Understanding and overcoming tumor heterogeneity in metastatic breast cancer treatment

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Abstract

Rational development of targeted therapies has revolutionised metastatic breast cancer outcomes, although resistance to treatment remains a major challenge. Advances in molecular profiling and imaging technologies have provided evidence for the impact of clonal diversity in cancer treatment resistance, through the outgrowth of resistant clones. In this review we focus on the genomic processes that drive tumoral heterogeneity, the mechanisms of resistance underlying metastatic breast cancer treatment and discuss implications for future treatment strategies.

Introduction

Breast cancer is the most common malignancy in women worldwide. Substantial improvements in survival of people with breast cancer over the last 30 years has been attributed to screening, enhanced locoregional treatment and increasingly effective systemic adjuvant therapies in early-stage disease (1). In the metastatic setting, rational development of targeted systemic therapies has improved median overall survival (Table 1), although many targeted therapies remain costly and can cause detrimental side effects. Promisingly, small subsets of patients with metastatic breast cancer (mBC) may be cured by combination therapy. In HER2 positive (HER2+) breast cancer (BC), cancers that overexpress HER2 or have amplification of the *ERBB2* (*HER2)* gene, the landmark CLEOPATRA trial demonstrated that 16% of patients remain progression free at 8 years and may be effectively cured (2). Similarly, combinations of CDK4/6 inhibitors and endocrine therapy for the treatment of hormone receptor positive and HER2 negative (HR+ HER2-) disease, improve overall survival(3, 4), and importantly increase the proportion of patients with long term responses that last for many years(2, 5). Yet resistance to treatment remains the major challenge, rendering most mBC incurable, claiming approximately 500,000 lives every year (6). Here, we review drivers of resistance to BC targeted therapies, in particular the role of intratumoral heterogeneity (ITH) in resistance, and discuss potential treatment strategies to further increase cure rates. The focus of this Review is proportionately reflective of the scientific advances in our understanding of heterogeneity in resistance of HR+ disease.

Advances in molecular technologies have allowed direct measurement of clonal diversity of cancer(7, 8), ITH is a result of this diversity that occurs within the same tumor. Substantial evidence now exists in support of ITH as a key contributor to therapeutic resistance, (Figure 1), especially in the metastatic setting. Clinical intrinsic resistance refers to the failure of cancer to show clinical/ radiological response to treatment, due to the presence of pre-existing resistance mechanisms, whereas clinical acquired resistance refers to an initial clinical/ radiological response followed by cancer progression on treatment, due to the eventual development of resistance mechanisms. The clinical pattern of resistance may not directly reflect the cellular origin of resistance. Intrinsic resistant subclones expand in the face of

powerful selection pressures imposed by potent anti-cancer therapies, which may initially manifest as a radiological mixed response, although ultimately uniform failure of therapy ensues. The clinical pattern of acquired resistance may reflect diverse cellular mechanisms, for instance the pre-existence of an intrinsically resistant rare subclone in the cancer that may be selected by therapy, or *de novo* development of a resistance mechanism in a clone that subsequently expands, or a phenotypic shift in the cancer that leads to resistance without needing to evoke Darwinian concepts of clonal evolution.

Histology- and immunohistochemistry (IHC)-based classification of BC has defined three clinical subtypes: HR+HER2-, HER2+ and triple negative breast cancer (TNBC). These represent a crude, indirect measure of inter-tumoral heterogeneity, and allow for the appropriate selection of subtype-targeted therapies. Molecular profiling has allowed further subdivision into four main classes: luminal oestrogen receptor positive (luminal A and B), HER2 enriched, and basal-like (9); moreover multiple transcriptomic subgroups of TNBC have also been defined(10). Gene expression profiling on tissue bulk sample analysis has become an invaluable tool in clinical practice for estimating outcomes in early BC, however such tissue bulk analysis provides more limited resolution of ITH within a tumor sample. Technological advances such as the analysis of genetic material from circulating tumor cells (CTCs) at a single-cell resolution provide a means to more precisely measure ITH from heterogeneous, longitudinally collected CTCs, for real time selection and monitoring of therapy which remains an unmet need.

Heterogeneity in metastatic breast cancer

ITH refers to the molecular variation within each tumor, both genomic and non-genomic, that leads to differences between populations of tumor cells. The extent of genetic heterogeneity as an underlying feature of mBC has been demonstrated, down to single cell resolution (11). As reviewed elsewhere (39), non-genomic heterogeneity, encompasses both stochastic heterogeneity, caused by spontaneous variations in biological processes between genetically identical cells whereby random diversity ensues(12), or deterministic heterogeneity caused by variations in epigenetic modifications or DNA methylation profile. While non-genomic and genomic ITH both likely contribute to treatment resistance, this Review focuses predominantly on genomic ITH in BC.

Multiple mutational processes from exogenous and endogenous sources, contribute to the somatic mutational landscape in cancer. Next generation sequencing (NGS) together with computational tools can delineate clonal and subclonal mutations, aiding the differentiation between “driver” mutations which actively contribute to cancer formation from “passenger” mutations which have no direct cancer promoting role, but represent an important historical record of the processes active during cancer formation (13). Although BC has the highest number of amplified driver genes (mean of 2.1), compared to other solid tumours (14), interrogation of the many thousand passenger mutations has even further resolved the mutational patterns and underlying mutational processes in tumorigenesis (13). In malignancy, the vast number of cancer cells also leads to ‘neutral drift’, with accumulation of passenger mutations in rare subclones of a cancer(15). When a cancer is treated with a therapy the selective pressure this exerts may switch some of these accumulating mutations from ‘passenger’ to ‘driver’, resulting in outgrowth of a resistant subclone (Figure 2).

In addition to large-scale efforts by the Tumor Cancer Genome Atlas (TCGA) and the International Cancer Genome Consortium (ICGC) that have enumerated primary BC genetics (16, 17), sequencing of 617 mBC samples uncovering nine established cancer genes (*TP53, ESR1, GATA3, KMT2C, NCOR1, AKT1, NF1, RIC8A,* and *RB1)* that were more frequently mutated in the metastatic setting compared with early BC (18). Genomic comparisons of matched primary and

metastatic samples have also revealed that metastatic breast tumors frequently possess a higher numbers of mutations (mutational load), including driver mutations, and somatic copy-number aberrations compared to matched primary tumors(19). In HR+ HER2- BC, this high mutational load in the metastatic tissue is likely to reflect the selection and outgrowth of resistant clones and acquired mechanisms of treatment resistance, as we discuss later.

In individual cancers, driver mutations found in metastasis may not be found in the matched primary tumours(18, 20). This may be in part due to limitations in sampling whereby small subclones are missed in the primary specimen, but are selectively expanded in the metastasis. Alternatively, it is likely that some driver mutations have occurred after the cells have disseminated from the breast (true acquisition). Breast cancer brain metastases may be particularly clonally distinct, characterised by a greater number of private mutations relative to other metastatic sites(21). This may indicate that certain driver mutations are specific to the organ to which they metastasize, and in turn contribute to heterogeneous response or resistance to therapies between distant metastases.

Processes that drive heterogeneity and evolution mBC

Genomic drivers

During metastatic dissemination and colonization in distant organs, and through the treatment of mBC, different mutational processes may be active. Thus, the genome of each mBC cell may be viewed as a combined evolutionary record from three sources: the ancestral “normal” cell type (which may differ between luminal and basal-like BC), the primary breast tumor, and the process of metastatic dissemination(22). This concept is illustrated by a landmark study using a mathematical approach to capture the signatures of mutational processes in each tumor, reflecting the combined accumulation of genetic mutations caused by chemical, physical or biological processes (23). Such studies have identified prominent signatures in BC indicative of DNA repair deficiencies, and endogenous mutagenic processes, such as those involving activated DNA cytidine deaminases (APOBECs), among others. APOBEC accounts for 15% of all mutagenesis in

sequenced human tumours (24). Whilst APOBEC enzymes play an important physiological role in restricting viral infections(25), aberrant enzymatic activity may promote oncogenic mutations such as *PIK3CA* mutations, and may also contribute to genomic instability and genomic heterogeneity within tumours, through the generation of localized clusters of mutations at DNA translocation sites known as "kataegis" (26).

Mutational signatures including APOBEC are amenable to detection in clinically relevant samples (13). The PlasmaMATCH multi-cohort platform trial has reinforced that APOBEC plays a vital role in shaping the subclonal diversity of HR+HER2- advanced BC, with recurrent second mutations observed in *PIK3CA* at APOBEC mutagenesis sites (27). Interestingly the enrichment of signature 17 may reflect prior chemotherapy exposure in HR+HER2- BC(28). Furthermore, it is apparent that there is a shift from age-related mutagenesis in primary BC toward more APOBEC-driven processes in HR+HER2- mBC (28). Thereby highlighting the role of mutational signatures in identifying the processes that promote heterogeneity.

Determinants of heterogeneity

Subclonal expansion, following selective outgrowth of any given cell clone, has been described to follow branched or linear evolution (Figure 2). The theory of cancer as an evolutionary process was first described by Peter Nowell in 1976, drawing implicit parallels to Darwin's theory of natural selection, a stepwise accumulation of somatic mutations along tumor progression, with sequential and subclonal selection of the fittest clones (Figure 1). ITH in the metastatic setting may manifest through acquisition of more fit clones in individual metastases, that may subsequently seed metastasize in pre-existing or new sites, and the microenvironment of different sites may differentially select fitter clones. Importantly the additional pressure of targeted therapies, result in selection of resistant clones, potentially driving further ITH in the cancer.

Another layer in understanding tumor-intrinsic determinants of ITH is the cancer stem cell (CSC) model, whereby a subpopulation of tumor cells are identified

as CSC due to their unique ability to initiate and sustain tumor growth. Similar to normal stem cells, CSC are often characterised by their expression of drug-efflux proteins, and thus may be less sensitive to therapies than the CSC progeny comprising the bulk of the tumor, and may drive therapy-resistant regrowth (29). Pre-clinical data showing the ability of human mammary epithelial cells to de-differentiate into CSC-like cells with tumorigenic capacity, has challenged the view of a strictly defined CSC population in BC (30), and correlated with higher mortality (31). Whether the CSC phenotype is an evolutionary outcome of clonal competition, a transient state or a distinct separate population, it nonetheless likely plays a key role in development of intrinsic and acquired resistance to targeted therapies, as reviewed extensively elsewhere(32).

The complex architecture of supportive stromal, immune, and endothelial cells that make up the tumor microenvironment (TME), can also contribute to ITH during tumor development, by exerting selective pressure for cells adapted to certain microenvironmental conditions (33). Patterns of TME gene expression appear to change with BC phenotypes (34, 35), and the ways in which paracrine signalling and TME crosstalk influence gene expression in BC is reviewed elsewhere (57). Single cell analyses of human BC, have revealed substantial transcriptomic heterogeneity both in the carcinoma cells, as well as the non-carcinoma microenvironmental cells (36-38). Multi-platform profiling of multi-regional metastasis in autopsy studies, have revealed that the immune TME is not uniform across metastatic sites within a single patient (21). In addition to the diversity of tumor-infiltrating lymphocytes (TILs) within each patient and between patients, PD1 and PDL1 expression was highly variable. Interestingly tumor phylogenetic trees appear to be correlated with TIL-TCR (T-Cell receptor) trees across metastases, suggesting co-evolution between tumor diversity and T cell response across metastases(21). This heterogeneity in the immune TME has important implications for understanding immune surveillance during tumor progression, and in responsiveness to immune checkpoint blockade in mBC.

Metastatic dissemination of BC

Extensive ITH within mBC leads to the question of how BC disseminates from the primary site of origin, and when in the process ITH originates. Phylogenetic analyses have been used to interrogate evidence for monoclonal and polyclonal models of mBC dissemination (Figure 1). Ideally, a direct comparison of multiple metastatic samples from the same patient, would establish if there are common events amongst metastatic clones that may act gatekeepers in metastatic progression; yet in practice obtaining multiple biopsies from each patient at different time points is very challenging, so studies of this design are rare. In two autopsy studies, each of 10 patients with mBC, examples of both monoclonal and polyclonal evolution (21) or predominantly polyclonal origins (19) were seen in individual patients. The former study also observed a metastasis-to-metastasis seeding pattern in the monoclonal model, whereas the latter concluded that metastases are initiated and maintained as groups of cellular clones, suggesting a polyclonal seeding pattern. Furthermore, WES data on treated and untreated mBC samples, has demonstrated that polyclonal seeding appears to be more prevalent in untreated metastasis than treated metastases, likely due to treatment pressures selecting for resistant subclones that manifest clinically as monoclonal metastases (39). Collectively, these phylogenetic patterns have, suggested that ITH is predominantly generated after the initiation of metastasis (19).

Subclonal divergence of individual metastases has been consistently observed in the vast majority of studies. Evidence exists that the seeding pattern may be dependent on BC phenotype, with a predominance of monoclonal seeding in primary luminal cancers, and both seeding patterns seen in non-luminal primary tumors (40). This notion of a phenotype-dependent seeding pattern is supported by data from two patients with metastatic TNBC, both exhibiting polyclonal seeding patterns (41).

The extent of subclonal divergence between multiple metastases within a patient is variable. Heterogenous population of driver and passenger mutations are apparent within each metastatic site, albeit specific subsets of metastases appear to be more closely related to each other than they are to others(21, 40). It is likely this geographical ITH, underlies the clinical phenomenon of differential response and progression, observed frequently in more heavily pre-treated patients. Receptor

subtype conversion from primary to metastatic disease, coincides with significant subclonal divergence (42).

While these studies have greatly advanced our understanding human mBC, they come with caveats that genetic phylogeny data in isolation provides an incomplete picture of how tumor cells evolve, as intermediate clones that do not persist are not observable, and this approach does not capture phenotypic and TME contributions. Consequently, the general mechanisms of tumor dissemination remain open for discovery. In particular, analysis of phylogenetic data requires model fitting, that may overestimate polyclonal seeding patterns especially where data is inferred from clustering mutations from bulk sequenced tumours. At the same time although it is evident that polyclonal dissemination does occur in BC, it is likely that these events are still being underestimated as under-sampling of the primary and/or metastatic lesions is likely to lead to monoclonal inferences. Therefore, cases demonstrating monoclonal dissemination requires further scrutiny.

Resistance to targeted therapies and the role of cancer heterogeneity in resistance

The theory of clonal evolution would support that targeted therapies represent a potent selection pressure leading to the outgrowth of resistant subclones(8) (Figure 1). The ability to differentiate whether resistant clones exist prior to treatment and are selected under treatment pressure, or whether they develop as a result of treatment, is paramount for assessing resistance and considering future therapeutic strategies. Here we describe key mechanisms of resistance and how they evolve on targeted treatment in the three main subtypes of BC.

HR positive mBC

HR+ BC, expressing oestrogen receptor (ER) and/ or progesterone receptor (PR), accounts for 70% of all BC(43), and is characterised by expression of ER and its downstream targets, one of which is PR (44,66). Endocrine therapy remains the standard of care for HR+ mBC, increasingly given in combination with other targeted therapies. Although almost all patients will eventually develop resistance to

treatment, loss of ER expression leading to endocrine insensitivity only accounts for up to 20% of patients(45).

Resistance to endocrine therapy

Aromatase Inhibitors (AI) work through depleting the oestrogen ligand, and resistance to AI often involves mutations to the oestrogen receptor gene (ESR1) resulting in ligand-independent constitutive activation. *ESR1* mutations can also alter the chromatin binding properties of the ER, resulting in differential ER-regulated gene expression, and a more pro-metastatic phenotype (46). While *ESR1* mutations are rare in primary BC (0-3% of patients) (47-49), and comparatively infrequent in patients who relapse following previous adjuvant AI therapy (7-12%) (50,51), in mBC previously treatment with AI *ESR1* mutations are present in 20- 55% of patients (47, 52).

ESR1 mutations are frequently subclonal in HR+ cancers, and strikingly, in a study of patients receiving AI between 28.6- 49.1% of patients with *ESR1* mutations detected in circulating tumor DNA (ctDNA), harboured polyclonal *ESR1* mutations (53). The presence of multiple *ESR1* mutations in one patient highlights the substantial selection pressure for these mutations during endocrine therapy, and the contribution of ITH in resistance processes. All of these observations likely suggest that the large tumor bulk of metastatic disease is important in *ESR1* mutation development, with the low tumor volume of micro-metastatic disease not allowing *ESR1* mutations to commonly develop (52). Such mutations may arise through clonal selection of low abundance intrinsic resistant clones, or alternatively these could arise through genuine acquisition due to error-prone replication taking place in the cancer during the course of endocrine-based combination therapies.

Once *ESR1* mutations have emerged that are detectable in ctDNA, this is indicative that a cancer is intrinsically resistant to further AI therapy. The endocrine therapy, fulvestrant has limited activity against *ESR1* mutations, exemplified by the phase III SoFEA and EFECT trials of advanced HR+ mBC, with two main treatment arms, fulvestrant +/- anastrozole and exemestane. Patients with an *ESR1* mutation benefited from slight improvement in PFS after taking a fulvestrant-containing regimen versus the AI exemestane (median PFS 3.9 months versus 2.4 months).

Furthermore, subgroup analysis of *ESR1* mutations demonstrated a worse one year overall survival of patients receiving exemestane (62%) compared to fulvestrant (54). Drugs aimed to have improved efficacy against activating *ESR1* mutations are in clinical development, including oral ER degraders (55-57).

Specific *ESR1* mutations may have important differences in function and resistance to therapy. *ESR1* Y537S mutations conferred enhanced resistance to fulvestrant in preclinical research, (46, 58), which is supportive of clinical data from PALOMA-3, a randomized phase III trial of palbociclib plus fulvestrant versus placebo plus fulvestrant, whereby a positive selection of *ESR1* Y537S was demonstrated through fulvestrant therapy, highlighting its role in resistance to fulvestrant therapy (6,59). *ESR1* mutations are also selected through AI and CDK4/6 combination therapy, confirming resistance to the endocrine partner remains relevant in this combination (60).

In sequencing data from 692 single site metastatic tumor biopsies from patients previously exposed to endocrine therapy, activating alterations in the mitogen-activated protein kinase (MAPK) pathway were found in 22% of all tumours, furthermore these were mutually exclusive with *ESR1* mutations (61), suggesting distinct routes of endocrine resistance. In contrast, in ctDNA data from the PlasmaMATCH trial, MAPK alterations were co-enriched in patients with *ESR1* mutations(62). These distinct findings likely reflect multiple geographical resistance mechanisms that are not apparent by sequencing individual tissue sites, but can be captured by ctDNA fragments that are continuously released to the bloodstream from potentially all metastatic sites. The presence of both *MAPK* and *ESR1* mutations in ctDNA identifies a patient population with adverse prognosis, potentially suggesting that cancers with high levels of ITH have poor outcome(62).

In HR+ BC, *PIK3CA* mutations have similar prevalence in the metastatic setting (53%) and early disease (40-45%)(63, 64), whereas *AKT1* mutations are likely enriched in mBC (62). Studies suggest that mutations in *PIK3CA* and *AKT1* are mutually exclusive(65-67). Interestingly neither *PIK3CA* (64, 68, 69) or *AKT1* (70) mutations are associated with worse outcomes in HR+ BC compared with *PIK3CA/AKT1* wild-type.

The BOLERO-2 phase III clinical trial was the first to demonstrate the synergistic activity in dual targeting of both ER and mTOR, with improved PFS for everolimus plus exemestane compared to exemestane alone (71). Subsequently, the SOLAR-I phase III clinical trial, demonstrated improved PFS for the alpha-selective PI3K inhibitor alpelisib, in *PIK3CA* mutated HR+ mBC. A superior PFS of 11 months was noted in those who received combination therapy compared to 5.7 months in the fulvestrant arm(72). Although, the results from these clinical trials indicate cross talk between the PI3K and ER pathway, further research is required to establish the likely complex cross talk between these pathways, as indeed clinical trials have failed to reliably validate an association between *PIK3CA* mutations with endocrine resistance, as seen in preclinical research (96)

Analysis of tumor biopsies has recently demonstrated that approximately 12-15% of patients with *PIK3CA* mutant mBC have multiple *PIK3CA* mutations, frequently occurring in *cis* on the same allele, leading to hyperactivation of PI3K and downstream signalling. Furthermore, it is apparent that the second hit *PIK3CA* mutations are selected at sites characteristic of APOBEC mutagenesis. APOBEC mutational patterns are enriched in HR+ BC exposed to prior endocrine therapy, suggesting APOBEC activity subsequently edits *PIK3CA* resulting in hyperactive PI3K signalling (NCT03182634). Other acquired mutations, such as *ESR1* mutations, do not occur clearly at APOBEC sites and this discrepancy is not understood.

Endocrine therapy also appears to exert selective pressure for alterations in key transcription factors, that may lead to transcriptional reprogramming related to resistance. For example, alterations in *ARID1A,* a gene involved in SWI/SNF signalling, were detected in 5% of primary cases, but increased in up to 12% of cases with treatment resistant HR+ BC(20, 73). Furthermore, CRISPR technology have demonstrated a critical role for SWI/SNF chromatin remodelling in *in vitro* studies with endocrine therapy resistance (74).

MYC amplification is found more frequency in advanced BC, and has long been associated with endocrine resistance and prior progression (87). A comparison

of 28 pairs of matched primary and metastatic tumor specimens demonstrated a higher expression of *c-MYC* in the metastatic tumours following the development of tamoxifen resistance in the same patient (75). Similarly, high levels of *MYCN* amplifications have also been identified in progression samples compared to baseline(76).

Resistance to CDK4/6 inhibitor in HR+ BC

In advanced HR+ BC the standard of care has shifted to combination endocrine therapy and CDK4/6 inhibitors (Table 1), which approximately double PFS compared to endocrine therapy alone and also improve overall survival. Clonal selection dominates has been linked to resistance to CDK4/6 inhibitors, by phylogenetic analysis detected in 85% of patients at progression in one study(5). Multiple genetic resistance mechanisms have been identified, many of which directly involve other cell cycle regulators. The tumor suppressor retinoblastoma (RB1) protein controls transition from G1-S phase in the cell cycle. During G1 several growth signals result in cyclin D binding to CDK4 or 6 causing inactivating hyper-phosphorylation of RB1, activation of E2F that promote the S phase transcription programme(77). Loss of function mutations of *RB1* cause intrinsic resistance to CDK4/6 inhibition in preclinical models (78-80), and may also be acquired subclonally in patient derived xenografts selecting by prior therapy (78). While rare in HR+ primary BC, *RB1* mutations are enriched in in up to 4% of metastatic cases (18). As both baseline *RB1* mutations (35) and acquired, often subclonal, *RB1* mutations have been associated with resistance to CDK4/6 inhibitor therapy, alternative treatments are begin sought after for these patients (5, 81). As one example, preclinical data has demonstrated synthetic lethality with aurora kinase inhibitors in RB1-deficient models(82).

Gene amplification of CCNE1 and overexpression of Cyclin E1 leading to bypass activation of CDK2, has been observed in some models with CDK4/6 inhibitor resistance (78), and in biopsies from patients who progressed on this therapy, likely reflecting a phenotypic shift in the cancer(83). CDK6 overexpression, mediated by CDK6 amplification or in some cases rare FAT1 mutations, have also been reported to promote resistance to CDK4/6 inhibitors(84), (85).

Selection of *PTEN* (suppressor of the PI3K/AKT pathway) loss has been observed in serial biopsies and rapid autopsies from patients treated with a combination of ribociclib and letrozole (86). Increased *AKT* activation and PTEN loss promoted resistance to CDK4/6 inhibition *in vitro* and *in vivo*, in a mechanism involving CDK4 and CDK2 activation *(86)*. Aberrant Fibroblast growth factor receptor (FGFR) activity has been associated with early relapse and shorter overall survival, specifically in HR+ BC(87). More recently, *FGFR1* gene amplification has also been associated with resistance to CDK4/6 inhibitor therapy leading to a shorter progression-free survival. *FGFR1* amplification in cell lines and xenografts display increased resistance to fulvestrant and palbociclib compared to FGFR1-nonamplified models. Interestingly this resistance was reversed with the FGFR tyrosine kinase inhibitor lucitanib (88). However, whether this could be used clinically for CDK4/6 inhibitor-resistant disease may be dependent on a high-level clonal *FGFR2* amplification, as low level or subclonal amplification does not respond to treatment(89).

HER2 positive mBC

HER2 is a member of the human epidermal growth factor receptor family and is encoded by *ERBB2*. This oncogene is overexpressed in approximately 15% of all mBC(90). HER2 forms homodimers and heterodimers with other family members such as *EGFR* or HER3, resulting in potent transduction of downstream signals, which can enhance tumor survival. Advent of anti-HER2 therapies such as trastuzumab have been transformative for women with HER2+ mBC (91), with 10-15% of patients achieving long term non-progression (2). However, due to common resistance to anti-HER2 therapy this remains an aggressive subtype, with brain metastasis occurring in 40-50% of all patients (92). Several mechanisms of resistance have been described which ultimately cause reactivation of the HER2 pathway or its downstream signalling.

Resistance to anti-HER2 therapies

Advances in molecular imaging has furthered our understanding of tumor heterogeneity in HER2+ BC and also has demonstrated clinical utility in identifying

individuals who may benefit from HER2 targeted treatment. The ZEPHIR trial revealed striking levels of inter- and intra- tumoral heterogeneity in HER2 expression, 29% of pre-treated advanced HER2+ mBC were considered HER2-PET/CT negative, with little or no trastuzumab-zirconium uptake. Patients with HER2-PET scan heterogeneity had a median time to treatment failure (TTF) of 2.8 months with the antibody drug conjugate trastuzumab-emtansine (T-DM1, Table 1) compared to 15 months of TTF with homogeneous HER2 overexpression (93). Heterogeneous *HER2* amplification and overexpression is also observed in a small number of primary HER2+ BC, associated with a poor response to T-DM1 in the neoadjuvant setting(94). It is likely that the degree of HER2 heterogeneity is magnified in mBC by selection for HER2-negative subclones following prior therapy, providing a possible explanation of the MARIANNE trial, which failed to show superiority of TDM1 in combination with taxane and trastuzumab in HER2+ mBC(95).

Although infrequent, *ERBB2* mutations have been identified to co-exist with *ERBB2* amplification(16, 96). Activating *ERBB2* mutations are enriched in mBC compared to early disease, however the clinical significance of this remains unclear(97). These mutations are selected with increasing lines of HER2 directed therapy, and may represent a means of subclonally-acquired resistance to trastuzumab based therapy(98). It has generally not been established whether *ERBB2* mutations and *ERBB2* amplification co-occur in the same cells, or parallel evolution of *ERBB2* mutations in non-amplified cancers. Activating *ERBB2* mutations, have been identified in extracellular, transmembrane and tyrosine kinase domains, are thought to activate HER2 signalling pathways, even in the absence of *ERBB2* amplification(96).

Alterations in the PI3K/AKT/mTOR pathway, including activating mutations in *PIK3CA* and or loss of the lipid phosphatase *PTEN*, are established resistance mechanisms to HER2 directed therapies (99-102). However, similar proportions of activating *PIK3CA* mutations have been found in both primary and metastatic biopsies, highlighting that these mutations are not necessarily selected during anti-HER treatment(103). BOLERO- 3, a phase III clinical trial in trastuzumab-resistant HER2+ mBC revealed that everolimus in combination with trastuzumab and

chemotherapy provided a modest improvement in median PFS of 7·0 months, vs 5·8 months with placebo(104). Combined biomarker analyses of the BOLERO-1 and BOLERO-3 trials demonstrated an improved PFS in patients harbouring *PIK3CA* mutations or PTEN loss when treated with everolimus (105).

Biomarker analysis of the CLEOPATRA trial, a phase III clinical trial investigating the role of pertuzumab plus trastuzumab plus docetaxel (THP) versus placebo plus trastuzumab plus docetaxel (TH) as first-line treatment for patients with HER2+ mBC, demonstrated that PIK3CA mutations are poor prognostic markers and predict poor PFS to both anti-HER2 therapy arms, although, the *PIK3CA* mutations did not predict a better response to THP than TH(106). In contrast the EMILIA trial, both *PIK3CA* and *PTEN* were associated with a poor response in the control arm (Capecitabine plus lapatinib), compared to the treatment arm with TDM1, indicating that *PIK3CA* and *PTEN* mutations may not result in resistance to the anti-Her2 ADCs(2, 107, 108). Interestingly down regulation of PI3K has been described as positive predictive factor in long-responders on anti-HER2 agents(109).

Cyclin D1- CDK4 is a multi-protein structure needed to drive cell-cycle progression from G1 to S phase. This pathway is regulated by several mechanisms including HER2(110). Several in vivo and in vitro models have demonstrated the role of cyclin D1/ CDK4 in growth of breast tumours driven by ERBB2(110-112), as well as the ability of CDK4/6 inhibition to overcome resistance to anti-HER2 therapy (110, 113). Cyclin E overexpression not only confers a worse prognosis, but is also prevalent in individuals who have not received previous anti-HER treatment, which may suggest utility as a biomarker for intrinsic resistance to HER2-targeted therapy (114).

PATRICIA is a phase II clinical trial investigating the role of combination therapy with palbociclib, trastuzumab +/- letrozole in HER2+ mBC patients who have already received 2-4 lines of anti-HER2 treatment. The combination treatment conferred superior PFS in patients with the PAM50 luminal subtype, compared with non-luminal disease (12.4 and 4.1 months respectively). This trial highlights the importance of defining molecular subtypes in clinical practice (115).

Approximately 50% of patients who are HER2+ are also classified as HR+. Tumours with co-expression of HR and HER2 are less sensitive to endocrine therapy compared to HR+ and HER2 negative tumours. This indicates cross communication between the HER2 and ER signalling pathway, which has been shown to be bi-directional (116). In the PATINA and MONARCHER trials, concurrent inhibition of ER and HER2 led to improved outcomes(117, 118). Multiplex assay technology has demonstrated that heterogeneity of HER2 expression appears to be more pronounced in HR+ and HER2+ tumours compared with HR- and HER2+ (119). This resultant ITH might contribute to a small extent to the inferior pathological cure rates commonly observed in the neo-adjuvant setting in HR+HER2+ tumours (26%) compared to HR-HER2+ (78%)(120).

TNBC

In comparison to HR+ BC, TNBC is a highly aggressive subtype with higher rates of metastasis, relapse, and poor overall survival(121, 122). Despite the molecular heterogeneity observed in TNBC, chemotherapy remains the standard of care. Increasing evidence suggest TNBC are more immunogenic than HR+ BC, characterised by higher levels of TILs and PDL1 expression(123), the significance of this in regards to disease evolution and treatment has been reviewed elsewhere(124, 125). Indeed, the IMPASSION130 trial has demonstrated an improvement in median PFS of 2.5 months with the addition of atezolizumab in PD-L1 positive TNBC, with PD-L1 expression more frequently on immune cells than tumor cells. The mechanisms of resistance to anti-PDL1 therapy in TNBC have so far not been robustly determined. Heterogeneity in PD-L1 expression levels between distinct metastatic lesions may shape the immune response and thereby likelihood of response to atezolizumab (126). In other tumor types, clonal selection of acquired resistance such as acquired JAK pathway mutations have been implicated(127), albeit further research is required in TNBC.

Tackling subclonal heterogeneity in the clinic

Defining heterogeneity in clinics

The genetic evolution of BC over time, particularly in response to treatment, highlights the need for longitudinal biopsies of metastases. Diagnostic technologies have transitioned from open surgical biopsies to minimally invasive techniques, including incisional, excisional, core needle, bite and vacuum-assisted (128). Fixed and paraffin-embedded tissues remain the main method of tissue preservation for clinical diagnosis, although their inability to capture the genetic diversity of solid tumours is apparent (129). Single-site sampling, whereby tissue taken from a single spatial location is currently used as a representation of the entire tumor, introduces substantial sampling bias.

Morphological assessment of tissue based on H&E-stained preparations would benefit from a standardised approach. Incorporation of multi-site tumor sampling is a better alternative that can represent different areas of the same tumor, which can have varying levels of gene expression and differentiation e.g. central core versus external borders, reviewed elsewhere(130, 131). Other sampling techniques include the analysis of the residual tumor in its entirety, as a means to represent ITH of the total tumor mass(132). Furthermore, although many gene expression tests are now available for clinical use in BC including: OncotypeDX, Mammoprint and PAM50, the accuracy of such tests relies on precise microdissection, whereby contamination with normal tissue introduces a strong source of bias in bulk genomic predictors (133). These aspects of ITH need to be incorporated into clinical practice in order to improve reproducibility, and representation of ITH in the molecular analysis of mBC.

The adoption of automated artificial intelligence-based extraction of morphological features based on H&E-stained preparations can be useful to incorporate sub-visual textural heterogeneity measurements(134), and overcome limitations in subjective visual assessments and in some instances improve performance when used in conjunction with standard detection and diagnostic protocols (135). Furthermore, multisite sampling in conjunction with a differentiation score such as the Gleason score for prostate cancer, may be a useful approach in measuring heterogeneity in clinical practice(136).

Advances in molecular imaging present the possibility of assessing gross intra-metastasis heterogeneity, although likely lack the precision for detecting intermixed heterogeneity in individual metastases. Ultimately current diagnostic histopathology techniques based on a single tissue biopsy is insufficient to establish the underlying complex genetic alterations and the biological events involved in BC.

Liquid biopsies, on the other hand, allow non-invasive and repeated sampling for assessment of genomic features and ITH, predominantly using two different sources of circulating genetic information, ctDNA and CTC, which are shed from tumours into the bloodstream, where a small proportion survive, extravasate, and colonize distant sites. Although liquid biopsies have been shown to be highly accurate in assessing tumor genotype, and potentially in predicting which mutations are subclonal, there is limited evidence that assessing subclonality of a mutation is useful in making treatment decisions (137, 138).

Primary and cultured CTC from 19 HR+ BC patients who developed multidrug resistant metastatic disease demonstrated that 82% acquired HER2 expression, highlighting the need to monitor tumor cell subpopulations using CTC which may interconvert, leading to striking consequences for disease progression and drug response (139). Furthermore, it has been demonstrated that CTC-derived quantitative RNA-based digital PCR scoring assay, individualised to cancer-type specific marker, offer a non-invasive means to inform BC treatment by using pharmacodynamics measurements(140).

Early intervention at molecular relapse

The Norton-Simon hypothesis models the growth of cancer and its regression after therapy(141), which is primarily based on the Gompertzian growth curve(142). This theory suggests that initially growth from an overall low tumor burden occurs at an exponential rate, then progresses into a phase of rapid proliferation, and eventually tumor growth reaches a plateau. Currently radiographic detection is only possible when the mass is nearing the final phase of tumor growth. Therefore, it is logical to assume that earlier detection allowing intervention prior to the rapid growth phase could lead to more successful disease eradication (Figure 3). Proof of principle of early intervention on molecular relapse has come from prostate cancer,

where early introduction of enzalutamide in patients without metastatic disease but rising PSA, improves overall survival(143).

Mutation tracking using ctDNA has allowed for the detection of molecular residual disease (MRD) in order to predict relapse in patients with early BC. Among patients who eventually relapsed, 50% had detectable ctDNA in a single post-operative sample drawn 2-4 weeks after completion of therapy for early BC(144). Furthermore, MRD-positive patients exhibited significantly worse disease-free survival than MRD negative patients(144). Serial mutation tracking beyond the postoperative period increased the sensitivity of relapse prediction to 80%-89%(144, 145), with ctDNA detected at a median of 7.9 to 8.9 months earlier than clinical relapse(144, 145). It has been demonstrated that tracking a greater number of individualised mutations can improve MRD detection, albeit sensitivity is driven by the number of tumor mutations available to track(146).

Currently routine surveillance of individuals with a high risk of BC recurrence does not involve regular imaging, nor surveillance with tumor markers, due to the lack of evidence of benefit enshrined in international guidelines. Detection of molecular relapse before macroscopic recurrence, allowing for earlier initiation of therapy whilst metastatic tumor burden (MTB) and clonal diversity are low(52, 144), has the potential to improve outcomes. Clinical trials are ongoing to evaluate this, for example the cTRAK trial in patients with TNBC (NCT03145961).

Metastatic tumor burden may have a key role in subclonal resistance. For example, *ESR1* mutations are only rarely selected during AI treatment of low tumor volume or micro-metastatic disease (52). The exact reasons for this lack of selection remains unknown. It is likely that mutant subclones are not pre-existent in micro-metastatic disease, and that low-level ongoing replication may allow for acquisition of the mutations, whilst micro-metastatic disease is overall more dormant.
Early intervention may be the ideal scenario to investigate therapies that aim to block the subsequent development of ITH in the cancer, for example drugs that aim to inhibit APOBEC enzymes in ER positive breast cancer (147). Timely clinical detection of APOBEC mutational signatures would be critical for this strategy, which may require longitudinal monitoring of high risk patients, via liquid biopsy(148).

Combination therapy to block clonal outgrowth

Combination therapies are a logical solution to target resistance mechanisms emerging from heterogenous cancer cell sub-populations. Indeed, clinical benefit of combining endocrine treatment with CDK4/6, PI3K and mTOR inhibitors is well established. As discussed earlier, preclinical research has helped identify potentially effective combinations and molecular biomarkers, such as the combination of PI3K and CDK4/6 inhibitors in the context of acquired RB1 mutation (78). If effective in the clinical setting, such combination therapy may address the issue of pre-existing heterogeneity and prevent the development of resistance secondary to on-target resistance mutations. Tolerability of such combinations remains one of the main barriers towards clinical implementation. For example, temporary withdrawal of targeted therapies can mitigate the selective advantage conferred upon the drug resistant cells, and enable repopulation of the tumor with drug sensitive cells (Figure 4). Although intermittent dose scheduling can temporarily supress clonal outgrowth of drug-resistant cells, it is unable to eliminate it in its entirety. Therefore, combination strategies that target a smaller population of pre-existing drug-resistant cells and a larger proportion of drug-sensitive cells are likely to be most effective.

Due to the heterogenous nature of mBC, a single tumor will likely contain a mixture of subclones which will be both resistant and sensitive to a particular treatment (Figure 4). Thus, elucidating each tumours genomic makeup, combined with computational based models, may help select the most appropriate combination regimen, and produce the optimal dosing schedules to account for this inherent heterogeneity. Mathematical models, based on data from gene expression profiles and biology networks show promising preliminary results(149-151).

Antibody drug-conjugates, monoclonal antibodies connected by a specified linkage to anti-tumor cytotoxic molecule, provide a unique form of combination therapy whereby a single molecule, can target two distinct populations. Trastuzumab-deruxtecan has a higher drug to antibody ratio compared with T-DM1, with a higher membrane permeability resulting in an increased bystander effect, death of surrounding cells without specified target(152). Results from early clinical trials suggest that Trastuzumab-deruxtecan may have superior efficacy in heavily

pre-treated patients with HER2+ BC(153, 154), with activity also in non-amplified cancers that express HER (HER2 low BC)(155). These studies suggest that the systematic development of ADCs with an innate ability to exert significant bystander effect may help ameliorate ITH.

Enhanced monitoring for evolution of resistance

Non-invasive strategies such as ctDNA analysis have substantial potential to monitor resistance and direct which patients require combination therapy, which may have additional advantage in avoiding treatment-related toxicity in patients unlikely to benefit. Failure to suppress the level of ctDNA early in treatment is linked to poor prognosis, and thus may triage patients as candidates for combination therapy. Similarly, sequential ctDNA analysis through therapy may detect the emergence of resistant clones and direct intervention to block the ongoing growth of the resistant subclone. For example, the PADA-1 study is monitoring for the emergency of *ESR1* mutations on AI+CDK4/6 and testing the early intervention of fulvestrant (NCT03079011).

Understanding curative responses

Within all subtypes of BC there appears to be a small subset of patients with durable responses, some of whom are almost certainly cured by treatment(156). Understanding the basis for sustained sensitivity in these patients will likely be important in developing clinical strategies for circumventing resistance in the remaining majority of patients. For example it is unknown whether a lack of genetic or non-genetic heterogeneity may underlie these responders, or whether these cancers have particularly singular oncogene addiction (157) which blocks routes to developing resistance. It is certainly clear that identifying mutations which occur early in tumorigenesis, or phenotypes unique to the cell of origin, that persist throughout evolution should be important in predicting long-term response. For example HR+ BC is considered positive in the presence of at least 1% tumor nuclei(158), yet cancers with infrequent ER staining are not those that likely achieve longer term responses either to single agent endocrine therapies or in combination.

Conclusion

Scientific and clinical achievements have already led to a substantial improvement in BC related morbidity and mortality. Despite encouraging pre-clinical data, the majority of targeted agents yield a transient response in the clinical setting. Emerging techniques continue to shed light on the complex interplay between genomic and non-genomic heterogeneity in BC and the role in mechanisms of therapeutic resistance. Refinement of BC classification, and studies exploring longitudinal data in the metastatic setting will be essential in elucidating the evolution of BC, highlighting opportunities for more sophisticated personalized medicine and progress toward curing mBC.

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Table 1- A table illustrating a selected range of targeted treatments available for the three main subtypes of mBC and their licenced indications of use as approved by the FDA. Cytotoxic agents have not been included.

Hormone receptor positive HER2 negative					
Treatment				Selected pivotal studies	FDA licensed indication of use
Endocrine therapy	Aromatase inhibitor	ER receptor (indirect)	Letrozole Anastrozole	TARGET(159) ILBCG(160)	Monotherapy: postmenopausal women, including prior progression on tamoxifen. Combination: taken with CDK4/6 in postmenopausal.
			Exemestane	Paridaens et al (161)	
	SERM	ER receptor	Tamoxifen	Borner et al 1994 (162)	Monotherapy: adults with mBC.
	SERD	ER receptor	Fulvestrant	CONFIRM(163)	Monotherapy: postmenopausal women. Combination: given with CDK4/6 in postmenopausal women, and prior progression on endocrine therapy.
inhibitors	CDK 4/6 inhibitor	G1 cell cycle transition	Palbociclib	PALOMA-3(164) MONARCH-3 (165) MONALEESA- 7(166)	Combination: given with AI/ fulvestrant in postmenopausal women and prior progression on endocrine therapy. Premenopausal, therapy should be combined with a LHRH.
			Abemaciclib		
			Ribociclib		
	PI3K inhibitor	Alpha subunit specific	Alpelisib*	SOLAR-1 (167)	Combination: given with fulvestrant, in men and postmenopausal women with a PIK3CA-mutation, following progression on or after an endocrine treatment.
	MTOR inhibitor	mTORC1	Everolimus	BOLERO-2 (168)	Combination: given with Exemestane, in postmenopausal women, and prior progression on AI.

Triple negative					
Monoclonal antibody	Antibody drug conjugate	Anti-TROP-2	Sacituzumab govitecan *	IMMU-132-01(169)	Monotherapy: following prior progression on at least 2 therapies (This indication is approved under accelerated approval)
	PD1 immune checkpoint	PDL-1	Atezolizumab	IMPassion130(170)	Combination: given with nab-paclitaxel whose tumours express PD-L1 (This indication is approved under accelerated approval).
	PD1 immune checkpoint	PD1	Pembrolizumab	KEYNOTE086(171)	Monotherapy: following prior progression, who have no satisfactory alternative treatment options, and microsatellite instability-high or mismatch repair deficient (This indication is approved under accelerated approval).

HER2 Positive					
Monoclonal antibodies	HER2 inhibition + ADCC	HER2 receptor	Trastuzumab	CLEOPATRA(172)	Monotherapy: following at least one chemotherapy regimen. Combination: given with pertuzumab and docetaxel, who have not received prior anti-HER2 therapy or chemotherapy for metastatic disease.
	HER2 inhibition	HER2 receptor	Pertuzumab	CLEOPATRA(172)	Combination: given with trastuzumab and docetaxel, who have not received prior anti-HER2 therapy or chemotherapy for metastatic disease.
	Antibody drug conjugate	HER2 receptor	Trastuzumab Emtansine	EMILIA(173)	Monotherapy: following prior progression on trastuzumab and a taxane.
			Trastuzumab deruxtecan	DESTINY-Breast01(174)	Monotherapy: following prior progression on at least 2 or more prior anti-HER2-based regimens in the metastatic setting.
Small molecule inhibitors	Tyrosine kinase inhibitor	HER2 receptor	Tucatinib	HER2CLIMB(175)	Monotherapy: following prior progression on at least one or more prior anti-HER2-based regimens in the metastatic setting.
		HER2 and EGFR	Neratinib	NALA(176)	Combination: given with capecitabine, following progression on two or more prior anti-HER2 based regimens in the metastatic setting.
			Lapatinib	EGF100151(177)	Combination: given with capecitabine, following progression on an anthracycline, a taxane, and trastuzumab.

BRCA1/2				
Small molecule inhibitors	PARP inhibitor	Olaparib	OlympiAD (178)	Monotherapy: following at least one chemotherapy regimen and with deleterious or suspected deleterious *gBRCAm*.

Abbreviations: SERM- Selective oestrogen receptor modulators, SERD- Selective oestrogen receptor degrader. ADCC- antibody-dependent cellular cytotoxicity. Note endocrine therapy can also be used for HR+/HER2+ BC. *pending approval by EMA at the time of writing.

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Figure 1- Illustrative representation of the clonal evolution of BC. Stepwise accumulation of somatic mutations with sequential and subclonal selection of the fittest clones due to selection pressure exerted by targeted treatment, resulting in a heterogeneous metastatic tumor sites. Metastatic site A demonstrates monoclonal dissemination from primary BC, whereby a single subclone with metastatic potential seeds the metastatic lesions. Metastatic site B demonstrates polyclonal dissemination whereby two subclone from primary seed the metastatic lesion. Metastatic site A demonstrates the development of new acquired resistance after two lines of different targeted therapies. Metastatic site B demonstrates intrinsic resistant clones which lead to the initial clonal outgrowth after first targeted treatment and the partial resolution of these clones during second line of therapy with the development of new acquired resistant subclones

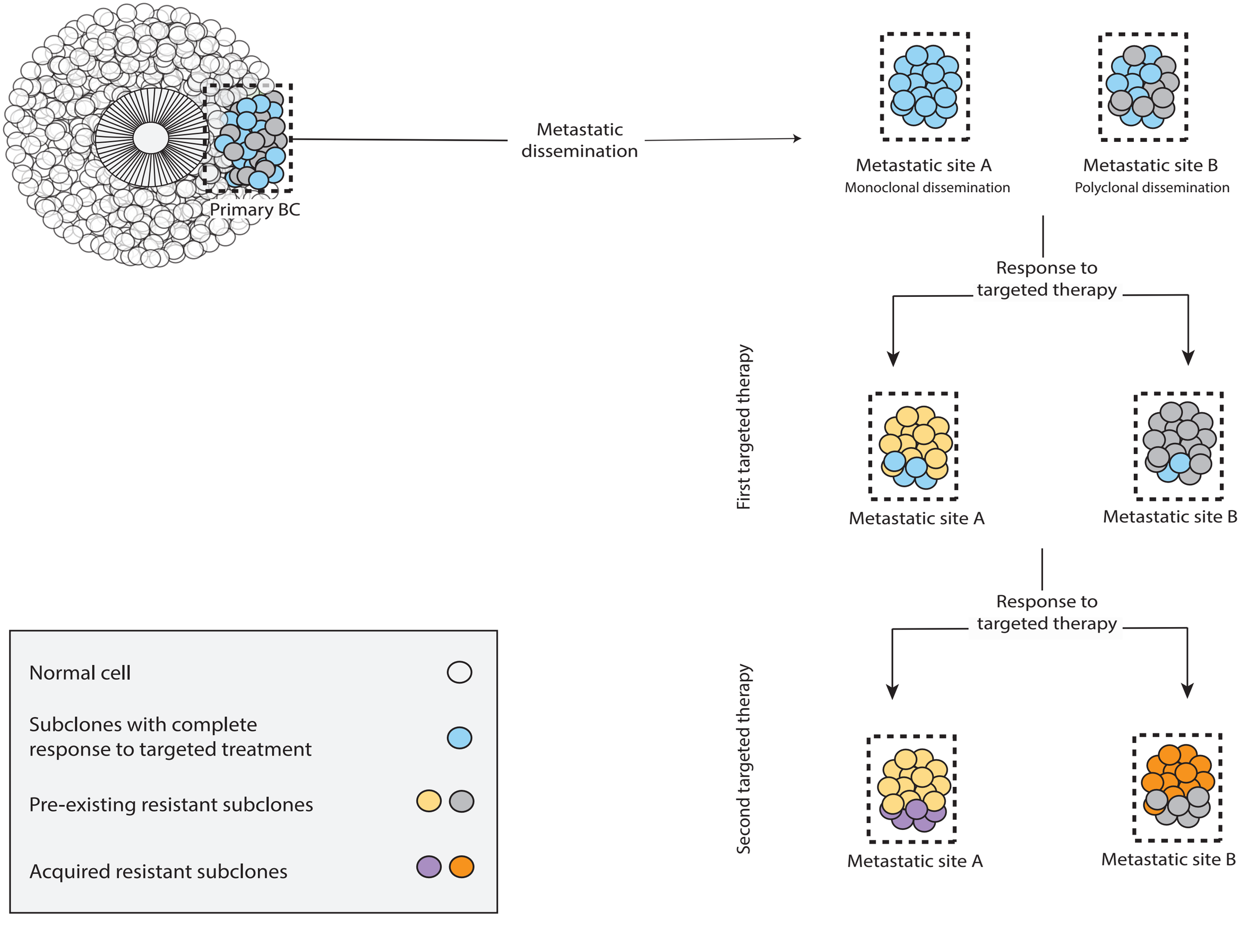
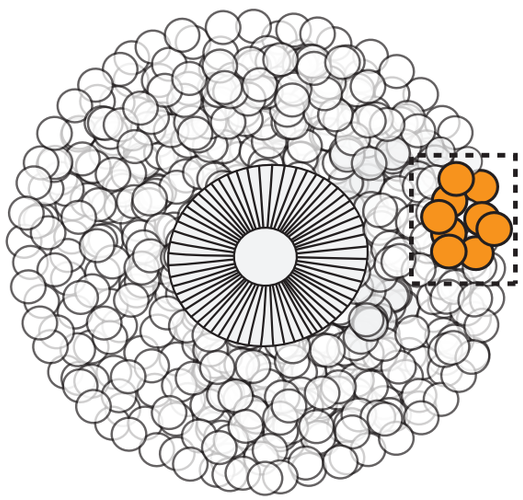


Figure 2- Models of tumour evolution- Tumor evolution is always branched as cells are constantly dividing and mutating. Therefore, at any given time point there will be cells originating from multiple cell lineages. Linear model of evolution suggests that only one cell lineage survives, often this is perceived to be the case due to sampling bias. Mutations are continually occurring however when an adaptive mutation occurs, there is expansion of the 'fittest clone', and this positive selection leads to a selective sweep driving tumour progression. Punctuated evolution occurs when this adaptive mutation expansion occur rapidly, rather than gradual evolution which occurs over a greater duration of time. Neutral evolution occurs when evolution occurs in the absence of positive selection, with secondary mutations and drift in tumour genomics.



Primary BC biopsy

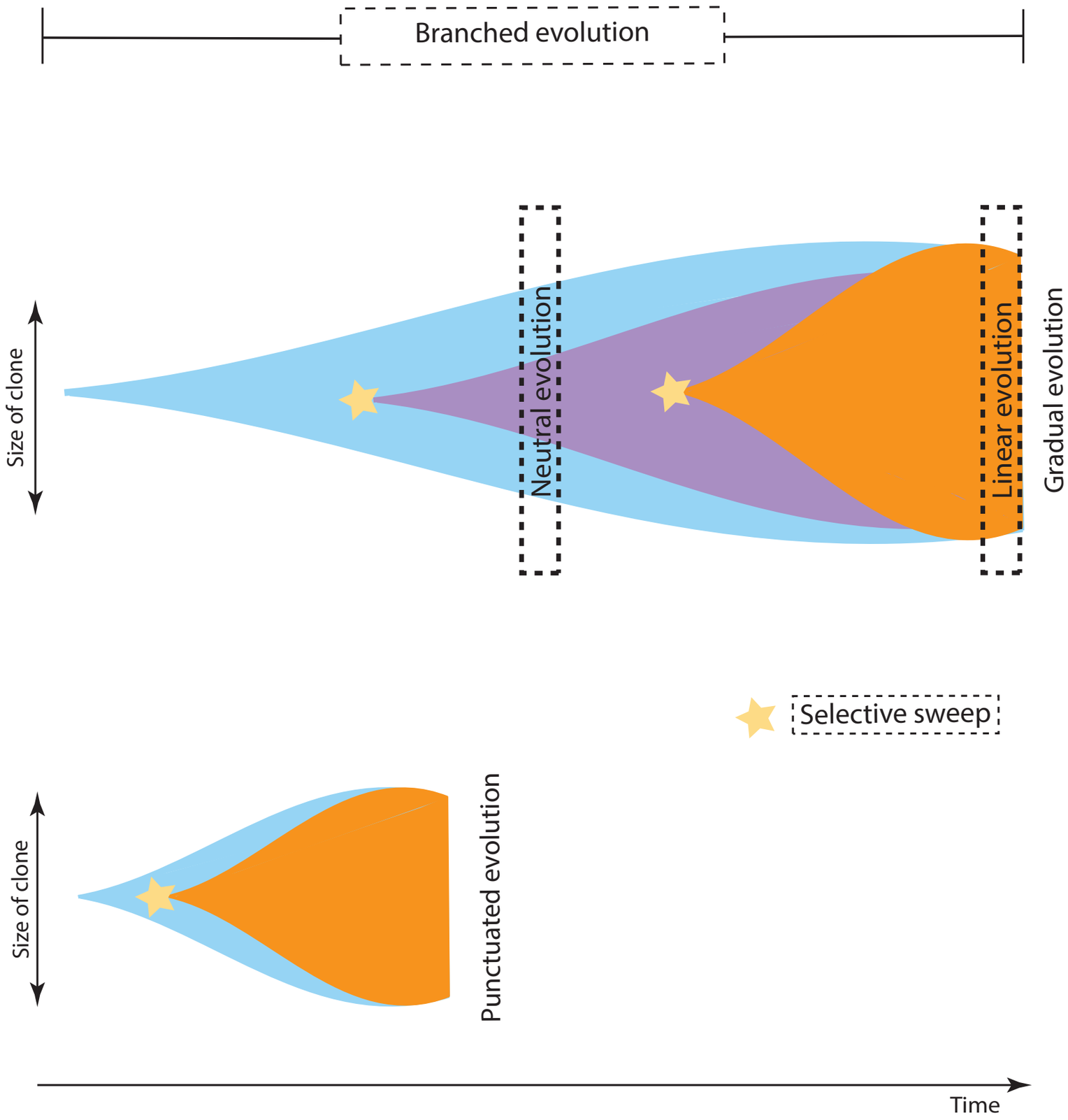
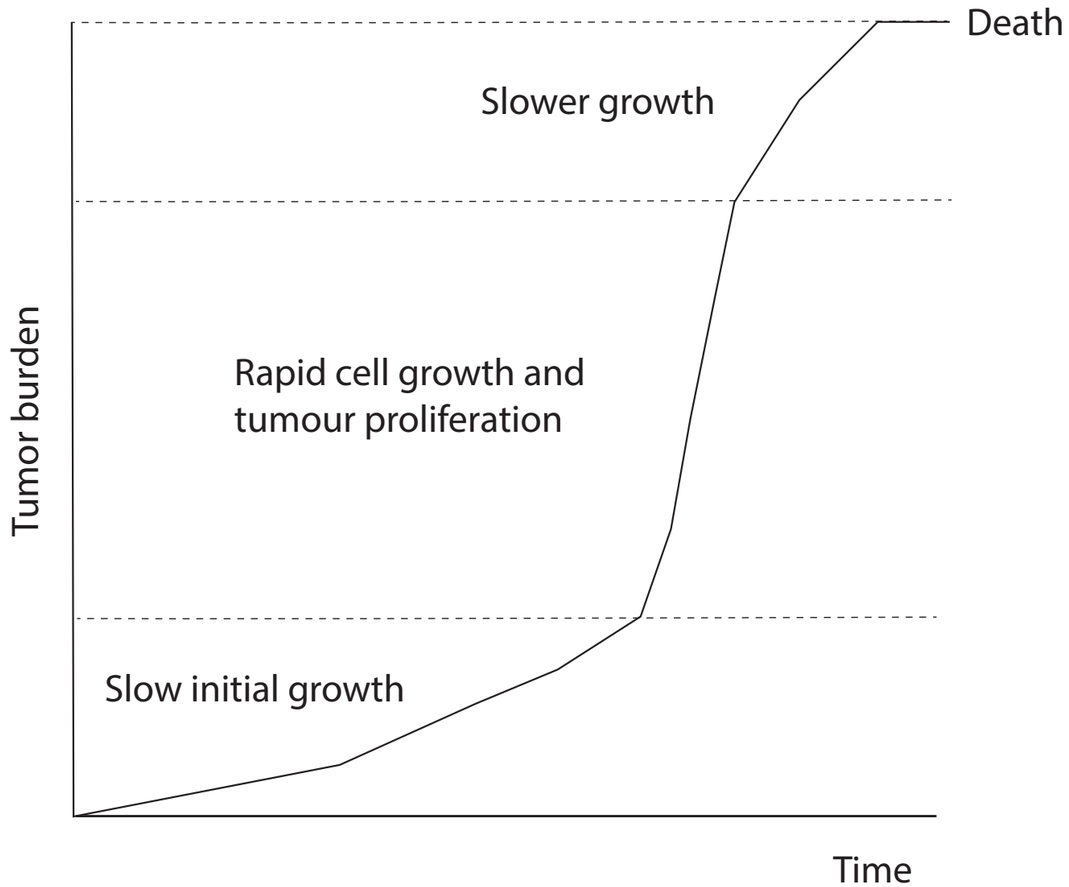
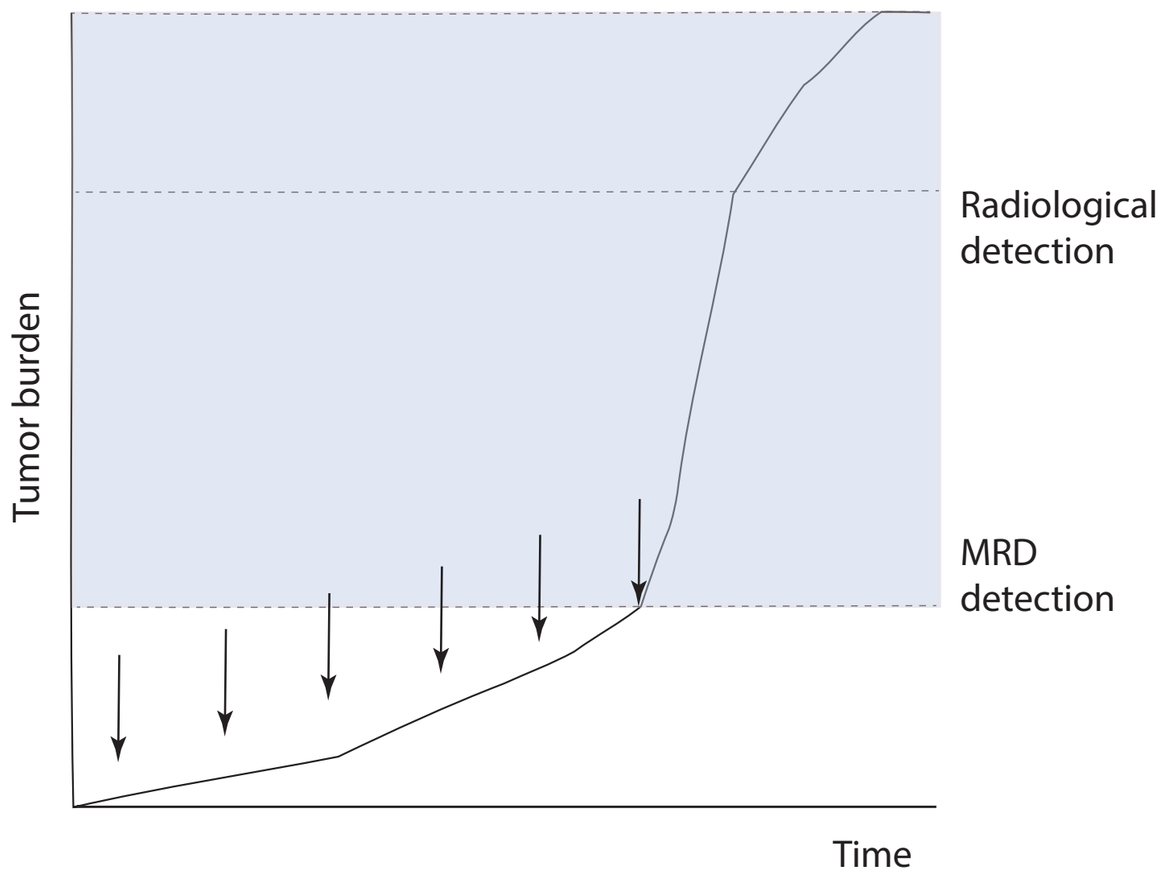
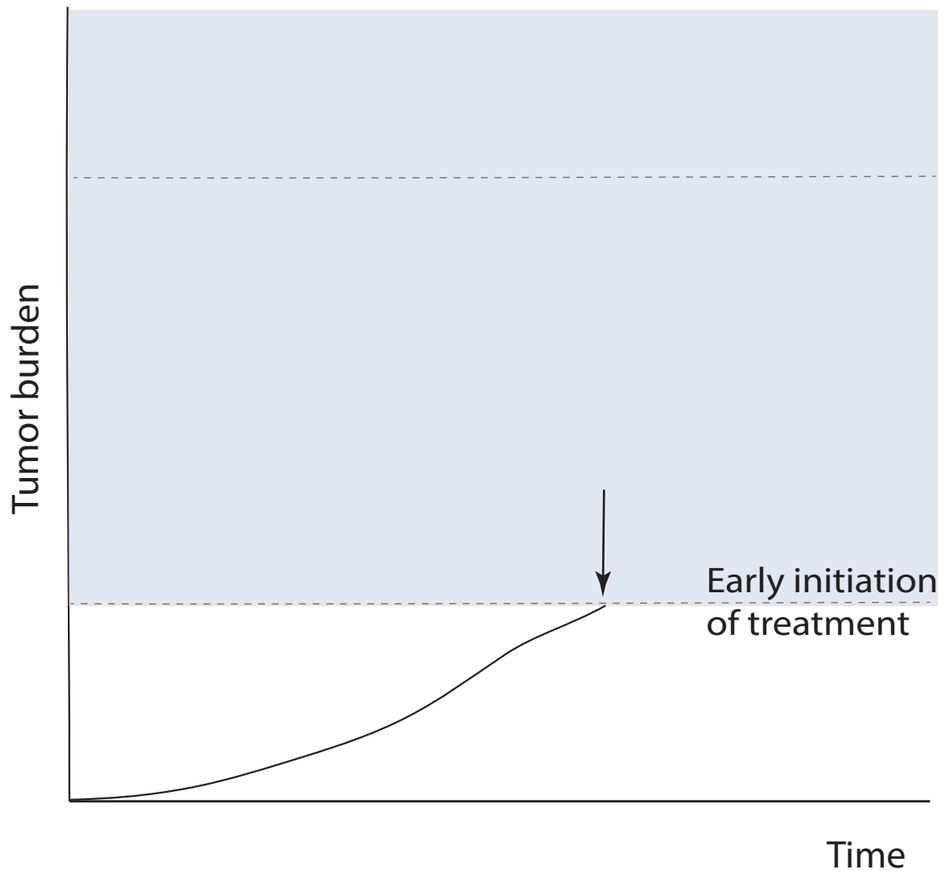


Figure 3- Gompertzian tumor growth curves, A illustrates the three phases of tumor growth, B Arrows illustrates the use of serial blood tests for the earlier detection of MRD, detection is considerably earlier than radiological detection and thus at a point where the tumor is less heterogeneous. C. Early initiation of targeted treatment D. Treatment continued until tumour eradication.







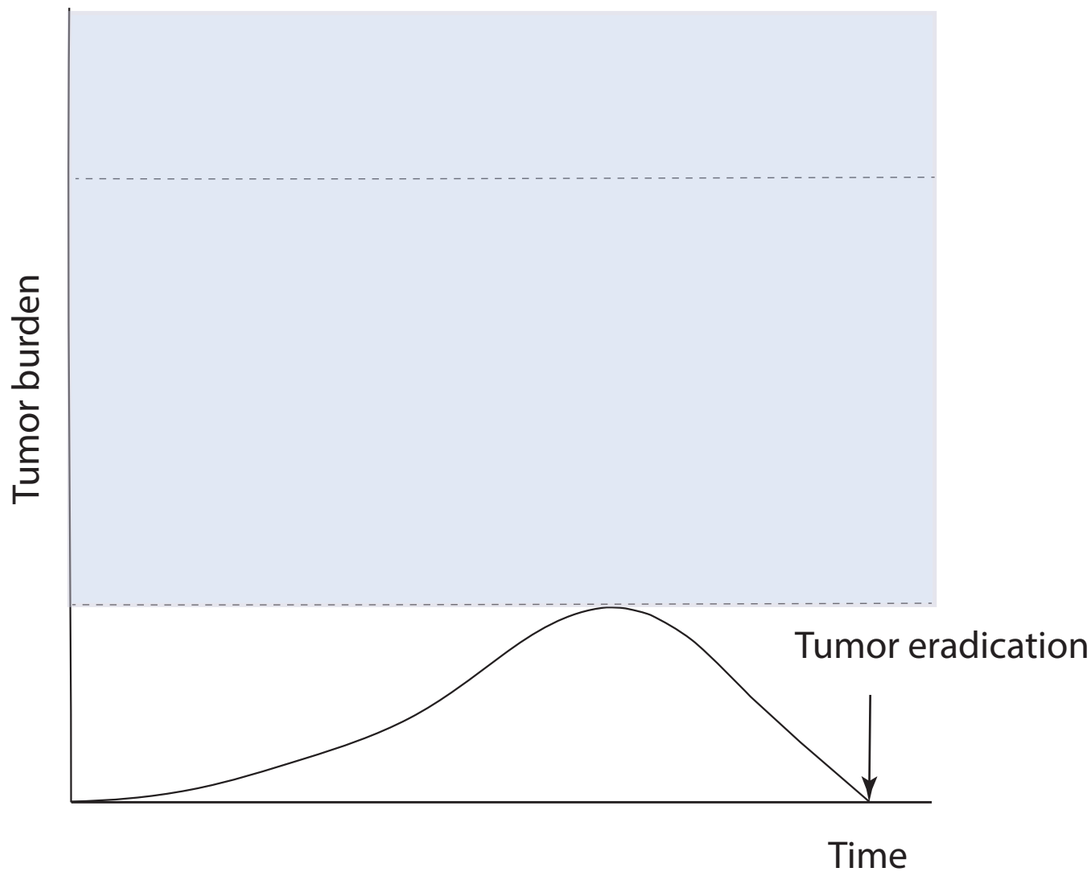


Figure 4- Illustrative representation on the effects of targeted therapy and treatment schedules on ITH. Pre-treatment subclones contain 4 distinct populations: sensitive to drug A and B, not targeted by drug therapy and pre-existing resistance to drug A. Targeted monotherapy, leads to regression of sensitive subclones, with the eventual outgrowth of untargeted and pre-existing resistant subclones. Targeted combination therapy, leads to the regression of sensitive subclones, with the eventual outgrowth of untargeted and pre-existing resistant clones, with eventual development of acquired resistant subclones due to the selective pressure of targeted treatment. Intermittent combined therapy, leads to initial regression of sensitive subclones, with outgrowth of sensitive subclones on treatment withdrawal and regression on treatment resumption. A slow but eventual outgrowth of pre-existing and acquired resistance subclones is also seen due to the selective pressure of targeted treatment.