VIEWPOINT

Brain metastasis

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Abstract [Au: Although Viewpoint abstracts are written by the Editor, please feel free to edit if necessary for scientific precision/clarity.]

Brain metastasis, which commonly arises in patients with lung cancer, breast cancer and melanoma, is associated with poor survival outcomes and poses distinct clinical challenges. The brain microenvironment, with its unique cell types, anatomical structures, metabolic constraints and immune environment, differs drastically from microenvironments of extracranial lesions, imposing a distinct and profound selective pressure on tumour cells that, in turn, shapes the metastatic process and therapeutic responses. Accordingly, the study of brain metastasis could uncover new therapeutic targets and identify novel treatment approaches to address the unmet clinical need. Moreover, such efforts could provide insight into the biology of primary brain tumours, which face similar challenges to brain metastases of extracranial origin, and vice versa. However, the paucity of robust preclinical models of brain metastasis has severely limited such investigations, underscoring the importance of developing improved experimental models that holistically encompass the metastatic cascade and/or brain microenvironment. In this Viewpoint, we asked four leading experts to provide their opinions on these important aspects of brain metastasis biology and management.

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How does the unique brain microenvironment influence brain metastases?

Adrienne Boire. Cancer represents the pathological 'dark side' of evolutionary biology. A cancer cell is, by definition, genetically unstable, which generates a dynamic and heterogeneous state that bestows the tumour cell with near-infinite adaptability to selective pressure. The microenvironment is the most important source of selective pressure in the system, sculpting the tumour's population dynamics. The clinical problem of brain metastasis brings these large concepts into sharp relief **[Au: We are unclear on your meaning of 'sharp relief' here (we suspect this will also be unclear to some readers) – is there an alternative phrase?]** — the brain is wholly unlike any other site of tumour growth in the body and, as such, asserts profound selective pressure on cancer cells.

The brain actually comprises two main microenvironments that differ in their composition — the densely cellular parenchyma and the cerebrospinal fluid (CSF)-filled leptomeninges **[Au: Please reference this statement (this will also cover the ensuing discussion on the two microenvironments). A Review would be ideal here.]** . **[Au: Edit OK?]** The brain parenchyma contains cell types that exist nowhere else in the body, such as astrocytes, microglia, oligodendrocytes and neurons, some of which are electrically active and exist within a vast connectome **[Au: Combined to improve flow, OK?]** . Thus, interactions between cancer cells and these brain-specific cell types are unique to brain metastases. In the case of the leptomeninges, the pia and arachnoid contain circulating CSF generated by the choroid plexi, which are structures unique to the brain.

Beyond these anatomical differences, the brain parenchyma and leptomeninges are markedly distinct from the rest of the body at the metabolic level **[Au: Please reference this statement (preferably the same reference request above).]**. In the case of the brain parenchyma, neurons take up the majority of oxygen and effectively 'outsource' a number of metabolic tasks to the liver, relying on glucose or ketones to supply sufficient fuel for oxidative metabolism. By contrast, the leptomeninges contain the circulating CSF, which is markedly hypoxic with low concentrations of **[Au:OK?]** metabolic intermediates and micronutrients. As cancer cells are quite metabolically active, they must 'solve' these metabolic problems to divide and grow. These metabolic constraints are also unique to brain metastases.

These parenchymal and leptomeningeal microenvironments are sufficiently different such that they select for distinct characteristics in the metastatic cancer cells that inhabit these

spaces[1]. Importantly, these two compartments are anatomically distinct from each other and from the systemic circulation, suggesting that cancer cells within each of these sites possess a distinct array of adaptations and, therefore, vulnerabilities.

Priscilla Brastianos. When metastatic tumour cells arrive **[Au:OK?]** in the brain, they encounter a remarkably complex microenvironment that differs from the niche of the primary site. Intracranial metastases need to adapt to this unique milieu to grow effectively. The brain itself consists of a range of cellular constituents, including neurons, astrocytes and microglia, which are not present in the extracranial organs from which the primary cancer originated. Consequently, the metabolic environment in the brain also varies considerably from the primary tumour site; for example, compelling data suggest that breast cancer cells adapt to this environment by adopting 'brain-like' properties, specifically a γ-aminobutyric acid (GABA)-ergic phenotype similar to neuronal cells, when they metastasize to the brain[1]. **[Au: Edit OK?]**

The immune environment is also vastly different in the brain compared with that of other extracranial sites. Historically, the brain has been considered an immune-privileged organ site, whereby immune cell access from the periphery is limited by the blood–brain barrier (BBB). However, data has emerged to suggest that, although the brain might be an immune sanctuary, blood-borne immune cells do in fact enter the brain, especially in the setting of brain metastases **[Au: Edit OK?]** . Indeed, the presence of tumour-infiltrating T lymphocytes has now been confirmed in brain metastases from patients with lung cancer, breast cancer, melanoma and renal cell carcinoma[2] **[Au: Edit OK?]** . Brain metastases also phenotypically adapt to the immune microenvironment. Notably, expression of cathepsin S, a protease that is usually expressed by leukocytes, was found to be expressed in brain metastases in patients with **[Au:OK?]** breast cancer, with preclinical experiments revealing that cathepsin S mediates the transmigration of breast cancer cells through the BBB[3]. **[Au: Combined sentences to clarify these observations were from the same study (and in patients versus in vivo experiments)]**

Crucially, seminal papers have demonstrated that modulation of the immune system with systemic administration of combined immune checkpoint blockade leads to a clinically meaningful benefit in ~50% of patients with melanoma that has metastasized to the brain[4,5]. Nevertheless, given that ~50% of treated patients do not benefit from combined immune checkpoint blockade **[Au: Clarified combined ICB (nivolumab + ipilimumab), rather than**

monotherapy, to improve link to previous sentence. OK?] , additional studies are needed to investigate the best approach for modulating the immune system for the optimal treatment of brain metastases.

Livia Garzia. Metastases involving the central nervous system (CNS) can be divided into parenchymal, leptomeningeal and epidural (lesions in the vertebral column **[Au: Clarified epidural met here, as parenchymal and leptomeningeal were defined above in A. Boire's response, OK?]**) metastases. These microenvironments all offer distinct challenges and opportunities to circulating tumour cells (CTCs) that travel through the blood stream, the brain lymphatics or the CSF. The best studied microenvironment is that of parenchymal brain metastases derived from adult metastatic cancers, such as lung cancer, breast cancer and melanoma **[Au:OK?]** .

[Au: Paragraph break to improve flow, OK?] The BBB and the blood–CSF barrier (BCSFB) are the gatekeepers of the CNS, protecting the brain from the potentially lethal consequences of massive inflammation (brain oedema) **[Au: Please reference this statement. (also to broadly cover the ensuing BBB and BCSFB discussion)]** . Unique in the body, the tight junctions that seal brain capillaries — comprised of endothelial cells (BBB) or specialized epithelial and leptomeningeal cells (BCSFB) — render the brain virtually impermeable to hydrophilic solutes **[Au: Edit OK? Have I retained your meaning?]** . In addition to the specialized endothelial cells, the BBB is composed of pericytes and astrocytes, as well as other cell types such as resident immune and non-immune cells (microglia and stem cells), which regulate its functions **[Au: Edit OK?]** . The BBB is a selective barrier in that it is only relatively impermeable to solutes and cells at homeostasis; however, when compromised, as occurs in the presence of a brain tumour, it can become pronouncedly leaky. Nonetheless, even a leaky BBB can still shield CTCs that have extravasated and established micrometastases from the action of chemotherapeutics or biologics.

[Au: Paragraph break to improve flow, OK?] Endothelial cells in the BBB are subject to preferential fusion with circulating exosomes generated by cancers with brain-specific tropism, and the integrins that characterize these brain-specific exosomes could be the targets of a brain metastasis prevention strategy (Hoshino et al., 2015) **[Au: Please add Hoshino et al. to the reference list.] [Au: The functional effect(s) of endothelial cell–exosome fusion is unclear and has not been stated — is this related to remodelling the BBB endothelial cells**

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Commented [A2]: To be honest in this cited paper the place in which the finding seems to fail was the brain. I would suggest to avoid presenting this possibility as that at the most, since it is very far from being demonstrated.

to enable extravasation into the brain during metastasis? If so, perhaps this statement can be deleted, as it is repeated in the next sentence.] . The mechanism that CTCs adopt to cross the BBB and BCSFB are similar to that of leukocyte transendothelial migration, but once they access areas permissive to blood flow, it is crucial that tumour cells remain in contact long enough to remodel endothelial cells for blood vessel escape (diapedesis) **[Au:OK?]** . In fact, brain areas with a low blood **[Au:OK?]** flow rate are emerging as preferred sites of parenchymal metastasis[1,2]. Solitary CTCs that cross the BBB are then challenged to transition from micrometastases to macrometastases. In vivo studies have shown that different cancers, such as non-small-cell lung cancer (NSCLC) or melanoma, display intrinsic differences in the steps required to transition to brain macrometastases **[Au: Please reference this statement.]** . NSCLC cells are subject to cell death and regression at the micrometastatic stage, whereas melanoma cells that reach the micrometastatic stage in a favourable position have a high likelihood of progression to a macrometastasis. Importantly, within the same cancer type, differences have been highlighted in the reciprocal **[Au: What is the meaning of reciprocal' here? Does it mean 'common' rather than 'shared'? (my understanding is that tumours tend to use one over the other of these mechanisms to obtain a blood supply).]** occurrence of vascular co-option and neoangiogenesis, which are more prevalent in parenchymal metastases and leptomeningeal masses, respectively[3]. This finding suggests that the subarachnoid space and the brain parenchyma are not equal soils, even to the same cancer type **[Au: Edit OK?]** .

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Together with the specialized endothelial cells of brain capillaries, astrocytes also are a brain-specific cell type that shape the niche of parenchymal brain metastases. Reactive astrocytes, which first come in contact with tumour cells following extravasation, produce plasmin as a defense against metastatic invasion **[Au: Edit for clarity, OK?]** . This process selects for tumour cells expressing proteins that dampen the activation of proteases, including serine protease inhibitors such as serpins, allowing tumour cells to survive despite plasmin production[4] **[Au: Edit OK?]** . Reactive astrocytes are also present and infiltrate the progressing brain metastases at the periphery of the lesion **[Au:OK?]** , where they influence metastatic progression by relaxing endothelial cell junctions through cytokine secretion and regulate tumour cell gene expression via exosomes or gap junctions[5,6]. Reactive astrocytes at the periphery of metastases also regulate innate and adaptive immune cells, inducing a switch from a metastasis-hostile brain parenchymal microenvironment to a cancer-promoting one[7].

Microglia are resident immune cells of the brain and, together with bone marrow-derived macrophages, constitute the major fraction of the immune microenvironment of brain metastases. Microglia can switch between resting and activated phenotypes in response to **[Au:OK?]** disease or injury; for example, activated microglia can repair the transient damage of the BBB that occurs during cancer cell transmigration **[Au:OK?]** , contributing to shielding of newly formed metastases from systemically administered drugs[8]. When macrometastases are established, microglia and macrophages loose phagocytic activity and adopt an indirect tumour-supportive and anti-inflammatory role through increased anti-inflammatory cytokine production, increased recruitment of peripheral monocytes and inhibition of T-cell proliferation. Furthermore, metastasis-associated microglia and macrophages also act directly on tumour cells through secretion of growth factors that enhance cell proliferation, such as transforming growth factor β (TGF-β), interleukin 6 (IL-6) and epidermal growth factor (EGF). The common consensus is that neurons in the brain are passive bystanders of metastatic colonization, bearing the consequences of astrocyte, microglial and macrophage activity in the tumour microenvironment and being subject to irreparable damage and extensive cell death **[Au: Please reference this statement.]** . Although the support for this hypothesis is certainly strong given that neural damage contributes to the vast majority of morbidity in patients with brain metastasis **[Au: Edit OK?]** , recent advances in our understanding of the interplay between neurons and primary brain cancers, with neural activity fuelling high-grade glioma progression[9], warrant a thorough investigation of the role of neural activity in brain metastasis. Breast cancer cells, in their path to adapt to the brain microenvironment, express GABAergic genes and use GABA and glutamate as oncometabolites[10].

Very little is known about the microenvironment of leptomeningeal metastasis. The BCSFB, constituted by the choroid plexus and the arachnoid, has a different structure than the BBB. The BCSFB capillaries are fenestrated, and solutes and cells freely access the choroid plexus stroma, but are then stopped from passively accessing the subarachnoid space and the CSF by epithelial cells connected by tight junctions, which constitute the second and impermeable layer of the choroid plexus. Breast and lung cancer are the two major contributors to leptomeningeal metastasis, but all cancers can spread to this compartment, albeit less frequently. The CSF is particularly devoid of nutrients and growth factors; for example, glucose, lipid and amino acid concentrations are ~10-fold lower than in blood

[Au:OK?] **[Au: Please reference this statement.]** . The CSF is not merely a 'thinned' ultrafiltrate of the blood — several substances, including insulin-like growth factor 1 (IGF-1), IGF-2, TGFβ1, IL-13, bone morphogenetic protein 6 (BMP6) and BMP7, are actively secreted into the CSF by the choroid plexus **[Au: Edit OK?]** . Global studies of the CSF metabolome before and during progression of brain metastasis and, more importantly, of leptomeningeal metastasis, are in their infancy[11], but it is conceivable that massive adaptation to growth in suspension and in the absence of the sheltering perivascular niche must occur for tumour cells to successfully colonize the subarachnoid space. Leptomeningeal-tropic breast cancer cells do educate their subarachnoid microenvironment by secreting complement component 3 (C3), which, in turn, opens the tight junctions of the epithelial cells of the BCSFB, allowing growth factors to enter the CSF and support metastatic growth **[Au: Please reference this statement.]** . Both the lack of primary human samples for analysis and the inaccessibility of the choroid plexus to in vivo time-lapse imaging has largely hampered our understanding of the initial phases of leptomeningeal colonization, but studies on leptomeningeal metastasis of primary brain tumours have revealed a major role for macrophages in supporting tumour cell proliferation in this compartment[12].

Manuel Valiente. The BBB is an initial and key filter to prevent invasion of metastatic cells into the brain, but it is not the only one. Extracranial **[Au:OK?]** [Yes] organs targeted by metastases share a relatively similar cellular composition with the organ in which the primary tumour originated, inferring a relatively simple process for metastasis-initiating cells to regrow the tumour[1]. By contrast, **[Au:OK?]** [Yes] most metastatic cells reaching the brain are not adapted to this microenvironment and, consequently, perish or remain unable to regrow the tumour[2]. The huge selective pressure imposed by the microenvironment is a major contributor in defining which of the initial clones seeding the brain will later succeed during organ colonization and give rise to clinically relevant macrometastases[3,4]. Those rare cells that are equipped with the appropriate 'tools', or that acquire these tools at the moment they are exposed to the brain parenchyma, will survive. However, it is important to note that surviving the action of natural brain defences during the initial moments of colonization does not directly involve the ability to grow in the brain. Surviving metastatic cells might need to evolve in situ to take advantage of specific brain resources that allow them to resume full growth capacity. The process by which cancer cells adapt to this highly specialized microenvironment

might involve the selection of additional genomic and epigenomic modifications ~~changes that will become incorporated by the metastasis~~ **[Au: Please clarify 'that will become incorporated' — do you mean 'that are essential for growth of the metastasis'?]** [We can avoid the controversial part]. Given that the nature of the selective **[Au:OK?]** [Yes] pressure acting on metastatic cells responds to the unique milieu of brain-resident cells, the mechanisms that cancer cells use to overcome this pressure and regrow the tumour in the brain would be, by definition, different from those required at the primary tumour site or even at extracranial metastatic sites.

Consequently, the brain microenvironment is a central aspect that dictates the development of brain metastases **[Au: Edit OK?]** [drives means that it always promote colonization, however it could also inhibit]. Thus, dissecting the mechanisms required for tumour cells to cross the BBB, block natural brain defences and interact with different components of the brain microenvironment will improve our understanding of key aspects of secondary brain tumour biology, including the specific molecular strategies driving the process of adaptation.

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In what ways might studying brain metastases provide insights into primary brain tumours?

A.B. Primary cancer cells face very similar challenges to their metastatic counterparts — both cell types must face enormous metabolic constraints, evade resident and invading immune cells and continue to grow within the closed CNS **[Au:OK?]** . Remarkably, this rather simple observation has enabled the discovery of conserved molecular processes between primary and metastatic brain tumours. For example, the gap junctions originally observed between metastatic cancer cells and astrocytes[2] have been observed between glioblastoma cells and astrocytes[3] and between diffuse intrinsic pontine glioma cells and astrocytes[4].

A second example is the vasculature supplying the tumour. Studies in both metastatic[5,6] and primary brain tumours[7] suggest that the interactions between cancer cells and blood vessels within the brain are highly heterogeneous, and do not constitute a normal BBB, but rather a blood–tumour barrier with its own regulatory system. In both of these examples, shared molecular mechanisms between primary and metastatic brain tumours suggest that certain therapeutic approaches might be applicable to both tumour types. **[Au: Edit OK?]**

P.B. The rapid advances made in genomic technologies and the recent surge in comprehensive genomic studies have demonstrated that many molecular pathways driving primary brain tumours are also frequently altered in brain metastases. For this reason, the development of therapeutic agents **[Au:OK? (agents could also theoretically be agonists, depending on the target/approach)]** with substantial activity in brain metastases might lead to improved therapies for primary brain tumours, and vice versa.

For example, BRAF and MEK inhibitors demonstrate efficacy in brain metastases from *BRAF*-mutant **[Au:OK?]** metastatic melanoma. In 2014, *BRAF*^{V600E} mutations were found to be present in 95% of papillary craniopharyngiomas[6], which are rare brain tumours that are located close to critical structures (such as the optic chiasm and hypothalamus) and strongly associated with morbidity **[Au: Edit OK?]** . Shortly after this genomic discovery, several case reports were published demonstrating dramatic responses to BRAF and MEK inhibitors in patients with papillary craniopharyngiomas[7]. Furthermore, an ongoing phase II trial (Alliance A071601; NCT03224767) is now investigating the efficacy of combined BRAF and MEK inhibition in patients with *BRAF*^{V600E}-mutant **[Au: Clarified this was in the *BRAF*^{V600E}-mutant**

setting (attained info from ClinicalTrials.gov entry), OK?] papillary craniopharyngiomas, in whom systemic therapy has historically played a limited role. Similarly, oncogenic fusions involving genes encoding receptor tyrosine kinases have been discovered in a broad range of tumour types, including brain metastases and primary brain tumours. Brain-penetrant agents that target fusion proteins **[Au:OK?]** (for example, anaplastic lymphoma kinase (ALK) and neurotrophic receptor tyrosine kinase (NTRK) fusions), including entrectinib, have shown high response rates in patients with intracranial metastases in phase I trials **[Au:OK?]** [8]. These exciting results are now being extended to primary brain tumours. Advancements in our understanding of brain metastases will certainly advance the field of primary brain tumour research.

L.G. Metastasis-initiating cells or relapse-driving clones are present early on in primary brain tumours (such as medulloblastoma and glioblastoma **[Au:OK? Since glioblastoma multiforme is no longer a recognized classification, for journal style we refer to this as just 'glioblastoma'.]**) and primary **[Au:OK? '…early on in *the development and/or progression of*…'?]** lung and breast cancers, indicating that tumour evolution at relapse and metastasis is a branched and intricate nonlinear phenomena in several cancer types[13–18]. Thus, it is conceivable that the tumour microenvironment drives the selection of clones that can successfully repopulate or metastasize and shapes the parallel evolution of cancers of different ontogenies that share a common microenvironment at relapse or metastasis. Beyond the differences in the mechanism of initial tumorigenesis, once tumours are established, the invasive behaviour and patterns of parenchymal spreading are similar between glioblastoma and brain metastasis of breast and lung cancer. For example, the extensive interaction between tumour cells and macrophages or microglia in the tumour microenvironment occurs in both brain metastases and subgroups of primary glioblastoma, and issues of drug delivery across the BBB are shared between different entities that colonize the brain **[Au: Please reference this statement.]** . Since one of the major challenges in our understanding of the biology of primary and secondary brain tumours is the paucity of human samples available for molecular profiling during the course of disease progression, there is a clear advantage in grouping brain cancers according to their **[Au:OK?]** molecular driver genes (for example, *BRAF* or *PTEN*/*PIK3CA*/*MTOR* **[Au:OK?]**) and patterns of invasion and/or metastasis (for example, parenchymal versus leptomeningeal), as this strategy will reveal

patterns of convergent evolution of different cancers that colonize similar microenvironments. Leptomeningeal brain metastases of breast cancer also show a convergence with medulloblastoma leptomeningeal metastases, whereby they both harbour upregulated expression of C-C motif chemokine ligand 2 (CCL2)[19,20]. **[Au: Please clarify the purpose of Ref 20, which deals with C3 (rather than CCL2) in leptomeningeal metastases.]**

M.V. Despite what one could imagine, there are many differences between brain metastases and primary brain tumours. **[Au:OK?]** [Yes]

The most remarkable difference is that glioblastoma, the most frequent primary brain tumour (but much less prevalent **[Au:OK?]** [Yes] than brain metastases), rarely metastasizes outside of the brain despite its strong invasive nature. Although the explanation for this phenomenon remains unknown, it could have important implications. By contrast, brain metastases are commonly found in patients that have additional extracranial systemic metastases, which seems to be important for response to immunotherapies[5]. Consequently, studying the crosstalk between brain metastases and the immune system might generate new opportunities to increase the sensitivity of primary brain tumours to immunotherapy.

There are also therapies that are efficacious in primary brain **[Au:OK?]** [Yes] tumours but not in brain metastases. Indeed, clinical trials with the alkylating agent **[Au:OK?]** [Yes] temozolomide in patients with brain metastasis have not been encouraging[6]. However, since glioblastoma responses to temozolomide are transient **[Au:OK?]** and every patient eventually develops resistance, dissecting the lack of sensitivity in brain metastases at the molecular level might offer new strategies to challenge glioblastoma relapse. This approach is especially interesting given that preclinical evidence has shown that temozolomide could be effective in a preventive scenario for brain metastases[7], suggesting that therapeutic sensitivity could be modulated in cancer cells.

In addition to therapeutic response, there are also clear differences regarding the underlying biology. Notably, glioblastoma has a more potent pro-angiogenic **[Au:OK?]** [Yes] nature than brain metastases **[Au: Please reference this statement.]** [PMID: 27861605], and further research on this topic might inspire the development of imaging approaches to noninvasively differentiate between primary brain tumours and brain metastases with high specificity **[Au:OK?]** [Yes]. There are also important similarities. Although glioblastoma is more invasive than an established brain metastasis, which remains well-circumscribed with discrete

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invasive fronts, the basic mechanism of local invasion is similar. Glioma cells and metastatic cells in the brain use pre-existing blood vessels to disseminate locally and increase their resistance to a variety of stressors[8]. Comparative analysis of the underlying regulatory mechanisms of this process of vascular co-option could identify common therapeutic targets for primary and secondary brain tumours. Additional common vulnerabilities in primary and secondary brain tumours include metabolic pathways[9]. The fact that metastatic cells need to alter their metabolism when growing in the brain also infers **[Au:OK?]** [Yes] that the initial metabolic profile present in the primary tumour might be incompatible with survival in the brain **[Au: Please reference this statement.]** [PMID: 25525878, PMID:25511375]. Thus, reverting the metabolic state acquired in the brain could be a new therapeutic strategy, not only for brain metastasis but also for primary brain tumours.

Increasing the number of comparative studies between primary brain tumours and brain metastases could offer a unique vision of the underlying biology of brain malignancies and generate unpredicted therapeutic opportunities. These comparative approaches will eventually reach well-known clinical scenarios for which there are currently no explanations, such as the higher incidence of epilepsy in patients with primary brain tumours than those with brain metastases.

How can we improve preclinical models of brain metastasis?

A.B. Preclinical models of brain metastasis are just that — models of human disease. There are no 'bad' models, only models that have simply been ill-used or findings that have perhaps been **[Au:OK?]** over-interpreted. The 'best' model for the study of brain metastasis really depends on the question that is being asked. If the question is related to cancer cell–immune cell interactions, then a syngeneic or immunocompetent system is really ideal. If the question is one of metastatic tumour–primary tumour interactions **[Au: I'm not quite sure what 'metastatic tumour–primary tumour interactions' means and wondered if it would be better phrased as 'how metastatic tumours evolve and progress from primary tumours' or something similar?]** , then a genetically engineered mouse model (GEMM) is ideal. If the question relates to tumour heterogeneity or human-specific genetic changes, xenograft systems are often the best option.

Importantly, as a community, we are undertaking these lines of inquiry in the 'post-omics' era. Improved sequencing capacity for DNA, RNA and protein (and soon metabolites) at the bulk and single-cell levels has been joined with corresponding revolutions in computational biology, such that we now have the capacity to cope with the frankly enormous datasets generated from human samples. Collaborative projects such as the Human Tumor Atlas Network (HTAN) will become foundational for our field **[Au: Can HTAN be referenced? Or alternatively, if there is a website link, please add this here in brackets (this will be added to a 'Related links' section)]** . In the near future, we may well ask ourselves whether we should be using mouse models as our primary tool for discovery and what kinds of questions we can reasonably expect to answer with them. **[Au: Edit OK?]**

P.B. The metastatic cascade to the brain involves growth within the primary tumour, intravasation into the blood stream, survival within the circulation, extravasation and invasion into the brain and angiogenesis and proliferation within the brain parenchyma. Preclinical models attempt to recapitulate these steps within the cascade. In vitro assays can be used to study the passage of cancer cells through the BBB[9]. In vivo, **[Au:OK?]** cancer cells implanted intracranially (via stereotactic injection) in murine models allow investigators to monitor growth within the brain parenchyma, and fluorescently labelled cells can be tracked using bioluminescent imaging[10]. These models are limited in that they only mimic one part of the

metastatic cascade. Intracarotid and intracardiac injection of cancer cells can model **[Au:OK?]** dissemination of the cancer cells into the blood stream and can be used to study extravasation through the BBB as well as colonization and growth within the brain. Kienast et al.[11] employed an intracranial window in nude mice to track individual fluorescently labelled cancer cells injected via the carotid artery; this model reflects colonization into the brain, including the interaction of the metastases with the vasculature of the brain. The vast majority of existing mouse models of brain metastases use immunodeficient mice and human cell lines, in which the lack of an immune system limits the ability to study the interaction of cancer cells with the immune microenvironment. Syngeneic mouse cell lines with brain tropism are also used, although many of the immunocompetent **[Au:OK?]** mice develop a high burden of extracranial metastases when these cell lines are injected into the bloodstream. Few GEMMs of brain metastases that recapitulate the metastatic cascade have been developed, but those few that have been generated **[Au:OK?]** also demonstrate a high frequency of extracranial metastases[12-14]. Improving preclinical models of brain metastases will expand our ability to study the biology of brain metastases and, more importantly, identify novel therapies for this disease. A concerted effort from the scientific community is needed to expand the number of cell lines available with brain tropism, as well as optimize models to better mimic the complex metastatic cascade.

L.G. Spontaneous (transgenic) or syngeneic models of brain metastasis are the best models to study the interaction between brain metastases and the brain microenvironment. However, one drawback of using transgenic models for the formulation of new hypotheses (when human samples are not available) or validation of candidate driver processes in brain metastases is their relative genetic simplicity; transgenic models have a clonal representation of few selected mutations, whereas human metastasis show a relevant subclonal heterogeneity that influences their evolution during progression and metastasis. **[Au: Split sentence to improve flow, OK?]** To this end, incorporating steps that create intratumour heterogeneity by random somatic mutagenesis with viruses or transposons will improve the predictive value of the model for metastasis[21,22]. Transplantation-based preclinical models such as patient-derived xenografts (PDXs) — which are preferred over xenografts established from highly passaged cell lines — **[Au:OK?]** have the advantage of recapitulating human genetic and epigenetic alterations and intratumour heterogeneity, but at the cost of an intact

immune system in the immunocompromised rodent host **[Au: Edit OK?]** . The recent development of immunocompetent **[Au:OK?]** humanized recipient mice for establishing xenografts of human origin **[Au:OK?]** seems to be a promising avenue to improve preclinical models of brain metastasis, although more research is needed to completely evaluate their limitations, especially in long-term studies in which graft versus host immune events **[Au: Edited to further clarify 'graft versus host immune events', OK? (I assume you are not referring to graft versus host disease (GVHD) specifically)]** might be an issue[23]. When developing a brain metastasis preventive approach or when studying the early steps of metastatic colonization to the brain, it is crucial that PDX models are implanted orthotopically in order to mimic as closely as possible the entirety of the metastatic cascade. As the vast majority of biologics and targeted therapies for brain metastasis will be in the context of heavily treated primary tumours, a further optimization step is to include rodent adjust therapeutic protocols **[Au: Please clarify: do you mean rodent-adjusted protocols? What does this entail? Treatment optimization in rodent models that recapitulate the clinical scenario (i.e. previous treatment)?]** , as discoveries made in untreated preclinical models could be of limited predictive value when translated to heavily treated patients.

M.V. The ideal in vivo model of **[Au:OK?]** [Yes] brain metastasis would completely recapitulate the metastatic cascade, have an intact immune system that co-evolves during cancer progression and be compatible with therapeutic interventions (for example, surgery, radiotherapy and chemotherapy). Despite the large amount of available GEMMs of lung cancer, breast cancer and melanoma (which are the main sources of brain metastasis), only three GEMMs have been shown to give rise to brain metastasis[10–12]. These GEMMs are infrequently used by brain metastasis investigators, the reasons for which might be related to the low and variable incidence of brain metastases, the lack of macrometastases derived from the primary tumour or extracranial metastases (due to early death), the absence of reporters that are compatible with non-invasive high-throughput imaging in cancer cells and the differences between the molecular profile of some of these experimental models and the human disease. It is also true that many other commonly used GEMMs have not been characterized for their incidence of brain metastasis. To cite two examples, the *Kras*^{G12D};*Trp53*-null lung adenocarcinoma[13] and ERBB2-activated breast cancer GEMMs[14] did not ~~report or discard~~ **[Au: Please clarify 'discard' in this context.]** [interrogate] the presence

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of brain metastases, although lung and breast cancer are among the cancer types with the highest rate of brain metastasis[15] **[Au: Edit OK?]** [Yes]. It seems appropriate to extend the characterization of these, and new, GEMMs to the brain in addition to incorporating systemic therapies that could increase the time available for brain metastasis to develop.

Alternatively, given the available information on molecular alterations present in brain metastases, which differ from those in the primary tumour and other extracranial metastasis, it is tempting to speculate whether engineering such alterations in GEMMs **[Au:OK?]** [Yes] could be a valuable strategy to generate improved models. In favour of this approach, a genetic manipulation of *Akt1* **[Au:OK?]** [Yes] introduced into a spontaneous melanoma mouse **[Au:OK?]** [Yes] model correlated with an impressive incidence of brain metastases[12]. Given the extensive data demonstrating the functional importance of the AKT pathway in human brain metastasis, this study could be considered the proof-of-concept for this approach. However, the genetic *Akt1* alteration introduced in mice is not prevalent in the human disease **[Au: Clarified you mean that the specific *Akt1* mutation is not prevalent in humans, OK?** [Yes] **(Or did you mean that the resultant AKT1-mutant tumours did not resemble the AKT1-mutant human disease?)]** , and the recent engineering of an *Akt1* mutation that is prevalent in human melanoma in mouse primary tumours **[Au:OK?]** [Yes] was shown to provide general pro-metastatic attributes favouring a pan-metastatic phenotype rather than brain-specific metastasis **[Au:OK?]** **[Yes. Basically the initial paper used an alteration that did not resemble any human mutations, but the second one used one that is "real"]** [16]. In fact, there are no highly abundant and recurrent mutations that are associated with brain metastasis in humans, which imposes a challenge for deciding which alteration should be prioritized into models. Gain-of-function screens containing clinically relevant candidates could perhaps be a solid strategy for development of GEMMs that metastasize to the brain.

Spontaneous models of brain metastasis from **[Au:OK?]** [Yes] a primary tumour are highly valuable for developing biomarkers or therapeutic strategies to prevent brain metastasis. However, whether they are strictly necessary to understand the biology of brain colonization and develop strategies aimed at impairing the viability of cancer cells once they have reached the brain is a matter of continuous debate. By contrast, the most popular experimental approach consists on injecting brain-tropic cell lines into the systemic circulation to induce metastasis in the brain. In favour of these models, there are publications validating the same mechanisms using brain-metastatic (BrM) **[Au:OK?]** [Yes] cell lines and GEMMs that

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develop brain metastasis[17]. In addition, work with BrM cell lines has provided new therapeutic opportunities that have been translated to patients, such as the inhibition of gap junctions between cancer cells and astrocytes or the inhibition of STAT3 in the same glial cell [18,19] **[Au: I suggest briefly providing an example, as this seems like an impactful statement.]** .

Alternative models for exploratory purposes in which multiple candidates can be tested do exist. One of the most popular strategies is brain organotypic cultures, which consist on an ex vivo approach to interrogate either early or advanced stages of brain colonization using genetic or pharmacological strategies[3,19].

What are unique therapeutic targets in the brain and how can we improve treatment of brain metastases?

A.B. There are two major categories of treatments tailored to the unique therapeutic targets in the brain — anti-neoplastic agents that overcome the BBB and BCSFB and targeted therapies that interrupt cancer cell–microenvironment interactions **[Au: Edit OK?]** .

In the first case, understanding these barrier systems in both states of health and malignancy (that is, both the BBB and blood–tumour barrier) is essential. Our understanding of these barrier systems in health is excellent. However, we are only just beginning to understand the function and regulation of the blood–tumour barrier. I hasten to add that the relationship between the parenchyma and the leptomeningeal space is far from completely understood. It is clear that the relationship between these compartments is complex, and is currently best captured by the 'glymphatic' hypothesis **[Au: Please briefly describe this hypothesis (this seems to be important contextually for the next sentence). Is this related to CSF flow between these compartments and waste clearance in the brain?]** [8–10]. At present, when studying a novel compound, it is essential that we concurrently collect blood, tumour tissue **[Au:OK?]** and CSF for pharmacokinetic analyses. Too often, CSF is collected as a proxy for drug delivery into the brain parenchyma.

In the second case, targeting cancer cell–microenvironment interactions in clinical practice is extraordinarily challenging. As patients with brain metastases very often harbour disease outside the brain, both intracranial and extracranial disease must be addressed. Clinical trials designed to presuppose a population with active disease exclusively within the brain will result in slow accrual and will not reflect current practice **[Au: Edit OK?]** . However, **[Au:OK?]** trials allowing enrollment of patients with active extracranial disease must be designed and interpreted with care. Consensus opinions, in particular those from the Response Assessment in Neuro-Oncology (RANO) collaborative group, have provided a roadmap to aid clinical investigators in addressing these issues **[Au:OK?]** [11].

Ultimately, I foresee that strategies targeted to brain metastases (for example, anti-connexin-43 antibodies **[Au:OK?]**) will be joined with tumour-directed therapies that can penetrate the brain and CSF (for example, the EGFR inhibitor **[Au:OK?]** osimertinib). Ideally, this approach of combining multiple orthogonal strategies will eliminate toxic untargeted

treatments such as radiotherapy, thereby both increasing the survival and improving the quality of life of patients with brain metastases.

P.B. Clinically, patients often have divergent therapeutic responses in intracranial and extracranial lesions **[Au: Edit OK?]** . Following progression of the brain lesions, >50% of patients die from their brain metastases **[Au: Edit OK?]** . The BBB and blood–tumour barrier play crucial roles in the decreased intracranial **[Au:OK?]** response rates that are often seen clinically with systemic therapies[15]. Whether genetic heterogeneity contributes to this divergence in therapeutic response in brain metastases remains an open question. In breast cancer, data has emerged showing shifts in the status **[Au: expression or mutational status?]** of hormone receptors and human epidermal growth factor receptor 2 (HER2; also known as ERBB2) between intracranial and extracranial metastases **[Au: Following what treatment?]** **[Au: Please reference this statement.]** . In another study, **[Au:OK?]** genomic characterization of 86 matched brain metastases, primary tumours and normal tissues **[Au: Clarified study detail, which seems important contextually. OK?]** demonstrated branched evolution, whereby brain metastases and primary tumours shared a common ancestor, yet the brain metastases and primary tumours diverged genomically[16]. As a result of this branched evolution, brain metastases harboured genetic alterations that were undetected in the primary tumour, including driver mutations for which targeted therapies are available. Clinically, this finding implies that genetic screening of the primary tumour, which is often done as standard in the clinic, might miss potentially actionable targets in the brain metastasis. Signalling **[Au:OK?]** pathways commonly altered in brain metastases include the PI3K, cyclin-dependent kinase (CDK) and EGFR pathways; compared to matched primary tumours from the same patient, many of these genetic alterations are exclusive to the brain metastasis **[Au: Please reference this statement.]** . A genomically guided phase II **[Au:OK?]** trial (Alliance A071701; NCT03994796) has now been initiated, in which intracranial and extracranial tissue from patients with progressive brain metastases will be sequenced for specific genetic mutations (CDK pathway, PI3K pathway and NTRK and/or **[Au:OK?]** ROS fusions), and patients will be treated with the corresponding CNS-penetrant inhibitor (abemaciclib, GDC-0084 or entrectinib). The objective of this unique precision medicine study is to determine whether targeting alterations observed in brain metastases will improve clinical outcomes.

L.G. The presence of the BBB and BCSFB constitutes a unique opportunity to develop targeted strategies to prevent metastasis by blocking the mechanisms that mediate extravasation. Several attractive targets have been identified and are subject of intensive study in parenchymal brain metastasis[4,24,25], whereas the identification of key factors for BCSFB transmigration in leptomeningeal disease is lagging behind, with very few targets identified[20]. Reactive astrocytes also offer a unique opportunity to manage brain metastasis, with signal transducer and activator of transcription 3 (STAT3) inhibitors at the forefront[7]. Modulators of neural activity, which have been targets of drug development to cure neurological conditions, are an untapped arsenal of compounds with favourable brain distribution that could be repurposed once the extent of crosstalk between brain metastases and neurons is further investigated. Inhibitors of neurotransmitter receptors and metabolism, as well as agents targeting brain-specific growth factor signalling **[Au:OK?]** , represent the most attractive targets **[Au: Please reference this statement with the intended '(ref)'.]** . The composition of the extracellular matrix (ECM) of brain tumours is also unique and could be targeted by biologics in the context of combination therapies. Indeed, targeting of tenascin C and hyaluronic acid, which are abundant in the brain ECM, has shown promise in preclinical studies[26] **[Au: Edit OK?]** . Exploiting the unique microenvironment of brain metastasis with immunotherapy approaches in combination with targeted agents directed against molecular drivers present at the time of treatment will have the greatest impact on patient therapies. In light of our deeper understanding of tumour evolution, a key aspect in the management of brain metastasis will be to promote the use of tissue biopsies to confirm the presence of the target when it is safe to do so, and also to prioritize agents that were efficacious in preclinical models closely mimicking patient care, especially when targeted agents are to be used in an adjuvant or second-line treatment scenario.

M.V. The pressure imposed on cancer cells by the microenvironment forces them to adapt or perish. Two main aspects of this biology have been identified and validated as potential anti-metastasis strategies — the anti-tumorigenic behaviour of the naïve brain and the pro-metastatic niche provided by the rewired tumour-associated microenvironment **[Au: Edit OK?]** [Yes]. The rules governing the crosstalk between cancer cells and the microenvironment are derived from the unique cellular compartments present in the brain. Astrocytes and

microglia have been shown to have the ability to kill cancer cells[3,4]; however, these defences could be circumvented by different molecular mechanisms hijacked by metastasis-initiating cells. Targeting these mechanisms or reinforcing the anti-tumorigenic nature of glial cells could be a valuable strategy to be explored. An additional strategy taken by cancer cells is to ~~transform~~ reprogram the brain microenvironment **[Au:OK?]** [Yes]. Indeed, **[Au:OK?]** the anti-tumorigenic nature of the brain is less evident once metastases have grown. Interestingly, this finding correlates with the activation **[Au:OK?]** [Yes] of signalling pathways that are not present in the naïve brain microenvironment[19,20]. Targeting these pathways terminates the pro-tumorigenic behaviour of the rewired brain microenvironment[19], but might also boost the anti-tumorigenic nature of the brain.

In addition to the unique targets present in the brain microenvironment, cancer cells themselves also respond to the new microenvironment by altering gene and protein expression patterns in situ[20,21] **[Au: Edit OK?]** [Yes]. Emerging evidences suggest that a new avenue for therapeutic intervention might be derived from the specific vulnerabilities created in the process of adapting to the brain microenvironment[17].

An area that remains unexplored given the emergency imposed by the limited survival of patients is the influence of brain metastasis on neurocognition. Although neurocognitive impairment is one of the most frequent brain-specific symptoms **[Au:OK?]** [Yes] among patients with brain metastases, at the experimental level, very limited research has been devoted to it. Recent findings in primary (Venkatesh et al. 2019, Venkataramani et al. 2019) and secondary (Zeng et al. 2019) brain tumors demonstrating that cancer cells integrate into brain circuits hijacking the synaptic machinery have opened the possibility to dissect their impact on neuronal processing. Improvements in patient survival outcomes will increase the importance of quality of life **[Au:OK?]** [Yes] and it will become more important to understand how metastatic cells influence neurons at the cellular level and also neural circuits **[Au: Edit OK?]** [Yes]. These questions will require new expertise and new models to explore a very unique aspect of metastases that grow in this organ.

In summary, treating brain metastases using the same systemic therapy given to the primary tumour has proved ineffective (with some exceptions for very small groups of patients; for example, ALK inhibitors), and the brain microenvironment is emerging as a potential resource to improve therapies directed against brain metastasis. These considerations suggest that it might be appropriate to consider organ-specific therapies. Thus,

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it is essential that preclinical efforts take this into account. In this sense, performing subcutaneous injections to test anti-metastasis compounds is never the best option **[Au: Please clarify – are you referring to subcutaneous xenograft models [Yes], or subcutaneous injection of an anti-metastasis compound?]** . Similarly, it is important to differentiate preventive experimental treatments from interventional ones that act on established metastases, which are closer to the clinical situation and include a rewired pro-tumorigenic brain microenvironment.

In addition to developing new therapeutic strategies, it is also very important to understand why available treatments fail. For instance, relapse after surgery has not been modelled in mice, but it is expected that interactions with the brain microenvironment are as important as they are during the initial colonization of the brain (such as, for instance, vascular co-option)[3,22] **[Au: Edit OK?]** [Yes]. In addition to relapse, the lack of efficacy of whole-brain radiotherapy suggests that clinicians should move away from this approach in favour of more localized treatments such as stereotactic radiosurgery. However, the reasons for these poor responses to radiotherapy in the context of brain metastasis have barely been addressed experimentally. Investing additional research into the molecular mechanisms of resistance will improve our knowledge of brain metastasis biology and also offer ways to improve available therapies **[Au: Edit OK?]** .

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Competing interests

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M.V. declares that he does not have any competing financial interests.