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<AT>Primary and metastatic brain cancer genomics and emerging biomarkers for immunomodulatory cancer treatment. , , F. Passiglia[1*], C. Caglevic[2*], E. Giovannetti[3], JA. Pinto[4], P. Manca[5], S. Taverna[1], A. Listì[1], I. Gil-Bazo[6], LE. Raez[7], A. Russo[1], C. Rolfo[8#<AU>]
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<ABS-HEAD>Abstract

<ABS-P>Recent studies with immunomodulatory agents targeting both cytotoxic T-lymphocyte protein 4 (CTLA4) and programmed cell death 1 (PD1)/ programmed cell death ligand 1 (PDL1) have shown to be very effective in several cancers revealing an unexpected great activity in patients with both primary and metastatic brain tumors. Combining anti-CTLA4 and anti-PD1 agents as upfront systemic therapy has revealed to further increase the clinical benefit observed with single agent, even at cost of higher toxicity. Since the brain is an immunological specialized area it‘s crucial to establish the specific composition of the brain tumors’ microenvironment in order to predict the potential activity of immunomodulatory agents. This review briefly summarizes the basis of the brain immunogenicity, providing the most updated clinical evidences in terms of immune-checkpoint inhibitors efficacy and toxicity in both primary and metastatic brain tumors with the final aim of defining potential biomarkers for immunomodulatory cancer treatment.
<KWD>Keywords: Brain; metastasis; immunotherapy; CTLA4; PD1/PDL1; biomarkers

<H1>1. Introduction

Primary malignant brain tumors and central nervous system (CNS) metastasis are associated with poor prognosis. Despite multimodality approaches, including local surgical and radiation treatments and systemic chemotherapies, morbidity and mortality remain still very high, reaching a median overall survival (OS) of about 12 months. The natural history of these patients is characterized by a progressive neurological deterioration and a rapid decline of their quality of life (QoL) because of the very aggressive pattern of growth associated with these tumors and the toxicity profile related to the combination therapies. Thus, we have an urgent clinical need of new effective treatment strategies which are able to extend the survival of patients affected by both primary and metastatic brain cancers preserving their QoL.
There are now several new biological drugs available that are effective for brain metastasis, like the third-generation epidermal growth factor receptor tyrosine kinase inhibitor (EGFR-TKI) osimertinib in lung cancer. This compound is able to selectively target both the EGFR activating and resistant

T790M mutations[1] and has shown a promising activity in T790M+ patients with CNS disease[2], likely due to its greater penetration in animal models' blood brain barrier (BBB), as compared to other TKIs, gefitinib, rociletinib, or afatinib[3]. Two randomized phase III AURA and FLAURA trials showed a significant survival benefit along with a more tolerable safety profile in favour of osimertinib over platinum-chemotherapy and first-generation TKIs, respectively, in both pre-treated and naïve patients with advanced T790M+ NSCLC and brain metastasis, suggesting it as the new standard of care in this special population. Similarly alectinib has shown systemic and CNS efficacy in ALK+ NSCLC patients included in two phase II trials[4] and the randomized phase III ALEX trial has recently confirmed a significant superior CNS activity versus crizotinib in patients with previously untreated advanced ALK+ NSCLC and brain metastasis, regardless of prior CNS radiotherapy[5]. Finally, dabrafenib plus trametenib combination has shown a great activity along with a tolerable safety profile in BRAF+ melanoma patients with brain metastasis included in the phase II COMBI-MB trial, supporting further investigation in this setting[6]. Overall these evidences suggest that new effective drugs will be available soon for the treatment of patients with oncogene addicted tumors and brain metastasis, offering the potential for long-term disease control together with improved QoL. However, these patients represent only a minority of the whole cancer population harboring CNS disease to whom the standard approach still remain the multimodality treatment usually associated with very poor outcomes. Because of their worse prognosis, these patients have been historically excluded from clinical trials. However recent studies with monoclonal antibodies (MoAbs) targeting both cytotoxic T-lymphocyte protein4 (CTLA4) and programmed cell death 1 (PD1)/ programmed cell death ligand 1 (PDL1) have shown to be very effective in several tumor types revealing also unexpected activity in patients with both primary and metastatic brain tumors, thus pushing the clinical investigation of immunomodulatory agents in this special population. This review briefly summarizes the basis of the brain immunogenicity, providing the most updated clinical evidences in terms of ICIs efficacy and toxicity in both primary and metastatic brain tumors with the final aim of defining potential biomarkers for immunomodulatory cancer treatment in clinical practice.

<H1>2. Biological basis of brain immunogenicity

The brain has been historically considered as an immune-privileged area lacking the potential for immune-surveillance, likely because of its peculiar anatomic features, including the BBB and the absence of a standard lymphatic system. Early pre-clinical evidences on murine-models showed that grafted tissue into the brain were not rapidly rejected by host as observed in all the other extracranial sites where they were implanted[7]. However subsequent studies revealed that immune-rejection of grafted tissue into the brain just required longer time than in other extracranial sites[8], suggesting that CNS is a specialized area characterized by specific both structural and functional limitations to the immune-system activity. Paradoxically, the limited penetration of some drugs into the brain could result in intracranial metastatic deposits that remain sensitive to these agents, even in the context of the development of drug resistance within the extracranial tumor compartments[9]. Conversely, exposure of intracranial tumor deposits to sub-therapeutic drug concentrations might promote the early development of drug resistance and isolated disease progression in the brain, while the extra-cranial disease remains sensitive to treatment.

Brain microenvironment is characterized by the co-existence of several immune cell types, including both peripherally-derived immune cells and specialized organ-resident cells, taking part to different biological processes, like crosstalk with tumor cells and glioma stem cells, tumor angiogenesis and metastatic spread, thus differently contributing to the biological background and the clinical behavior of CNS tumors[10]. Interesting evidences demonstrated that peripherally-derived immune cells can also assimilate to the resident cells by a tissue-specific reprogramming process following entry into the brain[11,12], and this may have significant therapeutic implications.

In contrast to the historical concept that the brain lacked a lymphatic drainage system, two recent paradigm-shifting studies revealed that leucocytes may traffic to the CNS and peculiar lymphatic vessels within the dural sinuses connect cerebrospinal fluid and peripheral cervical lymph-nodes

draining both brain antigens and leucocytes[13,14]. These data first suggest that the lymphatic system could represent a direct way to exchange fluids, immune cells and tumor cells between the cervical lymph nodes and the cerebral spinal fluid. However, the existence of these vessels in humans need to be confirmed and their potential role in brain tumor progression and metastasis need to be investigated in order to identify possible therapeutic applications. In addition to that, the antigen presentation process acts in the CNS differently than other extracranial areas, with several cell types including microglia, tumor-infiltrating dendritic cells, macrophages, astrocytes, and pericytes, all playing a potential role as antigen-presenting cells (APCs)[15-17]. To date it is not clear yet if such process take place within or outside the CNS with the brain antigens drained through the lymphatic vessels to the peripheral cervical lymph nodes for antigen presentation. The APCs ability into the brain could be clinically harnessed by the development of vaccines and this strategy is currently being investigated in multiple trials.

Recent studies elucidated some mechanisms of immune-suppression adopted by primary and metastatic brain tumors to escape systemic immune response. Glioblastoma (GBM) cells may induce immune-suppression both at tumor microenvironment and at systemic level by secreting several cytokines and soluble factors, including TGF-b, VEGF, IDO, IL-10, PGE2, PDL1, STAT3, periostin, which act inhibiting T-cells growth and proliferation, decreasing their response to pro-inflammatory signals, and favoring the recruitment of regulatory T cells (Tregs), tumor associated macrophages (TAMs), and myeloid-derived suppressive cells (MDSCs) at the tumor site[18-26]. Similarly, in the inflammatory tumor microenvironment of brain metastasis, microglia and macrophages were also shown to express immunosuppressive factors like PD-L1, favoring immune-escape[26].

Overall these evidences suggest that CNS is a specialized area characterized by a unique immune-suppressive microenvironment and a highly regulated immune-response, offering the biological rationale for effective immunomodulatory treatment strategies across different brain tumors.

<H1>3. Immune checkpoints inhibitors for primary brain tumors

<H2>3.1 Clinical Efficacy

Among primary brain tumors, GBM is the most frequent malignancy and its diagnosis involves a very bad prognosis. Maximal tumor resection followed by radiotherapy was the standard of care until 2005, then a randomized phase 3 clinical trial showed that adding temozolomide to radiotherapy followed by adjuvant temozolomide improved OS from 12.1 months for the radiotherapy alone group to 14.6 months for the combined radiotherapy–temozolomide group[27]. Since the first approval of Ipilimumab for metastatic melanoma in 2010, immunotherapy became an attractive and powerful weapon for several solid and hematological malignancies, including GBM.

In a single institution report, five recurrence GBM patients were treated with Ipilimumab alone or in combination with other drugs. One patient achieved progression free survival (PFS) longer than 19 months, but all the others had progression within the first 6 months of treatment. Toxicity grade 2 or higher was reported in all the patients[28].In another non-randomized experience, the combination of ipilimumab with bevacizumab was assessed in 16 patients that had GBM recurrence: 3 patients after chemoradiation therapy and one patient after a first line palliative radiotherapy were treated with this combination. Radiological responses assessed by Response Assessment in Neuro – Oncology (RANO) showed 31% partial responses (PR), 31% stable disease (SD) and 38% progression disease (PD). Only 2 patients experienced treatment-limiting toxicities[29].
In the KEYNOTE 028 phase 1 trial, a cohort of 26 patients with recurrent GBM with at least 1% of PD-L1 expression and not previous use of Bevacizumab, was assessed for response rate according to RECIST 1.1 criteria. Patients were treated with pembrolizumab 10 mg/kg every 2 weeks for 24 months or until disease progression, death, limiting toxicity or withdraw of consent. The median age was 55 years. After a median follow up of 60 weeks 84% of the patients were discontinued.

Among all patients only one partial response was reported and 12 (48%) patients achieved stable disease. Reported PFS was 2.8 months and median OS was 14.4 months. Grade 3-4 toxicity was reported in the 15% of this cohort with no deaths related to pembrolizumab[30].

The CheckMate 143 is an open label randomized trial, of nivolumab or nivolumab plus ipilimumab combination, in GBM patients including Karnofsky performance status (KPS) of 70% or greater with recurrence after chemoradiotherapy with temozolomide and not previous use of bevacizumab. Cohort 1 randomized two different groups: nivolumab 3 mg/kg every 2 weeks (10 patients) and nivolumab 1 mg/kg plus ipilimumab 3 mg/kg every 3 weeks for 4 doses followed by nivolumab 3 mg/kg every 2 weeks (10 patients). A third cohort named 1b included 20 patients to receive nivolumab 3 mg/kg and ipilimumab 1 mg/kg for 4 doses followed by nivolumab 3 mg/kg every 2 weeks. Radiological measurement of the tumor was done using RANO criteria at weeks 6, 12 and then after every 8 weeks. Methylated/unmethylated MGMT gene expression was present in the 20 and 40% of cohort 1 (nivolumab alone arm), 20 and 60% of cohort 1 (nivolumab- ipilimumab arm) and in the 35 and 50% of patients of cohort 1b. One percent or greater PD-L1 expression was present in the 70%, 50% and 77% of cohort 1 (nivolumab arm), cohort 1 (nivolumab- ipilimumab arm) and cohort 1b respectively. For cohort 1 (nivolumab), cohort 1 (nivolumab- Ipilimumab) and cohort 1b the main reasons for discontinuing treatment were disease progression in the 90%, 60%, 50% and toxicity in 0%, 30% and 5% of these cohorts respectively. No treatment – related deaths were reported in any of the cohorts[31,32]. No grade 3-4 toxicity was reported in the nivolumab arm; however, it was highly frequent in the nivolumab- Ipilimumab (cohort1) in the 90% of patients. ALT increased, colitis, diarrhea, and lipase increased was reported in 20% or more of the patients in the combination arm of cohort 1. In the cohort 1b (nivolumab 3 mg/kg plus Ipilimumab 1mg/kg) grade 3-4 toxicity was lower (25%) than the combination treatment arm of cohort 1. The most common grade3-4 toxicity for its cohort was fatigue (15%), ALT increased, AST increased (10% each), colitis, fatigue and dizziness (5% each). Serious grade 3 or higher treatment- related adverse events were reported in both combination nivolumab plus ipilimumab treatment arms in the 70% (cohort 1) and in the 10% (cohort 1b).Among the 3 arms not complete responses and only one partial response was reported in the nivolumab arm (cohort 1). Stable disease was the best response in 50% of patients in the nivolumab arm, 40% combination arm of cohort 1 and 50% of the cohort 1b. Progressive disease as the best overall response (ORR) was reported in the 30% of nivolumab arm, in the 60% of the combination arm of cohort 1 and in the 45% of patients of cohort 1b. Median PFS was greater in the cohort 1b (2.4 months) when compared with the combination arm of cohort 1 (2.1 months) and only 1.9 months for the nivolumab alone arm. Nevertheless, median OS was greater for the nivolumab arm (10.5 months), as compared with both combinations arms, 9.3 and 7.3 months for cohorts 1 and 1b respectively.

Cohort 2 of CA209-143 trial included GBM patients that had recurrence after chemo-radiation with temozolomide and KPS of 70% or higher regardless of MGMT status. Patients were randomized to receive nivolumab 3 mg/kg every 2 weeks (n= 184) or Bevacizumab 2 mg/kg every 2 weeks (n=185) in a proportion of 1:1. The primary end point was OS, secondary end points included ORR and PFS using RANO criteria, 12 months OS, safety and biomarkers. Primary end point was not met, reporting a median OS for nivolumab arm of 9.8 months and 10 months for bevacizumab group (p 0.76, HR 1.04). Most of patients were discontinued due to disease progression. PFS was higher for bevacizumab arm than for nivolumab arm (3.5 and 1.5 months respectively, $p < 0,0001$). ORR was higher in patients treated with bevacizumab (23%) when compared with patients treated with nivolumab (7,8%), however duration of responses was higher among patients treated with nivolumab (11.1 months versus 5.3 months). PD-L1 expression did not favor nivolumab arm, moreover there was a trend in favor of the bevacizumab arm. Treatment-related grade 3-4 toxicities were similar in both arms (18.1% for nivolumab, 15.2% for bevacizumab respectively)[33].

Other 2 cohorts of this trial, cohort 1c and cohort 1d focused on newly diagnosed GBM patients. Cohort 1c included patients regardless of the methylation status of MGMT (n=55, 12 methylated, 43 unmethylated). Patients received an induction treatment with nivolumab 3mg/kg per 2 weeks followed by nivolumab plus chemoradiation with temozolomide for weeks 3 to 9 and later nivolumab in combination with temozolomide during weeks 14 to 37. Cohort 1d only included unmethylated MGMT patients (n=58), using the same induction treatment with nivolumab as cohort 1c followed by nivolumab plus standard radiotherapy for weeks 3 to 9. This part of the trial excluded patients with chronic or escalating doses of corticosteroids, KPS < 70 and secondary GBM. Patients were treated until progression. Preliminary data updated at July 2017 has provided interesting results. Grade 3-4 toxicity was more frequent among methylated patients (58%) than in non- methylated patients from cohort 1c (44%) and cohort 1d (39%). Nevertheless, toxicity that leaded discontinuation of treatment was lesser in methylated patients than in unmethylated patients from cohort 1c and 1d (8.3%, 18.6% and 17.2% respectively). Reported 12 months OS was 100% for the methylated group, 81.3% and 73% for both unmethylated arms (cohort 1c and 1d respectively). Median PFS for unmethylated 1c and 1d cohort was 7.7 and 6.4 months while it was not reached for the methylated arm[34,35].

In a Canadian report, a very high mutational load of Biallelic Mismatch Repair Deficiency (BMRD) was found among brain tumors patients. Based on this, two siblings of 3.5 and 6.5 years old, both with recurrent glioblastoma were treated with nivolumab. They both also had neurofibromatosis type 1. After a 9 and 5 months period of treatment both patients had confirmed reduction of their tumors and resumed daily activities including normal schooling[36].

Beyond checkpoints inhibitors, other modalities of immunotherapy with fusions of dendritic and glioma cells have shown promising results in a phase 1-2 Japanese trial including newly diagnosed and recurrence GBM patients after failing treatment with temozolomide. In the group of patients after temozolomide recurrence (n=10) PFS was 10.3 months and OS 10.8 months; in the newly diagnosed group (n=22) reported PFS was 18.3 months and OS 30.5 months[37]. Another promising immunotherapeutic approach emerged from recent GBM cases who achieved durable clinical responses following intracranial delivery of IL13R2 directed chimeric antigen receptor (CAR) T cells therapy[38,39], while the addition of the EGFRvIII-peptide vaccine Rindopepimunt to the standard temozolomide chemoradiation failed to demonstrate any survival benefit in newly diagnosed EGFRvIII-positive GBM patients[40].

In addition to the aforementioned studies reported in Table 1, there are several other clinical trials currently looking for improving expectative of survival among GBM patients. These trials include recurrence and newly diagnosis patients, single and combination checkpoints inhibitors use, tumor vaccines in combination with checkpoints inhibitors or alone and also other promising modalities[41].

<H2>3.2 Emerging biomarkers

GBM, as previously mentioned, is a highly deadly malignancy. Tumor resection is often insufficient to prevent the recurrence, and 5-year survival, despite adjuvant or palliative temozolomide-based chemo-radiation, is low. Whole genome sequencing analysis of GBM cells provided us some knowledge about molecular pathways underlying tumorigenesis, thus both PIK3R1 and PIK3CA might be considered as potential targetable genes to treat this disease[42].

Identification of biomarkers related to immune checkpoint efficacy in brain tumors remains challenging considering that most of the available information come from clinical trials in other solid tumors, mainly melanoma and NSCLC[43].

PD-L1 expression is positively correlated with gliomas grading and is a potential marker for mesenchymal molecular subtype[44]. PD-L1 expression>1%was found in 61 % of GBM (38% with PD-L1 expression > 5%, 5% with PD-L1 expression >50%) and it can be detected from plasma samples

in more than 50% of high grade glioma patients[45]. PD-L1 and PD-1 positivity have been related with worse survival outcomes[46]. Unfortunately, as mentioned above, PD-L1 blockade, alone or in combination with ipilimumab or bevacizumab, has not achieved good results in patients with newly diagnosed or recurrent GBM, therefore PD-L1 expression would not seem to be a good predictive biomarker for this disease.

Brain tumors have a lower mutational load compared to other tumors, in fact CTLA-4, PDCD1 and IDO1 expression in GBM is lower than NSCLC, melanoma or bladder cancer. In a recent work, predicted-neoantigen burden, pre-existing or basal levels of tumor-infiltrating T lymphocytes (TILs), and differential expression of immune-checkpoints, exhibited inconsistent patterns of benefit or resistance when compared to NSCLC or melanoma[47], therefore the role of TILs among GBM patients remains still unclear.

Mismatch Repair Deficiency (MMRD) is highly related with different cancer types usually affecting younger GBM patients. First tested in colorectal cancer carrying MMRD, immunotherapy showed to be highly effective in these patients. Eighty-six patients with 12 different types of metastatic solid tumors with MMRD were treated with pembrolizumab, achieving a disease control rate (DCR) of 56% including 21% of complete responses, 2-year PFS of 53% and 2-year OS of 64%[48]. Hypermutant GBM biallelic mismatch repair deficiency (BMMRD) showed durable response to nivolumab where the predicted neoantigen load was between 7 to 16 times higher than in immune-responsive melanomas, lung cancers, or microsatellite-unstable GI cancers[36]. Despite isolated experiences among GBM patients carrying this mutation treated with immunotherapy, MMRD seems to be a biomarker able to predict response to immunotherapy in this malignancy, however, future research will clarify if this condition effectively predicts high responses also in GBM patients as already proven in other solid tumors.

Unique properties of the brain tissue make the tumor microenvironment different because the distinctive extracellular matrix and resident cells types that include microglia, astrocytes, intrinsic resident macrophages and MDSCs which inactivate effector immune cells[10]. CD8 T cells and NK cells infiltration in tumor site was related to resistance PD-1 blockade in murine models[49]. Immune cell repertoire in peripheral blood could be an interesting biomarker to checkpoint inhibitors. In a recent work, T-cell diversification assessed by NGS of TCRβ was related to significantly higher rates of control disease in several solid tumors[50], however its potential role in GBM remains unclear.

Recent findings have shown that a small proportion of GBM patients carry an ultramutaded somatic or germline mutations in the polymerase Ɛ gene (POLE). These patients seem to have benefit when treated with checkpoints inhibitors blockade. This mutation was associated in many of the carriers with the germline MSH6 mutation, therefore both POLE and MSH6 mutation are related with mismatch repair damage[51].

It is known that patients with colorectal cancer that address microsatellite instability (MSI) are good responders to checkpoints inhibitors. There is high relation between MSI and MMRD in solid tumors. Even though MSI-low has been described in the 25% of recurrence and in the 8.5% of newly diagnosed GBM patients[52], little is known about the response to checkpoint inhibitors and it is potential role as a novel biomarker in this disease.

Low grade gliomas are currently classified based on molecular profiles that include IDH and Ip/19q status. Neuroradiologists could distinguish T2-flair among some patients with low grade glioma tumors that correlates with both IDH mutation and 1p/19q non-co-deleted low-grade tumors. That report shows that imaging interpretation could probably correlates with mutations and therefore with possible treatments opening a door to study a new biomarker (imaging -biomarker) for low and high-grade gliomas[53].

It is expected that ongoing clinical trials could identify novel biomarkers to immune checkpoint inhibitors.

<H1>4. Immune checkpoints inhibitors for brain metastasis

<H2>4.1 Clinical efficacy

Brain metastasis occur more frequently than primary brain tumors, being CNS a common site of progression for several solid tumors, particularly melanoma and lung cancer. Because of their worse prognosis, patients with CNS disease have been excluded from initial clinical trials with immunomodulatory agents blocking both CTLA-4 and PD1/PDL1 immunosuppressive receptors. However, the great success obtained with these agents in a wide spectrum of tumor types has subsequently pushed the design of both retrospective analysis and prospective clinical trials aiming to investigate the clinical efficacy of such drugs in patients with brain metastasis. The first available data came from a retrospective analysis of the phase II CheckMate CA189007 trial showing a promising intracranial activity of the anti-CTLA4 ipilimumab in melanoma patients with asymptomatic pre-treated brain metastasis[54]. These preliminary evidences were subsequently confirmed in a phase II prospective study showing a great activity and a tolerable safety profile of ipilimumab in melanoma patients with a response rate of 18% and 5% and a median OS of 7.0 and 3.7 months in asymptomatic and symptomatic brain metastasis subgroups, respectively, suggesting a durable clinical benefit which was comparable to that observed in the whole population without brain metastases[55].Besides CTLA-4 inhibitors, immunomodulatory agents targeting PD1 have been also investigated in this setting. Pembrolizumab has first shown a promising activity with a 20% - 30% ORR and a tolerable safety profile in patients with NSCLC or melanoma and asymptomatic, untreated brain metastasis[56]. A recent retrospective analysis investigated the potential intracranial activity of nivolumab or pembrolizumab in patients with metastatic melanoma, confirming an ORR of about 20% and a median OS of 5.7 months and 13 months in presence of asymptomatic and symptomatic disease, respectively[57]. Finally pooled analysis of the checkmate 017/057 studies comparing nivolumab vs docetaxelin lung cancer patients with previously treated or untreated asymptomatic brain metastases demonstrated a longer median OS in favor of nivolumab in the subgroup of patients with pre-treated brain metastases, even if both frequency and time to new brain lesions were similar to chemo arm[58]. Overall these data suggested that ICI monotherapy could be considered as an effective treatment option in a subset of patients with CNS disease, particularly those with asymptomatic and untreated brain metastases. The encouraging activity observed with single agent immune-checkpoint therapy has prompted the design of prospective trials combining different immunomodulatory agents or different treatment approaches to further enhance the intracranial activity of these drugs. The Italian phase II NIBIT-M1 trial evaluated the combination of ipilimumab and fotemustine in melanoma patients with and without brain metastases, including about 25% with asymptomatic CNS disease, reaching a median PFS of 3.0 months, a median OS of 12.7 months, and a 3-year survival rate of 27.8% in patients with CNS disease, similar to the outcomes observed in the whole population[59,60]. The phase II CheckMate 204 trial evaluated nivolumab plus ipilimumab in melanoma patients with asymptomatic brain metastasis reaching more than 50% of intracranial ORR, with 21% complete response. However about 50% of patients experienced Grade (G) 3–4 toxicities and 30% discontinued treatment because of adverse events, consistently with the results associated with this combination in patients without CNS disease. The Australian ABC phase II trial reported similar activity and safety data, with an intracranial ORR of 42%and a 6-month intracranial PFS of 46%in the cohort of patients receiving nivolumab plus ipilimumab combination. Conversely the cohort of patients treated with single agent nivolumab obtained an intracranial ORR of 20% and 6-month intracranial PFS of 28%, while only 16% ORR was observed in patients previously treated with BRAF and MEK inhibitors and 6% ORR in the small subgroup receiving prior local therapies. The positive results of both these trials[61,62]reported in table 2 support the use of immunotherapy combinations as new standard upfront therapy in patients with metastatic melanoma and untreated brain metastasis. The NIBIT-M2 is a randomized, phase 3 trial currently comparing fotemustine vs its combination with ipilimumab or vs the ipilimumab and nivolumab combination in melanoma patients with asymptomatic and untreated

brain metastases. Several ongoing studies are currently investigating the clinical efficacy and safety profile of combination strategies also in NSCLC patients with untreated brain metastasis, but the results are not available yet. Finally, some retrospective series demonstrated that immune-checkpoint inhibitors may be safely combined with stereotactic radiation in the management of brain metastasis from melanoma and NSCLC patients, significantly improving survival as compared to radiotherapy alone[63-65], thus suggesting a synergistic effect between these two treatment strategies which need to be investigated in prospective clinical trials.

<H2>4.2 Emerging biomarkers

To date, although several methodological and biological limitations, the tumor PD-L1 expression assessment by IHC on tumor tissue represents the only predictive biomarker validated and approved for clinical use. Recently pembrolizumab revealed a significant superiority over platinum based chemotherapy as first-line treatment of non-oncogene addicted NSCLC patients whose tumors overexpressed PD-L1>50%[66], becoming the new backbone in this subgroup of patients. In light of these evidences, the PD-L1 testing has been incorporated within the international guidelines and it is now recommended together with the molecular testing for all patients with newly diagnosed advanced NSCLC[67].

However very few data are still available regarding PD-L1 expression in the brain. Preliminary evidence revealed a lack of PD-L1 expression in brain metastasis of patients with several solid tumors, with the greatest expression in melanoma and renal cell carcinoma[68]. Conversely the expression of PD-L1 in NSCLC-derived brain metastases seems to be significantly higher than the matched primary tumor[69]. Also, the correlation between brain PD-L1 expression and tumor infiltrating lymphocytes (TILs) is quite controversial as well as their association with patients' survival[68,70]. Both TILs density/composition and PD-L1 expression in the brain inflammatory microenvironment have shown to be significantly heterogeneous among different patients with brain metastasis, varying from high levels to low/absent expression[70]. Of course, such differences in the brain tumor microenvironment significantly influence both patients' prognosis and the response of brain metastasis to ICIs, thus the potential role as prognostic/predictive biomarker need to be further investigated in this special population. Another study has recently shown a significant disagreement of both PD-L1 expression and TILs density in the microenvironment of primary tumor and matched brain metastasis of lung cancer patients, highlighting the spatial-temporal heterogeneity associated with tumor PD-L1 expression which should be always taken into account when oncologists decide to treat patients with anti-PD1 agents[71]. Several evidences have recently revealed that a high tumor mutational burden (TMB) is significantly associated with an increased response to immunotherapy[72,73], favoring the creation of new foreign peptides defined as neoantigens which ultimately promote the immune-recognition of cancer. A recent work classified lung cancer along with melanoma among the tumors with the highest mutation burden[74], thus more likely to respond to ICIs. Similarly, colorectal cancer patients with defective mismatch repair (MMR) and microsatellite instability (MSI) received significant benefit from pembrolizumab[75], suggesting a potential role as biomarker of ICIs efficacy, likely to its correlation with TMB. The very few evidence currently available from literature suggested that such molecular alterations may be detected only in a minority of brain metastasis from melanoma[76], lung cancer[77], and colorectal cancer patients[78], but the differences in TMB between primary tumors and brain metastases as well as their predictive role in this special population need to be further explored in prospective trials.

Looking for molecular biomarkers, the retrospective analysis of randomized studies of ICIs in pre-treated NSCLC patients [79] have recently suggested that patients with oncogene-addicted tumors may not be good candidate to ICIs therapy, likely due to the very low TMB featuring this subgroup of cancers and the consequent reduced number of ``neo-antigens" triggering a protective immune response[80]. Similarly, molecular alteration in the JAK genes were associated with acquired resistance to ICIs in patients with metastatic melanoma[81,82]. Considering the high incidence of brain metastasis in this molecular defined subset of patients, it is crucial elucidate the genomic landscape

of brain metastasis and the differences with the matched primary tumors before to definitively exclude these patients from a potential effective treatment option.

Previous studies showed that oncogene mechanistically linked to the primary cancers are also implicated in driving the incidence of brain metastases, as described for the mutated expression of BRAF in metastases from melanoma, aberrant levels of HER2 in breast cancer brain metastases, and mutated EGFR in brain metastases from lung cancer[83,84]. However, other studies suggested that specific mutations of some oncogenes are more often represented in brain metastases[85,86], prompting a comprehensive genomic characterization of brain metastases compared to their primary cancers.

Despite the large number of patients afflicted, the characterization of specific genomic aberrations in brain metastases is still limited to retrospective studies that have been biased towards the activating mutations in oncogenes or tumor suppressors previously associated with the primary cancer[87]. For example, the analysis of ten melanoma metastases for a panel of BRAF, NRAS, AKT, PIK3CA and KIT activating mutations using a mass spectrometry approach showed that BRAF and NRAS mutations were the most frequent, similar to primary melanoma[88]. Similarly, BRAF and NRAS mutations were detected in primary melanoma and matched brain metastases from 44 patients with 80% consistency using Sanger sequencing[89]. In specimens from matched brain metastases and primary colorectal cancer high-resolution DNA melting analysis of 19 oncogenes, including BRAF, and KRAS showed that 9 of 10 matched brain metastases were 100% concordant with mutations observed in the primary cancer[90]. However, studies investigating EGFR mutations in lung cancer brain metastases from Caucasian patients revealed that the mutation markedly less frequent (i.e., around 2%), than in primary tumors[91]. However, where a mutation was discovered in the metastasis, it was concordant with a mutation in the primary cancer. Moreover, similar results for EGFR mutational status in brain metastases and primary tumors were detected in Japanese patients[92].

These results suggest that coding mutations in most investigated oncogenes are either equivalent or less represented in the brain metastases compared to the primary cancer. Conversely, other studies suggest that the incidence of mutations in onco-suppressor genes is increased in metastatic cancers[93].

More recently, the whole-exome sequencing of 86 matched brain metastases, primary tumors, and normal tissue, showed a branched evolution, where all metastatic and primary sites shared a common ancestor yet continued to evolve independently. Thus, additional potentially oncogenic alterations are present in brain metastases, and might contribute to differential therapeutic response. In particular, 53% of brain metastases cases harbored potentially clinically informative alterations that were not detected in the matched primary-tumor sample, as well as in regional lymph nodes, or extracranial metastases[94]. Finally, although genetically divergent from samples of their originator tumor, brain metastases are remarkably homogenous with respect to driver and/or potentially targetable alterations[94].

<H1>5. Exosomes as novel ``circulating'' biomarkers

The potential of exosomes as markers of prognosis or response to immunotherapy in patients with brain tumors is enthusiastically investigated. Exosome contents can help identify the cells of origin, thus offering the opportunity to identify biomarkers or therapeutic targets in body fluids[95]. Skog and colleagues showed that exosomes carrying glioma-associated proteins and angiogenic factors accumulated in the plasma of patients with glioma and proposed that exosomes could be used as disease biomarkers[96]. It was also suggested that in glioma, where few prognostic markers exist, the exosomal cargo determined at the time of diagnosis may be useful in predicting patients' outcomes[97]. Circulating exosomes in the body fluids of patients with brain tumors may be used to decode molecular features of the neoplasms or measure their responses to therapy[97]. Several tumor-related molecules with altered expression patterns have been found in circulating exosomes of glioma patients including EGFRvII, EGFR, podoplanin, mutant IDH1[98], PTEN[99]mRNA and miR-21[100]. Overall these data indicated that tumor brain-derived exosomes can be good candidate as

biomarkers for brain neoplasms. Tumor-derived exosomes are also considered as regulatory elements through which cancer cells can communicate with and re-program the immune cells population in the TME[101]. Exosomes isolated from plasma of head and neck squamous cell carcinomas (HNSCC) patients seem to have immunosuppressive properties and, as it was recently demonstrated, play a role in the regulation of tumor progression. Interestingly, these exosomes were shown to carry PD-L1 and PD-1, but it was not clear if PD-1 and PD-L1 shuttled by these exosomes were biologically active and were responsible for the reported immune-inhibitory effects[102]. Recently, Theodoraki and colleagues reported that exosomes collected by plasma of patients with HNSCC carried biologically active PD-L1 which induced T-cell dysfunction upon co-incubation of these exosomes with activated CD8+ T cells. While levels of soluble PD-L1 (sPD-L1) were elevated in patients' plasma, only exosome-bound PD-L1 levels correlated with disease activity and with the patients' clinic-pathological profiles. Anti-PD-1 antibodies reversed suppression induced by PD-L1+ exosomes in activated T cells. Circulating PD-L1+ exosomes emerge as promising potential markers of immune dysfunction and disease progression[103]. These findings encourage the researchers to investigate on the potential role of exosomes in patients undergoing immunomodulatory cancer therapies for brain neoplasms.

<H1>6. Future perspectives and conclusions

A deeper understanding of the molecular basis of brain immunogenicity and cancer immune-escape along with the impressive clinical benefit obtained with immunomodulatory agents in a significant subgroup of patients with different tumor types has pushed the clinical investigation of immunotherapy in patients with CNS disease. Although early clinical trials showed promising activity and tolerable safety profile, immunomodulatory agents have not reached regulatory approval for primary brain tumors yet. GBM emerged as a ``cold tumor'' characterized by a low mutational load and an immunosuppressive microenvironment. Thus, checkpoint blockade by PD1/CTLA4 single agent inhibitors in absence of pre-existing antitumor immunity could not be sufficient to treat these patients. Conversely combinations with antiangiogenics, vaccines, CAR-T cell therapy, or different immunomodulatory agents targeting the different contributors to the tumor immunosuppression could represent the best strategy to reactivate antitumor immune-response and is currently under investigation in ongoing trials. Encouraging intracranial activity of checkpoint blockers has been observed also in patients with metastatic brain tumors, particularly from melanoma and NSCLC, suggesting that ICI monotherapy could be considered as an effective treatment option in a subset of patients with asymptomatic and untreated brain metastases. Combining anti-CTLA4 and anti-PD1 agents as upfront systemic therapy in patients with melanoma brain metastases revealed to offer a significant control of both intracranial and extracranial disease, further increasing the clinical benefit observed with single agents at cost of increased grade 3-4 toxicities. Therefore, the next question to be addressed in clinical trials will be: there is still a role for upfront radiotherapy in this population? or it will be finally replaced by systemic therapies like immunomodulatory combinations? Preliminary evidences revealed that immune-checkpoint inhibitors may be safety combined with stereotactic radiation in the management of brain metastasis from melanoma and NSCLC patients, suggesting a synergistic effect between these two treatment strategies. However, questions regarding the appropriate doses and sequences of combinations need to be addressed in prospective clinical trials which should investigate also the biological interaction between these different treatment approaches. Could immunotherapy have a radio-sensitizing effect potentiating the intracranial activity of radiotherapy or can such combination increase also the control of extracranial disease as result of the ``abscopal effect''? are opened questions that remain to be addressed in upcoming studies.

Potential synergistic interactions between immunotherapy and systemic chemotherapy need to be also elucidated in clinical trials including patients with brain tumors. Pre-clinical data showed that cytotoxic drugs enhance tumor immunogenicity inducing neoantigens production, upregulating MHC molecules, and reducing immune suppressive cell, like Treg, TAMs, and MDSCs, in the tumor microenvironment[104]. Clinical studies have recently confirmed this biological rational in

patients with different metastatic solid tumors, such as melanoma and lung cancer, however data on brain metastasis are still lacking. Thus, new studies prospectively exploring optimal doses and timing of chemo-radio-immunotherapies combinations in this special population are largely awaited.

Furthermore, we should continue to monitor and documenting autoimmune response associated to ICIs combinations in an anatomically restricted and immunologically specialized area like the brain, because of the high risks of neurological side effects which have sometimes lead to treatments related deaths in clinical trials. A significant step forward in the treatment of brain tumors could be offered by the combination of checkpoint inhibitors and CAR T cells therapy. This is a cancer treatment infusing patients' own T-cells after have been engineered in the lab and modified to recognize and kill tumor cells[105]. Preliminary studies demonstrated an interesting activity of this strategy in several solid tumors, including lung cancer, and is now being extensively investigated in clinical trials. Regional delivery of CAR T cells is another promising approach to bypass the BBB and enhance antitumor activity in an anatomically restricted and immunologically specialized area like the brain while reducing systemic toxicity associated with systemic delivery, and is emerging as the frontier of immunotherapy for solid tumors. Generally, it was well tolerated and efficacious in selected GBM cases[38,39]. Preclinical studies have recently shown promising antitumor efficacy following loco-regional intracranial delivery of HER2-CAR T cells for the treatment of multifocal Her2+ brain metastases in xenograft models [106], suggesting a potential role for clinical setting. Exosomes can cross the BBB and can be used to deliver small biological or pharmaceutical molecules to brain tumours[107]. The use of exosomes for treatment of CNS tumours can be divided into three main categories: (I) exosomes for immunomodulation-based therapy, (II) exosomes as delivery vehicles for anti-tumour nucleotides, and (III) exosomes as drug delivery vehicles. Thus exosomes will likely play an important role in increasing the efficacy of immunotherapy for treatment of brain neoplasms.

The advent of immune-checkpoint inhibitors, including both single agent ICI and potential combinations could really produce a paradigm shift in the management of both primary and metastatic brain cancers, at least for those tumors harboring an immune active microenvironment that could be successfully targeted by immune-modulating agents (figure 1). Therefore, a deeper insight into both brain tumors and host biology is needed in order to elucidate the specific mechanisms in the highly regulated brain microenvironment. Identifying predictive biomarkers associated with clinical response/resistance to ICIs is a major challenge for translational research to help oncologists in selecting patients who may gain major benefit from immunotherapy and sparing others from an ineffective treatment and futile, life-threatening toxicities. Even if limited by a lack of standardization in testing methods the tumor PD-L1 assessment by IHC represents the only predictive biomarker currently approved for clinical use. However, because of its low diagnostic accuracy, PD-L1 alone is not appropriate to univocally select patients with different tumor types to treat with ICIs. A deeper understanding of the cancer immunity cycle has allowed to elucidate the complex interactions between cancer cells and immune system, favoring the identification of multiple factors which play a crucial role in modulating both intensity and timing of the antitumor immune response[108].Thus, beyond PD-L1 expression, other biological parameters including tumor genomic alterations, TMB, tumor neoantigens load, and TILs density in the tumor microenvironment are currently under investigation and validation as predictive biomarkers for clinical use(figure 2). Very few preliminary data are still available for primary brain tumors, while many studies demonstrated a significant correlation with checkpoint inhibitors activity in other metastatic cancers. However, since the brain is a specialized area with a unique genetic and immune landscape it remains crucial to establish the composition of the brain tumor microenvironment and its potential correlation with matched primary tumor in order to predict the potential activity of immunomodulatory in the CNS at single patient's level. Although genetically divergent from samples of their originator tumor, brain metastases seem to be remarkably homogenous with respect to driver and/or potentially targetable alterations. However differently from targeted therapy,

immunotherapy modulate a complex network of molecular and cellular pathways and immune response is a very dynamic process taking place at different sites other than primary tumor microenvironment, making the identification of reliable biomarkers a very hard and difficult process. In this scenario exosomes can represent interesting candidate as potential biomarkers. The cargo of exosomes mirrors the parent cell conditions and their nucleic acid and protein content is preserved from degradation, thus the idea that exosomes might travel useful information in the 'liquid biopsies' field[109]. Nowadays, further studies need to understand to role of exosomes in immunomodulatory tumor brain treatment, but thanks to their peculiar features, these vesicles are potentially excellent candidates for monitoring the clinical efficacy of Immune checkpoints inhibitors for primary brain tumors and metastasis.

Finally only the combination of different biological parameters integrating information from the brain tumor genomics and microenvironment, host immune system, and peripheral blood compartment could allow to define the immunological status of each patients, and consequently personalize the treatment strategy including immunomodulatory combinations. The scientific community should promote a new era of clinical trials specifically devoted to patients with both primary and metastatic brain tumors, including the availability of biological samples longitudinally collected in a systematic and standardized manner for the identification of reliable immune-related biomarkers for immunomodulatory cancer treatment.

Conflict of Interest Statement:

F.P, E.G, JA.P, I-G.B, A.R declare that there are no conflicts of interest.

C.C: MSD, GSK, Bayer, Boehringer Ingelheim, Astellas, Roche, Astrazeneca, BMS, Novartis, Lilly, Tecnofarma

L.R: BMS, Merck and Roche

C.R: MSD, Novartis

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Figures' legend:

<Figure>**Figure 1:** Therapeutic targets for immunomodulatory cancer therapies

<Figure>**Figure 2:** Emerging biomarkers for immunomodulatory cancer therapies

Tables

<Table>Table 1: Clinical trials concerning immunotherapy for patients with primary CNS tumors.

PD-L1: programmed death-ligand 1; GBM: glioblastoma; PFS: progression-free survival; OS: overall survival; PR: partial response; SD: stable disease; PD: progression disease; DOR: duration of response; RT: radiotherapy; TMZ: temozolomide. Findings are reported as ``experimental'' vs ``control''

TREATMENT	**PHASE**	**PATIENTS**	**FINDINGS**	**Ref.**
Ipilimumab	II	72 patients with brain metastatic melanoma: 51 patients were asymptomatic (group A) 21 patients had CNS symptoms (group B)	RR: 18% (A); 5% (B) OS: 7.0 months (A); 3.7 months (B)	Margolin et al. 2012
Pembrolizumab	II	36 patients with advanced melanoma (n=18) or NSCLC (n=18) with symptomatic untreated brain metastasis	Intracranial RR: 22% (melanoma), 33% (NSCLC).	Goldberg et al. 2016
Ipilimumab + Bevacizumab	II	86 patients with first line treatment	DCR: 46.5% DOR: 11.1 vs 3.3 months	Di Giacomo [illegible]
Nivolumab + RT + TMZ vs Nivolumab + RT	I	110 patients with untreated GBM	*Updated results will be presented*	Omuro et al. 2017
Autologous cultured glioma cells obtained from surgical specimens fused with autologous dendritic cells	I/II	10 patients with recurrent GBM initially diagnosed with glioma treated with TMZ (group R) 22 patients with newly diagnosed GBM (groupN)	PFS: 10.3 months (R); 18.3 months (N) OS : 18.0 months (R); 30.5 months (N)	Akasaki et al. 2016
Rindopepimut + oral TMZ vs Placebo + oral TMZ	III	745 patients with surgery and chemoradiation-treated GBM expressing EGFRvIII with no evidence of progression	OS: 20.1 vs 20.0 months	Weller et al. 2017

Fotemustine		metastatic melanoma 20 with CNS asymptomatic metastasis	50% (CNS metastasis)	et al. 2012
Ipilimumab + Nivolumab	-	90 patients with asymptomatic CNS metastatic melanoma	RR: 56%; CR: 20%.	Tawbi et al. 2017
Ipilimumab + Nivolumab vs Nivolumab	II	66 patients with asymptomatic CNS metastatic melanoma	Intracranial RR: 24.65% vs 7.41% and 0.3%	Long et al. 2017

<Table>Table 2: Clinical trials concerning immunotherapy for patients with CNS metastatic tumors

CNS: central nervous system; NSCLC: non-small cell lung cancer; RR: response rate; CR: complete response; DCR: disease control rate; Findings are reported as ``experimental" vs ``control"

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