

DOUTORAMENTO

MEDICINA E ONCOLOGIA MOLECULAR

Title: Molecular characterization of breast cancer in the neoadjuvant treatment setting

Título: Caracterização Molecular do carcinoma da mama em contexto de tratamento neoadjuvante

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JANEIRO DE 2024

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This PhD thesis has been submitted in fulfilment of the requirements for the PhD degree in Medicine and Molecular Oncology at the Medical School of the University of Porto.

Dissertação de candidatura ao grau de Doutor apresentado à Faculdade de Medicina da Universidade do Porto, no âmbito do Programa Doutoral em Medicina e Oncologia Molecular.

PERSONAL ACKNOWLEDGEMENTS

I would like to thank to all the patients who accepted to participate in this clinical study. Their motivation to participate and to help in breast cancer clinical investigation was very inspiring.

I thank my Supervisor and Co-Supervisor, Professor José Luís Costa and Professor Fernando Schmitt, for all the support and motivating words to overcome all the difficulties.

I thank all the persons involved in this project at IPATIMUP and Local Health Unit of Matosinhos.

I thank the Porto University, in particular its Medical School, for accepting me in their challenging PhD Program of Medicine and Molecular Oncology.

I thank my family, especially my parents, for all the support and love because they are responsible for who I am.

I thank my husband, for all the support and understanding.

I dedicate this work to my daughter, Francisca, who was born during this journey to illuminate my way.

LIST OF PUBLICATIONS

The work presented in this doctoral thesis is published in peer-reviewed scientific journals, as listed below:

Article number 1

Alexandra Mesquita, Anabela Ferro, José Carlos Machado, Fernando Schmitt. *Next generation sequencing of breast cancer in the neoadjuvant setting*. Pathobiology 1–7, 2023. DOI: 10.1159/000533810.

Q1 JCR and Scimago

Article number 2

Alexandra Mesquita, Fernando Schmitt, José Carlos Machado, José Luís Costa. *Utility of Circulating tumor DNA in different clinical scenarios of breast cancer*. Cancers 12(12), 3797, 2020. DOI: 10.3390/cancers12123797.

Q1 JCR and Scimago

Article number 3

Catarina Lopes Fernandes, Diogo Silva, Alexandra Mesquita *Novel HER2 targeted therapies in breast cancer*. Cancers 16, 87, 2024. DOI:org/10.3390/cancers16010087.

Q1 JCR and Scimago

Article number 4

Diogo Silva, Alexandra Mesquita *Evolving Evidence for the Optimization of Neoadjuvant Therapy in Triple- Negative Breast Cancer* Breast Cancer (Auckl), 16:11782234221107580, 2022.DOI: 10.1177/11782234221107580.

Article number 5

Élia Cipriano, Alexandra Mesquita *Emerging Therapeutic Drugs in Metastatic Triple-Negative Breast Cancer* Breast Cancer (Auckl), 15:11782234211002491, 2021. DOI: 10.1177/11782234211002491.

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ABSTRACT

Many patients with locally advanced breast cancer are proposed to neoadjuvant chemotherapy (NAT) before surgery, especially those with triple negative or Erb-B2 receptor tyrosine kinase 2 (*ERBB2*) positive breast cancer due to the better response to these treatments. In these molecular intrinsic subgroups of patients, many advances in treatment have occurred in the last years with the approval of new anti-HER2 targeted therapies and immunotherapy which were reviewed. Nevertheless, only some patients achieve a pathological complete response (pCR), after NAT and there is no biomarker to predict which patients will reach pCR and, therefore, have a better prognosis.

The determination of gene somatic alterations using next-generation sequencing (NGS) in the tumors is important in order to identify potential opportunities of treatment for the patients, if targeted therapies are available, such as those harboring HER2 and *PIK3CA* gene. There are also already some clinical trials addressing the use of some targeted therapies in neoadjuvant setting such as Poli-(ADP ribose) polymerases (*PARP*) inhibitors, for *BRCA 1* or *2* mutated patients. Taking this into consideration, 67 patients with locally advanced breast cancer were recruited for clinical evaluation during NAT. Tissue samples of some of these patients, collected before NAT, were analyzed using NGS. Besides *ERBB2* gene amplification, the most frequent gene alterations detected in HER2 positive tumors were in *MYC* gene, followed by repairing DNA genes involved in homologous recombination pathway such as *BRCA* and *ATM*. Luminal B HER2 negative breast tumors presented mainly *TP53* and *MYC* gene alterations. A specific pattern of genomic profile was not found for each group of tumors. Nevertheless, the presence of high number of *TP53* and *BRCA 1* or *2* gene alterations in triple negative breast cancer is reported according to the literature.

Twelve patients achieved pCR after NAT: *ERBB2* gene alterations were the most frequent in this cohort of pCR patients, probably due to the good response rates of anti-HER2 targeted therapies coupled with chemotherapy used in this scenario, followed by *BRCA 1* and *2*, *MYC*, *TP53*, *PIK3CA*, and *MET* alterations. Tumors that did not achieve a pCR were mainly triple negative; in this subgroup some *BRCA 1* and *2* and *PIK3CA* gene alterations were identified, as well as *TP53* mutations. Although not all of them have a targeted therapy available, the identification is important once it can have impact on prognosis and therefore allow a better treatment choice and closer follow-up. Therefore, detection of these somatic mutations, coupled with the availability of targeted therapies, holds promise as a potential therapeutic avenue to enhance tumor response rates during NAT, or as a complementary treatment following surgery.

Although tumor mutation burden (TMB) was lower in pCR samples, it was not possible to establish a correlation between TMB and pCR, probably due to sample size. Nevertheless, this evaluation in non-pCR samples could serve as a valuable criterion for selecting patients suitable for immunotherapy. It was also possible to determine microsatellite instability (MSI) in the samples. Although MSI high determination is rare in breast cancer, it might be of clinical value once immunotherapy is approved by Food and Drug Administration (FDA) in these patients. Further exploration through clinical trials is imperative to investigate these prospects.

Taking into consideration the importance for clinical practice of detecting tumors gene alterations during the disease, it would be important to do it in a less invasive way; therefore, the concept of «liquid biopsy» would be of interest to be studied in the neoadjuvant setting.

RESUMO

Grande parte dos doentes com carcinoma da mama localmente avançado são actualmente propostos para realização de quimioterapia neoadjuvante antes da cirurgia, particularmente doentes com carcinoma da mama triplo negativo e carcinoma da mama *HER2* positivo. Nestes subgrupos moleculares de tumores, vários avanços terapêuticos ocorreram nos últimos anos, tais como a aprovação de novas terapêuticas anti-*HER2* e imunoterapia e foram revistos. Apenas em alguns casos se consegue atingir resposta patológica completa do tumor e não existe biomarcador preditivo que indique quais os doentes que atingirão resposta patológica completa e consequentemente apresentarão melhor prognóstico.

A determinação de alterações genéticas somáticas nos tumores, utilizando técnicas de sequenciação de nova geração, sobretudo no caso de tumores que não alcançaram a resposta patológica completa após quimioterapia neoadjuvante é importante, pois poderá permitir a identificação de oportunidades de tratamento aos doentes, sobretudo se existirem terapêuticas alvo aprovadas, tais como terapêuticas anti-*HER2* e direccionadas ao gene *PIK3CA*. Existem já também alguns ensaios clínicos no contexto neoadjuvante utilizando os inibidores da *PARP* nos doentes com mutação *BRCA 1* ou *2*.

Desta forma, foram recrutamos para este estudo clínico 67 doentes com cancro da mama localmente avançado e foi efectuada a análise descritiva clínica durante o tratamento. O tecido tumoral de alguns desses doentes, obtido antes da realização de quimioterapia neoadjuvante, foi analisado molecularmente utilizando sequenciação de nova geração. Para além da amplificação do gene *ERBB2*, as alterações genéticas mais frequentemente encontradas nos tumores *HER2* positivos foram nos genes *MYC*, seguidos dos genes responsáveis pela reparação homóloga do DNA tais como *BRCA* e *ATM*. Os tumores da mama Luminal B *HER2* negativos apresentaram sobretudo alterações nos genes *TP53* e *MYC*. No entanto, não foi encontrado um padrão específico para cada subgrupo de tumores. No entanto, a presença de um elevado número de alterações nos *TP53* e *BRCA* no caso dos carcinomas da mama triplo negativos está de acordo com o revisto na literatura.

Doze doentes atingiram a resposta patológica completa após a quimioterapia neoadjuvante: as alterações no gene *ERBB2* foram as mais frequentes, provavelmente devido à utilização de terapêuticas anti-*HER2* associadas a quimioterapia neste contexto, seguidas de alterações no gene *BRCA 1* e *2*, *MYC*, *TP53*, *PIK3CA* e *MET*. No caso dos tumores que não atingiam resposta patológica completa, tratavam-se sobretudo de tumores triplos negativos; neste subgrupo as

alterações moleculares mais frequentes ocorreram nos genes *BRCA 1* e *2* e no *TP53*. Embora nem todas estas alterações possuam terapêutica alvo aprovada, a sua identificação poderá ter implicações prognósticas e como tal na escolha do melhor tratamento e realização de um seguimento mais apertado.

Este estudo realça a importância a aplicação de um painel genómico extenso no caso do carcinoma da mama, sobretudo devido à inexistência de painéis de sequenciação específicos para este tipo de tumores. A deteção de mutações somáticas, sobretudo nos casos em que existe uma terapêutica alvo disponível, torna-se, assim, promissora como potencial terapêutica co-adjuvante à resposta tumoral durante a quimioterapia neoadjuvante, ou como tratamento complementar após a cirurgia.

Apesar dos tumores que alcançaram resposta patológica completa apresentarem valores menos elevados de carga mutacional tumoral, não foi possível estabelecer uma relação estatisticamente significativa entre este dado e a probabilidade de se atingir resposta patológica completa, provavelmente devido à dimensão da amostra analisada. Contudo, esta avaliação, sobretudo no caso dos tumores que não atingiram resposta patológica completa, poderá ser utilizado como critério para seleção de doentes que poderão beneficiar de imunoterapia. Além disso, foi também possível proceder à determinação da instabilidade de microssatélites nas amostras. Apesar de ser rara a presença de elevada instabilidade de microssatélites no cancro da mama, a sua determinação poderá ser útil na prática clínica, uma vez que a utilização de imunoterapia está aprovada pela agência americana do medicamento neste contexto. É necessária a realização de ensaios clínicos que validem estes resultados.

Atendendo à importância crescente na prática clínica da detecção de alterações moleculares nos tumores ao longo da doença, vislumbra-se importante fazê-lo de uma forma menos invasiva; desta forma o conceito de «biópsia líquida» assume particular interesse a ser estudado no contexto neoadjuvante.

LIST OF ABBREVIATIONS

AC: Doxorubicin/Cyclophosphamide
ADC: Antibody drug conjugate
AJCC: American Joint Committee on Cancer
AF: Allele frequency
ATM: Ataxia telangiectasia mutated
BRCA 1: Breast Cancer gene 1
BRCA2: Breast Cancer gene 2
CA 15.3: CA 15.3 antigen
CA 125: CA 125 antigen
CHEK1: Checkpoint kinase 2
CHEK2: Checkpoint kinase 1
CNV: Copy number variation
Cp: Carboplatin
cfDNA: Cell free DNA
ChT: Chemotherapy
CT: Computed tomography
CTCs: Circulating tumor cells
ctDNA: Circulating tumor DNA
DCIS: Ductal *in situ* carcinoma
DNA: Deoxyribonucleic acid
DSBs: Double strand breaks
ERBB2: Human Epidermal growth factor Receptor 2
EGFR: Epidermal growth factor receptor
ER: Estrogen receptor
ESR 1: Estrogen receptor gene 1
ESMO: European Society for Medical Oncology
FDA: Food and Drug Administration
FFPE: Paraffin-embedded

FISH: Fluorescence in situ hybridization
FGFR1: Fibroblast growth factor receptor 1
HER2: Human epidermal growing factor
HGF: hepatocyte growth factor
IDH1: Isocitrate desidrogenase 1
LCIS: Lobular *in situ* carcinoma
LOH: Loss of heterozygosity
mCRPC: Castration-resistant prostate cancer
MET: Mesenchymal-epithelial transition
MYC: Myc protein
MRI: Magnetic resonance imaging
MSH2: MutS homolog 2
MSH6: MutS homolog 6
MSI: Microsatellite instability
MSI-H: Microsatellite instability high
MSI- L: Microsatellite instability low
MSS: Microsatellite stability
NAT: Neoadjuvant chemotherapy
NCCN: National Comprehensive Cancer Network
NGS: Next generation sequencing
NSCLC: Non-small cell lung cancer
NST: No special type
NTRK1: Neurotrophin receptor kinase 1
Pac: Paclitaxel
PALB2: Partner and Localizer of *BRCA2*
PARP: Poli-(ADP ribose) polymerases
pCR: Pathologic complete response
PD1: Programmed cell death protein 1
PDL1: Programmed death-ligand 1
PIK3CA: Phosphatidylinositol 3-kinase

PTEN: Phosphatase and tensin homolog

PR: Progesterone receptor

RAD51: *RAD51* homolog 1 protein

Rb: Retinoblastoma gene

RET: Rearranged during transfection

RNA: Ribonucleid acid

ROS1: ROS kinase 1

SNV: Single nucleotide variation

T: Docetaxel

TMB: Tumor mutation burden

TP53: Tumor protein 53

Ola: Olaparib

CHAPTER 1

INTRODUCTION

Data published in articles number 2,3, 4 and 5 is included in this chapter.

Clinical perspectives on breast cancer

Epidemiology, aetiology and diagnosis of breast cancer

Breast cancer is the most common cancer in women in developed regions (1). Regarding cancer-related mortality, it is the second cause of death. The incidence continues to increase mainly due to improvements in mammography screening (2). Several risk factors have been identified and histological and molecular classification are essential for treatment decision and clinical guiding.

The main risk factors for breast cancer are advanced age, gender, exposure to female hormones, reproductive history, family history and environmental factors such as alcohol abuse and excessive fat and salt intake (3). Family history is also a recognized risk factor. Women with a first degree relative have a risk 1.5-3 times higher to develop it (3,4). Besides the sporadic breast cancer tumors, it may rarely be associated with DNA repair associated BReast CAncer gene 1 and 2 (*BRCA1* and *BRCA2*) germline mutations which account for 5-10% of all cases. Besides sporadic cases of breast cancer, hereditary syndromes such as Li-Fraumeni, associated with tumor protein p53 (*TP53*) germline mutation, and Cowden Syndrome associated with phosphatase and tensin homolog (*PTEN*) germline mutation also increase the risk of developing the disease (3).

The diagnosis includes clinical examination and imaging using mammography and breast ultrasound and is confirmed by histopathological analysis of the breast tissue (2). After diagnosis, the stage of each breast cancer patient is determined in order to evaluate the disease extension and to define the correct treatment. The TNM system of American Joint Committee on Cancer (AJCC) is the most accepted and widely used for staging (5). This system comprises tumor size and extension (T), regional lymph node status (N) and presence of distant metastasis (M). Different combinations of T, N and M originate 5 stages (0, I, II, III and IV), used to stratify breast cancer patients and to predict prognosis and treatment (6). To evaluate the extension of the disease, imaging examinations such as computed tomography (CT) scan is required mainly in axillary node positive disease and bone scintigraphy may be of interest if clinical symptoms are present.

Histological Classification of breast cancer

Breast tumors histological classification is made according to the World Health Organization (WHO) classification (6). The complete WHO classification is complex and includes several histological types of breast cancer. The most important classification for clinical practice divides breast cancer in invasive and *in situ* carcinomas: invasive carcinomas comprise breast tissue cells' abnormal proliferation that invades through the duct wall into stroma, whereas *in situ* carcinomas are non-invasive lesions defined by the proliferation of epithelial cells confined to breast ducts or lobules. *In*

situ lesions can be ductal carcinoma in situ (DCIS) or lobular carcinoma in situ (LCIS), depending on cytoarchitectural features. Invasive ductal carcinoma also known as ductal carcinoma or carcinoma of no special type (NST) accounts for 70-80% of all breast tumors; Invasive lobular carcinoma is present in 5-15% of all patients.

Molecular classification

Besides histological classification, breast cancers are also classified according to the histological grade and proliferation marker ki67. Molecular characteristics are also important and are based on the presence or absence of endocrine receptors: estrogen receptors (ER) and progesterone receptors (PR) and also *human epidermal growth factor receptor 2* (HER2) (7). They are classified as luminal A tumors, luminal B, luminal B HER2 positive and triple negative breast cancers according to these molecular intrinsic features (table 1).

Subtype	Clinicalpathologic classification
Luminal A	«Luminal A like» ER positive PR positive Ki67 low HER2 negative
Luminal B	«Luminal B-like HER2 negative» ER positive PR negative Ki67 high HER2 negative
HER2 positive	HER2 positive «non luminal» HER2 positive ER and PR negative
«Basal like»	HER2 negative ER and PR negative

Table 1: Definitions of molecular intrinsic subtypes of breast cancer (adapted from Cardoso et al, *Early breast cancer: ESMO Clinical Practice Guidelines for diagnosis, treatment and follow-up*, Annals of Oncology 30: 1194–1220, 2019)

Luminal A is ER and PR positive, HER2 negative and the proliferation marker Ki-67 is low; luminal B is ER positive, HER2 negative and at least one of the following: Ki-67 high (generally defined as superior to 20%) and PR negative or low. In addition, luminal B breast cancers may also be ER positive, HER2 positive and independent of Ki67 and PR status; HER2 overexpression subtype is ER and PR negative with HER2 positive; basal-like breast cancer, which is commonly called triple negative is ER, PR and HER2 negative (8).

The ER is a nuclear transcription factor that stimulates breast normal cells growth by the binding of estrogen. This process is particularly important in cancer development since it induces tumor growth. Seventy-five per cent of breast tumors are ER positive, being well-differentiated, less aggressive, and associated with a better prognosis (9). ER positivity is a predictor of the response to endocrine therapies. Moreover, about 65-75% of the breast tumors are PR positive and its function is usually related to ER signaling activation (9). Accordingly, PR-positive tumors are frequently ER-positive, and associate with a therapy response rate of 75-85%. *ERBB2* or HER2 is a growth factor receptor expressed by breast epithelial cells membrane (7,9). Currently, it is evaluated by immunohistochemistry and/or fluorescence in situ hybridization (FISH), being overexpressed in 15% of all breast cancer cases. Therapies targeting *ERBB2*, such as trastuzumab and pertuzumab are currently available for patients, improving their outcome in all the treatment scenarios of breast cancer, such as neoadjuvant, adjuvant and metastatic treatment (9).

Gene expression profiles have been emerging to assist in adjuvant treatment decision (10). Oncotype DX® (21-gene panel), MammaPrint® (70-gene panel) and PAM50 (Prosigna) (50-gene panel) are different gene expression profiles that allow for tumors' classification and prognosis evaluation, and are useful in treatment decision making, especially in luminal B HER2 negative tumors, because in this subtype the benefit from chemotherapy may be not totally clear (10). Nevertheless, these profiles are not always used due to difficulty to be obtained, time consumption and cost, and clinicians have to base their decision on clinical, histological and molecular features of the tumors.

Systemic treatment of breast cancer

Early-stage disease

Early-stage disease of breast cancer may be defined as localized tumor to breast and/or axilla, excluding distant metastasis. Surgery of the breast tumor remains the *gold standard* for breast cancer treatment. Several other systemic treatments are possible such as chemotherapy, endocrine therapy, immunotherapy and targeted therapy.

The molecular classification described above in table number 1, helps guiding the correct treatment for the patients. According to European Society of Medical Oncology (ESMO) guidelines for early-stage breast cancer (8), patients with luminal A tumors must be preferentially treated with adjuvant endocrine therapy; luminal B HER2 negative tumors may benefit from chemotherapy besides endocrine therapy; HER2 positive tumors must be treated with chemotherapy and HER2 therapies

once they are larger than 5mm or have axillary involvement; triple negative breast cancer must be treated with chemotherapy (table 2).

Subtype	Recommended treatment	Observations
Luminal A	ET alone	ChT may be useful in tumors $\geq$ 5cm and/or with $\geq$ 3 axillary nodes involved
Luminal B HER2 negative	ChT followed by ET	
Luminal B HER2 positive	ChT with anti-HER2 therapy and ET	
HER2 positive (non luminal)	ChT with anti-HER2 therapy	If ChT is contraindicated, anti-HER2 therapy and ET may be considered
Triple negative	ChT *	If NAT add IT; gBRCA high risk add Ola

Table 2: Systemic treatment recommendations for early-stage breast cancer according to molecular subtype (adapted from Cardoso et al, *Early breast cancer: ESMO Clinical Practice Guidelines for diagnosis, treatment and follow-up*, Annals of Oncology 30: 1194–1220, 2019). ChT: chemotherapy; ET: endocrine therapy, IT: Immunotherapy; gBRCA: germline mutated *BRCA*; Ola: Olaparib. *except for some node-negative special histological subtypes such as secretory or adenoid cystic carcinomas or very low clinical risk (pT1a pN0) tumors.

Chemotherapy treatment may be classified as neoadjuvant or adjuvant depending on if it is done before or after surgery and its intent. Adjuvant chemotherapy is done after surgery of the tumor in order to reduce the probability of recurrence and eliminate micrometastasis.

Neoadjuvant chemotherapy (NAT) is provided to patients before surgery and in case of locally advanced breast cancers. Locally advanced breast cancer is defined as a breast cancer larger than 2cm and/or with lymph node involvement (11). These tumors are treated with NAT previous to surgery in order to reduce tumor size and, therefore, facilitate the surgery outcome, evaluate response of the tumor to chemotherapy and eliminate micrometastasis. Chemotherapy protocols in neoadjuvant and adjuvant setting are mainly based on anthracyclines, taxanes and/or platinum agents according to ESMO and National Comprehensive Cancer Network (NCCN) guidelines (8,11). Chemotherapy treatment is of specially value in HER2 positive breast cancers due to the availability of anti-HER2 targeted therapies such as trastuzumab and pertuzumab added to traditional chemotherapy and in triple negative breast cancer. Recently, the approval of immunotherapy in the neoadjuvant setting using anti programmed cell death protein 1 (PD1) agents such as pembrolizumab coupled with chemotherapy in triple negative breast cancer, has led to better results in terms of response rate for the patients submitted to NAT. These results are independent of programmed death ligand 1 (PDL1) determination and other breast biomarkers (11).

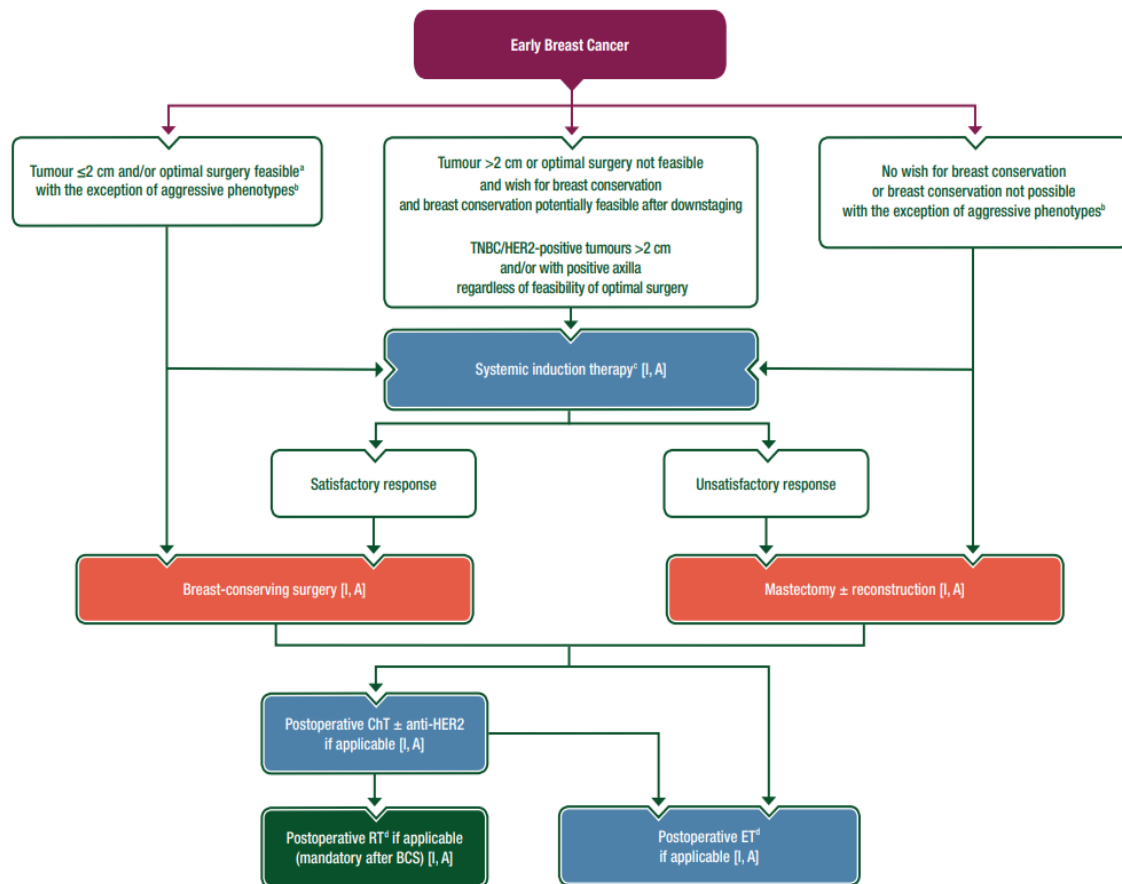


Figure 1: Treatment algorithm for early-breast cancer according to ESMO guidelines (adapted from Cardoso F et al, *Early breast cancer: ESMO Clinical Practice Guidelines for diagnosis, treatment and follow-up*, Annals of Oncology 30: 1194–1220, 2019). ChT: chemotherapy, BCS: breast cancer surgery, ET: endocrine therapy, RT: radiotherapy, [I,A]: category recommendation (I: evidence and/or general agreement that a given treatment or procedure is beneficial, useful, effective; A: data derived from multiple randomized clinical trials or meta-analysis).

Tumors responding to NAT have better prognosis mainly if pathological completed response (pCR) is achieved (no residual tumor cells detected after treatment). Although surgery is mandatory for this subgroup of patients, independently of response to chemotherapy, there is no biomarker to predict which patients will reach pCR and therefore have a better prognosis. Systemic treatment after surgery depends on the molecular intrinsic classification of the tumor as well as the tumor response to NAT. Luminal A and B HER2 negative tumors proceed with endocrine therapy; HER2 positive tumors are treated with trastuzumab or trastuzumab emtansine (T-DM1) during 1 year, depending on the pCR; triple negative breast cancers treated with immunotherapy during NAT maintain it for 1 year; those not treated with immunotherapy may benefit from oral capecitabine for 6 months; patients with germline mutated high risk *BRCA 1* or 2 germline mutations benefit from 1 year of adjuvant olaparib (8,11).

Metastatic disease

In metastatic setting, systemic treatment has a palliative intent and is much more complex. Systemic treatment is also based on the chemotherapy, endocrine therapy, immunotherapy or targeted therapies and depends on patient clinical status and comorbidities, symptoms related to the disease and molecular characteristics of the tumor. Chemotherapy is normally reserved for cases of visceral crisis disease (symptomatic visceral metastasis) and HER2 positive or triple negative breast cancers. As well as in the neoadjuvant setting, anti-HER2 targeted therapies (described below) coupled with chemotherapy in HER2 positive breast cancer tumors and immunotherapy coupled with chemotherapy in triple negative breast cancer tumors are the recommendation. Endocrine therapies coupled with cyclin inhibitors such as palbociclib, ribociclib and abemaciclib are also part of the recommendations in HER2 negative and endocrine positive tumors with impressive results in terms of progression free survival and overall survival and good clinical tolerance (8,11).

Targeted therapies for gene alterations approved in breast cancer

There are some targeted therapies approved for breast cancer patients when cancer tumors carry specific gene mutations and/or alterations, such as in *ERBB2* genes and Phosphatidylinositol 3-kinase gene (*PIK3CA*). Other targeted therapies are approved if a germline mutation is present such as in Breast Cancer gene 1 or 2 genes (*BRCA1* or *2*), (table 3).

Next generation sequencing (NGS) techniques developed in recent years have allowed for the identification of these gene mutations with targeted therapies available.

Gene alteration	Targeted therapy approved
<i>ERBB2</i> gene amplification	Monoclonal antibodies sub as trastuzumab and pertuzumab (NAT, adjuvant and palliative treatment); ADCs sub as T-DM1 and trastuzumab deruxtecan; tyrosine kinase inhibitors such as lapatinib, tucatinib and neratinib (metastatic breast cancer)
<i>PIK3CA</i> somatic mutations	Alpelisib (metastatic breast cancer)
<i>BRCA 1</i> and <i>BRCA 2</i> germline mutation	<i>PARP</i> inhibitors olaparib and talazoparib (metastatic breast cancer and adjuvant breast cancer therapy)
<i>ESR1</i> mutation	Elacestrant (metastatic breast cancer, only FDA approved)

Table 3: Correlation between gene alterations and targeted therapy available in breast cancer.

ADCs: antibody drug conjugates

The use of several anti-HER2 classes of therapies (figure 2) such as monoclonal antibodies (trastuzumab and pertuzumab) in first line metastatic treatment has led to a huge improvement in patient's overall survival (12). The approval of antibody drug conjugates such as T-DM1 (13) or trastuzumab deruxtecan have incredible results in terms of response rate of the tumors and progression free survival. Margetuximab is another antibody targeting HER2 but not approved in clinical practice in Portugal. Tyrosine kinase inhibitors harboring HER2, such as lapatinib, tucatinib and neratinib are also approved in breast cancer metastatic setting, with interesting results regarding tumor response rates and patient's progression free survival (13).

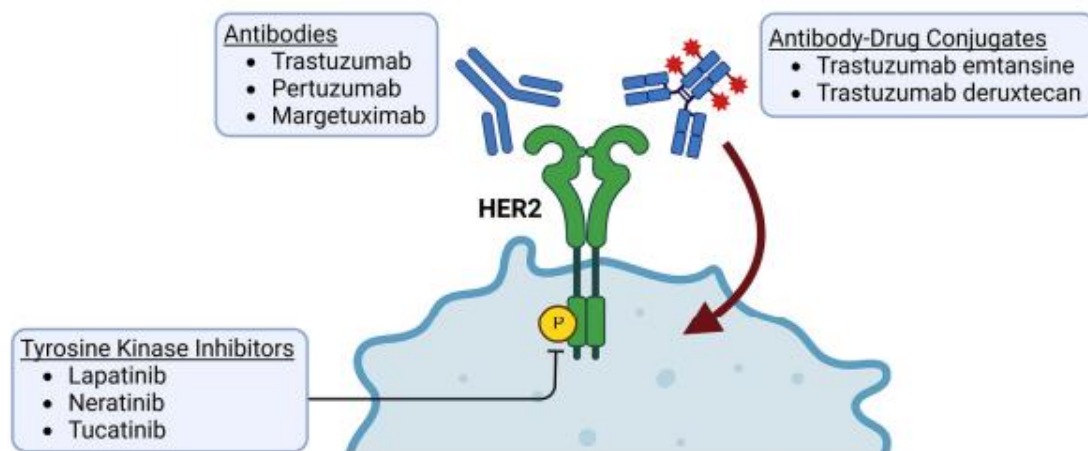


Figure 2: Anti-HER2 targeted therapies in breast cancer by different classes (adapted from Jacobs AT. et al, *Targeted therapy for breast cancer: an overview of drug classes and outcomes*, Biochemical Pharmacology 204 115209, 2022)

In patients with stage IV breast cancer whose tumors harbor a *PIK3CA* gene mutation and who progressed on or after an aromatase inhibitor and according to SOLAR 1 trial, alpelisib has showed benefit in terms of progression free survival when combined with endocrine therapy (14).

Recently elacestrant was approved by Food and Drug Administration (FDA) for metastatic breast cancer patients whose tumors carry an estrogen receptor 1 (*ERS1*) mutation and who have progressed on endocrine therapy, showing an improvement in response rate and progression free survival (15).

BRCA 1 and *2* gene mutations have also been extensively studied in recent years once targeted therapies such as Poly-(ADP ribose) polymerases (*PARP*) inhibitors are also approved in the metastatic setting in patients carrying a *BRCA* germline mutation and more recently as an adjuvant treatment too. These drugs have shown an improvement in progression free survival of patients carrying this mutation (16).

The use of *PARP* inhibitors in the metastatic setting is well established, nevertheless in the neoadjuvant setting is not totally clear: current trials suggest a role for neoadjuvant *PARP* inhibition in germline *BRCA* mutated patients. GeparOLA trial indicates that intensity de-escalation with olaparib instead of carboplatin maybe an option for germline *BRCA*-mutated patients. Furthermore, results from PREMETRAC trial suggest a possible benefit for the use of *PARP* inhibitors in treatment-naïve triple negative patients, which are corroborated by Litton et al. phase II clinical trial that showed comparable pCR rates between 24 weeks of monotherapy with talazoparib and standard chemotherapy regimens for germline *BRCA* mutated TNBC patients. Therefore, *PARP* inhibitors may represent an option in the neoadjuvant setting for treatment-naïve or minimally treated germline *BRCA* mutated triple negative breast cancer patients (Table 4).

Trial	Phase	n	Arms	pCR	Ref.
BrighTNess	3	A: 316	A: Veliparib+Cp+T ---AC	A:53%	Loibl et al
		B: 160	B: Placebo+Cp+T ---AC	B:58%	
		C: 158	C: Placebo+T ---AC	C: 61%	Geyer et al
Talazoparib	2	17	24 weeks talazoparib (without NAT)		Litton et al
NCT02282345					
GeparOla	2	107	Pac + Ola Pac + Cp ---AC	55% 48%	Fasching et al

Table 4: Neoadjuvant clinical trials of *PARP* inhibitors in early-stage triple negative breast cancer (adapted from Silva D. and Mesquita A. *Evolving evidence for the optimization of neoadjuvant treatment in triple negative breast cancer*, Breast Cancer (Auckl). 16: 11782234221107580, 2022). Cp: carboplatin; T: Docetaxel; AC: doxorubicin/cyclophosphamide, Pac: paclitaxel, Ola: Olaparib.

Although in breast cancer this approval is only in case of a germline mutation, in other malignancies such as ovarian and prostate cancer, the use of *PARP* inhibitors is also approved when somatic mutations are identified, making this mutation extremely relevant in clinical practice in terms of possibility of treatment, once it is well established in other malignancies (17, 18, 19).

Some clinical trials are trying to address if the use of *PARP* inhibitors may be useful in *BRCA 1* or *2* breast cancer somatic mutation, mainly in the metastatic setting are ongoing. Nevertheless, the somatic mutation identification is not usually done in clinical practice and there is not a NGS panel specific for these patients. Therefore, the identification of gene somatic mutations/alterations using a comprehensive genomic profile panel is important once it may identify potential targeted therapies for the patients in case of metastization but it also may be a key factor in the neoadjuvant setting for the patients who do not achieve pCR, making them a potential therapy. Clinical trials addressing these questions are needed.

Otherwise, obtaining biologic material from the patient in a less invasive way for NGS performing and detecting such gene alterations, would be of great value for the patients and clinicians.

Value of liquid biopsy in breast cancer

Molecular changes such as tumor DNA mutations, could, also theoretically be monitored during chemotherapy treatment of the disease. Nevertheless, biopsies would have to be made repeatedly, being an invasive procedure to the patients. In recent years, new methodologies of studying the dynamic range of molecular alterations of the tumor have emerged. Recent developments in sequencing have supported the clinical validation of circulating tumor DNA (ctDNA) as a «liquid biopsy». ctDNA is the genetic material released by the tumor to blood circulation. After collection of blood samples, multiple analysis using ctDNA, circulating tumor cells (CTCs), exosomes and tumor proteins may be performed. Analyzing the molecular profile, it is possible to obtain a molecular diagnosis, look for therapeutic resistance and make a personalized treatment (figure 3). ctDNA correlates with tumor burden and may provide a molecular profile of the tumor during the treatment (20). It contains alterations present in the tumor itself, such as mutations (21, 22,23).

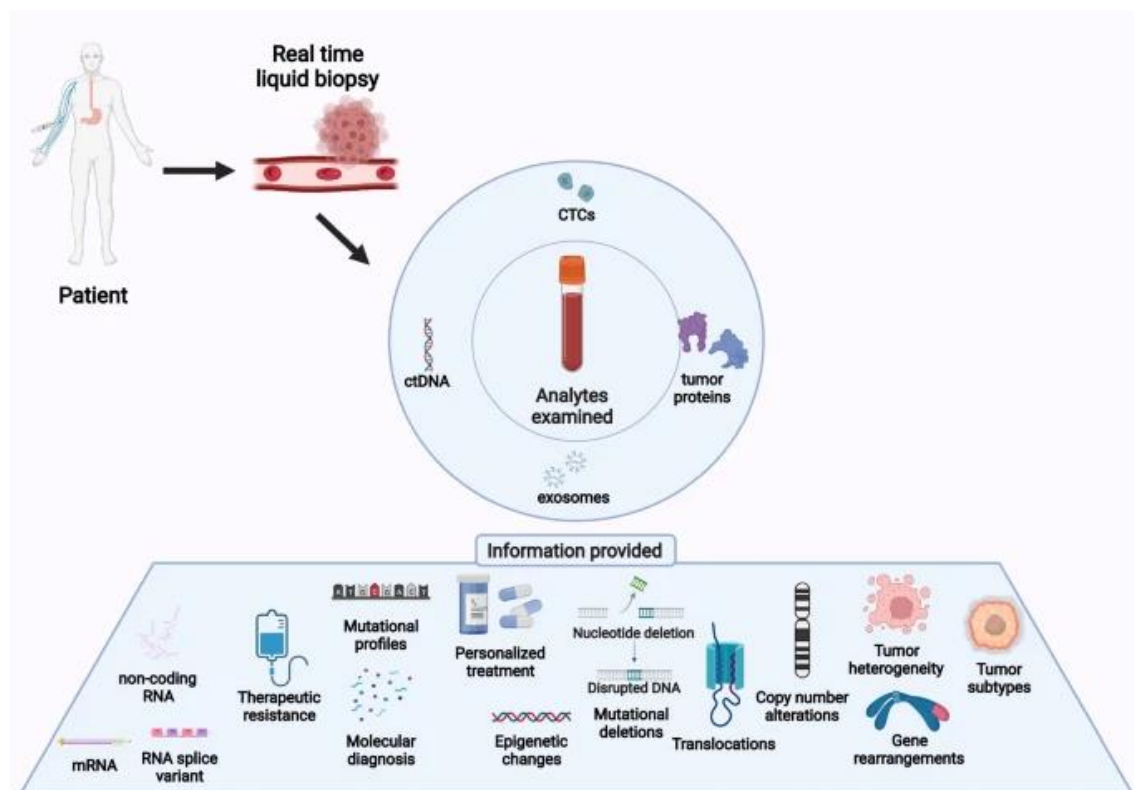


Figure 3: Value of liquid biopsy (adapted from Lone Sn et al, *Liquid biopsy: a step closer to transform diagnosis, prognosis and future of cancer treatments*, Mol Cancer,18;21(1):79, 2022). CTCs: circulating tumor cells.

As ctDNA acts as a repository of genetic material from throughout the body, it may provide material for more comprehensive mutation profiling and understanding of tumor heterogeneity. It also, allows the collection of tumor DNA material in a less invasive way for the patients using a blood sample collection, giving a dynamic photogram of the molecular profile of the tumor during

treatment. Therefore, it could be useful to use this tool to understand the tumor mutation profile and evolution during treatment.

There are some NGS panels approved using liquid biopsy for breast and /or pan-cancer approved by FDA) and used in clinical practice (table 5).

Liquid biopsy Assay	Clinical application	Genes analyzed
Gaurdant360® CDx assay	NSCLC, Pan-cancer	70 genes
Therascreen® (Qiagen) PI3KCA	Breast Cancer	11 mutations in <i>PI3KCA</i> gene
Oncomine® (Thermofisher)	Breast, Lung, Colon Cancer, Pan-cancer	52 genes
TS0500 ctDNA® (Illumina)	Pan-cancer	500+genes in pan-cancer
Avenio ctDNA® (Roche)	Breast, Lung, Colon-retal, Gastric, Melanoma, Pancreatic, Ovarian, Glioma, Thyroid cancers	17 genes

Table 5: FDA-approved liquid biopsies assays (adapted from Mesquita A. Et al, *Utility of circulating tumor DNA in different scenarios of breast cancer*, Cancers 12, 37, 2020). NSCLC: Non-small cell lung cancer.

NGS methods are used to examine ctDNA for the presence of gene variants, to detect early-stage disease, response to treatment, therapeutic resistance, and prognosis (24, 25). It also may detect disease progression and minimal residual disease after treatment. These advanced laboratory techniques have allowed for a daily collaboration between clinicians and laboratory teams since somatic mutations in tumors have gained a high clinical impact for patients due to the increasing targeted therapies available for some cancers. In 2016, the FDA firstly approved the use of ctDNA as a liquid biopsy test for patients with stage IV non-small cell lung cancer (NSCLC) to check Epidermal growing factor receptor (*EGFR*) mutations (26). This identification was often difficult in small lung tumor tissue samples available and repeating the lung biopsy was invasive for the patient. The identification of the mutation using ctDNA has allowed clinicians to overcome this difficulty and made it possible to treat patients with anti-*EGFR* targeted therapies available, today the standard of care. It has also allowed us to understand the resistance mechanisms of the tumors that stop responding to these treatments, and to select them for new therapies according to new mutations found in ctDNA. Spence, T. et al. analyzed the *EGFR* T790M status of 343 sequential patients with NSCLC using ctDNA collected during anti-*EGFR* treatment; 24% of the patients had the mutation and these patients were treated with osimertinib, a targeted drug approved in this scenario (27).

Therefore, a new paradigm of personalized medicine in which a targeted drug can be used in specific patients harboring a specific tumor mutation has emerged. Since this first clinical

application approval, the use of ctDNA is being investigated in different cancer types, including breast cancer. This has already been studied in different scenarios of breast cancer, such as early stage, metastatic and neoadjuvant setting (table 6).

In metastatic breast cancer, measuring treatment response in patients with metastatic breast cancer is usually done by clinical evaluation, assessment of CA15.3 levels, and radiographic imaging. However, serial radiographic imaging is often inconclusive, and CA 15.3 fluctuations do not necessarily reflect tumor response or progression (28). No marker for monitoring therapy responses in patients with metastatic breast cancer has yet reached wide clinical use, making it difficult for clinicians to anticipate a disease progression. The use of ctDNA in breast cancer was first established in metastatic disease, as tumor burden was related to the total amount of ctDNA (29). In metastatic breast cancer, ctDNA has shown promising results to monitor disease as reported by Dawson, S.J et al. (30). In this study, the authors compared the radiographic imaging of tumors with ctDNA, CA 15.3, and circulating tumor cells in 30 women with metastatic breast cancer who were receiving systemic therapy. They concluded that ctDNA levels showed a greater dynamic range and correlation with changes in tumor burden than CA 15-3 or circulating tumor cells. The authors mainly used the detection of mutations in the genes *PIK3CA* and *TP53* as surrogates for ctDNA. At the time, there was no mutation targeted therapy approved for metastatic breast cancer. Recently, targeted therapy for *PIK3CA* mutations has emerged. These mutations are found in 20–30% of breast cancer patients (31, 32). The use of ctDNA to identify *PIK3CA* mutations was also investigated in a subgroup analysis of SOLAR 1 trial by Juric, D. et al. and the treatment showed consistent clinically meaningful treatment benefit for patients with ctDNA mutant status (8). It made it possible to use a more comfortable and less invasive tool to detect a tumor mutation for which a targeted therapy is available. *BRCA* mutation also represents an intriguing scenario once there are targeted therapies such as *PARP* inhibitors available in case of germline mutation. Therefore, ctDNA-based assessment of somatic *BRCA* mutations using ctDNA could potentially expand the cohort of patients treatable with *PARP* inhibitors in the future (33).

Clinical Scenario	Clinical Use	Assay used	Findings
Early stage Disease	Early Detection	CancerSEEK	Detect cancers through the determination of mutations using the cfDNA. The median sensitivity of the test was 70% among the cancer types studied; 33% in breast cancer.
	Anticipating relapse	Ultra deep sequencing	ctDNA was detected before clinical or radio relapse in patients (sensitivity of 89%).
		dPCR analysis of ctDNA	Detection of ctDNA during follow-up is associated with high risk of relapse.
	Treatment resistance	dPCR analysis of ctDNA	<i>ESR1</i> mutations can predict resistance to endocrine therapy in early disease.
Metastatic disease	Monotoring disease	dPCR analysis of ctDNA	The ctDNA levels showed a greater dynamic range, and correlation with changes in tumor burden, than CA 15-3 or circulating tumor cells in patients with breast cancer receiving therapy.
	Treatment resistance	NGS and dPCR analysis of ctDNA	<i>ESR1</i> mutations can predict resistance to endocrine therapy; <i>ESR1</i> mutation analysis in plasma after progression can be a useful tool to guide the clinician's choice for subsequent endocrine therapies.
	Selecting targeted therapies	dPCR analysis of ctDNA	According to SOLAR 1 Trial, alpelisib was approved in patients with <i>PI3KCA</i> mutation; The use of ctDNA to identify <i>PI3KCA</i> mutation was validate.
		PLASMAmatch	ctDNA was able to identify patients with important targetable mutation.
Locally Advanced Disease	Detecting minimal residual disease	Dropped dPCR; Targeted digital sequencing (TARDIS)	ctDNA concentrations were lower in patients who achieved pCR compared to patients with residual disease; slow decrease of ctDNA levels during NAT was associated with poorer survival.
		Foundation One Liquid Assay	Detection of ctDNA in patients with early-stage triple-negative breast cancer after neoadjuvant chemotherapy was independently associated with disease recurrence.

Table 6: Clinical utility of using ctDNA in different breast cancer scenarios (adapted from from Mesquita A. Et al, *Utility of circulating tumor DNA in different scenarios of breast cancer*, Cancers, 12, 3797, 2020). *ESR1*: estrogen receptor 1 gene.

More recently the resistance mechanisms of cyclin inhibitor drugs using ctDNA have been studied. A study from Condorelli R. et al. has shown a retinoblastoma gene (*Rb*) mutation emerging after cyclin inhibitors treatment and failure. The mutation was detected in the ctDNA of an ER-positive, HER2-negative metastatic breast cancer patient. Cyclin inhibitors are approved in metastatic ER-

positive and HER2-negative breast cancer patients in first and second therapy lines. They act by inhibiting the cyclin D1-CDK4/6-retinoblastoma pathway (34,35).

There are some clinical trials currently addressing the question of using ctDNA to select targeted therapies for breast cancer patients. The PLASMAmatch trial is a phase 2 trial that included 1051 patients, with ctDNA available for 1034 of them. Patients were divided in different treatment cohort groups according to the mutation identified and target therapy used. The authors found that ctDNA testing was highly accurate, and showed high sensitivity for mutations identified in advanced breast cancer tissue biopsies. ctDNA was able to identify patients with important targetable mutations and therefore it could be used in routine clinical practice (36).

When looking at locally Advanced Breast Cancer, the results from the literature are not as well established as in the other clinical scenarios, such as the metastatic setting. There are some studies trying to correlate residual disease after NAT and ctDNA with interesting but premature results, and therefore there is the need of studying it in this subgroup of patients.

CHAPTER 2

THESIS OBJECTIVES

In the current Precision Medicine era reshaping Oncology, alongside the myriad of targeted therapies for solid malignancies, this study aims to harness this full potential by systematically identifying the landscape of somatic gene alterations in breast cancer, particularly in the neoadjuvant setting. Therefore 2 main objectives were designed:

1. Molecular characterization of a population of locally advanced breast cancer tumors before neoadjuvant chemotherapy using comprehensive genomic profiling (published data);
2. Identification of potential therapeutic strategies (pCR and non pCR samples), taking into consideration the molecular alterations detected (published data);

CHAPTER 3

MOLECULAR CHARACTERIZATION OF A POPULATION OF LOCALLY
ADVANCED BREAST CANCER TUMORS BEFORE NEOADJUVANT
CHEMOTHERAPY TREATMENT USING A COMPREHENSIVE GENOMIC
PROFILE

The content of this chapter includes original data published in article number 1 and data published in articles number 3, 4 and 5.

INTRODUCTION

Molecular characterization of breast cancer tumors is becoming an important issue in recent years. Several gene alterations with clinical interest are frequently detected in breast cancer tumors as described in some literature studies, such as *ERBB2*, *PI3KCA*, *BRCA1*, *BRCA2*, *ATM*, *PTEN*, *PALB2*, *RAD51*, *MYC*, *MET*, *NTRK1*, *FGFR1*, among others (37, 38, 39, 40). Regarding the neoadjuvant setting, most of the molecular studies focused on triple negative breast cancer (40). Some of them have tried to establish a correlation between some molecular alterations and response of the tumors to systemic treatment. In a study from Huang Li et al, the authors tried to correlate the presence of DNA repair mutation genes and probability of achieving pCR and responding to some chemotherapy protocols; nevertheless, the authors underlined the need of an extended follow up to validate their results (40). Other studies, focused in HER2 positive breast cancer, tried to understand the resistance to these therapies in the neoadjuvant setting and found that mutations in *PIK3CA* and *PTEN* genes were frequently found in these tumors (41). NGS techniques allow billions of DNA bases to be read at the same time (42,43). NGS may reveal the somatic genetic alterations in a cancer genome and provide information about potential treatments available. In the neoadjuvant scenario there is no molecular biomarker to predict which patients will achieve pCR. Therefore, studying the molecular landscape of the tumor is an important tool to provide insights in cancer treatment opportunities as well as monitoring it along time.

EXPERIMENTAL PLAN

Prospective and observational clinical study in patients with breast cancer candidates to neoadjuvant chemotherapy treated at Oncology Department, Local Health Unit of Matosinhos, Portugal.

Patient selection

Inclusion criteria

- Patients over 18 years of age with diagnosis of breast cancer candidates to NAT;
- Eastern Cooperative Oncology Group (ECOG) status grade 0 to 2;
- Measurable disease RECIST 1.1 criteria.

Exclusion criteria

- Personal history of another cancer diagnosed in the last 5 years, except patients treated with curative intent and no evidence of disease;
- Medical psychiatric disease or severe comorbidities.

Samples collection and storage

- Tumor tissue samples from documented breast cancer tumors were selected from the archive of Pathology Department, Local Health Unit of Matosinhos. Samples were selected by a certified Pathologist;
- Tissue samples were sent to Molecular Pathology and Immunology Institute of Porto University and revised by a certified Pathologist;
- Blood samples were collected by venous puncture using specific tubes such as vacutainer PTT tubes;
- For each patient, the blood samples were collected immediately before the administration of each chemotherapy cycle and 24h hours after;
- All samples were sent and stored to Molecular Pathology and Immunology Institute of Porto University.

Clinical Procedures during the study at the Oncology Department

A. Before starting treatment

- Informed consent;
- Revision of inclusion and exclusion criteria;
- Histological confirmation of diagnosis, and immunohistochemical report (endocrine receptors level expression, HER2, ki67);
- Clinical history report: symptoms, signs and physical examination of the patient;
- Laboratory blood tests results: hemogram, liver and renal function, tumor markers CA 15.3 and CA 125;
- Breast magnetic resonance imaging (MRI) and report;
- Radiological staging of the disease.

B. During the treatment

- Clinical evaluation and description of adverse events;

- Blood tests including hemogram, liver and renal function, tumor markers CA 15.3 and CA 125;
- Extra blood collection;
- Dose reductions report or discontinuation.

Samples processing

For each patient a formaldehyde-fixed, paraffin-embedded (FFPE) tumor biopsy sample was prepared by the Department of Pathology at Local Health Unit of Matosinhos and sent to Molecular Pathology and Immunology Institute of Porto University for further analysis:

DNA and RNA extractions - A certified pathologist evaluated histologically the hematoxylin/eosin slides and each tumor block was sliced in 10 x 10-µm slices. DNA was extracted from six full-FFPE slices with the Maxwell® RSC DNA FFPE kit, while the RNA was extracted from the remaining four full-FFPE slices with the Maxwell® RSC RNA FFPE kit, according to the manufacturer's instructions. DNA and RNA quantitation were accessed on Qubit®2.0 fluorometer with Qubit™ dsDNA HS Assay and Qubit™ RNA HS Assay kits, respectively.

Targeted NGS assays: All DNA and RNA samples were interrogated with OncoPrint™ Comprehensive Assay Plus (OCA-Plus). DNA and RNA libraries were prepared automatically with the Ion Chef™ system, according to the manufacturer's instructions recommended for FFPE samples (MAN0018490). The OCA-Plus assay allows the concurrent analysis of DNA- and RNA-FFPE derived samples, inquiring more than 500 cancer-associated variants, gene fusions, and includes biomarkers for tumor mutational burden (TMB), loss of heterozygosity (LOH) and microsatellite instability status (MSI).

For DNA library preparation, each sample (20 ng of FFPE-extracted DNA) was deaminated with uracil-DNA glycosylase (UDG) according to the manufacturer's protocol. For RNA library preparation, the FFPE-RNA samples (20 ng) were first reverse-transcribed to cDNA with SuperScript® VILO™ cDNA Synthesis kit, following the cDNA synthesis program: 10 min at 25 °C, 10 min at 50 °C, and 5 min at 85 °C.

Post-amplification, the DNA or RNA pooled libraries were quantified on Qubit®2.0 fluorometer using Qubit™ dsDNA HS Assay. Each DNA library pool was combined with the corresponding RNA library pool at a 90:10 ratio (DNA:RNA) and then loaded onto Ion 550 chips. High depth sequencing

was performed using the Ion S5™XL sequencer and data was assembled with Torrent Suite Software v.5.16.1.

Data analysis — Sequencing data were mapped to the Human Genome Build 38 in the Ion Reporter™ software (v. 5.18.4.0) and analyzed with the workflow “OncoPrint™ Comprehensive Plus – w2.3 – DNA and Fusions – Single Sample.” Additionally, coverage analysis reports from the Ion Reporter™ Software providing measurements of mapped reads, mean depth, uniformity, and alignment over a target region were used as quality assessment of the sequencing reactions.

Statistical Analysis and Data Visualization Mutation profile figures were generated using the OncoPrinter and MutationMapper data visualization tools available on the cBioPortal for Cancer Genomics platform (<https://www.Cbioportal.org/visualize>). Some categories of genetic alterations were considered to graphically display the mutation profiles: missense mutation, truncating mutation, other (including amplification). Mutation profiles were generated considering patients' tumor stage, subtype (ER and PR receptors, HER2 status), and pCR. Statistical analysis was performed using SPSS software (V.26 IBM®).

As mentioned before blood samples were stored at -20°C temperature at Molecular Pathology and Immunology Institute of Porto University, for further processing and analysis in the future.

RESULTS

Clinical results of general population recruited

Sixty-seven patients were recruited for this project. Sixty-six female and 1 male; median age 50 years old (25-72). All of the patients had locally advanced breast cancer invasive carcinoma at diagnosis. Thirty patients had stage II disease and the others stage III (figure 4).

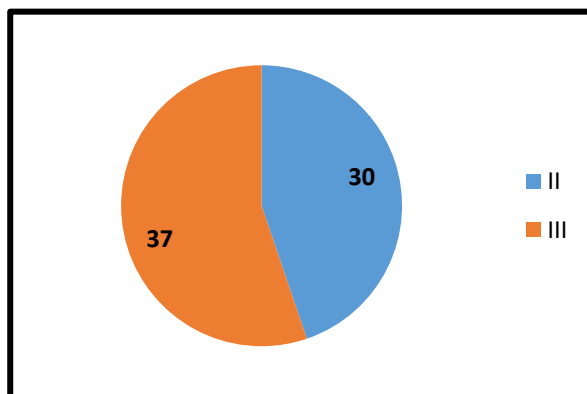


Figure 4: Clinical Stage Disease (TNM)

Regarding immunohistochemical characterization, most of the patients (33 patients) had HER2 positive disease; 23 had triple negative disease; 27 patients had ER positive disease and 22 PR positive. Median ki67 was 40% (10-100).

Patients with luminal B like HER2 positive breast cancer were treated with doxorubicin 60mg/mm2/cyclophosphamide 600mg /m2 (AC protocol) for 4 cycles, followed by docetaxel 75mg/m2 associated with trastuzumab and pertuzumab, for 4 cycles. This is the standard treatment used for these patients and recommended in all Oncology Guidelines. One patient was treated with Carboplatin AUC 6/Docetaxel 75mg/m2/Trastuzumab for 6 cycles, because the patient had already done anthracyclines in the past and therefore the cardiac anthracycline cumulative dose had been achieved. Triple negative breast cancer patients were all treated with AC protocol for 4 cycles, followed by Docetaxel 100mg/m2 for 4 cycles, as well as Luminal B HER2 negative breast cancer patients. Three patients did not complete all chemotherapy protocol due to cardiac complications, associated with anthracyclines and/or dual HER2 blockage. 1 patient died during chemotherapy due to liver metastization.

All patients had imaging response of the tumor on MRI. In 18 of them the tumor was not detected on MRI. Only 11 of the 67 patients had elevation of CA 15.3 before starting treatment and 2 patients had elevation of CA 125. These patients had progressive decrease of the tumor markers values during chemotherapy. The other patients had normal values of these tumor markers when the primary tumor was present and/or axillary involvement documented. After surgery, all patients had normal tumor markers values. Taking into consideration the subgroup of patients who achieved pCR, only 2 of them had positive tumor markers at the beginning of the treatment.

All the patients were submitted to surgery. Only 8 of them did a mastectomy mainly due to patients preferences and insufficient mammary tissue to achieve a visual acceptable result. The other patients were treated with conservative surgery followed by adjuvant radiotherapy. The tumor of 25 of the 68 patients (37,3%) submitted to surgery achieved pCR after NAT. Only one of them had a lobular invasive carcinoma. All of them had grade 3 tumors. Thirteen of these patients had HER2 positive disease and 9 had triple negative disease. Median ki67 was 40 (20-100). All the other patients had partial pathological response to chemotherapy. In 4 of these patients who achieved pCR, the tumor was described in the MRI after chemotherapy. In other 3 patients the tumor was not described in the MRI and it was detected in the mammary tissue after surgery.

Clinical results of the population whose breast cancer tissue samples were analyzed

Breast cancer tissue samples from 31 patients were analyzed (table 7). All patients had invasive breast carcinoma. 31 of them had NST carcinoma (1 of them with a micropapillary component). Regarding clinical stage characterization, most of the patients had stage III disease at diagnosis. Twelve patients achieved a pCR after neoadjuvant chemotherapy. Most of these tumors were HER2 positive and were treated with anti-HER2 therapy.

MRI evaluation showed no evidence of the tumor in 11 of the patients who achieved pCR. One of them had a residual image of 11mm in the MRI.

Immunohistochemical analysis identified: 16 of the patients had luminal HER 2 positive disease; 10 patients had triple negative breast cancer disease. The other patients had endocrine receptors positive disease (most of them luminal B HER 2 negative disease).

Only 5 of these 31 patients had elevated tumor marker CA 15.3 before chemotherapy. All of them had a decline in the value of the tumor markers during chemotherapy; nevertheless 2 of them maintained elevated values after neoadjuvant chemotherapy. Four patients presented with elevation of tumor marker CA 125 before chemotherapy, and only one maintained elevation after neoadjuvant chemotherapy.

		<i>n</i>
PATIENT LEVEL		
Sex	female	30
	male	1
Histology	NST (only)	30
	NST micropapillary component	1
Tumor Markers before NAT	positive	9
Tumor Markers after NAT	positive	3
SAMPLES LEVEL		
Subtype level (ER, PR, HER2)	ER+	14
	PR+	12
	HER2+	16
	Triple negative	10
Clinical Stage	II	11
	III	20
MRI after NAT	no evidence of the tumor	11
pCR	yes	12

Table 7: Clinical characterization of the population whose breast cancer samples were analyzed

(adapted from Mesquita A. et al, *Next generation sequencing of breast cancer in the neoadjuvant setting*, *Pathobiology* 1–7, 2023).

All patients were treated with chemotherapy including doxorubicin 60mg/m² /cyclophosphamide 600mg/m² protocol for 4 cycles, followed by docetaxel 75-100mg/m² for 4 cycles. HER2 positive patients were also treated with dual HER2 blockage with pertuzumab and trastuzumab before surgery of the tumor. One of the patients did not complete the chemotherapy protocol due to cardiac toxicity after 4 cycles of anthracyclines; despite this, the patient achieved a pCR. All of these 31 patients had a benefit from neoadjuvant chemotherapy, and 12 of them achieved a pCR.

Molecular characterization of breast cancer tumors using NGS

Tissue sample from 31 patients were analyzed, using Next Generation Sequencing. DNA from the tissue samples was isolated with RecoverAll Total Nucleic Acid Extraction kit.

Oncomine Comprehensive Plus – w2.3 – DNA and Fusions – Single Sample (Thermofisher Scientific®), panel was used. This panel analyzes more than 500 genes. It allows to identify:

- 165 genes with recurrent hotspot mutations
- 333 genes with focus copy number variants (CNV) gains or loss
- 227 genes with full coding DNA sequences
- More than 1mb exonic footprint for tumor mutation burden (TMB)
- Microsatellite instability high/microsatellite stability (MSI-H/MSS) markers for MSI
- 49 fusion driver genes
- *MET* exon skipping detection at DNA and RNA level – Cellularity calculation
- 46 genes with homologous recombination repair pathway
- Loss of heterozygosity (LOH) detection

The use of this panel allows not only the identification of gene mutations or CNV but also an extensive information about the tumor which can be useful in clinical practice, such as TMB and MSI status.

Several gene alterations were identified using this NGS panel in the different tumors. Some of these tumors had multiple gene alterations identified. Some of the genes identified in the different tissue samples, have a targetable therapy available. Therefore, the investigational team focused on describing these genes alterations due to the importance for clinical practice. These genes are: *ERBB2*, *PI3KCA*, *BRCA1*, *BRCA2*, *ATM*, *MYC*, *MET*, *NTRK1*, *FGFR1*, *ROS1*, *RAS* and *EGFR*. Nevertheless, other gene alterations identified, although not yet targetable, were also analyzed due

to the frequency that they are reported and will be analyzed. The gene alterations detected will be preferentially described in the order of the prevalence detected in the samples.

ERBB2 gene

HER2 belongs to the family of HER tyrosine kinase transmembrane receptors, which includes HER-1/epidermal growth factor (*EGFR*), HER2, HER3 and HER4. Whenever activated by a ligand, these receptors stabilize in the cell membrane through homo- or heterodimerization, and hence activate intracellular signaling pathways – mitogen-activated protein kinase (MAPK), phosphatidylinositol 3-kinase (PI3K), AKT and mammalian target of rapamycin-dependent (44). These pathways lead to cell division and growth, essential to cancer's proliferation. HER family of receptors were found to play an important role in physiological breast tissue growth and differentiation. HER2 is composed by an extracellular ligand-binding domain (subdivided in four sub-domains I-IV), a transmembrane domain and an intracellular tyrosine kinase domain. HER2 is the only HER family receptor that does not have a physiological ligand, but it plays an important role in heterodimerization with other HER receptors, particularly HER3. This receptor is encoded by the HER2 oncogene and was found to be overexpressed in a large number of solid tumors, such as breast, lung, colorectal and gastric cancers (45). Its overexpression and abnormal ligand-independent activation in cancer cells leads to uncontrolled cell growth, proliferation, evasion to apoptosis and high metastatic potential, thus fulfilling four of the fourteen described hallmarks of cancer.

ERBB2 gene alterations were detected in 16 tumors. In all of these tumors, the type of alteration was a CNV, with amplification. The copy numbers varied among the different tumors (figure 5). All of these 16 cases were described as HER2 (3+) in immunohistochemistry and were treated with anti-HER2 therapy. None of the HER2 (1+) or HER2 (2+) tumors had *ERBB2* gene amplification detected. The tumors which achieved pCR after NAT, had a median value of copy numbers of 41.15 (7.46-43.6). This value was higher than the other tumors 10,62 (5,8-45.65) which did not achieve pCR.

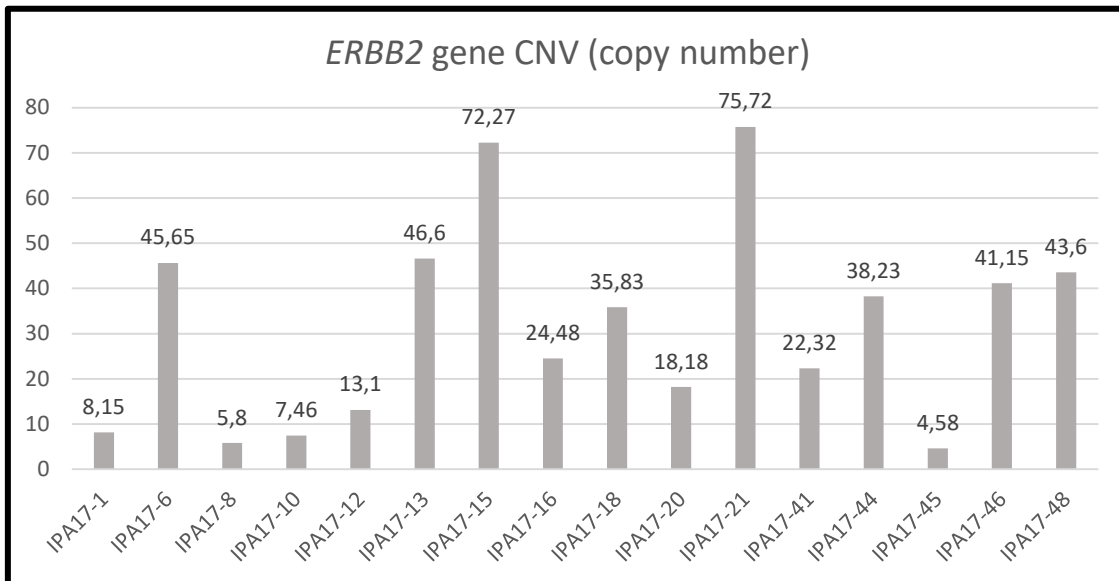


Figure 5: Type of *ERBB2* gene alteration and copy number in each tumor sample analyzed. IPA17 number: samples codification.

BRCA1 and 2 genes

BRCA1 and *BRCA2* genes are tumor-suppressors and their protein products have multiple functions, such as serving as key enzymes in the homologous recombination pathway, a mechanism for repairing DNA double strand breaks (DSB). Loss-of-function mutations of *BRCA1/2* disables the homologous recombination pathway, which forces cells to rely on lower fidelity more error-prone pathways for DNA repair, such as non-homologous end-joining, during which mutations and genetic instability may be introduced (46).

Sixteen patients had *BRCA* gene alterations in the tissue samples. Most of the tumors had LOH (14 cases). In one patient a *BRCA1* truncating mutation was detected (p.Leu2173ThrfsTer3) with an allele frequency of 6.24 mut/mb. In other patient a SNV mutation in *BRCA2* gene (p.Lys3326Ter) was detected with a higher allele frequency of 32.67 mut/mb. None of these patients achieved pCR after NAT. Most of these patients had triple negative disease as described in the literature.

LOH is also being studied as recent data suggests that in addition to the germline mutation, locus-specific LOH may also be necessary to predict sensitivity to DNA-damaging agents and therefore allow better outcomes for the patients (47). Clinical trials are prospectively evaluating different patterns of LOH, trying to identify the benefit of *PARP* inhibitors in this scenario (46,47). Therefore, using a NGS panel which allows us to obtain these results may be useful for the identification of the patients who might benefit from these therapies in the future.

TP53 gene

Tumor protein 53 (*TP53*) is a tumor suppressor gene and its somatic mutation is one of the most frequent somatic mutations in breast cancer (48). *TP53* mutations are identified in up to 37% of breast tumors especially in HER2 positive and basal-like subtype (49). *TP53* mutations were related to increased risk of breast cancer-specific death regardless of tumor size, nodal status and hormone receptor expression. Moreover, *TP53* mutation status has been associated with response to breast cancer treatment (49).

Twelve tissue samples had a *TP53* mutation identified (table 8). Median allele frequency of mutation was 29,09 mut/mb (2,89-74,95). Most of them were hotspot or truncating mutations. From these cohort of patients, 6 achieved a pCR. Median mutation allele frequency in patients who achieved pCR was 44,4 mut/mb (2,89-73,81), higher than in general population of the study and those who did not achieve pCR (median of 13,87).

Gene	Gene alteration	Type of mutation	Amino acid alteration
<i>TP53</i>	SNV	Truncating	p.Arg196Ter
<i>TP53</i>	Indel	Truncating	p.Met237ValfsTer2
<i>TP53</i>	SNV	Hotspot	p.Arg273Leu
<i>TP53</i>	Indel	Truncating	p.Cys242AlafsTer5
<i>TP53</i>	SNV	Hotspot	p.Tyr236Cys
<i>TP53</i>	SNV	Hotspot	p.Met237Ile
<i>TP53</i>	Indel	Truncating	p.Cys229TyrfsTer10
<i>TP53</i>	SNV	Hotspot	p.Gly266Arg
<i>TP53</i>	SNV	Hotspot	p.Cys275Tyr
<i>TP53</i>	Indel	Truncating	p.Arg209LysfsTer6
<i>TP53</i>	SNV	Hotspot	p.Arg213Gln
<i>TP53</i>	SNV	Hotspot	p.Tyr234Ter

Table 8: *TP53* gene alterations detected. SNV: single nucleotide variant.

MYC gene

MYC gene alterations were found in 14 tumor samples. *MYC* plays multiple roles in breast cancer development and progression. *MYC* amplification appears to be a strong prognostic marker of early recurrence in node negative breast cancer patients in some studies (50). Therefore, it is important to define amplification threshold in studies evaluating *MYC* amplification in breast cancer in order to establish a more intensive follow-up.

All of the 14 samples had CNV of *MYC* gene, with amplification and gain of function. The copy number variations ranged from 6.01 to 23.35 in the different tumors and, once again, it was higher in patients achieving a pCR (figure 6).

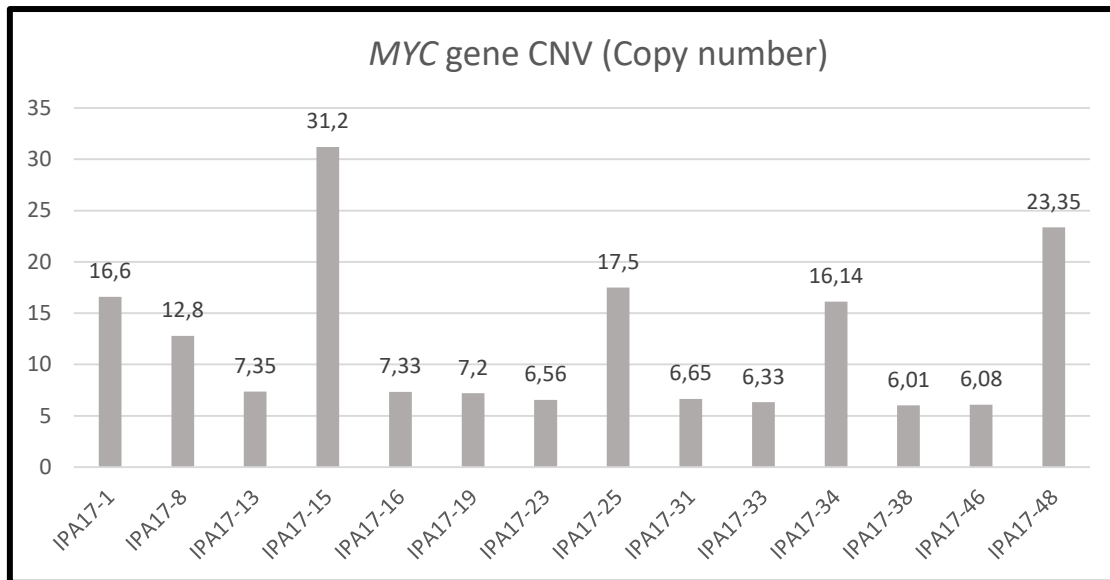


Figure 6: Type *MYC* gene alteration and copy number in each tumor sample analyzed.

ATM gene

Tissue samples from 6 patients had ataxia telangiectasia mutated (*ATM*) gene alteration (table 9). The *ATM* is a protein serine/ threonine kinase, which belongs to the core components of DNA-damage response machinery and acts as an intracellular sensor by recognizing DSBs (51). Most of *ATM* gene alterations detected in these tumors were LOH. Tumors with mutations in genes encoding proteins involved in DNA repair, such as *ATM*, may be more sensitive to treatments that induce cytotoxicity by inducing DNA damage or inhibiting DNA repair mechanisms, such as platinum treatments and *PARP* inhibitors. More recently, it was demonstrated that *ATM* and *CHK2* gene alterations are required to boost the efficacy of endocrine agents in luminal tumors (52).

Two of the patients achieved pCR, both of these tumors were HER2 positive and were treated with anti-HER2 therapies.

Gene	Gene alteration	Type of mutation	Amino acid alteration
<i>ATM</i>	SNV	Truncating	p.Arg2598Ter
<i>ATM</i>	SNV	Truncating	p.Arg1730Ter

Table 9: *ATM* gene alterations detected.

PIK3CA gene

Six patients had a Phosphatidylinositol 3-kinase (*PIK3CA*) gene alterations found in their breast cancer tissue samples (table 10). Most of them were SNV gene alterations. Only one patient achieved a pCR, the others did not, although they responded to chemotherapy.

PIK3CA mutations are common activating mutations associated with breast cancer (occurring in 20–30% of all cases) and as described before are potent predictive markers for responses to *PIK3CA* inhibitors (53). In these patients the results are interesting because there is a molecular target available for this population in the metastatic setting.

Although this is not the clinical scenario of this patient, this information may be useful in the future in case of metastization.

Gene	Gene alteration	Type of mutation	Amino acid alteration
<i>PIK3CA</i>	SNV	Hotspot	p.His1047Arg
<i>PIK3CA</i>	SNV	Hotspot	p.Lys111Asn
<i>PIK3CA</i>	SNV	Hotspot	p.Glu39Lys
<i>PIK3CA</i>	SNV	Hotspot	p.Tyr1021His
<i>PIK3CA</i>	CNV	amplification	-
<i>PIK3CA</i>	SNV	Hotspot	p.His1047Arg

Table 10: *PIK3CA* gene alterations detected.

MET gene

Mesenchymal-epithelial transition (*MET*) is an oncogene encoding for a trans-membrane tyrosine kinase receptor activated by the hepatocyte growth factor (HGF). In breast cancer, preclinical and clinical studies highlighted the role of *MET* deregulation on carcinogenesis and development of aggressive phenotypes. *MET* is over-expressed in at least 20-30% of cases. However, up-regulation of HGF/*MET* axis represents a strong and independent predictor of poor outcome and aggressiveness, irrespective of histological subtype (54). Two tissue samples presented with CNV and amplification of the gene. In some cancers, *MET* inhibitors are already approved in clinical practice. Although not yet approved in breast cancer, it is important to identify these gene alterations which may be useful in the future.

EGFR gene

EGFR extracellular domain leads to the phosphorylation of multiple tyrosine residues in the intracellular domain of *EGFR*. This *EGFR* activation induces cell proliferation, motility, and survival of the cells (55). The *EGFR* protein is variously overexpressed in breast cancer, especially in triple negative breast cancer, with a frequency ranging from 13% to 78%, depending on the primary

antibodies used to the determination (55). It is considered an attractive therapeutic target for *EGFR* inhibitors and therefore many clinical trials have been conducted, but response rates have been disappointing. (56)

One patient had a *EGFR* hotspot SNV. This patient had luminal B HER2 positive disease and it did not achieve pCR.

ROS1 gene

ROS1 kinase activity is constitutively activated when a *ROS1* mutation is present, leading to increased cell proliferation, survival and migration (57). *ROS1* mutation is determined in non-small cell lung cancer and patients whose tumors carry the gene mutation are able to be treated with targeted therapies such as crizotinib (58). A *ROS1* CNV was detected in tissue sample. This patient had HER2 positive disease and achieved a pCR.

RAS gene

A *KRAS* CNV was detected in one tissue sample. This patient had a triple negative breast cancer disease and did not achieve a pCR.

Dysregulation of *RAS* function is largely associated with tumorigenesis. *RAS* might also confer resistance to therapies in breast cancer. It induces resistance to cisplatin and induces resistance to lapatinib. It also affects response to tamoxifen but not chemotherapy in breast cancer patients (59).

In patients with metastatic colon cancer determination of *RAS* status is used to identify those who might benefit from monoclonal antibodies therapy such as cetuximab or panitumumab (wild type). Therefore, *RAS* determination is already used in some cancers. Taking this into consideration, its determination in breast cancer patients may play an important role in a near future.

FGFR1 gene

Two samples had a Fibroblast growth factor receptor 1 (*FGFR1*) gene alteration detected (CNV), with amplification and gain of function.

Fibroblast growth factor receptors (FGFRs) play key roles in promoting the proliferation, differentiation, and migration of cancer cell. Inactivation of FGFRs by tyrosine kinase inhibitors has achieved great success in tumor targeted therapy (60). Among *FGFR* alterations, gene

amplification has been found in an elevated percentage of cases of breast cancer patients (66%), however point mutations (26%) and chromosomal rearrangements (8%) have also been detected in a large study (61). Considering that many *FGFR* aberrations may lead to a gain-of-function, it has been hypothesized that *FGFR* inhibitors would be useful as anticancer agents in breast cancer patients as they are already approved in other malignancies.

NTRK1 gene

The neurotrophin receptor kinase 1, (*NTRK1*) plays an important role during the development of the nervous system (62). It is also oncogenic and there is accumulating evidence for its overexpression and involvement in cancer progression including in the form of TrkA fusion proteins as described in lung cancer. Furthermore, TrkA is a therapeutic target in oncology and Trk inhibitors are being tested in several clinical trials (62). In breast cancer, *NTRK1* has been shown to stimulate tumor cell invasion and metastasis. However, TrkA protein expression in a large clinically relevant cohort of breast tumors, as well as its overall clinical significance remain unclear. Two samples had a CNV in *NTRK1* gene.

IDH gene

Isocitrate deshydrogenase (*IDH*) is an important enzyme of the tricarboxylic acid cycle, whose mutated genes are associated with several tumors. The mutation may lead to the increase of 2-Hydroxoglutarate, and some studies have shown it is increased in some malignancies. Targeted drugs have been developed for these mutations and are being studied in several clinical trials and enasidenib and ivosidenib have already been approved for clinical use in some cancers (63). *IDH* gene had alterations in 2 patients, both of the gene alterations were CNVs.

Analyzing gene alterations found according to tumor molecular intrinsic classification

When analyzing the gene alterations found according to the intrinsic molecular subtype of breast cancer, we have concluded that the most frequent gene alterations detected in triple negative breast cancer were in *TP53*, *BRCA* and *MYC* genes (figure 7 and table 11). Besides *ERBB2* gene amplification, the most frequent gene alteration detected in HER2 positive tumors were in *MYC* gene, followed by repairing DNA genes involved in homologous recombination pathway such as *BRCA* and *ATM*. Luminal B HER2 negative breast tumors presented mainly *TP53* and *MYC* gene alterations. A specific pattern of genomic profile was not found for each group of tumors.

Nevertheless, the presence of high number of *TP53* and *BCRA* gene alterations in triple negative breast cancer is reported according to the literature.

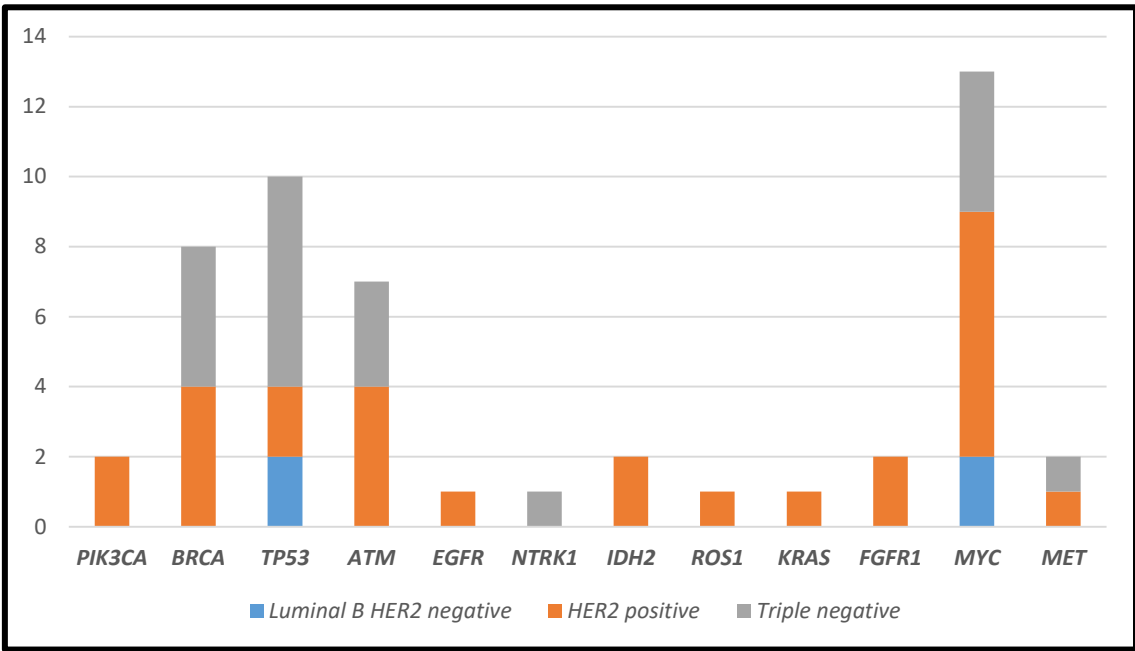


Figure 7: Gene alterations found according to breast cancer molecular intrinsic subtype.

	Luminal B HER2 negative	HER2 positive	Triple negative
PIK3CA	0	2	0
BRCA	0	4	4
ATM	0	4	3
TP53	2	2	6
EGFR	0	2	0
NTRK1	0	0	1
IDH2	0	2	0
ROS1	0	1	0
FGFR1	0	2	0
KRAS	0	1	0
MYC	2	7	4
MET	0	1	1

Table 11: Gene alterations found according to breast cancer molecular intrinsic subtype.

Other frequently reported genes in breast cancer detected

PALB 2 gene

A gene alteration in Partner and Localizer of *BRCA2* (*PALB 2*) was identified in 4 samples. *PALB 2* gene acts as a *BRCA2* gene binding partner, facilitating homologous recombination, essential for DSB repair pathway (64). Some large studies in breast cancer have identified mutations in *PALB2* gene patients, has at high risk for developing breast cancer (65).

The analysis of molecular characteristics of *PALB2*-associated tumors is essential for clinical management of breast cancer once that tumors with biallelic inactivation of the genes involved in homologous recombination are highly sensitive to some drugs and cancers arising by the haploinsufficiency mechanism are unlikely to respond to systemic treatments such as platins or *PARP* inhibitors (66). Taking into consideration the 4 cases where the somatic gene alteration was identified, one of them achieved a pCR. It was a triple negative breast cancer. All of the gene alterations detected were LOH.

PTEN gene

In 7 tissue samples, a *PTEN* gene alteration was identified. *PTEN* represents one of the most frequently mutated gene in some human cancers, including breast cancer (67).

Although germline mutations in *PTEN* gene can be identified in some genetic syndromes, somatic mutations in breast cancer tumors are more frequent. LOH at the *PTEN* locus was reported in nearly 40%–50% of breast tumors, whereas the loss of *PTEN* function due to *PTEN* mutations was detected in 5%–10% of breast cancer cases (68).

In this study, 7 samples had a *PTEN* gene alteration identified. Most of these alterations were LOH, with the exception of one sample, with a SNV truncating mutation identified (p.Gln87Ter). Only one of these patients achieved a pCR after NAT, the tumor had a LOH identified and was classified as a HER2 positive breast cancer.

CHEK 1 and CHEK 2 genes

Checkpoint kinase 1 (*CHEK1*) is a conserved protein kinase in the process of biological evolution, which exerts an essential influence in regulating cell cycle checkpoints caused by DNA damage. Its overexpression may serve a key function among a variety human cancer mechanisms (69). Some studies have determined the prevalence of *CHEK1* overexpression in breast cancer (70).

Some studies suggested that the metastasis of cancer cells was increased by *CHEK1* (69). Four samples had a LOH in *CHEK1* gene identified and none of them achieved a pCR.

Checkpoint kinase 2 (*CHEK2*) is a tumor suppressor gene that encodes a serine/threonine kinase, the CHK2. This gene is involved in several pathways such as DNA repair, cell cycle regulation and apoptosis in response to DNA damage (71, 72). Some clinical trials are trying to address if targeted therapies such as *PARP* inhibitors have some role in tumors with *CHEK2* gene mutations. Recently, a study showed that olaparib showed efficacy in different cancer types harboring somatic or germline mutations in the *BRCA1/2* genes but not in *ATM* and *CHEK2* (72).

In this study, we identified a *CHEK2* SNV, truncating mutation (p.Arg519Ter) in one sample. This is a case of triple positive breast cancer which did not achieve a pCR. Other 3 samples had LOH in this gene identified.

RAD 51 gene

Like in the cases of *BRCA 1* and *2* germline mutations, pathogenic germline variants in homologous recombination related genes, such as *RAD51C* and *RAD51D* confer an increased risk of breast and/or ovarian cancers (73,74). *RAD51* overexpression in tissue cancers has been found in a variety of cancer cell lines and has been associated with resistance to DNA-damaging treatment (75). Several studies have also demonstrated a relationship between *RAD51* protein expression and the biology and metastatic potential of malignant disease, where *RAD51* overexpression was significantly associated with lymph node and distant metastasis (76,77), in several malignancies such as breast cancer. Therefore, the identification of such gene alterations might be important as a prognostic factor in the future. Once again, clinical trial specifically designed to address this issue are necessary.

In this study we have identified some cases of *RAD51* gene alterations: a truncating mutation (pArg23Ter) was identified. This is a breast cancer HER2 positive tumor, which did not achieve a pCR. This tumor also presented other homologous recombination genes affected, such as *BRCA1* and *2* and *ATM*. We have identified LOH in other 12 samples.

MSH2 and 6 genes

In this study we have identified 2 cases with mutS homolog 2 and 6 (*MSH2* and *MSH6*) SNV mutations in two samples. These cases corresponded to a triple negative breast cancer and to a HER2 positive breast cancer. None of them achieved pCR. One of the cases corresponded to a

patient with past medical history of colon cancer, although Lynch Syndrome was not detected when germline mutations were analyzed.

RET gene

Rearranged during transfection gene (*RET*) is a proto-oncogene encoding a transmembrane tyrosine kinase receptor that is activated by one of its four ligands and one of its coreceptors. Loss-of-function germline mutations in *RET* are responsible for Hirschsprung disease, while gain-of-function mutations for multiple endocrine neoplasia type 2 (80).

Although *RET* gene somatic alterations are frequently reported in some malignancies such as lung, thyroid, and colon cancer, in breast cancer they are rare (81). In breast cancer tumors it is mainly related to ER positive disease and *RET* overexpression is related to endocrine therapy resistance (81). Therefore, the identification of this gene alteration might be useful to monitoring endocrine therapy in these patients. Otherwise, in some malignancies, there are targeted therapies for *RET* gene alterations approved, such as cabozantinib, sorafenib, lenvatinib, and vandetanib, most often used in the treatment of thyroid, liver, and renal cancers (82).

We have identified a *RET* gene alteration in one tumor sample. The gene alteration identified was a SNV hotspot mutation (gain of function), with p.Arg912Trp, amino acid alteration (allele frequency of 2.3mut/mb).

TMB ANALYSIS

TMB is defined as the total number of somatic mutations in a region of tumor genome and is associated with the immunogenicity of many different tumors, including breast cancer, once it correlates with tumor neoantigen and T cell infiltration (83).

In a study from McGrail DJ et al, high values of TMB (defined as more than 20 mutations per megabase of DNA – mut/mB) were not correlated to response to immunotherapy. For cancers with a strong correlation between TMB status and T cell infiltration, patients with a high TMB had improved clinical outcome. In contrast, TMB status was not predictive of outcome in a second class of tumors, which included breast cancer tumors. The authors concluded that more studies are needed to establish this correlation for all solid tumors (84).

Recently, in phase II KEYNOTE-158 study which included patients with advanced solid tumors, the anti-PD-1 antibody pembrolizumab produced an objective response rate (ORR) of 29% (95% confidence interval [CI]: 21–39%) in patients with TMB $\geq$ 10 mut/Mb compared to 6% (95% CI: 5–8%) in patients with TMB < 10 mut/Mb (85). While data from the KEYNOTE-158 study led to the approval of pembrolizumab to treat patients with TMB-high status ($\geq$ 10 mut/Mb as determined using the Foundation One CDx assay) regardless of tissue of tumor origin (tumor tissue-agnostic approval), this trial did not include any patients with metastatic breast cancer (85). Therefore, several questions remain unanswered about the true role of TMB as a predictive biomarker of benefit to immunotherapy therapy in breast cancer.

In fact, breast cancer is considered a cancer with an intermediate TMB, and triple negative disease is frequently the one with higher TMB (86, 87). Therefore, the approval of the use of immunotherapy in metastatic breast cancer, remains lying on the determination of PDL-1 and combined positive score of the tumor, which evaluates the number of PDL1–staining cells (tumor cells, lymphocytes, macrophages) relative to all viable tumor cells in the tumor sample; only triple negative tumors with a positive score (superior to 10) are candidates to immunotherapy in combination with chemotherapy in the metastatic setting, according to KEYNOTE 355 clinical trial (88).

Tissue samples presented a median TMB of 4.77 mut/mB (0.95–1,250.14). The patient with a higher TMB had a TMB of 1,250.14 mut/mB. A pCR was not achieved in this case. 304 different mutations were detected in this tumor sample, including a polymerase gene mutation.

Analyzing the patients who achieved a pCR, the median TMB was 4.75 mut/mB (2.85–95), as described in figure number 8; lower than in patients who did not achieve a pCR, 5.71 mut/mB (0.95–1,250.14). A statistically significant correlation between TMB values and pCR was not found ($p > 0.05$). This result is probably related to sample size and heterogeneity.

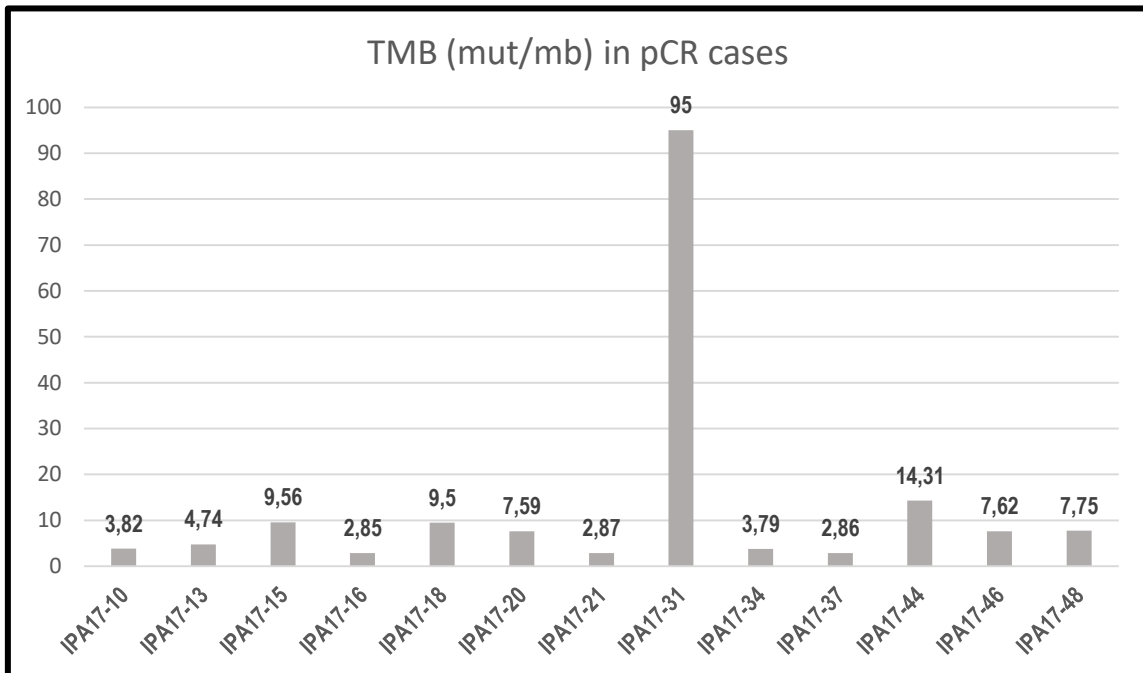


Figure 8: TMB in patients who achieved a pCR (mut/mb).

Taking into consideration 3 groups of patients, classifying the high TMB as a value higher than 20 mutations/mb, intermediate as 5-19 mutations/mb and low-TMB as a value lower than 5 mutation/mb, 4 tumors had high values of TMB (figure 9).

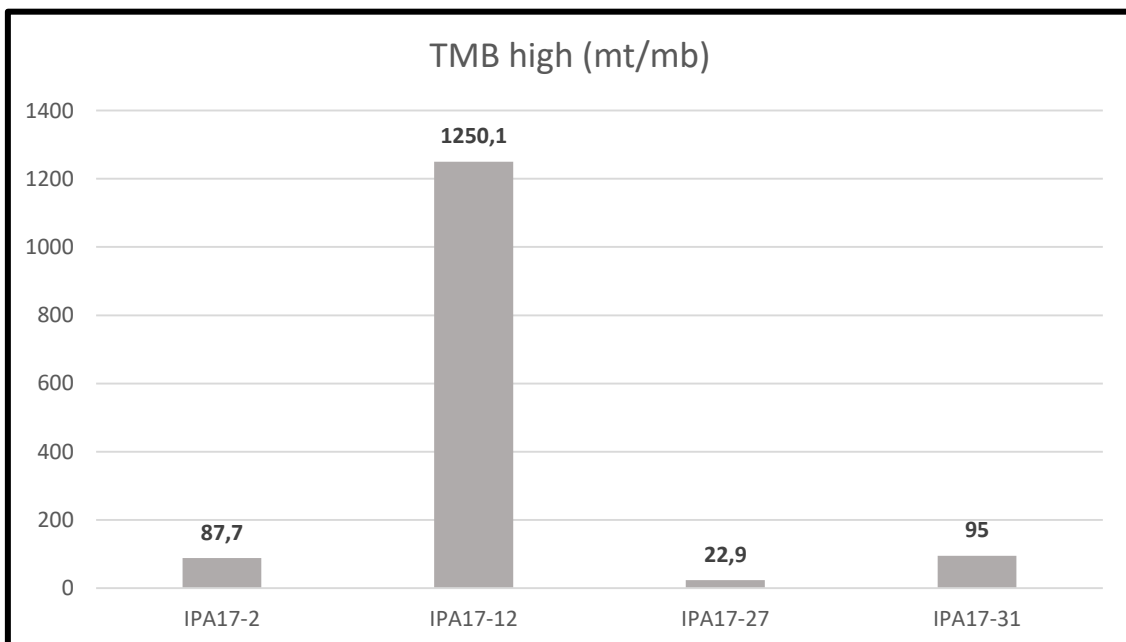


Figure 9: TMB high cases.

These 4 tumors had *BRCA1* and *BRCA 2* mutations detected in common, suggesting the importance of DNA damage repair proteins in maintaining DNA integrity and immune reaction in breast cancer patients. Three of them, had a DNA polymerase gene (*POLE*), which may, in theory justify the high mutation number in these samples.

Nevertheless, these results are important once they can help in the identification of the patients who might benefit from immunotherapy in the future, once that the use of these drugs is approved by FDA in TMB high cases.

MSI ANALYSIS

MSI is caused by defective DNA mismatch repair genes and is characterized by a decrease or increase in repeated nucleotide sequences, which can lead to evasion of apoptosis, development of malignant mutations, and tumorigenesis (89).

It can be distinguished into three types: high microsatellite instability (MSI-H), low microsatellite instability (MSI-L) and microsatellite stability (MSS). The prognosis of MSI -H patients with breast cancer is poor (90). MSI high breast cancer tumors are rare and this is more frequently detected in triple negative breast cancer (91). The clearest influencing factor is believed to be *BRCA1*. A study of Zhu et al. has shown that *BRCA1* expression function can affect the silencing mechanism of satellite DNA in chromatin, which will be damaged by *BRCA1* mutation, resulting in failure of normal cell replication process. At the same time, the mutation of *BRCA1*, which can make the repair function of DNA loss, will cause microsatellite instability and abnormal cells (92).

MSI-H tumors have been demonstrated as effective predictors of immunotherapy response, and have been approved by the FDA as biomarkers for the treatment of solid tumors with immune checkpoint inhibitors targeting PD-1, regardless of tumor origin, including breast cancer (93)

Tissue samples presented a median MSI score of 5,61 (0-18,45), as described in figure 10. The sample with a higher MSI score corresponded to a patient with luminal B disease who achieved pCR after NAT.

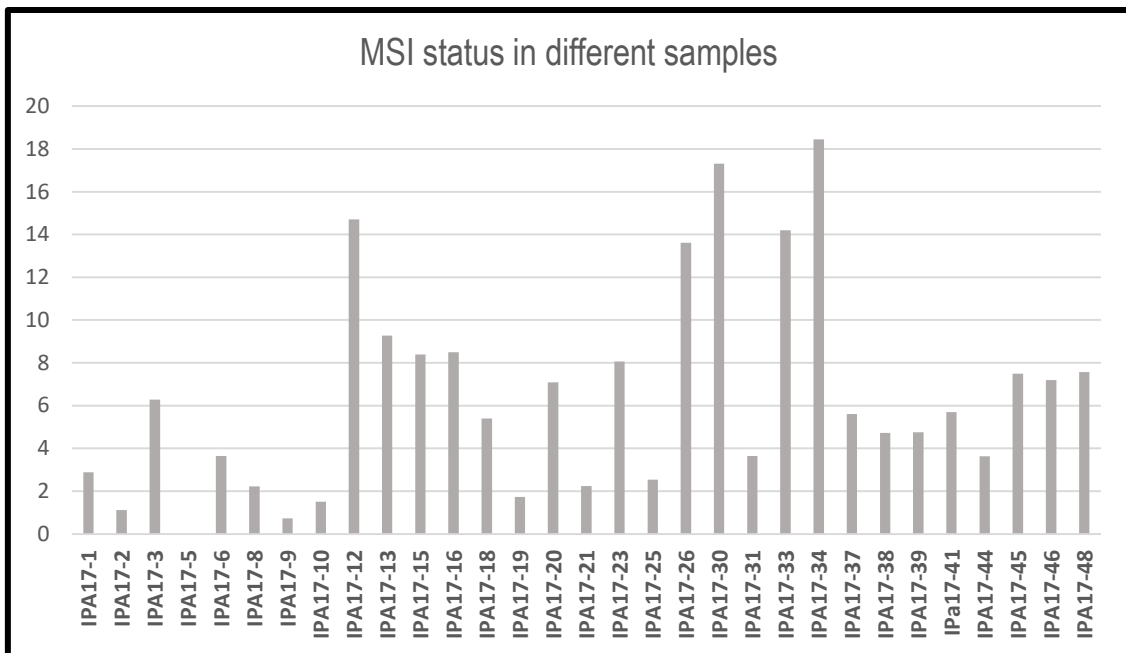


Figure 10: MSI status in different samples

In the subgroup of patients who achieved pCR, median MSI score was 7,07 (1,51-18,45), a value higher than in the general population of the study. Nevertheless, a correlation between MSI and pCR cannot be reached.

Once there is an opportunity of treatment approved by FDA for patients whose tumors express MSH-H, the determination of this value remains of clinical value. As said before, it is extremely rare in breast cancer patients and its clinical significance remains unclear. Therefore, clinical trials addressing this question are needed

The identification of such gene alterations described is extremely important in current days as it allows patients to be treated with targeted therapies available. In the same way, determination of TMB and MSI status of the tumor opens the opportunity of treatment with immunotherapy, a treatment that has revolutionized Oncology in recent years.

CHAPTER 4

IDENTIFICATION OF POTENTIALLY TARGETABLE MOLECULAR GENE ALTERATIONS

The content of this chapter includes full data published in article number 1, and data published in articles number 3,4 and 5.

INTRODUCTION

In a Precision Medicine Era in Oncology, several targeted therapies have been approved in the last years. In some malignancies such as lung cancer this molecular field is extensively developed and NGS techniques for analyzing tumor somatic mutations are crucial for the definition of the patient's treatment. Although in breast cancer patients this molecular evaluation is not so strongly established in clinical practice due to the lack of specific molecular panels and availability of much more therapeutic options regarding classic chemotherapy, there are some targeted therapies already approved, mainly in the metastatic setting, harboring gene alterations in *ERBB2*, *PIK3CA*, *BRCA 1* and *2* and *ERS1* genes, as described before. Studies in the neoadjuvant scenario are rare and they represent a real need in clinical practice once the curative intent of these treatment is the main goal. Some patients submitted to NAT achieve pCR but others do not. Although it is well established that some molecular intrinsic subtypes such as HER2 positive and triple negative breast cancer are good responders to NAT, there is not a predictive marker of pCR. Patients who do not achieve it are submitted to the same chemotherapy protocols but unfortunately some of them maintain large tumor lesions after NAT and rarely, some of them progress. Taking all these clinical difficulties faced by Medical Oncologists, there is a real need to investigate the molecular landscape of these tumors, mainly those who do not achieve pCR, in order to identify potentially gene alterations with targeted therapies approved for these patients during or after NAT.

SAMPLES GENOMIC PROFILE

The gene alterations mentioned above and considered with clinical potential impact were analyzed in pCR and non-pCR samples in order to identify potential treatment opportunities of treatment in the future. An Oncoprint figure was made to make it easier to visualize this correlation between gene alterations detected and pCR status (figure 11), as well as staging and immunohistochemical characterization.

As it can be observed in the Oncoprint figure (figure 12), one tumor had several somatic alterations identified.

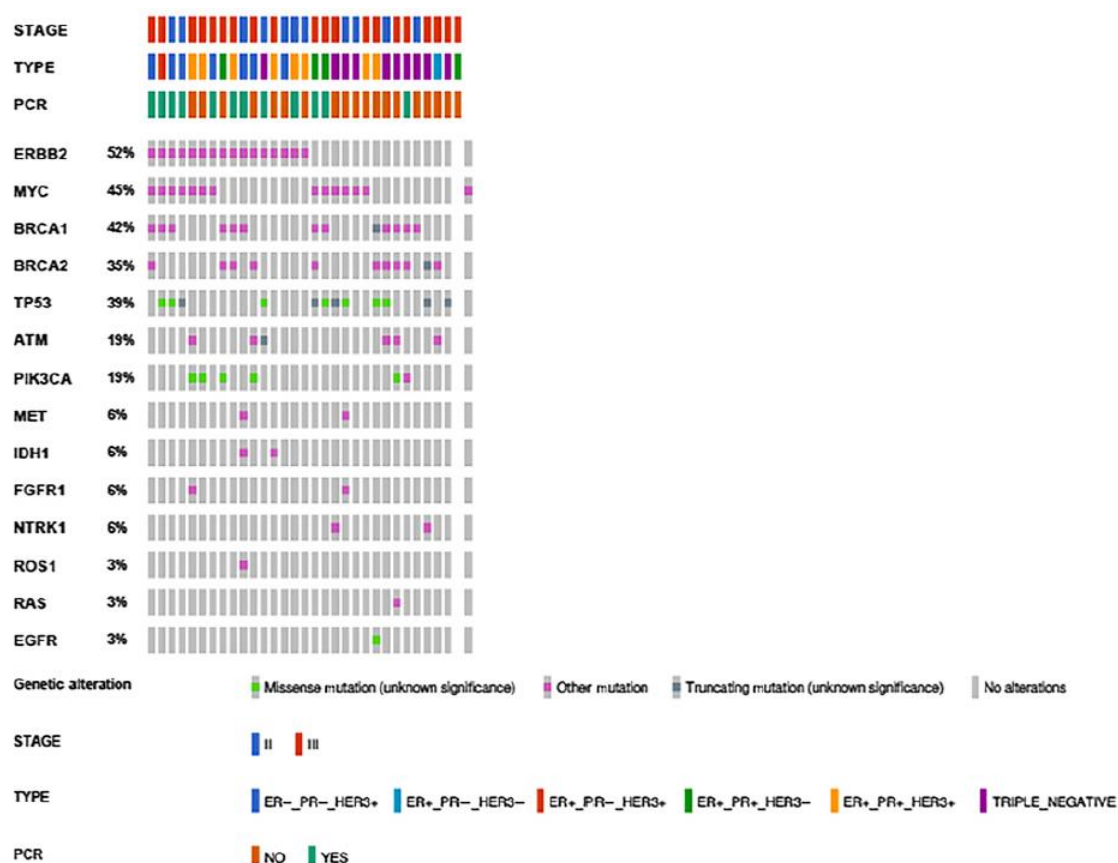


Figure 11: Oncoprint figure

pCR samples

ERBB2 gene

The *ERBB2* gene amplification was present in 8 of the 12 (67%) patients who achieved pCR. The determination of HER2 status is a gold standard in Medical Oncology, once the targeted therapies with anti-HER2 monoclonal antibodies such as trastuzumab pertuzumab and ADCs (table 12 and 13), have improved the response rate of the tumors in different settings of the disease. In the neoadjuvant setting, the use of trastuzumab and pertuzumab in combination with chemotherapy, improved pCR rates during NAT according to NEOSPHERE Trial. As mentioned before, according to the literature, patients who achieve a pCR after NAT have a better prognosis (11,13). The relevance of this determination is also critical in the adjuvant setting. In fact, in patients who achieve a pCR after NAT, according to international guidelines, patients must be submitted to adjuvant trastuzumab during 52 weeks, differently from patients who do not achieve pCR who might benefit from T-DM1 therapy (13). There are some clinical trials trying to address the impact of using

trastuzumab deruxtecan in neoadjuvant setting too, and studies with tyrosine kinase inhibitors in neoadjuvant setting were negative and these drugs approval relies on the metastatic setting.

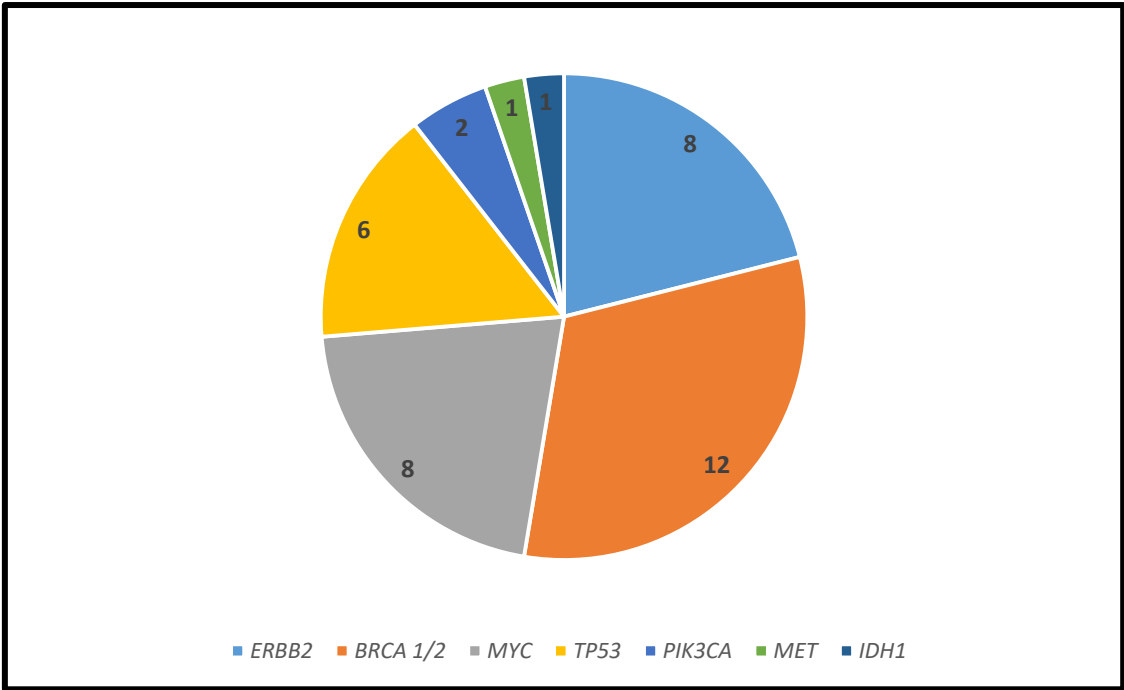


Figure 12: Genes alterations in pCR samples by frequency.

Monoclonal Antibody	Approved use
Trastuzumab	NAT (+ Pertuzumab+ChT) Adjuvancy (+ ChT or monotherapy) Palliative (+/- Pertuzumab; +/- ChT)
Pertuzumab	NAT/Adjuvancy (+ Trastuzumab+ ChT Palliative (+ Trastuzumab; + ChT in 1L)
Margetuximab	Palliative (+ChT ≥ 3L)

Table 12: Approved anti-HER2 monoclonal antibodies therapies (Adapted from Lopes Fernandes C., Silva D. and Mesquita A., *Novel HER2 targeted therapies in breast cancer*, Cancers 16, 87, 2024). 1L: first line therapy 3L: third line therapy.

ADC	Approved use
T-DM1	Adjuvancy HER2 positive early-stage Palliative HER2 positive ≥2L
Trastuzumab deruxtecan	Palliative HER2 positive ≥2L Palliative HER2 low

Table 13: Approved anti-HER2 ADCs in breast cancer (Adapted from Lopes Fernandes C., Silva D. and Mesquita A., *Novel HER2 targeted therapies in breast cancer*, Cancers 16, 87, 2024). 2L: second line therapy.

BRCA 1 and or BRCA2 genes

BRCA 1 and or *BRCA2* gene alterations were detected in 12 tumors. Most of the gene alterations were LOH. Clinical trials addressing the impact of this gene alterations are evaluating the use of targeted therapies already approved in patients with *BRCA1* or 2 germline mutations such as *PARP* inhibitors (94,95, 96,97,98). In the neoadjuvant setting there are several clinical trials such as GeparOla described before, trying to address the benefit of using these agents, as described before.

Therefore, using an NGS panel which allows us to obtain these results may be useful for the identification of the patients who might benefit from these therapies in the future.

MYC gene

Eight pCR tissue samples had a *MYC* gene amplification detected. *MYC* plays multiple roles in breast cancer development and progression. *MYC* amplification appears to be a strong prognostic marker of early recurrence in node negative breast cancer patients (99). Therefore, it is important to define amplification threshold in studies evaluating *MYC* amplification in breast cancer.

TP53 gene

Six samples had a *TP53* gene alteration detected. Median mutation allele frequency in patients who achieved pCR was 44,4 (2,89-73,81), higher than in those who did not. Most of these patients had triple negative disease. *TP53* is a suppressive tumor gene and its somatic mutation is one the most frequent in breast cancer. *TP53* mutation status has also been associated with response to breast cancer treatments and therefore its identification might be useful for the evaluation of probability of achieving pCR as well as prognosis of the disease and definition of a correct follow-up.

PIK3CA gene

Two patients of this cohort had a *PIK3CA* gene alterations. *PIK3CA* mutations are common activating mutations associated with breast cancer are potent predictive markers for response to *PIK3CA* inhibitors (14). As explained before, according to SOLAR 1 trial, alpelisib has showed benefit in terms of progression free survival in patients with stage IV breast cancer (14).

Although it is difficult to establish a relationship between the presence of this mutation and probability of achieving pCR (due to sample size and the heterogeneity of several mutations found in each sample), the availability of a therapeutic targeted for this gene alteration may underline the need for some clinical trials in these patients.

MET gene

MET gene amplification was identified in one sample. As it represents a predictor of poor outcome of the disease and in some cancers, *MET* inhibitors are already approved in clinical practice (100), although not yet approved in breast cancer and the gene alteration needed to use these drugs is not the one identified in this scenario it is important to identify these gene alterations which may be useful in the future.

IDH1 gene

IDH1 gene had alterations in 1 tissue sample. Targeted drugs have been developed for these mutations and are being studied (101). Once again, these drugs are not approved in case of gene CNV, nor in breast cancer.

Non pCR samples

ERBB2 gene

Seven patients had HER2 positive disease. Although the use of anti-HER2 targeted therapies improved the response of these tumors, some of them continue not to respond to chemotherapy protocols and therefore not achieve a pCR. This determination is important once that there are targeted therapies available in cases of patients not achieving pCR. Recently, KATHERINE trial showed that these patients benefit from complementary treatment with trastuzumab emtansine (T-DM1) instead of only trastuzumab (102). Therefore, this determination is crucial in clinical practice for the correct treatment of the patients.

BRCA 1 and/or 2 genes

BRCA 1 and/or *2* gene alterations were identified in 7 samples (figure 12). Although most of these gene alterations detected corresponded to LOH, in two samples SNV truncating mutations were identified, one case of triple negative breast cancer and the other HER2 positive disease. The identification of these mutations in breast cancer patients may be useful in the future for clinical practice, allowing the selection of patients who might benefit from a *PARP* inhibitors in case of metastization. It is also logical to think that patients whose tissue samples carry this gene alteration and who do not achieve a pCR, might benefit from a complementary treatment with these agents in the adjuvant setting or during the neoadjuvant treatment. Taking this into considerations clinical trials are needed in this scenario *BRCA* somatic mutations. In fact, in case of germline mutation, the use of olaparib is already approved after neoadjuvant treatment in high risk patients according

to OlympiA trial which showed that among patients with high-risk, HER2-negative early breast cancer and germline *BRCA1* or *BRCA2* pathogenic or likely pathogenic variants, adjuvant olaparib after completion of local treatment and neoadjuvant or adjuvant chemotherapy was associated with significantly longer survival free of invasive or distant disease than was placebo (103).

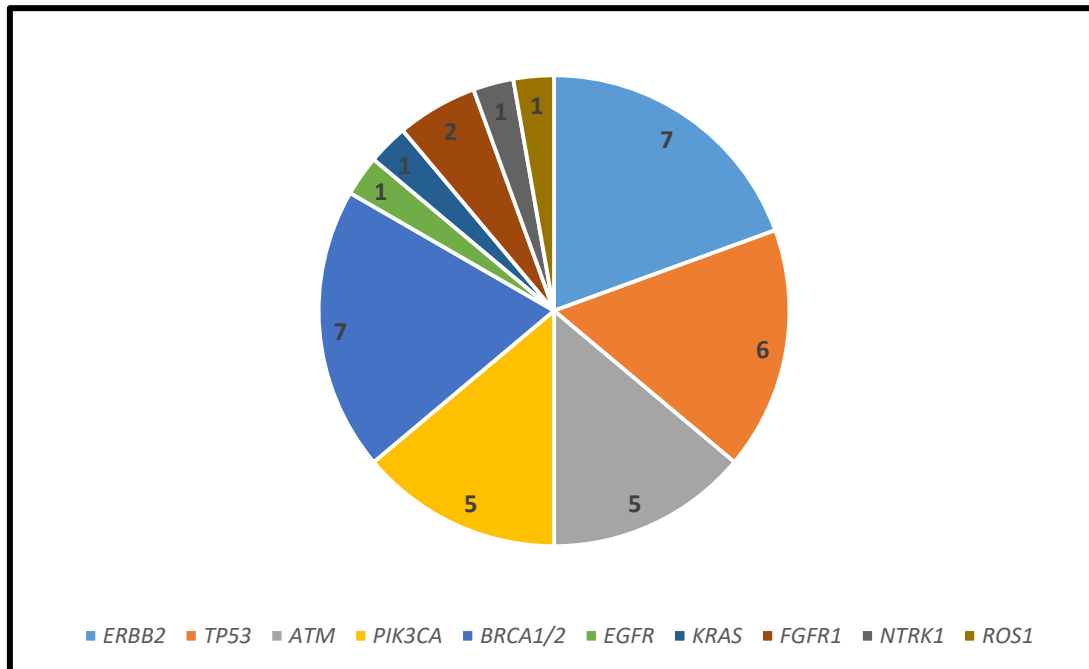


Figure 12: genes alterations in non-pCR samples by frequency

TP53 gene

Six tumors of this cohort carried a *TP53* gene alteration. Most of these patients had triple negative disease. Once there is a correlation described between the presence of this mutation in triple negative breast cancer and worse outcome, this information may be useful to chemotherapy protocols intensification or even complementary systemic treatment after NAT. The follow up of these patients might also be more intense once the prognosis is reserved. Once again, clinical trials are needed.

ATM gene

Five tissue samples carried an *ATM* gene alteration, most of them were LOH. These tumors may be more sensitive to treatments such as platinum agents and *PARP* inhibitors. Therefore, it may be import to identify them for the correct treatment selection, although in the current days the approval for using these drugs is only in case of *BRCA 1 or 2* germline mutation.

PIK3CA gene

Five patients carried a *PIK3CA* gene alteration. As described before, in these patients the results are interesting once there is a molecular targeted therapy available. Therefore, it is possible that in the future these cohort of patients, might benefit from supplementary treatment with these agents during NAT or as an adjuvant treatment. Clinical trials addressing this question may be interesting and open a new treatment opportunity for these patients.

EGFR gene

In one patient *EGFR* mutations was detected. As *EGFR* has a targeted therapy approved in some malignancies, some clinical trials have been conducted in breast cancer, especially in triple negative breast cancer, with disappointing results. Nevertheless, anti-*EGFR* therapies available are not approved in breast cancer scenario nor in this specific mutation, although a SNV hotspot mutation (p.Arg451Cys) was detected in this sample.

KRAS gene

A *KRAS* CNV was detected in one sample. This patient had a triple negative disease. In patients with metastatic colon cancer determination of *RAS* status is used to identify those who might benefit from monoclonal antibodies therapy. Taking this into consideration, its determination in breast cancer patients may play an important role in a near future.

FGFR1 gene

Two samples had a *FGFR1* gene alteration detected (CNV). *FGFR* inhibitors would be useful as anticancer agents in breast cancer patients as they are already approved in other malignancies (104). Nevertheless, the approval for these targeted therapies is not in case of CNV detection.

NTRK1 gene

The *NTRK1* gene was also identified. Trk inhibitors are being tested in several clinical trials (105). However, TrkA protein expression in a large clinically relevant cohort of breast tumors, as well as its overall clinical significance remain unclear. Two samples had a determination of CNV in *NTRK1* gene, not a gene alteration sufficient to allow a targeted therapy as gene fusion allows.

Therefore, the most frequent gene alterations detected in pCR samples were *ERBB2* gene alterations, followed by *BRCA 1* and *2*, *MYC*, *TP53*, *PIK3CA*, and *MET* alterations.

In the subgroup of patients who did not achieve a pCR, gene alterations in *ERBB2* were also the most frequent, followed by *BRCA*, *TP53*, *ATM*, *PIK3CA* gene alterations. Rare gene alterations such as in *FGFR1*, *NTRK1*, *KRAS* and *ROS 1* were also identified (figures 11 and 12).

The identification of these tumor somatic gene alterations particularly in non pCR samples, mainly if there is a truly SNV gene alteration, might be of extreme importance once there are targeted therapies available harboring these genes. In fact, some of the mutations identified such as in *ERBB2*, *PIK3CA* and *BRCA1* and 2 genes, have already targeted therapies approved and therefore their identification opens the possibility of using these therapies. Although some gene alterations in some genes such as in *TP53* do not have a targeted therapy available, it is known that tumors harboring this mutation generally have worse prognosis; therefore, chemotherapy intensification during or after NAT as well as a redefined follow up of these patients might be considered. On the other hand, it is also described that the presence of some gene mutations such as in *ATM* genes, related to homologous recombination pathway are more likely to respond to chemotherapy based on platinum agents as well as *PARP* inhibitors, making their identification important for clinical practice.

CHAPTER 5

CONCLUSIONS AND ONGOING WORK/FUTURE DIRECTIONS

CONCLUSIONS

This thesis showcases the significance of employing comprehensive genomic testing in breast cancer cases, primarily due to the scarcity of specific target assays for this type of cancer. Several somatic mutations were identified in each breast cancer tumor samples, important for clinical practice, such as *ERBB2*, *BRCA 1 and 2*, *PIK3CA*, *TP53*, *MYC*, *MET*, *EGFR*, *ROS1*, *FGFR*, *IDH*, *PALB2*, *CHEK 1 and 2*, *RAD51* and *MSH2 and 6* genes.

The detection of these somatic mutations, coupled with the availability of targeted therapies, holds promise as a potential therapeutic avenue to enhance tumor response rates during NAT, or as a complementary treatment following surgery in order to obtain better outcomes for the patients. Some of the mutations identified such as in *ERBB2*, *PIK3CA* and *BRCA1 and 2* genes, have already targeted therapies approved in breast cancer and therefore the study opens the possibility of using these therapies in this scenario once clinical trials are approved.

When focusing on patients who did not achieve pCR after NAT, the most common gene alterations identified were in *ERBB2* gene (due to the high incidence of HER2 positive tumors candidates to NAT), *BRCA 1 and 2*, *TP53*, *ATM*, *PIK3CA* genes; rare gene alterations mainly CNVs in *FGFR1*, *NTRK1*, *ROS 1*, *KRAS* were also identified as well as *EGFR* gene alterations. Although some gene alterations such as SNV truncating mutations in *TP53* do not have a targeted therapy available, it is known that tumors harboring this mutation generally have worse prognosis; therefore, chemotherapy intensification during or after NAT as well as a redefined follow up of these patients might be considered. On the other hand, it is also described that the presence of some gene mutations such as in *ATM* gene, related to homologous recombination pathway are more likely to respond to chemotherapy based on platins as well as *PARP* inhibitors.

When analyzing breast cancer tumors molecular intrinsic subgroups, it was possible to observe that *BRCA* and *TP53* gene alterations were the most frequently detected in triple negative breast cancers; in HER2 positive tumors, besides *ERBB2* gene amplifications, *TP53* and *MYC* gene alterations were the most frequent; Luminal B HER2 negative tumors rarely presented with gene alterations. It was not possible to establish a correlation between gene alterations and probability of achieving pCR due to sample size and heterogeneity of the alterations detected.

The NGS panel employed in this study also allowed for the determination TMB. We found that patients who achieved a pCR had lower values of TMB than those who did not. Nevertheless, it was not possible to establish a statistically significant correlation between TMB and pCR, probably due to sample size. Nevertheless, this evaluation in non-pCR samples could serve as a valuable criterion for selecting patients suitable for immunotherapy, once that these drugs are

approved in TMB high tumors by FDA. It could be potentially used as a complementary marker to select these patients.

It was also possible to determine MSI in the samples. Although MSI high determination is extremely rare in breast cancer, it might be of clinical value once immunotherapy is approved by FDA in these patients. Therefore, it may represent another opportunity for the patients to be submitted to these drugs. Further exploration through clinical trials is imperative to investigate these prospects and provide a robust implementation of these tools in clinical practice.

ONGOING WORK AND FUTURE DIRECTIONS

In breast cancer, approximately 30% of patients treated with NAT achieve pCR. Analysis of gene expression changes in ctDNA of sequential patient samples collected before and during treatment may be a promising way to analyze the molecular changes that occur during treatment in real-time and in a less invasive way. Comparing samples from patients who have a complete response to neoadjuvant treatment to samples from those who do not have it can provide a better understanding of the resistance mechanisms of the tumor, allowing for the definition of a better therapy strategy for the patients. Nevertheless, detection of ctDNA after NAT has been challenging in patients, even when the residual disease is observed in the surgery. Recent studies have found that ctDNA becomes undetectable in more than 90% of patients during NAT (106). Nevertheless, no correlation has been established between pCR and ctDNA. A recent study showed that after completion of NAT, ctDNA concentrations were lower in patients who achieved pCR compared to patients with residual disease. Further, patients with pCR showed a larger decrease in ctDNA concentrations during NAT (107). Previously, some studies already suggested that during NAT, ctDNA levels decreased and minimal residual disease of the tumor was undetectable after the surgery. A slow decrease of ctDNA levels during NAT was associated with poorer survival (108). A recent study from Radovich, M. et al. showed that detection of ctDNA in patients with early-stage triple negative breast cancer after NAT was independently associated with disease recurrence. The authors used the samples of a cohort of 196 patients with locally advanced breast cancer. At 24 months, disease-free survival probability was 56% for ctDNA-positive patients compared with 81% for ctDNA-negative patients (109).

Liquid biopsy is a promising option for detecting driver somatic mutations, and the low ctDNA levels found in these early-stages represent a challenge for sequencing techniques. More studies are needed in this specific clinical scenario to overcome this difficulty and to achieve a prognostic

molecular tool for these patients. This may allow us, in the future, to optimize the clinical follow-up of these patients and predict who are more likely to have recurrence of the disease.

Taking all of this into consideration, we collected blood samples of the patients included in this protocol. Blood samples were collected before starting NAT, immediately before each chemotherapy cycle, at the end of NAT and after surgery. Samples from all the 67 patients were collected.

A huge biologic database, with a total of more than 600 blood samples (at least 9 samples per patient) were collected and are stored at the laboratory. The team intends to analyze these samples using NGS in order to proceed to the molecular characterization of them.

Taking all this into consideration, we have established as **future directions** of this work:

1. Evaluate if liquid biopsy is representative of pCR to neoadjuvant chemotherapy in patients with locally advanced breast cancer (samples collected and data to be analyzed in the future);
2. Molecular characterization of liquid biopsies collected taking as a starting point the molecular alterations detected in tissue samples already analyzed and described in this work;
3. Implementation of a NGS panel specific for each patient, taking into consideration the tissue gene alterations detected and described in this work.

REFERENCES

Besides the publication list of the PhD candidate:

1. Ferlay J, et al. *Cancer incidence and mortality worldwide: sources, methods and major patterns in GLOBOCAN 2012*. International journal of cancer 136: E359386. 2, 2005.
2. Senkus E, et al. *Primary breast cancer: ESMO Clinical Practice Guidelines for diagnosis, treatment and follow-up*. Annals of oncology 26: v8-v30., 2005.
3. Govindan R. et al. *Principles & Practice of Oncology Review*. Lippincott Williams & Wilkins. 7, 2007.
4. Rojas K, Stuckey A. *Breast Cancer Epidemiology and Risk Factors*. Clinical obstetrics and gynecology 59: 651-672. 8, 2016.
5. Amin MB, et al., *AJCC Cancer Staging Manual* (8th edition), 2019.
6. Sunil R. Lakhani, *International Agency for Research on Cancer*. WHO Classification of Tumors of the Breast, 2019.
7. Yersal O, Barutca S, *Biological subtypes of breast cancer: Prognostic and therapeutic implications*. World J Clin Oncol. 10;5(3):412-2, 2014.
8. Cardoso F et al, *Early breast cancer: ESMO Clinical Practice Guidelines for diagnosis, treatment and follow-up*, Annals of Oncology 30: 1194–1220, 2019.
9. Dai X, Xiang L, Li T, Bai Z. *Cancer Hallmarks, Biomarkers and Breast Cancer Molecular Subtypes*. Journal of Cancer 7: 1281-1294, 2016.
10. Verma A, Kaur J, Mehta K. *Molecular oncology update: Breast cancer gene expression profiling*. Asian Journal of Oncology 1: 65, 2016.
11. *NCCN Clinical Practice Guidelines in Oncology*, National Cancer Institute, version 3.2023, Dec 2023.
12. Swan S. et al, *Pertuzumab, trastuzumab and docetaxel in HER2 positive metastatic breast cancer*, N Engl J Med, 372:724-734, 2015.
13. Jacobs AT et al, *Targeted therapy for breast cancer: an overview of drug classes and outcomes*. Biochemical Pharmacology 204115209, 2022.
14. André F et al, *Alpelisib for PIK3CA-Mutated, Hormone Receptor-Positive Advanced Breast Cancer*, N Engl J Med 16, 380(20):1929-1940, 2019.
15. Bidard FC. Et al, *Elacestrant versus endocrine therapy for estrogen receptor positive, HER2 negative advanced breast cancer: results from randomized phase II ESMERALD trial*, J Clin Oncol, 40(28):3246-3256, 2022.
16. MD Robson et al, *Olaparib for Metastatic Breast Cancer in Patients with a Germline BRCA Mutation*. N Engl J Med, 377:523-533, 2017.
17. Coleman, R. L. et al. *Rucaparib maintenance treatment for recurrent ovarian carcinoma after response to platinum therapy (ARIEL3): a randomised, double-blind, placebo-controlled, phase 3 trial*. Lancet 390, 1949–1961, 2017.
18. Bell, D. et al. *Integrated genomic analyses of ovarian carcinoma*. Nature 474, 609–615, 2011.
19. Bono G. et al, *Olaparib for metastatic castration resistance prostate cancer*, N Engl J Med 2020; 382:2091, 2102.
20. Crowley E et al, *Liquid biopsy: monitoring cancer genetics in the blood*. Nat Rev Clin Oncol., 10:472-484, 2013.
21. Arruda L., Caldas C, *Cell free circulating tumor DNA as a liquid biopsy in breast cancer*. Molecular Oncology 10 464-474, 2016.
22. Lone N. et al, *Liquid biopsy: a step closer to transform diagnosis, prognosis and future of cancer treatments*, Molecular Cancer volume 21, Article number: 79, 2022.

23. Alimirzage S. et al, *Liquid biopsy in breast cancer: A comprehensive review*. Clinical Genetics, 95(6), 643-660, 2009.
24. 100. Kaisaki, P.J. et al. *Targeted next-generation sequencing of plasma DNA from cancer patients: Factors influencing consistency with tumor DNA and prospective investigation of its utility for diagnosis*. PLoS ONE, 2016.
25. Chae, Y.K. et al, *Concordance of genomic alterations by next-generation sequencing in tumor tissue versus circulating tumor DNA in breast cancer*. Mol. Cancer Ther. 16, 1412–1420, 2017.
26. Lim, M. et al, *Liquid Biopsy in Lung Cancer: Clinical Applications of Circulating Biomarkers (CTCs and ctDNA)*. Micromachines 2018.
27. Spence, T. et al, *Clinical implementation of circulating tumour DNA testing for EGFR T790M for detection of treatment resistance in non-small cell lung cancer*. J. Clin. Pathol, 2020.
28. Duffy, M.J. et al, *CA 15-3: Uses and limitation as a biomarker for breast cancer*. Clin. Chim. Acta, 411, 1869–1874, 2010.
29. Lee, Y. et al, *Correlation between progression-free survival, tumor burden, and circulating tumor DNA in the initial diagnosis of advanced-stage EGFR-mutated non-small cell lung cancer*. Thorac. Cancer, 9, 1104–1110, 2018.
30. Dawson, S.-J. et al. *Analysis of Circulating Tumor DNA to Monitor Metastatic Breast Cancer*. N. Engl. J. Med. 2013, 368, Cancers, 2020.
31. Fritsch, C. et al. *Characterization of the novel and specific PI3K α inhibitor NVP-BYL719 and development of the patient stratification strategy for clinical trials*. Mol. Cancer Ther. 2014, 13, 1117–1129.
32. André, F. et al. *Apelisis for PIK3CA-Mutated, Hormone Receptor–Positive Advanced Breast Cancer*. N. Engl. J. Med., 380, 1929–1940, 2019.
33. Vidula, N. et al. Abstract PD1-13: Somatic BRCA mutation detection by circulating tumor DNA analysis in patients with metastatic breast cancer: Incidence and association with tumor genotyping results, germline BRCA mutation status, and clinical outcomes. Cancer Res., 78, PD1-13. 66, 2018.
34. Condorelli, R.; et al. *Polyclonal RB1 mutations and acquired resistance to CDK 4/6 inhibitors in patients with metastatic breast cancer*. Ann. Oncol., 29, 640–645, 2018.
35. Abbosh, C. et al, *Circulating tumor DNA analyses reveal novel resistance mechanisms to CDK inhibition in metastatic breast cancer*. Ann. Oncol., 28, 535–537, 2018.
36. Turner CN et al, *Circulating tumor DNA to direct therapy in advanced breast cancer (plasmamatch) trial*, Lancet Oncol, 21: 1296–308, 2020.
37. Zainal NC et al, *Landscape of somatic mutations in 560 breast cancer whole genome sequences*, Nature, 534(7605): 47–54, 2016.
38. Jameson JL, Longo DL. *Precision medicine personalized, problematic, and promising*. N Engl J Med, 372(23):2229–34, 2015.
39. Hodson R. *Precision medicine*. Nature, 537(7619):S49, 2016.
40. Huang Li et al, *A predictor of pathological complete response to neoadjuvant chemotherapy in triple negative breast cancer patients with DNA repair genes*, Ann Transl Med., 9(4): 301, 2021.
41. Cosgrove N. et al, *Predictive modelling of response to neoadjuvant therapy in HER2+ breast cancer*, npj Breast Cancer, 2023.
42. Hussen B et al. *The emerging roles of NGS in clinical oncology and personalized medicine*. Pathol Res Pract, 230:153760, 2020.
43. Desmedt C, Voet T, Sotiriou C, Campbell P. *Next generation sequencing in breast cancer: first take home messages*. Curr Opin Oncol., 24(6):597–604, 2012.
44. Xia W. et al, *Anti-tumor activity of GW572016: a dual tyrosine kinase inhibitor blocks EGF activation of EGFR/erbB2 and downstream Erk1/2 and AKT pathways*, Oncogene, volume 21, pages6255–6263, 2002.
45. Koepen HK et al, *Overexpression of HER2/neu in solid tumors: an immunohistochemical survey*, Histopathology 38(2):96-104, 2001.

46. Maxwell KN et al, *BRCA locus-specific loss of heterozygosity in germline BRCA1 and BRCA2 carriers*. Nat. Commun 8, 319, 2017.
47. Popova T. et al, *Ploidy and large-scale genomic instability consistently identify basal-like breast carcinomas with BRCA1/2 inactivation*. Cancer Res. 72, 5454–5462, 2012.
48. Cancer Genome Atlas Network. *Comprehensive molecular portraits of human breast tumours*. Nature 490:61–70, 2012.
49. Andrikoupolo A. et al, *P53 mutations determined by targeted NGS in breast cancer: a case-control study*. Oncotarget. 12:2206-2214, 2021.
50. Xu J et al, *MYC and Breast Cancer*, Genes & Cancer / vol 1 no 6 ,2010.
51. Foroughizadeh M et al, *Variation of ATM Gene Expression in Peripheral Blood Cells of Sporadic Breast Carcinoma in Iranian patients*, Avicenna J Med Biotech, 4(2): 95-10, 2012
52. Stucci L. et al, *The ATM gene in breast cancer: its relevance in clinical practice*. Genes (Basel), 12(5): 727, 2021.
53. Shimoi T et al, *PIK3CA mutation profiling in patients with breast cancer, using a highly sensitive detection system*, Cancer Science, 109: 2558–2566, 2018.
54. Elizabeth A. et al, *MET in human cancer: germline and somatic mutations*, Ann Transl Med 5(10):205, 2017.
55. Nakai K, Hung MC, Yamaguchi H. *A perspective on anti-EGFR therapies targeting triple-negative breast cancer*. Am J Cancer Res.6:1609–1623, 2016.
56. Kim A. Et al, *Mutations of the Epidermal Growth Factor Receptor Gene in Triple-Negative Breast Cancer*. J Breast Cancer., 20(2): 150–159, 2017.
57. Roskoski R. *ROS1 Protein-Tyrosine Kinase Inhibitors in the Treatment of ros1 Fusion Protein-Driven Non-Small Cell Lung Cancers*. Pharmacol. Res. 121:202–212, 2017.
58. D'Angelo A. Et al, *Focus on ROS1-Positive Non-Small Cell Lung Cancer (NSCLC): Crizotinib, Resistance Mechanisms and the Newer Generation of Targeted Therapies*. Cancers (Basel). 12(11): 3293, 2020.
59. Galie M. *RAS as Supporting Actor in Breast Cancer*. Front Oncol. 9: 1199, 2019.
60. Helsten T. et al. *The FGFR Landscape in Cancer: Analysis of 4,853 Tumors by Next-Generation Sequencing*. Clin. Cancer Res. 22:259–267, 2016.
61. Pezet S., McMahon S.B. *Neurotrophins: Mediators and modulators of pain*. Annu. Rev. Neurosci. 29:507–538, 2006.
62. Taishnavi A., Le A.T., Doebele R.C. *TRKing down an old oncogene in a new era of targeted therapy*. Cancer Discov. 5:25–34, 2015.
63. Gu J. et al, *Biological roles and therapeutic applications of IDH2 mutations in human cancer*. Front. Oncol., Sec. Cancer Metabolism, 2021.
64. Wu S. et al, *Molecular mechanisms of PALB2 function and its role in breast cancer management*, Front. Oncol., 28 February 2020
65. Yu-Hu Z. et al, *Germline PALB 2 mutations in cancers and its distinction from somatic mutations in breast cancer*. Front. Genet., Volume 11, 2020.
66. Lee JEA, et al. *Molecular analysis of PALB2-associated breast cancers*. J Pathol., 245: 53-60, 2018.
67. Carbognin L. et al, *Prognostic and predictive implications of PTEN in breast cancer: unfulfilled promises but intriguing perspectives*, Cancers (Basel), 11(9):1401 2019 Sep; 11(9): 1401, 2019.
68. Coughlin C.M. et al, *Approaches and Limitations of Phosphatidylinositol-3-Kinase Pathway Activation Status as a Predictive Biomarker in the Clinical Development of Targeted Therapy*. Breast Cancer Res. Treat. 124:1–11, 2010.
69. King C. et al, *LY2606368 causes replication catastrophe and antitumor effects through CHK1-dependent mechanisms*. Mol Cancer Ther. 14:2004–2013, 2015.
70. Wu Mai et al, *The clinical significance of CHEK 1 in breast cancer: high throughput data analysis and immunohistochemical study*, Int J Clin Exp Pathol. 12(1): 1–20, 2019.

71. Kim H et al, *Checkpoint Kinase 1 (CHK1) Functions as Both a Diagnostic Marker and a Regulator of Epithelial-to-Mesenchymal Transition (EMT) in Triple-Negative Breast Cancer*. *Curr. Issues Mol. Biol.* 44(12), 5848-5865, 2022.
72. Apostolou P., Paposotiri I, *Current perspectives on CHEK2 mutations in breast cancer*, *Breast Cancer* (Dove Med Press). 9: 331–335, 2017.
73. Li, N. et al. *Combined tumor sequencing and case/control analyses of RAD51C in breast cancer*. *J Natl Cancer Inst*, 2019.
74. Loveday, C. et al. *Germline mutations in RAD51D confer susceptibility to ovarian cancer*. *Nat. Genet* 43, 879–882 ,2011.
75. Ward A, Khanna KK, Wiegman AP. *Targeting homologous recombination, new pre-clinical and clinical therapeutic combinations inhibiting RAD51*. *Cancer Treat Rev*, 41: 35–45, 2015.
76. Sosinska-Mielcarek K. et al. *Immunohistochemical prediction of brain metastases in patients with advanced breast cancer: the role of Rad51*. *Breast* (Edinburgh, Scotland), 22: 1178–83, 2013.
77. Woditschka S. et al. *DNA double-strand break repair genes and oxidative damage in brain metastasis of breast cancer*. *J Natl Cancer Inst*, 2014.
78. Vasen HF, Watson P, Mecklin JP, Lynch HT. *New clinical criteria for hereditary nonpolyposis colorectal cancer (HNPCC, Lynch syndrome) proposed by the International Collaborative group on HNPCC*. *Gastroenterology*.
79. Schwartz C. et al, *Breast cancer in Lynch Syndrome patients: genomic and pathologic evidence in support of a potential causal relationship*, *Clin Cancer Res*. 28(2): 404–413, 2022.
80. Nigro C. et al, *RET in breast cancer: pathogenic implications and mechanisms of drug resistance*, *Cancer Drug Resist.* 2(4): 1136–1152, 2019.
81. Morandi A, et al. *GDNF-RET signaling in ER-positive breast cancers is a key determinant of response and resistance to aromatase inhibitors*. *Cancer Res*. 73(12):3783–95, 2013.
82. Pecar G. et al, *RET signaling in breast cancer therapeutic resistance and metastasis*, *Breast Cancer Research* volume 25, Article number: 26 ,2023).
83. Chalmers ZR, et al. *Analysis of 100,000 human cancer genomes reveals the landscape of tumor mutational burden*. *Genome Med*. 2017.
84. McGrail DJ et al, *High tumor mutation burden fails to predict immune checkpoint blockade response across all cancer types*, *Ann Oncol* 32(5):661-672, 2021.
85. Marabelle A.,et al, *Association of tumor mutational burden with outcomes in patients with advanced solid tumours treated with pembrolizumab: Prospective biomarker analysis of the multicohort, open-label, phase 2 KEYNOTE-158 study*. *Lancet Oncol.* 21:1353–1365, 2020.
86. Giannakis M.,et al, *Genomic Correlates of Immune-Cell Infiltrates in Colorectal Carcinoma*. *Cell Rep*.17:1206, 2016.
87. Barroso-Sousa R., et al. *Prevalence and mutational determinants of high tumor mutation burden in breast cancer*. *Ann. Oncol.* 31:387–394, 2020.
88. Cortes J. et al, *Pembrolizumab plus chemotherapy in advanced triple negative breast cancer*. *N Engl J*, 387:217-226, 2022.
89. Overman MJ et al, *Nivolumab in patients with metastatic DNA mismatch repair-deficient or microsatellite instability-high colorectal cancer (CheckMate 142): An open-label, multicentre, phase 2 study*. *Lancet Oncol*18 (9):1182–91, 2017.
90. Li K. et al, *Microsatellite instability: a review of what the oncologist should know*, *Cancer Cell Int* 20:16, 2020.
91. Ren XY et al, *Mismatch Repair Deficiency and Microsatellite Instability in Triple-Negative Breast Cancer: A Retrospective Study of 440 Patients*, *Front Oncol*,11:570623, 2021.
92. Zhu Q. et al, *BRCA1 tumor suppression occurs via heterochromatin-mediate silencing*. *Nature*. 477(7363):179–184, 2011.

93. Prasad V, Kaestner V, Mailankody S., *Cancer Drugs Approved Based on Biomarkers and Not Tumor Type-FDA Approval of Pembrolizumab for Mismatch Repair-Deficient Solid Cancers*. JAMA Oncol 4(2):157–8, 2018.
94. Tung N, Garber JE. *BRCA1/2 testing: therapeutic implications for breast cancer management*. Br J Cancer.119(2):141–52, 2018.
95. Byrski T. et al, *Results of a phase II open-label, non-randomized trial of cisplatin chemotherapy in patients with BRCA1-positive metastatic breast cancer*. Breast Cancer Res. 14(4):R110, 2012.
96. Robson MD et al. *Olaparib for metastatic breast cancer in patients with a germline BRCA mutation*. N Engl J Med.377(6):523–33, 2017.
- 97.Montemorano L, Lightfoot M, Bixel K. *Role of olaparib as maintenance treatment for ovarian cancer: the evidence to date*. Onco Targets Ther. 12:11497–506, 2019.
98. Maxwell KN, et al, *BRCA locus-specific loss of heterozygosity in germline BRCA1 and BRCA2 carriers*. Nat Commun.8(1):319, 2017.
99. Xu J, Chen Y, Olapade O. *MYC and breast cancer*. Genes Cancer.1(6):629–40, 2010.
100. ood G, Hockings H, Hilton D, Kermorgant S. *The role of MET in chemotherapy resistance*. Oncogene. 40(11):1927–41, 2021.
101. u J, Zhang R, Yang Z, Duan Z, Yin D, Zhou Y. *Biological roles and therapeutic applications of IDH2 mutations in human cancer*. Front Oncol. 11:644857, 2021.
102. Minchwitz GV. et al, *Trastuzumab Emtansine for Residual Invasive HER2-Positive Breast Cancer*, N Engl J Med 380:617-628, 2019.
103. Tut ANJ. Et al, *Adjuvant olaparib for patients with BRCA 1 or 2 mutated breast cancer*, N Engl J Med 384:2394-2405, 2021.
104. Kommalapati A. et al, *FGFR inhibitors in oncology: insight on the management of toxicities in clinical practice*. Cancers. 2021;13(12):2968.
105. Griffin N. et al, *The receptor Tyrosine kinase, TrKa is increased and targetable in Her2 positive breast cancer*. Biomolecules.10(9):1329, 2020.
106. Ma, M. et al, *Liquid biopsy- ctDNA detection with great potential and challenges*. Ann. Transl. 3, 235, 2005.
107. Contente-Cuomo et al, *Personalized circulating tumor DNA analysis to detect residual disease after neoadjuvant therapy in breast cancer*. Sci. Transl. Med.11, eaax7392, 2019.
108. Riva, F. et al, *Patient-Specific Circulating Tumor DNA Detection during Neoadjuvant Chemotherapy in Triple-Negative Breast Cancer*. Clin. Chem. 63, 691–699, 2017.
109. Radovich et al, *Association of Circulating Tumor DNA and Circulating Tumor Cells After Neoadjuvant Chemotherapy with Disease Recurrence in Patients With Triple-Negative Breast Cancer*, JAMA Oncol. S6(9): 1–6, 2020.

ANNEXES

(published articles will be present in the next pages)

1. Alexandra Mesquita, Anabela Ferro, José Carlos Machado, Fernando Schmitt. *Next generation sequencing of breast cancer in the neoadjuvant setting*. Pathobiology 1–7, 2023. DOI: 10.1159/000533810 .

Q1 JCR and Scimago

2. Alexandra Mesquita, Fernando Schmitt, José Carlos Machado, José Luís Costa. *Utility of Circulating tumor DNA in different clinical scenarios of breast cancer*. Cancers 12(12), 3797, 2020. DOI: 10.3390/cancers12123797

Q1 JCR and Scimago

3. Catarina Lopes Fernandes, Diogo Silva, Alexandra Mesquita *Novel HER2 targeted therapies in breast cancer*. Cancers 16, 87, 2024. DOI: org/10.3390/cancers16010087

Q1 JCR and Scimago

4. Diogo Silva, Alexandra Mesquita *Evolving Evidence for the Optimization of Neoadjuvant Therapy in Triple- Negative Breast Cancer* Breast Cancer (Auckl), 16:11782234221107580, 2022. DOI: 10.1177/11782234221107580.

5. Élia Cipriano, Alexandra Mesquita *Emerging Therapeutic Drugs in Metastatic Triple-Negative Breast Cancer* Breast Cancer (Auckl), 15:11782234211002491, 2021. DOI: 10.1177/11782234211002491.

Next-Generation Sequencing of Breast Cancer in the Neoadjuvant Setting

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Keywords

Breast cancer · Neoadjuvant chemotherapy · Next-generation sequencing

Abstract

Introduction: Many patients with locally advanced breast cancer are proposed to neoadjuvant chemotherapy (NAT) before surgery. Only some of them achieve a pathological complete response (pCR). The determination of gene somatic alterations using next-generation sequencing (NGS) in the non-pCR tumors is important, in order to identify potential opportunities of treatment for the patients, if targeted therapies are available. **Methods:** Breast cancer tissue samples of 31 patients, collected before NAT, were analyzed by NGS using the OncoPrint™ Comprehensive Assay Plus (OCA-Plus) panel. **Results:** Twelve patients achieved pCR after NAT. *ERBB2* gene alterations were the most frequent in this cohort of pCR patients, followed by *BRCA 1* and *2*, *MYC*, *TP53*, *PIK3CA*, and *MET* alterations. Tumors that did not achieve a pCR were mainly triple negative. In this subgroup some *BRCA 1* and *2* and *PIK3CA* gene alterations were identified, as well as *TP53* mutations. The NGS panel employed in this study also allowed for the determination of tumor mutation burden (TMB). **Conclusion:** This study showcases the significance of employing comprehensive

genomic testing in breast cancer cases, primarily due to the scarcity of specific target assays. The detection of somatic mutations, coupled with the availability of targeted therapies, holds promise as a potential therapeutic avenue to enhance tumor response rates during NAT, or as a complementary treatment following surgery. Moreover, evaluating the TMB in non-pCR samples could serve as a valuable criterion for selecting patients suitable for immunotherapy. Further exploration through clinical trials is imperative to investigate these prospects.

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Introduction

In recent years, specific molecular targeted treatments have been developed for many malignancies. This precision Medicine Era has revolutionized oncology treatments [1]. In breast cancer, there are a few somatic molecular alterations important in clinical practice [2], and their identification represents a new opportunity of cancer treatment.

Next-generation sequencing (NGS) techniques allow billions of DNA bases to be read at the same time [3, 4]. NGS may reveal the somatic genetic alterations in a cancer genome and provide information about potential treatments available. Therefore, studying the molecular

during chemotherapy; 2 of them maintained elevated values after NAT. Four patients presented with elevation of tumor marker CA125 before chemotherapy, and one case maintained elevated CA125.

Most of the 12 pCR samples had stage III disease ($n = 8$). Tumors were HER2 (3+) in 75% of the cases ($n = 8$); 2 patients had triple-negative disease. The remaining 2 patients had luminal B disease (positive hormone receptors and HER2 negative).

Tumors that not did achieve a pCR were mainly triple negative ($n = 8$) or positive for hormone receptors. Tumor markers remained positive after NAT in 2 patients.

Mutation Profile Generated by NGS

The NGS panel used in this protocol included gene alterations with an important impact in clinical practice. This is the largest panel with an extensive molecular information about the tumor available. Although it is not specific for breast cancer tumors, it provides the oncologists important information regarding potential targeted therapies approved. The number of gene alterations detected ranged from 6 to more than 300 in different tissue samples. The analysis will focus on the genes with a clinical impact already proved for patients: *ERBB2*, *PI3KCA*, *BRCA 1*, *BRCA 2*, *ATM*, *MYC*, *MET*, *NTRK1*, *FGFR1*, *ROS1*, *RAS*, and *EGFR*. The type of gene alterations detected is described in online supplementary Table 1 (for all online suppl. material, see <https://doi.org/10.1159/000533810>).

For an individual sample, it was possible to identify various clinical relevant gene alterations. Figure 1 (oncoprint figure) is made to visualize and correlate clinical features with the gene alterations found in each sample, as well as the histological response after NAT. It allows the visualization of each sample and to classify it according to TNM stage, immunohistochemical characteristics (ER, PR, HER2+; positive HER2 [3+], HER2-; negative HER2 [0, 1+ or 2+]); the result after NAT such as achieving pCR or not is also presented; the gene molecular changes are classified and the percentage of each gene alterations detected is also presented.

pCR Samples Mutation Profile

ERBB2 gene alterations were detected in 16 (52%) of this cohort. These tumors had a median value of gene copy numbers of 41.15 (7.46–43.6). This value was higher than the other tumors 10.62 (5.8–45.65) which did not achieve pCR. The *ERBB2* gene amplification was present in 8 of the 12 patients who achieved pCR, making it the most frequent gene alteration detected in this cohort. All

of the tumors with HER2 gene amplification were characterized as HER2 (3+) in immunochemistry. Six cases had HER2 low phenotype without *ERBB2* gene amplification: 2 of them had HER (2+) and 4 cases had HER2 (1+).

The determination of tumor HER2 status is a gold standard in Medical Oncology, once the targeted therapies with anti-HER2 monoclonal antibodies, have improved the response rate in the neoadjuvant setting [5]. According to the literature, patients who achieve a pCR after NAT have a better prognosis [6].

BRCA 1 and/or *BRCA 2* gene alterations were detected in 12 tumors. Most of the gene alterations were LOH. *BRCA1* and *BRCA2* genes are tumor-suppressors and they serve as key enzymes in the homologous recombination pathway, a mechanism for repairing DNA double-strand breaks [7].

High-response rates to platinum agents in women with *BRCA* germline metastatic breast cancer have been reported [8]. Targeted therapies such as *PARP* inhibitors [9, 10] are approved in the metastatic patients carrying a *BRCA* germline mutation as described in online supplementary Table 2.

LOH is also being studied as recent data suggests that it may also predict sensitivity to DNA-damaging agents [11]. Clinical trials are prospectively evaluating different patterns of LOH, trying to identify the benefit of *PARP* inhibitors in this scenario [11, 12]. Although in breast cancer these targeted drugs are only approved in germline mutated patients, in some cancers, such as ovarian cancer, *PARP* inhibitors are also approved in patients carrying a somatic mutation [13]. Therefore, using an NGS panel which allows us to obtain these results may be useful for the identification of the patients who might benefit from these therapies in the future.

Eight pCR tissue samples had a *MYC* gene amplification detected. *MYC* plays multiple roles in breast cancer development and progression. *MYC* amplification appears to be a strong prognostic marker of early recurrence in node negative breast cancer patients [14]. Therefore, it is important to define amplification threshold in studies evaluating *MYC* amplification in breast cancer.

Six samples (50%) had a *TP53* gene alteration detected. Median mutation allele frequency in patients who achieved pCR was 44.4 (2.89–73.81), higher than in those who did not. Most of these patients had triple-negative disease (50%). *TP53* is a suppressive tumor gene and its somatic mutation is one the most frequent in breast cancer. *TP53* mutation status has also been associated with response to breast cancer treatments [15].

drugs have been developed for these mutations and are being studied [20]. Once again, these drugs are not approved in case of gene CNV, nor in breast cancer.

No pCR Samples Mutation Profile

Seven patients had HER2 (3+) disease. Although the use of anti-HER2 targeted therapies improved the response of these tumors, some of them continue not to respond to chemotherapy protocols and therefore not achieve a pCR.

BRCA 1 and/or 2 gene alterations were identified in 2 samples. The identification of these mutations in breast cancer patients may be useful in the future for clinical practice, allowing the selection of patients who might benefit from a PARP inhibitor in case of metastatic disease. It is also logical to think that patients whose tissue samples carry this gene alteration and who do not achieve a pCR, might benefit from a complementary treatment with these agents in the adjuvant setting or during the neoadjuvant treatment. Taking this into considerations clinical trials are needed in this scenario *BRCA* somatic mutations.

Six tumors of this cohort carried a *TP53* gene alteration. Most of these patients had triple-negative disease. There is correlation described between the presence of this mutation in triple-negative breast cancer and worse outcome [21]. This information may be useful to chemotherapy protocols intensification or even complementary systemic treatment after NAT. Once again, clinical trials are needed.

Five tissue samples carried an *ATM* gene alteration, most of them were LOH. Numerous substrates involved in DNA repair are regulated by ATM protein [22–24]. These tumors may be more sensitive to treatments such as platinum agents and PARP inhibitors. Therefore, it may be important to study them.

Five patients carried a *PIK3CA* gene alteration. As described before, in these patients the results are interesting once there is a molecular-targeted therapy available. Therefore, it is possible that in the future, these cohorts of patients might benefit from supplementary treatment with these agents. Clinical trials addressing this question may be interesting.

In 1 patient, an *EGFR* mutation was detected. The *EGFR* protein is overexpressed in breast cancer, especially in triple-negative cases [25]. The tumor sample had a SNV hotspot mutation (COSM3266583; p.Arg451Cys). This patient had a HER2-positive (3+) disease with negative hormone receptors and did not achieve pCR after NAT.

As *EGFR* has a targeted therapy approved in some malignancies, some clinical trials have been conducted in breast cancer. Nevertheless, anti-EGFR therapies avail-

able are not approved in breast cancer scenario or in this specific mutation.

A *KRAS* CNV was detected in one sample. This patient had a triple-negative disease. Dysregulation of RAS function is largely associated with tumorigenesis; RAS might also confer resistance to therapies in breast cancer [26].

In patients with metastatic colon cancer determination of RAS status is used to identify those who might benefit from monoclonal antibodies therapy. Taking this into consideration, its determination in breast cancer patients may play an important role in a near future.

Two samples had a *FGFR1* gene alteration detected (CNV). Among *FGFR* alterations, gene amplification is the most common in breast cancer patients (66%) [27]. *FGFR* inhibitors would be useful as anticancer agents in breast cancer patients as they are already approved in other malignancies [28, 29]. Nevertheless, the approval for this targeted therapy is not in case of CNV detection.

The *NTRK1* gene plays an important role as an oncogenic gene [30]. Trk inhibitors are being tested in several clinical trials [31]. However, TrkA protein expression in a large clinically relevant cohort of breast tumors, as well as its overall clinical significance remains unclear.

Two samples had a determination of CNV in *NTRK1* gene, not a gene alteration sufficient to allow a targeted therapy as gene fusion allows. Both of the patients had triple-negative breast cancer disease and did not achieve a pCR after NAT.

Tumor Mutation Burden

TMB is defined as the total number of somatic mutations in a region of tumor genome and is associated with the immunogenicity of many different tumors [32].

Tissue samples presented a median TMB of 4.77 (0.95–1,250.14). The patient with a higher TMB had a TMB of 1,250.14 mb. A pCR was not achieved in this case. 304 different mutations were detected in this tumor sample, including a polymerase gene mutation.

Analyzing the patients who achieved a pCR, the median TMB was 4.75 (2.85–95), lower than in patients who did not achieve a pCR, 5.71 (0.95–1,250.14). A statistically significant correlation between TMB values and pCR was not found ($p > 0.05$). This result is probably related to sample size and heterogeneity.

Recently in a study from McGrail et al. [33], high values of TMB (tumors with ≥ 10 mutations/mb) were not correlated to response to immunotherapy. For cancers with a strong correlation between TMB status and T cell infiltration, patients with a high TMB had improved clinical outcome. In contrast, TMB status was not predictive of outcome in a second class of tumors, which

included breast cancer tumors. The authors concluded that more studies are needed to establish this correlation [33] for all solid tumors.

Discussion

This work demonstrates the value of comprehensive genomic testing in breast cancer due to the lack of specific target assays available for these patients. The genetic alterations identified in an early stage may provide insights on the biological behavior of these tumors and provide clinical insights for better manage these patients.

Therefore, the identification of somatic mutations with targeted therapies available in patients who do not achieve a pCR might be useful in the future as a therapeutic option after surgery, as well as in case of metastasis. *ERBB2* gene alterations are more common in the pCR subgroup of patients, which is probably explained by the use of anti-HER2 targeted therapies. We found a significant number of patients who did not achieve pCR whose tumors had *PIK3CA* and *BRCA* gene alterations. This information may be important once there are targeted therapies available for these patients approved in other scenarios, but that might be useful in this subgroup of patients in order to increase the probability of tumor response during NAT or as a complementary therapeutic after surgery. Clinical trials are needed in these scenarios. Determination of TMB, mainly in those tumors who do not achieve pCR can, in the future, be useful to select patients who may benefit from immunotherapy but once again, clinical trials are needed in this subset of patients.

References

- Jameson JL, Longo DL. Precision medicine: personalized, problematic, and promising. *N Engl J Med*. 2015 Jun 4;372(23):2229–34.
- Hodson R. Precision medicine. *Nature*. 2016 Sep 8;537(7619):549.
- Hussen B, Abdullah ST, Salih A, Sabir D, Siddiq K, Rasid MF, et al. The emerging roles of NGS in clinical oncology and personalized medicine. *Pathol Res Pract*. 2022 Feb;230:153760.
- Desmedt C, Voet T, Sotiriou C, Campbell P. Next generation sequencing in breast cancer: first take home messages. *Curr Opin Oncol*. 2012 Nov;24(6):597–604.
- Gianni L, Pienkowski P, Im YH, Roman L, Tseng LM, Liu MC, et al. Efficacy and safety of neoadjuvant pertuzumab, trastuzumab in women with locally advanced, inflammatory or early Her2 positive breast cancer (Neosphere), a randomized multicenter phase 2 trial. *Lancet Oncol*. 2012 Jan;13(1):25–32.
- Gass P, Lux MP, Rauh C, Hein A, Bani M, Fiessler C, et al. Prediction of pathological complete response and prognosis in patients with neoadjuvant treatment for triple negative breast cancer. *BMC Cancer*. 2018 Oct 29; 18(1):1051.
- Tung N, Garber JE. *BRCA1/2* testing: therapeutic implications for breast cancer management. *Br J Cancer*. 2018;119(2):141–52.
- Byrski T, Dent R, Bledharz P, Foszczynska-Kloda M, Gronwald J, Huzarski T, et al. Results of a phase II open-label, non-randomized trial of cisplatin chemotherapy in patients with *BRCA1*-positive metastatic breast cancer. *Breast Cancer Res*. 2012;14(4):R110.
- Robson MD, Im S, Senkus E, Xu B, Domchek S, Masuda N, et al. Olaparib for metastatic breast cancer in patients with a germline *BRCA* mutation. *N Engl J Med*. 2017;377(6): 523–33.
- Montemurro L, Lightfoot M, Bixel K. Role of olaparib as maintenance treatment for ovarian cancer: the evidence to date. *Onco Targets Ther*. 2019;12:1497–506.
- Maxwell KN, Wubbenhorst B, Wenz BM, De Sloover D, Pluta J, Emery L, et al. *BRCA* locus-specific loss of heterozygosity in germline *BRCA1* and *BRCA2* carriers. *Nat Commun*. 2017;8(1):319.
- Popova T, Manie E, Rieunier G, Caux-Moncoutier V, Tirapo C, Dubois T, et al. Ploidy and large-scale genomic instability consistently identify basal-like breast carcinomas with *BRCA1/2* inactivation. *Cancer Res*. 2012;72(21):5454–62.
- Dong F, Davinini PK, Howitt BE, Beck AH. A *BRCA1/2* mutational signature and survival in ovarian high-grade serous carcinoma. *Cancer Epidemiol Biomarkers Prev*. 2016; 25(11):1511–6.

Statement of Ethics

This study protocol was reviewed and approved by Ethics Committee of Local Health Unit of Matosinhos, Hospital Pedro Hispano, approval number 23/CE/JAS, on 8 March 2019. Written informed consent was obtained from participants to participate in the study.

Conflict of Interest Statement

All authors declare no conflict of interest.

Funding Sources

Reagents for laboratory use were partially supported by Thermo Fisher Scientific.

Author Contributions

Alexandra Sofia Amaral Mesquita is the Medical Oncologist of the patients recruited and responsible for clinical data assessment and article writing. Anabela Ferro was responsible for tumor samples processing and NGS sequencing. José Carlos Machado was responsible for article revision. Fernando Schmitt was responsible for article revision.

Data Availability Statement

All data generated or analyzed during this study are included in this article and its online supplementary material files. Further inquiries can be directed to the corresponding author.

- 14 Xu J, Chen Y, Olapade O. MYC and breast cancer. *Genes Cancer*. 2010 Jun;1(6):629–40.
- 15 Andrikopoulou A, Terpos E, Chatzidakou S, Apostolidou K, Ntanas-Stathopoulos I, Gavriatopoulou M, et al. TP53 mutations determined by targeted NGS in breast cancer: a case-control study. *Oncotarget*. 2021;12(21):2206–14.
- 16 Shimoi T, Hamada A, Yamagishi M, Hirai M, Yoshida M, Nishikawa T, et al. PIK3CA mutation profiling in patients with breast cancer, using a highly sensitive detection system. *Cancer Sci*. 2018;109(8):2558–66.
- 17 André F, Ciruelos E, Rubovszky G, Campone M, Loi S, Rugo HS, et al. Alpelisib for PIK3CA-mutated, hormone receptor-positive advanced breast cancer. *N Engl J Med*. 2019 May 16;380(20):1929–40.
- 18 Tovar EA, Gravel C. MET in human cancer: germline and somatic mutations. *Ann Transl Med*. 2017;5(10):205.
- 19 Wood G, Hockings H, Hilton D, Kermorgant S. The role of MET in chemotherapy resistance. *Oncogene*. 2021;40(11):1927–41.
- 20 Gu J, Zhang R, Yang Z, Duan Z, Yin D, Zhou Y. Biological roles and therapeutic applications of IDH2 mutations in human cancer. *Front Oncol*. 2021;11:644857.
- 21 Mitri Z, Abuhadra N, Goodyear SM, Hobbs E, Kaempfer A, Thompson AM, et al. Impact of TP53 mutations in triple negative breast cancer. *Npj Precis Oncol*. 2022;6(1):64.
- 22 Foroughizadeh M, Mozdaran H, Majidzadeh-A K, Kaviani A. Variation of ATM gene expression in peripheral blood cells of sporadic breast carcinomas in Iranian patients. *Avicenna J Med Biotechnol*. 2012 Apr-Jun; 4(2):95–101.
- 23 Stucci L, Interno V, Tucci M, Perrone M, Mannavola F, Palmirotta R, et al. The ATM gene in breast cancer: its relevance in clinical practice. *Genes*. 2021;12(5):727.
- 24 Haricharan S, Punturi N, Singh P, Holloway KR, Anurag M, Schmelz J, et al. Loss of MutL disrupts CHK2-dependent cell-cycle control through CDK4/6 to promote intrinsic endocrine therapy resistance in primary breast cancer. *Cancer Discov*. 2017;7(10):1168–83.
- 25 Nakai K, Hung MC, Yamaguchi H. A perspective on anti-EGFR therapies targeting triple-negative breast cancer. *Am J Cancer Res*. 2016;6(8):1609–23.
- 26 Galie M. RAS as supporting actor in breast cancer. *Front Oncol*. 2019;9:1199.
- 27 Helsten T, Elkin S, Arthur E, Tomson BN, Carter J, Kurzrock R. The FGFR landscape in cancer: analysis of 4,853 tumors by next-generation sequencing. *Clin Cancer Res*. 2016;22(1):259–67.
- 28 Kommalapati A, Tella S, Borad M, Javle M, Mahipal A. FGFR inhibitors in oncology: insight on the management of toxicities in clinical practice. *Cancers*. 2021;13(12):2968.
- 29 Miknyoczki SJ, Wan W, Chang H, Dobrzanski P, Ruggeri BA, Dionne CA, et al. The neurotrophin-trk receptor axes are critical for the growth and progression of human prostatic carcinoma and pancreatic ductal adenocarcinoma xenografts in nude mice. *Clin Cancer Res*. 2002;8(6):1924–31.
- 30 Griffin N, Marsland M, Roselli S, Oldmeadow C, Attia J, Walker MM, et al. The receptor Tyrosine kinase, TrkA is increased and targetable in Her2 positive breast cancer. *Biomolecules*. 2020;10(9):1329.
- 31 Lu J, Blakely CM, Barve M, et al. Entrectinib in NTRK fusion-positive (NTRK-fp) breast cancer: updated data from STARTRK-2. Presented at: 2022 ESMO Breast Cancer Congress; May 3–5, 2022. Berlin, Germany. Poster 173P.
- 32 Chalmers ZR, Connelly CE, Fabrizio D, Gay L, Ali SM, Ennis R, et al. Analysis of 100,000 human cancer genomes reveals the landscape of tumor mutational burden. *Genome Med*. 2017;9(1):34.
- 33 McGrail DJ, Filie PG, Rashid NU, Voorwerk L, Slagter M, Kok M, et al. High tumor mutation burden fails to predict immune checkpoint blockade response across all cancer types. *Ann Oncol*. 2021; 32(5):661–72.



Review

Utility of Circulating Tumor DNA in Different Clinical Scenarios of Breast Cancer

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Received: 3 November 2020; Accepted: 14 December 2020; Published: 16 December 2020



Simple Summary: This review is focused on the concept of a specific type of “liquid biopsy”, circulating cell-free tumor DNA (ctDNA). It explores the advantages and limitations of using this technique and the latest advances of using it in different clinical scenarios of breast cancer: early, metastatic, and locally advanced disease. It provides the latest advances in this area applied to clinical research and clinical practice, as well as the importance of the collaboration between clinicians and laboratory teams to fully grasp the potential of ctDNA in a precision medicine era.

Abstract: Breast cancer is a complex disease whose molecular mechanisms are not completely understood. Developing target therapies is a promising approach. Therefore, understanding the biological behavior of the tumor is a challenge. Tissue biopsy in the metastatic setting remains the standard method for diagnosis. Nevertheless, it has been associated with some disadvantages: It is an invasive procedure, it may not represent tumor heterogeneity, and it does not allow for treatment efficacy to be assessed or early recurrences to be detected. Analysis of circulating tumor DNA (ctDNA) may help to overcome this as it is a non-invasive method of monitoring the disease. In early-stage disease, it can detect early recurrences and monitor tumors’ genomic profiles, identifying the emergence of new genetic alterations which can be related to tumor-acquired resistance. In the metastatic setting, the analysis of ctDNA may also allow for the anticipation of clinical and radiological progression of the disease, selection of targeted therapies, and for a photogram of tumor heterogeneity to be provided. It may also detect disease progression earlier in locally advanced tumors submitted to neoadjuvant treatment, and identify minimal residual disease. ctDNA analysis may guide clinical decision-making in different scenarios, in a precision medicine era, once it acts as a repository of genetic tumor material, allowing for a comprehensive mutation profiling analysis. In this review, we focused on recent advances towards the implementation of ctDNA in a clinical routine for breast cancer.

Keywords: ctDNA; breast cancer; clinical utility; molecular biology; next generation sequencing

1. Introduction

Breast cancer is the most prevalent cancer in women worldwide [1]. Regarding cancer-related mortality, it is the second cause of death in developed regions [1]. The incidence has increased since the introduction of mammography screening, and continues to grow with the ageing of the population [2].

Tissue biopsy of the tumors remains the standard method for cancer diagnosis. Nevertheless, it has been associated with several disadvantages in metastatic and recurrent breast cancer cases: It may

not represent the tumor heterogeneity and it does not allow for the evaluation of treatment efficacy or detection of early recurrences or residual disease [3,4]. In addition, it cannot be used repeatedly due to the invasiveness of the procedure for the patient, cost, and time consumption [5], and so it cannot be used for monitoring the disease along time.

Therefore, new methodologies of studying the dynamic range of molecular alterations of the tumor have been developed. Recent findings in DNA sequencing and digital molecular techniques have supported the clinical use of circulating tumor DNA (ctDNA) as a “liquid biopsy” [6]. Liquid biopsies have emerged in a precision medicine era and they, already, play an important role in the decision making process in some cancers such as lung and colorectal cancer [7,8]. In breast cancer patients, clinical, histological, and immune-histochemical features of the tumor have guided the most appropriate treatment approaches for years. Breast cancer tumors are classified as luminal A, luminal B, luminal B Her2-positive, and triple-negative breast cancers according to the presence of estrogen and progesterone receptors, and HER2 high positivity or values of proliferation marker ki67 [9]. This classification helps to guide medical oncologists to choose the most appropriate treatment.

The heterogeneity of the breast cancer disease and variations between clinical and molecular classification was addressed by Dawson, S.J. et al. A classification based on molecular information on the genomic and transcriptomic landscapes of breast cancer was able to define 10 molecular clusters associated with different clinical outcomes. The integrated analysis of the somatic copy number aberrations (CNAs) and their effect on gene expression allowed novel molecular subgroups to be identified and, therefore, a better clarification of breast cancer heterogeneity [10].

In recent years, molecular characterization and identification of mutations of the tumors have gained more relevance in breast cancer patients, mainly in the metastatic disease, due to the development of targeted therapies.

2. ctDNA—The Concept

Circulating cell-free DNA (cfDNA) was firstly reported in 1948 in the blood of healthy people [11], and later in cancer patients [12].

Cell-free DNA is fragmented DNA that is found in the non-cellular blood components of healthy individuals. Among tumor patients, ctDNA is 150–200 base pair fragments that are released by tumor cells into the blood representing a small fraction of the total cfDNA. Therefore, ctDNA carries tumor-specific mutations that are similar to the tumor itself [8,13]. It acts as a liquid biopsy once it consists of only a blood collection which can identify similar genetic alterations of the tumor itself. The tumor evolves, and many clinical decisions are made based on the tissue biopsy at diagnosis. The analysis of ctDNA opens the possibility to study the tumor in every time points of its progression, making it possible to obtain more information for clinical decisions.

ctDNA is a subset of total cfDNA and its detection varies [14] depending on tumor stage, vascularization, burden, apoptotic rate, and metastatic potential of the cancer cells, and the factors affecting the patient’s blood volume [15]. The ctDNA is released through lysis of necrotic and apoptotic cells; it may also be released from the digestion of tumor cells by macrophages or by direct secretion of DNA by tumor cells [16]. However, a recent study demonstrated that apoptosis is the main source of ctDNA since the induction of tumor apoptosis after chemotherapy treatment increases in ctDNA levels [17].

Detection of ctDNA has been challenging for three main reasons: Discrimination of ctDNA from normal cfDNA; the presence of extremely low levels of ctDNA; and the accurate quantification of the number of mutant fragments in a sample. Therefore, this discrimination is done by the recognition of mutations in the ctDNA. These somatic mutations, which are present only in the genomes of cancer cells, are not present in the DNA of normal cells [16,17]. Therefore, ctDNA can be useful to monitor tumor alterations, because it has a high turnover rate and is representative of the genomics of the tumor’s mutations, epigenetic changes, and microsatellite instability [18–20]. Although the use of ctDNA is a promising approach in cancer patients, it has some limitations: It is an expensive

technique (mainly when using next-generation sequencing (NGS)); it does not provide information about morphological and tumor microenvironment status; in low tumor burden patients the chance of having informative results is low and it requires a previous knowledge of the targeted of interest mutation in most cases. Another limitation in the analysis of ctDNA is the extent to which the information obtained from the liquid biopsy sample reflects the tumor tissue; this is related to the fact that technical and biological factors can affect the concordance between tumor and plasma, generating false-negative and false-positive results in ctDNA analysis [21].

In breast cancer patients, plasma is the main fluid sample used for ctDNA analysis, while other body fluids are utilized for other cancer types [22] such as cerebrospinal fluid, urine, and saliva [23,24]. Next-generation sequencing (NGS) methods are used to examine ctDNA for the presence of gene variants, to detect early-stage disease, response to treatment, therapeutic resistance, and prognosis [25,26]. It also may detect disease progression and minimal residual disease after treatment [6]. It retains the genetic variant and epigenetic features of tumors, such as mutations, insertions, deletions, rearrangements, and DNA methylation of oncogenes or tumor suppressor genes, making it a potential biomarker [27]. Currently, NGS using molecular barcodes (unique molecular identifiers (UMIs)) can detect mutations at allele frequencies down to 0.1% and can be applied to highly sensitive detection of targeted ctDNA mutations. These methods include tagged-amplicon deep sequencing (Tam-seq), safe-sequencing system (Safe-SeqS), and cancer personalized profiling by deep sequencing (CAPP-Seq), with 97%, 98%, and nearly 100% sensitivity, respectively [28]. Methods using polymerase chain reaction (PCR) are also widely used, such as digital PCR (dPCR), which can detect minor allele frequencies less than 0.1% [28].

Whole genome sequencing (WGS) is usually used to get the whole genomic profile of tumor DNA including point mutations, rearrangements, and copy number variations (CNVs). It provides us with abundant information, but it is expensive and less sensitive. Whole exome sequencing (WES) may be an alternative. Nevertheless, both require high input sample volume, which hampers their application in routine testing and for screening and early diagnosis when the amount of ctDNA is low [28]. The development of these sequencing methods and novel exome sequencing kits led to the need for a robust statistical framework. According to the study published by Barbitoff, Y.A. et al. most of the observed bias in WES stems from mappability limitations of short reads, as well as exome probe design [29]. Due to the high complexity of the analysis, the lack of biological material, and the huge cost associated, neither WGS nor WES will be possible as routine molecular strategies used to analyze the ctDNA.

These advanced laboratory techniques have allowed for a daily collaboration between clinicians and laboratory teams since somatic mutations in tumors have gained a high clinical impact for patients due to the increasing targeted therapies available for some cancers. In 2016, the Food and Drug Administration (FDA) firstly approved the use of ctDNA as a liquid biopsy test for patients with stage IV non-small cell lung cancer (NSCLC) to check for epidermal growth factor receptor (EGFR) mutations [30]. This identification was often difficult in small lung tumor tissue samples available and repeating the lung biopsy was invasive for the patient. The identification of the mutation using ctDNA has allowed clinicians to overcome this difficulty and made it possible to treat patients with anti-EGFR targeted therapies available, today the standard of care. It has also allowed us to understand the resistance mechanisms of the tumors that stop responding to these treatments, and to select them for new therapies according to new mutations found in ctDNA. Spence, T. et al. analyzed the EGFR T790M status of 343 sequential patients with NSCLC using ctDNA collected during anti-EGFR treatment; 24% of the patients had the mutation and these patients were treated with osimertinib, a targeted drug approved in this scenario [31].

Therefore, a new paradigm of personalized medicine in which a targeted drug can be used in specific patients harboring a specific tumor mutation has emerged. Since this first clinical application approval, the use of ctDNA is being investigated in different cancer types, including breast cancer.

3. ctDNA—Clinical Application in Breast Cancer

Breast cancer is a complex disease whose molecular mechanisms are not completely understood. Developing targeted therapies is a promising approach in this area. Therefore, understanding the biological behavior of the tumor is the main challenge.

Liquid biopsies such as using ctDNA may help to overcome this, as they are a non-invasive method of monitoring the development of the disease during treatment and detecting emerging mutations [32]. They also allow tumor heterogeneity to be accessed, which can be related to acquiring therapy resistance [33]. Cancer is a heterogeneous disease, with different areas of the same tumor having different genetic profiles; likewise, heterogeneity exists between metastases within the same patient. A biopsy or tissue section from one part of a tumor may not represent the molecular intratumoral and intermetastatic heterogeneity [5,34,35]. As ctDNA from the primary tumor or metastasis is released into the blood, it may provide material for more comprehensive mutational profiling of the tumors in a less invasive way.

Although the first studies were in metastatic disease, the value of ctDNA in early disease has also been proven. Recent advances have also shown that ctDNA may also have a role in detecting residual disease after neoadjuvant chemotherapy and patient stratification due to genomic alterations, making it a valuable tool in locally advanced disease [6].

The concordance (same mutation status in plasma and tumor) between tumor tissue and ctDNA is being investigated, is not consistent across different studies, and has been established firstly in lung and colorectal cancers. In a study by Sung, J.S et al., 126 cases of non-small cell lung cancer patients were examined, and blood samples were analyzed for concordance of ctDNA and tumor tissue using ultra-deep sequencing and tissue genotyping. High concordance rates for *EGFR* mutations were observed [36]. In breast cancer, some studies have demonstrated concordance between breast cancer tissue and ctDNA. In a study conducted by Takeshita, T. et al., the authors compared *ESR1* mutation statuses of 35 cfDNA and matched tumor tissue in patients with metastatic breast cancer and found an overall concordance rate of 74.3% [37].

Woodhouse, R. et al. recently published their reports about clinical analytical validation of the FoundationOne® Liquid CDx assay which is based on the first FDA-approved FoundationOne® CDx tissue-based diagnostic tool analytically and clinically validated for solid tumors. The ability of the FoundationOne Liquid CDx assay to detect genomic alterations also detected in tumor tissue was demonstrated in this study. The test, recently approved by the FDA, consists of a cancer cfDNA-based comprehensive genomic profiling assay which targets 324 genes. It detects the major types of genomic alterations in addition to complex biomarkers such as microsatellite instability, tumor fraction, and blood tumor mutational burden [38].

The NILE study (Non-invasive versus Invasive Lung Evaluation; ClinicalTrials.gov; NCT03615443) is another FDA-approved study using cfDNA. The study has included 282 patients and has analyzed ctDNA in previously untreated metastatic NSCLC using this test. It allowed for the identification of tumor biomarkers with high concordance with tissue samples and in a more rapid way [39]. There are some assays using ctDNA approved for clinical use by FDA, and they have already been implemented in clinical practice. Table 1 describes the assays approved [40].

3.1. Early Diagnosis and Relapse

Early diagnosis of breast cancer is vital for reducing cancer-related mortality. It also allows for the selection of appropriate treatment approaches such as adjuvant therapy and therefore increases the chance of a patient's survival. Clinically proven biomarkers that can be used to diagnose and guide patient management earlier in the course of the disease are not available. Serum-based protein biomarkers such as cancer antigen-125 (CA-125) and cancer antigen-15.3 (CA 15.3) are commonly used for monitoring breast cancer patients, but these proteins are also found in the serum of individuals without cancer; therefore, they are not useful for diagnosis [41]. Therefore, there are not sensitive biomarkers to detect distant metastases before they are radiological evident. Coombes, R.C et al. used

the cfDNA of 49 patients collected after surgery and adjuvant therapy. The authors found that plasma ctDNA was detected before clinical or radiologic relapse in 16 of the 18 relapsed patients (sensitivity of 89%); metastatic progression was anticipated with a lead time of up to 2 years (median, 8.9 months; range, 0.5–24.0 months), providing a good opportunity for clinical intervention [42].

Table 1. Food and Drug Administration (FDA)-approved liquid biopsies assays.

Liquid Biopsy Assay	Clinical Application	Genes Analyzed
FoundationOne® Liquid CDx assay	NSCLC, mCRPC	70 genes + MSI-H (BRCA 1, 2, EGFR)
Guardant360® CDx assay	NSCLC, pan-cancer	70 genes using NGS
Therascreen® (Qiagen) PI3KCA	Breast cancer	11 mutation in PIK3CA gene
EpiproColon®	Colorectal cancer	PCR, methylation
Cobas® EGFR mutation test (Roche)	NSCLC	EGFR variants
In Vision First-Lung®	NSCLC	37 genes NSCLC
Oncobeam Lung-1®	NSCLC	EGFR
Oncobeam Lung-2®	NSCLC	EGFR, KRAS, BRAF
Oncomine® (Thermo Fisher Scientific)	Breast, lung, colon cancer, pan-cancer	52 genes cancer assay
TS0500 ctDNA® (Illumina)	Pan-cancer	500+ genes
Avenio ctDNA® (Roche)	Breast, lung, colorectal, gastric, melanoma, pancreatic, ovarian, glioma, thyroid cancers	17 genes

Non-small cell lung cancer (NSCLC); metastatic castration resistant prostate cancer (mCRPC); microsatellite instability (MSI-H).

Very low levels of ctDNA are usually not detectable in early disease [42]. Therefore, in the setting of early breast cancer, the clinical utility of ctDNA-based liquid biopsy remains unclear, as the percutaneous biopsy of breast tumors continues to be preferred for diagnosis. Nevertheless, a blood test (CancerSEEK) has already been studied for early detection of some cancers such as ovary, liver, stomach, pancreas, esophagus, colon, rectum, lung, and breast cancer [43]. According to this study, this multi-analyte blood test was able to detect some cancers through the determination of mutations and circulating proteins using the cfDNA. The median sensitivity of the test was 70% among the cancer types studied, but it was lower for breast cancer tumors (about 33%) [43].

Some studies have shown that the fraction of ctDNA detected in a breast cancer patient in early disease is directly correlated with disease stage and is significantly lower at earlier stages of the disease [44]. As an example, Board, R.E et al. showed that the mutation rates observed in ctDNA within genes such as PIK3CA were much higher in advanced-stage breast tumors compared to stage I and II tumors [45]. The authors also found a concordance of 95% between ctDNA and tissue samples of the tumors.

Several studies have already examined the potential of ctDNA for early detection of breast cancer at early stages [46,47]. In a study conducted by Phallen, J. et al., an evaluation of 200 patients with colorectal, breast, lung, or ovarian cancer detected somatic mutations in the plasma of 71%, 59%, 59%, and 68%, respectively, of patients with stage I/II disease. Analyses of mutations in blood using ctDNA revealed high concordance with alterations in the tumors of these patients (72%) [46].

A prospective and multicenter study of 101 women with early-stage breast cancer using circulating tumor DNA mutation tracking found that detection of circulating tumor DNA during follow-up had a median lead-time of 10.7 months compared with clinical relapse, anticipating relapse in all breast cancer subtypes [48]. This anticipation of clinical relapse may lead us to take clinical measures that allow for the detection of early recurrence and properly managing it.

Kim, C. et al. found that the low-level expression of estrogen receptor 1 (ESR1) in breast cancer tissue samples is correlated with endocrine therapy resistance in estrogen receptor (ER)-positive primary breast cancer and is associated with the treatment outcome [49]. ESR1 mutations can also be identified using ctDNA analysis and predict resistance to endocrine therapy in early disease. Nevertheless, they are rarely detected during adjuvant treatments [50].

Recently, a prospective study conducted by Liu M.C, et al. investigated the ability of target methylation analyses of cfDNA of different cancer types to detect and localize different cancer types. The authors concluded that cfDNA sequencing and analysis of methylation patterns detected more than 50 cancer types across different stages, including breast cancer [51].

Therefore, although not validated for diagnosis, ctDNA may play an important role in early breast cancer evaluation and decision making.

3.2. Metastatic Disease

Measuring treatment response in patients with metastatic breast cancer is usually done by clinical evaluation, assessment of CA15.3 levels, and radiographic imaging. However, serial radiographic imaging is often inconclusive, and CA 15.3 fluctuations do not necessarily reflect tumor response or progression [52].

No marker for monitoring therapy responses in patients with metastatic breast cancer has yet reached wide clinical use, making it difficult for clinicians to anticipate a disease progression. The use of ctDNA in breast cancer was first established in metastatic disease, as tumor burden was related to the total amount of ctDNA [53].

In metastatic breast cancer, ctDNA has shown promising results to monitor disease as reported by Dawson, S.J et al. [54]. In this study, the authors compared the radiographic imaging of tumors with ctDNA, CA 15-3, and circulating tumor cells in 30 women with metastatic breast cancer who were receiving systemic therapy. They concluded that ctDNA levels showed a greater dynamic range and correlation with changes in tumor burden than CA 15-3 or circulating tumor cells. The authors mainly used the detection of mutations in the genes PIK3CA and TP53 as surrogates for ctDNA. At the time, there was no mutation target therapy approved for metastatic breast cancer.

Some clinical studies have investigated ctDNA mutations associated with targeted therapy response in patients with HER2-positive breast cancer and endocrine therapy response in patients with ER-positive metastatic breast cancer [55,56]. These studies showed that ESR1 mutations normally arise following the treatment of metastatic disease and can predict resistance to endocrine therapy with aromatase inhibitors [55]. Acquired ESR1 missense alterations occur in about 30% of patients who have received prior endocrine therapies and are associated with an aggressive clinical phenotype of the disease [56]. Fribbes, C. et al. conducted a prospective-retrospective analysis, assessing ESR1 mutations in available archived plasma from the Study of Faslodex vs. Exemestane with or without Anastrozole (SoFEA) and Palbociclib Combined with Fulvestrant in Hormone Receptor-Positive HER2-Negative Metastatic Breast Cancer after Endocrine Failure (PALOMA3) trials. The authors concluded that ESR1 mutation analysis in plasma after progression can be a useful tool to guide the clinician's choice for subsequent endocrine therapies [56].

Detection of ctDNA nucleotide alterations to assess response to anti-HER2-targeted therapies has also been investigated [57]. As reported in Ye, Q. et al. study, 46 genes were detected from an assessment of 486 single-nucleotide variants; only 7 genes considered relevant to targeted therapy resistance were detected in the treatment-resistant group. In addition, two patients in whom HER2 p.S855I mutations were detected had benefited from anti-HER2 therapy. Therefore, the authors concluded that targeted NGS of ctDNA has a potential clinical utility to detect biomarkers from HER2-targeted therapies [57]. In a recent study from Guan, X. et al., the authors found that the level of HER2 amplification in ctDNA by NGS had a high concordance with the primary tumor (approximately 80%); moreover, HER2 copy numbers showed fluctuations during HER2-targeted therapies, and patients with positive levels after 6 weeks of treatment showed a reduction in progression-free survival [58]. With the widely recommended use of anti-HER2 target drugs for treatment of HER2-positive breast cancer patients in metastatic disease due to the benefits in overall survival and progression-free survival, it is important to explore the molecular mechanisms behind the good results of this therapy and monitor them. On the other hand, it is important to understand the reason why some tumors do not respond to anti-HER2

targeted therapies to be able to investigate the mechanisms of tumor resistance in this scenario and overcome them.

Recently, targeted therapy for PIK3CA mutations has emerged. These mutations are found in 20–30% of breast cancer patients [59,60]. Alpelisib is a targeted drug approved for treating metastatic breast cancer patients with positive endocrine receptors and PIK3CA mutations. It is an α -specific PI3K inhibitor that selectively inhibits p110 α approximately 50 times as strong as other isoforms [59]. In a phase 3 study (SOLAR 1 trial), alpelisib plus fulvestrant were compared with placebo plus fulvestrant in patients with HR-positive and HER2-negative advanced breast cancer who had previously been treated with endocrine therapy. The results showed that treatment with alpelisib and fulvestrant prolonged progression-free survival among these patients' population [60]. According to this trial, the use of alpelisib was approved in patients with metastatic HR-positive, HER2-negative, and PIK3CA-mutated tumors. The use of ctDNA to identify PIK3CA mutations was also investigated in a subgroup analysis of SOLAR 1 trial by Juric, D. et al. and the treatment showed consistent clinically meaningful treatment benefit for patients with ctDNA mutant status [61]. It made it possible to use a more comfortable and less invasive tool to detect a tumor mutation for which a targeted therapy is available.

Breast cancer disease carrying a BRCA gene (BRCA) mutation also represents an intriguing scenario. Poly (ADP-ribose) polymerase (PARP) inhibitors such as olaparib and talozaparib are approved in metastatic breast cancer patients carrying a germline BRCA1 or -2 mutation and HER2-negative tumors. These drugs are used taking into account the rationale of synthetic lethality mechanism, which means two conditions that independently would not cause cell death when used in combination are lethal. In patients carrying a germline BRCA1 or BRCA2 mutation, homologous recombination pathway is defective; the use of PARP inhibitors to hamper the base excision repair (BER) mechanism causes cell damage and consequently death [62]. In OlympiAD and EMBRACA trials, olaparib and talozaparib, respectively, showed a median progression-free survival and response rate significantly longer than in the standard chemotherapy group in this subgroup of germline BRCA1 or BRCA2-mutated patients [62–64]. Therefore, ctDNA-based assessment of somatic BRCA mutations using ctDNA could potentially expand the cohort of patients treatable with PARP inhibitors in the future [65].

More recently the resistance mechanisms of cyclin inhibitor drugs using ctDNA have been studied. A study from Condorelli R. et al. has shown a retinoblastoma gene (Rb) mutation emerging after cyclin inhibitors treatment and failure. The mutation was detected in the ctDNA of an ER-positive, HER2-negative metastatic breast cancer patient. Cyclin inhibitors are approved in metastatic ER-positive and HER2-negative breast cancer patients in first and second therapy lines. They act by inhibiting the cyclin D1-CDK4/6-retinoblastoma pathway [66,67].

There are some clinical trials currently addressing the question of using ctDNA to select targeted therapies for breast cancer patients. The PLASMAmatch trial is a phase 2 trial that included 1051 patients, with ctDNA available for 1034 of them. Patients were divided in different treatment cohort groups according to the mutation identified and target therapy used. The authors found that ctDNA testing was highly accurate, and showed high sensitivity for mutations identified in advanced breast cancer tissue biopsies. ctDNA was able to identify patients with important targetable mutations and therefore it could be used in routine clinical practice [68].

As described above, liquid biopsies are being investigated and applied in different cases mainly in metastatic breast cancer, with targeted therapies for specific mutations being constantly approved for this subgroup of patients. This application in clinical practice is increasing and has led to a constant need of molecular laboratories to develop new strategies to provide quick and quality molecular results to medical oncologists not only using ctDNA, but also tumor tissue. These results are currently fundamental to decision making in clinical practice; therefore, improving investigation and sharing results in this area is mandatory.

3.3. Locally Advanced Breast Cancer: Detecting Residual Minimal Disease

Patients with locally advanced breast cancer are commonly treated with neoadjuvant therapy (NAT) to reduce cancer size and study the treatment response to chemotherapy.

In breast cancer, ~30% of patients treated with NAT achieve pathological complete responses (pCR) with no histological evidence of invasive tumors in the resected breast tissue and lymph nodes [67]. Achieving pCR is correlated with a good prognosis of the disease. Patients with HER2-positive and triple-negative breast cancer tumors are the best candidates for neoadjuvant chemotherapy as they have the highest probability of achieving pCR [69]. Analysis of gene expression changes in ctDNA of sequential patient samples collected before and during treatment may be a promising way to analyze the molecular changes that occur during treatment in real-time. Comparing samples from patients who have a complete response to neoadjuvant treatment to samples from those who do not have it can provide a better understanding of the resistance mechanisms of the tumor, allowing for the definition of a better therapy strategy for the patients. Nevertheless, detection of ctDNA after NAT has been challenging in patients, even when the residual disease is observed in the surgery. Recent studies have found that ctDNA becomes undetectable in more than 90% of patients during NAT [70]. Nevertheless, no correlation has been established between pCR and ctDNA.

A recent study showed that after completion of neoadjuvant therapy, ctDNA concentrations were lower in patients who achieved pCR compared to patients with residual disease. Further, patients with pCR showed a larger decrease in ctDNA concentrations during neoadjuvant therapy [71]. Previously, some studies already suggested that during NAT, ctDNA levels decreased and minimal residual disease of the tumor was undetectable after the surgery. A slow decrease of ctDNA levels during NAT was associated with poorer survival [72].

A recent study from Radovich, M. et al. showed that detection of ctDNA in patients with early-stage triple-negative breast cancer after neoadjuvant chemotherapy was independently associated with disease recurrence. The authors used the samples of a cohort of 196 patients with locally advanced breast cancer submitted to neoadjuvant chemotherapy. At 24 months, disease-free survival probability was 56% for ctDNA-positive patients compared with 81% for ctDNA-negative patients [73].

Liquid biopsy is a promising option for detecting driver somatic mutations, and the low ctDNA levels found in these early stages represent a challenge for sequencing techniques. More studies are needed in this specific clinical scenario to overcome this difficulty and to achieve a prognostic molecular tool for these patients. This may allow us, in the future, to optimize the clinical follow-up of these patients and predict who are more likely to have recurrence of the disease.

4. Conclusions

ctDNA sequencing analysis is an important molecular tool option to inform on tumor mutational and molecular landscapes in a less invasive way. It could be used in early-stage disease, allowing for the detection of early recurrences; however, it is also a valuable method to longitudinally monitor tumors' genomic profiles and detect the emergence of genetic alterations in the metastatic scenario, anticipating clinical symptoms or radiological evidence of disease progression. Additionally, it allows for a better selection of targeted therapies and provides a more comprehensive photogram of tumor heterogeneity. Consequently, ctDNA analysis may guide clinical decision-making in the precision medicine era (Table 2).

Table 2. Clinical utility of using ctDNA in different breast cancer scenarios.

Breast Cancer Scenario	Clinical Use	Assay Used	Findings	Reference
Early-stage disease	Early detection	CancerSEEK	Detect cancers through the determination of mutations using the cfDNA. The median sensitivity of the test was 70% among the cancer types studied; 33% in breast cancer.	[43]
	Anticipating relapse	Ultra deep sequencing	ctDNA was detected before clinical or radiologic relapse in cancer patients (sensitivity of 89%).	[42]
		dPCR analysis of ctDNA	Detection of ctDNA during follow-up is associated with high risk of relapse.	[48]
	Treatment resistance	dPCR analysis of ctDNA	ESR1 mutations can predict resistance to endocrine therapy in early disease.	[50]
Metastatic disease	Monitoring disease	dPCR analysis of ctDNA	The ctDNA levels showed a greater dynamic range, and correlation with changes in tumor burden, than CA 15-3 or circulating tumor cells in patients with breast cancer receiving therapy.	[54]
	Treatment resistance	NGS and dPCR analysis of ctDNA	ESR1 mutations can predict resistance to endocrine therapy; ESR1 mutation analysis in plasma after progression can be a useful tool to guide the clinician's choice for subsequent endocrine therapies.	[55,56]
	Selecting targeted therapies	dPCR analysis of ctDNA	According to the SOLAR 1 trial, alpelisib was approved in patients with PI3KCA mutation; the use of ctDNA to identify PI3KCA mutation was validated.	[60,61]
		PLASMAmatch	ctDNA was able to identify patients with important targetable mutation.	[68]
Locally advanced disease	Detecting minimal residual disease	Dropped dPCR; Targeted digital sequencing (TARDIS)	ctDNA concentrations were lower in patients who achieved pCR compared to patients with residual disease; slow decrease of ctDNA levels during NAT was associated with poorer survival.	[71,72]
		Foundation One® Liquid Assay	Detection of ctDNA in patients with early-stage triple-negative breast cancer after neoadjuvant chemotherapy was independently associated with disease recurrence.	[73]

Author Contributions: A.M. did the research and writing of the article. The article was reviewed by J.L.C. and F.S. All authors have read and agreed to the published version of the manuscript.

Funding: This work resulted from “Institute for Research and Innovation in Health Sciences” (POCI-01-0145-FEDER-007274)- “GenomePT” (POCI-01-0145-FEDER-022184)-“Advancing cancer research: from knowledge to application” (NORTE-01-0145-FEDER-000029) supported by COMPETE 2020-Operational Programme for Competitiveness and Internationalisation (POCI)- Norte Portugal Regional Programme (NORTE 2020)-Lisboa Portugal Regional Operational Programme (Lisboa2020)-Algarve Portugal Regional Operational Programme (CRESC Algarve2020)- under the PORTUGAL 2020 Partnership Agreement- through the European Regional Development Fund (ERDF)- by FCT - Fundação para a Ciência e a Tecnologia (PTDC/MEC-ONC/32018/2017). The funding sources had no involvement in this review article data, writing of the review or the decision to submit it for publication.

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

References

1. Ferlay, J.; Soerjomataram, I.; Dikshit, R.; Eser, S.; Mathers, C.; Rebelo, M.; Parkin, D.M.; Forman, D.; Bray, F. Cancer incidence and mortality worldwide: Sources, methods and major patterns in GLOBOCAN 2012. *Int. J. Cancer* **2015**, *136*, E359–E386. [\[CrossRef\]](#) [\[PubMed\]](#)
2. Senkus, E.; Kyriakides, S.; Ohno, S.; Penault-Llorca, F.; Poortmans, P.; Rutgers, E.; Zackrisson, S.; Cardoso, F. Primary breast cancer: ESMO Clinical Practice Guidelines for diagnosis, treatment and follow-up. *Ann. Oncol.* **2013**, *6*, vi7–vi23. [\[CrossRef\]](#) [\[PubMed\]](#)
3. Cheng, F.; Su, L.; Qian, C. Circulating tumor DNA: A promising biomarker in the liquid biopsy of cancer. *Oncotarget* **2016**, *7*, 48832–48841. [\[CrossRef\]](#) [\[PubMed\]](#)
4. Heitzer, E.; Ulz, P.; Geigl, J.B. Circulating tumor DNA as a liquid biopsy for cancer. *Clin. Chem.* **2015**, *61*, 112–123. [\[CrossRef\]](#)
5. Murtaza, M.; Dawson, S.-J.; Tsui, D.W.Y.; Gale, D.; Forshew, T.; Piskorz, A.M.; Parkinson, C.; Chin, S.-F.; Kingsbury, Z.; Wong, A.S.C.; et al. Non-invasive analysis of acquired resistance to cancer therapy by sequencing of plasma DNA. *Nature* **2013**, *497*, 108–112. [\[CrossRef\]](#)
6. Arruda, L.; Caldas, C. Cell free circulating tumor DNA as a liquid biopsy in breast cancer. *Mol. Oncol.* **2016**, *10*, 464–474. [\[CrossRef\]](#)
7. Mayo-de-Las-Casas, C.; Jordana-Ariza, N.; Garzón-Ibañez, M.; Balada-Bel, A.; Bertrán-Alamillo, J.; Viteri-Ramírez, S.; Reguart, N.; Muñoz-Quintana, M.A.; Llanes-Barragan, P.; Camps, C.; et al. Large scale, prospective screening of EGFR mutations in the blood of advanced NSCLC patients to guide treatment decisions. *Ann. Oncol.* **2017**, *28*, 2248–2255. [\[CrossRef\]](#)
8. Bi, F.; Wang, Q.; Wang, Y.; Zhang, L.; Zhang, J. Circulating tumor DNA in colorectal cancer: Opportunities and challenges. *Am. J. Transl. Res.* **2020**, *12*, 1044–1055.
9. Cardoso, F.; Kyriakides, S.; Ohno, S.; Penault-Llorca, F.; Poortmans, P.; Rubio, I.; Zackrisson, S.; Senkus, E. Early breast cancer: ESMO Clinical Practice Guidelines for diagnosis, treatment and follow-up. *Ann. Oncol.* **2019**, *30*, 1194–1220. [\[CrossRef\]](#)
10. Dawson, S.-J.; Rueda, O.M.; Aparicio, S.; Caldas, C. A new genome-driven integrated classification of breast cancer and its implications. *EMBO J.* **2013**, *32*, 617–628. [\[CrossRef\]](#)
11. Mandel, P.; Metais, P. Les acides nucléiques du plasma sanguin chez l’homme. *CR Seances Soc. Biol. Fil.* **1948**, *142*, 241–243.
12. Leon, S.; Shapiro, B.; Sklaroff, D.M.; Yaros, M.J. Free DNA in the serum of cancer patients and the effect of therapy. *Cancer Res.* **1977**, *37*, 646–650. [\[PubMed\]](#)
13. Alimirzaie, S.; Bagherzadeh, M.; Akbari, M.R. Liquid biopsy in breast cancer: A comprehensive review. *Clin. Genet.* **2019**, *95*, 643–660. [\[CrossRef\]](#)
14. Diehl, F.; Schmidt, K.; Choti, M.A.; Romans, K.; Goodman, S.; Li, M.; Kinzler, K.W. Circulating mutant DNA to assess tumor dynamics. *Nat. Med.* **2008**, *14*, 985–990. [\[CrossRef\]](#) [\[PubMed\]](#)
15. Kidd, E.; Jeffrey, S.S. Circulating tumor cells versus tumor-derived cell-free DNA: Rivals or partners in cancer care in the era of single-cell analysis? *Genom. Med.* **2013**, *5*, 70. [\[CrossRef\]](#) [\[PubMed\]](#)
16. Diaz, L.A., Jr.; Bardelli, A. Liquid biopsies: Genotyping circulating tumor DNA. *J. Clin. Oncol.* **2014**, *32*, 579–586. [\[CrossRef\]](#)

17. Marques, J.; Junqueira-Neto, S.; Pinheiro, J.A.; Machado, J.C.; Costa, J.L. Induction of apoptosis increases sensitivity to detect cancer mutations in plasma. *Eur. J. Cancer* **2020**, *127*, 130–138. [\[CrossRef\]](#)
18. Nawroz-Danish, H.; Eisenberger, C.F.; Yoo, G.; Wu, L.; Koch, W.; Black, C.; Ensley, J.F.; Wei, W.-Z.; Sidransky, D. Microsatellite analysis of serum DNA in patients with head and neck cancer. *Int. J. Cancer* **2004**, *111*, 96–100. [\[CrossRef\]](#)
19. Lee, J.; Cho, S.-M.; Kim, M.S.; Lee, S.H.; Chung, Y.-J.; Jung, S.-H. Circulating tumor DNA in a breast cancer patient's plasma represents driver alterations in the tumor tissue. *Genomics Inform.* **2017**, *15*, 48–50. [\[CrossRef\]](#)
20. Ma, M.; Zhu, H.; Zhang, C.; Sun, X.; Gao, X.-S.; Chen, G. "Liquid biopsy"—ctDNA detection with great potential and challenges. *Ann. Transl. Med.* **2015**, *3*, 235.
21. Keller, L.; Belloum, Y.; Wikman, H.; Pantel, K. Clinical relevance of blood-based ctDNA analysis: Mutation detection and beyond. *Br. J. Cancer* **2020**. [\[CrossRef\]](#) [\[PubMed\]](#)
22. Sands, J.; Li, Q.; Hornberger, J. Urine circulating-tumor DNA (ctDNA) detection of acquired EGFR T790M mutation in non-small-cell lung cancer: An outcomes and total cost-of-care analysis. *Lung Cancer* **2017**, *110*, 19–25. [\[CrossRef\]](#) [\[PubMed\]](#)
23. De Mattos-Arruda, L.; Mayor, R.; Ng, C.K.Y.; Weigelt, B.; Martínez-Ricarte, F.; Torrejon, D.; Oliveira, M.; Arias, A.; Raventos, C.; Tang, J.; et al. Cerebrospinal fluid derived circulating tumor DNA better represents the genomic alterations of brain tumors than plasma. *Nat. Commun.* **2015**, *6*, 8839. [\[CrossRef\]](#) [\[PubMed\]](#)
24. Aro, K.; Wei, F.; Wong, D.T.; Tu, M. Saliva liquid biopsy for point-of-care applications. *Front. Public Heal.* **2017**, *5*, 77. [\[CrossRef\]](#)
25. Kaisaki, P.J.; Cutts, A.; Popitsch, N.; Herrero, C.C.; Pentony, M.M.; Wilson, G.; Page, S.; Kaur, K.; Vavoulis, D.; Henderson, S.; et al. Targeted next-generation sequencing of plasma DNA from cancer patients: Factors influencing consistency with tumor DNA and prospective investigation of its utility for diagnosis. *PLoS ONE* **2016**, *11*, e0162809. [\[CrossRef\]](#)
26. Chae, Y.K.; Davis, A.A.; Jain, S.; Santa-Maria, C.; Flaum, L.; Beaubier, N.; Platanius, L.C.; Gradishar, W.; Giles, F.J. Concordance of genomic alterations by next-generation sequencing in tumor tissue versus circulating tumor DNA in breast cancer. *Mol. Cancer Ther.* **2017**, *16*, 1412–1420. [\[CrossRef\]](#)
27. Esposito, A.; Criscitello, C.; Locatelli, M.; Milano, M.; Curigliano, G. Liquid biopsies for solid tumors: Understanding tumor heterogeneity and real time monitoring of early resistance to targeted therapies. *Pharmacol. Ther.* **2016**, *157*, 120–124. [\[CrossRef\]](#)
28. Chen, M.; Zhao, H. Next-generation sequencing in liquid biopsy: Cancer screening and early detection. *Hum. Genomics* **2019**, *13*, 19. [\[CrossRef\]](#)
29. Barbitoff, Y.A.; Polev, D.E.; Glotov, A.S.; Serebryakova, E.A.; Shcherbakova, I.V.; Kiselev, A.M.; Kostareva, A.A.; Glotov, O.S.; Predeus, A.V. Systematic dissection of biases in whole-exome and whole-genome sequencing reveals major determinants of coding sequence coverage. *Nat. Sci. Rep.* **2020**, *10*, 2057. [\[CrossRef\]](#)
30. Lim, M.; Kim, C.J.; Sunkara, V.; Kim, M.-H.; Cho, Y.-K. Liquid Biopsy in Lung Cancer: Clinical Applications of Circulating Biomarkers (CTCs and ctDNA). *Micromachines* **2018**, *9*, 100. [\[CrossRef\]](#)
31. Spence, T.; Perera, S.; Weiss, J.; Grenier, S.; Ranich, L.; Shepherd, F.; Stockley, T.L. Clinical implementation of circulating tumour DNA testing for EGFR T790M for detection of treatment resistance in non-small cell lung cancer. *J. Clin. Pathol.* **2020**, *1–7*. [\[CrossRef\]](#)
32. Pérez-Callejo, D.; Romero, A.; Provencio, M.; Torrente, M. Liquid biopsy based biomarkers in non-small cell lung cancer for diagnosis and treatment monitoring. *Transl. Lung Cancer Res.* **2016**, *5*, 455–465. [\[CrossRef\]](#) [\[PubMed\]](#)
33. Parikh, A.R.; Leshchiner, I.; Elagina, L.; Goyal, L.; Levovitz, C.; Siravegna, G.; Livitz, D.; Rhrissorrakrai, K.; Martin, E.E.; Van Seventer, E.E.; et al. Liquid versus tissue biopsy for detecting acquired resistance and tumor heterogeneity in gastrointestinal cancers. *Nat. Med.* **2019**, *25*, 1415–1421. [\[CrossRef\]](#) [\[PubMed\]](#)
34. De Mattos-Arruda, L.; Cortes, J.; Santarpia, L.; Vivancos, A.; Tabernero, J.; Reis-Filho, J.S.; Seoane, J. Circulating tumor cells and cell-free DNA as tools for managing breast cancer. *Nat. Rev. Clin. Oncol.* **2013**, *10*, 377–389. [\[CrossRef\]](#) [\[PubMed\]](#)
35. Diaz, L.A., Jr.; Williams, R.T.; Wu, J.; Kinde, I.; Hecht, J.R.; Berlin, J.; Allen, B.; Bozic, I.; Reiter, J.G.; Nowak, M.A.; et al. The molecular evolution of acquired resistance to targeted EGFR blockade in colorectal cancers. *Nature* **2012**, *486*, 537–540. [\[CrossRef\]](#)

36. Sung, J.S.; Chong, H.Y.; Kwon, N.-J.; Kim, H.M.; Lee, J.W.; Kim, B.; Lee, S.B.; Park, C.W.; Choi, J.Y.; Chang, W.J.; et al. Detection of somatic variants and EGFR mutations in cell-free DNA from non-small cell lung cancer patients by ultra-deep sequencing using the ion ampliseq cancer hotspot panel and droplet digital polymerase chain reaction. *Oncotarget* **2017**, *8*, 106901. [\[CrossRef\]](#)
37. Takeshita, T.; Yamamoto, Y.; Yamamoto-Ibusuki, M.; Tomiguchi, M.; Sueta, A.; Murakami, K.; Omoto, Y.; Iwase, H. Comparison of ESR1 Mutations in Tumor Tissue and Matched Plasma Samples from Metastatic Breast Cancer Patients. *Transl. Oncol.* **2017**, *10*, 766–771. [\[CrossRef\]](#)
38. Woodhouse, R.; Li, M.; Hughes, J.; Delfosse, D.; Skoletsky, J.; Ma, P.; Meng, W.; Dewal, N.; Milbury, C.; Clark, T.; et al. Clinical and analytical validation of FoundationOne® Liquid CDx assay a novel 324-Gene cfDNA-based comprehensive genomic profiling assay for cancers of solid tumor origin. *PLoS ONE* **2020**, *15*, e0237802. [\[CrossRef\]](#)
39. Leighl, N.B.; Page, R.D.; Raymond, V.M.; Daniel, D.B.; Divers, S.G.; Reckamp, K.L.; Villalona-Calero, M.A.; Dix, D.; Odegaard, J.L.; Lanman, R.B.; et al. Clinical utility of comprehensive cell free DNA analyses to identify genomic biomarkers in patients with newly diagnosed metastatic non-small cell lung cancers. *Clin. Cancer Res.* **2019**, *25*, 4691–4700. [\[CrossRef\]](#)
40. US Food and Drug Administration (FDA). Available online: www.fda.gov (accessed on 10 October 2020).
41. Mazzucchelli, R.; Colanzi, P.; Pomante, R.; Muzzonigro, G.; Montironi, R. Prostate tissue and serum markers. *Adv. Clin. Pathol. Off. J. Adriat. Soc. Pathol.* **2000**, *4*, 111–120.
42. Coombes, R.C.; Page, K.; Salari, R.; Hastings, R.K.; Armstrong, A.C.; Ahmed, S.; Ali, S.; Cleator, S.J.; Kenny, L.M.; Stebbing, J.; et al. Personalized detection of circulating tumor DNA antedates breast cancer metastatic recurrence. *Clin. Cancer Res.* **2019**, *25*, 4255–4263. [\[CrossRef\]](#) [\[PubMed\]](#)
43. Cohen, J.D.; Li, L.; Wang, Y.; Thoburn, C.; Afsari, B.; Danilova, L.; Douville, C.; Javed, A.A.; Wong, F.; Mattox, A.; et al. Detection and localization of surgically resectable cancers with a multi-analyte blood test. *Science* **2018**, *359*, 926–930. [\[CrossRef\]](#) [\[PubMed\]](#)
44. Chen, K.-Z.; Lou, F.; Yang, F.; Zhang, J.-B.; Ye, H.; Chen, W.; Guan, T.; Zhao, M.-Y.; Su, X.-X.; Shi, R.; et al. Circulating tumor DNA detection in early-stage non-small cell lung cancer patients by targeted sequencing. *Sci. Rep.* **2016**, *6*, 31985. [\[CrossRef\]](#) [\[PubMed\]](#)
45. Board, R.E.; Wardley, A.M.; Dixon, J.M.; Armstrong, A.C.; Howell, S.; Renshaw, L.; Donald, E.; Greystoke, A.; Ranson, M.; Hughes, A.; et al. Detection of PIK3CA mutations in circulating free DNA in patients with breast cancer. *Breast Cancer Res. Treat.* **2010**, *120*, 461–467. [\[CrossRef\]](#)
46. Phallen, J.; Sausen, M.; Adleff, V.; Leal, A.; Hruban, C.; White, J.; Anagnostou, V.; Fiksel, J.; Cristiano, S.; Papp, E.; et al. Direct detection of early-stage cancers using circulating tumor DNA. *Sci. Transl. Med.* **2017**, *9*, 403. [\[CrossRef\]](#)
47. Beaver, J.A.; Jelovac, D.; Balakrishna, S.; Cochran, R.L.; Croessmann, S.; Zabransky, D.J.; Wong, H.Y.; Toro, P.V.; Cidado, J.; Blair, B.G.; et al. Detection of cancer DNA in plasma of early stage breast cancer patients. *Clin. Cancer Res.* **2014**, *20*, 2643–2650. [\[CrossRef\]](#)
48. Garcia-Murillas, I.; Chopra, N.; Comino-Méndez, I.; Beaney, M.; Tovey, H.; Cutts, R.J.; Swift, C.; Kiplani, D.; Afentakis, M.; Hrebien, S.; et al. Assessment of Molecular Relapse Detection in Early-Stage Breast Cancer. *JAMA Oncol.* **2019**, *5*, 1473–1478. [\[CrossRef\]](#)
49. Kim, C.; Tang, G.; Pogue-Geile, K.L.; Costantino, J.P.; Baehner, F.L.; Baker, J.; Cronin, M.T.; Watson, D.; Shak, S.; Bohn, O.L.; et al. Estrogen receptor (ESR1) mRNA expression and benefit from tamoxifen in the treatment and prevention of estrogen receptor-positive breast cancer. *J. Clin. Oncol.* **2011**, *29*, 4160. [\[CrossRef\]](#)
50. Schiavon, G.; Hrebien, S.; Garcia-Murillas, I.; Cutts, R.J.; Pearson, A.; Tarazona, N.; Fenwick, K.; Kozarewa, I.; Lopez-Knowles, E.; Ribas, R.; et al. Analysis of ESR1 mutation in circulating tumor DNA demonstrates evolution during therapy for metastatic breast cancer. *Sci. Transl. Med.* **2015**, *7*, 313ra182. [\[CrossRef\]](#)
51. Liu, M.C.; Oxnard, G.R.; Klein, E.A.; Swanton, C.; Seiden, M.V.; Liu, M.C. On behalf of the CCGA Consortium. Sensitive and specific multi-cancer detection and localization using methylation signatures in cell-free DNA. *Ann. Oncol.* **2020**, *31*, 6. [\[CrossRef\]](#)
52. Duffy, M.J.; Evoy, D.; McDermott, E.W. CA 15-3: Uses and limitation as a biomarker for breast cancer. *Clin. Chim. Acta* **2010**, *411*, 1869–1874. [\[CrossRef\]](#) [\[PubMed\]](#)
53. Lee, Y.; Park, S.; Kim, W.S.; Lee, J.C.; Jang, S.J.; Choi, J.; Choi, C. Correlation between progression-free survival, tumor burden, and circulating tumor DNA in the initial diagnosis of advanced-stage EGFR-mutated non-small cell lung cancer. *Thorac. Cancer* **2018**, *9*, 1104–1110. [\[CrossRef\]](#) [\[PubMed\]](#)

54. Dawson, S.-J.; Tsui, D.W.; Murtaza, M.; Biggs, H.; Rueda, O.M.; Chin, S.-F.; Dunning, M.J.; Gale, D.; Forshew, T.; Mahler-Araujo, B.; et al. Analysis of Circulating Tumor DNA to Monitor Metastatic Breast Cancer. *N. Engl. J. Med.* **2013**, *368*, 13. [\[CrossRef\]](#) [\[PubMed\]](#)
55. Guttery, D.S.; Page, K.; Hills, A.; Woodley, L.; Marchese, S.D.; Rghebi, B.; Hastings, R.K.; Luo, J.; Pringle, J.H.; Stebbing, J.; et al. Noninvasive detection of activating estrogen receptor 1 (ESR1) mutations in estrogen receptor-positive metastatic breast cancer. *Clin. Chem.* **2015**, *61*, 974–982. [\[CrossRef\]](#) [\[PubMed\]](#)
56. Fribbens, C.; O’Leary, B.; Kilburn, L.; Hrebien, S.; Garcia-Murillas, I.; Beaney, M.; Cristofanilli, M.; Andre, F.; Loi, S.; Loibl, S.; et al. Plasma ESR1 mutations and treatment of estrogen receptor positive advanced breast cancer. *J. Clin. Oncol.* **2016**, *34*, 2961–2968. [\[CrossRef\]](#)
57. Ye, Q.; Qi, F.; Bian, L.; Zhang, S.-H.; Wang, T.; Jiang, Z. Circulating-free DNA Mutation Associated with Response of Targeted Therapy in Human Epidermal Growth Factor Receptor 2-positive Metastatic Breast Cancer. *Chin. Med. J.* **2017**, *130*, 522–529. [\[CrossRef\]](#)
58. Guan, X.; Liu, B.; Niu, Y.; Dong, X.; Zhu, X.; Li, C.; Li, L.; Yi, Z.; Sun, X.; Chen, H.; et al. Longitudinal HER2 amplification tracked in circulating tumor DNA for therapeutic effect monitoring and prognostic evaluation in patients with breast cancer. *Breast* **2020**, *49*, 261–266. [\[CrossRef\]](#)
59. Fritsch, C.; Huang, A.; Chatenay-Rivauday, C.; Schnell, C.; Reddy, A.; Liu, M.; Kauffmann, A.; Guthy, D.; Erdmann, D.; De Pover, A.; et al. Characterization of the novel and specific PI3K α inhibitor NVP-BYL719 and development of the patient stratification strategy for clinical trials. *Mol. Cancer Ther.* **2014**, *13*, 1117–1129. [\[CrossRef\]](#)
60. André, F.; Ciruelos, E.; Rubovszky, G.; Campone, M.; Loibl, S.; Rugo, H.S.; Iwata, H.; Conte, P.; Mayer, I.A.; Kaufman, B.; et al. Alpelisib for PIK3CA-Mutated, Hormone Receptor-Positive Advanced Breast Cancer. *N. Engl. J. Med.* **2019**, *380*, 1929–1940. [\[CrossRef\]](#)
61. Juric, D.; Ciruelos, E.; Rubovszky, G.; Campone, M.; Loibl, S.; Rugo, H.; Iwata, H.; Conte, P.; Mayer, I.; Kaufman, B.; et al. Abstract GS3-08: Alpelisib + fulvestrant for advanced breast cancer: Subgroup analyses from the phase III SOLAR-1 trial. *Cancer Res.* **2019**, *79*, GS3-08.
62. 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. [\[CrossRef\]](#) [\[PubMed\]](#)
63. Robson, M.; Im, S.-A.; Senkus, E.; Xu, B.; Domchek, S.M.; Masuda, N.; Delalogue, S.; Li, W.; Tung, N.; Armstrong, A.; et al. Olaparib for Metastatic Breast Cancer in Patients with a Germline BRCA Mutation. *N. Engl. J. Med.* **2017**, *377*, 523–533. [\[CrossRef\]](#) [\[PubMed\]](#)
64. Litton, J.; Rugo, H.S.; Ettl, J.; Hurvitz, S.A.; Goncalves, A.; Lee, K.-H.; Fehrenbacher, L.; Yerushalmi, R.; Mina, L.A.; Martin, M.; et al. Talazoparib in Patients with Advanced Breast Cancer and a Germline BRCA Mutation. *N. Engl. J. Med.* **2018**, *379*, 753–763. [\[CrossRef\]](#) [\[PubMed\]](#)
65. Vidula, N.; Isakoff, S.; Niemierko, A.; Malvarosa, G.; Park, H.; Abraham, E.; Spring, L.; Peppercorn, J.; Moy, B.; Ellisen, L.; et al. Abstract PD1-13: Somatic BRCA mutation detection by circulating tumor DNA analysis in patients with metastatic breast cancer: Incidence and association with tumor genotyping results, germline BRCA mutation status, and clinical outcomes. *Cancer Res.* **2018**, *78*, PD1-13.
66. Condorelli, R.; Spring, L.; O’Shaughnessy, J.; Lacroix, L.; Bailleux, C.; Scott, V.; Dubois, J.; Nagy, R.; Lanman, R.; Iafrate, A.; et al. Polyclonal RBT mutations and acquired resistance to CDK 4/6 inhibitors in patients with metastatic breast cancer. *Ann. Oncol.* **2018**, *29*, 640–645. [\[CrossRef\]](#)
67. Abbosh, C.; Swanton, C. Circulating tumour DNA analyses reveal novel resistance mechanisms to CDK inhibition in metastatic breast cancer. *Ann. Oncol.* **2018**, *28*, 535–537. [\[CrossRef\]](#)
68. Turner, N.C.; Kingston, B.; Kilburn, L.S.; Kernaghan, S.; Wardley, A.M.; MacPherson, I.R.; Baird, R.D.; Roylance, R.; Stephens, P.; Oikonomidou, O.; et al. Circulating tumour DNA analysis to direct therapy in advanced breast cancer (plasmaMATCH): A multicentre, multicohort, phase 2a, platform trial. *Lancet Oncol.* **2020**, *21*, 1296–1308. [\[CrossRef\]](#)
69. Cortazar, P.; Zhang, L.; Untch, M. Meta-analysis results from the collaborative trials in neoadjuvant breast cancer (CTNeoBC). *Cancer Res.* **2012**, *72*, S1–S11.
70. Rothé, F.; Silva, M.J.; Venet, D.; Campbell, C.; Bradbury, I.; Rouas, G.; De Azambuja, E.; Maetens, M.; Fumacalli, D.; Rodrik-Outmezguine, V.; et al. Circulating tumor DNA in HER2-amplified breast cancer

71. McDonald, B.R.; Contente-Cuomo, T.; Sammut, S.-J.; Odenheimer-Bergman, A.; Ernst, B.; Perdigones, N.; Chin, S.-F.; Farooq, M.; Mejia, R.; Cronin, P.A.; et al. Personalized circulating tumor DNA analysis to detect residual disease after neoadjuvant therapy in breast cancer. *Sci. Transl. Med.* **2019**, *11*, eaax7392. [[CrossRef](#)]
72. Riva, F.; Bidard, F.-C.; Houy, A.; Saliou, A.; Madic, J.; Rampanou, A.; Hego, C.; Milder, M.; Cottu, P.; Sablin, M.-P.; et al. Patient-Specific Circulating Tumor DNA Detection during Neoadjuvant Chemotherapy in Triple-Negative Breast Cancer. *Clin. Chem.* **2017**, *63*, 691–699. [[CrossRef](#)] [[PubMed](#)]
73. Radovich, M.; Jiang, G.; Hancock, B.A.; Chitambar, C.; Nanda, R.; Falkson, C.; Lynce, F.C.; Gallagher, C.; Isaacs, C.; Blaya, M.; et al. Association of Circulating Tumor DNA and Circulating Tumor Cells After Neoadjuvant Chemotherapy with Disease Recurrence in Patients with Triple-Negative Breast Cancer: Preplanned Secondary Analysis of the BRE12-158 Randomized Clinical Trial. *JAMA Oncol.* **2020**, *6*, 1410–1415. [[CrossRef](#)] [[PubMed](#)]

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Review

Novel HER-2 Targeted Therapies in Breast Cancer

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Simple Summary: HER-2-targeting drugs changed the paradigm of HER-2-positive breast cancer, improving survival rates of an aggressive and lethal disease. Recently, a plethora of new drugs have been approved in different settings, leading to a continuous change in the treatment algorithm of HER-2-positive breast cancer. This current review aims to summarize all the new approved therapeutic options and highlight the new therapeutic options under development.

Abstract: Human epidermal growth factor 2 (HER-2)-positive breast cancer represents 15–20% of all breast cancer subtypes and has an aggressive biological behavior with worse prognosis. The development of HER-2-targeted therapies has changed the disease's course, having a direct impact on survival rates and quality of life. Drug development of HER-2-targeting therapies is a prolific field, with numerous new therapeutic strategies showing survival benefits and gaining regulatory approval in recent years. Furthermore, the acknowledgement of the survival impact of HER-2-directed therapies on HER-2-low breast cancer has contributed even more to advances in the field. The present review aims to summarize the newly approved therapeutic strategies for HER-2-positive breast cancer and review the new and exploratory HER-2-targeted therapies currently under development.

Keywords: breast cancer; HER-2; novel therapies



Citation: Fernandes, C.L.; Silva, D.J.; Mesquita, A. Novel HER-2 Targeted Therapies in Breast Cancer. *Cancers* **2024**, *16*, 87. <https://doi.org/10.3390/cancers16010087>

Received: 18 November 2023

Revised: 13 December 2023

Accepted: 21 December 2023

Published: 23 December 2023



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1. Introduction

Breast cancer is estimated to be the type of cancer with the highest incidence in both sexes and the second most lethal all over the world in 2023, with an estimated 300,560 new cases and 43,700 deaths in the United States [1]. The human epidermal growth factor 2 (HER-2) overexpressing subtype accounts for 15–20% of all breast cancer cases [2] and, along with triple-negative, is historically associated with more aggressive behavior and a worse overall prognosis [3,4]. This paradigm has been changing ever since HER-2-targeted therapies were introduced in clinical practice, with excellent clinical responses and increasing survival rates.

HER-2 is one of the four transmembrane tyrosine kinase receptors in the HER family. Its activation leads to the activation of intracellular cell growth pathways, which results in abnormal cell proliferation and apoptosis evasion. Its overexpression in a subset of breast and ovary cancer patients was discovered in the late 1980s [5]. The search for an effective targeted therapy was promptly initiated, leading to the development of trastuzumab in the early 1990s and its later approval to treat HER-2-overexpressing metastatic breast cancer in 1998. Treatment with this targeted monoclonal antibody has been shown to almost double response rates and time to progression in this subset of patients [6]. Hence, ever since the approval of trastuzumab, the investigation of alternative HER-2 inhibitory agents has proliferated, mainly because trastuzumab demonstrated limited efficacy and resistance mechanisms started to come to light [7–9].

Regarding this, the search for better treatment options for HER-2-overexpressing breast cancer continues to this day. Current treatment options under investigation include not only

HER-2-targeted therapies [monoclonal antibodies, tyrosine kinase inhibitors (TKIs) and antibody-drug conjugates (ADCs)], but also combination therapies with agents from other classes, such as cyclin-dependent kinase (CDK) inhibitors, immune-checkpoint inhibitors (ICIs) and cell-therapies, among others. There are currently 662 ongoing interventional studies registered in clinicaltrials.gov for HER-2-positive breast cancer [10], and the number keeps growing.

The present review aims to summarize approved and in-development therapies for HER-2-overexpressing breast cancer and provide a comprehensive perspective on future directions in the treatment of this subset of patients.

2. HER-2 Biology and Cellular Mechanisms

2.1. HER-2 Receptor and Signaling Pathway

HER-2 belongs to the family of HER tyrosine kinase transmembrane receptors, which includes HER-1/epidermal growth factor (EGFR), HER-2, HER-3 and HER-4. Whenever activated by a ligand, these receptors stabilize in the cell membrane through homo- or heterodimerization, and hence activate intracellular signaling pathways—mitogen-activated protein kinase (MAPK), phosphatidylinositol 3-kinase (PI3K), AKT and the mammalian target of rapamycin-dependent [11]. These pathways lead to cell division and growth, essential to cancer's proliferation. The HER family of receptors was found to play an important role in physiological breast tissue growth and differentiation. HER-2 is composed of an extracellular ligand-binding domain (subdivided into four subdomains, I-IV), a transmembrane domain and an intracellular tyrosine kinase domain. Subdomain IV is the one closest to the cell membrane, making it essential to the stabilization of the receptor, while subdomain II is the one structurally more exposed, thus playing an important role in heterodimerization with other HER receptors [12]. HER-2 is the only HER family receptor that does not have a physiological ligand, so it relies on heterodimerization with other HER receptors, particularly HER-3, to activate downstream signaling pathways. The HER-2 receptor is encoded by the HER-2 (also known as *neu* or *c-erbB-2*) oncogene and was found to be overexpressed in a large number of solid tumors, such as breast, lung, colorectal and gastric cancers [13]. Its overexpression and abnormal ligand-independent activation in cancer cells leads to uncontrolled cell proliferation, evasion of apoptosis and high metastatic potential, thus fulfilling four of the fourteen described hallmarks of cancer [14].

2.2. HER-2 Pathological Classification

According to American Society of Clinical Oncology–College of American Pathologists (ASCO–CAP) guidelines, HER-2 positivity is defined as a 3+ score in immunohistochemistry (IHC) staining in $\geq 10\%$ of tumor cells or a 2+ score with HER-2 gene amplification through in situ hybridization (ISH) [15].

Recently, the concept of HER-2 low was established, regarding sensitivity to some anti-HER-2 therapies. It is defined as a 2+ score in IHC staining with absence of HER-2 amplification by ISH or simply a 1+ score in IHC, the latter with no need to search for ISH amplification [15].

3. Overview of Currently Approved HER-2-Targeted Therapies

Since the initial approval of trastuzumab in 1998, many other HER-2-targeted agents have been developed and some of them have been incorporated into clinical practice, integrating into an increasingly complex therapeutical algorithm.

3.1. Monoclonal Antibodies

3.1.1. Trastuzumab

Status: Approved worldwide for use in adjuvant, neoadjuvant and metastatic settings. Trastuzumab was the first-ever HER-2-targeted monoclonal antibody (mAb) to be developed. It consists of a humanized immunoglobulin (Ig) G1 mAb that inhibits HER-2 by binding to its extracellular subdomain IV. Besides HER-2 signaling pathway inhibition,

leading to cell cycle arrest, trastuzumab also induces antibody-dependent cell-mediated cytotoxicity (ADCC) [16,17] and antibody-dependent cell phagocytosis (ADCP) [18], both mediated by trastuzumab Fc-portion's interaction with Fcγ receptors in the membrane of immune effector cells, such as natural killer (NK) cells and macrophages.

Early studies demonstrated a synergistic effect between trastuzumab and chemotherapy [19], which led to the first clinical trials testing this combination, eventually resulting in trastuzumab approval in combination with chemotherapy in HER-2-overexpressing metastatic breast cancer [6] and later in early breast cancer [20] as well.

Trastuzumab is currently approved (see Table 1):

- (a) In the metastatic setting, in the first line in combination with docetaxel for 6 cycles and then maintenance with pertuzumab until disease progression, regarding its overall survival benefit when compared with trastuzumab only with docetaxel (56.5 versus 40.8 months, hazard ratio (HR) 0.68) [21], or in monotherapy when the patient has contraindication to taxanes and/or pertuzumab [16];
- (b) In the neoadjuvant setting in combination with pertuzumab and taxane for locally advanced, inflammatory or early high-risk tumors, regarding its advantage in 5-year progression-free survival (86% versus 81%, HR 0.69) and in 5-year disease-free survival (84% versus 81%), when compared with trastuzumab plus chemotherapy [22];
- (c) In the adjuvant setting in combination with a taxane for 12 weeks and then as maintenance to complete 17 cycles, which demonstrated a 3-year invasive disease-free survival of 98.7% [23] or in monotherapy for one year after neoadjuvant therapy in patients achieving pathological complete response (pCR), which showed a 5-year disease-free survival of 84% and a 5-year overall survival of 92% [20].

3.1.2. Pertuzumab

Status: Approved worldwide for use in neoadjuvant, adjuvant and metastatic settings.

Pertuzumab is also a humanized mAb but, unlike trastuzumab, it binds the HER-2 subdomain II, preventing HER-2 dimerization with other HER family receptors, specially HER-3 [9]. This precludes HER-2 subsequent mitogenic signaling and thus cell growth and tumor proliferation. Like trastuzumab, pertuzumab also induces ADCC and ADCP, which intensifies its antitumoral activity.

Phase 2 studies showed a good safety profile but limited efficacy with pertuzumab in monotherapy [24]. Pertuzumab and trastuzumab combination therapy was then tested, revealing a favorable safety profile as well as major survival benefits [21].

Pertuzumab is currently approved (see Table 1):

- (a) In the metastatic setting, in first line in combination with docetaxel for 6 cycles and maintenance with trastuzumab until disease progression [21], as mentioned above;
- (b) In the neoadjuvant setting, in combination with trastuzumab and docetaxel to treat HER-2-positive early breast cancer with ≥ 2 cm [22];
- (c) In the adjuvant setting, in combination with trastuzumab for 1 year after combination with taxane in node-positive or high-risk node-negative disease, in which it demonstrated a 3-year invasive disease-free survival of 92% [25].

There is an ongoing phase 2 clinical trial testing the safety of omitting chemotherapy associated with trastuzumab and pertuzumab in the neoadjuvant setting (NCT01817452).

3.1.3. Margetuximab

Status: Approved in the United States of America (USA) for use in the metastatic setting.

Margetuximab, previously called MGH22, is a mAb targeting subdomain IV of the HER-2 receptor, the same as trastuzumab. It has similar receptor affinity, but it differs in its engineered Fc-portion, which allows higher affinity to Fcγ receptor CD16A and lower affinity to inhibitory Fcγ receptor CD32B, leading to increased CD16A-mediated ADCC [26].

When compared to trastuzumab, margetuximab revealed a similar toxicity profile and progression-free survival (PFS) benefit, with no overall survival (OS) benefit in the third line and beyond in a phase 3 study for HER-2-overexpressing metastatic breast cancer [27].

Margetuximab is currently approved (see Table 1):

- (a) In the metastatic setting, in combination with chemotherapy in third line and beyond, showing a 9-month benefit in progression-free survival, although no benefit in overall survival when compared with trastuzumab [27].

An ongoing phase 2 study is testing the replacement of trastuzumab with margetuximab in the neoadjuvant setting, in association with pertuzumab and paclitaxel (NCT04425018).

Table 1. Summary of monoclonal antibodies (mAb) currently in use.

mAb	Target	Approved Use	Reference
Trastuzumab	HER-2 subdomain IV	Neoadjuvancy (+pertuzumab +ChT)	[22]
		Adjuvancy (+ChT or monotherapy)	[20,23]
		Palliative ($\pm$ pertuzumab $\pm$ ChT)	[21]
Pertuzumab	HER-2 subdomain II	Neoadjuvancy/Adjuvancy (+trastuzumab $\pm$ ChT)	[22,25]
		Palliative (+trastuzumab $\pm$ ChT) in 1L	[21]
Margetuximab	HER-2 subdomain IV	Palliative (+ChT) in $\geq$ 3L	[27]

ChT: Chemotherapy; 1L: First line; 3L: Third line.

3.2. Tyrosine Kinase Inhibitors (TKIs)

3.2.1. Lapatinib

Status: Approved worldwide for use in the metastatic setting.

Lapatinib is a potent and reversible tyrosine kinase inhibitor (TKI) of HER-2 and HER-1/EGFR. It competes with adenosine triphosphate (ATP) in its binding site to the two kinases, due to their similar structure, thereby inhibiting downstream signaling and cell proliferation [28]. Lapatinib also showed efficacy against truncated p95HER-2, which is resistant to trastuzumab extracellular binding and blockade [29].

Lapatinib proved to be effective in the treatment of HER-2-overexpressing metastatic breast cancer, in combination with capecitabine or trastuzumab, after progression with first-line trastuzumab [30]. It also showed benefits in association with endocrine therapy in hormonal receptors (HR)-positive and HER-2-positive breast cancer. Studies in the adjuvant setting showed no significant benefit and increased toxicity when adding or replacing trastuzumab with lapatinib [31,32]. In the neoadjuvant setting, lapatinib showed no benefit in the rate of pathological complete response (pCR) when compared with trastuzumab [33] but it showed a significant benefit when combined with trastuzumab and chemotherapy [34,35]. However, it is not yet approved in this setting.

Besides being active against part of trastuzumab-resistant tumor cells, lapatinib crosses the blood–brain barrier due to its small molecular size and has a significant intracranial antitumoral effect [36].

Lapatinib is currently approved (see Table 2):

- (a) In the metastatic setting, in combination with capecitabine, which proved a 4-month benefit in time to progression (8.4 versus 4.4 months, HR 0.49) and/or trastuzumab [30].

3.2.2. Neratinib

Status: Approved in the USA and Europe in the adjuvant setting and in the USA in the metastatic setting.

Neratinib, also called HKI-272, has an irreversible and pan-inhibitor effect on HER kinases, acting by covalently binding to a cysteine residue in the ATP binding site, specifically, residues Cys-773 in HER-2 and Cys-805 in HER-1 [37,38]. It also leads to the dissociation and degradation of HSP90.

Neratinib did not show any benefit in the metastatic setting in HER-2-overexpressing breast cancer when compared to lapatinib and capecitabine [39,40], but it was revealed to be effective when in combination with capecitabine [41], warranting its approval in the metastatic setting beyond third line. In the early-disease setting, neratinib showed a benefit in recurrence-free survival when used for one year in extended adjuvant therapy after 2-year neoadjuvant/adjuvant trastuzumab therapy, specifically in HR-positive patients [42]. In the neoadjuvant setting, neratinib showed benefits when compared to trastuzumab [43] and when added to it [44].

Neratinib is currently approved (see Table 2):

- (a) In the metastatic setting, in combination with capecitabine, in third line and beyond, showing a 2.2-month benefit in progression free-survival (8.8 versus 6.6 months, HR 0.76) [41].
- (b) In the adjuvant setting, for 1 year, after completing 1 year of adjuvant trastuzumab, regarding its 5-year invasive disease-free survival benefit of 2.5% (90.2% versus 87.7%, HR 0.73) [42].

Three phase 2 trials are currently evaluating neratinib in the neoadjuvant setting (NCT01042379; NCT04886531; NCT05919108) and one in the adjuvant setting in patients without pCR after neoadjuvant treatment (NCT05388149).

3.2.3. Tucatinib

Status: Approved worldwide for use in the metastatic setting.

Tucatinib is a reversible HER-2-selective TKI, with very little EGFR inhibition, which guarantees a more favorable tolerability profile than other TKIs [45]. Its exact mechanism of action is not yet clarified, but a molecular study points to a strong and stable binding to the ATP pocket in HER-2 tyrosine kinase [46]. Tucatinib demonstrated greater efficacy, in combination with capecitabine and trastuzumab, in third-line treatment of HER-2-overexpressing metastatic breast cancer with or without brain metastases [47].

Tucatinib is currently approved (see Table 2):

- (a) In the metastatic setting, beyond third-line treatment, in combination with capecitabine and trastuzumab. This combination proved to increase progression-free survival by 2.7 months (7.6 versus 4.9 months, HR 0.57) and overall survival by 5.5 months (24.7 versus 19.2 months, HR 0.73) when compared with trastuzumab plus capecitabine [47].

A phase 3 trial, HER2CLIMB-05, is currently evaluating the efficacy of tucatinib associated with trastuzumab and pertuzumab in patients not previously treated with a TKI (NCT05132582). There are four other phase 2 studies evaluating combinations of tucatinib, trastuzumab and different chemotherapy agents in the palliative setting (NCT05748834; NCT05458674; NCT05583110; NCT05955170). The previously mentioned phase 2 basket trial in the neoadjuvant setting also has one arm evaluating the efficacy of neoadjuvant tucatinib combined with trastuzumab and pertuzumab in HER-2-positive breast cancer patients (NCT01042379).

3.2.4. Pyrotinib

Status: Approved in China for use in the metastatic setting.

Pyrotinib is the most recently developed and approved TKI for HER-2-overexpressing metastatic breast cancer. It is an irreversible pan-HER TKI that covalently binds to the catalytic region of the HER-2 kinase [48]. It has proved, so far, to be effective in the metastatic setting in combination with capecitabine when compared to placebo or lapatinib in Chinese patients [49,50]. Pyrotinib is not yet approved for marketing in the European Union nor in the United States of America as a result of the lack of data on efficacy, tolerability and cost-effectiveness in a broader population [51].

Pyrotinib is currently approved (see Table 2):

- (a) In the metastatic setting in combination with capecitabine (only in China), showing a significantly higher progression-free survival (18.1 versus 7.0 months, HR 0.36) and

higher overall response rate (78.5% versus 57.1%) when compared with lapatinib and capecitabine [49,50].

There are multiple ongoing clinical trials involving pyrotinib, mostly held in China. There are five studies in the metastatic setting (NCT05346861, NCT05255523, NCT04605575, NCT05429294, NCT04246502), with one phase 3 study and two studies in first-line treatment. There are six ongoing studies in the adjuvant setting (NCT04254263, NCT05841381, NCT05861271, NCT04659499, NCT05880927, NCT05834764), two of them phase 3, and seven studies in the neoadjuvant setting (NCT04929548, NCT05659056, NCT06000917, NCT04917900, NCT05430347, NCT04900311, NCT04290793).

Table 2. Summary of tyrosine kinase inhibitors approved in clinical practice.

TKI	Target	Effect	Approved Use	Reference
Lapatinib	HER-1 and 2	reversible	Palliative (+capecitabine and/or trastuzumab)	[30]
Neratinib	HER-1, 2 and 4	irreversible	Adjuvant (after 1y trastuzumab ±pertuzumab) Palliative (+capecitabine)	[42] [41]
Tucatinib	HER-2	irreversible	Palliative (+capecitabine +trastuzumab)	[47]
Pyrotinib	HER-1, 2 and 4	irreversible	Palliative (+capecitabine)	[49,50]

3.3. Antibody-Drug Conjugates (ADCs)

3.3.1. Trastuzumab Emtansine

Status: Approved worldwide for use in the metastatic and adjuvant settings.

Trastuzumab emtansine (T-DM1) is the first-ever approved antibody–drug conjugate for solid tumors, approved for use in HER-2-positive metastatic breast cancer since 2013 [52] and in the adjuvant setting since 2019 [53]. It is composed of the IgG1 mAb trastuzumab connected to emtansine, a cytotoxic agent that acts by microtubule inhibition, through a non-cleavable linker [54].

Studies demonstrated T-DM1's superiority in terms of rate of response, survival and toxicity profile, when compared with the previous standard second-line treatment, lapatinib and capecitabine, leading to its early approval in this setting [52]. Later studies also showed its efficacy in the adjuvant setting [53].

T-DM1 is currently approved (see Table 3):

- In the metastatic setting, as second-line treatment for HER-2-overexpressing metastatic breast cancer after trastuzumab therapy, as the EMILIA trial showed a 3.2-month increase in progression-free survival (9.6 versus 6.4 months, HR 0.65) [52] and a 4-month increase in overall survival (29.9 versus 25.9 months, HR 0.75) [55] when compared with lapatinib plus capecitabine;
- In the adjuvant setting, for residual disease after neoadjuvant chemotherapy combined with trastuzumab and pertuzumab, showing an increase in invasive disease free survival (88.3% versus 77.0% at 3 years, HR 0.50) when compared with trastuzumab [53].

A recent and still unpublished phase 2 trial, ATEMPT, evaluated the efficacy of adjuvant T-DM1 in stage I disease, compared with paclitaxel and trastuzumab (NCT01853748).

3.3.2. Trastuzumab Deruxtecan

Status: Approved in the USA and Europe for use in the metastatic setting.

Trastuzumab deruxtecan (T-DXd) is composed of the mAb trastuzumab connected to deruxtecan, a topoisomerase I inhibitor that causes cell apoptosis and double-stranded DNA breaks. These two compounds are connected by a linker that is cleaved by cathepsins, leading to its selective cleavage inside tumor cells, where cathepsins are upregulated. The cleavability of the ADC also allows a bystander killer effect, a mechanism that expands the drug's cytotoxic effect to surrounding tumor cells. It also differs from T-DM1 in its higher antibody:drug ratio of 1:8 (T-DM1 has a 1:3.5) [56].

T-DXd proved to be effective in monotherapy in the treatment of HER-2-overexpressing refractory metastatic breast cancer [57,58], even when compared with T-DM1 [59]. More recent studies demonstrated that T-DXd is also effective in the treatment of HER-2-low metastatic breast cancer [60].

T-DXd is currently approved (see Table 3):

- (a) In the metastatic setting in HER-2-positive breast cancer, in the second line or further, regarding its benefit in delaying disease progression (75.8% of patients had no disease progression at 12 months versus 34.1% with T-DM1, HR 0.28) and delaying death (94.1% were alive at 12 months compared with 85.9%, HR 0.55) [59];
- (b) In the metastatic setting in HER-2-low breast cancer, regarding its 4.7-month benefit in progression-free survival when compared with physician's choice chemotherapy (HR 0.51) and its 6.4-month benefit in overall survival (HR 0.64) [60].

There are currently two ongoing trials (phase 2 and 3) evaluating T-DXd as a first-line treatment in metastatic HER-2-positive breast cancer and one study evaluating it as a first-line treatment in HER-2-low patients (see Table 4). A phase 3 study is testing T-DXd as an adjuvant therapy after neoadjuvant treatment with residual disease. Three other studies, one phase 3, are evaluating the role of T-DXd in the neoadjuvant setting (see Table 4).

Table 3. Summary of antibody–drug conjugates approved in clinical practice.

ADC	Cytotoxic	Linker	Drug-to-Antibody Ratio	Approved Use	Reference
T-DM1	Emtansine (DM1)	Non-cleavable	3.5:1	Adjuvant HER-2+ EBC HER-2+ MBC ≥ 2L	[53] [52]
T-DXd	Deruxtecan (DXd)	Cleavable	8:1	HER-2+ MBC ≥ 2L HER-2-low MBC	[58,59] [60]

EBC: Early breast cancer; MBC: Metastatic breast cancer; 2L: Second line; 3L: Third line.

Table 4. Summary of ongoing clinical trials involving HER-2-targeted agents already in use in clinical practice.

Anti-HER-2 Agent	Clinical Trials (Phase)	Population	Treatment in Study	Status
Trastuzumab	-	-	-	-
Pertuzumab	NCT01817452 (II)	HER-2+ EBC	Neoadjuvant P+T ± ChT	Recruiting
Margetuximab	NCT04262804 (II) NCT04425018 (II)	HER-2+ MBC ≥ 3L HER-2+ EBC	Margetuximab + ChT Neoadj margetuximab + P + ChT	Completed; unpublished Recruiting
Lapatinib	-	-	-	-
Neratinib	NCT01042379 (II) NCT04886531 (II) NCT05919108 (II) NCT05388149 (II)	HER-2+ EBC HER-2+ EBC HER-2m EBC HER-2+ EBC	Neoadj neratinib Neoadj neratinib + T + ET Neoadj neratinib Adjuvant neratinib + T-DM1	Recruiting Recruiting Not yet recruiting Recruiting
Tucatinib	NCT05132582 (III) NCT05748834 (II) NCT05458674 (II) NCT05583110 (II) NCT05955170 (II) NCT01042379 (II)	HER-2+ MBC HER-2+ MBC HER-2+ MBC HER-2+ MBC HER-2+ MBC HER-2+ EBC	Tucatinib + T + P Tucatinib + ChT Tucatinib + T + ChT Tucatinib + T + ChT Tucatinib + T + ChT Neoadj tucatinib + T + P	Recruiting Recruiting Recruiting Recruiting Not yet recruiting Recruiting
Pyrotinib	NCT05346861 (III) NCT05255523 (II) NCT04605575 (II) NCT05429294 (II) NCT04246502 (II) NCT04254263 (III) NCT05841381 (III) NCT05861271 (II) NCT04659499 (II) NCT05880927 (II) NCT05834764 (II)	HER-2+ MBC HER-2+ MBC HER-2+ MBC HER-2+ MBC HER-2+ MBC HER-2+ EBC HER-2+ EBC HER-2+ EBC HER-2+ EBC HER-2+ EBC HER-2+ EBC	Pyrotinib rechallenge Pyrotinib + T in ≥ 2L Pyrotinib + ChT Pyrotinib + T + ChT in 1L Pyrotinib + ChT in 1L Adj pyrotinib + T Adj pyrotinib + T + ChT Adj pyrotinib + ChT Adj pyrotinib + ChT Adj pyrotinib Adj pyrotinib	Recruiting Not yet recruiting Recruiting Recruiting Not yet recruiting Recruiting Not yet recruiting Not yet recruiting Not yet recruiting Recruiting Recruiting

Table 4. Cont.

Anti-HER-2 Agent	Clinical Trials (Phase)	Population	Treatment in Study	Status
Pyrotinib	NCT04929548 (II)	HER-2+ EBC	Neoadj pyrotinib + T + P	Not yet recruiting
	NCT05659056 (II)	HER-2+ EBC	Neoadj pyrotinib + T + ChT	Recruiting
	NCT06000917 (II)	HER-2+ EBC	Neoadj pyrotinib + T + ChT	Recruiting
	NCT04917900 (II)	HER-2+ EBC	Neoadj pyrotinib + T + ChT	Recruiting
	NCT05430347 (II)	HER-2+ EBC	Neoadj pyrotinib + T + ChT	Not yet recruiting
	NCT04900311 (II)	HER-2+ EBC	Neoadj pyrotinib + T + ChT	Not yet recruiting
T-DM1	NCT04290793 (II/III)	HER-2+ EBC	Neoadj pyrotinib + ChT	Not yet recruiting
	NCT01853748 (II)	HER-2+ EBC	Adj T-DM1	Active, not recruiting
T-DXd	NCT04784715 (III)	HER-2+ MBC	T-DXd ± P in 1L	Recruiting
	NCT05744375 (II)	HER-2+ MBC	T-DXd in 1L	Recruiting
	NCT04622319 (III)	HER-2+ EBC	Adj T-DXd	Recruiting
	NCT05113251 (III)	HER-2+ EBC	Neoadj T-DXd ± T + P + ChT	Recruiting
	NCT05900206 (II)	HER-2+ EBC	Neoadj T-DXd	Recruiting
	NCT05704829 (II)	HER-2+ EBC	Neoadj T-DXd	Not yet recruiting
	NCT05953168 (II)	HER-2-low MBC	T-DXd in 1L	Not yet recruiting

Adj: Adjuvant; Neoadj: Neoadjuvant; ChT: Chemotherapy; EBC: Early breast cancer; ET: Endocrine therapy; MBC: Metastatic breast cancer; P: Pertuzumab; T: Trastuzumab; 1L: First line; 2L: Second line.

4. Novel HER-2-Targeted Therapies

Despite the great evolution in HER-2-targeted therapies and the outstanding improvement in HER-2-overexpressing breast cancer prognosis, there is still a long way to go in HER-2 breast cancer treatment. In the early setting, there is still a considerable number of relapses, and, in the metastatic setting, most cases eventually progress under HER-2-targeted therapy, due to either suboptimal tumor cell growth inhibition or mechanisms of resistance. For these reasons, there is continuous research and new drug development in this area, which is summarized below.

4.1. Novel Monoclonal Antibodies

4.1.1. MM-302

Status: Phase 1 clinical trials.

MM-302 is a liposome containing approximately 20,000 molecules of doxorubicin, and it is coated by 45 surface anti-HER-2 antibodies [61]. Preclinical studies demonstrated the efficacy of this drug in HER-2-overexpressing breast and gastric cancer and showed synergy with trastuzumab. A phase 2 clinical trial testing MM-302 in combination with trastuzumab in heavily pretreated patients showed, however, no clinical benefit, leading to the study's early termination [62].

4.1.2. Inetetamab

Status: Phase 2 clinical trials.

Inetetamab, also called cipterbin, is a novel anti-HER-2 mAb directed against subdomain IV of HER-2, with a slightly different amino acid sequence in the Fc portion. Preclinical studies demonstrated efficacy against HER-2-overexpressing tumors [63,64]. A recently published retrospective study reported that inetetamab combined with vinorelbine and pyrotinib was effective in heavily pretreated HER-2-positive breast cancer patients [65].

An ongoing phase 2 trial is evaluating the role of inetetamab in the neoadjuvant setting, in combination with pertuzumab and chemotherapy. Five phase 2 studies are testing the combination of inetetamab with pyrotinib and chemotherapy in the metastatic setting, in first or subsequent lines (see Table 5).

Table 5. Summary of ongoing or recent clinical trials involving monoclonal antibodies (mAb) and bispecific antibodies (bsAb).

mAb/bsAb	Ongoing Clinical Trials (Phase)	Population	Treatment in Study	Status
MM-302	None	-	-	-
Inetetamab	NCT05749016 (II)	HER-2+ EBC	Neoadj inetetamab + P + ChT	Recruiting
	NCT05823623 (II)	HER-2+ MBC	Inetetamab + pyrotinib + ChT	Recruiting
	NCT04681911 (II)	HER-2+ MBC	Inetetamab + pyrotinib + ChT	Recruiting
	NCT05823623 (II)	HER-2+ MBC	Inetetamab + pyrotinib + ChT	Recruiting
	NCT05621434 (II)	HER-2+ MBC	Inetetamab + pyrotinib + ChT in 1L	Recruiting
	NCT04963595 (II)	HER-2+ MBC	Inetetamab + pyrotinib + ChT in 1L	Not yet recruiting
Zanidatamab (ZW25)	NCT05027139 (I)	HER-2+ MBC	ZW25	Concluded; unpublished
	NCT04224272 (II)	HER-2+ /HR+ MBC	ZW25 + palbociclib + fulvestrant	Concluded; unpublished
	NCT05027139 (Ib/II)	HER-2+ MBC	ZW25 + ALX148	Recruiting
	NCT05035836 (II)	HER-2+ EBC	Neoadj ZW25	Recruiting
	NCT01042379 (II)	HER-2+ EBC	Neoadj ZW25	Recruiting
MBS301	NCT03842085 (I)	HER-2+ MBC	MBS301	Recruiting
Anbenitamab (KN026)	NCT03847168 (I)	HER-2+ MBC	KN026	Concluded; unpublished
	NCT04165993 (II)	HER-2+ /low MBC	KN026 + ChT	Active, not recruiting
	NCT04881929 (II)	HER-2+ EBC	Neoadj KN026 + ChT	Recruiting
Zenocutuzumab (MCLA-128)	NCT02912949 (I)	HER-2+	MCLA-128	Published
	NCT03321981 (II)	HER-2+ /HER-2-low /HR+ MBC	MCLA-128 + T + ChT / + ET	Active, not recruiting
HER(Per)-S-Fab	None	-	-	-
HER2-2XCD16	None	-	-	-

Adj: Adjuvant; ChT: Chemotherapy; EBC: Early breast cancer; MBC: Metastatic breast cancer; Neoadj: Neoadjuvant; P: Pertuzumab; T: Trastuzumab.

4.2. Bispecific HER-2-Targeted Antibodies

Bispecific antibodies were developed with the aim of targeting different epitopes or domains in the HER-2 receptor, optimizing its blockade, or even targeting a different receptor besides HER-2, such as HER-3. This receptor plays an essential role in HER-2/HER-3 dimerization and downstream signaling of the PI3K pathway, one of the known mechanisms of resistance to HER-2-targeted therapy.

4.2.1. Zanidatamab

Status: Phase 2 clinical trials.

Zanidatamab (ZW25) is a humanized IgG1 mAb that simultaneously targets subdomains II and IV of the HER-2 extracellular component. Each HER-2 receptor can be targeted by two molecules of this drug, which enhances HER-2 internalization and downregulation [66].

Zanidatamab has proven its efficacy in second-line treatment of HER-2-positive MBC in two phase 1 studies, whether in monotherapy [67] or in combination with chemotherapy [68]. There are currently ongoing clinical trials testing zanidatamab's efficacy in combination with ADCs (NCT05027139), with CDK4/6 inhibitors (NCT04224272) and in monotherapy in the neoadjuvant setting (NCT05035836).

4.2.2. MBS301

Status: Phase 1 clinical trials.

MBS301 is a bispecific IgG1 antibody targeting extracellular subdomains II and IV. It is glycol-engineered from trastuzumab and pertuzumab, consisting of one-half of each of these mAbs. In preclinical studies, this drug showed greater efficacy when compared to the trastuzumab and pertuzumab association, mostly due to an increased ADCC stimulation [69].

A phase 1 clinical trial is still ongoing in China (NCT03842085) and no preliminary data are yet available.

4.2.3. Anbenitamab

Status: Phase 2 clinical trials.

Anbenitamab, also called KN026, is a bispecific antibody (bsAb) derived from trastuzumab and pertuzumab which showed efficacy in tumor cells resistant to these two drugs [70]. Phase 1 clinical trials showed a similar efficacy to the trastuzumab and pertuzumab combination even in heavily pretreated patients [71].

Preliminary data from two recently concluded phase 1 studies in HER-2-overexpressing breast and gastric cancer patients revealed promising antitumoral activity with good tolerability [71,72]. Ongoing clinical trials are evaluating KN026's efficacy in the neoadjuvant treatment of breast cancer and in the metastatic setting in combination with chemotherapy (see Table 5).

4.2.4. Zenocutuzumab

Status: Phase 1 clinical trials.

Zenocutuzumab (MCLA-128) is another IgG1 bsAb, but instead of targeting different subdomains of HER-2, it targets subdomain I of HER-2 and the ligand-binding site of HER-3, thereby impeding HER-2-HER-3 dimerization and downstream signaling. Besides blocking the HER-2 signaling pathway, zenocutuzumab also strongly stimulates ADCC [73].

Zenocutuzumab demonstrated efficacy in combination with trastuzumab and vinorelbine in the third line and beyond in HER-2-positive MBC [74]. A recently published phase 1 study testing this drug's efficacy in patients with NGR1 fusion demonstrated robust and durable efficacy with a good tolerability profile [75].

There is currently one ongoing phase 2 trial testing a combination with chemotherapy, trastuzumab or endocrine therapy in HER-2-positive or HER-2-low/hormonal receptor-positive breast cancer patients (NCT03321981).

4.2.5. HER2(Per)-S-Fab

Status: Preclinical studies.

HER2(Per)-S-Fab is a bispecific antibody composed of pertuzumab antigen-binding fraction (Fab), which binds the HER-2 subdomain II and an anti-CD16/FcγRIIIa antibody [76]. The anti-CD16/FcγRIIIa portion exerts an immune stimulatory effect, by recruiting natural killer (NK) cells, which express CD16, to the tumoral microenvironment (TME) and promoting its cytotoxic effects against target HER-2-positive cells [76]. There are no ongoing clinical trials with this drug.

4.2.6. HER2-2XCD16

Status: Preclinical studies.

HER2-2XCD16 is a tribody which integrates an anti-HER-2 single chain fused with IFN-γ, leading to IFN-dependent cell death, even in tumors resistant to HER-2-blockage [77]. Besides an antitumoral effect in HER-2-overexpressing breast cancer cells, this molecule demonstrated an ability to interfere with the TME, switching it to an antitumoral environment [78].

4.2.7. Discontinued Bispecific Antibodies

There are three other bispecific antibodies that showed an antitumoral effect in HER-2-positive breast cancer but were, meanwhile, discontinued, by decision of the sponsor.

MM-111 targets both the extracellular component of HER-2 and the heregulin-binding site of HER-3, inhibiting both HER-2 and PI3K pathways [79]. Preclinical studies showed efficacy against HER-2-overexpressing breast cancer cells, and when combined with trastuzumab, it demonstrated more efficacy than trastuzumab with pertuzumab [79].

Ertumaxomab targets HER-2 and CD3 and has a trimodal action through the recognition of not only tumor cells, but also stromal cells and T-cells [80,81]. Preclinical studies demonstrated a moderate antitumoral effect in both HER-2-overexpressing and HER-2-low breast cancer, with inferior efficacy when compared with standard of care [81]. Phase 2 trials of ertumaxomab were early-terminated.

ISB1302 or GBR1302 also targets CD3 in T-cells and directs them to HER-2-overexpressing tumor cells, where they bind HER-2. Phase 1 studies confirmed activation of a T-cell-mediated immune response [82], but no further studies were conducted.

4.3. Novel Tyrosine Kinase Inhibitors (TKIs)

4.3.1. Pozotinib

Status: Phase 2 clinical trials.

Pozotinib is an irreversible pan-HER TKI that inhibits EGFR, HER-2 and HER-4. Pre-clinical studies revealed an antitumoral effect in HER-2-overexpressing or HER-2-mutant breast, lung and gastric cancer lines [83], and demonstrated that pozotinib upregulates HER-2 expression in the cell membrane and potentiates the antitumoral effect of other anti-HER-2 therapies, such as T-DM1 [84]. Phase 1 studies confirmed the efficacy of pozotinib in the treatment of HER-2-positive tumors [85]. A phase 2 trial tested pozotinib's efficacy in the treatment of HER-2-overexpressing metastatic breast cancer patients in the third line and beyond, showing meaningful antitumoral efficacy with overall survival benefits and a toxicity profile similar to other TKIs [86,87]. A phase 1b (NCT03429101) and other phase 2 studies were recently conducted in metastatic breast cancer patients, in monotherapy or association with T-DM1, both in HER-2-positive (NCT02659514; NCT02418689) and HER-2-mutant patients (NCT02544997), and preliminary data is still awaited (see Table 6). There are no clinical trials currently ongoing.

Table 6. Summary of ongoing or recent clinical trials involving tyrosine kinase inhibitors (TKIs).

TKI	Target	Effect	Clinical Trials (Phase)	Population	Status
Pozotinib	Pan-HER (HER-1,2,4)	Irreversible	NCT02418689 (II)	HER2+ MBC	Published
			NCT02659514 (II)	HER+ MBC	Completed; unpublished
			NCT03429101 (Ib)	HER2+ MBC	Completed; unpublished
			NCT02544997 (II)	HER2m MBC	Completed; unpublished
DZD1516	HER-2	Reversible	NCT04509596 (I)	HER2+ MBC	Ongoing

MBC: Metastatic breast cancer; HER2m: HER-2-mutated; HER2+: HER-2 positive.

4.3.2. DZD1516

Status: Phase 1 clinical trials.

DZD1516 is a reversible HER-2-specific TKI with full blood–brain barrier penetration [88]. In preclinical studies, it revealed an antitumoral effect in HER-2-positive tumors [88]. A phase 1 study from the same author revealed good disease control in heavily pretreated HER-2-positive breast cancer patients with brain metastases [88]. Another phase 1 study is currently ongoing (NCT04509596) and preliminary data confirms good intracranial disease control with a favorable toxicity profile [89].

4.3.3. Discontinued TKIs

Epertinib, also called S-222611, is a reversible pan-HER TKI. In preclinical studies, it revealed a superior antitumoral effect in HER-2-overexpressing tumors when compared with lapatinib and a more favorable toxicity profile when compared with irreversible TKIs [90]. Phase 1 clinical trials confirmed an antitumoral effect and good tolerability in HER-2-overexpressing breast, upper digestive, head and neck and renal tumors in monotherapy [91], and in combination with trastuzumab with or without capecitabine [92]. Despite these encouraging results, no further trials were performed.

BDTX-189 is an irreversible EGFR/HER-2 TKI. Preliminary data from a recently concluded phase 1 trial, MasterKey-01, revealed an antitumoral effect in patients with HER-2/HER-3-mutated and HER-2-amplified solid tumors [93]. Despite the promising results, the drug's sponsor decided to discontinue further research.

4.4. Novel Antibody–Drug Conjugates (ADCs)

Despite the great efficacy of currently commercialized ADCs for HER-2-overexpressing breast cancer, mechanisms of resistance to T-DM1 and T-DXd have already been identified, and the development of further ADCs, with a greater therapeutic index and less toxicity, is needed, justifying continued research in this area.

4.4.1. Trastuzumab Duocarmycin (SYD985)

Status: Phase 3 clinical trials.

Trastuzumab duocarmycin is an IgG1 ADC composed of trastuzumab seco-duocarmycin-hydroxybenzamide-azaindole (seco-DUBA), a prodrug of DUBA, a DNA alkylator cytotoxic drug, in a drug-to-antibody ratio of 2.8:1, lower than both T-DM1 and T-DXd. After binding to HER-2 and being internalized, the cleavable linker binding both components of the ADC is cleaved in the lysosome, and the active drug DUBA is released in the cytoplasm [94]. SYD985 has considerable bystander killing effect for two reasons—DUBA itself is membrane-permeable after being released from the lysosome, and SYD985 cleaving proteases, like cathepsin B, are usually produced by tumor cells, meaning they are present in the extracellular milieu, thereby maximizing this ADC's effect in surrounding tumor cells [94].

Preclinical studies demonstrated SYD985's efficacy in HER-2-overexpressing breast cancer cells, with similar HER-2 binding, HER2-mediated internalization and ADCC stimulation when compared with T-DM1 [95]. Phase 1 studies confirmed efficacy not only in HER-2-overexpressing but also in HER-2-low metastatic breast cancer, with a greater efficacy in the HER-2-low subset of patients when compared with T-DM1, probably due to its potent bystander killing effect [94]. The results of the phase 3 clinical trial TULIP are still to be published but preliminary data reveal a significant improvement in progression-free survival with no significant increase in overall response rate or overall survival, when compared with physician's choice in third line and beyond HER-2-overexpressing metastatic breast cancer [96].

SYD985 is currently being tested along with other targeted therapies in the neoadjuvant setting (NCT01042379—see Table 7).

Table 7. Summary of antibody–drug conjugates (ADCs) and other antibody conjugates currently in study.

ADC/Antibody Conjugates	Payload	Antibody-to-Drug Ratio	Linker	Clinical Trial (Phase)	Setting	Status
Trastuzumab duocarmycin (SYD985)	Seco-DUBA	2.8:1	Cleavable	NCT03262935 (III) NCT01042379 (II)	HER-2+ MBC in $\geq 3L$ HER-2+ EBC (neoadj)	Closed; unpublished Recruiting
Trastuzumab rezetecan (SHR-A1811)	SHR9265	5.7:1	Cleavable	NCT06057610 (III) NCT05814354 (III) NCT05845138 (I/II) NCT05792410 (Ib/II) NCT05635487 (II)	HER-2+ MBC HER-2-low MBC HER-2-low MBC HER-2-low MBC HER-2+ EBC (neoadj)	Recruiting Recruiting Recruiting Recruiting Recruiting
ARX788	MMAF	1.9:1	Non-cleavable	NCT04829604 (II) NCT04983121 (II) NCT05426486 (II/III) NCT01042379 (II)	HER-2+ MBC HER-2+ EBC (neoadj) HER-2+ EBC (neoadj) HER-2+ EBC (neoadj)	Recruiting Recruiting Recruiting Recruiting
Disitamab vedotin (RC48)	MMAE	4:1	Cleavable	NCT03052634 (I/II) NCT05331326 (II) NCT03500380 (II/III) NCT04400695 (III) NCT05904964 (III) NCT06105008 (II) NCT05134519 (II) NCT05726175 (II)	HER-2+ /low MBC HER-2+ MBC HER-2+ MBC HER-2-low MBC HR+/HER-2-low MBC HR+/HER-2-low MBC HER-2+ EBC (neoadj) HER-2+ EBC (neoadj)	Closed; unpublished Recruiting Recruiting Recruiting Recruiting Not yet recruiting Not yet recruiting Not yet recruiting
Zanidatamab zovodotin (ZW49)	Zovodotin	2:1	Cleavable	NCT03821233 (I)	HER-2+ solid tumors	Closed; unpublished

Table 7. Cont.

ADC/Antibody Conjugates	Payload	Antibody-to-Drug Ratio	Linker	Clinical Trial (Phase)	Setting	Status
MRC002	MMAE	3.8:1	Cleavable	NCT05263869 (II) NCT04924699 (II/III) NCT04742153 (II)	HER-2+ MBC HER-2+ MBC HER-2-low MBC	Recruiting Recruiting Recruiting
A166	Duostatin-5	2:1	Cleavable	NCT03602079 (I/II) NCT05311397 (I)	HER-2+ solid tumors	Published Recruiting
ALT-P7	MMAE	2:1	Cleavable	NCT03281824 (I)	HER-2+ MBC	Closed; unpublished
XMT-1522	AF-HPA	12:1	Cleavable	NCT02952729 (I)	HER-2+ MBC	Closed; unpublished
BAY2701439	Thorium-227	Unknown	Unknown	NCT04147819 (I)	HER-2+ solid tumors	Closed; unpublished
BDC-1001	TLR-7/8 agonist	Unknown	Non-cleavable	NCT04278144 (II)	HER-2+ solid tumors Metastatic HER-2+ BC	Recruiting Recruiting

Adj: Adjuvant; EBC: Early breast cancer; MBC: Metastatic breast cancer; Neoadj: Neoadjuvant.

4.4.2. Trastuzumab Rezetecan

Status: Phase 2/3 clinical trials.

Trastuzumab rezetecan, also called SHR-A1811, is an ADC composed of trastuzumab and SHR9265, a novel topoisomerase I inhibitor derived from exatecan with a better liposolubility and cellular permeability. The two compounds are bonded by a stable and cleavable linker, in a drug-to-antibody ratio of 5.7:1 [97]. In preclinical studies, SHR-A1811 showed HER2-dependent antitumoral activity in breast and gastric cancer cell lines, resulting in a dramatic and sustained tumoral cell growth inhibition. It was also shown to have a significant bystander effect [97].

A recently published phase 1 study in HER2-expressing or -mutated solid tumors revealed that SHR-A1811 had a favorable antitumor effect with a manageable toxicity profile [98].

There are currently several ongoing trials testing SHR-A1811 in monotherapy or in combination with other agents—one phase 3 trial in HER-2-overexpressing metastatic breast cancer (NCT06057610), three in HER-2-low metastatic breast cancer (NCT05814354; NCT05845138; NCT05792410) and one in the neoadjuvant setting (NCT05635487).

4.4.3. ARX788

Status: Phase 2/3 clinical trials.

ARX788 is an ADC composed of an anti-HER-2 mAb conjugated with monomethyl auristatin F (MMAF), a highly potent synthetic auristatin derivative that acts as a microtubule inhibitor. These two components are connected by a non-cleavable linker, as in T-DM1, with a unique site-specific conjugation technology, unlike other ADCs, in which cytotoxic payloads are randomly assembled to antibodies. This ADC's drug-to-antibody ratio is 1.9:1, lower than existent ADCs [99].

Preclinical studies revealed efficacy in HER-2-overexpressing and HER-2-low breast tumor cells and even in cells resistant to T-DM1. It also showed a stronger antitumoral activity when compared with T-DM1 [99,100]. A phase 1 clinical trial confirmed its efficacy in HER-2-overexpressing metastatic breast cancer patients who progressed on previous HER-2-targeted therapies, with a manageable safety profile [101].

There are currently ongoing one phase 2 trial in the metastatic setting [(NCT04829604), and three phase 2/3 studies in the neoadjuvant setting, two in combination with the TKI pyrotinib (NCT04983121; NCT05426486) and one in monotherapy in a previously mentioned multi-arm study (NCT01042379).

4.4.4. Disitamab Vedotin (RC48)

Status: Phase 2/3 clinical trials; approved in China, not approved by FDA or EMA.

Disitamab vedotin is an ADC that combines hertuzumab (a new anti-HER-2 mAb that targets HER-2 subdomain IV in a different epitope than trastuzumab) with monomethyl-

auristatin E (MMAE), also known as vedotin, an inhibitor of tubuline polymerization during cell division [102,103]. This ADC integrates a cleavable linker and has an estimated drug-to-antibody ratio of 4:1.

In preclinical studies, RC48 demonstrated greater efficacy than trastuzumab and lapatinib against HER-2-overexpressing breast cancer cells and showed a greater antitumoral effect than T-DM1 in trastuzumab- and lapatinib-resistant tumor cells [102]. Ongoing phase 1 studies are assessing RC48's efficacy in both HER-2-overexpressing and HER-2-low breast cancer (NCT02881138; NCT03052634).

There are several ongoing phase 2/3 trials in China, testing the effect of disitamab vedotin in different subsets of breast cancer: two studies in HER-2-positive metastatic breast cancer (NCT05331326; NCT03500380); three studies in HER-2-low metastatic breast cancer (NCT04400695; NCT05904964; NCT06105008); and finally, two studies in the neoadjuvant setting of HER-2-positive breast cancer (NCT05134519; NCT05726175) (see Table 7).

Disitamab vedotin received approval in China in June 2021 for treatment of HER-2-positive locally advanced/metastatic breast cancer beyond the third line [104].

4.4.5. Zanidatamab Zovodotin (ZW49)

Status: Phase 1 clinical trials.

Zanidatamab zovodotin is an ADC that combines zanidatamab, a bsAb targeting domains II and IV of the HER-2 extracellular component, and N-acyl sulfonamide auristatin, also called zovodotin, an auristatin that inhibits microtubule polymerization during cell division and that is associated with a more favorable toxicity profile than other auristatins such as MMAE and MMAF. It is bonded by a cleavable linker and has a drug-to-antibody ratio of 2:1 [105].

A preclinical study documented the antitumoral activity of ZW49 against HER-2-overexpressing and HER-2-low breast cancer cells and registered an increased binding and internalization of this ADC when compared with trastuzumab-based ADCs [105]. A phase 1 study testing ZW49 in HER-2-overexpressing solid tumors has been recently completed (NCT03821233). Preliminary data confirmed its efficacy in monotherapy, with a manageable toxicity profile in heavily pretreated HER-2-positive solid tumors [106].

4.4.6. MRG002

Status: Phase 2/3 clinical trials.

MRG002 is an ADC composed of a recombinant humanized anti-HER-2 mAb similar to trastuzumab but with less ADCC stimulation, called MAB802, and MMAE, an auristatin previously mentioned. These compounds are connected by a protease-cleavable linker and the ADC's estimated drug-to-antibody ratio is 3.8:1 [107].

Preclinical studies showed an antitumoral effect in HER-2-overexpressing breast cancer cells and greater efficacy when compared with both trastuzumab and T-DM1, with an antitumoral effect in T-DM1-resistant cancer cells [107].

There are currently three phase 2/3 clinical trials ongoing in China, in the metastatic setting for HER-2-overexpressing (NCT05263869; NCT04924699) and HER-2-low (NCT04742153) breast cancer (see Table 7).

4.4.7. A166

Status: Preclinical studies.

A166 is a trastuzumab-based ADC that carries a duostatin-5 payload, a cytotoxic that acts by inhibiting microtubules. Duostatin-5 binds to trastuzumab by a protease-cleavable linker through a site-specific conjugation technology, in a drug-to-antibody ratio of 2:1.

Phase 1 studies revealed promising the efficacy and tolerable toxicity of A166 in HER-2-positive advanced solid tumors refractory to standard HER-2-targeted therapies [108]. There is another phase 1 trial currently ongoing in China in HER-2-overexpressing solid tumors (NCT05311397), but there are still no studies specifically in a breast cancer setting.

4.4.8. ALT-P7

Status: Phase 1 clinical trials.

ALT-P7 is another ADC composed by a trastuzumab biosimilar, HM2, and MMAE, a microtubule inhibitor, both connected by a cleavable linker in a ratio of 2:1 [109]. A phase 1 trial including HER-2 positive breast cancer patients that progressed under trastuzumab-based therapy was recently conducted (NCT03281824) and preliminary data reveals a favorable tolerability profile and indicates that there will probably be a phase 2 study [110].

4.4.9. XMT-1522

Status: Phase 1 clinical trials.

XMT-1522 is an ADC composed of an anti-HER-2 mAb, HT-19, which binds subdomain IV of HER-2 in a different epitope from trastuzumab, combined with a microtubule-inhibiting auristatin derivative, AF-HPA, in a proportion of 12:1. This ADC has this abnormally high drug-to-antibody ratio due to a biodegradable polymer-based conjugation platform that enables these high ratios without plasmatic aggregation or interference with the drug pharmacokinetics [109,111].

Preclinical studies demonstrated XMT-1522 to be effective against HER-2-overexpressing breast cancer cells, even in the presence of T-DM1 resistance, and with an approximately 100 times higher potency than T-DM1 [112]. It also showed a synergistic effect with trastuzumab and pertuzumab [113] and revealed an antitumoral effect in HER-2-low breast cancer cells [114]. A phase 1 clinical trial testing this ADC in the metastatic HER-2-overexpressing breast cancer setting (NCT02952729) was recently concluded, and preliminary data showed a good tolerability and antitumor activity [115].

4.4.10. Discontinued ADCs

PF-06804103 is composed of a trastuzumab-derived antibody associated with Aur0101, a potent microtubule polymerization inhibitor, with site-specific conjugation technology, in a proportion of 4:1 [116]. Preclinical studies showed antitumor activity against both HER-2-positive and HER-2-low breast cancer, showing greater response rates and more durable complete responses when compared with T-DM1 [117]. A recently published phase 1 trial confirmed antitumor activity in HER-2-positive and HER-2-low breast and gastric cancers but revealed significant toxicity in almost half of the patients [118], leading to the drug's discontinuation.

Alta-ADC is an ADC composed of pertuzumab and MMAE in a proportion of 2:1. It has a particularly low affinity for HER-2 at low pH, allowing its dissociation in the endosome, and subsequent pertuzumab release to bind a different HER-2 receptor in the cell membrane. This particularity allows Alta-ADC to be effective in lower doses, which warrants a more manageable toxicity profile [119]. Despite preclinical studies showing superior efficacy when compared with T-DM1 and also efficacy in HER-2-low tumors, no more studies were conducted.

MEDI4276 is an ADC that combines a HER-2-bispecific antibody, which binds HER-2 in two different epitopes from trastuzumab and pertuzumab, and a novel microtubule inhibitor called AZ13599185, both connected by a cleavable linker, in a ratio of 4:1 [120,121]. Preclinical studies revealed a 10-fold more potent antitumoral effect than T-DM1 and also efficacy in T-DM1-resistant and HER-2-low tumor cells [121]. A phase 1/2 study conducted in 2016–2017, in heavily pretreated HER-2-overexpressing breast and gastric cancer patients, showed unacceptable toxicity [122], leading to its discontinuation.

DHES0815A is composed of an anti-HER-2 mAb linked to the cytotoxic drug pyrrolo [2,1-c][1,4] benzodiazepine monoamide (PBD-MA), which creates crosslinks in DNA minor grooves, leading to DNA strand breaks [123]. A phase 1 study revealed a modest antitumoral effect with significant toxicity [124], which led to drug discontinuation in the setting of breast cancer.

4.5. Other Antibody Conjugates

4.5.1. Immune-Stimulating Antibody Conjugates (ISAC)

BDC-1001 is not an ADC, as it includes no cytotoxic molecule, but is an immune-stimulating antibody conjugate, as it carries an immune-stimulating agent. It is composed of a trastuzumab biosimilar and a TLR-7/8 agonist that stimulates antigen-presenting cells to recognize and kill HER-2-overexpressing tumor cells, promoting a durable adaptive immune response. The two components are bound by a non-cleavable linker.

In preclinical studies, BDC-1001 showed an antitumoral effect through a solid immune response [125].

There are currently ongoing two phase 2 studies, one in HER-2-overexpressing metastatic breast cancer in combination with pertuzumab (NCT05954143), and one in HER-2-overexpressing solid tumors in combination with nivolumab (NCT04278144).

4.5.2. Radionuclide–Antibody Conjugates

Thorium-227 conjugates (TTCs) are a combination of monoclonal antibodies with α -emitting radionuclides (thorium-227) that induce DNA double strand breaks, resulting in cell death, and they also have the capacity to stimulate an immunogenic response [126]. BAY2701439 is a TTC composed of an anti-HER-2, trastuzumab-derived antibody, conjugated with thorium-227. Preclinical studies demonstrated an antitumoral effect in HER-2-overexpressing tumor cells, including breast cancer cells, and in this set of patients, BAY2701439 also revealed efficacy in HER-2-low and T-DM1-resistant tumors [127]. A phase 1 clinical trial testing BAY2701439 in HER-2-overexpressing solid tumors was recently concluded, and preliminary data is awaited (NCT04147819).

4.6. Combinations of HER-2-Targeted and Other Agents

4.6.1. Combination of HER-2-Targeted and Other Targeted Agents

HER-2-positive breast cancer is a heterogeneous disease, with tumors having different levels of hormonal receptor expression and a wide range of genomic alterations. Therefore, there is a growing interest in assessing the benefit of combining HER-2-targeted agents with other targeted therapies. Table 8 lists the main ongoing trials testing the combination of HER-2-targeted and other targeted agents.

Table 8. Summary of ongoing clinical trials testing combinations of HER-2-targeted agents with other targeted agents.

Combination Agent	Clinical Trials (Phase)	Population	Treatment in Study	Status
CDK4/6 inhibitors	NCT05577442 (II)	HR+/HER-2+ MBC	Dalpiciclib + pyrotinib + ET	Not yet recruiting
	NCT05969184 (II)	HR+/HER-2+ MBC	Palbociclib + T + P + ET	Recruiting
	NCT03304080 (I/II)	HR+/HER-2+ MBC	Palbociclib + T + P + ET	Active, not recruiting
	NCT02448420 (II)	HR+/HER-2+ MBC	Palbociclib + T + ET	Active, not recruiting
	NCT03530696 (II)	HR+/HER-2+ MBC	Palbociclib + T-DM1	Completed; unpublished
	NCT03054363 (II)	HR+/HER-2+ MBC	Palbociclib + tucatinib + ET	Completed; unpublished
	NCT05076695 (II)	HR+/HER-2+ MBC	Palbociclib + pyrotinib + T + F	Recruiting
	NCT05076695 (II)	HR+/HER-2+ EBC	Neoadj palbociclib + pyrotinib + T + F	Recruiting
	NCT04858516 (II)	HR+/HER-2+ MBC	Neoadj palbociclib + pyrotinib + T + ET	Not yet recruiting
	NCT03913234 (II/III)	HR+/HER-2+ MBC	Ribociclib + T + ET	Recruiting
PARP inhibitors	NCT05319873 (I/II)	HR+/HER-2+ MBC	Ribociclib + tucatinib + T	Recruiting
	NCT02657343 (I/II)	HR+/HER-2+ MBC	Ribociclib + T/T-DM1	Completed; unpublished
PIK3CA inhibitors	NCT03368729 (I/II)	HER-2+ mBRCA MBC	Niraparib + T	Recruiting
	NCT03765983 (II)	HER-2+ mPIK3CA MBC	GDC-0084 + T	Recruiting
	NCT04108858 (I/II)	HER-2+ mPIK3CA MBC	Copanlisib + T + P	Recruiting
	NCT02705859 (I)	HER-2+ mPIK3CA MBC	Copanlisib + T	Completed
	NCT05230810 (I/II)	HER-2+ mPIK3CA MBC	Alpelisib + tucatinib	Recruiting
	NCT04208178 (III)	HER-2+ mPIK3CA MBC	Alpelisib + P + T in 1L	Active, not recruiting
ATK inhibitors	NCT05063786 (III)	HER-2+ mPIK3CA MBC	Alpelisib + T $\pm$ F	Recruiting
	NCT04253561 (I)	HER-2+ mAKT MBC	Ipatasertib + T + P	Recruiting

Adj: Adjuvant; Neoadj: Neoadjuvant; ChT: Chemotherapy; EBC: Early breast cancer; ET: Endocrine therapy; F: fulvestrant; HR: Hormone receptors; MBC: Metastatic breast cancer; mAKT: AKT mutated; mBRCA: BRCA mutated; mPIK3CA: PIK3CA mutated; P: Pertuzumab; T: Trastuzumab.

4.6.2. Combination of HER-2-Targeted Agents and Immunotherapy

Besides the direct inhibition of HER-2 signaling pathways through HER-2-targeted therapies, leading to cell cycle arrest and cell growth inhibition, there is also an important role for immune-mediated cell death in HER-2-overexpressing tumors. Ever since the development of the first HER-2-targeted drug, trastuzumab, the immune system proved to play an important role in tumor growth inhibition and apoptosis through ADCC and ADCP [17,18]. This cytotoxic mechanism is so important that its blockade by HLA class I (such as HLA-G)-mediated NK cells' inhibition constitutes an important resistance mechanism to trastuzumab [128]. Besides ADCC and ADCP, trastuzumab was also shown to stimulate the production of TGF β and IFN- γ by tumor cells and NK cells, which leads to the expression of PD-1 on NK cells [128].

These parallel mechanisms of action prove that immune stimulation may be of great importance in HER-2-positive tumor cells' death, and it may enhance HER-2 inhibition and help overcome resistance mechanisms to HER-2-targeted agents. Immune stimulation in this context has the additional advantage of triggering immune memory against HER-2 cancer cells and providing a more durable and sustained antitumoral response [129].

This premise has led to the conceptualization of novel immune-stimulating drugs and to the evaluation of the benefit of several combinations of immune checkpoint inhibitors (ICI) with HER-2-targeted agents. A phase 1 study published in 2019 confirmed that pembrolizumab in association with trastuzumab had antitumor efficacy in previously trastuzumab-resistant patients with both HER-2 and PD-L1 positivity [130]. A recently published phase 3 trial showed, however, no benefit in adding an ICI, atezolizumab, to neoadjuvant chemotherapy and double HER-2 blockade, regarding the rate of pCR [131]. Table 9 summarizes the main ongoing clinical trials testing combinations of ICI with HER-2-targeted agents.

Table 9. Summary of ongoing clinical trials evaluating the combination of HER-2-targeted agents with immune-checkpoint inhibitors (ICI).

Combination Agent	Clinical Trials (Phase)	Population	Treatment in Study	Status
Atezolizumab	NCT03199885 (III)	HER-2+ MBC	Atezolizumab + T + P + ChT	Active, not recruiting
	NCT03125928 (II)	HER-2+ MBC	Atezolizumab + T + P + ChT	Active, not recruiting
	NCT04759248 (II)	HER-2+ MBC	Atezolizumab + T + ChT	Recruiting
	NCT04740918 (III)	HER-2+ PD-L1+ MBC	Atezolizumab + T-DM1	Recruiting
	NCT04873362 (III)	HER-2+ EBC	Adj atezolizumab + T-DM1	Recruiting
	NCT03595592 (III)	HER-2+ EBC	Neoadj atezolizumab + T + P	Active, not recruiting
Durvalumab	NCT03820141 (II)	HER-2+ MBC	Durvalumab + T + P in 1L	Recruiting
	NCT05795101 (II)	HER-2+/low MBC	Durvalumab + T-DXd	Recruiting
	NCT02649686 (I)	HER-2+ MBC	Durvalumab + T	Completed
Pembrolizumab	NCT04789096 (II)	HER-2+ MBC	Pembrolizumab + tucatinib + T $\pm$ ChT	Recruiting
	NCT03032107 (Ib)	HER-2+ MBC	Pembrolizumab + T-DM1	Active, not recruiting
	NCT03988036 (II)	HER-2+ EBC	Neoadj pembrolizumab + T + P	Completed
	NCT03747120 (II)	HER-2+ EBC	Neoadj pembrolizumab + T + P + ChT	Recruiting

Adj: Adjuvant; Neoadj: Neoadjuvant; ChT: Chemotherapy; EBC: Early breast cancer; ET: Endocrine therapy; MBC: Metastatic breast cancer; P: Pertuzumab; T: Trastuzumab; 1L: First line.

5. Other exploratory Therapies in HER-2-Positive Breast Cancer

5.1. PROTACs

Proteolysis-targeting chimeras (PROTACs) are stimulators of the natural ubiquitin-proteasome system. They are composed of two ligands connected by a linker—one ligand that binds the target protein and another ligand that binds a ubiquitin ligase. The binding of PROTACs to their targets induces ubiquitylation and degradation of the target protein by the ubiquitin-proteasome system, after which the PROTAC regenerates to bind other targets [132].

Ab-PROTAC 3 is a trastuzumab-PROTAC conjugate, connected by a cleavable linker, that induces ubiquitin-proteasome-mediated degradation of bromodomain-containing 4 (BDR4), a protein that plays an essential role in DNA replication during cell division.

Preclinical studies showed that this conjugate can selectively target BDR4 in HER-2-positive tumor cells [133].

ORM-5029 is a pertuzumab–PROTAC conjugate that targets GSTP1, a protein important in protein synthesis in the ribosome and thus cell survival. Preclinical studies revealed a ten- to a thousand-fold greater potency than trastuzumab in suppressing HER-2-positive cancer cells, and it also showed an effect in HER-2-low cancer. There is a phase 1 ongoing trial testing ORM-5029 in HER-2-positive solid tumors (NCT05511844).

5.2. Cell Therapies

Cell-based immunotherapy is an innovative therapeutic modality, currently in use only in hemato-oncology, that uses genetically engineered immune cells from the patient to target and eliminate specific tags in tumor cells. After removing immune cells (T cells, NK cells or macrophages) from the patient's tumoral microenvironment to modify their receptors accordingly, these cells are expanded *ex vivo* and reimplanted in the patient's circulation to boost the patient's antitumoral immune response. Despite success in liquid tumors, implementation of cell-based therapies in solid tumors has been full of obstacles, regarding the increased difficulty of accessing immune cells in the tumor, the triggered systemic inflammatory response syndrome (SIRS) and the side effects profile [134,135].

There are currently a few ongoing phase 1 clinical trials in HER-2-positive tumors, including with CAR-T cells (NCT04511871), CAR-NK cells (NCT05385705) and with CAR-macrophages (NCT04660929).

5.3. Cancer Vaccines

Another early and exploratory therapeutical modality is cancer vaccines. Cancer vaccines grant the advantages of having a very mild and manageable toxicity profile when compared with any other antitumoral treatment, even targeted HER-2 therapies, a very favourable posology, with no need for frequent administrations, and of providing a long-term and durable immune response specifically targeted against the tumor [136,137]. Cancer vaccines can be peptide-based, protein-based, cell-based, made of dendritic cells, virus-based or made of recombinant DNA.

The first vaccine to show benefits in cancer was SipuleucelT, which proved to be effective in prostate cancer [138]. Neliipimut-S is a vaccine that combines E75, a peptide that mimics the extracellular domain of the HER-2 receptor, and granulocyte-macrophage colony-stimulating factor. Preclinical studies demonstrated E75 recognition by CD8+ lymphocytes and subsequent HER-2-positive cells death [139]. Phase 1 and 2 studies confirmed its safety and efficacy in triggering an immune response [140–142]. A phase 3 study failed, however, to show a disease-free survival benefit in HER-2-positive early breast cancer patients, leading to the study's early termination [143]. A vaccine integrating a different peptide, GLSI-100, revealed an effective immune response in a phase 2b trial [144] and is currently being evaluated in a phase 3 trial (NCT05232916).

There are several other molecules undergoing evaluation in ongoing phase 1 and 2 trials (for example, NCT04418219; NCT03384914; NCT03387553; NCT05378464; NCT05325632).

6. Conclusions and Future Directions

Ever since the discovery and description of HER-2, the landscape of HER-2-overexpressing breast cancer has been constantly evolving. Trastuzumab was the first-ever targeted drug developed, more than a quarter of a century ago, bringing a shift of paradigm in the prognosis of this subset of patients, so much that it is still part of the standard of care. Since then, several other effective targeted agents have been developed and successfully integrated into clinical practice, contributing to a continuous improvement in the treatment of this subset of patients. Despite this constant improvement, drug resistance still occurs, and the disease still progresses under HER-2-targeted therapy, mostly due to suboptimal HER-2 inhibition or to mechanisms of resistance. It makes it essential to keep investing in the development of new drugs and better therapeutical options.

Thanks to a joint effort of basic scientists, clinical investigators and the pharmaceutical industry, continued research in this area has been bringing to light multiple and promising novel HER-2-targeted drugs, which optimize HER-2 blockage and overcome some of the resistance mechanisms identified so far. There is a special focus on the recently discovered class of antibody–drug conjugates, which allow scientists to combine HER-2 targeting with a tailor-delivered cytotoxic molecule with limited toxicity, and progress in this area keeps growing. The focus has not only been on HER-2-targeted drugs; other classes of drugs have also shown a benefit and synergistic effect with anti-HER-2 therapies, thereby improving treatment efficacy and diversifying future therapeutical weapons. Even immunotherapy and other forms of immune stimulation have been showing a significant benefit in this disease previously considered “cold” and non-immune-sensitive.

Despite many encouraging discoveries in the last few years, there is still a long way to go towards improving HER-2-positive breast cancer patients’ lives. There are currently 662 ongoing clinical trials, and many more promising to come up soon. Other flourishing therapeutical possibilities, still very exploratory, include single-cell sequencing, transcriptomics, proteomics, theragnostics and even modulating the microbiome. Advances in molecular diagnosis could help identify tumor-specific resistance mechanisms and surrogate biomarkers, allowing for early resistance detection and the tailoring of therapeutical options.

Author Contributions: Original draft preparation and editing: C.L.F. and D.J.S.; Review: A.M. All authors have read and agreed to the published version of the manuscript.

Funding: This research received no external funding.

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

References

1. Siegel, R.L.; Miller, K.D.; Wagle, N.S.; Jemal, A. Cancer statistics, 2023. *CA Cancer J. Clin.* **2023**, *73*, 17–48. [CrossRef] [PubMed]
2. Giaquinto, A.N.; Sung, H.; Miller, K.D.; Kramer, J.L.; Newman, L.A.; Minihan, A.; Jemal, A.; Siegel, R.L. Breast Cancer Statistics, 2022. *CA Cancer J. Clin.* **2022**, *72*, 524–541. [CrossRef] [PubMed]
3. Slamon, D.J.; Clark, G.M.; Wong, S.G.; Levin, W.J.; Ullrich, A.; McGuire, W.L. Human breast cancer: Correlation of relapse and survival with amplification of the HER-2/neu oncogene. *Science* **1987**, *235*, 177–182. [CrossRef] [PubMed]
4. Pegram, M.D.; Pauletti, G.; Slamon, D.J. HER-2/neu as a predictive marker of response to breast cancer therapy. *Breast Cancer Res. Treat.* **1998**, *52*, 65–77. [CrossRef] [PubMed]
5. Slamon, D.J.; Godolphin, W.; Jones, L.A.; Holt, J.A.; Wong, S.G.; Keith, D.E.; Levin, W.J.; Stuart, S.G.; Udove, J.; Ullrich, A.; et al. Studies of the HER-2/neu proto-oncogene in human breast and ovarian cancer. *Science* **1989**, *244*, 707–712. [CrossRef] [PubMed]
6. Slamon, D.J.; Leyland-Jones, B.; Shak, S.; Fuchs, H.; Paton, V.; Bajamonde, A.; Fleming, T.; Eiermann, W.; Wolter, J.; Pegram, M.; et al. Use of chemotherapy plus a monoclonal antibody against HER2 for metastatic breast cancer that overexpresses HER2. *N. Engl. J. Med.* **2001**, *344*, 783–792. [CrossRef]
7. Mukohara, T. Mechanisms of resistance to anti-human epidermal growth factor receptor 2 agents in breast cancer. *Cancer Sci.* **2011**, *102*, 1–8. [CrossRef]
8. Garrett, J.T.; Arteaga, C.L. Resistance to HER2-directed antibodies and tyrosine kinase inhibitors: Mechanisms and clinical implications. *Cancer Biol. Ther.* **2011**, *11*, 793–800. [CrossRef]
9. 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. Oncogene* **2012**, *17*, 1–16. [CrossRef]
10. ClinicalTrials.gov. Available online: <https://clinicaltrials.gov/search?distance=50&cond=Breast%20Cancer&term=HER-2positive%20Breast%20Cancer&aggFilters=ages:adult%20older,status:rec%20act,studyType:int> (accessed on 7 November 2023).
11. Xia, W.; Mullin, R.J.; Keith, B.R.; Liu, L.H.; Ma, H.; Rusnak, D.W.; Owens, G.; Alligood, K.J.; Spector, N.L. Anti-tumor activity of GW572016: A dual tyrosine kinase inhibitor blocks EGF activation of EGFR/erbB2 and downstream Erk1/2 and AKT pathways. *Oncogene* **2002**, *21*, 6255–6263. [CrossRef]
12. Franklin, M.C.; Carey, K.D.; Vajdos, F.F.; Leahy, D.J.; de Vos, A.M.; Sliwkowski, M.X. Insights into ErbB signaling from the structure of the ErbB2-pertuzumab complex. *Cancer Cell* **2004**, *5*, 317–328. [CrossRef] [PubMed]
13. Koeppen, H.K.; Wright, B.D.; Burt, A.D.; Quirke, P.; McNicol, A.M.; Dybdal, N.O.; Sliwkowski, M.X.; Hillan, K.J. Overexpression of HER2/neu in solid tumours: An immunohistochemical survey. *Histopathology* **2001**, *38*, 96–104. [CrossRef] [PubMed]
14. Hanahan, D. Hallmarks of Cancer: New Dimensions. *Cancer Discov.* **2022**, *12*, 31–46. [CrossRef] [PubMed]

15. Wolff, A.C.; Somerfield, M.R.; Dowsett, M.; Hammond, M.E.H.; Hayes, D.F.; McShane, L.M.; Saphner, T.J.; Spears, P.A.; Allison, K.H. Human Epidermal Growth Factor Receptor 2 Testing in Breast Cancer: ASCO-College of American Pathologists Guideline Update. *J. Clin. Oncol.* **2023**, *41*, 3867–3872. [\[CrossRef\]](#) [\[PubMed\]](#)
16. Baselga, J. Clinical trials of Herceptin(R) (trastuzumab). *Eur. J. Cancer* **2001**, *37* (Suppl. S1), 18–24. [\[CrossRef\]](#) [\[PubMed\]](#)
17. Park, S.; Jiang, Z.; Mortenson, E.D.; Deng, L.; Radkevich-Brown, O.; Yang, X.; Sattar, H.; Wang, Y.; Brown, N.K.; Greene, M.; et al. The therapeutic effect of anti-HER2/neu antibody depends on both innate and adaptive immunity. *Cancer Cell* **2010**, *18*, 160–170. [\[CrossRef\]](#)
18. Shi, Y.; Fan, X.; Deng, H.; Brezski, R.J.; Ryczyn, M.; Jordan, R.E.; Strohl, W.R.; Zou, Q.; Zhang, N.; An, Z. Trastuzumab triggers phagocytic killing of high HER2 cancer cells in vitro and in vivo by interaction with Fcγ receptors on macrophages. *J. Immunol.* **2015**, *194*, 4379–4386. [\[CrossRef\]](#)
19. Baselga, J.; Norton, L.; Albanell, J.; Kim, Y.M.; Mendelsohn, J. Recombinant humanized anti-HER2 antibody (Herceptin) enhances the antitumor activity of paclitaxel and doxorubicin against HER2/neu overexpressing human breast cancer xenografts. *Cancer Res.* **1998**, *58*, 2825–2831.
20. 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 HER-2 positive breast cancer. *N. Engl. J. Med.* **2011**, *365*, 1273–1283. [\[CrossRef\]](#)
21. Swain, S.M.; Kim, S.B.; Cortes, J.; Ro, J.; Semiglazov, V.; Campone, M.; Ciruelos, E.; Ferrero, J.M.; Schneeweiss, A.; Knott, A.; et al. Pertuzumab, trastuzumab, and docetaxel for HER-2 positive metastatic breast cancer (CLEOPATRA study): Overall survival results from a randomised, double-blind, placebo-controlled, phase 3 study. *Lancet Oncol.* **2013**, *14*, 461–471. [\[CrossRef\]](#)
22. Gianni, L.; Pienkowski, T.; Im, Y.H.; Roman, L.; Tseng, L.M.; Liu, M.C.; Lluch, A.; Staroslawska, E.; Haba Rodriguez, J.; Im, S.A.; et al. Efficacy and safety of neoadjuvant pertuzumab and trastuzumab in women with locally advanced, inflammatory, or early HER-2 positive breast cancer (NeoSphere): A randomised multicentre, open-label, phase 2 trial. *Lancet Oncol.* **2012**, *13*, 25–32. [\[CrossRef\]](#) [\[PubMed\]](#)
23. Tolaney, S.M.; Barry, W.T.; Dang, C.T.; Yardley, D.A.; Moy, B.; Marcom, P.K.; Albain, K.S.; Rugo, H.S.; Ellis, M.; Chir, B.; et al. Adjuvant paclitaxel and trastuzumab for node-negative, HER-2 positive breast cancer. *N. Engl. J. Med.* **2015**, *372*, 134–141. [\[CrossRef\]](#) [\[PubMed\]](#)
24. Gianni, L.; Llado, A.; Bianchi, G.; Cortes, J.; Kellokumpu-Lehtinen, P.L.; Cameron, D.A.; Miles, D.; Salvagni, S.; Wardley, A.; Goeminne, J.C.; et al. Open-label, phase II, multicenter, randomized study of the efficacy and safety of two dose levels of Pertuzumab, a human epidermal growth factor receptor 2 dimerization inhibitor, in patients with human epidermal growth factor receptor 2-negative metastatic breast cancer. *J. Clin. Oncol.* **2010**, *28*, 1131–1137. [\[PubMed\]](#)
25. Von Minckwitz, G.; Procter, M.; de Azambuja, E.; Zardavas, D.; Benyunes, M.; Viale, G.; Suter, T.; Arahmani, A.; Rouchet, N.; Clark, E.; et al. Adjuvant Pertuzumab and Trastuzumab in Early HER-2 positive Breast Cancer. *N. Engl. J. Med.* **2017**, *377*, 122–131. [\[CrossRef\]](#) [\[PubMed\]](#)
26. Nordstrom, J.L.; Gorlatov, S.; Zhang, W.; Yang, Y.; Huang, L.; Burke, S.; Li, H.; Ciccarone, V.; Zhang, T.; Stavenhagen, J.; et al. Anti-tumor activity and toxicokinetics analysis of MGAH22, an anti-HER2 monoclonal antibody with enhanced Fcγ receptor binding properties. *Breast Cancer Res.* **2011**, *13*, R123. [\[CrossRef\]](#) [\[PubMed\]](#)
27. Rugo, H.S.; Im, S.A.; Cardoso, F.; Cortes, J.; Curigliano, G.; Musolino, A.; Pegram, M.D.; Bachelot, T.; Wright, G.S.; Saura, C.; et al. Margetuximab versus Trastuzumab in Patients with Previously Treated HER-2 positive Advanced Breast Cancer (SOPHIA): Final Overall Survival Results From a Randomized Phase 3 Trial. *J. Clin. Oncol.* **2023**, *41*, 198–205. [\[CrossRef\]](#) [\[PubMed\]](#)
28. Traxler, P. Tyrosine kinases as targets in cancer therapy—Successes and failures. *Expert Opin. Ther. Targets* **2003**, *7*, 215–234. [\[CrossRef\]](#) [\[PubMed\]](#)
29. Scaltriti, M.; Chandarlapaty, S.; Prudkin, L.; Aura, C.; Jimenez, J.; Angelini, P.; Sánchez, G.; Guzman, M.; Parra, J.L.; Ellis, C.; et al. Clinical benefit of lapatinib-based therapy in patients with human epidermal growth factor receptor 2-positive breast tumors coexpressing the truncated p95HER2 receptor. *Clin. Cancer Res.* **2010**, *16*, 2688–2695. [\[CrossRef\]](#)
30. Geyer, C.E.; Forster, J.; Lindquist, D.; Chan, S.; Romieu, C.G.; Pienkowski, T.; Jagiello-Gruszfeld, A.; Crown, J.; Chan, A.; Kaufman, B.; et al. Lapatinib plus capecitabine for HER-2 positive advanced breast cancer. *N. Engl. J. Med.* **2006**, *355*, 2733–2743. [\[CrossRef\]](#)
31. Goss, P.E.; Smith, I.E.; O'Shaughnessy, J.; Ejlertsen, B.; Kaufmann, M.; Boyle, F.; Buzdar, A.U.; Fumoleau, P.; Gradishar, W.; Martin, M.; et al. Adjuvant lapatinib for women with early-stage HER-2 positive breast cancer: A randomised, controlled, phase 3 trial. *Lancet Oncol.* **2013**, *14*, 88–96. [\[CrossRef\]](#)
32. Piccart-Gebhart, M.; Holmes, E.; Baselga, J.; de Azambuja, E.; Dueck, A.C.; Viale, G.; Zujewski, J.A.; Goldhirsch, A.; Armour, A.; Pritchard, K.; et al. Adjuvant Lapatinib and Trastuzumab for Early Human Epidermal Growth Factor Receptor 2-Positive Breast Cancer: Results From the Randomized Phase III Adjuvant Lapatinib and/or Trastuzumab Treatment Optimization Trial. *J. Clin. Oncol.* **2016**, *34*, 1034–1042. [\[CrossRef\]](#) [\[PubMed\]](#)
33. Untch, M.; Loibl, S.; Bischoff, J.; Eidtmann, H.; Kaufmann, M.; Blohmer, J.U.; Hilfrich, J.; Strumberg, D.; Fasching, P.A.; Kreienberg, R.; et al. Lapatinib versus trastuzumab in combination with neoadjuvant anthracycline-taxane-based chemotherapy (GeparQuinto, GBG 44): A randomised phase 3 trial. *Lancet Oncol.* **2012**, *13*, 135–144. [\[CrossRef\]](#) [\[PubMed\]](#)
34. Guarneri, V.; Dieci, M.V.; Grigolo, G.; Miglietta, F.; Girardi, F.; Bisagni, G.; Generali, D.G.; Cagossi, K.; Sarti, S.; Frassoldati, A.; et al. Trastuzumab-lapatinib as neoadjuvant therapy for HER-2 positive early breast cancer: Survival analyses of the CHER-Lob trial. *Eur. J. Cancer* **2021**, *153*, 133–141. [\[CrossRef\]](#) [\[PubMed\]](#)

35. De Azambuja, E.; Holmes, A.P.; Piccart-Gebhart, M.; Holmes, E.; Di Cosimo, S.; Swaby, R.F.; Untch, M.; Jackisch, C.; Lang, I.; Smith, I.; et al. Lapatinib with trastuzumab for HER-2 positive early breast cancer (NeoALTTO): Survival outcomes of a randomised, open-label, multicentre, phase 3 trial and their association with pathological complete response. *Lancet Oncol.* **2014**, *15*, 1137–1146. [\[CrossRef\]](#) [\[PubMed\]](#)
36. Lin, N.U.; Carey, L.A.; Liu, M.C.; Younger, J.; Come, S.E.; Ewend, M.; Harris, G.J.; Bullitt, E.; Abbeele, A.D.; Henson, J.W.; et al. Phase II trial of lapatinib for brain metastases in patients with human epidermal growth factor receptor 2-positive breast cancer. *J. Clin. Oncol.* **2008**, *26*, 1993–1999. [\[CrossRef\]](#) [\[PubMed\]](#)
37. Rabindran, S.K.; Discifani, C.M.; Rosford, E.C.; Baxter, M.; Floyd, M.B.; Golas, J.; Hallett, W.A.; Johnson, B.D.; Nilakantan, R.; Overbeek, E.; et al. Antitumor activity of HKI-272, an orally active, irreversible inhibitor of the HER-2 tyrosine kinase. *Cancer Res.* **2004**, *64*, 3958–3965. [\[CrossRef\]](#) [\[PubMed\]](#)
38. Segovia-Mendoza, M.; Gonzalez-Gonzalez, M.E.; Barrera, D.; Diaz, L.; Garcia-Becerra, R. Efficacy and mechanism of action of the tyrosine kinase inhibitors gefitinib, lapatinib and neratinib in the treatment of HER-2 positive breast cancer: Preclinical and clinical evidence. *Am. J. Cancer Res.* **2015**, *5*, 2531–2561.
39. Martin, M.; Bonnerette, J.; Geyer, C.E., Jr.; Ito, Y.; Ro, J.; Lang, I.; Kim, S.B.; Germa, C.; Vermette, J.; Wang, K.; et al. A phase two randomised trial of neratinib monotherapy versus lapatinib plus capecitabine combination therapy in patients with HER2+ advanced breast cancer. *Eur. J. Cancer* **2013**, *49*, 3763–3772. [\[CrossRef\]](#)
40. Freedman, R.A.; Gelman, R.S.; Wefel, J.S.; Melisko, M.E.; Hess, K.R.; Connolly, R.M.; Poznak, C.H.; Niravath, P.A.; Puhalla, S.L.; Ibrahim, N.; et al. Translational Breast Cancer Research Consortium (TBCRC) 022: A Phase II Trial of Neratinib for Patients with Human Epidermal Growth Factor Receptor 2-Positive Breast Cancer and Brain Metastases. *J. Clin. Oncol.* **2016**, *34*, 945–952. [\[CrossRef\]](#)
41. Saura, C.; Oliveira, M.; Feng, Y.H.; Dai, M.S.; Chen, S.W.; Hurvitz, S.A.; Kim, S.B.; Moy, B.; Delaloge, S.; Gradishar, W.; et al. Neratinib Plus Capecitabine versus Lapatinib Plus Capecitabine in HER-2 positive Metastatic Breast Cancer Previously Treated with ≥ 2 HER2-Directed Regimens: Phase III NALA Trial. *J. Clin. Oncol.* **2020**, *38*, 3138–3149. [\[CrossRef\]](#)
42. Martin, M.; Holmes, F.A.; Ejlertsen, B.; Delaloge, S.; Moy, B.; Iwata, H.; Minckwitz, G.; Chia, S.K.L.; Mansi, J.; Barrios, C.H.; et al. Neratinib after trastuzumab-based adjuvant therapy in HER-2 positive breast cancer (ExteNET): 5-year analysis of a randomised, double-blind, placebo-controlled, phase 3 trial. *Lancet Oncol.* **2017**, *18*, 1688–1700. [\[CrossRef\]](#) [\[PubMed\]](#)
43. Park, J.W.; Liu, M.C.; Yee, D.; Yau, C.; van 't Veer, L.J.; Symmans, W.F.; Paoloni, M.; Perlmutter, J.; Hylton, N.M.; Hogarth, M.; et al. Adaptive Randomization of Neratinib in Early Breast Cancer. *N. Engl. J. Med.* **2016**, *375*, 11–22. [\[CrossRef\]](#) [\[PubMed\]](#)
44. Jacobs, S.A.; Robidoux, A.; Abraham, J.; Perez-Garcia, J.M.; La Verde, N.; Orcutt, J.M.; Cazzaniga, M.E.; Piette, F.; Antolin, S.; Aguirre, E.; et al. NSABP B7-7: A phase II randomized neoadjuvant trial with paclitaxel + trastuzumab and/or neratinib followed by chemotherapy and postoperative trastuzumab in HER2(+) breast cancer. *Breast Cancer Res.* **2019**, *21*, 133. [\[CrossRef\]](#) [\[PubMed\]](#)
45. Kulukian, A.; Lee, P.; Taylor, J.; Rosler, R.; de Vries, P.; Watson, D.; Forero-Torres, A.; Peterson, S. Preclinical Activity of HER2-Selective Tyrosine Kinase Inhibitor Tucatinib as a Single Agent or in Combination with Trastuzumab or Docetaxel in Solid Tumor Models. *Mol. Cancer Ther.* **2020**, *19*, 976–987. [\[CrossRef\]](#) [\[PubMed\]](#)
46. Kaur, S.; Bhattacharya, R.; Banerjee, D.; Shukla, J. Application of Tucatinib and Trastuzumab: Dual Anti HER2 Therapy Against HER2 Positive Breast Cancer. *Indian J. Clin. Biochem.* **2021**, *36*, 124–125. [\[CrossRef\]](#) [\[PubMed\]](#)
47. Curigliano, G.; Mueller, V.; Borges, V.; Hamilton, E.; Hurvitz, S.; Loi, S.; Murthy, R.; Okines, A.; Paplomata, E.; Cameron, D.; et al. Tucatinib versus placebo added to trastuzumab and capecitabine for patients with pretreated HER2+ metastatic breast cancer with and without brain metastases (HER2CLIMB): Final overall survival analysis. *Ann. Oncol.* **2022**, *33*, 321–329. [\[CrossRef\]](#) [\[PubMed\]](#)
48. Li, X.; Yang, C.; Wan, H.; Zhang, G.; Feng, J.; Zhang, L.; Chen, X.; Zhong, D.; Lou, L.; Tao, W.; et al. Discovery and development of pyrotinib: A novel irreversible EGFR/HER2 dual tyrosine kinase inhibitor with favorable safety profiles for the treatment of breast cancer. *Eur. J. Pharm. Sci.* **2017**, *110*, 51–61. [\[CrossRef\]](#)
49. Ma, F.; Ouyang, Q.; Li, W.; Jiang, Z.; Tong, Z.; Liu, Y.; Li, H.; Yu, S.; Feng, J.; Wang, S.; et al. Pyrotinib or Lapatinib Combined with Capecitabine in HER-2 positive Metastatic Breast Cancer with Prior Taxanes, Anthracyclines, and/or Trastuzumab: A Randomized, Phase II Study. *J. Clin. Oncol.* **2019**, *37*, 2610–2619. [\[CrossRef\]](#)
50. Jiang, Z.; Yan, M.; Hu, X.; Zhang, Q.; Ouyang, Q.; Feng, J.; Yin, Y.; Sun, T.; Tong, Z.; Wang, X.; et al. Pyrotinib combined with capecitabine in women with HER2+ metastatic breast cancer previously treated with trastuzumab and taxanes: A randomized phase III study. *J. Clin. Oncol.* **2019**, *37*, 1001. [\[CrossRef\]](#)
51. Bao, Y.; Zhang, Z.; He, X.; Cai, L.; Wang, X.; Li, X. Cost-Effectiveness of Pyrotinib Plus Capecitabine versus Lapatinib Plus Capecitabine for the Treatment of HER2-Positive Metastatic Breast Cancer in China: A Scenario Analysis of Health Insurance Coverage. *Curr. Oncol.* **2022**, *29*, 6053–6067. [\[CrossRef\]](#)
52. Verma, S.; Miles, D.; Gianni, L.; Krop, I.E.; Welslau, M.; Baselga, J.; Pegram, M.; Oh, D.Y.; Diéras, V.; Guardino, E.; et al. Trastuzumab emtansine for HER-2 positive advanced breast cancer. *N. Engl. J. Med.* **2012**, *367*, 1783–1791. [\[CrossRef\]](#) [\[PubMed\]](#)
53. Von Minckwitz, G.; Huang, C.S.; Mano, M.S.; Loibl, S.; Mamounas, E.P.; Untch, M.; Wolmark, N.; Rastogi, P.; Schneeweiss, A.; Redondo, A.; et al. Trastuzumab Emtansine for Residual Invasive HER-2 positive Breast Cancer. *N. Engl. J. Med.* **2019**, *380*, 617–628. [\[CrossRef\]](#) [\[PubMed\]](#)

54. Lewis Phillips, G.D.; Li, G.; Dugger, D.L.; Crocker, L.M.; Parsons, K.L.; Mai, E.; Blattler, W.A.; Lambert, J.M.; Chari, R.V.J.; Lutz, R.J.; et al. Targeting HER-2 positive breast cancer with trastuzumab-DM1, an antibody-cytotoxic drug conjugate. *Cancer Res.* **2008**, *68*, 9280–9290. [\[CrossRef\]](#) [\[PubMed\]](#)
55. Dieras, V.; Miles, D.; Verma, S.; Pegram, M.; Welslau, M.; Baselga, J.; Krop, I.E.; Blackwell, K.; Hoersch, S.; Xu, J.; et al. Trastuzumab emtansine versus capecitabine plus lapatinib in patients with previously treated HER-2 positive advanced breast cancer (EMILIA): A descriptive analysis of final overall survival results from a randomised, open-label, phase 3 trial. *Lancet Oncol.* **2017**, *18*, 732–742. [\[CrossRef\]](#) [\[PubMed\]](#)
56. Yver, A.; Agatsuma, T.; Soria, J.C. The art of innovation: Clinical development of trastuzumab deruxtecan and redefining how antibody-drug conjugates target HER-2 positive cancers. *Ann. Oncol.* **2020**, *31*, 430–434. [\[CrossRef\]](#) [\[PubMed\]](#)
57. 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 HER-2 positive breast cancer previously treated with trastuzumab emtansine: A dose-expansion, phase 1 study. *Lancet Oncol.* **2019**, *20*, 816–826. [\[CrossRef\]](#) [\[PubMed\]](#)
58. 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 HER-2 positive Breast Cancer. *N. Engl. J. Med.* **2020**, *382*, 610–621. [\[CrossRef\]](#)
59. Cortes, J.; Kim, S.B.; Chung, W.P.; Im, S.A.; Park, Y.H.; Hegg, R.; Kim, M.H.; Tseng, L.M.; Petry, V.; Chung, C.F.; et al. Trastuzumab Deruxtecan versus Trastuzumab Emtansine for Breast Cancer. *N. Engl. J. Med.* **2022**, *386*, 1143–1154. [\[CrossRef\]](#)
60. Modi, S.; Jacot, W.; Yamashita, T.; Sohn, J.; Vidal, M.; Tokunaga, E.; Tsurutani, J.; Ueno, N.T.; Prat, A.; Chae, Y.S.; et al. Trastuzumab Deruxtecan in Previously Treated HER-2 low Advanced Breast Cancer. *N. Engl. J. Med.* **2022**, *387*, 9–20. [\[CrossRef\]](#)
61. Espelin, C.W.; Leonard, S.C.; Geretti, E.; Wickham, T.J.; Hendriks, B.S. Dual HER2 Targeting with Trastuzumab and Liposomal-Encapsulated Doxorubicin (MM-302) Demonstrates Synergistic Antitumor Activity in Breast and Gastric Cancer. *Cancer Res.* **2016**, *76*, 1517–1527. [\[CrossRef\]](#)
62. Miller, K.; Cortes, J.; Hurvitz, S.A.; Krop, I.E.; Tripathy, D.; Verma, S.; Rihani, K.; Reynolds, J.G.; Wickham, T.J.; Molnar, I.; et al. HERMIONE: A randomized Phase 2 trial of MM-302 plus trastuzumab versus chemotherapy of physician's choice plus trastuzumab in patients with previously treated, anthracycline-naïve, HER-2 positive, locally advanced/metastatic breast cancer. *BMC Cancer* **2016**, *16*, 352. [\[CrossRef\]](#) [\[PubMed\]](#)
63. Wang, T.; Zhang, P.; Di, L.; Wang, X.; Yang, J.; Tong, Z.; Liu, J.; Feng, J.; Liu, D.; Yu, Q.; et al. Efficacy and safety of inotamab in combination with chemotherapy as first-line treatment of HER-2 positive metastatic breast cancer: A subgroup analysis in the HOPES study. *Transl. Breast Cancer Res.* **2022**, *3*, 1136380. [\[CrossRef\]](#)
64. Bian, L.; Xu, B.H.; Di, L.J.; Wang, T.; Wang, X.J.; Jiao, S.C.; Yang, J.L.; Tong, Z.S.; Liu, J.; Feng, J.F.; et al. [Phase III randomized controlled, multicenter, prospective study of recombinant anti-HER2 humanized monoclonal antibody (Cipterbin) combined with vinorelbine in patients with HER2 positive metastatic breast cancer: The HOPES Study]. *Zhonghua Yi Xue Za Zhi* **2020**, *100*, 2351–2357. [\[PubMed\]](#)
65. Liu, X.; Zhang, P.; Li, C.; Song, X.; Liu, Z.; Shao, W.; Li, S.; Wang, X.; Yu, Z. Efficacy and safety of inotamab-containing regimens in patients with HER-2 positive metastatic breast cancer: A real-world retrospective study in China. *Front. Oncol.* **2023**, *13*, 1136380. [\[CrossRef\]](#) [\[PubMed\]](#)
66. Weissner, N.E.; Wickman, G.; Abraham, L.; O'Toole, J.; Harbourn, B.; Guedia, J.; Cheng, C.W.; Chan, P.; Browman, D.; Gold, M.R.; et al. The bispecific antibody zanidatamab's (ZW25's) unique mechanisms of action and durable anti-tumor activity in HER2-expressing cancers. In Proceedings of the AACR Annual Meeting 2021, Philadelphia, PA, USA, 9–14 July 2021; p. 1005.
67. Meric-Bernstam, F.; Hanna, D.; Beeram, M.; Lee, K.W.; Kang, Y.K.; Chaves, J.; Lee, J.; Goodwin, R.; Vaklavas, C.; Oh, D.Y.; et al. Safety, anti-tumour activity, and biomarker results of the HER2-targeted bispecific antibody ZW25 in HER2-expressing solid tumours. *Ann. Oncol.* **2019**, *30*, v167–v168. [\[CrossRef\]](#)
68. Bedard, P.L.; Im, S.A.; Elimova, E.; Rha, S.Y.; Goodwin, R.; Ferrario, C.; Lee, K.W.; Hanna, D.; Meric-Bernstam, F.; Mayordomo, J.; et al. Zanidatamab (ZW25), a HER2-targeted bispecific antibody, in combination with chemotherapy (chemo) for HER-2 positive breast cancer (BC): Results from a phase 1 study. In Proceedings of the San Antonio Breast Cancer Symposium 2021, San Antonio, TX, USA, 7–10 December 2021; Cancer Research: San Antonio, TX, USA, 2022.
69. Huang, S.; Li, F.; Liu, H.; Ye, P.; Fan, X.; Yuan, X.; Wu, Z.; Chen, J.; Jin, C.; Shen, B.; et al. Structural and functional characterization of MBS301, an afucosylated bispecific anti-HER2 antibody. *MAbs* **2018**, *10*, 864–875. [\[CrossRef\]](#) [\[PubMed\]](#)
70. Wei, H.; Cai, H.; Jin, Y.; Wang, P.; Zhang, Q.; Lin, Y.; Wang, W.; Cheng, J.; Zeng, N.; Xu, T.; et al. Structural basis of a novel heterodimeric Fc for bispecific antibody production. *Oncotarget* **2017**, *8*, 51037–51049. [\[CrossRef\]](#)
71. Zhang, J.; Ji, D.; Cai, L.; Yao, H.; Yan, M.; Wang, X.; Shen, W.; Du, Y.; Pang, H.; Lai, X.; et al. First-in-human HER2-targeted Bispecific Antibody KN026 for the Treatment of Patients with HER-2 positive Metastatic Breast Cancer: Results from a Phase I Study. *Clin. Cancer Res.* **2022**, *28*, 618–628. [\[CrossRef\]](#)
72. Ji, D.; Zhang, J.; Shen, W.; Du, Y.; Xu, J.; Yang, J.; Luo, X.; Kong, P.; Yang, F.; Hu, X. Preliminary safety, efficacy and pharmacokinetics (PK) results of KN026 (a HER2 bispecific antibody) monotherapy in advanced solid tumor patients with HER2 expression. *J. Clin. Oncol.* **2020**, *38*, 1041. [\[CrossRef\]](#)
73. Geuijen, C.A.W.; De Nardis, C.; Maussang, D.; Rovers, E.; Gallenne, T.; Hendriks, L.J.A.; Visser, T.; Nijhuis, R.; Logtenberg, T.; Kruijff, J.; et al. Unbiased Combinatorial Screening Identifies a Bispecific IgG1 that Potently Inhibits HER3 Signaling via HER2-Guided Ligand Blockade. *Cancer Cell* **2018**, *33*, 922–936.e10. [\[CrossRef\]](#)

74. Hamilton, E.P.; Petit, T.; Pistilli, B.; Goncalves, A.; Ferreira, A.; Dalenc, F.; Cardoso, F.; Mita, M.M.; Dezentje, V.O.; Manso, L.; et al. Clinical activity of MCLA-128 (zenocutuzumab), trastuzumab, and vinorelbine in HER2 amplified metastatic breast cancer (MBC) patients (pts) who had progressed on anti-HER2 ADCs. *J. Clin. Oncol.* **2020**, *38*, 3093. [\[CrossRef\]](#)
75. Schram, A.M.; Goto, K.; Kim, D.; Martin-Romano, P.; Ou, S.I.; O’Kane, G.M.; O’Reilly, E.M.; Umemoto, K.; Duruisseaux, M.; Neuzillet, C.; et al. Efficacy and safety of zenocutuzumab, a HER2 x HER3 bispecific antibody, in advanced NRG1 fusion-positive (NRG1+) cancer. *J. Clin. Oncol.* **2022**, *40*, 105. [\[CrossRef\]](#)
76. Deng, W.; Liu, J.; Pan, H.; Li, L.; Zhou, C.; Wang, X.; Shu, R.; Dong, B.; Cao, D.; Li, Q.; et al. A Bispecific Antibody Based on Pertuzumab Fab Has Potent Antitumor Activity. *J. Immunother.* **2018**, *41*, 1–8. [\[CrossRef\]](#) [\[PubMed\]](#)
77. Oberg, H.H.; Kellner, C.; Gonnermann, D.; Sebels, S.; Bauerschlag, D.; Gramatzki, M.; Kabelitz, D.; Peipp, M.; Wesch, D. Tribody [(HER2)(2)xCD16] Is More Effective Than Trastuzumab in Enhancing gamma delta T Cell and Natural Killer Cell Cytotoxicity Against HER2-Expressing Cancer Cells. *Front. Immunol.* **2018**, *9*, 814. [\[CrossRef\]](#) [\[PubMed\]](#)
78. Zhang, H.; Lam, L.; Nagai, Y.; Zhu, Z.; Chen, X.; Ji, M.Q.; Greene, M.I. A targeted immunotherapy approach for HER2/neu transformed tumors by coupling an engineered effector domain with interferon-gamma. *Oncotarget* **2018**, *7*, e1300739. [\[CrossRef\]](#) [\[PubMed\]](#)
79. McDonagh, C.F.; Huhlov, A.; Harms, B.D.; Adams, S.; Paragas, V.; Oyama, S.; Zhang, B.; Luss, L.; Overland, R.; Nguyen, S.; et al. Antitumor activity of a novel bispecific antibody that targets the ErbB2/ErbB3 oncogenic unit and inhibits heregulin-induced activation of ErbB3. *Mol. Cancer Ther.* **2012**, *11*, 582–593. [\[CrossRef\]](#)
80. Kiewe, P.; Hasmmuller, S.; Kahlert, S.; Heinrigs, M.; Rack, B.; Marme, A.; Korfel, A.; Jager, M.; Lindhofer, H.; Sommer, H.; et al. Phase I trial of the trifunctional anti-HER2 x anti-CD3 antibody ertumaxomab in metastatic breast cancer. *Clin. Cancer Res.* **2006**, *12*, 3085–3091. [\[CrossRef\]](#)
81. Jager, M.; Schoberth, A.; Ruf, P.; Hess, J.; Lindhofer, H. The trifunctional antibody ertumaxomab destroys tumor cells that express low levels of human epidermal growth factor receptor 2. *Cancer Res.* **2009**, *69*, 4270–4276. [\[CrossRef\]](#)
82. Wermke, M.; Alt, J.; Kauh, J.; Back, J.; Salhi, Y.; Reddy, V.; Bayever, E.; Ochsenreither, S. Preliminary biomarker and pharmacodynamic data from a phase I study of single-agent bispecific antibody T-cell engager GBR 1302 in subjects with HER-2 positive cancers. *J. Clin. Oncol.* **2018**, *36*, 69. [\[CrossRef\]](#)
83. Kim, H.J.; Kim, H.P.; Yoon, Y.K.; Kim, M.S.; Lee, G.S.; Han, S.W.; Im, S.A.; Kim, T.Y.; Oh, D.Y.; Bang, Y.J. Antitumor activity of HM781-36B, a pan-HER tyrosine kinase inhibitor, in HER2-amplified breast cancer cells. *Anticancer Drugs* **2012**, *23*, 288–297. [\[CrossRef\]](#)
84. Robichaux, J.P.; Elamin, Y.Y.; Vijayan, R.S.K.; Nilsson, M.B.; Hu, L.; He, J.; He, J.; Zhang, F.; Pisegna, M.; Poteete, A.; et al. Pan-Cancer Landscape and Analysis of ERBB2 Mutations Identifies Pozotinib as a Clinically Active Inhibitor and Enhancer of T-DM1 Activity. *Cancer Cell* **2020**, *37*, 420. [\[CrossRef\]](#) [\[PubMed\]](#)
85. Kim, T.M.; Lee, K.W.; Oh, D.Y.; Lee, J.S.; Im, S.A.; Kim, D.W.; Han, S.W.; Kim, Y.J.; Kim, T.Y.; Kim, J.H.; et al. Phase I Studies of Pozotinib, an Irreversible Pan-HER Tyrosine Kinase Inhibitor in Patients with Advanced Solid Tumors. *Cancer Res. Treat.* **2018**, *50*, 835–842. [\[CrossRef\]](#) [\[PubMed\]](#)
86. Park, Y.H.; Lee, K.H.; Sohn, J.H.; Lee, K.S.; Jung, K.H.; Kim, J.H.; Lee, K.H.; Ahn, J.S.; Kim, T.Y.; Kim, G.M.; et al. A phase II trial of the pan-HER inhibitor pozotinib, in patients with HER-2 positive metastatic breast cancer who had received at least two prior HER2-directed regimens: Results of the NOV120101-203 trial. *Int. J. Cancer* **2018**, *143*, 3240–3247. [\[CrossRef\]](#) [\[PubMed\]](#)
87. Park, Y.H.; Jung, K.H.; Sohn, J.; Lee, K.S.; Kim, J.H.; Yang, Y.; Beak, E.; Han, H.; Im, S.-A. Pozotinib for HER-2 positive metastatic breast cancer (MBC): Final clinical efficacy and safety results for long-term follow-up of the phase II NOV120101-203 trial. In *Annals of Oncology, Proceedings of the ESMO Congress 2022, Berlin, Germany, 3–5 May 2022*; Elsevier: Amsterdam, The Netherlands, 2022; pp. S207–S208.
88. Zhang, J.; McAndrew, N.P.; Wang, X.; Du, Y.; DiCarlo, B.; Wang, M.; Chen, K.; Yu, W.; Hu, X. Preclinical and clinical activity of DZD1516, a full blood-brain barrier-penetrant, highly selective HER2 inhibitor. *Breast Cancer Res.* **2023**, *25*, 81. [\[CrossRef\]](#) [\[PubMed\]](#)
89. Hu, X.; Zhang, J.; McAndrew, N.P. Early Clinical Safety and Pharmacokinetics Data of DZD1516, an BBB-Penetrant Selective HER2 Inhibitor for the Treatment of HER-2 positive Metastatic Breast Cancer. *J. Clin. Oncol.* **2022**, *40*, 1038. [\[CrossRef\]](#)
90. Tanaka, H.; Hirata, M.; Shinonome, S.; Wada, T.; Iguchi, M.; Dohi, K.; Inoue, M.; Ishioka, Y.; Hojo, K.; Yamada, T.; et al. Preclinical antitumor activity of S-222611, an oral reversible tyrosine kinase inhibitor of epidermal growth factor receptor and human epidermal growth factor receptor 2. *Cancer Sci.* **2014**, *105*, 1040–1048. [\[CrossRef\]](#) [\[PubMed\]](#)
91. Spicer, J.; Baird, R.; Suder, A.; Cresti, N.; Corbacho, J.G.; Hogarth, L.; Frenkel, E.; Matsumoto, S.; Kawabata, I.; Donaldson, K.; et al. Phase 1 dose-escalation study of S-222611, an oral reversible dual tyrosine kinase inhibitor of EGFR and HER2, in patients with solid tumours. *Eur. J. Cancer* **2015**, *51*, 137–145. [\[CrossRef\]](#)
92. Macpherson, I.R.; Spiliopoulou, P.; Rafii, S.; Saggese, M.; Baird, R.D.; Garcia-Corbacho, J.; Italiano, A.; Bonnetterre, J.; Campone, M.; Cresti, N.; et al. A phase I/II study of epertinib plus trastuzumab with or without chemotherapy in patients with HER-2 positive metastatic breast cancer. *Breast Cancer Res.* **2019**, *22*, 1. [\[CrossRef\]](#)
93. Schram, A.M.; Ahnert, J.R.; Patel, M.R.; Jauhari, S.; Sachdev, J.C.; Zhu, V.W.; LoRusso, P.; Nguyen, D.; Le, X.; O’Connor, M.; et al. Safety and preliminary efficacy from the phase 1 portion of MasterKey-01: A First-in-human dose-escalation study to determine the recommended phase 2 dose (RP2D), pharmacokinetics (PK) and preliminary antitumor activity of BDTX-189, an inhibitor of allosteric ErbB mutations, in patients (pts) with advanced solid malignancies. *J. Clin. Oncol.* **2021**, *39*, 3086.

94. Banerji, U.; van Herpen, C.M.L.; Saura, C.; Thistlethwaite, F.; Lord, S.; Moreno, V.; Macpherson, I.R.; Boni, V.; Rolfo, C.; de Vries, E.G.E.; et al. Trastuzumab duocarmazine in locally advanced and metastatic solid tumours and HER2-expressing breast cancer: A phase I dose-escalation and dose-expansion study. *Lancet Oncol.* **2019**, *20*, 1124–1135. [\[CrossRef\]](#)
95. Dokter, W.; Ubink, R.; van der Lee, M.; van der Vleuten, M.; van Achterberg, T.; Jacobs, D.; Loosveld, E.; van den Dobbelsteen, D.; Egging, D.; Matthaar, E.; et al. Preclinical profile of the HER2-targeting ADC SYD983/SYD985: Introduction of a new duocarmycin-based linker-drug platform. *Mol. Cancer Ther.* **2014**, *13*, 2618–2629. [\[CrossRef\]](#) [\[PubMed\]](#)
96. Manich, C.S.; O'Shaughnessy, J.; Aftimos, P.G.; van den Tweel, E.; Oesterholt, M.; Escrivá-de-Romani, S.I.; Tueux, N.Q.; Tan, T.J.; Lim, J.S.; Ladoire, S.; et al. Primary outcome of the phase III SYD985.002/TULIP trial comparing [vic]-trastuzumab duocarmazine to physician's choice treatment in patients with pre-treated HER-2 positive locally advanced or metastatic breast cancer. *Ann. Oncol.* **2021**, *32*, 1283–1346.
97. Zhang, T.; You, L.; Xu, J.; Yin, J.; Qu, B.; Mao, Y.; Fu, B.; Cao, D.; Zhao, L.; Feng, J.; et al. SHR-A1811, a novel anti-HER2 ADC with superior bystander effect, optimal DAR and favorable safety profiles. *Cancer Res.* **2023**, *83* (Suppl. S8), LB031. [\[CrossRef\]](#)
98. Yao, H.; Ryu, M.H.; Park, J.; Voskoboinik, M.; Kim, J.H.; Liu, K.; Barve, M.; Acuna-Villaorduna, A.; Im, S.-A.; Roy, A.C.; et al. The HER2-targeting ADC SHR-A1811 in HER2-expressing/mutated advanced non-breast solid tumors (STs): Results from the global phase I study. *Ann. Oncol.* **2023**, *34*, S458–S497. [\[CrossRef\]](#)
99. Skidmore, L.; Sakamuri, S.; Knudsen, N.A.; Hewet, A.G.; Milutinovic, S.; Barkho, W.; Biroc, S.L.; Kirtley, J.; Marsden, R.; Storey, K.; et al. ARX788, a Site-specific Anti-HER2 Antibody-Drug Conjugate, Demonstrates Potent and Selective Activity in HER-2 low and T-DM1-resistant Breast and Gastric Cancers. *Mol. Cancer Ther.* **2020**, *19*, 1833–1843. [\[CrossRef\]](#)
100. Barok, M.; Le Joncour, V.; Martins, A.; Isola, J.; Salmikangas, M.; Laakkonen, P.; Joensuu, H. ARX788, a novel anti-HER2 antibody-drug conjugate, shows anti-tumor effects in preclinical models of trastuzumab emtansine-resistant HER-2 positive breast cancer and gastric cancer. *Cancer Lett.* **2020**, *473*, 156–163. [\[CrossRef\]](#)
101. Zhang, J.; Ji, D.; Shen, W.; Xiao, Q.; Gu, Y.; O'Shaughnessy, J.; Hu, X. Phase I Trial of a Novel Anti-HER2 Antibody-Drug Conjugate, ARX788, for the Treatment of HER-2 positive Metastatic Breast Cancer. *Clin. Cancer Res.* **2022**, *28*, OF1–OF10.
102. Shi, F.; Liu, Y.; Zhou, X.; Shen, P.; Xue, R.; Zhang, M. Disitamab vedotin: A novel antibody-drug conjugates for cancer therapy. *Drug Deliv.* **2022**, *29*, 1335–1344. [\[CrossRef\]](#)
103. Yao, X.; Jiang, J.; Wang, X.; Huang, C.; Li, D.; Xie, K.; Xu, Q.; Li, H.; Li, Z.; Lou, L.; et al. A novel humanized anti-HER2 antibody conjugated with MMAE exerts potent anti-tumor activity. *Breast Cancer Res. Treat.* **2015**, *153*, 123–133. [\[CrossRef\]](#)
104. Deeks, E.D. Disitamab Vedotin: First Approval. *Drugs* **2021**, *81*, 1929–1935. [\[CrossRef\]](#)
105. Hamblett, K.J.; Barnscher, S.D.; Davies, R.H.; Hammon, P.W.; Hernandez, A.; Wickman, G.R.; Fung, V.K.; Ding, T.; Garnett, G.; Galey, A.S.; et al. ZW49, a HER2 targeted biparatopic antibody drug conjugate for the treatment of HER2 expressing cancers. In *Proceedings of the 2018 San Antonio Breast Cancer Symposium, San Antonio, TX, USA, 4–8 December 2018*; Cancer Research: San Antonio, TX, USA, 2019.
106. Jhaveri, K.; Dotan, E.; Oh, D.; Ferrario, C.; Tolcher, A.W.; Lee, K.; Liao, C.; Kang, Y.; Kim, Y.H.; Hamilton, E.P.; et al. Preliminary results from a phase I study using the bispecific, human epidermal growth factor 2 (HER2)-targeting antibody-drug conjugate (ADC) zanidatamab zovodotin (ZW49) in solid cancers. *Ann. Oncol.* **2022**, *33*, S197–S224. [\[CrossRef\]](#)
107. Li, H.; Zhang, X.; Xu, Z.; Li, L.; Liu, W.; Dai, Z.; Zhao, Z.; Xiao, L.; Li, H.; Hu, C. Preclinical evaluation of MRG002, a novel HER2-targeting antibody-drug conjugate with potent antitumor activity against HER-2 positive solid tumors. *Antib. Ther.* **2021**, *4*, 175–184. [\[PubMed\]](#)
108. Zhang, J.; Liu, R.; Gao, S.; Li, W.; Chen, Y.; Meng, Y.; Liu, C.; Jin, W.; Wu, J.; Wang, Y.; et al. Phase I study of A166, an antibody–drug conjugate in advanced HER2-expressing solid tumours. *NPJ Breast Cancer* **2023**, *9*, 28. [\[CrossRef\]](#) [\[PubMed\]](#)
109. Rinnerthaler, G.; Gampenrieder, S.P.; Greil, R. HER2 Directed Antibody-Drug-Conjugates beyond T-DM1 in Breast Cancer. *Int. J. Mol. Sci.* **2019**, *20*, 1115. [\[CrossRef\]](#) [\[PubMed\]](#)
110. Park, Y.H.; Kim, J.-Y.; Ahn, J.S.; Im, Y.-H.; Kim, S.-H.; Lee, S.; Chung, Y.S.; Park, S.J. First-in-human phase I study of ALT-P7, a HER2-targeting antibody-drug conjugate in patients with HER-2 positive advanced breast cancer. *J. Clin. Oncol.* **2020**, *38*, 3551. [\[CrossRef\]](#)
111. Yurkovetskiy, A.; Ter-Ovanesyan, E.; Conlon, P.; Devit, M.; Bu, C.; Bodyak, N.; Lowinger, T.; Bergstrom, D. Non-clinical pharmacokinetics of XMT-1522, a HER2 targeting auristatin-based antibody drug conjugate. *Cancer Res.* **2017**, *77* (Suppl. S13), 48. [\[CrossRef\]](#)
112. Le Joncour, V.; Martins, A.; Puhka, M.; Isola, J.; Salmikangas, M.; Laakkonen, P.; Joensuu, H.; Barok, M. A Novel Anti-HER2 Antibody-Drug Conjugate XMT-1522 for HER-2 positive Breast and Gastric Cancers Resistant to Trastuzumab Emtansine. *Mol. Cancer Ther.* **2019**, *18*, 1721–1730. [\[CrossRef\]](#) [\[PubMed\]](#)
113. Bodyak, N.; Park, P.; Gumerov, D.; DeVit, M.; Yin, M.; Thomas, J.D.; Qin, L.; Lowinger, T.B.; Bergstrom, D.A. Trastuzumab-Dolaflexin, a Highly Potent Fleximer-Based Antibody-Drug Conjugate, Demonstrates a Favorable Therapeutic Index in Exploratory Toxicology Studies in Multiple Species. *Cancer Res.* **2015**, *75*, 641. [\[CrossRef\]](#)
114. Traore, T.; Khattar, M. Synergy of an anti-HER2 ADC TAK-522 (XMT-1522) in combination with anti-PD1 monoclonal antibody (mAb) in a syngeneic breast cancer model expressing human HER2. *J. Immunol.* **2018**, *200*, 122–129. [\[CrossRef\]](#)
115. Hamilton, E.P.; Bardia, A.; Beeram, M.; Bendell, J.; Mosher, R.; Hailman, E.; Bergstrom, D.; Burris, H.; Soliman, H. Phase 1 dose escalation of XMT-1522, a novel HER2-targeting antibody-drug conjugate (ADC), in patients (pts) with HER2-expressing breast, lung and gastric tumors. *J. Clin. Oncol.* **2018**, *36*, 2546. [\[CrossRef\]](#)

116. Meric-Bernstam, F.; Calvo, E.; Moreno, V.; Chung, H.C.; Park, Y.H.; Bang, Y.-J.; Rosen, L.S.; Mita, M.M.; Garrido-Laguna, I.; Leung, A.C.F.; et al. A phase I dose escalation study evaluating the safety and tolerability of a novel anti-HER2 antibody-drug conjugate (PF-06804103) in patients with HER-2 positive solid tumors. *J. Oncol.* **2020**, *38*, 1039. [\[CrossRef\]](#)
117. Graziani, E.I.; Sung, M.; Ma, D.; Narayanan, B.; Marquette, K.; Puthenveetil, S.; Tumey, L.N.; Bikker, J.; Casavant, J.; Bennett, E.M.; et al. PF-06804103, A Site-Specific Anti-HER2 Antibody-Drug Conjugate for the Treatment of HER2-expressing Breast, Gastric, and Lung Cancers. *Mol. Cancer Ther.* **2020**, *19*, 2068–2078. [\[CrossRef\]](#) [\[PubMed\]](#)
118. Meric-Bernstam, F.; Calvo, E.; Lee, K.S.; Moreno, V.; Park, Y.H.; Rha, S.Y.; Chalasani, P.; Zhong, W.; Zhou, L.; Pirio-Shepherd, S.; et al. Safety and Tolerability of a Novel Anti-HER2 Antibody-Drug Conjugate (PF-06804103) in Patients with HER2-Expressing Solid Tumors: A Phase I Dose-Escalation Study. *Mol. Cancer Ther.* **2023**, *22*, 1191–1203. [\[CrossRef\]](#) [\[PubMed\]](#)
119. Kang, J.C.; Sun, W.; Khare, P.; Karimi, M.; Wang, X.; Shen, Y.; Ober, R.J.; Ward, E.S. Engineering a HER2-specific antibody-drug conjugate to increase lysosomal delivery and therapeutic efficacy. *Nat. Biotechnol.* **2019**, *37*, 523–526. [\[CrossRef\]](#) [\[PubMed\]](#)
120. Oganessian, V.; Peng, L.; Bee, J.S.; Li, J.; Perry, S.R.; Comer, F.; Xu, L.; Cook, K.; Senthil, K.; Clarke, L. Structural insights into the mechanism of action of a biparatopic anti-HER2 antibody. *J. Biol. Chem.* **2018**, *293*, 8439–8448. [\[CrossRef\]](#) [\[PubMed\]](#)
121. Li, J.Y.; Perry, S.R.; Muniz-Medina, V.; Wang, X.; Wetzel, L.K.; Rebelatto, M.C.; Hinrichs, M.J.; Bezabeh, B.Z.; Fleming, R.L.; Dimasi, N.; et al. A Biparatopic HER2-Targeting Antibody-Drug Conjugate Induces Tumor Regression in Primary Models Refractory to or Ineligible for HER2-Targeted Therapy. *Cancer Cell* **2016**, *29*, 117–129. [\[CrossRef\]](#) [\[PubMed\]](#)
122. Pegram, M.D.; Hamilton, E.P.; Tan, A.R.; Storniolo, A.M.; Balic, K.; Rosenbaum, A.I.; Liang, M.; He, P.; Marshall, S.; Scheuber, A.; et al. First-in-Human, Phase I Dose-Escalation Study of Biparatopic Anti-HER2 Antibody-Drug Conjugate MEDI4276 in Patients with HER-2 positive Advanced Breast or Gastric Cancer. *Mol. Cancer Ther.* **2021**, *20*, 1442–1453. [\[CrossRef\]](#) [\[PubMed\]](#)
123. Phillips, G.; Li, G.; Guo, J.; Yu, S.F.; Lee, G.; Zhang, D.; Dragovich, P.S.; Pillow, T.; Wei, B.; Lee, M.V.; et al. Preclinical development of DHES0815A: A HER2-directed antibody-drug conjugate comprised of a reduced potency PBD dimer linked to a domain I binding HER2 antibody. In Proceedings of the San Antonio Breast Cancer Symposium 2021, San Antonio, TX, USA, 7–10 December 2021; Cancer Research: San Antonio, TX, USA, 2022.
124. Krop, I.; Hamilton, E.; Jung, K.H.; Modi, S.; Kalinsky, K.M.; Phillips, G. A phase I dose-escalation study of DHES0815A, a HER2-targeting antibody-drug conjugate with a DNA monoalkylator payload, in patients with HER-2 positive breast cancer. In Proceedings of the San Antonio Breast Cancer Symposium 2021, San Antonio, TX, USA, 7–10 December 2021; Cancer Research: San Antonio, TX, USA, 2022.
125. Pegram, M.; Rasco, D.; Spira, A.; Wang, D.; Weinberg, B.A.; Alonso, M.; Fang, L.; Husain, A.; Kowanetz, M.; Perez, E.A.; et al. Preliminary results from a phase I/II study of BDC-1001, a novel HER2 targeting TLR7/8 immune-stimulating antibody conjugate (ISAC), in patients (pts) with advanced HER2-expressing solid tumors. In Annals of Oncology, Proceedings of the ESMO Congress 2021, Online, 16–21 September 2021; Elsevier: Amsterdam, The Netherlands, 2021.
126. Hagemann, U.B.; Ellingsen, C.; Schuhmacher, J.; Kristian, A.; Mobergslie, A.; Cruciani, V.; Wickstroem, K.; Schatz, C.A.; Kneip, C.; Goffier, S.; et al. Mesothelin-Targeted Thorium-227 Conjugate (MSLN-TTC): Preclinical Evaluation of a New Targeted Alpha Therapy for Mesothelin-Positive Cancers. *Clin. Cancer Res.* **2019**, *25*, 4723–4734. [\[CrossRef\]](#)
127. Karlsson, J. HER2-TTC: A Targeted thorium conjugate to treat HER2 expressing cancers with potent alpha radiation [abstract]. *Cancer Res.* **2021**, *81* (Suppl. S13), ND07. [\[CrossRef\]](#)
128. Zheng, G.; Guo, Z.; Li, W.; Xi, W.; Zuo, B.; Zhang, R.; Wen, W.; Yang, A.G.; Jia, L. Interaction between HLA-G and NK cell receptor KIR2DL4 orchestrates HER-2 positive breast cancer resistance to trastuzumab. *Signal Transduct. Target. Ther.* **2021**, *6*, 236. [\[CrossRef\]](#)
129. Yang, T.; Kang, L.; Li, D.; Song, Y. Immunotherapy for HER-2 positive breast cancer. *Front. Oncol.* **2023**, *13*, 1097983. [\[CrossRef\]](#) [\[PubMed\]](#)
130. Loi, S.; Giobbie-Hurder, A.; Gombos, A.; Bachelot, T.; Hui, R.; Curigliano, G.; Campone, M.; Biganzoli, L.; Bonnefoi, H.; Jerusalem, G.; et al. Pembrolizumab plus trastuzumab in trastuzumab-resistant, advanced, HER-2 positive breast cancer (PANACEA): A single-arm, multicentre, phase 1b-2 trial. *Lancet Oncol.* **2019**, *20*, 371–382. [\[CrossRef\]](#) [\[PubMed\]](#)
131. Huober, J.; Barrios, C.H.; Niikura, N.; Jarzab, M.; Chang, Y.C.; Huggins-Puhalla, S.L.; Pedrini, J.; Zhukova, L.; Graupner, V.; Eiger, D.; et al. Atezolizumab with Neoadjuvant Anti-Human Epidermal Growth Factor Receptor 2 Therapy and Chemotherapy in Human Epidermal Growth Factor Receptor 2-Positive Early Breast Cancer: Primary Results of the Randomized Phase III Impassion050 Trial. *J. Clin. Oncol.* **2022**, *40*, 2946–2956. [\[CrossRef\]](#) [\[PubMed\]](#)
132. Bekes, M.; Langley, D.R.; Crews, C.M. PROTAC targeted protein degraders: The past is prologue. *Nat. Rev. Drug Discov.* **2022**, *21*, 181–200. [\[CrossRef\]](#)
133. Maneiro, M.A.; Forte, N.; Shchepinova, M.M.; Kounde, C.S.; Chudasama, V.; Baker, J.R.; Tate, E.W. Antibody-PROTAC Conjugates Enable HER2-Dependent Targeted Protein Degradation of BRD4. *ACS Chem. Biol.* **2020**, *15*, 1306–1312. [\[CrossRef\]](#)
134. Sloas, C.; Gill, S.; Klichinsky, M. Engineered CAR-Macrophages as Adoptive Immunotherapies for Solid Tumors. *Front. Immunol.* **2021**, *12*, 783305. [\[CrossRef\]](#)
135. Marofi, F.; Al-Awad, A.S.; Sulaiman Rahman, H.; Markov, A.; Abdelbasset, W.K.; Ivanovna Enina, Y.; Mahmoodi, M.; Hassanzadeh, A.; Yazdanifar, M.; Stanley Chartrand, M.; et al. CAR-NK Cell: A New Paradigm in Tumor Immunotherapy. *Front. Oncol.* **2021**, *11*, 673276. [\[CrossRef\]](#)
136. Al-Awadhi, A.; Lee Murray, J.; Ibrahim, N.K. Developing anti-HER2 vaccines: Breast cancer experience. *Int. J. Cancer* **2018**, *143*, 2126–2132. [\[CrossRef\]](#)

137. Lollini, P.L.; Cavallo, F.; Nanni, P.; Forni, G. Vaccines for tumour prevention. *Nat. Rev. Cancer* **2006**, *6*, 204–216. [\[CrossRef\]](#)
138. Handy, C.E.; Antonarakis, E.S. Sipuleucel-T for the treatment of prostate cancer: Novel insights and future directions. *Future Oncol.* **2018**, *14*, 907–917. [\[CrossRef\]](#)
139. Dillon, P.M.; Brenin, C.M.; Slingluff, C.L., Jr. Evaluating Nelipecimut-S in the Treatment of Breast Cancer: A Short Report on the Emerging Data. *Breast Cancer* **2020**, *12*, 69–75. [\[CrossRef\]](#) [\[PubMed\]](#)
140. Murray, J.L.; Gillogly, M.E.; Przepiorka, D.; Brewer, H.; Ibrahim, N.K.; Booser, D.J.; Hortobagyi, G.N.; Kudelka, A.P.; Grabstein, K.H.; Cheever, M.A.; et al. Toxicity, immunogenicity, and induction of E75-specific tumor-lytic CTLs by HER-2 peptide E75 (369–377) combined with granulocyte macrophage colony-stimulating factor in HLA-A2+ patients with metastatic breast and ovarian cancer. *Clin. Cancer Res.* **2002**, *8*, 3407–3418. [\[PubMed\]](#)
141. Clifton, G.T.; Hale, D.; Vreeland, T.J.; Hickerson, A.T.; Litton, J.K.; Alatrash, G.; Murthy, R.K.; Qiao, N.; Philips, A.; Lukas, J.; et al. Results of a Randomized Phase IIb Trial of Nelipecimut-S + Trastuzumab versus Trastuzumab to Prevent Recurrences in Patients with High-Risk HER2 Low-Expressing Breast Cancer. *Clin. Cancer Res.* **2020**, *26*, 2515–2523. [\[CrossRef\]](#) [\[PubMed\]](#)
142. Mittendorf, E.A.; Clifton, G.T.; Holmes, J.P.; Schneble, E.; van Echo, D.; Ponniah, S.; Peoples, G.E. Final report of the phase I/II clinical trial of the E75 (nelipecimut-S) vaccine with booster inoculations to prevent disease recurrence in high-risk breast cancer patients. *Ann. Oncol.* **2014**, *25*, 1735–1742. [\[CrossRef\]](#)
143. Mittendorf, E.A.; Lu, B.; Melisko, M.; Price Hiller, J.; Bondarenko, I.; Brunt, A.M.; Sergii, G.; Petrakova, K.; Peoples, G.E. Efficacy and Safety Analysis of Nelipecimut-S Vaccine to Prevent Breast Cancer Recurrence: A Randomized, Multicenter, Phase III Clinical Trial. *Clin. Cancer Res.* **2019**, *25*, 4248–4254. [\[CrossRef\]](#)
144. Patel, S.; McWilliams, D.; Thompson, J.; Patel, M.; Daugherty, F.J. Evaluation of booster injections in maintaining peak immunity in a phase IIb study evaluating HER2/neu peptide GP2 (GLSF-100) versus GM-CSF alone after adjuvant trastuzumab in women with HER2-positive breast cancer [abstract]. 2022 ASCO Annual Meeting. *J. Clin. Oncol.* **2022**, *40*, LBA550. [\[CrossRef\]](#)

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Evolving Evidence for the Optimization of Neoadjuvant Therapy in Triple-Negative Breast Cancer

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Breast Cancer: Basic and Clinical Research
Volume 16: 1–12

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DOI: 10.1177/11782234221107580



ABSTRACT: Representing 15% to 20% of all invasive breast cancers, adjuvant systemic treatment for early-stage, high-risk triple-negative breast cancer (TNBC) is preferentially done in the neoadjuvant setting based on a chemotherapy backbone of anthracyclines and taxanes. Pathological complete response to neoadjuvant treatment constitutes the main objective, regarding its correlation with oncological outcomes. The optimal neoadjuvant regimen to achieve the highest rates of pathological complete response is still under investigation, with the increasing knowledge on the molecular pathways, genomic sequencing, and immunological profile of TNBC allowing for the development of a wide array of new therapeutic options. This review aims to summarize the current evidence and ongoing clinical trials of new therapeutic options for the neoadjuvant treatment of TNBC patients.

KEYWORDS: Triple negative, neoadjuvant, clinical trial, review

RECEIVED: July 24, 2021. **ACCEPTED:** May 27, 2022.

TYPE: Review

FUNDING: The author(s) received no financial support for the research, authorship, and/or publication of this article.

DECLARATION OF CONFLICTING INTERESTS: The author(s) declared no potential conflicts of interest with respect to the research, authorship, and/or publication of this article.

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Introduction

Triple-negative breast cancer (TNBC) occurs more frequently in younger women (<40 years) and has an aggressive behaviour with shorter relapse-free and overall survival (OS) rates. According to GLOBOCAN 2020, female breast cancer surpassed lung cancer as the leading cause of cancer globally in 2020, with 2.3 million new cases worldwide.^{1,2} Presenting symptoms can be classified into 3 different categories: breast lump, nonlump breast, and nonbreast symptoms. Breast lump is the most common presentation sign allowing for more frequent diagnosis at a curable stage.³

Expression of hormonal receptors and/or human epidermal growth factor receptor 2 (HER2) are the main elements guiding clinical management of breast cancer, allowing for the subclassification as hormone receptor positive, HER2 positive, or TNBC.¹

Patients with TNBC are usually diagnosed at an earlier median age and face a more aggressive disease's course with a peak in recurrence between 1 to 3 years after diagnosis.^{4,5} Integration of histological, molecular, and genetic profiles is crucial for a better clinical management of TNBC.

Lehmann et al.⁶ defined a molecular classification for TNBC of 6 subtypes with basal-like 1 (BL1), immunomodulatory (IM), and molecular (M) subtypes predominantly enriched with high-grade tumours; luminal androgen receptor (LAR) and mesenchymal stem-like (MSL) subtypes mostly associated with low-grade tumours. In 2016, Lehman et al.⁷ revised the proposed classification into 4 molecular subtypes: BL1, BL2, M, and LAR. Regarding clinical outcome, LAR subtype has worst prognosis, and IM has better outcomes.^{7,8} Recent reviews have correlated the different molecular and transcriptomic profiles of TNBC to better understand tumoral behaviour and develop new targeted therapies.⁹

In the last 5 years, the development of new drugs in the neoadjuvant setting such as immune-checkpoint inhibitors (ICIs), poly-ADP-ribosyl polymerase inhibitors (PARPi), phosphatidylinositol-4,5-bisphosphate 3-kinase catalytic subunit alpha (PIK3CA) inhibitors, androgen receptor (AR)-targeted therapies or anti-VEGF agents have been analysed in several trials.¹⁰

Rationale for Neoadjuvant Treatment on TNBC and Patient Selection

Neoadjuvant chemotherapy is standard of care for locally advanced/inoperable TNBC, with clinical response to treatment predicting clinical outcomes in this subset of patients.¹¹ Triple-negative breast cancers have clinicopathologic features that support its aggressive behaviour when compared to non-TNBC: high mean tumour size, higher grade, and high proliferation index at diagnosis.¹²

Patients with TNBC tend to present higher rates of polymerase chain reaction (pCR) after neoadjuvant chemotherapy compared to luminal subtypes.¹³ Triple-negative breast cancer are more commonly associated to breast cancer gene (BRCA) mutations with over 80% of BRCA1-mutated and almost 10% of BRCA2-mutated breast cancer classified within this subgroup.⁵ Breast cancer genes encode crucial components of DNA damage repair mechanisms such as homologous recombination deficiency (HRD) and DNA stability.⁵ Homologous DNA recombination defects render TNBC more susceptible to DNA-damaging agents including platinum-based cytotoxic agents or poly (ADP-ribose) polymerase (PARP) inhibitors.¹⁴ These agents cause DNA strand cross-links that are neither recognized nor repaired by the homologous recombination defective system, leading to cell death.

Table 1. Molecular pathways in TNBC subtypes as therapeutic targets.

MOLECULAR SUBTYPES	CELLULAR PATHWAYS	THERAPEUTIC TARGETS	REF.
BL1	Cell cycle DNA repair Proliferation	PARP inhibitors Platinum agents Conventional chemotherapy	Lehmann et al. ⁶ , Burstein et al. ²⁰ , Collignon et al. ²¹
BL2	Growth factor pathways Metabolic pathways (glycolysis and gluconeogenesis)	mTOR inhibitors Growth-factor inhibitors	
IM	Immune cell processes	Immune-checkpoint inhibitors	
M	Cell motility, differentiation, and growth factor signalling	mTOR inhibitors EMT-targeted therapy CSC-targeted therapy AXL inhibitor	
MSL	Low proliferation Angiogenesis	PI3K inhibitors Antiangiogenic therapy SRC antagonist	
LAR	Androgen receptor Luminal gene expression Molecular apocrine subtype	Antiandrogen blockade CDK4/6 inhibitors Immune-checkpoint inhibitors	

Source: Adapted from Lehmann et al.⁶ Collignon et al.²¹ and Burstein et al.²⁰

Abbreviations: AXL, tyrosine-protein kinase receptor; BL, basal-like; CDK, cyclin-dependent kinase; CSC, cancer stem cells; DNA, deoxyribonucleic acid; EGFR, epidermal growth factor receptor; EMT, epithelial-mesenchymal-transition; FGFR, fibroblast growth factor receptors; IGF-1R, insulin-like growth factor receptor; IL, interleukin; IM, immunomodulatory; LAR, luminal androgen receptor; MET, hepatocyte growth factor; MSL, mesenchymal stem like; mTOR, mammalian target of rapamycin; PARP, poly ADP-ribose polymerase; PD1, programmed cell death 1; PDGFR, platelet-derived growth factor receptors; PD-L1, programmed death-ligand 1; PI3K, phosphatidylinositol 3-kinase; SRC, Proto-oncogene tyrosine-protein kinase Src; TGF β , transforming growth factor beta; TNBC, triple-negative breast cancer.

The National Comprehensive Cancer Network (NCCN) 2022 Breast Cancer Guidelines standard of care for TNBC comprises a combination of anthracyclines plus taxanes in the neoadjuvant setting. Pathological complete response after neoadjuvant treatment is associated with improvement in disease-free survival (DFS).¹¹ Furthermore, patients who do not have a pathological complete response after neoadjuvant chemotherapy have a 20% to 30% higher risk of relapse. Therefore, the degree of pathological response after neoadjuvant chemotherapy allows the definition of a subset of TNBC patient which benefit from adjuvant chemotherapy, as stated in CREATE-X trial.¹⁵

Early TNBC cT1a (<0.5 cm) or cT1b (0.6–1.0 cm) without lymph node involvement has good prognosis without neoadjuvant treatment. Vaz-Luis et al performed an observational study of 363 TNBC patients with T1a–b N0 disease treated with adjuvant chemotherapy versus those without adjuvant therapy to assess the distant recurrence-free survival (DRFS). The subgroup of untreated T1a and T1b had a 5-year DRFS of 93% and 90% compared to treated T1a and T1b DRFS of 100% and 96%.¹⁶

The NCCN guidelines on breast cancer define candidates for neoadjuvant treatment patients with inoperable disease or with operable disease if one of the following is present: cT $\geq$ 2 or cN $\geq$ 1 HER2-positive or TNBC; large primary relative to breast size if breast conservation is desired; cN+ likely to become cN0 following neoadjuvant treatment; delay of definitive surgery. Therefore, for patients with TNBC tumours >1 cm N0/N+, systemic chemotherapy is recommended. Standard chemotherapy regimens for TNBC consist of a combination of anthracyclines, taxanes, and alkylating agents.

Sequential use of anthracyclines plus taxanes is mainly used based on retrospective evidence from subgroup analysis before 2010. First reports for anthracycline-based regimens had pCR rates ranging from 14% to 47% and sequential regimens of anthracyclines plus taxanes rates ranging from 17% to 39%.¹⁷ The phase III BCIRG-005 clinical trial assessed the efficacy and safety of sequential versus concurrent use of taxane-anthracycline-based adjuvant chemotherapy. Ten years follow-up analysis confirmed that doxorubicin, cyclophosphamide, and docetaxel (TAC) and sequential doxorubicin, cyclophosphamide plus docetaxel monotherapy (AC $\rightarrow$ T) had similar efficacy outcomes. Doxorubicin, cyclophosphamide, and docetaxel regimen with granulocyte colony-stimulating factor (G-CSF) support provided a shorter treatment duration with less acute toxicity.¹⁸

The GeparTrio trial assessed pCR rate after neoadjuvant anthracyclines, cyclophosphamide, and taxanes. The subgroup analysis of this cohort revealed rates of pCR up to 57%.¹⁹ Since these first trials, the main goal has been to define which agent or combination of agents achieves the highest pCR in this setting.

TNBC Molecular Heterogeneity as Therapeutic Target

Molecular heterogeneity of TNBC was first formally classified in 2011 by Lehman et al. with the proposal of 6 molecular subtypes, each presenting unique molecular characteristics that can be used as therapeutic targets (Table 1). Owing to the histological landscape of tumour specimens, IM and MSL subtypes were fused into the same molecular subtype reflecting infiltrating lymphocytes and tumour-associated mesenchymal

cells, originating a 4-subtype classification (BL1, BL2, M, and LAR).⁷ Each subtype has a specific molecular signature that contributes to the differential response to each treatment strategy. Basal-like 1 has increased expression of cell cycle and DNA damage response genes,⁸ contributing to an increased sensitivity to platinum agents and better response to neoadjuvant chemotherapy. Homologous recombination deficiency sensitizes tumour cells to platinum-induced cell death.

Differential subtype response to neoadjuvant therapy was first analysed in a retrospective study of 125 TNBC patients that analysed the addition of carboplatin to neoadjuvant chemotherapy with a combination of anthracyclines plus taxanes, anthracyclines, or taxanes. Basal-like 1 tumours had the highest pCR under carboplatin regimens than the remaining subtypes (BL2, M, IM, MLS, and LAR) summed (80% vs. 23%, $p = .027$), and LAR tumours had the lowest pCR despite treatment regimen (14.3% vs. 42.7%, $p = .045$).²⁴

Despite being chemo-resistant, LAR subtype has molecular evidence of oestrogen plus AR activation,²⁰ which seems to contribute to its lower proliferative index and response to anti-oestrogen or anti-androgen therapies.^{4,23}

Mesenchymal subtype (MES) was characterized by pathways of mismatch repair and DNA damage repair. Basal-like immunosuppressed (BLIS) showed downregulation of immune and cytokine pathways, being associated with worse outcomes. Basal-like immune-activated (BLIA) showed upregulation of immunoregulatory pathways and proved to be the subtype with best clinical outcomes.²⁴

Relevance of BRCA Status

DNA repair mechanisms play an important role on the stability and integrity of the genome. Homologous recombination, end joining, mismatch repair, nucleotide/base excision repair, and telomere metabolism are the most relevant DNA repair mechanisms.²⁵

From the wide range of lesions occurring to the DNA molecule, DNA double-strand break (DSB) poses the greatest threat to genome stability.²⁵ BRCA1 and BRCA2 are crucial for the homologous recombination repair, and inherited defects to one of these genes can lead to cancer. Breast cancer gene loss translates in genomic instability leading to oncogenic transformation of nontumorigenic cellular populations into cancer stem cells and tumour development. As classic tumour suppressor genes, BRCA genes tend to rely on the '2-hit' hypothesis to initiate cancer progression. Individuals predisposed to BRCA-associated hereditary cancers carry a germline mutation in the BRCA genes, needing a second hit to the wild-type BRCA allele to develop cancer.²²

Five percent of all breast cancers arise from BRCA1/BRCA2 germline mutations due to cellular impairment to repair DSB through homologous recombination.^{26,27} Germline mutations for BRCA genes occur in 10% to 20% of TNBC patients and somatic mutations in 3% to 5%.²⁸ Breast cancer

gene 1/2 status seems to affect tumoral phenotype, with germline-mutated BRCA1 carriers mainly developing TNBC at younger ages (median age at diagnosis of 47 years for BRCA1 versus 58 years for BRCA2) and BRCA2 carriers usually presenting with hormone receptor-positive disease.^{29,30}

Recently, the concept of BRCAness status has emerged comprising sporadic cancers that do not have germline BRCA mutation but share phenotypic characteristics and defective HR.³¹ Somatic mutations, large deletions, DNA hyper-methylation of BRCA1/2, germline PALB2 mutations, and RAD51C hyper-methylation are the most common associated mechanisms.³¹ Breast cancer gene 1 mutation increases sensitivity to interstrand crosslinking agents (platinum or alkylating agents), topo-isomerase II inhibitors (anthracyclines), or PARP inhibitors.^{32,33}

Neoadjuvant Platinum Agents for TNBC

Despite being put aside in the early studies on neoadjuvant treatment for TNBC, increasing knowledge on the association between BRCA mutation and triple-negative phenotype renewed the interest on this subset of drugs. Platinum salts have increased efficacy in cells with a defected DNA repair system, inducing single-strand (SSBs) and DSBs.³⁴ Several trials and retrospective studies demonstrated the effectiveness of platinum-based regimens for TNBC BRCA-mutated patients in a preoperative setting. A retrospective analysis of 6903 patients included 102 patients with BRCA1 germline mutation, which received neoadjuvant chemotherapy with cyclophosphamide, methotrexate, fluorouracil (14 patients), docetaxel plus doxorubicin (25 patients), doxorubicin without docetaxel (51 patients), or cisplatin (12 patients). Twenty-four of 102 patients achieved pCR, with highest pCR rate for gBRCA1 carriers who received neoadjuvant cisplatin therapy (83%; 10 out of 12 patients), no survival data was reported.³⁵

Platinum-based neoadjuvant regimens showed superior pCR rates when compared to standard chemotherapy regimens in several randomized clinical trials (Table 2). The CALGB 40603/Alliance was a randomized phase II trial designed to assess the addition of carboplatin and/or bevacizumab to neoadjuvant regimens in stage II-III TNBC patients. Patients were randomized to paclitaxel 80 mg/m² weekly for 12 weeks followed by dose-dense doxorubicin plus cyclophosphamide (ddAC) for 4 weeks or assigned to carboplatin (AUC 6) every 21 days for 4 weeks with/without bevacizumab 10 mg/kg each 2 weeks during 9 cycles. Carboplatin AUC 6 arm had a greater percentage of pCR (41%-54%; odds ratio [OR]:1.71; $p = .0029$). Despite better pCR, the addition of carboplatin did not improve long-term OS.^{36,37}

The GeparSixto/GBG 66, a phase II clinical trial, randomized previously untreated, nonmetastatic, stage II/III TNBC patients to 18 weeks of paclitaxel once a week, nonpegylated liposomal doxorubicin plus bevacizumab simultaneously to the backbone regimens, either carboplatin (AUC 1.5)

Table 2. Neoadjuvant trials of platinum-based chemotherapy in early-stage TNBC.

TRIAL	PHASE	N	ARMS	PCR	DFS	OS	REF.
CALGB 40603	3	433	T + AC vs T + Cbp + AC (± bevacizumab)	T + AC: 41% T + Cbp + AC: 54%	T + AC: 56.6% T + Cbp + AC: 85.5%	T + AC: 63.4% T + Cbp + AC: 87.9%	Sikov et al. ³⁶ Shepherd et al. ³⁷
GeparSixto	2	315	npDOX + T + Bev npDOX + T + Bev + Cbp	npDOX + T + Bev: 42% npDOX + T + Bev + Cbp: 57%	npDOX + T + Bev: 75.8% npDOX + T + Bev + Cbp: 86.1%	npDOX + T + Bev: 86% npDOX + T + Bev + Cbp: 91.9%	von Minckwitz et al. ³⁸ Loibl et al. ³⁹
GeparSepto GBG 69	3	276	NabT → ECPac → EC NabT → AC/EC/FEC	NabPac: 56% T: 37%	NabPac: 78.8% T: 68.9%	NabPac: 89.7% T: 87.2%	Untch et al. ⁴⁰
ETNA	3	219	NabT → AC/EC/FEC T → AC/EC/FEC	NabPac: 41% T: 37%	NabPac: 63.5% T: 61.0%	NabPac: 87.3% T: 84.8%	Gianni et al. ⁴¹
WSG-ADAPT	2	336	NabT + Gem NabT + Cbp	Nabpac + Gem: 28.7% Nabpac + Cbp: 45.9%	Not reported	Nabpac + Gem: 84.7% Nabpac + Cbp: 92.2%	Gluz et al. ⁴²
NCT01276769	2	91	T + Cbp → surgery → A EP → surgery → taxane	T + Cbp: 38.6% EP: 14%	T + Cbp: 71.1% EP: 52.8%	T + Cbp: 70.1% EP: 72.5%	Zhang et al. ⁴³
GEICAM/2006-03	2	94	EC-D EC-D + Cbp	EC-D: 30% EC-D + Cbp: 30%	Not reported	Not reported	Alba et al. ⁴⁴
NCT01090128	1	10	NabT + AC	100%	Not reported	Not reported	Werner et al. ⁴⁵
NcoSTOP	2	100	T + Cbp + AC Cbp + Docetaxel	T + Cbp + AC: 55% Cbp + Docetaxel: 52%	With pCR: 100% Without pCR: 81%	With pCR: 100% Without pCR: 86%	Sharma et al. ⁴⁶

Abbreviations: AC, anthracycline; Bev, bevacizumab; Cbp, carboplatin; D, docetaxel; DFS, disease-free survival; EC, epirubicin + cyclophosphamide; EP, epirubicin + paclitaxel; FEC, 5-fluorouracil + epirubicin + cyclophosphamide; Gem, gemcitabine; NabT, nab-paclitaxel; OS, overall survival; pCR, pathological complete response; T, paclitaxel; TNBC, triple-negative breast cancer.

each week or no carboplatin. First analysis showed that adding carboplatin resulted in better pCR rates (53.2% vs 36.9; $p = .005$).³⁸ The final report confirmed a significantly better DFS (hazard ratio [HR]: 0.56; 95% confidence interval [CI]: [0.34, 0.93]; $p = .022$) without statistically significant improvement of OS.³⁹

Efficacy and safety of combining taxanes and platinum agents in the neoadjuvant setting was assessed in a recent phase II trial that analysed 59 stage II/III TNBC that underwent 4 cycles of carboplatin AUC 6 and weekly nab-paclitaxel 100 mg/m² for 16 cycles. Patients with DNA repair defect achieved higher pCR rates (overall response rate [ORR]: 4.76; $p = .03$).⁴⁷

TBCRC 030 was an open-label, randomized, phase II trial aimed to evaluate the impact of HRD on pathologic response to single-agent cisplatin or paclitaxel. Patients were randomized to either cisplatin (75 mg/m² every 3 weeks) during 4 cycles or weekly paclitaxel (80 mg/m²) for 12 weeks, with a pCR rate of 15.3% for cisplatin and 11.9% for paclitaxel.⁴⁸

Regarding the influence of BRCA status on platinum sensitive and the new targeted drugs for BRCA-mutated TNBC, BrightTNess trial compared the impact on pCR of adding veliparib to carboplatin or carboplatin alone to standard neoadjuvant chemotherapy in TNBC. A phase III, randomized, placebo-controlled trial randomly assigned 634 patients (2:1:1) to receive 1 of 3 segment 1 regimens: paclitaxel (80 mg/m² weekly for 12 cycles) plus carboplatin AUC6 (every 3 weeks, 4 cycles) plus veliparib 50 mg *per os* twice a day; paclitaxel, carboplatin, and veliparib placebo (twice a day); paclitaxel, carboplatin placebo, and veliparib placebo. Following segment 1, all received doxorubicin plus cyclophosphamide every 3 weeks for 4 cycles. Results stated higher pCR rate when combining paclitaxel, carboplatin, and veliparib than in patients receiving paclitaxel alone (53% vs 31%; $p < .0001$). This was not true for the comparison with patients receiving paclitaxel plus carboplatin (58%; $p = .36$).⁴⁹

The phase III GeparOcto trial compared efficacy and safety of sequential treatment with dose-dense epirubicin, paclitaxel, and cyclophosphamide (iddEPC) or weekly paclitaxel plus nonpegylated liposomal doxorubicin with additional carboplatin (PMCb) in TNBC. Final analysis demonstrated a pCR of 48.3% for iddEPC and 48% for the carboplatin arm, showing that PMCb did not improve pCR rate compared to iddEPC.⁵⁰

GeparOLA trial documented a similar pCR rate to GeparSixto trial in HRD TNBC patients treated with neoadjuvant paclitaxel plus carboplatin (59.3% vs 63.5%).⁵¹

The phase II INFORM Trial aimed to compare pCR after neoadjuvant single-agent cisplatin (75 mg/m² for 3–4 cycles) or doxorubicin-cyclophosphamide (60 mg/m²–600 mg/m² for 3–4 cycles) every 2 (dose dense) or 3 weeks according to oestrogen receptor status in stage II/III BRCA carriers HER2-negative patients. For TNBC, dose-dense

Table 3. Neoadjuvant clinical trials of PARP inhibitors in early-stage TNBC.

TRIAL	PHASE	N	ARMS	PCR	REF.
BrightNess	3	A:316 B:160 C:158	A: Veliparib + Cp + T → AC B: Placebo + Cp + T → AC C: Placebo + T → AC	A: 53% B: 58% C: 31%	Loibl et al. ⁴⁰ Geyer et al. ⁶⁰
Talazoparib NCT02282345	2	17	24 weeks talazoparib (without neoadjuvant chemotherapy)	47%	Litton et al. ⁶¹
GeparOLA	2	107	Pac + Ola 1 Pac + Cp → EC	Pac + Ola: 55% Pac + Cp: 48%	Fasching et al. ⁵¹

Abbreviations: AC, doxorubicin + cyclophosphamide; Cp, carboplatin; EC, epirubicin + cyclophosphamide; Pac, paclitaxel; PARP, poly ADP-ribose polymerase; pCR, pathological complete response; T, docetaxel; TNBC, triple-negative breast cancer.

schedule was mandatory, with 23% achieving pCR with cisplatin and 29% with AC.⁵² Pathologic complete response rate for cisplatin versus AC was consistent with results from GeparSixto, GeparOcto, and BrightNess trials. Among BRCA carrier's addition of platin did not significantly improve pCR. The lack of additional benefit among BRCA carriers could be explained by the greater sensitivity of BRCA breast cancer carriers to DNA-damaging agents (CDDP or anthracyclines plus alkylating agents). Higher sensitivity to DNA-damaging agents leads to a better response to anthracyclines plus alkylating agents, reducing the potential benefit of adding a platinum agent. Therefore, gBRCA1/2 status and HRD may better predict chemosensitivity and higher pCR but not platinum benefit in the neoadjuvant setting.⁵³

Conclusion

Addition of a platinum agent to standard neoadjuvant anthracycline and taxane-based chemotherapy has been matter of extensive investigation, with most clinical trials showing a benefit in the rate of pathological complete response. Moreover, KEYNOTE-522 and BrightNess trial showed that improvement on pCR rate led to an increase on event-free survival (EFS) for TNBC patients treated with a platinum agent. Despite increases in EFS, no clinical trial demonstrated OS benefit. A recent meta-analysis of clinical trials comparing addition of carboplatin to the standard anthracycline-taxane regimen showed benefit in DFS (HR: 0.60; 95% CI: 0.47-0.78; $p < .001$) and OS (HR: 0.69, 95% CI: 0.50-0.95; $p = .02$).⁵⁴ Therefore, addition of a platinum agent to standard neoadjuvant chemotherapy should be a shared informed decision, integrating the grade of toxicity that patient is able/willing to tolerate and the clinicopathological features of the disease.

Neoadjuvant PARP Inhibitors for TNBC

In 2005, Farmer et al pivotal study demonstrated that BRCA1/BRCA2 dysfunction increased cell sensitivity to inhibition of PARP enzymatic activity, involved in base excision repair. This

repair mechanism represents a key pathway in DSB repair, and its inhibition leads to chromosomal instability and cell death by apoptosis.⁵⁵

A subsequent study used the concept of synthetic lethality to prove that, despite loss of one gene is not lethal, concurrent inactivation leads to cell death. Poly-ADP-ribosyl polymerase is involved in single-strand DNA breaks repair, and its inhibition alone is not lethal because SSBs can be repaired by other mechanisms such as homologous repair. In the absence of BRCA1/BRCA2, homologous repair mechanisms cannot compensate for PARP inhibition, leading to increased cytotoxicity and subsequent cell death.⁵⁶ The promising results of a proof-of-concept trial for Olaparib in germline BRCA1/2-mutated advanced breast cancer in 2009 was the first step for the development of PARPi as a valid therapeutic option for breast cancer (Table 3).⁵⁷ OlympiAD and EMBRACA trials showed positive results for olaparib and talazoparib in the metastatic setting for germline BRCA-mutated HER2-negative breast cancer patients.^{58,59} Following PARPi approval in this setting, several clinical trials aimed to compare pCR and long-term outcomes between PARPi and standard neoadjuvant chemotherapy.

I-SPY trial, a phase II platform trial, assessed the impact of adding novel agents or novel combinations to standard neoadjuvant chemotherapy in high-risk primary breast cancer. Addition of veliparib and carboplatin versus weekly paclitaxel improved pCR rate in TNBC patients (52% vs 26%).⁶²

Following these positive results, BrightNess trial assessed the association of veliparib plus carboplatin or carboplatin alone to neoadjuvant chemotherapy. Combination of paclitaxel, carboplatin, and veliparib did not achieve a higher pCR when compared to paclitaxel and carboplatin (53% vs 58%, $P = .36$).⁴⁹ Subgroup analysis stratifying pCR by germline BRCA mutation found no difference in favour of gBRCA mutation carriers (51% vs 48%).⁴⁹ Long-term outcomes presented in ESMO 2021 showed that at a median follow-up of 4.5 years after surgery, pCR and EFS were not improved by the addition of veliparib.⁶⁰

Both OlympiAD and I-SPY 2 led to the design of a trial to assess olaparib plus standard neoadjuvant chemotherapy in

early breast cancer. Multicentric phase II GeparOLA trial explored the combination of neoadjuvant chemotherapy plus olaparib or carboplatin, followed by epirubicin/cyclophosphamide in HRD nonmetastatic HER2-negative breast cancer. About 55.1% on paclitaxel plus olaparib versus 48.6% on paclitaxel plus carboplatin achieved pCR. Subgroup stratification showed that TNBC patients had a pCR rate of 56.0% with olaparib and of 59.3% with carboplatin.^{62,63}

A similar rate of pCR was observed for the use of PARPi for TNBC neoadjuvant approach both in BrighTNess and GeparOLA trials (53% vs 56%). However, monotherapy with PARPi in metastatic setting was assessed for patients with gBRCA1/2 mutation regardless of HR status and reported an improvement of overall response- and progression-free survival compared to nonplatin regimens.⁵¹ Results from the GeparOLA phase II study did not show evidence of benefit in pCR for the combination of olaparib plus conventional chemotherapy.⁵¹

Recent data have been published regarding the combination of olaparib plus platinum-based neoadjuvant regimens, with PARTNER phase II/III trial randomizing patients to neoadjuvant carboplatin AUC5 plus paclitaxel with or without olaparib 150 mg during 4 cycles followed by physician's choice of anthracyclines regimen. Preliminary data on safety supports the combination, with the most common adverse events (AEs) grade ≥ 3 being haematological. Neutropenia was reported in 19% with febrile neutropenia in 2% of patients. Other haematological AE reported were anaemia (15%) and thrombocytopenia (5%). Fatigue (7%), hypertension (3%), headache (3%), and diarrhoea (2%) were the most common grade ≥ 3 nonhaematological AEs.⁶⁴

The PETREMAC trial is a large phase II clinical trial evaluating the use of several treatment options. Olaparib monotherapy in 32 treatment-naïve TNBC patients had a high overall response rate (56%).⁶⁵

Talazoparib has been evaluated on the neoadjuvant setting since 2017, when the first feasibility study for early breast cancer with germline BRCA mutation was performed.⁶² The highest response rates and favourable toxicity profile led to the modification to a phase II design to assess pCR after 4 to 6 months of single-agent talazoparib. Litton et al designed a trial to assess pCR to monotherapy with talazoparib for 6 months in germline BRCA-positive patients. Twenty patients underwent 6 months of daily talazoparib followed by surgery, with TNBC patients achieving a pCR rate of 57% (95% CI: 29%–82%).⁶⁶

Results from a phase II, nonrandomized, open-label trial on the efficacy plus safety of talazoparib in the neoadjuvant setting for early gBRCA1/2-mutated HER2-negative breast cancer was terminated in September 2020. Forty-eight TNBC patients were treated with 24 weeks of neoadjuvant talazoparib monotherapy 1 mg/day followed by surgery, achieving a pCR

of 46%.⁶⁷ Efficacy data of niraparib monotherapy in the neoadjuvant setting was shown in a phase I clinical trial where 15 patients with somatic or germline BRCA-mutated TNBC received niraparib 200 mg once daily during 2 months before surgery plus additional cycles of niraparib up to 6 months after surgery or neoadjuvant chemotherapy at treating physician's discretion. Fifteen patients performed magnetic resonance imaging (MRI) and breast ultrasound after 2 months of treatment, with a response rate of 89% by MRI.⁶⁸

Conclusion

Although evidence for the use of PARP inhibitors in the neoadjuvant setting is not totally established, current trials suggest a role for neoadjuvant PARP inhibition in germline BRCA-mutated patients. GeparOLA trial indicates that intensity de-escalation with olaparib instead of carboplatin maybe an option for germline BRCA-mutated patients. Furthermore, results from PREMETRAC trial suggest a possible benefit for the use of PARP inhibitors in treatment-naïve TNBC patients, which are corroborated by Litton et al. phase II clinical trial that showed comparable pCR rates between 24 weeks of monotherapy with talazoparib and standard chemotherapy regimens for germline BRCA-mutated TNBC patients. Therefore, PARP inhibitors may represent an option in the neoadjuvant setting for treatment-naïve or minimally treated germline BRCA-mutated TNBC patients.

Neoadjuvant Immunotherapy for TNBC

Despite breast cancer being classified as a non-immunogenic cancer, TNBC IM subtype has characteristics like tumour-infiltrating lymphocytes (TILs), expression of immune evasion molecules (PD-L1) and genomic instability that turns this subtype into a 'hot' and immunogenic tumour.²⁰ Immune-checkpoint inhibitors have shown to improve outcomes compared to standard chemotherapy alone in the metastatic setting. Despite combination of atezolizumab plus paclitaxel did not improve PFS or OS in IMpassion131 clinical trial, results from KEYNOTE-355 and IMpassion130 led to the approval of the combination of conventional chemotherapy and ICIs as first-line treatment for PD-L1 $\geq 10\%$ and PD-L1 $\geq 1\%$ metastatic TNBC, respectively.^{69–71} Following these results in metastatic setting and the evidence that immune escape exponential increases with disease progression, several randomized trials have been studying the use of neoadjuvant chemo-immunotherapy.^{72,73}

Several clinical trials have been published recently, supporting the combination of ICI to standard neoadjuvant chemotherapy as a promising option in the neoadjuvant setting for TNBC (Table 4).

In KEYNOTE 522, TNBC patients were randomized to 4 cycles of pembrolizumab, weekly paclitaxel followed by AC. Final estimated pCR for standard neoadjuvant chemotherapy

Table 4. Neoadjuvant TNBC clinical trials with immunotherapy.

TRIAL	PHASE	N	ARMS	PCR	DFS	REF.
I-SPY2	2	250	Pembro + Pac → AC	60%	Not reported	Nanda et al. ⁷⁴
Keynote522	3	1174	Pembro + Pac + Cbp Placebo → Pac + Cbp	Pembro + Pac + Cbp: 64.8% Pac + Cbp + Placebo: 51.2%	Pembro + Pac + Cbp: 84.5% Pac + Cbp + Placebo: 76.8%	Schmid et al. ⁷⁵
GeparNuevo	2	174	Durva/Placebo + Nab-Pac → EC	Durva: 53.4% Placebo: 44.2%	Durva: 85.6% Placebo: 77.2%	Loibl et al. ⁷³
IMpassion131	3	333	Atezolizumab Nab-paclitaxel + Anthracyclines	Atezolizumab: 58% Nab-Pac + Ant: 41%	Not reported	Cortes et al. ⁷¹
NeoTRIPaPDL1	3	280	Atezolizumab Carboplatin Abraxane AC, EC or FEC	Atezolizumab: 48.6% Chemo: 44.4%	Not reported	Gianni et al. ⁷⁶
GeparDouze	3	1520	Atezolizumab + Cbp + Pac → EC/AC Placebo + Cbp + Pac → EC/AC	Not yet published	On going	Mittendorf et al. ⁷⁹

Abbreviations: AC, doxorubicin + cyclophosphamide; Cb, carboplatin; DFS, disease-free survival; EC, epirubicin + cyclophosphamide; FEC, 5-fluorouracil, epirubicin + cyclophosphamide; NabT, nab-paclitaxel; pCR, pathological complete response; T, paclitaxel; TNBC, triple-negative breast cancer.

versus pembrolizumab was 22% vs 60%, respectively.⁷⁵ Event-free survival was recently reported by Schmid et al.⁷⁸ with an estimated EFS of 84.5% (95% CI: [81.7, 86.9]) for pembrolizumab plus chemotherapy and of 76.8% (95% CI: [72.2, 80.7]) for the placebo-chemotherapy group at 36 months (HR for event or death: 0.63; 95% CI: [0.48, 0.82]; $P < .001$).

Pembrolizumab was further evaluated in a phase III trial that randomly assigned (2:1 ratio) stage II/III TNBC patients to receive neoadjuvant therapy with 4 cycles of pembrolizumab (200 mg), paclitaxel plus carboplatin or placebo each 3 weeks plus paclitaxel and carboplatin. Combination of pembrolizumab plus chemotherapy achieved a pCR of 64.8% (95% CI: [59.9, 69.5]) versus 51.2% (95% CI: [44.1, 58.3]) for the placebo-chemotherapy arm.⁷⁴ Pathological complete response rate was significantly higher with the combination of immunotherapy plus neoadjuvant chemotherapy in the TNBC subgroup.

GeparNuevo is a phase II double-blind placebo-controlled trial, stratifying TNBC patients by stromal TILs, to durvalumab/placebo every 4 weeks plus nab-paclitaxel followed by epirubicin/cyclophosphamide. In both arms, pCR rate was highest for PD-L1-positive tumours, with 53.4% of pCR (95% CI: [42.5%, 61.4%]) for durvalumab and 44.2% (95% CI: [33.5%, 55.3%]) for placebo.⁷³ NeoTRI-PaPDL1, a phase III trial, evaluated the combination of neoadjuvant carboplatin AUC2, nab-paclitaxel with or without atezolizumab followed by adjuvant anthracycline regimen with EFS as primary endpoint. In the intention to treat (ITT) population, addition of atezolizumab resulted in a pCR of 48.6% versus 44.4% (OR 1.18; 95% CI: [0.74, 1.89]; $p = .48$) in the chemotherapy only arm.⁷⁶ However, NeoTRIPaPDL1 was the only trial adopting a nonanthracycline-containing neoadjuvant regimen and not having pCR as primary endpoint.

IMpassion 031, phase III trial, compared efficacy and safety of atezolizumab 840 mg/placebo each 2 weeks plus nab-paclitaxel 125 mg/m² weekly up to 12 weeks followed by doxorubicin 60 mg/m² plus cyclophosphamide at 600 mg/m² each 14 days during 8 weeks in the neoadjuvant setting. A difference of 17% in pCR rate (95% CI: [6, 27]; $p = .0044$) was seen in favour of atezolizumab plus chemotherapy.⁷⁷

Efficacy of atezolizumab in the neoadjuvant setting is being further evaluated on the GeparDouze clinical trial. A phase III, placebo-controlled trial that randomized TNBC patients to receive atezolizumab/placebo 1200 mg IV every 3 weeks concurrently with both sequential regimens of weekly paclitaxel 80 mg/m² for 12 doses with every 3 weeks carboplatin AUC5 (4 cycles maximum) followed by AC or EC every 2 to 3 weeks for 4 cycles. Adjuvant therapy resumes atezolizumab/placebo 1200 mg each 21 days for 6 months.⁷⁹

Tarantino et al. performed a systematic review and meta-analysis evaluating the main clinical trials randomizing TNBC patients to neoadjuvant chemo-immunotherapy regimens. Results confirmed that combination of PD-L1 blockade plus neoadjuvant chemotherapy improves pCR rates. Moreover, in line with NeoTRIPaPDL1 trial, anthracycline-based regimens appeared to be the most efficient strategy to enhance tumour immunogenicity. TONIC is an adaptive, noncomparative, phase II trial that randomized metastatic TNBC patients with less than 4 lines of palliative chemotherapy to 1 of 5 arms: (1) 24(3 × 8) Gy irradiation of one metastatic lesion; (2) doxorubicin 15 mg weekly for 2 weeks; (3) cyclophosphamide 50 mg daily orally; (4) cisplatin 40 mg/m² weekly for 2 weeks; (5) no induction. Following this induction period, all received nivolumab 3 mg/kg until RECIST 1.1 progression.⁸⁰ Main results showed that short-term chemotherapy or irradiation followed by nivolumab lead to clinical benefit with higher

durable response rates. Chemotherapy regimens based on doxorubicin or cisplatin had higher response rate to nivolumab.⁸¹

Conclusion

KEYNOTE-522 results led to a fast change in clinical practice, supporting US Food and Drug Administration (FDA) approval for the combination of pembrolizumab plus standard chemotherapy for neoadjuvant treatment of high-risk, early-stage TNBC patients with CPS ≥ 1 . Despite this approval, evidence is lacking for the selection of responders to upfront pembrolizumab. KEYNOTE-522 subgroup analysis showed a consistent benefit regardless age, performance status, chemotherapy schedule, tumour size, PD-L1, or nodal status.

Regarding the results of KEYNOTE-522, combination of pembrolizumab plus standard chemotherapy followed by adjuvant pembrolizumab should be considered for high-risk, early-stage TNBC fit for the combination. Future investigation on predictive biomarkers to better tailor the use of chemo-immunotherapy and the role of neoadjuvant immunotherapy for oestrogen receptor low patients is needed.

Emerging Therapies for TNBC

New molecules aiming to provide new therapeutic options and tailored approaches are under investigation in the neoadjuvant setting for TNBC patients.

AR targeting in TNBC

Androgen receptor expression occurs in 10% to 40% of TNBC, and its targeting is an interesting therapeutic option because LAR subtype has been associated with better prognosis, less chemotherapy sensitivity, and lower pCR after neoadjuvant treatment.⁸² Inhibition of AR was first studied in the metastatic setting for AR-positive TNBC on a phase II clinical trial where patients received enzalutamide 160 mg id until disease progression with clinical benefit.⁸³ Also in the metastatic setting, abiraterone demonstrated a 6.7% response rate with 2.8 months of PFS and a clinical benefit rate of 20% for AR-positive TNBC over 6 months.⁸⁴ Despite not approved, bicalutamide, enzalutamide and abiraterone might offer a chemo-free alternative for chemo-resistant patients with TNBC LAR subtype.

Identifying chemotherapy-resistant TNBC patients during neoadjuvant treatment is being assessed by the phase II ARTEMIS trial, where patients were randomized to 4 cycles of doxorubicin-based neoadjuvant chemotherapy. For chemotherapy-insensitive TNBC, patients were offered the possibility to be randomized to one of the single-arm molecular profile defined clinical trials. From the 15 patients with TNBC LAR subtype offered enzalutamide (ZT) 160 or 120 mg daily plus paclitaxel 80 mg/m² weekly (maximum 12 cycles), 5 showed response (NCT02276443).

Phosphatidylinositol-3-kinase (PI3K)/AKT/mammalian target of rapamycin (mTOR)-targeted therapies

PI3K/AKT/mTOR signalling pathway represents the most activated cancer driver with PI3KCA, PTEN inactivating, and AKT1 activating mutations occurring in 25% of TNBC.⁸⁵⁻⁸⁷

One arm in the I-SPY 2 trial assessed the addition of AKT inhibitor (MK2206) to weekly paclitaxel followed by AC. In the TNBC subgroup, estimated pCR rate was 40% for the AKT inhibitor and 22% for chemotherapy alone.^{82,88} Addition of Everolimus 30 mg to paclitaxel was compared to paclitaxel, 5-fluorouracil, epirubicin and cyclophosphamide every 3 weeks for 4 cycles in a phase II clinical trial, not statistically improving pCR rate.⁸⁷

The FAIRLANE clinical trial aimed to evaluate the addition of ipatasertib to taxane-based regimens, with ipatasertib achieving 17% of pCR rate versus 13% in the placebo arm for the ITT population. Subgroup analysis showed that pCR rate was 16% in the PTEN-low group with ipatasertib (versus 13% without) and 18% (versus 12% without) in the PIK3CA/AKT1/PTEN-altered subgroup. Addition of ipatasertib to paclitaxel did not statistically significantly improve pCR but was higher for the biomarker-selected subgroup.⁸⁹

Epidermal growth factor (EGFR) inhibitors

Epidermal growth factor receptor is expressed in 45% to 70% of TNBC, playing an important role in TNBC and being associated with poor prognosis.⁹⁰⁻⁹² Epidermal growth factor receptor mutations are rarely observed in this subgroup, and its inhibition appears as an interesting option with several anti-EGFR monoclonal antibodies and tyrosine kinase inhibitors (TKIs) being developed.⁹³

Epidermal growth factor receptor inhibitors were first explored in the metastatic setting, with phase II clinical trials assessing the addition of gefitinib to standard treatment for hormone receptor positive or HER2-positive metastatic breast cancer (MBC). Arteaga et al.⁹⁴ designed a phase I-II trial assessing the addition of gefitinib to weekly trastuzumab in HER2 positive MBC, without clinical benefit comparing to monotherapy with trastuzumab. Ciardiello et al., assessed addition of gefitinib with docetaxel as first-line therapy for MBC in a phase II clinical trial. Final analysis suggested that the combination could be potentially active in untreated MBC patients.⁹⁵

The addition of cetuximab to docetaxel in the neoadjuvant setting was evaluated in a multicentric phase II trial for operable stage II-III TNBC patients, achieving a pCR of 22%.⁹⁶

Panitumumab was first considered in a 2014 multicentric trial. This multicentric phase II pilot study combined panitumumab with anthracycline-taxane-based chemotherapy in stage II-III TNBC patients with a 46.8% of pCR.⁹⁷ A more recent single-arm phase II trial reviewed the impact of combining

panitumumab plus neoadjuvant chemotherapy achieving a pCR of 42% (95% CI: [20%, 66%]).⁹⁸

Women's triple-negative first-line study, an ongoing phase II clinical trial, assessed the role of panitumumab in patients with chemo-insensitive TNBC (NCT02593175).

Anti-vascular endothelial growth factor therapies

Bevacizumab and apatinib are the most relevant anti-VEGF in the neoadjuvant setting for TNBC. Five trials evaluated the role of bevacizumab in the neoadjuvant setting, with a meta-analysis confirming the benefit of combining bevacizumab with standard chemotherapy. Improve in pCR rate was not reflected in DFS or definitive OS.⁹⁹

In 2020, a phase II multicentric trial randomized 17 stage IIB-III TNBC patients to apatinib 500mg daily or placebo added to standard chemotherapy for 6 cycles every 21 days. An increase of 22% in pCR rate was reported (72.7% versus 50%; $p = .600$).¹⁰⁰

Other agents and drug conjugates

Eribulin, a microtubule inhibitor, has been studied as a potential alternative to taxane-based regimens. As a microtubule inhibitor, eribulin inhibits microtubule growth and disrupts mitosis. A multicenter randomized phase II trial stratified by HRD status assessed the impact of adding eribulin to neoadjuvant chemotherapy. For high HRD score or germline BRCA-mutated TNBC, addition of eribulin to weekly paclitaxel plus carboplatin followed by anthracycline regimen resulted in a pCR rate of more than 40%.¹⁰¹

Sacituzumab govitecan (SG), an antibody-drug conjugate, was first evaluated in the metastatic setting for second and subsequent lines. Results from the ASCENT Trial showed an increase in OS from 6.7 months for the single-agent chemotherapy group to 12.1 months with SG (HR: 0.48; 95% CI: [0.38, 0.59]; $p < .001$).¹⁰² Based on this evidence, the role of SG in the early setting is being assessed by the NeoSTAR trial for neoadjuvant treatment and SASCIA trial in patients with residual disease after neoadjuvant therapy. The NeoSTAR clinical trial evaluates SG 10 mg/kg (days 1, 8 of each 21 day cycle) in the neoadjuvant setting. After 4 cycles, if biopsy-proven residual disease exist, patients will be allowed to receive standard neoadjuvant therapy at physician's discretion (NCT04230109).¹⁰³

Post-neoadjuvant strategies

Despite lack of solid evidence on the impact of neoadjuvant chemotherapy on disease-free or OS, this strategy enables a reduction on tumour burden and *in vivo* assessment of chemosensitivity. Moreover, rate of pathological response after neoadjuvant treatment has been consolidated as a surrogate for oncological outcomes. Assessment of pathological response

allows the stratification of patients regarding the need of treatment intensification or de-intensification in the adjuvant setting. Currently, 3 major strategies have been assessed – chemotherapy, immunotherapy, and PARPis.

CREATE-X, a randomized phase III clinical trial, proved the significant improvement on both DFS and OS with post-neoadjuvant capecitabine for TNBC patients versus control group at 5 years follow-up (DFS: 74.1% vs. 67.6%; OS: 89.2% vs. 83.6%; $p = .001$).¹⁵ The benefit of platinum agents in the adjuvant setting for stage II/III TNBC patients with residual disease was evaluated in the ECOG-ACRIN EA1131 phase III clinical trial, without improvement on DFS for cisplatin or carboplatin versus adjuvant capecitabine at 3 years follow-up (HR: 1.09; 95% CI: [0.62, 1.90]).¹⁰⁴

Increase on EFS at 36 months stated on the KEYNOTE-522 for the arm of pembrolizumab plus standard chemotherapy and adjuvant pembrolizumab, adds evidence to the potential benefit of ICIs in the adjuvant setting.

The multicentre phase III OlympiA trial demonstrated the benefit in mortality rate, disease, and distant-free survival ($p = .02$; $p < .001$, and $p < .001$) for 1 year of adjuvant olaparib for germline TNBC-mutated patients with residual disease after neoadjuvant treatment.¹⁰⁵

Results from these trials reinforce the value of neoadjuvant systemic treatment for TNBC, allowing for a personalized treatment strategy.

Conclusion and Future Directions

The growing clinical evidence on TNBC led to significant changes on management for both metastatic and neoadjuvant settings. Better definition and stratification of TNBC patients according to molecular characteristics allows for therapeutic tailoring and consequent improvements in response rates and survival outcomes. Recent clinical trials showing the benefit of adding ICI to neoadjuvant chemotherapy regimens led to clinical practice changes, reflecting the need to bring molecular classification of TNBC to the core of therapeutic decision. Stratification of TNBC patients according to molecular features, such as HRD, germline BRCA mutation or EGFR expression, will help to achieve better response rates to neoadjuvant treatment and improve oncological outcomes.

Future clinical trials designed to better define which subgroup of TNBC patients are best candidates to treatment with conventional cytotoxic agents or combination of cytotoxic agents plus targeted therapies are needed.

Author Contributions

DS: Literature review, manuscript writing, proof editing and submission.

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REFERENCES

- Harbeck N, Penault-Llorca F, Cortes J, et al. Breast cancer. *Nat Rev Dis Primers*. 2019;5:66. doi:10.1038/s41572-019-0111-2.
- Sung H, Ferlay J, Siegel RL, et al. Global cancer statistics 2020: GLOBOCAN estimates of incidence and mortality worldwide for 36 cancers in 185 countries. *CA Cancer J Clin*. 2021;71:209-249. doi:10.3322/caac.21660.
- Koo MM, von Wagner C, Abel GA, McPhail S, Rubin GP, Lyratzopoulos G. Typical and atypical presenting symptoms of breast cancer and their associations with diagnostic intervals: evidence from a national audit of cancer diagnosis. *Cancer Epidemiol*. 2017;48:140-146. doi:10.1016/j.canep.2017.04.010.
- Yeh J, Chun J, Schwartz S, et al. Clinical characteristics in patients with triple negative breast cancer. *Int J Breast Cancer*. 2017;2017:1796145. doi:10.1155/2017/1796145.
- Sporikova Z, Koudelakova V, Trojanec R, Hajdich M. Genetic markers in triple-negative breast cancer. *Clin Breast Cancer*. 2018;18:e841-e850. doi:10.1016/j.clbc.2018.07.023.
- Lehmann BD, Bauer JA, Chen X, et al. Identification of human triple-negative breast cancer subtypes and preclinical models for selection of targeted therapies. *J Clin Invest*. 2011;121:2750-2767. doi:10.1172/JCI45014.
- Lehmann BD, Jovanovic B, Chen X, et al. Refinement of triple-negative breast cancer molecular subtypes: implications for neoadjuvant chemotherapy selection. *PLoS ONE*. 2016;11:e0157368. doi:10.1371/journal.pone.0157368.
- Bareche Y, Venet D, Ignatiadis M, et al. Unravelling triple-negative breast cancer molecular heterogeneity using an integrative multiomic analysis. *Ann Oncol*. 2018;29:895-902. doi:10.1093/annonc/mdy024.
- Zhao S, Zuo WJ, Shao ZM, Jiang YZ. Molecular subtypes and precision treatment of triple-negative breast cancer. *Ann Transl Med*. 2020;8:499. doi:10.21037/atm.2020.03.194.
- Lee JS, Yoon SE, Yuan Y. Neoadjuvant treatment for triple negative breast cancer: recent progress and challenges. *Cancers (Basel)*. 2020;12:1404. doi:10.3390/cancers12061404.
- von Minckwitz G, Untch M, Blohmer JU, et al. Definition and impact of pathologic complete response on prognosis after neoadjuvant chemotherapy in various intrinsic breast cancer subtypes. *J Clin Oncol*. 2012;30:1796-1804. doi:10.1200/JCO.2011.38.8595.
- Anders C, Carey LA. Understanding and treating triple-negative breast cancer. *Oncology (Williston Park)*. 2008;22:1233-1239.
- Liedtke C, Mazouni C, Hess KR, et al. Response to neoadjuvant therapy and long-term survival in patients with triple-negative breast cancer. *J Clin Oncol*. 2008;26:1275-1281. doi:10.1200/JCO.2007.14.4147.
- Li ZY, Zhang Z, Cao XZ, Feng Y, Ren S-S. Platinum-based neoadjuvant chemotherapy for triple-negative breast cancer: a systematic review and meta-analysis. *J Int Med Res*. 2020;48:300060520964340. doi:10.1177/0300060520964340.
- Masuda N, Lee SJ, Ohtani S, et al. Adjuvant capecitabine for breast cancer after preoperative chemotherapy: phase III randomized GeparTrio trial. *J Natl Cancer Inst*. 2016;108:e12645. doi:10.1093/jnci/djv085.
- Vaz-Luis I, Ottesen RA, Hughes ME, et al. Outcomes by tumor subtype and treatment pattern in women with small, node-negative breast cancer: a multi-institutional study. *J Clin Oncol*. 2014;32:2142-2150. doi:10.1200/JCO.2013.53.1608.
- Bidard FC, Matthieu MC, Chollet P, et al. p53 status and efficacy of primary anthracycline/alkylating agent-based regimen according to breast cancer molecular classes. *Ann Oncol*. 2008;19:1261-1265. doi:10.1093/annonc/mdn039.
- Mackey JR, Pienkowski T, Crown J, et al. Long-term outcomes after adjuvant treatment of sequential versus combination docetaxel with doxorubicin and cyclophosphamide in node-positive breast cancer: BCIRG-005 randomized trial. *Ann Oncol*. 2016;27:1041-1047. doi:10.1093/annonc/mdw098.
- von Minckwitz G, Kummel S, Vogel P, et al. Neoadjuvant vinorelbine-capecitabine versus docetaxel-doxorubicin-cyclophosphamide in early nonresponsive breast cancer: phase III randomized GeparTrio trial. *J Natl Cancer Inst*. 2008;100:542-551. doi:10.1093/jnci/djn085.
- Burstein MD, Tsimelzon A, Poage GM, et al. Comprehensive genomic analysis identifies novel subtypes and targets of triple-negative breast cancer. *Clin Cancer Res*. 2015;21:1688-1698. doi:10.1158/1078-0432.CCR-14-0432.
- Collignon J, Lousberg L, Schroeder H, Jerusalem G. Triple-negative breast cancer: treatment challenges and solutions. *Breast Cancer (Dove Med Press)*. 2016;8:93-107. doi:10.2147/BCTT.S69488.
- Gorodetska I, Kozaretska I, Dubrovka A. BRCA genes: the role in genome stability, cancer stemness and therapy resistance. *J Cancer*. 2019;10:2109-2127. doi:10.7150/jca.30410.
- Narayanan R, Dalton JT. Androgen receptor: a complex therapeutic target for breast cancer. *Cancers (Basel)*. 2016;8:108. doi:10.3390/cancers8120108.
- Santoni A, Sánchez-Muñoz A, Lluch A, et al. Triple negative breast cancer subtypes and pathologic complete response rate to neoadjuvant chemotherapy. *Oncotarget*. 2018;9:26406-26416. doi:10.18632/oncotarget.25413.
- Rickman K, Smogorzewska A. Advances in understanding DNA processing and protection at stalled replication forks. *J Cell Biol*. 2019;218:1096-1107. doi:10.1083/jcb.201809012.
- Rehbeck TR, Mitra N, Wan F, et al. Association of type and location of BRCA1 and BRCA2 mutations with risk of breast and ovarian cancer. *JAMA*. 2015;313:1347-1361. doi:10.1001/jama.2014.5985.
- Roy R, Chun J, Powell SN. BRCA1 and BRCA2: different roles in a common pathway of genome protection. *Nat Rev Cancer*. 2011;12:68-78. doi:10.1038/nrc3181.
- Belli C, Duso BA, Ferraro E, Carignano G. Homologous recombination deficiency in triple negative breast cancer. *Breast*. 2019;45:15-21. doi:10.1016/j.breast.2019.02.007.
- Mavaddat N, Barrowdale D, Andrulis IL, et al. Pathology of breast and ovarian cancers among BRCA1 and BRCA2 mutation carriers: results from the Consortium of Investigators of Modifiers of BRCA1/2 (CIMBA). *Cancer Epidemiol Biomarkers Prev*. 2012;21:134-147. doi:10.1158/1055-9965.EPI-11-0775.
- Ellsworth DL, Turner CE, Ellsworth RE. A review of the hereditary component of triple negative breast cancer: high- and moderate-penetrance breast cancer genes, low-penetrance loci, and the role of nontraditional genetic elements. *J Oncol*. 2019;2019:4382606. doi:10.1155/2019/4382606.
- Turner N, Tutt A, Ashworth A. Hallmarks of 'BRCAness' in sporadic cancers. *Nat Rev Cancer*. 2004;4:814-819. doi:10.1038/nrc1457.
- De Talhouet S, Peron J, Vuilleumier A, et al. Clinical outcome of breast cancer in carriers of BRCA1 and BRCA2 mutations according to molecular subtypes. *Sci Rep*. 2020;10:7073. doi:10.1038/s41598-020-63759-1.
- Tarsounas M, Sung P. The antitumorigenic roles of BRCA1-BARD1 in DNA repair and replication. *Nat Rev Mol Cell Biol*. 2020;21:284-299. doi:10.1038/s41580-020-0218-z.
- Garutti M, Pelizzari G, Bartoletti M, et al. Platinum salts in patients with breast cancer: a focus on predictive factors. *Int J Mol Sci*. 2019;20:3390. doi:10.3390/ijms20143390.
- Byrski T, Gronwald J, Huzarski T, et al. Pathologic complete response rates in young women with BRCA1-positive breast cancers after neoadjuvant chemotherapy. *J Clin Oncol*. 2010;28:375-379. doi:10.1200/JCO.2008.20.7019.
- Sikov WM, Berry DA, Perou CM, et al. Impact of the addition of carboplatin and/or bevacizumab to neoadjuvant once-per-week paclitaxel followed by dose-dense doxorubicin and cyclophosphamide on pathologic complete response rates in stage II to III triple-negative breast cancer: CALGB 40603 (Alliance). *J Clin Oncol*. 2015;33:13-21. doi:10.1200/JCO.2014.57.0572.
- Shepherd JH, Ballman K, Polley M-YC, et al. CALGB 40603 (Alliance): long-term outcomes and genomic correlates of response and survival after neoadjuvant chemotherapy with or without carboplatin and bevacizumab in triple-negative breast cancer. *J Clin Oncol*. 2022;40:1323-1334. doi:10.1200/jco.21.01506.
- von Minckwitz G, Schneeweiss A, Lohbl S, et al. Neoadjuvant carboplatin in patients with triple-negative and HER2-positive early breast cancer (GeparSixto; GBG 66): a randomised phase 2 trial. *Lancet Oncol*. 2014;15:747-756. doi:10.1016/S1473-0166(14)70160-3.
- Loibl S, Weber KE, Timms KM, et al. Survival analysis of carboplatin added to an anthracycline/taxane-based neoadjuvant chemotherapy and HRD score as predictor of response-final results from GeparSixto. *Ann Oncol*. 2018;29:2341-2347. doi:10.1093/annonc/mdy460.
- Untch M, Jackisch C, Schneeweiss A, et al. Nab-paclitaxel versus solvent-based paclitaxel in neoadjuvant chemotherapy for early breast cancer (GeparSepto - GBG 69): a randomised, phase 3 trial. *Lancet Oncol*. 2016;17:345-356. doi:10.1016/S1473-0166(15)00542-2.
- Gianni L, Mansutti M, Anton A, et al. Comparing neoadjuvant nab-paclitaxel vs paclitaxel both followed by anthracycline regimens in women with ERBB2/HER2-negative breast cancer-the evaluating treatment with neoadjuvant abraxane (ETNA) trial: a randomized phase 3 clinical trial. *JAMA Oncol*. 2018;4:302-308. doi:10.1001/jamaoncol.2017.4612.
- Giluz O, Nitz U, Liedtke C, et al. Comparison of neoadjuvant nab-paclitaxel+carboplatin vs nab-paclitaxel+gemcitabine in triple-negative breast cancer: randomized WSG-ADAPT-TN trial results. *J Natl Cancer Inst*. 2018;110:628-637. doi:10.1093/jnci/djx258.
- Zhang P, Yin Y, Xu B, et al. Abstract P3-14-07: carboplatin plus paclitaxel compared with epirubicin plus paclitaxel as neoadjuvant chemotherapy for triple-negative breast cancer - A phase II clinical trial. *Cancer Res*. 2013;73:P3-14-07-P13-14-07. doi:10.1158/0008-5472.Sabcs13-p3-14-07.
- Alba E, Chacon JL, Lluch A, et al. A randomized phase II trial of platinum salts in basal-like breast cancer patients in the neoadjuvant setting. *Breast Cancer Res Treat*. 2012;136:487-493. doi:10.1007/s10549-012-2100-y.
- Werner TL, Ray A, Lamb JG, et al. A phase I study of neoadjuvant chemotherapy with nab-paclitaxel, doxorubicin, and cyclophosphamide in patients with stage II to III breast cancer. *Clin Breast Cancer*. 2017;17:503-509. doi:10.1016/j.clbc.2017.04.010.

46. Sharma P, Kimler BF, O'Dea A, et al. Results of randomized phase II trial of neoadjuvant carboplatin plus docetaxel or carboplatin plus paclitaxel followed by AC in stage I-III triple-negative breast cancer (NCT02413320). *J Clin Oncol*. 2019;37:516-516. doi:10.1200/JCO.2019.37.15_suppl.516.
47. Yuan Y, Lee JS, Yost SE, et al. Phase II trial of neoadjuvant carboplatin and nab-paclitaxel in patients with triple-negative breast cancer. *Oncologist*. 2021;26:e382-e393. doi:10.1002/onco.13574.
48. Mayer EL, Abramson VG, Jankowitz RC, et al. TBCRC 030: a randomized phase II study of preoperative cisplatin versus paclitaxel in TNBC—Evaluating the homologous recombination deficiency (HRD) biomarker. *J Clin Oncol*. 2019;37:507-507. doi:10.1200/JCO.2019.37.15_suppl.507.
49. Loibl S, O'Shaughnessy J, Untch M, et al. Addition of the PARP inhibitor veliparib plus carboplatin or carboplatin alone to standard neoadjuvant chemotherapy in triple-negative breast cancer (BrightTnes): a randomised, phase 3 trial. *Lancet Oncol*. 2018;19:497-509. doi:10.1016/s1470-2045(18)30111-6.
50. Schneeweiss A, Mühs V, Tesch H, et al. Intense dose-dense epirubicin, paclitaxel, cyclophosphamide versus weekly paclitaxel, liposomal doxorubicin (plus carboplatin in triple-negative breast cancer) for neoadjuvant treatment of high-risk early breast cancer (GeparOcto-GBG 84): a randomised phase III trial. *Eur J Cancer*. 2019;106:181-192. doi:10.1016/j.ejca.2018.10.015.
51. Fasching PA, Link T, Hauke J, et al. Neoadjuvant paclitaxel/olaparib in comparison to paclitaxel/carboplatin in patients with HER2-negative breast cancer and homologous recombination deficiency (GeparOLA study). *Ann Oncol*. 2021;32:49-57. doi:10.1016/j.annonc.2020.10.471.
52. Tung N, Arun B, Hacker MR, et al. TBCRC 031: randomized phase II study of neoadjuvant cisplatin versus doxorubicin-cyclophosphamide in germline BRCA carriers with HER2-negative breast cancer (the INFORM trial). *J Clin Oncol*. 2020;38:1539-1548. doi:10.1200/JCO.2019.03292.
53. Pohl-Rescigno E, Hauke J, Loibl S, et al. Association of germline variant status with therapy response in high-risk early-stage breast cancer: a secondary analysis of the GeparOcto randomized clinical trial. *JAMA Oncol*. 2020;6:744-748. doi:10.1001/jamaoncol.2020.0007.
54. Pathak N, Sharma A, Elavarasi A, et al. Moment of truth—adding carboplatin to neoadjuvant/adjuvant chemotherapy in triple negative breast cancer improves overall survival: an individual participant data and trial-level meta-analysis. *Breast*. 2022;64:7-18. doi:10.1016/j.breast.2022.04.006.
55. Farmer H, McCabe N, Lord CJ, et al. Targeting the DNA repair defect in BRCA mutant cells as a therapeutic strategy. *Nature*. 2005;434:917-921. doi:10.1038/nature03445.
56. Bryant HE, Schultz N, Thomas HD, et al. Specific killing of BRCA2-deficient tumours with inhibitors of poly(ADP-ribose) polymerase. *Nature*. 2005;434:913-917. doi:10.1038/nature03443.
57. Tutt A, Robson M, Garber JE, et al. Oral poly(ADP-ribose) polymerase inhibitor olaparib in patients with BRCA1 or BRCA2 mutations and advanced breast cancer: a proof-of-concept trial. *Lancet*. 2010;376:235-244. doi:10.1016/s0140-6736(10)60892-6.
58. Robson M, Im SA, Senkus E, et al. Olaparib for metastatic breast cancer in patients with a germline BRCA mutation. *N Engl J Med*. 2017;377:523-533. doi:10.1056/NEJMoa1706450.
59. Litton JK, Rugo HS, Ettl J, et al. Talazoparib in patients with advanced breast cancer and a germline BRCA mutation. *N Engl J Med*. 2018;379:753-763. doi:10.1056/NEJMoa1802905.
60. Geyer CE, Sikov WM, Huober J, et al. Long-term efficacy and safety of addition of carboplatin with or without veliparib to standard neoadjuvant chemotherapy in triple-negative breast cancer: 4-year follow-up data from BrightTnes, a randomized phase III trial. *Ann Oncol*. 2022;33:384-394. doi:10.1016/j.annonc.2022.01.009.
61. Litton JK, Scoggins M, Ramirez DL, et al. A feasibility study of neoadjuvant talazoparib for operable breast cancer patients with a germline BRCA mutation demonstrates marked activity. *NPJ Breast Cancer*. 2017;3:49. doi:10.1038/s41523-017-0052-4.
62. Wang H, Yee D. I-SPY 2: a neoadjuvant adaptive clinical trial designed to improve outcomes in high-risk breast cancer. *Curr Breast Cancer Rep*. 2019;11:303-310. doi:10.1007/s12609-019-00334-2.
63. Robson ME, Tung N, Conte P, et al. OlympiAD final overall survival and tolerability results: olaparib versus chemotherapy treatment of physician's choice in patients with a germline BRCA mutation and HER2-negative metastatic breast cancer. *Ann Oncol*. 2019;30:558-566. doi:10.1093/annonc/mdz012.
64. Alba KP, McMurtry E, Vallier A-L, et al. Abstract P3-10-05: preliminary safety data from stage 1 and 2 of the phase II/III PARTNER trial: addition of olaparib to platinum-based neoadjuvant chemotherapy in triple negative and/or germline BRCA mutated breast cancer patients. *Cancer Res*. 2020;80:P3-10-05-P3-10-05. doi:10.1158/1538-7445.Sabcs19-p3-10-05.
65. Eikesdal HP, Yndestad S, Elzawahry A, et al. Olaparib monotherapy as primary treatment in unselected triple negative breast cancer. *Ann Oncol*. 2021;32:240-249. doi:10.1016/j.annonc.2020.11.009.
66. Litton JK, Scoggins ME, Hess KR, et al. Neoadjuvant talazoparib for patients with operable breast cancer with a germline BRCA pathogenic variant. *J Clin Oncol*. 2020;38:388-394. doi:10.1200/JCO.2019.01304.
67. Litton JK, Beck JT, Jones JM, et al. Neoadjuvant talazoparib in patients with germline BRCA1/2 (gBRCA1/2) mutation-positive, early HER2-negative breast cancer (BC): results of a phase 2 study. *J Clin Oncol*. 2021;39:505-505. doi:10.1200/JCO.2021.39.15_suppl.505.
68. Han H, Liu MC, Hamilton E, et al. Abstract P3-11-03: pilot neoadjuvant study of niraparib in HER2-negative, BRCA-mutated resectable breast cancer. *Cancer Res*. 2020;80:P3-11-03-P3-11-03. doi:10.1158/1538-7445.Sabcs19-p3-11-03.
69. Schmid P, Adams S, Rugo HS, et al. Atezolizumab and nab-paclitaxel in advanced triple-negative breast cancer. *N Engl J Med*. 2018;379:2108-2121. doi:10.1056/NEJMoa1809615.
70. Cortes J, Cescon DW, Rugo HS, et al. Pembrolizumab plus chemotherapy versus placebo plus chemotherapy for previously untreated locally recurrent inoperable or metastatic triple-negative breast cancer (KEYNOTE-355): a randomised, placebo-controlled, double-blind, phase 3 clinical trial. *Lancet*. 2020;396:1817-1828. doi:10.1016/s0140-6736(20)32531-9.
71. Miles D, Gligorov J, André F, et al. Primary results from IMpassion131, a double-blind, placebo-controlled, randomised phase III trial of first-line paclitaxel with or without atezolizumab for unresectable locally advanced/metastatic triple-negative breast cancer. *Ann Oncol*. 2021;32:994-1004. doi:10.1016/j.annonc.2021.05.801.
72. Schmid P, Cortes J, Pusztai L, et al. Pembrolizumab for early triple-negative breast cancer. *N Engl J Med*. 2020;382:810-821. doi:10.1056/NEJMoa1910549.
73. Loibl S, Untch M, Barchardi N, et al. A randomised phase II study investigating durvalumab in addition to an anthracycline taxane-based neoadjuvant therapy in early triple-negative breast cancer: clinical results and biomarker analysis of GeparNuevo study. *Ann Oncol*. 2019;30:1279-1288. doi:10.1093/annonc/mdz158.
74. Nanda R, Liu MC, Yau C, et al. Effect of pembrolizumab plus neoadjuvant chemotherapy on pathologic complete response in women with early-stage breast cancer: an analysis of the ongoing phase 2 adaptively randomized I-SPY2 trial. *JAMA Oncol*. 2020;6:676-684. doi:10.1001/jamaoncol.2019.6650.
75. Schmid P, Cortes J, Dent R, et al. KEYNOTE-522: phase III study of pembrolizumab (pembro) + chemotherapy (chemo) vs placebo (pbo) + chemo as neoadjuvant treatment, followed by pembro vs pbo as adjuvant treatment for early triple-negative breast cancer (TNBC). *Ann Oncol*. 2019;30:v853-v854. doi:10.1093/annonc/mdz394.003.
76. Gianni L, Huang CS, Egle D, et al. Pathologic complete response (pCR) to neoadjuvant treatment with or without atezolizumab in triple-negative, early high-risk and locally advanced breast cancer: NeoTRIP Michelangelo randomized study. *Ann Oncol*. 2022;33:534-543. doi:10.1016/j.annonc.2022.02.004.
77. Mittendorf EA, Zhang H, Barrios CH, et al. Neoadjuvant atezolizumab in combination with sequential nab-paclitaxel and anthracycline-based chemotherapy versus placebo and chemotherapy in patients with early-stage triple-negative breast cancer (IMpassion031): a randomised, double-blind, phase 3 trial. *Lancet*. 2020;396:1090-1100. doi:10.1016/s0140-6736(20)31953-x.
78. Schmid P, Cortes J, Dent R, et al. Event-free survival with pembrolizumab in early triple-negative breast cancer. *N Engl J Med*. 2022;386:556-567. doi:10.1056/NEJMoa2112651.
79. Loibl S, Jackisch C, Rastogi P, et al. GeparDuo/NSABP B-59: a randomized double-blind phase III clinical trial of neoadjuvant chemotherapy with atezolizumab or placebo in patients with triple negative breast cancer (TNBC) followed by adjuvant atezolizumab or placebo. *Ann Oncol*. 2019;30:iii38. doi:10.1093/annonc/mdz097.014.
80. Kok M, Horlings HM, van de Vijver K, et al. Adaptive phase II randomized non-comparative trial of nivolumab after induction treatment in triple negative breast cancer: TONIC-trial. *Ann Oncol*. 2017;28:v608. doi:10.1093/annonc/mdz440.006.
81. Voorwerk L, Slagter M, Horlings HM, et al. Immune induction strategies in metastatic triple-negative breast cancer to enhance the sensitivity to PD-1 blockade: the TONIC trial. *Nat Med*. 2019;25:920-928. doi:10.1038/s41591-019-0432-4.
82. Gerratsen L, Basile D, Buono G, et al. Androgen receptor in triple negative breast cancer: a potential target for the targetless subtype. *Cancer Treat Rev*. 2018;68:102-110. doi:10.1016/j.ctrv.2018.06.005.
83. Traina TA, Miller K, Yardley DA, et al. Enzalutamide for the treatment of androgen receptor-expressing triple-negative breast cancer. *J Clin Oncol*. 2018;36:884-890. doi:10.1200/JCO.2016.71.3495.
84. Bonnefoi H, Grellety T, Tredan O, et al. A phase II trial of abiraterone acetate plus prednisone in patients with triple-negative androgen receptor positive locally advanced or metastatic breast cancer (UCBG 12-1). *Ann Oncol*. 2016;27:812-818. doi:10.1093/annonc/mdw067.
85. Levine DA, Bogomolny F, Yee CJ, et al. Frequent mutation of the PIK3CA gene in ovarian and breast cancers. *Clin Cancer Res*. 2005;11:2875-2878. doi:10.1158/1078-0432.Ccr-04-2142.

86. Pascual J, Turner NC. Targeting the PI3-kinase pathway in triple-negative breast cancer. *Ann Oncol*. 2019;30:1051-1060. doi:10.1093/annonc/mdz133.
87. Gonzalez-Angulo AM, Akcakanat A, Liu S, et al. Open-label randomized clinical trial of standard neoadjuvant chemotherapy with paclitaxel followed by FEC versus the combination of paclitaxel and everolimus followed by FEC in women with triple receptor-negative breast cancer. *Ann Oncol*. 2014;25:1122-1127. doi:10.1093/annonc/mdl124.
88. Wolf DM, Yau C, Wulfschlegel J, et al. Mechanism of action biomarkers predicting response to AKT inhibition in the I-SPY 2 breast cancer trial. *Nat Breast Cancer*. 2020;6:48. doi:10.1038/s41523-020-00189-2.
89. Oliveira M, Saura C, Nuciforo P, et al. FAIRLANE, a double-blind placebo-controlled randomized phase II trial of neoadjuvant ipatasertib plus paclitaxel for early triple-negative breast cancer. *Ann Oncol*. 2019;30:1289-1297. doi:10.1093/annonc/mdz177.
90. Masuda H, Zhang D, Bartholomew C, Doihara H, Hortobagyi GN, Ueno NT. Role of epidermal growth factor receptor in breast cancer. *Breast Cancer Res Treat*. 2012;136:331-345. doi:10.1007/s10549-012-2289-9.
91. Nakajima H, Ishikawa Y, Furuya M, et al. Protein expression, gene amplification, and mutational analysis of EGFR in triple-negative breast cancer. *Breast Cancer*. 2014;21:66-74. doi:10.1007/s12282-012-0354-1.
92. Tilch E, Scidens T, Cocciardi S, et al. Mutations in EGFR, BRAF and RAS are rare in triple-negative and basal-like breast cancers from Caucasian women. *Breast Cancer Res Treat*. 2014;143:385-392. doi:10.1007/s10549-013-2798-1.
93. Liu Y, Zhou Y, Huang KH, et al. Targeting epidermal growth factor-overexpressing triple-negative breast cancer by natural killer cells expressing a specific chimeric antigen receptor. *Cell Prolif*. 2020;53:e12858. doi:10.1111/cpr.12858.
94. Arteaga CL, O'Neill A, Moulder SL, et al. A phase I-II study of combined blockade of the ErbB receptor network with trastuzumab and gefitinib in patients with HER2 (ErbB2)-overexpressing metastatic breast cancer. *Clin Cancer Res*. 2008;14:6277-6283. doi:10.1158/1078-0432.CCR-08-0482.
95. Ciardiello F, Troiani T, Caputo F, et al. Phase II study of gefitinib in combination with docetaxel as first-line therapy in metastatic breast cancer. *Br J Cancer*. 2006;94:1604-1609. doi:10.1038/sj.bjc.6603141.
96. Nabholz JM, Chalabi N, Radosevic-Rubin N, et al. Multicentric neoadjuvant pilot phase II study of cetuximab combined with docetaxel in operable triple negative breast cancer. *Int J Cancer*. 2016;138:2274-2280. doi:10.1002/ijc.29952.
97. Nabholz JM, Abrial C, Mouret-Reynier MA, et al. Multicentric neoadjuvant phase II study of panitumumab combined with an anthracycline/taxane-based chemotherapy in operable triple-negative breast cancer: identification of biologically defined signatures predicting treatment impact. *Ann Oncol*. 2014;25:1570-1577. doi:10.1093/annonc/mdu183.
98. Matsuda N, Wang X, Lim B, et al. Safety and efficacy of panitumumab plus neoadjuvant chemotherapy in patients with primary HER2-negative inflammatory breast cancer. *JAMA Oncol*. 2018;4:1207-1213. doi:10.1001/jamaoncol.2018.1436.
99. Nahleh Z, Botrus G, Dwivedi A, Jennings M, Nagy S, Teyli A. Bevacizumab in the neoadjuvant treatment of human epidermal growth factor receptor 2-negative breast cancer: a meta-analysis of randomized controlled trials. *Mol Clin Oncol*. 2019;10:357-365. doi:10.3892/mco.2019.1796.
100. Liu Y, Zhang X, Wang L, et al. 270P Apatinib added to taxanes and platinum neoadjuvant chemotherapy for patients with triple-negative and HER2-positive breast cancer: a multicenter, randomized, phase II, open-label trial. *Ann Oncol*. 2020;31:S346. doi:10.1016/j.annonc.2020.08.079.
101. Masuda N, Bando H, Yamanaka T, et al. Erlotinib-based neoadjuvant chemotherapy for triple-negative breast cancer patients stratified by homologous recombination deficiency status: a multicenter randomized phase II clinical trial. *Breast Cancer Res Treat*. 2021;188:117-131. doi:10.1007/s10549-021-06184-w.
102. Bardia A, Hurvitz SA, Tolane SM, et al. Sacituzumab govitecan in metastatic triple-negative breast cancer. *N Engl J Med*. 2021;384:1529-1541. doi:10.1056/NEJMoa2028485.
103. Spring LM, Tolane SM, Desai N, et al. Abstract OT-03-06: phase 2 study of response-guided neoadjuvant sacituzumab govitecan (IMMU-132) in patients with localized triple-negative breast cancer (NeoSTAR). *Cancer Res*. 2021;81:OT-03-06. doi:10.1158/1538-7445.Sabcs20-ot-03-06.
104. Mayer IA, Zhao F, Arteaga CL, et al. Randomized phase III postoperative trial of platinum-based chemotherapy versus capecitabine in patients with residual triple-negative breast cancer following neoadjuvant chemotherapy: ECOG-ACRIN E.A1131. *J Clin Oncol*. 2021;39:2539-2551. doi:10.1200/jco.21.00976.
105. Tutt A, Garber JE, Kaufman B, et al. OlympiA: a phase III, multicenter, randomized, placebo-controlled trial of adjuvant olaparib after (neo)adjuvant chemotherapy in patients with germline BRCA1/2 mutations and high-risk HER2-negative early breast cancer. *J Clin Oncol*. 2021;39:LBA1. doi:10.1200/JCO.2021.39.15_suppl.LBA1.

Emerging Therapeutic Drugs in Metastatic Triple-Negative Breast Cancer

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Breast Cancer: Basic and Clinical Research
Volume 15: 1–14
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DOI: 10.1177/11782234211002491



ABSTRACT: Metastatic triple-negative breast cancer (TNBC) is a heterogeneous disease with a poor prognosis and currently with few treatment options. Treatment of these patients is highly based on systemic chemotherapy. Some targeted drugs were recently approved for these patients: two poly(ADP-ribose) polymerase inhibitors in patients with germline BRCA1/2 mutations (olaparib and talazoparib), immune checkpoint inhibitors in association with chemotherapy if programmed death-ligand 1 positive (atezolizumab plus nabpaclitaxel and pembrolizumab plus chemotherapy [nabpaclitaxel, paclitaxel, and carboplatin plus gemcitabine]), and an antibody-drug conjugate sacituzumab-govitecan in heavily pretreated patients (at least 2 previous lines for the metastatic setting). Combinations using these and other targeted treatment options are under investigation in early and late clinical trials, and we will probably have some practice-changing results in the new future. Other targeted drugs explored in phase II and phase III clinical trials are PI3K/AKT pathway inhibitors and androgen receptor antagonists in patients with alterations in these signaling pathways. The definition of molecular subtypes has been essential for the development of these treatment strategies. Soon, the treatment of metastatic TNBC could be based on personalized medicine using molecular testing for targeted drugs instead of only systemic chemotherapy. The authors present a review of emerging treatment options in metastatic TNBC, focusing on targeted drugs, including the recent data published in 2020.

KEYWORDS: Triple-negative breast cancer, platinum chemotherapy, PARP inhibitors, immune checkpoint inhibitors, androgen receptor antagonists, PI3K/AKT pathway, sacituzumab-govitecan

RECEIVED: October 28, 2020. **ACCEPTED:** February 16, 2021.

TYPE: Review

FUNDING: The author(s) received no financial support for the research, authorship, and/or publication of this article.

DECLARATION OF CONFLICTING INTERESTS: The author(s) declared no potential conflicts of interest with respect to the research, authorship, and/or publication of this article.

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Introduction

Breast cancer (BC) is associated with approximately 2.1 million new cases in 2018, mostly in women. BC is also the leading cause of cancer mortality in women.¹ The incidence and mortality of BC have disparities worldwide.² Triple-negative breast cancer (TNBC) is defined by the absence of the estrogen receptor, androgen receptor (AR), and human epidermal growth factor receptor 2 (HER2) and accounts for nearly 15% of BC. Patients are usually young (<50 years old), have a shorter time to relapse in the early stages, and are at higher risk for visceral metastasis, including brain metastasis.³ Most TNBC have a high pathological histological grade and high proliferation rates, especially invasive ductal carcinoma, metaplastic, apocrine, and medullary carcinoma. Rare histological subtypes, as salivary gland-type adenoid-cystic and secretory carcinoma, are low-grade neoplasms.⁴ The prevalence of germline BRCA1/2 mutations is higher in patients with TNBC.⁵ This tumor subtype is also associated with more tumor-infiltrating lymphocytes (TILs) than other subtypes.⁶ The survival curves in TNBC, different from other BC subtypes, are characterized by an increase in relapse and a decrease in survival during the first 3 to 5 years after diagnosis. Late recurrences are more frequent in estrogen-positive BC than in TNBC.^{7,8} Metastatic TNBC (mTNBC) is a heterogeneous and aggressive disease that responds differently to standard chemotherapy and targeted drugs.³

A molecular classification of TNBC described by Lehmann et al identified 6 subtypes using gene expression signatures: basal-like 1, basal-like 2, immunomodulatory (IM), mesenchymal (MES), mesenchymal stem-like, and luminal androgen receptor (LAR). Each subtype is enriched in distinct gene ontologies and gene expression patterns.⁹ The correlation between molecular subtype, gene expression, and cell pathway is essential to identify targeted agents.^{10,11} Regarding prognosis, the IM subtype is associated with a better prognosis, and the LAR subtype to a poor prognosis.¹² More recently, Burstein et al¹³ identified 4 TNBC subtypes: LAR, MES, basal-like immunosuppressed, and basal-like immune-activated. Claudin-low tumors are recently discussed. This phenotype involves various intrinsic subtypes associated with high immune and stromal infiltration levels and a low mutation burden, proliferation, and genomic instability.¹⁴

Historically, mTNBC used to be treated with cytotoxic chemotherapy.^{15,16} The growing knowledge of biology, molecular alterations, and genome sequencing has been essential in developing new drugs in recent years. Some targeted drugs, such as poly(ADP-ribose) polymerase (PARP) inhibitors, immune checkpoint inhibitors (ICIs), AR-targeted drugs, drugs targeting the PI3K/AKT pathway, or antibody-drug conjugates (ADCs), have demonstrated clinical benefit in these patients.¹⁷ The authors present in this article a review of the most recent drug advances in mTNBC, particularly in the last 5 years, including the new data published in the year 2020.



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BRCA1/2 Mutations and Homologous Recombination Deficiency

DNA damage in cells, caused by extracellular agents or endogenous events, is repaired by various DNA repair mechanisms. Those are base excision repair (BER), nucleotide excision repair, mismatch repair, and double-strand break (DSB) repair that includes either homologous recombination during the S and G2 cell cycle phases and nonhomologous end joining.¹⁸ BRCA1/2 is directly involved in DNA homologous recombination repair (HRR) and plays an essential role in genome stability. Mutations in BRCA1/2 or other homologous recombination defects result in growth defects and genetic instability.¹⁹ Germline BRCA1/2 mutations are present in approximately 10% to 20% of patients with TNBC, especially in those aged <60 years.²⁰⁻²³ The presence of these mutations is associated with hereditary breast and ovarian cancer (HBOC) syndrome.²⁴ Almost all the known BRCA1 mutations have gene expression patterns coincident with basal-like subtype.⁹ BRCA2 mutation is more associated with lobular histology.²⁵ Testing for germline BRCA1/2 mutations is essential in patients with mTNBC to predict future cancer risk and guide therapeutic strategies.²⁶

There are other multiple genetic alterations beyond BRCA1/2 mutations that are associated with HBOC syndrome. Some of these alterations are also involved in the DNA repair mechanisms, like PALB2, ATM, CHEK2, RAD51, and TP53.²⁷ The discovery of gene mutations that participate in DNA repair pathways supports the possibility for therapeutic targets by the synthetic lethality mechanism as occurs with the BRCA1/2 mutations tumors treated with PARP inhibitors. The evaluation of tumor tissue for loss of heterozygosity, HRR deficiency, or mutational signatures could support therapeutic strategies guided by the patient's functional status.²⁷ The analysis of somatic mutation genome from cancers permitted identifying more than 20 mutational signatures, including an identified signature 3 associated with the breast, ovarian, and pancreatic cancer and the inactivating BRCA1/2 mutations.^{28,29} A study performing a comprehensive characterization of signature 3 revealed that it is associated with the biallelic BRCA1/2 inactivation (germline, somatic, or epigenetic silencing). Nevertheless, many samples with signature 3 did not have the BRCA1/2 inactivation, suggesting that other HRR defects can contribute to this signature. The study verified an association with rare truncating variants in other HRR machinery components: germline PALB2 mutations, epigenetic silencing, and somatic mutations in RAD51C. The authors conclude that signature 3 could represent a biomarker for HRR status and could help design the therapeutic trials.³⁰

The Role of Platinum-Based Chemotherapy

In the last few years, chemotherapy was the only treatment strategy approved for mTNBC.^{15,16,31} The role of platinum drugs, by their mechanism of action—DNA adduct formation

with subsequent DSRs, has been questioned in patients with TNBC, as it is associated with a high percentage of BRCA1/2 mutations and other homologous recombination deficiency (HRD).³² In the absence of functional BRCA proteins, tumor cells are more sensitive to platinum drugs due to their mechanism of action.³³ A phase II multicentre clinical trial TBCRC009 evaluated the platinum monotherapy (cisplatin 75 mg/m² or carboplatin area under the curve [AUC] 6) in mTNBC. The results were published in 2015. The study included 86 patients, 69 as a first-line therapy, and 11 with BRCA1/2 mutations. The overall response rate (ORR) was 25.6% (95% confidence interval [CI]: 16.8%-36%) in the overall population, numerically higher with cisplatin (32.6%) versus carboplatin (18.7%) and was 54.5% in patients with BRCA1/2 mutations.³⁴ Three years later, a phase III trial was published, the TNT trial. This study compares carboplatin AUC 6 versus the standard of care, docetaxel 100 mg/m² in 376 patients with mTNBC. There were no differences in ORR between carboplatin and docetaxel (31.4% vs 34.0%, respectively) in the overall population. There were no differences in median progression-free survival (PFS) and overall survival (OS): 3.1 months (95% CI: 2.4-4.2) and 12.8 months (95% CI: 10.6-15.3) in carboplatin versus 4.4 months (95% CI: 4.1-5.1) and 12.0 months (95% CI: 10.2-13.0) in docetaxel treated group, $P = .40$ and $.96$, respectively. There were 43 patients with germline BRCA1/2 mutations included. In the BRCA-mutated patients, there was a significant difference in ORR between carboplatin treated (68%) versus docetaxel (33.3%), $P = .03$. There is also a significant difference in median PFS in BRCA-mutated: 6.8 months in the carboplatin group versus 4.4 months in the docetaxel group, $P = .002$. Despite that, there were no differences in the OS evaluation. The safety profile was expected for both drugs. The benefit observed in BRCA-mutated patients was not observed in BRCA1 methylation, BRCA1 mRNA-low tumors, and a high score in Myriad HRD assay.³⁵ In this trial, carboplatin demonstrated similar clinical benefit compared with docetaxel with a favorable toxicity profile and is currently an option for mTNBC.³⁶

Table 1 describes the main characteristics of these two studies. A Cochrane systematic review that included 4418 women evaluated the platinum-containing regimens for metastatic BC. The authors concluded that in women with mTNBC, there is preliminary low-quality evidence of a moderate survival benefit from platinum-based drugs. In contrast, in patients with non-TNBC metastatic disease, there is evidence of no survival benefit and high toxicity from platinum-based regimens.⁴⁴ Despite these results, platinum agents have been widely used for treating patients with mTNBC. Regarding the presented results, it will be important to develop and understand which biomarkers could predict clinical benefit with platinum chemotherapy in mTNBC beyond the germline BRCA1/2 mutations.

Table 1. Selected published platinum-based chemotherapy and PARP inhibitors clinical trials in the mTNBC population.

AUTHOR, STUDY	N	PHASE	POPULATION	TREATMENT (EXPERIMENTAL VS COMPARED DRUG)	TARGET	ORR (%)	PFS (MONTHS)	OS (MONTHS)
Platinum-based chemotherapy								
Isakoff et al. ³⁴ (TBCRC009)	86	II	First- or second-line in mTNBC	Cisplatin or carboplatin	DNA repair	Cisplatin: 32.6% Carboplatin: 18.7% BRCA1/2: 54.5%	2.9	11
Tutt et al. ³⁵ (TNT)	376	III	mTNBC (BRCA1/2 in 43 patients)	Carboplatin vs docetaxel	DNA repair BRCA1/2	NS (31.4% vs 34%) BRCA1/2: 68% vs 33.3%	NS (3.1 vs 4.4) BRCA1/2: 6.8 vs 4.4	NS (12.8 vs 12.0)
PARP inhibitors								
Robson et al. ³⁷ (OlympiAD)	302	III	Pretreated TNBC and hormone receptor-positive BC with germline BRCA1/2 mutations	Olaparib vs standard chemotherapy	BRCA1/2	59.9% vs 28.8%	7.0 vs 4.2	NS (19.3 vs 19.6)
Lifton et al. ³⁸ (EMBRACA)	431	III	Pretreated TNBC and hormone receptor-positive BC with germline BRCA1/2 mutations	Talazoparib vs standard chemotherapy	BRCA1/2	62.6% vs 27.2%	8.6 vs 5.6	NS (22.3 vs 19.5)
PARP inhibitors plus platinum-based chemotherapy								
Han et al. ³⁹ (BROCADE)	290	II	Locally recurrent or metastatic breast cancer with germline BRCA1/2 mutations (~40% TNBC)	Carboplatin/paclitaxel plus veliparib vs plus placebo Veliparib plus temozolomide vs placebo plus carboplatin/paclitaxel	BRCA1/2 DNA repair	78.0% vs 61.3% 28.6% vs 61.3%	NS (14.1 vs 12.3) 7.4 vs 12.3	NS (28.3 vs 25.9) 19.1 vs 25.9
Dieras et al. ⁴⁰ (BROCADE3)	509	III	HER2-negative advanced/metastatic BC with germline BRCA1/2 mutations (48% TNBC)	Carboplatin/paclitaxel plus veliparib vs plus placebo	BRCA1/2 DNA repair	75.8% vs 74.1%	14.5 vs 12.6	NS (33.5 vs 28.2)
Sharma et al. ⁴¹ (SWOG S1416)	335	II	Pretreated mTNBC (germline BRCA1/2, BRCA-like with HRD and non-BRCA-like groups)	Cisplatin plus veliparib vs plus placebo	BRCA1/2 HRD	BRCA-like: NS NS (45% vs 35%)	BRCA-like: 5.7 vs 4.3	BRCA-like: NS (13.7 vs 12.1)
PARP inhibitors plus immunotherapy								
Domchek et al. ⁴² (MEDIOA)	34	II	Metastatic BC with germline BRCA1/2 mutations	Olaparib plus durvalumab	BRCA1/2 PD-L1	63.3%	8.2	20.5
Vinayak et al. ⁴³ (TOPACIO/Keynote-162)	54	II	Pretreated mTNBC	Niraparib plus pembrolizumab	DNA repair PD-L1	29% (33% in PD-L1 pos vs 15% in PD-L1 neg)	8.1 (in BRCA-mutated)	-

Abbreviations: BC, breast cancer; HER2, human epidermal growth factor receptor 2; HRD, homologous recombination deficiency; mTNBC, metastatic triple-negative breast cancer; NS, not significant; ORR, overall response rate; OS, overall survival; PARP, poly(ADP-ribose) polymerase; PD-L1, programmed death-ligand 1; PFS, progression-free survival; TNBC, triple-negative breast cancer.
 Key results: Platinum-based chemotherapy showed greater OSR in patients with BRCA1/2 mutations. Poly(ADP-ribose) polymerase inhibitors olaparib and talazoparib showed higher ORR and median PFS than standard chemotherapy, with no difference in OS. Poly(ADP-ribose) polymerase inhibitors combined with platinum-based chemotherapy revealed conflicting results, with higher median PFS in a phase III trial, but without differences in OS. In combination with immunotherapy, PARP inhibitors only have shown few data from phase II studies, mostly in BRCA-mutated patients.

Poly(ADP-Ribose) Polymerase Inhibitors in mTNBC

Poly(ADP-ribose) polymerase 1 is a DNA damage sensor and a signal transducer that binds to DNA breaks. The PARP inhibitors impair the repair of single-strand breaks (SSBs) by the following mechanisms: disruption of the BER pathway and inhibiting the auto-PARYlation or PARP release from DNA (PARP1 trapping). The unresolved SSBs lead to DSBs that result in cell death in the HRR-deficient cells.⁴⁵ Some clinical trials have evaluated multiple PARP inhibitors in patients with BC and BRCA1/2 germline mutations. The first phase III published trial in this setting, demonstrating benefit, was OlympiAD trial in 2017. It is a randomized, open-label study that involves 302 patients with previously treated (≤ 2 previous chemotherapy drugs) metastatic HER2-negative BC with a germline BRCA1/2 mutation. The study compares olaparib 300 mg twice daily versus physician's choice chemotherapy (capecitabine, eribulin, or vinorelbine in the 21-day cycle) in a 2:1 ratio. The median PFS was meaningfully superior in the olaparib group: 7.0 versus 4.2 months, the hazard ratio (HR) 0.58 (95% CI: 0.43–0.80, $P < .001$). The ORR was almost double in the olaparib group: 59.9% versus 28.8% in standard chemotherapy. The OS was not significantly different in the two groups, numerically nearly 20 months. The safety profile was favorable in the olaparib group: adverse events (AEs) grade ≥ 3 Common Terminology Criteria for Adverse Events (CTCAE) were 36.6% in the olaparib group and 50.5% in the standard chemotherapy arm. The most frequent any-grade AEs associated with olaparib were anemia, nausea, vomiting, fatigue, and neutropenia.³⁷ The EMBRACA trial was published in the next year. This study had a similar design: a randomized, open-label trial, including 431 patients with previously treated advanced BC (≤ 3 cytotoxic agents) and germline BRCA1/2 mutations. Patients were randomized to talazoparib 1 mg once daily versus physician's choice chemotherapy (capecitabine, eribulin, gemcitabine, or vinorelbine) in a 2:1 ratio. The median PFS is significantly higher in the talazoparib group: 8.6 versus 5.6 months, HR: 0.54 (95% CI: 0.41–0.71, $P < .001$). The ORR was also almost double in talazoparib-treated patients: 62.6% versus 27.2% in the chemotherapy group, *odds ratio* 5.0 (95% CI: 2.9–8.8, $P < .001$). There was no difference in median OS, nearly 20 to 22 months. EMBRACA trial, unlike OlympiAD, permitted crossover to PARP inhibitor after progression, and 18% of patients in the standard group were treated with this drug. The safety profile was worse in talazoparib regarding hematological grade AEs grade ≥ 3 CTCAE: 55% versus 38%. The talazoparib group revealed a significant improvement in the estimated overall mean change from the baseline in the global quality-of-life (QoL) on the European Organization for Research and Treatment of Cancer Quality of Life Questionnaire (EORTC QLQ)-C30, compared with a deterioration in the chemotherapy group: 3.0 (95% CI: 1.2–4.8) versus -5.4 (95% CI: -8.8 to -2.0), $P < .001$.³⁸ These 2 PARP inhibitors are approved and

recommended in pretreated metastatic HER2-negative BC with germline BRCA1/2 mutations in the first- to third-line setting.^{16,36,46}

Combining PARP inhibitors and chemotherapy to achieve an enhanced efficacy is also under investigation. There are some recently reported data on this issue. Veliparib, a PARP inhibitor with minimal PARP trapping, alone or with chemotherapy, was studied in early phase trials and demonstrated efficacy and a safety profile.⁴⁷ A randomized phase II clinical trial (BROCADE) evaluated the combination of veliparib with carboplatin/paclitaxel or with temozolomide in patients with BRCA1/2 mutations in recurrent or metastatic BC with ≤ 2 previous therapeutic lines. Patients ($n = 290$) were randomized in a 1:1:1 ratio to veliparib plus carboplatin/paclitaxel, veliparib plus temozolomide, and placebo plus carboplatin/paclitaxel. The ORR was superior in the veliparib plus carboplatin/paclitaxel group (77.8%) versus placebo plus carboplatin/paclitaxel group (61.3%), $P = .027$. The median PFS and OS were numerically higher in the group containing veliparib than placebo, but without a statistically significant difference. There was no significant increase in toxicity, comparing the addition of veliparib in the two carboplatin/paclitaxel groups. The veliparib plus temozolomide group was compared with placebo plus carboplatin/paclitaxel. Temozolomide plus veliparib was inferior in terms of ORR, median PFS, and median OS.³⁹ The first results of the phase III trial (BROCADE3) were presented at the European Society for Medical Oncology (ESMO) 2019 Congress and published in 2020. The study included patients with HER2-negative advanced/metastatic BC with ≤ 2 previous treatment lines and a germline BRCA1/2 mutation. Patients ($n = 509$) were randomized in a 2:1 ratio to carboplatin AUC 6 on day 1 and paclitaxel 80 mg/m² on days 1, 8, and 15, with or without veliparib 120 mg bid on days 2 to 5 in a 21-day cycle. Patients with no progression during the chemotherapy phase maintained veliparib or placebo 300 to 400 mg/day. Prior platinum exposure was allowed. The median PFS was superior in the veliparib group: 14.5 months (95% CI: 12.5–17.7) versus 12.6 months (95% CI: 10.6–14.4), HR: 0.71 (95% CI: 0.57–0.88), $P = .0016$. The authors verified a durable benefit with a 3-year PFS rate of 25.7% (95% CI: 20.3–31.4) versus 10.7% (95% CI: 5.8–17.3). There were no significant differences in median OS (33.5 vs 28.2 months, HR: 0.95, $P = .67$), and the ORR was 75.8% and 74.1%, respectively. Emesis, neutropenia, anemia, and thrombocytopenia were the most frequent side effects, occurring similarly between the 2 arms.⁴⁰ A subgroup analysis of hormone receptor-positive and mTNBC was presented in ESMO Breast Cancer Congress 2020: 243 patients (48%) were TNBC, and in this subgroup, the median PFS was 16.6 months (95% CI: 12.3–22.7) in the veliparib arm versus 14.1 months (95% CI: 11.0–15.8) in placebo, HR: 0.72 (95% CI: 0.52–1.00), $P = .051$. The benefit was durable, with a PFS rate at 3 years of 35.3% (95% CI: 27.2–43.6) versus 13.0% (95% CI: 5.3–24.2). The median OS was 35.0 and 30.0 months,

respectively.⁴⁸ There were no significant differences in the two subgroups relating QoL evaluation through EORTC QLQ-C30, QLQ-BR23, EQ-5D-5L, and Brief Pain Inventory.⁴⁹

This year, the results from SWOG S1416 were presented at the American Society of Clinical Oncology 2020 annual meeting. This phase II study evaluated the combination of cisplatin 75 mg/m² with veliparib or placebo 400 mg bid days 1 to 14 in a 21-day-cycle in pretreated with <1 prior line. Three groups of mTNBC (n = 335) were analyzed: germline BRCA-mutated (n = 37), BRCA-like with HRD evaluated by 4 somatic or germline biomarkers (n = 101), and non-BRCA-like (n = 110). In germline BRCA-mutated, the median PFS difference was not statistically different, although with a better numerically median PFS. In non-BRCA-like patients, there was no benefit when veliparib was associated with cisplatin. In the BRCA-like group, the median PFS was higher in patients treated with veliparib plus cisplatin (5.7 months) in comparison with placebo (4.3 months), HR: 0.58, *P* = .023. The ORR and median PFS were numerically better but without significant difference. Grade 3 to 4 CTCAE hematological side effects were higher in veliparib groups.⁴¹ The last 3 trials evaluated the combination of a platinum agent with a PARP inhibitor. There are no data regarding the comparison of a PARP inhibitor and platinum drugs in mTNBC and specifically in patients with BRCA1/2 mutations. A study in this setting would be extremely relevant to guide the better treatment for this subgroup of patients, including the choice of drug, drug sequence, and to know whether it needs a combination approach. This knowledge is even more critical in countries where patients do not have access to the new targeted drugs like PARP inhibitors, and platinum drugs might have an essential role. So, it is crucial to know the specific role of platinum, PARP inhibitor, or their combination in the future.

Combining PARP inhibitors with ICIs is another combination strategy under investigation with promising results and an acceptable safety profile. The MEDIOLA trial is a phase II basket study that evaluated olaparib plus durvalumab in patients with advanced solid tumors. The metastatic BC with germline BRCA1/2 mutations cohort was presented. Patients (n = 34) were submitted to olaparib 300 mg bid for 4 weeks, and after that, 300 mg bid plus durvalumab 1.5 g every 4 weeks until disease progression. The disease control rate (DCR) was 80% (24 of 30) at 12 weeks and 50% (15 of 30) at 28 weeks. The ORR was 63.3% in the overall population, with better results in patients without previous lines and only 1 prior line. The median PFS was 8.2 months (95% CI: 4.6–11.8), and the median OS was 20.5 months (95% CI: 16.2–23.9). The most frequent grade ≥3 side effects were anemia, neutropenia, and pancreatitis.⁴² Another PARP inhibitor, niraparib 200 mg id, was evaluated in association with pembrolizumab 200 mg in a 21-cycle in TOPACIO/Keynote-162 phase II study. Preliminary results involving 54 patients (12 with BRCA1/2 mutation) revealed an ORR of 29% and a DCR of 49%. The

ORR was 33% in programmed death-ligand 1 (PD-L1) positive with a combined positive score (CPS) ≥1% and 15% in PD-L1 negative. Patients had durable responses with 13 patients (6 with BRCA1/2 mutations) with >6 months on treatment.⁴³ The DORA trial (NCT03167619) is a phase II study that evaluates olaparib's role alone or combined with durvalumab as maintenance therapy in patients with advanced TNBC treated with platinum drugs. The primary endpoint is PFS.⁵⁰ It warrants more data on these 2-drug combinations after these promising preliminary results.

The combination of PARP inhibitors with other targeted drugs or with radiation therapy is also under investigation. The VIOLETTE study (NCT03330847) is evaluating the efficacy and safety of olaparib in association with AZD1775 (a WEE1 checkpoint inhibitor) or with AZD6738 (an ataxia telangiectasia and Rad3-related protein inhibitor), two DNA damage repair agents, in patients with mTNBC with ≤2 prior lines.⁵¹ The RadioPARP study (NCT03109080) is evaluating the combination of olaparib plus radiation therapy in TNBC.⁵²

Immune Checkpoint Inhibitors

Triple-negative breast cancer is the BC subtype that has demonstrated the highest incidence of TILs.⁶ The high TILs are also associated with a better prognosis and better treatment response in the early stage in TNBC.⁵³ Tumors may suppress T-cell activity by activating inhibitory checkpoint pathways, limiting the antitumor immune response. The ICIs target the T-cells' regulatory pathways to enhance antitumor immune responses, as the examples of the cytotoxic T-lymphocyte antigen 4, programmed death-1, or lymphocyte antigen 3.⁵⁴ The associated immunogenicity related to high TILs led to clinical trials using ICIs in metastatic and early TNBC, with the essential characteristics described in Table 2. The safety and antitumor efficacy of pembrolizumab 10 mg/kg were initially evaluated in a phase Ib trial in patients with heavily treated TNBC with PD-L1 ≥1%. This trial included 32 patients, and pembrolizumab demonstrated an acceptable toxicity profile and an ORR of 18.5%.⁵⁵ A phase II clinical trial was then developed in patients with previously pretreated mTNBC (cohort A) and untreated mTNBC (cohort B). It evaluated the efficacy and safety of pembrolizumab 200 mg in a 21-day cycle. In cohort A, 170 patients were treated in second or later lines. The ORR was 5.3% (2.7–9.9) in all patients, and the median duration of response (DOR) was not reached when data were published. Grade 3 to 4 AEs occurred in 12.9%.⁵⁶ In cohort B, 84 patients with untreated mTNBC without central nervous system metastases and PD-L1 CPS ≥1% were treated with pembrolizumab. The ORR was 21.4% (95% CI: 13.9–31.4), and the DCR was 23.8% (95% CI: 15.9–34.0). The median DOR was 10.4 months, with 44.4% responses ongoing at the cutoff, and the median PFS was 2.1 months and median OS 18.0 months. Grade 3 AEs occurred in 9.5% of patients without grade 4 AEs, with a manageable safety profile.⁵⁷

Table 2. Selected published immune checkpoint inhibitor clinical trials in the mTNBC population.

AUTHOR, STUDY	N	PHASE	POPULATION	TREATMENT (EXPERIMENTAL VS COMPARED DRUG)	TARGET	ORR (%)	PFS (MONTHS)	OS (MONTHS)
Immune checkpoint inhibitors								
Nanda et al. ⁵⁶ (Keynote-012)	32	Ib	Heavily pretreated mTNBC	Pembrolizumab	PD-L1	18.5%	1.9	11.2
Adams et al. ⁵⁸ (Keynote-096 cohort A)	170	II	Pretreated mTNBC	Pembrolizumab	PD-L1	5.3%	2.0	9.0
Adams et al. ⁵⁷ (Keynote-096 cohort B)	84	II	Untreated mTNBC	Pembrolizumab	PD-L1	21.4%	2.1	18.0
Cortés et al. ⁵⁹ (Keynote-119)	622	III	Pretreated mTNBC	Pembrolizumab vs standard chemotherapy	PD-L1	ITT: 9.6% vs 10.6% CPS ≥ 1%; 12.3% vs 9.4% CPS ≥ 10%; 17.7% vs 9.2%	NS (2.1 vs 3.3)	NS (9.9 vs 10.8)
Immune checkpoint inhibitors plus chemotherapy								
Schmid et al. ⁵⁹ (Impassion130)	902	III	Untreated advanced TNBC	Nabpaclitaxel plus atezolizumab vs plus placebo	PD-L1	56.0% vs 45.9% PD-L1 ≥ 1%; 58.9% vs 42.6% 7.2 vs 5.5 PD-L1 ≥ 1%; 7.5 vs 5.0	7.2 vs 5.5 PD-L1 ≥ 1%; 7.5 vs 5.0	NS ITT: 21.0 vs 18.7 PD-L1 ≥ 1%; 25.4 vs 17.9
Cortés et al. ⁶⁰ (Keynote-355)	847	III	Untreated mTNBC	Chemotherapy (taxane or carboplatin/gemcitabine) plus pembrolizumab vs plus placebo	PD-L1	–	CPS ≥ 10%; 9.7 vs 5.6	–
Milas et al. ⁶¹ (Impassion131)	651	III	Untreated advanced TNBC	Paclitaxel plus atezolizumab vs plus placebo	PD-L1	–	NS PD-L1 ≥ 1%; 6.0 vs 5.7 ITT: 5.7 vs 5.6	NS PD-L1 ≥ 1%; 22.1 vs 28.3 ITT: 19.2 vs 22.8
Voorwerk et al. ⁶² (TONIC)	70	II	mTNBC	Nivolumab preceded by induction treatments: irradiation to a single lesion, low-dose cyclophosphamide, cisplatin, or doxorubicin, or a 2-week waiting period	PD-L1 Microenvironment	Overall population: 20% Cisplatin: 23% Doxorubicin: 35%	1.9	–

Abbreviations: CPS, combined positive score; ITT, intention to treat; mTNBC, metastatic triple-negative breast cancer; NS, not significant; ORR, overall response rate; OS, overall survival; PD-L1, programmed death-ligand 1; PFS, progression-free survival; TNBC, triple-negative breast cancer.
 Key results: Immune checkpoint inhibitors in monotherapy have demonstrated low ORR, without positive PFS and OS results compared with standard chemotherapy. Immune checkpoint inhibitors plus chemotherapy showed some conflicting results: atezolizumab plus nabpaclitaxel revealed higher ORR and PFS in PD-L1 positive. In contrast, atezolizumab plus paclitaxel did not reveal a benefit in survival compared with placebo plus chemotherapy. The PFS was also higher with pembrolizumab plus different backbone chemotherapy in PD-L1 positive, comparing with placebo. These trials did not prolong the OS.

A phase III trial (Keynote-119) compared pembrolizumab 200 mg in a 21-day cycle versus single-agent chemotherapy (capecitabine, eribulin, gemcitabine, or vinorelbine) in previously treated patients with mTNBC (1 or 2 previous lines, anthracycline and/or taxane). The study included 622 patients. The median OS and PFS were not significantly superior when treated with pembrolizumab, in intention to treat (ITT) population, in CPS $\geq 1\%$ or $\geq 10\%$. Regarding OS, although not significant, the HR was inferior per PD-L1 percentage (0.78 in CPS $\geq 10\%$, 0.86 in CPS $\geq 1\%$, and 0.97 in the ITT population). An exploratory analysis demonstrated benefit in OS in patients with CPS $\geq 20\%$ (14.9 vs 12.5 months, HR: 0.58 [95% CI: 0.38–0.88]). The duration of the response was higher with pembrolizumab. This trial demonstrated a trend toward pembrolizumab to respond accordingly to PD-L1 enrichment. The safety profile was favorable in pembrolizumab, with a grade 3 to 5 CTCAE AEs 14% in pembrolizumab versus 36% in the chemotherapy arm.⁵⁸ Immune checkpoint inhibitors have shown the role of PD-L1 positive in selecting patients for ICIs as it is associated with better clinical outcomes. Immune checkpoint inhibitors as monotherapy have demonstrated low ORR in previously pretreated patients with better results in first-line setting. Indeed, ICIs as monotherapy in later lines are currently not recommended outside a clinical trial.³⁶

The combination of ICIs with chemotherapy in first-line setting is under evaluation and has already been approved. The first phase III trial in this situation was IMpassion130. Patients ($n=902$) with untreated advanced TNBC, that relapsed >12 months after completion of early-stage treatment, were treated with nabpaclitaxel 100 mg/m² on days 1, 8, and 15 with atezolizumab or placebo 840 mg on days 1 and 15 (28-day cycle) in a 1:1 ratio. The median PFS was superior in the atezolizumab group: 7.2 versus 5.5 months in the placebo group, HR: 0.80 (95% CI: 0.69–0.92), $P=.002$. In the PD-L1-positive subgroup (the expression on tumor-infiltrating immune cells $\geq 1\%$), the median PFS was also higher in atezolizumab: 7.5 versus 5.0 months, HR: 0.62 (95% CI: 0.49–0.78), $P<.001$. The ORR was 56.0% in atezolizumab versus 45.9% in placebo, $P=.002$, and 58.9% in PD-L1 positive treated with atezolizumab and 42.6% when treated with placebo, $P=.002$. Although not statistically significant, the median OS was numerically higher in the immunotherapy group: 21.3 versus 17.6 months, HR: 0.84 (95% CI: 0.69–1.02), $P=.08$. In the PD-L1-positive subgroup, formal testing for OS was not performed. The median OS was 25.0 months in atezolizumab, and 15.5 months in placebo, HR: 0.62 (95% CI: 0.45–0.86). The most frequent AEs were similar between the two groups, with a higher hypothyroidism rate in the atezolizumab group.⁵⁹ The final OS analysis was presented recently with a median follow-up time of 18.8 months, and 73.8% died. In ITT, the median OS was 21.0 months in the atezolizumab arm versus 18.7 months in placebo, HR: 0.87 (95% CI: 0.75–1.02), $P=.0770$. Although not statistically significant, numerically median OS was superior in

the PD-L1 population with a 7.5-month median improvement: 25.4 versus 17.9 months, HR: 0.67 (95% CI: 0.53–0.86).⁶³ The nabpaclitaxel plus atezolizumab combination is already approved and recommended for mTNBC with PD-L1-positive patients.^{16,36} Another phase III trial (Keynote-355) compared pembrolizumab with chemotherapy (nabpaclitaxel, paclitaxel, or gemcitabine/carboplatin) versus placebo plus chemotherapy in the first line. The study included 847 patients with mTNBC, with >6 months from completion of early-staged treatment. This study allowed chemotherapy beyond taxanes, including carboplatin-based treatment, which has an important role in these patients. The PFS analysis was done by a hierarchical model by PD-L1 status using CPS. The median PFS was superior in the pembrolizumab arm in patients with CPS $\geq 10\%$, 9.7 versus 5.6 months, HR: 0.65 (95% CI: 0.49–0.86), $P=.0012$. The estimated PFS at 12 months was 39.1% in the pembrolizumab group and 23.0% in the placebo group. According to statistics prespecified methods, although with a numerically superior median PFS, PFS was not significantly superior in the CPS $\geq 1\%$ subgroup (7.6 and 5.6 months, $P=.0014$) and was not compared in the ITT population. The benefits of pembrolizumab plus chemotherapy were irrespective of chemotherapy protocol in a subgroup analysis. The OS analysis is ongoing. The rate of grade 3 to 5 AEs was comparable between the 2 arms (68.1% and 66.9%), with no new safety concerns.^{64,65} In November 2020, based on the Keynote-355 trial, the Food and Drug Administration (FDA) approved pembrolizumab plus chemotherapy for advanced irresectable or metastatic TNBC with CPS $\geq 10\%$.⁶⁵

The IMpassion130 and Keynote-355 have some concerns that might limit the use of their criteria in clinical practice. They excluded patients with early relapses, and IMpassion130 used nabpaclitaxel to avoid corticoid exposure. Still, this drug is no currently the standard of care, and it would be difficult to have access to this drug in some countries.⁶⁶ A phase III trial (IMpassion131) evaluates the combination of atezolizumab and paclitaxel in first-line treatment for mTNBC. A phase III trial (IMpassion132) evaluated the combination of atezolizumab and chemotherapy (carboplatin/gemcitabine or capecitabine) in patients with early relapses (within 12 months after completion of the curative intention treatment).^{67–69} The primary results of the IMpassion131 trial were presented at ESMO 2020 Congress. Patients ($n=651$) were randomized in a 2:1 ratio for atezolizumab 840 mg on days 1 and 15 or placebo plus paclitaxel 90 mg/m² on days 1, 8, and 15 every 28 days. Programmed death-ligand 1 status was evaluated through immune cell expression, positive if $\geq 1\%$, and the primary endpoint is the PFS test hierarchically first in the PD-L1 positive and then in the ITT population. The median PFS was not significantly different between the two arms in the PD-L1 positive or ITT population. In the PD-L1 positive, the median PFS was 5.7 months in the placebo and 6.0 months in atezolizumab, HR: 0.82 (95% CI: 0.60–1.12), $P=.20$. The median OS

was not significantly different between the 2 groups analyzed in PD-L1 and the ITT population. In PD-L1 positive, median OS was 28.3 months in the placebo arm and 22.1 months in the atezolizumab arm, HR: 1.12 (95% CI: 0.76-1.65). Grade ≥ 3 side effects were balanced in each arm.⁶¹ Contradictory to IMpassion130, combination of atezolizumab with paclitaxel did not improve survival in PD-L1 or in ITT population. The ways why the combination of atezolizumab with nabpaclitaxel improved survival but not with paclitaxel are still unknown. In the future, it will be necessary to understand this question.

The IM agents can also play a role when associated with ICIs. A phase II multicohort trial (TONIC) evaluated some strategies to improve the ICI efficacy. Patients ($n=70$) were randomized to 1 of the 5 arms with different induction treatments: irradiation to a single lesion, low-dose cyclophosphamide, cisplatin, or doxorubicin a 2-week waiting period, and after that were submitted to a biopsy and treated with nivolumab. The ORR was 20%, and the median DOR was 9 months (95% CI: 4.7-not reached). The induction cisplatin and doxorubicin arms were associated with a better ORR, 23% and 35%, respectively, which may induce a more favorable tumor microenvironment and increased probability nivolumab responses. The responders had higher levels of TILs and higher levels of CD8 and PD-L1, and low levels of Ca15-3. An increased T-cell infiltration after induction, a higher T-cell receptor clonality, and upregulation of inflammation-related signatures were observed in cisplatin and doxorubicin.⁶² This trial enhances the importance of microenvironment and other IM-associated agents.

Anti-AR Antagonists

The AR promotes cell proliferation in TNBC and is an emerging biomarker in mTNBC.⁷⁰ Some trials studied the role of anti-AR antagonists in advanced TNBC (Table 3). Bicalutamide, an oral AR antagonist, was the first drug studied in AR-positive mTNBC. In a phase II study (TBCRC011), AR was positive when immunohistochemistry (IHC) staining was $>10\%$, which corresponds to 12% of all screened patients ($n=51$). This trial included 28 patients. The clinical benefit rate (CBR) was the primary endpoint. The CBR at 6 months was 19% (95% CI: 7-39). The median PFS was 12 weeks. The most common AEs were fatigue, hot flashes, limb edema, and aspartate and alkaline aminotransferase elevations.⁷¹ Abiraterone is an irreversible and potent inhibitor of CYP17. Abiraterone 1000 mg plus prednisolone 5 mg bid was evaluated in a phase II study (UCBG 12-1) in patients with AR-positive ($\geq 10\%$ by IHC staining) advanced TNBC. The percentage of AR-positive was 37.6%, but only 34 patients entered the study. The CBR was 20% (95% CI: 7.7-38.6) at 6 months, and the ORR was 6.7% (95% CI: 0.8-22.1). The median PFS was 2.8 months (95% CI: 1.7-5.4). The most frequent AEs were fatigue, hypertension, hypokalemia, and nausea, mostly grades 1 and 2.⁷² Seviteronel,

another inhibitor of CYP17, was studied in a phase I trial with an acceptable safety profile.⁸⁰ A phase II clinical trial (TBCRC) in advanced TNBC with AR expression evaluated the enzalutamide, a potent AR inhibitor. AR was positive if $>0\%$, which was present in 80% of patients, and negative when 0%. The study involves 118 patients, with 78 of them with evaluable possible. At 16 weeks, the CBR was 33% (23-45) and 22% (19-39) at 24 weeks. The ORR was 8%, the median PFS was 3.3 months (95% CI: 1.9-4.1), and the median OS was 17.6 months (95% CI: 11.6-not reached) in the evaluable group. The most frequent AEs were fatigue, nausea, and decreased appetite.⁷³ The combination of bicalutamide with CDK4/6 palbociclib (NCT02605486) and ribociclib (NCT03090165) is studying in AR-positive metastatic BC in phase II trials.^{81,82}

PI3K/AKT Pathway in mTNBC

Genetic alterations in the phosphatidylinositol 3-kinase/AKT/mammalian target of the rapamycin (PI3K/AKT/mTOR) pathway are frequent in all BC subtypes, including TNBC.⁴¹ The Cancer Genome Atlas Network reported 7% PIK3CA mutations, 35% PTEN mutations/loss, and 30% INPP4B loss in basal-like BC subtype.⁸³ In patients with TNBC, 70% harbored a PIK3CA, AKT1, or a PTEN aberration, and PI3K mutation is more frequent in the LAR subtype.⁸⁴ Buparlisib is a pan-class PI3K inhibitor studied as first-line treatment with paclitaxel in HER2-negative advanced BC in phase II/III trial (BELLE-4). The addition of buparlisib did not improve PFS, and the trial was stopped at the end of the phase II part.⁸⁵

Ipatasertib is an oral AKT inhibitor studied in a randomized phase II study in advanced or metastatic untreated TNBC (LOTUS). Patients ($n=166$) were randomized in a 1:1 ratio to paclitaxel 80 mg/m² on days 1, 8, and 15 plus ipatasertib 400 mg or placebo on days 1 to 21 in a 28-day cycle. The median PFS was superior in the ipatasertib group: 6.2 versus 4.9 months, HR: 0.60 (95% CI: 0.37-0.98), $P=.037$. In the 48 PTEN-low tumor patients, the median PFS was 6.2 months in ipatasertib and 3.7 months in placebo, HR: 0.59 (95% CI: 0.26-1.32), $P=.18$. In PIK3CA/AKT1/PTEN-altered tumors, the median PFS was 9.0 months in ipatasertib versus 4.9 months in placebo, HR: 0.44 (95% CI: 0.20-0.99), $P=.041$. In ITT, the ORR was 40% in ipatasertib and 32% in placebo. The median DOR was comparable in ITT and PTEN-low but was higher in PIK3CA/AKT1/PTEN-altered tumors (11.2 vs 6.1 months). The most frequently documented grade 3 AEs were diarrhea and neutropenia, with a higher proportion of all grade ≥ 3 AEs (54% vs 42%). There was no difference in QoL using EORTC QLQ-C30.⁷⁴ Another AKT inhibitor, capivasertib, was evaluated in combination with paclitaxel as first-line mTNBC therapy in phase II randomized trial (PAKT). Patients ($n=140$) were randomized to paclitaxel 90 mg/m² on days 1, 8, and 15 plus capivasertib or placebo 400 mg bid on days 2 to 5, 9 to 12, and 16 to 19 in a 28-day cycle. The median PFS was 5.9 months

Table 3. Selected published clinical trials using targeted drugs in the mTNBC population.

AUTHOR, STUDY	N	PHASE	POPULATION	TREATMENT (EXPERIMENTAL VS COMPARED DRUG)	TARGET	ORR (%)	PFS (MONTHS)	OS (MONTHS)
AR antagonists								
Gucalp et al. ⁷¹ (TBCRC011)	28	II	Metastatic AR-positive TNBC	Bicalutamide	AR	CBR at 6 months: 19%	12 weeks	–
Bonneter et al. ⁷² (UCBG 12-1)	34	II	Metastatic AR-positive TNBC	Abiraterone plus prednisone	AR	CBR at 6 months: 20% ORR: 6.7%	2.8	–
Taina et al. ⁷³	78	II	Metastatic AR-positive TNBC	Enzalutamide	AR	CBR at 16 weeks: 33%	3.3	17.6
AKT inhibitors								
Kim et al. ⁷⁴ (LOTUS)	166	II	Metastatic untreated TNBC	Paclitaxel plus ipatasenib vs plus placebo	PI3K/AKT/ PTEN	ITT: 40% vs 32% PTEN-low: 48% vs 23% PIK3CA/AKT1/ PTEN-altered: 50% vs 44%	ITT: 6.2 vs 4.9 PTEN-low: NS (6.2 vs 3.7) PIK3CA/AKT1/ PTEN-altered: 9.0 vs 4.9	–
Schmid et al. ⁷⁵ (PAKT)	140	II	Metastatic untreated TNBC	Paclitaxel plus capivasertib vs plus placebo	PI3K/AKT/ PTEN	ITT: 34.8% vs 28.8% PIK3CA/AKT1/ PTEN-altered: 35.3% vs 18.2%	ITT: NS (5.9 vs 4.2) PIK3CA/AKT1/ PTEN-altered: 9.3 vs 3.7	ITT: 19.1 vs 12.5 PIK3CA/AKT1/ PTEN-altered: NS (NR vs 10.4)
Dent et al. ⁷⁶ (IPATunity130)	255	III	PIK3CA/AKT1/PTEN-altered advanced irresectable or metastatic TNBC	Paclitaxel plus ipatasenib vs plus placebo	PI3K/AKT/ PTEN	39% vs 35%	NS (7.4 vs 6.1)	–
PI3K/AKT pathway plus AR antagonists								
Lehmann et al. ⁷⁷ (TBCRC032)	17	Ib/II	Metastatic AR-positive TNBC	Enzalutamide with/without taselisib	AR PI3K/AKT/ PTEN	CBR at 16 weeks: 35.7%	3.4	–
Antibody-drug conjugate								
Bardia et al. ⁷⁸ (IMMU-132-01)	108	I/II	Heavily pretreated mTNBC	Sacituzumab-govitecan	Trop-2	33.3%	5.5	13.0
Bardia et al. ⁷⁹ (ASCENT)	529	III	Heavily pretreated mTNBC	Sacituzumab-govitecan vs standard chemotherapy	Trop-2	35% vs 5%	5.6 vs 1.7	12.1 vs 6.7

Abbreviations: AR, androgen receptor; CBR, clinical benefit rate; ITT, intention to treat; mTNBC, metastatic triple-negative breast cancer; NR, not reached; NS, not significant; ORR, overall response rate; OS, overall survival; PFS, progression-free survival; PI3K/AKT/PTEN, phosphatidylinositol 3-kinase/AKT/protein kinase B; TNBC, triple-negative breast cancer; Trop-2, tropoblast cell-surface antigen 2.

Key results: Androgen receptor antagonists showed preliminary data in a poor prognostic subpopulation, with a CBR between 20% and 33% between 4 and 6 months. In combination with paclitaxel, AKT inhibitors have shown better ORR and PFS in 2 phase II trials in patients with PIK3CA/AKT1/PTEN alterations. Nevertheless, a phase III trial was negative when ipatasenib was added to paclitaxel comparing with placebo in PIK3CA/AKT1/PTEN-altered advanced irresectable or metastatic TNBC. Sacituzumab-govitecan has demonstrated a higher ORR, PFS, and OS than standard chemotherapy in heavily pretreated patients.

in the capivasertib arm and 4.2 months in the placebo arm, HR: 0.74 (95% CI: 0.50–1.08), $P = .11$. In PIK3CA/AKT1/PTEN-altered subgroup, the median PFS was 9.3 months with capivasertib versus 3.7 months with placebo, HR: 0.30 (95% CI: 0.11–0.79), $P = .01$. The ORR in the ITT population was 34.8% in capivasertib, and 28.8% in placebo. In PIK3CA/AKT1/PTEN-altered subgroup, the ORR was 35.3% in capivasertib versus 18.2% in placebo. The median OS was significantly higher in the capivasertib arm in the ITT population: 19.1 versus 12.6 months, HR: 0.61 (95% CI: 0.37–0.99), $P = .04$. The median OS was not reached in capivasertib-treated patients with PIK3CA/AKT1/PTEN-alterations. Diarrhea, neutropenia, infection, rash, and fatigue were the most frequent AEs associated with capivasertib.⁷⁵

As we can see in these phase II referred trials (Table 3), the addition of an AKT inhibitor to paclitaxel in first line prolongs the PFS, especially in patients with PIK3CA/AKT1/PTEN-altered tumors. The phase III clinical trial (IPATUNITY130) evaluates the combination of ipatasertib and paclitaxel versus placebo and paclitaxel in PIK3CA/AKT1/PTEN-altered advanced irresectable or metastatic untreated TNBC (cohort A) or hormone receptor–positive/HER2-negative BC. The primary endpoint was investigator-assessed PFS.⁸⁶ The primary results from this trial were published at the end of 2020. In cohort A, 255 patients were enrolled, 51% with PIK3CA/AKT1 mutations and 49% with PTEN alterations. With a median follow-up of 8.3 months and 33% patients remaining on treatment, there was no difference in PFS between the 2 groups: median PFS 7.4 months (95% CI: 5.6–8.5) in the ipatasertib arm and 6.1 months (95% CI: 5.5–9.0) in the placebo arm, HR: 1.02 (95% CI: 0.71–1.45), $P = .9237$. The proportion of grade ≥ 3 AEs was similar in the 2 arms (46% and 44%). The most frequent AEs were diarrhea (80% and 31%), alopecia (46% and 44%), and nausea (36% and 23%).⁷⁶ These results did not corroborate the previous results from phase II trials. A phase III randomized trial is also investigating the clinical benefit and safety of capivasertib plus paclitaxel combination in first-line treatment in patients with mTNBC (CAPITello290).⁸⁷

PI3K/AKT pathway inhibitors are also under investigation in combination with other drugs. Luminal androgen receptor subtype is enriched in PIK3CA mutations (40%–50%). Enzalutamide combined with tasisib (PI3K inhibitor) was studied in a phase Ib/II study (TBCRC032) in AR-positive mTNBC. In phase II, 17 pretreated patients were randomized to receive enzalutamide 160 mg with or without tasisib 4 mg. Hyperglycemia and rash were the most frequent AEs. All the patients had disease progression at 16 weeks, and the CBR was 35.7% in the combination arm compared with no one in the enzalutamide arm. The median PFS was 3.4 months. There were no different results when patients were stratified using PIK3CA mutations. The LAR signature corresponded to a better clinical benefit (75.0%) compared with 12.5% in other

subtypes.⁷⁷ Ipatasertib is also under investigation combined with atezolizumab and paclitaxel in phase III randomized trial (IPATUNITY170) in untreated advanced or mTNBC.⁸⁸

Antibody-Drug Conjugates

The ADC is recently being studied in mTNBC and is showing promising results. There are some ADCs in ongoing trials, as the examples of sacituzumab-govitecan, ladiratumab-vedotin, trastuzumab-deruxtecan, AVID100, U3-1402, CAB-ROR2-ADC, Anti-CA6-DM4 immunoconjugate, and glembatumumab-vedotin. Almost all these ADCs are under investigation in early phase trials, except sacituzumab-govitecan and trastuzumab-deruxtecan. There are phase III clinical trials evaluating the last two drugs.⁸⁹

Sacituzumab-Govitecan

Sacituzumab-govitecan is an ADC that incorporates a humanized monoclonal antibody (hRS7) that targets the target human trophoblast cell-surface antigen 2 (Trop-2) and enables the internalization and delivery of SN-38 (govitecan). This is an active metabolite of irinotecan, a topoisomerase I inhibitor. Trophoblast cell-surface antigen 2 is highly overexpressed in mTNBC and has limited expression in normal tissues.⁹⁰ Sacituzumab-govitecan delivers more SN-38 than irinotecan, which may overcome RAD51-mediated HRR pathway in Trop-2-expressing tumors. Also, sacituzumab-govitecan delivers SN-38 in its most active nonglucuronidated form, explaining the lower incidence of diarrhea with sacituzumab-govitecan compared with irinotecan.⁹¹ A phase I/II basket study (IMMU-132-01) evaluated it in previously treated metastatic cancers. The metastatic BC cohort was published at the end of the year 2019. This trial included 108 heavily treated mTNBC patients with median previous lines of 3 (range: 2–10). They were treated with 10 mg/kg of the studied drug on days 1 and 8 in a 21-day cycle. The most frequent grade ≥ 3 side effects were anemia and neutropenia and febrile neutropenia. Nausea and diarrhea were frequent any-grade side effects. The ORR was 33.3% (95% CI: 24.6–43.1), and the median DOR was 7.7 months. Median PFS was 5.5 months (95% CI: 4.1–6.3) and the median OS 13.0 months (95% CI: 11.2–13.7).⁷⁸ A phase III confirmatory trial (ASCENT) in heavily pretreated mTNBC (≥ 2 lines) has compared sacituzumab-govitecan versus physician's choice chemotherapy (capecitabine, gemcitabine, vinorelbine, and eribulin). This study was stopped earlier this year due to significant potential benefits in these patients. The results of 529 patients enrolled were recently presented. The primary endpoint is PFS in the brain metastasis–negative population. The median PFS was 5.6 months in sacituzumab-govitecan versus 1.7 months in the physician's choice chemotherapy arm (HR: 0.41; $P < .0001$). The median OS was also higher in the experimental drug arm: 12.1 versus 6.7 months (HR: 0.48; $P < .0001$). The most frequent grade ≥ 3 side effects were neutropenia (51%)

vs 31%), diarrhea (10.5% vs <1%), anemia (8% vs 5%), and febrile neutropenia (6% vs 2%). No treatment-related deaths were reported.⁷⁹ A postbiomarker analysis revealed that the clinical benefit was independent of Trop-2 expression, assessed using IHC.⁸² The FDA approved the sacituzumab-govitecan for patients who have received at least 2 previous treatment lines in the mTNBC setting.⁹³ Ongoing multiple trials are testing the combination of sacituzumab-govitecan with chemotherapy, talazoparib, and immunotherapy.⁹¹

Other Targeted Pathways

Epidermal growth factor repair is highly expressed in basal TNBC subtypes. Epidermal growth factor repair inhibitors were previously evaluated but with disappointing results.⁹⁴ The role of angiogenesis is also being questioned in TNBC, mostly in neoadjuvant strategies and metastatic disease. Some trials have demonstrated a benefit in PFS, although without benefit in OS.⁹⁶ Ongoing trials are evaluating the combination of bevacizumab plus chemotherapy or ICIs. Cabozantinib, a multiple tyrosine kinase inhibitor, including MET and vascular endothelial growth factor receptor 2, was evaluated in mTNBC in a phase II study, including 35 patients. The CBR was 31% (95% CI: 17%-49%), and the median PFS was 1.9 months (95% CI: 1.3-3.3).⁹⁵ More recently, cabozantinib was evaluated in combination with nivolumab, but without achieving the primary endpoint of ORR.⁹⁶

Mitogen-activated protein kinase activity is 1 of the mechanisms of resistance to taxane-based chemotherapy. This pathway was evaluated in TNBC in a phase II study (COLET). Cobimetinib (MEK inhibitor) was combined with paclitaxel as first-line treatment in advanced TNBC in multiple cohorts. Cohort I compared paclitaxel plus cobimetinib versus paclitaxel plus placebo in 90 patients. The median PFS was 5.5 months for the combination strategy versus 3.8 months, HR: 0.73 (95% CI: 0.43-1.24), $P = .2$. The ORR was 38.3% versus 20.9%, and the safety profile was expected for both drugs.⁹⁷ Atezolizumab was combined with cobimetinib and taxane in cohorts II (paclitaxel, $n = 63$) and III (nabpaclitaxel, $n = 62$). The ORR was 34% in cohort II (44% in PD-L1 positive and 11% in PD-L1 negative) and 29% in cohort III (33% in PD-L1 positive and 27% in PD-L1 negative). The 6-month PFS was 40.5% in cohort II and 50.1% in cohort III.⁹⁸

Trilaciclib is a cell cycle selective inhibitor of cyclin-dependent kinases-4/6 that transiently maintain immune cells, hemopoietic stem, and progenitor cells in G1 arrest. So, it is believed to have the potential to optimize antitumor activity and minimize myelotoxicity. The drug was studied in a phase II clinical trial in recurrent or mTNBC with ≤ 2 previous lines. Patients ($n = 142$) were randomized in a 1:1:1 ratio to gemcitabine 100 mg/m² and carboplatin AUC 2 on days 1 and 8 (group 1), gemcitabine and carboplatin plus trilaciclib 240 mg/m² on days 1 and 8 (group 2) or on days 1, 2, 8, and 9 on a 21-day

cycle. No significant difference was observed in myelosuppression endpoints. Regarding efficacy, median OS was superior in trilaciclib groups: 17.8 months (8.8-not reached) in group 3, 20.1 months (9.4-not reached) in group 2, and 12.6 months in group 1. It was believed that the induced decreased activity of FOXM1, overexpressed in TNBC, induced by trilaciclib, leading to increased sensitivity for chemotherapy, contributed to a better outcome.⁹⁹

Conclusions

Metastatic TNBC is a heterogeneous disease that used to be treated only with chemotherapy-based strategies. The discovery of genomic alterations like BRCA1/2 mutations, HRD, and the molecular subtypes has led to the development of promising drugs in this disease. Indeed, it is changing the way how we treat mTNBC. Platinum-based chemotherapy is non-inferior to other systemic chemotherapy in mTNBC and has better outcomes than a taxane in BRCA1/2-mutated patients regarding ORR and PFS. A platinum drug should be considered in these patients. PARP inhibitors olaparib and talazoparib were both approved for patients with germline BRCA1/2 mutations and metastatic BC. The combinations of PARP inhibitors and chemotherapy or ICIs are under investigation with promising results. Questions remain on what patients should be treated with PARP inhibitors as monotherapy or in combination therapy. Perhaps, PARP inhibitors may have a role in maintenance therapy after platinum chemotherapy leading to chemotherapy-free interval for the patients. Immune checkpoint inhibitors demonstrated clinical benefit associated with chemotherapy in patients with PD-L1 positive. They should be used in the first-line setting, with the two combinations approved: atezolizumab plus nabpaclitaxel or pembrolizumab plus various chemotherapy protocols (nabpaclitaxel, paclitaxel, and carboplatin plus gemcitabine). Also, in the first-line setting, two AKT inhibitors—ipatasertib and capivasertib associated with paclitaxel—have demonstrated promising outcomes in patients with PI3K/AKT pathway alterations in phase II trials. Nevertheless, a phase III trial IPATunity was negative regarding PFS, with immature OS data and other studies ongoing. Androgen receptor antagonists in the LAR subtype showed encouraging results in a poor prognostic subpopulation, with the need for more data in this setting. The ADC sacituzumab-govitecan also showed clinical benefit in heavily pretreated patients and is already approved by the FDA for the third line or beyond in the mTNBC setting. The incorporation into earlier lines is awaited as well as in combination with other drugs. A proposed algorithm that includes approved therapies for treating mTNBC is described in Figure 1. Other targeted drugs and combinations of some referred drugs are under investigation and will bring us results shortly. Knowing the tumor's biology and targeting drugs in a personalized medicine era brings us new hope for patients with mTNBC.

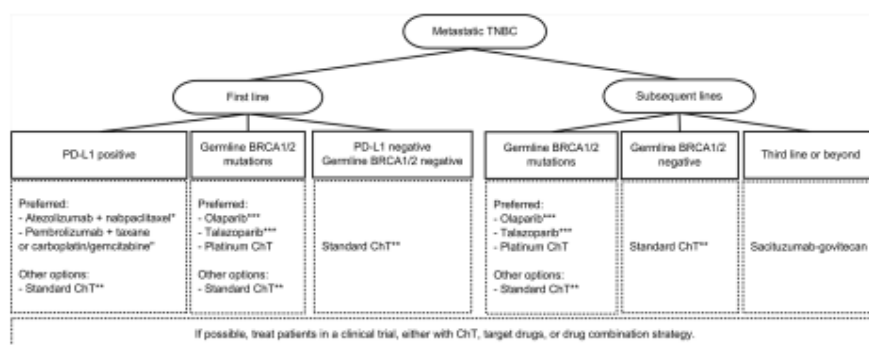


Figure 1. Proposed treatment algorithm for the treatment of mTNBC, including the approved drugs.

*If PD-L1 $\geq 1\%$ and disease-free survival from (neo)adjuvant ChT >12 months.

**If PD-L1 (CPS) $\geq 10\%$ and disease-free survival from (neo)adjuvant ChT >6 months.

***If both PD-L1 and germline BRCA1/2 are positive, there are no data on which is the best approach.

****Standard chemotherapy includes taxanes, anthracyclines, antimetabolites, and microtubule inhibitors. Carboplatin is an alternative with comparable efficacy and a better toxicity profile than docetaxel. A drug combination or single-agent chemotherapy could be used, depending on the disease's extension or rapid progression.

*****If previous anthracycline and a taxane in the (neo)adjuvant or metastatic setting.

ChT indicates chemotherapy; CPS, combined positive score; mTNBC, metastatic triple-negative breast cancer; PD-L1, programmed death-ligand 1; TNBC, triple-negative breast cancer.

Author Contributions

EC and AM contributed to the conception and design of the work; EC was responsible for the acquisition and analysis of data and the draft of the article; AM critically revised the article; and both authors approved the final version of the article.

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REFERENCES

- Bray F, Ferlay J, Soerjomataram I, Siegel RL, Torre LA, Jemal A. Global cancer statistics 2018: GLOBOCAN estimates of incidence and mortality worldwide for 36 cancers in 185 countries. *CA Cancer J Clin*. 2018;68:394-424. doi:10.3322/caac.21492.
- Henry NL, Shah PD, Haider I, Freer PE, Jagi R, Sabel MS. Cancer of the breast. In: Niederhuber JE, Armitage JO, Doroshow JH, Kastan MB, Tepper JE, eds. *Abeloff's Clinical Oncology*. 6th ed. Philadelphia, PA: Elsevier; 2020:1560-1603.e12. doi:10.1016/B978-0-323-47674-4.00088-8.
- Sharma P. Biology and management of patients with triple-negative breast cancer. *Oncologist*. 2016;21:1050-1062. doi:10.1634/theoncologist.2016-0067.
- Borri F, Granaglia A. Pathology of triple negative breast cancer [published online ahead of print June 13, 2020]. *Semin Cancer Biol*. doi:10.1016/j.semcancer.2020.06.005.
- Petrucelli N, Daly MB. BRCA1- | BRCA2-associated hereditary breast and ovarian cancer. *GeneReviews*. <https://www.ncbi.nlm.nih.gov/books/NBK1247/>. Updated 2016. Accessed December 31, 2020.
- Stanton SE, Adams S, Disis ML. Variation in the incidence and magnitude of tumor-infiltrating lymphocytes in breast cancer subtypes: a systematic review. *JAMA Oncol*. 2016;2:1354-1360. doi:10.1001/jamaoncol.2016.1061.
- Foulkes WD, Smith IE, Reis-Filho JS. Triple-negative breast cancer. *N Engl J Med*. 2010;363:1938-1948. doi:10.1056/NEJMra1001389.
- Dent R, Trudeau M, Pritchard KI, et al. Triple-negative breast cancer: clinical features and patterns of recurrence. *Clin Cancer Res*. 2007;13:4429-4434. doi:10.1158/1078-0432.CCR-06-3045.
- Lehmann BD, Bauer JA, Chen X, et al. Identification of human triple-negative breast cancer subtypes and preclinical models for selection of targeted therapies. *J Clin Invest*. 2011;121:2750-2767. doi:10.1172/JCI45014.
- Lehmann BD, Pietenpol JA. Clinical implications of molecular heterogeneity in triple negative breast cancer. *Breast*. 2015;24:S36-S40. doi:10.1016/j.breast.2015.07.009.
- Sporikova Z, Koudelakova V, Trojanec R, Hajdich M. Genetic markers in triple-negative breast cancer. *Clin Breast Cancer*. 2018;18:e841-e850. doi:10.1016/j.clbc.2018.07.023.
- Bareche Y, Venet D, Ignatiadis M, et al. Unravelling triple-negative breast cancer molecular heterogeneity using an integrative multiomic analysis. *Ann Oncol*. 2018;29:895-902. doi:10.1093/annonc/ndy024.
- Burstein MD, Tsimelzon A, Poage GM, et al. Comprehensive genomic analysis identifies novel subtypes and targets of triple-negative breast cancer. *Clin Cancer Res*. 2015;21:1688-1698. doi:10.1158/1078-0432.CCR-14-0432.
- Fougner C, Bergholtz H, Norum JH, Sævi T. Re-definition of Claudin-low as a breast cancer phenotype. *Nat Commun*. 2020;11:1-11. doi:10.1038/s41467-020-15574-5.
- Cardoso F, Senkus E, Costa A, et al. 4th ESO-ESMO international consensus guidelines for advanced breast cancer (ABC 4). *Ann Oncol*. 2018;29:1634-1657. doi:10.1093/annonc/ndy192.
- NCCN guidelines. Breast cancer. NCCN.org. https://www.nccn.org/professionals/physician_gls/pdf/breast.pdf. Updated 2020. Accessed July 11, 2020.
- Lyons TG, Traim TA. Emerging novel therapeutics in triple-negative breast cancer. *Adv Exp Med Biol*. 2019;1152:377-399. doi:10.1007/978-3-030-20301-6_20.
- Fleck O, Nielsen O. DNA repair. *J Cell Sci*. 2004;117:515-517. doi:10.1242/jcs.00952.
- Roy R, Chao J, Powell SN. BRCA1 and BRCA2: different roles in a common pathway of genome protection. *Nat Rev Cancer*. 2012;12:68-78. doi:10.1038/nrc3181.
- Gonzalez-Angulo AM, Timms KM, Liu S, et al. Incidence and outcome of BRCA mutations in unselected patients with triple receptor-negative breast cancer. *Clin Cancer Res*. 2011;17:1082-1089. doi:10.1158/1078-0432.CCR-10-2560.
- Armstrong N, Ryder S, Forbes C, Ross J, Quek RG. A systematic review of the international prevalence of BRCA mutation in breast cancer. *Clin Epidemiol*. 2019;11:543-561. doi:10.2147/CLEP.S206949.
- Fostira F, Tsiatlidou M, Papadimitriou C, et al. Prevalence of BRCA1 mutations among 403 women with triple-negative breast cancer: implications for genetic screening selection criteria: a Hellenic Cooperative Oncology Group Study. *Breast Cancer Res Treat*. 2012;134:353-362. doi:10.1007/s10549-012-2021-9.
- Rummel S, Varner E, Shriver CD, Ellsworth RE. Evaluation of BRCA1 mutations in an unselected patient population with triple-negative breast cancer. *Breast Cancer Res Treat*. 2013;137:119-125. doi:10.1007/s10549-012-2348-2.
- Petrucelli N, Daly MB, Pal T. BRCA1- and BRCA2-associated hereditary breast and ovarian cancer summary genetic counseling findings. *GeneReviews*. Seattle, WA: University of Washington; 1993:1-37.
- Hatano Y, Tamada M, Matsuo M, Hara A. Molecular trajectory of BRCA1 and BRCA2 mutations. *Front Oncol*. 2020;10:361. doi:10.3389/fonc.2020.00361.
- Tung NM, Garber JE. BRCA1/2 testing: therapeutic implications for breast cancer management. *Br J Cancer*. 2018;119:141-152. doi:10.1038/s41416-018-0127-5.

27. Nielsen FC, van Overbeek Hansen T, Sørensen CS. Hereditary breast and ovarian cancer: new genes in confined pathways. *Nat Rev Cancer*. 2016;16:599-612. doi:10.1038/nrc.2016.72.
28. Alexandrov LB, Nik-Zainal S, Wedge DC, et al. Signatures of mutational processes in human cancer. *Nature*. 2013;500:415-421. doi:10.1038/nature12477.
29. Nik-Zainal S, Davies H, Staaf J, et al. Landscape of somatic mutations in 560 breast cancer whole-genome sequences. *Nature*. 2016;534:47-54. doi:10.1038/nature17676.
30. Polak P, Kim J, Braunstein LZ, et al. A mutational signature reveals alterations underlying deficient homologous recombination repair in breast cancer. *Nat Genet*. 2017;49:1476-1486. doi:10.1038/ng.3934.
31. Thomassen C, Lüftner D, Untch M, et al. International consensus conference for advanced breast cancer, Lisbon 2019: ABC5 consensus—assessment by a German group of experts. *Breast Care*. 2020;15:82-95. doi:10.1159/000505957.
32. Gerrata L, Fanotto V, Pelizzari G, Agostinetti E, Puglisi F. Do platinum salts fit all triple negative breast cancers. *Cancer Treat Rev*. 2016;48:34-41. doi:10.1016/j.ctrv.2016.06.004.
33. Torrisi R, Zucarelli M, Agostinetti E, et al. Critical reviews in oncology / hematology platinum salts in the treatment of BRCA-associated breast cancer: a true targeted chemotherapy? *Crit Rev Oncol / Hematol*. 2019;135:66-75. doi:10.1016/j.critrev.2019.01.016.
34. Isakoff SJ, Mayer EL, He L, et al. TBCRC009: a multicenter phase II clinical trial of platinum monotherapy with biomarker assessment in metastatic triple-negative breast cancer. *J Clin Oncol*. 2015;33:1902-1909. doi:10.1200/JCO.2014.57.6660.
35. Tutt A, Tovey H, Cheang MCU, et al. Carboplatin in BRCA1/2-mutated and triple-negative breast cancer BRCAAnex subgroups: the TNT trial. *Nat Med*. 2018;24:628-637. doi:10.1038/s41591-018-0009-7.
36. Cardoso F, Paluch-Shimon S, Senkus E, et al. 5th ESO-ESMO international consensus guidelines for advanced breast cancer (ABC 5). *Ann Oncol*. 2020;31:1623-1649. doi:10.1016/j.annonc.2020.09.010.
37. Robson M, Im S-A, Senkus E, et al. Olaparib for metastatic breast cancer in patients with a germline BRCA mutation. *N Engl J Med*. 2017;377:523-533. doi:10.1056/NEJMoa1706450.
38. Litton JK, Rugo HS, Ettl J, et al. Talazoparib in patients with advanced breast cancer and a germline BRCA mutation. *N Engl J Med*. 2018;379:753-763. doi:10.1056/NEJMoa1802905.
39. Han HS, Diéras V, Robson M, et al. Veliparib with temozolomide or carboplatin/paclitaxel versus placebo with carboplatin/paclitaxel in patients with BRCA1/2 locally recurrent/metastatic breast cancer: randomized phase II study. *Ann Oncol*. 2018;29:154-161. doi:10.1093/annonc/mdx505.
40. Diéras VC, Han HS, Kaufman B, et al. LBA9—phase III study of veliparib with carboplatin and paclitaxel in HER2-negative advanced/metastatic gBRCA-associated breast cancer. *Ann Oncol*. 2019;30:v857-v858. doi:10.1093/annonc/mdx394.008.
41. Sharma P, Rodler E, Barlow WE, et al. Results of a phase II randomized trial of cisplatin +/- veliparib in metastatic triple-negative breast cancer (TNBC) and/or germline BRCA-associated breast cancer (SWOG S1416). *J Clin Oncol*. 2020;38:1001. doi:10.1200/JCO.2020.38.15_suppl.1001.
42. Domchek S, Postel-Vinay S, Im S-A, et al. Phase II study of olaparib (O) and durvalumab (D) (MEDIOA): updated results in patients (pts) with germline BRCA-mutated (gBRCAm) metastatic breast cancer (MBC). *Ann Oncol*. 2019;30:v477. doi:10.1093/annonc/mdx253.017.
43. Vinayak S, Tolane SM, Schwartzberg LS, et al. TOPACIO/Keynote-162: niraparib + pembrolizumab in patients (pts) with metastatic triple-negative breast cancer (TNBC), a phase 2 trial. *J Clin Oncol*. 2018;36:1011. doi:10.1200/JCO.2018.36.15_suppl.1011.
44. Egger SJ, Willson ML, Morgan J, et al. Platinum-containing regimens for metastatic breast cancer. *Cochrane Database Syst Rev*. 2017;6:CD003374. doi:10.1002/14651858.CD003374.pub4.
45. Mateo J, Lord CJ, Serra V, et al. A decade of clinical development of PARP inhibitors in perspective. *Ann Oncol*. 2019;30:1437-1447. doi:10.1093/annonc/mdz192.
46. Tung NM, Boughey JC, Pierce LJ, et al. Management of hereditary breast cancer: American Society of Clinical Oncology, American Society for Radiation Oncology, and Society of Surgical Oncology Guideline. *J Clin Oncol*. 2020;38:2080-2106. doi:10.1200/JCO.20.00299.
47. Somlo G, Frankel PH, Arun BK, et al. Efficacy of the PARP inhibitor veliparib with carboplatin or as a single agent in patients with germline BRCA1- or BRCA2-associated metastatic breast cancer: California Cancer Consortium Trial NCT01149083. *Clin Cancer Res*. 2017;23:4066-4076. doi:10.1158/1078-0432.CCR-16-2714.
48. Ayoub J-P, Friedlander ML, Diéras VC, et al. 140O veliparib plus carboplatin-paclitaxel in patients with HER2-negative advanced/metastatic gBRCA-associated breast cancer: results in hormone receptor-positive and triple-negative breast cancer subgroups from the phase III BROCADE3 trial. *Ann Oncol*. 2020;31:S65. doi:10.1016/j.annonc.2020.03.241.
49. Diéras V, Han HS, Kaufman B, et al. Veliparib with carboplatin and paclitaxel in (BRCA)-mutated advanced breast cancer (BROCADE3): a randomised, double-blind, placebo-controlled, phase 3 trial. *Lancet Oncol*. 2020;21:1269-1282. doi:10.1016/S1473-2045(20)30447-2.
50. Sammons S, Tan TJY, Traina TA, et al. Dora: a randomized phase II multicenter maintenance study of olaparib alone or olaparib in combination with durvalumab in platinum responsive advanced triple-negative breast cancer (aTNBC). *J Clin Oncol*. 2019;37:TPS1113. doi:10.1200/JCO.2019.37.15_suppl.TPS1113.
51. Tutt A, Stephens C, Frewer P, et al. VIOLETTE: a randomized phase II study to assess DNA damage response inhibitors in combination with olaparib (Ola) vs Ola monotherapy in patients (pts) with metastatic, triple-negative breast cancer (TNBC) stratified by alterations in homologous recombination. *J Clin Oncol*. 2018;36:TPS1116. doi:10.1200/JCO.2018.36.15_suppl.TPS1116.
52. Kinova YM, Loirat D, Berger F, et al. Radiopar: a phase I of olaparib with radiation therapy (RT) in patients with inflammatory, locoregionally advanced or metastatic triple-negative breast cancer (TNBC) or patient with operated TNBC with residual disease—preliminary results. *J Clin Oncol*. 2020;38:571. doi:10.1200/JCO.2020.38.15_suppl.571.
53. Denkert C, von Minckwitz G, Darb-Esfahani S, et al. Tumour-infiltrating lymphocytes and prognosis in different subtypes of breast cancer: a pooled analysis of 3771 patients treated with neoadjuvant therapy. *Lancet Oncol*. 2018;19:40-50. doi:10.1016/S1473-2045(17)30904-X.
54. Pennock GK, Chow LQM. The evolving role of immune checkpoint inhibitors in cancer treatment. *Oncologist*. 2015;20:812-822. doi:10.1634/theoncologist.2014-0422.
55. Nanda R, Chow LQM, Dees EC, et al. Pembrolizumab in patients with advanced triple-negative breast cancer: phase Ib keynote-012 study. *J Clin Oncol*. 2016;34:2460-2467. doi:10.1200/JCO.2015.64.8931.
56. Adams S, Schmid P, Rugo HS, et al. Pembrolizumab monotherapy for previously treated metastatic triple-negative breast cancer: cohort A of the phase II KEYNOTE-086 study. *Ann Oncol*. 2019;30:397-404. doi:10.1093/annonc/mdy517.
57. Adams S, Loi S, Toppmeyer D, et al. Pembrolizumab monotherapy for previously untreated, PD-L1-positive, metastatic triple-negative breast cancer: cohort B of the phase II KEYNOTE-086 study. *Ann Oncol*. 2019;30:405-411. doi:10.1093/annonc/mdy518.
58. Cortés J, Lipatov O, Im S-A, et al. KEYNOTE-119: phase III study of pembrolizumab (pembro) versus single-agent chemotherapy (chemo) for metastatic triple-negative breast cancer (mTNBC). *Ann Oncol*. 2019;30:v859-v860. doi:10.1093/annonc/mdx394.010.
59. Schmid P, Adams S, Rugo HS, et al. Atezolizumab and nab-paclitaxel in advanced triple-negative breast cancer. *N Engl J Med*. 2018;379:2108-2121. doi:10.1056/NEJMoa1809615.
60. Cortes J, Cescon DW, Rugo HS, et al. Pembrolizumab plus chemotherapy versus placebo plus chemotherapy for previously untreated locally recurrent inoperable or metastatic triple-negative breast cancer (KEYNOTE-355): a randomised, placebo-controlled, double-blind, phase 3 clinical trial. *Lancet*. 2020;396:1817-1828. doi:10.1016/S0140-6736(20)32531-9.
61. Miles DW, Gligorov J, André F, et al. LBA15 Primary results from IMpassion131, a double-blind placebo-controlled randomised phase III trial of first-line paclitaxel (PAC) ± atezolizumab (atezo) for unresectable locally advanced/metastatic triple-negative breast cancer (mTNBC). *Ann Oncol*. 2020;31:S1147-S1148. doi:10.1016/j.annonc.2020.08.2243.
62. Voorwerk L, Slagter M, Hurlings HM, et al. Immune induction strategies in metastatic triple-negative breast cancer to enhance the sensitivity to PD-1 blockade: the TONIC trial. *Nat Med*. 2019;25:920-928. doi:10.1038/s41591-019-0432-4.
63. Emens LA, Adams S, Barrios CH, et al. LBA16 IMpassion130: final OS analysis from the pivotal phase III study of atezolizumab + nab-paclitaxel vs placebo + nab-paclitaxel in previously untreated locally advanced or metastatic triple-negative breast cancer. *Ann Oncol*. 2020;31:S1148. doi:10.1016/j.annonc.2020.08.2244.
64. Cortes J, Cescon DW, Rugo HS, 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. doi:10.1200/JCO.2020.38.15_suppl.1000.
65. FDA grants accelerated approval to pembrolizumab for locally recurrent unresectable or metastatic triple negative breast cancer. <https://www.fda.gov/drugs/drug-approvals-and-databases/fda-grants-accelerated-approval-pembrolizumab-locally-resectable-or-metastatic-triple-negative-breast-cancer>. Accessed December 31, 2020.
66. Kang C, Syed YY. Atezolizumab (in combination with Nab-Paclitaxel): a review in advanced triple-negative breast cancer. *Drugs*. 2020;80:601-607. doi:10.1007/s40265-020-01295-y.
67. Cortés J, André F, Gonçalves A, et al. IMpassion132 phase III trial: atezolizumab and chemotherapy in early relapsing metastatic triple-negative breast cancer. *Future Oncol*. 2019;15:1951-1961. doi:10.2217/fon-2019-0059.

68. Dent R, Andre F, Gonçalves A, et al. IMpassion132: a double-blind randomized phase 3 trial evaluating chemotherapy (CT) ± atezolizumab (atezo) for early progressing locally advanced/metastatic triple-negative breast cancer (mTNBC). *J Clin Oncol*. 2018;36:TPS1115. doi:10.1200/JCO.2018.36.15_suppl.TPS1115.
69. Miles D, Andre F, Gligorov J, et al. 317TIP-IMpassion131: phase III study comparing 1L atezolizumab with paclitaxel vs placebo with paclitaxel in treatment-naïve patients with inoperable locally advanced or metastatic triple negative breast cancer (mTNBC). *Ann Oncol*. 2017;28:v105. doi:10.1093/annonc/mdx365.080.
70. Salvi S, Bonafè M, Bravaccini S. Androgen receptor in breast cancer: a wolf in sheep's clothing? A lesson from prostate cancer. *Semin Cancer Biol*. 2020;60:132-137. doi:10.1016/j.semcancer.2019.04.002.
71. Gucalp A, Tolancy S, Isakoff SJ, et al. Phase II trial of bicalutamide in patients with androgen receptor-positive, estrogen receptor-negative metastatic breast cancer. *Clin Cancer Res*. 2013;19:5505-5512. doi:10.1158/1078-0432.CCR-12-3327.
72. Bonnefili H, Grellety T, Tredan O, et al. A phase II trial of abiraterone acetate plus prednisone in patients with triple-negative androgen receptor positive locally advanced or metastatic breast cancer (UCBG 12-1). *Ann Oncol*. 2016;27:812-818. doi:10.1093/annonc/mdw067.
73. Traina TA, Miller K, Yardley DA, et al. Enzalutamide for the treatment of androgen receptor-expressing triple-negative breast cancer. *J Clin Oncol*. 2018;36:884-890. doi:10.1200/JCO.2016.71.3495.
74. Kim SB, Dent R, Im SA, et al. Ipatasertib plus paclitaxel versus placebo plus paclitaxel as first-line therapy for metastatic triple-negative breast cancer (LOTUS): a multicentre, randomised, double-blind, placebo-controlled, phase 2 trial. *Lancet Oncol*. 2017;18:1360-1372. doi:10.1016/S1470-2045(17)30450-3.
75. Schmid P, Abraham J, Chan S, et al. Capivasertib plus paclitaxel versus placebo plus paclitaxel as first-line therapy for metastatic triple-negative breast cancer: the PAKT trial. *J Clin Oncol*. 2020;38:423-433. doi:10.1200/JCO.19.00368.
76. Dent R, Kim S-B, Oliveira M, et al. Double-blind placebo-controlled randomized phase III trial evaluating first-line ipatasertib combined with paclitaxel for PIK3CA/AKT1/PTEN-altered locally advanced unresectable or metastatic triple-negative breast cancer (aTNBC): primary results from IPAT. In: *SABCS 2020*. <https://www.abstractsonline.com/pp8/#!/9223/presentation/674>. Updated 2020.
77. Lehmann BD, Abramson VG, Sanders ME, et al. TBCRC 032 IB/II multicenter study: molecular insights to AR antagonist and PI3K inhibitor efficacy in patients with AR+ metastatic triple-negative breast cancer. *Clin Cancer Res*. 2020;26:2111-2123. doi:10.1158/1078-0432.CCR-19-2170.
78. Bardia A, Mayer IA, Vahdat LT, et al. Sacituzumab govitecan-hyzi in refractory metastatic triple-negative breast cancer. *N Engl J Med*. 2019;380:741-751. doi:10.1056/NEJMoa1814213.
79. Bardia A, Tolancy SM, Loirat D, et al. LBA17 ASCENT: a randomized phase III study of sacituzumab govitecan (SG) vs treatment of physician's choice (TPC) in patients (pts) with previously treated metastatic triple-negative breast cancer (mTNBC). *Ann Oncol*. 2020;31:1149-S1150. doi:10.1016/j.annonc.2020.08.2245.
80. Bardia A, Gucalp A, DaCosta N, et al. Phase I study of seviteronel, a selective CYP17 lyase and androgen receptor inhibitor, in women with estrogen receptor-positive or triple-negative breast cancer. *Breast Cancer Res Treat*. 2018;171:111-120. doi:10.1007/s10549-018-4813-z.
81. Gucalp A, Proverbs-Singh TA, Corben A, et al. Phase I/II trial of palbociclib in combination with bicalutamide for the treatment of androgen receptor (AR)+ metastatic breast cancer (MBC). *J Clin Oncol*. 2016;34:TPS1103. doi:10.1200/JCO.2016.34.15_suppl.TPS1103.
82. Kwapisz D. Cyclin-dependent kinase 4/6 inhibitors in breast cancer: palbociclib, ribociclib, and abemaciclib. *Breast Cancer Res Treat*. 2017;166:41-54. doi:10.1007/s10549-017-4385-3.
83. Kobold DC, Fulton RS, McLellan MD, et al. Comprehensive molecular portraits of human breast tumours. *Nature*. 2012;490:61-70. doi:10.1038/nature11412.
84. Millia SZ, Gatalica Z, Winkler J, et al. Predictive biomarker profiling of > 6000 breast cancer patients shows heterogeneity in TNBC, with treatment implications. *Clin Breast Cancer*. 2015;15:473-481e3. doi:10.1016/j.clbc.2015.04.008.
85. Martin M, Chan A, Dirix L, et al. A randomized adaptive phase II/III study of buparlisib, a pan-class I PI3K inhibitor, combined with paclitaxel for the treatment of HER2+ advanced breast cancer (BELLE-4). *Ann Oncol*. 2017;28:313-320. doi:10.1093/annonc/mdw562.
86. Dent R, Kim S-B, Oliveira M, et al. IPATunity130: a pivotal randomized phase III trial evaluating ipatasertib (IPAT) + paclitaxel (PAC) for PIK3CA/AKT1/PTEN-altered advanced triple-negative (TN) or hormone receptor-positive HER2-negative (HR+/HER2-) breast cancer (BC). *J Clin Oncol*. 2018;36:TPS1117. doi:10.1200/JCO.2018.36.15_suppl.TPS1117.
87. Schmid P, Cortes J, Robson ME, et al. A phase III trial of capivasertib and paclitaxel in first-line treatment of patients with metastatic triple-negative breast cancer (CAPhella290). *J Clin Oncol*. 2020;38:TPS1109. doi:10.1200/JCO.2020.38.15_suppl.TPS1109.
88. A study of Ipatasertib in combination with atezolizumab and paclitaxel as a treatment for participants with locally advanced or metastatic triple-negative breast cancer. <https://clinicaltrials.gov/ct2/show/NCT04177108>. Accessed August 5, 2020.
89. Nagayama A, Vidula N, Ellis L, Bardia A. Novel antibody-drug conjugates for triple negative breast cancer. *Ther Adv Med Oncol*. 2020;12. doi:10.1177/1758835920915980.
90. Goldenberg DM, Stein R, Sharkey RM. The emergence of trophoblast cell-surface antigen 2 (TROP-2) as a novel cancer target. 2018;9:2899-2906.
91. Syed YY. Sacituzumab govitecan: first approval. *Drugs*. 2020;80:1019-1025. doi:10.1007/s40265-020-01337-5.
92. Hurvitz S, Tolancy S, Punie K, Bardia A. Biomarker evaluation in the phase 3 ASCENT study of sacituzumab govitecan versus chemotherapy in patients with metastatic triple-negative breast cancer. In: *SABCS 2020*. <https://www.abstractsonline.com/pp8/#!/9223/presentation/674>. Updated 2020.
93. Wahby S, Fashoyin-Aje L, Osgood CL, et al. FDA approval summary: accelerated approval of Sacituzumab govitecan-hyzi for third line treatment of metastatic triple-negative breast cancer (mTNBC). *Clin Cancer Res*. 2020;27:1-5. doi:10.1158/1078-0432.CCR-20-3119.
94. Nakai K, Hung MC, Yamaguchi H. A perspective on anti-EGFR therapies targeting triple-negative breast cancer. *Am J Cancer Res*. 2016;6:1609-1623.
95. Tolancy SM, Ziehr DR, Guo H, et al. A phase II study of cabozantinib for metastatic triple-negative breast cancer (TNBC). *J Clin Oncol*. 2015;33:1080. doi:10.1200/JCO.2015.33.15_suppl.1080.
96. Barroso-Sousa R, Trippa L, Li T, et al. Abstract P3-09-10: a phase II study of nivolumab in combination with cabozantinib for metastatic triple-negative breast cancer (mTNBC). *Cancer Res*. 2020;80:P30910. doi:10.1158/1538-7445.SABCS19-P3-09-10.
97. Brufsky A, Miles D, Zvirbulis Z, et al. Abstract P5-21-01: cobimetinib combined with paclitaxel as first-line treatment for patients with advanced triple-negative breast cancer (COLET study): primary analysis of cohort I. 2018. *Cancer Res*;78:P52101. doi:10.1158/1538-7445.SABCS17-P5-21-01.
98. Brufsky A, Kim S-B, Zvirbulis Z, et al. Phase II COLET study: atezolizumab (A) + cobimetinib (C) + paclitaxel (P)/nab-paclitaxel (nP) as first-line (1L) treatment (tx) for patients (pts) with locally advanced or metastatic triple-negative breast cancer (mTNBC). *J Clin Oncol*. 2019;37:1013. doi:10.1200/JCO.2019.37.15_suppl.1013.
99. Tan AR, Wright GS, Thummala AR, et al. Trilaciclib plus chemotherapy versus chemotherapy alone in patients with metastatic triple-negative breast cancer: a multicentre, randomised, open-label, phase 2 trial. *Lancet Oncol*. 2019;20:1587-1601. doi:10.1016/S1470-2045(19)30616-3.