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Review

Breast Cancer Brain Metastases: Clonal Evolution in Clinical Context

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Abstract: Brain metastases are highly evolved manifestations of breast cancer arising in a unique microenvironment, giving them exceptional adaptability in the face of new extrinsic pressures. The incidence is rising in line with population ageing, and use of newer therapies that stabilise metastatic disease burden with variable efficacy throughout the body. Historically, there has been a widely held view that brain metastases do not respond to circulating therapeutics because the blood-brain-barrier (BBB) restricts their uptake. However, emerging data are beginning to paint a far more complex picture where the brain acts as a sanctuary for dormant, subclinical proliferations that are initially protected by the BBB, but then exposed to dynamic selection pressures as tumours mature and vascular permeability increases. Here, we review key experimental approaches and landmark studies that have charted the genomic landscape of breast cancer brain metastases. These findings are contextualised with the factors impacting on clonal outgrowth in the brain: intrinsic breast tumour cell capabilities required for brain metastatic fitness, and the neural niche, which is initially hostile to invading cells but then engineered into a tumour-support vehicle by the successful minority. We also discuss how late detection, abnormal vascular perfusion and interstitial fluid dynamics underpin the recalcitrant clinical behaviour of brain metastases, and outline active clinical trials in the context of precision management.

Keywords: breast cancer; brain metastases; clonal evolution; precision medicine; genomics; tumour microenvironment

1. Clinico-epidemiologic profile of brain metastatic breast cancer

Around 30% of breast cancer (BC) patients develop metastatic (stage IV) disease, and at least 15% will experience symptomatic relapse in the brain [2], a serious complication that causes rapid neurological decline and death in virtually all patients within a few years. Receiving this diagnosis marks a significant downturn for patients, both physiologically and psychologically. In addition to the personal burden, this is also a major socioeconomic problem because it often affects women who are members of the tax-paying workforce, are of childbearing age and/or have dependent children. The direct economic impact is also substantial, with the annual cost of care estimated to be ~$100,000 USD/patient in 2006, more than double stage IV patients without brain metastases (BM) [3]. Young age, high histologic grade and tumour subtype are major risk factors [4,5].

BM are usually confirmed radiologically with dynamic, contrast enhanced magnetic resonance imaging (DCE-MRI) in patients who develop neurological symptoms (e.g. chronic headache, motor, cognitive or speech deficits and seizures). Symptoms are managed with steroid treatment, which produces iatrogenic Cushing's syndrome and contributes to the overall morbidity burden. Depending on the extent of disease, age and general health, local control measures can include surgical excision, stereotactic and/or whole brain radiotherapy (SRS, WBRT). These modalities have been mainstays of treatment since the 1950s, and while their precision and efficacy are improving,

ultimately they do not prevent recurrence. For BCBM, a combination of surgery and SRS seems to provide the most benefit, increasing median survival to ~22 months compared to 5.5 months for WBRT [4]. Cytotoxic chemotherapy is not routinely used as the toxicity burden often outweighs clinical benefit, however particular agents may add benefit in combination regimens.

2. Breast cancer cell-intrinsic features can drive breast cancer metastasis to the brain

The risk of developing BM is associated with primary tumour phenotype, highest for triple-negative (TN), HER2+, basal-like and claudin-low disease [5-7]. While systemic therapy is different between these groups and obviously impacts BM development, in most cases micrometastases are already present in the brain before primary cancer diagnosis. So to some degree, metastatic fitness and site predilection are (epi)genetically coded in the primary tumour, influenced by environmental and germline modifiers [8]. Specific examples of this include high expression of HER2, HER3, COX2, HB-EGF, neuroserpin, neurotrophin-3 and the glycosyltransferase ST6GALNAC5; and suppression of *PTEN* [9-14].

The requirements for initially colonising the brain are fundamentally different to those needed for sustained outgrowth, however some changes associated with risk of brain relapse have also been identified in BM surgical samples, implying a continuing requirement for outgrowth in the brain. For example, there are now robust data from independent studies showing that *ERBB2* over-expression and *PTEN* suppression in BM are underpinned by positive selection of hard-wired DNA copy-number alterations (CNAs) [12,13]. Other genes harbouring CNAs with corresponding changes in expression have recently been identified, but their significance remains to be elucidated. For example, the mitochondrial protein *TMEM65* is amplified and overexpressed in ~50% of BM from breast and lung cancers; *SOX11* is amplified and overexpressed in ~30% of breast and ~80% of lung cancer-BM; and *NRG1* is lost and suppressed in ~60% of BC-BM [12,15]. Large BC genome sequencing projects have not yet illuminated particular mutant alleles that bestow brain metastatic fitness, but hopefully these analyses will be forthcoming with the assembly of additional clinical data that includes site-specific relapse information.

Pre-programming of metastatic behaviour is also mediated systemically by tumour-derived microvessels that circulate miRNA, mRNA and protein cargoes throughout the body. Exosome cargoes can prime pre-metastatic niches to receive CTCs and create a favourable niche for their outgrowth. Mechanistically this can involve vascular permeabilisation, angiogenesis, and extracellular matrix remodelling [16-18]. Molecular profiling of exosomes from brain-seeking cell lines have implicated particular miRNA and protein cargoes, but the clinical significance of these candidates is yet to be determined [19]. With the exception of elegant experiments showing that exosomes carrying integrin-β3 can mediate brain-tropic behaviour [20], little is known about microvessel specification of brain relapse. In particular, the field eagerly awaits technological developments that will facilitate microvessel profiling from prospectively collected patient blood samples.

BM are usually associated with extracranial metastases, particularly the liver, as the two are likely trapping sites for circulating tumour cells (CTCs), but ~17% of BM are not associated with extracranial disease [21]. This restricted spread is associated with better prognosis, and is infrequent in African-American patients [22]. Interestingly, brain-only-metastasis (BoM) is not linked to HER2 or hormone receptor status [21], but is associated with expression of HER3 [10], which is the preferred oncogenic dimerization partner of HER2, and has ample access to neuregulin ligands in the brain [23-25]. Molecular profiling of BCs that exhibit BoM may identify new mechanisms and clinically informative biomarkers. Overall the frequency of BCs exhibiting BoM is only ~25 for every 1000 cases, and so assembling a sample cohort to look for (epi)genetic features associated with this behaviour could be logistically challenging, however the clinical homogeneity in this group may enable molecular discoveries using a relatively small cohort.

3. Extrinsic factors that drive clonal evolution in brain metastases

3.1. Microenvironment-driven selection pressure

The development of BM from CTCs is an incredibly complex process featuring continuous extrinsic selection pressure (depicted in Figure 1). This is initially driven by a requirement for CTCs to circumvent anoikis, tolerate shear stress in the circulation, then extravasate at distant sites, which ultimately favours cells most capable of co-opting the distant niche. Detailed analysis of BC patient CTC-derived cell lines implicated HER2, EGFR, heparanase (HPSE) and Notch1 in brain metastatic fitness [26]. Progression depends on actively crossing the blood-brain-barrier (BBB), the specialised vascular lining separating neural and vascular compartments of the central nervous system. The BBB features continuous tight junctions that oppose paracellular diffusion, diverting the passage of circulating metabolic substrates, xenobiotics and other solutes to selective transcellular transport systems. Its function depends on contact between endothelia, the vascular-astroglial basement membrane, pericytes and the glia limitans, an interconnected layer of astrocyte foot processes that is key to neurovascular regulation [27]. Ultrastructural and immunofluorescent imaging data show that a fraction of CTCs arrest in capillaries or venules [28], and occasionally extend filopodia that mechanically push through the endothelium and adhere to the basement membrane [29-31]. Filopodia then dynamically extend and retract into the parenchyma as cells migrate to a perivascular position [29,31]. Mediators of this process include specific adhesion molecules and proteases (e.g. integrins α5, β1, β3, Cathepsin-S, MMPs and E-selectin [30,32-36]).

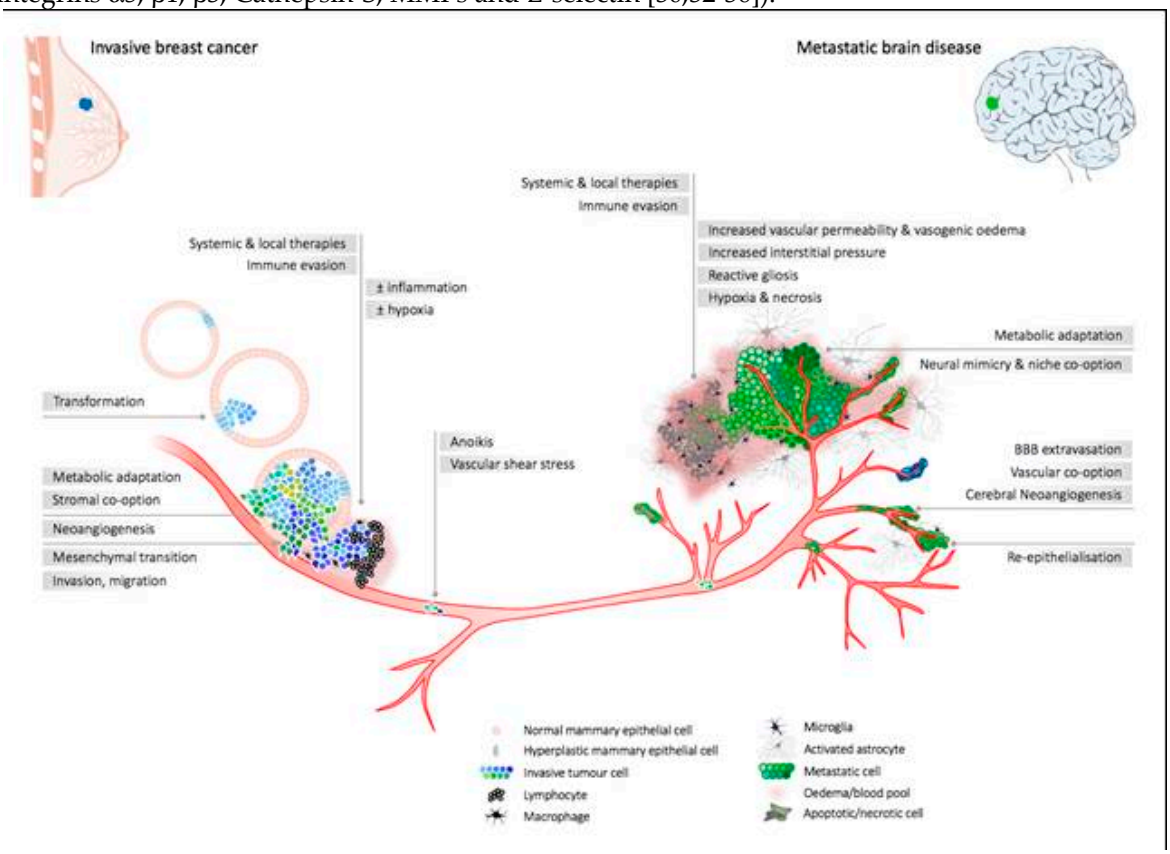

Figure 1: Schematic showing the breast cancer brain metastatic cascade. Requisite capabilities for metastatic fitness and extrinsic pressures driving clonal evolution are indicated in horizontal and vertical tracks, respectively.

The perivascular niche (PVN) is another critical point of tumour cell attrition – most are unable to tolerate the neuroinflammatory reaction that is rapidly instigated by microglia and astrocytes [11,28,31,37-44]. Cross-talk with resident pericytes and endothelia in the PVN seems to be key for tumour cell survival, quiescence and therapeutic resistance – capabilities that underpin dormancy[45]. Further progression can take months or years depending on the evolutionary distance

between BM-competent cells and their ancestors, and also their inherent adaptability. For example, aberrant DNA repair is common feature of BM, likely an adaptation to oxidative stress [46]. DNA repair is also defective in TNBC, which relapses in the brain earlier than other subtypes [47], suggesting that pre-existing DNA repair defects could underlie rapid clinical progression. In terms of 'late metastasising' BCs, the anti-angiogenic glycoprotein thrombospondin-1, secreted by endothelia lining stable microvessels, has been linked to dormancy [45]. Inducing neo-angiogenesis could be a key milestone for tumours escaping dormancy, as endothelial tip cells are a source of tumour-promoting TGF- β1 and periostin that seem to overcome the effects of thrombospondin.

In order for micrometastases to progress, the initially hostile neural niche must be transformed into one that promotes colonisation, which essentially involves transforming the glial compartment into a tumour support engine [35,37,48-50]. Cells succeeding to this stage seem to migrate along paths of least resistance, dividing as they go. Some proliferate in the perivascular pathway, forming sheaths as they co-opt the vasculature; others prefer interstitial tracks and form well-demarcated parenchymal lesions [11,51]. These patterns co-exist in any given tumour at different proportions [52], but whether achieved by co-option or angiogenesis, vascular proximity is critical for outgrowth [31,53].

Tumour cells use remarkable mechanisms to co-opt the neural niche. They cope with oxidative stress, repurpose neurotransmitters as metabolic substrates, recruit and promote the differentiation of neural progenitors into astrocyte support cells, mimic neural traits and effectively 'plug-and-play' with the new niche by inducing growth factor receptors (e.g. HER3, HER4, NTR3), particularly those that converge on the akt/PI3K/mTOR, MAPK and NF-κB [10,12,37,46,48,54-56]. Loss of the PI3K inhibitor *PTEN* is one of the only recurrent genomic features identified to date [12,13,55], but a recent pioneering study by Zhang, Yu and colleagues demonstrated that *PTEN* suppression can also occur as a reversible adaptation. They found that astrocyte-derived exosomes with cargoes from the miR-17/92 locus were able to silence *PTEN* in micrometastatic cells, which increased their secretion of microglia-activating CCL2 and promoted outgrowth [50].

3.2. Therapy-driven clonal selection

Historically there has been a widely held view that BM do not respond to circulating therapeutics because the BBB restricts their uptake. However, the idea that insufficient uptake is solely responsible is at odds with the fact that the standard clinical diagnostic modality is DCE-MRI, which relies on passive movement of injected contrast agents into the brain interstitium through vessel fenestrations in tumours larger than ~5mm. Drug uptake is certainly heterogeneous and lower than extracranial tumours [57-60], but large monoclonal antibodies (mAbs) accumulate to levels associated with efficacy at other sites [57,58,61,62], and so the treatment-refractory behaviour of BM must be due to more than permeability alone [58].

We can tend to relate clonal progression to clinical progression directly, on a patient or whole-tumour scale, but in reality, cycles of seeding, dormancy and regression or are likely occurring simultaneously throughout the cerebrum on staggered timelines. BM arise from convergence of perivascular and interstitial proliferations, including index/parent lesions as well as the progeny of tumour self-seeding, all encased in reactive brain parenchyma [38,63]. As small lesions that were once fully protected behind an intact BBB become increasingly permeable, for a time they may be exposed to sub-efficacious drug concentrations, providing an ideal milieu for selection and outgrowth of resistant clones. In understanding the developing of therapeutic resistance, it is important to consider how vascular permeability changes over time, the effects on haemodynamics and tissue architecture, and the implications for drug delivery (section 5.2).

Identifying the capabilities that are clonally selected in BM arising on a background of pre-treatment could inform the development of more holistic therapeutic strategies. For example, a landmark preclinical study from the Massagué team showed that protocadherin 7-expressing tumour cells form gap junctions with astrocytes, creating a feedback situation where cGAMP passed to astrocytes induce secretion of IFNα and TNF. In turn, the cytokines act as paracrine factors that promote tumour growth and chemoresistance through STAT1 and NF-κB [44]. These findings

prompted a clinical trial exploring the feasibility of using the gap junction modulator, meclofenamate, for BM treatment (Table 1). Another study assessing the efficacy of PI3K/mTOR therapy in HER2+ BM patient-derived xenografts (PdX) found that non-responders exhibited higher genomic instability and defective DNA repair compared to responders [55], consistent with a separate report on mTOR treatment resistance [64]. These findings raise the possibility of using PARP inhibitors to chemosensitise HER2+ BM with hypermutator phenotypes. There is also circumstantial evidence implicating HER3 in drug resistance – it is induced and activated in BM from breast and lung cancers, and has been separately implicated in resistance to anti-oestrogen, -HER2 and cytotoxic therapies [65-69]. Antibodies targeting HER3 (e.g. patritumab, AV-203, MM-121, AMG888 and HER2/3 bispecifics) are currently being assessed for treatment of various solid cancers, though so far, not specifically BM. Molecular profiling of experimental or clinical samples representing pre- and post-therapy clonality and transcriptome profiles is urgently needed to identify additional candidates.

4. The molecular portrait of breast cancer brain metastases

An increasing number of studies are reporting high-resolution analysis of clinical and experimental samples to address biological questions and clinical challenges associated with BC-BM. Key approaches, their strengths, limitations, and landmark findings are discussed below (Table 2).

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Table 1. Summary of current clinical trials of molecular-targeted agents for breast cancer patients with established brain metastases [114].

NCT-ID	Subtype	Phase	Experimental arm(s)	Comparator Arm	Approach	Primary Endpoints
02429570	All	O	Meclofenamate	NA	GAP junction modulator	ORR, PFS, safety
01621906	All	O	WBRT + Sorafenib + [18F]FLT PET at baseline	WBRT + [18F]FLT PET at baseline	XRT + VEGFR	RR (radiographic)
01386580	All	1/2	Glutathione pegylated liposomal doxorubicin	Glutathione pegylated liposomal doxorubicin + Trastuz	Carrier (CTx + HER2)	MTD, safety
01132664	HER2+	1/2	Buparlisib + Trastuz	Buparlisib + Trastuz+ Capecitabine	VEGFR+HER2+CTx	MTD, RR, PFS, safety
02154529	HER2+	1/2	Tesevatinib + Trastuz	Tesevatinib dose escalation+ Trastuz	Broad-spec RTKi	MTD, PFS, RR, safety
01921335	HER2+	1	ARRY-380 + Trastuz	ARRY-380 dose escalation+ Trastuz	HER2	MTD, RR and PFS
01332929	All	1	Bevacizumab + WRBT	Bevacizumab dose escalation+ WRBT	XRT + VEGFR	MTD, RR, PFS
02598427	HER2+	1	Intrathecal Pertuzumab + Trastuz	Pertuzumab dose escalation + Trastuz	HER2 (CSF delivery)	MTD, safety
02650752	HER2+	1	Lapatinib + Capecitabine	Lapatinib dose escalation + Capecitabine	CTx + HER2	MTD, RR, PFS
01276210	All	1	Sorafenib tosylate + SRS	Sorafenib tosylate dose escalation + SRS	VEGFR+Raf kinase	MTD, RR, PFS
00981890	All	1	Sunitinib + SRS	NA	XRT + VEGFR	Safety, MTD
00649207	All	1	Veliparib + WBRT	Veliparib dose escalation + WBRT	PARPi	MTD, safety
01724606	All	1	Sorafenib + WBRT	Sorafenib dose escalation + WBRT	XRT + VEGFR	MTD, safety
02308020	All	2	Abemaciclib	NA	CDK4/6i	RR, PFS, safety
02768337	All	2	Afatinib + 4 Gy XRT	Afatinib	XRT + HER2	Drug uptake
01441596	HER2+	2	Afatinib + vinorelbine	Afatinib	CTx + HER2	PFS
02048059	All	2	ANG1005 (formerly GRN1005)	NA	Carrier (CTx)	RR, PFS, OS
01898130	All	2	Bevacizumab	NA	VEGFR+HER2	RR, PFS, safety
02000882	All	2	Buparlisib + Capecitabine (+ Trastuz if HER2+)	NA	CTx + panPI3Ki	RR
01934894	HER2+	2	Cabazitaxel + Lapatinib	Cabazitaxel + Lapatinib (different doses)	CTx + HER2	RR, MTD, safety
02260531	All	2	Cabozantinib + Trastuz	Cabozantinib	c-met + VEGFR	RR, PFS, safety
02669914	All	2	Durvalumab (MEDI4736)	NA	PDL1i	RR, PFS, safety
01305941	HER2+	2	Everolimus + Vinorelbine + Trastuz	NA	CTx + HER2	RR, PFS, safety
01480583	HER2+	2	GRN1005 + Trastuz	GRN1005 alone	Carrier (CTx + HER2)	RR, PFS, safety
01494662	HER2+	2	Neratinib (HKI-272)	Neratinib (HKI-272) + Capecitabine	CTx + HER2	RR, PFS, safety
01173497	TNBC	2	Iniparib + Irinotecan	NA	CTx + PARPi	Efficacy, RR
01783756	HER2+	2	Lapatinib + Everolimus + Capecitabine	NA	CTx + HER2 + mTORi	RR, PFS, safety
01622868	HER2+	2	Lapatinib + WBRT or SRS	WBRT or SRS	XRT + HER2	RR, PFS, safety
01218529	All	2	Lapatinib + WRBT	NA	XRT + HER2	RR
02614794	HER2+	2	ONT-380 + Capecitabine + Trastuz	Placebo + Capecitabine + Trastuz	CTx + HER2	PFS, RR, safety
02774681	All	2	Palbociclib (+ Trastuz if HER2+)	NA	CDK4/6i	RR (radiographic), PFS, safety
02312622	All	2	Pegylated irinotecan (NKTR 102)	NA	Carrier (CTx)	Disease control rate, PFS
02536339	HER2+	2	Pertuzumab + Trastuz	NA	HER2	RR, PFS, OS, safety
01924351	HER2+	2	SRS + HER-2 directed therapy	NA	XRT + HER2	Relapse rate
02571530	HER2+	2	Intra-arterial cerebral infusion of Trastuz	May consider dose escalation	HER2	MTD, OS, PFS
00303992	HER2+	2	Trastuz + Irinotecan	NA	CTx + HER2	RR, disease progression
02185352	All	2	WBRT + Bevacizumab, Etoposide, Cisplatin	WBRT alone	XRT + VEGFR	RR, PFS
00820222	HER2+	3	Lapatinib + Capecitabine	Trastuzumab + capecitabine	CTx + HER2	PFS, RR
00073528	ER/HER2+	3	Lapatinib + Letrozole	Placebo + Letrozole	CTx (aromatase-i) + HER2	RR, PFS, safety

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Abbreviations: CSF, cerebrospinal fluid; CTx, chemotherapy; i, inhibitor; MTD, maximum tolerated dose; NCT-ID, Clinical Trials.gov identifier; OS, overall survival; RR, response rate; PARP, poly (ADP-ribose) polymerase; PDL1, programmed death-ligand 1; PFS, progression-free survival; RTK, receptor tyrosine kinase; SRS, stereotactic radiosurgery; trastuz, trastuzumab; WBRT, whole brain radiotherapy; XRT, radiotherapy; [18F]FLT PET, 3-deoxy-3-18F-fluorothymidine positron emission tomography

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Table 2. A catalogue of Brain Metastasis genomic studies.

Study	BCBM only?	Matched pairs?	Cohort size	FF or FFPE	GEX	CNA	Mutation Analysis	Exome	WGS	Targeted or Discovery	Key Findings
Bos 2009 [70]	Yes	No	1*	F	Array	No	No	No	No	D	- COX2, HBEGF (EGFR ligand), ST6GALNAC5 (a2,6-sialyltransferase) over-expressed, mediating BC cell passage through the BBB, with ST6GALNAC5 expression enhancing BC cell adhesion to brain endothelial cells
da Silva 2010 [56]	No	Some	78	FFPE	DASL (512 genes)	No	Onco-Carta	No	No	T/D	- Over-expression of ≥1 member of the HER family, in particular HER3 (relative to matched primary tumours) - Somatic mutations in *EGFR, HRAS, KRAS, NRAS, PIK3CA* - Increased activation of MAPK pathway in BM vs primary tumours
Ding 2010 [83]	Yes	Yes	1	FF	No	SNP	No	No	Yes	D	- Matched peripheral blood, the primary tumour, BM and PdX - BM acquired 2 private mutations and a large deletion, was enriched for 20 shared mutations (PdX was similar) - 2 overlapping large deletions (*CTNNA1*) in all 3 tumour samples - variation frequencies indicate that metastases arise from a minority of cells within the BC
Wikman 2012 [13]	Yes	Some	25	FF	*in silico*	aCGH/AI	GSS	No	No	T/D	- 9 chromosomal loci with significant differences, incl. amplification of EGFR (7p11.2) and loss of 10q22.3-qter - AI at *PTEN* was more frequent in BM (52%) and BC with a brain relapse (59%) compared with BC without relap (18%; P = 0.003) or relapse other than brain tumours (12%; P = 0.006). - Loss of *PTEN* was especially frequent in HER2-negative BM (64%) - *PTEN* mRNA expression was suppressed in BM compared with primary tumours - *PTEN* mutations were frequently found in BM.
McMullin 2014 [78]	Yes	No	19	FF	Array	No	GSS	No	No	T/D	- BRCA1 deficient-like GEX signature found in HER2+ BCBM in absence of *BRCA1* mutations - Values are significantly higher in HER2-/ER- primary tumours vs HER2+/ER + and HER2-/ER + tumours
Salhia 2014 [15]	Yes	No	35	FF	Array	aCGH^	No	No	No	D	- Frequent large chromosomal gains in 1q, 5p, 8q, 11q, and 20q; broad-level deletions at 8p, 17p, 21p and Xq - *ATAD2, BRAF, DERL1, DNMTRB* and *NEK2A* frequently amplified and overexpressed - *ATM, CRYAB* and *HSPB2* commonly deleted and down-regulated - Enrichment in cell cycle and G2/M transition pathways, which contain *AURKA, AURKB* and *FOXM1* - Defects in cell migration and adhesion due to hypermethylation + suppression of *PENK, EDN3* and *ITGAM*. - Hypomethylation + induction of *KRT8* likely affects adhesion and permeability
Bollig-Fischer 2015 [82]	Yes	No	10	FF & FFPE	No	aCGH	No	No	No	T/D	- Stem cell pluripotency pathway enrichment - Recurring significant amplification of *SOX2, PIK3CA, NTRK1, GNAS, CTNNB1,* and *FGFR1*
Brastianos 2015 [84]	No	Yes	86	FF & FFPE	No	No	No	Yes	No	D	- 86 trios of matched BM, primary tumours, and normal tissue - 53% of cases had potentially clinically informative alterations in BM - Individual brain metastasis deposits were genetically homogenous - Distal extracranial and regional lymph node metastases were highly divergent from BM. - Alterations associated with sensitivity to PI3K/AKT/mTOR, CDK, and HER2/EGFR inhibitors in BM
Lee 2015 [79]	Yes	Some	42	FFPE	No	No	Ion AmpliSeq Cancer	No	No	T	- Frequent somatic mutations included *TP53* (59.5%), *MLH1* (14.3%), *PIK3CA* (14.3%), and *KIT* (7.1%) - No significant differences in mutation profiles between BCBM and BC - *TP53* mutation frequency was higher in BCBM than in primary BC (59.5% vs 38.9%, respectively)

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Saunus 2015 [12]	No	No	36	FF	RNASeq	SNP	No	Yes	No	D	- Novel candidate genes significantly mutated *DSC2, ST7, PIK3R1* and *SMC5* - DNA repair, ERBB–HER signalling, axon guidance and protein kinase-A signalling pathways - Actionable genomic alterations identified in 31/36 BMs (86%) - Altered patient management (+trastuzumab) in a case of HER2 status conversion from BC to BCBM - *ERBB2* (HER2) expression correlated with *ERBB3* (HER3, r2=0.496; p < 0.0001) and, HER3 and HER4 were frequently activated in an independent cohort of 167 archival BM from seven primary cancer types - HER3 ligands *NRG1/2* were barely detectable by RNAseq, with *NRG1* (8p12) genomic loss in 63.6% BCBM, suggesting a microenvironmental source of ligand - Mutational signature analysis facilitated identification of the primary cancer type for two CUP
Vareslija 2015 [81]	Yes	Yes	7	U	RNASeq	No	No	No	No	D	- ER-specific metastatic pathways - Common functional pathways altered incl. extracellular matrix, cell adhesion and neuronal differentiation - *ANTRX1, THBS2, FAP, VCAN* and *TIMP2* part of the invasion and migration network that drives extravasation - EMT stemness signalling driven by *ANTRX1*, and WNT-driven *RUNX* prominent in cells acquiring migration abil
Lee 2016 [80]	Yes	Some	41	FFPE	Nanostring (252 genes)	No	No	No	No	T	- 22/252 genes found to be significantly differentially expressed between primary BC and BCBM - *CXCL12, MMP2, MMP11, VCAM1,* and *MME* were significantly upregulated in primary BC compared to BCBM - *SOX2* and *OLIG2* upregulated in BCBM - PAM50 molecular subtype conversion observed in 8/17 pairs (47.1%)

*pleural effusion sample with subsequent *in vivo* selection for brain seeking derivatives; ^study also performed whole genome methylation analysis using the Infinium HumanMethylation 27 BeadArray

Abbreviations: AI, allelic imbalance; BCBM, Breast cancer brain metastasis; BM, Brain metastasis; CUP, cancer of unknown primary; GEX, gene expression profiling; GSS, Gene Specific Sanger Sequencing; T, targeted; D, discovery; T/D, elements of both (i.e. study limited by panel, no alternate option at the time); U, unclear.

4.1. Analysis of 'brain-seeking' clonal cell line derivatives

To enrich for brain-tropic (epi)genomic traits, BC cell lines can be intravenously injected into experimental mice, developing BMs isolated, expanded *in vitro* and subjected to successive *in vivo* passage cycles. Brain-tropic cell line derivatives have been applied in various ways, for example, comparison to more heterogeneous parental cultures to identify genomic/transcriptomic traits associated with BM [70,71]. Use of these derivatives provides functional validation experiments with greater relevance than is provided by lines derived from primary breast tumours or pleural effusions and also guarantees a higher 'take rate' when generating cohorts of experimental BM. Brain-seeking derivatives of five BC cell lines and the parental lines have been exome profiled [72], with little evidence found for a definitive genetic driver. Indeed, Jacob et al [72] report that metastatic fitness can arise without *de novo* mutation, and can simply occur from further enrichment of particular mutant alleles.

The pioneering investigation of gene expression profiles in brain-seeking derivatives of two lines originally derived from TNBC pleural effusions (MDA-MB-231 and CN34) identified that *COX2*, *HBEGF*, and *ST6GALNAC5* are enhancers of BBB extravasation [70]; COX2 and HBEGF but not ST6GALNAC5, also likely function in extravasation in the lung. Interestingly, these genes were not differentially expressed in an independent cohort [73], nor could the role of ST6GALNAC5 in BBB extravasation be verified in a separate study [74]. There could be a technical or sampling-related basis for these discrepancies; for example, the independent studies exclusively analysed TNBCs while the original used unselected cases, and the BBB model was inherently different, comprising haematopoietic stem cell-derived endothelia and pericytes rather than umbilical vein endothelial cells and astrocytes. These inconsistencies do however highlight important limitations of model systems. As with any xenograft, a major limitation is that the brain-seeking system involves a mouse host supporting human cells in the absence of a full immune complement, a crucial impediment to the usual spread of cancer. On the other hand, syngeneic systems (e.g. 4T1.2) that model innate anti-tumour responses are also flawed because all components are mouse and may not be representative of human biology/physiology. The *in vitro* expansion steps in between successive *in vivo* passages also impart a selection pressure not encountered *in vivo*, and intra-tumoural heterogeneity is reduced in these models. Nonetheless, brain-tropic cell lines are reproducible models amenable to controlled hypothesis testing, and are very important experimental tools for BM research.

4.2. Analysis of human clinical samples

High-resolution molecular profiling of human tumours is essential to ensure relevance and make discoveries that lead to clinically translatable outcomes. Technological improvements have reduced the cost and vastly increased the volume and scale of cancer (epi)genome and transcriptome analysis. While The Cancer Genome Atlas (TCGA) and International Cancer Genome Consortium (ICGC) have made huge advances cataloguing BC genomes [75-77], currently they are not focussing on metastatic deposits. BM resection is not routinely performed and thus fresh frozen tissue samples are rare, however prospectively collected cohorts are beginning to be sequenced by independent groups, and methodological advances are now allowing analysis of the lower quality, fragmented DNA and RNA from more accessible FFPE samples. Considering the vast cohort sizes and volumes of genomic data generated for primary BC [75-77], much of which is on the precipice of clinical translation, there is significant scope for extending the depth and breadth of (epi)genomic and transcriptomic analysis of BM.

A plethora of 'omics studies with small BM cohorts and/or targeted gene panels have emerged in the last few years (Table 1 [13,15,78-82]). Unsurprisingly, genes frequently altered in BC were also commonly detectable in BM, though mutant allele frequencies (MAF) for some were enriched in BM (e.g. *TP53*, *PTEN*, *ATM*) [13,15,79]. The high frequency of potentially actionable mutations identified supports implementation of BM diagnostic profiling to inform targeted therapy. Interestingly, a 'BRCA1-deficient-like' signature was identified in HER2+ BM with wild-type *BRCA1*, raising the possibility of using PARP inhibitors [78]. Indeed, inipirib and veliparib are being assessed clinically

(Table 1). These trials are focusing on TNBC or unselected BC, but prospective assignment of HER2+ cases to PARPi therapy based on molecular profiling of any previously resected BM would be a rational next step to see whether the signature has predictive power in this group.

In terms of illuminating aspects of the biology, novel candidates have been identified in clinical sample discovery cohorts and validated, or are awaiting functional investigation in the field (Table 2). These include altered genes with corresponding changes in expression, functional gene networks with members collectively mutated more frequently than expected by chance, or that are differentially expressed between BM and matching primary tumours (section 4.3). A unifying observation from the sequencing of primary tumour genomes is that cancer is underpinned by vast heterogeneity, with a seemingly infinite number of roads leading to Rome. However, in metastatic disease the distant 'host organ' is a common denominator, and so based on the idea that the neural niche may surpass intrinsic alterations in BM with different histologies and treatment histories, our group profiled the genomic and transcriptomic landscapes of BM from melanoma, lung and breast cancers [12]. We integrated the data to identify recurrently altered, functionally interconnected gene networks (DNA repair, axon guidance, HER/*ERBB* and protein kinase-A signalling), and individualgenes harbouring an unusually high number of expressed mutations that were predicted to be deleterious (e.g. *DSC2*, *ST7*, *PIK3R1*, *SMC5).*

The main limitations of using clinical samples in this way are; firstly, a lack of full control over other variables like patient age, germline modifiers and treatment history. Second, sampling bias – profiling is usually performed on small pieces of larger tumours, which may not be representative of the whole tumour. Using larger amounts of tissue to circumvent sampling bias necessitates sequencing with more depth (thus more cost) in order detect subclonal alterations. Finally, clonal diversity reflects the growth requirements at the time of surgical excision, and so alterations that facilitate earlier stages of metastatic progression may be heavily diluted once selection pressure wanes – profiling human BM is essentially an endpoint analysis biased toward the latest stages of the metastatic cascade.

4.3. Subtractive analysis of breast cancer-brain met pairs

Comparative analysis of patient-matched pairs of primary tumour and normal tissue is essential to determine whether genetic variations are somatically acquired or inherited, and a 'trio' that also includes matching metastatic deposits is often considered the pinnacle for identifying metastasis-associated changes. In 2010, Ding and colleagues profiled the exomes and CNAs of a BM, primary tumour and a PdX model, and showed that BM are seeded from a minority of primary tumour cells [83]. The tumours were closely related, with 48/50 variants present in all three lesions. There was extensive heterogeneity in the primary, but a reduced MAF range in the BM and PdX, reflecting the selection processes involved in metastatic progression. Brastianos and colleagues recently sequenced the exomes of 86 trios, achieving impressive statistical power for deep analysis of clonal selection [84]. The majority of cases exhibited branching evolution, with a 'trunk' of shared changes and a series of 'private' mutations reflecting continual independent evolution. Alterations predicting sensitivity to PI3K/AKT/mTOR, CDK and HER2/EGFR inhibitors were identified; and importantly, in 53% of patients, these clinically informative or targetable mutations were not detected in the primary tumours. While extracranial disease deposits (e.g. lymph node mets, pleural effusions) may be more accessible for biopsy than BM, these were also highly divergent. These deep genomic data extend other findings from matched metastatic deposits (Table 2), which collectively indicate that management decisions should be based on BM diagnostic profiling wherever possible, rather than primary tumour or extracranial disease biomarkers.

Matched clinical sample pairs are often only available as archival specimens, with severely fragmented RNA. However, specialised technologies have been successfully applied to identify candidate mediators of BM development. DASL expression array profiling on 39 matched pairs revealed *ERBB3* (HER3) and its adaptor protein *GRB7* amongst genes most significantly induced in BM [56]. HER3 induction and activation have since been confirmed in independent, matched BM cohorts from breast and lung cancers, and unmatched BM from a wide range of primary cancer types,

suggesting this is an adaptation of carcinoma cells to the neuregulin-rich microenvironment [12,56,85,86]. Others found that double-strand DNA damage repair genes (including *BARD1* and *RAD51*) were over-represented in the BM compared to matching BCs [46], and in a third example, GEX profiling of 8 BM compared to unpaired but clinically matched BC found that overexpression of Hexokinase 2 (*HK2*) was associated with poor survival [87].

Anecdotally, there is often an expectation that identifying private alterations and differentially expressed genes will reveal the (epi)genetic history of metastatic disease and illuminate the biology underpinning progression, because the first studies of this kind were revolutionary (e.g. [56,70,83]), and according to classical scientific method, the most robust hypothesis testing involves test and control. But even the ostensibly 'controlled' design of matched pair/trio studies is limited by unavoidable issues that confound interpretation of any tissue profiling experiment – sampling bias, and the 'endpoint' nature of the analysis. Notwithstanding the insights that subtractive approaches can provide into the biology involved, they discount candidates that may have important roles at both primary and metastatic sites, and the candidates identified are not necessarily clinically relevant, as primary BCs are usually successfully treated months or years before clinical presentation of brain disease.

5. Factors underlying the recalcitrant behaviour of brain metastases

5.1. Late detection

Currently, surgical stump recurrence is common because BM are poorly demarcated, excising a margin of normal brain tissue is not appropriate, and the residual cells that persist in the post-irradiation tissue environment are very pervasive [88]. There is an urgent need for effective molecular-targeted therapies to augment local control measures [58,89], but prospective clinical data for established BM are lacking. This is largely because historically, a heavy co-morbidity burden and dismal prognosis restricted participation of BM patients in clinical trials. Where they were included but there was no/minimal impact on intracranial disease progression, this was often interpreted as an overall lack of efficacy, but we know that dosing is critical for achieving optimal delivery [90], and that the brain microenvironment impacts substantially on uptake and efficacy [58,60,89,91]. Some trials have assessed brain relapse as a secondary endpoint (e.g. [92]), though were essentially assessing prevention, or efficacy against small, asymptomatic BM with intact microvasculature. With time, rational modification of traditional clinical trial design [93], and recognition that BM are not completely impenetrable to circulating agents, this trend is beginning to change (Table 1).

Recurrence also occurs at new sites in the brain. Deep exome sequencing has shown that in any given patient, there is far less divergence amongst consecutive BM than between BM and matching primary or extracranial tumours [84], consistent with new lesions arising from stochastic DTC awakening and/or self-seeding. Even synchronous BM that presented months apart were found to be close relatives despite treatment in the interim. Thus suggesting that the next level of therapeutic resistance was attained with minimal additional genomic change, through epigenomic or non-coding alterations not captured by exome sequencing, and perhaps also that requisite changes can be dynamic, reversible adaptations mediated largely via microenvironmental cross-talk [50,94]. It is becoming clear that the role of the brain microenvironment in driving therapeutic resistance has been severely underestimated [58,95]. For example, neither trastuzumab alone nor in combination with pertuzumab reduce the incidence of BM, they do delay their development, reflecting a window of time in which resistant clones grow out after an initial debulking effect [92,96].

In any case, by the time they are symptomatic and detected clinically, BM are essentially highly evolved manifestations of BC that have already developed resistance to multiple lines of therapy, and can efficiently adapt to new extrinsic pressures. So long as they are identified at such a late stage, any treatment benefits will continue to be incremental [97]. Conventionally, diagnostic imaging is performed on symptomatic patients, not for screening those at high risk; yet animal imaging data show that that DCE-MRI grossly underestimates the true extent of disease [98], and we know that up to 75% of BM are asymptomatic [99]. If metastatic screening is to be considered in the future to see if

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we can treat small and/or dormant lesions with curative intent in at least a proportion of patients, we need to couple the transition to precision cancer care with a search for reliable diagnostic imaging targets [100], and develop clever conjugates that can cross the BBB to access small, dormant deposits with intact microvasculature.

5.2. Abnormal vascular perfusion and hypoxia lead to inadequate drug uptake and therapeutic resistance

At a cellular level, the neurological symptoms associated with BM are due to displacement and damage to normal brain tissue as tumours proliferate within the confines of the cerebrum, but vasogenic oedema and elevated intracranial pressure are also involved. Ongoing proliferation in solid tumours can fuel a perpetual cycle of hypoxia and unchecked neo-angiogenesis, creating chaotic, dysfunctional microvascular networks [101]. Constant vascular remodelling results in dyscoordinated vasoregulation, abnormal hydrostatic pressure gradients and blood flow patterns; which, paradoxically, creates areas of sluggish blood flow and poor drug penetration in an otherwise hypervascularised environment [28,101,102].

Abnormal fluid dynamics also reduces drug efficacy because it leads to patchy hypoxia. Radiotherapy and certain cytotoxics act by generating reactive oxygen species that damage DNA, but strand breaks are more readily repairable in the absence of oxygen, allowing cells to escape fatal chromosome aberrations and instead, erroneously repair DNA to increase genetic diversity. Hence there are strong links between tumour hypoxia, cancer stem cell activity and chemo-/radio-resistance [103]. Indeed, BM are characterised by abnormal DNA repair processes, and hypermutator phenotypes have been associated with drug resistance in PdX of BM [46,55,78].

6. Future directions and final comments

Given that inadequate and uneven delivery are critical factors limiting drug penetration, the field is beginning to explore alternative modes of drug delivery other than classic intravenous supply of naked compounds [104]. For example, there are several active clinical trials attempting to chemo/radiosensitise BM by targeting neoangiogenesis with VEGF signalling inhibitors (e.g. bevacizumab, sorafenib; Table 1). The intended effect of anti-angiogenic drugs was originally to starve tumours of oxygen, but in the context of combination regimens, blocking development of immature vessels may be beneficial because it reduces vascular tortuosity, normalises perfusion dynamics and *increases* oxygenation, thus enhancing drug delivery and the efficacy of DNA-damaging agents – the so-called 'vascular normalisation' effect [101,105].

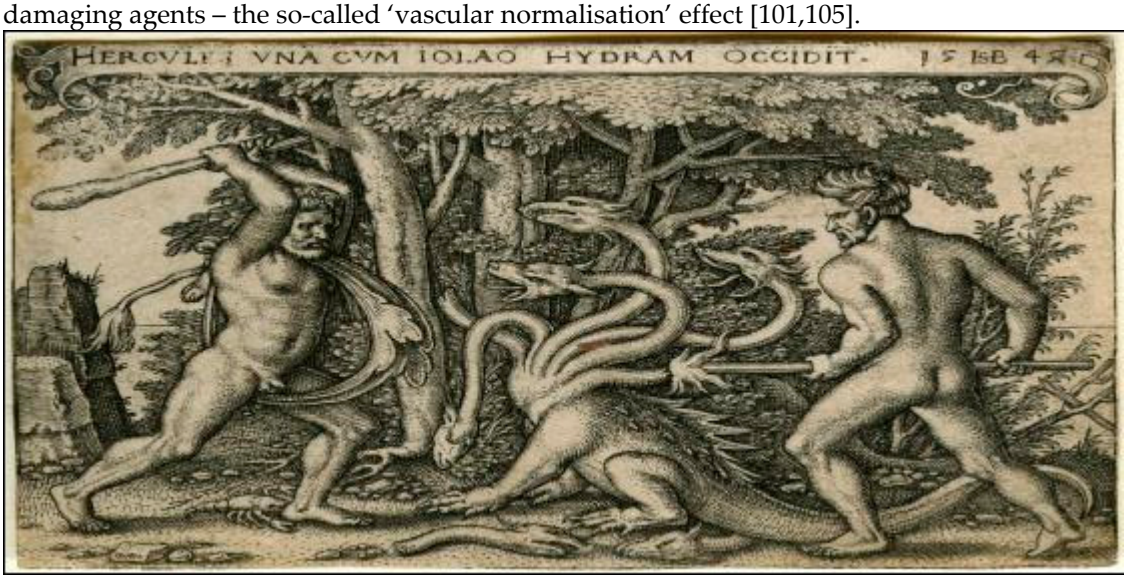

Figure 2: the ancient Greek/Roman fable of devine Hero, Heracles and his nephew (Iolaus) fighting the Hydra of Lerna, a serpentine water monster with heads that regenerated stronger and more numerous if severed [1].

Other innovative approaches being investigated include carrier- or receptor-mediated drug transport across the BBB [104], and injected microbubbles that oscillate and permeabilise the BBB when energised using anatomically focused, MRI-guided ultrasound [106,107], which could improve drug bioavailability and improve immune recognition. Others are focusing on the development of multifunctional nanoconjugates designed to overcome multiple biological and physiological barriers before releasing their therapeutic payloads [108-110]. This is a very attractive concept for cancer therapy generally, but particularly for brain tumours where the BBB represents a unique challenge. Moreover, when it is not feasible to obtain a tissue biopsy to inform management decisions (e.g. high comorbidity burden, oligometastatic disease or inoperable anatomical location), nanoconjugates could provide unprecedented theranostic capabilities [111] n ancient Roman and Greek mythology, the Lernean Hydra was a serpentine water monster, raised by the goddess Hera to kill her illegitimate stepson and son of Zeus, Heracles. Hydra had toxic breath, multiple heads with venomous fangs, and grew new heads for every one severed by its opponents (Figure 2). In a way, the myth is an analogy for metastatic brain disease, symbolising the perceived hopelessness of challenging an apparently immortal beast that becomes more empowered with every attempt to decapitate it. In the end, Hydra was defeated by Heracles, who used the serpent's own poisonous blood to burn each severed head so it could not regrow.

Numerous strategies have been proposed to improve the clinical management of BC patients with metastatic brain disease, including prevention; better risk prediction and diagnosis of small, more manageable BM using molecular imaging; targeted drug delivery vectors like nanoparticles; application of targeted agents to the neurosurgical cavity and externally activating sites of bioavailability to targeted drug conjugates (e.g. ultrasound BBB permeabilisation). Taking the Hydra analogy further, one idea gaining more support in the biomedical community is simultaneous targeting of tumour cell abnormalities and features of the neural niche on which growth and drug resistance depend [58,112,113]. Multiple studies have now verified the role of the metastatic brain tumour microenvironment in driving adaptation, outgrowth and drug resistance [12,37,40,48-50,94]. A noteworthy example of how these findings are being clinically translated is an ongoing Meclofenamate feasibility study (an NSAID traditionally used for treatment of pain but which also inhibits gap junction gating). The community eagerly awaits similar examples in the future.

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