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Review

Deciphering breast cancer: from biology to the clinic

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SUMMARY

Breast cancer remains a leading cause of cancer-related mortality in women, reflecting profound disease heterogeneity, metastasis, and therapeutic resistance. Over the last decade, genomic and transcriptomic data have been integrated on an unprecedented scale and revealed distinct cancer subtypes, critical molecular drivers, clonal evolutionary trajectories, and prognostic signatures. Furthermore, multi-dimensional integration of high-resolution single-cell and spatial technologies has highlighted the importance of the entire breast cancer ecosystem and the presence of distinct cellular "neighborhoods." Clinically, a plethora of new targeted therapies has emerged, now being rapidly incorporated into routine care. Resistance to therapy, however, remains a crucial challenge for the field.

INTRODUCTION

Breast cancer is a global problem: it is the most commonly diagnosed cancer in women, with an estimated 2.3 million new cases and >685,000 deaths reported in 2020 (Sung et al., 2021). Although survival rates have markedly improved over the past two decades, the incidence of this disease continues to rise worldwide. Improved outcomes have been largely attributable to mammographic screening and adjuvant therapies (Hashim et al., 2016); however, highly effective systemic therapies for advanced disease are now making an important impact. A combination of genetic and non-genetic factors influences breast cancer incidence. The latter includes age, reproductive risk factors (e.g., early menarche and late menopause), exogenous female hormones, lifestyle factors (e.g., post-menopausal obesity and alcohol consumption), radiation exposure, high mammographic density, and the presence of histologic lesions such as atypical hyperplasia, although some of these factors can also be underpinned by genetic predisposition (Danaei et al., 2005; Hankinson et al., 2004).

Breast cancer comprises multiple biological entities characterized by heterogeneity in pathology, genomic alterations, gene expression, and the tumor microenvironment (TME), which collectively influence clinical behavior and treatment response. However, the classic parameters of histopathology, tumor size and grade, nodal involvement, and marker expression currently being used to guide treatment decisions are imperfect, particularly in the case of advanced cancers, which eventually develop resistance. Hence, there is a pressing need to better predict response to therapy and a need to improve selection of optimized therapy. Over the past decade, the intrinsic molecular subtypes of breast cancer and predictive signatures have been further refined, while the genomics revolution has enabled the sequencing of vast numbers of breast tumors at unprecedented speed and resolution. Deep genomic analyses have also provided substantive insights into intratumoral heterogeneity and clonal evolution during disease progression and metastasis. Furthermore, it has become increasingly clear that the entire tumor ecosystem must be considered when dissecting the biology of breast cancer and improving therapeutic strategies. In this review, we focus on human disease and highlight recent developments in deciphering breast tumoral heterogeneity, genetic drivers, and cellular complexity within the whole tumor, much of which is being propelled through novel multi-modal platforms. Finally, we summarize the main players being incorporated into breast cancer therapy.

TRADITIONAL BREAST CANCER CLASSIFICATION

Human breast carcinomas are stratified according to a multi-dimensional framework that incorporates histopathological classification, clinical characteristics, and advanced molecular analysis. At diagnosis, tumors are broadly classified by histology as *in situ* carcinoma or invasive carcinomas, depending on the spread of malignant cells from breast lobules or ducts into the surrounding stroma (Figure 1) (reviewed in WHO Classification of Tumours). The most common form of pre-invasive breast cancer is ductal carcinoma *in situ* (DCIS), for which only 10%–30%

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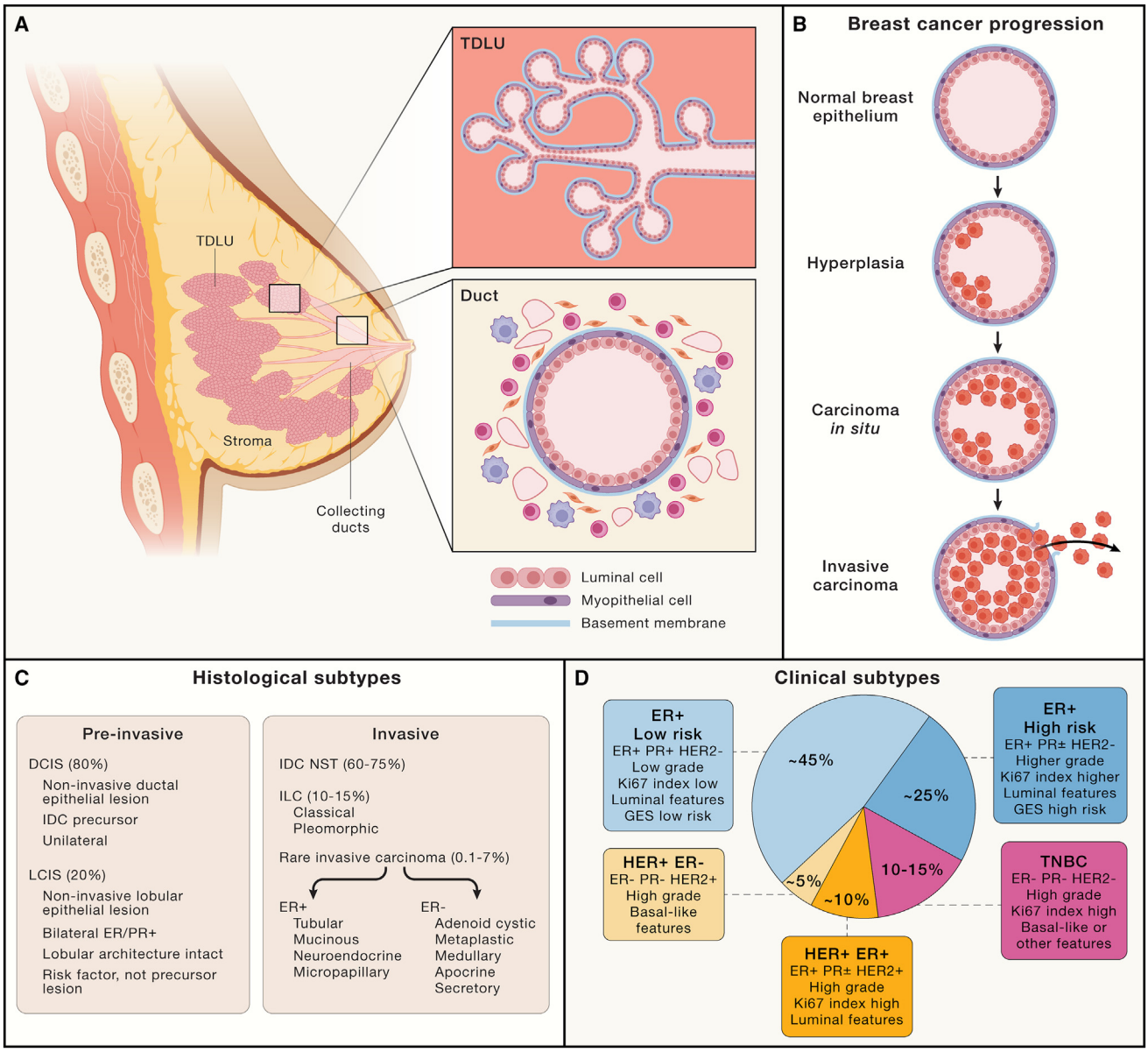

Figure 1. Breast structure and histopathological classification of breast cancer

(A) Schematic representation of the human breast, depicting the terminal ductal lobular unit (TDLU), the functional unit of the breast where the majority of tumors arise, and a cross-sectional view of the branched epithelial ductal tree. Breast ducts consist of a bi-layered epithelium of luminal and myoepithelial cells, surrounded by an immune, fibroblast, and adipocyte-rich stroma that influences both normal breast physiology and carcinogenesis.

(B) Simplified model of breast cancer pathogenesis. Proliferation of abnormal cells from the ductal or lobular epithelium can lead to pre-invasive lesions termed carcinoma *in situ*. Once tumor cells breach the basement membrane and infiltrate the surrounding stroma, the cancer is classified as invasive carcinoma.

(C) Overview of major histological subtypes of pre-invasive lesions and invasive breast carcinomas. Invasive ductal carcinoma "no special type" (NST) accounts for the large majority of breast tumors. Approximately 15%–25% of invasive cancers are characterized by distinctive growth patterns and cytological features.

(D) Comparison of the major clinical subtypes of breast cancer, based primarily on histological features and immunohistochemical expression of estrogen receptor (ER), progesterone receptor (PR), human epidermal growth factor receptor 2 (HER2), and the proliferation marker Ki67. ER+ tumors can be stratified as high or low risk, depending on tumor grade and proliferation (Ki67 score). HER2+ tumors can be subdivided on the basis of ER expression, identifying tumors with distinct molecular and prognostic features. ER is essential for clinical classification, while PR expression is an ancillary marker. DCIS, ductal carcinoma *in situ*; LCIS, lobular carcinoma *in situ*; IDC NST, invasive ductal carcinoma, no special type; ILC, invasive lobular carcinoma; TNBC, triple-negative breast cancer; GES, gene expression signature.

of cases progress to invasive cancer, but predictive biomarkers for progression to invasive or metastatic disease are suboptimal. Invasive carcinomas are a heterogeneous group of diseases that are further subdivided according to cell morphology: these are most commonly invasive ductal carcinoma (IDC), representing 60%–75% of cases, followed by invasive lobular carcinoma

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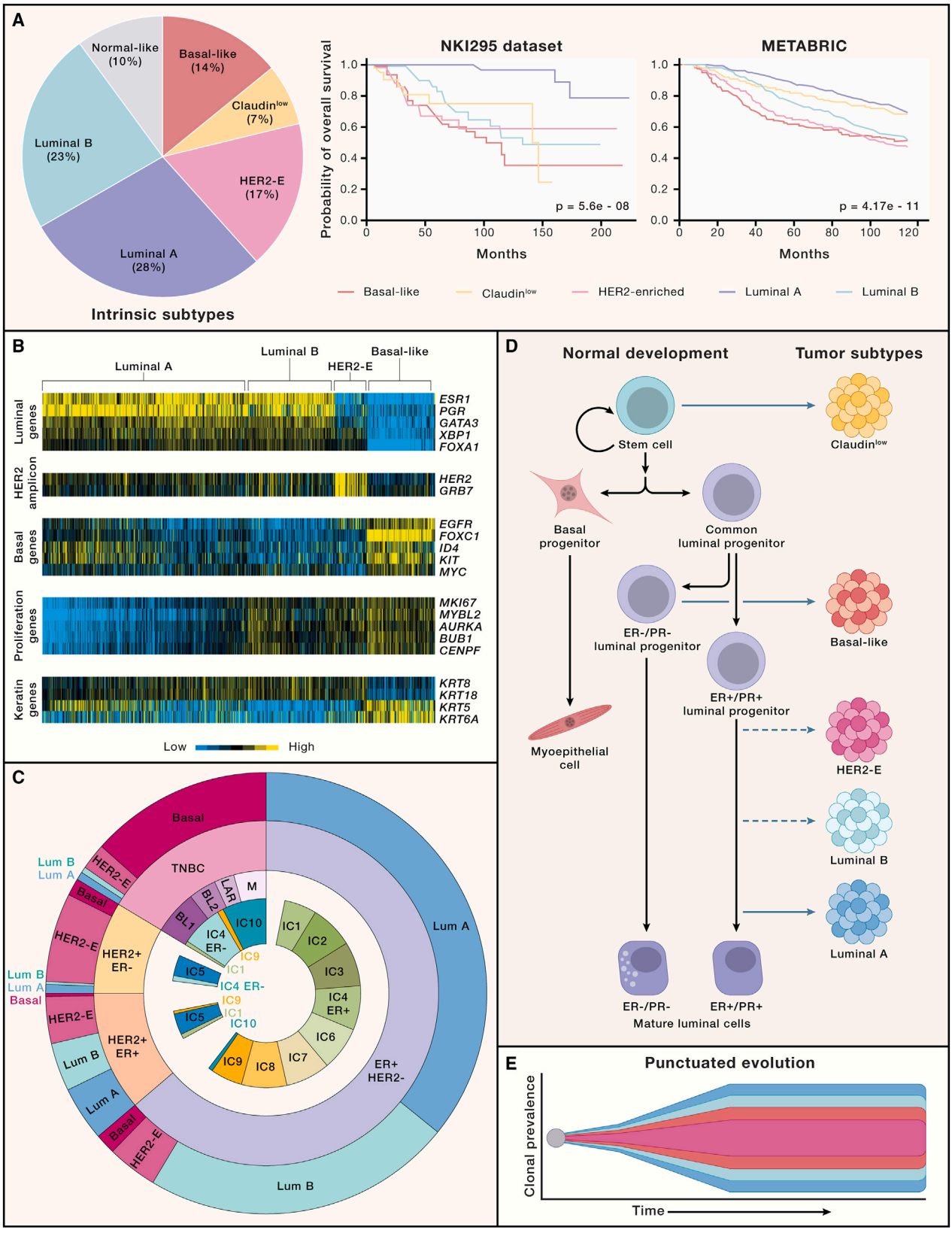

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(ILC) that comprises 10%–15% of tumors. IDC and ILC are distinct diseases, differing in their pathology, genomic profiles, metastatic organotropism, and treatment response. IDC can be stratified into numerous subtypes of which IDC of "no special type" (NST) is the most common. ILC is characterized by a striking dys-cohesive phenotype, with small neoplastic cells infiltrating the surrounding stroma in a single-file growth pattern. Aside from ILC, the remaining rare, special histological subtypes constitute 0.1%–7% of breast cancer (Figure 1) (Weigelt and Reis-Filho, 2009). A 3-tiered grading system (Bloom-Richardson), based on the degree of differentiation (growth pattern and nuclear pleomorphism) and proliferative activity (mitotic index), further subdivides invasive carcinomas for prognostic evaluation (Harbeck et al., 2019). A rare clinical variant characterized by dermal lymphatic involvement, termed inflammatory breast cancer, is associated with high metastatic propensity and poorer survival rates.

Beyond morphological classification, breast tumors are clinically stratified according to expression of the estrogen receptor (ER), progesterone receptor (PR), and human epithelial growth factor receptor 2 (HER2/ERBB2) into three broad clinical groups: ER+, HER2+, and triple-negative breast cancer (TNBC) (Figure 1). ER+ cancers account for approximately 70% of all breast cancers, where ER+ is defined as $\geq$1% ER-positive tumor cells (although $\geq$10% expression is considered to have greater clinical relevance), while HER2+ tumors can be further subdivided into the HER2+ER+ ($\sim$70%) and HER2+ER− ($\sim$30%) subgroups. HER2 status is reported by immunohistochemistry and further assessed via chromogenic or fluorescence *in situ* hybridization (CISH and FISH, respectively) to determine gene amplification. HER2+ tumors (defined as circumferential 3+ staining in >10% cells or *HER2/ERBB2* amplification) account for approximately 15% of breast cancers and exhibit extensive biological heterogeneity. Increasing attention is being paid to HER2-low breast cancer, where low (1+) to moderate (2+) HER2 expression is detectable by immunohistochemistry, but *ERBB2* amplification is absent (reviewed in Tarantino et al. (2020)). TNBC ($\sim$15% of breast cancers) lacks expression of ER, PR, and HER2 and encompasses diverse subtypes that are generally characterized by expression of EGFR and cytokeratins CK5 and CK14. These tumors frequently follow an aggressive clinical course associated with younger age, higher grade at diagnosis, and poor prognosis. TNBCs are prone to early recurrence and metastasis, particularly to lung and brain, and account for a disproportionately high fraction of breast cancer mortalities. Overall, these clinical-pathological variables, although not definitive biological parameters, continue to play a vital role in considering prognosis, treatment selection, and clinical trial design.

INTRINSIC MOLECULAR SUBTYPES OF BREAST CANCER

The advent of RNA-based molecular profiling has profoundly influenced our understanding of breast cancer heterogeneity and impacted patient stratification and treatment selection over the past two decades. Five primary intrinsic molecular subtypes have emerged as a result of pioneering microarray expression profiling studies (Herschkowitz et al., 2007; Perou et al., 2000; Sørlie et al., 2001): luminal A, luminal B, HER2-enriched, basal-like, and claudin-low, each exhibiting unique biological, prognostic, and clinical features (Figure 2). Luminal A tumors are typically low-grade ER+PR+ tumors; express a strong luminal gene signature that includes *ESR1*, *GATA3*, *XBP1*, and *FOXA1*; and show more favorable relapse-free survival and overall survival post-treatment, compared with all other breast cancer subtypes. Luminal B tumors are ER+ but exhibit lower luminal gene expression (e.g., *PGR*) and higher expression of proliferation genes. HER2-enriched (HER2-E) tumors represent 15%–20% of breast cancer cases and are distinguished by *HER2/ERBB2* amplification on chromosome 17q12 and intermediate expression of the luminal gene signature. Although HER2-E tumors largely overlap with HER2 positivity as determined by immunohistochemistry or FISH, not all clinically based HER2+ tumors are of the HER2-E molecular subtype. Conversely, the HER2-E molecular profile can align with HER2− tumors (TCGA, 2012). While the majority (70%) of HER2+ER− tumors are HER2-E, small subsets exist within the basal-like and luminal B subgroups, which may reflect distinct "cells of origin" (Prat et al., 2014). Basal-like tumors, representing $\sim$15% of patients, are highly proliferative and display augmented expression of basal cytokeratins and EGFR and low expression of the luminal A signature. Furthermore, they are characterized by high chromosomal instability and have a strong association with germline *BRCA1* mutations. Interestingly, these tumors are molecularly closer to high-grade serous ovarian cancer (HG-SOC) than luminal tumors (TCGA, 2012). While the majority of basal-like

Figure 2. Identification of intrinsic subtypes of breast cancer by molecular profiling

(A) Graphs depicting the frequency and prognosis of breast cancer intrinsic subtypes. Percentages are from the publicly available NKI-295 dataset (n = 295). Normal-like tumors have significant contamination with normal breast tissue. Prognostic outcomes for each subtype are shown as overall survival. The NKI survival curve is from Prat et al. (2010); the survival curve based on the METABRIC dataset (n = 1,608 tumors) was generated using cBioPortal.

(B) Gene expression data for key genes associated with the breast cancer intrinsic subtypes. Breast tumor samples from the TCGA (n = 792) were ordered according to the 4 main intrinsic subtypes for selected genes. Yellow, higher than median gene expression; black, median; blue, lower than median. Reproduced with permission from Hoadley et al. (2014).

(C) Schematic representation of the distribution of breast cancer subtypes, defined by histopathology and molecular analysis. Data for intrinsic subtypes and pathological classification are from Cejalvo et al. (2017), TNBC subtypes are from Lehmann et al. (2011) (subsequently refined to four groups), and the integrative cluster analysis is from Rueda et al. (2019), where a 5% threshold was applied for IntCluster association with each clinical subtype. IC, integrative cluster; ER, estrogen receptor; TNBC, triple-negative breast cancer; HER2, human epidermal growth factor receptor 2; BL1, basal-like 1; BL2, basal-like 2; LAR, luminal androgen receptor; M, mesenchymal.

(D) Schematic model depicting relationships between the normal breast differentiation hierarchy and the intrinsic subtypes. Arrows denote the closest normal epithelial cell type (putative cell of origin) for each tumor subtype, based on comparison of gene expression signatures (Lim et al., 2009) and mouse stem cell studies (Fu et al., 2020).

(E) Punctuated model of clonal evolution. A large number of genetic alterations occur early in tumor development via short bursts of evolution, followed by stable expansion of dominant clones.

tumors are triple negative (~80%), the terms are not strictly interchangeable. Interestingly, although TNBC patients have a proclivity for early relapse, compared with luminal tumors, some patients (~40%) with basal-like disease display exquisite sensitivity to chemotherapy and high rates of pathological complete response (pCR) (Rouzier et al., 2005). The least frequent subtype, claudin-low tumors, are typically triple-negative tumors that display low expression of proliferation markers, adhesion proteins, and luminal differentiation genes. The claudin-low profile also shows enrichment in mesenchymal features and immune cell infiltrate, high representation among metaplastic and medullary carcinomas, and poor sensitivity to chemotherapy (Prat et al., 2010). More recently, the integration of refined histology and molecular analyses for The Cancer Genome Atlas (TCGA) breast cancer dataset revealed 12 consensus subgroups, with unique molecular signatures identified for rare histological types (Thennavan et al., 2021).

Single-cell transcriptomic analyses have provided further insights into heterogeneity among the malignant population in the different subtypes of breast cancer. Interrogation of treatment-naive breast tumors has illuminated profound inter-patient variability across all major subtypes (Chung et al., 2017; Pal et al., 2021; Wu et al., 2021). Despite extensive intratumoral heterogeneity, tumors tended to show two broad clusters of cycling and non-cycling cells across the different clinical groups. Notably, intrinsic subtyping of single malignant cells indicated the presence of diverse phenotypes within individual tumors, with multiple intrinsic subtypes apparent in many tumors (Chung et al., 2017; Wu et al., 2021). For example, a small subset of basal-like cells was detectable in some luminal and HER-2E tumors. The contribution of these minor subsets to overall tumor heterogeneity remains unclear; however, single-cell profiling has the potential to identify clinically relevant subpopulations. The data further suggest that gene signatures defined by bulk RNA sequencing may not always accurately reflect tumor phenotype and relevant biological pathways. Further single-cell studies on carcinoma cells will be required for a deeper understanding of cellular state changes between early and advanced disease.

The delineation of intrinsic molecular subtypes and their refinement has led to a new era of molecular diagnostics to help refine therapy selection for breast cancer patients. First generation prognostic signatures include Oncotype DX, MammaPrint, and genomic grade index (GGI). Oncotype DX-gene recurrence score (RS) quantifies the mRNA expression of 21 tumor and housekeeping genes and provides robust prognostic information (Paik et al., 2004). It has been prospectively validated in large trials to identify patients with ER+ tumors who can safely avoid chemotherapy, although limited value was shown for pre-menopausal women with nodal involvement (Kalinsky et al., 2021; Sparano et al., 2019). MammaPrint, which measures mRNA expression of 70 genes (van't Veer et al., 2002), has been prospectively validated in the MINDACT trial, where a low genomic risk score successfully predicted the majority of patients who could safely avoid chemotherapy despite clinical criteria (including regional node involvement) placing them at high risk of distant relapse (Cardoso et al., 2016). Similar to Oncotype DX, the genomic prediction was more robust in women aged 50 years or older (Piccart et al., 2021). Second-generation assays such as Prosigna, Endopredict, and Breast Cancer Index offer differing utility but have not yet been prospectively validated (Andre et al., 2022). Prosigna incorporates the PAM50 intrinsic gene set (a 50-gene signature developed to define breast cancer subtypes), tumor size, nodal status, and proliferative index to predict the risk of recurrence and identify the tumor subtype in post-menopausal women. It has shown promise in retrospective clinical studies (Parker et al., 2009) and is currently undergoing prospective validation in the OPTIMA trial (ISRCTN42400492). Ongoing refinement of molecular signatures will be required to better enable chemotherapy selection for pre-menopausal patients.

Molecular profiling has provided an important foundation for understanding the etiology of breast cancer, since the intrinsic subtypes bear striking resemblance to normal cells within the mammary stem cell hierarchy (Lim et al., 2009) (Figure 2). The molecular similarities suggest that distinct breast epithelial cells serve as cells of origin for malignant transformation across subtypes, with additional heterogeneity attributable to intrinsic and extrinsic influences including mutation signatures, epigenetic events, and the microenvironment. Poorly differentiated claudin-low tumors have remarkably similar transcriptomes to the normal breast stem cell-enriched population, while at the opposite end of the differentiation spectrum, luminal A tumors align most closely with mature luminal cells. There is compelling evidence that luminal progenitor cells are the precursors for basal-like cancers, including for familial *BRCA1*-mutated tumors (Lim et al., 2009; Molyneux et al., 2010). These studies together with insights from the mouse mammary gland (Fu et al., 2020) highlight how normal stem cell biology and cancer biology are inextricably intertwined. In the future, it will be important to determine the differential sensitivity of normal stem and progenitor cells in human breast to ovarian hormones, given the central role they play in breast carcinogenesis.

DISSECTING THE GENOMIC LANDSCAPE OF BREAST CANCER

In a series of landmark studies based on next-generation sequencing, a combination of whole-genome sequencing (WGS), whole-exome sequencing, copy-number profiling, and/or transcriptomic analysis has comprehensively cataloged genetic diversity in breast tumors, leading to the identification of novel molecular drivers and providing clues for druggable targets (Banerji et al., 2012; Curtis et al., 2012; Ellis et al., 2012; Shah et al., 2012; Stephens et al., 2012; TCGA, 2012). These analyses have enabled the detection of a myriad of genetic abnormalities, including DNA sequence changes, copy-number changes, rearrangements, as well as detection of epigenetic modifications. The systematic identification of driver versus passenger mutations was an enormous challenge, given the vast number of changes and high degree of tumor heterogeneity. Mutations in the lipid kinase *PIK3CA* and the tumor suppressor *TP53* were found to dominate the mutation landscape in breast cancer, while only a handful of other genes harbored coding mutations in >5% of tumors. Indeed, only a small number of common aberrations were evident between patient tumors. In 100 patient breast tumors (mainly ER+), 73 different

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combinations of mutated driver genes were identified, with tumors harboring up to 6 mutations across a catalog of more than 40 distinct drivers (Stephens et al., 2012).

Through the large-scale integration of multi-platform datasets in TCGA, distinct genomic alterations have been linked to the molecular subtypes of breast cancer, implicating specific genetic events in the development of divergent tumor phenotypes (TCGA, 2012) (Figure 2). Interestingly, luminal A cancers harbor the highest number of recurrently mutated genes but the lowest overall number of mutations and copy-number alterations (CNAs). The top recurrently mutated genes in these tumors include *PIK3CA* (~45%), *GATA3*, *MAP3K1*, and *CDH1*. Lobular cancers are molecularly distinct and are characterized by loss of *CDH1* and *PTEN*, and enrichment for *TBX3* and *FOXA1* mutations (Ciriello et al., 2015). By contrast, luminal B tumor genomes have a higher frequency of *TP53* mutations, are substantially more complex with marked copy-number changes and focal amplifications, and often display a hypermethylated phenotype (TCGA, 2012). HER2-E tumors exhibit a high mutational burden, most commonly affecting *TP53* (75%) and *PIK3CA* (40%), and high levels of amplification of *ERBB2/HER2*. Interestingly, a heterogeneous distribution of drivers and CNAs was seen across HER2+ and HER2− regions in HER2-amplified tumors, implicating independent driver events in HER2− areas (Ng et al., 2015). Basal-like tumors exhibit complex genomic landscapes defined by CNAs, rearrangements, and a high mutational burden that encompasses frequent inactivating mutations in *TP53* (~80%). Notably, high intratumoral heterogeneity, based on mutation and copy-number profiles, was found to associate with worse outcome across the different breast cancer subtypes (Pereira et al., 2016).

Multi-omics analyses of approximately 2,000 patient tumors with long-term clinical outcome data (METABRIC dataset) have brought us a step closer toward the realization of precision medicine. A new molecular taxonomy for breast cancer termed "integrative subtypes" delineated 10 subgroups (IntClust 1–10), which are distinguished by distinct CNAs, expression signatures, known and putative driver mutations, and clinical outcomes (Curtis et al., 2012). ER+ tumors could be stratified into several integrative clusters of intermediate to poor prognosis, including four ER+ subgroups (IntClust 1, 2, 6, and 9) that hold a high risk of late relapse and harbor copy-number changes that may be targetable (Rueda et al., 2019). Identification of such patients is highly relevant given that ER+ cancers can recur up to two decades after diagnosis. In addition, multiple distinct molecular subtypes of TNBC emerged, differing in mutational and copy-number profiles, expression signatures, response to chemotherapy, and prognosis (Bareche et al., 2018; Lehmann et al., 2011). Four primary subgroups can be distinguished: two basal-like, mesenchymal, and luminal androgen receptor (LAR)-expressing. Potential relationships between the different molecular datasets are depicted in Figure 2.

A remarkable diversity and complexity of somatic mutational processes has been uncovered for breast cancer. Although mutations in driver genes are important, CNAs appear to dominate the genomic landscape of breast cancer (The ICGC/TCGA Pan-Cancer Analysis of Whole Genomes Consortium, 2020). Discrete mutational signatures were revealed through analysis of genomic rearrangements and mutations in 560 breast tumors (Nik-Zainal et al., 2016). Of note, a distinct signature was elucidated for TNBCs that exhibited a high homologous recombination deficiency (HRD) score but did not harbor detectable *BRCA1/2* mutations or *BRCA1* promoter hypermethylation, whereas tumors with *BRCA1/2* mutations were associated with a different rearrangement signature. These signatures provide an imprint of DNA damage processes during oncogenesis and may potentially represent better biomarkers of defective HRD than *BRCA1/2* mutations. Collectively, the integration of expression profiles and genomic alterations with clinico-pathological parameters should provide better predictive biomarkers of response, as exemplified in the study by Tanioka et al. (2018).

THE DYNAMICS OF CLONAL EVOLUTION DURING TUMOR PROGRESSION

Extensive subclonal diversification and spatial heterogeneity within tumors have been resolved through deep sequencing. At the earliest stages of the invasive process, reconstruction of the evolutionary history from DCIS to IDC at single-cell resolution has indicated that genomic evolution occurs prior to invasion of multiple clones into the surrounding tissue (Casasent et al., 2018). While subclonal diversification can be a late and rate-limiting step (Nik-Zainal et al., 2012), many common driver mutations tend to occur early in disease progression (Gerstung et al., 2020; Yates et al., 2015), although the extent and timing of subclonal diversification was variable. Moreover, TNBCs appear to maintain a large reservoir of subclones that continue to evolve CNAs during primary tumor expansion (Minussi et al., 2021). Highly multiplexed single-cell DNA analyses of TNBC indicated that most mutations, rearrangements, and copy-number changes were acquired during the early stages of tumor evolution in short bursts, implying a punctuated rather than gradual evolution model (Gao et al., 2016; Wang et al., 2014) (Figure 2).

Analysis of the genomic landscape of metastatic breast cancer has highlighted the clonal relatedness between primary tumors and metastatic lesions and indicated that lesions can derive from subclones within the primary tumor (Ding et al., 2010; Hoadley et al., 2016; Shah et al., 2012; Yates et al., 2015). As the majority of driver mutations and CNAs were widely represented among the lesions, they presumably occurred before emergence of the most recent common ancestor. Clones seeding metastases or recurrences also continue to acquire mutations and additional variants that are not detectable in the primary tumor (Angus et al., 2019; Bertucci et al., 2019). Interestingly, the presence of private "driver" mutations in metastases from treated versus untreated patients indicated that these changes are associated with drug resistance rather than driving metastasis, in line with systemic therapy promoting subclonal diversification (Hu et al., 2020; Yates et al., 2015). Moreover, single-cell genomics analysis of TNBC patients pre- and post-neoadjuvant treatment revealed that chemoresistance can occur through adaptive selection of pre-existing mutant clones via transcriptional reprogramming (Kim et al., 2018). The epigenetic landscape also plays a crucial role in determining heterogeneity and evolution in systemically treated patients with metastatic breast cancer (Patten et al., 2018). Large-scale studies of patient

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tumors with long-term follow-up data will be necessary to decipher the impact of genomic/epigenomic evolution on tumorigenesis.

FAMILIAL BREAST CANCER

An estimated 5%–10% of all breast cancer cases are attributable to germline pathogenic variants in breast cancer susceptibility genes, of which pathogenic variants in the highly penetrant genes *BRCA1/2* together account for approximately 15% of familial breast cancer risk (reviewed in Nielsen et al., 2016). *BRCA1* (17q21) and *BRCA2* (13q12–13) are critical tumor suppressors that serve as guardians of genomic integrity, largely facilitated by their role in high-fidelity HR-mediated repair of double-strand DNA breaks. The cumulative lifetime breast cancer risk is 72% for *BRCA1*-mutation carriers and 69% for *BRCA2* carriers, together with an increased ovarian cancer risk of 44% and 17%, respectively (Kuchenbaecker et al., 2017). Notably, *BRCA1*- and *BRCA2*-mutated tumors have distinct molecular, clinical, and histopathological features, implying different cellular origins and oncogenic mechanisms. *BRCA1*-mutated tumors typically manifest as aggressive, early-onset and high-grade triple-negative tumors, with a molecular signature that closely aligns with the sporadic basal-like subtype. *BRCA2*-mutated tumors are usually ER+ tumors of the luminal B subtype, although TNBC is not infrequent. Germline pathogenic variants in a number of other genes have been linked to breast cancer risk (Hu et al., 2021). Other high penetrance susceptibility genes include *TP53*, where pathogenic variants are associated with a multicancer disorder, Li-Fraumeni syndrome, and confer >100-fold risk increase for early-onset breast cancer, and *PALB2*, where variants in this BRCA2-interacting protein confer an increased risk of both breast and ovarian cancer. Mutations in *PTEN* (Cowden syndrome), *CDH1* (diffuse gastric cancer and ILC), and *STK11* (Peutz-Jeghers syndrome) represent important, albeit rare, germline pathogenic variants. Moderate risk pathogenic variants, including the DNA repair genes *CHEK2* and *ATM*, also account for a small proportion of familial breast cancer risk, further implicating the disruption of high-fidelity DNA repair as a crucial event in breast oncogenesis. Importantly, about 20% of familial breast cancer may be attributable to single-nucleotide polymorphisms (SNPs) identified through GWASs (Michailidou et al., 2017). More than 300 risk-modifier SNPs have been identified that have substantial multiplicative effects that are quantifiable using polygenic risk scores (Mavaddat et al., 2019).

MAJOR SIGNALING PATHWAYS IN BREAST ONCOGENESIS

Many of the somatic genomic aberrations occurring in breast cancer culminate in dysregulation of key signaling pathways that control cellular proliferation, survival, and/or differentiation pathways, thus identifying a number of potential biomarkers and therapeutic targets. In addition to commonly mutated genes, those mutated at a low frequency must be considered, as they may converge on common oncogenic pathways. Furthermore, aberrations in epigenetic regulators such as *KMT2C*, *KAT6A*, and *ARID1A* have the capacity to exert widespread effects. While decades of research have mapped out a compendium of genetic alterations in breast cancer and their potential function within signaling networks, there is still much to learn about pathway crosstalk, compensatory mechanisms, and the development of drug resistance. The following section focuses on dominant pathways perturbed in breast cancer, with emphasis on human studies (Figure 3). Other complicit pathways include the JAK-STAT, E-Cadherin-integrin, NF-κB, and NOTCH signaling nodes, for which data are steadily accumulating.

Steroid hormone signaling

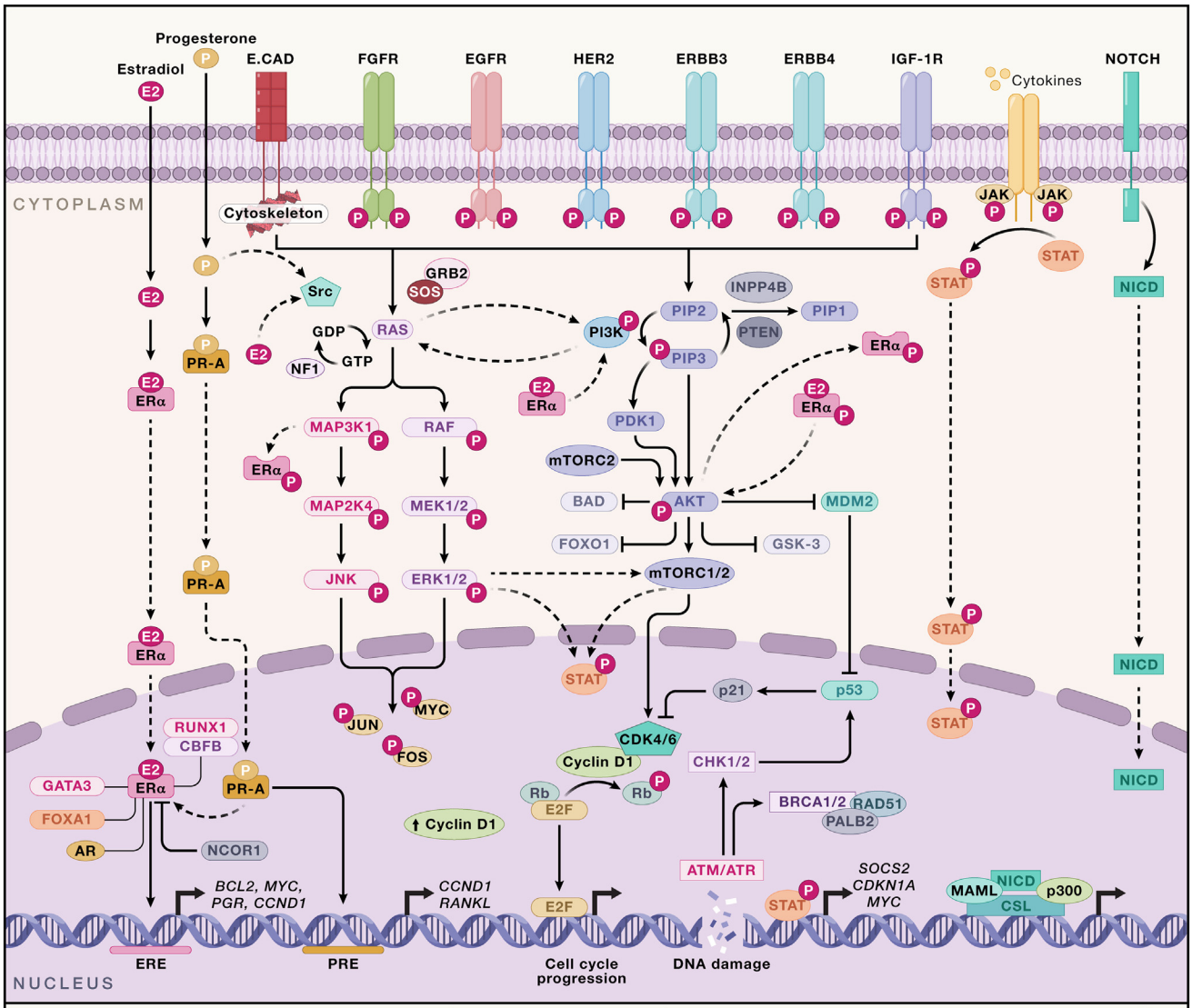
The ovarian steroid hormones estrogen and progesterone are intimately linked to normal development and breast carcinogenesis. Increased exposure to ovarian hormones, such as through early menarche, late menopause, and shorter menstrual cycles, are well-established breast cancer risk factors (Hankinson et al., 2004). ERα (encoded by the *ESR1* gene) is the key driver of tumorigenesis in ER+ luminal breast cancer, hence agents targeting the ER pathway are the cornerstone of treatment for ER+ disease. Within the ER transcriptional network, both GATA3 and the pioneering factor FOXA1 execute important cooperative functions. Loss-of-function mutations in *GATA3* occur frequently in luminal breast cancer (10%–15%), whereas *FOXA1* mutations are rare. Loss of *GATA3* has been linked to the epithelial to mesenchymal transition (EMT), tumor progression, and metastasis, and low GATA3 expression is strongly predictive of poor disease-free survival (Mehra et al., 2005).

Crosstalk between estrogen and growth factor receptor signaling, predominantly via the PI3K-AKT-mTOR or RAS-RAF-MAPK pathway, is well recognized for modulating ERα activity (Osborne et al., 2005). Furthermore, endocrine therapy resistance is underpinned by multiple mechanisms including the acquisition of *ESR1* mutations (Jeselsohn et al., 2015) and genetic activation of the PI3K-AKT and MAPK pathways. For example, genomic profiling of ER+ tumors and/or circulating tumor-DNA (ctDNA) in both early- and late-stage ER+ disease has revealed multiple subclonal resistance mutations in these pathways (Griffiths et al., 2021; Kingston et al., 2021; Razavi et al., 2018). Thus, multiple convergent phenotypes can convey acquired resistance to endocrine therapy (or combination therapy), with distinct genetic mutations occurring in different elements of collaborating pathways.

Progesterone and its receptors (PR-A and PR-B), which are themselves ER target genes, play an integral role in breast oncogenesis (Brisken, 2013). However, PR interaction with ER is complex, as it can associate with ERα on chromatin in the presence of ligand to attenuate breast cancer cell proliferation (Mohammed et al., 2015). The androgen receptor (AR), expressed in up to 90% of ER+ breast cancer, has been shown to repress ER-regulated cell-cycle genes and may play a tumor suppressor role in ER+ disease (Hickey et al., 2021).

ERBB receptor signaling network

The ERBB receptor tyrosine kinase ERBB2/HER2 is of critical importance in breast cancer, culminating in the development of HER2-targeting agents that have revolutionized treatment for this aggressive cancer type (Slamon et al., 2001). HER2 is an



	TP53	PIK3CA	GATA3	CDH1	KMT2C	MAP3K1	USH2A	PTEN	NCOR1	MAP2K4	RUNX1	ARID1A	NF1	TBX3	FOXA1	ERBB2	PIK3R1	AKT1	NOTCH2	RB1
All	33%	33%	12%	12%	9%	8%	6%	5%	5%	4%	4%	4%	4%	3%	3%	3%	3%	3%	2%	2%
Luminal A	11%	47%	15%	22%	12%	14%	4%	6%	6%	6%	5%	4%	4%	5%	5%	3%	3%	4%	2%	1%
Luminal B	36%	30%	20%	5%	10%	4%	6%	6%	5%	5%	4%	6%	3%	4%	1%	1%	2%	2%	2%	4%
HER2-E	71%	33%	4%	4%	10%	4%	8%	12%	3%	1%	4%	6%	4%	0%	3%	6%	6%	1%	5%	3%
Basal-like	89%	7%	0%	1%	6%	3%	10%	9%	4%	0%	1%	2%	5%	1%	1%	1%	4%	0%	4%	5%

Figure 3. Network of aberrant signaling pathways in breast cancer

Summary of major signaling pathways in breast cancer, highlighting pathway crosstalk and the intersection of common genetic alterations on key signaling nodes. The average mutation rate for 20 significantly mutated genes in each tumor subtype is indicated. Data are taken from analysis of the PanCancer dataset (n = 1,066 tumors) using cBioPortal. E2, estradiol; P (yellow), progesterone; P (pink), phosphorylation; ER, estrogen receptor; PR, progesterone receptor; ERE, estrogen response element; PRE, progesterone response element; ECAD, E-cadherin; FGFR, fibroblast growth factor receptor; EGFR, epidermal growth factor receptor; HER2, human epidermal growth factor receptor 2; IGF-1R, insulin-like growth factor type 1 receptor; NICD, Notch intracellular domain.

orphan receptor that is primarily activated by hetero-dimerization with other ERBB family members, although ligand-independent homo-dimerization is a feature of HER2-overexpressing cells (Yarden and Sliwkowski, 2001). Diverse mechanisms of resistance to anti-HER2 therapy have been elucidated, including loss or concealment of the extracellular domain, activating

mutations in *HER2* or *HER3*/*ERBB3*, and activating mutations in downstream pathways, including the MEK/ERK (often through loss of *NF1*), PI3K/AKT/mTOR, and cyclin D1/CDK4/6 pathways, thus providing targetable avenues for therapy (reviewed in Swain et al. [2023]). In contrast to HER2, EGFR is overexpressed in approximately 60% of TNBCs, but EGFR-targeting therapies have yielded variable clinical benefit in patients to date.

PI3K-AKT pathway

The phosphatidylinositol-3-kinase (PI3K)-AKT pathway encompasses a complex family of lipid kinases, which mediate signaling by receptor tyrosine kinases and other kinases through stimulation of AKT and mTOR signaling (Hoxhaj and Manning, 2020). Following pathway activation, the amplitude of transduction is controlled by negative regulators, most notably PTEN. Aberrations occur in multiple components of this pathway: gain-of-function mutations in the p110α catalytic subunit of PIK3CA are most prevalent in luminal A and HER2-amplified tumors, with other low-frequency mutations in *AKT1*, *PTEN*, and *PIK3R1* resulting in pathway stimulation (TCGA, 2012). Focal amplification of *PIK3CA* was detectable across all tumor types. Basal-like tumors often harbor deletion/mutation of *PTEN* (∼35%) and *INPP4B* (30%) but also exhibit amplification of *AKT3* (∼11%) and less prevalent mutations in *PIK3CA* (8%). In a striking example of parallel genetic evolution in response to PI3K inhibitor treatment in the metastatic setting, convergent loss of PTEN function was observed at multiple sites of metastasis, each with different *PTEN* alterations, underscoring hyperactivation of the PI3K-AKT axis as a major cause of acquired resistance (Juric et al., 2015). As PI3K is a significantly deregulated pathway across all subtypes of breast cancer, the identification of robust biomarkers of PI3K pathway activation and targetable components is paramount.

MAPK signaling pathway

The RAS family of proto-oncogenes is activated by an array of cell surface receptors, including receptor tyrosine kinases, with stimulation leading to sequential activation of the MAPK and ERK signaling cascades. Although *RAS* genes are among the most frequently mutated oncogenes in human cancer, *RAS* mutations are rarely observed in breast cancer (<5% patients). Nonetheless, aberrant RAS pathway signaling occurs in around 50% of breast tumors as a consequence of constitutive upstream signaling (such as via HER2 overexpression). Oncogenic activation of RAS/MAPK is associated with a significant reduction in tumor-infiltrating lymphocytes (TILs) and lower survival in TNBC patients, while concomitant MEK inhibition and anti-PD1/PD-L1 immunotherapy augmented anti-tumor immunity in preclinical TNBC models (Loi et al., 2016). In addition to the ERK pathway, MAP3K1 can regulate MAPK-JNK signaling through phosphorylation of substrates such as MAP2K4. Relatively frequent somatic mutations occur in *MAP2K4* and *MAP3K1* in luminal cancers (∼12% in total), often coincident with *PIK3CA* mutations. While JNK pathway activation is predictive of chemotherapy sensitivity in TNBC, recent evidence suggests JNK signaling fosters an immunosuppressive TME to drive tumor progression, implicating JNK-targeted immunotherapy as a potential treatment strategy for TNBC (Semba et al., 2022).

Cyclin D1-CDK4/6-RB axis

The cyclin D1-CDK4/6-RB axis is the pivotal regulator of the G1-S transition of the cell cycle. Several mitogenic pathways relevant to breast cancer, including estrogen and HER2-PI3K signaling, augment CDK4/6 activity via transcriptional upregulation of *CCND1* (encoding cyclin D1) or post-translational stabilization of this cyclin. Furthermore, amplification of *CCND1* and *CDK4* are frequently observed in luminal B (58% and 25%, respectively) and HER2+ breast cancers (38% and 24%, respectively). Extensive crosstalk between ER and cyclin D-CDK4/6 signaling underpins the use of CDK4/6 inhibitors to treat patients with advanced ER+ breast cancer. Additional benefits of CDK4/6 inhibitors have been reported, such as enhanced efficacy of immunotherapy through chemokine-mediated T cell recruitment (Uzhachenko et al., 2021). Unlike luminal cancers, cell-cycle activation in basal-like tumors is achieved through loss of *RB1* (TCGA, 2012), thereby rendering them unresponsive to CDK4/6 inhibitors and underscoring the importance of determining the mode of RB pathway inactivation. CDK4/6 inhibitor resistance arises through pleiotropic mechanisms including RB loss, cyclin E-CDK2 activation, the co-opting of other CDKs such as CDK7, and activation of other oncogenic pathways including RAS/MAPK and PI3K/AKT (Goel et al., 2022).

FGFR pathway

Amplification of members of the fibroblast growth factor tyrosine kinase receptor family, receptors *FGFR1* and *FGFR2*, are relatively common in breast cancer (10%–15%), where they induce downstream oncogenic signaling cascades including the RAS-MAPK pathway. For example, *FGFR1* amplification is a recognized driver in luminal B cancers (Turner et al., 2010), and C-terminal *FGFR2* truncation was recently identified as a potent and clinically actionable single-driver alteration in multiple cancers including breast cancer (Zingg et al., 2022). Moreover, aberrant FGFR signaling has been linked to resistance to endocrine therapy and CDK4/6 inhibitors (Formisano et al., 2019).

THE TUMOR MICROENVIRONMENT: OBLIGATE PARTNERS IN TUMOR PROGRESSION

Breast carcinoma cells exist within a complex ecosystem comprising diverse cell types that include infiltrating immune cells, fibroblasts, endothelial cells, pericytes, adipocytes, and parenchymal cells. Dynamic and multi-layered crosstalk between malignant and non-malignant cells within the TME plays a fundamental role in breast cancer initiation and progression, with tumor cells shaping their environment by reprogramming tissue-resident and recruited cells to support their survival, growth, and dissemination. In many breast cancer patients, effective anti-tumor immunity is hindered by the immunosuppressive nature of the TME, in which the expression of immune checkpoint receptors and inhibitory cytokine production restrains the recruitment and function of cytotoxic immune cells. The rapid development of scRNA sequencing and high-dimensional imaging technologies has paved the way for deconvolution of the breast TME (Figure 4). These studies have uncovered profound heterogeneity in the immune and stromal compartments, particularly within the more abundant T cell, myeloid, and fibroblast populations.

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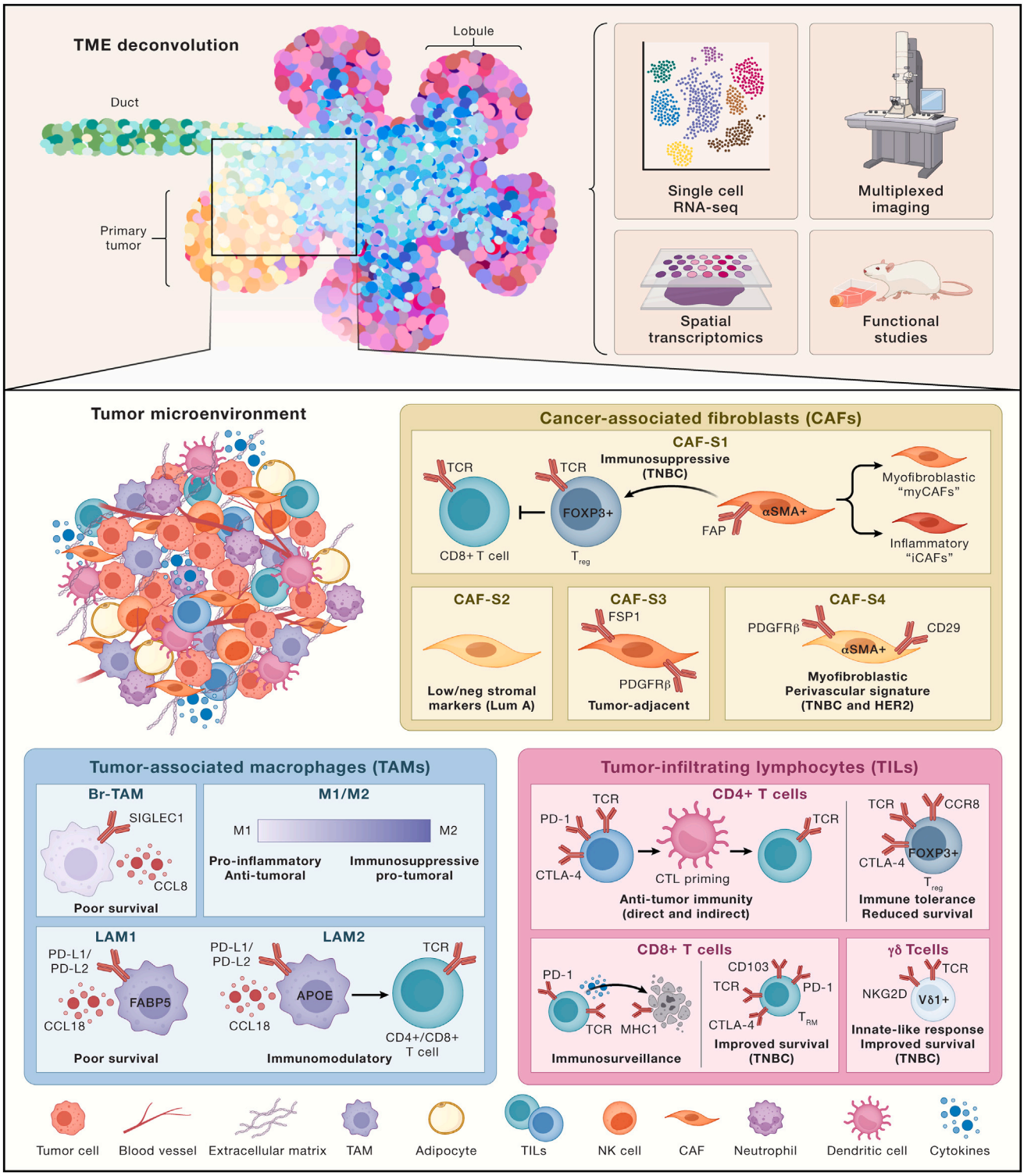

Figure 4. Dissection of the breast tumor microenvironment

Schematic representation of the complexity and diversity within the breast tumor microenvironment (TME), resolved through the integration of advanced transcriptomics, high-resolution imaging platforms, and preclinical models. The lower panels highlight key subsets identified within the immune and stromal compartments of breast tumors, each associated with distinct cellular functions, tumor subtypes, and clinical outcomes. Four distinct CAF subsets (CAF-S1–S4) are defined by the expression of fibroblast markers αSMA, FAP, FSP1, PDGFR-B, and CD29. CAF-S1 fibroblasts stimulate immunosuppression via CD4+ FOXP3+ Tregs and can be further refined into eight subgroups that are broadly characterized as myofibroblastic or inflammatory (Kieffer et al., 2020). TILs

(legend continued on next page)

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The stromal compartment

Cancer-associated fibroblasts (CAFs), a collective term for tumor-educated mesenchymal cells that exhibit extensive phenotypic and functional heterogeneity, are a key feature of breast cancers (reviewed in Kalluri (2016)). Recent molecular studies have begun to elucidate the dynamic nature of CAF composition during breast tumor progression. Four distinct subtypes have been defined, CAF-S1 to CAF-S4, each with distinct transcriptional programs related to cell adhesion, ECM organization and immune response, and enrichment of immunosuppressive CAFs (S1) observed in TNBC (Costa et al., 2018). Interestingly, CAF-S1 has been linked with resistance to anti-HER2 therapy and immune exclusion (Rivas et al., 2022). Single-cell studies have further resolved CAFs into 8 states encompassing immunosuppressive cells associated with regulatory T cell (Treg)-mediated immunosuppression and immunotherapy resistance (Kieffer et al., 2020) or into five CAF states, identifying inflammatory-like and myofibroblast-like CAFs (Wu et al., 2021). Two additional immune-modulatory populations, pCAFs and sCAFs (based on expression of *Pdpn* and *S100a4*), have been linked to clinical outcomes, with the pCAF/sCAF ratio significantly associated with *BRCA1/2* mutations and recurrence-free survival in TNBC (Friedman et al., 2020). The precise relationships between the different reported CAF states, however, remain to be determined.

The immune compartment

The immune tumor environment has been the subject of intense interest due to advances in cancer immunotherapy. The TME hosts a plethora of immune cell subsets, including tumor-infiltrating lymphocytes (TILs), innate lymphoid cells, and myeloid cells. These have been deconvoluted at unprecedented resolution in recent single-cell studies of breast tumors. The gene expression landscape evident within the T cell compartment indicates a continuum of diverse cellular states rather than a restricted number of differentiation subtypes (Azizi et al., 2018; Wu et al., 2021). These activation states partly reflect T cell receptor diversity and are likely to be influenced by local niches within the TME. In TNBC, tissue-resident memory $CD8^+$ T cells express high levels of immune checkpoint molecules and are predictive of improved patient outcome (Savas et al., 2018), while a highly proliferative subset of these cells featured in TNBC and HER2+ tumors but not in "immune-cold" ER+ tumors (Pal et al., 2021). In addition to $CD8^+$ T cells, tumor immunogenicity in TNBC has been linked to distinct subsets of breast-resident gamma-delta T cells (Wu et al., 2019) and an epigenetically regulated interplay between natural killer (NK) and $CD4^+$ T cells (Zhang et al., 2021b). Extending the power of single-cell analyses to tracking patient response to immune checkpoint blockade has recently uncovered specific immunophenotypes and gene signatures that accompany T cell clone expansion in TNBC patients receiving treatment (Bassez et al., 2021) and also distinguished responders from non-responders (Zhang et al., 2021a). B cell populations have been under-explored at the single-cell level thus far; however, plasma cell clusters were prominent in TNBC and HER2+ cancers (Pal et al., 2021). Interestingly, B cell signatures were recently demonstrated to have higher prognostic and predictive value than TILs in early-stage HER2+ breast cancer (Fernandez-Martinez et al., 2023). Taken together, these studies have profound implications for immunotherapy.

Tumor-associated macrophages (TAMs) constitute a major component of the immune infiltrate in breast cancer. There is abundant evidence from mouse models underscoring the pro-tumoral behavior of TAMs. In response to tumor- and microenvironment-derived signals, recruited monocytes and tissue-resident macrophages undergo functional reprogramming to fuel cancer cell proliferation and metastasis and to suppress anti-tumor immunity (reviewed in Pollard (2004)). Notably, a signature recently derived for human TAMs provides evidence that macrophages undergo breast tumor-specific transcriptional reprogramming and predicts poor clinical outcome (Cassetta et al., 2019). There is much interest in exploiting macrophage plasticity to unleash their tumoricidal potential, with evidence suggesting that TAM reprogramming may synergize with immunotherapy to deliver a robust anti-tumor response (Guerriero et al., 2017). Profound heterogeneity was uncovered in the myeloid compartment through single-cell profiling (Azizi et al., 2018; Wu et al., 2021), identifying multiple cell types and states, including novel lipid-associated macrophages (LAMs) that express immunoregulatory molecules such as PD-L1 and PD-L2, and predicting worse clinical outcome in large patient cohorts (Wu et al., 2021). Contrary to the macrophage polarization model that proposes mutually exclusive M1 and M2 states, overlapping expression of anti-tumor M1- and pro-tumor M2-associated genes often occurred in the same cells (Azizi et al., 2018). Notably, the high degree of diversity within the T cell and macrophage compartments across breast cancer subtypes has been corroborated by single-cell proteomics using approximately 70 markers (Wagner et al., 2019). Neutrophils are the second key myeloid population in the TME. A high circulating neutrophil-to-lymphocyte ratio serves as a robust biomarker of poor outcome in breast cancer, particularly within TNBC (Ethier et al., 2017). Most information on neutrophils in breast cancer has emanated from mouse models, revealing that these cells demonstrate remarkable plasticity and are the main drivers of metastatic colonization in distant sites (Wculek and Malanchi, 2015).

ELUCIDATION OF SPATIAL CELLULAR ORGANIZATION AND NEIGHBORHOODS

Dissecting tissue architecture is fundamental to understanding heterotypic interactions that promote the tumorigenic state

predominantly comprise cytotoxic CD8+ T cells, CD4+ helper T cells, immunosuppressive FOXP3+CD4+ regulatory T cells (Treg), and CD19+ B cells. Two additional T cell subsets, tissue-resident memory T cells (T_{RM}), and Vδ1-expressing γδ T cells are associated with positive clinical outcomes in TNBC. The immunosuppressive milieu within the breast TME, largely attributable to immune checkpoint receptors and inhibitory cytokines, impairs anti-tumor T cell immunity. TAMs are associated with a spectrum of polarization states ranging from anti-tumor M1-like to pro-tumor M2-like, although increased TAM complexity has emerged from single-cell studies. Br-TAMs refer to the breast tumor-specific TAM signature that is highly enriched in aggressive breast cancer subtypes (Cassetta et al., 2019), while LAM1 and LAM2 are CCL18-expressing lipid-associated macrophage subsets associated with poor survival (Wu et al., 2021). TME, tumor microenvironment; TILs, tumor-infiltrating lymphocytes; CAFs, cancer-associated fibroblasts; TAMs, tumor-associated macrophages; NK, natural killer; TCR, T cell receptor; Treg, regulatory T cell; CTL, cytotoxic T cell; T_{RM}, tissue-resident memory T cells.

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and enhance or impede an immune response. The spatial mapping of cellular constituents within the breast tumor ecosystem has been enabled by multiplexed imaging platforms as well as spatial transcriptomics. In one of the first high-dimensional imaging studies based on multiplexed ion beam imaging (MIBI), the presence of a structured immune environment was reported in TNBC, with ordered immune structures apparent along the tumor-immune border (Keren et al., 2018). In parallel, spatial resolution together with molecular profiling of microdissected regions could stratify TNBCs based on their immune microenvironment (Gruosso et al., 2019). More recently, high-resolution mapping has delineated prognostic cellular communities. Low-risk ER+ tumors were found to be devoid of the exclusive immune-hot stromal environments found in TNBCs and instead comprised a vascularized and T cell-involved community associated with poor outcome (Jackson et al., 2020). Spatial mapping coupled with multi-platform genomics of breast tumors from the METABRIC cohort further indicated that genomic alterations influence both TME architecture and phenotype (Ali et al., 2020). For example, luminal B tumors of the IntClust 9 group that frequently carry *TP53* mutations were the only ER+ tumors in which enrichment for macrophage-T cell neighborhoods was apparent. In studies to correlate tissue organization with functional states, the co-localization of regulatory and dysfunctional T cells within "suppressed expansion" structures in the TME was shown to predict poor outcome in patients with ER+ breast disease (Danenberg et al., 2022). Finally, through the application of spatial transcriptomics to visualize global mRNA distribution and context, LAMs with an immunosuppressive phenotype were often seen juxtaposed to PD1+ lymphocytes (Wu et al., 2021), while inflammatory-like and myofibroblast-like CAFs were spatially segregated in independent patient cohorts, with myoCAFs found closely associated with cancer cells (Andersson et al., 2021; Wu et al., 2021). The organization of heterotypic cells (both malignant and non-malignant) into tumor zones or "ecotypes" that show distinct cellular compositions and clinical outcomes suggests that phenotypes are shaped by diverse local niches in tumors (Wu et al., 2021). Further integrative analysis of spatial architecture and expression data at single-cell resolution will help resolve the presence of conserved structural and functional domains in breast tumors.

CURRENT THERAPIES FOR BREAST CANCER

While clinico-pathologic features continue to underpin the diagnostic workup of a newly diagnosed breast cancer, approaches to therapy are increasingly informed by the intrinsic subtype (Figure 5). Phenotyping is being extended to incorporate newer biomarkers (e.g., Ki67, PD-L1, TIL score) and genomic assays (e.g., *BRCA1/2* germline status, RNA-based prognostic assays) due to their prognostic and predictive utility. RNA-based assays are proving most helpful for patients with luminal tumors in the post-menopausal setting, where the primary goal is to identify patients with luminal B tumors who require chemotherapy. Tumor sequencing is also being used to identify "actionable" mutations in the pathways described above or for the presence of HRD to help guide treatment. As outlined below, combination therapy targeting the "driver" and co-operating pathway is an emerging theme, an approach that is now extending to the TME with immunotherapy. Collectively, these interventions have changed the disease trajectory across all tumor subtypes.

ER+ breast cancer

Endocrine therapy with the selective ER modulator tamoxifen or an aromatase inhibitor (AI), which blocks estradiol synthesis, has long been the mainstay for early and advanced ER+ disease (Burstein, 2020) (Figure 6). Since AIs (exemestane, letrozole, and anastrozole) can only be used in the post-menopausal setting, ovarian ablation through surgery or suppression is increasingly being incorporated into endocrine regimens for younger women, who exhibit increased risk of early and late relapse (Francis et al., 2018). Both tamoxifen and aromatase inhibitors can also be used for prevention of ER+ tumors (Cuzick, 2017). Fulvestrant, which has dual properties as an ER inhibitor and selective ER degrader (SERD), is also efficacious and generally used for advanced/metastatic disease. Intense efforts are underway to identify more potent oral SERDS or proteolysis-targeting chimeric (PROTAC)-mediated degraders of ER.

Over the last decade, the importance of incorporating combinatorial therapy in the metastatic setting has become apparent for ER+ disease. The known cooperative role for estrogen signaling and cyclin D1 in G1/S cell-cycle progression has led to a number of landmark studies, and the subsequent incorporation of a CDK4/6 inhibitor (e.g., palbociclib, ribociclib, or abemaciclib) with endocrine therapy as gold standard therapy for metastatic disease (McAndrew and Finn, 2022). The significant improvement in progression-free survival (PFS) spurred studies in the adjuvant setting, where abemaciclib has thus far demonstrated benefit for patients with high-risk disease (Johnston et al., 2020). As resistance to CDK4/6 inhibitors in the advanced disease setting is inevitable, a growing number of early-phase studies are underway to address this major challenge. Potential targets include PIK3CA and AKT (see below), CDK2/4/6 (e.g., PF-06873600), CDK2 (e.g., BLU-222, PF-07104091), and CDK7 (e.g., samuraciclib).

The acquisition of *ESR1* mutations, rarely evident in primary disease, in 30%–40% of tumors following prolonged aromatase inhibitor therapy represents a major clinical challenge. Use of a SERD may help to overcome resistance, with recent data pointing to benefits of monitoring occult disease for *ESR1* mutations present in ctDNA, with an early switch to fulvestrant prior to disease progression (Bidard et al., 2022). To combat other dominant resistance mechanisms linked to activation of PI3K-ATK-mTOR signaling in luminal tumors, combination therapy with exemestane and everolimus (an allosteric inhibitor of mTORC1) was one of the first targets to show benefit (Piccart et al., 2014). This led to a large number of pan-PI3K inhibitor studies that yielded disappointing results. However, targeting *PIK3CA*-activating mutations with the α-selective inhibitor alpelisib has produced favorable outcomes (André et al., 2019), including in the post-CDK4/6 inhibitor setting. One challenge, however, is that "on-target" inhibition of p110α impacts insulin receptor signaling, leading to elevated blood glucose and insulin levels. Late-phase studies are also underway exploring the AKT

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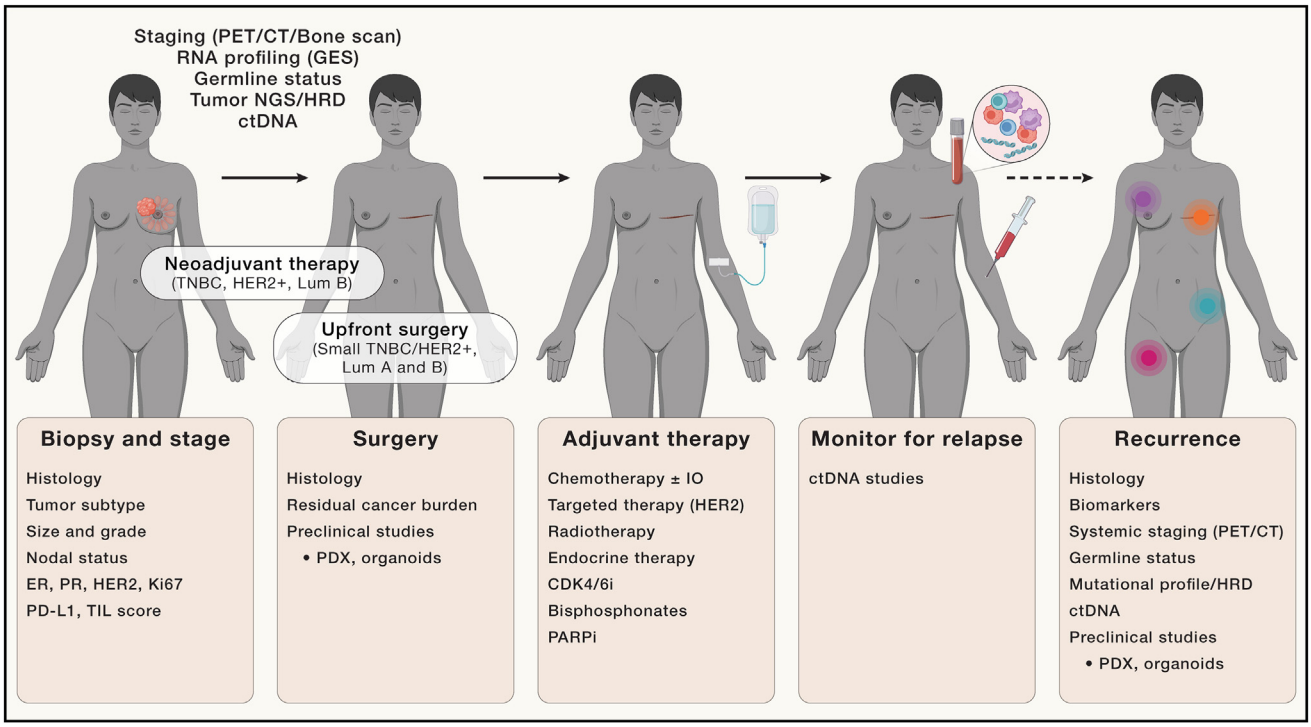

Figure 5. Clinical management of breast cancer and current therapies

Schematic diagram depicting treatment pathways in the clinic or deployed in clinical trials. Biopsy and staging are essential for treatment selection and full clinicopathological assessment. Genomic studies can include RNA-profiling, germline, and somatic tumor sequencing. Upfront surgery is conducted for lower risk disease, while neoadjuvant chemotherapy is often applied for TNBC and HER2+ tumors with the goal of achieving a pathologic complete response (pCR) or reduced residual cancer burden (RCB) score and of downstaging luminal B tumors. In a research setting, ctDNA assessment and preclinical modeling using patient-derived xenografts (PDX) or tumor organoids can be undertaken. Adjuvant therapy is selected on the basis of tumor phenotype, genotype, and surgery. Monitoring for relapse is usually confined to clinical examination and breast imaging but, in a research setting, can include serial imaging and ctDNA assays. If recurrence occurs, restaging and evaluation of histopathologic and mutational features of the tumor are important. Functional assays (PDXs and organoids) can potentially guide therapeutic options. PET, positron emission tomography; CT, computed tomography; GES, gene expression signature; NGS, next-generation sequencing; HRD, homologous recombination deficiency; ctDNA, circulating tumor DNA; TIL, tumor-infiltrating lymphocyte.

inhibitors capivasertib and ipatasertib. Indeed, recent findings for the first-in-class inhibitor capivasertib indicate that this strategy will be effective, particularly where tumors harbor an AKT pathway mutation (Howell et al., 2022).

HER2-amplified disease

Targeting HER2 amplification with the humanized monoclonal antibody trastuzumab is the exemplar for targeted therapy in breast cancer (Slamon et al., 2001). Trastuzumab, which targets an extracellular domain in HER2, attenuates intracellular RAS/MEK/ERK and PI3K/AKT/mTOR signaling and induces Fc-receptor-mediated antibody-dependent cell-mediated cytotoxicity (ADCC) (Figure 6). Its introduction has radically improved survival rates, which had been similarly dismal to that for TNBC. As a result of trastuzumab/chemotherapy regimens and newer potent anti-HER2 inhibitors, survival rates now exceed 90% in the early disease setting, and durable responses are usually achieved in the advanced setting, although therapeutic resistance is frequently seen. Pertuzumab was the second FDA approved humanized anti-HER2 agent. It targets a different extracellular domain and can prevent dimerization with other ERBB receptors (EGFR, HER2, and HER4), synergizing the arrest of downstream signaling (Agus et al., 2002). Dual HER2 blockade with trastuzumab and pertuzumab (and new analogs) has rapidly become a new standard of care following demonstration of greater efficacy in neoadjuvant, adjuvant, and first-line relapsed HER2+ cancer. Other HER2 inhibitors include small molecule tyrosine kinase inhibitors such as neratinib (which irreversibly inhibits EGFR, HER2, and HER4) and tucatinib (HER2-specific), which can cross the blood-brain barrier (reviewed in Swain et al. (2023)). Multiple mechanisms of resistance to anti-HER2 therapy have emerged, such as CDK4/6 activation, which can be targeted by dual inhibition using trastuzumab and a CDK4/6 inhibitor (Tolaney et al., 2020).

Anti-HER2 antibody drug conjugates (ADCs) have recently taken center stage, where potent killer (cytotoxic) payloads are covalently bound to the monoclonal antibody via a cleavable synthetic linker. While the ADC is usually internalized, the cleavable linkers can also release the cytotoxic payload to adjacent tumor cells resulting in a potent "bystander effect." The first in class ADC was T-DM1 comprising the killer payload emtansine (Verma et al., 2012), which elicited responses in brain metastases in a subset of patients. Recently, a novel ADC T-DXd (DS-8201), which incorporates the topoisomerase I inhibitor deruxtecan as its payload, was also shown to be highly active, including in T-DM1 refractory disease (Cortés et al., 2022; Modi et al., 2020).

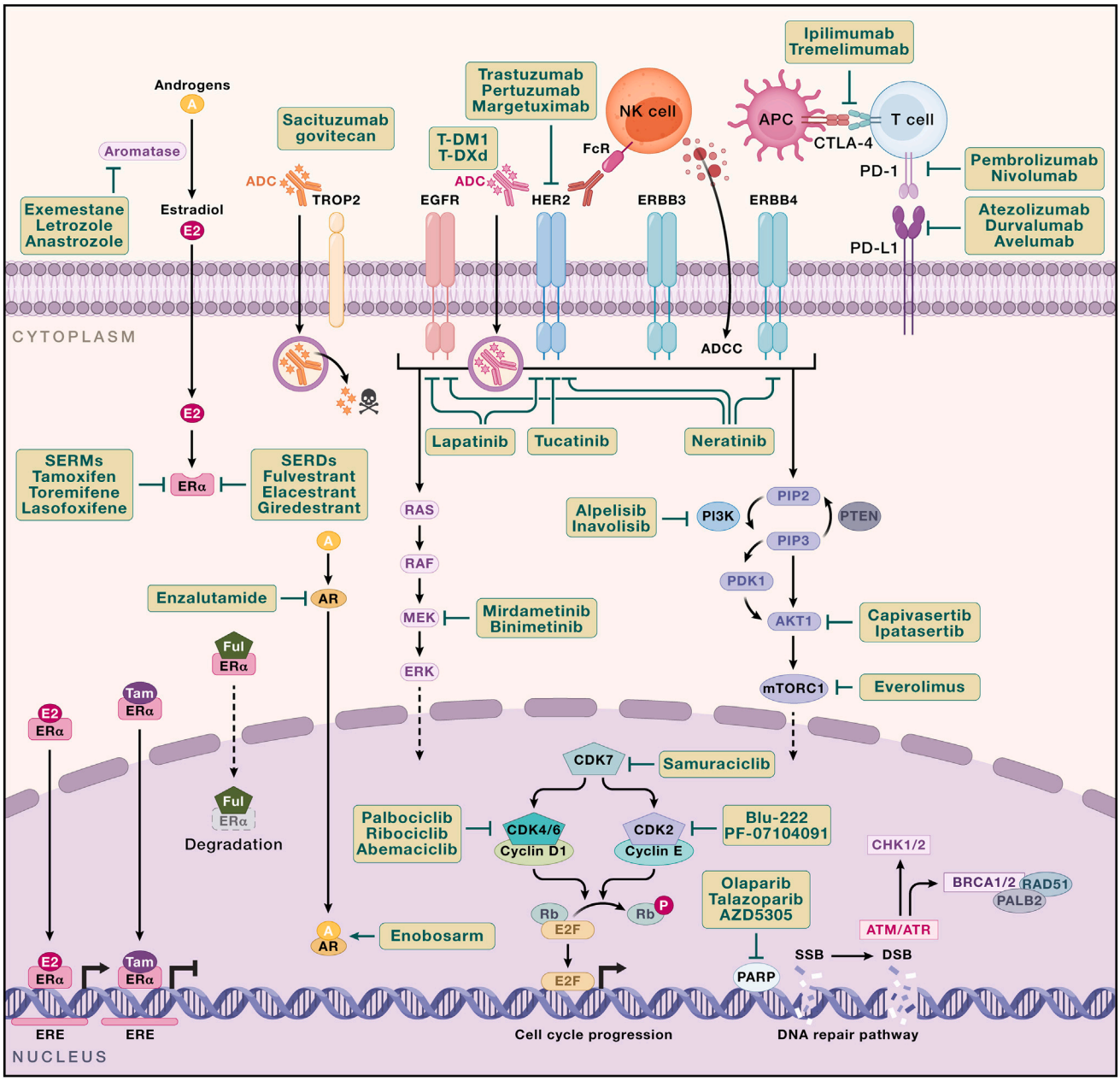

Figure 6. Key signaling nodes targeted in breast cancer, with examples of current and emerging therapies

Schematic showing key cell surface receptors that have been successfully targeted, including by anti-HER2 monoclonal antibodies (trastuzumab, pertuzumab, and margetuximab) or antibody-drug conjugates (ADCs), trastuzumab emtansine (T-DM1), and trastuzumab deruxtecan (T-DXd). Trop2 has also been targeted by ADCs (sacituzumab govitecan). Monoclonal immune checkpoint inhibitors against PD-L1 (atezolizumab, durvalumab, and avelumab) or cognate ligands on immune cells such as PD1 (pembrolizumab and nivolumab) or CTLA-4 (ipilimumab and tremelimumab) are also targets. Tyrosine kinase inhibitors such as lapatinib, neratinib, and tucatinib target HER2 to block downstream signaling through the RAS/RAF/MEK/ERK and PI3K/AKT/mTOR pathways. MEK, PI3K, and AKT inhibitors are under investigation; the PIK3CA inhibitor alpelisib and mTORC1 inhibitor everolimus are approved. For ER+ breast cancer, selective ER modulators (SERMS) or aromatase inhibitors (AIs) to block estradiol production have transformed clinical practice. Selective ER degrader fulvestrant and an emerging class of oral SERDS are beneficial in relapsed disease. Targeting AR is under investigation with AR-activators for ER+ disease and AR inhibitors for the LAR TNBC subtype. Combination therapy targeting CDK4/6 has proven beneficial for ER+ breast cancer and is showing promise in HER2+ disease. The targeting of single-strand DNA breaks in *BRCA1*/*BRCA2* mutation carriers with PARP inhibitors is in clinical practice; drugs targeting other germline mutations or homologous recombination deficiency are also under investigation. E2, estradiol; ER, estrogen receptor; ERE, estrogen response element; A, androgen; AR, androgen receptor; TAM, tamoxifen; Ful, fulvestrant; EGFR, epidermal growth factor receptor; HER2, human epidermal growth factor receptor 2; ADCC, antibody-dependent cell cytotoxicity; APC, antigen-presenting cell; SSB, single-strand break; DSB, double-strand break.

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Notably, a small subset of patients with HER2-low tumors was found to respond to trastusumab in early studies, suggesting that benefit may extend beyond HER2+ disease (Paik et al., 2008). This observation has been substantiated with the finding that T-DXd significantly improves outcomes, compared with standard chemotherapy, in heavily pre-treated patients (Modi et al., 2022). Most patients had ER+ disease, underscoring HER2 signaling as a key driver of resistance in ER+ breast cancer. These findings have enormous clinical significance and will likely alter clinical practice algorithms. Deciphering how these tumors are positioned within the luminal subtype could prove helpful for future treatment algorithms.

Based on the promising activity of immune checkpoint inhibitors (ICI) in TNBC (see below), attention has turned to HER2+ disease, given the notable levels of PD-L1 expression and TIL infiltrates and importance of ADCC for innate immune response to trastuzumab. To date, early-phase trials have yielded mixed results. While atezolizumab (anti-PD-L1) failed to improve responses to T-DM1 in patients with previously treated HER2+ disease, a trend toward better PFS was observed for PD-L1+ tumors (Emens et al., 2020). Atezolizumab and T-DM1 are now being investigated for early PD-L1+HER2+ disease (NCT04740918). These and other findings have led to a large number of other immunomodulatory approaches, currently under investigation.

TNBC

The landscape for management of TNBC has radically shifted over the last few years. Important biomarkers recognized in this context include *BRCA* mutation status, PD-L1 immune checkpoint expression, TIL content, and somatic genomic signatures indicating HRD (Figure 6) (reviewed in Savas et al. (2016)). These reflect the growing importance of immune checkpoint inhibitors (ICIs) for this subtype of breast cancer, where heightened genomic instability can be associated with increased neoantigens and immune infiltrates. A high concentration of TILs in the TME is a robust predictor of neoadjuvant chemotherapy response and is strongly associated with a favorable prognosis in TNBC and HER2+ patients (Savas et al., 2016). Unlike melanoma and lung cancer, monotherapy with ICIs has shown limited benefit in breast cancer. However, combining PD-1 or PD-L1 ICIs with various backbone chemotherapies has demonstrable benefit, albeit primarily in the first-line metastatic disease setting, where immune function is less likely to be "exhausted" (Cortes et al., 2020; Schmid et al., 2018). Here, PD-L1 expression is both prognostic and predictive of response.

Remarkable outcomes have been observed in treatment-naive patients when ICIs are combined with neoadjuvant chemotherapy, with improved pCR rates and subsequent PFS (Mittendorf et al., 2020; Schmid et al., 2022). In this setting, patients with PD-L1-negative tumors also derived benefit from ICIs, bringing into question the value of PD-L1 as a biomarker in treatment-naive disease. It remains to be determined whether other immune biomarkers (such as TILs or IFNγ) or the spatial scoring of the proximity of T cells to tumor cells could be helpful. The intriguing finding that pathological responses can sometimes be observed with ICIs alone has led to studies investigating immune induction prior to chemotherapy.

In TNBC with germline or somatic mutations in *BRCA1/2* (about 15%–20%), synthetic lethality can be triggered by PARP inhibitors that block single-strand DNA repair in the HRD setting. Both olaparib and talazoparib were highly effective in pre-treated patients with a germline mutation (Litton et al., 2018; Robson et al., 2017). Subsequent evaluation of olaparib in the early (adjuvant) setting has shown remarkable improvements in disease-free and overall survival for patients with high-risk disease (Tutt et al., 2021). Although wild-type TNBC does not appear to respond to single-agent PARP inhibitors, a broader application for tumors that exhibit "BRCAness" or HRD is an area of increased interest, including in combination with ICIs. The targeting of other DNA damage response proteins, such as selective inhibitors of ATM, ATR, Aurora kinase A, CHK1/2, RAD51, and WEE1, often in combination therapy with PARP inhibitors, is being explored.

The remarkable developments in ADC therapy, as noted for HER2+ breast cancer, have now been emulated in TNBC with the recent development of sacituzumab govitecan (SG). SG is a humanized IgG1κ monoclonal antibody targeting trophoblast cell surface antigen 2 (TROP2), which is a glycoprotein overexpressed in 80% of poor prognosis breast cancers. This ADC, coupled to the topoisomerase I inhibitor SN-38 through a hydrolyzable linker, has elicited impressive activity in heavily pre-treated patients with TNBC (Bardia et al., 2021), leading to rapid FDA approval. Its utility is being extended to ER+ disease, where it has improved PFS and overall survival in HER2-low and HER2-negative subtypes in heavily pre-treated patients progressing on endocrine/CDK4/6 inhibitors and chemotherapy (Rugo et al., 2022).

The pressing need to identify targeted therapies for TNBC has catalyzed a number of early clinical trials targeting pathways that include PI3K/AKT/mTOR, EGFR, RAS/MAPK, and JAK/STAT (reviewed in Garrido-Castro et al. (2019)). The LAR subtype of TNBC (often presenting with apocrine features) is characterized by expression of AR and downstream targets. In contrast to ER+ disease, AR is more likely to drive tumor growth in TNBC due to the absence of ER activity. This has prompted studies on AR inhibitors such as bicalutamide and enzalutamide, albeit with modest clinical activity observed to date. Epigenetic alterations are common in TNBC, but therapies targeting epigenetic regulators have thus far yielded disappointing results. However, several inhibitors are currently in the early stages of clinical testing (Garrido-Castro et al., 2019), including BET/-bromodomain inhibitors. Finally, novel strategies aimed at targeting chromosomal instability in TNBC may be feasible through inhibition of IL-6-STAT3 and downstream cGAS-STING signaling with agents such as tocilizumab (Hong et al., 2022).

Conclusions

The last decade has witnessed a transformative leap in our understanding of the molecular landscape of breast cancer and tumor heterogeneity. The emerging consensus is that a small number of dominant genetic drivers act in concert with individual rare mutations and copy-number alterations to fuel tumorigenesis. Given the massively parallel sequencing of 1,000s of breast tumors to date, it seems probable that most driver genes have been elucidated. In TNBC, there appears to

be a notable lack of recurrently mutated and targetable pathways. For such cancers where chromosomal instability is a driving force, targeting mechanisms that underlie genomic instability may be necessary. The functional importance of many aberrations and genetic dependencies across the different breast cancer subtypes remain to be determined. This demands comprehensive functional screening (e.g., using CRISPR-Cas9 editing) combined with deeper analyses and data integration to deconvolve pivotal molecular pathways and interconnecting nodes. Despite the wealth and depth of genetic information now available for breast cancer, translation into precision medicine and routine clinical practice remains an ongoing challenge. Nevertheless, the continuing refinement and integration of genomic and expression signatures with clinico-pathological features has dramatically expanded our understanding of mechanisms underpinning breast cancer and should ultimately lead to improved biomarker tools to guide treatment escalation and de-escalation. Novel approaches to personalizing therapy should also benefit further from the application of multi-omics machine learning, recently shown to predict response to therapy (Sammut et al., 2022).

Revolutionary advances in single-cell technologies and computational analysis have paved the way for dissection of tissue heterogeneity and clonal evolution for breast cancer at remarkable resolution. However, the true extent and impact of heterogeneity on clinically relevant parameters such as prognosis and therapy prediction and on tumor evolution are yet to be determined. A further caveat to single-cell RNA-seq studies is that different stringencies have been applied for cluster analysis, leading to variable data. It is important to note that without a functional readout, the relevance of the different clusters/subsets remains unresolved. The emergence of drug resistance continues to pose a major barrier to the efficacy of therapies. Branching evolution is a major source of clonal diversification in breast cancer, with subclonal populations playing a prominent role in treatment failure and disease recurrence. As the majority of resistant clones may be pre-existing, it will be crucial to develop better strategies to detect and target these cells before they undergo multi-step adaptation.

Multi-dimensional integration of an array of single-cell sequencing and spatial technologies has begun to unravel the importance of the entire tumor ecosystem. However, it will take considerable time to decipher how spatial cellular organization, together with genomic alterations, influences tumor phenotype and progression. The diverse niches that are being uncovered may reflect the recruitment of specific cell subsets or cell differentiation. The wide spectrum of phenotypes and states displayed by immune and stromal cells, based on single-cell studies, has confounded our understanding of extrinsic drivers of oncogenesis and the parameters that allow tumor cells to evade the immune system. Nevertheless, the generation of single-cell atlases that include spatial architecture and integration with longitudinal patient data should enable the elucidation of conserved tumor "neighborhoods" to provide vital information on cellular interactions and niches. Collectively, multi-dimensional data may help prime the next phase of clinical trials through the integration of diagnostic and predictive biomarkers and the development of new therapies.

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