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Immunotherapeutic early-phase clinical trials and malignant gliomas: A single-center experience and comprehensive immunophenotyping of circulating leukocytes

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Abstract

Background. Immunotherapeutic early-phase clinical trials (ieCTs) increasingly adopt large expansion cohorts exploring novel agents across different tumor types. High-grade glioma (HGG) patients are usually excluded from these trials.

Methods. Data of patients with recurrent HGGs treated within multicohort ieCTs between February 2014 and August 2019 (experimental group, EG) at our Phase I Unit were retrospectively reviewed and compared to a matched control group (CG) of patients treated with standard therapies. We retrospectively evaluated clinical, laboratory, and molecular parameters through univariate and multivariate analysis. A prospective characterization of circulating leukocyte subpopulations was performed in the latest twenty patients enrolled in the EG, with a statistical significance cutoff of $P < .1$.

Results. Thirty HGG patients were treated into six ieCTs. Fifteen patients received monotherapies (anti-PD-1, anti-CSF-1R, anti-TGFβ, anti-cereblon), fifteen patients combination regimens (anti-PD-L1 + anti-CD38, anti-PD-1 + anti-CSF-1R). In the EG, median progression-free survival and overall survival (OS) from treatment initiation were 1.8 and 8.6 months; twelve patients survived more than 12 months, and two of them more than 6 years. Univariate analysis identified O^6-methylguanine DNA methyltransferase (*MGMT*) promoter methylation and total protein value at six weeks as significantly correlated with a better outcome. Decreased circulating neutrophils and increased conventional dendritic cells levels lead to significantly better OS.

Conclusions. A subgroup of EG patients achieved remarkably durable disease control. *MGMT* promoter methylation identifies patients who benefit more from immunotherapy. Monitoring dynamic changes of innate immune cell populations may help to predict clinical outcomes.

Key Points

- A subgroup of HGG patients treated into ieCTs achieved a durable clinical benefit.
- *MGMT* methylation may identify patients more likely to benefit from immunotherapy.
- Dynamic changes of circulating neutrophils and dendritic cells may predict clinical outcomes.

Importance of the Study

Although few effective therapeutic options exist to date, clinical trials accrual of recurrent high-grade glioma (HGG) patients still remains poor. The present study is the first that evaluated a population of recurrent HGG patients treated into immunotherapeutic early-phase clinical trials (ieCTs). Our analysis showed a surprisingly durable disease control reached only by a small proportion of patients and confirmed O^6-methylguanine DNA methyltransferase promoter methylation as a potential predictive biomarker of clinical benefit. Monitoring dynamic changes of circulating innate immune cells populations identified decreased total neutrophils and increased conventional dendritic cells levels as factors significantly correlated to a better outcome. Recruiting HGG patients into multicohort ieCTs exploring novel immunotherapeutics or combination approaches should be strongly encouraged, allowing us to push forward only strategies worthy of further clinical development, and to perform ancillary biomarker studies to identify factors useful to select the small subgroup of patients most likely to benefit from immunotherapy.

Prognosis of patients with a diagnosis of glioblastoma (GBM) remains extremely poor, with only a few effective therapeutic options available and minor improvements in survival over the past decades.[1] While many factors contribute to this lack of progress, one of the major hurdles is represented by the poor clinical trial accrual.[2,3]The advent of immunotherapy has recently revolutionized the therapeutic management of several "historically" resistant cancers.[4] The remarkable clinical successes of the immunomodulating agents led to an unprecedented evolution of the traditional drug development paradigm of anticancer new drugs, particularly involving the nature and goals of phase I trials.[5,6] Immunotherapeutic early-phase clinical trials (ieCTs) increasingly adopt innovative "adaptive" designs characterized by a rapid dose-escalation to explore the safety of novel agents and to determine the recommended dose and schedule, followed by large multiple expansion cohorts evaluating antitumor activity across different tumor types.[7,8] Only a few of these "new wave" multicohort pharmacological ieCTs allow the inclusion of patients with a diagnosis of malignant glioma. Reasons for this exclusion are many. First, ieCTs provide almost ever pharmacodynamics ancillary studies with the collection of serial bioptic tissue sampling for biomarkers analysis, simply not feasible in brain tumor patients. Second, given the well-recognized detrimental effect impairing immunotherapy efficacy,[9] the use of corticosteroids is often not allowed into ieCTs or permitted at very low-dose, while glioma patients frequently need corticosteroids to control brain edema. Finally, the disappointing results of the first randomized trials with immune-checkpoint inhibitors have further dampened the enthusiasm for immunotherapy as potential therapeutic breakthrough in the glioma field.[10,11] Critical barriers to an effective antitumor immunity are still represented by the peculiar central nervous system (CNS) immunological milieu, the variety of systemic and local immunosuppressive forces, the broad intratumoral heterogeneity, and the low immunogenicity configuring GBM as the paradigm of "immune-desert" cancers.[12]

Nevertheless, recent data from a small pilot randomized trial of neoadjuvant anti-programmed cell death protein 1 (PD-1) blockade in recurrent, surgically resectable GBM showed that the glioma microenvironment is susceptible to the immunomodulatory effects of preoperative immune-checkpoint inhibition, with a significantly improved survival compared to patients receiving pembrolizumab only as adjuvant.[13] In the present paper, we reviewed data of all consecutive patients with a diagnosis of recurrent high-grade gliomas (HGGs; WHO grade III-IV) treated into multicohort ieCTs in the early-drug development Unit of the Humanitas Cancer Center between 2014 and 2019. Different prognostic scores for patients with solid tumors treated into ieCTs have been built so far, but none of these include patients with glioma.[14,15] With that purpose, we investigated the prognostic and predictive value of a large series of clinical, laboratory, and molecular variables. In a subset of these patients, we also prospectively monitored different circulating immune cell populations, correlating their baseline values and dynamic changes during treatment with clinical outcome.

Materials and Methods

This is a retrospective, single-center study evaluating patients diagnosed with recurrent HGGs enrolled within ieCTs at Humanitas Cancer Center between February 2014 and August 2019. The study protocol design was approved by the institutional review board of our Center and was conducted according to national and local regulations.

Patients and Treatments

We evaluated all consecutive adult (≥18 years old) patients diagnosed with recurrent HGGs enrolled and treated within multicohort ieCTs at Humanitas Cancer Center Phase I Unit between February 2014 and August 2019. All patients who had received at least one administration of experimental treatment were included in the analysis representing our experimental group (EG). HGGs were considered as all histologically confirmed grade III and IV astrocytic gliomas according to 2016 WHO classification, recurred or progressed at least after surgery, radiotherapy, and/or chemotherapy with temozolomide.[16] Multicohort ieCTs were defined as phase I-II trials exploring the safety and the antitumor activity of novel immunomodulating agents (given as monotherapy or within combinations) in different tumor types (at least three malignancies).

A large series of clinical, laboratory, and molecular data were collected retrospectively for each patient of the EG at the time of entry into the ieCT. Clinical variables studied were age, gender, Eastern Cooperative Oncology Group performance status (ECOG PS), number and type of previous treatments, steroid use at baseline and during experimental treatment, tumor location, the extent of first surgical resection (partial or complete), type of ieCT (monotherapy or combination), and target of the experimental therapeutic intervention. Laboratory parameters were evaluated as baseline values and as dynamic six weeks (+/−1 week) changes after experimental treatment initiation and included: lactate dehydrogenase, albumin, total protein, hemoglobin, platelet, neutrophil, eosinophil, monocyte, and lymphocyte count, neutrophil to lymphocyte ratio (NLR). Molecular information collected were isocitrate dehydrogenase (*IDH*) 1/2 mutational status, O^6-methylguanine DNA methyltransferase (*MGMT*) promoter methylation status, programmed death-ligand 1 (PD-L1) expression, and mismatch repair (MMR) proteins.

As part of a larger prospective immunological translational study evaluating circulating and infiltrating leukocytes in patients with diffuse malignant gliomas active at our Institution (ONC/OSS-04/2017 study), a quantitative and immunophenotypical characterization of circulating leukocyte subpopulations was performed in a subset of patients of the EG.

Efficacy and Safety Endpoints

We assessed: overall response rate (ORR), defined as the proportion of patients with complete (CR) or partial response (PR) according to Response Assessment in Neuro-Oncology (RANO) criteria[17] by investigator review, disease control rate (DCR) defined as the percentage of patients with PR or CR, or stable disease (SD) ≥12 weeks, duration of response (DOR) defined as the time from first documented PR/CR to progressive disease (PD), progression-free survival (PFS; time from treatment initiation to the first documented PD or death from any cause), time to treatment failure (TTF; time from treatment initiation to discontinuation for any reason), overall survival (OS; time from treatment initiation to death from any cause).

Clinical and radiological evaluations were performed according to trial requirements in EG and according to standard-of-care guidelines in control group (CG). If allowed by the ieCT, in the presence of clinical benefit and/or when a pseudoprogression was suspected, patients continued to receive experimental treatment until PD confirmation. To better differentiate pseudoprogression from true progression we used functional and metabolic imaging (eg. perfusion MRI and 11C-methionine PET) and proper multidisciplinary tumor board discussion as per local practice. For the purpose of the present analysis, we registered as PD the date of the first radiological progression.

Treatment-related adverse events (TRAEs) were reported according to Common Terminology Criteria for Adverse Events (CTCAE) version 5.0.

Sample Staining and Flow Cytometry Data Acquisition

Whole blood samples were incubated with monoclonal antibodies (mAbs) under standard conditions. A complete list of the mAbs used to identify immune cells is reported in Supplementary Table S1. NK cells were identified as $CD45^+$ cells lacking the expression of CD3, CD14, and CD19. Among them, different subpopulations of NK cells were identified according to the expression of CD56 and CD16.[18] According to previous reports,[19,20] dendritic cells (DCs) were identified as $CD45^+$ cells lacking the expression of lineage markers (CD3, CD14, CD16, CD19, CD20, CD56) while constitutively expressing HLA-DR. Conventional DCs (cDCs) were identified as $CD123^-/CD11c^+$ DCs expressing either CD141 (cDC1s) or CD1c (cDC2s). Plasmacytoid DCs (pDCs) were identified as $CD123^+/CD11c^-$ DCs. Erythrocyte lysis was performed with Ammonium–Chloride–Potassium (ACK) lysing buffer, and dead cell exclusion was performed using the Fixable Viability Stain 780 (BD Biosciences, New Jersey, USA). All operations were done at 4°C in the dark. Monocytes were identified as $CD45^+$, $CD3^-$, $CD19^-$, $CD56^-$, $CD11b^+$, $CD66b^-$, HLA-DR^+ cells and were divided into three major subpopulations based on CD16 and CD14 expression: classical ($CD14^+CD16^-$), intermediate ($CD14^+CD16^+$), and nonclassical ($CD14^{dim}CD16^+$) monocytes.[21] Neutrophils were defined as $CD45^+$, $CD3^-$, $CD19^-$, $CD56^-$, $CD11b^+$, HLA-DR^-, $CD66b^+$ cells. Based on the expression of CD16 and CD62L, three neutrophil subsets were identified: immature or band ($CD16^{low}CD62L^+$), mature or classical ($CD16^+CD62L^+$) and activated or aged ($CD16^+CD62L^{low}$) neutrophils.[22] For monocytes and neutrophils analysis dead cell exclusion was performed using the Zombie Aqua™ Fixable Viability Kit (Biolegend, San

Diego, USA), and all incubations were performed at room temperature. Staining conditions for each mAb were preliminarily determined in titration assays, as previously described.[23] All samples were acquired on fluorescence-activated cell sorting (FACS) Symphony A5 flow cytometer (BD Biosciences, New Jersey, USA) and were analyzed with FlowJo software version 9.9.6 and 10.6.2 (FlowJo LLC, Ashland, Oregon). Gating strategies used to identify all these immune populations are shown in Supplementary Figure S1.

Immunological analyses were prospectively performed at baseline (T0), at the time of first tumor assessment after treatment initiation (T1), and at the end of treatment (TF).

Statistical Methods

Data were described as numbers and percentages or as median and range. Differences in the distribution of categorical variables were tested using χ^2 test, while differences in continuous data using median test.

Immunological parameters variation was defined according to the formula *T1 or TF—T0/T0 * 100,* in order to make individual patient variations comparable.

Follow-up time was estimated with the inverse Kaplan–Meier method. Survival curves were generated using the Kaplan–Meier method. Differences between groups were evaluated using the log-rank test.

The Cox proportional hazards regression model was used to calculate the hazard ratios (HRs) and their 95% confidence intervals (CIs) in univariable and multivariable evaluations.

We selected from the historical database of our institution a CG of 30 recurrent HGG patients treated in the same time frame with standard therapies (temozolomide, fotemustine, lomustine, and procarbazine, bevacizumab) matched (1:1) for sex, age (greedy algorithm with a maximum difference of 5 years), *IDH* mutational status, ECOG PS, and line of treatment with patients of the EG. A descriptive analysis was performed comparing survival in the two groups.

Since the high number of immunological parameters taken into consideration for the statistical analyses, the small sample size and the explorative nature of the study, we arbitrary decided to consider a *P*-value of .1 as critical value for reporting these results. A *P*-value of .05 was considered for all other results regarding clinical, demographical, and molecular parameters in descriptive and survival analyses.

All analyses were carried out with SAS software, version 9.4 (SAS Institute, Cary, NC).

Results

Patients and Treatments

Between February 2014 and August 2019, six out of 25 multicohort ieCTs conducted at Humanitas Cancer Center Phase I Unit allowed the inclusion of primary brain tumor (PBT) patients. Overall, thirty patients with recurrent HGG were treated into these six ieCTs, representing our EG (M/F: 20/10; median age: 50 years; ECOG PS 0/1: 22/8). Twenty-seven patients (90%) had a diagnosis of GBM (WHO grade IV), three of anaplastic astrocytoma (WHO grade III). Molecular analyses were performed on tumor tissue collected at the time of first diagnosis (63%) or after a second surgery when available (37%). Most tumors (21, 72%) were *IDH* wild-type (WT) and had the *MGMT* promoter methylated (20, 71%), PD-L1 was expressed in 20% of cases, whereas MMR deficiency was found only in two cases (7%). At the time of enrollment, twenty patients (66%) needed steroid therapy, with a median dexamethasone dose of 2 mg (range 1–6), whereas only ten patients were off steroids. Twenty-five (83%) patients have been treated into ieCTs at first recurrence after the standard Stupp regimen, while five (16%) as third-line therapy. Half of patients received experimental monotherapies [anti-PD-1 (NCT02054806, NCT02628067), anti-colony-stimulating factor 1 receptor (CSF-1R; NCT02829723), anti-transforming growth factor beta (NCT02937272), anti-cereblon (NCT01421524)], and half experimental combination regimens [anti-PD-L1 + anti-CD38 (NCT03637764), anti-PD-1 + anti-CSF-1R (NCT02829723)]. After discontinuation of ieCTs, eleven patients (37%) received one further systemic therapy (fotemustine, depatuxizumab mafodotin, dose-intensified temozolomide), whereas two patients (7%) were treated with two more lines (fotemustine, procarbazine, and lomustine); three patients (10%) underwent reirradiation and two (10%) re-surgery. Clinical and demographic characteristics of the EG and the CG are summarized in Table 1. Targets of ieCTs, numbers of treated patients, and the main immunological effects of experimental agents are shown in Table 2. Patients in our CG were treated with standard treatments, including fotemustine (77%), lomustine and procarbazine (20%), and bevacizumab (3%).

Clinical Outcomes and Biomarkers Analysis

At the time of data cutoff (15 December 2020), 24 patients (80%) had died, six patients (20%) were still alive, of which two currently progression-free. Three patients received experimental immunotherapeutic agents beyond one year, and one is still on treatment at the time of writing. Among patients in the EG, the ORR according to RANO criteria was 10%, including 1 CR (*IDH*-WT anaplastic astrocytoma) and 2 PR (1 *IDH* mutant GBM, 1 *IDH*-WT GBM). Among responders, the median time to response and median DOR were 5 months and 25 months, respectively. The DCR by investigator review was 40% (1 CR + 2 PR + 9 SD ≥ 12 weeks). Reasons for discontinuation of experimental treatments included radiological disease progression or clinical worsening (26 cases; 87%), TRAEs (7%; 2 cases), and accidental overdose (3%; 1 case). With a median follow-up of 55.6 months (range 1.2–81.8), median PFS, TTF, and OS of the EG were 1.8 months, 3.0 months, and 8.6 months, respectively. Twelve patients (40%) survived more than 12 months after the experimental treatment initiation, and two of them (*IDH*-WT, *MGMT* methylated, 1p/19q-non codeleted anaplastic astrocytoma, enrolled 16 months after the initial diagnosis; *IDH*-mutated, *MGMT* methylated GBM, enrolled 7 months after the initial diagnosis) for more than 6 years (81 and 79 months, respectively).

Table 1. Patient Demographics and Clinical Characteristics

Characteristics	EG ($n = 30$)	CG ($n = 30$)
Median age (range)	51 (26–71)	49 (25–69)
Male	20 (64%)	20 (64%)
Female	10 (36%)	10 (36%)
GBM	27 (90%)	21 (70%)
Anaplastic astrocytoma	3 (10%)	9 (30%)
ECOG 0	22 (73%)	22 (73%)
ECOG 1	8 (27%)	8 (27%)
Unifocal disease	13 (43%)	16 (53%)
Multifocal disease	17 (57%)	14 (47%)
MGMT methylated	20 (71%)	18 (60%)
MGMT unmethylated	8 (28%)	11 (37%)
MGMT missing	2 (1%)	1 (3%)
IDH mutated	8 (27%)	8 (27%)
IDH wild-type	21 (72%)	22 (73%)
IDH missing	1 (1%)	
PD-L1 ≥ 1%	6 (20%)	NA
PD-L1 < 1%	19 (63%)	NA
PD-L1 missing	5 (17%)	
MMR proficient	26 (86%)	NA
MMR deficient	2 (7%)	NA
MMR missing	2 (7%)	
Adjuvants treatments received		
Radiotherapy	30 (100%)	30 (100%)
Concomitant TMZ	30 (100%)	28 (93%)
Maintenance TMZ	29 (93%)	30 (100%)
Median n° of previous systemic treatments (range)	1 (1–2)	1 (1–2)
Needing steroids	20 (66%)	22 (73%)
Median dose of dexamethasone(mg, range)	2 (1–6)	4 (2–24)

Abbreviations: CG, control group; EG, experimental group; GBM, glioblastoma; *IDH*, isocitrate dehydrogenase; *MGMT*, O^6-methylguanine DNA methyltransferase; MMR DNA, mismatch repair; NA, not assessed; PD-L1, programmed death-ligand 1; TMZ, temozolomide.

Table 2. Targets of Immunomodulating Agents

Targets	N treated Patients (%)	Immunological Effects
Monotherapies	15 (50%)	
Anti-PD-1	3 (10%)	Activation of T-cell-mediated immune responses
Anti-cereblon	7 (23%)	T-cell activation and proliferation through enhancing of IL-2 production
Anti-CSF-1R	3 (10%)	Reduction of TAM-mediated immune suppression
Anti-TGF-βRI	2 (7%)	Rescue of suppressed T-cell activity and proliferation
Combination therapies	15 (50%)	
Anti-CSF-1R + anti-PD-1	5 (17%)	
Anti-CD38 + anti-PD-L1	10 (33%)	Anti-CD38: Tregs decrease, T-cell clonality increase Anti-PD-L1: activation of T-cell-mediated immune responses

Abbreviations: CD38, cluster of differentiation 38; CSF-1R, colony-stimulating factor 1 receptor; IL-2, interleukin 2; TAM, tumor-associated macrophages; PD-1, programmed cell death protein 1; PD-L1, programmed death-ligand 1; TGF-βRI, transforming growth factor beta type I receptor; Tregs, T regulatory cells.

The Univariate analysis identified two parameters associated with a better OS: *MGMT* promoter methylation (HR 0.36, CI: 0.14–0.91; $P = .03$), and total protein value at six weeks (HR 0.84, CI: 0.73–0.97; $P = .02$). *IDH* mutation status was close to statistical significance (HR 0.38, CI: 0.14–1.037; $P = .06$). The steroid dosage at 3 months was associated with worse survival (HR: 1.17; CI: 1.05–1.30; $P = .004$). Patients with methylated *MGMT* had significantly superior six-months PFS (PFS-6) and OS-12 rate compared to those with unmethylated *MGMT* (40% vs 12.5% $P = .015$; 55% vs 12.5% $P = .024$) (Figure 1A). *IDH*-mutated patients similarly had significantly superior PFS-6 and OS-12 rate compared to those with wild-type *IDH* tumors (62.5% vs 14.3% $P = .02$ and 75% vs 23.8% $P = .050$) (Figure 1B). A bivariable model including methylated *MGMT* and *IDH* mutation confirmed their effect (HR: 0.31, 95%CI: 0.11–0.82, $P = .019$; HR: 0.35, 95%CI: 0.12–1.01, $P = .053$), albeit the second is not statistically significant at level .050. Interestingly, after data cutoff, an *IDH*-WT GBM patient achieved a PR according to RANO criteria 15 months after experimental treatment discontinuation without receiving any other therapeutic interventions meanwhile (Figure 2A and B). During the course of experimental treatment, this patient had a slight increment of target lesions overtime, however not reaching the PD definition per RANO criteria, and was withdrawn from the trial due to clinical worsening.

Data of our EG were compared with those of the matched CG selected from the historical database of our institution and treated with standard approved treatments. Patients in the EG had a better OS compared to those in the CG, albeit this result did not reach the statistical significance ($P = .124$) (Figure 1C). OS-6, OS-12, OS-18 were 70%, 40%, 29.6%, and 53.3%, 23.3%, 13.3%, in the EG and CG respectively ($P = .124$) (Figure 1C).

Safety

In the entire EG, ten patients (33%) experienced ≥1 of any grade TRAE. The most common TRAEs were nausea, transaminases elevation, diarrhea, rash, and hyperuricemia. Serious TRAEs have been reported in five cases (17%) consisting of one grade 3 neutropenia, one grade 3 interstitial pneumonia, two grade 3–4 transaminases elevation, and one grade 3 fatigue. Lung and liver toxicities have been

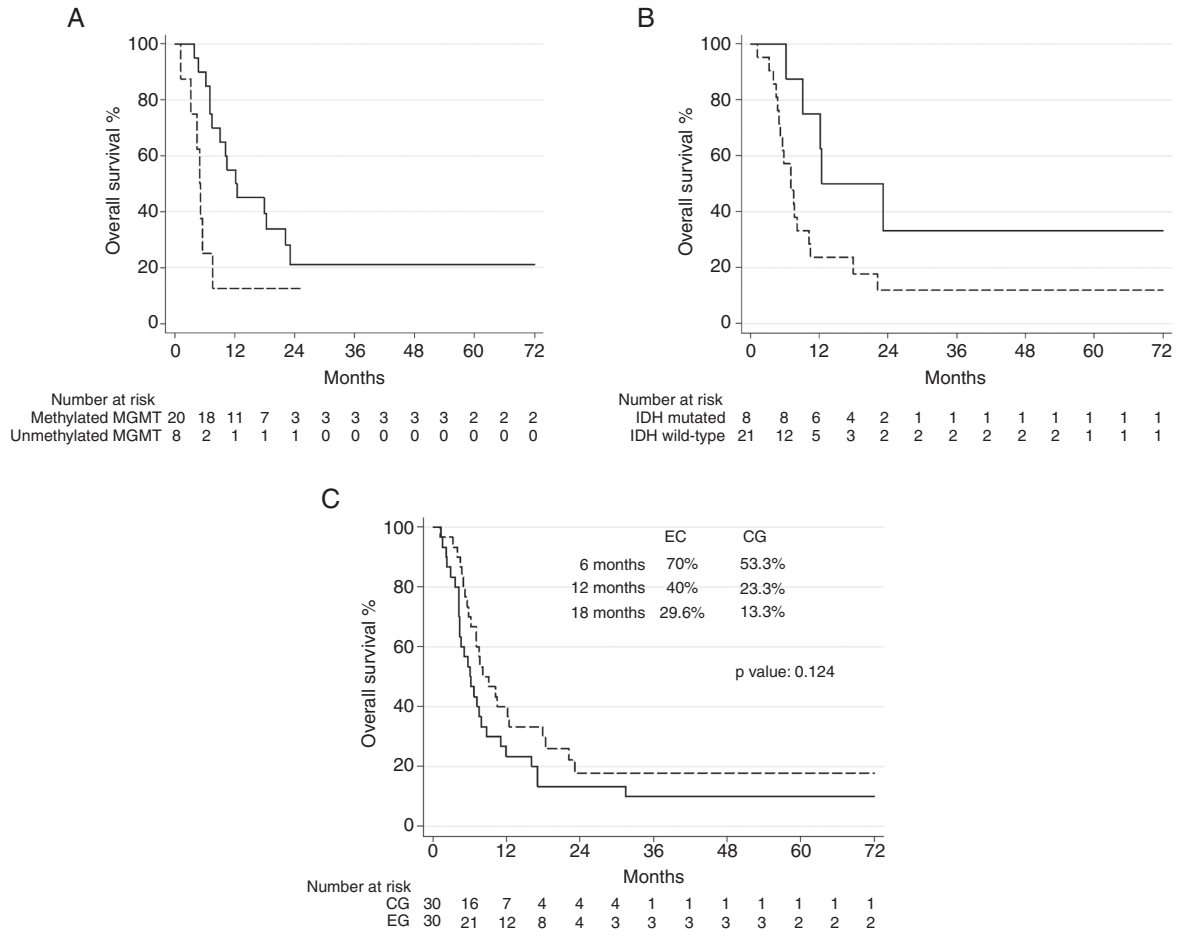

Figure 1. (A) Kaplan–Meier survival curves of experimental group according to MGMT methylation status. Solid line indicates MGMT methylated patients, dashed line indicates MGMT unmethylated patients. (B) Kaplan–Meier survival curves of experimental group according to IDH mutation status. Solid line indicates IDH-mutated patients, dashed line indicates IDH wild-type patients. (C) Kaplan–Meier survival curves of EG and CG. Solid line indicates CG, dashed line indicates EG.

considered as immune-relted advers events (irAEs), led to treatment withholding in one case, and to discontinuation in two cases. IrAEs were managed with high-dose steroids according to institutional guidelines. No drug-related worsening of neurological deficits and treatment-related deaths were reported in the EG.

Peripheral Blood Immunophenotyping

To identify immunological predictive or prognostic parameters for OS, a prospective and comprehensive peripheral blood FACS analysis was performed on the latest twenty consecutive patients included in our EG.

Absolute count of all immune cells and their subpopulations, evaluated at baseline before study entry (T0), did not significantly correlate with survival (data not shown). No correlation with clinical outcome was reported even when considering dynamic changes of circulating lymphocyte counts (CD4 T cells, CD8 T cells, and B cells), whereas some interesting findings emerged from the analysis of innate immunity leukocytes.

Patients with a decreased number of total circulating neutrophils during treatment (14 evaluable cases) had a statistically significantly higher OS ($P = .043$; Figure 3A and B). Considering circulating neutrophil subpopulations at T1, a higher ratio of $CD16^{+}CD62L^{low}$ aged neutrophils (Na) to T lymphocytes (NaTR) and an increased number of Na were associated with an increased risk of death (HR = 3.12, $P = .085$, Figure 4A; HR = 1.21, $P = .079$, Figure 4B). On the contrary, a high ratio between $CD16^{low}CD62L^{+}$ immature neutrophils (Ni) and aged neutrophils (Ni/Na) at T1 correlated with better survival (HR = 0.83, $P = .095$) (Figure 4A). A higher absolute count of Ni at TF (HR = 0.99, $P = .018$) seemed to be associated with better survival (Figure 4C). An overall decrease of circulating total NK cells, DCs, and their subpopulations was observed during treatment in most cases. However, as shown in Kaplan–Meier curves in Figure 3C and D, those patients with an increase from baseline of cDCs (15 evaluable cases) presented a significantly

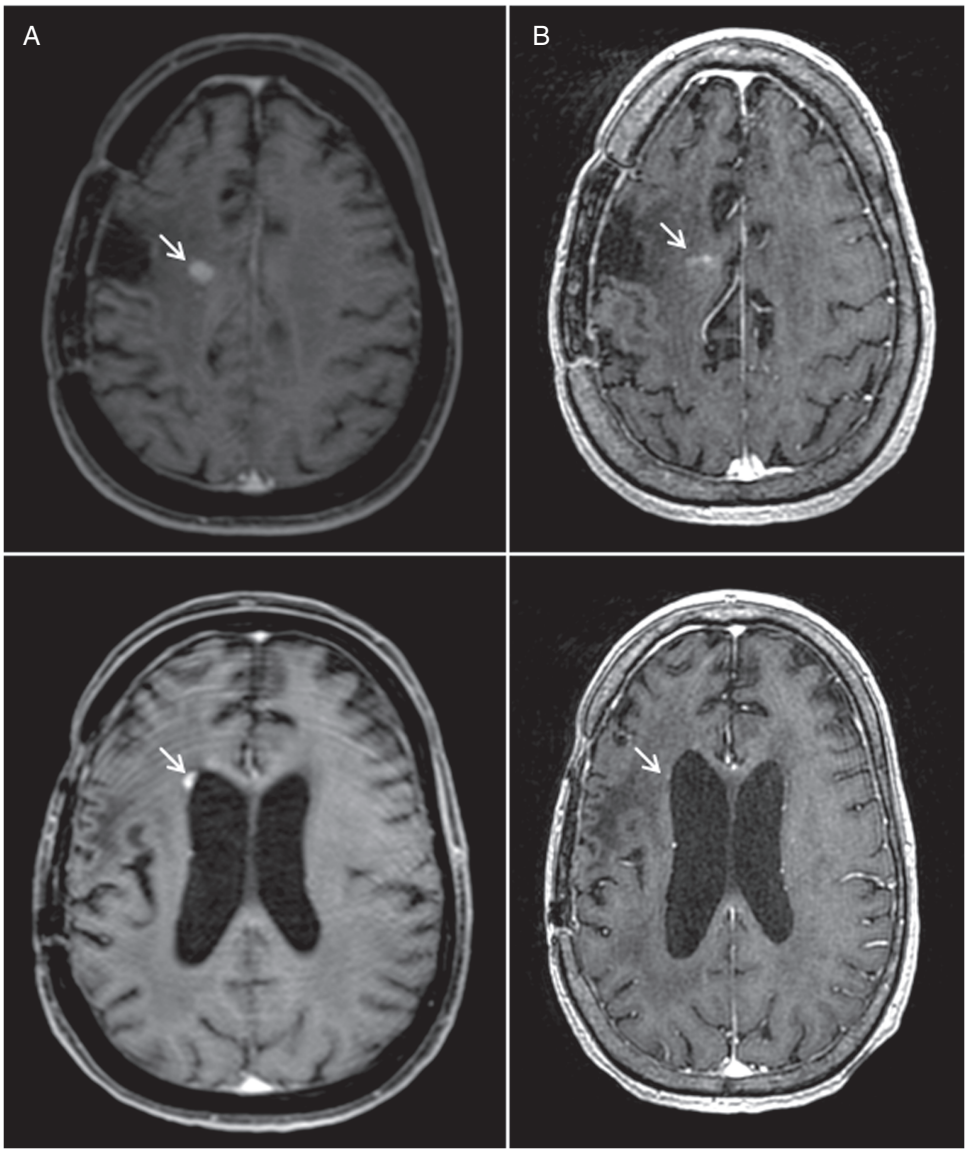

Figure 2. (A, top and bottom panel) Axial postcontrast T1-weighted brain MRI images of patient n°18 (*IDH*-wt GBM) at the time of treatment discontinuation. (B, top and bottom panel) Postcontrast T1-weighted brain MRI images of the same patient 15 months after treatment discontinuation, showing partial response. White arrows indicate enhancing lesions.

better outcome ($P = .035$). An increase of cDCs at ΔT1 and a higher number of $CD56^{bright}$ NK cells at T1 correlated with better survival (HR = 0.25, $P = .063$, Figure 4B; HR = 0.81, $P = .045$, Figure 4A).

Discussion

Despite decades of intensive clinical research, GBM remains an urgent unmet clinical need. With its own concept of boosting tumor-specific adaptive immunity, cancer immunotherapy has recently emerged as a cornerstone of modern oncology achieving regulatory approvals for several different malignancies. With the aim of bringing such novel and effective therapeutic weapons to patients in a timely manner, significant changes in the design and scope of phase I trials have been made during the latest years. ieCTs are now larger studies, exploring antitumor activity along with the safety and tolerability of novel agents across different malignancies through the use of multiple expansion cohorts.[7,8] PBTs patients are mostly excluded from such type of trials due to the unavailability of serial bioptic sampling, the frequent need of corticosteroids, concerns regarding the activity of immunotherapy in CNS, and a frequent rapid clinical deterioration. This paper presented data of a series of recurrent HGGs patients enrolled and treated with first and second-generation immunomodulating agents into multicohort ieCTs at our institution. Our

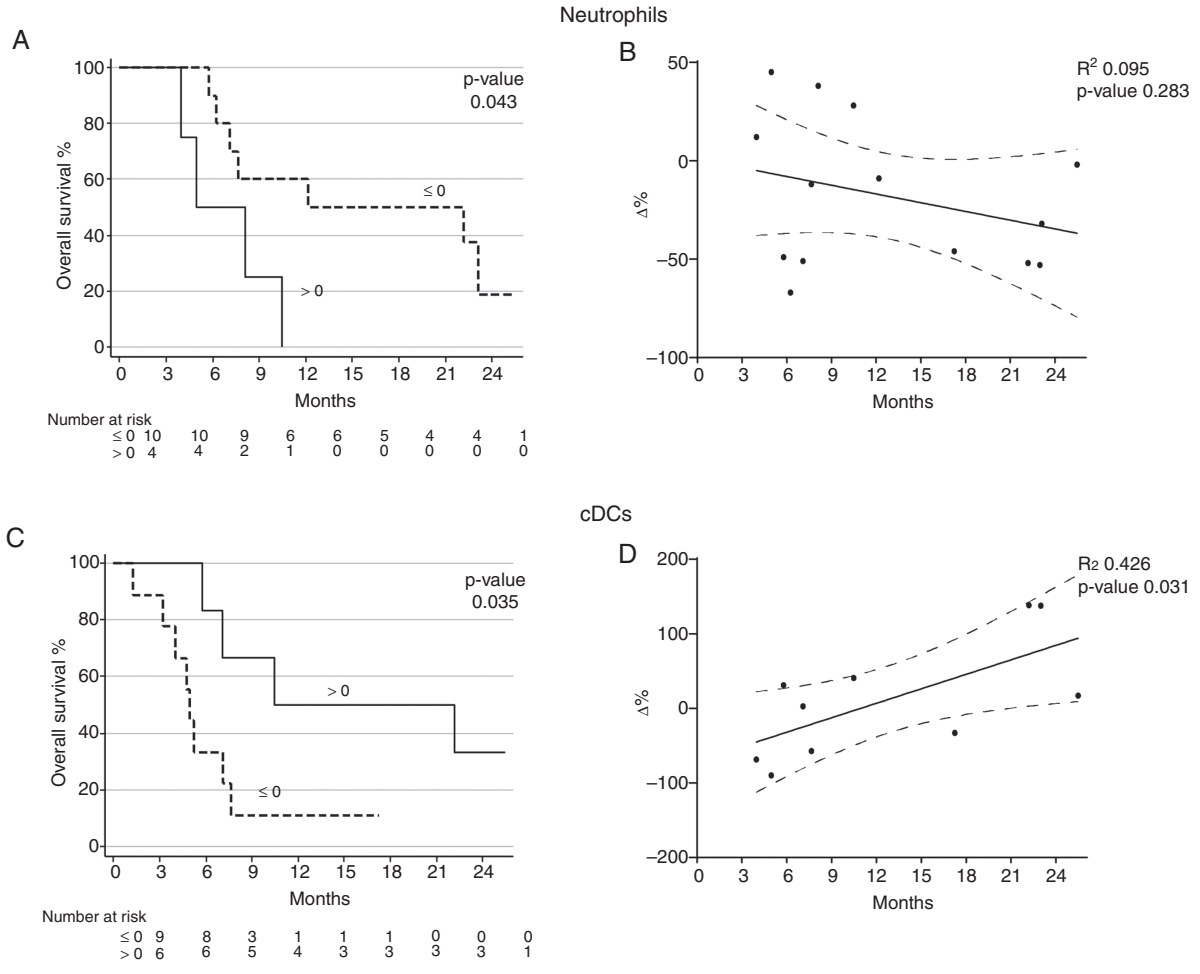

Figure 3. (A–B) Kaplan–Meier survival curves and linear regression analysis of total neutrophils at ΔTF stratified according to the cutoff level of 0. Size of each patient group and the *P*-value are given below the figure. (C–D) Kaplan–Meier survival curves and linear regression analysis of dendritic cells at ΔTF stratified according to the cutoff level of 0. Size of each patient group and the *P*-value are given below the figure.

analysis seems to confirm that only a small proportion of patients achieves therapeutic benefit from immunotherapy, but it can be of noteworthy duration. The ORR of 10% of our EG reflects the one previously observed in the CheckMate 143 with nivolumab[10] and in phase I trials of other anti-PD-1/PD-L1 agents (pembrolizumab and atezolizumab) in the same setting.[24,25] Responses are seen in both *IDH* wild-type and mutated tumors, the latter commonly considered as less immunogenic.[26] With a median OS of 8.6 months, patients treated into ieCTs showed a better outcome compared to a historical matched CG receiving standard therapeutic options. Importantly, our EG does not represent a particularly selected population since most patients had a multifocal disease presentation at the time of study entry and received steroids for symptom control. Recently published data suggested that the use of corticosteroids, even at low doses, can negatively affect both adaptive and innate immune responses, leading to poorer survival in GBM patients receiving anti-PD-1/PD-L1 blockade.[27] According to other previous reports,[10] a share of patients in our EG achieved a surprisingly durable disease control over time. 40% of patients lived more than 12 months after immunotherapy initiation, 13% more than 24 months, and 7% more than six years. Interestingly, six patients (20%) obtained a significantly prolonged survival (>12 months) even after treatment discontinuation because of apparent radiological PD, clinical deterioration, or toxicity. Moreover, an *IDH*-WT GBM patient showed a radiological major partial response more than one year after treatment discontinuation, without having received further treatment in the meantime. These findings highlight the challenges in interpreting treatment response with conventional brain MRI for GBM patients receiving immunotherapy into clinical trials. As in other tumor types, it is conceivable that inflammatory responses caused by immunotherapeutic agents would lead to changes in the permeability of the blood-brain barrier and contrast extravasation, mimicking an early progression and causing premature therapy discontinuation. PD-L1 was overall low-expressed in our EG and, according to the recent literature, showed no predictive significance. *MGMT* methylated patients had a

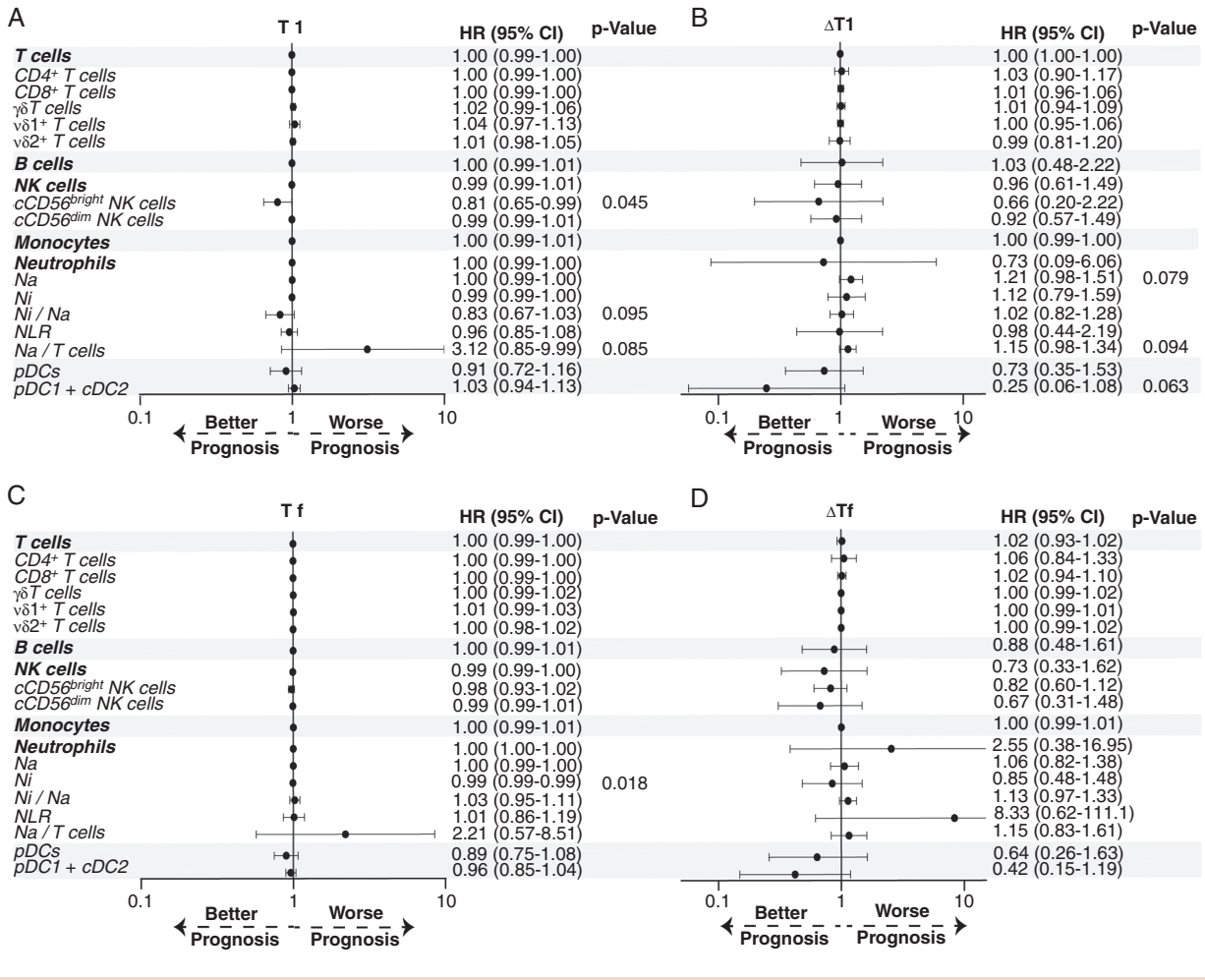

Figure 4. Forest plot of the association between immune cell subpopulations and patient's overall survival at T1 (A) and ΔT1 (B) and at TF (C) and ΔTF (D). Circles represent the hazard ratio (HR) and the lines the 95% confidence interval (CI). Only significant *P*-values are displayed (*P*-value < 0.1).

significantly longer survival in our EG. This finding is consistent with what emerged from larger phase 3 studies of nivolumab and dendritic cell vaccination,[10,28] suggesting that *MGMT* promoter methylation could represent a potential biomarker to select patients for inclusion in future immunotherapy trials. Moreover, the univariate analysis showed total protein value after six weeks from experimental treatment initiation as a positive prognostic factor. This finding may be explained as a nonspecific measure of good nutritional status identifying a patient subset with preserved clinical conditions.

Unfortunately, the multivariate analysis failed to identify a number of clinical, laboratory, and molecular parameters useful to develop a specific prognostic score, probably due to the small sample size.

We prospectively investigated whether circulating immune cell subpopulations and their dynamic changes during treatment may provide useful correlations of patient outcomes in a subset of twenty cases part of our EG. Though CD4+ T cells counts have been positively associated with the prognosis in glioma patients treated either with radiation, temozolomide, and vaccine therapy,[29,30] we did not find any significant correlation between peripheral T-cell populations and patient outcomes in our EG. Instead, interesting findings have emerged from the study of innate immunity compartment.

Significant evidence demonstrated that the number of circulating neutrophils and a high NLR were associated with a worse prognosis in GBM patients.[31] Moreover, NLR as a single threshold value has a well-known negative prognostic significance for patients with advanced cancers treated with immune-checkpoint inhibitors,[32] whereas its dynamic changes are reported to be a nonlinear predictor of patient outcomes.[33] In our series, an overall reduction in the absolute circulating neutrophils counts during the course of ieCT correlated with significantly better survival. We found that an increase of aged neutrophils at first tumor assessment correlates with a worse prognosis, while a higher absolute number of immature neutrophils is associated with better clinical outcomes. Immature neutrophils are immunostimulatory, promoting T-cell survival, and enhancing proliferation and interferon gamma (IFN-γ)

production by T cells, while aged neutrophils are immunosuppressive inhibiting T-cell response via CD18-mediated contact-dependent arginase-1 release.[34] Recent work demonstrated suppression of the number, phenotype, and function of circulating DC subsets in patients with GBM, likely due to tumor-induced immunosuppression and dexamethasone use.[35] Moreover, GBM patients exhibiting increased DC levels over time seemed to have a more favorable prognosis.[36] A high number of activated NK cells at diagnosis are associated with a more favorable prognosis in GBM patients[37] as well as a persistent activation of NK cells induced by DC vaccination correlated with prolonged survival.[38] Most of the cases in our EG showed a progressive decline of both DC and NK cell counts as an expression of a general scenario of cancer immune escape. Conversely, those patients deriving a prolonged clinical benefit from immunotherapy presented an increase in circulating cDCs during treatment. Even a higher count of $CD56^{bright}$ NK cells at the time of first tumor assessment correlated with a better outcome in our cohort. DCs and NK cells are highly sophisticated players of the innate immune system that function as a bridge between innate- and antigen-specific immunity. cDCs are professional antigen-presenting cells orchestrating adaptive immune responses by antigen uptake, presentation, and costimulation of effector T cells. NK cells mediate immune-surveillance via cytotoxic effector-functions and serve as regulatory lymphocytes interacting with both innate and adaptive immune cells, such as monocytes/macrophages, DCs, and T lymphocytes. Particularly, $CD56^{bright}$ NK subset shows little cytotoxic activity and is functionally characterized by distinctive immunoregulatory properties, being a significant source of proinflammatory cytokines and chemokines including IFN-γ, tumor necrosis factor alpha, granulocyte-macrophage colony-stimulating factor, interleukin 10, and interleukin 13.[39] Our findings are in accordance with previous reports on patients affected by lung cancer and advanced thymoma treated with immune-checkpoint blockade[40–42] and emphasize the relevance of innate immune cells activation in achieving benefit from immunomodulating agents. We speculate that monitoring the dynamic changes of neutrophil, NK, and DC subpopulations may identify HGG patients most likely to achieve durable clinical benefit from immunotherapy.

To the best of our knowledge, the current study is the first to have evaluated a population of HGG patients treated into ieCTs. Although the retrospective and monocentric nature of our analysis inevitably implies a series of inherent biases, some important considerations can be derived. Recruiting HGG patients into ieCTs, represents, in our opinion, not only a reasonable option but something to strongly encourage. Enrolling GBM patients in such types of trials will allow us to catch early signs of activity, pushing forward only promising strategies worthy of further clinical development, and to implement different ancillary biomarker studies. In this analysis, we focused on circulating leukocytes evaluating changes in functional subsets over time. Our findings suggested the potential ability of different immunotherapeutic strategies to activate innate immunity as the crucial step for developing robust, specific immune responses capable of effectively attacking GBM cells. Our experience with the share of long survivors sustains the impression that we are yet far from disclosing the full potential of immune-based treatments for glioma patients. Identifying the small subset of patients more likely to achieve clinical benefit and understanding what we fail to trigger in most cases that do not respond, are critical aspects to address in the near future if we want to raise the bar beyond current disappointing results.

Supplementary Material

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

Keywords

circulating leukocytes | early-phase clinical trials | glioblastoma | gliomas | immunotherapy

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