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# Pembrolizumab activity in recurrent high-grade gliomas in partial or complete loss of mismatch repair protein expression: a monocentric, observation and prospective study

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## Abstract

### Background

Pembrolizumab, an anti PD-1 immune checkpoint inhibitor, has shown important activity in several cancers with hypermutated phenotype. Expression loss of mismatch repair (MMR) protein on immunohistochemical analysis appears to be associated with hypermutation in high-grade gliomas. This study evaluated the efficacy and safety of pembrolizumab in patients with HGGs and immunohistochemical loss of at least one MMR protein. In addition, potential molecular biomarkers predicting pembrolizumab activity were evaluated

### Materials and Methods

We prospectively enrolled patients with recurrent HGG and partial or complete loss of MMR protein expression. Pembrolizumab was administered by intravenous infusion at the standard dose of 200 mg once every 3 weeks until unacceptable toxicity or disease progression. Primary end point was disease control rate (DCR). As exploratory post hoc analyzes, next generation sequencing (NGS) for the evaluation of tumor mutational burden (TMB) and immunostaining for CD8 + T-cells and CD68 + macrophages were performed.

### Results:

310 patients with recurrent HGG were screened; 13 of them with MMR expression loss were enrolled and treated with pembrolizumab. Of these 13 cases, eight were glioblastoma, four anaplastic astrocytoma, and one anaplastic oligodendroglioma. Median age was 43 years. DCR was 31% with four patients showing stable disease as the best response and none with partial or complete response. TMB ranged between 6.8 and 23.4 mutations/megabase. Mutations found in treated patients, TMB, CD8 + T-Cell and CD68 + macrophage do not appear to be associated with pembrolizumab activity.

### Conclusions

Pembrolizumab demonstrated no benefit in this patient population and no molecular biomarkers associated with pembrolizumab activity were found.

# INTRODUCTION

## *Epidemiology and Classification of brain tumors*

Primary malignant brain tumors account for 1.6% of all cancers and are the cause of 2% of all cancer deaths in Western countries[1]. The average annual age-adjusted incidence rate of all primary (malignant and non-malignant) brain and other Central Nervous System (CNS) tumors for the years 2013-2017 was 23.8 per 100.000; this rate was higher in non-hispanic compared to hispanic (24.2 Vs 21.4 per 100.000), was higher in females compared to males (26.3 Vs 21 per 100.000) and was similar in blacks compared to whites (23.88 Vs 23.83 per 100.000). Non-malignant tumors (70.3%) were twice as common as malignant tumors (29.7%); incidence rates were highest for meningiomas (8.81 per 100,000 population), tumors of the pituitary (4.20 per 100,000 population), glioblastomas (3.23 per 100,000 population), and nerve sheath tumors (2.03 per 100,000 population). The average annual age-adjusted incidence rate for primary malignant brain and others CNS tumors was 7.08 per 100.000. For malignant tumors, the incidence rate was highest for glioblastoma (3.23 per 100,000 population), followed by glioma malignant, NOS (0.51 per 100,000), diffuse astrocytoma (0.45 per 100,000 population) and lymphoma (0.43 per 100,000 population). The most commonly primary malignant brain and other CNS tumor was glioblastoma (14.5% of all tumors and 48.6% of malignant tumors)[1].

Regarding survival, the estimated 5-year survival rate following malignant brain and CNS tumors was 36%; this value was highest in children age 0-14 years (75.4%) Vs to those ages 15-39 years (72.5%) ore 40+ years (21-5%). However, there is a large variation in survival estimates depending upon tumor histologies. For example, five-

year survival rates are 94% for pilocytic astrocytomas but are less than 5% for glioblastomas[1].

The gliomas category represents 25.1% of all primary brain and other CNS tumors and 80.8% of malignant tumors[1](Figure 1). The 2016 World Health Organization (WHO) system[2] is the used classification and it is based not only on histological characteristics (like the previous one from 2007[3]) but integrates this information with molecular characteristics in order to better classify the different entities that belong to this category.

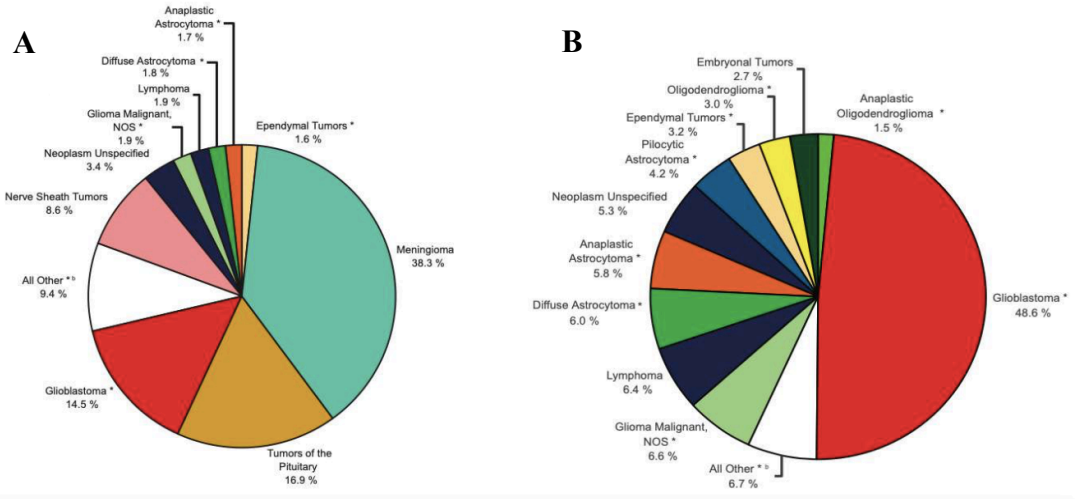


Figure 1: Distribution of All Primary Brain and Other CNS Tumors (Malignant and Non-Malignant) (A); Distribution of Malignant Primary Brain and Other CNS Tumors (B)[1]

This classification can limit the diagnostic discrepancy among neuropathologists, which can often reach up to 20%, mainly linked to the experience of the single specialist in the neuro-oncology field[4]. Historically, gliomas are classified into "low-grade" (WHO grade I and II) and "high-grade" (WHO grade III and grade IV) with substantial

differences in terms of clinical course and prognosis. Low-grade gliomas (WHO grade I-II) are more common in the 20s and 40s, while so-called anaplastic gliomas (WHO grade III) and glioblastoma (WHO grade IV) generally have a later onset, from 40 to 70 years; over 70 years old, the most common diagnosis is glioblastoma (WHO grade IV). The 2016 WHO classification defines how brain tumors are diagnosed on the basis of molecular alterations. Diffuse gliomas are in fact classified into mutated IDH (Astrocytomas grade II and grade III characterized by ATRX mutation and p53 mutation and grade II and III oligodendrogliomas defined by the presence of codelection 1p / 19q and absence of mutation of ATRX and p53) and IDH non-mutated which, in the vast majority of cases, include glioblastomas (WHO grade IV) showing EGFR amplification and PTEN mutation, anaplastic astrocytomas and anaplastic oligodendrogliomas (WHO grade III) characterized by more aggressiveness and poor prognosis (Figure 2).

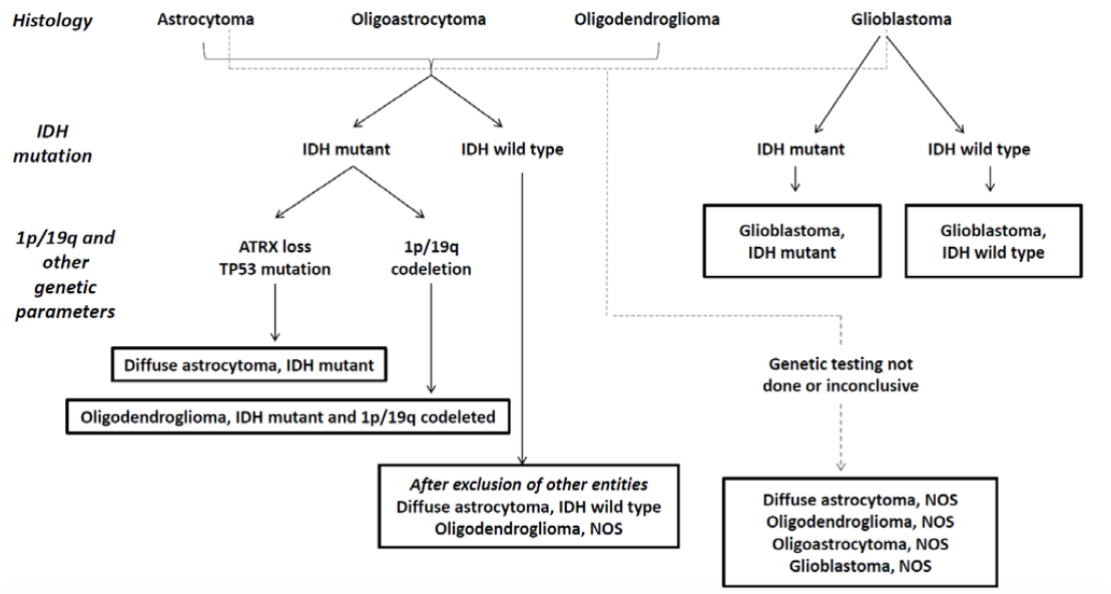


Figure 2: An algorithm for classification of gliomas based og histological and molecular features[2]

***Diagnosis***

The signs and symptoms caused by brain neoplasms can be highly variable and are usually associated with the location of the tumor and its growth pattern. If the tumor grows rapidly and the localization is limited, focal neurological deficits will normally occur, while a slow-growing diffuse mass can more frequently lead to cognitive and compartmental deficits as well as seizures. The most frequent symptoms associated with brain tumor are headache unresponsive to analgesic therapy, focal neurological deficits, nausea, vomiting and new onset seizures; however, these symptoms are not specific and therefore a careful evaluation of the clinical context and the modalities of onset remains of fundamental importance. Since a diagnostic suspicion for brain tumor is raised, neuroradiological investigation with contrast-enhanced magnetic resonance (MRI) is considered "gold-standard". MRI study in a patient with brain tumor should include multiple (in 3D acquisition) T1-weighted sequences without and with gadolinium as well as with T2-weighted (usually axial and / or coronal) FLAIR (Fluid Attenuated Inversion Recovery) sequences. At least one sequence in Diffusion mode (DWI) would be useful for the microstructural analysis of the tissue by measuring the displacement of water molecules (protons). With rare exceptions (e.g. for WHO grade I glioma), gadolinium enhancement is typical of high-grade gliomas (WHO grade III-IV), and the tumor area is measured as the product of the two largest perpendicular diameters of the enhancement that appear in T1-weighted images after gadolinium[5,6]. For low-grade gliomas (WHO grade I-II), usually without gadolinium enhancement, the definition of the tumor diameter is more controversial, and generally performed with the perpendicular diameter method on the areas of impaired signal on T2 or FLAIR scans, even if the border between tumor and edema is often not easily recognizable.

Magnetic resonance spectroscopy (MRS) of hydrogen is a non-invasive diagnostic method that allows to obtain in vivo metabolic information of the brain tissue analyzed by recording signals according to specific molecules present at the tissue level; the metabolites normally analyzed are N-acetylaspartate (NAA) which is normally present in healthy brain tissue, and Choline (Cho), which is normally present in high concentrations in tumor tissue. The use of Positron Emission Tomography (PET) with 18F-deoxyglucose can be a diagnostic tool to identify systemic tumors with brain metastases, but, due to the metabolic characteristics of the brain and of the tracer, it cannot be considered as an aid in the diagnosis of primary brain tumors. The situation is different in the case of using marked amino acids for the PET examination. Amino acid PET, in particular with 18F-fluoroethyltyrosine (FET) which is a particularly specific tracer for gliomas, is recommended for discerning neoplastic from non-neoplastic tissue, establish lesion extension to plan surgical resection, hot spot localization for biopsy planning, postresection assessment, radiation therapy planning and baseline monitoring for chemo-radiation. At the moment it is not used in common clinical practice but reserved only for centers that have this method available, but it is particularly useful in such a complex area as neuroradiology[7]. The evaluation of the response to oncological treatments has always been extremely difficult in neuroncology due to the presence of complex radiological aspects such as pseudoprogressions or the evaluation of the response to treatment with antiangiogenic drugs (which can reduce the intensity of gadolinium enhancement regardless of the size of tumor lesions). For this reason, response evaluation criteria have been proposed that take into consideration not only the radiological aspect of the enhancing lesions but also the extent of the alteration of the FLAIR sequences, the general clinical conditions of the patient and the use of corticosteroids. These criteria (RANO criteria[8]) have substantially integrated and

replaced the previous criteria used in neuroradiology (MacDonald criteria[5]) as they are able to be more precise and applicable in the neuroncological field.

***Treatments for gliomas***

The gold standard of treating brain tumors, and particularly high-grade gliomas, is a multidisciplinary approach that includes surgical resection followed by radiotherapy and chemotherapy alone or in combination. The choice of the type of treatment depends on the histology, the tumor grade according to WHO 2016, the location of the tumor and the general clinical condition of the patient. Recent advances in the understanding of the molecular pathways that regulate progression, invasiveness, tumor growth and resistance to treatments, as well as greater clarity on predictive and prognostic biomarkers in these cancers, now make it possible to use personalized therapies in order to offer the patient a precision treatment.

- *Surgery*

The first therapeutic approach in the radiological suspicion of glial lesion is surgery, which not only aims to confirm the diagnosis but provides the possibility of obtaining tumor tissue for molecular analysis and can improve neurological symptoms by reducing mass effect and edema. Surgery is not always possible because, for gliomas involving the brain stem, a surgical approach is not feasible. The data available in the literature confirm that the extent of surgical resection is able to influence the outcome for all gliomas, in particular for high-grade gliomas: a near-total or gross-total resection is statistically significantly associated to longer survivals[9,10]. In these studies, the extent of resection was generally defined by the presence or absence of residual contrast enhancement on MRI performed within 48 hours of resection. Currently, there is no common consensus on the role of subsequent resection in the management of patients

with recurrent glioblastoma. Several studies provide longer overall survival for selected patients with recurrent glioblastoma who receive a second surgery, while other studies report a limited impact of the second surgery on the natural history of disease. In a review of the literature, 2279 patients were selected from 28 clinical trials to evaluate the role of re-surgery in patients with glioblastoma recurrence and its impact on overall survival from diagnosis, progression free survival and quality of life (QoL)[11,12]. The median OS from diagnosis and from the second surgery was 18.5 months and 9.7 months, respectively. The extension of the resection to re-surgery seemed to improve OS even in patients who had received a subtotal resection at the first surgery. Preoperative PS and age were important predictors of better survival: carefully selected patients with good preoperative PS are those who could benefit most from a second surgery.

- *Radiation*

Radiation therapy is usually the most common post-surgical treatment for all grades of glioma and is often given in combination with chemotherapy in high-grade gliomas. It is usually delivered in a limited field that affects the surgical bed (or residual tumor) with a margin of 1 to 3 cm. The total delivered dose increases with the grade of glioma to be treated; for glioblastoma and high-grade gliomas, daily fractions of 1.8-2 Gy are administered over 4-6 weeks for a total dose of about 40-60 Gy. There is no evidence of greater benefit with the use of higher doses or alternative fractionation schemes[13]. The possibility of using re-irradiation for the treatment of relapses of high-grade gliomas is currently still controversial. The evidence available at the moment, which derives from systematic meta-analyzes and reviews given the absence of randomized controlled clinical trials, suggests that re-irradiation could be considered in selected

patients and would show encouraging data regarding disease control and survival rates[14].

- *Chemotherapy*

Chemotherapy plays a vital role in the treatment of high-grade gliomas. Several chemotherapy agents have been evaluated over the years, but, to date, the most commonly used drug is temozolomide, an oral cytotoxic DNA alkylating agent. Regarding glioblastoma, the benefit of temozolomide was demonstrated in a phase III study in newly diagnosed patients who were randomized after surgery to receive treatment with external beam radiotherapy alone or radiotherapy and concomitant temozolomide[15]. This study demonstrated that concomitant chemoradioterapy with adjuvant temozolomide resulted in a significant improvement in median overall survival compared with radiation only (14.6 months versus 12.1 months) and a significant increase in 2-year survival (26.5% versus 10.4%) with a benefit that was maintained at 5 years of follow-up[16]. A companion study showed that patients with glioblastoma containing a methylated MGMT promoter (the enzyme that repairs DNA damage due to temozolomide) benefited from temozolomide, whereas those who did not have a methylated MGMT promoter did not have such a benefit[17]. Regarding grade III gliomas, for the treatment of newly diagnosed patients with anaplastic astrocytoma (1p/19q non-codeleted), the final data from the CATNON trial[18] were recently published and outlined the cornerstones of the treatment of this type of cancer. In this phase III, open-label randomized controlled trial, patients with newly diagnosed anaplastic astrocytoma (WHO 2016 grade III) were randomized (1: 1: 1: 1) to receive radiotherapy alone (59 4 Gy in 33 fractions), radiotherapy with concurrent oral temozolomide (75 mg / m2 per day) , radiotherapy with adjuvant oral temozolomide (4-week cycles of 150–200 mg / m2 temozolomide given on days 1–5), or radiotherapy

with both concurrent and adjuvant temozolomide. The authors concluded that adjuvant temozolomide chemotherapy, but not concurrent temozolomide chemotherapy, was associated with a survival benefit in patients with 1p / 19q non-co-deleted anaplastic astrocytoma. Clinical benefit was dependent on IDH1 and IDH2 mutational status: in IDH1/2 wild-type tumors, neither concurrent nor adjuvant temozolomide improved survival compared with radiotherapy while in IDH1/2 mutated tumors, adjuvant temozolomide improved survival compared with no-adjuvant temozolomide, but no overall survival benefit was observed after concurrent radiochemotherapy with temozolomide compared with no concurrent radiochemotherapy. Patients with grade III oligodendrogliomas have increased response to therapy and length of survival compared with patients with grade III astrocytomas. These improved outcomes are strongly associated with the loss of heterozygosity of chromosome 1p and 19q, which is found in about 80% of cases[19]. In high-grade gliomas (and especially in glioblastoma) disease recurrence / progression is an inevitable event and the choice of the best treatment to propose at the time of recurrence is of crucial importance. To date, several clinical trials have been conducted on molecularly targeted drugs such as EGFR (gefitinib and erlotinib), multi-target inhibitors (vandetanib, sunitinib, dasatinib) without obtaining clear evidence of efficacy[20–25] superior to cytotoxic treatments with nitrosurea. The randomized trial of phase II Regoma[26] was published in 2019 and evaluated the use of regorafenib (a multi-kinase inhibitor with also anti-angiogenetic activity) in patients with recurrent glioblastoma, compared to lomustine: for the first time in many years, a target therapy has been shown to be able to improve survival in this category of patients compared to standard treatment.

***Predictive and prognostic factors in gliomas***

The 2016 WHO[2] classification integrated histological and strictly morphological assessments molecular characteristics in way to better predict outcome, to better stratify patients included in clinical trials and, finally, to tailor specific treatment to individual tumor types or patients. To date, three biomarkers have been identified as potent prognostic factors in gliomas: codeletion of chromosome arms 1p and 19q, O6-methylguanine-DNA methyltransferase (MGMT), isocitrate dehydrogenase (IDH)[27].

- *Codeletion of chromosome arms 1p and 19q*

The simultaneous loss of a copy of chromosomal arms 1p and 19q usually occurs in oligodendrogliomas and is now defined as a characteristic of this type of diagnosis, capable of helping in the differential diagnosis between oligodendroglioma and astrocytoma; it is also a signature that is considered as a positive prognostic factor and predictor of response to chemotherapy and radiotherapy treatment[19]. The 1p/19q codeletion is mutually exclusive with TP53 mutation and EGFR amplification, frequently associated with MGMT promoter methylation, and always associated with IDH1 or IDH2 mutation. MGMT promoter hypermethylation is significantly more frequent and the percentage of methylated CpG site was significantly higher in 1p/19q codeletion tumors compared with 1p and/or 19q intact. The high frequency of MGMT promoter methylation in 1p/19q codeletion gliomas might partly explain their chemosensitivity.

- *O6-methylguanine-DNA methyltranseferase (MGMT) promoter methylation*

MGMT is a DNA repair protein that normally catalyzes the transfer of a methyl group from the O6 position of the guanine nucleotide to a cysteine residue at the

145 position of the DNA chain. The silencing of the MGMT gene through the methylation of the promoter induces a low expression of the MGMT protein with a consequent decrease in the DNA repair activity; this has as a consequence the increased sensitivity to alkylating agents, such as temozolomide. Hegi et al.[17], to test the relationship between the methylation status of the MGMT promoter and the survival outcome of the patients enrolled in the EORTC 26981/22981-NCICStudio CE3 study, analyzed, by methylation-specific polymerase chain reaction (MS- PCR), the methylation status of the MGMT promoter in 206 evaluable samples, which was found to be methylated in 45% of the samples. Regardless of the type of treatment, MGMT promoter methylation was found to be an independent favorable prognostic factor ($p < 0.001$ from log-rank test; HR = 0.45; 95% CI 0.32-0.61). Among patients with the methylated MGMT promoter, a survival benefit was observed in the arm receiving concomitant treatment with TMZ and RT while in non-methylated patients, the difference in survival was not statistically significant between the two treatment groups (TMZ + RT Vs RT). Thus, MGMT methylation status was found to be a strong prognostic and predictive marker in patients with GBM treated with TMZ and RT. Two other phase III[28,29] studies have suggested that MGMT promoter methylation status could guide treatment decisions in elderly patients with GBM. (28,37). Both the NOA-08[28] and NORDIC[29] trials indicated that treatment with temozolomide alone was at least equally effective as treatment with RT alone in elderly patients with high-grade glioma and methylated MGMT promoter providing strong evidence that methylation status of the MGMT promoter has an important role in predicting response to treatment in elderly patients.

- *Isocitrate dehydrogenase mutations (IDH 1-2)*

The IDH1 / 2 genes encode Krebs cycle enzymes that produce CO2 and α-ketoglutarate (αKG) through the oxidative decarboxylation of isocitrate. IDH1 encodes a cytosolic protein, while IDH2 encodes a mitochondrial protein. Mutations of IDH1 [R132H] and IDH2 [172] are the most common types of IDH mutations observed in high-grade gliomas (> 90% samples with IDH1 / 2 mutation) and result in increased production of D-2-hydroxyglutarate, an oncometabolite, which can alter the DNA methylation pattern in the glioma cell and lead to alterations in gene transcription on a large number of targets as well as that they can reduce the synthesis of NADPH, with a consequent increase in oxidative stress, oxidation of DNA, overcoming the DNA repair mechanisms and induction of any damage on the DNA molecule itself[30,31]. Several studies have confirmed the prognostic role of IDH mutations in high-grade gliomas, while the predictive value still remains unclear despite some more recent data may confirm the validity of the IDH mutation as a positive predictor factor[18]. In a 2009 study[32] the IDH1 mutation was analyzed in 404 patients with glioma of which 184 (45%) were patients with GBM in which the IDH1 mutation was found to be present in 6% of cases. The presence of the IDH1 mutation was associated with a better outcome in all gliomas of all grades. For GBM patients with IDH mutation, OS was 27.4 months versus 14 months in the absence of the IDH mutation (IDH WT - $p < 0.01$). After adjustment for grade, age, MGMT status, genomic profile, and treatment type, multivariate analysis confirmed that the IDH1 mutation was an independent favorable prognostic factor (HR = 0.297; 95% CI 0.15 to 0.56, $p < 0.001$). In another study, 395 GBM samples were analyzed; the IDH mutation was shown to be associated with a significant improvement in survival (26.6 months in patients with IDH mutations vs 14.5 months in IDH WT)[33].

***Mismatch Repair Proteins***

DNA mismatch repair (MMR) is a system that aims to identify and repair any errors in the DNA chain (insertions, deletions and incorrect incorporations) in order to guarantee genomic stability and integrity. The MMR system substantially depends on four key genes: mutS homologue 2 (MSH2), mutS homologue 6 (MSH6), mutL homologue 1 (MLH1) and post-meiotic segregation increased 2 (PMS2). These proteins are usually identified in the clinical setting by immunohistochemical analyzes on tumor tissue. In the event that these MMR proteins are not expressed or are dysfunctional / inactivated, it could induce a hypermutated profile in tumor cells causing 10 to 100 times more somatic mutations than in tumor cells without dysfunction in the MMR mechanism; this type of profile is usually associated with the generation of numerous neo-antigens capable of activating the immune system more and promoting its anti-tumor activity. In this regard, Hodges et al.[34] showed that the loss of expression of at least one of the proteins of the MMR complex on immunohistochemical analysis was associated with a hypermutated profile in patients with glioma. This type of correlation has also been confirmed by more recent studies[35–37]. In a recent and interesting paper[38], for the first time, the frequency and prognostic role of the loss of MMR expression at immunohistochemistry in patients with high-grade glioma were investigated: MMR proteins were analyzed in the tumor tissue of 355 consecutive patients by dividing the levels of expression in "present" (+ / +) in the case of unequivocal nuclear labeling in tumor cells with staining intensity comparable to that of internal control, "partial loss" (+/−) in the case of visible nuclear labeling in tumor cells, but with an intensity weaker than the internal control or only comparable to the intensity of the inert stromal cells, and "complete loss" (- / -) in the case of no visible nuclear labeling in tumor cells. In conclusion, in 43/355 samples (12.1%) an alteration (partial or complete) of the

expression of at least one of the proteins of the MMR complex was found: this alteration was statistically more frequent in anaplastic glioma (WHO 2016 grade III), in recurrent disease, in IDH-mutant gliomas and in patients treated with temozolomide. After adjustment for relevant clinical confounders, this molecular alteration was not associated with survival.

***Immune-Checkpoint Inhibitors***

The Programmed Cell Death (PD) and Cytotoxic T-Lymphocyte Antigen 4 (CTLA4) pathways are just some of the mechanisms put in place by the cancer cell (including high-grade gliomas) to inhibit the activity of T lymphocytes cytotoxic (CD8 +) within tumor tissue[39,40]. In case of PD-1 or PD-L1 inhibition, a block of the interaction between PD-L1 (ligand) and its receptor (PD1) is established which overcomes the inhibition of the activity of the T-Cells and thus promote a robust and effective immune response against cancer cells. In this regard, some monoclonal antibodies have been developed, called immune-checkpoint inhibitors, directed against the PD-1 receptor or against its PD-L1 ligand with the aim of turning off the inhibitory signal and allowing to enhance the immune response. In the oncology field, the use of these drugs has led to a real revolution, obtaining exciting results in different types of cancer[41–50]. In early studies using immune-checkpoint inhibitors in several types of cancer, patients with brain metastases were excluded[51]. Subsequently, thanks to the efforts for the study of the interactions between the tumor microenvironment of brain metastases and the immune system, the immunological aspects of the CNS were partly clarified and it was understood that it would turn out to be completely different from other tissues[52]. This improved knowledge and the availability of immune-checkpoint inhibitors (anti-CTLA4, anti-PD1, anti-PD-L1) have made it possible to extend the use of these drugs to patients with brain metastases and primary brain tumors. In fact, there have been

numerous studies that have evaluated the use of immune-checkpoint inhibitors in patients with brain metastases deriving from different types of cancer (melanoma[53–56], lung cancer[54,57], renal cancer[58]) obtaining interesting results in terms of survival and intracranial disease control. Despite these results, the choice of immune-checkpoint inhibitors therapy in patients with primary brain metastases for whom immunotherapy is the standard requires attention, careful patient selection and case-by-case decisions[59]. Nivolumab and pembrolizumab, two of the most widely used anti-PD1 immune-checkpoint inhibitors in oncology have been tested in some cancers with deficient MMR protein expression[60–65], demonstrating therapeutic efficacy in this patient setting; based on these findings, pembrolizumab received US Food and Drug Administration (FDA) approval for the treatment of solid tumors with MMR deficiency[66,67]. Regarding high-grade gliomas, there are few prospective studies evaluating the activity of immune checkpoint inhibitors. Nivolumab was evaluated in three phase III studies, in different settings, in patients with glioblastoma: CheckMate 498 and CheckMate 548 in newly diagnosed glioblastoma patients MGMT un-methylated and methylated respectively, while CheckMate143 in recurrent glioblastoma patients. Unfortunately, these studies did not demonstrate the ability of nivolumab to extend overall survival compared to standard treatment[68–70]. Similarly, pembrolizumab was evaluated in the Keynote-028 study, a phase Ib trial that enrolled 28 patients with PD-L1 positive (≥ 1%) recurrent glioblastoma patients (only one third of patients were treated after the first relapse), showing poor results in this patient category[71]. A retrospective observational study by Memorial Sloan Kettering Cancer Center evaluated the use of pembrolizumab in 25 heavily-pretreated recurrent high-grade glioma patients and reported a low response rate although few patients had prolonged PFS[72]. Pembrolizumab was also evaluated as monotherapy or in combination with bevacizumab in a phase II study enrolling

bevacizumab-naive recurrent glioblastoma patients[73]. Also in this case, pembrolizumab was ineffective both in monotherapy and in combination with bevacizumab, regardless of the tumor biomarkers analyzed in the study population (PD-L1, Tumor Infiltrated Lymphocytes -TIL, VEGF, PlGF, VEGF-C, VEGF-D, soluble VEGFR1, bFGF and sTie-2). Unfortunately, in all these mentioned studies, neither the expression status of the proteins of the MMR complex, nor the Tumor Mutational Burden (TMB) were analyzed.

For a better clarification regarding the role of MMR status as a potential biomarker of pembrolizumab activity, we performed this prospective and observational study in which pembrolizumab was administered to patients with recurrent high-grade glioma and loss of expression of MMR proteins, used a surrogate marker for hypermutation.

## MATERIALS AND METHODS

### *Patients*

High-grade glioma patients with complete or partial loss of at least one MMR protein expression were prospectively enrolled in this single-center observational study; the trial was approved by the local Ethics Committee (IOV EC n.6.18) and complied with International Ethical Guidelines for Biomedical Research Involving Human Subject, good clinical practice guidelines and the Declaration of Helsinki. Inclusion criteria. The inclusion criteria were:

- Histologically confirmed diagnosis of high-grade glioma (glioblastoma, anaplastic astrocytoma, oligodendroglioma and anaplastic oligodendroglioma)
- Age ≥ 18 years;
- Recurrent disease according to RANO criteria[8];
- Failure of both radiotherapy and chemotherapy with temozolomide;
- No prior immunotherapy
- ECOG Performance Status 0-2
- Complete or partial loss of at least 1 MMR protein assessed by immunochemistry at diagnosis or recurrence
- Dexamethasone dosage ≤ 4 mg/day for 7 days prior to start pembrolizumab
- Written informed consent
- Prior chemotherapy discontinued at least 4 weeks prior to starting pembrolizumab
- Bidimensionally measurable enhancing lesion (10 mm) on brain MRI
- Adequate hematological, renal, hepatic function

- Absence of autoimmune diseases

***Primary and Secondary Endpoints***

The primary endpoint was Disease Control Rate (DCR) defined as the proportion of patients with confirmed complete response (CR), partial response (PR) and stable disease (SD). Among the secondary endpoints we evaluated the Progression Free Survival (PFS) defined as the time from start of pembrolizumab to disease progression or death from any cause; Overall Survival, defined as the time from start of pembrolizumab to the date of death from any cause; and Duration of Response (DOR), defined as the time from first documented evidence of partial / complete response or stable disease until the first documented progression of disease or death from any cause; and Safety.

***Procedures***

MMR protein status was evaluated with immunohistochemistry to assess the expression of the 4 main proteins of the MMR complex (MLH1, PMS2, MSH2, MSH6; Dako, Glostrup, Denmark). For each of the MMR proteins analyzed, the expression on immunohistochemistry was classified as: retained, partial loss (heterogeneous pattern of staining with coexistence of positive and of at least 30% negative tumor cells), and complete loss. Stained slides were jointly evaluated by two pathologists. All enrolled patients received the standard flat dose of intravenous pembrolizumab (200 mg once every 3 weeks) until progression according to immunotherapy response assessment in neuro-oncology (iRANO) criteria[74]. Before starting treatment with pembrolizumab, a baseline brain MRI was performed within 2 weeks of starting treatment; subsequent brain MRI for disease assessments were performed every 8 weeks or when clinically

indicated. Adverse events were rated according to National Cancer Institute Common Terminology Criteria for Adverse Events (CTCAE v4).

***Mutational and Copy Number Variation Status***

Two different targeted next-generation sequencing (NGS) panels were used. The first was the Oncomine Tumor Mutational Load (TML) assay (Thermofisher), which covers 1.65Mb of genomic space for the assessment of tumor mutational burden and includes all exons of 409 cancer-related genes for mutational and copy number assessment. The second panel, named ACC GBM panel, which was designed with the contribution of the Italian Alliance Against Cancer (ACC) and explores the mutational asset of 53 glioma-associated gene.

***Tumor Mutation Burden, Mutational Signature and Microsatellite Instability***

Tumor mutational burden and mutational spectrum were evaluated using Oncomine TML 5.10 plugin available on IonReporter software (Thermofisher).Microsatellite instability was assessed using TitanoKit (DiatechPharmacogenetics) which analyzes 6 poly-a microsatellites (BAT25, BAT26, BAT40, NR21, NR24 TGFβRII) and 4 dinucleotide markers (D2S123, D17S250, D5S346, D18S58).

***MGMT methylation status, PD-L1 and MHC-I immunohistochemistry***

MGMT promoter methylation status was assessed by pyrosequencing using a commercial kit (MGMT plus, DiatechPharmacogenetics) on a PyroMark Q96ID system equipped with PyroMark CpG (Qiagen) software. Immunohistochemistry with anti-PD-L1 (clone 22C3; Dako) and anti-MHC class I (clone ES05; Dako) primary antibodies was performed using the BOND-MAX system (Leica Biosystems).

***Macrofages and CD8+ cell density***

A multispectral imaging analysis was performed using three markers: CD8, which recognizes cytotoxic T lymphocytes, CD68 for the total macrophage fraction, and glial fibrillary acidic protein GFAP as a tissue architecture marker. The stained tissue slide was accompanied by an unstained control slide to subtract the background tissue autofluorescence signal. Cell density/$mm^2$ was chosen as the election parameter to quantitively characterize the immune cell infiltrate.

# RESULTS

### *Patients and Treatment*

From May 2017 to May 2019, 310 patients with HGG were screened by immunohistochemistry for MMR protein expression at Veneto Institute of Oncology - IRCCS. For 260 patients (84%) the analysis was performed on the tissue sample resulting from the first surgery while in 50 patients (16%) on the tissue obtained from the second surgery at recurrence. Of the entire population screened, 37 patients (12%) had a partial or complete loss of at least one of the proteins of the MMR complex but, among these, 17 had poor clinical conditions (ECOG PS> 2) and 7 were taking a cortisone dose> 4 mg / day. Ultimately, 13 patients were enrolled in the study and treated with pembrolizumab (patients characteristics reported in Table 1); of these 8 had a diagnosis of glioblastoma, 4 of anaplastic astrocytoma and 1 of anaplastic oligodendroglioma. Nine tumors (69%) were MGMT methylated and four (31%) IDH mutated. Six HGGs had concurrent partial loss of MSH2 and MSH6, one had complete loss of both MSH2 and MSH6; one had complete loss of MSH6 alone, two had partial loss of both MLH1 and PMS2, two partial loss of MSH2 alone and one partial loss of MSH6 alone (Table 2). In four cases, immunohistochemistry and molecular analysis were performed on the primary tumor while in 9 cases in recurrent tumor. In patients enrolled, median prior chemotherapy lines were two (range 1-5) and the entire patients population received radiotherapy and temozolomide as first line treatment.

### *Safety and Clinical Activity*

At the time of analysis, median follow-up was 20.6 months, and two patients were still alive. Median number of cycles of pembrolizumab was 3 (range 1-23) and all patients discontinued treatment due to disease progression. Therapy was well tolerated: only

| **Patient characteristics** | **N (%)** |
|---|---|
| Patients | 13 (100%) |
| Median Age | 43 (range 21-65) |
| Gender | |
| - *Male* | 7 (54) |
| - *Female* | 6 (46) |
| ECOG PS | |
| - *0* | 3 (23) |
| - *1* | 9 (69) |
| - *2* | 1 (8) |
| Histology | |
| - *Glioblastoma* | 8 (61) |
| - *Anaplastic Astrocytoma* | 4 (31) |
| - *Anaplastic Oligodendroglioma* | 1 (8) |
| Surgery at recurrence | 9 (69) |
| Prior Radiotherapy | 13 (100) |
| Prior Chemotherapy (temozolomide) | 13 (100) |
| Median previous CT lines | (2 (range 1-5) |
| Steroids | |
| - *Yes* | 5 (38) |
| - *No* | 8 (62) |
| MGMT status | |
| - *Methylated* | 9 (69) |
| - *Unmethylated* | 4 (31) |
| IDH status | |
| - *Wild - type* | 9 (69) |
| - *Mutated* | 4 (31) |

Table1: Patient characteristics

one patient (8%) reported a grade 3 maculo-papular rash as pembrolizumab-related adverse event. Regarding pembrolizumab activity in this patient cohort, DCR was 31%

with 4 patients achieved stable disease and none with partial or complete response. Nine patients (69%) achieved progression of disease as the best response (Table 3).

| **Characteristics** | **N (%)** |
|---|---|
| Status of MMR protein expression | |
| Complete loss of *MSH2+MSH6* | 1 (8) |
| Complete loss of *MSH6* | 1 (8) |
| Partial loss of *MSH2+MSH6* | 6 (46) |
| Partial loss of *MLH1+PMS2* | 2 (15) |
| Partial loss of *MSH6* | 1 (8) |
| Partial loss of *MSH2* | 2 (15) |

Table2: MMR expression

The four patients with stable disease were two with diagnosis of anaplastic astrocytoma, one glioblastoma and one anaplastic oligodendroglioma; median duration of stable disease was 7.7 months (range 5.2 - 16.7). Two cases had partial loss of both MSH2 and MSH6 protein expression, one had partial loss of MSH2 alone and one had partial loss of MSH 6 alone. Only one patient had IDH mutated tumor. Median PFS was 2.2 months (95%CI 1.6 - 2.8); the 6-months PFS was 23 %. Median OS was 5.6 months (95%CI 0.1 - 11.9) with 12-months OS of 38% (Figure 3).

| | |
|---|---|
| Complete response | 0/13 (0%) |
| Partial Response | 0/13 (0%) |
| Stable Disease | 4/13 (31%) |
| Disease Control Rate | 4/13 (31%) |
| Progression Disease | 9/13 (69%) |
| mPFS | 2.2 months (95%CI 1.6-2.8) |
| - 6months-PFS | 23% |
| mOS | 5.6 months (95%CI 0.1-11.9) |
| - 12 months-OS | 38% |

Table 3: Pembrolizumab activity and efficacy

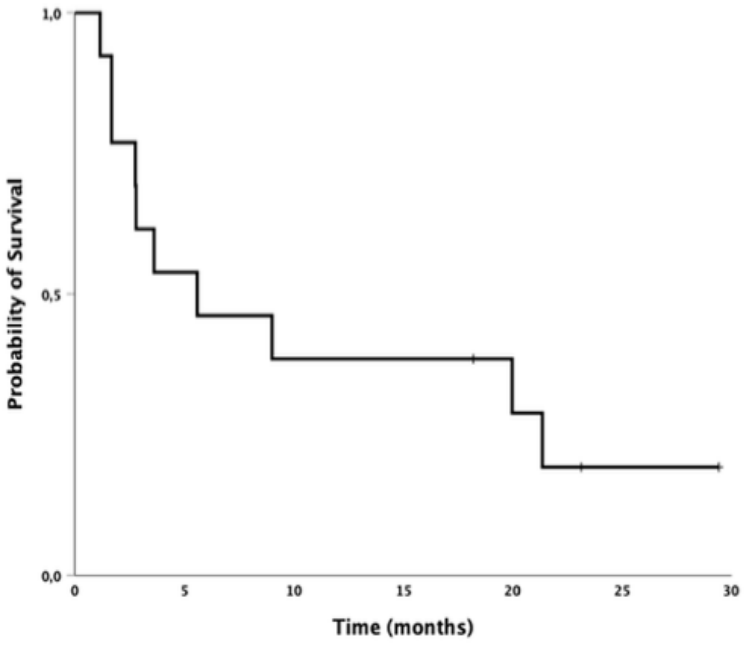


Figure 3: Kaplan-Meier Survival Analysis (OS). mOS was 5.6 (95%CI 0.1-11.9)

***Multigene Mutation Status***

Molecular and tumor microenvironment analysis were performed on samples from 12 enrolled patients for whom tumor tissue was available; for one patient, tissue was not

available for these analyses. Average sequencing coverage obtained with tumor mutational load (TML) next generation sequencing (NGS) panel was 277x (120-556x) in tumor and 274x (125-651x) in normal tissue; overall, 29 mutations were identified in 14 genes and mutations were found in at least one gene in all 12 cases (Figure 4). The most frequent somatic mutations were in *TP53* (8/12; 67%) and *IDH1*(4/12; 33%). *ATRX, NF1, PTPN11* and *RET* mutations were found in two cases (17%). T*P53, IDH1, ATRX* and *NF1* mutation were confirmed by using the ACC GBM capture-based custom panel. A truncating somatic mutation of the *MSH6* MMR gene was found in a sample of a patient diagnosed with glioblastoma and was also confirmed by the ACC GBM panel: this patient had disease progression during treatment with pembrolizumab.

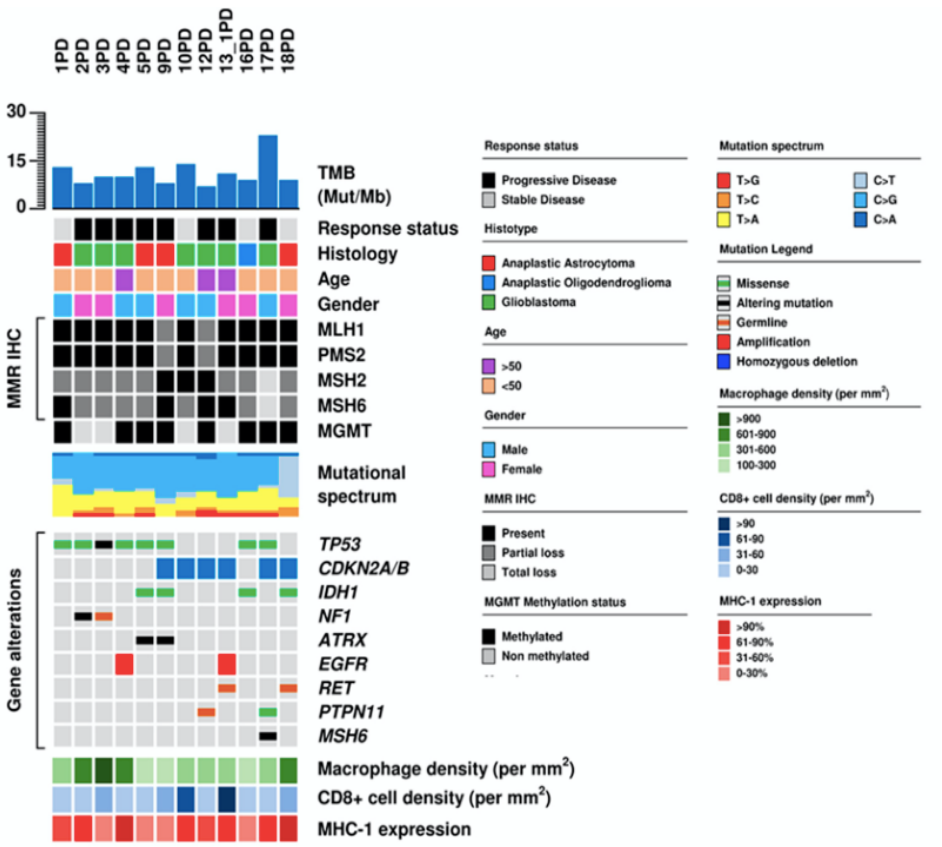


Figure 4: Genomic and Immunological characteristics of 12 HGG patients

(In the matrix we show the characteristics of each patient and matched tumor sample. Gene mutation, immunohistochemical analysis of MMR gene, are correlated to histology, treatment response, tumor mutational burden (TMB) and immunological characteristics)

Germline mutations were found in five patients: one patient had the *PTPN11* Asp61Tyr mutation (rs397507510), reported as pathogenic in the ClinVar database; one had *RET* Arg982Cys (rs17158558) mutation and three had mutation in NF1 (rs769087878), PMS1 (rs2066459), RET (rs149238501) respectively, classified as of uncertain clinical significance in the ClinVar database. Tumor Mutational Burden (TMB) in the 12 HGG ranged between 6.7 and 26.9 mutations/Mb (median 10.02) (Figure 4). In 8 out of 12 cases, a number of mutations> 9 mut / Mb was found, thus being able to consider these tumors as hypermutated[75] but all tumors were microsatellite stable. Only one case had a pathogenic MSH6 somatic mutation; just this one had the highest TMB (26.9 mutations / Mb), a CD8 + density of 30.6/mm$^2$ and a macrophage density of 483.1/mm$^2$. All patients analyzed had low (≤ 5%) or absent PD-L1 expression. Analyzing separately the two patient populations of the study, divided into those who had a PD as best response and those who had instead SD, we found that: in the PD group, the median value of TMB was 10.02 mutations / Mb, the median value of expression of MHC-I was 60%, the median value of the macrophage density was 438.05/mm$^2$ and the density of CD8 + was 25.9/mm$^2$. In the SD group, the median TMB was 11.36 mutations/Mb, the median expression of MHC-I was 55%, the median macrophage density was 407.45/mm$^2$ and CD8+ density was 30.05/mm$^2$. All these characteristics, together with the complete or partial loss of MMR protein expression, the methylation status of the MGMT promoter and the IDH mutation status do not appear to be significantly different between the PD and SD populations (Table 4). Regarding the gene and chromosomal Copy Number Variants (CNV), focal gene amplification was found in seven genes: EGFR in two cases, PDGFRA and CDK4 in four cases and KIT, KDR, MDMR4 and PIK3C2B in one case. Two genes (CDKN2A and CDKN2B) showed homozygous deletions in 6 of 12 cases (50%). The most frequent whole chromosome

alterations were gains of chromosome 7 and 8 (2/12, 17%) and loss of chromosome 9 (6/12, 50%).

| **Variables** | ***p* Value** |
|---|---|
| Loss of (yes Vs no) | |
| - *MSH2* | 0.7 |
| - *MSH6* | 0.4 |
| - *PMS2* | 0.5 |
| - *MLH1* | 0.5 |
| MMR protein loss (complete Vs partial) | 0.9 |
| MGMT status (methylated Vs unmethylated) | 0.2 |
| IDH status (mutated Vs wild-type) | 0.9 |
| TMB | 0.5 |
| MHC-I expression | 0.9 |
| Macrophage density | 0.9 |
| CD8+ density | 0.9 |

Table 4: Association of molecular and immunological variables with Disease Control Rate

## DISCUSSION

Our prospective and observational study demonstrated that pembrolizumab does not appear to lead clinical benefit in patients with high-grade glioma who have partial or complete loss of expression of at least one of the MMR proteins. Treatment with immune checkpoint inhibitors pembrolizumab or nivolumab has already been tested in patients with recurrent and newly diagnosed high-grade glioma, but MMR status was not assessed. Two recent and international phase III trial evaluated the use of nivolumab (anti-PD1) in patients with newly diagnosed methylated and unmethylated MGMT glioblastoma respectively, associated with chemoradiotherapy treatment according to the STUPP protocol. Both completed enrollment and neither was a survival advantage confirmed for the addition of nivolumab[76,77]. Another phase III trial (CheckMate 143) evaluated use of nivolumab in recurrent glioblastoma patients showing a 6-months PFS of 15.7% and a 12-months OS of 41.8%; in terms of activity, the disease control rate (DCR) was found to be 29.4% with SD in 21.6% patients, PR in 6.5% and CR in 1.3%[70]. Regarding the use of pembrolizumab, two major studies have evaluated its activity and efficacy in patients with brain tumors. The Phase Ib study, Keynote-028 trial[71], evaluated the safety and efficacy of pembrolizumab in 25 patients with PD-L1 positive recurrent glioblastoma; of these, 31% had already received at least two lines of chemotherapy. The DCR was found to be 52% with SD in 48% of patients and PR in 4%; the 6-months PFS was 44% and the 12-months OS was 74%. A more recent phase II basket trial, Keynote-158[63], evaluated pembrolizumab in several types of noncolorectal cancer with immunoistochemical loss of at least one MMR protein or on the presence of microsatellite instability (MSI) at PCR. Of all the tumors treated, only in patients with brain tumor there were no radiological responses to treatment (the histology of these tumors was not specified). Of all the tumors treated, only in patients

with brain tumors there were no radiological responses to treatment (the histology of these tumors was not specified). In the same cohort, the median PFS was 1.1 months (95% CI 0.7-2.1) while the median OS was 5.6 month (95% CI 1.5-16-2), similar data to those of the sample analyzed in our study. In patients diagnosed with a different tumor than brain tumors, the Objective Response Rate ORR (CR + PR) was found to be between 18% and 57%. Like our study, these data also confirm that immunohistochemical loss of MMR proteins cannot be considered a predictive biomarker for the efficacy of immune checkpoint inhibitors treatment in patients with high-grade glioma. Although all patients in our study had partial or complete loss of expression of at least one of the MMR complex proteins, none of them were found to have microsatellite instability (MSI). This data had already been confirmed previously by a study[78] with the observation that, despite the partial or total loss of expression in the immunohistochemical analysis of the MMR proteins, the presence of MSI could not be present. The same study had in fact demonstrated by single cell whole genome sequencing that MSI could be present in cellular subclones and therefore would not be detectable by the MSI assay. This finding could explain why none of the samples analyzed in our study were MSI. In the sample analyzed by our study, we found 8 cases (67%) that were hypermutated (> 9mus / Mb) and no cases were ultramutated (> 100 muts / Mb). Of the hypermutated cases, 3 were treatment-naïve while 5 were previously treated with temozolomide; the hypermutated phenotype could be related to previous temozolomide therapy in 5/8 cases in which TMB was evaluated in recurrent tumor. The possible correlation between MMR status and hypermutation is currently unclear in gliomas[35,36,79]. Compared to the data present in the literature[34], the high frequency of hypermutated gliomas in our population suggests that the loss of expression of MMR proteins could be useful for detecting hypermutated tumors, as well as McCord et al.[35]

proposed a previous work. In the latter study, 8/9 hypermutated gliomas had a loss of MMR expression on immunohistochemistry and MMR mutations, but the microsatellite status was not analyzed as it has not been elucidated whether these hypermutated patients identified with MMR immunohistochemical loss are responsive. to immune checkpoint inhibitors[35]. Our study showed that patients with high-grade hypermutated glioma and immunohistochemical loss of MMR treated with pembrolizumab had stable disease (SD) as the best response to treatment and that TMB values were similar both in patients with stable disease (SD) than with progression disease (PD). Since none of the cases included in our study had MSI and an MMR mutation was found in one case, it can be inferred that MMR immunoistochemical loss may not be correlated with MMR deficiency (dMMR) in gliomas and that, furthermore , cannot represent a predictive biomarker for the selection of patients with glioma to be candidates for treatment with immune checkpoit inhibitors. A further retrospective analysis by Touat et al.[78] demonstrated the lack of efficacy of anti-PD1 treatment in 11 patients with hypermutation e loss of MMR immunoistochemical expression; this data can strengthen the observation that, although immunohistochemical loss MMR may be a surrogate for the state of hypermutation, it cannot certainly predict the efficacy of anti-PD1 treatment in glioma patients. TMB analysis has always attracted a lot of attention, since the advent of immune checkpoint inhibitors. There is a study in the literature that demonstrated that a high TMB is associated with a better survival in patients with several types of cancer who received therapy with immune checkpoint inhibitors, with the sole exception of high-grade gliomas[80]. In the study it was shown that there is an extreme variability between the different types of cancer regarding the cut-off value to define a disease with "high TMB": in fact, as regards gliomas this value stands at 5.9 muts / Mb (compared for example to 52.2 muts / Mb in colorectal cancer).

Precisely for this reason, as reported in patients with MMR germline mutations[81], we hypothesized that patients with high-grade glioma can benefit from treatment with immune checkpoint inhibitors only if ultramutated (> 100muts / Mb). The analysis of the tumor microenvironment in patients undergoing treatment with immune checkpoint inhibitors is of fundamental importance as it is known that it can significantly influence the activity of this category of pharmacy. This is of the most importance in patients with high-grade glioma. similar to our study, a trial published in 2019[82] showed how TMB in 66 patients with glioblastoma treated with pembrolizumab or nivolumab was not different between responders and non-responders; the same study showed that, in non-responders' patients, in addition to an immunosuppressive expression signature, a high level of CD68 + macrophage infiltration was also present. In line with this data and with previously published data in which a particularly immunosuppressive environment is a characteristic of high-grade gliomas[83], the patients treated in our study who showed a stable disease (SD) were those with lower grade of glioma and, in case of glioblastoma histology, an high mutational load, low presence of immune suppressive macrophages CD68 +, high number of CD8 + T cells and high expression of MHC class I molecules. These aspects suggest that a high tumor mutational load and a tumor microenvironment that is not strongly immunosuppressive may be the basis of the efficacy and activity of immune checkpoint inhibitors in patients with high-grade glioma. The possibility of combining treatment with immune checkpoint inhibitors with other drugs that are able to inhibit immunosuppressive tumor macrophages could be the turning point in the treatment of this patient population: regorafenib, an oral multi-kinase inhibitor could be a candidate. valid as, in the REGOMA trial[26] , it was also shown to improve OS in recurrent glioblastoma patients. A phase II basket trial is active on this aspect (NCT04704154) which is evaluating the association of regorafenib and

nivololumab in several types of cancer, including glioblastoma. However, no results are available now.

This study certainly has several limitations: first of all, the sample size. In fact, we analyzed only 12 high-grade glioma patients but, this being a monocentric pilot study and considering the rarity of the loss of expression of MMR proteins in gliomas, the enormous effort made to enroll this number of patients and to perform this type of molecular analysis, must be considered. Secondly, this patient cohort appears to be not perfectly homogeneous; in fact, both patients with complete and partial loss of MMR protein expression were enrolled and, moreover, both grade III and grade IV glioma patients were enrolled. We hypothesized, as reported by previous experiences[34], that even patients with partial loss of MMR expression could be hypermutated. Regarding histology, however, this limitation makes it difficult to compare with the other phase II and III studies that used ICI, but that only enrolled patients with glioblastoma (grade IV). However, these results are similar to a previous retrospective study that included 25 recurrent, non-hypermutated HGG (10 grade III gliomas), treated with pembrolizumab[72].

The project of this PhD thesis produced a publication in the journal "Cancers (Basel)" in August 2020[84].

# BIBLIOGRAPHY


1. Ostrom, Q. T. *et al.* CBTRUS Statistical Report: Primary Brain and Other Central Nervous System Tumors Diagnosed in the United States in 2013-2017. *Neuro-Oncol.* **22**, iv1–iv96 (2020).

2. Louis, D. N. *et al.* The 2016 World Health Organization Classification of Tumors of the Central Nervous System: a summary. *Acta Neuropathol. (Berl.)* **131**, 803–820 (2016).

3. Louis, D. N. *et al.* The 2007 WHO classification of tumours of the central nervous system. *Acta Neuropathol. (Berl.)* **114**, 97–109 (2007).

4. Aldape, K. *et al.* Discrepancies in diagnoses of neuroepithelial neoplasms: the San Francisco Bay Area Adult Glioma Study. *Cancer* **88**, 2342–2349 (2000).

5. Macdonald, D. R., Cascino, T. L., Schold, S. C. & Cairncross, J. G. Response criteria for phase II studies of supratentorial malignant glioma. *J. Clin. Oncol. Off. J. Am. Soc. Clin. Oncol.* **8**, 1277–1280 (1990).

6. Wood, J. R., Green, S. B. & Shapiro, W. R. The prognostic importance of tumor size in malignant gliomas: a computed tomographic scan study by the Brain Tumor Cooperative Group. *J. Clin. Oncol. Off. J. Am. Soc. Clin. Oncol.* **6**, 338–343 (1988).

7. Overcast, W. B. *et al.* Advanced imaging techniques for neuro-oncologic tumor diagnosis, with an emphasis on PET-MRI imaging of malignant brain tumors. *Curr. Oncol. Rep.* **23**, 34 (2021).

8. Albert, N. L. *et al.* Response Assessment in Neuro-Oncology working group and European Association for Neuro-Oncology recommendations for the clinical use of PET imaging in gliomas. *Neuro-Oncol.* **18**, 1199–1208 (2016).

9. Sanai, N. & Berger, M. S. Extent of resection influences outcomes for patients with gliomas. *Rev. Neurol. (Paris)* **167**, 648–654 (2011).

10. Ammirati, M., Vick, N., Liao, Y. L., Ciric, I. & Mikhael, M. Effect of the extent of surgical resection on survival and quality of life in patients with supratentorial glioblastomas and anaplastic astrocytomas. *Neurosurgery* **21**, 201–206 (1987).

11. Montemurro, N., Perrini, P., Blanco, M. O. & Vannozzi, R. Second surgery for recurrent glioblastoma: A concise overview of the current literature. *Clin. Neurol. Neurosurg.* **142**, 60–64 (2016).

12. Brandes, A. A., Bartolotti, M. & Franceschi, E. Second surgery for recurrent glioblastoma: advantages and pitfalls. *Expert Rev. Anticancer Ther.* **13**, 583–587 (2013).

13. Prados, M. D. *et al.* Phase III trial of accelerated hyperfractionation with or without difluromethylornithine (DFMO) versus standard fractionated radiotherapy

with or without DFMO for newly diagnosed patients with glioblastoma multiforme. *Int. J. Radiat. Oncol. Biol. Phys.* **49**, 71–77 (2001).

14. Kazmi, F., Soon, Y. Y., Leong, Y. H., Koh, W. Y. & Vellayappan, B. Re-irradiation for recurrent glioblastoma (GBM): a systematic review and meta-analysis. *J. Neurooncol.* **142**, 79–90 (2019).

15. Stupp, R. *et al.* Radiotherapy plus concomitant and adjuvant temozolomide for glioblastoma. *N. Engl. J. Med.* **352**, 987–996 (2005).

16. Stupp, R. *et al.* Effects of radiotherapy with concomitant and adjuvant temozolomide versus radiotherapy alone on survival in glioblastoma in a randomised phase III study: 5-year analysis of the EORTC-NCIC trial. *The Lancet.Oncology* **10**, 459–466 (2009).

17. Hegi, M. E. *et al.* MGMT gene silencing and benefit from temozolomide in glioblastoma. *N. Engl. J. Med.* **352**, 997–1003 (2005).

18. van den Bent, M. J. *et al.* Adjuvant and concurrent temozolomide for 1p/19q non-co-deleted anaplastic glioma (CATNON; EORTC study 26053-22054): second interim analysis of a randomised, open-label, phase 3 study. *Lancet Oncol.* **22**, 813–823 (2021).

19. Cairncross, J. G. *et al.* Specific genetic predictors of chemotherapeutic response and survival in patients with anaplastic oligodendrogliomas. *J. Natl. Cancer Inst.* **90**, 1473–1479 (1998).

20. van den Bent, M. J. *et al.* Randomized phase II trial of erlotinib versus temozolomide or carmustine in recurrent glioblastoma: EORTC brain tumor group study 26034. *J. Clin. Oncol. Off. J. Am. Soc. Clin. Oncol.* **27**, 1268–1274 (2009).

21. Franceschi, E. *et al.* Gefitinib in patients with progressive high-grade gliomas: a multicentre phase II study by Gruppo Italiano Cooperativo di Neuro-Oncologia (GICNO). *Br. J. Cancer* **96**, 1047–1051 (2007).

22. Franceschi, E. *et al.* EORTC 26083 phase I/II trial of dasatinib in combination with CCNU in patients with recurrent glioblastoma. *Neuro-Oncol.* **14**, 1503–1510 (2012).

23. Rich, J. N. *et al.* Phase II trial of gefitinib in recurrent glioblastoma. *J. Clin. Oncol. Off. J. Am. Soc. Clin. Oncol.* **22**, 133–142 (2004).

24. Kreisl, T. N. *et al.* Continuous daily sunitinib for recurrent glioblastoma. *J. Neurooncol.* **111**, 41–48 (2013).

25. Kreisl, T. N. *et al.* A phase I/II trial of vandetanib for patients with recurrent malignant glioma. *Neuro-Oncol.* **14**, 1519–1526 (2012).

26. Lombardi, G. *et al.* Regorafenib compared with lomustine in patients with relapsed glioblastoma (REGOMA): a multicentre, open-label, randomised, controlled, phase 2 trial. *The Lancet.Oncology* **20**, 110–119 (2019).

27. Ducray, F. *et al.* Predictive and prognostic factors for gliomas. *Expert Rev. Anticancer Ther.* **11**, 781–789 (2011).

28. Wick, A. *et al.* Superiority of temozolomide over radiotherapy for elderly patients with RTK II methylation class, MGMT promoter methylated malignant astrocytoma. *Neuro-Oncol.* **22**, 1162–1172 (2020).

29. Malmstrom, A. *et al.* Temozolomide versus standard 6-week radiotherapy versus hypofractionated radiotherapy in patients older than 60 years with glioblastoma: the Nordic randomised, phase 3 trial. *The Lancet.Oncology* **13**, 916–926 (2012).

30. Karsy, M. *et al.* A practical review of prognostic correlations of molecular biomarkers in glioblastoma. *Neurosurg. Focus* **38**, E4 (2015).

31. Krell, D. *et al.* IDH mutations in tumorigenesis and their potential role as novel therapeutic targets. *Future Oncol. Lond. Engl.* **9**, 1923–1935 (2013).

32. Sanson, M. *et al.* Isocitrate dehydrogenase 1 codon 132 mutation is an important prognostic biomarker in gliomas. *J. Clin. Oncol. Off. J. Am. Soc. Clin. Oncol.* **27**, 4150–4154 (2009).

33. Labussiere, M. *et al.* Combined analysis of TERT, EGFR, and IDH status defines distinct prognostic glioblastoma classes. *Neurology* **83**, 1200–1206 (2014).

34. Hodges, T. R. *et al.* Mutational burden, immune checkpoint expression, and mismatch repair in glioma: implications for immune checkpoint immunotherapy. *Neuro-Oncol.* **19**, 1047–1057 (2017).

35. McCord, M. *et al.* The efficacy of DNA mismatch repair enzyme immunohistochemistry as a screening test for hypermutated gliomas. *Acta Neuropathol. Commun.* **8**, 15-020-0892–2 (2020).

36. Indraccolo, S. *et al.* Genetic, Epigenetic, and Immunologic Profiling of MMR-Deficient Relapsed Glioblastoma. *Clin. Cancer Res. Off. J. Am. Assoc. Cancer Res.* **25**, 1828–1837 (2019).

37. Barresi *et al.* Ultra-Mutation in IDH Wild-Type Glioblastomas of Patients Younger than 55 Years is Associated with Defective Mismatch Repair, Microsatellite Instability, and Giant Cell Enrichment. *Cancers* **11**, 1279 (2019).

38. Caccese, M. *et al.* Mismatch-Repair Protein Expression in High-Grade Gliomas: A Large Retrospective Multicenter Study. *Int. J. Mol. Sci.* **21**, 6716 (2020).

39. Wintterle, S. *et al.* Expression of the B7-related molecule B7-H1 by glioma cells: a potential mechanism of immune paralysis. *Cancer Res.* **63**, 7462–7467 (2003).

40. Chen, L. & Han, X. Anti-PD-1/PD-L1 therapy of human cancer: past, present, and future. *J. Clin. Invest.* **125**, 3384–3391 (2015).

41. Weber, J. S. *et al.* Nivolumab versus chemotherapy in patients with advanced melanoma who progressed after anti-CTLA-4 treatment (CheckMate 037): a randomised, controlled, open-label, phase 3 trial. *The Lancet.Oncology* **16**, 375–384 (2015).

42. Larkin, J. *et al.* Combined Nivolumab and Ipilimumab or Monotherapy in Untreated Melanoma. *N. Engl. J. Med.* **373**, 23–34 (2015).

43. Brahmer, J. *et al.* Nivolumab versus Docetaxel in Advanced Squamous-Cell Non-Small-Cell Lung Cancer. *N. Engl. J. Med.* **373**, 123–135 (2015).

44. Reck, M. *et al.* Pembrolizumab versus Chemotherapy for PD-L1-Positive Non-Small-Cell Lung Cancer. *N. Engl. J. Med.* **375**, 1823–1833 (2016).

45. Motzer, R. J. *et al.* Nivolumab versus Everolimus in Advanced Renal-Cell Carcinoma. *N. Engl. J. Med.* **373**, 1803–1813 (2015).

46. Ansell, S. M. *et al.* PD-1 blockade with nivolumab in relapsed or refractory Hodgkin's lymphoma. *N. Engl. J. Med.* **372**, 311–319 (2015).

47. Borghaei, H. *et al.* Nivolumab versus Docetaxel in Advanced Nonsquamous Non-Small-Cell Lung Cancer. *N. Engl. J. Med.* **373**, 1627–1639 (2015).

48. Eggermont, A. M. M. *et al.* Adjuvant Pembrolizumab versus Placebo in Resected Stage III Melanoma. *N. Engl. J. Med.* **378**, 1789–1801 (2018).

49. Garon, E. B. *et al.* Pembrolizumab for the treatment of non-small-cell lung cancer. *N. Engl. J. Med.* **372**, 2018–2028 (2015).

50. Antonia, S. J. *et al.* Durvalumab after Chemoradiotherapy in Stage III Non-Small-Cell Lung Cancer. *N. Engl. J. Med.* **377**, 1919–1929 (2017).

51. Kaufman, H. L. *et al.* The promise of Immuno-oncology: implications for defining the value of cancer treatment. *J. Immunother. Cancer* **7**, 129 (2019).

52. Quail, D. F. & Joyce, J. A. The Microenvironmental Landscape of Brain Tumors. *Cancer Cell* **31**, 326–341 (2017).

53. Margolin, K. *et al.* Ipilimumab in patients with melanoma and brain metastases: an open-label, phase 2 trial. *Lancet Oncol.* **13**, 459–465 (2012).

54. Goldberg, S. B. *et al.* Pembrolizumab for patients with melanoma or non-small-cell lung cancer and untreated brain metastases: early analysis of a non-randomised, open-label, phase 2 trial. *The Lancet.Oncology* **17**, 976–983 (2016).

55. Harriet M. Kluger , Sarah B. Goldberg , Mario Sznol , John Tsiouris , Alexander Vortmeyer , Lucia JilaveanuAmanda L. Ralabate , Angel L. Rivera , Matthew M. Burke , Upendra P. Hegbe , Justine Vanessa Cohen , Xiaopan Yao , Stephanie Speaker , Matthew Madura , Elizabeth Knapp-Perry , Amit Mahajan , Veronica Chiang. Safety and activity of pembrolizumab in melanoma patients with untreated brain metastases.

56. Tawbi, H. A. *et al.* Combined Nivolumab and Ipilimumab in Melanoma Metastatic to the Brain. *N. Engl. J. Med.* **379**, 722–730 (2018).

57. Goldberg, S. B. *et al.* Pembrolizumab for management of patients with NSCLC and brain metastases: long-term results and biomarker analysis from a non-randomised, open-label, phase 2 trial. *Lancet Oncol.* **21**, 655–663 (2020).

58. Lombardi, G. *et al.* Immune-checkpoint inhibitors in brain metastases from renal cell carcinoma: a battle was lost but not the war. *Ann. Transl. Med.* **7**, S222–S222 (2019).

59. Di Giacomo, A. M. *et al.* Immunotherapy of brain metastases: breaking a 'dogma'. *J. Exp. Clin. Cancer Res. CR* **38**, 419 (2019).

60. Le, D. T. *et al.* Mismatch repair deficiency predicts response of solid tumors to PD-1 blockade. *Science* **357**, 409–413 (2017).

61. Azad, N. S. *et al.* Nivolumab Is Effective in Mismatch Repair–Deficient Noncolorectal Cancers: Results From Arm Z1D—A Subprotocol of the NCI-MATCH (EAY131) Study. *J. Clin. Oncol.* **38**, 214–222 (2020).

62. Le, D. T. *et al.* PD-1 Blockade in Tumors with Mismatch-Repair Deficiency. *N. Engl. J. Med.* **372**, 2509–2520 (2015).

63. Marabelle, A. *et al.* Efficacy of Pembrolizumab in Patients With Noncolorectal High Microsatellite Instability/Mismatch Repair-Deficient Cancer: Results From the Phase II KEYNOTE-158 Study. *J. Clin. Oncol. Off. J. Am. Soc. Clin. Oncol.* **38**, 1–10 (2020).

64. Overman, M. J. *et al.* Durable Clinical Benefit With Nivolumab Plus Ipilimumab in DNA Mismatch Repair–Deficient/Microsatellite Instability–High Metastatic Colorectal Cancer. *J. Clin. Oncol.* **36**, 773–779 (2018).

65. Andre, T. *et al.* Pembrolizumab versus chemotherapy for microsatellite instability-high/mismatch repair deficient metastatic colorectal cancer: The phase 3 KEYNOTE-177 Study. *J. Clin. Oncol.* **38**, LBA4–LBA4 (2020).

66. FDA grants accelerated approval to pembrolizumab for first tissue/site agnostic indication.

67. Lemery, S., Keegan, P. & Pazdur, R. First FDA Approval Agnostic of Cancer Site - When a Biomarker Defines the Indication. *N. Engl. J. Med.* **377**, 1409–1412 (2017).

68. https://news.bms.com/news/details/2020/Bristol-Myers-Squibb-Announces-Update-on-Phase-3-CheckMate--548-Trial-Evaluating-Patients-with-Newly-Diagnosed-MGMT-Methylated-Glioblastoma-Multiforme/default.aspx.

69. https://news.bms.com/news/corporate-financial/2019/Bristol-Myers-Squibb-Announces-Phase-3-CheckMate--498-Study-Did-Not-Meet-Primary-Endpoint-of-Overall-Survival-with-Opdivo-nivolumab-Plus-Radiation-in-Patients-with-Newly-Diagnosed-MGMT-Unmethylated-Glioblastoma-Multiforme/default.aspx.

70. Reardon, D. A. *et al.* Effect of Nivolumab vs Bevacizumab in Patients With Recurrent Glioblastoma: The CheckMate 143 Phase 3 Randomized Clinical Trial. *JAMA Oncol.* **6**, 1003–1010 (2020).

71. Reardon, D. A. *et al.* Treatment with pembrolizumab in programmed death ligand 1-positive recurrent glioblastoma: Results from the multicohort phase 1 KEYNOTE-028 trial. *Cancer* **127**, 1620–1629 (2021).

72. Reiss, S. N., Yerram, P., Modelevsky, L. & Grommes, C. Retrospective review of safety and efficacy of programmed cell death-1 inhibitors in refractory high grade gliomas. *J. Immunother. Cancer* **5**, 99-017-0302–x (2017).

73. Nayak, L. *et al.* Randomized Phase II and Biomarker Study of Pembrolizumab plus Bevacizumab versus Pembrolizumab Alone for Patients with Recurrent Glioblastoma. *Clin. Cancer Res. Off. J. Am. Assoc. Cancer Res.* **27**, 1048–1057 (2021).

74. Okada, H. *et al.* Immunotherapy response assessment in neuro-oncology: a report of the RANO working group. *Lancet Oncol.* **16**, e534–e542 (2015).

75. Campbell, B. B. *et al.* Comprehensive Analysis of Hypermutation in Human Cancer. *Cell* **171**, 1042-1056.e10 (2017).

76. Bristol-Myers Squibb - Press Release. Bristol-Myers Squibb Announces Phase 3 CheckMate -498 Study Did Not Meet Primary Endpoint of Overall Survival with Opdivo (nivolumab) Plus Radiation in Patients with Newly Diagnosed MGMT-Unmethylated Glioblastoma Multiforme.

77. Bristol Myers Squibb Announces Update on Phase 3 CheckMate -548 Trial Evaluating Patients with Newly Diagnosed MGMT-Methylated Glioblastoma Multiforme.

78. Touat, M. *et al.* Mechanisms and therapeutic implications of hypermutation in gliomas. *Nature* **580**, 517–523 (2020).

79. Wang, J. *et al.* Clonal evolution of glioblastoma under therapy. *Nat. Genet.* **48**, 768–776 (2016).

80. Samstein, R. M. *et al.* Tumor mutational load predicts survival after immunotherapy across multiple cancer types. *Nat. Genet.* **51**, 202–206 (2019).

81. Bouffet, E. *et al.* Immune Checkpoint Inhibition for Hypermutant Glioblastoma Multiforme Resulting From Germline Biallelic Mismatch Repair Deficiency. *J. Clin. Oncol. Off. J. Am. Soc. Clin. Oncol.* **34**, 2206–2211 (2016).

82. Zhao, J. *et al.* Immune and genomic correlates of response to anti-PD-1 immunotherapy in glioblastoma. *Nat. Med.* **25**, 462–469 (2019).

83. Pinton, L. *et al.* The immune suppressive microenvironment of human gliomas depends on the accumulation of bone marrow-derived macrophages in the center of the lesion. *J. Immunother. Cancer* **7**, 58-019-0536–x (2019).

84. Lombardi, G. *et al.* Pembrolizumab Activity in Recurrent High-Grade Gliomas with Partial or Complete Loss of Mismatch Repair Protein Expression: A Monocentric, Observational and Prospective Pilot Study. *Cancers* **12**, (2020).