Universidade de Lisboa
Faculdade de Farmácia

Tumor immune evasion in brain metastases of breast cancer

Carolina Leitão Machado Oliveira

Monografia orientada pela Professora Doutora Helena Isabel Fialho Florindo, Professora Catedrática e coorientado pela Doutora Rita Acúrcio, Investigadora Júnior

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Trabalho Final de Mestrado Integrado em Ciências Farmacêuticas apresentado à Universidade de Lisboa través da Faculdade de Farmácia

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Declaro ter desenvolvido e elaborado o presente trabalho em consonância com o Código de Conduta e de Boas Práticas da Universidade de Lisboa. Mais concretamente, afirmo não ter incorrido em qualquer das variedades de fraude académica, que aqui declaro conhecer, e que atendi à exigida referenciação de frases, extratos, imagens e outras formas de trabalho intelectual, assumindo na íntegra as responsabilidades da autoria.

Resumo

O cancro da mama é a doença maligna mais frequentemente diagnosticada em mulheres em todo o mundo, com o número de casos a aumentar anualmente. Este aumento é atribuído tanto aos avanços na deteção precoce como à crescente prevalência de fatores de risco na população. Os diversos subtipos de cancro da mama – positivo para o Recetor 2 do Fator de Crescimento Epidérmico Humano (HER2+), positivo para o Recetor Hormonal (HR+) e cancro da mama triplo negativo - têm um impacto significativo no desfecho e prognóstico dos pacientes. Foram identificados biomarcadores prognósticos e preditivos para melhorar a compreensão dos resultados clínicos e fornecer informações acerca da resposta à terapêutica.

O microambiente tumoral desempenha um papel crucial na progressão do cancro, com várias células imunológicas e mutações genéticas a contribuir para o desenvolvimento do cancro da mama. Dependendo do estadio e da classificação do tumor, as opções terapêuticas incluem cirurgia, quimioterapia, hormonoterapia e radioterapia. Os doentes com cancro da mama correm risco de metastização, sendo os locais mais comuns cérebro, gânglios linfáticos axilares, osso, pulmão e fígado. As metástases cerebrais ocorrem em 15-25% dos doentes, principalmente naqueles com os subtipos HER2+ e triplo negativo. Estas metástases estão associadas a um prognóstico desfavorável, sintomas neurológicos significativos e uma sobrevivência média de 2 a 25 meses.

O desenvolvimento de metástases cerebrais é um processo complexo em que as células tumorais têm de romper a barreira hematoencefálica, formando a barreira hemato-tumoral, com apenas algumas células a conseguirem sobreviver no microambiente cerebral. As opções de tratamento para as metástases cerebrais dependem do número e localização das lesões, sendo estas limitadas devido à dificuldade de penetração de medicamentos através da barreira hematoencefálica. Os tratamentos disponíveis incluem cirurgia, radioterapia, quimioterapia, hormonoterapia e imunoterapia.

A imunoterapia está a ganhar reconhecimento e tem sido intensamente investigada pelo seu potencial uso, com base no estadio da doença. A investigação atual foca-se nos inibidores do checkpoint, que têm como alvo proteínas-chave no ciclo celular, anticorpos monoclonais que visam o recetor HER2 e na Terapia de Células T com Recetor de Antigénio Quimérico (CAR-T), que reengenharia as células T do paciente para aumentar a sua capacidade de reconhecer e destruir células cancerígenas.

Os doentes com metástases cerebrais de cancro da mama têm sido amplamente excluídos de ensaios clínicos, o que resulta em barreiras significativas nos critérios de

elegibilidade. Este trabalho tem como objetivo explorar as opções terapêuticas mais recentes e promissoras para estes doentes, na esperança de que esses tratamentos se tornem prática comum, melhorando, no mínimo, a qualidade de vida dos mesmos.

Palavras-chave: Cancro da Mama; Metástases Cerebrais do Cancro da Mama; Imunoterapia

Abstract

Breast cancer is the most frequently diagnosed malignancy in women worldwide, with cases rising annually. This increase is attributed to both advancements in early screening and the growing prevalence of risk factors in the population. The various subtypes of breast cancer— Human Epidermal Growth Factor Receptor 2-positive ($HER2^+$), Hormone Receptor-positive (HR^+), and triple negative breast cancer (TNBC)—significantly impact patient outcomes and prognosis. Prognostic and predictive biomarkers have been identified to improve understanding of clinical outcomes and provide insights into therapy responses, respectively.

The tumor microenvironment (TME) plays a crucial role in cancer progression, with various immune cells and genetic mutations contributing to breast cancer development. Depending on the tumor's stage and classification, treatment options include surgery, chemotherapy, hormone therapy, and radiotherapy. Breast cancer patients are at risk for metastasis, with common sites including the brain, axillary lymph nodes, bone, lung, and liver. Brain metastases occur in 15-25% of patients, primarily in those with $HER2^+$ and TNBC subtypes. These metastases are associated with poor prognosis, significant neurological symptoms, and a median survival of 2 to 25 months.

The development of brain metastases is a complex process in which tumor cells must disrupt the blood-brain barrier (BBB), forming the blood-tumor barrier, with only a few cells able to survive in the brain's microenvironment. Treatment options for brain metastases are limited due to difficulties in drug penetration across the BBB and depend on the number and location of lesions. Available treatments include surgery, radiotherapy, chemotherapy, hormone therapy, and immunotherapy.

Immunotherapy is gaining recognition and has been investigated intensively for its potential use based on disease status. Current research focuses on immune checkpoint inhibitors, which target key checkpoint proteins in the cell cycle, monoclonal antibodies that target the HER2 receptor and Chimeric Antigen T-Cell Therapy, which re-engineers the patient's T cells to enhance their ability to recognize and destroy cancer cells.

Patients with breast cancer brain metastasis have largely been excluded from clinical trials, leading to significant barriers in trial eligibility criteria. This study aims to explore the latest and most promising therapeutic options for these patients, with the hope that these treatments will become standard practice to improve, at the very least, their quality of life.

Keywords: Breast Cancer; Breast Cancer Brain Metastasis; Immunotherapy

Abbreviatures

- AI – Aromatase Inhibitors
- APC – Antigen-presenting Cell
- ASC – Adipose-derived stem cells
- BBB – Brain Blood Barrier
- BC – Breast Cancer
- BBM – Breast cancer Brain metastases
- BCS – Breast-Conserving Surgery
- BTB – Brain Tumor Barrier
- CAR-T cell – Chimeric Antigen Receptor T cell
- CARs – Chimeric Antigen Receptors
- CDK – Cyclin-Dependent Kinase
- CNS – Central Nervous System
- CSF – Cerebrospinal Fluid
- CTLA-4 – Cytotoxic T-lymphocyte-Associated Antigen 4
- ER – Estrogen Receptor
- ESR1 – Estrogen Receptor 1
- FDA – Food and Drug Administration
- HDI – Human Development Index
- HER2 – Human Epidermal Growth Factor Receptor 2
- HRT – Hormone-Replacement Therapy
- IARC – International Agency for Research on Cancer
- IBC – Inflammatory Breast Cancer
- ICD – Immune Cell Death
- mAbs – Monoclonal Antibodies
- MBC – Metastatic Breast Cancer
- MHC – Major Histocompatibility Complex
- MRI – Magnetic Resonance Imaging
- MSC – Mesenchymal Stem Cell
- OS – Overall Survival
- PD-1 – Programmed cell-death protein 1
- PD-L1 – programmed death-ligand 1
- PR – Progesterone Receptor
- QoL – Quality of Life
- SLNB – Sentinel Lymph Node Biopsy
- SRS – Stereotactic Radiosurgery
- TIL – Tumor-Infiltrating Lymphocyte
- TME – Tumor Microenvironment
- TNBC – Triple Negative Breast Cancer
- WBRT – Whole-Brain Radiation Therapy

Índice

Introduction

Breast cancer is the most commonly diagnosed cancer among women worldwide. It is estimated to be the fourth cause of cancer deaths in the world, with approximately 2.3 million new cases and 666.000 deaths in 2022. According to the International Agency for Research on Cancer (IARC), breast cancer is the most common type of cancer in 158 countries and the leading cause of death in 111 countries. (1) It is expected to rise from 2.1 million new cases registered annually in 2018 to 4.4 million new cases diagnosed by 2070. (2) Although it is a public health challenge worldwide, its incidence, mortality, and survival rates vary considerably among different parts of the globe. (3) It is known that women living in transitioned countries have significantly higher incidence rates when compared with those in transitioning countries but in contrast, lower mortality rates are registered. (1) Despite all the intrinsic population factors, such as its structure, environment, and genetic factors, there are several risk factors associated with lifestyle including physical inactivity, obesity, and alcohol consumption (4), which are increasing and therefore leading to a higher prevalence of breast cancer. In addition, hereditary factors such as mutations in autosomal dominant genes represent between 5–10% of all breast cancer cases in women.

Breast cancer has a significantly high survival rate when compared to other types of cancer and is often treatable when diagnosed at an early stage. Following the latest developments in multimodal therapy, the chances of a patient surviving after treatment is between 70 to 80%. In addition, if detected early, the survival rate increases to >90%. The disease has several sub-types and stages (**Figure 1, 2**), influencing the treatment and the outcome.

According to breast cancer subtypes, breast cancer is managed with preoperative and postoperative systemic therapies, which include chemotherapy, hormone therapy, immunotherapy with monoclonal antibodies (mAbs) targeting tumor receptors, as well as surgery and radiation. (5) At later stages, the treatments aim to minimize symptoms, extend life, and maintain quality of life (QoL). Metastatic breast cancer (MBC) represents the major cause of death in breast cancer. The most common metastatic sites include the brain, bone, lung, liver, and axillary lymph nodes. (6) Data shows that 15-25% of breast cancer patients can develop brain metastases. (7) Brain metastases treatment constitutes a major therapeutic challenge since many drugs cannot cross the blood-brain barrier. A limited number of therapies are available with poor clinical outcomes. Nevertheless, several research efforts have been made to develop new targeted and effective therapeutics which will be discussed in this report. (8)

Breast Cancer

Staging and Classification

Breast cancer staging is assessed based on tumor size, lymph node involvement, the presence of metastases, and specific biomarkers. To determine the tumor stage, it is used the TNM classification by the American Joint Committee on Cancer (AJCC), with T, standing for the extent of the tumor, N for the extent of spread to lymph nodes and M for the presence of metastasis. It helps determine how aggressive the cancer is. There are 4 stages of the disease (**Figure 1**), with stage 0 corresponding to ductal carcinoma *in situ* (DCIS), noninvasive breast cancer, and stage IV to MBC. On a histological level, breast cancer can be categorized into two main subtypes: *in-situ* carcinoma and invasive carcinoma. The *in-situ* subtype is less common than the invasive subtype. Over 80% of invasive breast cancers are invasive ductal carcinomas (IDCs), while the remaining cases are invasive lobular carcinomas (ILCs).

ANATOMIC STAGE/PROGNOSTIC GROUPS			
Stage 0	Tis	N0	M0
Stage IA	T1*	N0	M0
Stage IB	T0	N1mi	M0
	T1*	N1mi	M0
Stage IIA	T0	N1**	M0
	T1*	N1**	M0
	T2	N0	M0
Stage IIB	T2	N1	M0
	T3	N0	M0
Stage IIIA	T0	N2	M0
	T1*	N2	M0
	T2	N2	M0
	T3	N1	M0
	T3	N2	M0
Stage IIIB	T4	N0	M0
	T4	N1	M0
	T4	N2	M0
Stage IIIC	Any T	N3	M0
Stage IV	Any T	Any N	M1

Figure 1 – Staging of breast cancer according to the TNM classification (9)

Based on specific breast cancer biomarkers (estrogen receptors (ER), progesterone receptors (PR), and the HER2 receptors, breast cancer can be divided into 3 groups: breast cancer-expressing hormone receptor (HR), breast cancer-expressing HER2 and triple-negative breast cancer (TNBC), when none of the previous receptors are expressed (ER^-, PR^-, $HER2^-$).

Stage II tumors are more likely to be of the $ER^+/PR^+/HER2^-$ and $ER^+/PR^+/HER2^+$ subtypes and stage IV tumors are 47% more likely to be $ER^+/PR^+/HER2^+$ than stage I tumors. Higher-stage tumors significantly decreased the odds of being of the TN subtype(10)

A study evaluated the 5-year relative survival in patients with breast cancer. The study included 8 combinations of ER/PR/HER2 tumors (11) Whilst it is known that women with breast cancer positive for both hormone receptors (PR^+/ER^+) have the best survival rate(12) and $HER2^+$ tumors are associated with higher risk of cancer recurrence and cancer-related death (13), in this study it was shown that women with $ER^+/PR^+/HER2^+$ tumors have the best 5-year survival (**Table 1**) when compared to the other subtypes. The worst survival rates were encountered in all ER- tumors.

Table 1 - Frequency Distribution and 5-Year Relative Survival of the ER/PR/HER2 Subtypes. Source: Breast Cancer Subtypes as Defined by the Estrogen Receptor (ER), Progesterone Receptor (PR), and the Human Epidermal Growth Factor Receptor 2 (HER2) among Women with Invasive Breast Cancer in California, 1999–2004 (10)

ER/PR/HER2	*n* (%)	5-Year relative survival (%)	95% CI
ER+/PR+/HER2−	32,624 (53.2)	96.4	(95.8, 97.0)
ER+/PR−/HER2−	6,081 (9.9)	91.9	(90.1, 93.7)
ER+/PR+/HER2+	6,979 (11.4)	91.3	(89.9, 92.7)
ER+/PR−/HER2+	2,157 (3.5)	88.0	(85.3, 92.7)
ER−/PR+/HER2−	661 (1.1)	82.7	(77.8, 87.6)
ER−/PR+/HER2+	349 (0.6)	78.8	(71.9, 85.7)
ER−/PR−/HER2−	8,022 (13.1)	76.2	(74.4, 78.0)
ER−/PR−/HER2+	4,436 (7.2)	75.9	(73.6, 78.3)
Total	61,309		

CI, confidence interval; ER, estrogen receptor; HER2, human epidermal growth factor receptor 2; PR, progesterone receptor.

HR+

Breast cancer-expressing hormone receptors, PR^+ or ER^+ are the most common breast cancer type. (14) The ER is a crucial diagnostic marker, with approximately 70–75% of invasive breast carcinomas exhibiting high ER expression. (15) This type of tumor is well-differentiated and is related to a better outcome after surgery as it is less aggressive. (16)

PR^+ tumors comprise 65-75% of all breast cancers. (17) The PR is expressed in over 80% of ER+ breast cancer patients (18), and is rarely found in ER^- cases, since PR expression is regulated by the ER. (19) PR+ tumors also show high response rates to hormone therapy, and

patients usually have better clinical outcomes. Therefore, the measurement of ER and PR is fundamental in both newly diagnosed breast cancer cases and metastatic lesions. (20)

HR^+ tumors can be further divided into two subcategories – luminal A (ER^+ and/or PR^+, $HER2^-$, and Ki67 ^{low}) and luminal B (ER^+ and/or PR^+, $HER2^-$, and Ki67 ^{high}). (6) Luminal A tumors correspond to 40% of all cancers. It tends to be grade I or II and typically has the best prognosis with relatively high survival rates and low recurrence rates of breast cancer. (21) Luminal B tumors are often diagnosed in younger women(22) and comprise between 15-20% of all breast cancer cases. This type of tumor tends to have a worse prognosis as it is more likely to have larger size, lymph nodes involved, and poorer tumor grade. Luminal A tumors present higher survival rates than luminal B. (23) However, luminal B shows a better response to neoadjuvant chemotherapy. (24)

Double-positive tumors (ER^+PR^+) represent 55-65% of all breast cancers. (25) When compared with other subtypes, this type of tumor tends to be diagnosed in older women and usually are smaller in size and lower in grade. Consequently, they show lower mortality rates. In contrast, double negative tumors (ER^-PR^-), which comprehend 18-25% of the tumors(26) are associated with a worse prognosis and do not fully respond to hormone therapy. (27)

HER2-Enriched

On average, 13-20% of all breast cancers overexpress HER2, encoded by the ERBB2 gene. (28) ERBB2 amplification is associated with a poor prognosis outcome due to the high metastatic potential of $HER2^+$ tumors (Biomarkers in Breast Cancer). The majority of this tumor subtype are ER^-PR^- and are poorly differentiated and associated with a more aggressive behavior and a worse outcome. (16,29) Those who are $ER^+PR^+HER2^+$ (triple positive) may benefit from hormonal therapy as they are positive for both progesterone and estrogen receptors. (30) A combination of anti-HER2 therapy and chemotherapy could also be an option. (31)

Triple Negative Breast Cancer (TNBC)

TNBC is characterized by the absence of ER, PR, and HER2 gene (32) comprising nearly 20% of all breast cancers (33) Additionally, TNBC can be further classified into 6 subcategories - basal-like (BL1 and BL2), claudin-low, mesenchymal (MES), luminal

androgen receptor (LAR), and immunomodulatory (IM). Among these, the basal-like are the most common type of tumors. (32)

TNBC is known for its aggressiveness, early relapse, and likely greater likelihood of being diagnosed at advanced stages. It is, histologically, poorly differentiated and has a high proliferative rate. These tumors are more prone to be diagnosed in younger age women, tend to be bigger, have higher grades, and higher probability of lymph node involvement. (34) In addition, they present a higher probability of recurrence within the first three years after diagnosis, as well as of develop brain metastases. (32) Therefore, compared to the ER^+ subtypes, the TNBC presents a lower survival rate.

Epidemiology

Incidence

Breast cancer is the most prevalent cancer in women worldwide. In 2022, it was estimated 2.3 million new cases all over the globe, comprising 11,6% of all cancer cases. Breast cancer was the most diagnosed cancer in 158 countries (**Figure 2**), according to IARC's Global Cancer Observatory. (1) It is estimated that in 4 cases of cancer, 1 is breast cancer.

The highest incidence rates were seen in France, Australia, New Zealand, Northern America, and Northern Europe. (1) Compared with South-Central Asia and Middle Africa, the rates in the regions previously mentioned, were 4 times higher. These data demonstrate that developed countries have a higher cancer incidence rate when compared to less developed countries (**Figure 3**). This may be related to a higher prevalence of reproductive risk factors, such as early age at menarche, later age at menopause, fewer children, less breastfeeding, hormone-replacement therapy, and oral contraceptives. (35)

In the past few years, developed countries have been increasing levels of sedentary lifestyle, obesity and alcohol consumption, which can also be related to higher cancer incidence rates. Despite having high incidence rates, developed countries show a better survival rate when compared to those less developed (36), due to great improvement in early detection by screening and increased breast cancer awareness in the past few decades.

In terms of prevention, incidence rates would tend to decrease if risk factors were modified. However, as there are few established modifiable risk factors, the World Health Organization is focusing on early diagnosis by recommending organized, population-based mammography screening every 2 years for women between 40 and 54 years at average risk for breast cancer. (37) In countries with fewer opportunities and where breast cancer is diagnosed

at a late stage and mammography screening is not cost-effective, the focus is on treating women with symptomatic lesions. (1)

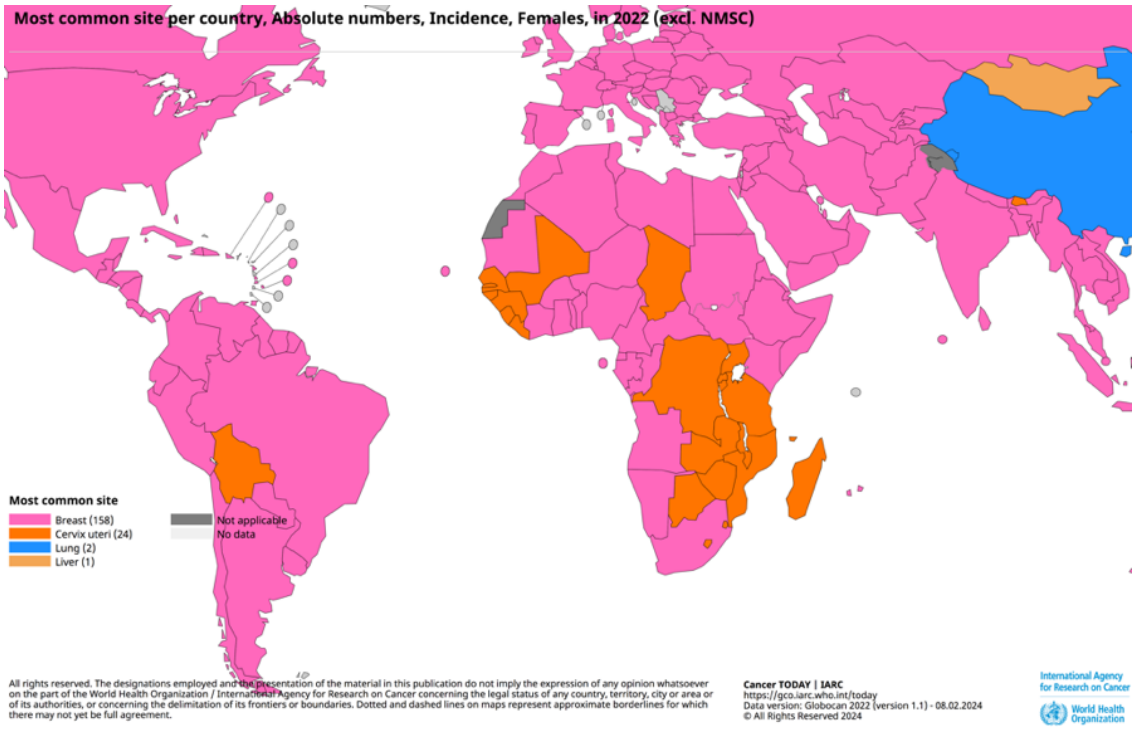

Figure 2 - Global maps present the most common type of cancer incidence by country in 2022 among women. The numbers of countries represented in each ranking group are included in the legend. (1)

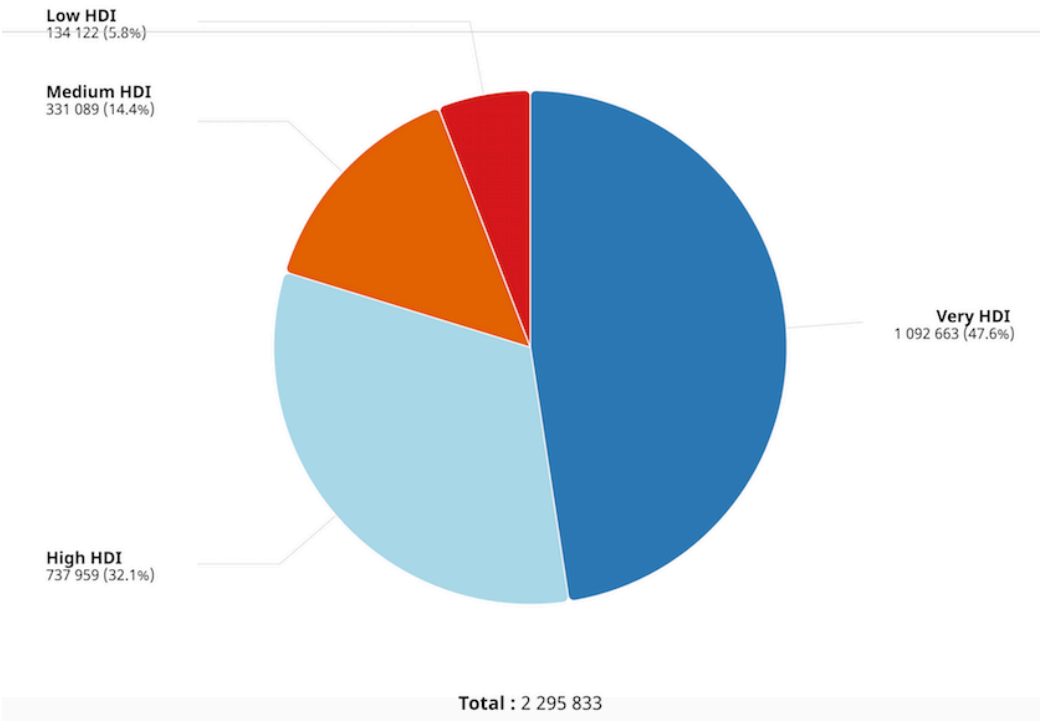

Figure 3 - Incidence of breast cancer in both sexes in 2022 associated with HDI levels all over the globe. HDI – human development index. (1)

Mortality

Breast cancer is yet the fourth cause of cancer deaths. In 2022, it was estimated that 666.000 women died from breast cancer, corresponding to 6.9% of all cancer deaths. It was the leading cause of cancer deaths in 111 countries (**Figure 4**). (1) Mortality rates are not uniform worldwide and are influenced by socioeconomic factors (**Figure 5**). The IARC GLOBOCAN database within the Global Cancer Observatory estimates that in countries with a high human development index (HDI), 1 in 71 women will die from breast. While in low HDI countries, the mortality rate is around 1 in 48 women.

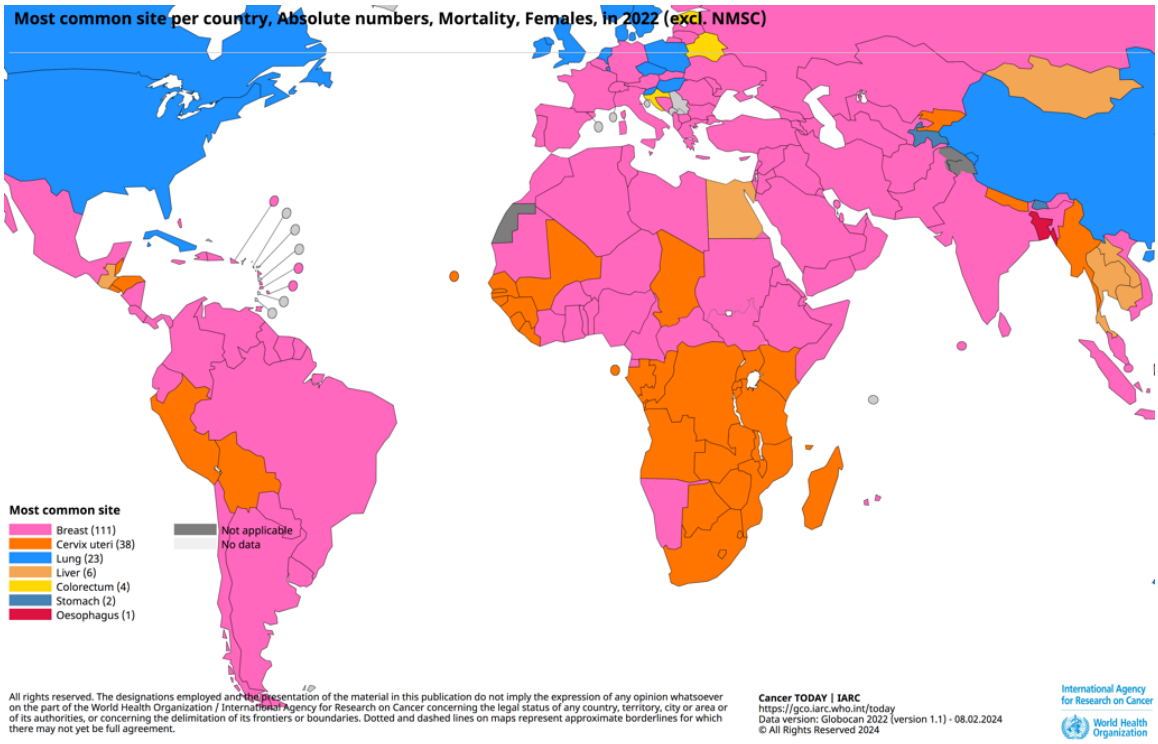

Figure 4 - Global maps present the most common type of cancer mortality by country in 2022 among women. The numbers of countries represented in each ranking group are included in the legend. (1)

In high-income countries, where breast cancer incidence rates are higher, mortality rates decreased in the early 1990s. This was due to huge efforts in early detection by screening and several treatment breakthroughs. European countries considerably reduced the mortality rates over the last three decades, and a greater reduction was observed in Northern Europe. Poland and Romania are examples of countries that do not follow the trend. In fact, Central and Eastern countries tend to have higher mortality rates since many political, economic, and social changes have influenced their perception of public health. In addition, these countries have limited access to the latest pharmacological treatments. In these countries, the average

time from breast cancer diagnosis to the start of treatment goes from 10 to 38 weeks in extreme cases. The average European time is 8 weeks.

Apart from Europe, mortality rates show great variation in different regions which appear to be linked to the economic level of the country. Sub-Saharan African countries are among the ones with the most noteworthy breast cancer mortality around the world.

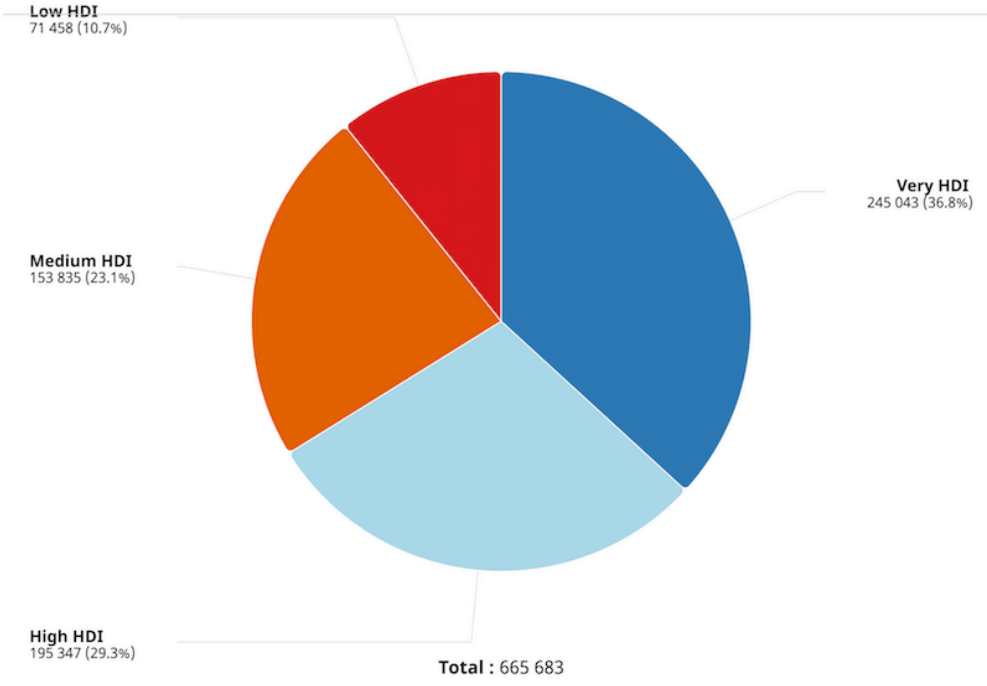

Figure 5 – Mortality rates of breast cancer in both sexes in 2022 associated with human development index (HDI) levels all over the globe. (1)

Risk Factors

Breast cancer is a multifactorial disease with many contributing factors. Its prevalence is increasing every day worldwide. Among factors, the population's lifestyle, including alcohol consumption, exposure to tobacco, physical inactivity, and high-fat diet contributes to breast cancer development. In 2022, the World Health Organization estimated that 46% of adults were overweight and 16% were obese. (38) According to many studies, it is established that the high levels of insulin and insulin-like growth factors in response to obesity, along with increased estrogen levels, have been found to stimulate the development and growth of cancer cells in both premenopausal and postmenopausal women. (39)

Gender is another risk factor, as females present an increased chance of developing breast cancer. Breast cells are particularly susceptible to hormones, such as estrogen and progesterone. High levels of circulating estrogen and androgen have also been associated with a higher risk of breast cancer in older age. Since premenopausal and postmenopausal women

face significant alterations in sex hormone levels, this stage of their lives represents a higher probability of developing breast cancer. (19)

Breast cancer in men only represents 1% of all cases. (40) When this occurs, it is usually in older or in those who face a hormonal imbalance or are exposed to radiation.

Additional hormonal factors, such as the excessive use of hormone replacement therapy (HRT) and oral contraceptives have also been considered risk factors. A heightened or prolonged exposure to estrogen by HRT or oral contraceptives is one of the causes of susceptibility to breast cancer. (40) Studies showed that breast cancer risk decreases in women who stop using oral contraceptives for 5-10 years. (41) For women who stop HRT for more than 2 years, the risk diminishes significantly. (42) In addition, breast density is also considered an independent risk factor for breast cancer. (43)

Women with a family history of breast cancer are more prone to develop it. There are gene mutations that contribute even more to the development of the disease, including the BRCA gene mutation. Mutations in the BRCA1 and BRCA2 genes are inherited through the dominant autosomal method and approximately 40% of hereditary breast cancer are due to this. For women with a family history of two or more cases of breast cancer and without BRCA mutations, they are 11 times more likely to develop it. Those who have cases of ovarian cancer with BRCA gene mutation in the family may also have a higher risk of breast carcinogenesis. (44) Nevertheless, hereditary breast cancer represents 5 to 10% of all cases. (45)

Biomarkers in Breast Cancer

Biomarkers can be categorized into two groups: prognostic and predictive. Prognostic biomarkers provide information regarding the overall cancer outcome for a patient, whereas predictive biomarkers offer insights into the likely effects of a therapeutic intervention and can be used as a target for therapy. (46)

Predictive Breast Cancer Biomarkers

Molecular

Regarding the predictive biomarkers, 4 main ones should always be considered which are: ER, PR, HER2, and Ki67. These biomarkers are used to predict a patient outcome and to select the best therapeutic strategy as well as to predict the tumor's possible resistance to treatments. (14,47)

For every breast cancer diagnosis, it is mandatory to evaluate the expression of ER, PR, and HER2. ER and PR expression will determine which patients can undergo hormone therapy,

while HER2 expression indicates those who are more likely to benefit from anti-HER2 therapy. (48) ER expression is a favorable prognosis when compared to ER-negative cancers. Generally, ER^- tumors have high resistance to hormone therapy. (49) When assessing both hormone therapy and HER2, tumors that are $ER^+PR^+HER2^-$ have the best patient outcome and the best response to hormone therapy. $ER^-PR^-HER2^+$ and TNBC tumors are the ones that show the worst prognosis, with aggressive behavior and a bad response to hormone therapy. (16)

Ki67 is another biomarker used in breast cancer. (50) It is used as a proliferation marker and predicts neoadjuvant therapy response or the outcome of adjuvant chemotherapy and hormone therapy for ER^+ tumors. (51,52) The luminal A and luminal B breast cancers are characterized by the absence or presence of Ki67, respectively. The luminal B has a poorer outcome in terms of response to systemic therapy (52) as it is more proliferative. (53)

Gene-related

<u>BRCA 1/2</u>

BRCA1 (located on the 17th number chromosome) and BRCA2 (located on the 13th number chromosome) are the most mutated genes in breast cancer. Women who have this mutation are 70% more prone to develop this disease in their lifetime when compared to those who do not have it. (54) These genes are involved in DNA repairing and transcriptional regulation in response to DNA damage (24), which is why they are considered tumor suppressor genes. (55) The protein encoded by the BRCA gene is involved in repairing double-strand DNA breaks through homologous recombination and so, mutations in these genes lead to high sensitivity to DNA-damaging drugs. (56)

Mutations in these genes are inherited in an autosomal dominant manner and sporadic mutations have already been reported. (57) BRCA genes are not only related to breast cancer but also to ovarian cancer. (58) Patients with these mutations undergo annual screening with magnetic resonance imaging (MRI) and mammography (59) as a prevention measure against breast cancer. Despite all these possible ways of detecting breast cancer at an early stage, bilateral prophylactic mastectomy remains the gold standard of prevention as BRCA carriers have a high risk of developing contralateral breast cancer. (60)

BRCA1-associated breast cancer tends to have a more aggressive behavior, and it is usually HR^-. (61) On the other hand, BRCA2-associated breast cancer is predominantly HR^+. (62)A study comparing the survival rates between BRCA2 carriers and non-carriers, concluded that BRCA2 carriers who were ER^+ had poorer survival rates than those with ER^- tumors. (63,64)

HER2

ERBB2 encodes the human epidermal growth factor receptor 2 (HER2). This is a protooncogene located in chromosome 17, encoding the human epidermal growth factor receptor family (HER1/2/3/4) with tyrosine kinase activity. (65) HER2 signaling promotes proliferation, cell survival, metastasis, and adhesion through multiple pathways, such as the Ras pathway which is involved in cell proliferation and survival. (33)

HER2 overexpression is present in 15-20% of all breast cancers and is associated with a poor outcome. $HER2^+$ tumors are linked to a worse prognosis since they have a high chance of recurrence and mortality. (66) The assessment of HER2 status by immunohistochemistry (IHC) is also a predictive biomarker for the selection of a precise treatment for breast cancer, as HER2-targeting agents are only effective in HER2-overexpressed tumors. (65) This status measurement is required for cases of invasive breast cancer and is recommended for cases of metastasis and recurrence. (20)

EGFR

For the last decades, research in molecular oncology focused on discovering of new targeted therapies. Epidermal growth factor receptor (EGFR) was one of the first identified targets promoting tumor survival. (67) The EGFR is encoded by the ERBB1 gene, also known as HER1. When activated, the ligands promote cell proliferation and protect them against apoptosis. Additionally, it promotes invasion and angiogenesis, resulting in the overexpression of EGFR, which promotes tumor growth. (68) Breast cancer overexpressing EGFR is detected in all breast cancer subtypes but is more frequently seen in TNBC and inflammatory breast cancer (IBC). Over 50% of TNBC overexpresses EGFR and, therefore, this type of tumor benefits from anti-EGFR therapy with EGFR inhibitors. (34) Approximately 30% of IBCs are associated with EGFR overexpression. IBC, in which cancer cells obstruct lymph capillaries in the skin, is the most aggressive type of breast cancer, and this overexpression is correlated to a worse prognosis, worse survival rate, and an increased risk of recurrence when compared to EGFR-negative tumors. (69) As chemotherapeutic agents are not sufficient for the treatment of IBC, targeted therapies, namely, agents targeting EGFR pathways have been fundamental to patients. (70)

c-Myc

c-Myc is a transcription factor encoded by the MYC gene, located in chromosome 8, which is amplified in several types of cancer. (71) It plays a role in cell growth, proliferation, metabolism, differentiation, and apoptosis. c-Myc regulates up to 15% of cancers and it is a potent activator of tumorigenesis. (71,72) MYC is involved in cell cycle progression and cell immortalization as telomerase, the one responsible for maintaining telomere length, is its direct target. A study demonstrated that MYC is essential for angiogenesis, and it has an important role when it comes to angiogenic switch in tumors. (73) The MYC gene overexpression has been reported in breast cancer and it has been associated with the most aggressive subtypes. (74)MYC and BRCA1 are both contributing to tumorigenesis, and it has been shown that BRCA1 is one of the genes activated by MYC. (75)

RAS

RAS genes were the first identified mutated genes in human cancer. (76) RAS oncogenes are a family of genes that code for proteins located on the inner surface of the cell membrane, known as RAS G-proteins, which mediate signal transduction through transmembrane receptors. (77) RAS mutations are present in only 5% of breast cancer patients. They are 4 RRAS isoforms: HRAS, NRAS, and KRAS. Among these, KRAS is the most frequently mutated in breast cancer, and it is associated with a poor prognosis. (78)

The KRAS gene encodes GTPases that, when activated by upstream signals (e.g., HR and cytokines), play a crucial role in controlling several pathways and cellular processes, including cell proliferation and growth, apoptosis, differentiation, cytoskeletal rearrangements, motility, and adhesion. (79) Mutations in this gene will originate dysfunctional GTPases that will lead to the activation of pathways that are related to tumorigenesis, including MAPK, PI3K, and RAF signaling pathways. Despite the lack of knowledge on the role of KRAS mutations in breast cancer progression, it is well known that the PIK3/AKT/PTEN pathway controlled by KRAS activity increases breast tumor growth.

NRAS and HRAS were found to be overexpressed in basal-like and HER2-positive breast cancer subtypes (80), while KRAS is more commonly associated with TNBC. (81)
HER2, as well as EGFR, are connected to RAS signaling by interacting with a RAS GDP-GTP exchange factor. Consequently, the overexpression of these receptors activates RAS signaling pathways, leading to tumorigenesis. (82)

Additionally, RAS is also associated with therapy resistance. Several efforts have been made to develop therapeutic approaches targeting RAS itself or its downstream signaling pathways to block these pathways and overcome resistance. (83)

TP53

p53 is a tumor suppressor protein that is encoded by the TP53 gene, which is a major transcription factor involved in multiple cellular processes. (84) When under genotoxic stress, p53 is activated and promotes DNA repair, cell cycle arrest, and apoptosis. (85) p53 is tightly regulated by MDM2, an E3 ligase that promotes its degradation via the ubiquitin-proteasome-dependent pathway (**Figure 6**). When mutated or inactivated by the overexpression of MDM2, by negative feedback, p53 is directly linked to tumorigenesis.

Mutations in this gene are found in 30-35% of primary invasive cases of breast cancer, but their occurrence is subtype-dependent. These mutations are most prevalent in HR^- tumors, while HR^+ and luminal subtypes typically retain the wild-type (*wt*) p53. Mutations in p53 are most common in TNBC (80%) and $HER2^+$ (70%) subtypes. (86)

Due to its high incidence in breast cancer, p53 is an important biomarker in clinical practice and a therapeutic target. Recent studies have identified compounds that can restore *wt* p53 properties, offering new anticancer treatment options.

The majority of TP53 mutations are missense, involving a single amino acid substitution in the p53 protein, which results in the loss of its DNA binding ability. Several compounds, including quinuclidines, pyrazoles, 2-sulfonylpyrimidines, and thiosemicarbazones, have been developed to restore mutant p53 to functions similar to the wild-type p53. Quinuclidine-based prodrugs are metabolized in vivo into the active compound 2-methylene-3-quinuclidinone (MQ), a nucleophile acceptor that modifies thiol groups via Michael addition. MQ targets exposed cysteine residues in the core domain of mutant p53, correcting its folding and restoring DNA binding. Similarly, the 2-sulfonylpyrimidine compound PK11007 selectively alkylates cysteine residues, enhancing the thermal stability of mutant p53's DNA binding domain, particularly in Y220C and V143A mutations. Thiosemicarbazones, another class of compounds, act as zinc chelators, promoting zinc binding to mutant p53, which is crucial for restoring its DNA binding function in cases where zinc-coordinating residues are mutated. (85)

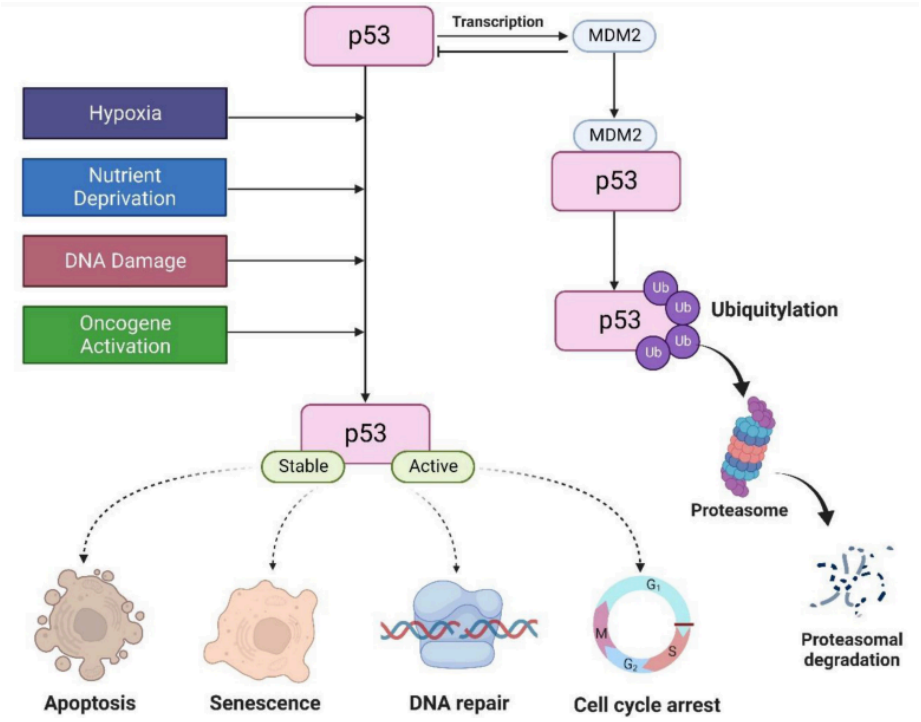

Figure 6 – p53 regulation. MDM2 regulates the p53 protein under normal conditions. Genotoxic stress such as hypoxia, activation of oncogenes, DNA damage, and nutrient deprivation, p53 is activated to regulate cell cycle, apoptosis, DNA repair, and senescence. (85)

Prognostic Breast Cancer Biomarkers

The most used prognostic biomarkers in breast cancer are tumor grade, tumor size, and presence of lymph node metastasis. (87) The presence and quantity of lymph node metastases (LNM) have been, and continue to be, the most significant prognostic factor in breast cancer (88). Breast cancer LNM can be categorized as follows: N0 (no cancer cells are present in nearby lymph nodes); N1 (cancer has spread to 1–3 axillary lymph nodes, or a small number of cancer cells have been detected in lymph nodes near the breastbone during a sentinel lymph node biopsy (SLNB); N2 (cancer has spread to 4–9 axillary lymph nodes, or the internal mammary lymph nodes are enlarged); and N3 (cancer has spread to 10 or more axillary lymph nodes, with at least one node larger than 2 mm, or cancer is found in lymph nodes under the clavicle, with at least one node larger than 2 mm). (18) Patients with four or more involved lymph nodes will have a worse prognosis than those with fewer affected. Despite not being fully correlated, it is possible to predict lymph node involvement by the tumor size. (89)

Accurate tumor sizing is crucial for guiding treatment options. Imaging exams such as MRI and mammography are used to determine tumor size. (18) Tumor size is also critical in evaluating the prognosis of breast cancer, as the likelihood of metastasis increases with larger

tumor size, and there is a positive correlation between tumor size and the number of involved lymph nodes. (89,90)

The histological tumor grade provides important information related to the clinical behavior of breast cancer. (91) According to the Nottingham Grading System, there are three histological grades in BC: well-differentiated (grade I); moderately differentiated (grade II); and poorly differentiated/most aggressive (grade III). (18) Higher-grade tumors are linked to reduced survival rates. (92) Usually, half of breast cancers are grade I or III. Grade II is associated with an intermediate risk and is usually divided into 2 groups – one more likely to grade I and another more similar to grade III. (93)

Available treatments

Nowadays, there are several therapeutic options to treat breast cancer selected according to its subtype and stage. Among therapies, the classical approaches include surgery, chemotherapy, hormone therapy, and radiotherapy. (94)

For surgery, there are two possible approaches, mastectomy and breast-conserving surgery (BCS). Mastectomy has been the most used treatment, where the whole breast is removed, including the nipple, areola, fascia of the pectoralis major muscle, and skin. Typically, this procedure is accompanied by a SLNB, where selected lymph nodes are excised. These are the nodes where the cancer is more likely to spread. In cases where the tumor has metastasized to the lymph nodes, an axillary lymph node dissection (ALND) is warranted to remove additional lymph nodes. To prevent high-risk women, such as those with a BRCA gene mutation, a double mastectomy can also be performed.

Another approach is the BCS. This procedure excises the tumor while preserving as much of the breast tissue as possible. Women undergoing BCS typically require adjuvant radiotherapy and, in some cases, hormone therapy and chemotherapy. BCS is suitable for women with early-stage cancers, particularly when the tumor is smaller than 5 cm and relatively small in proportion to the overall breast size. Women who are unable to go through radiation treatment cannot be treated with BCS and must go through mastectomy.

It is important to understand that choosing BCS and adjuvant radiation over mastectomy, or vice versa, does not affect overall survival (OS) outcomes. Mastectomy may reduce the risk of a second breast cancer in the same breast but does not decrease the likelihood of cancer recurrence in other parts of the body or the contralateral breast.

Chemotherapy has been used for decades and continues to have good outcomes. (94) Chemotherapeutic agents interfere with DNA replication and, therefore, help suppress tumor growth and, at the same time, promote an immune response against the tumor. One of the main mechanisms of chemotherapeutic agents is immune cell death (ICD), a form of cell death that activates immune responses. (14) However, the non-specific targeting of chemotherapeutic agents results in many adverse effects, such as hair loss and diarrhea. (94)

Radiotherapy is usually used in combination with surgery or chemotherapy and has a particular target, the tumor, leaving the adjacent tissue intact (5) There are two types of treatment - external beam radiation therapy (EBRT) and brachytherapy, an internal radiation. For women who had breast-conserving surgery, usually, EBRT is necessary for the entire breast, called whole breast radiation. Brachytherapy can also be used as a form of accelerated partial breast irradiation. (95) When used as an adjuvant therapy, it is used after surgery to eradicate the eventual remaining disease in the surroundings of the excision. Postmastectomy radiotherapy significantly reduces mortality rates in women with positive lymph nodes but not in those with negative nodes.

Hormone therapy is used for tumors that are HR-positive. Certain types of breast cancer are influenced by hormones such as estrogen and progesterone. The cancer cells possess receptors that bind to these hormones, promoting tumor growth.

There are several types of hormone therapy, and they can be either used as adjuvant or neoadjuvant therapy. Most types of hormone therapy either reduce estrogen levels in the body or inhibit estrogen from promoting the growth of breast cancer cells. It is recommended to use hormone therapy for 5 years.

In addition, modern approaches, including, targeted- and immuno-therapies (e.g., immune checkpoint inhibitors) have also been demonstrated to be effective. (96) Immunotherapy is a treatment modality that enhances the body's immune response against cancer. Immunotherapy modalities include mAbs, cytokines, or cancer vaccines. This therapeutic strategy has undergone significant advancements over the past decade and will be further discussed in subsequent sections.

Despite the progress in breast cancer treatments, it is often necessary to use a combination of multiple therapies to maximize the remission chance. In addition, late-stage tumors have been demonstrated to be refractory to most of the therapeutic strategies herein highlighted.

Breast Cancer Microenvironment-related Mechanisms

While breast cancer initiation is primarily driven by genetic alterations, the tumor microenvironment (TME) plays a crucial role in tumor evasion and progression. Breast tissue is predominantly composed of mammary adipose tissue, making it the primary microenvironment for breast cancer. (97)

Cancer cells can alter the surrounding adipose tissue by activating genes related to tumor growth, stemness, and progression. This change leads to the release of bioactive factors such as growth factors (HGF and IGF1), cytokines (ITNF-α and IL-12), chemokines (CCL5), and components of the extracellular matrix (ECM) (collagen I and IV) by both adipocytes and stromal cells, which further drives cancer progression. As a result, the TME is shaped by cancer-affected adipocytes and stromal cells, along with soluble factors and ECM proteins they secrete. (97) The TME is dynamic and recruits several stromal cell types, including endothelial progenitors, immune cells, fibroblasts, adipose-derived stem cells (ASCs), and mesenchymal stem cells (MSCs). This environment is crucial enabling cancer adaptability and immune evasion.

ASCs and MSCs are key players in remodeling the TME and promoting breast cancer progression. ASCs and MSCs fuse with tumor cells in a process that promotes malignancy (**Figure 7**). This communication can occur by different mechanisms such as cell-cell fusion, formation of tunneling nanotubes by the MSCs under stress conditions, binding of membrane-bound ligands to receptors, or via the release of the bioactive factors mentioned above, and extracellular vesicles (EVs) such as exosomes and microvesicles.

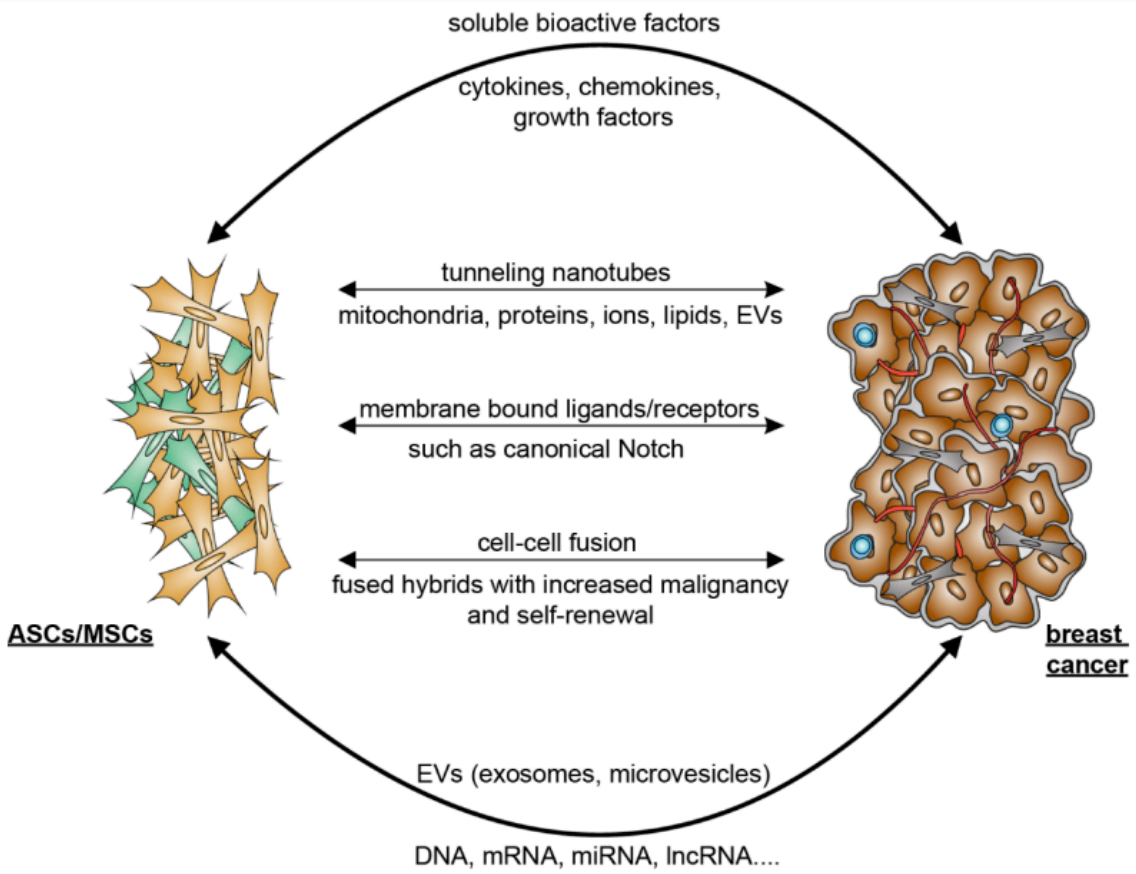

Figure 7 – Crosstalk between ASCs, MSCs, and breast tumor cells. ASCs/MSCs and breast cancer cells can communicate via direct or indirect methods. This communication can occur directly via cell-cell contact, through TNTs, cell-cell fusion or by the binding of membrane-bound ligands to their receptors, or indirectly by the release of bioactive factors such as cytokines, chemokines and growth factors and EVs. Abbreviations: EV, extracellular vesicles; ASCs, adipose tissue-derived mesenchymal stromal/stem cells; MSCs, mesenchymal stromal/stem cells; mRNA, messenger RNA; miRNA, microRNA; lncRNA, long non-coding RNA; TNTs, tunneling nanotubes. (97)

Tumor cell mutations and signaling pathways

Tumor cells exhibit rapid proliferation, genomic instability, and apoptosis evasion. This is promoted by several modifications in different cell signaling pathways, mutations in oncogenes, and inactivation of tumor suppressor genes. (98)

The most studied and direct signal pathways involved in breast cancer progression are those mediated by the ER and HER2. The activity of HER2 receptors triggers the signaling of other pathways, including MAPKs, key cellular components like glycogen synthase kinase-3 (GSK-3), and the PI3K/Akt/mTOR pathways. These interactions highlight the crucial role of signal integration and transduction in the progression and development of breast cancer. (99)

The PI3K/Akt/mTOR signaling pathway is involved in growth, proliferation, survival, metabolism, and the regulation of immune responses. This pathway is altered in several human tumors, including breast tumors, and its dysregulation is associated with several cancer

hallmarks, including uncontrolled proliferation, genomic instability, and metabolic reprogramming in tumor cells. It is also believed to be a major cause of resistance to therapies, making it a critical focus of research for understanding disease progression and exploring the potential of this pathway as a therapeutic target. (100)

Stromal cells (endothelial cells, pericytes, fibroblasts, bone-marrow mesenchymal stromal cells)

The TME components such as fibroblasts, endothelial cells, and MSCs have a vital role in tumor development and progression. It is known that tumors are not only composed of malignant cells but also by an altered surrounding stroma. (101)

The molecular events by which reactive stromal cells influence cancer cells are fundamental to enable the discovery of biomarkers and therapeutic targets. Cancer-associated fibroblasts (CAFs) constitute the majority of the cancer stroma and modulate the TME in ways that promote cancer initiation, angiogenesis, invasion, and metastasis. In breast cancer, CAFs not only drive tumor progression but also contribute to chemotherapy resistance.

CAFs play a significant role in promoting tumor initiation and progression through several mechanisms. These include influencing estradiol (E2) levels, secreting factors like HGF, TGF-β, SDF-1, VEGF, IL-6, and matrix metalloproteinases (MMPs), and inducing processes such as stemness, epigenetic changes, and epithelial-mesenchymal transition (EMT). (102) A study showed that CAFs can also enhance the growth of pre-cancerous breast epithelial cells (MCF10A) and inhibit their differentiation via aromatase-mediated estrogen synthesis. (103)Contrarily, another study showed that both normal fibroblasts (NAFs) and CAFs can inhibit MCF10A growth with NAFs having a stronger inhibitory effect, suggesting that the suppressive ability of fibroblasts is diminished as breast cancer progresses**.** (104)

Beyond E2 level modulation, CAFs contribute to breast cancer progression through increased growth factors and loss of tumor suppressor genes. IL-6, which is highly expressed in CAFs compared to NAFs, promotes cell migration and induces EMT, further driving breast cancer invasion. (105)

Recently, growing evidence suggests that CAFs can induce resistance to chemotherapy. (106) Collagen type I secreted by CAFs contributes to reduced uptake of chemotherapeutic drugs in tumors and plays a significant role in regulating tumor sensitivity to various chemotherapies. Moreover, chemotherapy and radiation-induced DNA damage in fibroblasts

stimulate the secretion of the gene WNT16B, which subsequently leads to breast cancer cell proliferation, invasion. (107)

Therefore, targeting CAFs offers a promising approach to control tumors and overcome therapeutic resistance. (108)

MSCs are multipotent stem cells that have the potential to differentiate into several types of progenitor cells involved in maintaining tissue homeostasis, regulating local immune responses, and regenerating damaged tissues. (97) Once in the TME, MSCs undergo polarization and interact with various cell populations, including immune cells, CAFs, cancer stem cells (CSCs), and breast cancer cells. In most cases, MSCs are associated with therapeutic resistance, nevertheless, there is also evidence that they can sensitize cancer cells to chemotherapy and radiotherapy. Due to their inherent regenerative and homing properties, MSCs are considered promising candidates for cell-based therapies(109)

In the context of brain metastases, the brain microenvironment created by the cells of the neurovascular unit also plays a role in the poor prognosis associated with these secondary tumors, with pericytes being among the involved cell types. Brain pericytes exhibited a strong chemoattractant effect on breast cancer cells and formed direct contact with them. By secreting large amounts of extracellular matrix proteins (collagens, elastins, fibronectins, and laminins), pericytes increase the adhesion of TNBC cells, which is likely crucial for the perivascular growth of these tumor cells. Additionally, pericytes secreted insulin-like growth factor 2 (IGF2), which significantly promotes the growth of breast cancer cells. (110)

Immune cell compartment

The immune cells are the most important elements throughout breast carcinogenesis. Normal breast tissue contains a significantly high immune cell population, such as $CD8^+$ and $CD4^+$ T cells, B cells, dendritic cells, macrophages, and natural killer (NK) cells in the ductal layer. (111) These cells, together, guarantee adaptive and innate immunity to the epithelial layer for protection against exogenous and endogenous pathogens, as well as the elimination of transformed cells. When there is a carcinogenic development, there are quantitative and qualitative changes in the nature and location of these immune cells, as well as an increase of these in both parenchymal and stromal compartments.

DCs play a key role in tumor-related immune responses as powerful antigen-presenting cells. They recognize, process, and present tumor antigens via major histocompatibility complexes (MHCs), initiating a naïve T cell response and connecting innate and adaptive

immunity. Immature DCs, derived from bone marrow, exhibit high endocytic activity but low T cell activation, potentially promoting antigen-specific tolerance rather than immunity. Upon encountering antigens, they mature and migrate to lymphoid organs, where they activate T cells, generating an immune response. Due to their heterogeneity, DCs can either enhance T cell responses, improve outcomes, or induce T cell tolerance, supporting tumor progression. (112)

In the breast tumor microenvironment, tumor-associated macrophages (TAMs) are the most prevalent immune cells and play a key role in regulating breast cancer progression. During tumor development, TAMs promote breast tumor growth by enhancing angiogenesis, aiding cancer cell metastasis, inducing cancer stemness, regulating energy metabolism, and contributing to immune system suppression. (113)

NK cells are cytotoxic lymphocytes known for recognizing and eliminating tumor cells, making them early responders against cancer. While cytokine-stimulated NK cells can contribute to antitumor responses, their function depends on their activation and maturation. NK cells in the TME can control tumor growth through cytotoxic activity, but this is influenced by their maturation and location. In humans, mature CD56dimCD16^{+} NK cells in peripheral blood are cytotoxic, while immature CD56brightCD16^{-} NK cells in lymphoid tissues have reduced cytotoxicity. Despite evidence of reduced-function NK cells infiltrating tumors like lung cancer, their role in BC progression remains largely unexplored. (114)

Several studies have identified tumor-infiltrating lymphocytes (TILs) as a favorable prognostic biomarker capable of predicting therapy response. TIL areas are commonly detected in hematoxylin and eosin-stained histological slides via light microscopy (101) and higher numbers of TILs are connected to a better clinical outcome. Specifically, CD4^{+} T-helper 1 (Th1) cells aid in antigen presentation by secreting cytokines and activating antigen-presenting cells, while CD8^{+} cytotoxic T-cells play a crucial role in tumor destruction, even though most of the time they are not functional.

Breast Cancer Brain metastases

MBC is one of the main causes of mortality associated with breast cancer. The most common sites of metastasis include the brain, axillary lymph nodes, bone, lung, and liver. (115) In the past two decades, breast cancer brain metastases (BBM) have been increasing due to better control of metastasis outside the central nervous system (CNS) and enhanced detection techniques for brain metastases. (116)

BBM are developed in 15-25% of breast cancer patients (7) and are predominantly found in $HER2^+$ tumors and TNBC. (117) BBM are considered the second most frequent type of brain metastase, only surpassed by from lung cancer.

When clinically suspected, BBM are confirmed by computed tomography (CT), which will show mass lesions with surrounding vasogenic edema associated. MRI provides a better understanding of the lesions, including their size, number, and distribution. (118) Unfortunately, BBM is associated with a poor QoL as patients will experience secondary effects such as neurologic deficit, headaches and epileptic manifestations, and a reduced OS(119) The prognosis remains poor, with a median survival rate ranging from 2 to 25.3 months. TNBC has the shortest survival (3 to 4 months) (120), while $HER2^+$ tumors have the longest (17 to 19 months), despite having the highest incidence rates.

The development of brain metastases is a long and complex process, taking about 32 months since the first diagnosis (121), as cancer cells require time to penetrate the blood-brain barrier (BBB) and to invade the brain, through extravasation. The brain microenvironment is complex with unique cell types, metabolic pathways, anatomical structures, and immune environment. In the brain, tumor cells tend to prefer the cerebellum, indicating that different brain areas have unique properties influencing the metastatic site.

Primary breast cancer cells need to invade surrounding tissue and blood vessels, adhere to the brain endothelium, penetrate the BBB, and finally invade the endothelial layer to grow in the brain parenchyma. (122) After this process, only a few tumor cells will have the capacity to survive in the brain environment. These cells will either grow within already existing blood vessels in a process called perivascular growth or start creating new blood vessels through neoangiogenesis to support their proliferation (**Figure 8**). The disruption of the BBB will lead to a newly permeable barrier known as the blood-tumor barrier (BTB). Subsequently, macroscopic brain metastases will form, indicating that the tumor has now spread into the brain. (8)

When it comes to treatment approaches, BBM can be classified as either limited or extensive. Extensive refers to more than 4 lesions, while limited refers to 3 or fewer lesions. (123) The treatment options vary based on the number of brain metastases present. Currently, BBM treatments are limited, since BBB penetration remains a challenge despite advancements to overcome it. Nevertheless, patients who develop BBM can undergo surgery, radiotherapy, chemotherapy, hormone therapy, and immunotherapy, with significant progress being made in targeted therapy, depending on the status of the disease.

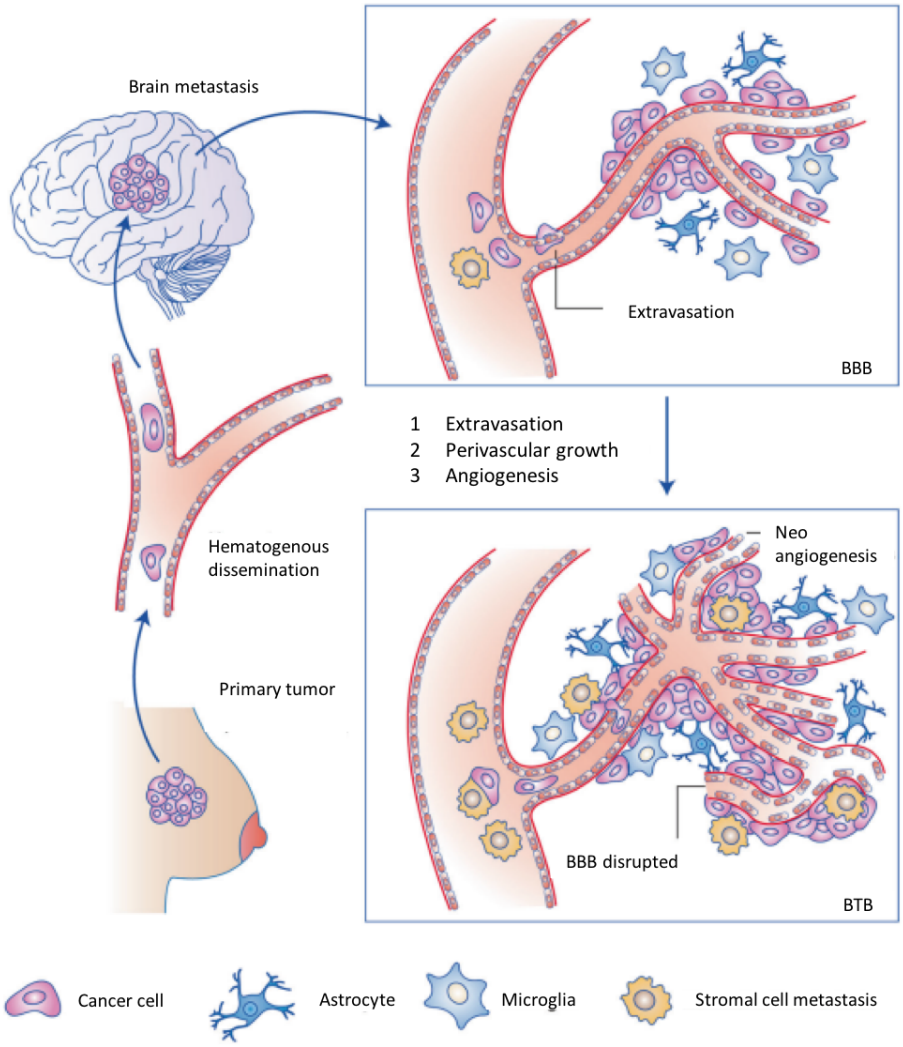

Figure 8 - Formation of brain metastases. Breast cancer cells metastasize into the brain through extravasation. When already at the brain parenchyma, they can either go through perivascular growth, keeping the BBB intact or become macroscopic through neoangiogenesis, disrupting the blood-brain barrier (BBB) and forming the blood-tumor barrier (BTB). (124)

Current treatments

Limited effective therapeutic options are available for BBM, and often therapeutic regimens involve a combination of therapies. The US National Comprehensive Cancer Network (NCCN) guidelines' treatment recommendations are based on the number and volume of metastases, location, primary tumor subtype, and the patient's performance status and prognosis. (123)

A better understanding of the BBB and the development for targeted therapies have been instrumental to improve the treatment and prevention of brain metastases. Currently, the gold standard treatment for BBM includes local surgery combined with radiotherapy and systemic treatment for the control of CNS disease. (125)

Surgery

Surgery is the therapeutic option for BBM patients presenting a good performance status, according to the Karnofsky Performance Status (KPS). Patients presenting a performance status of ≥70, suggest a good prognosis, those with single or few (≤3) lesions, and those with larger symptomatic lesions (≥3 cm). (126) The presence of clinical symptoms, peri-focal edema, and associated hydrocephalus are also selection criteria for surgical resection. Patients with a single metastasis, a good prognosis, and no extended extracranial disease, according to the guidelines published by the Congress of Neurological Surgeons for the surgical treatment of metastatic brain tumors, undergo surgery, followed by whole-brain radiation therapy (WBRT) to improve OS. Patchell et al. (127) evaluated patients' responses to surgery of single BBM. They either went through surgical resection followed by WBRT or needle biopsy followed by WBRT. The results showed that brain metastases recurrence was less frequent in the surgery group (20%) compared to the needle biopsy group (52%). Stereotactic radiosurgery (SRS) to the tumor bed can also be performed following surgery to improve local control. (118)

Patients with multiple brain metastases are not selected for surgery. This first-line treatment is used if the lesions are symptomatic and cause mass effects or obstructive hydrocephalus. (128) In alternative, surgical resection followed by SRS is used in patients with three or fewer lesions with >3 cm in diameter.

Surgery provides immediate relief from intracranial hypertension. In addition, it allows a direct histological diagnosis, when tissue diagnosis is needed. However, the location of the metastatic lesions may pose a limitation, and the accessibility is also important to take into consideration. (126) Surgery is not recommended by the NCCN for patients with limited brain metastases with disseminated systemic disease and few systemic treatment options. For those with five or more brain lesions, surgical resection is not the first-line treatment option and will only be performed on those for whom surgery will bring any survival benefit. Among breast cancer patients, leptomeningeal metastases (LM) are a concern, affecting around 5% of them, and are associated with a poor prognosis since cancer cells have achieved the cerebrospinal fluid (CSF) that surrounds the brain and spinal cord. (129)

Radiotherapy

Radiotherapy is one of the fundamental treatments for brain metastases. The local therapy WBRT is the most common. WBRT is used in patients with multiple lesions, as it aims to control macroscopic metastases and eradicate the microscopic ones to control the BBM-

associated symptoms. Most patients undergo conventional WBRT in a dose of 30Gy in 10 fractions, and these doses should not be exceeded unless the patient shows a poor prognosis and a lower performance status. (126) Even though it shows good results in patients with a poor prognosis, widely spread brain metastases, poor performance status, and uncontrolled systemic disease. Currently, WBRT is mostly used as an adjuvant therapy, especially after surgery for a single brain metastasis. Several clinical trials showed that patients who received adjuvant WBRT had fewer recurrences compared to those who did not receive radiation, both at the operative site (10 vs. 46%) and at other brain locations (10 vs. 37%). However, changes in OS were limited. (130)

Despite all the benefits of WBRT, its late toxicity and the new targeted therapeutic approaches have limited the use of WBRT. (131) The North Central Cancer Treatment Group (NCCTG) evaluated the WBRT-associated toxicity. Patients with 1 to 3 metastases were randomized into two groups: SRS *vs.* SRS followed by WBRT. Despite better brain control, the group submitted to WBRT showed a decline in cognitive function compared to those who did not receive the adjuvant therapy. (132) According to the WHO recommendations, WBRT is not recommended for patients with performance status 0-2. The treatment option is either surgery or radiosurgery. (132)

Radiotherapy has been linked to immunotherapy, demonstrating a synergistic effect. Radiotherapy can directly induce genotoxic effects in tumor cells, leading to DNA damage and subsequent cell death. In addition to apoptosis, radiotherapy causes necrosis and ICD, which elicits pro-inflammatory responses and modulates the behavior of surviving cells. These mechanisms suggested that radiotherapy can modulate the TME. (133) Breast tumors and brain tumors are typically classified as ‘cold’ tumors due to their highly immunosuppressive nature, characterized by a high content of myeloid cells and a low number of dendritic cells (DC) and T cells. In contrast, ‘hot’ tumors have a higher concentration of DC and T cells (**Figure 9**).

Radiation can recruit immune cells such as DCs, which are crucial for an effective immune response, thereby transforming "cold" tumors into "hot" tumors that are more likely to be recognized by the immune system—a significant advantage for immunotherapy. Additionally, radiotherapy disrupts the BBB, facilitating the entrance of immune cells, inducing pro-inflammatory responses, and making tumors more susceptible to immunotherapy. (134)

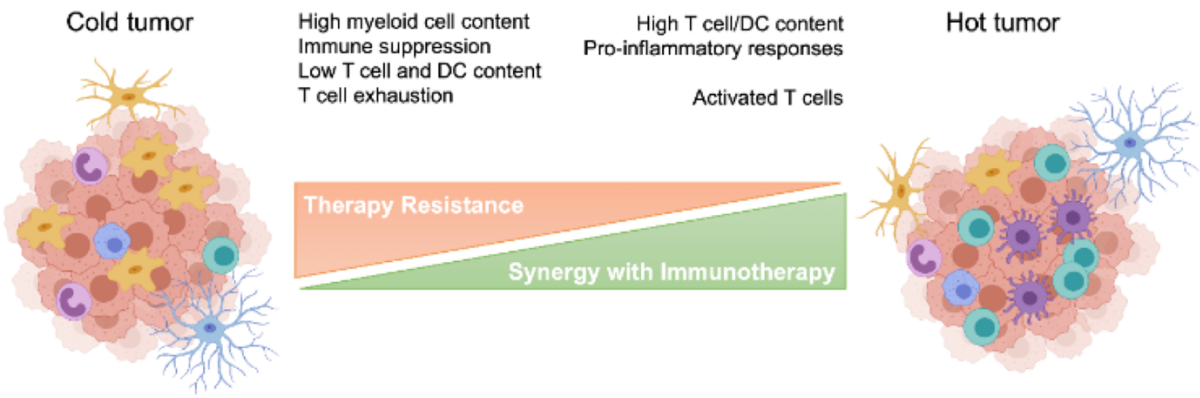

Figure 9 - Turning 'cold' tumors into 'hot' tumors via radiation. Radiotherapy can modulate the 'cold' TME to a 'hot' TME. 'Cold' tumors have a high concentration in myeloid cells and low content in DCs and T cells. In contrast, 'hot' tumors there is a high content in DCs and T cells, demonstrating a pro-inflammatory response, which can be harnessed for immunotherapy. (134)

***Stereotactic radiosurgery** (SRS)*

SRS is a precise radiation technique that does not involve an incision or opening and causes less damage to the surrounding tissue. It works as any other type of radiology treatment, as its x-ray beams destroy abnormal cells, preventing them from growing or reproducing. (135) The precision allows SRS to reach challenging sites in the brain, which has shown an advantage in patients who are poor candidates for surgery or have lesions in non-resettable locations. (126) Depending on the lesion size and patients' performance status, single fraction SRS or multiple fraction SRS can be considered.

SRS can be used as a neoadjuvant or as adjuvant therapy. Its safety after surgery is enhanced by its precision, which reduces the risk of infection. SRS shows an advantage over WBRT in that it does not cause the neurocognitive decline associated with the latter therapy. Adjuvant SRS, however, is associated with a higher risk of radio-necrosis generally associated with surrounding edema and can either be symptomatic or asymptomatic (136) when compared to adjuvant WBRT. (137) Preoperative SRS has the advantage of decreasing the risk of LMD compared to postoperative SRS, having a similar OS.

Kondziolka et al (138)conducted a study on patients with two to four brain metastases to compare the efficacy between WBRT and WBRT plus SRS. This trial demonstrated improved local control of the disease, with the time to local failure extending from 6 months with WBRT to 36 months with WBRT plus SRS. However, OS remained unchanged, likely due to the extent of extracranial disease. The Radiation Therapy Oncology Group (RTOG) also confirmed the efficacy of this combined therapy in patients with one to three brain metastases, demonstrating improved local control and an increase in OS by 1.6 months in patients with a

single unresectable lesion. (139) Currently, no findings support the use of SRS without WBRT in patients with over 4 lesions.

As mentioned above, for patients with lesions larger than 3 cm and associated symptoms, surgery is the treatment of choice. SRS can be considered after surgical resection in patients with a few lesions (fewer than 3) and those with lesions over 3 cm, as it reduces the risk of recurrence and increases survival. (137) In patients with single brain metastasis who undergo surgery plus WBRT, SRS can be added to the tumor bed to improve local control. (139)

Chemotherapy

Chemotherapy is a treatment option against BBM. However, traditional chemotherapeutic agents often show limited efficacy in treating brain metastases due to the unique characteristics of the brain and the presence of the BBB formed by brain capillaries and glial cell walls. (140) Most of these cytotoxic drugs are unable to cross the BBB and, therefore, cannot effectively target tumors within the brain. (141)

Currently, the drugs used as chemotherapeutic agents for breast cancer that can cross the BBB include cisplatin, etoposide, irinotecan, capecitabine, temozolomide, and intra-arterial carboplatin. (141) Capecitabine is often the first drug attempted for patients with HR^+ BBM, as it is thought to penetrate the BBB. These chemotherapeutic agents have been combined with radiation therapy, showing efficacy. WBRT can disrupt the BBB, allowing adjuvant chemotherapy to enhance the treatment effect. (142) A prospective study compared the impact on QoL in BBM patients treated with WBRT alone or WBRT plus carboplatin. It was demonstrated that OS (15.9 vs. 11.3 months) and progression-free survival (PFS) (10.2 vs. 6.8 months) were longer in the group treated with WBRT plus chemotherapy when compared to the WBRT-alone group. (141)

A non-randomized prospective study demonstrated that treatment with CMF (cyclophosphamide, methotrexate, and fluorouracil) or FAC (5-fluorouracil, doxorubicin, and cyclophosphamide) regimens resulted in a 59% CNS response. (143)

For HR^+ patients, cyclin-dependent kinases (CDKs) have been extensively studied for their potential to cross the BBB. CDKs regulate the transition between stages of the cell cycle, making them powerful therapeutic targets for cancer treatment. CDK4 and CDK6 inhibitors, such as palbociclib, ribociclib, abemaciclib, and dalpiciclib, are new drugs that inhibit cell cycle progression. While these inhibitors have demonstrated potent activity against $HR^+/HER2^-$ breast tumors, there are limited studies on their efficacy in BBM. Among them,

abemaciclib has shown a greater ability to cross the BBB compared to the others, leading to its inclusion in several clinical studies focused on BBM. (144)

Hormonal Therapy

Generally, most BBM patients are not ideal candidates for hormonal therapy, particularly those with rapidly growing tumors, HR^-, and/or $HER2^+$ subtypes(142,145). However, patients who are HR^+ may benefit from hormonal therapy, although its efficacy is considerably limited to those who are naïve to prior endocrine therapies and affected by ESR1 mutations. (142) A retrospective study involving 198 patients with HR^+ BBM (146) showed that those receiving hormonal therapy had a longer OS of 15 months compared to 4 months for those who did not receive hormonal therapy.

Tamoxifen and aromatase inhibitors (AIs) can be used in BBM patients. Tamoxifen is a selective estrogen receptor modulator, which blocks the effect of estrogen. AIs, such as anastrozole, letrozole, and exemestane are potent treatments for brain metastases, as they effectively lower serum and CSF concentrations of estradiol. (142)

Fulvestrant is the only FDA-approved selective estrogen receptor degrader (SERD) for breast cancer. Although it does not readily cross the BBB in animal models, a case study has demonstrated its activity in patients with brain metastases. (145)

ESR1 mutations are commonly acquired in HR^+ metastatic breast cancer following treatment with AIs. (147) A study has demonstrated that ESR1 mutations can be detected at a high rate in the plasma, specifically in the circulating tumor DNA (ctDNA), of patients following progression on AI therapy. (148) To study this mutation in patients who receive hormonal therapy, patients were treated either with fulvestrant or exemestane. The study demonstrated that patients with ESR1 mutations had improved PFS after taking fluvestrant when compared to exemestane. This analysis shows that patients with ESR1 mutated are at elevated risk of early death if treated with exemestane. For patients displaying ESR1 mutations, AI therapy is not appropriate. Therefore, when selecting the appropriate endocrine therapy backbone for patients, ctDNA analysis is recommended. (142)

Immunotherapy

Immunotherapy aims to use the patient's own immune system to specifically attack and eliminate tumor cells. The development of immunotherapeutic approaches advanced, in part due to the lack of effective treatment options against brain metastases. The brain has previously been described as an immune-privileged organ but is now seen as a target of

immunotherapeutic drugs. (149) Multiple lines of evidence have demonstrated that tumor cells manipulate immune regulatory mechanisms in the brain to ensure their survival and growth. (150) Several immunotherapeutic approaches, including checkpoint inhibitors and adoptive cell therapy (CAR-T) have shown promising results in BBM treatment. These and other immunotherapies will be discussed in detail in the following section.

Monoclonal Antibodies against HER-2 Receptor Protein

In general, breast cancer-expressing HER2 is resistant to chemotherapy and hormonal therapy. Therefore, anti-HER2-targeted therapy has been developed over the past decades, not only for primary breast cancer but also for BBM. A significant number of breast cancer patients (30-50%) expressing HER2 can develop brain metastases during their clinical course.

Given the limited treatment options for BBM from breast cancer, mAbs have been used to inhibit the HER2-overexpression. Current treatment guidelines for patients with $HER2^+$ BBM recommend a taxane in combination with trastuzumab and pertuzumab, followed by trastuzumab emtansine (T-DM1) for patients who have disease progression. (151)

Trastuzumab

Trastuzumab was the first mAb approved for metastatic breast cancer in the United States and the European Union in 1998 and 2000, respectively. In $HER2^+$ breast cancer, trastuzumab is widely used as a first-line therapy. In BBM, current evidence and future directions also emphasize the role of trastuzumab. Multiple studies have demonstrated that trastuzumab can prolong the time until CNS metastases develop in patients. (144) Although it is significantly effective in controlling systemic disease, trastuzumab's poor BBB penetration limits its ability to control intracranial disease. This limitation poses a challenge in effectively treating brain metastases in $HER2^+$ breast cancer patients.

Olson et al. showed that the incidence of brain metastases, as the first site of recurrence, was higher in patients receiving adjuvant trastuzumab compared to those who did not receive trastuzumab. (152) Therefore, trastuzumab is not yet an option in BBM treatment.

In the past two decades, antibody-drug conjugates (ADC) have altered the therapeutic landscape for metastatic solid tumors. ADCs are a combination of an antibody with a cytotoxic drug and one of the examples is trastuzumab deruxtecan (T-DXd), which combines an anti-HER2 antibody, a topoisomerase I inhibitor, and a cleavable tetrapeptide-based linker. (153)

For years, it has been assumed that mAbs, specifically ADCs, would not have activity in the CNS due to poor penetration across the BBB. However, a phase III study showed efficacy against $HER2^+$ BBM with ADCs. (154)

Another study demonstrated T-DXd efficacy in both high and low HER2 expression BBM. T-DXd inhibited tumor growth and prolonged survival, in which patients showed a CNS objective response rate of 73% and an extracranial response rate of 45%. (155) Given these findings, ADCs appear to be a viable option for treating solid brain tumors. Further trials, including those involving trastuzumab, will be conducted due to its therapeutic potential against BBM.

Tucatinib

Tucatinib is an oral tyrosine kinase inhibitor (TKI), approved for BBM treatment. It is approved in combination with trastuzumab and capecitabine for patients with $HER2^+$ metastatic breast cancer, including those with brain metastases. (151)

Tucatinib is highly selective for the HER2 receptor, with minimal inhibition of other HER family receptors, including EGFR, which is often an issue with other TKIs. In a previous study, tucatinib combined with trastuzumab and capecitabine showed promising antitumor activity in patients with $HER2^+$ metastatic breast cancer, including BBM. The pivotal HER2CLIMB trial (156) assessed the efficacy of tucatinib versus placebo, both in combination with trastuzumab and capecitabine, for treating $HER2^+$ metastatic breast cancer. The trial included 612 patients with and without brain metastases. With an additional 15.6 months of follow-up, tucatinib combined with trastuzumab and capecitabine continues to show a significant improvement in OS, offering a median survival benefit of 5.5 months. Patients treated with the tucatinib combination had a median OS of 24.7 months, compared to 19.2 months for those in the placebo combination group. (151) This final analysis confirmed that the tucatinib combination continues to provide a substantial survival benefit for patients with $HER2^+$ metastatic breast cancer. These survival results are particularly, striking considering that almost half of the HER2CLIMB trial participants had brain metastases, including some with active brain metastases at the beginning of the study. This is representative of the real-world situation, where up to 50% of patients with $HER2^+$ metastatic breast cancer will develop brain metastases at some point during their illness. (157)

Despite the described advancements in HER2-targeted treatments, patients with BBM still face a poor prognosis. While clinical trials for $HER2^+$ metastatic breast cancer have often excluded patients with brain metastases, both the American Society of Clinical Oncology's

Friends of Cancer Research Brain Metastases Working Group and the 2019 FDA guidance on "Cancer Clinical Trial Eligibility Criteria: Brain Metastases" have advocated for the inclusion of patients with brain metastases in clinical trials. (158,159)

Advanced in Immunotherapy for Brain Metastases of Breast Cancer

The incidence of BBM has been increasing and the treatment options must evolve to achieve a better outcome for patients. The traditional therapeutic approaches, including chemotherapy, radiotherapy, and hormonal therapy have shown limited efficacy against brain metastases. Immunotherapy has been described as an effective solution for patients at early stage and even for those with resistant metastasis. Studies have demonstrated that brain tumors present an immune microenvironment that can be targeted via immunotherapy. CNS works as an immune specialized environment with a tight control network that links microglia, astrocytes, and lymphocytes. Among immunotherapeutic approaches, the major focus has been directed to block immune checkpoint molecules.

Immune Checkpoint Inhibitors

The brain has a great variety of immune cells (e.g., tissue-resident macrophage, microglia, astrocytes). Therefore, immunotherapy, such as checkpoint inhibition, has been considered a treatment strategy for brain metastases. (134) Immune checkpoints are critical to avoid autoimmunity and attacking the host as they keep the cytotoxic activity of T cells under control. Tumor cells can block T cell activity and establish an immunosuppressive TME by up-regulating checkpoint components. Immune checkpoint inhibitors aim to reverse the inactivity of T cells and induce anti-tumor responses. (133)

In contrast to tumors with inflammatory phenotype and containing TILs, or ‘hot’ tumors, breast cancer and BBM are designated as ‘cold’ tumors, which means an immune desert and immune-excluded phenotype. Therefore, ‘cold’ tumors are thought to be resistant to checkpoint inhibition, and turning them into ‘hot’ tumors with a pro-inflammatory TME has been a strategy to sensitize them to immune checkpoint inhibition. The most relevant checkpoint inhibitors have been antibodies blocking the inhibitory checkpoint proteins cytotoxic T-lymphocyte antigen 4 (CTLA-4) and programmed cell death protein 1 (PD-1) that

are expressed in T cells and programmed death-ligand 1 (PDL-1) that is expressed in other immune cells (DC, TAM) and tumor cells. (160)

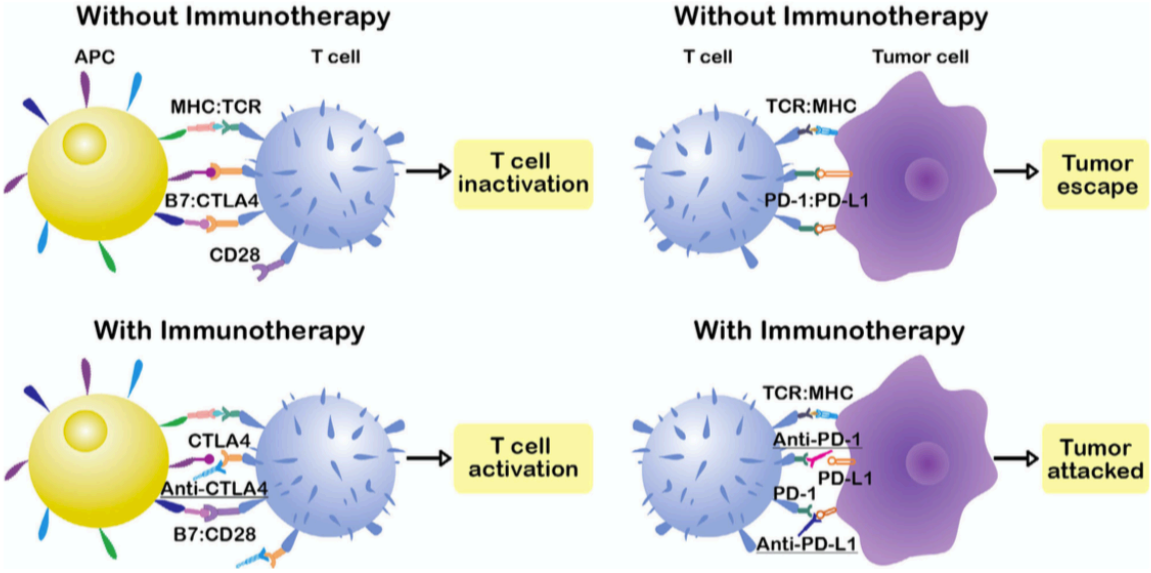

Figure 10 - Mechanism of action of immune checkpoint inhibitors for cancer immunotherapy. Immune checkpoints work to prevent immune responses. T cells recognize checkpoint proteins such as CTLA-4 and PD-1 and this binding works as a turn off sign for the T cells, disactivating it. Tumor cells can coopt this system by presenting checkpoints such as PD-L1 which, in turn will inhibit T-cell–mediated immunity. Immunotherapy will then work as a system that prevents the binding of T-cells to their protein pair. Immune checkpoint inhibitors will bind to checkpoint proteins, keeping T cells activated and ready to recognize and destroy cancer cells.
Abbreviations: APC, antigen-presenting cell; CD, cluster of differentiation; CTLA-4, cytotoxic T-lymphocyte–associated antigen 4; MHC, major histocompatibility complex; PD-1, programmed cell death protein 1; PD-L1, programmed death-ligand 1; TCR, T-cell receptor (161)

When T cells recognize checkpoint ligands in other cells, such as antigen-presenting cells (APC), they are turned off. In addition, tumor cells can inactivate T cells, as they can coopt this system by presenting checkpoints and binding their major histocompatibility complex (MHC) to the T-cell receptor (TCR). The use of checkpoint inhibitors works as a protective mechanism to activate T cells and drive effective anti-tumor responses (**Figure 10**). (161) Immune checkpoint inhibitors are effective in BBM, but are currently limited to TNBC patients.

CTLA4

CTLA-4 is a checkpoint protein found in the intracellular compartment in resting T cells that is transported to the cell surface when the T cell is stimulated. When binding to its ligand (B7) located in APC, the T cell is deactivated and unable to recognize and eliminate other cells, including cancer cells. Under normal circumstances, this mechanism is essential to prevent immune responses from over-amplification. To keep T cells activated, immune

checkpoint inhibitors have been developed. Anti-CTLA-4 therapy prevents the binding of CTLA-4 to B7. Instead, this protein will bind to an anti-CTLA-4 antibody, activating the T cell to recognize tumor cells. CTLA-4 is rapidly upregulated following T-cell antigen exposure and activation. Tremelimumab and Ipilimumab are both examples of human mAb antagonists of CTLA-4 that upregulate the cytotoxic activity of T cells. (162)

Recent studies have shown efficacy in patients with breast cancer who are treated with Ipilimumab in combination with other therapies such as RT and SRS. However, patients with BBM were not included. A study in $HER2^+$ patients assessed the efficacy of anti-CTLA4 therapy with brain radiotherapy in breast cancer patients. However, the study did not demonstrate satisfactory efficacy for further investigation, as most patients experienced rapid disease progression. Nonetheless, the combination of Tremelimumab with brain RT, and potentially Trastuzumab, will be considered for future treatment of patients with $HER2^+$ BBM. (163)

PD-1 and PD-L1

PD-1 is a cell surface receptor found on activated T and B cells, binding to PD-L1 and PD-L2, which play a role in regulating the immune system's response. It also suppresses T cell inflammatory activity, thereby promoting self-tolerance. While this process is generally beneficial, in the context of cancer, it can inhibit the immune system from effectively targeting and destroying tumor cells. PD-1 is an immune checkpoint that functions by promoting the apoptosis of antigen-specific T-cells in lymph nodes, while simultaneously reducing apoptosis in regulatory T cells. Since this mechanism blocks the system's ability to kill cancer cells, PD-1 inhibitors are a class of drugs designed to activate the immune system to attack tumors and are used in the treatment of breast cancer. (84)

Currently, PD-1 inhibitors, such as pembrolizumab and nivolumab, are being actively investigated in clinical trials for non-small cell lung cancer and melanoma with brain metastases. However, there is a lack of data on the use of anti-PD-1 therapies specifically for BBM. This gap highlights the need to explore the potential of enhancing the host's antitumor immune response in BBM by inhibiting these immune checkpoints.

PD-L1 is a transmembrane protein that plays a crucial role in suppressing immunity during certain events. PD-L1 is notably overexpressed in breast cancer, making it a significant prognostic marker and a target for cancer immunotherapy. Higher levels of PD-L1 expression are associated with more aggressive tumors. There are two hypotheses explaining PD-L1 expression in tumors: the innate model and the adaptive model. The innate model proposes that

PD-L1 expression is independent of the tumor microenvironment and is instead driven by intrinsic cellular signaling pathways within the tumor cells. On the other hand, the adaptive model suggests that TILs are the key drivers of PD-L1 expression, with tumor cells upregulating PD-L1 as a defense mechanism in response to the body's natural antitumor activity. Numerous PD-L1 inhibitors are currently being developed as immunotherapies showing promising results in clinical trials. The analysis of PD-L1 is essential in TNBC patients to determine their eligibility for immunotherapy. (164)

Several studies suggest that the immune response to malignant processes in the brain may vary depending on the type of cancer. A deeper understanding of the local immune response associated with brain metastases could lead to the development of new preventive and therapeutic strategies for breast cancer patients. A study was conducted to understand how the immune response affects the development of brain metastases. (165) The study included 84 patients with breast cancer who underwent excision of BBM. TIL ($CD4^+$, $CD8^+$) and macrophage/microglia ($CD68^+$) infiltration were determined in 96 %, 98 %, and 92 % of cases, respectively. PD-1 was expressed on TILs in 17% of the patients, more frequently in older patients or in those with $HER2^+$ tumors. Patients who were PD-1^+ had a longer OS after the surgical removal of BBM compared to patients who were PD-1^-. Specifically, the median OS for PD-1^+ patients was 27.9 months, whereas for PD-1^- patients, it was 13.9 months. However, no correlation was found between the expression of PD-1 on TILs and the expression of PD-1 ligands (such as PD-L1 and PD-L2) in BBM. PD-L1 was expressed in 53% of the cases (41 out of 77), and PD-L2 in 36% of the cases (28 out of 77), but this expression was not linked to any specific BBM phenotype.

Pembrolizumab has shown promising effects and a favorable safety profile in PD-L1-positive advanced TNBC and heavily pretreated $ER^+/HER2^-$ breast cancer. Additionally, PD-L1 inhibitors such as atezolizumab and avelumab have demonstrated particular efficacy in TBNC. Several ongoing clinical trials are further investigating various immune checkpoint inhibitors in both locally advanced and metastatic breast cancer, as well as in the adjuvant setting. (166)

Chimeric Antigen Receptor (CAR) T-Cell Therapy

CAR-T cells have been mostly used in hematologic malignancies. CAR-T cell therapy is a personalized treatment in which T cells are collected from the patient and reintroduced.

T cells are the main killers of the adaptive immune system but often cannot eliminate cancer cells. To enhance their effectiveness, they are isolated and genetically modified using viral vectors that introduce genes encoding Chimeric Antigen Receptors (CARs) (**Figure 11**). (161) These CARs are then expressed on the surface of the modified T cells and are designed to recognize and bind to specific proteins, or antigens, found on the surface of cancer cells. The modified T cells are then expanded to millions and infused back into the patient. Once inside the body, the CAR-T cells are intended to proliferate and, guided by their engineered receptors, target and destroy cancer cells that display the specific antigen on their surfaces. (167)

The CAR-T cell therapies approved by the FDA are those that either target the antigen CD19 or the antigen BCMA on B cells. The ones targeting CD19 are now being developed against brain metastases.

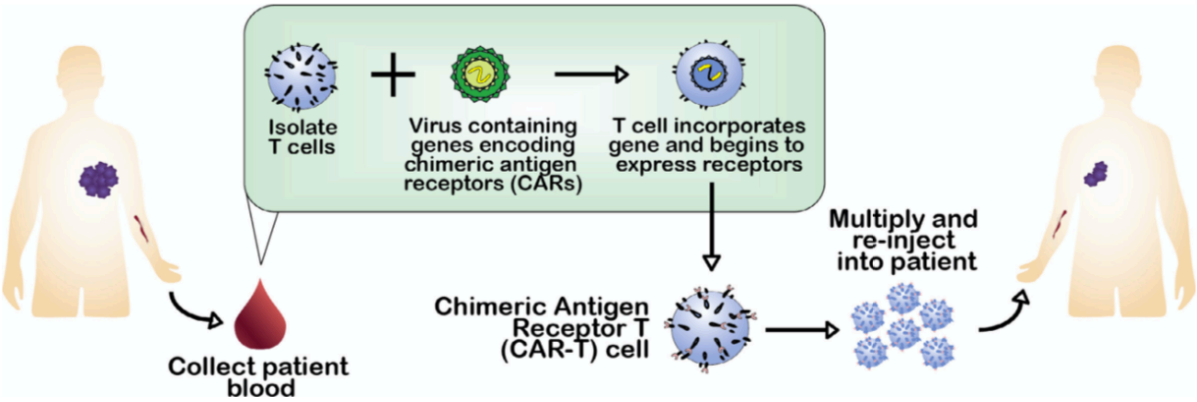

Figure 11 – CAR-T cell therapy involves collecting T cells from the patient, isolating them, and returning the remaining blood to the body. The isolated T cells are then genetically modified using viral vectors that introduce genes encoding Chimeric Antigen Receptors (CARs). These CARs are designed to bind specifically to proteins or antigens present on the surface of cancer cells. After the genetic modification, the redesigned T cells are multiplied to produce millions of copies, which are then reintroduced into the patient. Once back in the body, these CAR-T cells are expected to continue multiplying and target tumor cells for destruction. (161)

In 2018, a trial conducted by Brown et al. (168) explored CAR-T cell therapy in patients with glioblastoma, a type of brain tumor. The trial showed positive results, marking the beginning of the application of this therapy in treating brain tumors. In BBM, preclinical investigations using human xenograft mouse models have demonstrated that CAR T-cell therapy may effectively target $HER2^{+}$ brain metastases. This research suggests the potential for CAR T-cell therapy to be an effective treatment option for patients with these specific types of brain metastases. (169) Even though these trials demonstrated efficacy in treating brain metastases and brain tumors, the recurrence of disease and the development of cerebral edema remain significant concerns for patients undergoing CAR-T cell therapy. (170) Ongoing research is focused on better understanding these challenges and finding ways to reduce the

side effects. Currently, some trials are exploring the combination of immune checkpoint inhibitors, targeting PD-1 and PD-L1, with CAR-T cell therapy. This combination could potentially open new treatment avenues for patients with BBM. (161)

Breast Cancer Vaccines

Cancer vaccines aim to activate the patient's immune system to recognize and target tumor cells. These vaccines elicit $CD4^+$ and $CD8^+$ T cell responses against tumor-associated antigens (TAAs) and/or tumor-specific antigens (TSAs). (171) Whereas mAbs are considered a form of passive immunotherapy, breast cancer vaccines fall into active immunotherapy, inducing a therapeutic effect.

Currently, the most common type of vaccine for breast cancer consists of peptides derived from tumor antigens. In addition to peptides, there are other breast cancer vaccine formulations, including protein, plasmid, carbohydrate, and tumor cell-based vaccines (**Figure 12**).

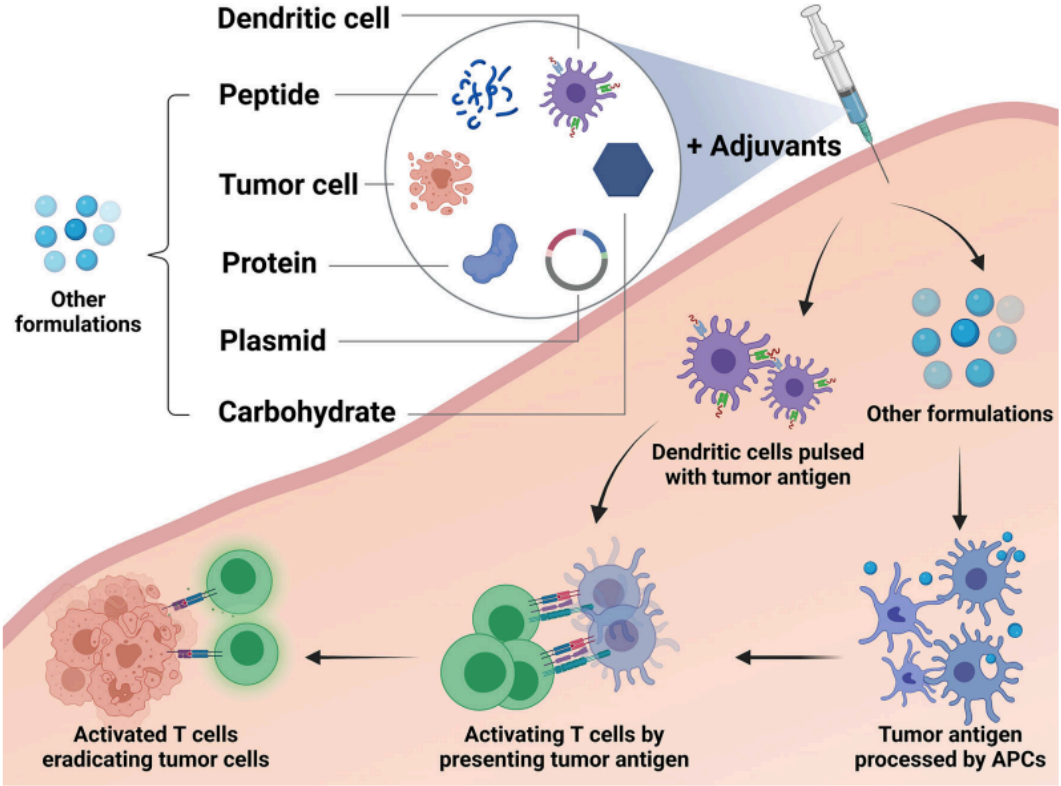

Figure 12 – Types of breast cancer vaccines and respective mechanisms. Breast cancer vaccines can be categorized into different types based on their formulation: peptide vaccines, protein-based vaccines, carbohydrate antigen vaccines, dendritic cell-based vaccines, and tumor cell vaccines. Unlike dendritic cell-based vaccines, which present antigens directly to T cells, all the other formulations require stimulation of antigen-presenting cells (APCs) to activate effector immune cells. (162)

All these formulations aim to stimulate autologous APCs, which in turn activate effector immune cells to enhance the anti-tumor response. Dendritic cell-based vaccines involve the *ex vivo* generation of DC that are either loaded with tumor antigens or transfected to express them. These cells process the antigens and directly present them to T cells, thereby initiating an immune response.

Currently, vaccines in combination with immune checkpoint inhibitors are under active investigation. Some preclinical studies suggest that cancer vaccines can also increase the expression of inhibitory receptors on the surface of T cells during activation. One reason for this is that tumor-specific T cells release more IFN-γ, which leads to an increase in PD-L1 expression. This upregulation is a natural mechanism to prevent excessive immune responses in the body. Immune checkpoint inhibitors can help counteract this immunosuppressive effect, which may otherwise weaken the anti-tumor immunity triggered by vaccines. Combining breast cancer vaccines with immune checkpoint inhibitors is a promising strategy that could potentially enhance and prolong the immune response, leading to significant clinical benefits. Additionally, combining vaccines with other therapies such as anti-HER2 mAbs has shown a synergetic effect. (162)

On December 6th, 2023, the results of a phase I clinical trial for an innovative breast cancer vaccine were presented at the San Antonio Breast Cancer Symposium. (172)This latest study focused on evaluating the vaccine's efficacy and dosage in patients with early TNBC. The vaccine is based on the hypothesis that certain proteins are produced in the body only at specific times and in specific tissues. The protein targeted by this vaccine is α-lactalbumin, which is typically produced in lactating breasts but not in other tissues or at other times, making it undetectable in normal aging breast tissue. However, it was found at elevated levels in over 70% of TNBC tumors. The vaccine was developed to target α-lactalbumin, aiming to attack cancer cells that overproduce this protein. The trial involved patients with early-stage TNBC who had completed treatment but were at risk of recurrence. More than half of the trial participants generated T-cell responses against α-lactalbumin, which was the anticipated outcome. The research team is now enrolling two new groups of patients for further study. The first group consists of cancer-free individuals with BRCA1 and BRCA2 mutations, who will undergo prophylactic surgery and receive the vaccine. The second group includes patients with early-stage TNBC who have completed chemoimmunotherapy and surgery and are receiving adjuvant pembrolizumab. Given the high risk of recurrence in these patients, they will receive

both pembrolizumab and the vaccine concurrently. Looking ahead, the research team plans to launch a preventive trial. (162)

Conclusion and future perspectives

In 2021, breast cancer became the most common cancer globally and the incidence of BBM is rising. Brain metastases develop in 15-25% of breast cancer patients, with nearly half of those having $HER2^+$ tumors. The high survival rates observed in the early stages contrast with the poor prognosis of BBM. Therefore, BBM represents a significant and growing medical challenge.

Recent studies have delved into the mechanisms of therapy resistance in BBM, revealing that tumor cells often adapt by altering signaling pathways, and modifying their microenvironment to evade treatment. These resistance mechanisms underscore the complexity of BBM and the need for therapies that can overcome these barriers. The TME in BBM is characterized by significant immunosuppression, driven by various cells whose role is not yet fully understood. These cells contribute to a hostile immune landscape that protects tumor cells from immune-mediated destruction, further complicating treatment efforts. In addition, a major challenge is the limited ability of drugs to cross the BBB and BTB to target tumor cells effectively.

Ongoing research is exploring innovative treatments, including ADCs and mAbs, which are showing promise in targeting BBM. In addition, the recent insights into the brain immune microenvironment offered a new avenue for the development of effective immunotherapies targeting the brain immune landscape.

The exclusion of BBM patients from many clinical trials further complicates the development of new therapies, perpetuating the lack of targeted treatments. Nevertheless, there is a strong push to include BBM patients in more clinical studies, which is essential for advancing therapeutic options.

Addressing these challenges through continued research, including studies on therapy resistance and tumor immunosuppression, as well as clinical trial inclusion, is critical to improving outcomes for patients with BBM and meeting this urgent medical need.

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