal analysis, investigation, visualization, methodology. **R. Stupp:** Formal analysis, supervision, investigation, methodology. **B. Neyns:** Resources, data curation, supervision, writing–review and editing. **A.M. Sonabend:** Conceptualization, resources, supervision, funding acquisition, investigation, writing–original draft, project administration, writing–review and editing.

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Note

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