MECHANISMS AND THERAPY FOR CANCER METASTASIS TO THE CENTRAL NERVOUS SYSTEM

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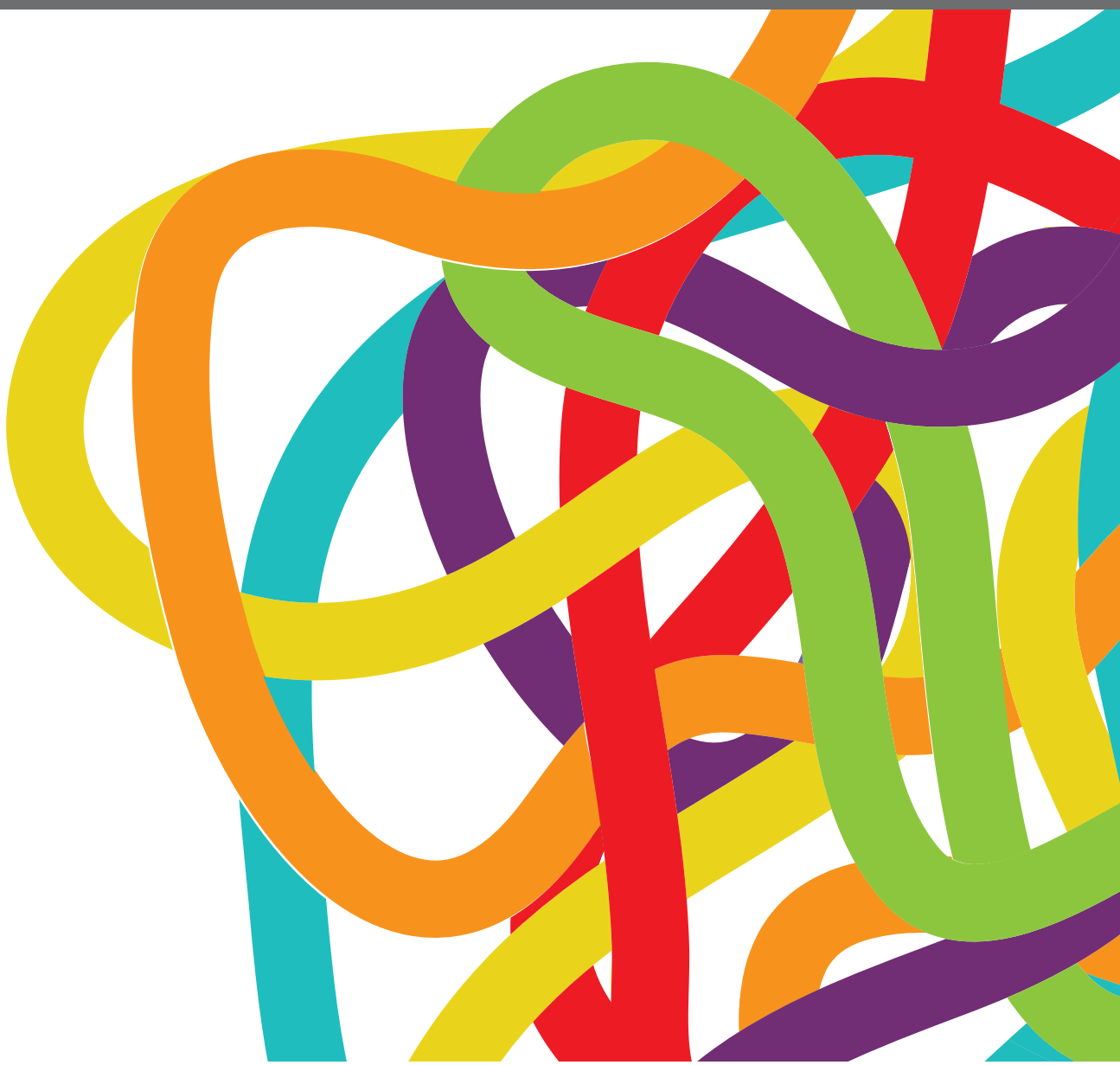

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MECHANISMS AND THERAPY FOR CANCER METASTASIS TO THE CENTRAL NERVOUS SYSTEM

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EDITORIAL
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Editorial: Mechanisms and Therapy for Cancer Metastasis to the Central Nervous System

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Editorial on the Research Topic

Mechanisms and Therapy for Cancer Metastasis to the Central Nervous System

Metastasis of malignancies to the brain, including parenchymal and leptomeningeal disease, represents a common neurological complication in cancer patients (1, 2). Currently, ∼10% of all cancer patients experience involvement of the central nervous system (CNS) (3, 4). Though ∼40% of patients with metastatic cancers are affected by brain metastases (1, 5), commonly used treatment options for brain metastases such as surgery or radiotherapy are associated with only modest benefits (6).

The study of tumor cell metastasis to the brain or leptomeninges has developed into an intensely researched area, as it has the potential to provide selective targets for developing therapeutic strategies for brain metastases. In this Research Topic, we have organized a collection of review and original research articles that will hopefully help the readers gain insight into different aspects of cancer metastases to the CNS. In an overview of brain metastasis, Franchino et al. describe different detection methods, including magnetic resonance imaging (MRI), computed tomography, positron emission tomography (PET) as well as advanced imaging techniques commonly used in the diagnosis, treatment planning, and follow-up of patients with brain metastases. The authors also introduce more sophisticated methods of tumor analysis to detect circulating biomarkers in body fluids such as blood and cerebrospinal fluid, and their use in monitoring treatment response and tumor progression. The benefits and impacts on prognosis of different commonly used therapeutic approaches are discussed, including more recent clinical trials featuring immunotherapies such as checkpoint inhibitors.

In the era of precision medicine, choice of cancer treatment has been increasingly prescribed based on the molecular or genomic properties of the individual cancer. Han and Brastianos describe novel approaches in genomic testing such as the use of cell-free circulating tumor DNA in the cerebrospinal fluid in the study of brain metastases. The authors focus on recent advances in genomic profiling of brain metastases and current knowledge of targeted therapies in the management of brain metastases from cancers of the breast, lung, colorectum, kidneys, and ovaries as well as melanoma. Identification of genomic alterations found in brain metastases and targeted therapies against these mutations represent an important research area that could potentially bring improved outcomes for patients with brain metastases.

Brain metastasis and leptomeningeal spread are process that can be partially recapitulated through *in vitro* assays. Despite their extensive use, these assays have limitations in the study of complex host/tumor cell interactions throughout metastasis. In this respect, animal models represent a versatile tool to examine anatomical barriers (such as the blood brain barrier), stromal/environmental factors, genetic factors, and the immune response. Singh et al. discuss the development of patient-derived xenograft (PDX) models for brain and leptomeningeal metastasis through injection of patient tumor cells into an appropriate microenvironment. These PDX models

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have been shown to maintain their molecular signatures and recapitulate the heterogeneous nature of the original patient tumor, providing some advantages over genetically engineered mouse models. These models have contributed significantly to our understanding of CNS metastasis, and will play a pivotal role in the identification and testing of potential therapeutic agents.

Despite improvements in systemic therapies, brain metastasis of breast cancer is associated with dismal survival with limited and non-specific treatment options. Study of breast cancer brain metastases and preclinical tests have relied mostly on injection of a few breast cancer cell lines; however, this approach suffers from a lack of tumor heterogeneity observed clinically and limited use for therapeutic studies due to their rapid progression following transplantation. Contreras-Zárate et al. developed and characterized a PDX model of breast cancer brain metastasis. The investigators obtained freshly resected brain metastases from breast cancer and implanted tumor cells in the mammary fat pad of female immune-deficient recipients. The xenografts retained critical clinical markers and gene expression profiles of parental tumors. Importantly, intra-cardiac injection of dissociated cells from xenografts led to tumors in the brain parenchyma within 8–12 weeks, suggesting that xenografts derived from brain metastases maintain the capacity to colonize the brain. These novel xenografts represent heterogeneous and clinically relevant models to study the biology of brain metastasis and to test drugs in therapeutically relevant settings.

Indeed, PDX models recapitulate many characteristics of breast cancer brain metastases including active angiogenesis and astroglial activation. Conversely, unlike other sites for tumor dissemination such as liver or bone, astrocytes represent a major cell type that come into contact with cancer cells during brain metastasis. In response to cancer cells that metastasize to the brain, astrocytes undergo further differentiation and activation to affect the survival and growth of disseminated cancer cells within the CNS. Wasilewski et al. discuss our current understanding of the contribution of reactive astrocytes to brain metastasis. Emphasis is placed on the signaling pathways and interactions that play a crucial part in the communication with metastatic cancer cells (7). In addition to astrocytes, local macrophages and microglia in the CNS constitute important components of the immune response to metastatic growth. Accumulation of microglial cells surrounding metastatic tumors has been described for both experimental and human brain metastases. Andreou et al. examined microglial/macrophage activation in a mouse model of breast cancer brain metastasis. Microglia can differentiate along the proinflammatory pathway to upregulate cytokine levels and acquire the ability to mediate an immune response against tumor cells. Alternatively, microglia can develop an anti-inflammatory phenotype that promotes angiogenesis and tumor growth. The authors identified populations of both proinflammatory and anti-inflammatory microglia/macrophages. The investigators further demonstrated that selective depletion of this microglia/macrophage population significantly reduced metastatic tumor burden and increased apoptosis. These findings suggest that microglia/macrophages are important effectors of the inflammatory response in brain metastases. Hence targeting the anti-inflammatory pathway in microglia/macrophages may offer therapeutic opportunities for patients with brain metastases.

Treatment options for intracerebral seeding of cancer cells are limited and lacking specificity. The molecular and cellular makeup of brain metastases usually differs from that of the primary tumors, as well as from metastases at other sites. Molecular detailing of metastatic cancer cell penetration, seeding, and outgrowth in the brain will contribute to the discovery of innovative cancer therapies.

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All authors listed have made a substantial, direct and intellectual contribution to the work, and approved it for publication.

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REFERENCES

1. Gavrilovic IT, Posner JB. Brain metastases: epidemiology and pathophysiology. *J Neuro Oncol.* (2005) 75:5–14.doi: 10.1007/s11060-004-8093-6
2. Hall WA,. Djalilian HR, Nussbaum ES, Cho KH. Long-term survival with metastatic cancer to the brain. *Med Oncol.* (2000) 17:279-86.
3. Barnholtz-Sloan JS, Sloan AE, Davis FG, Vigneau FD, Lai P, Sawaya RE. Incidence proportions of brain metastases in patients diagnosed (1973 to 2001) in the metropolitan detroit cancer surveillance system. *J Clin Oncol.* (2004) 22:2865–72. doi: 10.1200/JCO.2004.12.149
4. Davis FG, Dolecek TA, McCarthy BJ, Villano JL. Toward determining the lifetime occurrence of metastatic brain tumors estimated from 2007 United States cancer incidence data. *Neuro Oncol.* (2012) 14:1171–7. doi: 10.1093/neuonc/nos152
5. Tabouret E, Chinot O, Metellus P, Tallet A, Viens P, Goncalves A. Recent trends in epidemiology of brain metastases: an overview. *Anticancer Res.* (2012) 32:4655–62.
6. Arvold ND, Lee EQ, Mehta MP, Margolin K, Alexander BM, Lin NU, et al. Updates in the management of brain metastases. *Neuro Oncol.* (2016) 18:1043–65. doi: 10.1093/neuonc/now127
7. Priego N, Zhu L, Monteiro C, Mulders M, Wasilewski D, Bindeman W, et al. STAT3 labels a subpopulation of reactive astrocytes required for brain metastasis. *Nat Med.* (2018) 24:1024–35. doi: 10.1038/s41591-018-0044-4

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REVIEW
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Mechanisms and Therapy for Cancer Metastasis to the Brain

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Advances in chemotherapy and targeted therapies have improved survival in cancer patients with an increase of the incidence of newly diagnosed brain metastases (BMs). Intracranial metastases are symptomatic in 60–70% of patients. Magnetic resonance imaging (MRI) with gadolinium is more sensitive than computed tomography and advanced neuroimaging techniques have been increasingly used in the detection, treatment planning, and follow-up of BM. Apart from the morphological analysis, the most effective tool for characterizing BM is immunohistochemistry. Molecular alterations not always reflect those of the primary tumor. More sophisticated methods of tumor analysis detecting circulating biomarkers in fluids (liquid biopsy), including circulating DNA, circulating tumor cells, and extracellular vesicles, containing tumor DNA and macromolecules (microRNA), have shown promise regarding tumor treatment response and progression. The choice of therapeutic approaches is guided by prognostic scores (Recursive Partitioning Analysis and diagnostic-specific Graded Prognostic Assessment-DS-GPA). The survival benefit of surgical resection seems limited to the subgroup of patients with controlled systemic disease and good performance status. Leptomeningeal disease (LMD) can be a complication, especially in posterior fossa metastases undergoing a "piecemeal" resection. Radiosurgery of the resection cavity may offer comparable survival and local control as postoperative whole-brain radiotherapy (WBRT). WBRT alone is now the treatment of choice only for patients with single or multiple BMs not amenable to surgery or radiosurgery, or with poor prognostic factors. To reduce the neurocognitive sequelae of WBRT intensity modulated radiotherapy with hippocampal sparing, and pharmacological approaches (memantine and donepezil) have been investigated. In the last decade, a multitude of molecular abnormalities have been discovered. Approximately 33% of patients with non-small cell lung cancer (NSCLC) tumors and epidermal growth factor receptor mutations develop BMs, which are targetable with different generations of tyrosine kinase inhibitors (TKIs: gefitinib, erlotinib, afatinib, icotinib, and osimertinib). Other "druggable" alterations seen in up to 5% of NSCLC patients are the rearrangements of the "anaplastic lymphoma kinase" gene TKI (crizotinib, ceritinib, alectinib, brigatinib, and lorlatinib). In human epidermal growth factor receptor 2-positive, breast cancer targeted therapies have been widely used (trastuzumab, trastuzumab-emtansine, lapatinib-capecitabine, and neratinib). Novel targeted and immunotherapeutic agents have also revolutionized the systemic management of melanoma (ipilimumab, nivolumab, pembrolizumab, and BRAF inhibitors dabrafenib and vemurafenib).

Keywords: brain metastases, chemotherapy, neuroimaging, neuropathology, surgery, stereotactic radiosurgery, whole-brain radiotherapy, targeted therapy

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INTRODUCTION

Recent advances in chemotherapy and targeted therapies have improved survival in cancer patients. In this context of a better-controlled systemic disease, brain metastases (BMs) are emerging as a new challenge for the oncologist.

Brain metastases are the most frequent intracranial tumors: the incidence of newly diagnosed BMs is 3–10 times the incidence of newly diagnosed primary malignant brain tumors (1) and is still increasing. This trend could be explained by improvement in the quality of neuroimaging [magnetic resonance imaging (MRI)] and increased survival of patients with solid tumors (2).

In adults, lung (36–64%), breast (15–25%), and skin (melanoma) (5–20%) are the most frequent sources of BMs. Less frequent are cancers from colon-rectum, kidney, prostate, testis, ovary, sarcomas, and unknown tumors (3).

Brain metastases occur more frequently in patients with advanced disease. However, in some subgroups of patients, such as HER2+ breast cancer receiving trastuzumab, the brain represents now the first, often solitary, site of metastatic relapse (4).

The biology of BM remains poorly understood. Interactions between circulating tumor cells (CTCs) and blood–brain barrier (BBB) components are required. Some cytokines may act as CTC attractants and promote BM formation. BM development involves several steps (extravasation through non-fenestrated capillaries, local proliferation, and neoangiogenesis). Recently, clinical studies have detected specific genomic alterations in BMs but not in the primary tumor or extracranial metastases (5–7). How and when metastatic cells that spread to the brain evolve in a divergent way remains elusive: answering these questions could open the way for novel-specific therapies.

BIOLOGY AND PATHOPHYSIOLOGY OF BMs

Metastatization from systemic cancer requires several steps and complex genetic, epigenetic and biological changes in tumor cells, globally defined as "metastatic cascade," beginning with detachment from the primary tumor and invasion of the surrounding tissue, intravasation into blood vessels and hematogenous dissemination, arrest in brain capillaries, and extravasation. Ultimately, neoplastic cells have to colonize surrounding tissue induce angiogenesis and proliferate in response to local growth factors (8). Those cells that survive find microenvironments that are conducive to their growth and development, also known as "niches"(9); they then form micrometastases that may, in turn, establish clinically significant lesions after a fairly variable period of dormancy, in which requirements for cell division are acquired (10).

Tumor cells have the capacity to evade growth suppressors and inhibitors of cell proliferation *via* mechanisms that include the resistance of apoptosis by overexpression of Bcl-2, Bcl-xL and downregulation of pro-apoptotic Bax and Bim. Through a phenomenon called epithelial–mesenchymal transition, activated by intrinsic (gene mutations) or extrinsic factors (growth factor signaling), epithelial tumor cells can de-differentiate, migrate to a distant focus, survive to apoptosis, disseminate, and then re-differentiate to the original cell (11, 12). Activation of cells in the adjacent stroma (endothelial cells, cancer-associated fibroblasts, pericytes, and leukocytes) *via* paracrine signaling with pro-tumorigenic factors (transforming growth factor beta, hepatocyte growth factor, epidermal growth factor, fibroblast growth factor, and IL-6) sustain tumor growth, enhancing genomic instability and epigenetic dysregulation (8, 13, 14).

Invading tumor cells show a downregulation of proteins preserving structural tissue integrity, such as E-cadherins, integrins, and catenins, lose cell–cell adhesion, secrete proteolytic enzymes that degrade the epithelial basement membrane, penetrate the endothelial basement membrane of vessels, and enter the circulation. Tumor cells, which arrest in capillary beds adhering to the endothelium of target tissue, behave like macrophages, creating pseudopodia and penetrating the cell–cell junctions, and then gain access to the tissue parenchyma by activating angiogenic programs to develop a new vascular supply. Circulating cancer cells attract platelets because of their expressed surface tissue proteins, which protect them from the immune system (15, 16).

The BBB is a functional and anatomic barrier, which plays a central role in interacting with brain microenvironment and influencing metastatic colonization. Several components can be subjected to adaptions by metastatic tumors to breach this barrier. Studies have found a role of cell–cell adhesion factors, including cyclo-oxygenase 2, heparan-binding epidermal growth factor, and alpha-2,6 sialyltransferase (ST6GALNAC5). As the tumor cells adhere to the BBB, infiltrative and transmigratory processes allow the tumor cells to breach the BBB. Later, the tumor cells use the inflamed brain microenvironment as a *niche*. Tumor cells interact with activated microglia and astrocytes, which provide support for neuronal function (17). Studies have found that metastatic tumor cells secrete cytokines involved in the MAP kinase pathway: the hyperactivation of this pathway permits breast cancer cells to overexpress MMP2, a proinflammatory enzyme that helps in tissue remodeling and angiogenesis. Metastatic lung cancer cells have been found to produce IL-8, macrophage inhibitory factor, and plasminogen activator inhibitor 1. These factors activate astrocytes to produce growth factors (IL-6, IL-1B, and tumor necrosis factor), resulting in a perpetuation of cancer cell growth in the neural niche (18). Activation of VEGF, Notch pathways and secretion of BMP-2 (a growth factor that differentiates neuronal stem cells into astrocytes) sustain neoangiogenesis in BMs and tumor–astrocyte interactions (19).

Tumor-driven activation of the mitogen-activated protein kinase (MAPK) and AKT pathways promotes endothelin-driven astrocytic survival and upregulate the antiapoptotic genes BCL2L1, GSTA5, and TWIST1 (20). Tumor cells have also been observed to possess double-stranded DNA repair mechanisms, which protect from reactive oxidative species that are the primary mechanisms of microglial killing of tumor cells. Tumor metastatic cells activate mechanisms in the brain to escape immuno-surveillance [recruitment of myeloid-derived suppressor cells, reduced expression of transporter associated with antigen processing 1 (TAP1), thereby reducing the effect of T-cell-mediated cell death] (21).

Metastatic cells can also take advantage of other mechanisms in the neural niche, such as nerve growth factor–tropomyosin

receptor kinase B interaction on breast brain metastatic cells amplifying oncogenic signaling (22). A subset of metastatic breast cancer tissue has been observed to possess an upregulation of GABA receptors and transporters compared with their primary tumor tissue counterparts: after transporting synaptic GABA into their own cytosol, it is converted to NADH in the cellular mitochondria enhancing energy production, cellular respiration, and finally metastatic cell function and survival (23).

Understanding the mechanisms of metastatic cell invasion and survival within the neural niche could elucidate solutions for metastatic cancer prevention and cure.

DIAGNOSIS: CLINICAL AND NEUROIMAGING FEATURES

Intracranial metastases are most frequently diagnosed in patients with an established primary site of malignancy (metachronous presentation). To a lesser extent, BMs are discovered at the same time of primary tumor (synchronous presentation in up to 30%) or may be the initial presentation as an occult malignancy (precocious presentation) in up to 10% of patients (24).

Intracranial metastases are symptomatic in 60–70% of patients with neurologic symptoms including headache (40–50%), focal neurological deficits (40%), and seizures (15–20%). Impaired cognition and altered mental status are frequent in patients with multiple metastases and/or increased intracranial pressure. Another 5–10% of patients present with acute "stroke like" symptoms due to an intratumoral hemorrhage (especially in melanoma, kidney cancer, and choriocarcinoma). However, symptoms and signs at presentation can be subtle. As a general rule, BMs should be suspected in any patient with known systemic cancer in whom new neurologic findings develop (25).

In both asymptomatic and symptomatic patients, imaging of the brain has a primary role in the diagnosis and is important for subsequent patient management (**Table 1**).

Computed tomography (CT) can provide information on intracranial hemorrhage, herniation, mass effect, and hydrocephalus. MRI with intravenous contrast is preferable for the greater sensitivity than CT, particularly for lesions in the posterior fossa or multiple punctate metastases (26). There are no specific features on MRI that characterize BMs; however, a peripheral location, spherical shape, ring enhancement with extensive peritumoral edema, and multiple lesions suggest a metastatic disease (27).

TABLE 1 | Comparison of neuroimaging techniques in diagnosis of BMs.

Technique		Advantages	Disadvantages
CT		– First-line approach – Fast information on intracranial hemorrhage, herniation, mass effect, and hydrocephalus – Useful in non compliant patients	– Low resolution and low sensibility for differential diagnosis
MRI (T1/T2/FLAIR sequences)		– Major resolution – Better lesion characterization and localization (cortical/subcortical)	– Difficult resolution of calcifications – Longer time for image acquisition and processing
MRI with Gad		Better characterization	– Longer time – Allergic reactions to Gad
Advanced MRI	Spectroscopy	Useful in DD glioma vs BM	– Longer time
	Perfusion	Useful in DD HGG vs BM (rCBV value in peritumoral FLAIR hyperintense region)	– Longer time – Variable target definition for measurement – Unclear rCBV cutoff values in DD HGG vs BM
	DTI images	– Evaluate the structural organization of white matter tracts and spatial relationships with brain lesions – Useful in surgical planning	– Low specificity
	DWI and ADC images	– DD HGG/BM and abscesses (restricted diffusion with DWI+ is more typical in abscesses) – DD HGG/BM edema in peritumoral region (higher ADC signal in BM) – DD PCNSL/HGG and BM (DWI + ADC maps)	– Longer time
PET-CT	18F-FDG PET	– Metabolic and functional information	– Low specificity in DD with others brain lesions (inflammatory disease, abscesses, and granulomatous lesions) and other tumors (HGG)
	Amino acids PET	– More useful for the differentiation of tumor and non-tumoral processes, as tumors have significantly higher uptake	– Increased uptake in acute inflammatory lesions – Not sufficient discrimination from HGG and some non-neoplastic lesions

CT, computed tomography; MRI, magnetic resonance imaging; Gad, gadolinium; DTI, diffusion tensor imaging; DWI diffusion-weighted imaging; DD, differential diagnosis; BMs, brain metastases; HGG, high-grade glioma; rCBV, relative cerebral blood volume; ADC, apparent diffusion coefficient; PET, positron emission tomography; 18F FDG, 18F-fluorodeoxyglucose; FLAIR, fluid attenuated inversion recovery.

Differential diagnosis (DD) includes primary brain tumors [high-grade gliomas (HGGs), primary CNS lymphomas] and non-neoplastic conditions (abscesses, infections, hemorrhages, subacute infarcts, demyelinating diseases, granulomatous diseases, and radiation necrosis).

Advanced neuroimaging techniques have been increasingly used in the detection, treatment planning, and follow-up of BMs. The most frequently used techniques include MR proton spectroscopy (MRS), MR perfusion, diffusion-weighted imaging (DWI), and diffusion tensor imaging (DTI). MRS more often shows a lower choline to creatinine ratio in BMs than in HGGs (28). When employing MR perfusion (dynamic contrast-enhanced), although the relative cerebral blood volume (rCBV) ratio may not be reliable for a differentiation of the enhancing portion of HGGs from metastases, the evaluation of the peritumoral fluid attenuated inversion recovery (FLAIR) hyperintensity has shown lower rCBV in case of metastases compared with HGG: this is likely due to the presence of infiltrative cells and neoangiogenesis in HGGs, while metastatic lesions are surrounded by pure vasogenic edema. Peritumoural perfusion-weighted imaging can assist in preoperative differentiation between a glioma and a solitary metastasis, but to date there is no a definite cutoff value useful for radiological DD (29). In addition, evaluation of the DSC (dynamic susceptibility contrast MRI) perfusion signal changes over time in the contrast-enhancing mass has shown that metastasis has a vascular permeability or "leakiness" of contrast which is higher compared with an HGG but lower compared with PCNSL. DWI evaluates the mean diffusivity of water molecules in a given region of brain parenchyma: highly cellular tumor results in a "restricted diffusion" pattern, with hyperintensity on highly diffusion-weighted DWI sequences and lower signal intensity on apparent diffusion coefficient (ADC) images. Because of the various degrees of cellularity in different metastatic tumor types, DWI is highly variable (30). ADC can be helpful in distinguishing the peritumoral edema surrounding metastases, which usually demonstrates higher ADC signal, from the non-enhancing peritumoral area of HGGs, which demonstrates relatively lower ADC values (31). Diffusion-weighted MR imaging may be useful in the diagnosis of ring-enhancing lesions: restricted diffusion is more typical in abscesses compared with unrestricted diffusion in necrotic metastases or glioblastomas, but the findings are not specific (32). DTI is an MRI technique, which is able to evaluate the structural organization of the brain, particularly white matter tracts. It is useful in surgical planning to localize the relationships of a metastasis to the major white matter tracts. DTI has been also employed to differentiate metastases from HGGs (33, 34).

The capability of 18F-fluorodeoxyglucose (FDG) positron emission tomography (PET) in differentiating BMs from HGGs is limited, since a considerable overlap of standardized uptake values (SUV_{max}) exists. 18F-FDG PET also has limited specificity for distinguishing metastases from non-neoplastic lesions, such as brain abscesses, demyelinating tumefactive ("tumor-like") lesions, fungal infections, and neurosarcoidosis due to the increased glucose metabolism in inflammatory tissues (35).

Amino acid PET seems more useful for the differentiation of tumor and non-tumoral processes, as tumors have significantly higher uptake than non-neoplastic tissues. However, a moderate increase of uptake can also be seen in acute inflammatory lesions. In conclusion, in BMs, both 18F-FDG and amino acid PET do not provide sufficient discrimination from high-grade glial tumors and some non-neoplastic lesions (36, 37). Overall, no advanced imaging techniques can reliably identify the nature of an enhancing brain lesion, and hence histopathological analysis remains the gold standard.

A tissue diagnosis by biopsy should then be considered in patients with either unknown primary tumor or absent well-controlled systemic cancer, especially if a long interval has elapsed since the initial cancer diagnosis, or, seldom, in patients with active systemic cancer when the radiographic appearance is highly atypical and life expectancy is not too short.

When a brain mass is discovered on MRI and there is no prior history of cancer, in most cases the primary tumor is located in the lung (38, 39): thus, chest CT is recommended, while CT of the abdomen only occasionally shows an unsuspected cancer. Further search for a primary tumor is almost never fruitful in asymptomatic patients (40). Whole-body FDG PET is a sensitive tool for detecting a "probable" primary tumor by visualizing foci of abnormal uptake, more often in the lung (41, 42), but the specificity in differentiating malignant tumors from benign or inflammatory lesions is relatively low.

Regarding BMs from an undetected primary site after the first investigations, serial CT scans of the thorax during follow-up in asymptomatic patients can discover the primary tumor (a non-small cell lung carcinoma in the majority), but few patients only benefit in terms of survival from early detection and treatment (43). However, there are no data dealing with this issue in molecular subgroups with available targeted therapies [i.e., EGFR mutated or anaplastic lymphoma kinase (ALK)-rearranged tumors].

In patients with BMs, a CSF examination is not indicated, unless there are coexistent symptoms or signs or neuroimaging findings that suggest an associated leptomeningeal carcinomatosis.

DIAGNOSIS: NEUROPATHOLOGY

Routine hematoxylin–eosin stain of biopsy specimens usually allows the distinction of metastases from malignant gliomas, meningiomas, lymphomas, and more rare entities. Immunohistochemical markers may be useful for a further characterization of the tumor type.

The immunohistochemical expression of glial cell markers, such as glial fibrillary acidic protein or oligodendrocyte transcription factor (OLIG2), suggests a glial tumor.

Usually, BMs mimic the histological appearance of the primary tumor, but sometimes with a lesser degree of differentiation. Overall, the vast majority of BMs are adenocarcinomas. The presence of glandular architecture and mucous allows the distinction of an adenocarcinoma from a small or non-small cell carcinoma with neuroendocrine differentiation, squamous carcinoma, or unspecified non-small cell carcinoma (44). Apart from the morphological analysis, the most effective tool for characterizing a BM is immunohistochemistry (45).

Lung cancers are the most common cause of BM. The brain tropism of non-small cell lung cancer (NSCLC) is higher for adenocarcinoma (54.8%) and poorly differentiated carcinoma (31.7%) than for squamous cell carcinoma (46). Pulmonary

adenocarcinomas express cytokeratin 7 in the absence of expression of cytokeratin 20 (CK7+/CK20− phenotype). However, most adenocarcinomas from breast or other origins behave similarly, thus other markers are needed (47). Thyroid transcription factor (TTF1) is a marker of lung and thyroid origin, and is expressed in 80% of primary non-mucinous lung adenocarcinomas. TTF1 is also expressed in 90% of small cell and 50% of non-small cell neuroendocrine pulmonary carcinomas, thus the pathologist should search for the expression of neuroendocrine markers (CD56, chromogranin A, and synaptophysin) and pancytokeratin AE1/AE3. Focal expression of TTF1 has been described in breast carcinoma, while 20% of primary pulmonary adenocarcinomas do not express TTF1. Anti-Napsin A expression may help to identify a small fraction of these TTF1-negative adenocarcinomas (48). A molecular analysis searching for molecular abnormalities should include mutations of epidermal growth factor receptor (EGFR), Kirsten rat sarcoma viral oncogene homolog (KRAS), BRAF, human epidermal growth factor receptor 2 (HER2), c-MET abnormalities, translocations of ALK, rearranged during transfection (RET), or repressor of silencing 1 (ROS1).

When a breast cancer metastasis is suspected, the expression of hormonal receptors [estrogen receptors (ERs) and progesterone receptors (PgR)] must be investigated, although their expression is not specific as observed in ovarian, endometrial adenocarcinomas, and bronchopulmonary adenocarcinomas (47, 49). Furthermore, breast cancers frequently express mammaglobin, gross cystic disease fluid protein-15 (GCDFP15), and trans-acting T-cell-specific transcription factor (GATA3) (50). An immunohistochemical overexpression of HER2 has prognostic and therapeutic significance (51). "Triple negative" breast cancers (absence of expression of HER2, ER, and PR) have the highest risk of BM and a poor prognosis.

The diagnosis of BM from colorectal (CRC) adenocarcinoma is often done based on the morphological appearance and is supported by the immunohistochemical profile (CK20+/CK7−). The expression of CDX2, a transcription factor expressed in the nuclei of intestinal epithelial cells, also favors the diagnosis, such as the finding of an activating mutation of the RAS genes. In CRC adenocarcinoma KRAS mutations are found in approximately 40% of patients, resulting in the activation of the RAS/RAF/ERK pathway, rendering EGFR inhibitors ineffective (52–54). KRAS and neuroblastoma RAS viral oncogene homolog (NRAS) are closely related, and mutations tend to be mutually exclusive. Few data are available on the molecular subsets of BM from CRC adenocarcinoma. In addition to RAS, activating mutations in the BRAF gene have been found in 8–10% of CRC cancers, and they are almost always mutually exclusive with KRAS mutations (55–57). They are typically associated with right-sided tumors, high-grade mucinous histology, high frequency of lymph node and peritoneal metastasis, and microsatellite instability (58, 59).

The diagnosis of squamous cell carcinoma is based on conventional histology (presence of intercellular bridges and keratinization). The immunohistochemical expression of cytokeratins 5/6 or p40 (more specific than p63 which is expressed in 26–65% of adenocarcinomas) can confirm the epidermoid differentiation (50, 60): however, no marker can specifically determine the primary site.

Other markers of squamous differentiation are CK34bE12 and desmocollin-3, while TTF1 is not expressed. The coexistence of morphological and immunohistochemical features of carcinoma and adenocarcinoma suggests the possibility of an adenosquamous carcinoma of pulmonary or gynecological origin.

Most of the patients with BM from an unknown primary site can be diagnosed by searching for mucins and TTF1 and p40. When a primary breast cancer is suspected, TTF1 and GATA3 are the most relevant markers (50). CK7 and CK20 are less helpful, since many cancers with brain tropism, such as NSCLC or breast cancer, have a CK7+/CK20− phenotype.

MOLECULAR BIOLOGY

In BM, molecular alterations do not always reflect those of the primary tumor (**Table 2**). In BM of NSCLC, fibroblast growth factor receptor 1 (FGFR1) amplifications are found in 19% of squamous cell carcinomas, and in 15% of adenocarcinomas. A positive correlation of ALK and FGFR1 gene amplification status exists in BMs (61). Discoidin domain-containing receptor 2 (DDR2) mutation was reported in 1–4% of Sq-NSCLC, and its sensitivity to dasatinib inhibition was demonstrated both *in vitro* and *in vivo* (62).

With regard to EGFR status, lung adenocarcinomas carrying EGFR mutations have a higher risk of developing BM (64%), probably as a consequence of the prolonged survival due to treatment with EGFR-tyrosine kinase inhibitors (TKIs) (63, 64). In BM, EGFR status was evaluated in few studies. Porta et al. evaluated EGFR mutation status in 69 NSCLC-BM patients treated with erlotinib: 17 had EGFR mutation with an intracranial response rate of 82.4% (65, 66). ALK rearrangements occur in 3–7% of patients with NSCLC, but no data are available about the incidence in BM (67). Approximately 1.4% of NSCLCs harbor ROS1 rearrangements. ROS is strongly homologous to ALK, so that ALK inhibitors can be efficacious against ROS1-positive tumors (68). In NSCLC, multiple mechanisms of c-MET activation have been reported, including gene amplifications and exon 14 skip mutations (69).

In melanoma, BRAF mutations (mainly BRAF V600E) are found in 50% of melanoma BM, leading to a constitutive activation of the MAPK signaling pathway, promoting cellular growth, invasion, and metastasis (70).

The identification of BRAF mutations is relevant for therapeutic management, but it is not a diagnostic tool, given the fact that it is observed in numerous CNS tumors, notably in 10% of glioblastomas (in 50% of epithelioid glioblastomas), in over 60% of gangliogliomas and pleiomorphic xanthoastrocytomas, in 5% of BM of colon cancers, and in 1–4% of BM of NSCLC (71).

In breast cancer, 62–75% of patients maintain the same receptor status between the primary tumor and the brain metastasis, whereas discordance rates of 25–37.5% have been found. The rate of ER and PgR expression was 41.6% in the primary tumors and decreased to 12.5 and 16.6% in the BMs. By contrast, the rate for Her2+ tumors increased from 41.6% in primary breast cancer to 65.2%, respectively, in the BMs. All anti-estrogen treated breast tumors lost the ER expression in the BMs, whereas a Her2/neu conversion did not occur after treatment with trastuzumab.

TABLE 2 | Neuropathological and molecular markers of brain metastases.

Morphology	Histochemistry and IHC		Molecular biology	Standard detection method	Targeted therapy
Undifferentiated tumor	Desmin	Sarcoma			
	HMB45 MelanA	Melanoma	BRAF/NRAS mutations	ARMS-PCR/targeted NGS Real-time allele-specific PCR	Dabrafenib + trametinib Vemurafenib + cobimetinib
			cKIT mutations	Targeted NGS	Imatinib (not registered in this indication)
	CKAE1AE3	Undifferentiated carcinoma	EGFR, KRAS, BRAF, HER2, ALK, ROS1, RET, c-MET	ARMS-PCR/targeted NGS Real-time allele-specific PCR, IHC, FISH or RNA seq	
Adenocarcinoma (glandular ± mucosecretion)	Mucins, TTF1, p40, CK7, CK20	Unknown primary TTF1−, CK 7+/−, CK20−			
		Broncopulmonary adenocarcinoma (NSCLC) TTF1+, CK 7+, CK20−	EGFR-activating mutations	Targeted NGS or real-time allele-specific PCR (FDA-approved COBAS© EGFR mutation test v2, ARMS-PCR)	Gefitinib, erlotinib, afatinib
			EGFR resistant mutation: T790M	COBAS© EGFR mutation test v2 (tumor tissue and plasma) Dropet digital PCR (plasma), targeted NGS	Osimertinib
			ALK translocation	IHC, FISH, or RNA seq (for IHC+)	Crizotinib
			ROS1 translocation	IHC, FISH, or RNA seq (for IHC+)	Crizotinib, ceritinib, brigatinib, lorlatinib (not registered)
			RET translocation	FISH or RNA seq	Cabozantinib, vandetanib (not registered in this indication)
			c-MET amplification	FISH or targeted NGS	Crizotinib (not registered in this indication)
			c-MET splice-mutation (exon 14 skip mutation)	Targeted NGS	Crizotinib
			BRAF V600E or V600K mutations	Targeted NGS Real-time allele-specific PCR IHC (V600E)	Dabrafenib + trametinib Vemurafenib + cobimetinib
		Breast adenocarcinoma TTF1−, RE/RP+/− (GATA3+, mammaglobin+), CK 7+, CK20− (triple negative: CK5/6/14+)	HER2 amplifications	FISH, IHC	Trastuzumab, pertuzumab, lapatinib, trastuzumab-emtansine (TDM1)
		Colorectal adenocarcinoma CK 7−, CK20+, CDX2+	KRAS, NRAS mutations	ARMS-PCR/targeted NGS Allele-specific PCR-COBAS© KRAS mutation test	EGFR mAb (cetuximab, panitumumab) resistance
		Renal adenocarcinoma CK 7−, CK20−, vim+, CD10+			
		Rare cancers PSA, CA125, TG, PLAP, alpha-fetoprotein, β HCG			
Squamous cell carcinoma		Keratinization, intercellular bridges, p40+, CK5/6+, TTF1	FGFR1 amplification	FISH	Not registered drugs
			DDR2 mutations	Targeted NGS	Dasatinib (not registered in this indication)
Neuroendocrine carcinoma	CK+, TTF1+/−, chromogranin+, synapthophysin+	Small cell lung carcinoma (SCLC)	TTF1+		Not registered drugs
		Large cell neuroendocrine tumor	TTF1+/−		

CK, cytokeratins; TTF1, thyroid transcription factor; IHC, immunohistochemistry; ARMS-PCR, amplification refractory mutation system-polymerase chain reaction; FISH, fluorescent in situ hybridization, NGS, next-generation sequencing; RNA seq, RNA sequencing; EGFR, epidermal growth factor; KRAS, Kirsten rat sarcoma viral oncogene homolog; BRAF, v-raf murine sarcoma viral oncogene homolog B; NRAS, rat sarcoma oncogene; HER2, human epidermal growth factor receptor 2; ALK, anaplastic lymphoma kinase; ROS1, repressor of silencing 1; RET, rearranged during transfection; c-MET, tyrosine-protein kinase Met or hepatocyte growth factor receptor; cKIT, tyrosine-protein kinase Kit or CD117; FGFR1, fibroblast growth factor receptor 1; DDR2, discoidin domain-containing receptor 2.

Therefore, the receptor status of the primary tumor is invalid for planning a targeted therapy against BMs, especially after hormone therapy. In these cases, new tissue collection by biopsy or resection should be required for more accurate decision-making (72).

BIOMARKERS AND LIQUID BIOPSY

The diagnosis and management of BMs still rely on histopathology and neuroimaging. However, considering tumor complex genetic profiles, more sophisticated methods of tumor analysis are under investigations in fluids (so-called liquid biopsy). Circulating biomarkers, including tumor nucleic acids, tumor cells (CTCs), and extracellular vesicles, which contain tumor DNA as well as other macromolecules, such as microRNA, have shown promise to provide information regarding tumor evolution over time, specifically for monitoring treatment response and disease progression (73, 74).

Although the technical aspects of biomarker detection still require optimization, these tools have already demonstrated their diagnostic, prognostic, and predictive value in several tumor types, including breast, CRC, non-small cell lung, prostate cancers, and melanoma. In addition, they can be used in the laboratory to probe mechanisms of acquired drug resistance and tumor invasion and dissemination (75, 76).

Genomic alterations can be detected in the CSF by microarrays, digital or real-time polymerase chain reaction (qPCR) and targeted amplicon sequencing, and whole exome sequencing (77, 78). Although a lumbar puncture is a more invasive procedure than a blood draw, the possible lack of representative tumor DNA in the plasma may lead to inconclusive results. CSF likely represents a preferable source of liquid biopsy in brain metastatic lesions featuring meningeal carcinomatosis, at least when no extra-CNS localizations are evident (79). It remains unclear in patients with metastatic solid tumors diagnosed with leptomeningeal carcinomatosis whether tumor DNA detection in the CSF compartment always reflects the local presence of cells or whether this DNA may be derived from tumor cells circulating in the blood or even from distant extracerebral metastases (80–82). Yang and colleagues have evaluated EGFR gene status in tumor-derived free DNA in CSF to test correspondence with the molecular pattern of the primary tumor and to guide the clinical use of EGFR-TKI. Indeed, between the paired primary tumor and tumor-derived DNA in CSF, 75% of samples had a concordant EGFR status (83). Findings on two patients by Marchiò and colleagues corroborate the notion that CSF represents a preferable source for liquid biopsy in brain metastatic lesions featuring meningeal carcinomatosis, at least when no extra-CNS localizations are evident (72). Liquoral liquid biopsy can allow the identification of either actionable genetic alterations or a mutation correlated to resistance to targeted therapies leading to crucial changes in the treatment decision.

THERAPY

The choice of therapeutic approaches, both in clinical trials and daily practice, is guided by the knowledge of the natural prognostic factors. Karnofsky performance status (KPS), age, primary/systemic tumor activity, neurocognitive function, number of BMs, primary tumor type, and time from primary tumor diagnosis to the brain lesion all have shown an individual prognostic significance in patients with BMs (84, 85). Of these, the KPS is the major determinant of survival. On the basis of most powerful factors, prognostic indices have been developed to distinguish subgroups of patients with different outcomes. The first prognostic categorization [Recursive Partitioning Analysis (RPA)], developed in 1997 by the Radiation Therapy Oncology Group (RTOG) (84), subdivided patients treated with whole-brain radiotherapy (WBRT) into three classes: class I including patients with a KPS greater than 70, controlled primary tumor/number of extracranial metastases and age below 65 years (median survival 7.7 months); class III including patients with KPS less than 70 (median survival 2.3 months); and class II including the rest of patients (median survival 4.5 months).

A new prognostic index, the diagnostic-specific Graded Prognostic Assessment (DS-GPA), derived from an updated RTOG database, has been proposed more recently, considered as prognostic as the RPA, less subjective and more quantitative (86). A further analysis has shown that the prognostic factors vary according to the tumor type (87). The DS-GPA uses four factors (age, KPS, status of extracranial disease, and number of BMs) to subdivide patients into one of four categories with median survivals ranging from 2.6 to 11 months. A new updated GPA, including molecular markers, has been proposed to better estimate survival in patients with BM: lung-mol-GPA is an update of the DS-GPA, which incorporates EGFR and/or ALK mutation status in addition to the well-known prognostic factors such as KPS, age, presence of extracranial metastases, and number of BMs (88). For melanoma there are five significant prognostic factors: age, KPS, extracranial metastases, number of BMs, and BRAF status (89). Conversely, significant prognostic factors for breast cancer are KPS, age and tumor subtype (classified HER2, ER, and PgR status) (90), but not the number of BMs and extracranial metastases.

SURGERY

Three randomized trials have compared surgical resection followed by WBRT with WBRT alone (91–93). The American and European trials have shown a survival advantage for patients receiving the combined treatment (median survival 9–10 vs 3–6 months). In the American study, patients who received surgery displayed a lower rate of local relapses (20 vs 52%) and a longer time of functional independence. The Canadian study, which included more patients with an active systemic disease and a low performance status, did not show any benefit with the addition of surgery to WBRT. Therefore, the survival benefit of surgical resection seems limited to the subgroup of patients with controlled systemic disease and good performance status. Surgical resection allows a relief of symptoms of intracranial hypertension, a reduction of neurological deficits and seizures, and a rapid steroid taper.

Gross total resection of a brain metastasis can be achieved with lower morbidity using advanced image-guided systems, such as preoperative functional MRI and DTI, intraoperative neuronavigation and cortical mapping (94, 95).

Leptomeningeal disease (LMD) can be a complication, especially for patients with posterior fossa metastases undergoing a "piecemeal" resection (13.8%) compared with "en-bloc" resection (5–6%) (96).

Surgery may be considered for patients with two to three surgically accessible BMs in good neurological condition and controlled systemic disease: a complete resection yields results comparable to those obtained in single lesions (97).

The use of carmustine wafers in the resection cavity in newly diagnosed brain metastasis has not been tested in prospective trials (98).

Another technique is the GliaSite Radiation Therapy System, an intracavitary high-activity 125-I brachytherapy, performed with a balloon placed in surgical cavity and filled with a radioactive solution, delivering highly localized doses of radiation to the resection margins (60 Gy to 1 cm depth). However, this technique has not been further developed after some interesting preliminary results (99).

STEREOTACTIC RADIOSURGERY (SRS)

Stereotactic radiosurgery is a single, high-dose radiation treatment with precise localization of a target of 3–3.5 cm of maximum diameter by using gamma-knife (multiple cobalt sources) or linear accelerator (Linac) through a stereotactic device. The steep dose fall-off minimizes the risk of damage to the normal nervous tissue. Most BMs are an ideal target for SRS, owing to the small size, spherical shape, and distinct radiographic and pathologic margins (100). The dose is inversely related to tumor diameter and volume (101). In general local tumor control decreases as the size of metastasis increases and the dose that could be given decreases. Following SRS for newly diagnosed BMs a decrease of symptoms and a local tumor control (shrinkage or arrest of growth) at 1 year of 80–90% and a median survival of 6–12 months have been reported (102). Metastases from radioresistant tumors, such as melanoma and renal cell carcinoma, respond to SRS as well as do metastases from radiosensitive tumors (103). Radiosurgery allows the treatment of BMs in almost any location, including brainstem (104). The type of radiosurgical procedure, gamma-knife or Linac-based, does not have an impact on the outcome. SRS combined with WBRT (radiosurgical boost) is superior to WBRT alone in terms of survival (105) in patients with single lesions only. Survival following radiosurgery is comparable to that achieved with surgery (106). SRS is less invasive and can be performed in an outpatient setting, and offers cost effectiveness advantages over surgery; on the other hand, patients with larger lesions may require chronic steroid administration.

Acute (early) and chronic (late) complications following radiosurgery are reported in 10–40% of patients (107). Acute reactions (due to edema) occur more often within 2 weeks of treatment, and include headache, nausea and vomiting, seizures, and worsening of preexistent neurological deficits. These reactions are generally reversible with corticosteroids. Chronic complications consist of hemorrhages and radionecrosis (1–17%). Radiographically, a transient increase in the size of the irradiated lesion, with increasing edema and mass effect, with or without radionecrosis, cannot be distinguished from a tumor progression: MR spectroscopy and perfusion and PET techniques can give additional information (108, 109).

Hypofractionated radiosurgery is used for larger metastases (two to five fractions of smaller doses) to decrease the risk of radionecrosis and other neurological complications (110–112).

Most studies comparing SRS with surgical resection have reported similar outcomes in terms of survival for patients with oligometastases (113, 114). However, these are retrospective studies and are likely to be affected by selection bias.

Overall, the choice between surgery and SRS should be made considering many factors, such as number, location and size of BMs, neurologic symptoms, patient preference and physician expertise.

WBRT FOLLOWING SURGERY OR RADIOSURGERY

The rationale of WBRT in conjunction with surgery or SRS is that of destroying microscopic disease at the original tumor site or at distant intracranial locations: however, it has been questioned by recent trials (115). Three phase III trials (116–118) in patients with single brain metastasis have reported that the addition of WBRT to either surgery or SRS reduces the risk of local and distant relapses in the brain but does not improve overall survival. The American (116) and the Japanese (117) trials included patients with progressive systemic disease, while the EORTC 22952-26001 trial recruited patients with stable systemic disease, i.e., only those who could maximally benefit from the addition of early WBRT (118).

An individual patient data meta-analysis of three randomized trials assessing SRS with or without WBRT has challenged our current understanding of the effect of adding WBRT (119). The investigators reported a survival advantage for SRS alone in those patients presenting with one to four metastases, KPS of 70 or higher and age of 50 years or younger. Moreover, in the subgroup of patients with <50 years, a reduction in the risk of new BMs with adjuvant WBRT was not noted, while, in older patients (aged >50 years), WBRT decreased the risk of new BMs, but did not affect survival.

A secondary analysis of the Japanese trial has stratified patients by GPA score and suggested that a subgroup of patients with NSCLC with higher GPA scores (2.5–4.0) have a survival benefit from SRS + WBRT compared with SRS alone (median survival 16.7 vs 10.7 months) (120). However, a similar secondary analysis of the EORTC trial has not confirmed this finding (121).

The impact of adjuvant WBRT on cognitive functions and quality of life (QoL) has been analyzed in few studies (**Table 3**). Aoyama et al. compared, by using the Mini-Mental State Evaluation, the neurocognitive function of patients who underwent SRS alone or SRS + WBRT and showed a deterioration of neurocognitive function in long-term survivors (up to 36 months) after WBRT (120). Chang et al. in a small randomized trial have shown that patients treated with SRS plus WBRT were at greater risk of a decline in learning and memory function at 4 months after treatment compared with those receiving SRS alone (122).

A randomized phase III trial (Alliance trial) has compared SRS alone vs SRS + WBRT in patients with one to three BMs using a primary neurocognitive endpoint, defined as decline from baseline in any six cognitive tests at 3 months. The decline was significantly more frequent after SRS + WBRT vs SRS alone (88 vs 61.9%) (class

TABLE 3 | Phase III trials of SRS ± whole brain radiotherapy for brain metastases (BMs) with cognitive endpoints.

Reference	Inclusion criteria	Number of Pts	Primary endpoint	OS (months)	Outcome	Distant brain metastases and secondary endpoint
Chang et al. (122) (MDACC)	1–3 BMs	58	5-point drop on HVLT-R total recall at 4 months	SRS + WBRT: 5.7 SRS alone: 15.2 ($p = 0.003$)	Neurocognitive decline at 4 months: SRS + WBRT 52% SRS alone: 24%	SRS + WBRT: 73% SRS alone: 45% (p = 0.02) (at 1 year)
Brown et al. (287) (Alliance)	1–3 BMs	213	>1 SD in any of the six cognitive tests at 3 months	SRS + WBRT: 7.5 SRS alone: 10.7 (p = NS)	Neurocognitive decline at 4 months: SRS + WBRT 88% ($p = 0.02$) SRS alone: 62%	SRS + WBRT: 85% SRS alone: 51% ($p < 0.01$) (at 1 year)
Brown et al. (123)	Pts with 1–3 BM	213: – SRS alone, $n = 111$ – SRS plus WBRT, $n = 102$	Primary endpoint: cognitive deterioration (decline >1 SD from baseline on at least 1 cognitive test at 3 months) Secondary endpoint: time to intracranial failure, QoL, FI, long-term cognitive status, OS	m OS: – 10.4 ms for SRS alone – 7.4 months for SRS + WBRT ($p = 0.92$)	Cognitive deterioration at 3 months: – After SRS alone: 40/63 Pts (63.5%) – SRS + WBRT: 44/48 Pts (91.7%) Less deterioration with SRS alone ($p < 0.001$)	– Time to intracranial failure shorter for SRS alone compared with SRS + WBRT ($p < 0.001$) – For long-term survivors, cognitive deterioration was less after SRS alone at 3 months (45.5 vs 94%, $p = 0.07$) and at 12 months (60 vs 94%; $p = 0.04$) – QoL was higher at 3 months with SRS alone
Brown et al. (126) (NCCTG N107C/CEC·3)	1 resected BM and a resection cavity <5 cm	194 – SRS alone 98 Pts – WBRT 96 Pts	Cognitive-deterioration-free survival and OS	SRS: 12.3 months WBRT: 11.6 months	Median cognitive-deterioration-free survival: – SRS: 3.7 months – WBRT: 3 months – Cognitive deterioration at 6 months: SRS: 52% (28/54 Pts); WBRT: 85% (41/48 Pts)	

MDACC, M.D. Anderson Cancer Center; Alliance, alliance for clinical trials in Oncology; Pts, patients; HVLT-R, Hopkins verbal learning test-Recall; SRS, stereotactic radiosurgery; WBRT, whole-brain radiotherapy; NS, not significant; OS, overall survival; BMs, brain metastases; QoL, quality of life; FI, functional independence.

I) with more deterioration in immediate recall (31 vs 8%), delayed recall (51 vs 20%), and verbal fluency (19 vs 2%) (123).

A QoL analysis of the European Organisation for Research and Treatment of Cancer (EORTC) 22952-26001 trial has shown over 1 year of follow-up that patients receiving adjuvant WBRT had transient lower physical functioning and cognitive functioning scores and more fatigue (124). Based on the results of these trials, the American Society for Radiation Oncology (ASTRO) has recommended in its Choose Wisely campaign not to routinely add adjuvant WBRT to SRS for patients with a limited number of BMs, and the same has been done in the European Association of Neuro-Oncology guidelines (25). Importantly, in case of omission of WBRT, following either SRS or surgery, close monitoring with MRI is mandatory.

SRS FOLLOWING SURGERY

Surgery alone in brain metastasis is associated with a high risk of local recurrence (118). Postoperative SRS is an approach to decrease local relapse and avoid the cognitive sequelae of WBRT. SRS has other advantages over WBRT, such as reduced number of fractions and a shorter break for chemotherapy during radiation, thus reducing the risk of systemic recurrences. Based on retrospective cohort studies, the results of a meta-analysis by Lamba and colleagues (125), suggest that SRS of the resection cavity may offer comparable survival and similar local and distant control as adjuvant WBRT. A phase III randomized trial has been conducted to evaluate postoperatively SRS vs WBRT in brain metastasis patients (126). The co-primary endpoints were cognitive-deterioration-free survival and overall survival; secondary endpoints were QoL, adverse events, and functional independence. This trial concluded that SRS to the surgical cavity results in improved cognitive outcomes, better preservation of QoL and functional independence compared with WBRT. Despite worse intracranial control, there was no difference in overall survival. In conclusion, after resection of a brain metastasis, SRS should be considered one of the standard of care, as a less toxic alternative to WBRT (123, 127, 128).

However, postoperative SRS can be associated with a higher risk of developing leptomeningeal disease (LMD). Some recent studies have reported an incidence of LMD in patients receiving SRS to the resection cavity around 12–14% and breast cancer patients can have a particularly high rate of LMD (129–131). Several questions remain regarding SRS after surgery, such as major risk of radionecrosis, the optimal dose and fractionation schedule, especially for large metastases (>3 cm). The risk of radionecrosis following postoperative SRS is higher (between 9 and 15%) than that reported in an EORTC study with WBRT following surgery or SRS alone (2.6%) and could be reduced by modifying the treatment schedule with multifraction SRS (132). The actual incidence over time is 5.2% at 6 months, 17.2% at 12 months and 34.0% at 24 months (133). Preoperative SRS could be an alternative to reduce the risk of radionecrosis (134).

Overall, the true incidence of biopsy proven radionecrosis is unknown, as many studies have reported a combination of pathologically proven and MRI suspected radionecrosis. Advanced neuroimaging techniques (MRI perfusion, MR spectroscopy, and PET with amino acids) may help diagnosis (109, 135). Treatment options for radionecrosis include corticosteroids and bevacizumab that could reduce edema and symptoms (136).

There are still few data regarding the impact of postoperative SRS on health related quality of life. Caveats of available studies comparing postoperative SRS and WBRT for BMs include the heterogeneity regarding primary tumor histology (with varying proportion of melanoma, colon, renal, breast, and unknown histologies), numbers of BMs and treatment modalities, which were not always clearly reported (125). Some studies described outcomes of intraoperative radiotherapy instead of SRS (137).

WBRT ALONE

Whole-brain radiotherapy alone is now the treatment of choice for patients with single or multiple BMs not amenable to surgery or radiosurgery, or, more in general, patients with poor prognostic factors and limited life expectancy. Tumor volume reduction after WBRT seems to be associated with better neurocognitive function preservation and prolonged survival (138). Median survival following WBRT alone ranges from 3 to 6 months, with 10–15% of patients alive at 1 year. A meta-analysis of 39 trials, involving 10.835 patients, concluded that altered WBRT dose fractionation schemes are not superior in terms of overall survival, neurologic function or symptom control as compared with standard fractionation (30 Gy in 10 fractions or 20 Gy in 5 fractions) (139). Currently, radiosensitizers, such as motexafin gadolinium (Gad) or efaproxiral, have not provided any additional benefit over conventional treatment in BMs from NSCLC or breast cancer (140, 141). Recently a phase III randomized trial (QUARTZ) randomly assigned 538 NSCLC patients with BMs, unsuitable for surgical resection or stereotactic radiotherapy, either to optimal supportive care (OSC) plus WBRT (20 Gy in five daily fractions) or OSC alone. The primary outcome measure was quality-adjusted life-years (QALYs): QALYs was generated combining overall survival and patients' weekly completion of the EQ-5D questionnaire. An absence of a difference in QALYs and overall survival between the two groups was reported, suggesting that WBRT provides little additional benefit, over supportive care (i.e., dexamethasone), for this patient group (142).

The cognitive decline following WBRT has been linked to leukoencephalopathy, whose incidence is higher after WBRT vs SRS (143, 144). A study, evaluating 59 patients treated with primary SRS, found that even after multiple courses of SRS, QoL, measured with the EQ-5D instrument, was preserved in 77% of patients at 12 month follow-up (145). Thus SRS seems to be a valid treatment option that can help maintain neurocognition and QOL.

BRAIN DAMAGE FOLLOWING WBRT

Radiation-induced brain injury can be categorized as acute, early-delayed or late-delayed reactions (146). Acute injury is very rare and occurs hours to weeks after radiation therapy and consists of somnolence, drowsiness, and headache. Early delayed injury occurs from 1 to 6 months post-irradiation and is characterized by drowsiness and transient demyelination. Both acute and early-delayed reactions are normally reversible with corticosteroids. Late delayed effects, characterized by demyelination, vascular

abnormalities and ultimately white matter necrosis, are generally observed with a latency of 6 months or more post-irradiation, and are irreversible and progressive (147). Giving the prolonged survival of many cancer patients, long-term complications of WBRT could negatively impact the QoL of survivors. Currently, no treatments to prevent or reverse these effects are available.

Radiation-induced dementia with ataxia and urinary incontinence developed in up to 30% of patients by 1 year after receiving unconventional large size fractions of WBRT (6–8.5 Gy) (148). A diffuse hyperintensity of the periventricular white matter on T2-weighted and FLAIR images with associated hydrocephalus was observed on MRI. When using more conventional size fractions (up to 3 or 4 Gy per fraction) the risk is that of milder cognitive dysfunction, consisting of learning and memory impairment with changes in the white matter and cortical atrophy on MRI. Patients with advanced age and vascular disease (such as arterial hypertension) are at higher risk of developing cognitive dysfunction. The pathogenesis of this radiation-damage consists of an injury of the endothelium of microvessels, which leads to atherosclerosis and chronic ischemia: the clinical–radiological pictures are similar to those of small vessel disease.

To reduce the neurocognitive sequelae of WBRT pharmacological approaches have been investigated. The RTOG 0614 was a phase III trial investigating memantine, an *N*-methyl-D-aspartate receptor blocker, shown to be effective in vascular dementia, and included 508 patients with BM who were randomized to either WBRT plus placebo vs WBRT plus daily memantine for 6 months (149). Memantine has the potential to block excessive NMDA stimulation following ischemia, which causes damage of the normal brain. The study narrowly missed statistical significance for the primary endpoint of a lesser decline in delayed recall at 6 months. However, the memantine arm showed a longer time to cognitive decline, with probability of cognitive decline at 6 months being 54% in the memantine arm and 65% in the placebo arm. Other treatments, including donepezil and cognitive rehabilitation, have been tested in the population at risk for dementia, although they have not been adequately studied in patients with BM, following cranial radiotherapy (150). An innovative approach could be the use of intranasal metabolic stimulants, such as low dose insulin, which could be valuable in improving cognition and memory, by reversing impaired brain metabolic activity (151).

Radiation-induced cognitive deficits may result, at least in part, from a radiation injury to the neuronal stem cells in the subgranular zone of the hippocampus (152, 153), that are responsible for maintaining neurogenesis, and preserving memory functions. Preclinical and clinical studies have shown that radiation-induced injury to the hippocampus correlates with neurocognitive decline following WBRT. Hippocampal sparing WBRT uses intensity modulated radiotherapy (IMRT) to conformally reduce the radiation dose to the hippocampus, while applying the usual higher dose to the whole brain (154). A potential concern is whether hippocampal avoidance could lead to loss of control of metastases in the limbic structures: however, the hippocampus does not seem to be frequently involved in the metastatic process (155, 156). The single-arm phase II RTOG 0933 study has suggested that conformal avoidance of the hippocampus during WBRT is associated with some sparing of memory and QoL: performance on standardized memory tests declined 7% from baseline to 4 months in patients treated with HS-WBRT, as compared with 30% in historical control group (157).

The potential combined neuroprotective effects of hippocampal avoidance in addition to memantine during WBRT for BMs are being investigated in a phase III trial (158).

TREATMENT OF RECURRENT BRAIN METASTASIS

Treatment of recurrent BMs with salvage surgery or SRS is used in an increasing number of patients. Reoperation can afford a neurological improvement and prolongation of survival in patients with a local accessible brain lesion, high performance status, stable extracranial disease and relatively long time to recurrence (>6 months) (159, 160). Arbit et al. analyzed 109 patients with recurrent BMs of NSCLC. Thirty-two patients, who underwent surgery at relapse, had significantly longer median survival (15 months) than 77 patients in whom surgical intervention was omitted (10 months, $p < 0.001$) (161).

Salvage SRS after WBRT has been widely used during the initial development of SRS: several retrospective studies have reported reasonable local control and survival rates (162–165). A population-based study has suggested similar survival outcomes following either salvage SRS or boost SRS (166). Reirradiation with SRS after local recurrence of an initial SRS has been employed in a limited number of patients thus far, and the risk of long-term radionecrosis should be considered against the benefit (167). Multiple courses of SRS for new BMs after an initial course of SRS to defer WBRT could yield high rates of local control, low risk of toxicity, and favorable duration of overall and neurologic progression-free survival (PFS) (168, 169). When using multiple courses of SRS the aggregate volume, but not the cumulative number of BMs, and the GPA score, as recalculated at the second course of SRS, are seen as critical for survival (170). Salvage WBRT following previous WBRT or SRS is rarely employed.

CHEMOTHERAPY AND TARGETED THERAPIES

Unfortunately, few clinical trials of systemic agents have been conducted to date in patients with BM, and this population has frequently been excluded from clinical trials of emerging investigational drugs (171). Historically, the use of systemic therapy in patients with BMs has been limited by the presence of the BBB, which limits the access of hydrophilic and/or large agents into the CNS. However, the BBB is disrupted in macroscopic BMs resulting in an increased exposure to systemic drugs. An additional increase in BBB permeability can be induced by radiotherapy, even if unpredictably. The sensitivity of tumor cells to cytotoxic chemotherapy appears to be as important as the BBB: response rates are high in BMs from small cell lung cancer (30–80%), intermediate in breast cancer (30–50%) and NSCLC (10–30%), and low in melanoma (10–15%), and responses in the brain do not always parallel that of the systemic disease (172, 173). Because active BMs often coexist with active systemic disease, antitumor agents that can control both intracranial and extracranial disease are needed.

The combination of radiotherapy and chemotherapy may improve response rate, but not survival (174). Various chemotherapeutic agents have been employed to treat BM, often used in a combination of two to three agents along with WBRT. Temozolomide alone has a modest therapeutic effect with some improvement when used in conjunction with WBRT and/or other anticancer agents (175).

Brain metastases differ from metastases to other organs from a biologic and clinical perspective. The brain has a unique micro environment and an immune system distinct from other organs (176). Other considerations, which could explain the disappointing results of chemotherapy in BM, are the fact that metastatic cells in the brain often develop after multiple rounds of prior chemotherapies for the systemic disease, allowing for the development of resistance through the accumulation of different mutations. Second, the breakdown of BBB is heterogeneous in BM, preventing an optimal drug distribution. Finally, BM patients may develop neurologic deficits and seizures, which could require the use of medications (i.e., enzyme-inducing anticonvulsants, corticosteroids), that accelerate the metabolism of antitumor agents (177).

Recent advances in understanding the molecular basis of tumor growth in many solid tumors have allowed the development of agents targeting molecular pathways both in the extracranial and intracranial disease (172, 178–180). Encouraging results have emerged for tyrosine kinase inhibitors and monoclonal antibodies in subgroups of patients with BM (**Table 4**). As CNS immune-accessibility has become accepted, and immunotherapy (IT) gains greater understanding within trials for primary brain tumors, there is an increasing interest in immunotherapeutic approaches to BMs with immune checkpoint inhibitors (181–183) (**Table 5**).

Overall, the response rate to targeted agents in specific molecular subtypes of BMs seems now higher than those observed after conventional chemotherapy. However, even if the majority of targeted agents are small molecules, still the passage across the BBB is critical, as most of these new compounds, similar to the old chemotherapeutics, have been shown to be a substrate of one or more active efflux transporters (i.e., PgP signaling).

BMs From NSCLC

The survival of the general metastatic NSCLC population is approximately 12 months, with a median PFS ranging from 3 to 6 months. BMs are associated with poor prognosis, and the median survival ranges from 2.4 to 4.8 months for patients who receive palliative radiotherapy.

Platinum compounds (cisplatin and carboplatin) and pemetrexed, alone or in association (etoposide, vinorelbine, and radiotherapy) are the most commonly used chemotherapeutics against BMs from NSCLC (184). Temozolomide has shown some activity.

In the last decades, a multitude of molecular abnormalities have been discovered in NSCLC.

Approximately 33% of patients with NSCLC tumors and EGFR-TKI-sensitizing mutations develop BM (185). A pooled analysis including 464 patients from 16 trials to study the efficacy of first generation EGFR-TKIs (gefitinib, erlotinib) in NSCLC patients with BM showed significant beneficial effects, with a higher response rate (85 vs 45.1%) for EGFR mutated vs wild-type tumors and a median PFS of 7.4 months, and OS of 11.9 months in the EGFR mutation group (186). These data suggest that EGFR-TKIs are an effective treatment for NSCLC patients with BMs harboring activating EGFR mutations. However, even in EGFR wild-type patients, EGFR-TKIs seem to represent a potential second-line therapy with a response rate of about 10% (187). Evidence suggests that first generation EGFR-TKIs have limited BBB penetration (188, 189).

Afatinib is a second-generation irreversible covalent inhibitor of the EGFR tyrosine kinase including ErbB-2 (HER2) and ErbB-4. Subgroup analysis from LUX-LUNG 3 and LUX-LUNG 6 studies demonstrated a significant overall survival benefit for afatinib compared with chemotherapy in stage IIIB/IV lung adenocarcinoma patients with 19del-EGFR mutation (190). In a combined *post hoc* analysis of both studies, PFS was significantly improved with afatinib vs chemotherapy in patients with BM (8.2 vs 5.4 months; $p = 0.0297$) (191). Afatinib has reported good results in some cases of LM in stage IV exon 19-del-EGFR-mutant lung adenocarcinoma in association with WBRT, resulting in an almost complete regression of neurological symptoms as well as good, durable radiological responses (192). A good brain response in a patient with EGFR-mutant lung adenocarcinoma and multiple BMs who switched from erlotinib to afatinib due to hepatotoxicity, has been reported (193).

Based on the high intracranial response rates, TKIs have been hypothesized to be used alone as initial treatment in patients harboring activating EGFR mutations and asymptomatic small BMs (64, 194, 195). The main advantages of using TKIs alone are that patients can potentially avoid the adverse effects of WBRT as long as the intracranial disease is well controlled. The disadvantages could be that the discordance rate of EGFR mutations between the primary tumor and BMs can be as high as 32%, and the CSF penetration rate of gefitinib (1–10%) and erlotinib (2.5–13%) is limited. A meta-analysis has suggested that cranial radiotherapy (SRS or WBRT) associated with TKIs is more effective in improving response rate and disease control rate than radiotherapy alone or chemotherapy. Moreover, radiotherapy plus EGFR-TKIs significantly prolonged the median overall survival but significantly increased adverse events of any grade, especially rash and dry skin (196).

On the other hand, clinical trials offering a combination of erlotinib with radiation therapy (SRS or WBRT), in patient cohorts not specifically selected for target expression (197–199), has failed to demonstrate a superiority over radiotherapy alone. The use of up-front icotinib was recently evaluated in untreated patients in comparison with first-line chemotherapy (cisplatin plus pemetrexed) (200) or WBRT followed by chemotherapy (201). PFS was significantly longer for icotinib in both trials: PFS of 11.2 months in the icotinib group vs 7.9 in the chemotherapy group, an intracranial PFS of 10 months with icotinib vs 4.8 months with WBRT and CT.

Osimertinib, a third-generation EGFR-TKI, that targets activating mutations (EGFRm) and resistance mutations (T790M), has demonstrated robust systemic activity and a better CNS penetration with sustained tumor regression of BM. In the phase I BLOOM study, two third-generation EGFR-TKIs, osimertinib and AZD3759, were studied in patients with EGFR

TABLE 4 | Targeted agents in brain metastases (BMs).

Main studies on targeted therapies in BMs				Results
Reference	**Agent tested**	**Number of Pts**	**Clinical trial characteristics**	
NSCLC				
Sperduto et al. (199)	Erlotinib	126	• Phase III multicenter trial (RTOG 0320) • Three arms: arm 1: WBRT + SRS; arm 2: WBRT + SRS + TMZ; arm 3: WBRT + SRS + erlotinib • EGFR mutation not tested	• MST: arm 1 (44 Pts): 13.4 months; arm 2 (40 patients): 6.3 months; arm 3 (41 Pts): 6.1 months • CNS mPFS: arm 1: 8.1 months; arm 2: 4.6 months; arm 3: 4.8 months
Welsh et al. (197)	Erlotinib	40	Phase II study of erlotinib plus WBRT • Erlotinib started 1 week before WBRT	• ORR 86% (n = 36) • MST 11.8 months • 9 of 17 patients tested for EGFR mutation were positive • MST in EGFR mutant: 19.1 months • MST in EGFR wild-type 9.3 months
Rosell et al. (288)	Erlotinib + bevacizumab	109	Phase II trial, single-arm, multicentre	• Overall median PFS: 13.2 months in T790M-positive group • Median PFS: 16.0 months in the T790M-positive group while it was 10.5 months in T790M-negative
Ceresoli et al. (289)	Gefitinib	41	• Phase II single-arm study • No concurrent local therapy for BM • Both squamous and adenocarcinoma included • EGFR mutation not tested	• ODC 27% • mPFS 3 months • Disease control better in patients who received prior WBRT and who had adenocarcinoma histology
Costa et al. (205)	Crizotinib		Retrospective analysis of trials	Poor activity against CNS metastasis in NSCLC as evidenced by low concentrations detected in CNS samples
Gadgeel et al. (290)	Alectinib	50	Pooled analysis of two phase II studies • 136 patients had BM, 50 had measurable disease • ALK-positive NSCLC, previously treated with crizotinib	• CNS ORR 64% • CNS DOR 10.8 months
Shaw et al. (291)	Ceritinib	122	Phase I study in advanced ALK-positive NSCLC	• The ORR was 58% • Among the 80 patients who failed crizotinib, the response rate was 56% • NSCLC who received ceritinib with doses 400 mg or higher, the mPFS was 7.0 months
Crinò et al. (292)	Ceritinib	140 (100 with BM)	Phase I	• Intracranial overall response rate: 45.0% (95% CI, 23.1–68.5%) • Ceritinib treatment provided clinically meaningful and durable responses with manageable tolerability in chemotherapy- and crizotinib-pretreated patients, including those with brain metastases
Gettinger et al. (293)	Brigatinib (dual inhibitor of ALK and EGFR)	137	Phase I/II single-arm, open-label, multicenter study in patient Pts with advanced NSCLC	• 50 Pts with BM • Eight (53%) of 15 assessable patients with measurable BM had an intracranial response • 11 (35%) of 31 assessable patients with only non-measurable BM had complete disappearance of lesions on imaging • Median intracranial PFS for these Pts is 97 weeks
Yang et al. (294)	Osimertinib		Phase II AURA trial	• Activity in patients with CNS metastases: 16 (64%) of 25 evaluable Pts had an objective response, including 4 complete responses • Median PFS in Pts with CNS metastases was encouraging, although it was shorter than in those without (7.1 vs 13.7 months, respectively) • Benefit was observed across all predefined subgroups, including patients with asymptomatic CNS metastases at baseline (PFS: 8.5 vs 4.2 months)

(Continued)

TABLE 4 | **Continued**

Main studies on targeted therapies in BMs				Results
Reference	**Agent tested**	**Number of Pts**	**Clinical trial characteristics**	
Planchard et al. (295)	Dabrafenib and trametinib	57 total (only one patient with asymptomatic BM)	Phase II, multicentre, non-randomized, open-label study	Dabrafenib plus trametinib is a promising new therapy for patients with BRAFV600E-mutant NSCLC, with high overall response, a prolonged duration of response, and manageable toxicity. Few data on efficacy on BM
Breast cancer (HER2-positive)				
Bachelot et al. (231)	Lapatinib + capecitabine	45	• Single-arm phase II study • No prior WBRT, or lapatinib or capecitabine	CNS ORR 65.9% • CNS mPFS 5.5 months • mOS 17 months
Lin et al. (230)	Lapatinib + capecitabine	50	Phase II trial	CNS response of 20%PFS not reported
Cortes et al. (296)	Afatinib	121	Phase II, multicenter, randomized trial, open-label, three-arm study • Arm A: afatinib, arm B: afatinib +vinorelbine, arm C: investigator choice	• Arm A: PB = 30% • Arm B: PB = 34.2% • Arm C: PB = 41.9%
Freedman et al. (232)	Neratinib	40	• Multicenter, open-label phase II study • Patients who had progressed after one or • More lines of CNS directed therapy	• CNS ORR: 8% • mPFS 1.9 months • mOS 8.7 months
Melanoma				
Long et al. (243)	Dabrafenib	172	• Multicenter, open-label phase II study • V600E or V600K BRAF-mutant patients • Two cohorts: cohort A: BM treatment naive, cohort B: previously treated BM	• Cohort A, V600E (74 patients) – CNS ORR 39.2% – CNS PFS 16.1 weeks – OS 33.1 weeks • Cohort A, V600K (15 patients) – CNS ORR 6.7% – CNS PFS 8.1 weeks – OS 31.4 weeks • Cohort B, V600E (65 patients) – CNS ORR 30.8% – CNS PFS 16.6 weeks – OS 16.3 weeks • Cohort B, V600K (18 patients) – CNS ORR 22.2% – CNS PFS 15.9 weeks – OS 21.9 weeks
McArthur et al. (245)	Vemurafenib	146	• Open-label, phase II study • Two cohorts: – Cohort-1: previously untreated (90 Pts) – Cohort-2: previously treated (56 Pts)	• Cohort-1: – CNS ORR 18% – CNS PFS 3.7 months – OS 8.9 months • Cohort-2: – CNS ORR 23% – CNS PFS 4.0 months – OS 9.6 months

WBRT, whole-brain radiation therapy; SRS, stereotactic radiation; TMZ, temozolomide; Pts, patients; mPFS, median progression-free survival; EGFR, epidermal growth factor receptor; MST, median survival times; CNS, central nervous system; ODC, overall disease control; ORR, overall response rate; DOR, duration of response; mOS, median overall survival; PB, patient benefit (defined as intracranial or extracranial progression-free survival, no new neurologic signs or symptoms related to tumor, increase corticosteroid use) at 12 weeks; BM brain metastases; CT, chemotherapy.

mutation-positive advanced NSCLC with BM and LM, showing improved BBB penetration and preliminary interesting results (202, 203).

Other "druggable" alterations seen in up to 5% of NSCLC patients are the rearrangements of the ALK gene. In particular, ALK translocations have been found in 3% of BMs from NSCLC and seem to be maintained in brain metastasis (204). NSCLC with ALK activating translocations are sensitive to the ALK inhibitor crizotinib. A recent study on BMs from ALK-rearranged NSCLC (205) has reported that crizotinib was associated with more than

TABLE 5 | Immunotherapy (IT) in BMs.

Main studies on IT (checkpoint inhibitors) in BMs					Results
Reference	**Agent tested**	**Primary tumor**	**Number of pts**	**Clinical trial characteristics**	
Margolin et al. (244)	Ipilimumab	Melanoma BM	72	Single-agent, open-label phase II study	• Cohort A: 51 asymptomatic Pts with active BM who were not on corticosteroids; cohort B: 21 Pts with symptomatic melanoma-derived BM taking a corticosteroid • Immune-related response criteria: the RR of 25% (13 Pts) and 10% (2 Pts) in cohorts A and B, respectively • mOS: 7.0 months in cohort A and 3.7 months in cohort B
Di Giacomo et al. (252)	Ipilimumab and fotemustine	Melanoma BM	20/83 with asymptomatic BM	Open-label, single-arm, phase II trial	• The mPFS in Pts with BM was 3.0 months • 3-year follow-up of group with BM: mOS of 12.7 months
Patel et al. (297)	SRS vs SRS + ipilimumab	Melanoma BM	SRS: 34 SRS + ipilimumab: 20	Restrospective comparative analysis	• SRS: 1 year LC 91%; 12 months OS: 38% • SRS + ipilimumab: 1 year LC 71%; 12 months OS 37%
Weber et al. (298)	Ipilimumab/ budesonide (asymptomatic BM)	Melanoma BM	12	Retrospective analysis of phase II trial	• Intracranial RR: 41.6% • mOS: 14
Knisely et al. (261)	SRS vs SRS + ipilimumab	Melanoma BM	SRS: 17 SRS + ipilimumab: 11	Retrospective comparative analysis	• SRS: OS 4 months • SRS + ipilimumab: OS 21.3 months
Silk et al. (299)	SRS vs SRS + ipilimumab	Melanoma BM	SRS: 37 SRS + ipilimumab: 33	Retrospective comparative analysis	• SRS: PFS 3.3 months; OS 5.3 months • SRS + ipilimumab: PFS 2.7 months; OS 8.3 months
Mathew et al. (300)	SRS vs SRS + ipilimumab	Melanoma BM	SRS: 33 SRS + ipilimumab: 25	Retrospective comparative analysis	• SRS: 6-month OS 56% • SRS + ipilimumab: 6-month OS 45%
Ahmed et al. (265)	SRS/nivolumab	Melanoma BM	26	Retrospective analysis	• OS 11.8 months
Goldberg et al. (221)	Pembrolizumab	NSCLC, melanoma BM	36 (18 NSCLC, 18 melanoma)	Phase II trial	• Brain metastasis response was achieved in 4 (22%) of 18 Pts with melanoma and 6 (33%) of 34 Pts with NSCLC. Responses were durable
Schachter et al. (301)	Pembrolizumab	Melanoma BM	834		• Median follow-up was 22.9 months • Median overall survival was not reached in either pembrolizumab group and was 16 months with ipilimumab • Pembrolizumab continued to provide superior overall survival vs ipilimumab
Parakh et al. (302)	Nivolumab and pembrolizumab	Melanoma BM	66	Retrospective analysis	• The IC overall RR was 21% and disease control rate 56%. Responses occurred in 21% of Pts with symptomatic BM. The median OS was 9.9 months (95% CI 6.93–17.74). Pts with symptomatic BM had shorter PFS than those without symptoms (2.7 vs 7.4 months, $p = 0.035$) and numerically shorter OS (5.7 vs 13.0 months, $p = 0.068$). Pts requiring corticosteroids also had a numerically shorter PFS (3.2 vs 7.4 months, $p = 0.081$) and OS (4.8 vs 13.1 months, $p = 0.039$)

Pts, patients; BMs, brain metastases; NSCLC, non-small-cell lung cancer; RR, response rate; mOS, median overall survival; mPFS, median progression-free survival; 1 year LC, local control; IC RR, intracranial response rate.

55% disease control within the CNS at 3 months of therapy in both RT-naïve and RT-pretreated patients; moreover, crizotinib was associated with a moderate (18–33%) RECIST-confirmed response rate. However, the CNS is a common site of progression in NSCLC receiving crizotinib (206). Crizotinib, in addition of blocking ALK and ROS1, is also a potent c-MET inhibitor: clinical

trials using crizotinib are ongoing in patients with NSCLC and c-MET mutations (207).

The US Food and Drug Administration approval of ceritinib and alectinib (second-generation of ALK inhibitors) for patients failing crizotinib means that ALK TKIs with improved CNS penetration are now available. The recently reported ASCEND-4 study (208) compared ceritinib with chemotherapy for treatment-naive patients with advanced ALK-positive NSCLC; this trial allowed both untreated as well as symptomatic BM to be accrued. Ceritinib achieved superior PFS and RR, with a similar trend seen in the subset of patients with BM. In the 22 patients with measurable CNS disease, ceritinib achieved a 73% CNS RR, with 86% of patients without CNS progression at 24 weeks. The J-ALEX study compared alectinib with crizotinib in patients with TKI-naive advanced ALK-positive NSCLC and demonstrated an overall PFS improvement: while only 21% of the patients had BM, the PFS benefit in these patients was dramatic (hazard ratio, 0.08) (209).

Third-generation ALK inhibitors, such as brigatinib and lorlatinib (a selective brain-penetrant ALK/ROS1TKI active against most known resistance mutations) have shown efficacy on BM in initial studies (210–212).

Another compound investigated in association with WBRT in BMs from breast and NSCLC is veliparib, a PARP 1–2 inhibitor. Despite preliminary encouraging safety and efficacy results (213); a phase II, randomized study evaluating the efficacy and safety of veliparib in combination with WBRT vs WBRT plus placebo in patients with BMs from NSCLC did not show significant differences in OS, intracranial response rate, and time to progression (214).

c-MET is an oncogenic driver and is implicated in the resistance to targeted therapies, including EGFR and VEGFR inhibitors. Increased c-MET expression may predict response to c-MET-targeted drugs (215, 216). Since c-MET amplification can contribute to acquired resistance to EGFR-TKI therapy, combined inhibition of EGFR and c-MET is being investigated (217).

Nivolumab, a checkpoint inhibitor, is a human IgG4 anti-PD-1 monoclonal antibody active in the second-line treatment of metastatic NSCLC after progression on a platinum-based chemotherapy (218). Intracranial activity and responses to nivolumab in a small cohort of five patients with NSCLC and untreated/progressive BMs suggested a role for this molecule in brain disease control. Importantly, no grade 3/4 adverse events were seen. Systemic responses and intracranial responses were largely concordant. However, no firm conclusions can be drawn due to the small sample size of the cohort (219).

An analysis presented at American Society of Clinical Oncology (ASCO) 2016 pooled data of patients with advanced NSCLC and pretreated asymptomatic CNS metastases from CheckMate 063 (phase II), 017 (phase III), and 057 (phase III). The best response in the nivolumab arm with CNS metastases arm was CR/PR in 28%, SD in 33%, and PD in 39% compared with CR/PR in 19%, SD in 31%, and PD in 43% in the docetaxel arm. Among patients with pretreated CNS metastases (74% had prior radiotherapy and 85% had ≥2 extra-CNS sites of metastases), median OS was a little longer in the nivolumab group (8.4 months) compared with the docetaxel group (6.2 months). Nivolumab was well tolerated, with low-grade toxicities. One-third of patients had no evidence of CNS progression at time of PD/last assessment. Additional results (including OS and CNS progression rates in patients with/without pretreated CNS metastases and safety/efficacy of nivolumab in patients with untreated CNS metastases from CheckMate 012) will be presented shortly (220). Overall, these results support further investigation of nivolumab monotherapy in patients with NSCLC and asymptomatic CNS metastases.

Pembrolizumab, a fully human anti-PD-1 monoclonal antibody, is approved in first- and second-line treatment of metastatic NSCLC. Early data in NSCLC demonstrated that there is a response in BMs similar to that of the systemic disease (221). The median OS was 7.7 months to date. The available data for the use of anti-PD-1 agents in the treatment of BM do not yet include information on PD-L1 status.

BMs From SCLC

Various combinations of etoposide, teniposide, cisplatinum, or carboplatinum are active against BMs (173). So far, no targeted agents are available.

BMs From Breast Cancer

Chemotherapy regimens that combine cyclophosphamide, 5-FU, methotrexate, vincristine, cisplatin, and etoposide are active in patients with BMs from breast cancer (173). Capecitabine, belonging to the class of fluoropyrimidines, is an active drug (222). Likewise, high-dose methotrexate is effective in recurrent BMs (223), but a risk of leukoencephalopathy exists when administered after WBRT.

In HER2-positive, breast cancer targeted therapies have been widely used. Trastuzumab, which crosses a disrupted BBB within established BMs, could be active (224). Several case reports and small patients' series indicate an activity of the antibody-drug conjugate T-DM1 (trastuzumab-emtansine) (225, 226). Few data are available on the combination of different anti-HER2 agents. The use of pertuzumab, a monoclonal antibody, that binds HER2 on another epitope than trastuzumab, in combination with trastuzumab and docetaxel, leads to a substantial improvement in progression-free and overall survival and may delay CNS disease onset (227, 228).

The dual EGFR and HER2 tyrosine kinase inhibitor lapatinib has shown moderate antitumor activity in HER2-positive BM (229). In a phase II study in HER 2+ breast cancer patients with BMs, following trastuzumab-based systemic chemotherapy and WBRT (230), CNS objective responses to lapatinib were observed in 6% of patients, and 21% experienced ≥20% volumetric reduction. A recent phase II single-arm study (LANDSCAPE) has shown that the association of lapatinib and capecitabine in patients with previously untreated BMs from HER-positive metastatic breast cancer yields durable responses in up to 65% of patients (231). Based on these results a randomized trial comparing lapatinib and capecitabine vs WBRT has been launched.

A single-arm phase II trial on neratinib, an irreversible TKI of HER 2, has shown a response rate of 8% with an OS of 8.7 months in patients with BMs, pretreated either with WBRT or SRS (232). Despite the advances in treating HER2-positive breast cancer, many questions remain unanswered, such as how to impact prior resistance or affect a sanctuary site, and the optimal use of

these novel compounds with regard to disease setting, treatment sequence, and combination regimens (233).

There are no available data on the efficacy on BM of endocrine therapies. Another druggable pathway in breast BM is the phosphatidylinositol 3 kinase/Akt/mammalian target of rapamycin (mTOR) pathway, which is dysregulated in a significant number of HR-positive breast cancers (234).

Everolimus, an mTOR inhibitor, approved for the management of HR-positive, postmenopausal breast cancer patients, in combination with an aromatase inhibitor (235) has also shown CNS penetration with activity against subependymal giant-cell astrocytomas associated with tuberous sclerosis (236), and ongoing studies are testing the activity of everolimus in BM. Other small-molecule inhibitors, like abemaciclib (CDK 4/6 inhibitor), are being evaluated in breast cancer BM (237). PARP inhibitors are being investigated in BMs from triple negative breast cancer.

BMs From Melanoma

Approximately half of advanced melanoma patients will develop BM (MBMs). The median OS for patients with MBMs is 4–5 months (87, 238).

Fotemustine (response rate of 5–25%) and temozolomide (response rate 6–10%), either as single agents or in combination with WBRT, are the most active chemotherapeutics against BMs from melanoma (173, 239). Novel targeted and immunotherapeutic agents have revolutionized the systemic management of melanoma. A number of prospective clinical trials have demonstrated that these agents, either alone or in combination, can prolong PFS and OS (240–242). Several studies have assessed the impact of these agents in patients with MBMs in a prospective setting (243–245).

The immune checkpoint inhibitors ipilimumab [targeting the cytotoxic T-lymphocyte antigen 4 (CTLA-4)] and nivolumab have led to astonishing results and unexpected long-term survival gains in advanced/unresectable melanoma (246, 247). It is of relevance that both compounds interfere with T-cell signals (248).

Ipilimumab showed activity in melanoma BMs, particularly if asymptomatic, and improved OS (244, 249, 250). An open-label phase II multicenter US trial (244) has shown that ipilimumab has activity in those patients with melanoma BMs, who do not need corticosteroids: disease control (CR + PR + SD) after 12 weeks of treatment was 16% in the cohort of asymptomatic patients without corticosteroids compared with 5% in the cohort of symptomatic patients receiving corticosteroids. Importantly, the investigators did not report any neurological adverse event as an effect of an inflammatory response to treatment in the CNS, even in patients who received prior radiation therapy. The possibility remains that steroid treatment at initiation of ipilimumab could abrogate or downmodulate the immune response. One of the larger studies to investigate ipilimumab evaluated 127 patients and demonstrated an OS benefit (93 vs 42 weeks, $p < 0.0028$) for patients who received concomitant IT and RT (251).

A single-arm phase II trial of ipilimumab in combination with fotemustine in patients with melanoma and asymptomatic BMs showed intracranial disease control in 50% of patients and a median OS of 13.4 months (252). A phase III trial of this combination is currently ongoing. A triple-arm phase III clinical trial will compare the OS at 2 years of fotemustine monotherapy, ipilimumab and fotemustine, and ipilimumab and nivolumab in patients with metastatic melanoma with BMs. This study (NCT02460068) is currently recruiting participants and is not expected to reach completion until 2020.

The anti-PD-1 antibodies nivolumab and pembrolizumab have demonstrated highly durable response rates (41 and 38%, respectively) in large phase I trials (253, 254), that were confirmed in subsequent phase III trials (255) and in the second-line setting after failure of anti-CTLA-4 therapy (240, 256). These agents in combination with ipilimumab are currently investigated in several ongoing phase II trials in advanced melanoma patients with BM (257, 258).

Barker and colleagues reviewed the clinical outcomes of the combination of ipilimumab and RT in melanoma, including BM (259). Radiographically, it was noted that an increase of brain metastasis size >150% occurred in 40% of the tumors treated with SRS before or during ipilimumab, while this occurred in 10% of metastases treated with SRS after ipilimumab. Hemorrhage was also noted after SRS during ipilimumab in 42% of treated BMs. Preliminary results, that need further study, suggest an interaction between IT and RT. Also the reported "abscopal effect" in melanoma patients, in whom radiotherapy for one lesion induced a shrinkage of non-irradiated lesions, probably depending on the activation of an antitumor immune response, supports the potential of combining radiotherapy and IT in the treatment of melanoma (260).

Other several small, retrospective series have evaluated patients with melanoma BMs treated with IT and SRS reporting successful outcomes in terms of OS (261, 262). Qian et al. investigated melanoma BM patients treated concurrently (within 4 weeks of IT) with immune checkpoint inhibitors and SRS (defining "concurrent" when SRS was administered): the median percentage of the reduction in lesion volume was significantly greater for the concurrent group without hemorrhagic complications (263). In contrast to this report, the preliminary data reported by Shen et al. showed an increase in lesion size in 13 of 26 lesions treated concurrently (defined as IT starting "prior to or with SRS") (264). A recent phase I prospective study explored the maximum tolerable dose and safety of ipilimumab with SRS or WBRT in patients with BMs from melanoma, demonstrating the safety of combining SRS with either ipilimumab 3 or 10 mg/kg.

The higher rate of increasing lesions as well as radionecrosis among patients receiving SRS or WBRT in combination with immune checkpoints inhibitors is a matter of debate, but many authors believe that these findings could be an expression of a greater local immune reactions. Ahmed et al. retrospectively analyzed data from two prospective nivolumab trials in patients with advanced disease treated at a single institution, selecting patients with BMs who were treated with SRS within 6 months of receiving nivolumab. Local brain control rate was 91 and 85%, respectively, at 6 and 12 months, with a median survival time of 12 months and a distant BM control rate of 53%. These preliminary results suggest a better intracranial control with nivolumab compared with ipilimumab, probably related to a higher clinical activity and a lower toxicity profile; however, these initial findings should be confirmed in prospective trials (265). Combinations of

ipilimumab and new molecular agents, such as trametinib (MEK inhibitor), are under investigation (266).

Dabrafenib and vemurafenib are potent kinase inhibitors of BRAF V600E-mutated melanoma cells, with substantial activity in BRAF-mutated melanoma BM (267). The activity of dabrafenib in BMs was demonstrated in a large multicenter, open-label, phase II trial which enrolled V600E-V600K-mutated-BRAF metastatic melanoma patients and at least one measurable brain lesion. An overall intracranial objective response rate of 31%, with little difference between patients who progressed after prior CNS therapy and patients who were treatment naïve (243). Dabrafenib was well tolerated, and the number of spontaneous intracranial hemorrhages was lower than those reported after ipilimumab.

The activity of vemurafenib is also meaningful, as both retrospective (268) and phase II trials (269) have reported an intracranial response rate of 16–50%, even if the improvement of OS was disappointing. Acquired resistance to single-agent BRAF inhibitors develops within 6–7 months of therapy (270) and is mainly driven by MAPK reactivation. The combination of a BRAF inhibitor and a MEK inhibitor, in comparison with BRAF inhibitor alone, significantly improved response rates, median PFS/OS in phases I/II (dabrafenib/trametinib vs dabrafenib) (241, 271) and in phase III studies (vemurafenib/cobimetinib vs vemurafenib) (272).

Preliminary clinical reports evaluated the efficacy and safety of RT plus vemurafenib and dabrafenib in patients with BRAF V600E-mutated melanoma BMs (273, 274). These studies indicated a potential synergistic effect, resulting in 75% response rate, 65% of symptomatic relief, and median survival of 13.7 months. Ahmed et al. also showed good local control rates after vemurafenib and SRS with low toxicity (273).

Activating mutations in cKIT have been identified in up to 6% of some cutaneous melanomas subtypes. BRAF wild-type patients, which harbor cKIT mutations, could benefit from TKI inhibitors such as imatinib, an oral cKIT inhibitor that has demonstrated dramatic clinical responses (275, 276).

Dasatinib, a second-generation drug developed for CML (chronic myeloid leukemia) that also acts on cKIT, has better CNS penetration and perhaps more toxicity than imatinib.

Supportive Care

Corticosteroids

Corticosteroids are used to control cerebral edema and mass effect. Two evidence-based guidelines on the role of steroids have been published in Europe (277) and US (278). Dexamethasone is recommended for patients who are symptomatic, with a starting dose of 4–8 mg/day up to higher doses of 16–32 mg/day in patients with severe symptoms. Dexamethasone is the steroid of choice because of its minimal mineral corticoid effect and long half-life, although any other corticosteroid can be effective if given in equipotent doses. A neurological improvement within 24–72 h after beginning of treatment is seen in up to 75% of patients. When used as the sole form of treatment, dexamethasone produces about 1 month's remission of symptoms and slightly increases the 4- to 6-week median survival of patients who receive no treatment at all (278). To minimize side effects from chronic dexamethasone administration, including proximal myopathy, tapering of steroid dosing within 1 week of starting therapy and discontinuation within 2 weeks is encouraged. By contrast, asymptomatic patients do not need corticosteroids, even during radiotherapy.

The need for anticonvulsant medication is clear in patients who have experienced a seizure by the time their brain tumor is diagnosed. The evidence does not support prophylaxis with antiepileptic drugs (AEDs) in patients with brain tumors, including metastases. Twelve studies, either randomized trials or cohort studies, investigating the ability of prophylactic AEDs (phenytoin, phenobarbital, and valproic acid) to prevent first seizures, have been examined, and none have demonstrated efficacy (279). Subtherapeutic levels of anticonvulsants were extremely common and the severity of side effects appeared to be higher (20–40%) in brain tumor patients than in the general population receiving anticonvulsants, probably because of drug interactions. Phenytoin, carbamazepine, phenobarbital and to a lesser extent oxcarbazepine stimulate the cytochrome P450 system and accelerate the metabolism of corticosteroids and chemotherapeutic or targeted agents, such as nitrosoureas, paclitaxel, cyclophosphamide, topotecan, irinotecan, thiotepa, adriamycin, methotrexate, imatinib, erlotinib, and other TKIs, and thus reduce their efficacy. The role of prophylactic anticonvulsants remains to be addressed in some subgroups of patients, who have a higher risk of developing seizures, such as those with metastatic melanoma, hemorrhagic lesions, and multiple metastases (277, 280). A recent meta-analysis in patients with BM concluded that primary prevention with AEDs might not reduce the risk of seizures, and it is associated with frequent adverse effects (281). For patients who underwent a neurosurgical procedure the efficacy of prophylaxis has not been proven. The efficacy of newer AEDs (levetiracetam, topiramate, gabapentin, lamotrigine, lacosamide, and perampanel) has not been extensively investigated but in some retrospective studies their use in patients with seizures related to BMs, significantly reduce seizure frequency (independently of systemic treatment), produce few side effects and appear not to affect life expectancy (282).

Anticoagulation

Anticoagulation is the standard treatment for acute venous thromboembolism (VTE) in cancer patients. The ASCO published updated evidence-based guidelines for the treatment and prevention of VTE in patients with cancer based on a systematic review of the literature. These guidelines address the treatment and prevention of VTE in hospitalized medical and surgical cancer patients and in ambulatory patients receiving cancer therapy. They also concern immediate and extended secondary prophylaxis in patients with established VTE, the potential role of anticoagulation in the treatment of patients with cancer without other recognized indication, and the importance of VTE risk assessment in cancer patients [(283), https://www.asco.org/sites/new-www.asco.org/files/content-files/practice-and-guidelines/documents/VTE-Summary-of-Recs.pdf].

LMWH is preferred over UFH for the initial 5–10 days of anticoagulation for the cancer patient with a newly diagnosed VTE who does not have severe renal impairment (defined as creatinine clearance <30 mL/min). For long-term anticoagulation, LMWH for at least 6 months is preferred due to improved efficacy over

vitamin K antagonists. Prolongation of anticoagulation over 6 months has to be considered for select patients with active cancer, such as metastatic disease or those receiving chemotherapy. The insertion of an inferior vena cava filter is only indicated for patients with contraindications to anticoagulant therapy or as an adjunct to anticoagulation in patients with progression of thrombosis (recurrent VTE or extension of existing thrombus) despite optimal therapy with LMWH (284).

In patients with CNS malignancies and VTE anticoagulation is recommended as described for other patients with cancer. Careful monitoring is necessary to evaluate the risk of intracerebral hemorrhage on one side and, on the other, the effect of anticoagulation on survival. These patients merit individualized discussions of the risk and benefit of anticoagulation therapy (284, 285).

Use of novel oral anticoagulants for either prevention or treatment of VTE in cancer patients is not recommended at this time. An open-label, non-inferiority trial, randomly assigned patients with cancer and acute symptomatic or incidental VTE to receive either low-molecular-weight heparin followed by oral edoxaban at a dose of 60 mg once daily (edoxaban group) or subcutaneous dalteparin at a dose of 200 IU/kg of body weight once daily for 1 month followed by dalteparin at a dose of 150 IU/kg once daily (dalteparin group) for 6–12 months. This study concluded that oral edoxaban was non-inferior to subcutaneous dalteparin with respect to the composite outcome of recurrent VTE or major bleeding. The rate of recurrent VTE was lower but the rate of major bleeding was higher with edoxaban than with dalteparin (286).

Although several new studies are ongoing, some important questions remain regarding the relationship between thrombosis and cancer and the optimal care of patients at risk for VTE, in particular with CNS malignancies.

EXECUTIVE SUMMARY

- Advances in chemotherapy and targeted therapies have improved survival in cancer patients with an increase of the incidence of newly diagnosed BMs representing a stimulating challenge for development of new therapies.
- Advanced neuroimaging techniques have been increasingly used in the detection, treatment planning, and follow-up of BM.
- Better understanding of mechanisms of brain metastatization and molecular characterization of BM could help finding more selected and effective targeted therapies.
- More sophisticated methods of tumor analysis, including detection of circulating biomarkers in fluids (liquid biopsy), have shown promising information regarding tumor treatment response and progression.
- The therapeutic choice is guided by prognostic index, reserving surgical approaches to the subgroup of patients with controlled systemic disease and good performance status. Radiosurgery of the resection cavity may offer comparable survival and local control as postoperative WBRT. WBRT alone is now the treatment of choice for patients with single or multiple BMs not amenable to surgery or radiosurgery, or with poor prognostic factors, also considering the neurocognitive sequelae of WBRT. Technological advances, such as IMRT with hippocampal sparing and pharmacological approaches (memantine and donepezil), could reduce the risk of cognitive sequelae.
- Leptomeningeal disease (LMD) can be a complication, especially in posterior fossa metastases undergoing a "piecemeal" resection.
- In the last decades, a multitude of molecular abnormalities have been discovered representing potential druggable alterations, such as mutations of EGFR, ALK in NSCLC and HER2-mutations in breast cancer. Novel targeted and immunotherapeutic agents have also revolutionized the systemic management of melanoma.

AUTHOR CONTRIBUTIONS

FF contributed to the revision of the literature and to the writing of the manuscript with input from all authors revised. RR and RS supervised development of work and helped in data interpretation and manuscript final revision.

REFERENCES

1. Davis FG, Dolecek TA, McCarthy BJ, Villano JL. Toward determining the lifetime occurrence of metastatic brain tumors estimated from 2007 United States cancer incidence data. *Neuro Oncol* (2012) 14(9):1171–7. doi:10.1093/neuonc/nos152
2. Tabouret E, Bauchet L, Carpentier AF. Brain metastases epidemiology and biology. *Bull Cancer* (2013) 100(1):57–62. doi:10.1684/bdc.2012.1681
3. Taillibert S, Le Rhun É. Epidemiology of brain metastases. *Cancer Radiother* (2015) 19(1):3–9. doi:10.1016/j.canrad.2014.11.001
4. Tonyali O, Coskun U, Yuksel S, Inanc M, Bal O, Akman T. Risk factors for brain metastasis as a first site of disease recurrence in patients with HER2 positive early stage breast cancer treated with adjuvant trastuzumab. *Breast* (2016) 25:22–6. doi:10.1016/j.breast.2015.11.006
5. Brastianos PK, Carter SL, Santagata S, Cahill DP, Taylor-Weiner A, Jones RT, et al. Genomic characterization of brain metastases reveals branched evolution and potential therapeutic targets. *Cancer Discov* (2015) 5(11):1164–77. doi:10.1158/2159-8290.CD-15-0369
6. Saunus JM, Quinn MC, Patch AM, Pearson JV, Bailey PJ, Nones K, et al. Integrated genomic and transcriptomic analysis of human brain metastases identifies alterations of potential clinical significance. *J Pathol* (2015) 237(3):363–78. doi:10.1002/path.4583
7. Paik PK, Shen R, Won H, Rekhtman N, Wang L, Sima CS, et al. Next-generation sequencing of stage IV squamous cell lung cancers reveals an association of PI3K aberrations and evidence of clonal heterogeneity in patients with brain metastases. *Cancer Discov* (2015) 5(6):610–21. doi:10.1158/2159-8290.CD-14-1129
8. Svokos SA, Salhia B, Toms SA. Molecular biology of brain metastasis. *Int J Mol Sci* (2014) 15:9519–30. doi:10.3390/ijms15069519
9. Winkler F. The brain metastatic niche. *J Mol Med* (2015) 93(11):1213–20. doi:10.1007/s00109-015-1357-0
10. Luzzi KJ, MacDonald IC, Schmidt EE, Kerkvliet N, Morris VL, Chambers AF, et al. Multistep nature of metastatic inefficiency: dormancy of solitary cells after successful extravasation and limited survival of early micrometastases. *Am J Pathol* (1998) 153:865–73. doi:10.1016/S0002-9440(10)65628-3
11. Yilmaz M, Christofori G. EMT, the cytoskeleton, and cancer cell invasion. *Cancer Metastasis Rev* (2009) 28:15–33. doi:10.1007/s10555-008-9169-0
12. Thiery JP, Acloque H, Huang RY, Nieto MA. Epithelial-mesenchymal transitions in development and disease. *Cell* (2009) 139:871–90. doi:10.1016/j.cell.2009.11.007

13. Pietras K, Ostman A. Hallmarks of cancer: interactions with the tumor stroma. *Exp Cell Res* (2010) 316:1324–31. doi:10.1016/j.yexcr.2010.02.045
14. Qian BZ, Pollard JW. Macrophage diversity enhances tumor progression and metastasis. *Cell* (2010) 141:39–51. doi:10.1016/j.cell.2010.03.014
15. Kumar S, Weaver VM. Mechanics, malignancy, and metastasis: the force journey of a tumor cell. *Cancer Metastasis Rev* (2009) 28:113–27. doi:10.1007/s10555-008-9173-4
16. Beasley KD, Toms SA. The molecular pathobiology of metastasis to the brain: a review. *Neurosurg Clin N Am* (2011) 22(1):7–14, v. doi:10.1016/j.nec.2010.08.009
17. Custódio-Santos T, Videira M, Brito MA. Brain metastasization of breast cancer. *Biochim Biophys Acta* (2017) 1868(1):132–47. doi:10.1016/j.bbcan.2017.03.004
18. Seike T, Fujita K, Yamakawa Y, Kido MA, Takiguchi S, Teramoto N, et al. Interaction between lung cancer cells and astrocytes via specific inflammatory cytokines in the microenvironment of brain metastasis. *Clin Exp Metastasis* (2011) 28(1):13–25. doi:10.1007/s10585-010-9354-8
19. Reid H, Rahul J. The role of the neural niche in brain metastasis. *Clin Exp Metastasis* (2017) 34:369–76. doi:10.1007/s10585-017-9857-7
20. Kim SW, Choi HJ, Lee HJ, He J, Wu Q, Langley RR, et al. Role of the endothelin axis in astrocyte- and endothelial cell-mediated chemoprotection of cancer cells. *Neuro Oncol* (2014) 16(12):1585–98. doi:10.1093/neuonc/nou128
21. Liu Y, Komohara Y, Domenick N, Ohno M, Ikeura M, Hamilton RL, et al. Expression of antigen processing and presenting molecules in brain metastasis of breast cancer. *Cancer Immunol Immunother* (2012) 61(6):789–801. doi:10.1007/s00262-011-1137-9
22. Termini J, Neman J, Jandial R. Role of the neural niche in brain metastatic cancer. *Cancer Res* (2014) 74(15):4011–5. doi:10.1158/0008-5472.CAN-14-1226
23. Neman J, Termini J, Wilczynski S, Vaidehi N, Choy C, Kowolik CM, et al. Human breast cancer metastases to the brain display GABAergic properties in the neural niche. *Proc Natl Acad Sci U S A* (2014) 111(3):984–9. doi:10.1073/pnas.1322098111
24. Kralik SF, Kamer AP, Ho CY. Diagnostic imaging of intracranial metastasis. *Curr Probl Cancer* (2015) 39(2):99–112. doi:10.1016/j.currproblcancer.2015.03.003
25. Soffietti R, Abacioglu U, Baumert B, Combs SE, Kinhult S, Kros JM, et al. Diagnosis and treatment of brain metastases from solid tumors: guidelines from the European Association of neuro-Oncology (EANO). *Neuro Oncol* (2017) 19(2):162–74. doi:10.1093/neuonc/now241
26. Sze G, Milano E, Johnson C, Heier L. Detection of brain metastases: comparison of contrast-enhanced MR with unenhanced MR and enhanced CT. *AJNR Am J Neuroradiol* (1990) 11(4):785–91.
27. Schellinger PD, Meinck HM, Thron A. Diagnostic accuracy of MRI compared to CCT in patients with brain metastases. *J Neurooncol* (1999) 44(3):275–81. doi:10.1023/A:1006308808769
28. Server A, Josefsen R, Kulle B, Maehlen J, Schellhorn T, Gadmar Ø, et al. Proton magnetic resonance spectroscopy in the distinction of high-grade cerebral gliomas from single metastatic brain tumors. *Acta Radiol* (2010) 51(3):316–25. doi:10.3109/02841850903482901
29. Sparacia G, Gadde JA, Iaia A, Sparacia B, Midiri M. Usefulness of quantitative peritumoural perfusion and proton spectroscopic magnetic resonance imaging evaluation in differentiating brain gliomas from solitary brain metastases. *Neuroradiol J* (2016) 29(3):160–7. doi:10.1177/1971400916638358
30. Duygulu G, Ovali GY, Calli C, Kitis O, Yünten N, Akalin T, et al. Intracerebral metastasis showing restricted diffusion: correlation with histopathological findings. *Eur J Radiol* (2010) 74(1):117–20. doi:10.1016/j.ejrad.2009.03.004
31. Chiang IC, Kuo YT, Lu CY, Yeung KW, Lin WC, Sheu FO, et al. Distinction between high-grade gliomas and solitary metastases using peritumoral 3-T magnetic resonance spectroscopy, diffusion, and perfusion imagings. *Neuroradiology* (2004) 46(8):619–27. doi:10.1007/s00234-004-1246-7
32. Xu XX, Li B, Yang HF, Du Y, Li Y, Wang WX, et al. Can diffusion-weighted imaging be used to differentiate brain abscess from other ring-enhancing brain lesions? A meta-analysis. *Clin Radiol* (2014) 69:909–15. doi:10.1016/j.crad.2014.04.012
33. Tan Y, Wang X-C, Zhang H, Wang J, Qin JB, Wu XF, et al. Differentiation of high-grade-astrocytomas from solitary-brain-metastases: comparing diffusion kurtosis imaging and diffusion tensor imaging. *Eur J Radiol* (2015) 84:2618–24. doi:10.1016/j.ejrad.2015.10.007
34. Jiang R, Du FZ, He C, Gu M, Ke ZW, Li JH. The value of diffusion tensor imaging in differentiating high-grade gliomas from brain metastases: a systematic review and meta-analysis. *PLoS One* (2014) 9(11):e112550. doi:10.1371/journal.pone.0112550
35. Omuro AM, Leite CC, Mokhtari K, Delattre JY. Pitfalls in the diagnosis of brain tumours. *Lancet Neurol* (2006) 5(11):937–48. doi:10.1016/S1474-4422(06)70597-X
36. Chung JK, Kim YK, Kim SK, Lee YJ, Paek S, Yeo JS, et al. Usefulness of 11C-methionine PET in the evaluation of brain lesions that are hypo- or isometabolic on 18F-FDG PET. *Eur J Nucl Med Mol Imaging* (2002) 29(2):176–82. doi:10.1007/s00259-001-0690-4
37. Rapp M, Heinzel A, Galldiks N, Stoffels G, Felsberg J, Ewelt C, et al. Diagnostic performance of 18F-FET PET in newly diagnosed cerebral lesions suggestive of glioma. *J Nucl Med* (2013) 54(2):229–35. doi:10.2967/jnumed.112.109603
38. Le Chevalier T, Smith FP, Caille P, Constans JP, Rouesse JG. Sites of primary malignancies in patients presenting with cerebral metastases. *Arch Intern Med* (1985) 149(5):880–2.
39. Merchut MP. Brain metastases from undiagnosed systemic neoplasms. *Arch Intern Med* (1989) 149(5):1076–80. doi:10.1001/archinte.1989.00390050066013
40. Van de Pol M, van Aalst VC, Wilmink JT, Twijnstra A. Brain metastases from an unknown primary tumour: which diagnostic procedures are indicated? *J Neurol Neurosurg Psychiatry* (1996) 61(3):321–3. doi:10.1136/jnnp.61.3.321
41. Klee B, Law I, Højgaard L, Kosteljanetz M. Detection of unknown primary tumors in patients with cerebral metastases using whole-body 18F-fluorodeoxyglucose positron emission tomography. *Eur J Neurol* (2002) 9(6):657–62. doi:10.1046/j.1468-1331.2002.00467.x
42. Weller D. Screening, resilience, patient navigation and information needs – key areas in cancer control. *Eur J Cancer Care (Engl)* (2016) 25(1):3–5. doi:10.1111/ecc.12439
43. Rudà R, Borgognone M, Benech F, Vasario E, Soffietti R. Brain metastases from unknown primary tumor: a prospective study. *J Neurol* (2001) 248(5):394–8. doi:10.1007/s004150170180
44. Bekaert L, Emery E, Levallet G, Lechapt-Zalcman E. Histopathologic diagnosis of brain metastases: current trends in management and future considerations. *Brain Tumor Pathol* (2017) 34:8–19. doi:10.1007/s10014-016-0275-3
45. Becher MW, Abel TW, Thompson RC, Weaver KD, Davis LE. Immunohistochemical analysis of metastatic neoplasms of the central nervous system. *J Neuropathol Exp Neurol* (2006) 65(10):935–44. doi:10.1097/01.jnen.0000235124.82805.2b
46. Jin J, Zhou X, Liang X, Huang R, Chu Z, Jiang J, et al. Brain metastases as the first symptom of lung cancer: a clinical study from an Asian medical center. *J Cancer Res Clin Oncol* (2013) 139(3):403–8. doi:10.1007/s00432-012-1344-6
47. Pekmezci M, Perry A. Neuropathology of brain metastases. *Surg Neurol Int* (2013) 4(Suppl 4):245–55. doi:10.4103/2152-7806.111302
48. Kim MY, Go H, Koh J, Lee K, Lee K, Min HS, et al. Napsin A is a useful marker for metastatic adenocarcinomas of pulmonary origin. *Histopathology* (2014) 65(2):195–206. doi:10.1111/his.12383
49. Preusser M, Capper D, Ilhan-Mutlu A, Berghoff AS, Birner P, Bartsch R, et al. Brain metastases: pathobiology and emerging targeted therapies. *Acta Neuropathol* (2012) 123(2):205–22. doi:10.1007/s00401-011-0933-9
50. Kawaguchi KR, Lu FI, Kaplan R, Liu YF, Chadwick P, Chen Z, et al. In search of the ideal immunopanel to distinguish metastatic mammary carcinoma from primary lung carcinoma: a tissue microarray study of 207 cases. *Appl Immunohistochem Mol Morphol* (2014) 22(4):266–74. doi:10.1097/PAI.0b013e318297cc0b
51. Amir E, Clemons M, Purdie CA, Miller N, Quinlan P, Geddie W, et al. Tissue confirmation of disease recurrence in breast cancer patients: pooled analysis of multi-centre, multi-disciplinary prospective studies. *Cancer Treat Rev* (2012) 38(6):708–14. doi:10.1016/j.ctrv.2011.11.006
52. Lee HS, Kim WH, Kwak Y, Koh J, Bae JM, Kim KM, et al. Molecular testing for gastrointestinal cancer. *J Pathol Transl Med* (2017) 51(2):103–21. doi:10.4132/jptm.2017.01.24
53. Dahabreh IJ, Terasawa T, Castaldi PJ, Trikalinos TA. Systematic review: anti-epidermal growth factor receptor treatment effect modification by KRAS mutations in advanced colorectal cancer. *Ann Intern Med* (2011) 154(1):37–49. doi:10.7326/0003-4819-154-1-201101040-00006

54. Sorich MJ, Wise MD, Rowland A, Kichenadasse G, McKinnon RA, Karapetis CS. Extended RAS mutations and anti-EGFR monoclonal antibody survival benefit in metastatic colorectal cancer: a meta-analysis of randomized, controlled trials. *Ann Oncol* (2015) 26(1):13–21. doi:10.1093/annonc/mdu378
55. Bahrami A, Hesari A, Khazaei M, Hassanian SM, Ferns GA, Avan A. The therapeutic potential of targeting the BRAF mutation in patients with colorectal cancer. *J Cell Physiol* (2018) 233(3):2162–9. doi:10.1002/jcp.26041
56. Cohen R, Svrcek M, Dreyer C, Cervera P, Duval A, Pocard M, et al. New therapeutic opportunities based on DNA mismatch repair and BRAF status in metastatic colorectal cancer. *Curr Oncol Rep* (2016) 18(3):18. doi:10.1007/s11912-016-0504-2
57. Pietrantonio F, Petrelli F, Coinu A, Di Bartolomeo M, Borgonovo K, Maggi C, et al. Predictive role of BRAF mutations in patients with advanced colorectal cancer receiving cetuximab and panitumumab: a meta-analysis. *Eur J Cancer* (2015) 51(5):587–94. doi:10.1016/j.ejca.2015.01.054
58. Cohen R, Cervera P, Svrcek M, Pellat A, Dreyer C, de Gramont A, et al. BRAF-mutated colorectal cancer: what is the optimal strategy for treatment? *Curr Treat Options Oncol* (2017) 18(2):9. doi:10.1007/s11864-017-0453-5
59. Tran B, Kopetz S, Tie J, Gibbs P, Jiang ZQ, Lieu CH, et al. Impact of BRAF mutation and microsatellite instability on the pattern of metastatic spread and prognosis in metastatic colorectal cancer. *Cancer* (2011) 117(20):4623–32. doi:10.1002/cncr.26086
60. Travis WD, Brambilla E, Noguchi M, Nicholson AG, Geisinger K, Yatabe Y, et al. International Association for the Study of Lung Cancer/American Thoracic Society/European Respiratory Society: international multidisciplinary classification of lung adenocarcinoma: executive summary. *Proc Am Thorac Soc* (2011) 8(5):381–5. doi:10.1513/pats.201107-042ST
61. Preusser M, Berghoff AS, Berger W, Ilhan-Mutlu A, Dinhof C, Widhalm G, et al. High rate of FGFR1 amplifications in brain metastases of squamous and non-squamous lung cancer. *Lung Cancer Amst Neth* (2014) 83(1):83–9. doi:10.1016/j.lungcan.2013.10.004
62. Payne LS, Huang PH. Discoidin domain receptor 2 signaling networks and therapy in lung cancer. *J Thorac Oncol* (2014) 9(6):900–4. doi:10.1097/JTO.0000000000000164
63. Sekine A, Satoh H. Paradigm shift of therapeutic management of brain metastases in EGFR-mutant non-small cell lung cancer in the era of targeted therapy. *Med Oncol* (2017) 34(7):121. doi:10.1007/s12032-017-0978-2
64. Burel-Vandenbos F, Ambrosetti D, Coutts M, Pedeutour F. EGFR mutation status in brain metastases of non-small cell lung carcinoma. *J Neurooncol* (2013) 111(1):1–10. doi:10.1007/s11060-012-0990-5
65. Porta R, Sanchez-Torres JM, Paz-Ares L, Massuti B, Reguart N, Mayo C, et al. Brain metastases from lung cancer responding to erlotinib: the importance of EGFR mutation. *Eur Respir J* (2011) 37(3):624–31. doi:10.1183/09031936.00195609
66. Shonka N, Venur VA, Ahluwalia MS. Targeted treatment of brainmetastases. *Curr Neurol Neurosci Rep* (2017) 17(4):37. doi:10.1007/s11910-017-0741-2
67. Costa DB, Kobayashi S, Pandya SS, Yeo WL, Shen Z, Tan W, et al. CSF concentration of the anaplastic lymphoma kinase inhibitor crizotinib. *J Clin Oncol* (2011) 29(15):443–5. doi:10.1200/JCO.2010.34.1313
68. Bergethon K, Shaw AT, Ou SHI, Katayama R, Lovly CM, McDonald NT, et al. ROS1 rearrangements define a unique molecular class of lung cancers. *J Clin Oncol* (2012) 30(8):863–70. doi:10.1200/JCO.2011.35.6345
69. Onozato R, Kosaka T, Kuwano H, Sekido Y, Yatabe Y, Mitsudomi T. Activation of MET by gene amplification or by splice mutations deleting the juxta membrane domain in primary resected lung cancers. *J Thorac Oncol* (2009) 4(1):5–11. doi:10.1097/JTO.0b013e3181913e0e
70. Flaherty KT, Hodi FS, Fisher DE. From genes to drugs: targeted strategies for melanoma. *Nat Rev Cancer* (2012) 12(5):349–61. doi:10.1038/nrc3218
71. Berghoff AS, Preusser M. BRAF alterations in brain tumours: molecular pathology and therapeutic opportunities. *Curr Opin Neurol* (2014) 27(6):689–96. doi:10.1097/WCO.0000000000000146
72. Timmer M, Werner JM, Röhn G, Ortmann M, Blau T, Cramer C, et al. Discordance and conversion rates of progesterone-, estrogen-, and HER2/neu-receptor status in primary breast cancer and brain metastasis mainly triggered by hormone therapy. *Anticancer Res* (2017) 37(9):4859–65. doi:10.21873/anticanres.11894
73. Wang J, Bettegowda C. Applications of DNA-based liquid biopsy for central nervous system neoplasm. *J Mol Diagn* (2017) 19(1):24–34. doi:10.1016/j.jmoldx.2016.08.007
74. Lin X, Fleisher M, Rosenblum M, Lin O, Boire A, Briggs S, et al. Cerebrospinal fluid circulating tumor cells: a novel tool to diagnose leptomeningeal metastases from epithelial tumors. *Neuro Oncol* (2017) 19(9):1248–54. doi:10.1093/neuonc/nox066
75. Polivka J Jr, Pesta M, Janku F. Testing for oncogenic molecular aberrations in cell-free DNA-based liquid biopsies in the clinic: are we there yet? *Expert Rev Mol Diagn* (2015) 15(12):1631–44. doi:10.1586/14737159.2015.1110021
76. Gold B, Cankovic M, Furtado LV, Meier F, Gocke CD. Do circulating tumor cells, exosomes, and circulating tumor nucleic acids have clinical utility? A report of the Association for Molecular Pathology. *J Mol Diagn* (2015) 17:209–24. doi:10.1016/j.jmoldx.2015.02.001
77. Pentsova EI, Shah RH, Tang J, Boire A, You D, Briggs S, et al. Evaluating cancer of the central nervous system through next-generation sequencing of cerebrospinal fluid. *J Clin Oncol* (2016) 34(20):2404–15. doi:10.1200/JCO.2016.66.6487
78. Magbanua MJ, Roy R, Sosa EV, Hauranieh L, Kablanian A, Eisenbud LE, et al. Genome-wide copy number analysis of cerebrospinal fluid tumor cells and their corresponding archival primary tumors. *Genom Data* (2014) 2:60–2. doi:10.1016/j.gdata.2014.04.003
79. De Mattos-Arruda L, Mayor R, Ng CK, Weigelt B, Martínez-Ricarte F, Torrejon D, et al. Cerebrospinal fluid-derived circulating tumour DNA better represents the genomic alterations of brain tumours than plasma. *Nat Commun* (2015) 6:8839. doi:10.1038/ncomms9839
80. Magbanua MJ, Melisko M, Roy R, Sosa EV, Hauranieh L, Kablanian A, et al. Molecular profiling of tumor cells in cerebrospinal fluid and matched primary tumors from metastatic breast cancer patients with leptomeningeal carcinomatosis. *Cancer Res* (2013) 73(23):7134–43. doi:10.1158/0008-5472.CAN-13-2051
81. Lee JS, Melisko ME, Magbanua MJ, Kablanian AT, Scott JH, Rugo HS, et al. Detection of cerebrospinal fluid tumor cells and its clinical relevance in leptomeningeal metastasis of breast cancer. *Breast Cancer Res Treat* (2015) 154(2):339–49. doi:10.1007/s10549-015-3610-1
82. Le Rhun E, Weller M, Brandsma D, Van den Bent M, de Azambuja E, Henriksson R. EANO-ESMO Clinical Practice Guidelines for diagnosis, treatment and follow-up of patients with leptomeningeal metastasis from solid tumours. *Ann Oncol* (2017) 28(Suppl 4):iv84–99. doi:10.1093/annonc/mdx221
83. Yang H, Cai L, Zhang Y, Tan H, Deng Q, Zhao M, et al. Sensitive detection of EGFR mutations in cerebrospinal fluid from lung adenocarcinoma patients with brain metastases. *J Mol Diagn* (2014) 16(5):558–63. doi:10.1016/j.jmoldx.2014.04.008
84. Gaspar L, Scott C, Rotman M, Asbell S, Phillips T, Wasserman T, et al. Recursive partitioning analysis (RPA) of prognostic factors in three Radiation Therapy Oncology Group (RTOG) brain metastases trials. *Int J Radiat Oncol Biol Phys* (1997) 37:745–51. doi:10.1016/S0360-3016(96)00619-0
85. Lagerwaard FJ, Levendag PC, Nowak PJ, Eijkenboom WM, Hanssens PE, Schmitz PI. Identification of prognostic factors in patients with brain metastases: a review of 1292 patients. *Int J Radiat Oncol Biol Phys* (1999) 43(4):795–803. doi:10.1016/S0360-3016(98)00442-8
86. Sperduto PW, Berkey B, Gaspar LE, Mehta M, Curran W. A new prognostic index and comparison to three other indices for patients with brain metastases: an analysis of 1,960 patients in the RTOG database. *Int J Radiat Oncol Biol Phys* (2008) 70:510–4. doi:10.1016/j.ijrobp.2007.06.074
87. Sperduto PW, Chao ST, Sneed PK, Luo X, Suh J, Roberge D, et al. Diagnosis-specific prognostic factors, indexes, and treatment outcomes for patients with newly diagnosed brain metastases: a multi-institutional analysis of 4,259 patients. *Int J Radiat Oncol Biol Phys* (2010) 77(3):655–61. doi:10.1016/j.ijrobp.2009.08.025
88. Sperduto PW, Yang TJ, Beal K, Pan H, Brown PD, Bangdiwala A, et al. Estimating survival in patients with lung cancer and brain metastases: an update of the graded prognostic assessment for lung cancer using molecular markers (lung-molGPA). *JAMA Oncol* (2017) 3(6):827–31. doi:10.1001/jamaoncol.2016.3834
89. Sperduto PW, Jiang W, Brown PD, Braunstein S, Sneed P, Wattson DA, et al. Estimating survival in melanoma patients with brain metastases: an update of the graded prognostic assessment for melanoma using molecular markers (melanoma-molGPA). *Int J Radiat Oncol Biol Phys* (2017) 99(4):812–6. doi:10.1016/j.ijrobp.2017.06.2454

90. Venur VA, Ahluwalia MS. Prognostic scores for brain metastasis patients: use in clinical practice and trial design. *Chin Clin Oncol* (2015) 4(2):18. doi:10.3978/j.issn.2304-3865.2015.06.01
91. Patchell RA, Tibbs PA, Walsh JW, Dempsey RJ, Maruyama Y, Kryscio RJ, et al. A randomized trial of surgery in the treatment of single metastases to the brain. *N Engl J Med* (1990) 322(8):494–500. doi:10.1056/NEJM199002223220802
92. Vecht CJ, Haaxma-Reiche H, Noordijk EM, Padberg GW, Voormolen JH, Hoekstra FH, et al. Treatment of single brain metastasis: radiotherapy alone or combined with neurosurgery? *Ann Neurol* (1993) 33(6):583–90. doi:10.1002/ana.410330605
93. Mintz AH, Kestle J, Rathbone MP, Gaspar L, Hugenholtz H, Fisher B, et al. A randomized trial to assess the efficacy of surgery in addition to radiotherapy in patients with a single cerebral metastasis. *Cancer* (1996) 78(7):1470–6. doi:10.1002/(SICI)1097-0142(19961001)78:7<1470::AID-CNCR14>3.0.CO;2-X
94. Vogelbaum MA, Suh JH. Resectable brain metastases. *J Clin Oncol* (2006) 24(8):1289–94. doi:10.1200/JCO.2005.04.6235
95. Patel AJ, Suki D, Hatiboglu MA, Rao VY, Fox BD, Sawaya R. Impact of surgical methodology on the complication rate and functional outcome of patients with a single brain metastasis. *J Neurosurg* (2015) 122(5):1132–43. doi:10.3171/2014.9.JNS13939
96. Suki D, Abouassi H, Patel AJ, Sawaya R, Weinberg JS, Groves MD. Comparative risk of leptomeningeal disease after resection or stereotactic radiosurgery for solid tumor metastasis to the posterior fossa. *J Neurosurg* (2008) 108(2):248–57. doi:10.3171/JNS/2008/108/2/0248
97. Pollock BE, Brown PD, Foote RL, Stafford SL, Schomberg PJ. Properly selected patients with multiple brain metastases may benefit from aggressive treatment of their intracranial disease. *J Neurooncol* (2003) 61(1):73–80. doi:10.1023/A:1021262218151
98. Ewend MG, Brem S, Gilbert M, Goodkin R, Penar PL, Varia M, et al. Treatment of single brain metastasis with resection, intracavitary carmustine polymer wafers, and radiation therapy is safe and provides excellent local control. *Clin Cancer Res* (2007) 13(12):3637–41. doi:10.1158/1078-0432.CCR-06-2095
99. Schwarz SB, Thon N, Nikolajek K, Niyazi M, Tonn JC, Belka C, et al. Iodine-125 brachytherapy for brain tumours-a review. *Radiat Oncol* (2012) 7:30. doi:10.1186/1748-717X-7-30
100. Baumert BG, Rutten I, Dehing-Oberije C, Twijnstra A, Dirx MJ, Debougnoux-Huppertz RM, et al. A pathology-based substrate for target definition in radiosurgery of brain metastases. *Int J Radiat Oncol Biol Phys* (2006) 66(1):187–94. doi:10.1016/j.ijrobp.2006.03.050
101. Shaw E, Scott C, Souhami L, Dinapoli R, Kline R, Loeffler J, et al. Single dose radiosurgical treatment of recurrent previously irradiated primary brain tumors and brain metastases: final report of RTOG protocol 90-05. *Int Radiat Oncol Biol Phys* (2000) 47(2):291–8. doi:10.1016/S0360-3016(99)00507-6
102. Mehta MP, Tsao MN, Whelan TJ, Morris DE, Hayman JA, Flickinger JC, et al. The American Society for Therapeutic Radiology and Oncology (ASTRO) evidence-based review of the role of radiosurgery for brain metastases. *Int J Radiat Oncol Biol Phys* (2005) 63(1):37–46. doi:10.1016/j.ijrobp.2005.05.023
103. Manon R, O'Neill A, Knisely J, Werner-Wasik M, Lazarus HM, Wagner H, et al. Phase II trial of radiosurgery for one to three newly diagnosed brain metastases from renal cell carcinoma, melanoma, and sarcoma: an Eastern Cooperative Oncology Group study (E 6397). *J Clin Oncol* (2005) 23(34):8870–6. doi:10.1200/JCO.2005.01.8747
104. Fuentes S, Delsanti C, Metellus P, Peragut JC, Grisoli F, Regis J. Brainstem metastases: management using gamma knife radiosurgery. *Neurosurgery* (2006) 58(1):37–42. doi:10.1227/01.NEU.0000190655.95669.5C
105. Andrews DW, Scott CB, Sperduto PW, Flanders AE, Gaspar LE, Schell MC, et al. Whole brain radiation therapy with or without stereotactic radiosurgery boost for patients with one to three brain metastases: phase III results of the RTOG 9508 randomised trial. *Lancet* (2004) 363(9422):1665–72. doi:10.1016/S0140-6736(04)16250-8
106. Rades D, Kueter JD, Veninga T, Gliemroth J, Schild SE. Whole brain radiotherapy plus stereotactic radiosurgery (WBRT+SRS) versus surgery plus whole brain radiotherapy (OP+WBRT) for 1-3 brain metastases: results of a matched pair analysis. *Eur J Cancer* (2009) 45(3):400–4. doi:10.1016/j.ejca.2008.10.033
107. Williams BJ, Suki D, Fox BD, Pelloski CE, Maldaun MV, Sawaya RE, et al. Stereotactic radiosurgery for metastatic brain tumors: a comprehensive review of complications. *J Neurosurg* (2009) 111(3):439–48. doi:10.3171/2008.11.JNS08984
108. Menoux I, Armspach JP, Noël G, Antoni D. Imaging methods used in the differential diagnosis between brain tumour relapse and radiation necrosis after stereotactic radiosurgery of brain metastases: literature review. *Cancer Radiother* (2016) 20(8):837–45. doi:10.1016/j.canrad.2016.07.098
109. Menoux I, Noël G, Namer I, Antoni D. PET scan and NMR spectroscopy for the differential diagnosis between brain radiation necrosis and tumour recurrence after stereotactic irradiation of brain metastases: place in the decision tree. *Cancer Radiother* (2017) 21(5):389–97. doi:10.1016/j.canrad.2017.03.003
110. Aoyama H, Shirato H, Onimaru R, Kagei K, Ikeda J, Ishii N, et al. Hypofractionated stereotactic radiotherapy alone without whole-brain irradiation for patients with solitary and oligo brain metastasis using noninvasive fixation of the skull. *Int J Radiat Oncol Biol Phys* (2003) 56(3):793–800. doi:10.1016/S0360-3016(03)00014-2
111. Minniti G, D'Angelillo RM, Scaringi C, Trodella LE, Clarke E, Matteucci P, et al. Fractionated stereotactic radiosurgery for patients with brain metastases. *J Neurooncol* (2014) 117(2):295–301. doi:10.1007/s11060-014-1388-3
112. Eaton BR, LaRiviere MJ, Kim S, Prabhu RS, Patel K, Kandula S, et al. Hypofractionated radiosurgery has a better safety profile than single fraction radiosurgery for large resected brain metastases. *J Neurooncol* (2015) 123(1):103–11. doi:10.1007/s11060-015-1767-4
113. O'Neill BP, Iturria NJ, Link MJ, Pollock BE, Ballman KV, O'Fallon JR. A comparison of surgical resection and stereotactic radiosurgery in the treatment of solitary brain metastases. *Int J Radiat Oncol Biol Phys* (2003) 55(5):1169–76. doi:10.1016/S0360-3016(02)04379-1
114. Muacevic A, Kreth FW, Horstmann GA, Schmid-Elsaesser R, Wowra B, Steiger HJ, et al. Surgery and radiotherapy compared with gamma-knife radiosurgery in the treatment of solitary brain metastases of small diameter. *J Neurosurg* (1999) 91(1):35–43. doi:10.3171/jns.1999.91.1.0035
115. Hart MG, Grant R, Walker M, Dickinson H. Surgical resection and whole brain radiation therapy versus whole brain radiation therapy alone for single brain metastases. *Cochrane Database Syst Rev* (2005) 25(1):CD003292. doi:10.1002/14651858.CD003292.pub2
116. Patchell RA, Tibbs PA, Regine WF, Dempsey RJ, Mohiuddin M, Kryscio RJ, et al. Postoperative radiotherapy in the treatment of single metastases to the brain: a randomized trial. *JAMA* (1998) 280(17):1485–9. doi:10.1001/jama.280.17.1485
117. Aoyama H, Shirato H, Tago M, Nakagawa K, Toyoda T, Hatano K, et al. Stereotactic radiosurgery plus whole-brain radiation therapy vs stereotactic radiosurgery alone for treatment of brain metastases: a randomized controlled trial. *JAMA* (2006) 295(21):2483–91. doi:10.1001/jama.295.21.2483
118. Kocher M, Soffietti R, Abacioglu U, Villà S, Fauchon F, Baumert BG, et al. Adjuvant whole-brain radiotherapy versus observation after radiosurgery or surgical resection of one to three cerebral metastases: results of the EORTC 22952-26001 study. *J Clin Oncol* (2011) 29(2):134–41. doi:10.1200/JCO.2010.30.1655
119. Sahgal A, Aoyama H, Kocher M, Neupane B, Collette S, Tago M, et al. Phase 3 trials of stereotactic radiosurgery with or without whole-brain radiation therapy for 1 to 4 brain metastases: individual patient data meta-analysis. *Int J Radiat Oncol Biol Phys* (2015) 91(4):710–7. doi:10.1016/j.ijrobp.2014.10.024
120. Aoyama H, Tago M, Shirato H; Japanese Radiation Oncology Study Group 99-1 (JROSG 99-1) Investigators. Stereotactic radiosurgery with or without whole-brain radiotherapy for brain metastases: secondary analysis of the JROSG 99-1 randomized clinical trial. *JAMA Oncol* (2015) 1(4):457–64. doi:10.1001/jamaoncol.2015.1145
121. Churilla TM, Ballman KV, Brown PD, Twohy EL, Jaeckle K, Farace E, et al. Stereotactic radiosurgery with or without whole-brain radiation therapy for limited brain metastases: a secondary analysis of the north central cancer treatment group N0574 (Alliance) randomized controlled trial. *Int J Radiat Oncol Biol Phys* (2017) 99(5):1173–8. doi:10.1016/j.ijrobp.2017.07.045
122. Chang EL, Wefel JS, Hess KR, Allen PK, Lang FF, Kornguth DG, et al. Neurocognition in patients with brain metastases treated with radiosurgery or radiosurgery plus whole-brain irradiation: a randomised controlled trial. *Lancet Oncol* (2009) 10(11):1037–44. doi:10.1016/S1470-2045(09)70263-3

123. Brown PD, Jaeckle K, Ballman KV, Farace E, Cerhan JH, Anderson SK, et al. Effect of radiosurgery alone vs radiosurgery with whole brain radiation therapy on cognitive function in patients with 1 to 3 brain metastases: a randomized clinical trial. *JAMA* (2016) 316(4):401–9. doi:10.1001/jama.2016.9839
124. Soffietti R, Kocher M, Abacioglu U, Villa S, Fauchon F, Baumert BG, et al. A European organisation for research and treatment of cancer phase III trial of adjuvant whole-brain radiotherapy versus observation in patients with one to three brain metastases from solid tumors after surgical resection or radiosurgery: quality-of-life results. *J Clin Oncol* (2013) 31(1):65–72. doi:10.1200/JCO.2011.41.0639
125. Lamba N, Muskens IS, DiRisio AC, Meijer L, Briceno V, Edrees H, et al. Stereotactic radiosurgery versus whole-brain radiotherapy after intracranial metastasis resection: a systematic review and meta-analysis. *Radiat Oncol* (2017) 12(1):106. doi:10.1186/s13014-017-0840-x
126. Brown PD, Ballman KV, Cerhan JH, Anderson SK, Carrero XW, Whitton AC, et al. Postoperative stereotactic radiosurgery compared with whole brain radiotherapy for resected metastatic brain disease (NCCTG N107C/CEC·3): a multicentre, randomised, controlled, phase 3 trial. *Lancet Oncol* (2017) 18(8):1049–60. doi:10.1016/S1470-2045(17)30441-2
127. Scheitler-Ring K, Ge B, Petroski G, Biedermann G, Litofsky NS. Radiosurgery to the postoperative tumor bed for metastatic carcinoma versus whole brain radiation after surgery. *Cureus* (2016) 8(11):e885. doi:10.7759/cureus.885
128. Rao G, Ahmed S, Hess K, Mahajan A. Postoperative stereotactic radiosurgery vs observation for completely resected brainmetastases: results of a prospective randomized study. *Neurosurgery* (2016) 63(Suppl 1):184. doi:10.1227/01.neu.0000489784.83922.17
129. Atalar B, Modlin LA, Choi CY, Adler JR, Gibbs IC, Chang SD, et al. Risk of leptomeningeal disease in patients treated with stereotactic radiosurgery targeting the postoperative resection cavity for brain metastases. *Int J Radiat Oncol Biol Phys* (2013) 87:713–8. doi:10.1016/j.ijrobp.2013.07.034
130. Johnson MD, Avkshtol V, Baschnagel AM, Meyer K, Ye H, Grills IS, et al. Surgical resection of brain metastases and the risk of leptomeningeal recurrence in patients treated with stereotactic radiosurgery. *Int J Radiat Oncol Biol Phys* (2016) 94:537–43. doi:10.1016/j.ijrobp.2015.11.022
131. Ojerholm E, Lee JY, Thawani JP, Miller D, O'Rourke DM, Dorsey JF, et al. Stereotactic radiosurgery to the resection bed for intracranial metastases and risk of leptomeningeal carcinomatosis. *J Neurosurg* (2014) 121(Suppl):75–83. doi:10.3171/2014.6.GKS14708
132. Minniti G, Scaringi C, Paolini S, Lanzetta G, Romano A, Cicone F, et al. Single-fraction versus multifraction (3x9 Gy) stereotactic radiosurgery for large (>2 cm) brain metastases: a comparative analysis of local control and risk of radiation-induced brain necrosis. *Int J Radiat Oncol Biol Phys* (2016) 95(4):1142–8. doi:10.1016/j.ijrobp.2016.03.013
133. Kohutek ZA, Yamada Y, Chan TA, Brennan CW, Tabar V, Gutin PH, et al. Long-term risk of radionecrosis and imaging changes after stereotactic radiosurgery for brain metastases. *J Neurooncol* (2015) 125(1):149–56. doi:10.1007/s11060-015-1881-3
134. Patel KR, Burri SH, Boselli D, Symanowski JT, Asher AL, Sumrall A, et al. Comparing pre-operative stereotactic radiosurgery (SRS) to post-operative whole brain radiation therapy (WBRT) for resectable brain metastases: a multi-institutional analysis. *J Neurooncol* (2017) 131(3):611–8. doi:10.1007/s11060-016-2334-3
135. Ceccon G, Lohmann P, Stoffels G, Judov N, Filss CP, Rapp M, et al. Dynamic O-(2-18F-fluoroethyl)-L-tyrosine positron emission tomography differentiates brain metastasis recurrence from radiation injury after radiotherapy. *Neuro Oncol* (2017) 19(2):281–8. doi:10.1093/neuonc/now149
136. Boothe D, Young R, Yamada Y, Prager A, Chan T, Beal K. Bevacizumab as treatment for radiation necrosis of brain metastases post stereotactic radiosurgery. *Neuo Oncol* (2013) 15(9):1257–63. doi:10.1093/neuonc/not085
137. Hsieh J, Elson P, Otvos B, Rose J, Loftus C, Rahmathulla G, et al. Tumor progression in patients receiving adjuvant whole-brain radiotherapy vs localized radiotherapy after surgical resection of brain metastases. *Neurosurgery* (2015) 76:411–20. doi:10.1227/NEU.0000000000000626
138. Li J, Bentzen SM, Renschler M, Mehta MP. Regression after whole-brain radiation therapy for brain metastases correlates with survival and improved neurocognitive function. *J Clin Oncol* (2007) 25(10):1260–6. doi:10.1200/JCO.2006.09.2536
139. Tsao M, Xu W, Sahgal A. A meta-analysis evaluating stereotactic radiosurgery, whole-brain radiotherapy, or both for patients presenting with a limited number of brain metastases. *Cancer* (2012) 118(9):2486–93. doi:10.1002/cncr.26515
140. Mehta MP, Shapiro WR, Phan SC, Gervais R, Carrie C, Chabot P, et al. Motexafin gadolinium combined with prompt whole brain radiotherapy prolongs time to neurologic progression in non-small-cell lung cancer patients with brain metastases: results of a phase III trial. *Int J Radiat Oncol Biol Phys* (2009) 73(4):1069–76. doi:10.1016/j.ijrobp.2008.05.068
141. Scott C, Suh J, Stea B, Nabid A, Hackman J. Improved survival, quality of life, and quality-adjusted survival in breast cancer patients treated with efaproxiral (Efaproxyn) plus whole-brain radiation therapy for brain metastases. *Am J Clin Oncol* (2007) 30(6):580–7. doi:10.1097/COC.0b013e3180653c0d
142. Mulvenna P, Nankivell M, Barton R, Faivre-Finn C, Wilson P, McColl E, et al. Dexamethasone and supportive care with or without whole brain radiotherapy in treating patients with non-small cell lung cancer with brain metastases unsuitable for resection or stereotactic radiotherapy (QUARTZ): results from a phase 3, non-inferiority, randomised trial. *Lancet* (2016) 388(10055):2004–14. doi:10.1016/S0140-6736(16)30825-X
143. Patel KR, Prabhu RS, Kandula S, Oliver DE, Kim S, Hadjipanayis C, et al. Intracranial control and radiographic changes with adjuvant radiation therapy for resected brain metastases: whole brain radiotherapy versus stereotactic radiosurgery alone. *J Neurooncol* (2014) 120:657–63. doi:10.1007/s11060-014-1601-4
144. Trifiletti DM, Lee CC, Schlesinger D, Larner JM, Xu Z, Sheehan JP. Leukoencephalopathy after stereotactic radiosurgery for brain metastases. *Int J Radiat Oncol Biol Phys* (2015) 93:870–8. doi:10.1016/j.ijrobp.2015.07.2280
145. Kotecha R, Damico N, Miller JA, Suh JH, Murphy ES, Reddy CA, et al. Three or more courses of stereotactic radiosurgery for patients with multiply recurrent brain metastases. *Neurosurgery* (2017) 80(6):871–9. doi:10.1093/neuros/nyw147
146. Tofilon P, Fike J. The radioresponse of the central nervous system: a dynamic process. *Radiat Res* (2000) 153:357–70. doi:10.1667/0033-7587(2000)153[0357:TROTCN]2.0.CO;2
147. Monaco EA, Faraji AH, Berkowitz O, Parry PV, Hadelsberg U, Kano H, et al. Leukoencephalopathy after whole-brain radiation therapy plus radiosurgery versus radiosurgery alone for metastatic lung cancer. *Cancer* (2013) 19(1):226–32. doi:10.1002/cncr.27504
148. DeAngelis LM, Delattre JY, Posner JB. Radiation-induced dementia in patients cured of brain metastases. *Neurology* (1989) 39(6):789–96. doi:10.1212/WNL.39.6.789
149. Brown PD, Pugh S, Laack NN, Wefel JS, Khuntia D, Meyers C, et al. Memantine for the prevention of cognitive dysfunction in patients receiving whole-brain radiotherapy: a randomized, double-blind, placebo-controlled trial. *Neuro Oncol* (2013) 15(10):1429–37. doi:10.1093/neuonc/not114
150. Rapp SR, Case LD, Peiffer A, Naughton MM, Chan MD, Stieber VW, et al. Donepezil for irradiated brain tumor survivors: a phase III randomized placebo-controlled clinical trial. *J Clin Oncol* (2015) 33(15):1653–9. doi:10.1200/JCO.2014.58.4508
151. Dye NB, Gondi V, Mehta MP. Strategies for preservation of memory function in patients with brain metastases. *Chin Clin Oncol* (2015) 4(2):24. doi:10.3978/j.issn.2304-3865.2015.05.05
152. Monje ML, Palmer T. Radiation injury and neurogenesis. *Curr Opin Neurol* (2003) 16(2):12–34. doi:10.1097/00019052-200304000-00002
153. Gibson E, Monje M. Effect of cancer therapy on neural stem cells: implications for cognitive function. *Curr Opin Oncol* (2012) 24(6):672–8. doi:10.1097/CCO.0b013e3283571a8e
154. Zhao R, Kong W, Shang J, Zhe H, Wang YY. Hippocampal-sparing whole-brain radiotherapy for lung cancer. *Clin Lung Cancer* (2017) 18(2):127–31. doi:10.1016/j.cllc.2016.09.007
155. Ghia A, Tomé WA, Thomas S, Cannon G, Khuntia D, Kuo JS, et al. Distribution of brain metastases in relation to the hippocampus: implications for neurocognitive functional preservation. *Int J Radiat Oncol Biol Phys* (2007) 68(4):971–7. doi:10.1016/j.ijrobp.2007.02.016
156. Marsh JC, Herskovic AM, Gielda BT, Hughes FF, Hoeppner T, Turian J, et al. Intracranial metastatic disease spares the limbic circuit: a review of 697 metastatic lesions in 107 patients. *Int J Radiat Oncol Biol Phys* (2010) 76(2):504–12. doi:10.1016/j.ijrobp.2009.02.038
157. Gondi V, Pugh SL, Tome WA, Caine C, Corn B, Kanner A. Preservation of memory with conformal avoidance of the hippocampal neural stem-cell

compartment during whole-brain radiotherapy for brain metastases (RTOG 0933): a phase II multi-institutional trial. *J Clin Oncol* (2014) 32(34):3810–6. doi:10.1200/JCO.2014.57.2909
158. Suh JH. Hippocampal-avoidance whole-brain radiation therapy: a new standard for patients with brain metastases? *J Clin Oncol* (2014) 32(34):3789–91. doi:10.1200/JCO.2014.58.4367
159. Vecil GG, Suki D, Maldaun MV, Lang FF, Sawaya R. Resection of brain metastases previously treated with stereotactic radiosurgery. *J Neurosurg* (2005) 102(2):209–15. doi:10.3171/jns.2005.102.2.0209
160. Bindal RK, Sawaya R, Leavens ME, Hess KR, Taylor SH. Reoperation for recurrent metastatic brain tumors. *J Neurosurg* (1995) 83(4):600–4. doi:10.3171/jns.1995.83.4.0600
161. Arbit E, Wroński M, Burt M, Galicich JH. The treatment of patients with recurrent brain metastases. A retrospective analysis of 109 patients with nonsmall cell lung cancer. *Cancer* (1995) 76(5):765–73. doi:10.1002/1097-0142(19950901)76:5<765::AID-CNCR2820760509>3.0.CO;2-E
162. Chao ST, Barnett GH, Vogelbaum MA, Angelov L, Weil RJ, Neyman G, et al. Salvage stereotactic radiosurgery effectively treats recurrences from whole-brain radiation therapy. *Cancer* (2008) 113(8):2198–204. doi:10.1002/cncr.23821
163. Caballero JA, Sneed PK, Lamborn KR, Ma L, Denduluri S, Nakamura JL, et al. Prognostic factors for survival in patients treated with stereotactic radiosurgery for recurrent brain metastases after prior whole brain radiotherapy. *Int J Radiat Oncol Biol Phys* (2012) 83(1):303–9. doi:10.1016/j.ijrobp.2011.06.1987
164. Kurtz G, Zadeh G, Gingras-Hill G, Millar BA, Laperriere NJ, Bernstein M, et al. Salvage radiosurgery for brain metastases: prognostic factors to consider in patient selection. *Int J Radiat Oncol Biol Phys* (2014) 88(1):137–42. doi:10.1016/j.ijrobp.2013.10.003
165. Lucas JT Jr, Colmer HG IV, White L, Fitzgerald N, Isom S, Bourland JD, et al. Competing risk analysis of neurologic versus nonneurologic death in patients undergoing radiosurgical salvage after whole-brain radiation therapy failure: who actually dies of their brain metastases? *Int J Radiat Oncol Biol Phys* (2015) 92(5):1008–15. doi:10.1016/j.ijrobp.2015.04.032
166. Hsu F, Kouhestani P, Nguyen S, Cheung A, McKenzie M, Ma R, et al. Population-based outcomes of boost versus salvage radiosurgery for brain metastases after whole brain radiotherapy. *Radiother Oncol* (2013) 108(1):128–31. doi:10.1016/j.radonc.2013.04.025
167. Kim DH, Schultheiss TE, Radany EH, Badie B, Pezner RD. Clinical outcomes of patients treated with a second course of stereotactic radiosurgery for locally or regionally recurrent brain metastases after prior stereotactic radiosurgery. *J Neurooncol* (2013) 115(1):37–43. doi:10.1007/s11060-013-1191-6
168. Chen JC, Petrovich Z, Giannotta SL, Yu C, Apuzzo ML. Radiosurgical salvage therapy for patients presenting with recurrence of metastatic disease to the brain. *Neurosurgery* (2000) 46(4):860–6. doi:10.1227/00006123-200004000-00017
169. Kondziolka D, Kano H, Harrison GL, Yang HC, Liew DN, Niranjan A, et al. Stereotactic radiosurgery as primary and salvage treatment for brain metastases from breast cancer. Clinical article. *J Neurosurg* (2011) 114(3):792–800. doi:10.3171/2010.8.JNS10461
170. Shultz DB, Modlin LA, Jayachandran P, Von Eyben R, Gibbs IC, Choi CYH, et al. Repeat courses of stereotactic radiosurgery (SRS), deferring whole-brain irradiation, for new brain metastases after initial SRS. *Int J Radiat Oncol Biol Phys* (2015) 92(5):993–9. doi:10.1016/j.ijrobp.2015.04.036
171. Tsimberidou AM, Letourneau K, Wen S, Wheler J, Hong D, Naing A, et al. Phase I clinical trial outcomes in 93 patients with brain metastases: the MD Anderson Cancer Center experience. *Clin Cancer Res* (2011) 17(12):4110–8. doi:10.1158/1078-0432.CCR-10-3095
172. Venur VA, Ahluwalia MS. Novel terapeutica agents in the management of brain metastases. *Curr Opin Oncol* (2017) 29(5):395–9. doi:10.1097/CCO.0000000000000393
173. Soffietti R, Rudà R, Trevisan E. Brain metastases: current management and new developments. *Curr Opin Oncol* (2008) 20(6):676–84. doi:10.1097/CCO.0b013e32831186fe
174. Mehta MP, Paleologos NA, Mikkelsen T, Robinson PD, Ammirati M, Andrews DW, et al. The role of chemotherapy in the management of newly diagnosed brain metastases: a systematic review and evidence-based clinical practice guideline. *J Neurooncol* (2010) 96(1):71–83. doi:10.1007/s11060-009-0062-7
175. Zhu W, Zhou L, Qian JQ, Qiu TZ, Shu YQ, Liu P. Temozolomide for treatment of brain metastases: a review of 21 clinical trials. *World J Clin Oncol* (2014) 5:19–27. doi:10.5306/wjco.v5.i1.19
176. Preusser M, Berghoff AS, Schadendorf D, Lin NU, Stupp R. Brain metastasis: opportunity for drug development? *Curr Opin Neurol* (2012) 25(6):786–94. doi:10.1097/WCO.0b013e328359320d
177. Arvold ND, Lee EQ, Mehta MP, Margolin K, Alexander BM, Lin NU, et al. Updates in the management of brain metastases. *Neuro Oncol* (2016) 18(8):1043–65. doi:10.1093/neuonc/now127
178. Lin NU. Targeted therapies in brain metastases. *Curr Treat Options Neurol* (2014) 16(1):276. doi:10.1007/s11940-013-0276-z
179. Lin J, Jandial R, Nesbit A, Badie B, Chen M. Current and emerging treatments for brain metastases. *Oncology* (2015) 29(4):250–7.
180. Soffietti R, Trevisan E, Rudà R. Targeted therapy in brain metastasis. *Curr Opin Oncol* (2012) 24(6):679–86. doi:10.1097/CCO.0b013e3283571a1c
181. Farber SH, Tsvankin V, Narloch JL, Kim GJ, Salama AK, Vlahovic G. Embracing rejection: immunologic trends in brain metastasis. *Oncoimmunology* (2016) 5(7):e1172153. doi:10.1080/2162402X.2016.1172153
182. Johanns T, Waqar SN, Morgensztern D. Immune checkpoint inhibition in patients with brain metastases. *Ann Transl Med* (2016) 4:S9. doi:10.21037/atm.2016.09.40
183. Leal T, Chang JE, Mehta M, Robins HI. Leptomeningeal metastasis: challenges in diagnosis and treatment. *Curr Cancer Ther Rev* (2011) 7:319–27. doi:10.2174/157339411797642597
184. Schuette W. Treatment of brain metastases from lung cancer: chemotherapy. *Lung Cancer* (2004) 45(Suppl 2):S253–7. doi:10.1016/j.lungcan.2004.07.967
185. Ballard P, Yates JW, Yang Z, Kim DW, Yang JC, Cantarini M, et al. Preclinical comparison of osimertinib with other EGFR-TKIs in EGFR-mutant NSCLC brain metastases models, and early evidence of clinical brain metastases activity. *Clin Cancer Res* (2016) 22(20):5130–40. doi:10.1158/1078-0432.CCR-16-0399
186. Fan Y, Xu X, Xie C. EGFR-TKI therapy for patients with brain metastases from non-small-cell lung cancer: a pooled analysis of published data. *Onco Targets Ther* (2014) 7:2075–84. doi:10.2147/OTT.S67586
187. Berger LA, Riesenberg H, Bokemeyer C, Atanackovic D. CNS metastases in non-small-cell lung cancer: current role of EGFR-TKI therapy and future perspectives. *Lung Cancer* (2013) 80(3):242–8. doi:10.1016/j.lungcan.2013.02.004
188. Broniscer A, Panetta JC, O'Shaughnessy M, Fraga C, Bai F, Krasin MJ, et al. Plasma and cerebrospinal fluid pharmacokinetics of erlotinib and its active metabolite OSI-420. *Clin Cancer Res* (2007) 13(5):1511–5. doi:10.1158/1078-0432.CCR-06-2372
189. Zhao J, Chen M, Zhong W, Zhang L, Li L, Xiao Y, et al. Cerebrospinal fluid concentrations of gefitinib in patients with lung adenocarcinoma. *Clin Lung Cancer* (2013) 14(2):188–93. doi:10.1016/j.cllc.2012.06.004
190. Yang JC, Wu YL, Schuler M, Sebastian M, Popat S, Yamamoto N, et al. Afatinib versus cisplatin-based chemotherapy for EGFR mutation-positive lung adenocarcinoma (LUX-Lung 3 and LUX-Lung 6): analysis of overall survival data from two randomised, phase 3 trials. *Lancet Oncol* (2015) 16(2):141–51. doi:10.1016/S1470-2045(14)71173-8
191. Schuler M, Wu Y-L, Hirsh V, O'Byrne K, Yamamoto N, Mok T, et al. First-line afatinib versus chemotherapy in patients with non–small cell lung cancer and common epidermal growth factor receptor gene mutations and brain metastases. *J Thorac Oncol* (2016) 11(3):380–90. doi:10.1016/j.jtho.2015.11.014
192. Eze C, Hegemann NS, Roengvoraphoj O, Dantes M, Manapov F. Concurrent afatinib and whole-brain radiotherapy in exon 19-del-EGFR mutant lung adenocarcinoma: a case report and mini review of the literature. *Front Oncol* (2017) 10(7):88. doi:10.3389/fonc.2017.00088
193. Yamanaka Y, Sekine A, Kato T, Yamakawa H, Ikeda S, Baba T, et al. Afatinib therapy for brain metastases aggravated by a reduction in the dose of Erlotinib due to the development of hepatotoxicity. *Intern Med* (2017) 56(21):2895–8. doi:10.2169/internalmedicine.8638-16
194. Jamal-Hanjani M, Spicer J. Epidermal growth factor receptor tyrosine kinase inhibitors in the treatment of epidermal growth factor receptor-mutant non-small cell lung cancer metastatic to the brain. *Clin Cancer Res* (2012) 18(4):938–44. doi:10.1158/1078-0432.CCR-11-2529
195. Zimmermann S, Dziadziuszko R, Peters S. Indications and limitations of chemotherapy and targeted agents in non-small cell lung cancer brain metastases. *Cancer Treat Rev* (2014) 40(6):716–22. doi:10.1016/j.ctrv.2014.03.005

196. Jiang T, Min W, Li Y, Yue Z, Wu C, Zhou C. Radiotherapy plus EGFR TKIs in non-small cell lung cancer patients with brain metastases: an update meta-analysis. *Cancer Med* (2016) 5(6):1055–65. doi:10.1002/cam4.673
197. Welsh JW, Komaki R, Amini A, Munsell MF, Unger W, Allen PK, et al. Phase II trial of erlotinib plus concurrent whole-brain radiation therapy for patients with brain metastases from non-small-cell lung cancer. *J Clin Oncol* (2013) 31(7):895–902. doi:10.1200/JCO.2011.40.1174
198. Lee SM, Lewanski CR, Counsell N, Ottensmeier C, Bates A, Patel N, et al. Randomized trial of erlotinib plus whole-brain radiotherapy for NSCLC patients with multiple brain metastases. *J Natl Cancer Inst* (2014) 106(7):dju151. doi:10.1093/jnci/dju151
199. Sperduto PW, Wang M, Robins HI, Schell MC, Werner-Wasik M, Komaki R, et al. A phase 3 trial of whole brain radiation therapy and stereotactic radiosurgery alone versus WBRT and SRS with temozolomide or erlotinib for non-small cell lung cancer and 1 to 3 brain metastases: radiation therapy oncology group 0320. *Int J Radiat Oncol Biol Phys* (2013) 85(5):1312–8. doi:10.1016/j.ijrobp.2012.11.042
200. Shi YK, Wang L, Han BH, Li W, Yu P, Liu YP, et al. First-line icotinib versus cisplatin/pemetrexed plus pemetrexed maintenance therapy for patients with advanced EGFR mutation-positive lung adenocarcinoma (CONVINCE): a phase 3, open-label, randomized study. *Ann Oncol* (2017) 28(10):2443–50. doi:10.1093/annonc/mdx359
201. Yang JJ, Zhou C, Huang Y, Feng J, Lu S, Song Y, et al. Icotinib versus whole-brain irradiation in patients with EGFR-mutant non-small-cell lung cancer and multiple brain metastases (BRAIN): a multicentre, phase 3, open-label, parallel, randomised controlled trial. *Lancet Respir Med* (2017) 5(9):707–16. doi:10.1016/S2213-2600(17)30262-X
202. Yang Z, Guo Q, Wang Y, Chen K, Zhang L, Cheng Z, et al. AZD3759, a BBB-penetrating EGFR inhibitor for the treatment of EGFR mutant NSCLC with CNS metastases. *Sci Transl Med* (2016) 8(368):368ra172. doi:10.1126/scitranslmed.aag0976
203. Tan CS, Cho BC, Soo RA. Treatment options for EGFR mutant NSCLC with CNS involvement-Can patients BLOOM with the use of next generation EGFR TKIs? *Lung Cancer* (2017) 108:29–37. doi:10.1016/j.lungcan.2017.02.012
204. Preusser M, Berghoff AS, Ilhan-Mutlu A, Magerle M, Dinhof C, Widhalm G, et al. ALK gene translocations and amplifications in brain metastases of non-small cell lung cancer. *Lung Cancer* (2013) 80(3):278–83. doi:10.1016/j.lungcan.2013.01.019
205. Costa DB, Shaw AT, Ou SH, Solomon BJ, Riely GJ, Ahn MJ, et al. Clinical experience with crizotinib in patients with advanced ALK-rearranged non-small-cell lung cancer and brain metastases. *J Clin Oncol* (2015) 33(17):1881–8. doi:10.1200/JCO.2014.59.0539
206. Dagogo-Jack I, Gill CM, Cahill DP, Santagata S, Brastianos PK. Treatment of brain metastases in the modern genomic era. *Pharmacol Ther* (2016) 170:64–72. doi:10.1016/j.pharmthera.2016.10.011
207. Rodig SJ, Shapiro GI. Crizotinib, a small-molecule dual inhibitor of the c-Met and ALK receptor tyrosine kinases. *Curr Opin Investig Drugs* (2010) 11(12):1477–90.
208. Soria JC, Tan DSW, Chiari R, Wu YL, Paz-Ares L, Wolf J, et al. First-line ceritinib versus platinum-based chemotherapy in advanced ALK-rearranged non-small-cell lung cancer (ASCEND-4): a randomised, open-label, phase 3 study. *Lancet* (2017) 389(10072):917–29. doi:10.1016/S0140-6736(17)30123-X
209. Hida T, Nokihara H, Kondo M, Kim YH, Azuma K, Seto T, et al. Alectinib versus crizotinib in patients with ALK-positive non-small-cell lung cancer (J-ALEX): an open-label, randomised phase 3 trial. *Lancet* (2017) 390(10089):29–39. doi:10.1016/S0140-6736(17)30565-2
210. Kim D-W, Tiseo M, Ahn M-J, Reckamp KL, Hansen KH, Kim SW, et al. Brigatinib in patients with crizotinib-refractory anaplastic lymphoma kinase-positive non-small-cell lung cancer: a randomized, multicenter phase II trial. *J Clin Oncol* (2017) 35(22):2490–8. doi:10.1200/JCO.2016.71.5904
211. Shaw AT, Felip E, Bauer TM, Besse B, Navarro A, Postel-Vinay S, et al. Lorlatinib in non-small-cell lung cancer with ALK or ROS1 rearrangement: an international, multicentre, open-label, single-arm first-in-man phase 1 trial. *Lancet Oncol* (2017) 18(12):1590–9. doi:10.1016/S1470-2045(17)30680-0
212. Hochmair MJ, Schwab S, Prosch H. Complete remission of intrathecal metastases with lorlatinib therapy in a heavily pretreated ALK-positive lung cancer patient. *Anticancer Drugs* (2017) 28(8):928–30. doi:10.1097/CAD.0000000000000525
213. Mehta MP, Wang D, Wang F, Kleinberg L, Brade A, Robins HI, et al. Veliparib in combination with whole brain radiation therapy in patients with brain metastases: results of a phase 1 study. *J Neurooncol* (2015) 122(2):409–17. doi:10.1007/s11060-015-1733-1
214. Chabot P, Hsia TC, Ryu JS, Gorbunova V, Belda-Iniesta C, Ball D, et al. Veliparib in combination with whole-brain radiation therapy for patients with brain metastases from non-small cell lung cancer: results of a randomized, global, placebo-controlled study. *J Neurooncol* (2017) 131(1):105–15. doi:10.1007/s11060-016-2275-x
215. Hochart A, Leblond P, Le Bourhis X, Meignan S, Tulasne D. MET receptor inhibition: hope against resistance to targeted therapies? *Bull Cancer* (2017) 104(2):157–66. doi:10.1016/j.bulcan.2016.10.014
216. Gelsomino F, Facchinetti F, Haspinger ER, Garassino MC, Trusolino L, De Braud F, et al. Targeting the MET gene for the treatment of non-small-cell lung cancer. *Crit Rev Oncol Hematol* (2014) 89(2):284–99. doi:10.1016/j.critrevonc.2013.11.006
217. Nakagawa T, Takeuchi S, Yamada T, Nanjo S, Ishikawa D, Sano T, et al. Combined therapy with mutant-selective EGFR inhibitor and Met kinase inhibitor for overcoming erlotinib resistance in EGFR-mutant lung cancer. *Mol Cancer Ther* (2012) 11(10):2149–57. doi:10.1158/1535-7163.MCT-12-0195
218. Brahmer J, Reckamp KL, Baas P, Crinò L, Eberhardt WE, Poddubskaya E, et al. Nivolumab versus docetaxel in advanced squamous-cell non-small-cell lung cancer. *N Engl J Med* (2015) 373(2):123–35. doi:10.1056/NEJMoa1504627
219. Dudnik E, Yust-Katz S, Nechushtan H, Goldstein DA, Zer A, Flex D, et al. Intracranial response to nivolumab in NSCLC patients with untreated or progressing CNS metastases. *Lung Cancer* (2016) 98:114–7. doi:10.1016/j.lungcan.2016.05.031
220. Goldman JW, Crino L, Vokes EE, Holgado E, Reckamp K, Pluzanski A, et al. P2.36: nivolumab (nivo) in patients (pts) with advanced (adv) NSCLC and central nervous system (CNS) metastases (mets): track: immunotherapy. *J Thorac Oncol* (2016) 11(10S):S238–9. doi:10.1016/j.jtho.2016.08.107
221. Goldberg SB, Gettinger SN, Mahajan A, Chiang AC, Herbst RS, Sznol M, et al. Pembrolizumab for patients with melanoma or nonsmall-cell lung cancer and untreated brain metastases: early analysis of a nonrandomised, open-label, phase 2 trial. *Lancet Oncol* (2016) 17:976–83. doi:10.1016/S1470-2045(16)30053-5
222. Rivera E, Meyers C, Groves M, Valero V, Francis D, Arun B, et al. Phase I study of capecitabine in combination with temozolomide in the treatment of patients with brain metastases from breast carcinoma. *Cancer* (2006) 107(6):1348–54. doi:10.1002/cncr.22127
223. Lassman AB, Abrey LE, Shah GD, Panageas KS, Begemann M, Malkin MG, et al. Systemic high-dose intravenous methotrexate for central nervous system metastases. *J Neurooncol* (2006) 78(3):261. doi:10.1007/s11060-006-9155-8
224. Larsen PB, Kümler I, Nielsen DL. A systematic review of trastuzumab and lapatinib in the treatment of women with brain metastases from HER2-positive breast cancer. *Cancer Treat Rev* (2013) 39(7):720–7. doi:10.1016/j.ctrv.2013.01.006
225. Bartsch R, Berghoff AS, Vogl U, Rudas M, Bergen E, Dubsky P, et al. Activity of T-DM1 in Her2-positive breast cancer brain metastases. *Clin Exp Metastasis* (2015) 32(7):729–37. doi:10.1007/s10585-015-9740-3
226. De Vries CL, Linn SC, Brandsma D. Response of symptomatic brain metastases from HER-2 overexpressing breast cancer with T-DM1. *J Neurooncol* (2016) 127(2):401–3. doi:10.1007/s11060-015-2045-1
227. Swain SM, Baselga J, Miles D, Im YH, Quah C, Lee LF, et al. Incidence of central nervous system metastases in patients with HER2-positive metastatic breast cancer treated with pertuzumab, trastuzumab, and docetaxel: results from the randomized phase III study CLEOPATRA. *Ann Oncol* (2014) 25(6):1116–21. doi:10.1093/annonc/mdu133
228. Swain SM, Baselga J, Kim SB, Ro J, Semiglazov V, Campone M, et al. Pertuzumab, trastuzumab, and docetaxel in HER2-positive metastatic breast cancer. *N Engl J Med* (2015) 372(8):724–34. doi:10.1056/NEJMoa1413513
229. Petrelli F, Ghidini M, Lonati V, Tomasello G, Borgonovo K, Ghilardi M, et al. The efficacy ofl apatinib and capecitabinein HER-2 positive breast cancerwith

brain metastases: a systematic review and pooled analysis. *Eur J Cancer* (2017) 84:141–8. doi:10.1016/j.ejca.2017.07.024

230. Lin NU, Carey LA, Liu MC, Younger J, Come SE, Ewend M, et al. Phase II trial of lapatinib for brain metastases in patients with human epidermal growth factor receptor 2-positive breast cancer. *J Clin Oncol* (2008) 26(12):1993–9. doi:10.1200/JCO.2007.12.3588
231. Bachelot T, Romieu G, Campone M, Dieras V, Cropet C, Dalenc F, et al. Lapatinib plus capecitabine in patients with previously untreated brain metastases from HER2-positive metastatic breast cancer (LANDSCAPE): a single-group phase 2 study. *Lancet Oncol* (2013) 14(1):64–71. doi:10.1016/S1470-2045(12)70432-1
232. Freedman RA, Gelman RS, Wefel JS, Melisko ME, Hess KR, Connolly RM, et al. Translational breast cancer research consortium (TBCRC) 022: a phase II of neratinib for patients with human epidermal growth factor receptor 2-positive breast cancer and brain metastases. *J Clin Oncol* (2016) 34(9):945–52. doi:10.1200/JCO.2015.63.0343
233. Kümler I, Tuxen MK, Nielsen D. A systematic review of dual targeting in HER2-positive breast cancer. *Cancer Trat Rev* (2014) 40(2):259–70. doi:10.1016/j.ctrv.2013.09.002
234. Lu YS, Chen TW, Lin CH, Yeh DC, Tseng LM, Wu PF, et al. Bevacizumab preconditioning follone by etoposide and cisplatin is highly effective in treating brain metastases of breast cancer progressing from whole-brain radiotherapy. *Clin Cancer Res* (2015) 21(8):1851–8. doi:10.1158/1078-0432.CCR-14-2075
235. Piccart M, Hortobagyi GN, Campone M, Pritchard KI, Lebrun F, Ito Y, et al. Everolimus plus exemestane for hormone-receptor-positive, human epidermal growth factor receptor-2-negative advanced breast cancer: overall survival results from BOLERO-2. *Ann Oncol* (2014) 25(12):2357–62. doi:10.1093/annonc/mdu456
236. Franz DN, Belousova E, Sparagana S, Bebin EM, Frost M, Kuperman R, et al. Efficacy and safety of everolimus for subependymal giant cell astrocytomas associated with tuberous sclerosis complex (EXIST-1): a multicentre, randomised, placebo-controlled phase 3 trial. *Lancet* (2013) 381(9861):125–32. doi:10.1016/S0140-6736(12)61134-9
237. Leone JP, Leone BA. Breast cancer brain metastases: the last frontier. *Exp Hematol Oncol* (2015) 4:33. doi:10.1186/s40164-015-0028-8
238. Davies MA, Liu P, McIntyre S, Kim KB, Papadopoulos N, Hwu WJ, et al. Prognostic factors for survival in melanoma patients with brain metastases. *Cancer* (2011) 117:1687–96. doi:10.1002/cncr.25634
239. Atkins MB, Sosman JA, Agarwala S, Logan T, Clark JI, Ernstoff MS, et al. Temozolomide, thalidomide, and whole brain radiation therapy for patients with brain metastasis from metastatic melanoma: a phase II Cytokine Working Group study. *Cancer* (2008) 113(8):2139–45. doi:10.1002/cncr.23805
240. Weber JS, D'Angelo SP, Minor D, Hodi FS, Gutzmer R, Neyns B, 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. *Lancet Oncol* (2015) 16:375–84. doi:10.1016/S1470-2045(15)70076-8
241. Long GV, Stroyakovskiy D, Gogas H, Levchenko E, de Braud F, Larkin J, et al. Dabrafenib and trametinib versus dabrafenib and placebo for Val600 BRAF-mutant melanoma: a multicentre, double-blind, phase 3 randomised controlled trial. *Lancet* (2015) 386:444–51. doi:10.1016/S0140-6736(15)60898-4
242. Long GV, Flaherty KT, Stroyakovskiy D, Gogas H, Levchenko E, de Braud F, et al. Dabrafenib plus trametinib versus dabrafenib monotherapy in patients with metastatic BRAF V600E/K-mutant melanoma: long-term survival and safety analysis of a phase 3 study. *Ann Oncol* (2017) 28(7):1631–9. doi:10.1093/annonc/mdx176
243. Long GV, Trefzer U, Davies MA, Kefford RF, Ascierto PA, Chapman PB, et al. Dabrafenib in patients with Val600Glu or Val600Lys BRAF-mutant melanoma metastatic to the brain (BREAK-MB): a multicentre, open-label, phase 2 trial. *Lancet Oncol* (2012) 13(11):1087–95. doi:10.1016/S1470-2045(12)70431-X
244. Margolin K, Ernstoff MS, Hamid O, Lawrence D, McDermott D, Puzanov I, et al. Ipilimumab in patients with melanoma and brain metastases: an open-label, phase 2 trial. *Lancet Oncol* (2012) 13(5):459–65. doi:10.1016/S1470-2045(12)70090-6
245. McArthur GA, Maio M, Arance A, Nathan P, Blank C, Avril MF, et al. Vemurafenib in metastatic melanoma patients with brain metastases: an open-label, single-arm, phase 2, multicentre study. *Ann Oncol* (2017) 28(3):634–41. doi:10.1093/annonc/mdw641
246. Schadendorf D, Hodi FS, Robert C, Weber JS, Margolin K, Hamid O, et al. Pooled analysis of long term survival data from phase II and phase III trials of ipilimumab in unresectable or metastatic melanoma. *J Clin Oncol* (2015) 33:1889–94. doi:10.1200/JCO.2014.56.2736
247. Davey RJ, Westhuizen AV, Bowden NA. Metastatic melanoma treatment: combining old and new therapies. *Crit Rev Oncol Hematol* (2016) 98:242–53. doi:10.1016/j.critrevonc.2015.11.011
248. Pardoll DE. The blockade of immune checkpoints in cancer immunotherapy. *Nat Rev* (2012) 12:252–64. doi:10.1038/nrc3239
249. Robert C, Thomas L, Bondarenko I, O'Day S, Weber J, Garbe C, et al. Ipilimumab plus dacarbazine for previously untreated metastatic melanoma. *N Engl J Med* (2011) 364:2517–26. doi:10.1056/NEJMoa1104621
250. Hodi FS, O Day SH, McDermott DF, Weber RW, Sosman JA, Haanen JB, et al. Improved survival with ipilimumab in patients with metastatic melanoma. *N Engl J Med* (2010) 363:711–23. doi:10.1056/NEJMoa1003466
251. Theurich S, Rothschild SI, Hoffmann M, Fabri M, Sommer A, Garcia-Marquez M, et al. Local tumor treatment in combination with systemic ipilimumab immunotherapy prolongs overall survival in patients with advanced malignant melanoma. *Cancer Immunol Res* (2016) 4:744–54. doi:10.1158/2326-6066.CIR-15-0156
252. Di Giacomo AM, Ascierto PA, Queirolo P, Pilla L, Ridolfi R, Santinami M, et al. Three-year follow-up of advanced melanoma patients who received ipilimumab plus fotemustine in the Italian Network for Tumor Biotherapy (NIBIT)-M1 phase II study. *Ann Oncol* (2015) 26(4):798–803. doi:10.1093/annonc/mdu577
253. Hamid O, Robert C, Daud A, Hodi FS, Hwu WJ, Kefford R, et al. Safety and tumor responses with lambrozilumab (anti-PD-1) in melanoma. *N Engl J Med* (2013) 369:134–44. doi:10.1056/NEJMoa1305133
254. Topalian SL, Hodi FS, Brahmer JR, Gettinger SN, Smith DC, McDermott DF, et al. Safety, activity, and immune correlates of anti-PD-1 antibody in cancer. *N Engl J Med* (2012) 366:2443–54. doi:10.1056/NEJMoa1200690
255. Robert C, Long GV, Brady B, Dutriaux C, Maio M, Mortier L, et al. Nivolumab in previously untreated melanoma without BRAF mutation. *N Engl J Med* (2015) 372:320–30. doi:10.1056/NEJMoa1412082
256. Larkin J, Minor D, D'Angelo S, Neyns B, Smylie M, Miller WH Jr, et al. Overall survival in patients with advanced melanoma who received Nivolumab versus investigator's choice chemotherapy in CheckMate 037: a randomized, controlled, open-label phase III trial. *J Clin Oncol* (2017) 36(4):383–90. doi:10.1200/JCO.2016.71.8023
257. Carlino MS, Long GV. Ipilimumab combined with Nivolumab: a standard of care for the treatment of advanced melanoma? *Clin Cancer Res* (2016) 22(16):3992–8. doi:10.1158/1078-0432.CCR-15-2944
258. Spain L, Schmid T, Gore M, Larkin J. Efficacy of the combination of ipilimumab and nivolumab following progression on pembrolizumab in advanced melanoma with poor risk features. *Eur J Cancer* (2017) 75:243–4. doi:10.1016/j.ejca.2016.12.040
259. Barker A, Postow MA, Khan SA, Beal K, Parhar PK, Yamada Y, et al. Concurrent radiotherapy and ipilimumab immunotherapy for patients with melanoma. *Cancer Immunol Res* (2013) 1(2):92–8. doi:10.1158/2326-6066.CIR-13-0082
260. Stamell EF, Wolchok JD, Gnjatic S, Lee NY, Brownell I. The abscopal effect associated with a systemic anti-melanoma immune response. *Int J Radiat Oncol Biol Phys* (2013) 85(2):293–5. doi:10.1016/j.ijrobp.2012.03.017
261. Knisely JP, Yu JB, Flanigan J, Sznol M, Kluger HM, Chiang VL. Radiosurgery for melanoma brain metastases in the ipilimumab era and the possibility of longer survival. *J Neurosurg* (2012) 117:227–33. doi:10.3171/2012.5.JNS111929
262. Kiess AP, Wolchok JD, Barker CA, Postow MA, Tabar V, Huse JT, et al. Stereotactic radiosurgery for melanoma brain metastases in patients receiving ipilimumab: safety profile and efficacy of combined treatment. *Int J Radiat Oncol Biol Phys* (2015) 92(2):368–75. doi:10.1016/j.ijrobp.2015.01.004
263. Qian JM, Yu JB, Kluger HM, Chiang VL. Timing and type of immune checkpoint therapy affect the early radiographic response of melanoma brain metastases to stereotactic radiosurgery. *Cancer* (2016) 122:3051–8. doi:10.1002/cncr.30138
264. Shen C, Lin DD, Redmond KJ, Link K, Kummerlowe M, Douglass J, et al. Imaging and clinical profile following concurrent stereotactic radiation and immune therapy for melanoma brain metastases: preliminary results. *Int J Radiat Oncol Biol Phys* (2016) 96:E134. doi:10.1016/j.ijrobp.2016.06.927

265. Ahmed KA, Stallworth DG, Kim Y, Johnstone PA, Harrison LB, Caudell JJ, et al. Clinical outcomes of melanoma brain metastases treated with stereotactic radiation and anti-PD-1 therapy. *Ann Oncol* (2016) 27:434–41. doi:10.1093/annonc/mdv622
266. Robert C, Karaszewska B, Schachter J, Rutkowski P, Mackiewicz A, Stroiakovski D, et al. Improved overall survival in melanoma with combined dabrafenib and trametinib. *N Engl J Med* (2015) 372(1):30–9. doi:10.1056/NEJMoa1412690
267. Ramanujam S, Schadendorf D, Long GV. Systemic therapies for melanoma brain metastases: which drug for whom and when? *Chin Clin Oncol* (2015) 4(2):25. doi:10.3978/j.issn.2304-3865.2015.06.06
268. Harding JJ, Catalanotti F, Munhoz RR, Cheng DT, Yaqubie A, Kelly N, et al. A retrospective evaluation of vemurafenib as treatment for BRAF-mutant melanoma brain metastases. *Oncologist* (2015) 20(7):789–97. doi:10.1634/theoncologist.2014-0012
269. Dummer R, Goldinger SM, Turtschi CP, Eggmann NB, Michielin O, Mitchell L, et al. Vemurafenib in patients with BRAF(V600) mutation-positive melanoma with symptomatic brain metastases: final results of an open-label pilot study. *Eur J Cancer* (2014) 50(3):611–21. doi:10.1016/j.ejca.2013.11.002
270. Flaherty KT, Infante JR, Daud A, Gonzalez R, Kefford RF, Sosman J, et al. Combined BRAF and MEK inhibition in melanoma with BRAF V600 mutations. *N Engl J Med* (2012) 367:1694–703. doi:10.1056/NEJMoa1210093
271. Eroglu Z, Ribas A. Combination therapy with BRAF and MEK inhibitors for melanoma: latest evidence and place in therapy. *Ther Adv Med Oncol* (2016) 8(1):48–56. doi:10.1177/1758834015616934
272. Larkin J, Ascierto PA, Dréno B, Atkinson V, Liszkay G, Maio M, et al. Combined vemurafenib and cobimetinib in BRAF-mutated melanoma. *N Engl J Med* (2014) 371:1867–76. doi:10.1056/NEJMoa1408868
273. Ahmed KA, Freilich JM, Sloot S, Figura N, Gibney GT, Weber JS, et al. LINAC-based stereotactic radiosurgery to the brain with concurrent vemurafenib for melanoma metastases. *J Neurooncol* (2015) 122(1):121–6. doi:10.1007/s11060-014-1685-x
274. Narayana A, Mathew M, Tam M, Kannan R, Madden KM, Golfinos JG, et al. Vemurafenib and radiation therapy in melanoma brain metastases. *J Neurooncol* (2013) 113:411–6. doi:10.1007/s11060-013-1127-1
275. Postow MA, Carvajal RD. Therapeutic implications of KIT in melanoma. *Cancer J* (2012) 18(2):137–41. doi:10.1097/PPO.0b013e31824b2404
276. Hodi FS, Friedlander P, Corless CL, Heinrich MC, Mac Rae S, Kruse A, et al. Major response to imatinib mesylate in KIT-mutated melanoma. *J Clin Oncol* (2008) 26(12):2046–51. doi:10.1200/JCO.2007.14.0707
277. Rudà R, Franchino F, Soffietti R. Treatment of brain metastasis: current status and future directions. *Curr Opin Oncol* (2016) 28(6):502–10. doi:10.1097/CCO.0000000000000326
278. Ryken TC, McDermott M, Robinson PD, Ammirati M, Andrews DW, Asher AL, et al. The role of steroids in the management of brainmetastases: a systematic review and evidence-based clinical practice guideline. *J Neurooncol* (2010) 96(1):103–14. doi:10.1007/s11060-009-0057-4
279. Weston J, Greenhalgh J, Marson AG. Antiepileptic drugs as prophylaxis for post-craniotomy seizures. *Cochrane Database Syst Rev* (2015) 4(3):CD007286. doi:10.1002/14651858.CD007286.pub2
280. Mikkelsen T, Paleologos NA, Robinson PD, Ammirati M, Andrews DW, Asher AL, et al. The role of prophylactic anticonvulsants in the management of brain metastases: a systematic review and evidence-based clinical practice guideline. *J Neurooncol* (2010) 96(1):97–102. doi:10.1007/s11060-009-0056-5
281. Lobos-Urbina D, Kittsteiner-Manubens L, Peña J. Is primary prevention with antiepileptic drugs effective in brain tumors or brainmetastases? *Medwave* (2017) 17(Suppl1):e6871. doi:10.5867/medwave.2017.6871
282. Maschio M, Dinapoli L, Gomellini S, Ferraresi V, Sperati F, Vidiri A, et al. Antiepileptics in brain metastases: safety, efficacy and impact on life expectancy. *J Neurooncol* (2010) 98(1):109–16. doi:10.1007/s11060-009-0069-0
283. Lyman GH, Bohlke K, Khorana AA, Kuderer NM, Lee AY, Arcelus JI, et al. Venous thromboembolism prophylaxis and treatment in patients with cancer: American Society of Clinical Oncology clinical practice guideline update 2014. *J Clin Oncol* (2015) 33(6):654–6. doi:10.1200/JCO.2014.59.7351
284. Kreuziger LB. Balancing bleeding in brain metastases. *Blood* (2015) 126(4):432–3. doi:10.1182/blood-2015-06-648089
285. Hunter BD, Minichiello T, Bent S. Anticoagulation for the treatment of venous thromboembolism in patients with brain metastases: a meta-analysis and systematic review. *J Thromb Thrombolysis* (2017) 44(3):392–8. doi:10.1007/s11239-017-1536-7
286. Raskob GE, Van Es N, Verhamme P, Carrier M, Di Nisio M, Garcia D, et al. Edoxaban for the treatment of cancer-associated venous thromboembolism. *N Engl J Med* (2018) 378(7):615–24. doi:10.1056/NEJMoa1711948
287. Brown PD, Asher AL, Ballman KV, Farace E, Cerhan JH, Keith Anderson S, et al. NCCTG N0574 (alliance): a phase III randomized trial of whole brain radiation therapy (WBRT) in addition to radiosurgery (SRS) in patients with 1 to 3 brain metastases. *J Clin Oncol* (2015) 33(Suppl):abstrLBA4. doi:10.1200/jco.2015.33.18_suppl.lba4
288. Rosell R, Dafni U, Felip E, Curioni-Fontecedro A, Gautschi O, Peters S, et al. Erlotinib and bevacizumab in patients with advanced non-small-cell lung cancer and activating EGFR mutations (BELIEF): an international, multicentre, single-arm, phase 2 trial. *Lancet Respir Med* (2017) 5(5):435–44. doi:10.1016/S2213-2600(17)30129-7
289. Ceresoli GL, Cappuzzo F, Gregorc V, Bartolini S, Crino L, Villa E. Gefitinib in patients with brain metastases from non-small-cell lung cancer: a prospective trial. *Ann Oncol* (2004) 15(7):1042–7. doi:10.1093/annonc/mdh276
290. Gadgeel SM, Gandhi L, Riely GJ, Chiappori AA, West HL, Azada MC, et al. Safety and activity of alectinib against systemic disease and brain metastases in patients with crizotinib-resistant ALK-rearranged non-small-cell lung cancer (AF-002JG): results from the dose-finding portion of a phase 1/2 study. *Lancet Oncol* (2014) 15(10):1119–28. doi:10.1016/S1470-2045(14)70362-6
291. Shaw AT, Kim D-W, Mehra R, Tan DSW, Felip E, Chow LQM, et al. Ceritinib in ALK-rearranged non-small-cell lung cancer. *N Engl J Med* (2014) 370(13):1189–97. doi:10.1056/NEJMoa1311107
292. Crinò L, Ahn MJ, De Marinis F, Groen HJ, Wakelee H, Hida T, et al. Multicenter phase II study of whole-body and intracranial activity with ceritinib in patients with ALK-rearranged non-small-cell lung cancer previously treated with chemotherapy and crizotinib: results from ASCEND-2. *J Clin Oncol* (2016) 34:2866–73. doi:10.1200/JCO.2015.65.5936
293. Gettinger SN, Bazhenova LA, Langer CJ, Salgia R, Gold KA, Rosell R, et al. Activity and safety of brigatinib in ALK-rearranged non-small-cell lung cancer and other malignancies: a single-arm, open-label, phase 1/2 trial. *Lancet Oncol* (2016) 17(12):1683–96. doi:10.1016/S1470-2045(16)30392-8
294. Yang JC, Ahn MJ, Kim DW, Ramalingam SS, Sequist LV, Su WC, et al. Osimertinib in pretreated T790M-positive advanced non-small-cell lung cancer: AURA study phase II extension component. *J Clin Oncol* (2017) 35(12):1288–96. doi:10.1200/JCO.2016.70.3223
295. Planchard D, Besse B, Groen HJ, Souquet PJ, Quoix E, Baik CS, et al. Dabrafenib plus trametinib in patients with previously treated BRAF(V600E)-mutant metastatic non-small cell lung cancer: an open-label, multicentre phase 2 trial. *Lancet Oncol* (2016) 17(7):984–93. doi:10.1016/S1470-2045(16)30146-2
296. Cortes J, Dieras V, Ro J, Barriere J, Bachelot T, Hurvitz S, et al. Afatinib alone or afatinib plus vinorelbine versus investigator's choice of treatment for HER2-positive breast cancer with progressive brain metastases after trastuzumab, lapatinib, or both (LUXbreast 3): a randomised, open-label, multicentre, phase 2 trial. *Lancet Oncol* (2015) 16(16):1700–10. doi:10.1016/s1470-2045(15)00373-3
297. Patel KR, Shoukat S, Oliver DE, Chowdhary M, Rizzo M, Lawson DH. Ipilimumab and stereotactic radiosurgery versus stereotactic radiosurgery alone for newly diagnosed melanoma brain metastases. *Am J Clin Oncol* (2017) 40(5):444–50. doi:10.1097/COC.0000000000000199
298. Weber JS, Amin A, Minor D, Siegel J, Berman D, O'Day SJ. Safety and clinical activity of ipilimumab in melanoma patients with brain metastases: retrospective analysis of data from a phase 2 trial. *Melanoma Res* (2011) 21(6):530–4. doi:10.1097/CMR.0b013e32834d3d88
299. Silk AW, Bassetti MF, West BT, Tsien CI, Lao CD. Ipilimumab and radiation therapy for melanoma brain metastases. *Cancer Med* (2013) 2:899–906. doi:10.1002/cam4.140
300. Mathew M, Tam M, Ott PA, Pavlick AC, Rush SC, Donahue BR, et al. Ipilimumab in melanoma with limited brain metastases treated with stereotactic radiosurgery. *Melanoma Res* (2013) 23:191–5. doi:10.1097/CMR.0b013e32835f3d90
301. Schachter J, Ribas A, Long GV, Arance A, Grob JJ, Mortier L, et al. Pembrolizumab versus ipilimumab for advanced melanoma: final overall survival results of a multicentre, randomised, open-label phase 3 study (KEYNOTE-006). *Lancet* (2017) 390(10105):1853–62. doi:10.1016/S0140-6736(17)31601-X

302. Parakh S, Park JJ, Mendis S, Rai R, Xu W, Lo S, et al. Efficacy of anti-PD-1 therapy in patients with melanoma brain metastases. *Br J Cancer* (2017) 116(12):1558–63. doi:10.1038/bjc.2017.142

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REVIEW
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Reactive Astrocytes in Brain Metastasis

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Brain metastasis, the secondary growth of malignant cells within the central nervous system (CNS), exceeds the incidence of primary brain tumors (i.e., gliomas) by tenfold and are seemingly on the rise owing to the emergence of novel targeted therapies that are more effective in controlling extracranial disease relatively to intracranial lesions. Despite the fact that metastasis to the brain poses a unmet clinical problem, with afflicted patients carrying significant morbidity and a fatal prognosis, our knowledge as to how metastatic cells manage to adapt to the tissue environment of the CNS remains limited. Answering this question could pave the way for novel and more specific therapeutic modalities in brain metastasis by targeting the specific makeup of the brain metastatic niche. In regard to this, astrocytes have emerged as the major host cell type that cancer cells encounter and interact with during brain metastasis formation. Similarly to other CNS disorders, astrocytes become reactive and respond to the presence of cancer cells by changing their phenotype and significantly influencing the outcome of disseminated cancer cells within the CNS. Here, we summarize the current knowledge on the contribution of reactive astrocytes in brain metastasis by focusing on the signaling pathways and types of interactions that play a crucial part in the communication with cancer cells and how these could be translated into innovative therapies.

Keywords: brain metastasis, reactive astrocytes, metastases therapy, microenvironment heterogeneity, astrocyte signaling

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INTRODUCTION

Brain metastasis defines the secondary tumor formation within the brain and typically results from metastases of lung cancer, breast cancer and melanoma together with other primary tumors that less frequently metastasize in the brain, such as colorectal cancer (1). We will focus on metastatic cells invading the brain parenchyma in contrast to the less frequent invasion of the leptomeninges by cancer cells, which has a very different biology derived from its location (meningeal space filled with cerebrospinal fluid) and cellular components of the microenvironment (2). Brain metastasis accounts for the major part of intracranial malignancies (3) and its incidence has been suggested to be on the rise owing to: improved imaging modalities as well as a generally lower threshold to schedule MRI imaging by physicians nowadays, extension of overall survival time of patients being treated with targeted antibody-based therapies (e.g., trastuzumab) or small molecule inhibitors (e.g., the small molecule ALK kinase inhibitor crizotinib), thus increasing likelihood for recurrence with central nervous system (CNS) lesions accounting for a main part of relapses, "sanctuary site levels" of pharmacological agents because of poor drug penetration as demonstrated for

trastuzumab (4–7). Upon diagnosis of brain metastasis, affected patients suffer from significantly increased overall morbidity and mortality (1). Aside from being recognized as a serious obstacle to the care of cancer patients, only recently new insights into the molecular mechanisms accounting for metastatic spread to and growth within the brain have been made and new trials for assessing treatments in brain metastasis have been initiated to avoid traditional exclusion of this patient collective (8). Over the past decade, metastasis research with regard to the use of experimental mouse models of brain metastasis shed some light into the molecular and cellular events inherent to cancer cell dissemination and growth in the brain, which likely depends on the evolution of a series of cancer cell traits that are not necessarily required and exploited in other extracranial locations and that continue to be characterized (9–18). Though metastatic organotropism (site-specific metastasis) to different organs seems to employ some shared molecular mechanisms involved in cancer cell–host cell interactions across different tumor entities, metastasis to the brain as such is unprecedented in that the brain microenvironment harbors unique cellular and non-cellular elements and a higher degree of isolation and protection mediated by the blood–brain barrier (BBB) from both circulating molecules and cells found in the systemic circulation. Therefore, it is conceivable that cancer cells that are able to trespass the BBB and extravasate from brain capillaries face a complete different and unfamiliar tissue microenvironment subjecting cancer cells to strong selective forces (10, 19). Accordingly, cancer cells that are able to generate macrometastasis correspond to those seeds with the highest ability to integrate in such a demanding microenvironment, arguing against the BBB as the solely impediment to colonize and initiate outgrowth in the brain. The brain includes not only neurons but also glia. The glial compartment is involved in responding to any type of brain injury, such as astrocytes and microglia, the two main glial cell types together with oligodendrocytes, have been reported surround brain metastases (10, 20). Although the role of oligodendrocytes and, to a less extent, microglia has been poorly studied (21–23) in the context of brain metastasis, relatively abundant bibliography have considered brain metastasis-associated astrocytes. In this regard, recent discoveries provide compelling evidence that astrocytes, the major glial cell in the CNS, play an intricate role in brain metastasis by engaging different modes of interactions with incoming cancer cells. Although our knowledge on the crosstalk between astrocytes and cancer cells is still insufficient, recent seminal findings indicate that interactions with astrocytes occur at both early and late stages of the colonization process. Given that these interactions could provide both anti- and prometastatic stimuli to cancer cells characterizing them might aid in dissecting the molecular machinery in order to explore innovative targeted therapeutics in brain metastasis. Here, we summarize them to expose the importance of astrocytes in the biology of brain metastasis. We envision that understanding the impact of astrocytes, as one of the key host cell type in the pathogenesis of brain metastasis, may serve not only to understand the functional importance of the microenvironment in the development of this secondary tumor growth in the brain, but also to explore additional implications related to biomarkers and therapies.

WHAT ARE REACTIVE ASTROCYTES (RAs) AND HOW HAVE THEY BEEN STUDIED?

Reactive astrocytes are ubiquitously present in any brain injury (24, 25). As such they have been extensively described surrounding brain tumors including brain metastasis (10, 12, 20, 26, 27). Usually they are identified by their profound alterations including the gain of a hypertrophic phenotype as well as the upregulation of the cytoskeletal intermediate filament protein glial fibrillary acidic protein (GFAP) (24, 25). However, the word *reactive* indicates a more extensive number of changes (24) to be able to face a situation in which homeostasis has been compromised. There are many stimuli that could be informative to astrocytes of such a situation and which are commonly classified as danger-associated molecular patterns (DAMPs) and pathogen-associated molecular patterns (PAMPs) (28). PAMPs are generated by microbial infections (e.g., LPS) and usually provoke a primary immune response in the CNS through microglial cells and perivascular macrophages. In contrast, the exact identity and origin of DAMPs responsible to activate the reactive program in astrocytes in the context of brain metastasis remains unknown. The fact that very limited number of cancer cells, independently of the source of the primary tumor or oncogenomic profiles, from very early stages of colonization (i.e., when lodged with the brain capillaries during the process of extravasation) (10, 20) are able to trigger this response might indicate that, at least at these initial phases, tissue injury induced by cancer cells rather than DAMPs produced by cancer cells, would be responsible for triggering the activation. Throughout cancer cell evolvement and proliferation in the CNS the stimuli influencing the reactive state in astrocytes might underlie changes. In this sense different phases related to the behavior of RAs toward insults or tissue injuries have been described encompassing an acute phase and a chronic one, which is usually referred as to glial scar (24). The acute phase is usually responsible for limiting the extension of the damage (29), however, if this cannot be achieved the response becomes chronic, which usually impairs the ability of the CNS tissue to recover from the damage completely (30, 31). Additionally, different types of brain injuries have been associated with different transcriptomic changes in RAs (32, 33), which has lead to the proposal of a dichotomy similar to the one initially applied to macrophages and microglia (34). A similar situation seems to take place in the context of brain metastasis. Early on, RAs acting as a primary host defense efficiently limit the progression of incoming metastatic cells (10), whereas later RAs have been extensively described to promote the growth of cancer cells (9, 35–38).

A significant proportion of publications considering RAs in the field of brain metastasis research are based on data generated *in vitro* exclusively, using primary mouse astrocytes or an immortalized astrocyte cell line (27, 39–43). Techniques for *in vitro* culture of astrocytes were described long time ago (44), however, recent data have demonstrated important considerations that must be taken into account. Most common protocols use early postnatal brains to obtain primary cultures of astrocytes (44). Since young and aged astrocytes could differ molecularly (45, 46) these astrocytes might not mimic those coexisting with cancer

cells in the brain. Another caveat of working with astrocytes *in vitro* is that under regular culture conditions they instantly become reactive. In fact, the most widely applied method to assure the purity of the culture is to evaluate that >90% of the cells are GFAP+ (47). Since inducers of the reactive state *in vitro* likely differ from those present in secondary brain tumors, *in vitro* asytrocyte cultures used in these studies unlikely reproduce the disparity of phenotypes associated with RAs *in vivo* (48). Thus, validation of *in vitro* findings using *in vivo* approaches is an absolute requirement (a *sine qua non* condition) to generate reliable data aimed to develop potential therapeutics to target astrocytes in disease.

More advanced cultures including the addition of other cell types from the brain (10, 33), *ex vivo* brain organotypic cultures (10, 49) or brain organoids (50) are excellent platforms since they recapitulate closer the *in vivo* situation. Importantly, when applying these more sophisticated *in vitro* approaches it was found that the antimetastatic behavior of RAs, occurring during the early stages of colonization *in vivo*, was reproduced (10, 49). Alternatively, novel methodologies based on immunopanning allow avoiding the default reactive state of this cell type in culture (45). Interspecies variability and cross-species differences in cell–cell interactions need to be considered when working with non-syngeneic *in vitro* or *in vivo* systems. Differences have been reported between murine and human astrocytes regarding different aspects of their biology including the complexity of arborization, calcium response properties and transcriptomic profiles (47). Consequently findings obtained with mouse astrocytes, require validation in human samples if knowledge generated is aimed to be translated in a bench-to-bedside manner.

Up to now, studies involving RAs *in situ* are based on fixed tissue samples that evaluate GFAP+ cells (9, 10, 20, 37, 51) and none of them have reported the use of engineered astrocytes *in vivo* in the context of brain metastasis. However, as the interest in brain metastasis-associated RAs is gaining momentum and their examination will presumably be expanded toward the use of available and widely validated tools such as genetically engineered mouse models (GEMMs) that could drive reporters and/or genes of interest (29, 52, 53) in astrocytes or alternative approaches such as adeno-associated virus that target astrocytes (54) as well as *in vivo* electroporation with Star Track technology (55). Such experimental resources will need to be combined with brain metastasis models in order to determine the impact of the modifications introduced in astrocytes in the process of brain colonization by cancer cells. Spontaneous brain metastases from orthotopic injections of cancer cells (injection in the organ source of the primary tumor from which brain metastasis models were established) or GEMM that develop primary tumors are rare events and difficult to study (18, 56, 57). In contrast, models in which brain metastases are induced upon inoculation of metastatic cells in the circulation (9–12, 58–61) are compatible to study the interaction between metastatic cells and RAs during brain colonization. In these models functional experiments to dissect these interactions can be performed and analyzed using a variety of techniques such as non-invasive molecular imaging, intravital imaging and detailed histology. Whether reported differences between mice and human astrocytes (47) are relevant in the context of brain metastasis will require specific validation of experimental findings in human samples. Given the broad diversity of brain metastasis models including different tumor types, oncogenomic profiles and different species of cancer cells (human and mouse), the use of several available experimental models to confirm potential mediators of the interaction between cancer cells and astrocytes will be a good strategy to reach relevant conclusions with higher possibilities to be translated to patients (**Table 1**).

Thus, a growing number of resources to study RAs will certainly help to understand the complexity underlying their reciprocity with cancer cells in brain metastasis.

ASTROCYTES AS A SOURCE OF SECRETED MOLECULES

Main findings related to reactive astrocytes in the context of brain metastasis usually include the secretory nature of this glial cell type (**Table 2**). Upon the first encounter with metastatic cells RAs produce plasminogen activators (PAs), including secreted tissue PA. PAs have the ability to transform plasminogen into the protease plasmin which is responsible for the elimination of many cancer cells that cross the BBB (10). Consequently, the secretory ability of RAs during the initial stages of colonization limit metastatic progression (**Figure 1**). However, few cancer cells produce anti-PA serpins and consequently block the antitumor program derived from RAs (10). Cancer cells with the ability to counteract the innate defense of RAs will continue colonizing the brain. Conversely, upon the development and growth of metastasis, RAs have been shown to generate a protumorigenic niche through various mechanisms involving secreted molecules. For instance, increased expression of *COX2* in brain metastatic cancer cells

TABLE 1 | Research goals for brain metastasis-associated reactive astrocytes.

1	Incorporate novel approaches to manipulate astrocytes *in vitro* (i.e., immunopanning) and *in vivo* (i.e., GEMM).
2	Apply unbiased genomic and proteomic analysis of reactive astrocytes (single cell level and population level) associated with brain metastasis from different primary sources (i.e., lung cancer, breast cancer, melanoma).
3	Comparison of brain metastasis-associated reactive astrocytes with those present in other brain injuries (i.e., primary brain tumors, neurodegenerative disorders, ischemia, traumatic brain injury, autoimmune disorders).
4	Identify and characterize specific subpopulations within brain metastasis-associated reactive astrocytes and evaluate their potential therapeutic implications.
5	Dissect the biology behind antimetastatic and prometastatic reactive astrocytes: Are they different subpopulations? Do they coexist in time? Could prometastatic reactive astrocytes be transformed into antimetastatic astrocytes?
6	Does systemic disease (primary tumor and extracranial metastases) influence the brain microenvironment acting on reactive astrocytes before metastases are established in the brain?
7	Do brain metastasis-associated reactive astrocytes influence systemic disease outside the brain and/or organismal homeostasis as shown in other brain disorders?
8	Could reactive astrocytes associated with brain metastasis be the source of biomarkers for early diagnosis or response to therapy?

TABLE 2 | Secreted molecules by brain metastasis-associated reactive astrocytes.

RA secreted molecules	Phenotype in cancer cells	Cancer type	Reference
ET-1	Induction of cancer cell growth and stemness through activation of MAPK and AKT	Breast and lung cancer	Kim et al. (35)
IL-23	Increase invassiveness by inducing MMP2	Melanoma	Klein et al. (65)
HGF/SCF	Induction of proangiogenic cytokines by activating c-Met	Breast cancer	Xing et al. (63).
BDNF	Induction of cancer cell growth through activation of TrKB-HER2	Breast cancer	Choy et al. (36)
CCL7	Induction of tumor initiating potential in cancer cells	Breast cancer	Wu et al. (62)
MMP2/MMP9	Increase invassiveness of cancer cells	Breast cancer	Wang et al. (67)
miR-19a	Induction of cancer cell growth by targeting PTEN	Breast cancer and melanoma	Zhang et al. (37)
Hyaluronic acid	Induction of cancer cell growth and stemness through activation of MAPK and AKT	Lung cancer	Stevens et al. (38)
IFNα/TNFα	Induction of cancer cell growth and chemoresistance by STAT1 and NFκB	Breast and lung cancer	Chen et al. (9)
PA-Plasmin-FasL	Decrease the viability of non-brain-adapted cancer cells	Breast and lung cancer	Valiente et al. (10)

(MDA231BrM) has been linked to astrocyte activation and the production of CCL7 by this glial cell type (62). Astrocyte-derived CCL7 was associated with an increase in CD24low-CD44high-ESAhigh subpopulation of MDA231BrM cells. Additional paracrine cytokine signaling loops between tumor-associated astrocytes and cancer cells in breast cancer brain metastasis have been described (**Table 2**). Astrocytes secrete hepatocyte growth factor/scatter factor (HGF/SF) under the influence of cancer cell-derived IL1β (**Figure 1**). Targeting this mutual c-Met-HGF crosstalk between cancer cells and tumor-associated astrocytes by using the BBB-permeable compound Pterostilbene, a resveratrol analog, diminished the stem-like cell phenotype dependent upon this feed-forward signaling loop both *in vitro* and *in vivo* (63). Another growth factor, namely BDNF, was suggested to be linked to the interplay between astrocytes and breast cancer cells in brain metastasis. Astrocyte-derived BDNF can favor cancer cell proliferation by engaging heterodimerization of both Her2/NEU and TrkB receptors *in vitro*. Importantly, inhibition of this crosstalk by means of knocking down TrkB in cancer cells abrogated brain metastasis *in vivo* (36). In line with these studies, a previous one reported that brain metastatic cancer cells significantly up regulate IL-1β, which again seems to be embedded in a mutual signaling loop between cancer cells and astrocytes. This was associated with a cancer cell-mediated activation of astrocytes, reflected by a heightened expression and production of astrocytic Jagged1 in a NFκβ-dependent manner (64) (**Figure 1**). Accordingly, the resulting paracrine interaction between Jagged1 + astrocytes and cancer cells was able to increase the stem-like phenotype in cancer cells *via* the Notch-Hes5 pathway (64). In the context of melanoma-to-brain metastasis evidences exist arguing about the ability of cancer cells to reprogramme astrocytes (understood as the induction of transcriptional modifications providing prometastatic functions). Cancer cells were able to induce the production of IL-23 in RAs. This proinflammatory cytokine was shown to be of importance in the up-regulation of cancer cell-derived MMP2, which in turn mediates invasiveness of brain metastatic melanoma cells *in vitro*. Blocking this paracrine interaction either by pharmacological inhibition of IL-23 or by knocking down cancer cell MMP2 resulted in inhibition of melanoma invasion *in vitro* (65). A recent study further supports a potential role of MMP2 in breast-to-brain metastasis, as it was found to belong to 5-gene expression signature (together with *CXCL12*, *MMP11*, *VCAM1*, *MME*) discriminating between primary breast cancer and breast cancer brain metastases (66). To sum up, it seems to be evident that cancer cells get assistance originating from astrocytes after hijacking those and/or transforming them into passive bystanders sustaining migration and growth as well as tumor-initiating capabilities of cancer cells (**Figure 2**).

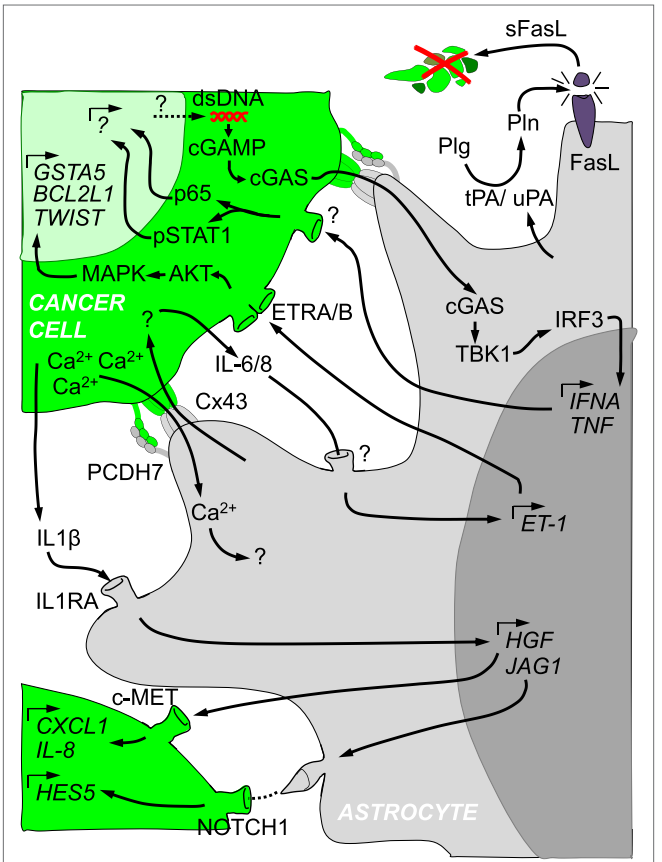
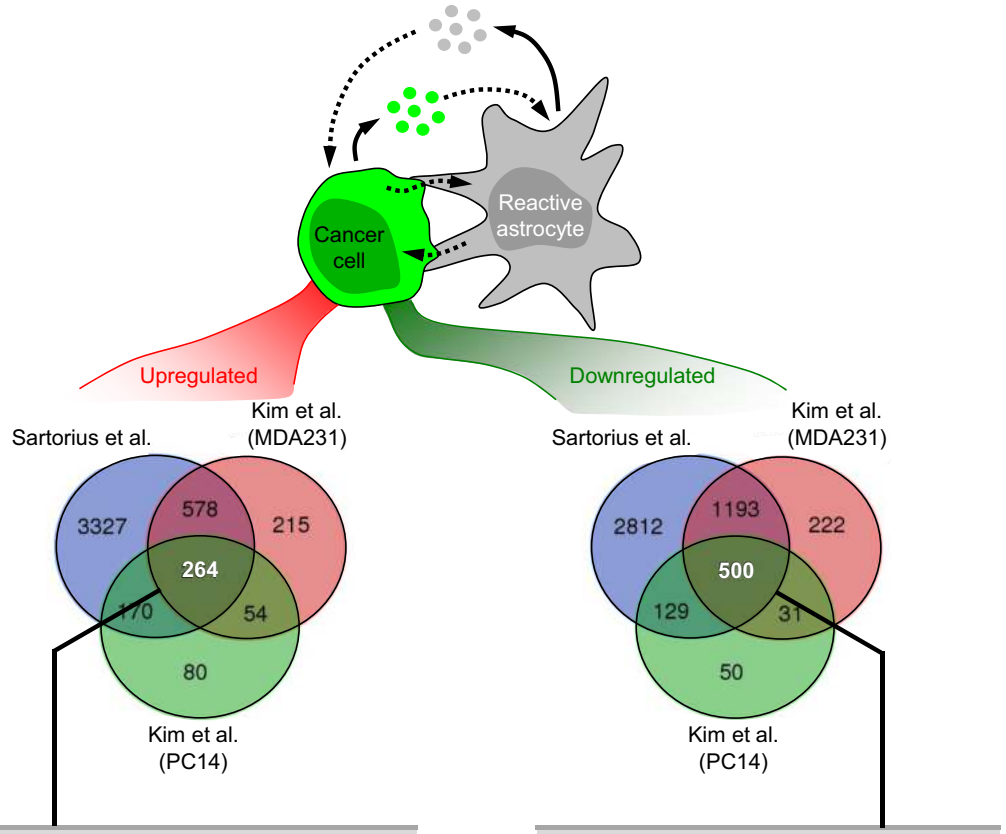


FIGURE 1 | Crosstalk between cancer cells and reactive astrocytes in brain metastasis. Cancer cells (in green) and astrocytes (in gray) are depicted with several of the molecular mechanisms described in their reciprocal crosstalk. The initial ability of reactive astrocytes to kill cancer cells through the production of Plasminogen activators is later modified into a supportive niche that involves secreted molecules, gap junctions, protocadherins, Notch receptor and ligands, among other components. Such a complex interactome influences each other cell type at the gene expression level.



GO terms	*p value*
tissue migration (GO:0090130)	*1.08E-05*
positive regulation of growth (GO:0045927)	*4.15E-05*
regeneration (GO:0031099)	*3.22E-06*
stem cell proliferation (GO:0072089)	*2.83E-04*
reg. of developmental growth (GO:0048638)	*1.98E-03*
positive regulation of cell division (GO:0051781)	*3.67E-03*

GO terms	*p value*
negative regulation of locomotion (GO:0040013)	*1.71E-12*
negative regulation of growth (GO:0045926)	*7.97E-09*
cell fate commitment (GO:0045165)	*1.29E-08*
aging (GO:0007568)	*1.31E-16*
negative regulation of growth (GO:0045926)	*7.97E-09*
reg. of extr. apoptotic sign. path. (GO: 2001236)	*7.57E-04*
reg. of intr. apoptotic sign. path. (GO:2001242)	*1.30E-03*

FIGURE 2 | Pathway analysis on the influence of astrocytes on cancer cells *in vitro*. Bioinformatic analysis of available datasets reporting transcriptome of cancer cells upon coculture with astrocytes (27, 68) allowed us to obtain commonly 264 upregulated and 500 downregulated pathways. Some of these pathways are shown. Reg, regulation; Extr, extrinsic; Intr, intrinsic; Sign, signaling; Path, pathway.

CANCER CELL–ASTROCYTE INTERACTIONS THROUGH GAP JUNCTIONS

Astrocytes form an interconnected network that allows signal transduction in a coordinated manner (69). Key players in this communication are gap junctions. Gap junctions are composed of connexins (Cxs). Out of the 21 reported Cxs, Cx43 (also referred to as GJA1) and Cx30 (GJB6) are the most abundant in the adult brain (69). In physiology, astrocytic gap junctions are required for proper neuronal activity, synaptic transmission, energy supply and control of blood flow (69). When astrocytes become reactive their functional and spatial domains can be altered which might also modify their connectivity (70). Interestingly, initially *in vitro* (27, 40) and later *in vivo* (9), brain metastatic cells have been shown to be able to establish gap junctions with RAs. Why do brain metastatic cells have developed this ability? The Fidler lab addressed whether besides secreted molecules from RAs additional interactions involving physical contact with cancer cells could benefit them (27, 40). Their rationale was based on the conspicuous proximity between some cancer cells from established metastasis and RAs *in vivo* (41). Their series of articles probed the physical interaction between them, its dependency on Cxs and the benefit it provided to cancer cells (27, 40). Through this cell–cell interaction, astrocytes induced the expression of 205 genes in different cancer cell lines from breast

and lung cancer (27). Although this gene program was expected to be Cx-dependent, the authors did not clarify this aspect since replicas including carbenoxolone, a gap junction inhibitor, or shRNA against Cx43 were not part of the transcriptomic profile. Hence, the resulting assumptions drawn from this gene list warrant further evaluation in respect to the dependency on Cx43. This concern was further enlarged given their findings by which calcein (a gap junction permeable molecular dye) is also transferred between fibroblasts and cancer cells although, in contrast to astrocytes, this cell type did not potentiate cancer cell survival in the presence of chemotherapies (27). Consequently, these results seem to be inconclusive regarding the dependence of the gene program induced in cancer cells by the influence of astrocytes. However, this point was partially clarified later when IL-6 and IL-8 production from cancer cells was shown to be dependent on the establishment of gap junctions with astrocytes (35). These cytokines influence both cancer cells and astrocytes, by inducing the expression of both endothelin receptors (ETAR and ETBR) on cancer cells and endothelin ligand (ET-1) on astrocytes (35) (**Figure 1**). Few of the initially deregulated genes upon cancer cell–astrocyte interaction were probed to be dependent on ET-1 (35). A number of these genes, including mesenchymal genes (*TWIST1),* inducers of resistance to stress (*GSTA5),* and antiapoptotic genes (*BCL2L1)*, were validated in human brain metastasis (35). Based on these findings, they provided evidence that chemotherapeutic drugs including paclitaxel, 5-fluorouracil (5-FU), and cisplatin killed less cancer cells than when the gap junction inhibitor carbenoxolone was added to cocultures or Cx43 was knocked down in astrocytes (27, 40). Yet, there were neither validations of the roles of these genes *in vivo* nor a molecular explanation on how gap junctions were established in the first place between the different cell types. Further, it remains to be seen whether the genes identified are main players in the brain metastasis phenotype. In contrast, the molecular mechanisms underlying the establishment of gap junctions between cancer cells and RAs has been recently reported (9). A gene list initially reported as commonly deregulated in brain tropic cancer cells from lung and breast cancer, included the protocadherin 7 (*PCDH7*) (10). Besides brain metastatic cells, *PCDH7* was also expressed in RAs *in vitro* and *in vivo* but not in other brain cell types (9). When *PCDH7* was downregulated from brain tropic cells, gap junction mediated transfer of calcein to RAs was severely impaired. Detailed analysis of the PCDH7-dependency of Cx43 mediated gap junction communication probed that the protocadherin is required to establish gap junctions between cancer cells and astrocytes (**Figure 1**). Once the gap junction channel connects both cell types, they exchange at least two types of molecules that have been described in brain metastasis: the ion Ca^{2+} and the secondary messenger cGAMP. Calcium is usually exchanged between astrocytes within the neural network to synchronize their activity and coordinate their responses under homeostatic conditions (69). Cancer cells from multiple brain metastastasis models were shown to co-opt gap junction communication with astrocytes to reduce their excessive calcium load (**Figure 1**). Excessive amounts of calcium could be detrimental for cancer cells since it is a known trigger of DNA damage and inducer of apoptosis (71). As a consequence, a decrease in the intracellular concentration of calcium seems to be a requirement to maintain an aggressive brain colonization pattern with marked resistance to chemotherapy. Intriguingly, use of gap junctions by cancer cells includes mechanisms reminiscent to antiviral cellular responses as shown previously (72, 73). Genomic instability is a frequent finding in advanced metastatic cancer (74) and as such has been reported in brain metastasis (75, 76). Although beneficial for cancer cells by boosting the generation of genetic variants that might be better fitted to colonize the brain, genomic instability also generates toxic byproducts such a double-stranded DNA (dsDNA) (77). Cytosolic dsDNA is sensed by cGAS which upon activation generates the second messenger cGAMP, driving an interferon response upon activation of STING (78). Transfer of cGAMP from cells infected with viruses to surrounding cells through Cx43 dependent gap junctions was described as a mechanism to prevent viral expansion (79). Hence, brain metastatic cells have managed to co-opt and utilize this ancient molecular mechanisms for their own benefit. Initiated by the transfer of the second messenger to RAs, cGAMP activates STING at the endoplasmic reticulum, which leads to TBK1 mediated phosphorylation of IRF3 as described in other cellular contexts (78). Thereby, phosphorylated IRF3 enters the nuclei where it induces the expression and secretion of TNFα and INFα. These two cytokines in turn can activate NFκβ and STAT1 in brain metastatic cells, which contributes to an increase in their proliferative potential and resistance to chemotherapeutic stress (9) (**Figure 1**). Interestingly, dsDNA is abundant in exosomes (80). Given the reported transfer of dsDNA between cells through exosomes in which the presence of Cx43 facilitates the entry into the recipient cell (81), additional mechanisms, which do not require juxtacrine, direct cell–cell contact, might also play a role *in vivo*.

INFLUENCE OF ASTROCYTES ON NON-CANCER CELLS

As delineated above, astrocytes, as the most abundant cell type confined to the CNS, will statistically (by means of localization) account for a majority of the interactions that brain-homing clones of cancer cells will be exposed to during early but also late stages of brain metastasis. However, other cell types of the brain metastasis environment such as endothelial cells, pericytes and resident microglia as well as incoming myeloid cells (i.e., macrophages) have been shown to interact and respond to the presence cancer cells (10, 17, 20, 22, 43, 58, 82, 83). Hence, astrocytes might also influence not only cancer cells but also other adjacent cell types in brain metastasis in a direct or indirect fashion. For example, astrocytes transfer exosome-enpacked microRNA-19a (miR-19a) to cancer cells. miR-19a silences the major tumor suppressor phosphatase and tensin homolog deleted on chromosome 10 (PTEN) in cancer cells. As a result of this interaction a more favorable adaptation of cancer cells to the new tissue environment is achieved by increasing their growth rate but also by inducing the secretion of the chemokine (C-C motif) ligand 2 (CCL2). Cancer cell secreted CCL2 participates in the generation of a protumorigenic niche by inducing an influx of brain metastasis-promoting Iba1+/CCR2+ myeloid cells. Importantly, higher CCL2 scores as determined by immunohistochemistry

were more frequently seen in brain metastatic tissue than in matched primary tumor tissue and additionally CCL2 expression correlated with PTEN loss in brain metastatic tissue (37). These results were corroborated by a more recent study (43). Although insights into astrocyte-mediated influence on other cell types in the context of brain metastasis is ill-defined, recent insights into phenotypic and genotypic signatures of astrocytes in other neurological diseases and other fields of neuroscience may aid in elucidating these potential implications in regard to brain metastasis (33). Emerging evidence has also reported the influence of RAs beyond the brain (84). Secretion of extracellular vesicles by RAs, including exosomes, could reach the systemic circulation in experimental models of inflammatory brain damage. Astrocyte-derived extracellular vesicles gain access to different organs (liver, lungs and spleen) where they induce an acute cytokine response characterized by the secretion of IL-17, IL-1β, IL-6, TNF-α, and CCL2. This acute cytokine response leads to the mobilization of Ly6b+ leukocytes that will infiltrate the brain to resolve the damage (84). Whether a similar mechanism is occurring in brain metastasis remains to be addressed. Thus, future studies in brain metastasis should consider not only the local influence of RAs but also their potential contribution to other symptoms which might contribute to the deterioration of patient health state during brain metastasis (**Table 1**).

In contrast to RAs in other disease conditions such as stroke or traumatic brain injury, brain metastasis is a continuously progressing insult (i.e., growth and evolution of cancer cells). In this given context astrocytes are unable to resolve the insult and over time cancer cells hijack some of their functions and prompting to astrocytes to work for their own benefit. Thus, astrocytes convert to a dubious fellow companion to cancer cells aiding them in remodeling their new habitat, potentially influencing the behavior of other CNS cell types.

ASTROCYTE HETEROGENEITY IN BRAIN METASTASIS

The brain is highly complex in respect to its cellular (and acellular) composition. The main cell type, the neuron, can be classified in two main classes (excitatory and inhibitory) and within them multiple subclasses are required to maintain the fine-tuning and wiring of neural circuits (85, 86). This complexity has remained exclusive to the neuronal compartment. However, evidence as to heterogeneity in non-neuronal components is steadily increasing. Recent findings have probed that subtypes of microglia reside in specific locations in the brain (87) which might be linked to subpopulations that emerge in and drive brain disorders of experimental models and humans (88). Astrocyte heterogeneity is of emerging interest given the potentially important implications in homeostasis (89–92) and disease (93–96). In brain metastasis in particular, a good body of evidence points toward opposite behaviors of astrocytes, which seem to be dependent on the disease stage. Initially, astrocytes act as a innate host defense system limiting the progression of the disease (10), while later on astrocytes favor it (9). Whether they belong to different subtypes of astrocytes or whether a consequence of the influence of cancer cells on them remains an issue of dispute. In view of the findings reported under homeostatic conditions and other CNS disorders, astrocytes are likely to include different subpopulations (48). Although heterogeneity in RAs associated with brain metastasis has not been formally probed, there are published observations that might be indicative of this possibility. During the colonization of the brain RAs surround brain metastatic cells (9, 10, 12, 20, 26). Besides GFAP other markers identifying this cell type have been reported in this glial cell type. However, these markers did not fully colocalize with each other, so that many GFAP+ RAs were negative for them, as in the case for Nestin. Nestin labels neural stem cells (97). The finding that Nestin is only present in a subset of RAs associated with brain metastasis (20) could suggest that heterogeneity among brain metastasis-associated astrocytes might have deeper implications at the functional level. In one study Xing et al. reported that Jagged1+ RAs were actively inducing Notch activity in brain metastatic cells (64). Again, the Jagged1 colocalization with GFAP was only partial (64), suggesting that within the population of RAs there could be also a Jagged1-subset as well. The same applies to endothelin receptor, which has been shown to be present in a heterogeneous pattern among RAs in the context of brain metastasis (98). Interestingly, endothelin receptor and Notch have been reported in reactive astrocytes in other brain injuries (95, 99). A more unambiguous example of the presence of RAs subpopulations associated with brain metastasis corresponds to the identification of p751-PDGFRβ+ astrocytes (100). Phosphorylation of Tyr751 was used to label this subpopulation of RAs associated with brain metastasis, preferentially located close to capillaries. This finding was expanded to human brain metastasis with breast and lung cancer. The inhibitor pazopanib, a multityrosine kinase inhibitor, including PDGFRβ, was used to evaluate the functional implications of this subpopulation. Pazopanib used *in vivo* in experimental brain metastasis models significantly prevented their development. Yet, given the unspecific inhibitory nature of this inhibitor and the previous report showing that another pazopanib target present in cancer cells was required for brain metastasis, makes it difficult to conclude about the potential involvement of p751-PDGFRβ+ RAs in brain metastasis. Authors probed that the phosphorylation of the PDGFRβ receptor in astrocytes was induced upon coculture with brain metastatic cancer cells, indicating that PDGFRβ+ RAs might represent a brain metastasis-specific subpopulation (**Table 1**).

Consequently, exploiting the molecular characterization of brain metastasis-associated RAs is an emerging area of research that will facilitate the understanding of their biology and which could also offer innovative ways to target this particular condition.

GENOMICS AND SIGNALING PATHWAYS IN BRAIN METASTASIS-ASSOCIATED ASTROCYTES

In contrast to existing examples in cancer cells (9, 40, 68) (**Figure 2**), there are no genomic data regarding RAs associated with brain metastasis. Instead, several publications have reported specific signaling pathways to be involved in the crosstalk (**Figure 1**). A reactive astrocytic phenotype observed in a melanoma brain metastasis model (51) was linked to an

earlier proposed gene signature for RAs in stroke or LPS treatment (32). Although the authors did not undertake an unbiased astrocyte-specific profiling in their experiments gliosis-related genes such as *Gfap, Cxcl10, Lcn-2, Serpina3n, Serpine1*, and *Timp-1* were significantly upregulated in the group of mice in which melanoma cells were coinjected together with astrocytes as compared to melanoma cells injected alone (51). Besides these gene expression changes additional signaling pathways have been reported (**Figure 1**) including Cx43/cGAS/TBK1/IRF3/IFNα. TNF (9), IL-6.IL-8/ET-1 (35), IL1B/IL1RA/HGF.JAG1 (63, 64). Increasing numbers of genomic studies on astrocytes are being performed in other neurological disorders (32, 52, 90) which will be an extraordinary repository for comparative analyses between different brain disorders to interrogate common and different aspects of the underlying biology of RAs in different scenarios as well as to evaluate the possibility to apply drug repurposing (**Table 1**).

ASTROCYTE-BASED THERAPIES

Although limited in number, some studies have tested the impact of targeting certain aspects of RAs associated with brain metastasis. Since these therapeutic efforts are aimed to block pro-metastatic components of the microenvironment, they have been applied to advanced stages of the disease. All of these preclinical studies have shown great potential thus opening the possibility of treating brain metastasis by targeting the microenvironment (9, 98, 100). In principle, these innovative therapies might be applied to a broader number of patients, given that all brain metastases harbour RAs associated independently of the source of the primary tumor. Such therapies might also involve less secondary effects, given that the target will not be attributed to normal brain tissue.

Macitentan

Macitentan is a FDA-approved BBB permeable inhibitor targeting endothelin receptor A and B (101) that is being used for treatment of pulmonary arterial hypertension. Macitentan has been repurposed to evaluate its potential effect in primary and secondary experimental brain tumor models (98, 102). Treatment of established experimental brain metastasis from lung (PC-14) and breast cancer (MDA231) in the preventive (micrometastasis) and interventional (macrometastasis) settings dramatically reduced brain metastasis and increased survival in mice, but only when combined with chemotherapy (Paclitaxel). Since these receptors are also present in cancer cells and endothelial cells, the therapeutic benefit cannot be assigned to targeting RAs alone. Nevertheless, given the contribution of astrocytes to endothelin receptor signaling (35) a part of it might be derived by the inhibitory effect in astrocytes. Although Macitentan alone induced a massive reduction in pAKT and pMAPK, this did not translate into a detectable phenotype with non-invasive bioluminescence monitoring. However, combination with Paclitaxel dramatically decreased the number of tumor-associated vessels, limiting the access of nutrients to cancer cells, which suffer from massive induction in cleaved caspase 3. Interestingly, initially described genes upregulated in cancer cells upon coculture with astrocytes (*BCL2L1*, *GSTA5*, and *TWIST1*) (27) were downregulated by Macitentan alone.

Given the finding of a similar phenotype in glioma models, a key contribution of the endothelin axis in brain tumors seems to be probable. The clinical trial initiated in recurrent glioma based on these findings (NCT01499251) was concluded recently and results should be publicly available soon. If positive results being reported, this therapeutic effort should be extended to brain metastasis patients.

Gap Junction Inhibitors

Gap junction intercellular communication (GJIC) is more and more seen as a potential target in different disease conditions such as different heart pathologies, seizures and cancer (103). There is a good amount of *in vitro* studies dedicated toward the characterization of pharmacological modulators of GJIC either *via* acute uncoupling or enhancement of signaling or their influence on gene expression, biosynthesis and turnover (103). Yet, until recently there have been only a few studies published on the potential usefulness of inhibition of GJIC in the setting of brain metastasis (27, 40). Initial work probed the protective effect of astrocytes against different chemotherapeutic drugs used in the clinic (paclitaxel, cisplatin, and 5-FU) on different melanoma cell lines *in vitro* (40). Inhibition of GJC channels pharmacologically by using the pan-Cx inhibitor carbenoloxone (CBX) or genetically by knocking down gap junctions in astrocytes during coculturing of cancer cells with astrocytes was able to render cancer cells chemosensitive (40). The therapeutic value of targeting gap junctions in experimental brain metastasis models was recently reported (9). Instead of CBX the authors used two drugs for this purpose: the anti-inflammatory compound meclofenamate, which was previously shown to inhibit Cx43 gap junction gating (104), and the benzopyrane derivative tonabersat, which was previously shown to have specific activity for binding to astrocytes and inhibit gap-junction-mediated processes (105–107). Both were used in brain-related disorders before (107, 108) and are FDA approved. The use of either meclofenamate or tonabersat in breast or lung brain metastasis from human or mouse cancer cells induced a significant decrease in brain tumor burden even after metastatic cells have seed and grew in this organ (9). Interestingly, given the brain specific mechanism targeted, none of the drugs show any effect when applied to orthotopic injections in the breast or in the lung (9). Based on these results an ongoing clinical trial (NCT02429570) has been launched to apply meclofenamate to recurring or progressing brain metastasis from multiple primary tumors.

Pazopanib

This orally bioavailable multiple tyrosine kinase inhibitor targeting VEGFR1–3, PDGFRα-β, c-kit, and B-Raf was initially found to target cancer cells in experimental HER2+ brain metastasis models (109). Later it was found that it also decreases tyrosine phosphorylation of PDGFRβ in RAs present in lung and breast cancer brain metastasis, as well as from astrocyte primary cultures obtained from craniotomies of five patients with brain metastases. As with Macitentan, the specific contribution of inhibition of astrocyte PDGFRβ receptor is not known. Thus, in

order to conclude about the contribution of this therapy, more specific genetic strategies targeting PDGFRβ in RAs are necessary. However, the ability of pazopanib to decrease the phosphorylation of the PDGFRβ receptor in astrocytes was correlated with reduced proliferative capacity of an immortalized astrocyte cell line (100). *In vivo*, targeting of Tyr phosphorylation of PDGFRβ did not decrease the GFAP population of RAs suggesting that pazopanib targets PDGFRβ+ RAs without killing them.

Compound E

As brain metastatic cancer cells co-opt physiological pathways to adapt to and to thrive within the CNS, the same applies to the acquisition of a stemness phenotype (64). RAs are involved in the regulation of the cancer stem-like cell phenotype in a Notch-dependent manner (64). Subsequently, Compound E, a gamma secretase inhibitor, has shown to impair the interaction between the astrocytic Jagged1 and cancer cells expressing Notch (64). When Compound E was administered to mice injected with a triple negative breast cancer model metastatic to the brain (MDA231-BrM) a significant decrease in the growth of cancer cells was observed (64).

Lenalidomide

Currently, no predictive biomarkers are clinically available to help in identifying those cancer patients which likely will experience brain metastasis during the course of their disease. Ongoing efforts to identify brain metastasis biomarkers found FN14 as a gene differentially expressed in brain metastasis as compared to primary tumors (110). This finding was later validated in a multicenter study involving 318 breast cancer patients, in which 138 developed brain metastases (13). Intriguingly, the presence of FN14, a TNFR family receptor member, in primary breast tumors was associated with a 5.24-fold increase in brain metastasis incidence (13). FN14 ligands include astrocyte growth factors TWEAK and TNF-alpha, where the former is mainly produced by astrocytes and microglial cells in the CNS (13, 111). Tackling FN14/TWEAK axis by using the thalidomide derivative lenalidomide (LND) impaired brain metastasis presumably through an effect on RA reactivity (13). Based on its anti-inflammatory properties demonstrated in other CNS conditions such as multiple sclerosis and the relative success of lenalidomide used in the framework of a first line regimen for multiple myeloma, upcoming studies should verify the reported beneficial effect in targeting brain metastasis, possibly in combination with other first line drugs.

DISCUSSION

Whilst brain metastasis remains a major threat to cancer patients, its annual incidence being on the rise, extracranial disease is becoming targeted more and more efficiently owing to the advent of molecular therapeutics as exemplified most recently by immune checkpoint inhibitors (112). New insights deriving from *in vitro* and *in vivo* preclinical models of brain metastasis have enabled researchers to have a more precise picture of the biology of coevolution of brain colonizing cancer cells and their surrounding microenvironment, *per se* offering ways for therapeutic exploitation. By analogy with insights derived from the field of neuroinflammation and neurodegeneration, where RAs are increasingly seen as main disease elements by means of modulating neurotoxic effects *via* non-cell autonomous mechanisms, more in-depth and comprehensive characterization of the RA phenotype on a genomic or proteomic scale in brain metastasis [employing techniques such as population or single cell RNA-seq, TRAP, or MS-based proteome analysis (32, 33, 52, 90)] might soon become a reality and enable researchers to validate potential candidates in brain metastasis models. It would be interesting to determine whether there is a difference, and if yes to what extent RAs found in brain metastasis distinguish from their counterparts seen in other disease conditions (32, 33, 52). Additionally, insights into the role of specific astrocyte subpopulations, their evolution during disease progression as well as their manipulation would provide a valuable means of targeting astrocyte-cancer cell interactions. Importantly, one must ask whether there is any specific therapeutic window as to which time point during brain metastasis might represent the most effective way of modulating and targeting this vicious cross-talk or even promote the antimetastatic behavior of RAs. Taken together, insights gained from recent research have undoubtedly put astrocytes in brain metastasis into perspective turning them from passive bystanders to active key players in brain metastasis.

In addition to that, new avenues of research will allow studying RAs in brain metastasis on a more systemic scale, an aspect which is believed to be underestimated in the context of gliosis in other CNS diseases such as infectious diseases, where proinflammatory molecules released from peripheral tissue sites of infection likely influence far-distant RAs (28). Interestingly, emerging studies have proposed the influence of the primary tumor on the brain environment facilitating the colonization of cancer cells (113, 114). The systemic influence of RAs in models of neuroinflammation has been also reported (84, 115). Given the strong response of RAs to brain metastasis during the course of brain colonization, it is temping to speculate that the identification of secreted molecules might represent putative biomarkers of early diagnosis or response to therapy, even more if specific prometastatic subpopulations of RAs could be identified to be targeted.

In sum, RAs are largely involved in reciprocal interactions with metastatic cells and govern distinct cancer cell phenotypic features required during the process of colonization such as invasive capacity, survival and stemness. The focus of future studies will likely shift toward the specific makeup of the brain microenvironment appreciating its complexity and heterogeneity as well as its role to serve as a putative future therapeutic target in combating brain metastasis more efficiently and successfully (**Table 1**).

AUTHOR CONTRIBUTIONS

MV, NP, and DW conceptualized the work and wrote the manuscript. CF-T performed the bioinformatic analysis.

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REFERENCES

1. Gavrilovic IT, Posner JB. Brain metastases: epidemiology and pathophysiology. *J Neurooncol* (2005) 75:5–14. doi:10.1007/s11060-004-8093-6
2. Boire A, Zou Y, Shieh J, Macalinao DG, Pentsova E, Massagué J. Complement component 3 adapts the cerebrospinal fluid for leptomeningeal metastasis. *Cell* (2017) 168:1101–13.e13. doi:10.1016/j.cell.2017.02.025
3. Maher EA, Mietz J, Arteaga CL, DePinho RA, Mohla S. Brain metastasis: opportunities in basic and translational research. *Cancer Res* (2009) 69: 6015–20. doi:10.1158/0008-5472.CAN-08-4347
4. Costa DB, Shaw AT, Ou S-HI, Solomon BJ, Riely GJ, Ahn M-J, et al. Clinical experience with crizotinib in patients with advanced ALK-rearranged non-small-cell lung cancer and brain metastases. *J Clin Oncol* (2015) 33:1881–8. doi:10.1200/JCO.2014.59.0539
5. Steeg PS, Camphausen KA, Smith QR. Brain metastases as preventive and therapeutic targets. *Nat Rev Cancer* (2011) 11:352–63. doi:10.1038/nrc3053
6. Osswald M, Blaes J, Liao Y, Solecki G, Gömmel M, Berghoff AS, et al. Impact of blood-brain barrier integrity on tumor growth and therapy response in brain metastases. *Clin Cancer Res* (2016) 22:6078–87. doi:10.1158/1078-0432.CCR-16-1327
7. Stemmler H-J, Schmitt M, Willems A, Bernhard H, Harbeck N, Heinemann V. Ratio of trastuzumab levels in serum and cerebrospinal fluid is altered in HER2-positive breast cancer patients with brain metastases and impairment of blood-brain barrier. *Anticancer Drugs* (2007) 18:23–8. doi:10.1097/01.cad.0000236313.50833.ee
8. Eichler AF, Chung E, Kodack DP, Loeffler JS, Fukumura D, Jain RK. The biology of brain metastases-translation to new therapies. *Nat Rev Clin Oncol* (2011) 8:344–56. doi:10.1038/nrclinonc.2011.58
9. Chen Q, Boire A, Jin X, Valiente M, Er EE, Lopez-Soto A, et al. Carcinoma-astrocyte gap junctions promote brain metastasis by cGAMP transfer. *Nature* (2016) 533:493–8. doi:10.1038/nature18268
10. Valiente M, Obenauf AC, Jin X, Chen Q, Zhang XH-F, Lee DJ, et al. Serpins promote cancer cell survival and vascular co-option in brain metastasis. *Cell* (2014) 156:1002–16. doi:10.1016/j.cell.2014.01.040
11. Bos PD, Zhang XH-F, Nadal C, Shu W, Gomis RR, Nguyen DX, et al. Genes that mediate breast cancer metastasis to the brain. *Nature* (2009) 459:1005–9. doi:10.1038/nature08021
12. Nguyen DX, Chiang AC, Zhang XH-F, Kim JY, Kris MG, Ladanyi M, et al. WNT/TCF signaling through LEF1 and HOXB9 mediates lung adenocarcinoma metastasis. *Cell* (2009) 138:51–62. doi:10.1016/j.cell.2009.04.030
13. Martínez-Aranda A, Hernández V, Guney E, Muixí L, Foj R, Baixeras N, et al. FN14 and GRP94 expression are prognostic/predictive biomarkers of brain metastasis outcome that open up new therapeutic strategies. *Oncotarget* (2015) 6:44254–73. doi:10.18632/oncotarget.5471
14. Li B, Wang C, Zhang Y, Zhao XY, Huang B, Wu PF, et al. Elevated PLGF contributes to small-cell lung cancer brain metastasis. *Oncogene* (2013) 32:2952–62. doi:10.1038/onc.2012.313
15. Jilaveanu LB, Parisi F, Barr ML, Zito CR, Cruz-Munoz W, Kerbel RS, et al. PLEKHA5 as a biomarker and potential mediator of melanoma brain metastasis. *Clin Cancer Res* (2015) 21:2138–47. doi:10.1158/1078-0432.CCR-14-0861
16. Wrage M, Hagmann W, Kemming D, Uzunoglu FG, Riethdorf S, Effenberger K, et al. Identification of HERC5 and its potential role in NSCLC progression. *Int J Cancer* (2015) 136:2264–72. doi:10.1002/ijc.29298
17. Sevenich L, Bowman RL, Mason SD, Quail DF, Rapaport F, Elie BT, et al. Analysis of tumour- and stroma-supplied proteolytic networks reveals a brain-metastasis-promoting role for cathepsin S. *Nat Cell Biol* (2014) 16:876–88. doi:10.1038/ncb3011
18. Cho JH, Robinson JP, Arave RA, Burnett WJ, Kircher DA, Chen G, et al. AKT1 activation promotes development of melanoma metastases. *Cell Rep* (2015) 13:898–905. doi:10.1016/j.celrep.2015.09.057
19. Kienast Y, von Baumgarten L, Fuhrmann M, Klinkert WEF, Goldbrunner R, Herms J, et al. Real-time imaging reveals the single steps of brain metastasis formation. *Nat Med* (2010) 16:116–22. doi:10.1038/nm.2072
20. Lorger M, Felding-Habermann B. Capturing changes in the brain microenvironment during initial steps of breast cancer brain metastasis. *Am J Pathol* (2010) 176:2958–71. doi:10.2353/ajpath.2010.090838
21. Pukrop T, Dehghani F, Chuang H-N, Lohaus R, Bayanga K, Heermann S, et al. Microglia promote colonization of brain tissue by breast cancer cells in a Wnt-dependent way. *Glia* (2010) 58:1477–89. doi:10.1002/glia.21022
22. Louie E, Chen XF, Coomes A, Ji K, Tsirka S, Chen EI. Neurotrophin-3 modulates breast cancer cells and the microenvironment to promote the growth of breast cancer brain metastasis. *Oncogene* (2013) 32:4064–77. doi:10.1038/onc.2012.417
23. Brantley EC, Guo L, Zhang C, Lin Q, Yokoi K, Langley RR, et al. Nitric oxide-mediated tumoricidal activity of murine microglial cells. *Transl Oncol* (2010) 3:380–8. doi:10.1593/tlo.10208
24. Sofroniew MV. Molecular dissection of reactive astrogliosis and glial scar formation. *Trends Neurosci* (2009) 32:638–47. doi:10.1016/j.tins.2009.08.002
25. Liddelow SA, Barres BA. Reactive astrocytes: production, function, and therapeutic potential. *Immunity* (2017) 46:957–67. doi:10.1016/j.immuni.2017.06.006
26. Fitzgerald DP, Palmieri D, Hua E, Hargrave E, Herring JM, Qian Y, et al. Reactive glia are recruited by highly proliferative brain metastases of breast cancer and promote tumor cell colonization. *Clin Exp Metastasis* (2008) 25:799–810. doi:10.1007/s10585-008-9193-z
27. Kim S-J, Kim J-S, Park ES, Lee J-S, Lin Q, Langley RR, et al. Astrocytes upregulate survival genes in tumor cells and induce protection from chemotherapy. *Neoplasia* (2011) 13:286–98. doi:10.1593/neo.11112
28. Burda JE, Sofroniew MV. Reactive gliosis and the multicellular response to CNS damage and disease. *Neuron* (2014) 81:229–48. doi:10.1016/j.neuron.2013.12.034
29. Wanner IB, Anderson MA, Song B, Levine J, Fernandez A, Gray-Thompson Z, et al. Glial scar borders are formed by newly proliferated, elongated astrocytes that interact to corral inflammatory and fibrotic cells via STAT3-dependent mechanisms after spinal cord injury. *J Neurosci* (2013) 33:12870–86. doi:10.1523/JNEUROSCI.2121-13.2013
30. Silver J, Miller JH. Regeneration beyond the glial scar. *Nat Rev Neurosci* (2004) 5:146–56. doi:10.1038/nrn1326
31. Hara M, Kobayakawa K, Ohkawa Y, Kumamaru H, Yokota K, Saito T, et al. Interaction of reactive astrocytes with type I collagen induces astrocytic scar formation through the integrin-N-cadherin pathway after spinal cord injury. *Nat Med* (2017) 23:818–28. doi:10.1038/nm.4354
32. Zamanian JL, Xu L, Foo LC, Nouri N, Zhou L, Giffard RG, et al. Genomic analysis of reactive astrogliosis. *J Neurosci* (2012) 32:6391–410. doi:10.1523/JNEUROSCI.6221-11.2012
33. Liddelow SA, Guttenplan KA, Clarke LE, Bennett FC, Bohlen CJ, Schirmer L, et al. Neurotoxic reactive astrocytes are induced by activated microglia. *Nature* (2017) 541:481–7. doi:10.1038/nature21029
34. Ransohoff RM. A polarizing question: do M1 and M2 microglia exist? *Nat Neurosci* (2016) 19:987–91. doi:10.1038/nn.4338
35. Kim SW, Choi HJ, Lee H-J, He J, Wu Q, Langley RR, et al. Role of the endothelin axis in astrocyte- and endothelial cell-mediated chemoprotection of cancer cells. *Neuro Oncol* (2014) 16:1585–98. doi:10.1093/neuonc/nou128
36. Choy C, Ansari KI, Neman J, Hsu S, Duenas MJ, Li H, et al. Cooperation of neurotrophin receptor TrkB and Her2 in breast cancer cells facilitates brain metastases. *Breast Cancer Res* (2017) 19:51. doi:10.1186/s13058-017-0844-3
37. Zhang L, Zhang S, Yao J, Lowery FJ, Zhang Q, Huang W-C, et al. Microenvironment-induced PTEN loss by exosomal microRNA primes brain metastasis outgrowth. *Nature* (2015) 527:100–4. doi:10.1038/nature15376
38. Stevens LE, Cheung WK, Adua SJ, Arnal-Estapé A, Zhao M, Liu Z, et al. Extracellular matrix receptor expression in subtypes of lung adenocarcinoma

potentiates outgrowth of micrometastases. *Cancer Res* (2017) 77(8):1905–17. doi:10.1158/0008-5472.CAN-16-1978
39. Seike T, Fujita K, Yamakawa Y, Kido MA, Takiguchi S, Teramoto N, et al. Interaction between lung cancer cells and astrocytes via specific inflammatory cytokines in the microenvironment of brain metastasis. *Clin Exp Metastasis* (2011) 28:13–25. doi:10.1007/s10585-010-9354-8
40. Lin Q, Balasubramanian K, Fan D, Kim S-J, Guo L, Wang H, et al. Reactive astrocytes protect melanoma cells from chemotherapy by sequestering intracellular calcium through gap junction communication channels. *Neoplasia* (2010) 12:748–54. doi:10.1593/neo.10602
41. Langley RR, Fan D, Guo L, Zhang C, Lin Q, Brantley EC, et al. Generation of an immortalized astrocyte cell line from H-2Kb-tsA58 mice to study the role of astrocytes in brain metastasis. *Int J Oncol* (2009) 35:665–72. doi:10.3892/ijo_00000378
42. Mendes O, Kim H-T, Lungu G, Stoica G. MMP2 role in breast cancer brain metastasis development and its regulation by TIMP2 and ERK1/2. *Clin Exp Metastasis* (2007) 24:341–51. doi:10.1007/s10585-007-9071-0
43. Hohensee I, Chuang H-N, Grottke A, Werner S, Schulte A, Horn S, et al. PTEN mediates the cross talk between breast and glial cells in brain metastases leading to rapid disease progression. *Oncotarget* (2017) 8:6155–68. doi:10.18632/oncotarget.14047
44. McCarthy KD, de Vellis J. Preparation of separate astroglial and oligodendroglial cell cultures from rat cerebral tissue. *J Cell Biol* (1980) 85:890–902. doi:10.1083/jcb.85.3.890
45. Foo LC, Allen NJ, Bushong EA, Ventura PB, Chung W-S, Zhou L, et al. Development of a method for the purification and culture of rodent astrocytes. *Neuron* (2011) 71:799–811. doi:10.1016/j.neuron.2011.07.022
46. Soreq L; UK Brain Expression Consortium, North American Brain Expression Consortium, Rose J, Soreq E, Hardy J, et al. Major shifts in glial regional identity are a transcriptional hallmark of human brain aging. *Cell Rep* (2017) 18:557–70. doi:10.1016/j.celrep.2016.12.011
47. Zhang Y, Sloan SA, Clarke LE, Caneda C, Plaza CA, Blumenthal PD, et al. Purification and characterization of progenitor and mature human astrocytes reveals transcriptional and functional differences with mouse. *Neuron* (2016) 89:37–53. doi:10.1016/j.neuron.2015.11.013
48. Anderson MA, Ao Y, Sofroniew MV. Heterogeneity of reactive astrocytes. *Neurosci Lett* (2014) 565:23–9. doi:10.1016/j.neulet.2013.12.030
49. Chuang H-N, van Rossum D, Sieger D, Siam L, Klemm F, Bleckmann A, et al. Carcinoma cells misuse the host tissue damage response to invade the brain. *Glia* (2013) 61:1331–46. doi:10.1002/glia.22518
50. Dezonne RS, Sartore RC, Nascimento JM, Saia-Cereda VM, Romão LF, Alves-Leon SV, et al. Derivation of functional human astrocytes from cerebral organoids. *Sci Rep* (2017) 7:45091. doi:10.1038/srep45091
51. Schwartz H, Blacher E, Amer M, Livneh N, Abramovitz L, Klein A, et al. Incipient melanoma brain metastases instigate astrogliosis and neuroinflammation. *Cancer Res* (2016) 76:4359–71. doi:10.1158/0008-5472.CAN-16-0485
52. Anderson MA, Burda JE, Ren Y, Ao Y, O'Shea TM, Kawaguchi R, et al. Astrocyte scar formation aids central nervous system axon regeneration. *Nature* (2016) 532:195–200. doi:10.1038/nature17623
53. Srinivasan R, Lu T-Y, Chai H, Xu J, Huang BS, Golshani P, et al. New transgenic mouse lines for selectively targeting astrocytes and studying calcium signals in astrocyte processes in situ and in vivo. *Neuron* (2016) 92:1181–95. doi:10.1016/j.neuron.2016.11.030
54. Deverman BE, Pravdo PL, Simpson BP, Kumar SR, Chan KY, Banerjee A, et al. Cre-dependent selection yields AAV variants for widespread gene transfer to the adult brain. *Nat Biotechnol* (2016) 34:204–9. doi:10.1038/nbt.3440
55. Figueres-Oñate M, García-Marqués J, López-Mascaraque L. UbC-StarTrack, a clonal method to target the entire progeny of individual progenitors. *Sci Rep* (2016) 6:33896. doi:10.1038/srep33896
56. Kato M, Takahashi M, Akhand AA, Liu W, Dai Y, Shimizu S, et al. Transgenic mouse model for skin malignant melanoma. *Oncogene* (1998) 17:1885–8. doi:10.1038/sj.onc.1202077
57. Meuwissen R, Linn SC, Linnoila RI, Zevenhoven J, Mooi WJ, Berns A. Induction of small cell lung cancer by somatic inactivation of both Trp53 and Rb1 in a conditional mouse model. *Cancer Cell* (2003) 4:181–9. doi:10.1016/S1535-6108(03)00220-4
58. Lyle LT, Lockman PR, Adkins CE, Mohammad AS, Sechrest E, Hua E, et al. Alterations in pericyte subpopulations are associated with elevated blood-tumor barrier permeability in experimental brain metastasis of breast cancer. *Clin Cancer Res* (2016) 22:5287–99. doi:10.1158/1078-0432.CCR-15-1836
59. Pencheva N, Buss CG, Posada J, Merghoub T, Tavazoie SF. Broad-spectrum therapeutic suppression of metastatic melanoma through nuclear hormone receptor activation. *Cell* (2014) 156:986–1001. doi:10.1016/j.cell.2014.01.038
60. Morsi A, Gaziel-Sovran A, Cruz-Munoz W, Kerbel RS, Golfinos JG, Hernando E, et al. Development and characterization of a clinically relevant mouse model of melanoma brain metastasis. *Pigment Cell Melanoma Res* (2013) 26:743–5. doi:10.1111/pcmr.12114
61. Malladi S, Macalinao DG, Jin X, He L, Basnet H, Zou Y, et al. Metastatic latency and immune evasion through autocrine inhibition of WNT. *Cell* (2016) 165:45–60. doi:10.1016/j.cell.2016.02.025
62. Wu K, Fukuda K, Xing F, Zhang Y, Sharma S, Liu Y, et al. Roles of the cyclooxygenase 2 matrix metalloproteinase 1 pathway in brain metastasis of breast cancer. *J Biol Chem* (2015) 290:9842–54. doi:10.1074/jbc.M114.602185
63. Xing F, Liu Y, Sharma S, Wu K, Chan MD, Lo H-W, et al. Activation of the c-Met pathway mobilizes an inflammatory network in the brain microenvironment to promote brain metastasis of breast cancer. *Cancer Res* (2016) 76:4970–80. doi:10.1158/0008-5472.CAN-15-3541
64. Xing F, Kobayashi A, Okuda H, Watabe M, Pai SK, Pandey PR, et al. Reactive astrocytes promote the metastatic growth of breast cancer stem-like cells by activating Notch signalling in brain. *EMBO Mol Med* (2013) 5:384–96. doi:10.1002/emmm.201201623
65. Klein A, Schwartz H, Sagi-Assif O, Meshel T, Izraely S, Ben Menachem S, et al. Astrocytes facilitate melanoma brain metastasis via secretion of IL-23. *J Pathol* (2015) 236:116–27. doi:10.1002/path.4509
66. Lee JY, Park K, Lee E, Ahn T, Jung HH, Lim SH, et al. Gene expression profiling of breast cancer brain metastasis. *Sci Rep* (2016) 6:28623. doi:10.1038/srep28623
67. Wang L, Cossette SM, Rarick KR, Gershan J, Dwinell MB, Harder DR, et al. Astrocytes directly influence tumor cell invasion and metastasis in vivo. *PLoS One* (2013) 8(12):e80933. doi:10.1371/journal.pone.0080933
68. Sartorius CA, Hanna CT, Gril B, Cruz H, Serkova NJ, Huber KM, et al. Estrogen promotes the brain metastatic colonization of triple negative breast cancer cells via an astrocyte-mediated paracrine mechanism. *Oncogene* (2016) 35:2881–92. doi:10.1038/onc.2015.353
69. Giaume C, Koulakoff A, Roux L, Holcman D, Rouach N. Astroglial networks: a step further in neuroglial and gliovascular interactions. *Nat Rev Neurosci* (2010) 11:87–99. doi:10.1038/nrn2757
70. Oberheim NA, Tian G-F, Han X, Peng W, Takano T, Ransom B, et al. Loss of astrocytic domain organization in the epileptic brain. *J Neurosci* (2008) 28:3264–76. doi:10.1523/JNEUROSCI.4980-07.2008
71. Roderick HL, Cook SJ. Ca2+ signalling checkpoints in cancer: remodelling Ca2+ for cancer cell proliferation and survival. *Nat Rev Cancer* (2008) 8:361–75. doi:10.1038/nrc2374
72. Gentili M, Kowal J, Tkach M, Satoh T, Lahaye X, Conrad C, et al. Transmission of innate immune signaling by packaging of cGAMP in viral particles. *Science* (2015) 349:1232–6. doi:10.1126/science.aab3628
73. Bridgeman A, Maelfait J, Davenne T, Partridge T, Peng Y, Mayer A, et al. Viruses transfer the antiviral second messenger cGAMP between cells. *Science* (2015) 349:1228–32. doi:10.1126/science.aab3632
74. Negrini S, Gorgoulis VG, Halazonetis TD. Genomic instability – an evolving hallmark of cancer. *Nat Rev Mol Cell Biol* (2010) 11:220–8. doi:10.1038/nrm2858
75. Woditschka S, Evans L, Duchnowska R, Reed LT, Palmieri D, Qian Y, et al. DNA double-strand break repair genes and oxidative damage in brain metastasis of breast cancer. *J Natl Cancer Inst* (2014) 106:1–13. doi:10.1093/jnci/dju145
76. Brastianos PK, Carter SL, Santagata S, Cahill DP, Taylor-Weiner A, Jones RT, et al. Genomic characterization of brain metastases reveals branched evolution and potential therapeutic targets. *Cancer Discov* (2015) 5:1164–77. doi:10.1158/2159-8290.CD-15-0369
77. Srivastava M, Raghavan SC. DNA double-strand break repair inhibitors as cancer therapeutics. *Chem Biol* (2015) 22:17–29. doi:10.1016/j.chembiol.2014.11.013
78. Wu J, Sun L, Chen X, Du F, Shi H, Chen C, et al. Cyclic GMP-AMP is an endogenous second messenger in innate immune signaling by cytosolic DNA. *Science* (2013) 339:826–30. doi:10.1126/science.1229963
79. Ablasser A, Schmid-Burgk JL, Hemmerling I, Horvath GL, Schmidt T, Latz E, et al. Cell intrinsic immunity spreads to bystander cells via the

intercellular transfer of cGAMP. *Nature* (2013) 503:530–4. doi:10.1038/nature12640
80. Kalluri R, LeBleu VS. Discovery of double-stranded genomic DNA in circulating exosomes. *Cold Spring Harb Symp Quant Biol* (2016) 81:275–80. doi:10.1101/sqb.2016.81.030932
81. Soares AR, Martins-Marques T, Ribeiro-Rodrigues T, Ferreira JV, Catarino S, Pinho MJ, et al. Gap junctional protein Cx43 is involved in the communication between extracellular vesicles and mammalian cells. *Sci Rep* (2015) 5:13243. doi:10.1038/srep13243
82. Rippaus N, Taggart D, Williams J, Andreou T, Wurdak H, Wronski K, et al. Metastatic site-specific polarization of macrophages in intracranial breast cancer metastases. *Oncotarget* (2016) 7:41473–87. doi:10.18632/oncotarget.9445
83. Siam L, Bleckmann A, Chaung H-N, Mohr A, Klemm F, Barrantes-Freer A, et al. The metastatic infiltration at the metastasis/brain parenchyma-interface is very heterogeneous and has a significant impact on survival in a prospective study. *Oncotarget* (2015) 6:29254–67. doi:10.18632/oncotarget.4201
84. Dickens AM, Tovar-Y-Romo LB, Yoo S-W, Trout AL, Bae M, Kanmogne M, et al. Astrocyte-shed extracellular vesicles regulate the peripheral leukocyte response to inflammatory brain lesions. *Sci Signal* (2017) 10(473):eaai7696. doi:10.1126/scisignal.aai7696
85. Custo Greig LF, Woodworth MB, Galazo MJ, Padmanabhan H, Macklis JD. Molecular logic of neocortical projection neuron specification, development and diversity. *Nat Rev Neurosci* (2013) 14:755–69. doi:10.1038/nrn3586
86. DeFelipe J, López-Cruz PL, Benavides-Piccione R, Bielza C, Larrañaga P, Anderson S, et al. New insights into the classification and nomenclature of cortical GABAergic interneurons. *Nat Rev Neurosci* (2013) 14:202–16. doi:10.1038/nrn3444
87. Prinz M, Erny D, Hagemeyer N. Ontogeny and homeostasis of CNS myeloid cells. *Nat Immunol* (2017) 18:385–92. doi:10.1038/ni.3703
88. Keren-Shaul H, Spinrad A, Weiner A, Matcovitch-Natan O, Dvir-Szternfeld R, Ulland TK, et al. A unique microglia type associated with restricting development of Alzheimer's disease. *Cell* (2017) 169:1276–90.e17. doi:10.1016/j.cell.2017.05.018
89. Ben Haim L, Rowitch DH. Functional diversity of astrocytes in neural circuit regulation. *Nat Rev Neurosci* (2017) 18:31–41. doi:10.1038/nrn.2016.159
90. Chai H, Diaz-Castro B, Shigetomi E, Monte E, Octeau JC, Yu X, et al. Neural circuit-specialized astrocytes: transcriptomic, proteomic, morphological, and functional evidence. *Neuron* (2017) 95:531–49.e9. doi:10.1016/j.neuron.2017.06.029
91. Tsai H-H, Li H, Fuentealba LC, Molofsky AV, Taveira-Marques R, Zhuang H, et al. Regional astrocyte allocation regulates CNS synaptogenesis and repair. *Science* (2012) 337:358–62. doi:10.1126/science.1222381
92. Martín R, Bajo-Grañeras R, Moratalla R, Perea G, Araque A. Circuit-specific signaling in astrocyte-neuron networks in basal ganglia pathways. *Science* (2015) 349:730–4. doi:10.1126/science.aaa7945
93. Bardehle S, Krüger M, Buggenthin F, Schwausch J, Ninkovic J, Clevers H, et al. Live imaging of astrocyte responses to acute injury reveals selective juxtavascular proliferation. *Nat Neurosci* (2013) 16:580–6. doi:10.1038/nn.3371
94. Ben Haim L, Ceyzériat K, Carrillo-de Sauvage MA, Aubry F, Auregan G, Guillermier M, et al. The JAK/STAT3 pathway is a common inducer of astrocyte reactivity in Alzheimer's and Huntington's diseases. *J Neurosci* (2015) 35:2817–29. doi:10.1523/JNEUROSCI.3516-14.2015
95. LeComte MD, Shimada IS, Sherwin C, Spees JL. Notch1-STAT3-ETBR signaling axis controls reactive astrocyte proliferation after brain injury. *Proc Natl Acad Sci U S A* (2015) 112:8726–31. doi:10.1073/pnas.1501029112
96. Booth HDE, Hirst WD, Wade-Martins R. The role of astrocyte dysfunction in Parkinson's disease pathogenesis. *Trends Neurosci* (2017) 40:358–70. doi:10.1016/j.tins.2017.04.001
97. Lendahl U, Zimmerman LB, McKay RD. CNS stem cells express a new class of intermediate filament protein. *Cell* (1990) 60:585–95. doi:10.1016/0092-8674(90)90662-X
98. Lee HJ, Hanibuchi M, Kim S-J, Yu H, Kim MS, He J, et al. Treatment of experimental human breast cancer and lung cancer brain metastases in mice by macitentan, a dual antagonist of endothelin receptors, combined with paclitaxel. *Neuro Oncol* (2016) 18:486–96. doi:10.1093/neuonc/now037
99. Shimada IS, Borders A, Aronshtam A, Spees JL. Proliferating reactive astrocytes are regulated by Notch-1 in the peri-infarct area after stroke. *Stroke* (2011) 42:3231–7. doi:10.1161/STROKEAHA.111.623280
100. Gril B, Palmieri D, Qian Y, Anwar T, Liewehr DJ, Steinberg SM, et al. Pazopanib inhibits the activation of PDGFRβ-expressing astrocytes in the brain metastatic microenvironment of breast cancer cells. *Am J Pathol* (2013) 182:2368–79. doi:10.1016/j.ajpath.2013.02.043
101. Clozel M. Endothelin research and the discovery of macitentan for the treatment of pulmonary arterial hypertension. *Am J Physiol Regul Integr Comp Physiol* (2016) 311:R721–6. doi:10.1152/ajpregu.00475.2015
102. Kim S-J, Lee HJ, Kim MS, Choi HJ, He J, Wu Q, et al. Macitentan, a dual endothelin receptor antagonist, in combination with temozolomide leads to glioblastoma regression and long-term survival in mice. *Clin Cancer Res* (2015) 21:4630–41. doi:10.1158/1078-0432.CCR-14-3195
103. Salameh A, Dhein S. Pharmacology of gap junctions. New pharmacological targets for treatment of arrhythmia, seizure and cancer? *Biochim Biophys Acta* (2005) 1719:36–58. doi:10.1016/j.bbamem.2005.09.007
104. Harks EG, de Roos AD, Peters PH, de Haan LH, Brouwer A, Ypey DL, et al. Fenamates: a novel class of reversible gap junction blockers. *J Pharmacol Exp Ther* (2001) 298:1033–41.
105. Chan WN, Evans JM, Hadley MS, Herdon HJ, Jerman JC, Parsons AA, et al. Identification of (-)-cis-6-acetyl-4S-(3-chloro-4-fluoro-benzoylamino)-3,4-dihydro-2,2-dimethyl-2H-benzo[b]pyran-3S-ol as a potential antimigraine agent. *Bioorg Med Chem Lett* (1999) 9:285–90. doi:10.1016/S0960-894X(98)00728-8
106. Herdon HJ, Jerman JC, Stean TO, Middlemiss DN, Chan WN, Vong AK, et al. Characterization of the binding of [3H]-SB-204269, a radiolabelled form of the new anticonvulsant SB-204269, to a novel binding site in rat brain membranes. *Br J Pharmacol* (1997) 121:1687–91. doi:10.1038/sj.bjp.0701331
107. Read SJ, Smith MI, Hunter AJ, Upton N, Parsons AA. SB-220453, a potential novel antimigraine agent, inhibits nitric oxide release following induction of cortical spreading depression in the anaesthetized cat. *Cephalalgia* (2000) 20:92–9. doi:10.1046/j.1468-2982.2000.00022.x
108. Jin M, Dai Y, Xu C, Wang Y, Wang S, Chen Z. Effects of meclofenamic acid on limbic epileptogenesis in mice kindling models. *Neurosci Lett* (2013) 543:110–4. doi:10.1016/j.neulet.2013.03.029
109. Gril B, Palmieri D, Qian Y, Smart D, Ileva L, Liewehr DJ, et al. Pazopanib reveals a role for tumor cell B-Raf in the prevention of HER2+ breast cancer brain metastasis. *Clin Cancer Res* (2011) 17:142–53. doi:10.1158/1078-0432.CCR-10-1603
110. Sanz-Pamplona R, Aragüés R, Driouch K, Martín B, Oliva B, Gil M, et al. Expression of endoplasmic reticulum stress proteins is a candidate marker of brain metastasis in both ErbB-2+ and ErbB-2- primary breast tumors. *Am J Pathol* (2011) 179:564–79. doi:10.1016/j.ajpath.2011.04.037
111. Desplat-Jégo S, Varriale S, Creidy R, Terra R, Bernard D, Khrestchatisky M, et al. TWEAK is expressed by glial cells, induces astrocyte proliferation and increases EAE severity. *J Neuroimmunol* (2002) 133:116–23. doi:10.1016/S0165-5728(02)00368-5
112. Couzin-Frankel J. Breakthrough of the year 2013. Cancer immunotherapy. *Science* (2013) 342:1432–3. doi:10.1126/science.342.6165.1432
113. Fong MY, Zhou W, Liu L, Alontaga AY, Chandra M, Ashby J, et al. Breast-cancer-secreted miR-122 reprograms glucose metabolism in premetastatic niche to promote metastasis. *Nat Cell Biol* (2015) 17:183–94. doi:10.1038/ncb3094
114. Liu Y, Kosaka A, Ikeura M, Kohanbash G, Fellows-Mayle W, Snyder LA, et al. Premetastatic soil and prevention of breast cancer brain metastasis. *Neuro Oncol* (2013) 15:891–903. doi:10.1093/neuonc/not031
115. Verkhratsky A, Matteoli M, Parpura V, Mothet J-P, Zorec R. Astrocytes as secretory cells of the central nervous system: idiosyncrasies of vesicular secretion. *EMBO J* (2016) 35:239–57. doi:10.15252/embj.201592705

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Development of Novel Patient-Derived Xenografts from Breast Cancer Brain Metastases

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Brain metastases are an increasing burden among breast cancer patients, particularly for those with HER2$^+$ and triple negative (TN) subtypes. Mechanistic insight into the pathophysiology of brain metastases and preclinical validation of therapies has relied almost exclusively on intracardiac injection of brain-homing cells derived from highly aggressive TN MDA-MB-231 and HER2$^+$ BT474 breast cancer cell lines. Yet, these well characterized models are far from representing the tumor heterogeneity observed clinically and, due to their fast progression *in vivo*, their suitability to validate therapies for established brain metastasis remains limited. The goal of this study was to develop and characterize novel human brain metastasis breast cancer patient-derived xenografts (BM-PDXs) to study the biology of brain metastasis and to serve as tools for testing novel therapeutic approaches. We obtained freshly resected brain metastases from consenting donors with breast cancer. Tissue was immediately implanted in the mammary fat pad of female immunocompromised mice and expanded as BM-PDXs. Brain metastases from 3/4 (75%) TN, 1/1 (100%) estrogen receptor positive (ER$^+$), and 5/9 (55.5%) HER2$^+$ clinical subtypes were established as transplantable BM-PDXs. To facilitate tracking of metastatic dissemination using BM-PDXs, we labeled PDX-dissociated cells with EGFP-luciferase followed by reimplantation in mice, and generated a BM-derived cell line (F2-7). Immunohistologic analyses demonstrated that parental and labeled BM-PDXs retained expression of critical clinical markers such as ER, progesterone receptor, epidermal growth factor receptor, HER2, and the basal cell marker cytokeratin 5. Similarly, RNA sequencing analysis showed clustering of parental, labeled BM-PDXs and their corresponding cell line derivative. Intracardiac injection of dissociated cells from BM-E22-1, resulted in magnetic resonance imaging-detectable macrometastases in 4/8 (50%) and micrometastases (8/8) (100%) mice, suggesting that BM-PDXs remain capable of colonizing the brain at high frequencies. Brain metastases developed 8–12 weeks after ic injection, located to the brain parenchyma, grew around blood vessels, and elicited astroglia activation characteristic of breast

cancer brain metastasis. These novel BM-PDXs represent heterogeneous and clinically relevant models to study mechanisms of brain metastatic colonization, with the added benefit of a slower progression rate that makes them suitable for preclinical testing of drugs in therapeutic settings.

Keywords: patient-derived xenograft, brain metastases models, breast cancer, brain colonization, triple negative, HER2$^+$

INTRODUCTION

Brain metastases are the most common form of brain cancer, exceeding the number of primary brain tumors by at least four times, and occurring in about 25% of all patients with cancer (1). Breast cancer is the second most common primary tumor responsible for brain metastasis (2, 3), especially from women with HER2$^+$ and triple negative [TN, estrogen receptor negative (ER$^-$), progesterone receptor negative (PR$^-$), and HER2$^-$] tumors (4–6). Brain metastases remain incurable and more than 80% of patients will die within a year of their brain-metastasis diagnosis (7, 8). Treating brain metastases has been particularly challenging due to unique anatomical and functional features in the brain. Therapies used to treat systemic metastases [e.g., trastuzumab for the treatment of breast tumors overexpressing HER2$^+$, or chemotherapies used to treat triple negative breast cancers (TNBCs)] have limited ability to cross the blood–brain barrier (BBB) at effective doses, and often fail to decrease brain metastatic burden (8, 9). Thus, there is an urgent need for improved therapeutic approaches for breast cancer brain metastases.

A critical limitation to achieve better therapeutic strategies for brain metastasis has been the narrow set of experimental models to study brain metastasis pathophysiology. Development of symptomatic brain metastasis requires cancer cells to disseminate from the primary tumor, intravasate into blood vessels, survive in circulation, extravasate through the BBB, survive the neuroinflammatory response in the brain, and outgrow into large metastasis (10–12). Studying this complex process requires *in vivo* animal models that mimic early and late stages of brain metastatic colonization, produce brain metastases at high frequencies, and demonstrate moderate tumor progression necessary for the preclinical screening of drugs that could be used in preventive and therapeutic settings (13, 14). Until recently, brain metastasis studies relied primarily on intracardiac (ic) injection of brain-homing cells derived from murine 4T1 (4T1BR5), human TN MDA-MB-231 (231Br, 231/LM2-4) (15–17), and HER2$^+$BT474 (BT474BR) cell lines (18). These models were developed by performing successive rounds of ic injection of breast cancer cell lines, which were then reisolated, cultured *in vitro* and then reinjected into nude mice (19). Although these brain metastatic cell lines are well characterized and produce brain metastases at high frequencies (20, 21), the rapid progression of metastatic burden in these models limits their usability for therapeutic testing of drugs. More importantly, these models do not fully represent the heterogeneity observed in breast tumors and their metastasis, which have emerged as critical factor in defining populations of patients that are likely to respond to a particular therapy.

During the past several years, researchers have developed transplantable models to grow primary breast tumors in the mammary fat pad of NOD/SCID/ILIIrg$^{-/-}$ (NSG) mice, with the long-term goal of personalizing medicine (22–24). These PDXs retain intratumoral heterogeneity and have become a clinically relevant alternative to cell lines (23, 25, 26). Here, we report the development and characterization of eight novel human breast cancer patient-derived xenografts (BM-PDXs) from ER$^+$, HER2$^+$, and TN subtypes and a matching TN cell line, which retain tumor heterogeneity and brain metastatic potential. We demonstrate that ic injection of cells dissociated from BM-PDXs produce brain metastases at high frequencies, with metastases that elicit astroglia activation and growth around vessels in a similar fashion to breast cancer brain metastasis. These novel BM-PDXs represent heterogeneous and clinically relevant models to study mechanisms of brain metastatic colonization, with the added benefit of a slower progression rate that makes them suitable for preclinical testing of drugs in therapeutic settings.

MATERIALS AND METHODS

Brain Metastases Transplantation and Establishment of Patient-Derived Xenografts

De-identified brain metastases and their clinical-pathological information (age, ER, PR, and HER2 status at the time of metastases resection, prior therapies, and survival) were obtained from consenting breast cancer patients undergoing neurosurgery. These samples were collected under approved IRB protocols at University of Colorado Anschutz Medical Campus. All animal studies were performed under approved University of Colorado Institutional Animal Care and Use Committee (IACUC) protocols.

Freshly removed brain metastasis samples were placed on sterile ice-cold DMEM and transported to the laboratory for transplantation into mice. Specimens that could not be immediately implanted were maintained at 4°C for no longer than 8 h. Female NSG mice, 6–8 weeks old were purchased from Jackson laboratories or bred at the UC Denver Center for Comparative Medicine breeding facility. Brain metastases were partitioned into 5–10 mm^3 pieces, dipped into cultrex, and implanted in the fourth mammary fat pad of anesthesized mice using a 10-gage trochar. In one case, brain metastatic cells were collected from cerebrospinal

fluid from a patient diagnosed with meningeal carcinomatosis. Here, cancer cells were collected by centrifugation and divided into two aliquots. One mouse was injected ic with cancer cells suspended in 100 µl PBS, another recipient was injected into the mammary fat pad with cancer cells resuspended in 50 µl cultrex. All mice were implanted with a silastic pellet providing slow release of 17β-estradiol (E2), as prior experience showed that E2 increases tumor uptake of breast cancer PDXs irrespective of tumor subtype. Tumors were palpated weekly to assess tumor take for up to 8 months postimplantation. Once palpable, tumor size was assessed weekly using caliper and volume estimated as length × width^2/2. When tumors reached ~1.5 cm in any direction, mice were euthanized and tumors removed. Tumors were divided into several 10 mm^3 pieces and reimplanted in the mammary fat pad of NSG mice, cryopreserved in 10% DMSO/90% FBS in liquid nitrogen, stored in trizol RNA extraction, and fixed in 10% formalin for paraffin embedding. BM-PDXs were considered established if they grew over two generations.

Labeling of BM-PDXs

A subset of BM-PDXs were labeled with lentiviral particles expressing EGFP-luciferase as we described previously (27). Briefly, >1 cm^3 tumors were resected from euthanized mice and digested in Accumax (Stemcell Tech) for 3 h at 30°C. Human cancer cells were separated from mouse stromal cells using a lineage cell depletion kit (MACS) and isolated breast cancer cells were plated in six-well ultralow attachment plates in DMEM-F12 media. Tumor cells were transduced with 30 MOI of lentiviral pHAGE-EF1aL-luciferase-UBC-GFP-W and GFP expression monitored for up to 48 h. Labeled tumor cells were then collected, washed, resuspended in 100 µl Cultrex basement membrane extract and injected in the mammary fat pad of NSG mice. Efficiency of transduction was assessed using luciferase activity imaging (IVIS) or GFP expression when tumors reached >1 cm^3. Labeled BM-PDX were cryopreserved, fixed for immunohistological analysis, stored in trizol for RNA extraction, or transplanted into a new recipient.

BM-F2-7 Cell Line Derivation and Culture

A cell line (F2-7) was derived from triple-negative BM-PDX. For this, tumor cells were dissociated from BM-PDX F2-7 using Accumax, and dissociated cells were plated in ultralow attachment six-well plates in DMEM-F12 supplemented with 10% of FBS, 1 µg/ml hydrocortisone, 100 ng/ml of cholera toxin, and 1 nM of insulin. After 4 weeks of growth in suspension, human cells free of fibroblasts were plated in collagen-I coated dishes, and purity validated by immunohistochemistry. F2-7 cells were labeled with GFP-luciferase as described for BM-PDXs. Short-Tandem Repeat analysis was performed in the established cell line and deposited at University of Colorado Tissue Culture Core facility for validation and future reference.

RNA Sequencing of BM-PDX and F2-7 Cell Line

High-throughput RNA sequencing from a cell line derivative (F2-7) and a selected set of BM-PDXs before and after labeling with EGFP-luciferase was performed. RNA was isolated from tumor samples using trizol followed by RNA cleanup using RNEeasy MinElute Cleanup kit (Qiagen), and RNA concentration was measured in a Nanodrop 2000 (Thermo Scientific). The Genomics and Microarray core facility at the University of Colorado AMC performed RNA quality control using an Agilent 2100 Bioanalyzer, and prepared RNA-seq libraries using the Illumina TruSeq Stranded mRNA LT Sample Prep Kit. The resulting libraries were sequenced on an Illumina HiSeq 2500 system (1 × 125 bp). After demultiplexing, the resulting reads were trimmed with cutadapt to remove 3′ adaptor sequences and low quality 3′ bases (Q < 10). The trimmed reads were then aligned to both the human (hg19/GRCh37) and mouse (mm10) genomes using Tophat2 (28). Reads were then assigned to either the human or mouse genome using disambiguate (29) and ambiguous reads were discarded. Unambiguous reads were assigned to features using Rsubread (30) and normalized read counts were produced using the rlog function in DESeq2 (31). The GEO Accession number for this data is GSE104020.

Experimental Brain Metastasis Using BM-PDXs

Two tumors from E22-1 BM-PDXs grown in the mammary fat pad were excised at necropsy, cut into 2 mm^2 pieces and dissociated using Accumax for 3 h. The digestion was stopped using DMEM/F12 10% FBS, and single cells isolated by filtering through 100 and 70 µm mesh filters. Viable cells were counted using trypan blue exclusion and 250,000 cells resuspended in 100 µl PBS were injected in the left cardiac ventricle of recipient female NSG mice ($n = 8$). Brain metastatic burden was assessed using T1/T2 contrast magnetic resonance imaging (MRI) 8 weeks after ic injection, and mice were euthanized under CO_2 asphyxiation at 8 or 12 weeks after ic injection as mice developed signs of CNS metastatic burden. In all cases, brains were removed at necropsy and brain hemispheres embedded in OCT and stored at −80°C until sectioning. Micrometastases were visualized with H&E and/or pan cytokeratin (PanCK) staining in six serial sections (10 µm thick), one every 300 µm in a sagittal plane through the right hemisphere of the brain.

Magnetic Resonance Imaging

To non-invasively detect and quantify brain metastatic colonization, brain MR scans were acquired using a Bruker 4.7 T PharmaScan and a bird-cage radio frequency 36 mm coil (Bruker Medical, MA, USA). Animals were injected *via* tail vain with 0.4 mmol/kg gadolinium contrast Multihance (gadobenate dimeglumine, Bracco Diagnostic) and anesthetized with 2–2.5% isoflurane. High-resolution rapid acquisition with relaxation enhancement (RARE) T2-weighted images with fat suppression were obtained (TR/TE = 4,000/80 ms) followed by a multislice multiecho (MSME) T1-weighted sequence (TR/TE = 700/11 ms). All images were obtained in the axial plane, with the field of view of 3 cm, slice thickness 1 mm, number of slices 16, matrix size 256 × 256. In-plane resolution was 90 µm. T1-weighted MSME images were acquired as well to confirm metastatic lozation. All

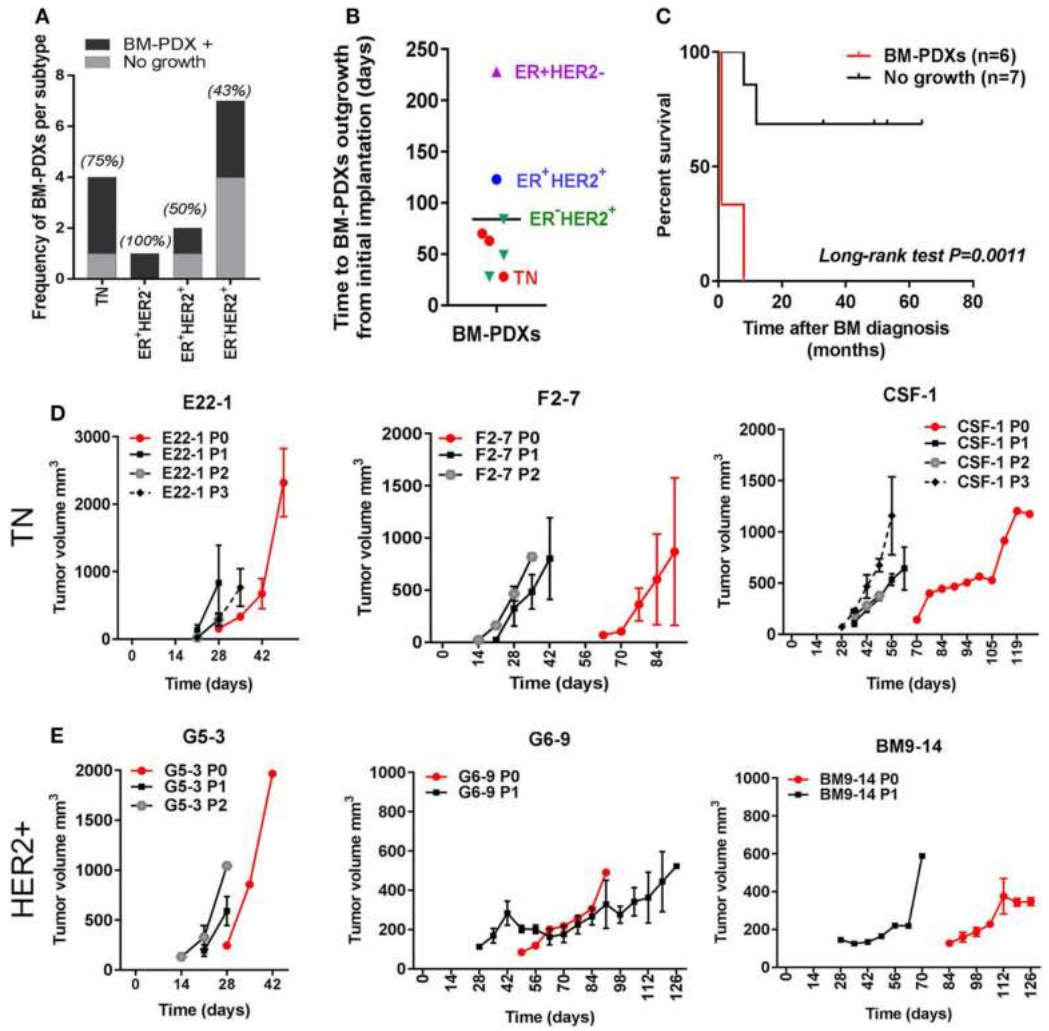

FIGURE 1 | Establishment of brain-metastases-patient-derived xenografts (BM-PDXs) from breast cancer. PDXs were established in NOD/SCID/ILIIrg$^{-/-}$ mice, and subsequently propagated *via* direct transplantation of solid tumor pieces into new recipient mice. Tumors were grown under continuous estrogen supplementation. **(A)** BM-PDXs established as a function of breast cancer subtypes. Graph depicts the number (bars) and percentage of BM-PDXs established (BM-PDXs^{+}) per tumor subtype compared to tumors that did not grow after 8 months of implantation. **(B)** Time in days from initial implantation to outgrowth as measurable tumors (~62.5 mm^3) for all PDXs, colors indicate breast cancer subtypes. **(C)** Survival (months) after brain metastases diagnosis of patients with breast cancer whose surgical samples had *in vivo* tumorigenic potential ($n = 6$), or not ($n = 7$). Log-rank (Mantel-Cox) test P value is shown. **(D,E)** Tumor growth after implantation (P0, red lines), and subsequent passaging (P1, P2, P3, black/gray lines) in **(D)** TN and **(E)** ER^{-}HER2^{+} BM-PDXs. For P0, 1–2 tumors were implanted in a single recipient. For P1-P3, data shows average tumor volume from two tumors in 1–2 recipient mice.

images were acquired and analyzed (T2-MRI for lesion numbers and diameters) using Bruker ParaVision (v4.3) software.

Immunohistochemistry in PDXs and Experimental Brain Metastasis

Tumors were removed from animals and fixed in 10% buffered formalin. Tissue was processed, paraffin embedded, and cut into 5-µm sections. After high-temperature antigen retrieval in citrate buffer, sections were stained with rabbit anti-epidermal growth factor receptor (anti-EGFR, Cell Signaling), rabbit monoclonal anti-C-erB-2 (SP3, Neomarkers), mouse monoclonal antibody anti-cytokeratin 5 (anti-CK5, Vector), rabbit polyclonal anti-PR (DAKO, Carpinteria, CA, USA), and rabbit polyclonal anti-ERα (SP1, Thermofisher). Sections were counterstained with hematoxylin and mounted. Representative photographs were taken under a light microscope at ×20 magnification.

Dual immunofluorescence of brain metastasis was performed 10-µm sections from frozen unfixed-OCT embedded brains. Sections were fixed in acetone and stained with a mouse monoclonal antibody specific for human cytokeratins (Pan-CK, MNF116, Dakocytomation, Glostrup Denmark); in combination with rat anti-GFAP (Invitrogen, CA, USA); rabbit anti-collagen IV (Millipore). Secondary antibodies were anti-mouse Alexafluor-488 or anti-rabbit Alexafluor-565 or

TABLE 1 | Clinical–pathological characteristics of BM-PDXs.

Clinical features of BM-PDXs						
			At brain metastasis diagnosis			
PDX	**Primary tumor Dx**	**BM-Dx**	**Age**	**ER**	**PR**	**HER2**
E22-1	TN	TN	58	0	0	0
F2-7	TN	TN	38	0	0	0
BM14-9	ER$^-$HER2$^+$	ER$^-$HER2$^+$	43	0	0	95% (3+)
G5-3	TN[a]	ER$^-$HER2$^+$	63	0	5% (1+)	30% (2+), FISH$^+$
G6-9	TN[a]	ER$^-$HER2$^+$	53	0	0	40% (2+)
G7-1	ER$^+$HER2$^+$	ER$^+$HER2$^+$	59	65% (2+)	70% (2+)	45% (2+), FISH$^+$
G13-1	ER$^+$HER2$^-$	ER$^+$HER2$^-$	63	70% (3+)	0	0
CSF-1[b]	TN	TN	ND	ND	ND	ND

Numbers represent % of positive cells followed by intensity score (in brackets). HER2$^+$ is defined as >10% and ≥(2+). ER$^+$ is defined as > 1%.

[a]*Primary tumors with history of TNBC that had converted to HER2$^+$ at brain metastasis.*

[b]*Sample obtained from cerebrospinal fluid, no pathology report at metastatic site.*

All patients had been treated with taxanes by the time of brain metastases.

anti-Rat-Alexafluor-594 (all from Invitrogen/Thermofisher, CA, USA). Nuclei were stained with 1 μg/ml 4′,6-diamidino-2-phenylindole (DAPI) in methanol for 10 min at room temperature. Photographs were taken under ×20 magnification for the same field using the UV, FITC, and TRITC filters.

RESULTS

Establishment of BM-PDXs in Relation to Breast Cancer Subtypes

We implanted a total of 14 brain metastases specimens from TN ($n = 4$), ER$^+$HER2$^-$ ($n = 1$), ER$^+$HER2$^+$ ($n = 2$), and ER$^-$HER2$^+$ ($n = 7$) breast cancer subtypes. From these, 8 (57.2%) successfully established as BM-PDXs, defined as those tumors that grew as xenografts in NSG mice at least in one consecutive passage and maintained expression of clinical markers from original patient sample. The frequency of BM-PDXs uptake varied among subtypes with a non-significant trend toward highest take rate in TNBC (3/4 BM-PDXs, 75%) and lower take rate in ER$^-$HER2$^+$ (3/7, 43%) (**Figure 1A**). The overall clinical–pathological characteristics of the brain metastases successfully established as BM-PDXs are presented in **Table 1**. Among HER2$^+$BM-PDXs, two specimens had prior history of TNBC but their brain metastasis were diagnosed as HER2$^+$; these were classified as HER2$^+$BM-PDXs. Time to xenograft tumor formation for each BM-PDX at initial implantation ranked between 28 and 223 days, with an average of 84 days (**Figure 1B**). Similar to prior reports, *in vivo* tumorigenic potential of explanted brain metastases (tumors that grew as BM-PDXs) was correlated with decreased survival of their donor patients ($P = 0.0011$) (**Figure 1C**). Tumor progression in initial PDX (P0) and subsequent *in vivo* passaging (P1 to P3) for a TN and HER2$^+$BM-PDXs is shown in **Figures 1D,E**. In one case, an ER-HER2$^+$ brain metastases was implanted but an inguinal tumor developed suddenly after 70 days, away from the implantation site. This tumor lacked HER2, EGFR, or CK5 and upon transplantation grew into a large mass within 2 weeks (data not shown). As this suggested either loss of human epithelial markers or most likely, outgrowth of a murine tumor, we did not consider this a successful PDX and excluded it from further analysis.

Preservation of Clinical Markers in BM-PDXs Over Multiple Passaging, Cell Dissociation, and Viral-Mediated Transduction

To determine whether outgrowth of BM-PDXs in the mammary fat pad retained key clinical features of brain metastases donors, we stained sections of BM-PDXs (P0-P1) for ER, PR, and HER2, and—when available—we compared them to clinical specimens at the time of implantation. TNBC brain metastases lack these markers but frequently express EGFR and CK5 (32). Thus, we added these to our validation panel. As show in **Figure 2A**, TN BM-PDXs expressed EGFR and CK5. For these samples there was no matching donor sample to compare, but lacked ER, PR, and HER2 as expected from TN tumors. ER-HER2$^+$BM-PDXs retained HER2, EGFR, and CK5 (**Figure 2B**), and ER$^+$BM-PDXs retained ER, HER2, EGFR, and CK5 expression similar to the donor sample (**Figure 2C**). Surprisingly the two ER$^+$BM-PDXs (G7-1, G13-1), showed increased PR expression as compared to the donor samples (**Figure 2C**), suggesting that ER is functional in these BM-PDXs and that E2-supplementation in mice upregulates PR in ER$^+$BM-PDXs.

To assess whether GFP and luciferase labeling would allow *in vivo* imaging of these BM-PDXs, we dissociated cells from >1 cm^3 xenografts BM-PDXs F2-7, CSF-1, and G5-3 and transduced them with high titer viral particles of a GFP-luciferase vector as described (27). Labeled cells were regrown in the mammary fat pad of NSG mice and tumor labeling was assessed by measuring luciferase activity (IVIS) and GFP expression (**Figure 3A**). Spontaneous metastases to surrounding areas were detected in some mice during tumor excision (**Figure 3B**), but without spontaneous metastases to brain or other organs. Since tumor cell dissociation and cell transduction with lentiviral vector might results in selection of subclones of the original tumor, we assessed whether labeled PDXs recapitulated the heterogeneity observed in parental PDXs. IHC staining showed that labeled BM-PDXs retained expression of EGFR, CK5, and HER2 (**Figure 3C**), suggesting that dissociated/labeled cells are capable of reconstituting tumor heterogeneity of BM-PDXs.

As tumor-dissociated cells survived short-term *in vitro* culture during labeling, we sought to determine whether dissociated brain-metastatic cells could be cultured as cell lines. We cultured dissociated cells from F2-7, E22, and G5-3 BM-PDXs in plates coated with collagen-I or ultralow attachment plates. In either condition, only cells from F2-7 BM-PDXs survived *in vitro* culture and remained proliferative after multiple passages of repeated freezing and thawing (**Figure 4A**). This F2-7 cell line-derivative retained expression of EGFR as its BM-PDXs counterpart (**Figure 4B**), and retained tumor initiating capability *in vitro* (measured as ability to form colonies in the absence of extracellular matrix in mammosphere assays, not shown). To further assess whether BM-PDXs labeling or cell line derivation

maintained features of parental BM-PDXs, we performed RNA sequencing followed by hierarchical gene clustering analysis of BM-PDX before and after GFP-luciferase labeling, and F2-7 BM-PDX and its cell line-derivative (**Figure 5**). Key genes (EGFR, KRT5, NTRK2) were expressed at similar levels in parental PDXs (i.e., E22-1 PDX-P0) compared to its labeled counterpart (E22-1 PDX-P0-I1), and in the F2-7 cell line (F2-7 CL) compared to its BM-PDXs parental (F2-7 P5). Taken together, these data suggests maintenance of clinical markers through passaging and manipulation of BM-PDXs.

BM-PDXs Retain Their Ability to Colonize the Brain at High Frequencies

Breast cancer PDXs grown in the mammary fat pad rarely metastasize to distant organs, but dissociated cells can colonize lung, bones and brain after ic injection (27). To assess whether BM-PDXs retain their ability to colonize the brain, we induced experimental brain metastasis using dissociated cells from TN E22-1 BM-PDX. For this, 250,000 dissociated cells were injected in the left ventricle of 8–week-old female NSG mice ($n = 8$) supplemented with estradiol, and metastases were allowed to grow

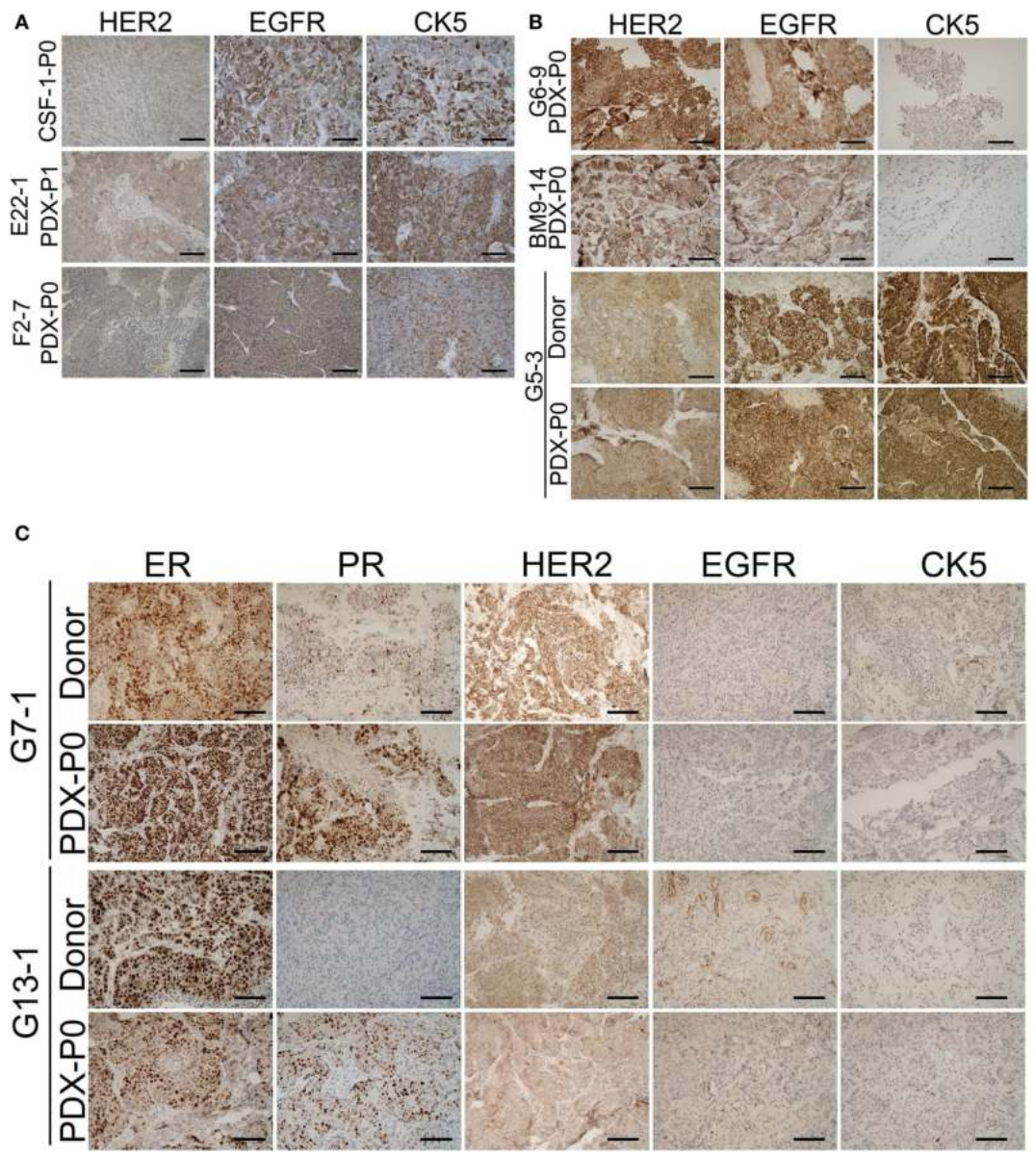

FIGURE 2 | Retention of estrogen receptor (ER), progesterone receptor (PR), HER2, epidermal growth factor receptor (EGFR), and cytokeratin (CK) expression in brain-metastases-patient-derived xenografts (BM-PDXs). Sections of BM-PDXs were stained by IHC for ER, PR, HER2, EGFR, and CK5 at first passage (P0) and compared to donor tumor (when available). **(A)** Expression of EGFR, HER2, and CK5 in triple negative (TN) BM-PDXs (ER, PR, negative, not shown). **(B)** Expression of HER2, EGFR, and CK5 in HER2$^+$BM-PDXs in donor and BM-PDXs P0. **(C)** Expression of ER, PR, HER2, EGFR, and CK5 in ER$^+$HER2$^+$BM-PDXs and their donor counterparts. Scale bars, 100 µm.

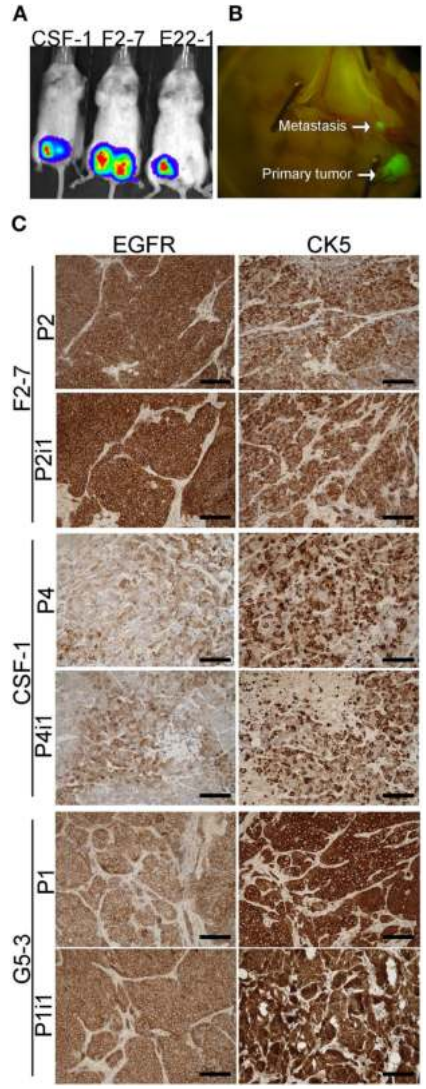

FIGURE 3 | GFP-Luciferase-labeled brain-metastases-patient-derived xenografts (BM-PDXs) retain expression of epidermal growth factor receptor (EGFR), cytokeratin 5 (CK5), and HER2. **(A)** Luciferase imaging (IVIS) of NSG mice carrying GFP-luciferase BM-PDXs F2-7, CSF-1, and G5-3 in the mammary fat pad. **(B)** Spontaneous metastases to lymph node in G5-3 GFP-luc carrying mice. **(C)** Expression of EGFR and CK5 in sections of F2-7, CSF-1, and G5-3 BM-PDXs before (P1, P2) and after GFP-luciferase labeling (P2-I1, P1-I1). Scale bars, 100 μm.

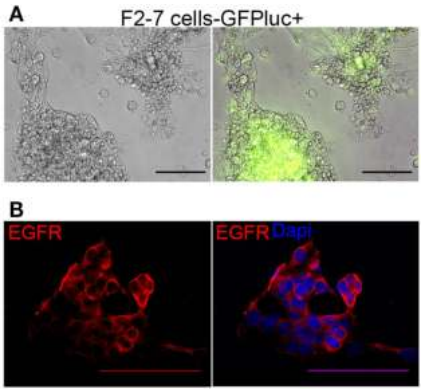

FIGURE 4 | Characteristics of F2-7 BM-cell line *in vitro*. **(A)** F2-7 cells were labeled *in vitro* with GFP-luciferase and cultured in collagen-I coated plates. *Left:* Brightfield, *right:* GFP expression of live cells. **(B)** Immunofluorescence staining shows epidermal growth factor receptor (EGFR) (red) expression in F2-7 cells grown in coverslips. 4′,6-diamidino-2-phenylindole (DAPI) labels nuclei. Scale bars are 100 μm.

until mice showed >15% weight loss or neurological impairment. Nine weeks after ic injection mice were imaged using T1/T2 MRI and mice without symptomatic metastases were left alive for 3 additional weeks. MRI-detectable brain metastases were found in 4/8 (50%) of injected mice, with metastatic lesions ranking from 0.29 to 0.62 mm in size (**Figure 6A**, top). One additional mouse was injected with E22^{-}BM-PDX dissociated cells but MRI was performed 14 weeks after ic injection. This mouse showed multiple large MRI-detectable metastases (**Figure 6A**, bottom). Histological analysis showed 8/8 mice (100%) harboring micro-metastases (defined as >50 μm cancer cell foci counted in six sagittal brain sections 300 μm apart) with a 10.25 median number of micrometastasis per mouse (**Figure 6B**). To determine whether brain metastases formed by BM-PDXs showed pathophysiological features similar to those encountered in humans, we performed double immunofluorescence staining of brain metastatic cells (pan-cytokeratin^{+}, green) and reactive astrocytes (GFAP^{+}, red) or blood vessels (Col-IV, red) in brain sections from mice injected with E22-1 BM-PDXs. Brain metastatic clusters were located to the brain parenchyma (**Figure 6C**), were associated with blood vessels (**Figure 6D**) and were surrounded by GFAP^{+} reactive astrocytes (**Figure 6E**); all of these characteristics of breast cancer brain metastases. Taken together, these studies demonstrate that BM-PDXs retain their ability to form large brain metastases and micro metastases at high frequencies, making them suitable models for studies of brain metastatic colonization and preclinical testing of drugs in preventive and therapeutic settings.

DISCUSSION

The increased incidence of brain metastasis in breast cancer patients and its dismal prognosis, has prompted the urgency to better understand the pathophysiology of brain metastases and to test novel therapeutic strategies for these patients. PDXs have emerged as required tools to validate *in vitro* studies in cells lines and to decipher the role of tumor heterogeneity in tumor progression and response to treatments (33–35). Therefore, we addressed whether PDX derived from brain-metastatic breast cancer are suitable models to study the pathophysiology of brain metastasis and to provide clinically relevant platforms for therapeutic drug testing. A diagram showing the overall procedure to achieve this from tumor implantation to ic injection of labeled cells is presented in **Figure 7**. By implanting fresh tumor samples in the mammary fat pad of NSG mice, we developed BM-PDXs from TN, ER^{-}HER2^{+}, ER^{+}HER2^{+}, and ER^{+}HER2^{-} subtypes. Consistent with the diverse incidence of brain metastasis among breast cancers subtypes (36–38), most specimens collected for

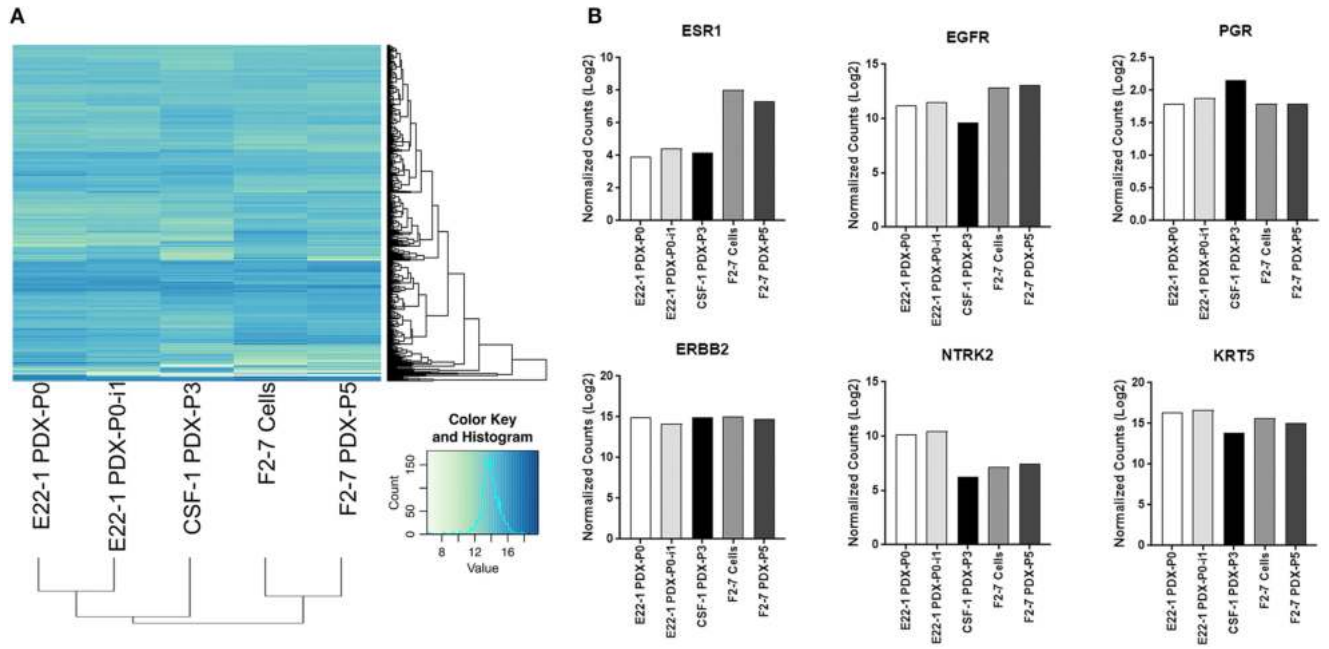

FIGURE 5 | RNA expression profiles of F2-7 cell line and brain-metastases-patient-derived xenografts (BM-PDXs) before and after labeling. RNA sequencing of BM-PDX E22-1 prior to (E22-1-PDX-P0) and after GFP-luciferase labeling (E22-1 PDX-P0-I1), BM-PDX CSF-1 P3, and F2-7 cell-line compared to F2-7 BM-PDX-P5. **(A)** Unsupervised hierarchical clustering of samples using the 500 most variable genes across all samples. **(B)** Normalized expression (log 2) plots of clinically relevant genes (ESR1, EGFR, PGR, ERBB2, NTRK2, KRT5). Differential expression was calculated using cufflinks (cuffdiff), and hierarchical clustering was performed in R.

implantation originated from TN and HER2$^+$ tumors, and 6 of 8 established BM-PDXs were from TN and ER$^-$HER2$^+$ subtypes. Similarly, BM-PDXs from ER$^+$ patients (who show the lowest incidence of BMs) (39) showed the slowest progression when implanted as xenografts (**Figure 1B**), despite the fact that mice were supplemented with estradiol. Of clinical importance, two specimens had prior history of TNBC but their brain metastasis were reclassified as HER2$^+$ by either immunohistochemistry or FISH. This is in agreement with recent reports of ERBB3/HER2 amplifications and mutations in breast cancer brain metastasis that are absent in primary tumors (40). This also highlights how changes in cancer cells occurring within the brain microenvironment modify tumor progression and impact their therapeutic alternatives.

Our BM-PDXs share characteristics of PDX models derived from primary tumors and other cancers. For example, our engraftment rate of 57.3% was similar to rates reported for engraftment of brain metastases from lung cancer (41). We also observed that the *in vivo* tumorigenic potential of patient-derived cancer cells was correlated with worse clinical outcome of patients. This is consistent with the idea that more aggressive/proliferating tumors are more likely to engraft as PDXs (24, 35). Unfortunately, these data also suggest that the potential use of personalized BM-PDX to test drug responses and guide clinical treatment, will not be feasible given the extremely short survival of those brain metastatic patients whose tumors grew as BM-PDXs (**Figure 1C**).

Similar to other studies, once established as PDXs, tumor cells appear to gain the ability to grow *in vivo* (22, 35), as demonstrated by the shorter time for BM-PDXs to develop into palpable tumors (**Figures 1D,E**). While no apparent gain or loss of critical cell makers were observed between donor and TN and ER$^-$HER2$^+$BM-PDXs (**Figures 2** and **5**), it is possible that the increased growth rate represents differences in the initial number of cancer cells that proliferated to give rise to a PDX, rather than the selection of a subset of rapidly proliferating tumor cells. While we observed a high proportion of cells expressing the basal marker CK5 (a marker associated with a stem-like phenotype in breast cancer) (32, 42, 43), we did not observe enrichment of CK5$^+$ after passaging or cell dissociation, which could be interpreted as a selection of a more aggressive tumor clone. However, only genetic tracing of clonal populations within the tumors would allows to answer this question definitively. Our RNA sequencing data showing conserved expression of critical genes after PDX-cell dissociation (**Figure 5**) suggests that BM-PDXs can be manipulated *in vitro* (i.e., using CRISPR-cas9). This opens the window to use PDXs in mechanistic studies previously limited to cell line models.

Despite being expanded in the mammary fat pad, our BM-PDXs remain capable of colonizing the brain at high frequencies, suggesting that passaging tumors in the mouse does not decrease their brain metastatic potential. While the incidence of MRI-detectable metastases and micrometastases after ic injection were only measured in a cohort of mice injected with the E22-1 BM-PDXs, ongoing experiments in our laboratory suggest that this finding can be extended to the F2-7 cell line and G3-5 BM-PDXs (not shown). Importantly, brain metastases from E22-1 BM-PDX elicit astroglia activation (marked by expression of GFAP$^+$ astrocytes) and brain metastatic outgrowth around vessels in the brain parenchyma. Therefore, experimental metastases

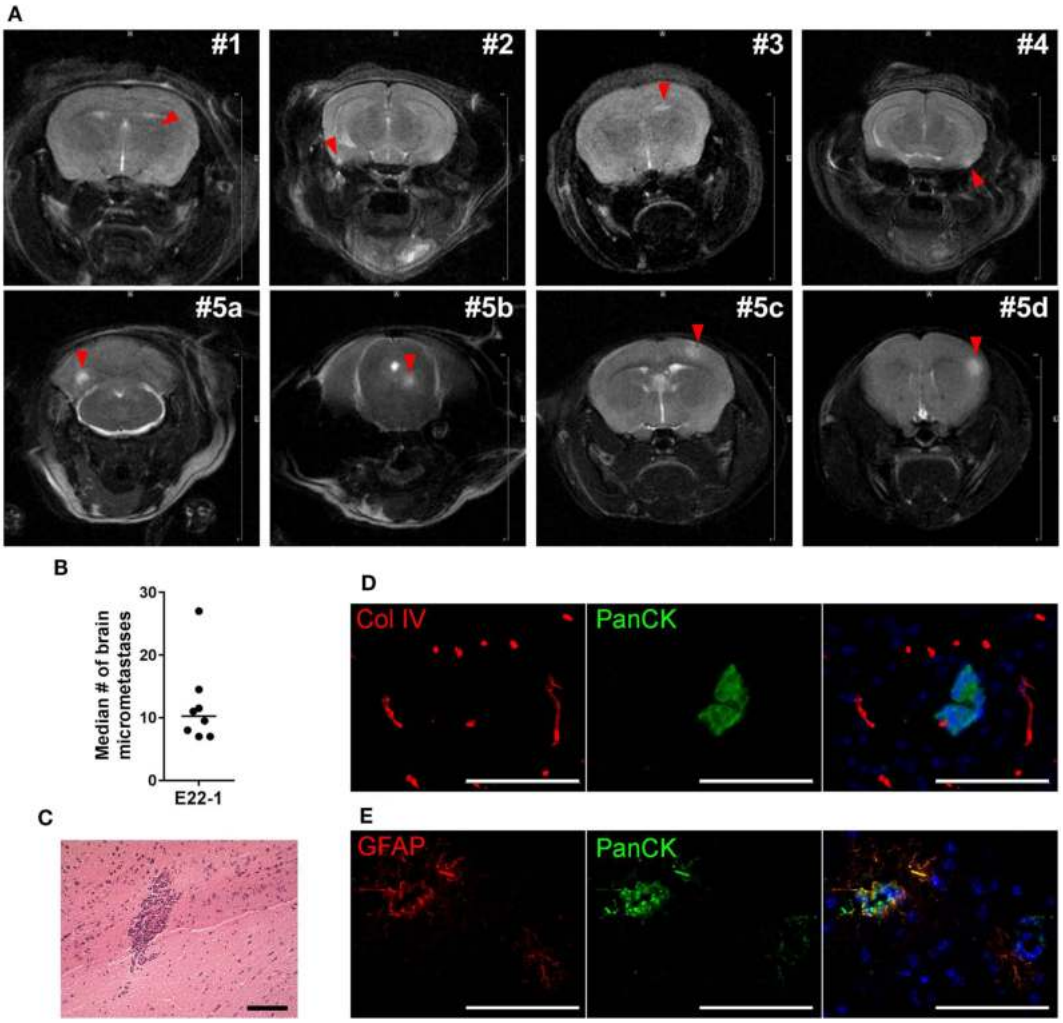

FIGURE 6 | Experimental metastases using brain-metastases-patient-derived xenografts (BM-PDXs). **(A)** Dissociated cells (250,000/100 µl PBS) from BM-PDX E22-1 were injected intracardially in female NSG mice ($n = 8$). Representative T2-weigthed rapid acquisition with relaxation enhancement (RARE) magnetic resonance imaging (MRI) image shows large metastases in 4/10 mice, 8 weeks after injection (top panel). Lower panel shows large and multiple metastases brain metastases in one mouse at 13 weeks postinjection. The summed diameter of all metastases in this mouse was 3.7 mm. **(B)** Median number of micrometastases per mouse, counted in six brain sections, 300 µm apart. **(C)** H&E staining shows micrometastasis in brain section from mice injected with E22-1 BM-PDX. **(D)** Double immunofluorescence staining shows metastatic E22-1 cells (Pan-cytokeratin, PanCK, green) surrounded by reactive astrocytes ($GFAP^+$, red) (20×). **(E)** Double immunofluorescence staining shows $PanCK^+$E22-1 cells outside of blood vessels (Col-IV) (20×).

with BM-PDXs recapitulate interactions with the brain microenvironment recognized as critical for brain metastatic success. Since we can genetically manipulate BM-PDXs dissociated cells or our F2-7 cell line, these novel models are now available to mechanistically assess how diverse breast tumors subtypes adapt to the brain microenvironment. More importantly, since PDXs show a slower progression rate than cell lines, these models are better suited for preclinical testing of drugs in a therapeutic setting, a task difficult to achieve in models where mice become moribund 3–4 weeks after injection.

It has been shown that breast cancer PDXs from primary tumors can colonize the brain if injected ic, suggesting that the intrinsic ability of tumor cells to colonize multiple organs is present in all PDXs regardless of site of origin. While our results indicate that BM-PDXs retain brain tropism, we observed spontaneous metastases of BM-PDXs from the orthotopic site to nearby vessels (**Figure 4B**) and in a few cases, metastases to bone and lungs after ic injection of BM-PDXs dissociated cells (not shown). This suggests, that similar to brain-homing cell lines and other PDXs, BM-PDXs maintain their ability to disseminate and colonize multiple metastatic sites (44). This also implies that ic injection of BM-PDXs might result in "undesired" metastases to other organs, which will limit our ability to measure brain-metastases-associated survival in these models. Recently, brain-metastatic PDX from lung cancer (41), melanoma (45) and $HER2^+$ breast cancer (46) were developed by direct intracranial injection of tumor samples in the brains of mouse or rats. Therefore, direct injection of dissociated cells from BM-PDXs might be an

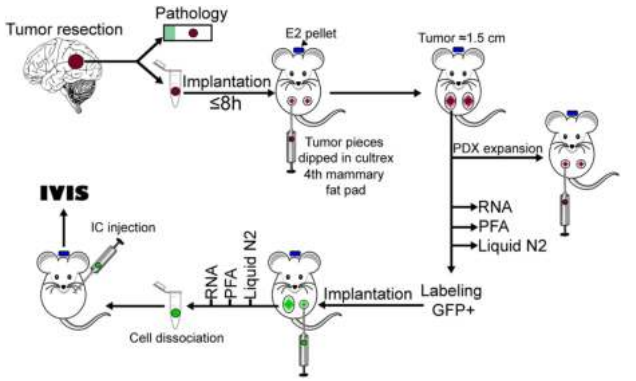

FIGURE 7 | Diagram of procedures used to develop brain-metastases-patient-derived xenografts (BM-PDXs) in this study.

alternative to induce a high frequency of brain metastases while minimizing the confounding effects of peripheral metastases in therapeutic studies. However, direct injection of cancer cells in the brain bypasses critical stages of brain metastastic colonization (hematogenous dissemination, intravasation, neuroinflammatory response, growth around vessels), which are hallmarks of breast cancer brain metastases. Intracarotid artery injection of cancer cells is a suitable alternative to ic injection for the production of brain-only metastasis-bearing mice with similar growth rates and mortality (47). Thus, we propose that intracarotid artery delivery of F2-7 cell line or BM-PDXs dissociated cells will enable the use of these heterogeneous models of brain metastatic breast cancer in mechanistic studies relevant to the pathophysiology of brain metastases, as well as to testing drug efficacy in preventive and therapeutic settings.

In conclusion, we developed and characterized eight novel PDX from breast cancer brain metastases from ER^+, $HER2^+$, and TN subtypes, derived a matching cell line from one TN BM-PDX and demonstrated their brain metastatic potential. While all animal models harbor advantages and limitations, these novel BM-PDXs represent clinically relevant models that can be used to study how the heterogeneity of cancer cells affects brain colonization as well as for validation of therapies.

ETHICS STATEMENT

This study was carried out in accordance with the recommendations of the Department of Health and Human Services (HHS) regulations at 45 CFR 46 (also known as the "Common Rule") and the Food and Drug Administration (FDA) regulations at 21 CFR 50 and 21 CFR 56; as well as Department of Veterans Affairs policies for human research protection, including the regulations at 38 CFR 16, and the VHA Handbook 1200.05., with written consent from all subject. All subjects gave written informed consent in accordance with the Declaration of Helsinki. The protocol was approved by the Colorado Multiple Institutional Review Board (COMIRB), protocol #13-3007 and the University of Colorado Denver Central Nervous System Biorepository Protocol, Steering Committee.

AUTHOR CONTRIBUTIONS

Conception and design: DC. acquisition of data (provided animals, acquired and managed patients, provided facilities, etc.): MC-Z, DO, AG, PK, CH, ND, KL, MG, VB, BJ, SE, AT, and DC. Analysis and interpretation of data (e.g., statistical analysis, biostatistics, imaging, computational analysis): MC-Z, AG, PK, NS, and DC. Administrative, technical, or material support: MC-Z, ND, CH, SE, DO, KL, MG, AT, PK, and DC. Study supervision: DC. All authors contributed to writing, review, and/or revision of the manuscript.

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REFERENCES

1. Kaal ECA, Niël CGJH, Vecht CJ. Therapeutic management of brain metastasis. *Lancet Neurol* (2005) 4(5):289–98. doi:10.1016/S1474-4422(05)70072-7
2. Lin NU, Bellon JR, Winer EP. CNS metastases in breast cancer. *JCO* (2004) 22(17):3608–17. doi:10.1200/JCO.2004.01.175
3. Anders C, Carey LA. Understanding and treating triple-negative breast cancer. *Oncology (Williston Park)* (2008) 22(11):1233–9; discussion 9-40, 43.
4. Evans AJ, James JJ, Cornford EJ, Chan SY, Burrell HC, Pinder SE, et al. Brain metastases from breast cancer: identification of a high-risk group. *Clin Oncol (R Coll Radiol)* (2004) 16(5):345–9. doi:10.1016/j.clon.2004.03.012
5. Tham Y-L, Sexton K, Kramer R, Hilsenbeck S, Elledge R. Primary breast cancer phenotypes associated with propensity for central nervous system metastases. *Cancer* (2006) 107(4):696–704. doi:10.1002/cncr.22041
6. Hicks DG, Short SM, Prescott NL, Tarr SM, Coleman KA, Yoder BJ, et al. Breast cancers with brain metastases are more likely to be estrogen receptor negative, express the basal cytokeratin CK5/6, and overexpress HER2 or EGFR. *Am J Surg Pathol* (2006) 30(9):1097–104. doi:10.1097/01.pas.0000213306.05811.b9
7. Morris PG, Murphy CG, Mallam D, Accordino M, Patil S, Howard J, et al. Limited overall survival in patients with brain metastases from triple negative breast cancer. *Breast J* (2012) 18(4):345–50. doi:10.1111/j.1524-4741.2012.01246.x

8. Braccini AL, Azria D, Thezenas S, Romieu G, Ferrero JM, Jacot W. Prognostic factors of brain metastases from breast cancer: impact of targeted therapies. *Breast* (2013) 22(5):993–8. doi:10.1016/j.breast.2013.05.011
9. Tomasello G, Bedard PL, de Azambuja E, Lossignol D, Devriendt D, Piccart-Gebhart MJ. Brain metastases in HER2-positive breast cancer: the evolving role of lapatinib. *Crit Rev Oncol Hematol* (2010) 75(2):110–21. doi:10.1016/j.critrevonc.2009.11.003
10. Ghajar CM, Peinado H, Mori H, Matei IR, Evason KJ, Brazier H, et al. The perivascular niche regulates breast tumour dormancy. *Nat Cell Biol* (2013) 15(7):807–17. doi:10.1038/ncb2767
11. Eichler AF, Chung E, Kodack DP, Loeffler JS, Fukumura D, Jain RK. The biology of brain metastases-translation to new therapies. *Nat Rev Clin Oncol* (2011) 8(6):344–56. doi:10.1038/nrclinonc.2011.58
12. Hu G, Kang Y, Wang XF. From breast to the brain: unraveling the puzzle of metastasis organotropism. *J Mol Cell Biol* (2009) 1(1):3–5. doi:10.1093/jmcb/mjp005
13. Marino N, Woditschka S, Reed LT, Nakayama J, Mayer M, Wetzel M, et al. Breast cancer metastasis: issues for the personalization of its prevention and treatment. *Am J Pathol* (2013) 183(4):1084–95. doi:10.1016/j.ajpath.2013.06.012
14. Palmieri D, Smith QR, Lockman PR, Bronder J, Gril B, Chambers AF, et al. Brain metastases of breast cancer. *Breast Dis* (2006) 26:139–47. doi:10.3233/BD-2007-26112
15. Palmieri D, Bronder JL, Herring JM, Yoneda T, Weil RJ, Stark AM, et al. Her-2 overexpression increases the metastatic outgrowth of breast cancer cells in the brain. *Cancer Res* (2007) 67(9):4190–8. doi:10.1158/0008-5472.CAN-06-3316
16. Munoz R, Man S, Shaked Y, Lee CR, Wong J, Francia G, et al. Highly efficacious nontoxic preclinical treatment for advanced metastatic breast cancer using combination oral UFT-cyclophosphamide metronomic chemotherapy. *Cancer Res* (2006) 66(7):3386–91. doi:10.1158/0008-5472.CAN-05-4411
17. Bos PD, Zhang XHF, Nadal C, Shu W, Gomis RR, Nguyen DX, et al. Genes that mediate breast cancer metastasis to the brain. *Nature* (2009) 459(7249):1005–9. doi:10.1038/nature08021
18. Zhang S, Huang WC, Zhang L, Zhang C, Lowery FJ, Ding Z, et al. SRC family kinases as novel therapeutic targets to treat breast cancer brain metastases. *Cancer Res* (2013) 73(18):5764–74. doi:10.1158/0008-5472.CAN-12-1803
19. Francia G, Cruz-Munoz W, Man S, Xu P, Kerbel RS. Mouse models of advanced spontaneous metastasis for experimental therapeutics. *Nat Rev Cancer* (2011) 11(2):135–41. doi:10.1038/nrc3001
20. Chen EI, Hewel J, Krueger JS, Tiraby C, Weber MR, Kralli A, et al. Adaptation of energy metabolism in breast cancer brain metastases. *Cancer Res* (2007) 67(4):1472–86. doi:10.1158/0008-5472.CAN-06-3137
21. Valastyan S, Weinberg RA. Tumor metastasis: molecular insights and evolving paradigms. *Cell* (2011) 147(2):275–92. doi:10.1016/j.cell.2011.09.024
22. Kabos P, Finlay-Schultz J, Li C, Kline E, Finlayson C, Wisell J, et al. Patient-derived luminal breast cancer xenografts retain hormone receptor heterogeneity and help define unique estrogen-dependent gene signatures. *Breast Cancer Res Treat* (2012) 135(2):415–32. doi:10.1007/s10549-012-2164-8
23. DeRose YS, Wang G, Lin YC, Bernard PS, Buys SS, Ebbert MT, et al. Tumor grafts derived from women with breast cancer authentically reflect tumor pathology, growth, metastasis and disease outcomes. *Nat Med* (2011) 17(11):1514–20. doi:10.1038/nm.2454
24. Lum DH, Matsen C, Welm AL, Welm BE. Overview of human primary tumorgraft models: comparisons with traditional oncology preclinical models and the clinical relevance and utility of primary tumorgrafts in basic and translational oncology research. *Curr Protoc Pharmacol* (2012) 14:14.22. doi:10.1002/0471141755.ph1422s59
25. Tentler JJ, Tan AC, Weekes CD, Jimeno A, Leong S, Pitts TM, et al. Patient-derived tumour xenografts as models for oncology drug development. *Nat Rev Clin Oncol* (2012) 9(6):338–50. doi:10.1038/nrclinonc.2012.61
26. Zhang H, Cohen AL, Krishnakumar S, Wapnir IL, Veeriah S, Deng G, et al. Patient-derived xenografts of triple-negative breast cancer reproduce molecular features of patient tumors and respond to mTOR inhibition. *Breast Cancer Res* (2014) 16(2):R36. doi:10.1186/bcr3640
27. Hanna C, Kwok L, Finlay-Schultz J, Sartorius CA, Cittelly DM. Labeling of breast cancer patient-derived xenografts with traceable reporters for tumor growth and metastasis studies. *J Vis Exp* (2016) 117:e54944. doi:10.3791/54944
28. Kim D, Pertea G, Trapnell C, Pimentel H, Kelley R, Salzberg SL. TopHat2: accurate alignment of transcriptomes in the presence of insertions, deletions and gene fusions. *Genome Biol* (2013) 14(4):R36. doi:10.1186/gb-2013-14-4-r36
29. Ahdesmaki MJ, Gray SR, Johnson JH, Lai Z. Disambiguate: an open-source application for disambiguating two species in next generation sequencing data from grafted samples. *F1000Res* (2016) 5:2741. doi:10.12688/f1000research.10082.1
30. Liao Y, Smyth GK, Shi W. The subread aligner: fast, accurate and scalable read mapping by seed-and-vote. *Nucleic Acids Res* (2013) 41(10):e108. doi:10.1093/nar/gkt214
31. Love MI, Huber W, Anders S. Moderated estimation of fold change and dispersion for RNA-seq data with DESeq2. *Genome Biol* (2014) 15(12):550. doi:10.1186/s13059-014-0550-8
32. Shao MM, Liu J, Vong JS, Niu Y, Germin B, Tang P, et al. A subset of breast cancer predisposes to brain metastasis. *Med Mol Morphol* (2011) 44(1):15–20. doi:10.1007/s00795-010-0495-2
33. Rosfjord E, Lucas J, Li G, Gerber HP. Advances in patient-derived tumor xenografts: from target identification to predicting clinical response rates in oncology. *Biochem Pharmacol* (2014) 91(2):135–43. doi:10.1016/j.bcp.2014.06.008
34. Siolas D, Hannon GJ. Patient-derived tumor xenografts: transforming clinical samples into mouse models. *Cancer Res* (2013) 73(17):5315–9. doi:10.1158/0008-5472.CAN-13-1069
35. Dobrolecki LE, Airhart SD, Alferez DG, Aparicio S, Behbod F, Bentires-Alj M, et al. Patient-derived xenograft (PDX) models in basic and translational breast cancer research. *Cancer Metastasis Rev* (2016) 35(4):547–73. doi:10.1007/s10555-016-9653-x
36. Tseng LM, Hsu NC, Chen SC, Lu YS, Lin CH, Chang DY, et al. Distant metastasis in triple-negative breast cancer. *Neoplasma* (2013) 60(3):290–4. doi:10.4149/neo_2013_038
37. Sihto H, Lundin J, Lundin M, Lehtimaki T, Ristimaki A, Holli K, et al. Breast cancer biological subtypes and protein expression predict for the preferential distant metastasis sites: a nationwide cohort study. *Breast Cancer Res* (2011) 13(5):R87. doi:10.1186/bcr2944
38. Weil RJ, Palmieri DC, Bronder JL, Stark AM, Steeg PS. Breast cancer metastasis to the central nervous system. *Am J Pathol* (2005) 167(4):913–20. doi:10.1016/S0002-9440(10)61180-7
39. Hung MH, Liu CY, Shiau CY, Hsu CY, Tsai YF, Wang YL, et al. Effect of age and biological subtype on the risk and timing of brain metastasis in breast cancer patients. *PLoS One* (2014) 9(2):e89389. doi:10.1371/journal.pone.0089389
40. Priedigkeit N, Hartmaier RJ, Chen Y, Vareslija D, Basudan A, Watters RJ, et al. Intrinsic subtype switching and acquired ERBB2/HER2 amplifications and mutations in breast cancer brain metastases. *JAMA Oncol* (2017) 3(5):666–71. doi:10.1001/jamaoncol.2016.5630
41. Lee HW, Lee JI, Lee SJ, Cho HJ, Song HJ, Jeong DE, et al. Patient-derived xenografts from non-small cell lung cancer brain metastases are valuable translational platforms for the development of personalized targeted therapy. *Clin Cancer Res* (2015) 21(5):1172–82. doi:10.1158/1078-0432.CCR-14-1589
42. Sartorius CA, Harvell DM, Shen T, Horwitz KB. Progestins initiate a luminal to myoepithelial switch in estrogen-dependent human breast tumors without altering growth. *Cancer Res* (2005) 65(21):9779–88. doi:10.1158/0008-5472.CAN-05-0505
43. Cittelly DM, Finlay-Schultz J, Howe EN, Spoelstra NS, Axlund SD, Hendricks P, et al. Progestin suppression of miR-29 potentiates dedifferentiation of breast cancer cells via KLF4. *Oncogene* (2013) 32(20):2555–64. doi:10.1038/onc.2012.275
44. Giuliano M, Herrera S, Christiny P, Shaw C, Creighton CJ, Mitchell T, et al. Circulating and disseminated tumor cells from breast cancer patient-derived xenograft-bearing mice as a novel model to study metastasis. *Breast Cancer Res* (2015) 17:3. doi:10.1186/s13058-014-0508-5
45. Wang J, Daphu I, Pedersen PH, Miletic H, Hovland R, Mork S, et al. A novel brain metastases model developed in immunodeficient rats closely mimics the growth of metastatic brain tumours in patients. *Neuropathol Appl Neurobiol* (2011) 37(2):189–205. doi:10.1111/j.1365-2990.2010.01119.x
46. Ni J, Ramkissoon SH, Xie S, Goel S, Stover DG, Guo H, et al. Combination inhibition of PI3K and mTORC1 yields durable remissions in mice bearing orthotopic patient-derived xenografts of HER2-positive breast cancer brain metastases. *Nat Med* (2016) 22(7):723–6. doi:10.1038/nm.4120

47. Zhang C, Lowery FJ, Yu D. Intracarotid cancer cell injection to produce mouse models of brain metastasis. *J Vis Exp* (2017) 120:e55085. doi:10.3791/55085

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Anti-inflammatory Microglia/Macrophages As a Potential Therapeutic Target in Brain Metastasis

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Brain metastasis is a common complication of cancer patients and is associated with poor survival. Histological data from patients with brain metastases suggest that microglia are the major immune population activated around the metastatic foci. Microglia and macrophages have the ability to polarize to different phenotypes and to exert both tumorigenic and cytotoxic effects. However, the role of microglia/macrophages during the early stages of metastatic growth in the brain has not yet been determined. The aim of this study was to profile microglial/macrophage activation in a mouse model of breast cancer brain metastasis during the early stages of tumor growth, and to assess the role of the anti-inflammatory microglial/macrophage population, specifically, during this phase. Following intracerebral injection of 5×10^3 4T1-GFP mammary carcinoma cells into female BALB/c mice, robust microglial/macrophage activation around the 4T1 metastatic foci was evident throughout the time-course studied (28 days) and correlated positively with tumor volume ($R^2 = 0.67$). Populations of classically (proinflammatory) and alternatively (anti-inflammatory) activated microglia/macrophages were identified immunohistochemically by expression of either induced nitric oxide synthase/cyclooxygenase 2 or mannose receptor 1/arginase 1, respectively. Temporally, levels of both pro- and anti-inflammatory cells were broadly stable across the time-course. Subsequently, selective depletion of the anti-inflammatory microglia/macrophage population by intracerebral injection of mannosylated clodronate liposomes significantly reduced metastatic tumor burden ($p < 0.01$). Moreover, increased levels of apoptosis were associated with tumors in clodronate liposome treated animals compared to controls ($p < 0.05$). These findings suggest that microglia/macrophages are important effectors of the inflammatory response in the early stages of brain metastasis, and that targeting the anti-inflammatory microglial/macrophage population may offer an effective new therapeutic avenue for patients with brain metastases.

Keywords: microglia, macrophages, brain metastasis, anti-inflammatory, mouse models

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INTRODUCTION

Brain metastasis is a common complication in cancer. It is estimated that 20–40% of cancer patients will develop metastases in the brain; this percentage is increasing due to better control of the systemic disease and improved diagnosis. Current treatment options include radiotherapy and surgical resection, while conventional chemotherapy is often offered as adjuvant therapy and

is beneficial when used early in the course of the disease (1, 2). Nevertheless, brain metastasis is associated with poor survival even after whole-brain radiotherapy (3). Thus, a better understanding of the molecular and cellular mechanisms governing the early stages of metastasis development within the brain could provide new targets for therapeutic intervention with the aim of developing multidisciplinary approaches to better manage the disease.

The CNS resident macrophages, microglia, appear to be important components of the immune response to metastatic growth in the brain. Clustering of microglial cells around metastatic tumors has been described for both experimental and human brain metastases (4–6). Moreover, histological analysis of autopsy samples from patients with brain metastases suggests that microglia are the major immune population activated around the metastatic foci. HLA-DR$^+$ microglia/macrophages infiltrate the intracranial metastatic lesions in the cases of breast, melanoma, small cell lung, and non-small cell lung cancers (5). The histological study of another cohort of patients with breast cancer brain metastasis revealed the presence and close association of CD68$^+$ microglia/macrophages with GFAP expressing astrocytes between clusters of carcinoma cells (7). In contrast, low numbers of scattered B and T lymphocytes were associated with human brain metastases (5), and minimal neutrophil infiltration of brain metastases in the mouse 4T1 mammary carcinoma model has been reported (8).

In recent years, it has been established that microglia, such as macrophages, are versatile cells of the adaptive immune response, which have the ability to express distinct functional programs depending on stimuli from the local microenvironment (9). Microglia can polarize to either a "classical" proinflammatory phenotype, characterized by increased levels of proinflammatory cytokines, induced nitric oxide synthase (iNOS), and the ability to elicit a T cell immune response against neoplastic cells, or to an "alternative" anti-inflammatory phenotype, which promotes angiogenesis and tumor growth. It is now recognized, however, that in fact a spectrum of inflammatory macrophage phenotypes exist and that the historical bipolar classification is an oversimplification (10). Interestingly, glioma-infiltrating microglia have been shown to acquire a predominantly anti-inflammatory phenotype, which possibly accounts for their immunosuppressive effect (11). In experimental gliomas, tumor infiltrating microglia and macrophages upregulate their anti-inflammatory molecular signatures; notably arginase 1 (Arg1), transforming growth factor β, matrix metalloprotease 2, and interleukin 10 (IL-10) (12, 13). However, the role and phenotype of microglia in the early stages of metastatic outgrowth within the brain, i.e., once the tumor cells have already extravasated across the endothelium, has not yet been determined.

In systemic tumors, the growth promoting properties of tumor-associated macrophages (TAMs) is now well established, and recent efforts have focused either on targeting their recruitment and proliferation, or on re-educating them toward tumor rejection. Notably, small-molecule inhibitors of two important signaling axes, CSF-1/CSF-1R and CCL2/CCR2, are undergoing clinical trials for breast and other types of solid cancers (14–16). Given the emerging functional and phenotypic heterogeneity of TAMs, novel approaches to target trophic subsets of macrophages and microglia in a cancer specific context may improve existing regimes of chemotherapy and radiotherapy and enhance personalized treatments (15).

Based on the above, the initial aim of the current study was to determine the temporal and spatial profile of microglial/macrophage activation in a mouse model of breast cancer brain metastasis, with the overall goal of elucidating their role in promoting or suppressing tumor growth. Subsequently, the effect of selectively depleting the anti-inflammatory microglial/macrophage population, which may be expected to be tumor promoting, was assessed *in vivo* as a potential therapeutic approach for brain metastasis.

MATERIALS AND METHODS

Brain Metastasis Model

All animal procedures were carried out in accordance with the UK Animals (Scientific Procedures) Act 1986 and with the University of Oxford (Clinical Medicine) Ethical Committee approval.

Female BALB/c mice, 6–9 weeks old (Charles River Laboratories, Kent, UK) were anesthetized with 3–4% isoflurane in 70:30% N_2O:O_2, placed on a stereotactic frame and anesthesia maintained with 2% isoflurane. Using a finely drawn glass microcapillary, 70 μm diameter tip, 5×10^3 syngeneic mouse mammary carcinoma 4T1-GFP cells were stereotactically injected into the left striatum, through a burr hole in the skull, in 0.5 μl PBS (coordinates + 0.5mm/1.9 mm lateral relative to bregma, 2.8 mm deep). Following injection, the scalp incision was sutured and animals allowed to recover from anesthesia. Animals were transcardially perfusion-fixed on days 7, 10, 14, 21, or 28 after 4T1-GFP cell injection under terminal anesthesia using 0.9% heparinised saline followed by PLP_{light} fixative (75 mM L-lysine monohydrochloride, 10 mM Na_2HPO_4, 2% w/v PFA, 1 mM sodium metaperiodate in phosphate buffer, 0.025% w/v glutaraldehyde, pH 7.2). Brains were excised, cryoprotected in 30% w/v sucrose, embedded in OCT and frozen in isopentane at −80°C. Intracerebral injections of vehicle (sterile PBS) were performed as above in control animals.

CNS Inflammation Model

An anti-inflammatory model of CNS inflammation was induced by intracerebral microinjection of 100 ng murine recombinant interleukin-4 (IL-4; Peprotech, UK) in 0.5 μl sterile Milli-Q H_2O using a 50 μm tipped glass microcapillary (coordinates + 0.5/1.9 mm lateral relative to bregma, 2.8 mm deep). Animals were sacrificed at 6, 24, 48, or 72 h after cytokine injection by transcardial perfusion-fixation, as described above for the brain metastasis model.

Microglia/Macrophage Depletion Studies

Mannosylated control (PBS) or clodronate (10 mg/ml) liposomes (Encapsula Nanosciences, USA) in PBS were gently rocked to yield a homogeneous suspension before intracerebral injection using a 50 μm tipped glass microcapillary. Using the IL-4 model of neuroinflammation above, control or clodronate liposomes (0.5 μl) were intracerebrally injected 24 h after

intracerebral injection of IL-4 (schematic in Figure S1A in Supplementary Material) at the same coordinates. Mice were transcardially perfusion-fixed 48 h after liposome injection, as described above. Subsequently, mice injected intracerebrally with 4T1-GFP cells (as above) were injected intracerebrally with control or clodronate liposomes (0.5 µl) 12 days later (schematic in Figure S1B in Supplementary Material) at the same coordinates. Animals were transcardially perfusion-fixed 3, 9, or 16 days after liposome injection. Two further control groups of metastasis bearing mice were injected intracerebrally with either 0.5 µl PBS or 0.5 µg of dichloromethylenediphosphonic acid disodium salt (clodronate salt; $CH_2O_6Cl_2Na_2P_2$; Sigma-Aldrich, UK) in 0.5 µl Milli-Q H_20 (pH 7).

Immunohistochemistry

Immunohistochemistry was performed on 10 µm thick tissue sections mounted on glass slides (Superfrost Plus, Menzel Gläzer, Braunschweig, Germany). Initially, slides were allowed to come to room temperature before rehydration in PBS (Thermo Fisher Scientific, UK; pH 7.4). When necessary, antigen retrieval was performed using sodium citrate buffer containing 0.1% Tween 20 (pH 6.0). Tissue was permeabilized in PBS containing 0.05% Tween 20 and endogenous peroxidases were quenched in 1% (v/v) hydrogen peroxide (30% w/w) (Sigma, Aldrich) in methanol. Brain sections were blocked for non-specific antibody binding in 10% serum (in PBS) for an hour and incubated overnight at 4°C with primary antibodies (1% serum in PBS): anti-Iba1 (Abcam ab5076), anti-iNOS (M-19) (SantaCruz sc650), anti-Arg1 (Novus Biologicals NBP1-54621), anti-MRC1 (AbD Serotec MCA2235), anti-COX2 (Abcam ab15191), anti-CC3 (Asp175) (Cell Signaling Technology 9661), and anti-CD31 (R&D AF3628). After rinsing in PBS, tissue sections were incubated with appropriate secondary antibodies (Vector Labs, CA, USA) for an hour: biotinylated horse anti-goat IgG, biotinylated goat anti-rabbit IgG, biotinylated rabbit anti-rat (mouse absorbed) IgG. This step was followed by incubation with the avidin/biotin complex-horseradish peroxidase (HRP) system (VECTASTAIN Elite ABC Kit Standard, Vector Laboratories, CA, USA). Peroxidase was detected using 3,3′-diaminobenzidine (DAB) and tissue was counterstained with 0.5% cresyl violet. Tissue was dehydrated using increasing concentration of ethanol, cleared in xylene and mounted in DPX mountant (Fisher Scientific, UK). Immunostained slides were imaged on ScanScope CS slide scanner (Aperio, Vista, CA, USA) and analyzed using ImageScope (Aperio, USA).

Quantitative analysis of the volumes of metastatic areas and microglial/macrophage infiltration was performed by manual demarcation of the areas of interest on brain sections 50 µm apart spanning the metastatic lesions: tumors were defined as cresyl violet-positive foci and microglial/macrophage infiltration area by the outer limit of reactive $Iba1^+$ cells. Quantitation of DAB for inflammatory markers was performed using the Positive Pixel Count Algorithm (Aperio, USA) and converted to area (μm^2) of immunostaining. Positive and strong positive pixels within the areas of interest were included for the analysis. In the IL-4 *in vivo* study, numbers of $Iba1^+$ cells were quantified across different fields of view from multiple sections, as specified.

Image Coregistration

An in-house Matlab/ImageJ based approach to coregister immunostains on sequential sections was developed. 10 µm thick sequential brain sections from mice bearing 4T1-GFP metastases were stained for different biomarkers: Iba1 for microglia/macrophage; iNOS and COX2 for proinflammatory phenotype; Arg1 and MRC1 for anti-inflammatory phenotype and counterstained with cresyl violet. Sequential IHC images were coregistered by choosing landmark points that correspond to the same areas on both images based on metastatic lesion and brain morphology (corpus callosum, ventricle). Coregistered images were subsequently thresholded by color to extract maps of the different stains and merged into a single overlap map. The overlap maps show colocalization of Iba1 with either polarization marker in white, and immunostained pixels for the polarization markers that did not colocalize with Iba1 pixels on the sequential section in red. The white pixels (double labeling) corresponded to the immunostained object for the polarization markers, rather the microglia/macrophage marker, to yield a percentage of Iba1-positive pixels that were positive for each of the polarization markers.

Immunofluorescence

Immunofluorescent detection of antigens was performed on 10–20 µm thick tissue sections mounted on glass slides (Superfrost Plus, Menzel Gläzer, Braunschweig, Germany). Tissue was permeabilized with PBS Tween 20 (0.05%), endogenous peroxidase activity was quenched in 1% H_2O_2 in PBS and endogenous biotin was blocked using the streptavidin/biotin blocking kit (Vector Laboratories, CA, USA). Tissue was blocked in TNB buffer (PerkinElmer, UK) for 1 h and primary antibodies were applied overnight at 4°C: anti-Iba1 (Abcam ab5076), anti-iNOS (M-20) (sc651), anti-Arg1(H-52 sc20150), anti-MRC1 (AbD MCA2235), anti-CC3 (Asp175) (CST 9661), and anti-GFP (Abcam ab13970). Next day, sections were washed in PBS and incubated with the appropriate fluorophore conjugated secondary antibodies for an hour: anti-goat TexasRed or antigoat DyLight 594 (Vector Labs, CA, USA), anti-rabbit TexasRed (Vector Labs, CA, USA), and anti-chicken CFTM488A (Sigma-Aldrich, UK). Alternatively, biotinylated secondary antibodies were applied on sections for 1 h followed by streptavidin-conjugated fluorophores (AMCA or Dylight 488) (Vector Labs, CA, USA) for an additional hour. iNOS and Arg1 signals were amplified using the TSA Biotin System (PerkinElmer LAS, UK). Briefly, after the incubation with anti-rabbit biotinylated secondary antibody, streptavidin–HRP (1:200 in TNB buffer) was applied to the tissue for 30 min followed by a 10 min incubation with biotinylated TSA (1:100 in amplification buffer). Streptavidin-conjugated AMCA was used as the final step for the amplification protocol.

A colocalization analysis was performed between the anti-inflammatory marker MRC1 and Iba1 using an in-house ImageJ plugin. The parameters measured by the plugin were the percentage area of marker A's signal that is above a user set threshold, the percentage area of marker B's signal that is above a user set threshold, the percentage area of the image where both markers are above the user set threshold (the colocalized image area), and the percentage of each marker's signal that

has overlaps with the colocalized image area. The plugin also calculated the Pearson's correlation coefficient and the Mander's overlap coefficient.

Immunofluorescent staining was also performed on cells grown on coverslips and treated appropriately (see below). Cells were fixed in 4% PFA (in PBS, pH 7.2) for 10 min and washed in PBS twice. Cells were permeabilized with PBS Tween 20 (0.05%) and blocked for endogenous biotin using the streptavidin/biotin blocking kit (Vector Laboratories, CA, USA). Subsequently, cells were incubated with TNB buffer (PerkinElmer) for 1 h in a humidified chamber to block non-specific antibody binding. Appropriate primary antibodies were applied to the cells overnight at 4°C: anti-rat MRC1 (AbD MCA2235), anti-mouse F4/80 Biotin (eBioscience, 13-4801-81), anti-iNOS (M-20) (sc651). Next day, cells were washed in PBS and appropriate fluorophore-conjugated fluorophores (anti-rat Texas Red, anti-rabbit Texas Red, streptavidin-conjugated Dylight 488) were applied to the cells for 1 h at room temperature. Cell nuclei were counterstain with DAPI (Vector Laboratories, CA, USA).

In Vitro Experiments

All cell lines used were routinely grown in DMEM (Invitrogen, UK) supplemented with 10% fetal bovine serum (Labtech, International, UK) and 1% L-glutamine (PAA, UK). Equal numbers of BV2 microglia or RAW 264.7 macrophages were seeded onto 12- or 96-well plates (Greiner Bio-one, CellStar). Cells were treated with 100 ng/ml LPS (*E. coli* derived, 0111:B4) (Sigma-Aldrich, UK) or 20 ng/ml murine recombinant IL-4 (Peprotech, UK) in DMEM for polarization to the pro- or anti-inflammatory phenotypes, respectively, or left untreated for the unpolarized control phenotype. At the end of the experiment, cells were fixed in 4% PFA (in PBS, pH 7.2) for immunofluorescence staining.

4T1-GFP, BV2, and RAW 264.7 cells were seeded onto 96-well plates and when cell confluency reached 60–70%, cells were treated with mannosylated control (PBS) or clodronate (10 mg/ml) liposomes (Encapsula Nanosciences, USA) diluted 1:50 or 1:100 in culture medium for 24 h. Alternatively, BV2 and RAW 264.7 cells seeded onto 96-well plates were treated with IL-4 (20 ng/ml) or LPS (100 ng/ml) for polarization when cell confluency reached 40–50%. 24 h after treatment with the polarizing agents, cells were treated with mannosylated control or clodronate liposomes diluted 1:50 in culture medium for 24 h. At the end of the experiment cells were subjected to the MTT viability assay.

MTT Cell Viability Assay

BV2, RAW 264.7, and 4T1-GFP cells treated as above were subjected to an MTT assay (CellTiter 96® Non-Radioactive Cell Proliferation Assay; Promega, UK) according to the manufacturer's instructions. Briefly, 15 µl of MTT dye solution was added per well in the dark. Plates were incubated at 37°C in a humidified, 5% CO_2 atmosphere for 3–4 h, depending on the cell line. After the development of the violet formazan product, 100 µl of solubilization/stop solution was added per well and plates were left shaking on a platform for 1 h at room temperature. Absorbance was recorded at 570 nm wavelength using a 96-well plate reader (Tecan Infinite® Pro, Switzerland).

Statistics

For assessment of microglial/macrophage activation over time and colocalization of pro- and anti-inflammatory markers one-way ANOVA was used, with *post hoc* Tukey's or Newman–Keuls multiple comparison tests. For *in vitro* assessment of liposome effects one-way ANOVA with *post hoc* Dunnett's multiple comparison tests were used to determine significant differences between groups. For the *in vivo* microglia/macrophage depletion studies two tailed unpaired *t*-tests were used to determine differences between groups.

RESULTS

Microglial/Macrophage Infiltration in 4T1-GFP Brain Metastases

The spatial and temporal profile of microglial/macrophage infiltration in the early stages of metastatic growth in the brain was assessed in the intracerebral syngeneic 4T1-GFP model. $Iba1^+$ macrophages/microglia were found in close vicinity to the metastatic foci, as well as in the wider tumor microenvironment (**Figure 1A**). Qualitatively, sustained microglial/macrophage infiltration of the 4T1-GFP metastatic foci was observed at all time points throughout the 28-day study (**Figure 1B**). Both ramified and amoeboid Iba1 positively stained cells were identified, with amoeboid cells that could be either microglia or perivascular macrophages (17) mostly associated closely with the 4T1-GFP foci, while ramified microglia were found both close to the tumor foci and further away in the broader tumor microenvironment (**Figure 1A**). Quantitatively, microglial/macrophage infiltration increased significantly over time as intracranial tumor volume increased (ANOVA $p < 0.001$; **Figure 1C**) and a strong positive correlation was evident between the Iba1 immunostained area and the volume of the 4T1-GFP metastases ($R^2 = 0.671$; **Figure 1D**).

Pro- and Anti-inflammatory Microglia/Macrophages in the Metastatic Brain

Since it is well established that microglia and macrophages can exert different functions in the tumor microenvironment depending on their molecular phenotype, the polarization state of microglia/macrophages infiltrating the 4T1-GFP metastatic foci was investigated. Both proinflammatory (iNOS and COX2) and anti-inflammatory (MRC1 and Arg1) phenotype markers were expressed in the metastatic brain at all time-points (**Figure 2A**). All four markers were specifically upregulated in response to brain metastasis, as indicated by the negligible expression in ipsilateral and contralateral hemispheres of mice injected intracranially with PBS as control (Figure S2 in Supplementary Material).

Double immunofluorescence was employed to specifically identify $Iba1^+$ cells bearing a pro- or anti-inflammatory phenotype; iNOS was used as the prototype marker for the proinflammatory phenotype, whilst Arg1 was used for the anti-inflammatory phenotype (**Figure 2B**). Both pro- and anti-inflammatory microglia/macrophages were evident in close vicinity to the 4T1-GFP metastatic foci at all time-points after intracerebral injection of

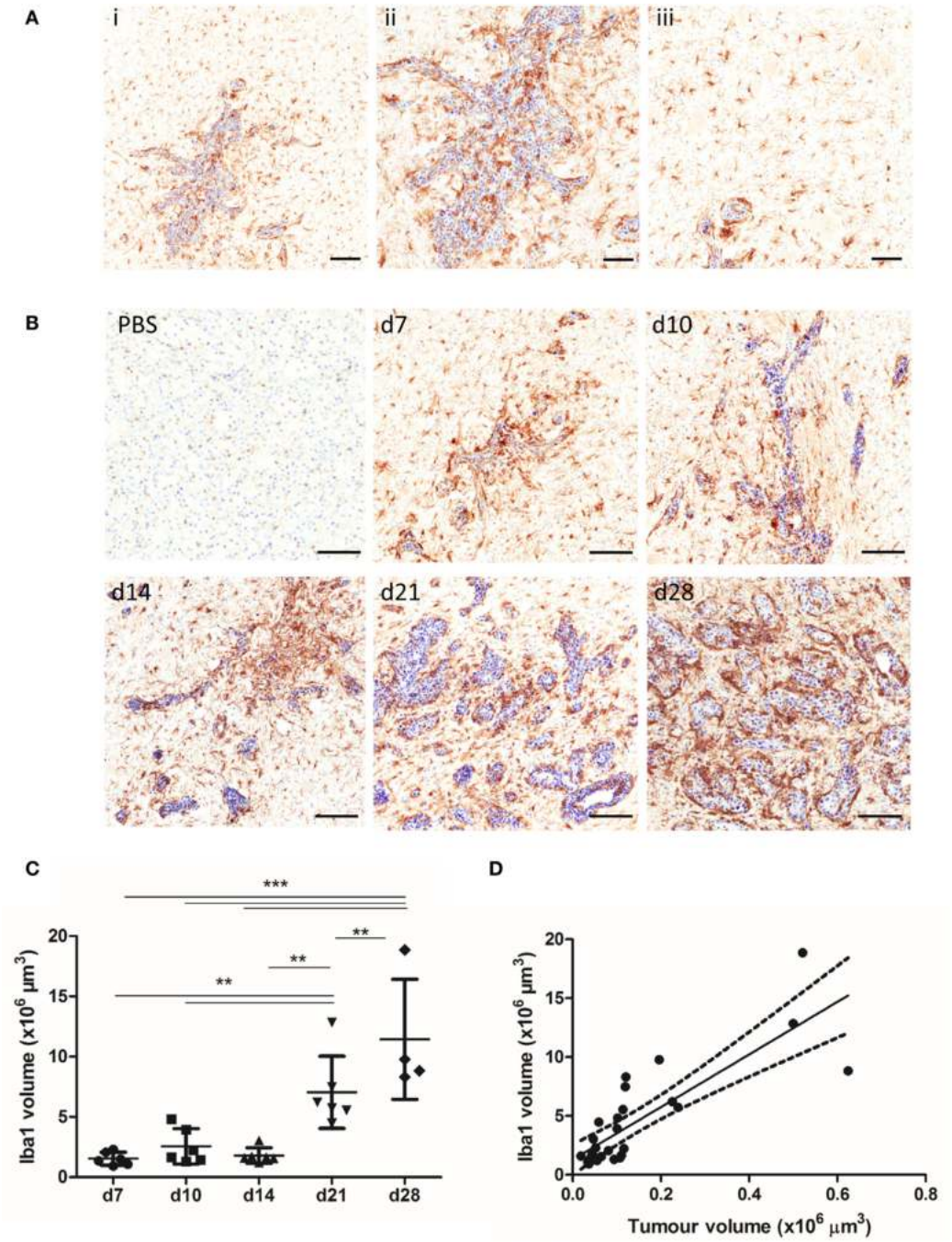

FIGURE 1 | **(A)** Iba1$^+$ microglia/macrophages (brown) are detected in close association with the tumor foci (ii) and within the broader tumor microenvironment (iii) in the 4T1-GFP metastatic brains, as shown by a representative image of a brain section (counterstained with cresyl violet) at day 7 after intracerebral injection of 5×10^3 4T1-GFP cells. **(B)** Representative immunohistochemical images showing increased expression of Iba1 at days 7, 10, 14, 21, and 28 after intracerebral injection of 5×10^3 4T1-GFP cells compared to intracerebral PBS injection (d7). **(C)** Quantitation of the Iba1$^+$ immunostained area in the metastatic brain showed a significant increase over time ($n = 4$–6 per group). $^{**}p < 0.01$, $^{***}p < 0.001$; one-way ANOVA with Tukey's multiple comparison test. **(D)** Pearson correlation plot of microglial/macrophage infiltration (Iba1$^+$) area against tumor volume (cresyl violet foci) revealed a strong positive correlation ($R^2 = 0.671$). Scale bars 100 µm [**(A)**, i, **(B)**] or 50 µm [**(A)**, ii, iii].

the tumor cells. Arg1 and iNOS were also expressed to some extent by other cells in the tumor microenvironment (**Figure 2B**).

Using the sequential section coregistration approach in Matlab (Figure S3A), the spatiotemporal expression of differently polarized microglia/macrophages during the early stages of brain metastasis was analyzed (**Figure 3**). Proinflammatory microglia/macrophages were identified as iNOS$^+$Iba1$^+$ or COX2$^+$Iba1$^+$ cells, and anti-inflammatory microglia/macrophages as MRC1$^+$Iba1$^+$

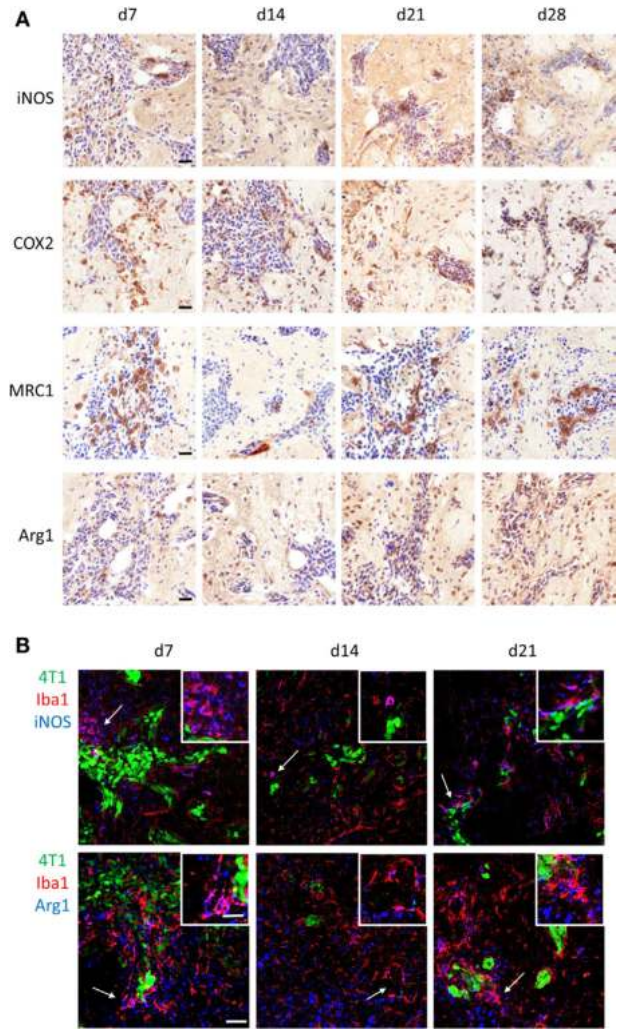

FIGURE 2 | **(A)** Immunohistochemical detection (brown stain) of the proinflammatory markers induced nitric oxide synthase (iNOS) and cyclooxygenase 2, and the anti-inflammatory markers mannose receptor 1 and arginase 1 (Arg1) at days 7, 10, 14, and 21 after intracerebral injection of 5×10^3 4T1-GFP cells (sections counterstained with cresyl violet). **(B)** Detection of pro- and anti-inflammatory microglia/macrophages in the metastatic brain by immunofluorescent colocalization of Iba1 with either iNOS (top panel) or Arg1 (bottom panel) at days 7, 14, and 21 after intracerebral injection of 5×10^3 4T1-GFP cells. Arrows show areas of colocalization, which are magnified in the insets. Scale bars 20 μm **(A)**, 50 μm **(B)**, or 25 μm [**(B)** insets].

or $Arg1^+Iba1^+$ cells. As for the double immunofluorescence, populations of both pro- and anti-inflammatory cells were found in close association with the tumor foci at days 7, 10, 14, and 21 after intracerebral injection of 4T1-GFP cells (**Figure 3A**). Quantitation of the colocalization of each marker with Iba1 as a percentage of the microglial/macrophage activation area, on representative sections from the central section of the 4T1-GFP tumors, revealed no significant differences in each phenotype over time. However, all of the markers appeared to show a trend toward a decrease in colocalization from day 7 to day 10 followed by an increase at days 14 and 21, with the exception of MRC1, which appeared to drop again at day 21 (Figures S3B–E in Supplementary Material). The strongest expression of both pro- and anti-inflammatory markers was evident at the core of the metastatic region, in close association with the tumor foci, compared to the broader tumor microenvironment. Thus, further quantitative analysis of the regions circumscribed by the tumor foci alone was performed (**Figure 3B**) and showed a significant increase in $iNOS^+$ microglia/macrophages at day 21 compared to all other time-points (ANOVA $p < 0.01$; **Figure 3B**). Similar trends toward increased $COX2^+$ and $Arg1^+$ microglia/macrophages at day 21 were also seen, but these did not reach significance (**Figure 3B**). Moreover, when data for the two proinflammatory markers (iNOS and COX2) and the two anti-inflammatory markers (MRC1 and Arg1) were normalized to their respective day 7 values and combined, both pro- and anti-inflammatory microglial/macrophage populations showed a significant increase over time: Pearson correlations $r = 0.49$, $p < 0.01$ for iNOS and COX2 combined; $r = 0.40$, $p < 0.05$ for MRC and Arg1 combined.

Effect of Mannosylated Clodronate Liposomes on Cell Viability *In Vitro*

Treatment of either the BV2 microglial cells, or RAW 264.7 macrophages with mannosylated control liposomes conferred no significant changes in cell viability (**Figures 4A,B**). In contrast, a statistically significant decrease in cell viability was observed for both cell lines after 24 h of treatment with mannosylated clodronate liposomes (1:50), compared to either untreated cells or cells treated with control liposomes (ANOVA $p < 0.001$; **Figures 4A,B**). No difference in effect was observed between treatment with 1:50 and 1:100 dilutions of clodronate liposomes (Figures S4A,B in Supplementary Material). 4T1-GFP mammary carcinoma cells do not express the mannose receptor MRC1 (Figure S4C in Supplementary Material) and, as expected, treatment with mannosylated clodronate liposomes at both concentrations (1:50 and 1:100 dilution) did not affect viability of 4T1-GFP cells (Figure S4D in Supplementary Material).

The specificity of the mannosylated liposomes for polarized microglial cells and macrophages was also investigated, by treatment of BV2 and RAW 264.7 cells with either LPS or IL-4 to induce a pro- or anti-inflammatory phenotype, respectively. As expected, iNOS was induced following LPS treatment and IL4 treatment led to a significant increase in MRC1 expression compared to no treatment or LPS treatment (unpaired *t*-test, $p < 0.001$) (Figures S5A,B in Supplementary Material). Both BV2 and RAW 264.7 cells treated with IL-4, to induce an anti-inflammatory phenotype, showed a significant decrease in cell viability upon treatment with mannosylated clodronate liposomes for 24 h (ANOVA $p < 0.01$; **Figures 4A,B**, blue bars). In contrast, the reduction in cell viability seen in BV2 and RAW 264.7 cells treated with LPS, to induce a proinflammatory phenotype, following incubation with mannosylated clodronate liposomes was not significant (**Figures 4A,B**; green bars). Thus, the effect of the clodronate liposomes observed in unstimulated cells appeared to be partially reversed in those polarized toward a proinflammatory phenotype.

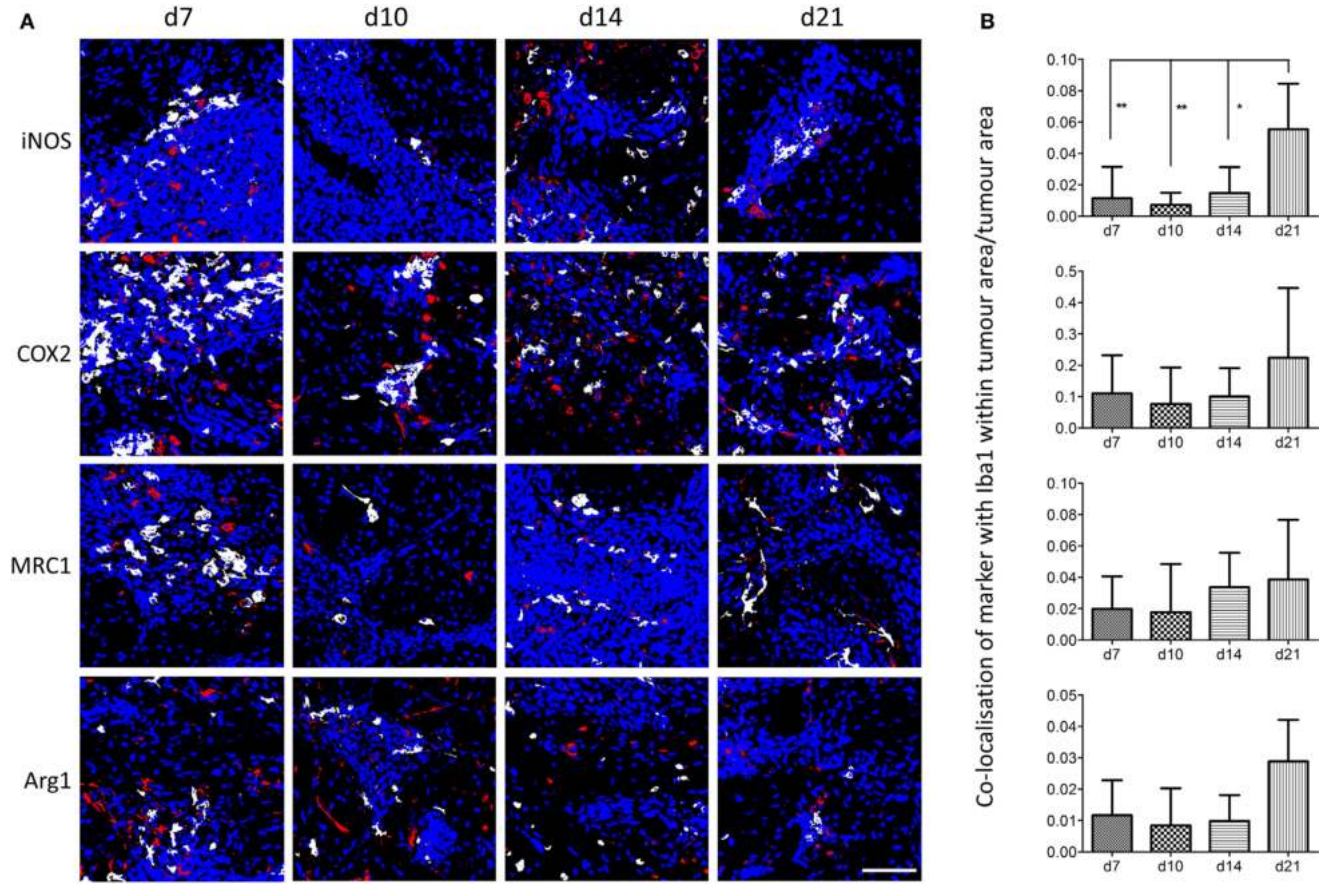

FIGURE 3 | **(A)** Matlab generated images of iNOS$^+$, COX2$^+$, MRC1$^+$, or Arg1$^+$ microglia/macrophages (Iba1$^+$) at days 7, 10, 14, and 21 after intracerebral injection of 5×10^3 4T1-GFP cells. White = colocalized pixels, blue = tumor cells, and red = inflammatory markers. **(B)** Quantitative analysis of colocalized pixels for pro- and anti-inflammatory markers with Iba1 within the tumor area only, normalized to tumor area (n = 3–6 per time point; $^*p < 0.05$, $^{**}p < 0.01$; one-way ANOVA with Tukey's multiple comparison test). Scale bar 50 μm.

Depletion of MRC1$^+$ Cells in an IL-4 Induced Neuroinflammatory Model

In order to validate the effect of mannosylated clodronate liposomes in targeting MRC1 expressing cells *in vivo*, a CNS inflammation model of anti-inflammatory cell polarization was employed as a proof-of-principle experiment. IL-4 injected intracerebrally into the left striatum induced a robust anti-inflammatory microglial/macrophage response, as indicated by a significant (ANOVA $p < 0.001$) increase in the number of Iba1$^+$ cells compared to the contralateral hemisphere ($n = 3$ per group, nine fields of view across three sections per animal; **Figure 5A**) and upregulation of MRC1 expression ($n = 3$ per group, nine sections per animal 50 μm apart; **Figures 5B,C**). Iba1 activation and MRC1 upregulation showed similar temporal and spatial changes across the time-course, reaching a peak at 48 h after IL-4 injection (**Figures 5A,B**). On this basis, the efficiency of MRC1$^+$ cell depletion *in vivo* using the mannosylated clodronate liposomes was assessed in IL-4 challenged brains; control or clodronate liposomes were intracerebrally injected 24 h after intracerebral injection of IL-4. Immunohistochemically, a significant decrease (ca. 60%; unpaired t-test $p < 0.05$) in MRC1 expression was observed 48 h after injection with mannosylated clodronate liposomes compared to mice injected with control liposomes ($n = 3$ per group, nine sections per animal 50 μm apart; **Figures 5D,E**).

Depletion of MRC1$^+$ Cells in the Metastatic 4T1-GFP Brain

Since the percentage of MRC1$^+$ microglia/macrophage in the metastatic brain was greatest at day 14 (Figure S3D in Supplementary Material), liposomes were administered 12 days after 4T1-GFP injection to deplete this cell population. Three days after intracerebral injection of mannosylated clodronate liposomes in the metastatic brain, a decrease in MRC1 expression around the tumor foci was observed (**Figure 6A**), which was statistically significant (unpaired t-test $p < 0.05$) compared to control liposome injected mice ($n = 4$ per group; **Figure 6B**). Depletion of MRC1$^+$ microglia/macrophages in the 4T1-GFP metastatic brain was also qualitatively demonstrated by double immunofluorescence (**Figure 6C**). Quantitation of the colocalization of

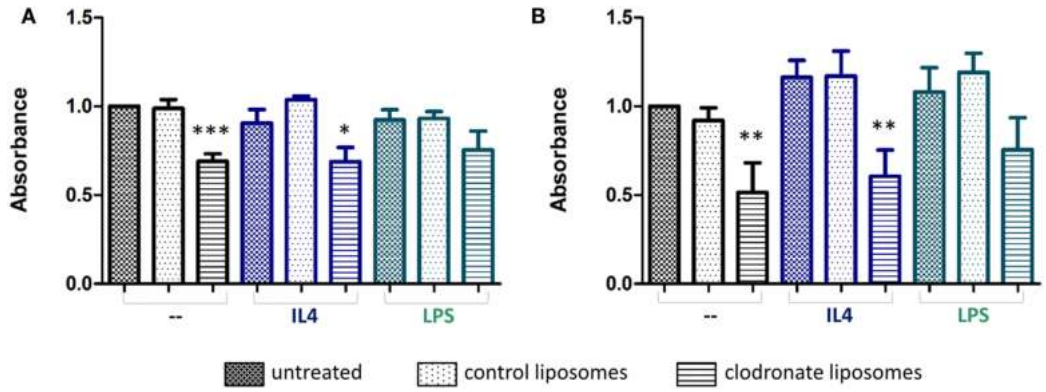

FIGURE 4 | Formazan absorbance as a measure of viability of non-polarized (black bars) or polarized (blue and green bars) BV2 **(A)** and RAW 264.7 **(B)** cells treated with mannosylated control or clodronate liposomes (1:50) for 24 h. Polarization of cells was achieved by treatment with either 20 ng/ml interleukin 4 (IL-4, anti-inflammatory) or 100 ng/ml LPS (proinflammatory) 24 h prior to the liposome treatment ($n = 3$, $^{*}p < 0.05$, $^{**}p < 0.01$, $^{***}p < 0.001$; one-way ANOVA with Dunnett's multiple comparison test for each polarization group). Absorbances of untreated non-polarized cells are normalized to 1.

MRC1 and Iba1 immunostains showed a significant decrease (unpaired t-test $p < 0.05$) in MRC1^{+} microglia/macrophages in the clodronate liposome injected brains compared to the control liposome injected brains ($n = 4$ per group; **Figure 6D**).

Effect of MRC1 Depletion on Intracranial Metastases

Quantitation of tumor volumes 9 days after mannosylated clodronate liposome administration (day 21 of metastasis time-course) showed a slight trend toward decreased tumor volume between the control and clodronate-injected animals (**Figure 7A**). However, the difference in tumor burden between the two experimental groups became statistically significant 16 days after liposome injection (day 28 of metastasis time-course; unpaired t-test $p < 0.01$; **Figure 7A**). Although, microglial/macrophage infiltration of the 4T1-GFP metastatic foci was evident in both the control liposome and clodronate liposome injected animals, this was greatly reduced in the clodronate liposome treated mice (**Figure 7B**). As controls for the intracerebral administration of the liposomes *per se*, PBS or free clodronate was intracerebrally injected into the metastatic brain as above. No significant changes in tumor volume were observed in either control group compared to the mannosylated control liposome injected or untreated 4T1-GFP groups, respectively (Figures S6A,B in Supplementary Material).

In support of the above findings, increased levels of CC3, a biomarker for apoptotic cell death, were evident in the clodronate liposome injected animals compared to the control liposome injected animals (**Figure 7C**). Quantitation of CC3 expression in close association with the metastatic foci revealed a significant increase of CC3 in the clodronate liposome injected animals compared to the control liposome injected animals at days 15 and 21 of the metastatic time-course (unpaired t-tests $p < 0.05$; **Figure 7D**). This effect had subsided to some degree by day 28, when the expression of CC3 was no longer significantly higher in the clodronate liposome injected animals than the controls (**Figure 7D**). CC3 expression was localized to GFP-positive 4T1 tumor cells in the clodronate liposome injected animals at day 21 (**Figure 7E**).

Given that anti-inflammatory macrophages are considered to be proangiogenic, the vascularity of the tumors in the metastatic brains intracerebrally injected with either mannosylated control or mannosylated clodronate liposomes was also assessed. The endothelial marker CD31 was immunohistochemically detected in the brains of both control and clodronate liposome injected mice (Figure S7A in Supplementary Material). No significant differences in the number of CD31-positive vessels associated with 4T1-GFP tumor foci were evident between animals treated with either control or clodronate liposomes at either time point (Figures S7B,C in Supplementary Material).

DISCUSSION

The inflammatory response to brain metastasis is poorly understood. Although the homing of microglia/macrophages in human and murine brain metastases has been reported, a more detailed understanding of the microglial/macrophage response to brain metastases in the early stages of development is still lacking. Here, we have shown a sustained microglial/macrophage infiltration of metastatic foci within the brain over an extended early time-course (28 days) after tumor induction, which was positively correlated with tumor burden. Although this activated microglia/macrophage population showed both pro- and anti-inflammatory phenotypes across the time-course, selectively depleting the anti-inflammatory phenotype significantly reduced metastatic burden.

Metastasis Growth and Microglial/Macrophage Activation

Our results demonstrate a dynamic association between microglia/macrophages and tumor cells during the early stages of metastatic outgrowth in the brain. These findings are in accord with a recent report from Rippaus et al. showing microglial and macrophage infiltration of experimental brain metastases,

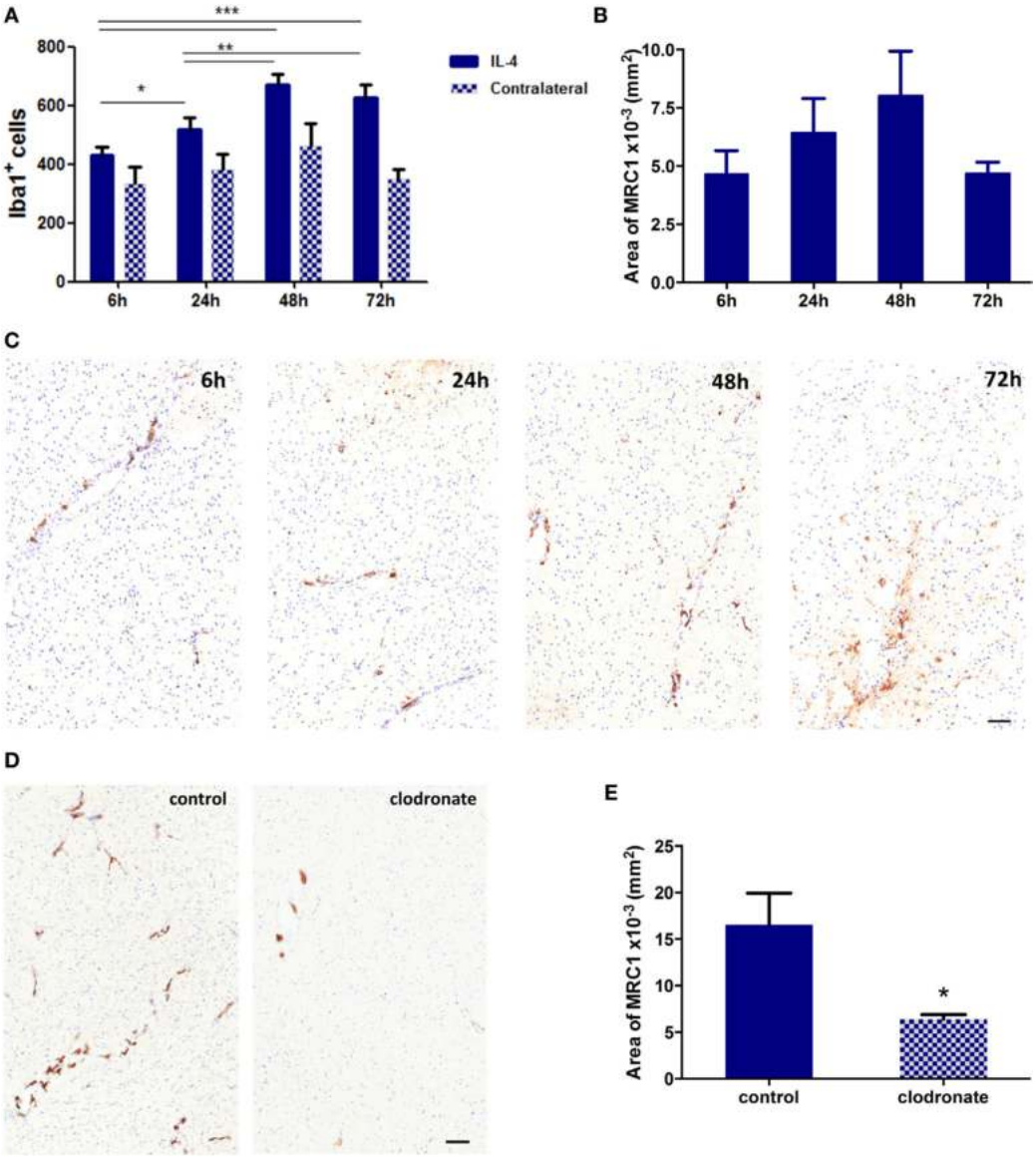

FIGURE 5 | **(A)** Quantitation of Iba1+ cells in the striatum of mice injected intracerebrally with 100 ng of murine recombinant interleukin 4 (IL-4, $n = 3$ per group; $^{*}p < 0.05$, $^{**}p < 0.01$, $^{***}p < 0.001$; one-way ANOVA with Newman–Keuls multiple comparison test for IL-4 injected hemispheres). **(B)** Quantitation of MRC1 expression in hemispheres injected with IL-4 ($n = 3$ per group). **(C)** Representative striatal IHC images for MRC1 expression (brown stain) in mice intracerebrally challenged with 100 ng IL-4. **(D)** Representative IHC images for MRC1 expression (brown stain) from striatal areas of mice injected with 100 ng IL-4 and either control or clodronate liposomes 24 h later. **(E)** Quantitation of MRC1 expression in hemispheres injected with mannosylated control or clodronate liposomes 24 h after intracerebral injection of 100 ng IL-4 ($n = 3$ per group; two tailed unpaired t-test, $^{*}p < 0.05$). Scale bars 50 μm.

although at a single time point after intracarotid injection of different breast cancer cell lines (4T1, PyMT, or MDA-MB-231) (18). Similarly, Zhai et al. have demonstrated sustained microglial infiltration in experimental gliomas (19). Thus, the microglial/macrophage response to malignant cells within the brain appears to be a robust feature of the tumor microenvironment.

Phenotypic analysis of microglia/macrophages in the metastatic brain revealed the presence of both proinflammatory (iNOS+, COX2+) and anti-inflammatory (Arg1+, MRC1+) cells during the early stages of metastatic outgrowth. These findings are similar to previous flow cytometry findings from dissociated tissue bearing parenchymal 4T1 metastases where both proinflammatory and anti-inflammatory CD11b+/CD45^{high} macrophages were identified (18). In that study, however, the authors specifically assessed the polarization of circulating macrophages recruited to the metastatic brain from the periphery. Here, we have demonstrated that a similar polarization is evident across the entire monocyte-derived population (resident microglia and peripheral macrophages) found within the metastatic microenvironment. In the context of cancer, proinflammatory cells can

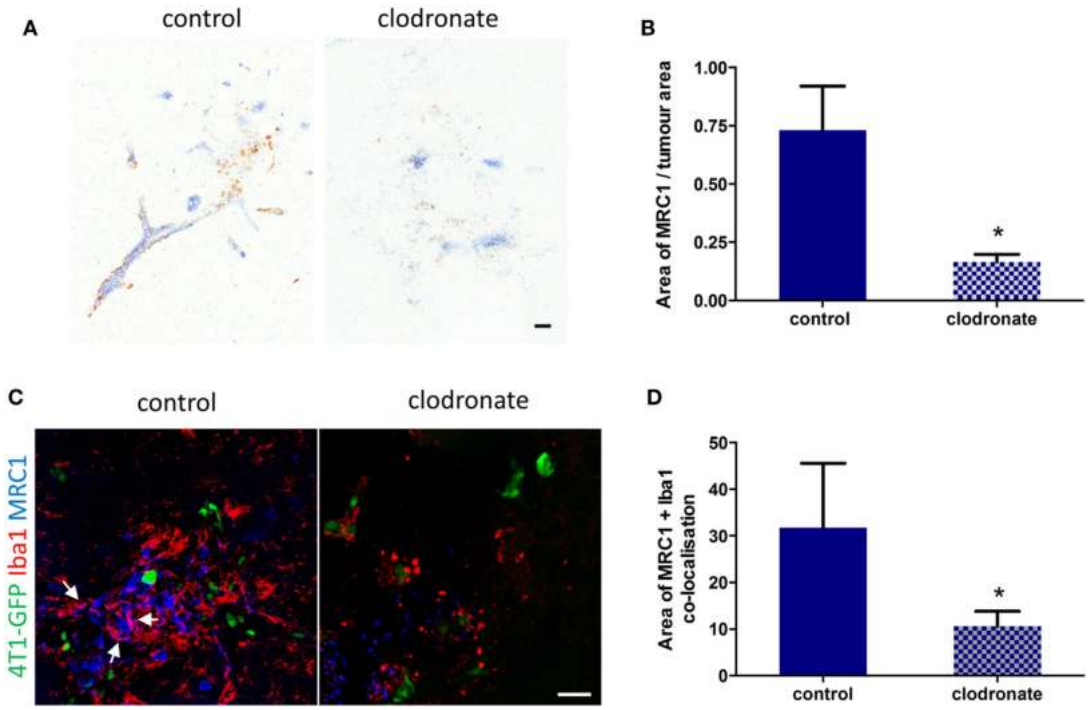

FIGURE 6 | **(A)** Representative IHC images for MRC1 expression (brown stain) from striatal regions of metastatic brains three days after injection with mannosylated control or clodronate liposomes. **(B)** Quantitation of MRC1 expression normalized to tumor area in brains bearing 4T1-GFP metastases 3 days after intracerebral injection of mannosylated control or clodronate liposomes ($n = 4$ per group; $^*p < 0.05$, two tailed unpaired t-test). **(C)** Representative immunofluorescent images of MRC1$^+$ microglia/macrophages in brains of mice injected with mannosylated control or clodronate liposomes. Arrows show colocalization of Iba1 and MRC1 immunostains. **(D)** Quantitation of colocalization of MRC1 with Iba1 three days after intracerebral injection of mannosylated liposomes ($n = 4$ per group; $^*p < 0.05$, two tailed unpaired t-test). Scale bar 50 μm.

exert cytotoxicity whilst anti-inflammatory cells can promote progression *via* their prosurvival and proangiogenic functions. Thus, the balanced coupling of the pro- and anti-inflammatory microglial/macrophage response during the early stages of metastatic progression may be permissive for the establishment of macrometastases. Although it has been shown that both pro- and anti-inflammatory microglia/macrophages exist in the metastatic brain, the possibility that microglia/macrophages acquire one phenotype bearing both pro- and anti-inflammatory characteristics cannot be excluded given that macrophage polarization consists of a dynamic equilibrium *in vivo* (20, 21). In support of this concept, a recent elegant study of human glioblastoma-associated microglia reports a continuum between pro- and anti-inflammatory phenotypes, a finding that further supports the presence of myeloid cells with simultaneous pro- and anti-inflammatory characteristics in the context of brain tumors (22).

Depletion of Anti-inflammatory Microglia/Macrophages

In order to test the hypothesis that (primarily) anti-inflammatory cells sustain metastatic outgrowth in the brain, mannosylated clodronate liposomes were used to selectively deplete the anti-inflammatory (MRC1 expressing) microglial/macrophage population. Mannosylated clodronate liposomes bind to MRC1 and, consequently, are taken up by MRC1-expressing microglia/macrophages and induce apoptosis within 2–3 days *via* clodronate-mediated depletion of intracellular iron (23). MRC1$^+$ microglia/macrophages have previously been successfully targeted *in vivo* in a preclinical model of multiple sclerosis using intracranial administration of mannosylated clodronate liposomes (24). This approach was further validated here, first *in vitro* and secondly *in vivo*. *In vitro*, the viability of microglia and macrophages, both in an unpolarized and anti-inflammatory polarized (IL-4 treated) state, was shown to be significantly reduced on incubation with mannosylated clodronate liposomes. The response of apparently unpolarized cells to the clodronate liposomes likely reflects the fact that cultured microglia/macrophages are rarely in a truly quiescent state and express MRC1 to some degree, as verified by immunofluorescence histochemistry (Figures S4C, S5B in Supplementary Material). This effect of clodronate liposomes on cell viability was partially ameliorated in cells treated with LPS, reflecting a shift toward a more proinflammatory state (also indicated by iNOS induction in some but not all cells; Figure S5A in Supplementary Material), and thus, a reduction in MRC1 expression. Subsequently, significant depletion of MRC1$^+$ cells *in vivo* following intracerebral injection of mannosylated clodronate liposomes was demonstrated in an IL-4 induced model of neuroinflammation.

Clodronate liposomes are widely used in preclinical models for depleting macrophages specifically, hence the potential

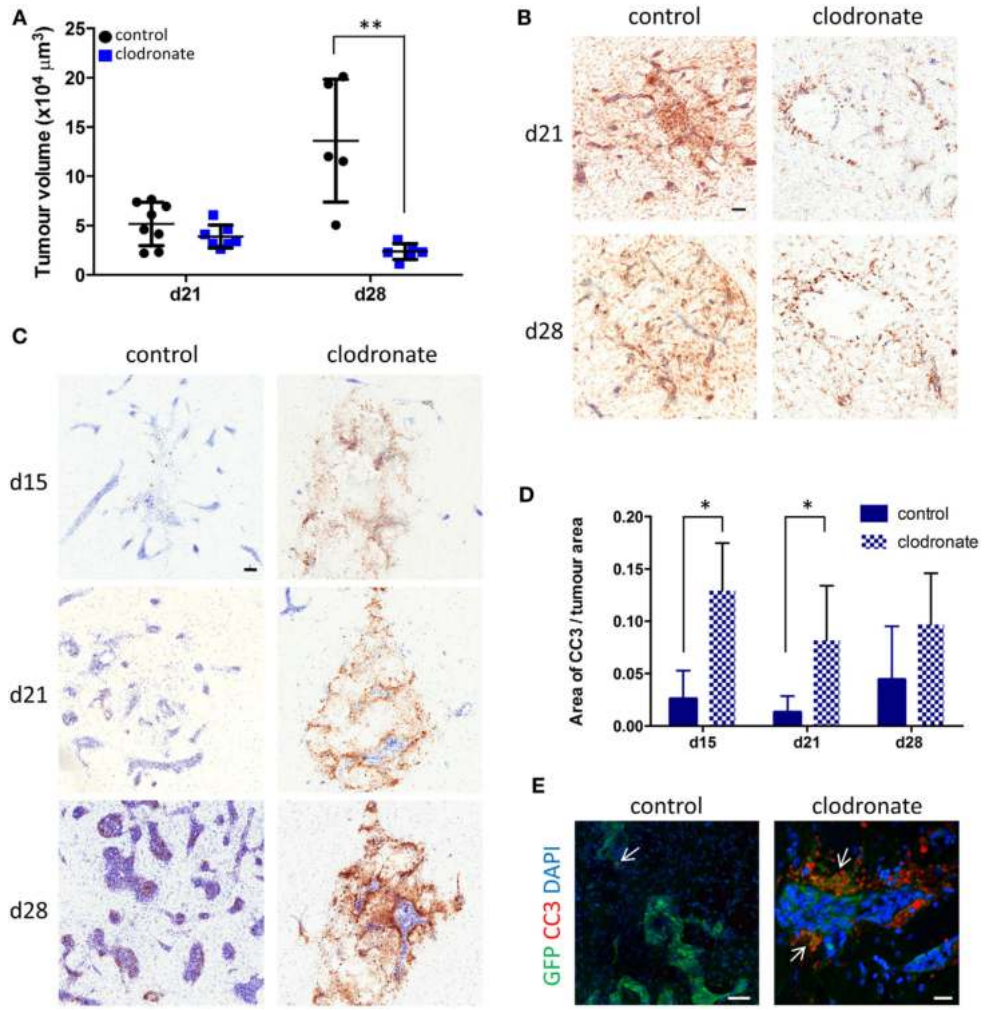

FIGURE 7 | **(A)** Quantitation of intracranial tumor burden for mice injected intracerebrally with mannosylated control or clodronate liposomes 12 days after intracerebral 4T1-GFP tumor cell injection (n = 5–8 per group; $^{**}p < 0.01$, two tailed unpaired t-test). Time points shown are relative to 4T1-GFP cell injections. **(B)** Representative immunohistochemical images for Iba1 expression (brown stain) for the control and clodronate liposome injected animals at days 21 and 28 after tumor cell injection. **(C)** Representative striatal immunohistochemical images for cleaved caspase 3 expression (brown stain) at days 15, 21, and 28 after intracerebral 4T1-GFP cell injection, in mice injected with either control or clodronate liposomes at day 12. **(D)** Quantitation of apoptosis as indicated by CC3 expression at days 15, 21, and 28 in metastatic brains intracerebrally injected with mannosylated control or clodronate liposomes (n = 3–5 per group; $^{*}p < 0.05$, two tailed unpaired t-test). **(E)** Immunofluorescent images showing colocalization (arrows) of CC3 (red) with GFP^{+} tumor cells (green) from representative control and clodronate liposomes injected animals at day 21 of the time-course study. Scale bars 50 µm [**(B,C,E)** control] or 20 µm [**(E)** clodronate].

effect of clodronate liposomes on other phagocytic cells, such as neutrophils or dendritic cells, was not investigated here. Data from experimental gliomas suggest that neither neutrophil nor dendritic cell infiltration is a robust feature of the inflammatory/immune response in the brain under the influence of tumors (8, 25). Moreover, it has been shown that clodronate liposome treatment depletes monocytes, but not neutrophils, *in vivo* in lung tissue (26, 27). Successful depletion of spleen F4/80^{+} macrophages, but not CD11c^{+} dendritic cells, has also been demonstrated after intraperitoneal administration of clodronate liposomes (28). Finally, the recruitment of blood-derived dendritic cells in the inflamed rat brain even after depletion of macrophages by intracerebroventricular clodronate liposome injection has been reported (29).

Once the action of mannosylated clodronate liposomes on anti-inflammatory polarized microglia/macrophages had been established, mice injected intracerebrally with metastatic 4T1-GFP cells were treated with these liposomes. In these mice intracranial tumor burden was found to be significantly reduced compared to mice treated with the control liposomes. Since none of the control groups, injected with (i) control liposomes, (ii) PBS, or (iii) free clodronate, showed any reduction in tumor burden compared to untreated metastasis bearing mice, these findings suggest that depletion of MRC1^{+} microglia/macrophages significantly reduces metastasis growth in the brain. Increased expression of CC3 following depletion of MRC1^{+} microglia/macrophages was strongly correlated with GFP-positive tumor cells, indicating tumor cell apoptosis. Although some CC3-positive

microglia/macrophages were also evident, this was to a much lesser degree than GFP/CC3 colocalization. Moreover, it should be noted that CC3 expression was spatially correlated with the tumor foci, whereas microglia within the tumor microenvironment were not CC3 positive. These findings support the concept that anti-inflammatory polarized microglia/macrophages promote and sustain metastatic outgrowth in the brain.

MRC1$^+$ microglia and macrophages are associated with a proangiogenic phenotype, and resident microglia have recently been shown to produce potent proangiogenic factors in glioma including vascular endothelial growth factor and CXCL2 (30). However, unlike a previous report indicating a reduction in vessel density in a subcutaneous mouse tumor model after treatment with clodronate liposomes (28), no changes in vascularity in clodronate liposome treated animals were found in the current study. This observation suggests that this subpopulation of microglia/macrophages also induce other mechanisms to support tumor growth in the brain. For example, a recent study reported a functional crosstalk between tumor cells and anti-inflammatory metastasis infiltrating microglia/macrophages that is mediated by the inflammatory molecule lymphotoxin-β (18). Alternatively, Miron et al. observed an increase in proinflammatory cells following MRC1$^+$ microglial depletion (24), which could also account for the decreased intracranial tumor burden observed in the clodronate liposome-injected animals.

In conclusion, our data indicate an active role for microglia/macrophages in the development of brain metastases and suggest that targeting the anti-inflammatory subpopulation or switching these cells to a more proinflammatory phenotype may be a promising route for therapeutic intervention. Importantly, harnessing the microglial/macrophage response to brain metastasis could be used in combination with other frontline therapies, such as radiotherapy, as an effective multimodal anticancer therapy (31).

ETHICS STATEMENT

All animal procedures were carried out in accordance with the UK Animals (Scientific Procedures) Act 1986 and with the University of Oxford (Clinical Medicine) Ethical Committee approval.

AUTHOR CONTRIBUTIONS

NS and KA conceived and designed the work. All authors contributed to the acquisition, analysis and/or interpretation of data for the work. All authors contributed to drafting the work and revising it critically for important intellectual content. All authors approved the version to be published and agree to be accountable for all aspects of the work.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at http://www.frontiersin.org/article/10.3389/fonc.2017.00251/full#supplementary-material.

REFERENCES

1. Eichler AF, Loeffler JS. Multidisciplinary management of brain metastases. *Oncologist* (2007) 12(7):884–98. doi:10.1634/theoncologist.12-7-884
2. Lin X, DeAngelis LM. Treatment of brain metastases. *J Clin Oncol* (2015) 33(30):3475–84. doi:10.1200/JCO.2015.60.9503
3. Pease NJ, Edwards A, Moss L. Effectiveness of whole brain radiotherapy in the treatment of brain metastases: a systematic review. *Palliat Med* (2005) 19(4):288–99. doi:10.1191/0269216305pm1017oa
4. Lorger M, Felding-Habermann B. Capturing changes in the brain microenvironment during initial steps of breast cancer brain metastasis. *Am J Pathol* (2010) 176(6):2958–71. doi:10.2353/ajpath.2010.090838
5. Berghoff AS, Lassmann H, Preusser M, Höftberger R. Characterization of the inflammatory response to solid cancer metastases in the human brain. *Clin Exp Metastasis* (2013) 30(1):69–81. doi:10.1007/s10585-012-9510-4
6. O'Brien ER, Kersemans V, Tredwell M, Checa B, Serres S, Sarmiento Soto M, et al. Glial activation in the early stages of brain metastasis: TSPO as a diagnostic biomarker. *J Nucl Med* (2014) 55(2):275–80. doi:10.2967/jnumed.113.127449
7. Fitzgerald DP, Palmieri D, Hua E, Hargrave E, Herring JM, Qian Y, et al. Reactive glia are recruited by highly proliferative brain metastases of breast cancer and promote tumor cell colonization. *Clin Exp Metastasis* (2008) 25(7):799–810. doi:10.1007/s10585-008-9193-z
8. Granot Z, Henke E, Comen EA, King TA, Norton L, Benezra R. Tumor entrained neutrophils inhibit seeding in the premetastatic lung. *Cancer Cell* (2011) 20(3):300–14. doi:10.1016/j.ccr.2011.08.012
9. Saijo K, Glass CK. Microglial cell origin and phenotypes in health and disease. *Nat Rev Immunol* (2011) 11(11):775–87. doi:10.1038/nri3086
10. Perry VH, Holmes C. Microglial priming in neurodegenerative disease. *Nat Rev Neurol* (2014) 10(4):217–24. doi:10.1038/nrneurol.2014.38
11. Li W, Graeber MB. The molecular profile of microglia under the influence of glioma. *Neuro Oncol* (2012) 14(8):958–78. doi:10.1093/neuonc/nos116
12. Gabrusiewicz K, Ellert-Miklaszewska A, Lipko M, Sielska M, Frankowska M, Kaminska B. Characteristics of the alternative phenotype of microglia/macrophages and its modulation in experimental gliomas. *PLoS One* (2011) 6(8):e23902. doi:10.1371/journal.pone.0023902
13. Szulzewsky F, Pelz A, Feng X, Synowitz M, Markovic D, Langmann T, et al. Glioma-associated microglia/macrophages display an expression profile different from M1 and M2 polarization and highly express Gpnmb and Spp1. *PLoS One* (2015) 10(2):e0116644. doi:10.1371/journal.pone.0116644
14. Panni RZ, Linehan DC, DeNardo DG. Targeting tumor-infiltrating macrophages to combat cancer. *Immunotherapy* (2013) 5(10):1075–87. doi:10.2217/imt.13.102
15. Noy R, Pollard JW. Tumor-associated macrophages: from mechanisms to therapy. *Immunity* (2014) 41(1):49–61. doi:10.1016/j.immuni.2014.06.010
16. Williams CB, Yeh ES, Soloff AC. Tumor-associated macrophages: unwitting accomplices in breast cancer malignancy. *NPJ Breast Cancer* (2016) 2:15025. doi:10.1038/npjbcancer.2015.25
17. Perry VH, Teeling J. Microglia and macrophages of the central nervous system: the contribution of microglia priming and systemic inflammation to chronic neurodegeneration. *Semin Immunopathol* (2013) 35(5):601–12. doi:10.1007/s00281-013-0382-8

18. Rippaus N, Taggart D, Williams J, Andreou T, Wurdak H, Wronski K, et al. Metastatic site-specific polarization of macrophages in intracranial breast cancer metastases. *Oncotarget* (2016) 7(27):41473–87. doi:10.18632/oncotarget.9445
19. Zhai H, Heppner FL, Tsirka SE. Microglia/macrophages promote glioma progression. *Glia* (2011) 59(3):472–85. doi:10.1002/glia.21117
20. Pollard J. Trophic macrophages in development and disease. *Nat Rev Immunol* (2009) 9(4):259–70. doi:10.1038/nri2528
21. Sica A, Mantovani A. Macrophage plasticity and polarization: in vivo veritas. *J Clin Invest* (2012) 122(3):787–95. doi:10.1172/JCI59643
22. Gabrusiewicz K, Rodriguez B, Wei J, Hashimoto Y, Healy LM, Maiti SN, et al. Glioblastoma-infiltrated innate immune cells resemble M0 macrophage phenotype. *JCI Insight* (2016) 1(2):e85841. doi:10.1172/jci.insight.85841
23. van Rooijen N, Sanders A, van den Berg TK. Apoptosis of macrophages induced by liposome-mediated intracellular delivery of clodronate and propamidine. *J Immunol Methods* (1996) 193(1):93–9. doi:10.1016/0022-1759(96)00056-7
24. Miron VE, Boyd A, Zhao JW, Yuen TJ, Ruckh JM, Shadrach JL, et al. M2 microglia and macrophages drive oligodendrocyte differentiation during CNS remyelination. *Nat Neurosci* (2013) 16(9):1211–8. doi:10.1038/nn.3469
25. Candolfi M, King GD, Yagiz K, Curtin JF, Mineharu Y, Muhammad AK, et al. Plasmacytoid dendritic cells in the tumor microenvironment: immune targets for glioma therapeutics. *Neoplasia* (2012) 14(8):757–70. doi:10.1593/neo.12794
26. Wilson MR, O'Dea KP, Zhang D, Shearman AD, van Rooijen N, Takata M. Role of lung-marginated monocytes in an in vivo mouse model of ventilator-induced lung injury. *Am J Respir Crit Care Med* (2009) 179(10):914–22. doi:10.1164/rccm.200806-877OC
27. Narasaraju T, Yang E, Samy RP, Ng HH, Poh WP, Liew AA, et al. Excessive neutrophils and neutrophil extracellular traps contribute to acute lung injury of influenza pneumonitis. *Am J Pathol* (2011) 179(1):199–210. doi:10.1016/j.ajpath.2011.03.013
28. Zeisberger SM, Odermatt B, Marty C, Zehnder-Fjällman AH, Ballmer-Hofer K, Schwendener RA. Clodronate-liposome-mediated depletion of tumour-associated macrophages: a new and highly effective antiangiogenic therapy approach. *Br J Cancer* (2006) 95(3):272–81. doi:10.1038/sj.bjc.6603240
29. Newman TA, Galea I, van Rooijen N, Perry VH. Blood-derived dendritic cells in an acute brain injury. *J Neuroimmunol* (2005) 166(1–2):167–72. doi:10.1016/j.jneuroim.2005.04.026
30. Brandenburg S, Muller A, Turkowski K, Radev YT, Rot S, Schmidt C, et al. Resident microglia rather than peripheral macrophages promote vascularisation in brain tumours and are source of alternative pro-angiogenic factors. *Acta Neuropathol* (2016) 131:365–78. doi:10.1007/s00401-015-1529-6
31. De Palma M, Lewis CE. Macrophage regulation of tumor responses to anticancer therapies. *Cancer Cell* (2013) 23(3):277–86. doi:10.1016/j.ccr.2013.02.013

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REVIEW
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Genetic Characterization of Brain Metastases in the Era of Targeted Therapy

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In the current era of molecularly targeted therapies and precision medicine, choice of cancer treatment has been increasingly tailored according to the molecular or genomic characterization of the cancer the individual has. Previously, the clinical observation of inadequate control of brain metastases was widely attributed to a lack of central nervous system (CNS) penetration of the anticancer drugs. However, more recent data have suggested that there are genetic explanations for such observations. Genomic analyses of brain metastases and matching primary tumor and other extracranial metastases have revealed that brain metastases can harbor potentially actionable driver mutations that are unique to them. Identification of genomic alterations specific to brain metastases and targeted therapies against these mutations represent an important research area to potentially improve survival outcomes for patients who develop brain metastases. Novel approaches in genomic testing such as that using cell-free circulating tumor DNA (ctDNA) in the cerebrospinal fluid (CSF) facilitate advancing our understanding of the genomics of brain metastases, which is critical for precision medicine. CSF-derived ctDNA sequencing may be particularly useful in patients who are unfit for surgical resection or have multiple brain metastases, which can harbor mutations that are distinct from their primary tumors. Compared to the traditional chemotherapeutics, novel targeted agents appear to be more effective in controlling the CNS disease with better safety profiles. Several brain metastases-dedicated trials of various targeted therapies are currently underway to address the role of these agents in the treatment of CNS disease. This review focuses on recent advances in genomic profiling of brain metastases and current knowledge of targeted therapies in the management of brain metastases from cancers of the breast, lung, colorectum, kidneys, and ovaries as well as melanoma.

Keywords: brain metastases, genomics, targeted therapy, sequencing, cancer heterogeneity

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INTRODUCTION

Brain metastases are the most common central nervous system (CNS) tumors in adults but represent an unmet need in current oncologic practice. The reported incidence of brain metastases is 9–17%; however, the true incidence in the current era of modern cancer therapies is thought to be higher (1). The incidence of brain metastases is rising due, in part, to improved diagnostic techniques and

increased patient survival through advanced systemic treatment approaches. Some types of cancers have greater tropism of metastasizing to the brain with most common cancers being lung cancer (39–56%), breast cancer (13–30%), melanoma (6–11%), gastrointestinal cancers (3–8%), and renal cell carcinoma (2–4%) (2). The underlying mechanisms of this organotropism toward specific secondary sites remain poorly understood.

Prognosis remains poor with a median survival ranging from 3 to 27 months after developing brain metastases (3). Treatment options are limited and usually involve multimodality approaches that include radiotherapy, surgery, and sometimes systemic therapy, depending on number of lesions, location, and primary tumor type. Historically, patients with brain metastases were uniformly treated with whole-brain radiotherapy, especially if multiple lesions are present, or surgical resection followed by radiotherapy for a solitary or large symptomatic lesion. More recently, stereotactic radiosurgery has been increasingly used for smaller lesions (<3 cm) in oligometastatic disease, which is commonly defined as up to four brain metastases. CNS-directed systemic treatment options are limited, and patients with brain metastases are commonly excluded from clinical trials, including those investigating novel targeted therapies. Differential responses to systemic treatments between brain metastases and extracranial disease are often observed, particularly with the traditional chemotherapy agents, where systemic disease is adequately controlled while brain disease progresses. This is at least partly explained by inadequate blood–brain barrier (BBB) penetration by systemic therapies (4). Although the BBB is frequently compromised by brain metastases as shown by brain imaging contrast enhancement, the residual BBB permeability limits drug delivery to subtherapeutic concentrations in brain metastases compared with extracranial tumors. With molecularly targeted therapies, there has been improved control of both intracranial and extracranial metastases. In the past decade, significant efforts have been made to characterize the genetic drivers in brain metastases using modern sequencing techniques. Better understanding of the genomic complexity and heterogeneity of brain metastases will lead to improved treatment strategies and research directions. This review focuses on recent advances in genomic profiling of brain metastases and current knowledge of targeted therapies in the management of brain metastases from cancers of the breast, lung, colorectum, kidneys, and ovaries as well as melanoma.

GENETIC HETEROGENEITY OF CANCER

Intratumoral heterogeneity within the primary tumor was shown in a multiregion spatial genetic analysis of four metastatic renal cell carcinoma (5). Genetic heterogeneity between primary and secondary tumors in the same patient was also shown in a study comparing the genetic profiling of 15 primary colorectal cancer and matched liver metastases (6). Similarly, genetic heterogeneity between brain metastases and their corresponding primary tumors was shown recently in a genomic analysis of matched brain metastases, primary tumors, and normal tissue in 86 patients (7). This study demonstrated that brain metastases harbor distinctive potentially actionable mutations not detected in paired primary tumors in 53% of the cases. This finding suggests that this genomic heterogeneity or divergent evolution of brain metastases from primary tumors may also contribute to the disparities in intracranial and extracranial disease response to systemic therapies, which were previously thought to be solely due to the inadequate BBB penetration of systemic drugs. In the same study, it was found that alterations associated with sensitivity to cyclin-dependent kinase (CDK) inhibitors were common in brain metastases (7). These include *CDKN2A* loss and *CDK4/6* amplifications. Alterations in the PI3K/AKT/mTOR pathway were also common as previously reported by others (8–10). Interestingly, brain metastases from different intracranial sites in the same patient shared nearly all of the potentially actionable driver mutations, suggesting that brain metastases are homogeneous within an individual (7). Prospective clinical trials using targeted therapies that cross the BBB are required to demonstrate that these potentially actionable mutations found in brain metastasis samples are indeed targetable.

GENOMIC PROFILING OF BRAIN METASTASES

In the current era of molecularly targeted therapies and precision medicine, choice of cancer treatment has been increasingly tailored according to the molecular or genomic characterization of the cancer the individual has. Identification of genomic alterations specific to brain metastases and targeted therapies against these mutations represent an important research area to improve survival outcomes for patients who develop brain metastases. Given spatial and temporal intratumoral heterogeneity within the same patient, repeated biopsies are often necessary to adequately characterize the somatic genetic alterations in human cancers. Genomic profiling of brain metastases poses a particular challenge as obtaining tissue samples of brain metastases is invasive and often difficult, especially in patients who are poor candidates for brain resections or have tumors in inaccessible sites. In addition, regional lymph node and other distal extracranial metastasis samples are not reliable surrogates for detecting these mutations present in brain metastases (7).

There has been great interest in developing alternative methods of genomic profiling of cancer that are clinically practical and non-invasive. Information collected by these techniques not only help refine systemic treatment decisions but also monitor response to treatment and identify emergent drug-resistant mutations by tracking the evolution of cancer genome to guide further therapy. For example, analysis of circulating tumor DNA (ctDNA) in plasma has been shown to be useful not only in characterizing cancers but also in monitoring disease response to therapy (11–13). However, it was recently shown that tumor DNA was either absent or present in a very little amount in the plasma of patients with primary brain tumors or brain metastases with no or little extracranial disease from solid tumors (12). In the same study, mutations that were present only in the brain metastases and not in the extracranial tumors were more represented in the cell-free ctDNA from the cerebrospinal fluid (CSF) compared to plasma. As seen with plasma ctDNA in previous studies, CSF ctDNA was also observed to change

throughout treatments (12). Mutant allelic frequency of CSF ctDNA decreased with tumor response to treatments and increased with progression.

Brain metastases can harbor drug-resistant mutations that result in restoration of signaling downstream of the target kinase, activation of an alternative signaling pathway, or alteration in the drug activating site (14). And these mutations may not be present in the primary tumor or extracranial metastases (7), therefore, genotyping primary tumor or other extracranial disease alone can miss actionable oncogenic driver mutations and targeted therapy opportunities for brain metastases. Routine brain biopsies are invasive with associated risks and may not be feasible in many patients with brain metastases. CSF ctDNA analysis may provide the ability to identify the drug-resistance mechanisms in patients who progressed in the CNS after initial response on targeted therapy without invasive procedures like a brain biopsy. In a recent study, CSF as a source of ctDNA was evaluated by sequencing 341 cancer-associated genes in cell-free DNA isolated from the CSF of 53 patients with brain metastases of solid tumors or primary brain tumors (14). Mutations, which are known to cause drug resistance to oncogenic kinases, were identified in 4 of 12 patients who progressed in the brain whilst on such therapy. Although this needs to be verified in a larger cohort of patients, these findings suggest that CSF ctDNA may be a useful biomarker that facilitates genome-directed treatments to target brain metastases and for monitoring CNS disease on treatment or during surveillance. Moving forward, more studies evaluating ctDNA extracted from CSF should be carried out using next-generation sequencing techniques that are capable of detection all classes of mutations including base substitutions/insertions/deletions, gene fusions, and gene copy number alterations. The fraction of cell-free ctDNA in CSF is higher than in plasma due to the relative absence of background normal DNA in CSF. This allows the detection of somatic mutations even with modest sequence coverage, whereas plasma cell-free ctDNA sequencing requires very deep sequence coverage to achieve sufficient sensitivity for detecting mutations occurring at low allele frequencies. CSF ctDNA analysis appears promising, and there are ongoing studies prospectively evaluating CSF ctDNA as a surrogate for brain metastasis biopsy sample. Furthermore, the use of additional circulating biomarkers, such as exosomes, will need to be prospectively explored.

NON-SMALL CELL LUNG CANCER (NSCLC)

Non-small cell lung cancer is the most common type of lung cancer, accounting for approximately 85% of all lung cancer cases (15). NSCLC, adenocarcinoma subtype in particular, is the most common cancer to metastasize to the brain (1). The risk of developing brain metastases during the course of disease in patients with treated stage III NSCLC is approximately 30–50% (15). Even in patients with surgically treated early stage (stage I–II) NSCLC, the 5-year actuarial risk of developing brain metastases is reported to be around 10% with the brain being the sole site of failure in 43% of these patients (15, 16). A large retrospective study of 975 patients with stage I–II NSCLC identified younger age, larger tumor size, lymphovascular space invasion, and hilar lymph node involvement to be associated with an increase in the risk of brain metastases in this population (15). Prognosis remains poor even with a multimodality treatment approach of brain metastases. Reported 1-year mortality rate after developing brain metastases ranges from 81 to 90% depending on the initial clinical stage of lung cancer (16). Discovery of NSCLC oncogenic driver mutations and molecularly targeted therapies have significantly improved survivals in the subset of patients with metastatic NSCLC harboring these targetable genomic aberrations.

Epidermal Growth Factor Receptor (EGFR) Mutations

An activating *EGFR* mutation is present in approximately 10–15% of Caucasians and 40% of East-Asian NSCLC patients (2). When the EGFR status was evaluated in paired brain metastasis and corresponding primary lung tumor samples, discordance rates up to 33% were observed (2). In a recent retrospective, population-based study, there was a higher cumulative incidence of brain metastases in patients with *EGFR*-mutant NSCLC compared to those with *EGFR* wild type (39.2 versus 28.2%) (17). *EGFR* mutations render these tumors sensitive to EGFR tyrosine kinase inhibitors (TKIs) resulting in significantly improved survival outcomes. Several phase III randomized trials compared the efficacy of first-generation EGFR TKIs, gefitinib and erlotinib, and an irreversible ErbB family inhibitor, afatinib, to the platinum-containing combination chemotherapy as first-line treatment in *EGFR*-mutant NSCLC patients. Progression-free survival (PFS) was significantly longer in the EGFR TKI group compared to the chemotherapy group, with hazard ratios for progression or death ranging from 0.38 to 0.58 (18–20).

Prospective data on the efficacy of EGFR TKIs in treating brain metastases from NSCLC are limited. However, currently available data suggest that these agents have CNS activity. *Post hoc* subgroup analyses of combined data from two phase III randomized trials of first-line afatinib in *EGFR*-mutant NSCLC patients, which allowed patients with asymptomatic brain metastases to enroll, showed survival benefit from treatment with afatinib compared to chemotherapy (21). PFS (8.2 versus 5.4 months) and objective response rate (ORR) (70–75 versus 20–28%) were significantly better with afatinib than chemotherapy. Pretreated patients with brain metastases may also benefit from afatinib as suggested by a report of 31 patients, of whom 35% had an objective response with an overall disease control rate of 66% (22). Other retrospective and small phase II studies also showed survival benefit from first-generation TKIs in patients with *EGFR*-mutant NSCLC that had metastasized to the brain (23–26). In these studies, ORR was 58–83%, and median PFS was in the range of 7–15 months.

The majority of patients who had initial response to EGFR TKIs had disease progression due to an acquired resistance within 1–2 years (27). The development of an additional *EGFR* mutation, *EGFR* T790M, is responsible for approximately 60% of this clinically observed acquired resistance (28). A third-generation T790M mutant specific EGFR TKI, osimertinib, has been shown to be highly active in patients who have progressed during prior

therapy with EGFR TKIs due to T790M mutation (27). Moreover, a recent preclinical study in animal models demonstrated superior penetration of the BBB with osimertinib than gefitinib or afatinib and sustained tumor regression in an *EGFR*-mutant mouse brain metastasis model (29).

Together, positive CNS activity observed with these EGFR TKIs suggests that incorporation of EGFR TKIs in the treatment of asymptomatic brain metastases from *EGFR*-mutant NSCLC appears reasonable and these agents may be utilized upfront and radiation therapy reserved until progression to avoid radiation related neurotoxicity. Prospective evaluations of EGFR TKIs in active brain metastases and sequential approaches with brain radiotherapy are warranted.

Anaplastic Lymphoma Kinase (ALK) Rearrangement

A fusion gene comprising portions of echinoderm microtubule like protein 4 (*EML4*) and *ALK* genes with resultant chimeric protein with constitutive kinase activity is found in approximately 2–7% of NSCLC (30, 31). Since its first discovery in 2007, there have been rapid advances in the development of several TKIs targeting this genomic aberration. A first-generation ALK TKI, crizotinib, was shown to significantly improve PFS and ORR in both first- and second-line settings in patients with *ALK*-rearranged NSCLC, compared to chemotherapy (32, 33). For example, in a phase III randomized trial of first-line crizotinib, PFS was 10.9 with crizotinib compared to 7 months with chemotherapy and ORR was 74 and 45%, respectively (33). Despite initial response, drug resistance invariably develops. Next-generation ALK TKIs, including ceritinib and alectinib, have been shown to be effective in patients with crizotinib-resistant disease (34, 35).

Brain metastasis is a relatively common complication of *ALK*-rearranged NSCLC, especially at progression. In a retrospective study, incidence rates of brain metastases were 23.8% at diagnosis, 45.5% at 2 years, and 58.4% at 3 years (36). Crizotinib has been shown to be effective in treating brain metastases, at least initially, as suggested by a large retrospective pooled analysis of patients with asymptomatic brain metastases treated with this agent on the PROFILE 1005 and 1007 trials (37). In this study, an intracranial disease control rate was 56% at 12 weeks in patients with previously untreated brain metastases with a median time to CNS progression of 7 months. In a phase III randomized trial of first-line crizotinib versus platinum containing chemotherapy, patients with stable treated brain metastases were allowed to enroll (38). Intracranial efficacy assessment was part of this study. This study showed that among patients with brain metastases CNS disease control rate was significantly higher with crizotinib at 12 weeks (85 versus 45%), and PFS was also significantly longer with crizotinib (9 versus 4 months). Recently two phase II trials of alectinib have shown remarkable CNS activity against brain metastases with CNS response rates up to 75% and median CNS disease response durations of 10–11 months (39, 40). Interestingly, the cumulative CNS progression rate at 12 months was lower than the cumulative non-CNS progression rate (24.8 versus 33.2%) (40).

Immunotherapy

Recently, there has been a significant development in immunotherapy to treat various types of cancer including NSCLC. Immune checkpoints, which exist to suppress immune response to protect against detrimental autoimmunity and inflammation, can be co-opted by tumors. Engagement of programmed death 1 (PD-1) receptor on activated T cells by programmed death ligand 1 (PD-L1) on tumor cells leads to T-cell inactivation, which in turn results in immune tolerance and subsequent progression of tumor. PD-1 inhibitors, including nivolumab and pembrolizumab, have shown to significantly improve ORR and survival outcomes in patients with metastatic NSCLC without other targetable mutations such as *EGFR* or *ALK* mutations. Both pembrolizumab and nivolumab were superior to docetaxel in previously treated patients (41, 42). Pembrolizumab was shown to be superior to platinum-containing doublet chemotherapy as first-line therapy in patients with NSCLC with more than 50% of tumor cells staining positive for PD-L1 (43). In another first-line trial, pembrolizumab combined with carboplatin and pemetrexed in PD-L1 unselected NSCLC patients was better than chemotherapy alone (44). These trials, however, excluded patients with active brain metastases. An early analysis of an ongoing trial (NCT02085070) investigating the activity and safety of pembrolizumab in NSCLC or melanoma patients with untreated or progressive brain metastases showed encouraging results (45). In this analysis, 6 (33%) of 18 patients with NSCLC had brain response that was durable. There were no grade 3 or 4 neurological toxic effects.

Squamous Cell Carcinoma

Squamous cell carcinoma accounts for approximately 20% of all lung cancers. A genomic analysis of primary squamous cell lung cancer in 79 patients showed that *PI3K* aberrant tumors had significantly worse overall survival (OS) (8.6 versus 18.8 months) and a higher incidence of brain metastases (27 versus 0%) compared to *FGFR1* aberrant tumors (9). The authors then analyzed six brain metastasis samples by whole-exome sequencing, four of these patients had matched samples of their primary lung cancer. This analysis showed heterozygous loss of *PTEN* in all of the brain metastasis samples with a pattern of gene expression consistent with *PTEN* loss. A deeper genomic analysis in this study demonstrated clonal heterogeneity with a low degree of shared events (as little as 15%) between the brain metastases and their corresponding primary lung tumor. Further studies examining the exact role of aberrant PI3K pathway in the development of brain metastases and potential targeted therapy are warranted.

BREAST CANCER

Breast cancer is the most common cancer in women and second most common cancer to metastasize to the brain. The exact incidence of brain metastases from breast primary in the current era of modern therapies is unknown; however, a 10-year cumulative incidence of CNS relapse in patients presenting with early stage breast cancer is estimated to be around 4–5% (46, 47). Triple receptor negative disease, basal-like subtype, and human

epidermal growth factor receptor 2 (HER2) positive breast cancer have an increased risk of developing brain metastases (46, 48). Early (<2 years after primary diagnosis) occurring brain metastases from breast primary are associated with early onset tumor, negative estrogen receptor (ER) status, HER2 overexpression, and triple receptor negative tumor (49). In comparison, many late occurring brain metastases are from ER positive primary tumors (50). Survival outcomes differ between different subtypes of breast cancer. The median OS after developing brain metastases was longer in patients with HER2-positive breast cancer at 11.5 months compared to luminal HER2 negative breast cancer at 9.3 months and triple receptor negative breast cancer at 4.9 months in a retrospective study of 1,256 patients (51).

Divergence of brain metastases from primary breast tumor has been suggested by several studies, in particular with regards to hormone receptor status. For example, retrospective studies reported a loss of hormone receptor expression in brain metastases compared to their corresponding breast primaries (52, 53), highlighting the importance of multisite tumor characterization, if clinically feasible, in the treatment decision making process. Molecular profiling of paired brain metastases and corresponding primary breast tumors by whole-exome sequencing revealed that brain metastases harbored genomic aberrations in the CDK pathway and PI3K/AKT/mTOR pathways, many alterations of which were not detected in the corresponding primary tumor (7).

A bioinformatics screen of genome-wide breast tumor methylation data available at The Cancer Genome Atlas (TGCA) and analysis of 11 pairs of primary breast tumors and their corresponding brain metastases identified three genes, *GALNT9*, *CCDC8*, and *BNC1*, that were frequently methylated (55, 73, and 71%, respectively) and silenced in brain metastases (50). Of these, *GALNT9* and *BNC1* were infrequently methylated in primary breast tumors, suggesting that they may be metastasis virulence genes and dysregulation of these occur as late events involved in brain colonization of these cancer cells. GALNT9, which is expressed most abundantly in the brain, plays an important role in *O*-glycosylation and, thereby, in cell adhesion and cell–cell communication. Cancer cells with aberrant *GALNT9* that reach the brain are perhaps favored to proliferate in the new microenvironment through dysregulated cell–cell interaction. *BNC1* encodes a zinc finger transcription factor that is involved in expression of a broad range of genes. Further studies are required to better understand the roles these mutations play in the development of brain metastases and to determine if they represent therapeutic targets.

HER2 Overexpression

Human epidermal growth factor receptor 2 protein overexpression and/or *HER2* oncogene amplification is found in approximately 20% of all breast cancer patients (54). HER2 overexpression is associated with an increased risk of recurrence and death in the absence of adjuvant HER2-directed therapy, and it predicts response to anti-HER2 therapies. HER2-positive breast cancer carries an increased risk of brain metastases, and approximately 30–50% of patients with HER2-positive breast cancer will develop brain metastases during the course of disease (55), with a cumulative incidence of around 12% at 10 years and 14% at 15 years (46, 56). The propensity of HER2-positive breast cancer to metastasize to the brain may be related to the improved survival of patients with HER2-directed therapy, the limited CNS penetration of HER2-directed agents, and the neurotropism of HER2-positive breast cancer (57). HER2-directed agents, including trastuzumab, pertuzumab, lapatinib, neratinib and T-DM1, significantly improve PFS and OS of patients with HER2-positive breast cancer. However, in a retrospective study, 24% of 182 patients with HER2-positive primary breast cancer had HER2 negative metastatic disease, and this discordance was associated with decreased survival (58). If feasible, HER2 status should be repeated in the metastatic disease, including brain metastasis, at relapse.

Trastuzumab, a monoclonal antibody against the HER2 receptor, has limited CNS activity due to its inability to cross the intact BBB. However, there is some evidence of an improved CNS penetration of trastuzumab after disruption of the BBB by radiation therapy. For example, a pharmacokinetic study showed that the ratio of the CSF to plasma levels of trastuzumab in patients with brain metastasis improved significantly from 1:420 before radiotherapy to 1:76 after radiotherapy (59). In patients with concomitant leptomeningeal carcinomatosis, the CSF to plasma ratio after radiotherapy was 1:49. Pertuzumab, another monoclonal antibody against the HER2 receptor, has a significant synergistic antitumor activity in combination with trastuzumab and docetaxel as shown in a randomized phase III placebo-controlled trial of pertuzumab, the CLEOPATRA trial (60). In exploratory analyses of this trial, the median time to development of brain metastases as first site of disease progression was significantly longer in the pertuzumab arm compared to the placebo arm (15.0 versus 11.9 months), and the median OS was 34.4 months in the pertuzumab arm, compared to 26.3 month in the placebo arm (61). Pertuzumab with high-dose trastuzumab in HER2-positive breast cancer patients with brain metastases after radiotherapy is currently under clinical evaluation in a phase II trial (NCT02536339). There is also an ongoing trial of intrathecal pertuzumab and trastuzumab in patients with new untreated asymptomatic or low symptomatic brain metastasis in HER2-positive breast cancer (NCT02598427). Lapatinib is a TKI that acts against both HER2 and EGFR receptors. It has been shown that this agent is able to cross the BBB. In the presence of brain metastases, the brain to plasma concentration of lapatinib was higher at 26% compared to the normal brain parenchyma where the concentration was low at 1.3–2.8% (62). However, the intracranial response to lapatinib alone has been shown to be low at 3–6% (63, 64). In a multicenter phase II study, 45 patients with previously untreated brain metastases from HER2-positive breast cancer were treated with lapatinib and capecitabine combination (55). The addition of capecitabine to lapatinib resulted in an encouraging intracranial response rate of 66% with a median time to intracranial progression of 5.5 months.

PI3K/AKT/mTOR Pathway

Actionable mutations in PI3K/AKT/mTOR pathway are frequent in breast cancer brain metastases (7). The addition of everolimus, an mTOR inhibitor, to an aromatase inhibitor in patients with hormone receptor positive metastatic breast cancer and to

trastuzumab and vinorelbine in patients with HER2-positive breast cancer led to improved survival outcomes in randomized placebo-controlled phase III trials (65, 66). Everolimus crosses the BBB as shown in patients with primary brain tumors. The role of everolimus in the management of patients with breast cancer brain metastases is currently being investigated in clinical trials (NCT01305941, NCT01783756).

Cyclin D-Cyclin-Dependent Kinase 4/6-INK4-Rb Pathway

Activation of cyclin-dependent kinase 4 and 6 (CDK4 and CDK6) by cyclin D leads to cell proliferation by initiating transition from the G1 phase to the S phase in cell cycle *via* phosphorylation of retinoblastoma protein (Rb). Alterations in this pathway are frequent in various cancer types. CDK inhibitors, such as palbociclib, abemaciclib, and ribociclib, have shown high efficacy in patients with hormone receptor positive breast cancer (67). Good CNS penetration of abemaciclib was recently shown (68). There are ongoing trials investigating these agents in patients with breast cancer brain metastases (NCT02896335, NCT02774681, NCT02308020).

MELANOMA

Melanoma is the third most common malignancy to metastasize to the brain. It is estimated that at least 50% of patients with stage IV melanoma will develop brain metastases during the course of disease (69). With supportive care alone, the median survival from developing brain metastases is only around 2 months (70).

Mitogen Activated Protein Kinase (MAPK) Pathway

About half of melanoma patients have an activating mutation in *BRAF*, an oncogene involved in the MAPK pathway that is a key regulator of cell growth, division, and differentiation (71). The most common *BRAF* mutation is the substitution of valine for glutamic acid (VAL600Glu or V600E) accounting for approximately 90% of all *BRAF* mutations seen in melanoma. *BRAF* V600E mutant melanoma tends to be more aggressive. BRAF inhibitors, including dabrafenib and vemurafenib, significantly improve survival in patients with metastatic *BRAF* mutant melanoma (72, 73). In a multicenter phase II trial, 172 patients with *BRAF* mutant melanoma with at least one asymptomatic brain metastasis were treated with dabrafenib (74). Patients were divided into two cohorts: those with and without previous local treatments for brain disease. Dabrafenib was active in both cohorts with ORR of 39.2% in patients without previous local therapy and 30.8% in those with previously treated brain metastases and median OS of 33 and 31 weeks, respectively. A retrospective study of 27 patients treated with vemurafenib for their *BRAF* mutant melanoma that had metastasized to the brain reported intracranial ORR of 50% and extracranial ORR of 71% (75). The median intracranial PFS was 4.6 months, and median OS was 7.5 months. Next-generation sequencing analysis of poorly responding brain metastases revealed co-occurring mutations in genes predicted to activate the PI3K/AKT pathway.

MEK kinase, which is downstream of BRAF, is activated by CRAF or members of the PI3K pathway as an escape mechanism from BRAF inhibition. When BRAF inhibitors were combined with MEK inhibitors, the efficacy was further improved in patients with extracranial disease, as evidenced by prolonged PFS and OS shown in phase III trials (76–78). This approach of dual BRAF and MEK inhibition therapy in patients with brain metastases is currently being evaluated in clinical trials (NCT02039947, NCT02537600).

PI3K/AKT/mTOR Pathway

A molecular analysis of 16 matched pairs of melanoma brain metastases and extracranial metastases showed that activation of the PI3K/AKT/mTOR pathway was enriched in the brain metastases (8). Preclinical cell culture and animal studies of a PI3K inhibitor, BKM120, demonstrated that this agent inhibited the growth of cell lines derived from melanoma brain metastases with inhibition rates of up to 80% and potently induced apoptosis and significantly inhibited the tumor growth of human brain metastatic melanoma cells in the brain of nude mice (79). These findings suggest the potential of PI3K inhibitors as adjunct targeted therapy in the treatment of advanced melanoma that has metastasized to the brain.

Immunotherapy

There has been dramatic improvement in survival of patients with advanced melanoma in recent years with the development of modern immunotherapy including cytotoxic T lymphocyte antigen-4 (CTLA-4) inhibitors and programmed death-1 (PD-1) checkpoint inhibitors. CTLA-4 plays an important role in the regulation of immune activation and tolerance (80). Its signaling inhibits T-cell activation. Ipilimumab is an inhibitor of CTLA-4 that was shown to improve OS in patients with metastatic melanoma compared to a peptide vaccine in a phase III trial (81). A phase II study of ipilimumab was conducted in 72 melanoma patients with brain metastases (82). The disease control rate was 24% in patients who were asymptomatic from their brain disease and were not on corticosteroid treatment. One- and two-year survival rates were 31 and 26% in this steroid-independent cohort. Interaction between PD-1 receptor and PD-ligand 1 (PD-L1) inhibits cytotoxic T-cell activity. PD-1 inhibitors, including nivolumab and pembrolizumab, have resulted in significantly improved survival outcomes in patients with advanced melanoma (83, 84). Pembrolizumab is currently under clinical evaluation in patients with brain metastases from melanoma (NCT02085070). Interim analysis from a phase II trial reported an intracranial ORR of 22% (45). In a randomized phase III trial, nivolumab and ipilimumab combination was shown to be superior to either drug alone in patients with advanced melanoma without brain metastases (85). Median PFS was 11.5 months in the combination arm compared to 6.9 months in the nivolumab alone arm and 2.9 months in the ipilimumab alone arm. There are ongoing trials evaluating the dual CTLA-4 and PD-1 inhibition therapy in melanoma patients with brain metastases (NCT02320058, NCT02621515).

Table 1 summarizes actionable mutations in brain metastases of NSCLC, breast cancer and melanoma, and their targeted therapies discussed earlier.

TABLE 1 | Overview of actionable mutations in brain metastases of non-small cell lung cancer, breast cancer and melanoma, and potential targeted therapies.

Cancer type	Mutation	Targeted therapy	Objective response rate (%)	Progression-free survival (months)	Reference or clinicaltrials.gov number
Non-small cell lung cancer	Activating *EGFR* mutation	First-generation EGFR TKIs: gefitinib, erlotinib	58–83	7–15	(23–26)
		ErbB family inhibitor: afatinib	70–75	8	(21)
	EGFR T790M	T790M specific EGFR TKI: osimertinib	NA	NA Superior blood–brain barrier penetration than gefitinib or afatinib demonstrated in preclinical study	(29)
	ALK rearrangement	First-generation ALK TKI: crizotinib	56–85	7–9	(37, 38)
		Second-generation ALK TKI: alectinib	75	10–11	(39, 40)
	(No specific mutation)	PD-1 inhibitor: pembrolizumab	NA Early analysis showed 33% intracranial response rate	NA	NCT02085070 (45)
Breast cancer	HER2 overexpression/*HER2* oncogene amplification	Dual anti-HER2 inhibition: pertuzumab plus trastuzumab	NA	NA	Intravenous: NCT02536339 Intrathecal: NCT02598427
		HER2/EGFR TKI: lapatinib (in combination with capecitabine)	66	5.5	(55)
	Mutation in PI3K/AKT/mTOR pathway	mTOR inhibitor: everolimus	NA	NA	NCT01305941 NCT01783756
	Mutation in CDK4/6 pathway	CDK inhibitors	NA	NA	NCT02896335 NCT02774681 NCT02308020
Melanoma	Activating *BRAF* mutation	BRAF inhibitors: dabrafenib	39	16	(74)
		Vemurafenib	50	4.6	(75)
		Dual BRAF/MEK inhibition: dabrafenib + trametinib	NA	NA	NCT02039947
		Vemurafenib + cobimetinib	NA	NA	NCT02537600
	Mutation in PI3K/AKT/mTOR pathway	PI3K inhibitor: BKM120	NA Efficacy demonstrated in preclinical study	NA	(79)
	(No specific mutation)	CTLA-4 inhibitor: ipilimumab	24	2.7	(82)
		PD-1 inhibitor: pembrolizumab	NA	NA	NCT02085070
		Dual CTLA-4/PD-1 inhibition: ipilimumab + nivolumab	NA	NA	NCT02320058 NCT02621515

EGFR, epidermal growth factor receptor; TKI, tyrosine kinase inhibitor; ALK, anaplastic lymphoma kinase; PD-1, programmed death 1; HER2, human epidermal growth factor receptor 2; PI3K, phosphoinositide 3-kinase; mTOR, mammalian target of rapamycin; CDK4/6, cyclin-dependent kinase 4 and 6; CTLA-4, cytotoxic T lymphocyte antigen-4; NA, not available.

COLORECTAL CANCER

While colorectal cancer is the third most common cancer in men and second in women, brain metastases are relatively rare with an incidence rate of 1.55% (86). A systematic review and pooled data analysis from 16 relevant studies showed that rectal location was associated with an increased risk of brain metastases compared to other colon locations. The presence of pulmonary metastases was also associated with an increased risk. The incidence of brain metastases from colorectal cancer may be increasing as a result of improved systemic therapy and survival of patients. The prognosis is poor with a reported median OS of 2.6–7.4 months after developing brain metastases (86).

RAS Mutation

KRAS and *NRAS* mutations are the most extensively investigated somatic mutations in metastatic colorectal cancers, and they predict tumor resistance to anti-EGFR therapy, such as cetuximab and panitumumab. There have been several studies examining these mutations in patients with brain metastases from colorectal cancer. A retrospective analysis of 918 patients with metastatic colorectal cancer who were genotyped for *RAS* mutations showed a significantly higher cumulative incidence of brain metastases at 2 years in patients with *RAS* mutation compared to those without (1.4 versus 0.2%) (87). Nearly two-thirds of the brain metastasis cases occurred in patients with *RAS* mutant colorectal cancer. Currently, there are no RAS inhibitors available for clinical use.

BRAF Mutation

BRAF activation mutations (most commonly V600E) occur in less than 10% of metastatic colorectal cancer and are associated with poorer survival. *BRAF* V600E mutations confer resistance to anti-EGFR therapy. To date, no clear association between *BRAF* mutation and development of brain metastases has been observed (88, 89). A clinical characterization of 524 metastatic colorectal cancer patients with known *BRAF* mutation status, where 57 patients had *BRAF* mutation, found no difference in

the incidence of brain metastases between patients with *BRAF* mutant tumors and those with wild-type tumors (88).

PIK3CA Mutation

PIK3CA mutations are found in approximately 10–15% of primary colorectal cancers. In a genomic analysis of colorectal metastases from different sites, *PIK3CA* mutation frequency was higher in brain and lung metastases compared with liver metastases (23.9% in brain, 20% in lung, 7.7% in liver) (89) Another study also found an association between *PIK3CA* mutations and brain metastases with a 2-year cumulative incidence of 1.4%, compared to 0.8% in patients with no *PIK3CA* mutation (87). However, this observation is difficult to interpret as many of those patients with *PIK3CA* mutation and brain metastases also had *RAS* mutations.

RENAL CELL CARCINOMA

Approximately 3.5–17% of patients with renal cell carcinoma develop brain metastases (69). Reported median OS after developing brain metastases ranges from 4.1 to 15 months (69). Patients with bone and thoracic metastases were found to have a higher rate of brain metastases compared to those with abdominal metastases (16 versus 2%) (90). An association between the loss of chromosome 9 and brain metastases was also observed (91). Registration-directed clinical trials investigating therapies for angiogenesis, mTOR signaling, and immunotherapy have excluded patients with brain metastases. Patients with brain metastases were included in the European Advanced RCC Sorafenib expanded-access study in which sorafenib, a VEGF TKI, was given to patients with previously treated advanced renal cell carcinoma. It was shown that the sorafenib safety profile in patients with brain metastases was similar to the overall study population (92). In a prospective open-label non-interventional study in a broad population of patients with advanced renal cell carcinoma treated with sorafenib in routine clinical practice, 115 patients (5% of total study population) had brain metastases (93). The median duration of therapy for this subgroup was similar to that of the total study population (7.0 versus 7.3 months), suggesting that sorafenib has activity in the CNS. In a retrospective study, treatment with TKI was associated with an improved median OS from developing brain metastases (94). However, in prospective studies, the response to these drugs has been more modest. A reported median PFS and OS were only 5.3 and 8.2 months, respectively, in patients with brain metastases treated with sunitinib as part of the global expanded-access protocol (95).

Genetic divergence of brain metastases from primary renal cell carcinoma has been shown recently in a genomic study of paired brain and primary tumors (7). In this study, some brain metastases from renal cell carcinoma had *PTEN*, *PIK3CA*, and *CDKN2A* mutations that were not detected in the corresponding primary tumors. Mutations in PI3K/ATK/mTOR pathway may be potential drivers in brain metastases development and therefore warrant further investigations.

In a randomized phase III trial, the treatment with nivolumab improved survival outcomes compared with everolimus in patients with previously treated advanced renal cell carcinoma (96). Unfortunately, patients with brain metastases were excluded. Clinical trials investigating the role of nivolumab or pembrolizumab in patients with brain metastases is currently underway (NCT02978404, NCT02596035, NCT02886585).

OVARIAN CANCER

Brain metastases from ovarian cancer are very rare with reported incidence rates ranging from 0.29 to 5%; however, the incidence has been rising since the introduction of platinum-based chemotherapy (97). In a recent next-generation sequencing-based genomic analysis of eight brain metastases of primary ovarian cancer, all eight brain metastasis samples harbored mutations in at least one DNA repair gene with seven of eight samples revealing either a *BRCA1* or *BRCA2* mutation (98). Other commonly observed mutations include *TP53*, *ATM*, and *CHEK2* mutations. These findings suggest that BRCA and DNA repair malfunction may possibly play a role in ovarian cancer metastasizing to the brain. A mutation in *ATM*, a regulator of DNA damage detection and repair *via* phosphorylation of a wide variety of downstream proteins including TP53 and BRCA1, may also play a role in the development of brain metastases (98). In cancers that lack homologous repair capacity due to BRCA1/2 or ATM dysfunction or loss, PARP inhibitors can lead to cancer cell death *via* mechanisms of synthetic lethality (99). PARP inhibitors, olaparib and veliparib, have been shown to have activity in the CNS (100, 101), therefore they represent a potential targeted therapy in these cancers with genetic alterations in genes involved in DNA damage response and repair.

CONCLUSION

The incidence of brain metastases is rising as modern cancer therapy is improving the survival of patients with advanced cancer. There is mounting evidence of genomic heterogeneity intratumorally as well as across different metastatic sites. Recently, genomic analyses of brain metastases and matching primary tumors have revealed that brain metastases can harbor actionable driver mutations that are not present in the primary tumors or other extracranial metastases. This genomic divergence of brain metastases from their primary tumors may contribute to the clinically observed treatment response disparities. Cancer genomic analysis using novel and far less invasive approaches, such as cell-free ctDNA in the CSF obtained *via* a lumbar puncture in the outpatient clinics, is very promising in providing critical information required for personalized genomic-directed therapy in patients with brain metastases. Moreover, these new less invasive genomic analysis techniques are more feasible even in patients who are not surgical candidates and can be repeated to determine tumor response to treatment or detect early progression during surveillance. Compared to the traditional chemotherapeutics, next-generation targeted agents appear to be more effective in controlling the CNS disease with better safety profiles. Several brain metastases-dedicated trials of various targeted therapies are currently underway to address the role of these agents in the treatment of CNS disease.

AUTHOR CONTRIBUTIONS

All the authors contributed to the manuscript.

REFERENCES

1. Nayak L, Lee EQ, Wen PY. Epidemiology of brain metastases. *Curr Oncol Rep* (2012) 14(1):48–54. doi:10.1007/s11912-011-0203-y
2. Berghoff AS, Bartsch R, Wohrer A, Streubel B, Birner P, Kros JM, et al. Predictive molecular markers in metastases to the central nervous system: recent advances and future avenues. *Acta Neuropathol* (2014) 128(6):879–91. doi:10.1007/s00401-014-1350-7
3. Brastianos PK, Curry WT, Oh KS. Clinical discussion and review of the management of brain metastases. *J Natl Compr Canc Netw* (2013) 11(9):1153–64. doi:10.6004/jnccn.2013.0133
4. Lockman PR, Mittapalli RK, Taskar KS, Rudraraju V, Gril B, Bohn KA, et al. Heterogeneous blood-tumor barrier permeability determines drug efficacy in experimental brain metastases of breast cancer. *Clin Cancer Res* (2010) 16(23):5664–78. doi:10.1158/1078-0432.CCR-10-1564
5. Gerlinger M, Rowan AJ, Horswell S, Larkin J, Endesfelder D, Gronroos E, et al. Intratumor heterogeneity and branched evolution revealed by multiregion sequencing. *N Engl J Med* (2012) 366(10):883–92. doi:10.1056/NEJMoa1113205
6. Lee SY, Haq F, Kim D, Jun C, Jo HJ, Ahn SM, et al. Comparative genomic analysis of primary and synchronous metastatic colorectal cancers. *PLoS One* (2014) 9(3):e90459. doi:10.1371/journal.pone.0090459
7. Brastianos PK, Carter SL, Santagata S, Cahill DP, Taylor-Weiner A, Jones RT, et al. Genomic characterization of brain metastases reveals branched evolution and potential therapeutic targets. *Cancer Discov* (2015) 5(11):1164–77. doi:10.1158/2159-8290.CD-15-0369
8. Chen G, Chakravarti N, Aardalen K, Lazar AJ, Tetzlaff MT, Wubbenhorst B, et al. Molecular profiling of patient-matched brain and extracranial melanoma metastases implicates the PI3K pathway as a therapeutic target. *Clin Cancer Res* (2014) 20(21):5537–46. doi:10.1158/1078-0432.CCR-13-3003
9. Paik PK, Shen R, Won H, Rekhtman N, Wang L, Sima CS, et al. Next-generation sequencing of stage IV squamous cell lung cancers reveals an association of PI3K aberrations and evidence of clonal heterogeneity in patients with brain metastases. *Cancer Discov* (2015) 5(6):610–21. doi:10.1158/2159-8290.CD-14-1129
10. Wikman H, Lamszus K, Detels N, Uslar L, Wrage M, Benner C, et al. Relevance of PTEN loss in brain metastasis formation in breast cancer patients. *Breast Cancer Res* (2012) 14(2):R49. doi:10.1186/bcr3150
11. Dawson SJ, Tsui DW, Murtaza M, Biggs H, Rueda OM, Chin SF, et al. Analysis of circulating tumor DNA to monitor metastatic breast cancer. *N Engl J Med* (2013) 368(13):1199–209. doi:10.1056/NEJMoa1213261
12. De Mattos-Arruda L, Mayor R, Ng CK, Weigelt B, Martinez-Ricarte F, Torrejon D, et al. Cerebrospinal fluid-derived circulating tumour DNA better represents the genomic alterations of brain tumours than plasma. *Nat Commun* (2015) 6:8839. doi:10.1038/ncomms9839
13. Murtaza M, Dawson SJ, Tsui DW, Gale D, Forshew T, Piskorz AM, et al. Non-invasive analysis of acquired resistance to cancer therapy by sequencing of plasma DNA. *Nature* (2013) 497(7447):108–12. doi:10.1038/nature12065
14. Pentsova EI, Shah RH, Tang J, Boire A, You D, Briggs S, et al. Evaluating cancer of the central nervous system through next-generation sequencing of cerebrospinal fluid. *J Clin Oncol* (2016) 34(20):2404–15. doi:10.1200/JCO.2016.66.6487
15. Hubbs JL, Boyd JA, Hollis D, Chino JP, Saynak M, Kelsey CR. Factors associated with the development of brain metastases: analysis of 975 patients with early stage nonsmall cell lung cancer. *Cancer* (2010) 116(21):5038–46. doi:10.1002/cncr.25254
16. Consonni D, Pierobon M, Gail MH, Rubagotti M, Rotunno M, Goldstein A, et al. Lung cancer prognosis before and after recurrence in a population-based setting. *J Natl Cancer Inst* (2015) 107(6):djv059. doi:10.1093/jnci/djv059
17. Hsu F, De Caluwe A, Anderson D, Nichol A, Toriumi T, Ho C. EGFR mutation status on brain metastases from non-small cell lung cancer. *Lung Cancer* (2016) 96:101–7. doi:10.1016/j.lungcan.2016.04.004
18. Mok TS, Wu YL, Thongprasert S, Yang CH, Chu DT, Saijo N, et al. Gefitinib or carboplatin-paclitaxel in pulmonary adenocarcinoma. *N Engl J Med* (2009) 361(10):947–57. doi:10.1056/NEJMoa0810699
19. Rosell R, Carcereny E, Gervais R, Vergnenegre A, Massuti B, Felip E, et al. Erlotinib versus standard chemotherapy as first-line treatment for European patients with advanced EGFR mutation-positive non-small-cell lung cancer (EURTAC): a multicentre, open-label, randomised phase 3 trial. *Lancet Oncol* (2012) 13(3):239–46. doi:10.1016/S1470-2045(11)70393-X
20. Sequist LV, Yang JC, Yamamoto N, O'Byrne K, Hirsh V, Mok T, et al. Phase III study of afatinib or cisplatin plus pemetrexed in patients with metastatic lung adenocarcinoma with EGFR mutations. *J Clin Oncol* (2013) 31(27):3327–34. doi:10.1200/JCO.2012.44.2806
21. Schuler M, Wu YL, Hirsh V, O'Byrne K, Yamamoto N, Mok T, et al. First-line afatinib versus chemotherapy in patients with non-small cell lung cancer and common epidermal growth factor receptor gene mutations and brain metastases. *J Thorac Oncol* (2016) 11(3):380–90. doi:10.1016/j.jtho.2015.11.014
22. Hoffknecht P, Tufman A, Wehler T, Pelzer T, Wiewrodt R, Schutz M, et al. Efficacy of the irreversible ErbB family blocker afatinib in epidermal growth factor receptor (EGFR) tyrosine kinase inhibitor (TKI)-pretreated non-small-cell lung cancer patients with brain metastases or leptomeningeal disease. *J Thorac Oncol* (2015) 10(1):156–63. doi:10.1097/JTO.0000000000000380
23. Porta R, Sanchez-Torres JM, Paz-Ares L, Massuti B, Reguart N, Mayo C, et al. Brain metastases from lung cancer responding to erlotinib: the importance of EGFR mutation. *Eur Respir J* (2011) 37(3):624–31. doi:10.1183/09031936.00195609
24. Song Z, Zhang Y. Gefitinib and erlotinib for non-small cell lung cancer patients who fail to respond to radiotherapy for brain metastases. *J Clin Neurosci* (2014) 21(4):591–5. doi:10.1016/j.jocn.2013.05.022
25. Wu YL, Zhou C, Cheng Y, Lu S, Chen GY, Huang C, et al. Erlotinib as second-line treatment in patients with advanced non-small-cell lung cancer and asymptomatic brain metastases: a phase II study (CTONG-0803). *Ann Oncol* (2013) 24(4):993–9. doi:10.1093/annonc/mds529
26. Park SJ, Kim HT, Lee DH, Kim KP, Kim SW, Suh C, et al. Efficacy of epidermal growth factor receptor tyrosine kinase inhibitors for brain metastasis in non-small cell lung cancer patients harboring either exon 19 or 21 mutation. *Lung Cancer* (2012) 77(3):556–60. doi:10.1016/j.lungcan.2012.05.092
27. Janne PA, Yang JC, Kim DW, Planchard D, Ohe Y, Ramalingam SS, et al. AZD9291 in EGFR inhibitor-resistant non-small-cell lung cancer. *N Engl J Med* (2015) 372(18):1689–99. doi:10.1056/NEJMoa1411817
28. Yu HA, Arcila ME, Rekhtman N, Sima CS, Zakowski MF, Pao W, et al. Analysis of tumor specimens at the time of acquired resistance to EGFR-TKI therapy in 155 patients with EGFR-mutant lung cancers. *Clin Cancer Res* (2013) 19(8):2240–7. doi:10.1158/1078-0432.CCR-12-2246
29. Ballard P, Yates JW, Yang Z, Kim DW, Yang JC, Cantarini M, et al. Preclinical comparison of osimertinib with other EGFR-TKIs in EGFR-mutant NSCLC brain metastases models, and early evidence of clinical brain metastases activity. *Clin Cancer Res* (2016) 22(20):5130–40. doi:10.1158/1078-0432.CCR-16-0399
30. Kwak EL, Bang YJ, Camidge DR, Shaw AT, Solomon B, Maki RG, et al. Anaplastic lymphoma kinase inhibition in non-small-cell lung cancer. *N Engl J Med* (2010) 363(18):1693–703. doi:10.1056/NEJMoa1006448
31. Soda M, Choi YL, Enomoto M, Takada S, Yamashita Y, Ishikawa S, et al. Identification of the transforming EML4-ALK fusion gene in non-small-cell lung cancer. *Nature* (2007) 448(7153):561–6. doi:10.1038/nature05945
32. Shaw AT, Kim DW, Nakagawa K, Seto T, Crino L, Ahn MJ, et al. Crizotinib versus chemotherapy in advanced ALK-positive lung cancer. *N Engl J Med* (2013) 368(25):2385–94. doi:10.1056/NEJMoa1214886
33. Solomon BJ, Mok T, Kim DW, Wu YL, Nakagawa K, Mekhail T, et al. First-line crizotinib versus chemotherapy in ALK-positive lung cancer. *N Engl J Med* (2014) 371(23):2167–77. doi:10.1056/NEJMoa1408440
34. Gainor JF, Tan DS, De Pas T, Solomon BJ, Ahmad A, Lazzari C, et al. Progression-free and overall survival in ALK-positive NSCLC patients treated with sequential crizotinib and ceritinib. *Clin Cancer Res* (2015) 21(12):2745–52. doi:10.1158/1078-0432.CCR-14-3009
35. Shaw AT, Kim DW, Mehra R, Tan DS, Felip E, Chow LQ, et al. Ceritinib in ALK-rearranged non-small-cell lung cancer. *N Engl J Med* (2014) 370(13):1189–97. doi:10.1056/NEJMoa1311107
36. Rangachari D, Yamaguchi N, VanderLaan PA, Folch E, Mahadevan A, Floyd SR, et al. Brain metastases in patients with EGFR-mutated or ALK-rearranged non-small-cell lung cancers. *Lung Cancer* (2015) 88(1):108–11. doi:10.1016/j.lungcan.2015.01.020
37. Costa DB, Shaw AT, Ou SH, Solomon BJ, Riely GJ, Ahn MJ, et al. Clinical experience with crizotinib in patients with advanced ALK-rearranged

non-small-cell lung cancer and brain metastases. *J Clin Oncol* (2015) 33(17):1881–8. doi:10.1200/JCO.2014.59.0539
38. Solomon BJ, Cappuzzo F, Felip E, Blackhall FH, Costa DB, Kim DW, et al. Intracranial efficacy of crizotinib versus chemotherapy in patients with advanced ALK-positive non-small-cell lung cancer: results from PROFILE 1014. *J Clin Oncol* (2016) 34(24):2858–65. doi:10.1200/JCO.2015.63.5888
39. Shaw AT, Gandhi L, Gadgeel S, Riely GJ, Cetnar J, West H, et al. Alectinib in ALK-positive, crizotinib-resistant, non-small-cell lung cancer: a single-group, multicentre, phase 2 trial. *Lancet Oncol* (2016) 17(2):234–42. doi:10.1016/S1470-2045(15)00488-X
40. Ou SH, Ahn JS, De Petris L, Govindan R, Yang JC, Hughes B, et al. Alectinib in crizotinib-refractory ALK-rearranged non-small-cell lung cancer: a phase II global study. *J Clin Oncol* (2016) 34(7):661–8. doi:10.1200/JCO.2015.63.9443
41. Borghaei H, Paz-Ares L, Horn L, Spigel DR, Steins M, Ready NE, et al. Nivolumab versus docetaxel in advanced nonsquamous non-small-cell lung cancer. *N Engl J Med* (2015) 373(17):1627–39. doi:10.1056/NEJMoa1507643
42. Herbst RS, Baas P, Kim DW, Felip E, Perez-Gracia JL, Han JY, et al. Pembrolizumab versus docetaxel for previously treated, PD-L1-positive, advanced non-small-cell lung cancer (KEYNOTE-010): a randomised controlled trial. *Lancet* (2016) 387(10027):1540–50. doi:10.1016/S0140-6736(15)01281-7
43. Reck M, Rodriguez-Abreu D, Robinson AG, Hui R, Csoszi T, Fulop A, et al. Pembrolizumab versus chemotherapy for PD-L1-positive non-small-cell lung cancer. *N Engl J Med* (2016) 375(19):1823–33. doi:10.1056/NEJMoa1606774
44. Langer CJ, Gadgeel SM, Borghaei H, Papadimitrakopoulou VA, Patnaik A, Powell SF, et al. Carboplatin and pemetrexed with or without pembrolizumab for advanced, non-squamous non-small-cell lung cancer: a randomised, phase 2 cohort of the open-label KEYNOTE-021 study. *Lancet Oncol* (2016) 17(11):1497–508. doi:10.1016/S1470-2045(16)30498-3
45. Goldberg SB, Gettinger SN, Mahajan A, Chiang AC, Herbst RS, Sznol M, 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. *Lancet Oncol* (2016) 17(7):976–83. doi:10.1016/S1470-2045(16)30053-5
46. Arvold ND, Oh KS, Niemierko A, Taghian AG, Lin NU, Abi-Raad RF, et al. Brain metastases after breast-conserving therapy and systemic therapy: incidence and characteristics by biologic subtype. *Breast Cancer Res Treat* (2012) 136(1):153–60. doi:10.1007/s10549-012-2243-x
47. Pestalozzi BC, Zahrieh D, Price KN, Holmberg SB, Lindtner J, Collins J, et al. Identifying breast cancer patients at risk for Central Nervous System (CNS) metastases in trials of the International Breast Cancer Study Group (IBCSG). *Ann Oncol* (2006) 17(6):935–44. doi:10.1093/annonc/mdl064
48. Shen Q, Sahin AA, Hess KR, Suki D, Aldape KD, Sawaya R, et al. Breast cancer with brain metastases: clinicopathologic features, survival, and paired biomarker analysis. *Oncologist* (2015) 20(5):466–73. doi:10.1634/theoncologist.2014-0107
49. Sanna G, Franceschelli L, Rotmensz N, Botteri E, Adamoli L, Marenghi C, et al. Brain metastases in patients with advanced breast cancer. *Anticancer Res* (2007) 27(4C):2865–9.
50. Pangeni RP, Channathodiyil P, Huen DS, Eagles LW, Johal BK, Pasha D, et al. The GALNT9, BNC1 and CCDC8 genes are frequently epigenetically dysregulated in breast tumours that metastasise to the brain. *Clin Epigenetics* (2015) 7:57. doi:10.1186/s13148-015-0089-x
51. Niikura N, Hayashi N, Masuda N, Takashima S, Nakamura R, Watanabe K, et al. Treatment outcomes and prognostic factors for patients with brain metastases from breast cancer of each subtype: a multicenter retrospective analysis. *Breast Cancer Res Treat* (2014) 147(1):103–12. doi:10.1007/s10549-014-3090-8
52. Bachmann C, Grischke EM, Fehm T, Staebler A, Schittenhelm J, Wallwiener D. CNS metastases of breast cancer show discordant immunohistochemical phenotype compared to primary. *J Cancer Res Clin Oncol* (2013) 139(4):551–6. doi:10.1007/s00432-012-1358-0
53. Hoefnagel LD, van de Vijver MJ, van Slooten HJ, Wesseling P, Wesseling J, Westenend PJ, et al. Receptor conversion in distant breast cancer metastases. *Breast Cancer Res* (2010) 12(5):R75. doi:10.1186/bcr2645
54. Owens MA, Horten BC, Da Silva MM. HER2 amplification ratios by fluorescence in situ hybridization and correlation with immunohistochemistry in a cohort of 6556 breast cancer tissues. *Clin Breast Cancer* (2004) 5(1):63–9. doi:10.3816/CBC.2004.n.011
55. Bachelot T, Romieu G, Campone M, Dieras V, Cropet C, Dalenc F, et al. Lapatinib plus capecitabine in patients with previously untreated brain metastases from HER2-positive metastatic breast cancer (LANDSCAPE): a single-group phase 2 study. *Lancet Oncol* (2013) 14(1):64–71. doi:10.1016/S1470-2045(12)70432-1
56. Kennecke H, Yerushalmi R, Woods R, Cheang MC, Voduc D, Speers CH, et al. Metastatic behavior of breast cancer subtypes. *J Clin Oncol* (2010) 28(20):3271–7. doi:10.1200/JCO.2009.25.9820
57. Venur VA, Leone JP. Targeted therapies for brain metastases from breast cancer. *Int J Mol Sci* (2016) 17(9):1543. doi:10.3390/ijms17091543
58. Niikura N, Liu J, Hayashi N, Mittendorf EA, Gong Y, Palla SL, et al. Loss of human epidermal growth factor receptor 2 (HER2) expression in metastatic sites of HER2-overexpressing primary breast tumors. *J Clin Oncol* (2012) 30(6):593–9. doi:10.1200/JCO.2010.33.8889
59. Stemmler HJ, Schmitt M, Willems A, Bernhard H, Harbeck N, Heinemann V. Ratio of trastuzumab levels in serum and cerebrospinal fluid is altered in HER2-positive breast cancer patients with brain metastases and impairment of blood-brain barrier. *Anticancer Drugs* (2007) 18(1):23–8. doi:10.1097/01.cad.0000236313.50833.ee
60. Swain SM, Baselga J, Kim SB, Ro J, Semiglazov V, Campone M, et al. Pertuzumab, trastuzumab, and docetaxel in HER2-positive metastatic breast cancer. *N Engl J Med* (2015) 372(8):724–34. doi:10.1056/NEJMoa1413513
61. Swain SM, Baselga J, Miles D, Im YH, Quah C, Lee LF, et al. Incidence of central nervous system metastases in patients with HER2-positive metastatic breast cancer treated with pertuzumab, trastuzumab, and docetaxel: results from the randomized phase III study CLEOPATRA. *Ann Oncol* (2014) 25(6):1116–21. doi:10.1093/annonc/mdu133
62. Taskar KS, Rudraraju V, Mittapalli RK, Samala R, Thorsheim HR, Lockman J, et al. Lapatinib distribution in HER2 overexpressing experimental brain metastases of breast cancer. *Pharm Res* (2012) 29(3):770–81. doi:10.1007/s11095-011-0601-8
63. Lin NU, Carey LA, Liu MC, Younger J, Come SE, Ewend M, et al. Phase II trial of lapatinib for brain metastases in patients with human epidermal growth factor receptor 2-positive breast cancer. *J Clin Oncol* (2008) 26(12):1993–9. doi:10.1200/JCO.2007.12.3588
64. Lin NU, Dieras V, Paul D, Lossignol D, Christodoulou C, Stemmler HJ, et al. Multicenter phase II study of lapatinib in patients with brain metastases from HER2-positive breast cancer. *Clin Cancer Res* (2009) 15(4):1452–9. doi:10.1158/1078-0432.CCR-08-1080
65. Andre F, O'Regan R, Ozguroglu M, Toi M, Xu B, Jerusalem G, et al. Everolimus for women with trastuzumab-resistant, HER2-positive, advanced breast cancer (BOLERO-3): a randomised, double-blind, placebo-controlled phase 3 trial. *Lancet Oncol* (2014) 15(6):580–91. doi:10.1016/S1470-2045(14)70138-X
66. Baselga J, Campone M, Piccart M, Burris HA III, Rugo HS, Sahmoud T, et al. Everolimus in postmenopausal hormone-receptor-positive advanced breast cancer. *N Engl J Med* (2012) 366(6):520–9. doi:10.1056/NEJMoa1109653
67. Turner NC, Ro J, Andre F, Loi S, Verma S, Iwata H, et al. Palbociclib in hormone-receptor-positive advanced breast cancer. *N Engl J Med* (2015) 373(3):209–19. doi:10.1056/NEJMoa1505270
68. Sahebjam S, Rhun EL, Kulanthaivel P, Turner PK, Klise S, Wang HT, et al. Assessment of concentrations of abemaciclib and its major active metabolites in plasma, CSF, and brain tumor tissue in patients with brain metastases secondary to hormone receptor positive (HR+) breast cancer. *J Clin Oncol* (2016) 34(15_suppl):526. doi:10.1200/JCO.2016.34.15_suppl.526
69. Dagogo-Jack I, Gill CM, Cahill DP, Santagata S, Brastianos PK. Treatment of brain metastases in the modern genomic era. *Pharmacol Ther* (2017) 170:64–72. doi:10.1016/j.pharmthera.2016.10.011
70. Fife KM, Colman MH, Stevens GN, Firth IC, Moon D, Shannon KF, et al. Determinants of outcome in melanoma patients with cerebral metastases. *J Clin Oncol* (2004) 22(7):1293–300. doi:10.1200/JCO.2004.08.140
71. Long GV, Menzies AM, Nagrial AM, Haydu LE, Hamilton AL, Mann GJ, et al. Prognostic and clinicopathologic associations of oncogenic BRAF in metastatic melanoma. *J Clin Oncol* (2011) 29(10):1239–46. doi:10.1200/JCO.2010.32.4327
72. Chapman PB, Hauschild A, Robert C, Haanen JB, Ascierto P, Larkin J, et al. Improved survival with vemurafenib in melanoma with BRAF V600E mutation. *N Engl J Med* (2011) 364(26):2507–16. doi:10.1056/NEJMoa1103782
73. Sosman JA, Kim KB, Schuchter L, Gonzalez R, Pavlick AC, Weber JS, et al. Survival in BRAF V600-mutant advanced melanoma treated with vemurafenib. *N Engl J Med* (2012) 366(8):707–14. doi:10.1056/NEJMoa1112302

74. Long GV, Trefzer U, Davies MA, Kefford RF, Ascierto PA, Chapman PB, et al. Dabrafenib in patients with Val600Glu or Val600Lys BRAF-mutant melanoma metastatic to the brain (BREAK-MB): a multicentre, open-label, phase 2 trial. *Lancet Oncol* (2012) 13(11):1087–95. doi:10.1016/S1470-2045(12)70431-X
75. Harding JJ, Catalanotti F, Munhoz RR, Cheng DT, Yaqubie A, Kelly N, et al. A retrospective evaluation of vemurafenib as treatment for BRAF-mutant melanoma brain metastases. *Oncologist* (2015) 20(7):789–97. doi:10.1634/theoncologist.2014-0012
76. Long GV, Stroyakovskiy D, Gogas H, Levchenko E, de Braud F, Larkin J, et al. Combined BRAF and MEK inhibition versus BRAF inhibition alone in melanoma. *N Engl J Med* (2014) 371(20):1877–88. doi:10.1056/NEJMoa1406037
77. Long GV, Stroyakovskiy D, Gogas H, Levchenko E, de Braud F, Larkin J, et al. Dabrafenib and trametinib versus dabrafenib and placebo for Val600 BRAF-mutant melanoma: a multicentre, double-blind, phase 3 randomised controlled trial. *Lancet* (2015) 386(9992):444–51. doi:10.1016/S0140-6736(15)60898-4
78. Robert C, Karaszewska B, Schachter J, Rutkowski P, Mackiewicz A, Stroiakovski D, et al. Improved overall survival in melanoma with combined dabrafenib and trametinib. *N Engl J Med* (2015) 372(1):30–9. doi:10.1056/NEJMoa1412690
79. Meier FE, Niessner H, Schmitz J, Schmid A, Calaminus C, Pichler B, et al. The PI3K inhibitor BKM120 has potent antitumor activity in melanoma brain metastases in vitro and in vivo. *J Clin Oncol* (2013) 31(15_suppl):e20050. doi:10.1200/jco.2013.31.15_suppl.e20050
80. Wolchok JD, Saenger Y. The mechanism of anti-CTLA-4 activity and the negative regulation of T-cell activation. *Oncologist* (2008) 13(Suppl 4):2–9. doi:10.1634/theoncologist.13-S4-2
81. Hodi FS, O'Day SJ, McDermott DF, Weber RW, Sosman JA, Haanen JB, et al. Improved survival with ipilimumab in patients with metastatic melanoma. *N Engl J Med* (2010) 363(8):711–23. doi:10.1056/NEJMoa1003466
82. Margolin K, Ernstoff MS, Hamid O, Lawrence D, McDermott D, Puzanov I, et al. Ipilimumab in patients with melanoma and brain metastases: an open-label, phase 2 trial. *Lancet Oncol* (2012) 13(5):459–65. doi:10.1016/S1470-2045(12)70090-6
83. Robert C, Ribas A, Wolchok JD, Hodi FS, Hamid O, Kefford R, et al. Anti-programmed-death-receptor-1 treatment with pembrolizumab in ipilimumab-refractory advanced melanoma: a randomised dose-comparison cohort of a phase 1 trial. *Lancet* (2014) 384(9948):1109–17. doi:10.1016/S0140-6736(14)60958-2
84. Robert C, Schachter J, Long GV, Arance A, Grob JJ, Mortier L, et al. Pembrolizumab versus ipilimumab in advanced melanoma. *N Engl J Med* (2015) 372(26):2521–32. doi:10.1056/NEJMoa1503093
85. Larkin J, Chiarion-Sileni V, Gonzalez R, Grob JJ, Cowey CL, Lao CD, et al. Combined nivolumab and ipilimumab or monotherapy in untreated melanoma. *N Engl J Med* (2015) 373(1):23–34. doi:10.1056/NEJMoa1504030
86. Christensen TD, Spindler KL, Palshof JA, Nielsen DL. Systematic review: brain metastases from colorectal cancer – incidence and patient characteristics. *BMC Cancer* (2016) 16:260. doi:10.1186/s12885-016-2290-5
87. Yaeger R, Cowell E, Chou JF, Gewirtz AN, Borsu L, Vakiani E, et al. RAS mutations affect pattern of metastatic spread and increase propensity for brain metastasis in colorectal cancer. *Cancer* (2015) 121(8):1195–203. doi:10.1002/cncr.29196
88. Tran B, Kopetz S, Tie J, Gibbs P, Jiang ZQ, Lieu CH, et al. Impact of BRAF mutation and microsatellite instability on the pattern of metastatic spread and prognosis in metastatic colorectal cancer. *Cancer* (2011) 117(20):4623–32. doi:10.1002/cncr.26086
89. Tie J, Lipton L, Desai J, Gibbs P, Jorissen RN, Christie M, et al. KRAS mutation is associated with lung metastasis in patients with curatively resected colorectal cancer. *Clin Cancer Res* (2011) 17(5):1122–30. doi:10.1158/1078-0432.CCR-10-1720
90. Bianchi M, Sun M, Jeldres C, Shariat SF, Trinh QD, Briganti A, et al. Distribution of metastatic sites in renal cell carcinoma: a population-based analysis. *Ann Oncol* (2012) 23(4):973–80. doi:10.1093/annonc/mdr362
91. Chang EL, Selek U, Hassenbusch SJ III, Maor MH, Allen PK, Mahajan A, et al. Outcome variation among "radioresistant" brain metastases treated with stereotactic radiosurgery. *Neurosurgery* (2005) 56(5):936–45; discussion-45. doi:10.1227/01.NEU.0000158324.20757.AC
92. Beck J, Procopio G, Bajetta E, Keilholz U, Negrier S, Szczylik C, et al. Final results of the European Advanced Renal Cell Carcinoma Sorafenib (EU-ARCCS) expanded-access study: a large open-label study in diverse community settings. *Ann Oncol* (2011) 22(8):1812–23. doi:10.1093/annonc/mdq651
93. Jager D, Ma JH, Mardiak J, Ye DW, Korbenfeld E, Zemanova M, et al. Sorafenib treatment of advanced renal cell carcinoma patients in daily practice: the large international PREDICT study. *Clin Genitourin Cancer* (2015) 13(2):156–64.e1. doi:10.1016/j.clgc.2014.07.007
94. Verma J, Jonasch E, Allen PK, Weinberg JS, Tannir N, Chang EL, et al. The impact of tyrosine kinase inhibitors on the multimodality treatment of brain metastases from renal cell carcinoma. *Am J Clin Oncol* (2013) 36(6):620–4. doi:10.1097/COC.0b013e31825d59db
95. Gore ME, Szczylik C, Porta C, Bracarda S, Bjarnason GA, Oudard S, et al. Final results from the large sunitinib global expanded-access trial in metastatic renal cell carcinoma. *Br J Cancer* (2015) 113(1):12–9. doi:10.1038/bjc.2015.196
96. Motzer RJ, Escudier B, McDermott DF, George S, Hammers HJ, Srinivas S, et al. Nivolumab versus everolimus in advanced renal-cell carcinoma. *N Engl J Med* (2015) 373(19):1803–13. doi:10.1056/NEJMoa1510665
97. Hardy JR, Harvey VJ. Cerebral metastases in patients with ovarian cancer treated with chemotherapy. *Gynecol Oncol* (1989) 33(3):296–300. doi:10.1016/0090-8258(89)90515-5
98. Balendran S, Liebmann-Reindl S, Berghoff AS, Reischer T, Popitsch N, Geier CB, et al. Next-generation sequencing-based genomic profiling of brain metastases of primary ovarian cancer identifies high number of BRCA-mutations. *J Neurooncol* (2017) 133(3):469–76. doi:10.1007/s11060-017-2459-z
99. Mateo J, Boysen G, Barbieri CE, Bryant HE, Castro E, Nelson PS, et al. DNA repair in prostate cancer: biology and clinical implications. *Eur Urol* (2017) 71(3):417–25. doi:10.1016/j.eururo.2016.08.037
100. Forster MD, Dedes KJ, Sandhu S, Frentzas S, Kristeleit R, Ashworth A, et al. Treatment with olaparib in a patient with PTEN-deficient endometrioid endometrial cancer. *Nat Rev Clin Oncol* (2011) 8(5):302–6. doi:10.1038/nrclinonc.2011.42
101. Su JM, Thompson P, Adesina A, Li XN, Kilburn L, Onar-Thomas A, et al. A phase I trial of veliparib (ABT-888) and temozolomide in children with recurrent CNS tumors: a pediatric brain tumor consortium report. *Neuro Oncol* (2014) 16(12):1661–8. doi:10.1093/neuonc/nou103

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REVIEW
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Preclinical Modeling and Therapeutic Avenues for Cancer Metastasis to the Central Nervous System

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Metastasis is the dissemination of cells from the primary tumor to other locations within the body, and continues to be the predominant cause of death among cancer patients. Metastatic progression within the adult central nervous system is 10 times more frequent than primary brain tumors. Metastases affecting the brain parenchyma and leptomeninges are associated with grave prognosis, and even after successful control of the primary tumor the median survival is a dismal 2–3 months with treatment options typically limited to palliative care. Current treatment options for brain metastases (BM) and disseminated brain tumors are scarce, and the improvement of novel targeted therapies requires a broader understanding of the biological complexity that characterizes metastatic progression. In this review, we provide insight into patterns of BM progression and leptomeningeal spread, outlining the development of clinically relevant *in vivo* models and their contribution to the discovery of innovative cancer therapies. *In vivo* models paired with manipulation of *in vitro* methods have expanded the tools available for investigators to develop agents that can be used to prevent or treat metastatic disease. The knowledge gained from the use of such models can ultimately lead to the prevention of metastatic dissemination and can extend patient survival by transforming a uniformly fatal systemic disease into a locally controlled and eminently more treatable one.

Keywords: leptomeningeal metastasis, brain metastasis, *in vivo* models, metastasis, brain metastasis therapies

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INTRODUCTION

A metastatic tumor is a secondary tumor formed from cells that have escaped from a primary tumor elsewhere in the body. Metastases are the most frequent neoplasm to affect the adult central nervous system (CNS), occurring 10 times more than primary brain tumors (1). Metastases commonly arise within the brain parenchyma, where tumor cells travel *via* the arterial circulation and are deposited at terminal "watershed areas" (2). Metastatic spread to the leptomeninges is a rare presentation of CNS metastasis, developing from the infiltration of metastatic cells into cerebrospinal fluid (CSF) encasing the brain and spine. Approximately 40% of cancer patients will develop brain metastases (BM) (3), and 5–8% will develop leptomeningeal metastases (LM) throughout the progression of the disease (4). The primary cancers that have the highest propensity to develop BM are lung (40–60%), breast (15–30%), and melanoma (5–15%) (5). Up to 24% of hematological malignancies result in LM, 10–32% from primary CNS tumors, and of solid tumors the most common origins

are lung (14–29%), breast (11–64%), melanoma (6–18%), and the gastrointestinal tract (4–14%) (6–10). Both BM and LM are associated with poor clinical outcome. The median survival rate of untreated BM and LM patients is 4–6 weeks, yet even when receiving standard interventions (palliative radiotherapy and intrathecal chemotherapy) survival is merely increased to 8–16 weeks (6, 11). The incidence of BM and LM has increased in recent years due to both the improved efficacy of primary tumor interventions, which in turn increases survival and time for metastatic development, as well as the lack of available treatments that are capable of penetrating the blood–brain barrier (BBB) to target the metastatic cells (7, 12). Moreover, administration of current genotoxic treatments can select for increasingly chemoresistant metastatic populations, contributing to their growing resistance over time (7, 12, 13).

THE METASTATIC PROCESS

Migration

A tumor cell can obtain a metastatic phenotype through several mechanisms. Briefly, this process requires a cell to undergo a (1) loss of cell–cell adhesion, (2) acquisition of motility, and (3) ability to digest through surrounding tissues to enter/exit the circulation. Different strategies can be employed by tumor cells to achieve invasive phenotypes. When intercellular junctions have been lost, cells can migrate as single entities (14). These single cells can adopt two main morphological types to promote their motility, amoeboid, and mesenchymal. Amoeboid cells are rounded or abnormally shaped and produce "bleb"-like protrusions to aid migration (13). A typically accepted mechanism to achieve a mesenchymal phenotype is through the epithelial–mesenchymal transition (EMT) (13). Initiated by external and internal factors (environmental cues, transcription factors, etc.), this transition induces a shift from an epithelial state to a more motile mesenchymal phenotype, characterized by an elongated, spindle-like form (15). Conversely, recent studies have addressed the necessity of EMT in metastasis where, due to the transient, non-linear of the process, tumor cells may not require full completion of EMT to become metastatic. Studies have shown that forced induction of EMT through overexpression of EMT-regulating transcription factors causes a loss of tumor-initiating properties in the mesenchymal tumor cell (16–18). As such, EMT has been proposed to include a spectrum of phenotypes, where a tumor cell will undergo partial phase shifts to promote migration as well as maintain their tumor-initiation capacity (19). Cells can migrate collectively with intact cell–cell contacts, where either a single cell or multiple cells will serve as a leader to a line or sheet of follower cells. Collectively migrating cells can be of either epithelial or mesenchymal phenotypes, which may differ between the leader and follower cells (15). In some cases, EMT may not be required at all to achieve migration. Utilizing a lineage-tracing Cre system, Fischer et al. determined that the majority of cells forming lung metastases were of an epithelial phenotype (20). Similar results were found by Zheng et al., using a genetically engineered model of pancreatic cancer and EMT inhibited by *SNAIL1* or *TWIST* deletion (21).

Invasion into the Circulation

These metastasizing cells can either secrete various matrix metalloproteinases and enzymes to remodel surrounding tissue or, in the case of amoeboid cells activate contractile actin:myosin core networks to squeeze between intercellular spaces, allowing them to intravasate into the surrounding tissue, and invade adjacent blood or lymphatic vessels (22). Once in the circulation, the majority of metastasizing cells will succumb to a myriad of lethal barriers, ranging from host's immune response to shearing forces within the vessel (23). These metastasizing cells, otherwise known as circulating tumor cells (CTCs), have adopted successful defensive strategies. One of the methods employed by single CTCs involves platelet aggregation, where the CTC will express thrombin to collect platelets and form protective layer from immune surveillance and hemodynamic shearing forces (24). Metastasizing cells that have invaded the circulation as collective groups can form clusters of CTCs or microemboli, arising from oligoclonal tumor cells and are rarer but appear to have a higher metastatic potential than single CTCs (25). These clusters provide protection similar to platelet shields but also an added benefit of avoiding anchorage-dependent apoptosis (26).

Colonization

As the cell arrests at the new site, both through homing mechanisms as well as physical restraints (27), the cell will extravasate into the tissue. Depending on the environmental cues the cell will either remain in a dormant state or colonize the tissue where initial seeding of the brain will form micrometastases and subsequent development of tumor-associated vasculature (neoangiogenesis) will give rise to macrometastases (28). The clonality of the resulting metastasis is dependant on the nature of the seeding cell. A single cell can give rise to a clonal metastasis, whereas a polyclonal metastasis can develop from a CTC cluster or seeding of the same region by multiple single cells (29). Recent studies have identified metastases to be primarily polyclonal, including prostate (30), breast (31), and pancreatic (32), which is consistent with the concept of enhanced survival and metastatic seeding potential by CTC clusters over single metastatic cells (29).

To enter the CNS, metastasizing cells must overcome additional barriers during extravasation and colonization: the BBB and brain–CSF barrier (BCSFB). It is thought that the properties required to exit the circulation are rate limiting; though millions of cells can be shed into the circulation, only a very small percentage are able to colonize the secondary environment (33). However, once these obstacles are overcome, the CNS becomes a sanctuary site for these metastasizing cells, allowing their escape and protection from typical cytotoxic agents and immune surveillance that are unable to cross an intact BBB and BCSFB (7).

The flow of arterial blood largely determines the spread of metastatic cells throughout the brain parenchyma: 85% of BM arise within the cerebrum, 5–10% within the cerebellum, and 3–5% within the brainstem (2). The type of primary tumor can also dictate the distribution and number of metastases within the brain. For instance, lung cancers typically result in multiple lesions within the occipital lobe and cerebellum, whereas breast cancer results in metastases within the brain parenchyma,

leptomeninges, cerebellum, and brain stem (5, 34). Within the meninges, LM are generated either by diffuse, non-adherent single cells or clusters or nodules (35).

Brain metastases typically develop from solid and hematological tumors, whereas LM can arise from primary CNS tumors (e.g., medulloblastoma, glioma, and PNET), systemic cancers (lymphoma and leukemia), or solid tumors. In LM, metastatic cells gain access to the CSF (and subsequently the leptomeninges) through several methods: the most common route is hematogenous or lymphatic systems; however, cells are also able to enter the CSF by escaping from adjacent bone tumors (i.e., the skull or spine) into the dural sinus or epidural plexus (36). Once in the CSF these cells can travel throughout the CNS, either remaining within the leptomeninges or invading the brain parenchyma, spinal cord, or nerve roots (**Figure 1**).

A significant controversy in cancer research surrounds the origin of metastatic spread; do metastases develop in a linear fashion after primary tumor formation, or in parallel to the primary tumor? The linear model of dissemination is intimated in cancers where there are close genetic similarities between the primary and secondary tumors, whereas the parallel model is suggested in cases of genetic diversity. A third theory suggests metastasis-to-metastasis seeding (37). Although from several studies the general thought appears to lean toward parallel progression of metastatic dissemination, recent phylogenetic studies have shown multiple modes of dissemination (38–42).

CONSIDERATIONS FOR THE DEVELOPMENT OF BM AND LM MODELS

The complex nature of the metastatic process has led to the generation of several 2- and 3-dimensional *in vitro* assays that strive to recapitulate the various stages of metastasis under more stable conditions. For instance angiogenesis or neovascularization, the process of forming new blood vessels branching from existing vasculature, can be recognized through a tube formation assay, where cells plated on an extracellular matrix layer that mimics the *in vivo* environment and will form tubule-like structures that resemble vessels (43). Cancer cell migration, an integral component of metastatic cells, can be modeled in multiple assays including transwell or Boyden chambers, scratch wound, zone exclusion, or microfluidics. These assays have been invaluable as tools to not only delineate the intricacies of the mechanistic regulation of metastasis but also serve as screening platforms for therapeutic targets, unfortunately they also face several limitations. The involvement of complex host/cell interactions throughout BM development are more accurately examined *in vivo*, such as anatomical barriers (BBB and BCSFB), stromal/environmental determinants, immune signaling and response, and cytokines/growth factors (44). As such, animal models represent a vital tool in a scientist's repertoire for translational research. A clinically relevant *in vivo* model can enable researchers to identify the genetic events that contribute to metastatic development within the CNS and provide a platform to identify and screen novel therapeutics (44).

Although the genetic mouse models have become an important tool in studying the functional significance of defined mutations in the development of BM and LM, such models lack the ability to recapitulate the genetic heterogeneity of primary human tumors. Furthermore, the genetically engineered mouse models (GEMMs) are limited by complex breeding schemes, incomplete tumor penetrance, and variable tumor onset (45). In contrast, patient-derived xenograft (PDX) models for many cancer subtypes (46–51) have been generated through injection of patient tumor cells into an appropriate microenvironment. Tumors generated through PDX models have been shown to retain the

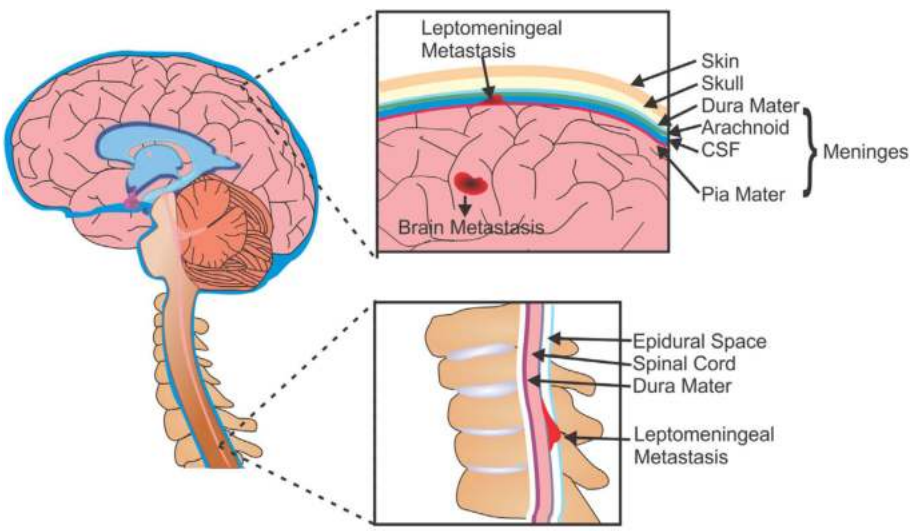

FIGURE 1 | Metastases of the brain and leptomeninges. Cells from primary tumors are able to metastasize to the brain and can form secondary tumors within the parenchyma (BM), or can enter the CSF and spread to the membranes surrounding the brain and spine (LM). CSF, cerebrospinal fluid; BM, brain metastasis; LM, leptomeningeal metastasis.

molecular identity and recapitulate the complex heterogeneity of the original patient tumor. In addition, PDX models allow for a more accurate evaluation of tumor growth patterns, metastatic properties, and their changes in response to therapeutic intervention (52, 53). Currently, various xenograft models have been developed that are capable of reproducing specific individual stages of metastasis, providing a more detailed understanding of the intricacies involved in the process. For instance, the avian embryo provides a unique model support system for many metastatic features, including growth, invasion, and angiogenesis. The chorioallantoic membrane, a vascularized embryonic tissue, shows easy engraftment of human cells, and the embryo itself provides an immunodeficient environment (54–56). The use of zebrafish xenograft models has also risen over the last few years, providing a novel high throughput and inexpensive platform for drug discovery and *in vivo* imaging (57, 58). Despite the novelty of these unique models, the use of mice and rats (murine) has remained a standby host species in modeling metastasis, providing highly reproducible disease development and easy to manipulate/inject due to size (59). The advent of transgenic and immunodeficient strains significantly increased the success rate of tumor transplantation and human–mouse xenograft model development (60).

When developing an appropriate *in vivo* model for LM and BM, several biological and technical factors must be considered.

1. Material injected: commercial cells are a commonly utilized cell source, providing an unlimited number of cells for cost-effective use. Unfortunately, these lines have undergone significant selective pressures from years of culturing that they rarely represent the genotypic or phenotypic profile of the original patient sample and are sometimes even misidentified or cross-contaminated (61). Conversely, patient-derived cell lines provide a much more accurate representation of clonal heterogeneity existing within the original tumor, as the length of culture remains minimal, though these lines also face difficulties due to poor growth and engraftment, and a limited life span (61). Moreover, a significant concern with patient-derived cell lines is that the majority of patients have received some form of therapy, thus there are very few samples that have not faced a selection pressure from exposure to a chemotherapeutic (62).
2. The number of cells delivered, a property easily controlled by the researcher, can also play a large role in the time it takes for engraftment. A larger the cell number injected may permit for a shorter incubation period; however, this may not accurately represent the slower growth observed with the clinical presentation of metastatic progression. Conversely, a low cell number may not be engrafted easily, reducing the success rate of engraftment or cell collection (62).
3. Host selection: the choice of host when establishing a metastasis model can be key to successful engraftment rates. Murine models can be divided into two broad categories: (1) syngeneic and (2) xenogeneic. Syngeneic models utilize cancer cell lines of the same genetic background as the host and are typically generated through chemical or spontaneous induction. These models offer researchers the ability to study oncogenesis and metastatic progression in the presence of a functioning immune response and potential to identify therapeutics that can target the immune system. Unfortunately, this model is solely mouse related, which can have difficulties with correlations to human disease. On the contrary, xenograft models are developed from the administration of human cancer cells into an immunocompromised host. The lack of an immune response, which would otherwise attack the foreign cells injected and limit engraftment, permits a high rate of human tumor transplantation and study of human cancer cell behavior in a live host but lacks information on the interaction between the immune system and tumor cells.
4. The route of injection (**Figure 2**): the location of cell delivery and subsequent tumor engraftment and metastatic progression is another decision vital to model development. Due to circulation patterns, some locations for metastatic spread are more likely to over others, such as tail vein injections resulting primary in lung metastases (62). Certain hosts do not possess the proper/compatible physiology to represent clinical disease progression, whereas injections in some areas may not even be feasible for a particular host due to anatomical differences. Another criteria is host size, where a larger animal may allow for easy and safe repeated access to the injection route (59).

When modeling metastasis, the best route of injection would replicate tumor formation at the primary site first and subsequent metastatic development. Several such models have been established with commercial mouse and human cell lines, unfortunately this method can be laden with difficulties in capturing the metastatic cells at the desired secondary site. To overcome this, successive rounds of *in vivo* selection are performed with cells harvested from the secondary site and reinjected, selecting for cells that are aggressively metastatic with each round (63, 64).

(a) Intracardiac/intracarotid: a common method for BM development is direct injection of tumor cells into the circulation. This method is more of an assessment of brain colonization and not full metastasis, as it selectively ignores the ability of cells to undergo EMT and intravasate into the circulation. In addition, the number of cells injected into the circulation is several folds higher than the number of cells that would typically escape from the primary tumor. Nonetheless, this method allows for selection of highly metastatic populations that are able to cross the BBB and BCSF to engraft into the brain. Intracardiac injections (*via* the left ventricle) allow cells to freely enter the circulation and have indiscriminate access to all organs of the body, allowing cells to seed metastases in different areas (65). Injection of cells into the intracarotid artery allows cells to travel directly to the brain, and primarily produces BM and LM (66–69).
(b) Orthotopic: orthotopic injections place cells directly into the originating environment of the primary tumor. For BM development, the most common route of inoculation of tumor cells derived from a BM or primary brain tumor is directly into the brain parenchyma (intracranial). This surpasses all barriers encountered in the initial and mid

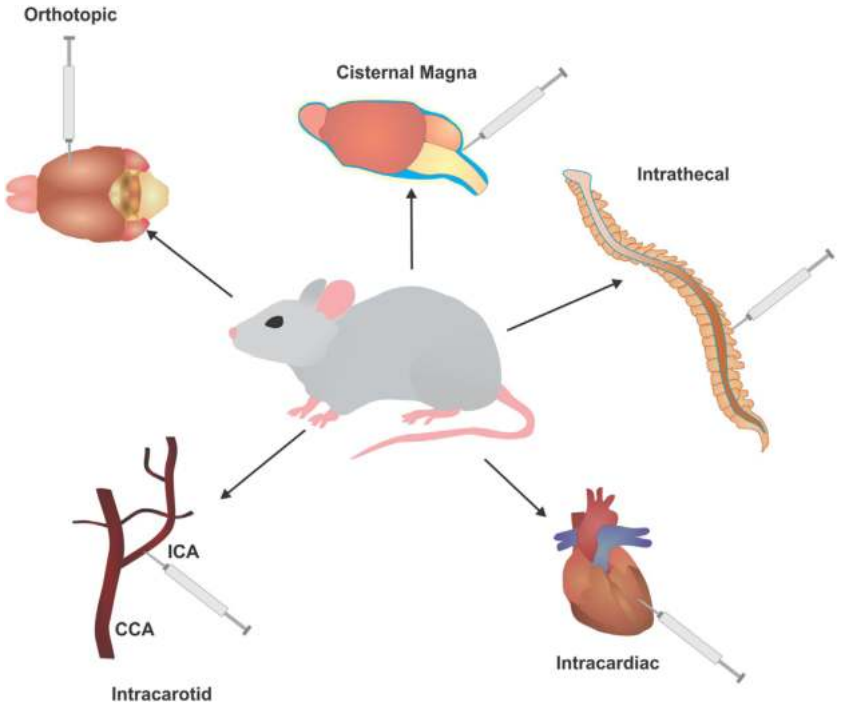

FIGURE 2 | Injection routes utilized to develop *in vivo* models of leptomeningeal metastases (LM) and brain metastases (BM). Common injection routes used in murine models to develop BM and LM, typically involving injection of cells of directly into the circulation or cerebrospinal fluid to bypass the initial stages of metastasis. ICA, intracarotid artery; CCA, common carotid artery.

stages of metastasis, allowing the cells to begin colonization, but creating a significant selection bias by giving cells that may not be capable of surviving the metastatic cascade an opportunity to engraft. When utilizing tumor cells from other primary cancers, the injection site will follow accordingly for better representation of the metastatic cascade. For instance, melanoma cells can be injected subcutaneously, lung cancer cells injected intrathoracically, and breast cancer cells injected into the fatpad (70–72).

(c) Intrathecal: a common method for LM modeling is intrathecal, allowing direct entry of cells into the CSF *via* the cisterna magna or spinal canal (73, 74). The larger size of a rat provides safe, easy, and repeated access to the CSF through these methods. For instance, an arachnoid catheter can be implanted into the great cistern and passed along the spinal cord (75).

Keeping these factors in mind, several groups have proven successful in developing clinically relevant models of LM and BM. Massague et al. performed several rounds of selection on human and mouse cancer lines through injection into the cisterna magna, allowing the cells to propagate within the leptomeningeal space before collecting the cells from the basilar meninges and thecal sac. After the third round of selection, cells were then injected intracardially, where hematogenously disseminated cells were found to consistently form LM as opposed to BM, faithfully replicating many clinical and histopathological aspects of LM (76). A more recent study preformed by this group utilized LM models to dissect the molecular processed involved in leptomeningeal dissemination of breast and lung cancers. They determined that cancer cells within the CSF express complement component 3 to promote disruption of the BCSF and is predictive of leptomeningeal relapse (77).

Sandén et al. were able to propagate a cell line from a primary patient sample of Group 3 MB with overexpression of c-Myc, a gene that the worst clinical outcome for MB. Intracerebellar injection of this cell line resulted in primary brain tumors that recapitulated both epigenetic and phenotypic characteristics of the original patient sample. In addition, similar to the clinical progression of the disease, researchers were able to observe metastatic spread to the meninges and down the spinal axis even before it was observed in the surviving patient, thus providing an opportunity to study early stages of spinal dissemination and allow for preclinical evaluation of targeted therapeutic interventions (45).

Recently, Singh et al. successfully established cell lines from primary patient samples of lung BMs, where specific *in vitro* culture conditions were utilized to enrich for a metastatic subpopulation of cancer stem cells within these BM, termed brain metastasis-initiating cells (BMICs). Using these BMICs, they developed a novel PDX model of lung-to-brain metastasis, where through intracardiac injections the researchers were able to obtain macrometastases, whereas intrathoracic injection of BMICs not only reformed tumors within the lung but also developed micrometastatic growths within the brain (78).

MODELING THERAPY RESPONSE

Originally described in 1800s (79, 80), few substantial advancements have been made since then in understanding the progression of LM, and this progress has added very little to the improvement upon the dismal survival rates for patients. A similar lack of therapeutic progress is seen with BMs. The typical treatment strategy for BM and LM is palliative care for poor risk patients, as treatment options offering any significant extension of survival are presently not available. Poor risk patients may receive radiation therapy, analgesics and corticosteroids for persistent pain and headaches, antidepressants, antinauseants, and anticonvulsants (36). Good risk patients will receive treatments tailored to control the tumor, including stereotactic radiosurgery, whole-brain radiotherapy, surgical resection, and systemic treatments (81). Systemic therapies include chemotherapies, small molecules (**Table 1**), and immunotherapies and can be administered depending on various patient factors, such as tumor histology and the patient's prior treatment history to theoretically target both the active systemic disease as well as the LM and BM (7). Intrathecal administration (direct injection into the spinal canal) of anticancer agents guarantees the treatment will enter the CSF; however, this route can have limited efficacy. An alternative intraventricular administration shows improved CSF drug levels, especially in bulky tumors, and less variability between patients (7).

1. Chemotherapy: various chemotherapeutic agents have been employed to treat LM and BM, often used in a combination of 2–3 along with whole-brain radiotherapy. For BM, standard chemotherapies are administered based on the primary cancer. For instance cisplatin, cyclophosphamide, etopside, prednisone, and irinotecan have all been administered for BM and LM of lung, breast, and melanoma cancers (81). Capecitabine, belonging to the class of fluoropyrimidines of chemotherapies, is administered for several cancer types (106), and a phase II trial has shown the combination of lapatinib and capecitabine as a first line treatment for HER2-positive breast cancer (89). Temozolomide has shown a modest therapeutic effect when administered alone, however shows much more promise when used in conjunction with whole-brain radiotherapy and/or other anticancer agents, as discussed in a thorough review by Zhu et al. (107). For LM, standard chemotherapies administered are methotrexate, cytarabine (Ara-C), thiotepa, all safe for intrathecal administration (108).
2. Small molecules: the mutational status of the primary tumor can determine the type of small molecule administered to BM and LM. Tyrosine kinase inhibitors (TKI) such as gefitinib, osimertinib, and erlotinib target EGFR mutations found in lung cancers, lapatinib is a dual TKI that targets HER2/neu and EGFR mutations and is applied to advanced or metastatic breast cancer, vemurafenib and dabrafenib target BRAF mutations in melanoma, and crizotinib, ceritinib, and alectinib target anaplastic lymphoma kinase fusions in lung cancers (7, 81).
3. Biologics: recent studies have shown promising results with the administration of monoclonal antibodies and immune-modulating therapies, activating T-cell responses to target BM and LM in a non-cytotoxic manner. Bevacizumab is a monoclonal antibody that targets high levels of VEGF in several cancers to inhibit angiogenesis (109). Rituximab and trastuzumab are non-cytotoxic monoclonal antibodies that are administered intrathecally, targeting CD20 and HER2/neu, respectively (7); however, trastuzumab has been linked with increased incidence of brain metastasis. Checkpoint inhibitors such as ipilimumab and nivolumab are a new class of immunotherapies showing great promise in phase trials, targeting CTLA-4 and PD-L1 in metastasis of kidney, melanoma, and non-small cell lung cancer (6, 110). A more extensive coverage of the ongoing research on immunotherapies directed at BM is comprehensively discussed in the review by Farber et al. (111).

One of the major hurdles in developing novel therapies for brain tumors has been the paucity of overlapping actionable targets between treatment-naïve and recurrent tumors. Using a sleeping beauty transposon system in Ptch+/− mice with mutated *Trp53*, Wu et al. were able to generate GEMM of sonic hedgehog MB with increased tumor penetrance and reduced latency period (112). The introduction of humanized therapy protocols combining surgical resection and fractionated craniospinal irradiation led to generation of a mouse model that recapitulated both tumor initiation as well as disease progression, including rise of local and distal metastasis (113). The development of novel therapeutics is further complicated by the hurdles encountered throughout delivery of anticancer drugs to the brain. The BBB and BCSFB are substantial obstacles that need to be overcome to identify feasible cancer therapies. Both barriers differ in composition, permeability, and function. The BBB is a barrier formed by the endothelial cells of the brain capillaries, closely associated with pericytes, perivascular astrocytes, and microglia, and separates the circulating blood from the brain interstitial fluid (114). The BCSFB is composed of modified cuboidal epithelium of the choroid plexus, serving to secrete and separate CSF from circulating blood (114). Both barriers express transporters, multi-specific carriers, receptors, and enzymes that help to regulate diffusion and transport of polar molecules, essential nutrients, and wastes, and restrain the passage of anticancer compounds into the brain (114). Several factors within the composition of a drug also impede its ability to penetrate the BBB and BCSFB, including lipid solubility, molecular weight, polarity, and protein binding (7).

Various methods have been employed when designing drugs and delivery systems to increase drug efficacy in crossing the BBB and BCSF and targeting BM and LM. One method utilized to avoid these barriers is the use of transporters, where a drug that is not able to cross the BBB is coupled to a substance that can. This process can improve the peripheral pharmacokinetics, yet results in a hybrid compound that may not be recognized by the transporter or is destroyed as a foreign body (115). Another method is the formulation of a compound to be highly lipid soluble and with low molecular weight, increasing the likelihood of drug transport by transmembrane diffusion. Typical clinical therapeutic drugs are small, lipid soluble molecules.

TABLE 1 | List of selected chemotherapies, small molecules, and immunotherapies administered in the treatment of brain metastases (BM) and leptomeningeal metastases.

Drug	Mechanism of action	Mode of delivery	Clinical uses	Reference
CHEMOTHERAPY				
Cisplatin	Inhibitor of DNA replication	Intravenous infusion	Treatment of BM in patients with breast cancer, NSCLC, and melanoma	Franciosi et al. (82)
Cyclophosphamide	Inhibitor of DNA replication	Intravenous infusion; oral administration	Treatment of BM in patients with breast cancer	Rosner et al. (83)
Irinotecan	Inhibitor of DNA replication and transcription	Intravenous infusion	Treatment of BM in patients with small cell lung cancer	Sevinc et al. (84)
Methotrexate	S-phase specific cytotoxic chemotherapy	Intraventricular or intrathecal infusion	Treatment of patients with neoplastic meningitis	Grossman et al. (8)
Cytarabine (Ara-C)	Antimetabolite, blocks activity of DNA polymerase Disruption of PI3K/Akt/mTOR pathway	Intraventricular or intrathecal infusion	Targeting leptomeningeal dissemination of patients with glioma, breast cancers, and NSCLC	Zhao et al. (85) Niwińska et al. (86) Nagpal et al. (87)
Thiotepa	Non-specific cell cycle inhibitor	Intraventricular or intrathecal infusion	Treatment of patients with neoplastic meningitis	Grossman et al. (8)
Capecitabine	Antimetabolite, blocks activity of DNA polymerase	Oral administration	Treatment of BM in patients with HER2-positive breast cancer	Petrelli et al. (88) Bachelot et al. (89)
SMALL MOLECULES				
Gefitinib	Inhibitor of EGFR-associated tyrosine kinase	Oral administration	Targeting brain metastasis in patients with NSCLC	Ceresoli et al. (90)
Osimertinib	Inhibitor of EGFR-activating mutations and EGFR with T790M mutation	Oral administration	Targeting brain metastasis in NSCLC patients with EGFR T790M mutation	Koba et al. (91)
Erlotinib	Inhibitor of EGFR-associated tyrosine kinase	Oral administration	Targeting brain metastasis in patients with NSCLC	Sperduto et al. (92)
Lapatinib	Inhibitor of EGFR and HER2/neu	Oral administration	Targeting brain metastasis in patients with HER2+ breast cancer	Lin et al. (93) Lin et al. (94)
Vemurafenib	Inhibitor of BRAF, resulting in disruption of BRAF/MEK/ERK pathway	Oral administration	Targeting brain metastasis in patients with V600E *BRAF* mutation-positive melanoma	Dummer et al. (95)
Dabrafenib	Inhibitor of BRAF, resulting in disruption of BRAF/MEK/ERK pathway	Oral administration	Targeting brain metastasis in patients with V600E *BRAF* mutation-positive melanoma	Long et al. (96)
Crizotinib	Inhibitor of anaplastic lymphoma kinase (ALK) and c-ros oncogene 1 (ROS1)	Oral administration	Targeting brain metastasis in patients with EML4-ALK fusion NSCLC	Yoshida et al. (97)
Ceritinib	Inhibitor of ALK	Oral administration	Targeting brain metastasis in patients with ALK-positive NSCLC	Melosky et al. (98)
Alectinib	Inhibitor of ALK	Oral administration	Targeting brain metastasis in patients with ALK-positive NSCLC that are resistant to crizotinib	Gadgeel et al. (99)
BIOLOGICS				
Bevacizumab	Monoclonal antibody, blocks angiogenesis though inhibition of VEGF-A	Intravenous infusion	Targeting brain metastasis in patients with NSCLC	Besse et al. (100)
Rituximab	Monoclonal antibody targeting CD20	Intraventricular or intrathecal infusion	Targeting leptomeningeal dissemination of patients with lymphoma	Schulz et al. (101)
Trastuzumab	Monoclonal antibody targeting HER2/neu	Intravenous infusion or subcutaneous injection	Preventing brain metastasis development in HER2-overexpressing metastatic breast cancer	Park et al. (102)
Ipilimumab	Monoclonal antibody targeting CTLA-4	Intravenous infusion	Targeting brain metastasis in patients with melanoma	Margolin et al. (103)
Nivolumab	Monoclonal antibody targeting PD-1	Intravenous infusion	Targeting brain metastasis in patients with NSCLC	Dudnik et al. (104)
Pembrolizumab	Monoclonal antibody targeting PD-1	Intravenous infusion	Targeting brain metastasis in patients with melanoma or NSCLC	Goldberg et al. (105)

Unfortunately, once across the BBB the drug must then enter the surrounding aqueous interstitial fluid of the brain to be effective, resulting in drugs that are too lipid soluble being sequestered to the capillary bed and unable to reach areas beyond the BBB (116).

The evidence of exosome-based communication in neural cells (117) opened up a possibility of potentially developing therapies that deliver short interfering RNA (siRNA) against specific targets to the brain. Despite a lack of clinical trial testing the

efficacy of exosome-based therapies for cancer has been initiated, a study published by Alvarez-Erviti et al. (118) demonstrated a prominent reduction of both mRNA and protein levels of BACE1 within multiple brain cell lineages post siRNA delivery to the brain (118). Other delivery strategies include targeting peptides, regulatory proteins, and oligonucleotides (115).

CONCLUSION

Brain metastases and LM are a common complication of cancer progression, associated with poor survival and limited treatment options. Elucidation of the molecular mechanisms underlying brain metastasis development, which includes the shifting microenvironment and interactions with the immune system the disseminated cell encounters, is supremely difficult to capture with an *in vitro* system. Consequently, experimental *in vivo* models are heavily relied on to serve as platforms to explore the nature of metastatic dissemination in a more comprehensive manner. Unique models have been generated with fish, mice, chicks, and companion animals (cats and dogs), all providing much needed knowledge to the field. However, there are several shortcomings associated with *in vivo* models, including the lack of feasible models that recapitulate the clinical progression of BM development in its entirety, and the obvious dissimilarities between the biological makeup of an animal and human. As such one must be aware of the benefits and caveats associated with available models to properly interpret results. Nonetheless, these animal models have provided significant knowledge of the characterization of metastatic disease progression in a live host and are a fundamental component to the identification, study, and testing of new cancer regimens.

AUTHOR CONTRIBUTIONS

Conceptualization of review, drafting of work, revision of manuscript, figure design, final approval of manuscript, and agreement for accountability for content of work: MS. Conceptualization of review, revision of manuscript, final approval of manuscript, and agreement for accountability for content of work: DB, CV, and SS.

REFERENCES

1. Sjøbakk TE, Vettukattil R, Gulati M, Gulati S, Lundgren S, Gribbestad IS, et al. Metabolic profiles of brain metastases. *Int J Mol Sci* (2013) 14:2104–18. doi:10.3390/ijms14012104
2. Patchell RA. The management of brain metastases. *Cancer Treat Rev* (2003) 29:533–40. doi:10.1016/S0305-7372(03)00105-1
3. Gavrilovic IT, Posner JB. Brain metastases: epidemiology and pathophysiology. *J Neurooncol* (2005) 75:5–14. doi:10.1007/s11060-004-8093-6
4. Glantz MJ, Hall WA, Cole BF, Chozick BS, Shannon CM, Wahlberg L, et al. Diagnosis, management, and survival of patients with leptomeningeal cancer based on cerebrospinal fluid-flow status. *Cancer* (1995) 75:2919–31. doi:10.1002/1097-0142(19950615)75:12<2919::AID-CNCR2820751220>3.0.CO;2-9
5. Palmieri D, editor. *Central Nervous System Metastasis, the Biological Basis and Clinical Considerations Vol. 18 Cancer Metastasis – Biology and Treatment*. (Chap. 1). Netherlands: Springer (2012). p. 1–13.
6. Leal T, Chang JE, Mehta M, Robins HI. Leptomeningeal metastasis: challenges in diagnosis and treatment. *Curr Cancer Ther Rev* (2011) 7:319–27. doi:10.2174/157339411797642597
7. Groves MD. New strategies in the management of leptomeningeal metastases. *Arch Neurol* (2010) 67:305–12. doi:10.1001/archneurol.2010.18
8. Grossman SA, Finkelstein DM, Ruckdeschel JC, Trump DL, Moynihan T, Ettinger DS. Randomized prospective comparison of intraventricular methotrexate and thiotepa in patients with previously untreated neoplastic meningitis. Eastern Cooperative Oncology Group. *J Clin Oncol* (1993) 11:561–9. doi:10.1200/JCO.1993.11.3.561
9. Nugent JL, Bunn PA Jr, Matthews MJ, Ihde DC, Cohen MH, Gazdar A, et al. CNS metastases in small cell bronchogenic carcinoma: increasing frequency and changing pattern with lengthening survival. *Cancer* (1979) 44:1885–93. doi:10.1002/1097-0142(197911)44:5<1885::AID-CNCR2820440550>3.0.CO;2-F
10. Kaplan JG, DeSouza TG, Farkash A, Shafran B, Pack D, Rehman F, et al. Leptomeningeal metastases: comparison of clinical features and laboratory data of solid tumors, lymphomas and leukemias. *J Neurooncol* (1990) 9:225–9. doi:10.1007/BF02341153
11. Soffietti R, Ruda R, Mutani R. Management of brain metastases. *J Neurol* (2002) 249:1357–69. doi:10.1007/s00415-002-0870-6
12. Langley RR, Fidler IJ. The biology of brain metastasis. *Clin Chem* (2013) 59:180–9. doi:10.1373/clinchem.2012.193342
13. Heerboth S, Housman G, Leary M, Longacre M, Byler S, Lapinska K, et al. EMT and tumor metastasis. *Clin Transl Med* (2015) 4:6. doi:10.1186/s40169-015-0048-3
14. Friedl P, Alexander S. Cancer invasion and the microenvironment: plasticity and reciprocity. *Cell* (2011) 147:992–1009. doi:10.1016/j.cell.2011.11.016
15. Clark AG, Vignjevic DM. Modes of cancer cell invasion and the role of the microenvironment. *Curr Opin Cell Biol* (2015) 36:13–22. doi:10.1016/j.ceb.2015.06.004
16. Celià-Terrassa T, Meca-Cortés O, Mateo F, Martínez de Paz A, Rubio N, Arnal-Estapé A, et al. Epithelial-mesenchymal transition can suppress major attributes of human epithelial tumor-initiating cells. *J Clin Invest* (2012) 122:1849–68. doi:10.1172/JCI59218
17. Ocaña OH, Córcoles R, Fabra A, Moreno-Bueno G, Acloque H, Vega S, et al. Metastatic colonization requires the repression of the epithelial-mesenchymal transition inducer Prrx1. *Cancer Cell* (2012) 22:709–24. doi:10.1016/j.ccr.2012.10.012
18. Tsai JH, Donaher JL, Murphy DA, Chau S, Yang J. Spatiotemporal regulation of epithelial-mesenchymal transition is essential for squamous cell carcinoma metastasis. *Cancer Cell* (2012) 22:725–36. doi:10.1016/j.ccr.2012.09.022
19. Lambert AW, Pattabiraman DR, Weinberg RA. Emerging biological principles of metastasis. *Cell* (2017) 168:670–91. doi:10.1016/j.cell.2016.11.037
20. Fischer KR, Durrans A, Lee S, Sheng J, Li F, Wong ST, et al. Epithelial-to-mesenchymal transition is not required for lung metastasis but contributes to chemoresistance. *Nature* (2015) 527:472–6. doi:10.1038/nature15748
21. Zheng X, Carstens JL, Kim J, Scheible M, Kaye J, Sugimoto H, et al. Epithelial-to-mesenchymal transition is dispensable for metastasis but induces chemoresistance in pancreatic cancer. *Nature* (2015) 527:525–30. doi:10.1038/nature16064
22. Friedl P, Wolf K. Tumour-cell invasion and migration: diversity and escape mechanisms. *Nat Rev Cancer* (2003) 3:362–74. doi:10.1038/nrc1075
23. Luzzi KJ, MacDonald IC, Schmidt EE, Kerkvliet N, Morris VL, Chambers AF, et al. Multistep nature of metastatic inefficiency: dormancy of solitary cells after successful extravasation and limited survival of early micrometastases. *Am J Pathol* (1998) 153:865–73. doi:10.1016/S0002-9440(10)65628-3
24. Palumbo JS, Talmage KE, Massari JV, La Jeunesse CM, Flick MJ, Kombrinck KW, et al. Tumor cell-associated tissue factor and circulating hemostatic factors cooperate to increase metastatic potential through natural killer cell-dependent and-independent mechanisms. *Blood* (2007) 110:133–41. doi:10.1182/blood-2007-01-065995
25. Aceto N, Bardia A, Miyamoto DT, Donaldson MC, Wittner BS, Spencer JA, et al. Circulating tumor cell clusters are oligoclonal precursors of breast cancer metastasis. *Cell* (2014) 158:1110–22. doi:10.1016/j.cell.2014.07.013
26. Liotta LA, Saidel MG, Kleinerman J. The significance of hematogenous tumor cell clumps in the metastatic process. *Cancer Res* (1976) 36:889–94.

27. Ramakrishna R, Rostomily R. Seed, soil, and beyond: the basic biology of brain metastasis. *Surg Neurol Int* (2013) 4:S256–64. doi:10.4103/2152-7806.111303
28. Singh M, Manoranjan B, Mahendram S, McFarlane N, Venugopal C, Singh SK. Brain metastasis-initiating cells: survival of the fittest. *Int J Mol Sci* (2014) 15:9117–33. doi:10.3390/ijms15059117
29. Cheung KJ, Ewald AJ. A collective route to metastasis: seeding by tumor cell clusters. *Science* (2016) 352:167–9. doi:10.1126/science.aaf6546
30. Gundem G, Van Loo P, Kremeyer B, Alexandrov LB, Tubio JMC, Papaemmanuil E, et al. The evolutionary history of lethal metastatic prostate cancer. *Nature* (2015) 520:353–7. doi:10.1038/nature14347
31. Cheung KJ, Padmanaban V, Silvestri V, Schipper K, Cohen JD, Fairchild AN, et al. Polyclonal breast cancer metastases arise from collective dissemination of keratin 14-expressing tumor cell clusters. *Proc Natl Acad Sci U S A* (2016) 113:E854–63. doi:10.1073/pnas.1508541113
32. Maddipati R, Stanger BZ. Pancreatic cancer metastases harbor evidence of polyclonality. *Cancer Discov* (2015) 5:1086–97. doi:10.1158/2159-8290.CD-15-0120
33. Vanharanta S, Massague J. Origins of metastatic traits. *Cancer Cell* (2013) 24:410–21. doi:10.1016/j.ccr.2013.09.007
34. Barnholtz-Sloan JS, Sloan AE, Davis FG, Vigneau FD, Lai P, Sawaya RE. Incidence proportions of brain metastases in patients diagnosed (1973 to 2001) in the Metropolitan Detroit Cancer Surveillance System. *J Clin Oncol* (2004) 22:2865–72. doi:10.1200/JCO.2004.12.149
35. Mack F, Baumert BG, Schäfer N, Hattingen E, Scheffler B, Herrlinger U, et al. Therapy of leptomeningeal metastasis in solid tumors. *Cancer Treat Rev* (2016) 43:83–91. doi:10.1016/j.ctrv.2015.12.004
36. DeAngelis LM, Boutros D. Leptomeningeal metastasis. *Cancer Invest* (2005) 23:145–54. doi:10.1081/CNV-50458
37. Naxerova K, Jain RK. Using tumour phylogenetics to identify the roots of metastasis in humans. *Nat Rev Clin Oncol* (2015) 12:258–72. doi:10.1038/nrclinonc.2014.238
38. Sanborn JZ, Chung J, Purdom E, Wang NJ, Kakavand H, Wilmott JS, et al. Phylogenetic analyses of melanoma reveal complex patterns of metastatic dissemination. *Proc Natl Acad Sci U S A* (2015) 112:10995–1000. doi:10.1073/pnas.1508074112
39. Krøigård AB, Larsen MJ, Brasch-Andersen C, Lænkholm AV, Knoop AS, Jensen JD, et al. Genomic analyses of breast cancer progression reveal distinct routes of metastasis emergence. *Sci Rep* (2017) 7:43813. doi:10.1038/srep43813
40. Brown D, Smeets D, Székely B, Larsimont D, Szász AM, Adnet PY, et al. Phylogenetic analysis of metastatic progression in breast cancer using somatic mutations and copy number aberrations. *Nat Commun* (2017) 8:14944. doi:10.1038/ncomms14944
41. Budczies J, von Winterfeld M, Klauschen F, Bockmayr M, Lennerz JK, Denkert C, et al. The landscape of metastatic progression patterns across major human cancers. *Oncotarget* (2015) 6:570–83. doi:10.18632/oncotarget.2677
42. Klein CA. Parallel progression of primary tumours and metastases. *Nat Rev Cancer* (2009) 9:302–12. doi:10.1038/nrc2627
43. DeCicco-Skinner KL, Henry GH, Cataisson C, Tabib T, Gwilliam JC, Watson NJ, et al. Endothelial cell tube formation assay for the in vitro study of angiogenesis. *J Vis Exp* (2014) 91:e51312. doi:10.3791/51312
44. Huszthy PC, Daphu I, Niclou SP, Stieber D, Nigro JM, Sakariassen PØ, et al. In vivo models of primary brain tumors: pitfalls and perspectives. *Neuro Oncol* (2012) 14:979–93. doi:10.1093/neuonc/nos135
45. Sandén E, Dyberg C, Krona C, Gallo-Oller G, Olsen TK, Enríquez Pérez J, et al. Establishment and characterization of an orthotopic patient-derived group 3 medulloblastoma model for preclinical drug evaluation. *Sci Rep* (2017) 7:46366. doi:10.1038/srep46366
46. Joo KM, Kim J, Jin J, Kim M, Seol HJ, Muradov J, et al. Patient-specific orthotopic glioblastoma xenograft models recapitulate the histopathology and biology of human glioblastomas in situ. *Cell Rep* (2013) 3:260–73. doi:10.1016/j.celrep.2012.12.013
47. Wakimoto H, Mohapatra G, Kanai R, Curry WT Jr, Yip S, Nitta M, et al. Maintenance of primary tumor phenotype and genotype in glioblastoma stem cells. *Neuro Oncol* (2012) 14:132–44. doi:10.1093/neuonc/nor195
48. Zhao X, Liu Z, Yu L, Zhang Y, Baxter P, Voicu H, et al. Global gene expression profiling confirms the molecular fidelity of primary tumor-based orthotopic xenograft mouse models of medulloblastoma. *Neuro Oncol* (2012) 14:574–83. doi:10.1093/neuonc/nos061
49. Yu L, Baxter PA, Voicu H, Gurusiddappa S, Zhao Y, Adesina A, et al. A clinically relevant orthotopic xenograft model of ependymoma that maintains the genomic signature of the primary tumor and preserves cancer stem cells in vivo. *Neuro Oncol* (2010) 12:580–94. doi:10.1093/neuonc/nop056
50. Shu Q, Wong KK, Su JM, Adesina AM, Yu LT, Tsang YT, et al. Direct orthotopic transplantation of fresh surgical specimen preserves CD133+ tumor cells in clinically relevant mouse models of medulloblastoma and glioma. *Stem Cells* (2008) 26:1414–24. doi:10.1634/stemcells.2007-1009
51. Singh SK, Hawkins C, Clarke ID, Squire JA, Bayani J, Hide T, et al. Identification of human brain tumour initiating cells. *Nature* (2004) 432:396–401. doi:10.1038/nature03128
52. Hoffman RM. Patient-derived orthotopic xenografts: better mimic of metastasis than subcutaneous xenografts. *Nat Rev Cancer* (2015) 15:451–2. doi:10.1038/nrc3972
53. Talmadge JE, Singh RK, Fidler IJ, Raz A. Murine models to evaluate novel and conventional therapeutic strategies for cancer. *Am J Pathol* (2007) 170:793–804. doi:10.2353/ajpath.2007.060929
54. Lokman NA, Elder AS, Ricciardelli C, Oehler MK. Chick chorioallantoic membrane (CAM) assay as an in vivo model to study the effect of newly identified molecules on ovarian cancer invasion and metastasis. *Int J Mol Sci* (2012) 13:9959–70. doi:10.3390/ijms13089959
55. Palmer TD, Lewis J, Zijlstra A. Quantitative analysis of cancer metastasis using an avian embryo model. *J Vis Exp* (2011) (51):e2815. doi:10.3791/2815
56. Wilson SM, Chambers AF. Experimental metastasis assays in the chick embryo. *Curr Protoc Cell Biol* (2004) 19:19.16. doi:10.1002/0471143030.cb1906s21
57. Brown HK, Schiavone K, Tazzyman S, Heymann D, Chico TJ. Zebrafish xenograft models of cancer and metastasis for drug discovery. *Expert Opin Drug Discov* (2017) 12:379–89. doi:10.1080/17460441.2017.1297416
58. Liu C, Zhang Y, Lim S, Hosaka K, Yang Y, Pavlova T, et al. A zebrafish model discovers a novel mechanism of stromal fibroblast-mediated cancer metastasis. *Clin Cancer Res* (2017) 23(16):4769–79. doi:10.1158/1078-0432.CCR-17-0101
59. Schabet M, Herrlinger U. Animal models of leptomeningeal metastasis. *J Neurooncol* (1998) 38:199–205. doi:10.1023/A:1005936304256
60. Shultz LD, Ishikawa F, Greiner DL. Humanized mice in translational biomedical research. *Nat Rev Immunol* (2007) 7:118–30. doi:10.1038/nri2017
61. Hughes P, Marshall D, Reid Y, Parkes H, Gelber C. The costs of using unauthenticated, over-passaged cell lines: how much more data do we need? *Biotechniques* (2007) 43:575, 577–8, 581–572 passim. doi:10.2144/000112598
62. Francia G, Cruz-Munoz W, Man S, Xu P, Kerbel RS. Mouse models of advanced spontaneous metastasis for experimental therapeutics. *Nat Rev Cancer* (2011) 11:135–41. doi:10.1038/nrc3001
63. Bos PD, Zhang XH, Nadal C, Shu W, Gomis RR, Nguyen DX, et al. Genes that mediate breast cancer metastasis to the brain. *Nature* (2009) 459:1005–9. doi:10.1038/nature08021
64. Fidler IJ. Metastasis: quantitative analysis of distribution and fate of tumor embolilabeled with 125 I-5-iodo-2'-deoxyuridine. *J Natl Cancer Inst* (1970) 45:773–82.
65. Song HT, Jordan EK, Lewis BK, Liu W, Ganjei J, Klaunberg B, et al. Rat model of metastatic breast cancer monitored by MRI at 3 tesla and bioluminescence imaging with histological correlation. *J Transl Med* (2009) 7:88. doi:10.1186/1479-5876-7-88
66. Kircher DA, Silvis MR, Cho JH, Holmen SL. Melanoma brain metastasis: mechanisms, models, and medicine. *Int J Mol Sci* (2016) 17:E1468. doi:10.3390/ijms17091468
67. Daphu I, Sundstrøm T, Horn S, Huszthy PC, Niclou SP, Sakariassen PØ, et al. In vivo animal models for studying brain metastasis: value and limitations. *Clin Exp Metastasis* (2013) 30:695–710. doi:10.1007/s10585-013-9566-9
68. Martinez-Aranda A, Hernandez V, Picon C, Modolell I, Sierra A. Development of a preclinical therapeutic model of human brain metastasis with chemoradiotherapy. *Int J Mol Sci* (2013) 14:8306–27. doi:10.3390/ijms14048306
69. Zhang Z, Hatori T, Nonaka H. An experimental model of brain metastasis of lung carcinoma. *Neuropathology* (2008) 28:24–8. doi:10.1111/j.1440-1789.2007.00826.x
70. Sakamoto S, Inoue H, Ohba S, Kohda Y, Usami I, Masuda T, et al. New metastatic model of human small-cell lung cancer by orthotopic transplantation in mice. *Cancer Sci* (2015) 106:367–74. doi:10.1111/cas.12624

71. Cruz-Munoz W, Man S, Xu P, Kerbel RS. Development of a preclinical model of spontaneous human melanoma central nervous system metastasis. *Cancer Res* (2008) 68:4500–5. doi:10.1158/0008-5472.CAN-08-0041
72. Marsden CG, Wright MJ, Carrier L, Moroz K, Pochampally R, Rowan BG. A novel in vivo model for the study of human breast cancer metastasis using primary breast tumor-initiating cells from patient biopsies. *BMC Cancer* (2012) 12:10. doi:10.1186/1471-2407-12-10
73. Choi SA, Kwak PA, Kim SK, Park SH, Lee JY, Wang KC, et al. In vivo bioluminescence imaging for leptomeningeal dissemination of medulloblastoma in mouse models. *BMC Cancer* (2016) 16:723. doi:10.1186/s12885-016-2742-y
74. Reijneveld JC, Taphoorn MJ, Voest EE. A simple mouse model for leptomeningeal metastases and repeated intrathecal therapy. *J Neurooncol* (1999) 42:137–42. doi:10.1023/A:1006237917632
75. Janczewski KH, Chalk CL, Pyles RB, Parysek LM, Unger LW, Warnick RE. A simple, reproducible technique for establishing leptomeningeal tumors in nude rats. *J Neurosci Methods* (1998) 85:45–9. doi:10.1016/S0165-0270(98)00115-0
76. Boire A, DeAngelis L, Massagué J. Development of a mouse model of leptomeningeal metastasis (P7.014). *Neurology* (2014) 82:7.014. doi:10.1093/neuonc/nou240.6
77. Boire A, Zou Y, Shieh J, Macalinao DG, Pentsova E, Massagué J. Complement component 3 adapts the cerebrospinal fluid for leptomeningeal metastasis. *Cell* (2017) 168:1101–13.e1113. doi:10.1016/j.cell.2017.02.025
78. Singh M, Venugopal C, Tokar T, Brown KR, McFarlane N, Bakhshinyan D, et al. RNAi screen identifies essential regulators of human brain metastasis-initiating cells. *Acta Neuropathol* (2017). doi:10.1007/s00401-017-1757-z
79. Eberth C. Zur entwick pung des ephlheliomas (cholesteatomas) der pia and der lung. *Virchow S Arch* (1870) 49:51–63. doi:10.1007/BF02214196
80. Cantillo R, Jain J, Singhakowinta A, Vaitkevicius VK. Blindness as initial manifestation of meningeal carcinomatosis in breast cancer. *Cancer* (1979) 44:755–7. doi:10.1002/1097-0142(197908)44:2<755::AID-CNCR2820440249>3.0.CO;2-E
81. Ahluwalia MS, Vogelbaum MV, Chao ST, Mehta MM. Brain metastasis and treatment. *F1000Prime Rep* (2014) 6:114. doi:10.12703/P6-114
82. Franciosi V, Cocconi G, Michiara M, Di Costanzo F, Fosser V, Tonato M, et al. Front-line chemotherapy with cisplatin and etoposide for patients with brain metastases from breast carcinoma, nonsmall cell lung carcinoma, or malignant melanoma: a prospective study. *Cancer* (1999) 85:1599–605. doi:10.1002/(SICI)1097-0142(19990401)85:7<1599::AID-CNCR23>3.3.CO;2-R
83. Rosner D, Nemoto T, Lane WW. Chemotherapy induces regression of brain metastases in breast carcinoma. *Cancer* (1986) 58:832–9. doi:10.1002/1097-0142(19860815)58:4<832::AID-CNCR2820580404>3.0.CO;2-W
84. Sevinc A, Kalender ME, Altinbas M, Ozkan M, Dikilitas M, Camci C, et al. Irinotecan as a second-line monotherapy for small cell lung cancer. *Asian Pac J Cancer Prev* (2011) 12:1055–9. doi:10.4172/2476-2253.1000102
85. Zhao KH, Zhang C, Bai Y, Li Y, Kang X, Chen JX, et al. Antiglioma effects of cytarabine on leptomeningeal metastasis of high-grade glioma by targeting the PI3K/Akt/mTOR pathway. *Drug Des Devel Ther* (2017) 11:1905–15. doi:10.2147/DDDT.S135711
86. Niwińska A, Rudnicka H, Murawska M. Breast cancer leptomeningeal metastasis: the results of combined treatment and the comparison of methotrexate and liposomal cytarabine as intra-cerebrospinal fluid chemotherapy. *Clin Breast Cancer* (2015) 15:66–72. doi:10.1016/j.clbc.2014.07.004
87. Nagpal S, Riess J, Wakelee H. Treatment of leptomeningeal spread of NSCLC: a continuing challenge. *Curr Treat Options Oncol* (2012) 13:491–504. doi:10.1007/s11864-012-0206-4
88. Petrelli F, Ghidini M, Lonati V, Tomasello G, Borgonovo K, Ghilardi M, et al. The efficacy of lapatinib and capecitabine in HER-2 positive breast cancer with brain metastases: a systematic review and pooled analysis. *Eur J Cancer* (2017) 84:141–8. doi:10.1016/j.ejca.2017.07.024
89. Bachelot T, Romieu G, Campone M, Diéras V, Cropet C, Dalenc F, et al. Lapatinib plus capecitabine in patients with previously untreated brain metastases from HER2-positive metastatic breast cancer (LANDSCAPE): a single-group phase 2 study. *Lancet Oncol* (2013) 14:64–71. doi:10.1016/S1470-2045(12)70432-1
90. Ceresoli GL, Cappuzzo F, Gregorc V, Bartolini S, Crinò L, Villa E. Gefitinib in patients with brain metastases from non-small-cell lung cancer: a prospective trial. *Ann Oncol* (2004) 15:1042–7. doi:10.1093/annonc/mdh276
91. Koba T, Kijima T, Takimoto T, Hirata H, Naito Y, Hamaguchi M, et al. Rapid intracranial response to osimertinib, without radiotherapy, in nonsmall cell lung cancer patients harboring the EGFR T790M mutation: two case reports. *Medicine (Baltimore)* (2017) 96:e6087. doi:10.1097/MD.0000000000006087
92. Sperduto PW, Wang M, Robins HI, Schell MC, Werner-Wasik M, Komaki R, et al. A phase 3 trial of whole brain radiation therapy and stereotactic radiosurgery alone versus WBRT and SRS with temozolomide or erlotinib for non-small cell lung cancer and 1 to 3 brain metastases: Radiation Therapy Oncology Group 0320. *Int J Radiat Oncol Biol Phys* (2013) 85:1312–18. doi:10.1016/j.ijrobp.2012.11.042
93. Lin NU, Carey LA, Liu MC, Younger J, Come SE, Ewend M, et al. Phase II trial of lapatinib for brain metastases in patients with human epidermal growth factor receptor 2-positive breast cancer. *J Clin Oncol* (2008) 26:1993–9. doi:10.1200/JCO.2007.12.3588
94. Lin NU, Diéras V, Paul D, Lossignol D, Christodoulou C, Stemmler HJ, et al. Multicenter phase II study of lapatinib in patients with brain metastases from HER2-positive breast cancer. *Clin Cancer Res* (2009) 15:1452–9. doi:10.1158/1078-0432.CCR-08-1080
95. Dummer R, Goldinger SM, Turtschi CP, Eggmann NB, Michielin O, Mitchell L, et al. Vemurafenib in patients with BRAF(V600) mutation-positive melanoma with symptomatic brain metastases: final results of an open-label pilot study. *Eur J Cancer* (2014) 50:611–21. doi:10.1016/j.ejca.2013.11.002
96. Long GV, Trefzer U, Davies MA, Kefford RF, Ascierto PA, Chapman PB, et al. Dabrafenib in patients with Val600Glu or Val600Lys BRAF-mutant melanoma metastatic to the brain (BREAK-MB): a multicentre, open-label, phase 2 trial. *Lancet Oncol* (2012) 13:1087–95. doi:10.1016/S1470-2045(12)70431-X
97. Yoshida T, Oya Y, Tanaka K, Shimizu J, Horio Y, Kuroda H, et al. Clinical impact of crizotinib on central nervous system progression in ALK-positive non-small lung cancer. *Lung Cancer* (2016) 97:43–7. doi:10.1016/j.lungcan.2016.04.006
98. Melosky B, Agulnik J, Albadine R, Banerji S, Bebb DG, Bethune D, et al. Canadian consensus: inhibition of ALK-positive tumours in advanced non-small-cell lung cancer. *Curr Oncol* (2016) 23:196–200. doi:10.3747/co.23.3120
99. Gadgeel SM, Gandhi L, Riely GJ, Chiappori AA, West HL, Azada MC, et al. Safety and activity of alectinib against systemic disease and brain metastases in patients with crizotinib-resistant ALK-rearranged non-small-cell lung cancer (AF-002JG): results from the dose-finding portion of a phase 1/2 study. *Lancet Oncol* (2014) 15:1119–28. doi:10.1016/S1470-2045(14)70362-6
100. Besse B, Le Moulec S, Mazières J, Senellart H, Barlesi F, Chouaid C, et al. Bevacizumab in patients with nonsquamous non-small cell lung cancer and asymptomatic, untreated brain metastases (BRAIN): a nonrandomized, phase II study. *Clin Cancer Res* (2015) 21:1896–903. doi:10.1158/1078-0432.CCR-14-2082
101. Schulz H, Pels H, Schmidt-Wolf I, Zeelen U, Germing U, Engert A. Intraventricular treatment of relapsed central nervous system lymphoma with the anti-CD20 antibody rituximab. *Haematologica* (2004) 89:753–4.
102. Park YH, Park MJ, Ji SH, Yi SY, Lim DH, Nam DH, et al. Trastuzumab treatment improves brain metastasis outcomes through control and durable prolongation of systemic extracranial disease in HER2-overexpressing breast cancer patients. *Br J Cancer* (2009) 100:894–900. doi:10.1038/sj.bjc.6604941
103. Margolin K, Ernstoff MS, Hamid O, Lawrence D, McDermott D, Puzanov I, et al. Ipilimumab in patients with melanoma and brain metastases: an open-label, phase 2 trial. *Lancet Oncol* (2012) 13:459–65. doi:10.1016/S1470-2045(12)70090-6
104. Dudnik E, Yust-Katz S, Nechushtan H, Goldstein DA, Zer A, Flex D, et al. Intracranial response to nivolumab in NSCLC patients with untreated or progressing CNS metastases. *Lung Cancer* (2016) 98:114–7. doi:10.1016/j.lungcan.2016.05.031
105. Goldberg SB, Gettinger SN, Mahajan A, Chiang AC, Herbst RS, Sznol M, 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. *Lancet Oncol* (2016) 17:976–83. doi:10.1016/S1470-2045(16)30053-5
106. Walko CM, Lindley C. Capecitabine: a review. *Clin Ther* (2005) 27:23–44. doi:10.1016/j.clinthera.2005.01.005

107. Zhu W, Zhou L, Qian JQ, Qiu TZ, Shu YQ, Liu P. Temozolomide for treatment of brain metastases: a review of 21 clinical trials. *World J Clin Oncol* (2014) 5:19–27. doi:10.5306/wjco.v5.i1.19
108. Gleissner B, Chamberlain MC. Neoplastic meningitis. *Lancet Neurol* (2006) 5:443–52. doi:10.1016/S1474-4422(06)70443-4
109. Shih T, Lindley C. Bevacizumab: an angiogenesis inhibitor for the treatment of solid malignancies. *Clin Ther* (2006) 28:1779–802. doi:10.1016/j.clinthera.2006.11.015
110. Johanns T, Waqar SN, Morgensztern D. Immune checkpoint inhibition in patients with brain metastases. *Ann Transl Med* (2016) 4:S9. doi:10.21037/atm.2016.09.40
111. Farber SH, Tsvankin V, Narloch JL, Kim GJ, Salama AK, Vlahovic G, et al. Embracing rejection: immunologic trends in brain metastasis. *Oncoimmunology* (2016) 5:e1172153. doi:10.1080/2162402X.2016.1172153
112. Wu X, Northcott PA, Dubuc A, Dupuy AJ, Shih DJ, Witt H, et al. Clonal selection drives genetic divergence of metastatic medulloblastoma. *Nature* (2012) 482:529–33. doi:10.1038/nature10825
113. Morrissy AS, Garzia L, Shih DJ, Zuyderduyn S, Huang X, Skowron P, et al. Divergent clonal selection dominates medulloblastoma at recurrence. *Nature* (2016) 529:351–7. doi:10.1038/nature16478
114. Redzic Z. Molecular biology of the blood-brain and the blood-cerebrospinal fluid barriers: similarities and differences. *Fluids Barriers CNS* (2011) 8:3. doi:10.1186/2045-8118-8-3
115. Banks WA. Characteristics of compounds that cross the blood-brain barrier. *BMC Neurol* (2009) 9(Suppl 1):S3. doi:10.1186/1471-2377-9-S1-S3
116. Oldendorf WH. Lipid solubility and drug penetration of the blood brain barrier. *Proc Soc Exp Biol Med* (1974) 147:813–5. doi:10.3181/00379727-147-38444
117. Fauré J, Lachenal G, Court M, Hirrlinger J, Chatellard-Causse C, Blot B, et al. Exosomes are released by cultured cortical neurones. *Mol Cell Neurosci* (2006) 31:642–8. doi:10.1016/j.mcn.2005.12.003
118. Alvarez-Erviti L, Seow Y, Yin H, Betts C, Lakhal S, Wood MJ. Delivery of siRNA to the mouse brain by systemic injection of targeted exosomes. *Nat Biotechnol* (2011) 29:341–5. doi:10.1038/nbt.1807

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