Review

Breast Cancer Heterogeneity and Response to Novel Therapeutics

Mariona Baliu-Piqué¹, Atanasio Pandiella² and Alberto Ocana¹,³,*

¹ Experimental Therapeutics Unit, Medical Oncology Department, Hospital Clínico San Carlos (HCSC), Instituto de Investigación Sanitaria San Carlos and CIBERONC, 28040 Madrid, Spain; maria.baliu@salud.madrid.org
² Instituto de Biología Molecular y Celular del Cáncer and CIBERONC, CSIC-IBSAL, 37007 Salamanca, Spain; atanasio@usal.es
³ Translational Oncology Laboratory, Centro Regional de Investigaciones Biomedicas, Castilla-La Mancha University (CRIB-UCLM), 02008 Albacete, Spain

* Correspondence: alberto.ocana@salud.madrid.org

Received: 26 September 2020; Accepted: 2 November 2020; Published: 5 November 2020

Simple Summary: Breast cancer is a heterogeneous disease that is driven by genetic, epigenetic and phenotypic modifications and is also affected by the microenvironment and the metabolism. In this article we review genetic and non-genetic causes of tumor heterogeneity focusing on the impact that heterogeneity has on resistance to therapy. We will provide examples of personalized medicines and their translation to the clinic.

Abstract: Targeted cancer therapies against oncogenic drivers are actively being developed and tested in clinical trials. Targeting an oncogenic driver may only prove effective if the mutation is present in most tumoral cells. Therefore, highly heterogeneous tumors may be refractory to these therapies. This makes tumor heterogeneity a major challenge in cancer therapy. Although heterogeneity has traditionally been attributed to genetic diversity within cancer cell populations, it is now widely recognized that human cancers are heterogeneous in almost all distinguishable phenotypic characteristics. Understanding the genetic variability and also the non-genetic influences of tumor heterogeneity will provide novel insights into how to reverse therapeutic resistance and improve cancer therapy.

Keywords: breast cancer; heterogeneity; drug resistance; targeted therapies

1. Phenotypic Features of Tumor Heterogeneity

Tumors cannot be considered as homogeneous and static entities. Tumor heterogeneity can be assumed for virtually all distinguishable phenotypic features of a tumor, that is, cellular morphology, gene expression, hormonal receptors, growth factors, cell surface markers, metabolism, motility, immunogenicity, proliferation capacity and the potential to metastasize and to promote angiogenesis [1–4]. It is recognized that tumor heterogeneity is associated with poor prognosis and survival [5,6] and is one of the leading determinants of therapeutic resistance and treatment failure [7,8].

Tumor heterogeneity comes in different flavors. First, heterogeneity may arise among the cells of one individual tumor, the so-called intratumoral heterogeneity [9,10]. Intratumoral heterogeneity may exist across different regions of the primary tumor, spatial heterogeneity and as variations of a primary tumor over time, temporal heterogeneity [11]. Second, intermetastatic heterogeneity is the variety between different metastatic lesions of the same patient [9,10] and can arise even when tumor cells in distant sites share a common ancestor, since specific factors from each metastatic site, for example,
tumor microenvironment, may induce divergence after initial colonization of distant different sites [11]. Third, heterogeneity may also be present within the cells of an individual metastasis. Intrametastatic heterogeneity may or may not impact the initial response to therapy but is likely to be responsible for disease recurrence after an initial response. Such recurrences result from mutations present in a small fraction of the cells within each metastasis either prior to treatment or as a consequence of it. Usually, the larger the lesion, the more likely that such resistant cells will exist or evolve [10]. Each metastasis is established by a single cell (or small group of cells) with a set of founder genetic alterations and it acquires new mutations as it grows [9,10]. Finally, heterogeneity also exists among the tumors of different patients, which requires personalized treatments adapted to each individual [10]. Because cancer is a heterogeneous dynamic disease, individual patients, lesions and sites should be characterized over time to assure personalized treatments adapted to target the molecular drivers.

In this article, we review genetic and non-genetic causes of tumor heterogeneity focusing on the impact that heterogeneity has on resistance to therapy. We discuss in detail how breast cancer heterogeneity is driven at the genetic, epigenetic and phenotypic level, the influence of the microenvironment and the metabolism and its specific role in resistance to anti-cancer therapies.

2. Genomic Indications of Tumor Heterogeneity

Early histologic and biochemical studies in breast cancer showed that breast tumors are composed of different subpopulations. Breast tumors were, therefore, classified according to the expression of several clinical biomarkers, such as estrogen (ER) and progesterone (PR) receptors and the human epidermal growth factor receptor 2 (HER2) gene amplification [12–14]. This subclassification stands as one of the major determinants dictating current therapy of breast tumors.

Transcriptomic studies classified breast tumors into four intrinsic subtypes with distinct clinical outcomes, namely luminal A, luminal B, HER2-enriched and basal-like [15]. Alternative classifications also exist; for example, some authors subdivided triple negative breast cancers (TNBC) into six subgroups: basal-like 1, basal-like 2, immunomodulatory, mesenchymal, mesenchymal stem-like and luminal androgen receptor subtype, demonstrating that heterogeneity at a transcriptomic level is clearly evident [16]. Additional data in this breast cancer subtype, using single cell level studies with a nanogrid single-nucleus RNA-sequencing technology, showed that even most cells displayed a basal-like subtype, a significant fraction of cells were HER2+, luminal A, luminal B and normal-like, reflecting breast tumor heterogeneity even within a single breast cancer subtype [17]. Recent studies based on genome sequencing, such as the Molecular Taxonomy of Breast Cancer International Consortium (METABRIC) [18,19] and The Cancer Genome Atlas Network (TCGA) [20] refined the genomic characterization of breast cancers. Moreover, sequencing studies highlighted the genetic differences between primary tumors and metastases [21]. Genomic studies at single cell level showed profound genetic heterogeneity and extensive clonal diversity in breast cancer [22,23], as well as heterogeneity in copy number alterations of genes and regions with known biological relevance in breast cancer, such as genes associated with metastasis and therapeutic response [24].

These studies and others not reviewed here demonstrate the heterogeneity of breast tumors and emphasize the importance of molecular-based stratification of breast cancer patients in an intent to select the right therapeutic strategy.

3. Genetic Heterogeneity and Personalized Medicine

Driver-molecular alterations are those that directly promote tumor generation/progression. Heterogeneity of driver-gene alterations, either intratumoral, intermetastatic or intrametastatic, determine the capability of a tumor to respond to a given targeted therapeutic agent. If a single clone lacks the driver-gene mutation being targeted, the clone will likely continue to grow even when the therapy is initiated. Analyses of several genomic databases have suggested that there are around 40–60 recurrent driver-alterations in breast cancer [25]. Breast tumors may present more than ten
somatic mutations. Of these mutations, only a few are present in the same genes in two different breast cancer patients. In most cases, these shared mutations correspond to driver-gene mutations [20,26,27].

Recent advances in the detection of genomic alterations and the increasing availability of genomic tests have provided relevant information about tumor heterogeneity. Such advances aid in the prediction of clinical treatment benefit and the identification of treatment resistance [28]. For example, the presence of genomic alterations in genes such as ERBB2, PIK3CA, AKT1, ESR1 and NTRK in advanced breast cancer patients help to stratify patients for targeted therapies [28]. The European Society for Medical Oncology (ESMO) has created the ESMO Scale for Clinical Actionability of Molecular Targets (ESCAT) and classified molecular abnormalities in six levels according to the evidence of clinical benefit demonstrated by targeting the specific alteration [29]. The alteration-targeted therapy which is associated with a clinical benefit based on improved outcome in clinical trials is ranked in the 1st level. Patients harboring level 1 alterations should have access to the targeted therapy as standard of care [25]. ERBB2 amplification, germline BRCA1/2 mutations, PIK3CA mutations, NTRK translocations and microsatellite instability (MSI) were included in the 1st tier of evidence (Table 1) [25].

Table 1. List of alterations ranked as level 1 by Condorelli et al. [25].

| Gene     | Type of Alteration                                      | Drug                          |
|----------|--------------------------------------------------------|-------------------------------|
| ERBB2    | Amplification (DNA copy number ≥ 6; size ≤ 10 Mb)      | Anti-HER2 monoclonal antibody (Trastuzumab) |
| BRCA 1/2 | Germline mutations: Truncated mutations (InDel, splice-site, non-sense (except BRCA2 K3326X)) and rare known inactivating missense mutations | PARP inhibitor (Olaparib)      |
| PIK3CA   | Major hot-spot activating missense mutations           | Alpha-specific PI3K inhibitor (Alpelisib) |
| NTRK     | Translocations                                         | Pan-Trk inhibitor (Larotrectinib)    |
| -        | Microsatellite instability (MSI)                       | Anti-PD1 antibody (Pembrolizumab)  |

4. Heterogeneity and Resistance to Anti-Cancer Therapies

4.1. Heterogeneity in Target Expression

The incorporation of targeted therapies into clinical practice has improved the prognosis of certain subgroups of patients with solid tumors, however, relapse may occur due to the presence of resistance mechanisms. Resistance present in the initial tumor is known as intrinsic or primary resistance. When resistance appears after an initial response, it is named secondary resistance. Two forms of secondary resistance are acknowledged. Acquired resistance refers to secondary resistance that raises during treatment, due to acquisition of additional molecular alterations, such as mutations, activation of bypass signaling pathways and cell lineage changes [11,30]. Another type of secondary resistance, termed intrinsic resistance, appears by the outgrowth of resistant clones present pre-treatment and selected to grow by pressure on other therapy-sensitive tumoral cells [11,31,32].

Most of the targets considered as druggable are not expressed homogeneously within the tumor. For instance endocrine therapy, such as Fulvestrant or Tamoxifen, is an effective targeted therapy for ER-positive breast cancer. However, its activity depends on the fraction of ER positive cells within a tumor and this varies widely. The recommended cutoff to distinguish ER-positive patients which will receive endocrine therapy is ≥ 1% ER positive tumor cells [33]. ER expression is a critical predictor of response to endocrine therapy, therefore it is not surprising that lack of ER expression by some clones results in resistance to therapy [34].
Target heterogeneity is also a main issue in antibody–drug conjugate (ADC) therapy. ADCs are constituted by an antibody linked to a cytotoxic agent. Heterogeneity in the expression of the target may lead to ADC resistance due to the inability of the ADC to kill cells that do not express the target [35]. Patients with breast cancers overexpressing HER2 or with amplification of \(ERBB2\), which include 20–25% patients, clinically benefit of regimens combining chemotherapy and HER2-targeted agents [36]. Trastuzumab Emtansine (T-DM1), a HER2-targeted-antibody–drug conjugate with a tubulin inhibitor payload (DM1), is an effective therapy for advanced metastatic disease as well as in the adjuvant setting. However, T-DM1 is only effective against cells expressing HER2. T-DM1 resistance appears through the selection of clones with no or limited expression of HER2 [37]. Moreover, a subgroup of HER2-overexpressing tumors may express truncated forms of HER2, which lack the region recognized by Trastuzumab, therefore impeding binding of T-DM1 [37,38]. To overcome such resistances, ADCs may be designed to exert a bystander killing effect, that is to kill cells which express the target molecule as well as surrounding cells irrespective of the expression of the target [35]. The bystander killing effect is of particular importance since it allows the delivery of the payload in areas where resistance clones outgrow due to the lack of inhibitory pressure.

A different Trastuzumab-derived ADC, Trastuzumab deruxtecan (Enhertu, DS-8201) has recently been granted an accelerated FDA approval for the treatment of unresectable or metastatic HER2-positive breast cancer patients who have received at least 2 prior lines of anti-HER2-based regimens in the metastatic setting [39,40]. DS-8201 is an HER2-targeted ADC with a potent topoisomerase I inhibitor payload which presents bystander killing [41] and showed antitumor efficacy against several breast cancer models with low HER2 expression [42]. Another interesting feature of ADCs is that their bystander killing effect can be modulated by the chemical properties of the ADCs. For example, newly designed ADCs deliver the drug in response to changes in the pH [43].

Another example of target heterogeneity is the heterogeneous expression of PD-L1 in breast cancer, which can vary up to 4-fold in different areas of the same biopsy [44,45]. Patients with metastatic or locally advanced TNBC with PD-L1 expression on immune cells occupying ≥1% of tumor area have demonstrated survival benefit with combined therapy of Atezolizumab and Nab-Paclitaxel [46]. Similar results were obtained recently with Pembrolizumab, confirming that patients expressing PD-L1 obtain better outcomes [47]. Moreover, it has been shown that PD-L1 expression remains largely unaltered after neoadjuvant chemotherapy [48,49], raising the possibility that adjuvant immune checkpoint inhibitor therapy could improve survival in this patient population. In this regard, recent data from neoadjuvant therapy in combination with Pembrolizumab has shown an increase in pathological complete response, compared with combinations without the immune checkpoint inhibitor [50].

### 4.2. Clonal Selection and Resistance

Exposure to targeted therapies often gives rise to mutations or genomic modifications in metastases that justify the progression or the appearance of recurrences. The classic example of resistance to targeted therapies is the introduction as first-line treatment of the Bcr-Abl selective tyrosine kinase inhibitor (TKI) Imatinib Mesylate for chronic myeloid leukemia (CML). Although initial responses were high, therapy failed in a substantial proportion of patients and initial responses were lost within 2 years in approximately half of patients due to the development of resistances. Resistance to this TKI depends on genomic mechanisms, such as point mutations in the Bcr-Abl kinase domain, as well as on Bcr-Abl-independent mechanisms, including activation of alternative signaling pathways or insensitivity of pre-existing clones which promotes their selection [51,52].

For breast cancer, there are also several examples of clonal selection and resistance. Single-cell DNA-sequencing of 20 TNBC before and after neoadjuvant chemotherapy showed that resistant cells are present in the tumor before the initiation of therapy and that adaptive resistance to neoadjuvant chemotherapy largely comes from the selection of pre-existent clones [53]. Similarly, DNA and RNA sequencing and live cell imaging plus single-cell RNA-sequencing of metastatic ER+ breast cancer patients showed that pre-existing minor subclones become dominant after chemotherapy,
indicating selection for resistance phenotypes [54,55], similarly to what was initially described for CML, lung cancer and melanoma [56].

Approximately 30% of breast cancer patients treated with Tamoxifen become refractory within 2–5 years or develop resistance to the drug along treatment [57]. Acquired mutations in the ESR1 gene, which encodes the ERα, alter the steroid hormone ligand-binding domain, restore the pro-oncogenic function of the ER and reduce or abrogate the therapeutic effect [58,59]. Moreover, aberrant methylation of CpG islands located in the 5′ regulatory regions of ESR1 gene has also been associated with loss of ER expression and, hence, with acquired resistance [34].

4.3. Presence of Compensatory Signaling Pathways

Acquired resistance to targeted drugs may also occur via the activation of alternative signaling pathways [37,60]. One of the Trastuzumab and T-DM1 mechanisms of action is the inhibition of the PI3K signaling pathway. Therefore PIK3CA and PTEN mutations may contribute to the acquisition of resistance to these agents [37,60,61]. Moreover, EGFR, HER3, HER4, IGF-1R, MET upregulation and heterodimerization may confer resistance to anti-HER2 therapies by restoring the original downstream signaling pathways activated by HER2-overexpression and amplification [61–63].

4.4. Genomic Instability

Defects in DNA repair pathways, for example through mutations in BRCA1, BRCA2 and PALB2, enable cancer cells to accumulate genomic alterations that contribute to their aggressive phenotype [64]. However, tumors rely on residual DNA repair capacities to survive DNA damage. Members of the poly-adenosine diphosphate–ribose polymerase (PARP) family of enzymes are central to the repair of single-strand DNA breaks. Inhibitors targeting PARP have shown promising clinical activity in tumors carrying germline mutations in either BRCA1 or BRCA2 [65–68]. The PARP inhibitor Olaparib is approved by the FDA for the treatment of BRCA-mutated breast, ovarian and pancreatic cancers. Olaparib inhibits PARP enzymes and traps PARP1 on DNA at single-strand breaks, leading to replication-induced DNA damage. Such damage needs to be repaired by homologous recombination, which makes cancer cells defective in DNA repair pathways highly sensitive to Olaparib [69,70].

As is the case for many other treatments, tumors frequently acquire resistance to PARP inhibitors [71]. Four distinct categories of resistance mechanisms have been described: (i) restoration of the homologous recombination mechanisms; (ii) decreased availability of PARP, for example, via point mutations in PARP1 [72]; (iii) increased drug efflux; and (iv) restoration of replication fork stability [73]. The restoration of the homologous recombination mechanisms mainly arises from reverting mutations in BRCA1 or BRCA2 genes that restore the open reading frame of the genes. Such mutations also cause clinical resistance to platinum-based chemotherapy [74,75]. The fact that polyclonality of multiple reverting mutations emerges within one patient illustrates the profound selection pressure exerted on these tumors to restore BRCA1/2 protein activity and acquire resistance [73].

4.5. Epigenetic Modifications

Besides genetic heterogeneity, human tumors usually contain epigenetic changes. Epigenetic modulation plays a critical role in regulating where and when genes are expressed during tumor development [76]. Gene promoter methylation, general hypomethylation and histone methylation and deacetylation are common in cancer. Such epigenetic alterations exert a selective effect on clones presenting a specific epigenetic event, such as the inactivation of tumor suppressor genes by promoter methylation [77]. Moreover, epigenetic heterogeneity also allows for reversible transitions from drug-sensitivity to drug-resistance [30].

In basal-like breast cancer, tumor cell populations, which persist following treatment with the MEK inhibitor, Trametinib, and the PI3K/mTOR inhibitor, BEZ235, showed increased activity of BRD4, KDM5B and EZH2 [78]. In addition, the genes involved in SWI/SNF chromatin remodeling complex, including ARID1A, ARID1B and ARID2, have been found to be mutated in metastatic
recurrences of treatment-resistant breast cancer patients [79]. These observations provide evidence that epigenetic changes can generate drug-resistant states that allow for the survival of small subpopulations within otherwise treatment-sensitive cancer cells. Therefore, drugs targeting epigenetic enzymes may decrease intratumoral cellular heterogeneity and reverse treatment resistance, when combined with chemotherapy or targeted therapies.

A beautiful example of the role of epigenetic modifications in drug resistance is the modulation of epigenetics to generate drug sensitization. In animal models of breast and ovarian cancer, the inhibition of Bromodomain and Extra-Terminal motif (BET) proteins impairs the transcription of BRCA1 and RAD51, two genes essential for homologous recombination and increases the tumor sensitivity to Olaparib [80]. Other example from our laboratory is the synergy observed when treating PLK1-resistant cells with the BET inhibitor JQ1 [81].

4.6. Tumor Microenvironment and Immune System

The tumor microenvironment (TME)—the space surrounding the tumor composed of immune cells, stroma and vasculature—also plays a role in resistance to therapy [82]. To understand tumor progression and the appearance of resistances to targeted therapies it is important to recognize the multiple components of the TME and the interactions between the tumor and its surrounding. The TME may create different selective pressures in distinct tumor areas and this may give rise to intratumor heterogeneity and favor the outgrowth of specific clones [83,84]. Hypoxia, inflammation and the fibrotic state of the tissue can directly and indirectly influence tumor heterogeneity [30,83]. Hypoxia is one of the most well-known examples, hypoxic conditions may trigger a set of adaptive transcriptional responses, including cell metabolism, invasion, survival, angiogenesis, differentiation and self-renewal, that seem to be involved in tumor progression and in the expression of drug-resistance genes [85–88]. Furthermore, hypoxia and inflammatory cytokines released by stromal or immune cells can induce epigenetic modifications that subsequently alter gene expression [8].

Fibroblasts constitute one of the most abundant cell types in the stroma. It is recognized that fibroblasts can apply suppressive functions on tumor cells. However, during tumor progression, fibroblasts lose their suppressive effect and allow tumor growth [89]. Cancer-associated fibroblasts (CAFs) are a heterogeneous cellular population that can alter the tumor response to therapy and the immune response. Four subsets of CAFs have been characterized in breast cancer [90]. The CAF-S1 subset, which is enriched in TNBC, is known to support an immunosuppressive environment [90].

The presence of tumor infiltrating lymphocytes (TILs) is associated with favorable outcome in breast cancer [91–93]. However, there is heterogeneity in subset composition, functional status and spatial location of immune cells within the tumor [94–96]. ‘Cold’ tumors have few immune cells, largely macrophages; mixed tumors harbor immune cells and tumor cells mixed together. In compartmentalized tumors, the immune and tumor cells are spatially segregated [95]. Around 70% of breast tumors are infiltrated with TILs, with a median TIL count of around 10% [48,49]. In breast tumors, PD-L1 is mainly expressed in stromal cells, including TILs, macrophages and morphologically fibroblast-like cells, while cancer cells express PD-L1 only in half the cases [48,97]. This pattern of expression observed in breast cancer implies PD1/PD-L1 signaling between tumor cells and immune cells, as well as between various types of immune cells, and is important in the action of PD1/PD-L1 targeting antibodies [98]. Recently, several transcriptomic signatures associated with the presence of immune infiltrates in breast tumors have been described [99,100]. Such signatures identify ‘hot’ tumors what could potentially predict response to immunotherapies.

The TME also differs between primary tumor and metastases, which influences the phenotype of tumor cells at distal sites [101]. Metastatic lesions in breast cancers have been shown to be less immunologically active [102]. Metastasis may escape from immune surveillance by down regulating chemotactic and immune activating cytokines and their receptors, decreasing antigen presentation and upregulating immunosuppressive mechanisms [102]. The presence of TILs and the expression of PD-L1 is substantially lower in metastases compared with primary tumors [102], a situation
which could impair the response of metastatic lesions to immune checkpoint inhibition with anti PD1/PD-L1 antibodies.

Moreover, it is now well recognized that tumor-normal cell fusions, the so called tumor-normal hybrids, are potent inducers of genomic instability and heterogeneity and that they contribute to resistance to therapy [103,104]. Cell fusion has been observed between breast tumor cells themselves [105], between tumor cells and normal breast epithelium [106], endothelial [107], stromal cells, stem cells [108] and macrophages [109,110].

Heterogeneity in the TME, the immune system and the presence of tumor-normal cell hybrids may directly and indirectly affect response to therapy and resistance to drugs. Immunosuppressive cancer microenvironments and immune desert tumors are the major impediments to checkpoint inhibitors. Some immunotherapy-resistant tumors have a low mutational burden, which translates to low antigen presentation and low tumor immunogenicity [111,112]. Other mechanisms of resistance to immune checkpoint blockade include the loss of β2-microglobulin, which impairs antigen presentation, and JAK1 or JAK2 mutations which make tumor cells insensitive to interferon gamma [113]. Recent studies have identified genomic correlates of response to immune checkpoint blockade which may help identify immunotherapy responsive tumors [114–116].

4.7. Metabolism

Besides its heterogeneous composition, the TME may also be a metabolic barrier to effector T cells and immunotherapy [117]. The TME can be metabolically hostile (hypoxic, depleted of nutrients and with accumulation of waste products) for T cells and lead to metabolic dysfunction. T cells require an adequate nutrient uptake to mount a proper immune response and the lack of sufficient nutrients or the failure to activate the right metabolic pathways may prevent effector T cell activation [117]. Effective antitumoral responses by T cells require optimal T cell fitness and circumstances to avoid exhaustion. PD1 signaling on T cells may induce the metabolic switch from glycolysis to fatty acid oxidation which impairs the effector function of T cells [118–121]. These observations suggest that anti-PD1 treatment may be able to restore T cell glycolysis and, subsequently, the effector function of T cells.

Moreover, tumor cells also secrete byproducts that may be harmful for T cells. For example, indoleamine 2,3-dioxygenase (IDO) catalyzes tryptophan, an essential amino acid for immune responses and produces kynurenine, which induces the generation of immunosuppressive regulatory T cells [122,123]. Moreover, high lactate concentrations have been shown to suppress the effector functions of CD8+ T cells [124].

In patients with advanced melanoma and renal cell carcinoma treated with Nivolumab, an anti-PD1 antibody, it has been shown that the serum kynurenine/tryptophan ratio increases upon treatment as an adaptive resistance mechanism associated with worse overall survival [125]. Increased kynurenine/tryptophan ratio inhibits T cell proliferation [126–128], and, therefore, may promote tumor immune resistance to PD1 blockade. These observations highlight the importance of metabolism in resistance to targeted therapies and advocates for metabolic monitoring of patients during immunotherapy.

5. Beyond Tumor Heterogeneity

Despite tumor heterogeneity, the identification of driver vulnerabilities and the development of novel targeted therapies has a meaningful clinical impact. Tumor-agnostic drugs are treatments that target a specific genetic characteristic irrespective of tumor histology (Table 2). Regardless the histologic origin, tumors presenting mismatch repair deficiency (MMRd) which exhibit MSI are highly sensitive to immune checkpoint blockade, with an overall response rate (ORR) of 36–46% across 15 different histologies and with 78% of responses after 6 months [129–131]. Therefore, Pembrolizumab has been the first FDA approved tumor-agnostic drug for the treatment of MSI-positive tumors [131–133]. Both the genomics and the TME of MSI-positive tumors contribute to the remarkable response rates: (i) MSI-positive tumors generate a great amount of neo-epitopes [129–131,134], which tend
to be subclonal since MMRd-induced mutations are predominantly subclonal and derive in highly heterogeneous tumors [135]; (ii) MSI-positive cancers are highly infiltrated with CD8+ T cells [136]; and (iii) MSI-positive cancers express high levels of multiple immune checkpoint molecules, including PD1 and PD-L1 [137].

Larotrectinib, a tropomyosin receptor kinase (TRK) inhibitor, was the second agnostic drug approved by the FDA [133]. In patients presenting neurotrophic receptor tyrosine kinase (NTRK) fusions, Larotrectinib has shown an ORR of 80% across 17 tumor types, with 16% of patients having a complete response [138]. Larotrectinib regulatory approval was followed by the approval of Entrectinib, another TRK inhibitor [133]. Despite the remarkable ORR achieved by Larotrectinib treatment, resistances have already been reported, including solvent front mutations, gatekeeper mutations and mutations in the xDFG motif [139]. These mutations modify the binding site of Larotrectinib, decreasing its inhibitory potency [139]. Preclinical and in silico modelling were able to predict which mutations of the TRK kinase domain would drive resistance to Larotrectinib and enabled the development of novel TRK inhibitors that targeted the kinase-domain of TRK mutants [140].

Future work in tumor-agnostic drug development should look for therapeutic agents that target true driver mutations and take in consideration coexisting or newly acquired mutations that can drive resistance. Approaches to predict mutations that drive resistances, such as preclinical and in silico modelling, will be of clinical relevance and will likely be introduced to predict resistances to new drugs.

Table 2. Molecular alterations with agnostic indications approved or in development [133,138].

| Gene       | Type of Alteration | Drug              | Development Phase |
|-----------|--------------------|-------------------|-------------------|
| Microsatellite instability–High (MSI-H) | Pembrolizumab | FDA approved |
| NTRK      | Gene fusions       | Larotrectinib, Entrectinib | FDA approved |
| RET       | Gene fusions | Selpercatinib, Pralsetinib, RXDX-105, TPX-0046 | Phase I/II, Phase I/II, Phase I/Ib, Phase I/I |
| NRG1      | Gene fusions       | Zenocutuzumab, Tarloxtinib | Phase I/II basket |
| FGFR (fibroblast growth factor receptor) | Gene fusions | Debio 1347, TAS-120 | Phase II basket |
| KRASG12C  | Mutations          | AMG 510, MRTX849  | Phase I |
| TRK, ROST, ALK | Mutations     | Repotrectinib  | Phase I/II |
| BRAF      | Mutations          | PLX8394           | Phase I/II |

6. Conclusions and Perspectives

There is a wide range of sources of biological heterogeneity including tumor immune profiles and TME, tumor mutational burden and development of tumoral cell polyclonality along therapy. Common patterns of resistance to driver-gene targeted therapies and the presence of low-frequency drivers must be anticipated and intercepted in order to achieve a durable clinical response. We believe that in the near future cancer patients will be treated with a rationally designed combination therapy of targeted agents, rather than with a single drug. These therapies may combine several drugs aimed at targeting different oncogenic drivers present in distinct tumoral cell populations. Moreover, these therapies may be combined with others, such as those that boost immune responses against tumors. Although examples of this approach are the ongoing studies combining immunotherapies with targeted agents in selected populations, much work is still needed to achieve a personalized treatment for each patient.
**Author Contributions:** Conceptualization, M.B.-P. and A.O.; formal analysis, M.B.-P. and A.O.; investigation, M.B.-P. and A.O.; writing—original draft preparation, M.B.-P., A.P. and A.O.; writing—review and editing, M.B.-P.; A.P. and A.O.; supervision, M.B.-P. and A.O.; funding acquisition, A.O. All authors have read and agreed to the published version of the manuscript.

**Funding:** This work has been supported by CRIS Cancer Foundation, Instituto de Salud Carlos III (PI19/00808), ACEPAIN, ALMOM, Diputación de Albacete and CIBERONC.

**Conflicts of Interest:** The authors declare no conflict of interest.

**References**

1. Fidler, I.; Hart, I. Biological diversity in metastatic neoplasms: Origins and implications. *Science* 1982, 217, 998–1003. [CrossRef] [PubMed]
2. Dick, J.E. Stem cell concepts renew cancer research. *Blood* 2008, 112, 4793–4807. [CrossRef] [PubMed]
3. Nicolson, G.L. Generation of phenotypic diversity and progression in metastatic tumor cells. *Cancer Metastasis Rev.* 1984, 3, 25–42. [CrossRef] [PubMed]
4. Heppner, G.H. Tumor heterogeneity. *Cancer Res.* 1984, 44, 2259–2265. [PubMed]
5. Landau, D.A.; Carter, S.L.; Stojanov, P.; McKenna, A.; Stevenson, K.; Lawrence, M.S.; Sougnez, C.; Stewart, C.; Sivachenko, A.; Wang, L.; et al. Evolution and Impact of Subclonal Mutations in Chronic Lymphocytic Leukemia. *Cell* 2013, 152, 714–726. [CrossRef]
6. Zhang, J.; Fujimoto, J.; Zhang, J.; Wedge, D.C.; Song, X.; Zhang, J.; Seth, S.; Chow, C.-W.; Cao, Y.; Gumbs, C.; et al. Intratumor heterogeneity in localized lung adenocarcinomas delineated by multiregion sequencing. *Science* 2014, 346, 256–259. [CrossRef]
7. Marusyk, A.; Almendro, V.; Polyak, K. Intra-tumour heterogeneity: A looking glass for cancer? *Nat. Rev. Cancer* 2012, 12, 323–334. [CrossRef]
8. Cajal, S.R.Y.; Sesé, M.; Capdevila, C.; Aasen, T.; De Mattos-Arruda, L.; Diaz-Cano, S.J.; Hernández-Losa, J.; Castellvi, J. Clinical implications of intratumor heterogeneity: Challenges and opportunities. *J. Mol. Med.* 2020, 98, 161–177. [CrossRef] [PubMed]
9. Reiter, J.G.; Baretti, M.; Gerold, J.M.; Makohon-Moore, A.P.; Daud, A.; Iacobuzio-Donahue, C.A.; Azad, N.S.; Kinzler, K.W.; Nowak, M.A.; Vogelstein, B. An analysis of genetic heterogeneity in untreated cancers. *Nat. Rev. Cancer* 2019, 19, 639–650. [CrossRef]
10. Vogelstein, B.; Papadopoulos, N.; Velculescu, V.E.; Zhou, S.; Diaz, L.A.; Kinzler, K.W. Cancer Genome Landscapes. *Science* 2013, 339, 1546–1558. [CrossRef] [PubMed]
11. Dagogo-Jack, I.; Shaw, A.T. Tumour heterogeneity and resistance to cancer therapies. *Nat. Rev. Clin. Oncol.* 2018, 15, 81–94. [CrossRef]
12. Nassar, A.; Radhakrishnan, A.; Cabrero, I.A.; Cotsonis, G.A.; Cohen, C. Intratumoral Heterogeneity of Immunohistochemical Marker Expression in Breast Carcinoma: A tissue microarray-based study. *Appl. Immunohistochem. Mol. Morphol.* 2010, 18, 433–441. [CrossRef] [PubMed]
13. Pertschuk, L.P.; Axiotis, C.A.; Feldman, J.G.; Kim, Y.-D.; Karavattayhayyil, S.J.; Braithwaite, L. Marked Intratumor Heterogeneity of the Proto-Oncogene Her-2/neu Determined by Three Different Detection Systems. *Breast J.* 1999, 5, 369–374. [CrossRef]
14. Davis, B.W.; Zava, D.T.; Locher, G.W.; Goldhirsch, A.; Hartmann, W.H. Receptor heterogeneity of human breast cancer as measured by multiple intratumoral assays of estrogen and progesterone receptor. *Eur. J. Cancer Clin. Oncol.* 1984, 20, 375–382. [CrossRef]
15. Perou, C.M.; Sorlie, T.; Eisen, M.B.; Van De Rijn, M.; Jeffrey, S.S.; Rees, C.A.; Pollack, J.R.; Ross, D.T.; Johnsen, H.; Akslen, L.A.; et al. Molecular portraits of human breast tumours. *Nature* 2000, 406, 747–752. [CrossRef] [PubMed]
16. Lehmann, B.D.; Bauer, J.A.; Chen, X.; Sanders, M.E.; Chakravarty, A.B.; Shyr, Y.; Pietenpol, J.A. Identification of human triple-negative breast cancer subtypes and preclinical models for selection of targeted therapies. *J. Clin. Investig.* 2011, 121, 2750–2767. [CrossRef]
17. Gao, R.; Kim, C.; Sei, E.; Foukakis, T.; Cirosetto, N.; Chan, L.-K.; Srinivasan, M.; Zhang, H.; Meric-Bernstam, F.; Navin, N.E. Nanogrid single-nucleus RNA sequencing reveals phenotypic diversity in breast cancer. *Nat. Commun.* 2017, 8, 228. [CrossRef]
18. Curtis, C.; Shah, S.P.; Chin, S.; Turashvili, G.; Rueda, O.M.; Dunning, M.J.; Speed, D.; Lynch, A.G.; Samarajiva, S.; Yuan, Y.; et al. The genomic and transcriptomic architecture of 2000 breast tumours reveals novel subgroups. *Nature* 2012, 486, 346–352. [CrossRef]

19. Pereira, B.; Chin, S.; Rueda, O.M.; Vollan, H.M.; Provenzano, E.; Bardwell, H.A.; Pugh, M.; Jones, L.; Russell, R.; Sammut, S.; et al. The somatic mutation profiles of 2433 breast cancers refine their genomic and transcriptomic landscapes. *Nat. Commun.* 2016, 7, 11479. [CrossRef]

20. The Cancer Genome Atlas Network. Comprehensive molecular portraits of human breast tumours. *Nature* 2012, 490, 61–70. [CrossRef]

21. Bertucci, F.; Ng, C.K.Y.; Patsouris, A.; Droin, N.; Piscuoglio, S.; Carubbia, N.; Soria, J.C.; Dien, A.T.; Adnani, Y.; Kamal, M.; et al. Genomic characterization of metastatic breast cancers. *Nature* 2019, 569, 560–564. [CrossRef]

22. Navin, N.; Kendall, J.; Trote, J.; Andrews, P.; Rodgers, L.; McIndoo, J.; Cook, K.; Stepansky, A.; Levy, D.; Esposito, D.; et al. Tumour evolution inferred by single-cell sequencing. *Nature* 2011, 472, 90–94. [CrossRef]

23. Wang, Y.; Waters, J.; Leung, M.L.; Unruh, A.; Roh, W.; Shi, X.; Chen, K.; Scheet, P.; Vattathil, S.; Liang, H.; et al. Clonal evolution in breast cancer revealed by single nucleus genome sequencing. *Nature* 2014, 512, 155–160. [CrossRef]

24. Baslan, T.; Kendall, J.; Volyanskyy, K.; McNamara, K.; Cox, H.; D’Italia, S.; Ambrosio, F.; Riggs, M.; Rodgers, L.; Leotta, A.; et al. Novel insights into breast cancer copy number genetic heterogeneity revealed by single-cell genome sequencing. *eLife* 2020, 9, e51480. [CrossRef]

25. Condorelli, R.; Mosele, F.; Verret, B.; Bachelot, T.; Bedard, P.L.; Cortes, J.; Hyman, D.M.; Juric, D.; Krop, I.; Bieche, I.; et al. Genomic alterations in breast cancer: Level of evidence for actionability according to ESMO Scale for Clinical Actionability of molecular Targets (ESCAT). *Ann. Oncol.* 2019, 30, 365–373. [CrossRef]

26. Wood, L.D.; Parsons, D.W.; Jones, S.; Lin, J.; Sjoblom, T.; Leary, R.J.; Shen, D.; Boca, S.M.; Barber, T.; Ptak, J.; et al. The Genomic Landscapes of Human Breast and Colorectal Cancers. *Science* 2007, 318, 1108–1113. [CrossRef]

27. Stephens, P.J.; Tarpey, P.S.; Davies, H.; Van Loo, P.; Greenman, C.; Wedge, D.C.; Nik-Zainal, S.; Martin, S.; Varela, I.; Bignell, G.R.; et al. The landscape of cancer genes and mutational processes in breast cancer. *Nature* 2012, 486, 400–404. [CrossRef]

28. Kratz, J.; Burkard, M.; O’Meara, T.; Pusztai, L.; Weitch, Z.; Bedard, P.L. Incorporating Genomics Into the Care of Patients With Advanced Breast Cancer. *Ann. Soc. Clin. Oncol. Educ. Book* 2018, 38, 56–64. [CrossRef] [PubMed]

29. Mateo, J.; Chakravarty, D.; Dienstmann, R.; Jezdic, S.; Gonzalez-Perez, A.; Lopez-Bigas, N.; Ng, C.K.Y.; Bedard, P.L.; Tortora, G.; Douillard, J.-Y.; et al. A framework to rank genomic alterations as targets for cancer precision medicine: The ESMO Scale for Clinical Actionability of molecular Targets (ESCAT). *Ann. Oncol.* 2018, 29, 1895–1902. [CrossRef]

30. Hinohara, K.; Polyak, K. Intratumoral Heterogeneity: More Than Just Mutations. *Trends Cell Biol.* 2019, 29, 569–579. [CrossRef]

31. Hata, A.N.; Niederst, M.J.; Archibald, H.L.; Gomez-Caraballo, M.; Siddiqui, F.M.; Mulvey, H.E.; Maruvka, Y.E.; Ji, F.; Bhang, H.C.; Radhakrishna, V.K.; et al. Tumor cells can follow distinct evolutionary paths to become resistant to epidermal growth factor receptor inhibition. *Nat. Med.* 2016, 22, 262–269. [CrossRef]

32. Spira, A.; Yurgelun, M.B.; Alexandrov, L.; Rao, A.; Bejar, R.; Polyak, K.; Giannakis, M.; Shilatifard, A.; Finn, O.J.; Dhodapkar, M.; et al. Precancer Atlas to Drive Precision Prevention Trials. *Cancer Res.* 2017, 77, 1510–1541. [CrossRef] [PubMed]

33. Hammond, M.E.H.; Hayes, D.F.; Dowsett, M.; Allred, D.C.; Hagerty, K.L.; Badve, S.; Fitzgibbons, P.L.; Francis, G.; Goldstein, N.S.; Hayes, M.; et al. American Society of Clinical Oncology/College of American Pathologists Guideline Recommendations for Immunohistochemical Testing of Estrogen and Progesterone Receptors in Breast Cancer. *J. Clin. Oncol.* 2010, 28, 2784–2795. [CrossRef]

34. Fan, W.; Chang, J.; Fu, P. Endocrine therapy resistance in breast cancer: Current status, possible mechanisms and overcoming strategies. *Future Med. Chem.* 2015, 7, 1511–1519. [CrossRef]

35. García-Alonso, S.; Ocaña, A.; Pandiella, A. Resistance to Antibody–Drug Conjugates. *Cancer Res.* 2018, 78, 2159–2165. [CrossRef] [PubMed]

36. Slamon, D.; Eiermann, W.; Robert, N.; Pienkowski, T.; Martin, M.; Press, M.; Mackey, J.; Glaspy, J.; Chan, A.; Pawlicki, M.; et al. Adjuvant Trastuzumab in HER2-Positive Breast Cancer. *N. Engl. J. Med.* 2011, 365, 1273–1283. [CrossRef] [PubMed]
37. Garcia-Alonso, S.; Ocaña, A.; Pandiella, A. Trastuzumab Emtansine: Mechanisms of Action and Resistance, Clinical Progress, and Beyond. Trends Cancer 2020, 6, 130–146. [CrossRef] [PubMed]
38. Scaltriti, M.; Rojo, F.; Ocaña, A.; Anido, J.; Guzman, M.; Cortes, J.; Di Cosimo, S.; Matias-Guiu, X.; Cajal, S.R.Y.; Arríbas, J.; et al. Expression of p95HER2, a Truncated Form of the HER2 Receptor, and Response to Anti-HER2 Therapies in Breast Cancer. J. Natl. Cancer Inst. 2007, 99, 628–638. [CrossRef]
39. Modi, S.; Saura, C.; Yamashita, T.; Park, Y.H.; Kim, S.B.; Tamura, K.; Andre, F.; Iwata, H.; Ito, Y.; Tsurutani, J.; et al. Trastuzumab Deruxtecan in Previously Treated HER2-Positive Breast Cancer. N. Engl. J. Med. 2020, 382, 610–621. [CrossRef] [PubMed]
40. Tamura, K.; Tsurutani, J.; Takahashi, S.; Iwata, H.; Krop, I.E.; Redfern, C.; Sagara, Y.; Doi, T.; Park, H.; Murthy, R.K.; et al. Trastuzumab deruxtecan (DS-8201a) in patients with advanced HER2-positive breast cancer previously treated with trastuzumab emtansine: A dose-expansion, phase 1 study. Lancet Oncol. 2019, 20, 816–826. [CrossRef]
41. Ogitani, Y.; Hagihara, K.; Oitate, M.; Naito, H.; Agatsuma, T. Bystander killing effect of DS-8201a, a novel anti-human epidermal growth factor receptor 2 antibody-drug conjugate, in tumors with human epidermal growth factor receptor 2 heterogeneity. Cancer Sci. 2016, 107, 1039–1046. [CrossRef] [PubMed]
42. Ogitani, Y.; Aida, T.; Hagihara, K.; Yamaguchi, J.; Ishii, C.; Harada, N.; Soma, M.; Okamoto, H.; Oitate, M.; Arakawa, S.; et al. DS-8201a, A Novel HER2-Targeting ADC with a Novel DNA Topoisomerase I Inhibitor, Demonstrates a Promising Antitumor Efficacy with Differentiation from T-DM. Clin. Cancer Res. 2016, 22, 5097–5118. [CrossRef]
43. Wang, T.; Wang, D.; Liu, J.; Feng, B.; Zhou, F.; Zhang, H.; Zhou, L.; Yin, Q.; Zhang, Z.; Cao, Z.; et al. Acidity-Triggered Ligand-Presenting Nanoparticles to Overcome Sequential Drug Delivery Barriers to Tumors. Nano Lett. 2017, 17, 5429–5436. [CrossRef] [PubMed]
44. Wimberly, H.; Brown, J.R.; Schalper, K.; Haack, H.; Silver, M.R.; Nixon, C.; Bossuyt, V.; Pusztai, L.; Lannin, D.R.; Rimm, D.L. PD-L1 Expression Correlates with Tumor-Infiltrating Lymphocytes and Response to Neoadjuvant Chemotherapy in Breast Cancer. Cancer Immunol. Res. 2015, 3, 326–332. [CrossRef]
45. Dill, E.A.; Gru, A.A.; Atkins, K.A.; Friedman, L.A.; Moore, M.E.; Bullock, T.N.; Cross, J.V.; Dillon, P.M.; Mills, A.M. PD-L1 Expression and Intratumoral Heterogeneity Across Breast Cancer Subtypes and Stages. Am. J. Surg. Pathol. 2017, 41, 334–342. [CrossRef] [PubMed]
46. Schmid, P.; Adams, S.; Rugo, H.S.; Schneeweiss, A.; Barrios, C.H.; Iwata, H.; Dieras, V.; Hegg, R.; Im, S.-A.; Wright, G.S.; et al. Atezolizumab and Nab-Paclitaxel in Advanced Triple-Negative Breast Cancer. N. Engl. J. Med. 2018, 379, 2108–2121. [CrossRef] [PubMed]
47. Cortes, J.; Cescon, D.W.; Rugo, H.S.; Nowecki, Z.; Im, S.-A.; Yusof, M.M.; Gallardo, C.; Lipatov, O.; Barrios, C.H.; Holgado, E.; et al. KEYNOTE-355: Randomized, double-blind, phase III study of pembrolizumab + chemotherapy versus placebo + chemotherapy for previously untreated locally recurrent inoperable or metastatic triple-negative breast cancer. J. Clin. Oncol. 2020, 38, 1000. [CrossRef]
48. Pelekanou, V.; Barlow, W.E.; Nahleh, Z.A.; Wasserman, B.; Lo, Y.-C.; Von Wahlde, M.-K.; Hayes, D.; Hortobagyi, G.N.; Gralow, J.; Tripathy, D.; et al. Tumor-Infiltrating Lymphocytes and PD-L1 Expression in Pre- and Posttreatment Breast Cancers in the SWOG S0800 Phase II Neoadjuvant Chemotherapy Trial. Mol. Cancer Ther. 2018, 17, 1324–1331. [CrossRef]
49. Li, X.; Warren, S.; Pelekanou, V.; Wali, V.; Cesano, A.; Liu, M.; Danaher, P.; Elliott, N.; Nahleh, Z.A.; Hayes, D.F.; et al. Immune profiling of pre- and post-treatment breast cancer tissues from the SWOG S0800 neoadjuvant trial. J. Immunother. Cancer 2019, 7, 88. [CrossRef]
50. Schmid, P.; Cortes, J.; Pusztai, L.; McArthur, H.; Kümmel, S.; Bergh, J.; Denkert, C.; Park, Y.H.; Hui, R.; Harbeck, N.; et al. Pembrolizumab for Early Triple-Negative Breast Cancer. N. Engl. J. Med. 2020, 382, 810–821. [CrossRef] [PubMed]
51. Hochhaus, A.; Erben, P.; Ernst, T.; Mueller, M.C. Resistance to Targeted Therapy in Chronic Myelogenous Leukemia. Semin. Hematol. 2007, 44, 15–24. [CrossRef] [PubMed]
52. Lussana, F.; Intermesoli, T.; Stefanoni, P.; Rambaldi, A. Mechanisms of Resistance to Targeted Therapies in Chronic Myeloid Leukemia. In Mechanisms of Drug Resistance in Cancer Therapy; Handbook of Experimental Pharmacology; Springer International Publishing: Cham, Switzerland, 2017; Volume 249, pp. 231–250.
53. Kim, C.; Gao, R.; Sei, E.; Brandt, R.; Hartman, J.; Hatschek, T.; Crosetto, N.; Foukakis, T.; Navin, N.E. Chemoresistance Evolution in Triple-Negative Breast Cancer Delineated by Single-Cell Sequencing. Cell 2018, 173, 879–893.e13. [CrossRef]
54. Brady, S.W.; McQuerry, J.A.; Qiao, Y.; Piccolo, S.R.; Shrestha, G.; Jenkins, D.F.; Layer, R.M.; Pedersen, B.S.; Miller, R.H.; Esch, A.; et al. Combating subclonal evolution of resistant cancer phenotypes. *Nat. Commun.* 2017, 8, 1231. [CrossRef] [PubMed]

55. Hong, S.P.; Chan, T.E.; Lombardo, Y.; Corleone, G.; Rotmensz, N.; Bravaccini, S.; Rocca, A.; Pruner, G.; McEwen, K.R.; Coombes, R.C.; et al. Single-cell transcriptomics reveals multi-step adaptations to endocrine therapy. *Nat. Commun.* 2019, 10, 3840. [CrossRef]

56. Burrell, R.A.; Swanton, C. Tumour heterogeneity and the evolution of polyclonal drug resistance. *Mol. Oncol.* 2014, 8, 1095–1111. [CrossRef]

57. Riggins, R.B.; Schrecengost, R.S.; Guerrero, M.S.; Bouton, A.H. Pathways to tamoxifen resistance. *Cancer Lett.* 2007, 256, 1–24. [CrossRef]

58. Robinson, D.R.; Wu, Y.-M.; Vats, P.; Su, F.; Lonigro, R.J.; Cao, X.; Kalyana-Sundaram, S.; Wang, R.; Ning, Y.; Hodges, L.; et al. Activating ESR1 mutations in hormone-resistant metastatic breast cancer. *Nat. Genet.* 2013, 45, 1446–1451. [CrossRef] [PubMed]

59. Toy, W.; Shen, Y.; Won, H.; Green, B.; Sakr, R.A.; Will, M.; Li, Z.; Gala, K.; Fanning, S.; King, T.A.; et al. ESR1 ligand-binding domain mutations in hormone-resistant breast cancer. *Nat. Genet.* 2013, 45, 1439–1445. [CrossRef] [PubMed]

60. Pagliarini, R.; Shao, W.; Sellers, W.R. Oncogene addiction: Pathways of therapeutic response, resistance, and road maps toward a cure. *EMBO Rep.* 2015, 16, 280–296. [CrossRef]

61. Chandarlapaty, S.; Sakr, R.A.; Giri, D.; Patil, S.; Heguy, A.; Morrow, M.; Modi, S.; Norton, L.; Rosen, N.; Hudis, C.; et al. Frequent mutational activation of the PI3K-AKT pathway in trastuzumab-resistant breast cancer. *Clin. Cancer Res.* 2012, 18, 6784–6791. [CrossRef]

62. Rexer, B.N.; Arteaga, C.L. Intrinsic and Acquired Resistance to HER2-Targeted Therapies in HER2 Gene-Amplified Breast Cancer: Mechanisms and Clinical Implications. *Crit. Rev. Oncog.* 2012, 17, 1–16. [CrossRef]

63. Gandullo-Sánchez, L.; Capone, E.; Ocaña, A.; Iacobelli, S.; Sala, G.; Pandiella, A. HER3 targeting with an antibody-drug conjugate bypasses resistance to anti-HER2 therapies. *EMBO Mol. Med.* 2020, 12, e11498. [CrossRef] [PubMed]

64. Turner, N.C. Signatures of DNA-Repair Deficiencies in Breast Cancer. *N. Engl. J. Med.* 2017, 377, 2490–2492. [CrossRef]

65. Lord, C.J.; Ashworth, A. PARP inhibitors: Synthetic lethality in the clinic. *Science* 2017, 355, 1152–1158. [CrossRef] [PubMed]

66. Turner, N.; Tutt, A.; Ashworth, A. Hallmarks of ‘BRCAness’ in sporadic cancers. *Nat. Rev. Cancer* 2004, 4, 814–819. [CrossRef]

67. Lord, C.J.; Ashworth, A. Mechanisms of resistance to therapies targeting BRCA-mutant cancers. *Nat. Med.* 2013, 19, 1381–1388. [CrossRef]

68. Turner, N.; Tutt, A.; Ashworth, A. Targeting the DNA repair defect of BRCA tumours. *Curr. Opin. Pharmacol.* 2005, 5, 388–393. [CrossRef] [PubMed]

69. Bryant, H.E.; Schultz, N.; Thomas, H.D.; Parker, K.M.; Flower, D.; Lopez, E.; Kyle, S.; Meuth, M.; Curtin, N.J.; Helleday, T. Specific killing of BRCA2-deficient tumours with inhibitors of poly(ADP-ribose) polymerase. *Nature* 2005, 434, 913–917. [CrossRef] [PubMed]

70. Farmer, H.; McCabe, N.; Lord, C.J.; Tutt, A.N.J.; Johnson, D.A.; Richardson, T.B.; Santarosa, M.; Dillon, K.J.; Knights, C.; et al. Targeting the DNA repair defect in BRCA mutant cells as a therapeutic strategy. *Nature* 2005, 434, 917–921. [CrossRef] [PubMed]

71. Davar, D.; Beumer, J.H.; Hamieh, L.; Tawbi, H. Role of PARP Inhibitors in Cancer Biology and Therapy. *Curr. Med. Chem.* 2012, 19, 3907–3921. [CrossRef]

72. Pettitt, S.J.; Krastev, D.B.; Brandsma, I.; Dréan, A.; Song, F.; Aleksandrov, R.; Harrell, M.I.; Menon, M.; Brough, R.; Campbell, J.; et al. Genome-wide and high-density CRISPR-Cas9 screens identify point mutations in PARP1 causing PARP inhibitor resistance. *Nat. Commun.* 2018, 9, 1849. [CrossRef] [PubMed]

73. Noordermeer, S.M.; Van Attikum, H. PARP Inhibitor Resistance: A Tug-of-War in BRCA-Mutated Cells. *Trends Cell Biol.* 2019, 29, 820–834. [CrossRef]

74. Edwards, S.L.; Brough, R.; Lord, C.J.; Natrajan, R.; Vatcheva, R.; Levine, D.A.; Boyd, J.; Reis-Filho, J.S.; Ashworth, A. Resistance to therapy caused by intragenic deletion in BRCA. *Nature* 2008, 451, 1111–1115. [CrossRef] [PubMed]
75. Feinberg, A.P. The Key Role of Epigenetics in Human Disease Prevention and Mitigation. *N. Engl. J. Med.* 2018, 378, 1323–1334. [CrossRef] [PubMed]

76. Feinberg, A.P. The Key Role of Epigenetics in Human Disease Prevention and Mitigation. *N. Engl. J. Med.* 2018, 378, 1323–1334. [CrossRef] [PubMed]

77. Jones, P.A.; Baylin, S.B. The Epigenomics of Cancer. *Cell* 2007, 128, 683–692. [CrossRef]

78. Risom, T.; Langer, E.M.; Chapman, M.P.; Ranta, J.; Fields, A.J.; Boniface, C.; Alvarez, M.J.; Kendsersky, N.D.; Pelz, C.R.; Johnson-Camacho, K.; et al. Differentiation-state plasticity is a targetable resistance mechanism in basal-like breast cancer. *Nat. Commun.* 2018, 9, 3815. [CrossRef] [PubMed]

79. Yates, L.R.; Knappskog, S.; Wedge, D.; Farmery, J.H.R.; Gonzalez, S.; Martincorena, I.; Alexandrov, L.B.; Van Loo, P.; Haugland, H.K.; Lilleng, P.K.; et al. Genomic Evolution of Breast Cancer Metastasis and Relapse. *Cancer Cell* 2017, 32, 169–184.e7. [CrossRef]

80. Yang, L.; Zhang, Y.; Shan, W.; Hu, Z.; Yuan, J.; Pi, J.; Wang, Y.; Fan, L.; Tang, Z.; Li, C.; et al. Repression of BET activity sensitizes homologous recombination–proficient cancers to PARP inhibition. *Sci. Transl. Med.* 2017, 9, eaal1645. [CrossRef] [PubMed]

81. Black, J.C.; Atabakhsh, E.; Kim, J.; Biette, K.M.; Van Rechem, C.; Ladd, B.; Burrowes, P.D.; Donado, C.; Mattoo, H.; Kleinstiver, B.P.; et al. Hypoxia drives transient site-specific copy gain and drug-resistant gene expression. *Genes Dev.* 2015, 29, 1018–1031. [CrossRef]

82. Widmer, D.S.; Hoek, K.S.; Cheng, P.F.; Eichhoff, O.M.; Biedermann, T.; Raaijmakers, M.I.G.; Hemmi, S.; Dummer, R.; Levesque, M.P. Hypoxia Contributes to Melanoma Heterogeneity by Triggering HIF1α-Dependent Phenotype Switching. *J. Investig. Dermatol.* 2013, 133, 2436–2443. [CrossRef] [PubMed]

83. Keith, B.; Johnson, R.S.; Simon, M.C. HIF1α and HIF2α: Sibling rivalry in hypoxic tumour growth and progression. *Nat. Rev. Cancer* 2012, 12, 9–22. [CrossRef]

84. Pistollato, F.; Abbadi, S.; Rampazzo, E.; Persano, L.; Della Puppa, A.; Frasson, C.; Sarto, E.; Scienza, R.; D’Avella, D.; Basso, G. Intratumoral Hypoxic Gradient Drives Stem Cells Distribution and MGMT Expression in Glioblastoma. *Stem Cells* 2010, 28, 851–862. [CrossRef] [PubMed]

85. Alkasalas, T.; Moyano-Galceran, L.; Arsenian-Henriksson, M.; Lehti, K. Fibroblasts in the Tumor Microenvironment: Shield or Spear? *Int. J. Mol. Sci.* 2018, 19, 1532. [CrossRef] [PubMed]

86. Costa, A.; Kieffer, Y.; Scholer-Dahirel, A.; Pelon, F.; Bourachot, B.; Cardon, M.; Sirven, P.; Magagna, I.; Fuhrmann, L.; Bernard, C.; et al. Fibroblast Heterogeneity and Immunosuppressive Environment in Human Breast Cancer. *Cancer Cell* 2018, 33, 463–479.e10. [CrossRef] [PubMed]

87. Adams, S.; Gray, R.J.; DeMaria, S.; Goldstein, L.; Perez, E.A.; Shulman, L.N.; Martino, S.; Wang, M.; Jones, V.E.; Saphner, T.J.; et al. Prognostic Value of Tumor-Infiltrating Lymphocytes in Triple-Negative Breast Cancers From Two Phase III Randomized Adjuvant Breast Cancer Trials: ECOG 2197 and ECOG. *J. Clin. Oncol.* 2014, 32, 2959–2966. [CrossRef]

88. Ali, H.R.; Provenzano, E.; Dawson, S.-J.; Blows, F.M.; Liu, B.; Shah, M.; Earl, H.M.; Poole, C.J.; Hiller, L.; Dunn, J.A.; et al. Association between CDB8+ T-cell infiltration and breast cancer survival in 12 439 patients. *Ann. Oncol.* 2014, 25, 1536–1543. [CrossRef] [PubMed]

89. Denkert, C.; Loibl, S.; Neske, A.; Roller, M.; Müller, B.M.; Komor, M.; Budczies, J.; Darb-Esfahani, S.; Kronenwett, R.; Hansch, C.; et al. Tumor-Associated Lymphocytes As an Independent Predictor of Response to Neoadjuvant Chemotherapy in Breast Cancer. *J. Clin. Oncol.* 2010, 28, 105–113. [CrossRef]
94. Gruossø, T.; Gigoux, M.; Manem, V.S.K.; Bertos, N.; Zuo, D.; Perlitch, I.; Saleh, S.M.I.; Zhao, H.; Souleimanova, M.; Johnson, R.M.; et al. Spatially distinct tumor immune microenvironments stratify triple-negative breast cancers. J. Clin. Investig. 2019, 129, 1785–1800. [CrossRef]

95. Keren, L.; Bosse, M.; Marquez, D.; Angoshvari, R.; Jain, S.; Varma, S.; Yang, S.-R.; Kurian, A.; Van Valen, D.; West, R.; et al. A Structured Tumor-Immune Microenvironment in Triple-Negative Breast Cancer Revealed by Multiplexed Ion Beam Imaging. Cell 2018, 174, 1373–1387.e19. [CrossRef]

96. Egelston, C.A.; Avalos, C.; Tu, T.Y.; Rosario, A.; Wang, R.; Solomon, S.; Srinivasan, G.; Nelson, M.S.; Huang, Y.; Lim, M.H.; et al. Resident memory CD8+ T cells within cancer islands mediate survival in breast cancer patients. JCI Insight 2019, 4, e130000. [CrossRef]

97. Cimino-Mathews, A.; Thompson, E.; Taube, J.M.; Ye, X.; Lu, Y.; Meeker, A.; Xu, H.; Sharma, R.; Lecksell, K.; Andrés-Pretel, F.; Galán-Moya, E.M.; Amir, E.; Pandiella, A.; Győrffy, B.; et al. Expression of MHC class I, HLA-A and HLA-B identifies immune-activated breast tumors with favorable outcome. OncolImmunology 2019, 8, e1629780. [CrossRef]

98. Tang, F.; Zheng, P. Tumor cells versus host immune cells: Whose PD-L1 contributes to PD-1 cellular interaction. BMC Cancer 2018, 29, 2223–2239. [CrossRef]

99. Comen, E.A. Tracking the seed and tending the soil: Evolving concepts in metastatic breast cancer. Discov. Med. 2012, 14, 97–104. [PubMed]

100. Szekely, B.; Bossuyt, V.; Li, X.; Wali, V.B.; Patwardhan, G.A.; Frederick, C.; Silber, A.; Park, T.; Harigopal, M.; Weiler, J.; Dittmar, T. Cell Fusion in Human Cancer: The Dark Matter Hypothesis. Discov. Med. 2013, 15, 371–377. [PubMed]

101. Lu, X.; Kang, Y. Efficient acquisition of dual metastasis organotropism to bone and lung through stable spontaneous fusion between MDA-MB-231 variants. Proc. Natl. Acad. Sci. USA 2009, 106, 9385–9390. [CrossRef]

102. Berndt, B.; Zanker, K.S.; Dittmar, T. Cell fusion is a potent inducer of aneuploidy and drug resistance in tumor cell/normal cell hybrids. Crit. Rev. Oncog. 2013, 18, 97–113. [CrossRef]

103. Mortensen, K.; Lichtenberg, J.; Thomsen, P.D.; Larsson, L.-I. Spontaneous fusion between cancer cells and endothelial cells. Cell. Mol. Life Sci. 2004, 61, 2125–2131. [CrossRef] [PubMed]

104. Noubissi, F.K.; Harkness, T.; Alexander, C.M.; Ogle, B.M. Apoptosis-induced cancer cell fusion: A mechanism of breast cancer metastasis. FASEB J. 2015, 29, 4036–4045. [CrossRef] [PubMed]

105. Shabo, I.; Midtbø, K.; Andersson, H.; Åkerlund, E.; Olsson, H.; Wegman, P.; Gunnarsson, C.; Lindström, A. Macrophage traits in cancer cells are induced by macrophage-cancer cell fusion and cannot be explained by cellular interaction. BMC Cancer 2015, 15, 922. [CrossRef]

106. Rizvi, N.A.; Hellmann, M.D.; Snyder, A.; Kvistborg, P.; Makarov, V.; Havel, J.J.; Lee, W.; Yuan, J.; Wong, P.; Ho, T.S.; et al. Mutational landscape determines sensitivity to PD-1 blockade in non–small cell lung cancer. Science 2015, 348, 124–128. [CrossRef] [PubMed]

107. Snyder, A.; Makarov, V.; Merghoub, T.; Yuan, J.; Zaretsky, J.M.; Desrichard, A.; Walsh, L.A.; Postow, M.A.; Wong, P.; Ho, T.S.; et al. Genetic Basis for Clinical Response to CTLA-4 Blockade in Melanoma. N. Engl. J. Med. 2014, 371, 2189–2199. [CrossRef]
113. Zaretsky, J.M.; Garcia-Diaz, A.; Shin, D.S.; Escuin-Ordinas, H.; Hugo, W.; Hu-Lieskovská, S.; Torrejon, D.Y.; Abril-Rodriguez, G.; Sandoval, S.; Barthly, L.; et al. Mutations Associated with Acquired Resistance to PD-1 Blockade in Melanoma. *N. Engl. J. Med.* 2016, 375, 819–829. [CrossRef] [PubMed]

114. Keenan, T.E.; Burke, K.P.; Van Allen, E.M. Genomic correlates of response to immune checkpoint blockade. *Nat. Med.* 2019, 25, 389–402. [CrossRef] [PubMed]

115. Miao, D.; Margolis, C.A.; Vokes, N.I.; Liu, D.; Taylor-Weiner, A.; Wankowicz, S.M.; Adeegbe, D.; Keliher, D.; Schilling, B.; Tracy, A.; et al. Genomic correlates of response to immune checkpoint blockade in microsatellite-stable solid tumors. *Nat. Genet.* 2018, 50, 1271–1281. [CrossRef] [PubMed]

116. Cimas, F.J.; Manzano, A.; Baliu-Piquet, M.; García-Gil, E.; Pérez-Segura, P.; Nagy, Á.; Pandiella, A.; Győrrfy, B.; Ocana, A. Genomic Mapping Identifies Mutations in RYR2 and AHNAK as Associated with Favorable Outcome in Basal-Like Breast Tumors Expressing PD1/PD-L. *Cancers 2020*, 12, 2243. [CrossRef]

117. Lim, A.R.; Rathmell, W.K.; Rathmell, J.C. The tumor microenvironment as a metabolic barrier to effector T cells and immunotherapy. *eLife* 2020, 9, 1–13. [CrossRef]

118. Saeidi, A.; Zandi, K.; Cheok, Y.Y.; Saeidi, H.; Wong, W.F.; Lee, C.Y.Q.; Cheong, H.C.; Yong, Y.K.; Larsson, M.; Shankar, E.M. T-Cell Exhaustion in Chronic Infections: Reversing the State of Exhaustion and Reinvigorating Optimal Protective Immune Responses. *Front. Immunol.* 2018, 9, 2569. [CrossRef] [PubMed]

119. Parry, R.V.; Chemnitz, J.M.; Frauwirth, K.A.; Lanfranco, A.R.; Braunstein, I.; Kobayashi, S.V.; Linsley, P.S.; Saeidi, A.; Zandi, K.; Cheok, Y.Y.; Saeidi, H.; Wong, W.F.; Lee, C.Y.Q.; Cheong, H.C.; Yong, Y.K.; Larsson, M.; Shankar, E.M. T-Cell Exhaustion in Chronic Infections: Reversing the State of Exhaustion and Reinvigorating Optimal Protective Immune Responses. *Front. Immunol.* 2018, 9, 2569. [CrossRef] [PubMed]

120. Patsoukis, N.; Bardhan, K.; Chatterjee, P.; Sari, D.; Liu, B.; Bell, L.N.; Karoly, E.D.; Freeman, G.J.; Petkova, V.; Seth, P.; et al. PD-1 alters T-cell metabolic reprogramming by inhibiting glycolysis and promoting lipolysis and fatty acid oxidation. *Nat. Commun.* 2015, 6, 6692. [CrossRef]

121. Zhang, C.; Yue, C.; Herrmann, A.; Song, J.; Egelston, C.; Wang, T.; Zhang, Z.; Li, W.; Lee, H.; Aftabizadeh, M.; et al. STAT3 Activation-Induced Fatty Acid Oxidation in CD8+ T Effector Cells Is Critical for Obesity-Promoted Breast Tumor Growth. *Cell Metab.* 2020, 31, 148–161.e5. [CrossRef]

122. Munn, D.H.; Mellor, A.L. Indoleamine 2,3 dioxygenase and metabolic control of immune responses. *Trends Immunol.* 2013, 34, 137–143. [CrossRef] [PubMed]

123. Mezrich, J.D.; Fehner, J.H.; Zhang, X.; Johnson, B.P.; Burlington, W.J.; Bradfield, C.A. An Interaction between Kynurenine and the Aryl Hydrocarbon Receptor Can Generate Regulatory T Cells. *J. Immunol.* 2010, 185, 3190–3198. [CrossRef] [PubMed]

124. Fischer, K.; Hoffmann, P.; Voelkl, S.; Meidenbauer, N.; Ammer, J.; Edinger, M.; Gottfried, E.; Schwarz, S.; Rothe, G.; Hoves, S.; et al. Inhibitory effect of tumor cell-derived lactic acid on human T cells. *Blood* 2007, 109, 3812–3819. [CrossRef] [PubMed]

125. Li, H.; Bullock, K.; Gurao, C.; Braun, D.; Shukla, S.A.; Bossé, D.; Lalani, A.-K.A.; Gopal, S.; Jin, C.; Horak, C.; et al. Metabolomic adaptations and correlates of survival to immune checkpoint blockade. *Nat. Commun.* 2019, 10, 4346. [CrossRef]

126. Munn, D.H.; Shafizadeh, E.; Attwood, J.T.; Bondarev, I.; Pashine, A.; Mellor, A.L. Inhibition of T Cell Proliferation by Macrophage Tryptophan Catabolism. *J. Exp. Med.* 1999, 189, 1363–1372. [CrossRef]

127. Hwu, P.; Du, M.X.; Lapointe, R.; Do, M.; Taylor, M.W.; Young, H.A. Indoleamine 2,3-Dioxygenase Production by Human Dendritic Cells Results in the Inhibition of T Cell Proliferation. *J. Immunol.* 2000, 164, 3596–3599. [CrossRef]

128. Munn, D.H.; Sharma, M.D.; Lee, J.R.; Jhaver, K.G.; Johnson, T.S.; Keskin, D.B.; Marshall, B.; Chandler, P.; Antonia, S.J.; Burgess, R.; et al. Potential Regulatory Function of Human Dendritic Cells Expressing Indoleamine 2,3-Dioxygenase. *Science 2002*, 297, 1867–1870. [CrossRef]

129. Le, D.T.; Durham, J.N.; Smith, K.N.; Wang, H.; Bartlett, B.R.; Aulakh, L.K.; Lu, S.; Kemberling, H.; Wilt, C.; Luber, B.S.; et al. Mismatch repair deficiency predicts response of solid tumors to PD-1 blockade. *Science 2017*, 357, 409–413. [CrossRef]

130. Le, D.T.; Uram, J.N.; Wang, H.; Bartlett, B.R.; Kemberling, H.; Eyring, A.D.; Skora, A.D.; Luber, B.S.; Azad, N.S.; Laheru, D.; et al. PD-1 Blockade in Tumors with Mismatch-Repair Deficiency. *N. Engl. J. Med.* 2015, 372, 2509–2520. [CrossRef]

131. Havel, J.J.; Chowell, D.; Chan, T.A. The evolving landscape of biomarkers for checkpoint inhibitor immunotherapy. *Nat. Rev. Cancer 2019*, 19, 133–150. [CrossRef] [PubMed]
132. FDA U.S. Food and Drug Administration. FDA Approves First Cancer Treatment for Any Solid Tumor with a Specific Genetic Feature. Available online: https://www.fda.gov/newsevents/newsroom/pressannouncements/ucm560167.htm (accessed on 10 September 2020).

133. Looney, A.-M.; Nawaz, K.; Webster, R.M. Tumour-agnostic therapies. Nat. Rev. Drug Discov. 2020, 19, 383–384. [CrossRef]

134. Germano, G.; Lamba, S.; Rospo, G.; Barault, L.; Magri, A.; Maione, F.; Russo, M.; Crisafulli, G.; Bartolini, A.; Lerda, G.; et al. Inactivation of DNA repair triggers neoantigen generation and impairs tumour growth. Nature 2017, 552, 116–120. [CrossRef]

135. Alexandrov, L.B.; Nik-Zainal, S.; Wedge, D.C.; Aparicio, S.A.J.R.; Behjati, S.; Biankin, A.V.; Bignell, G.R.; Bolli, N.; Borg, A.; Børresen-Dale, A.-L.; et al. Signatures of mutational processes in human cancer. Nature 2013, 552, 116–120. [CrossRef]

136. Pestana, R.C.; Sen, S.; Hobbs, B.P.; Hong, D.S. Histology-agnostic drug development—considering issues beyond the tissue. Nat. Rev. Clin. Oncol. 2020, 17, 555–568. [CrossRef]

137. Drilon, A.; Laetsch, T.W.; Kummer, S.; Dubois, S.G.; Lassen, U.N.; Demetri, G.D.; Nathenson, M.; Doebele, R.C.; Farago, A.F.; Pappo, A.S.; et al. Efficacy of Larotrectinib in TRK Fusion–Positive Cancers in Adults and Children. N. Engl. J. Med. 2018, 378, 731–739. [CrossRef]

138. Pestana, R.C.; Sen, S.; Hobbs, B.P.; Hong, D.S. Histology-agnostic drug development—considering issues beyond the tissue. Nat. Rev. Clin. Oncol. 2020, 17, 555–568. [CrossRef]

Publisher’s Note: MDPI stays neutral with regard to jurisdictional claims in published maps and institutional affiliations.

© 2020 by the authors. Licensee MDPI, Basel, Switzerland. This article is an open access article distributed under the terms and conditions of the Creative Commons Attribution (CC BY) license (http://creativecommons.org/licenses/by/4.0/).