

# Universidade de Lisboa

## Faculdade de Farmácia



## **Targeted therapies in breast cancer**

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Mestrado Integrado em Ciências Farmacêuticas

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## **Abbreviations Index**

ADC – Antibody-Drug Conjugate

AR – Androgen Receptor

BER – Base excision Repair

BC – Breast Cancer

CAIX – Carbonic Anhydrase IX

CDR – Complementarity-determining regions

CPP – Cell-penetrating Peptides

CRHC – Cancer-Related Health Cost

DNA - Deoxyribonucleic acid

EBV – Epstein-Barr virus

EGFR – Epidermal Growth Factor Receptor

EPR – Enhanced Permeability and Retention

ER – Estrogen Receptor

GDP – Gross Domestic Product

HER2 – Human Epidermal growth factor Receptor 2

HHV-8 - Human Herpes virus 8

HPV - Human Papillomaviruses

IARC – International Agency for Research on Cancer

mAb – Monoclonal Antibody

MDM2 – Murine double minute two

mTOR – Mammalian Target of Rampamycin

PAMAM - Poly(amido)amine

PEG – Polyethylene Glycol

PR – Progesterone Receptor

STAT3 - Signal Transducer and Activators of Transcription 3

TNBC – Triple Negative Breast Cancer

WHO – World Health Organization

## **Resumo**

O cancro da mama é uma doença responsável por milhões de mortes anualmente. É uma doença muito heterogênea devido às diferentes mutações existentes em cada caso. O cancro da mama não tem uma cura existente, ele é tratado através de uma série de esquemas terapêuticos que envolve radioterapia, quimioterapia e cirurgia. Tanto a radio como a quimioterapia são unicamente adjuvantes de modo a permitir a remoção do cancro através de uma lumpectomia ou mastectomia. A quimioterapia corrente usada não consegue distinguir células malignas de células saudáveis e isso remete para reações adversas sentidas pelo doente que tornam a terapia dolorosa. Existe, portanto, uma demanda de novas opções terapêuticas mais eficazes e seletivas. Contudo, muito investigadores referem descobrir novas terapêuticas seletivas que acabam por não o ser. Faz-se uma crítica a potenciais novos fármacos em investigação, mas também se propõe a síntese de fármacos mais eficazes e viáveis na cura do cancro da mama.

## **Abstract**

Breast cancer is a disease responsible for millions of deaths annually. It's a very heterogeneous disease due to the different mutations on each case. Breast cancer doesn't have existing cure, and it's currently approached through therapeutic schemes involving radiotherapy, chemotherapy and surgical procedure. Both radiotherapy as chemotherapy act adjunctly allowing further removal through lumpectomy or mastectomy. Current chemotherapy can't distinguish between healthy and malignant cells, causing patient suffering through adverse effects. So, there's a demand to find out novel therapies more efficient e selective. However, many researchers claim to found novel selective approaches, that ultimately aren't. Potentially new drugs on research will be analysed, and an efficient and viable drug design is proposed to cure breast cancer.

# Introduction

## Cancer Statistics

### Worldwide

According to the World Health Organization (WHO), cancer is the second leading cause of death globally, being responsible for 8.8 million deaths in 2015 meaning that 1 in every 6 deaths is due to cancer (1).

Only in 2012, approximately 14 million new cases were reported. This number is expected to rise about 70% over the next two decades representing a serious threat to global human life (1).

The most common worldwide type of cancer, in 2012, was: lung, breast (women only), colorectal, prostate and stomach in decreasing order; and the number of new cases diagnosed (thousands): 1,825; 1,677; 1,361; 1,112; 952 respectively (2).

The total annual economic cost of cancer in 2010 was estimated nearly 1.1 trillion euros, this value is expected to keep increasing (1).

A study conceived by the American Cancer Society and LIVESTRONG organization, reported that in 2008 the total economic impact of cancer worldwide was \$895 billion, being the most expending world cause of death. The value includes premature death and disability which accounts for productivity losses, representing 1.5% of gross domestic product (GDP). The study also shows that the top three most global economic impact cancers are lung (\$188 billion), colorectal (\$99 billion) and breast (\$88 billion) (3).

### Europe

The overall view in Europe alone is very similar and equally concerning. Cancer is also the second most important cause of death and morbidity in Europe, with about 1.9 million death cases each year, and more than 3.7 million new cases (4).

Also, in 2012, the most common type of cancer in Europe in decreasing order was: breast (women only), colorectal, lung, prostate, bladder, and the respective values (thousands) : 463,8; 446,7; 416,7; 409,9; 151,2 (5).

In 2009, a study evaluated the economic burden of cancer in the European Union reporting that over 126,205 billion euros are spent in cancer-related issues. This value covers both cancer-related health costs (CRHC) and productivity losses. 50,994 M € are spent only in CRHC and the expenditure with drugs accounted for 13,604 M € which represents more than a quarter of the value invested to overcome cancer. They've also shown that breast, prostate, colorectal and lung cancer were the highest CRHC, representing 43% of all cancer types (6).

Since cancer carries a great impact in both health and economy all over the globe, understanding it's prevention, occurrence, and state of the art therapies, grants the possibility of evaluate current barriers to optimal drug designs, allowing new drugs developments with higher efficiency, largely increasing the quality-adjusted life year (QALY) of the population or even by eliminating the disease; but also, educates the population to reducing risk exposure factors contributing to cancer development.

## **Carcinogenesis**

### **Induction Theories**

There are several theories which lead to today's knowledge of cancer development.

The cellular theory defends that the origin of cancer derives from a unique normal cell. Many researchers claim other discoveries and/or evidence that support this theory. One of which was from Weiberg in 1998 which claimed that the risk to contract cancer was 10% or 1 in  $10^{15}$  based on the average human body cell divisions in a lifetime ( $10^{16}$ ). Another research by Hockenbery et al. 1990 showed that transfecting normal B-lymphocytes with gene *bcl-2*, involved in lymphomas, turned these cells resistant to apoptosis, granting hypothesis to turn



malignant. These researches prove that to promote carcinogenesis, there must be a gap in the cell growth control and also in the cell death prevention, on normal healthy cells (7).

The noxious theory stands for exogenous damaging agents: chemicals, radiation and virus, that lead to tumorigenesis. This is the current theory that is easily correlated with nowadays worldwide cancer impact. Due to the overpopulation and the need to extract resources enough to achieve a healthy life-style for everyone, many strategies were adopted, an example of one is the use of pesticides to increase food quantity. The exposure to these chemicals have been already correlated with the development of carcinogenesis in the human body. One of the most common associations with chemicals and carcinogenesis, is the usage of tobacco, which has been greatly correlated with lung cancer by many researchers (8,9). Of its compounds, polycyclic aromatic hydrocarbons are the most proved to induce cancer, through DNA adducts formation, which damages DNA. If not repaired or if the cell doesn't initiate apoptosis, the cell could replicate and lead to carcinogenesis (10,11).

The International Agency for Research on Cancer (IARC) is an organization belonging to WHO, and for the past 30 years has been testing many chemicals that may induce carcinogenesis in the human body (12).

Another noxious induced category is radiation. While the mechanisms underlying radiation promotion of carcinogenesis are not quite yet established, studies that followed the individuals exposed to gamma radiance from atomic bombs showed that they have higher chance of developing cancer rather than individuals not exposed to it (13–15).

Besides atomic bombs, high sunlight exposure is correlated with nonmelanoma skin cancer development. UV radiation is divided by wavelength intervals into subgroups A, B and C. The UV-C is filtered in the atmosphere while A and B pass it. While there's evidence correlating melanoma with UV-B radiation; UV-A is also starting to be considered cancerous after chronic exposure (16).

UV radiation promotes, in the cell's DNA, malignant dimer formation as the main lesion, but also other non-dimer products that can also damage the DNA,

and can act by simple breaking single-strand DNA. These products will lead to carcinogenesis if not managed by the cell (17).

The last category in the noxious theory are viruses. Several viruses can lead to cancer.

Hepatitis B has been proved to develop hepatocellular carcinoma, although the mechanism in which occurs are not quite yet defined. There are multiples ways this virus could cause carcinogenesis: through the inflammatory process as an immune response, which could inflict damage on the cellular DNA; integration of the virus genome into the hosts DNA; epigenetic modifications; or inducing oxidative stress (18–20).

Human Papillomaviruses (HPV) consist in a double-stranded DNA that has tropism to epithelial cells. HPV induces cervical cancer in women, being the second most common cancer development in this gender. There are two risk types of HPV; high-risk viruses (type 16 and 18 for example) that act by integrating its own genome into the target cell genome, while the low-risk infect the cell leaving its genome as an episome. The former group is responsible for 70% of the cervical cancers. Although the carcinogenesis induction mechanism is correlated with E6/E7 HPV genes expression levels, without other external viral factors (e.g. a low estrogen concentration) there's no progression in cervical cancer (21–24).

Carcinogenesis induced by Epstein-Barr virus (EBV) is associated with T and B lymphomas, Hodgkin's disease and nasopharyngeal carcinoma (25,26). Although the association between the virus and the malignant pathologies have been studied, there's not yet evidence revealing how does the virus induces tumorigenesis in different types of cells (27).

The somatic theory attributes carcinogenesis to chromosomal disorder. A major hallmark in this theory was the discovery of the Philadelphia chromosome in chronic myeloid leukaemia, resulting in a reciprocal translocation between of chromosome 22 and 9. The abnormal protein generated, stimulated the appearance of this disease (28).

Another evidence supporting the somatic theory are aneuploidies, higher or lower changes in the DNA quantity. It's reported that higher changes are more often associated to tumorigenesis (29,30).

In all the previous theories, a common characteristic is shared. The disturbance of the human cell genome is present in all tumorigenesis-mechanism induction theories. Having this evidence, it's important to understand how the human body naturally responds to prevent the evolution of a damaged cell into a tumour.

### **DNA repair mechanisms**

When treating an existing tumour, many cytotoxic drugs act by targeting the DNA and induce mutations to lead the malignant cell to death. However, some endogenous repair mechanisms activate to prevent the cytotoxic effect. The repairing mechanisms could also be a future target for new to come cancer-reduction or cancer-elimination therapies.

*Table 1 – Endogenous repair mechanism according to a certain lesion type and the respective compounds in study to target them.*

<b>Repair Mechanism</b>	<b>Lesion Type</b>	<b>Inhibitors compound in study</b>
Base Excision Repair (BER)	Oxidative lesions	ML-199 NCS-666715, AR03
Non-homologous end joining (NHEJ)	Double-strand breaks	CC-115, CC-122, A12B4C3
Mismatch repair (MMR)	Nucleotide mismatch, Deletion loops	Pol $\beta$ inhibitor in MSH2- deficient cells, Methotrexate
Single strand break repair (SSBR)	Single strand breaks	Olaparib, Iniparib
Homologous Recombination (HR)	Double strand breaks	Mirin, RI-1
Nucleotide Excision Repair (NER)	Double strand breaks	UCN-01, Trabectedim
DNA interstrand crosslink repair pathway	Interstrand crosslinks	Trastuzumab

Table 1 briefly shows DNA repair mechanisms, specialized into different types of DNA lesions. These mechanisms are suspected to increase chemo and radiotherapy resistance in cancer therapies. To increase therapy success, DNA repair inhibitors are being tested as adjuvant in DNA-damage cancer therapies (31–35).

### **Tumor promotion**

In normal healthy cells, the previous DNA repairing mechanisms act by keeping the cell intact to harmful exogenous compounds, capable of inducing malignant transformation. However, there is still a great percentage of people that at some point develop cancer. If these repairing mechanisms fail, the cell notices an abnormality, signals itself and triggers an apoptosis state to prevent turning malignant. So how can cancer development happen?

A genetic approach explains how a mutation can lead to a tumour that keeps growing, mostly due to the disturbance on two gene classes: oncogenes and tumour suppression genes.

Oncogenes are dominant genes that encode proteins that are responsible for cell proliferation and for the regulation of the apoptosis pathway. Oncogenes are derived from protooncogenes when the later suffers structural alterations (mutation, gene fusion, juxtaposition to enhancer elements, amplification). Since protooncogenes are also dominant, it means they only have one *allele* needed to be targeted to promote tumour progression, which makes it easier to turn a normal cell into a malignant one. One oncogene example is the epidermal growth factor receptor (EGFR) gene that is expressed in several squamous carcinomas (head, neck, lung, etc...). EGFR is a receptor that's part of the tyrosine kinase family. Its activation induces signalling transduction cascades like RAS/RAF/MAPK pathway and PI3K/AKT pathway that allows cell proliferation and cell survival(36). Due to the EGFR pathway role in certain tumour proliferation, many cancer therapies have been developed to target it (37,38).

Tumour suppression genes counters the action of oncogenes, by intercepting the tumour progression pathway to stop cell proliferation. They are recessive genes, meaning both *alleles* of the gene need to be targeted to lose

function, adding an additional barrier on cancer prevention. An example of a tumour suppression gene class is the protein p53. The p53 levels are maintained low due to a ubiquitin ligase murine double minute two (MDM2), which is activated in the presence of high p53 levels. This protein is expressed due to some stressing cellular environment, causing the arrestment of the cell-cycle, leading to apoptosis or senescence. Due to the MDM2-p53 signal relevance in regulation of cell-cycle (39), these molecules are also another cancer therapy target (40,41).

Since an overbroad explanation about carcinogenesis process has been introduced, and the worldwide social and economic impact of breast cancer has also been demonstrated, it's important to understand the cellular malfunctions that lead to breast cancer and the current therapies aimed to treat this disease.

## **Breast Cancer**

As demonstrated before, this pathology affects largely more women than men, therefore, this subject will be focused on the female gender only.

### **Breast Cancer Subtyping**

Breast cancer (BC) is a very heterogenous disease. However, by analysing each case, it was concluded that some malignant cells share similarities amongst themselves, allowing a division according to their molecular portraits or gene expression, without compromising the diagnostic accuracy. Due to the purpose of this thesis, five main subtypes will be briefly discussed according to their molecular portraits: Luminal A, Luminal B, HER2-enriched, Basal-like and Claudin-low (42).

Luminal A and B subtypes received their name following the similarity in the gene expression on the luminal epithelium breast cells. They are distinguished from the other subtypes by analysing the high expressed levels of estrogen receptor (ER), which was found to be equal among them, and progesterone receptor (PR) levels, being lower in luminal B subtype (42,43). Luminal A subtype, contrary to the luminal B, has an upregulated expression of genes involved in cell differentiation and cell adhesion (eg. *Jun* proto-oncogene). Luminal B subtype has an upregulated expression of genes involved in cell-cycle

(eg. Cyclin B1), found as downregulated in subtype A, and activation of growth factor receptor signalling pathways as IGF-1R PI3K/AKT/mTOR, which explains its faster levels of proliferation (44). On the DNA level luminal A shown an overall lower mutation number than luminal B subtype (42).

HER2 - enriched Human Epidermal growth factor Receptor 2 (HER2) – enriched subtype has high expression levels of genes and proteins related to the HER2 and cell proliferation (eg, ERBB2, GRB7), low expression of basal-like-related genes and proteins (eg, FOX1, keratin 5) and normal levels of luminal genes (ESR1 and PGR) (42). HER2-enriched also presents high levels of receptor tyrosine kinase genes like FGFR4 and EGFR both responsible for cell proliferation and differentiation (37,45,46). HER-2 is the subtype with the most mutations across the genome.

Basal-like subtype, is characterized at the RNA and protein expression, with high levels of proliferation-related genes (eg. MKI67) and keratins which are found in the basal layer of the skin with low expression of luminal-related genes, and moderate expression of HER2-enriched-related genes. This subtype is the second with most abnormalities in the genome (42). Basal-like subtype it's often referred as Triple Negative Breast Cancer (TNBC), due to the negative values of ER, PGR and HER2 by immunohistochemical staining, but not all TNBC have the molecular portrait discussed in Basal-Like subtypes (47,48).

Besides intrinsic molecular classification, expression of ER, PGR, and HER2 has been used to group BC patterns. The below image helps understand the correlation between intrinsic molecular and gene expression subtyping of BC.

Intrinsic subtype		ER and/or PR	HER2
Luminal A-like	(LumA)	+	-
Luminal B/HER2 negative-like	(LumB/HER2 neg.)	+	-
Luminal B/HER2 positive-like	(LumB/HER2 pos.)	+	+
HER2-type	(HER2)	both-	+
Triple negative	(TN)	both-	-

Figure 1- Correlation between Molecular portrait (Intrinsic subtype) classification and immunohistochemical staining classification of ER, PR and HER2. Adapted from *Intrinsic Subtype and gene expression correlation* disclaimed in the *St Gallen International Expert Consensus of the primary therapy of Early Breast Cancer 2011*.

Although all the above subtypes have been classified and studied for decades, only in 2007 a new molecular subtype was found, called Claudin-low. This subtype is characterized by the low gene expression of claudins, and E-cadherin, both responsible for tight junctions in epithelial or endothelial cells. Claudin-low subtype showed irregular expression of keratins and low expression genes regarding HER2-enriched, Luminal A and B subtypes, meaning they also share the phenotype of TNBC. Conversely, Claudin-low revealed high expression levels of genes related to: immune response, cell communication, cell migration, cell differentiation, extracellular matrix and angiogenesis (49).

### **Breast Cancer Subtypes Prognosis**

To prioritize and define new BC drugs, and due to the large heterogeneity of this disease, there's a need to evaluate patients' outcome. Although age and tumour nodes are important to evaluate patients' prognosis, the focus will be kept of the cancer subtypes.

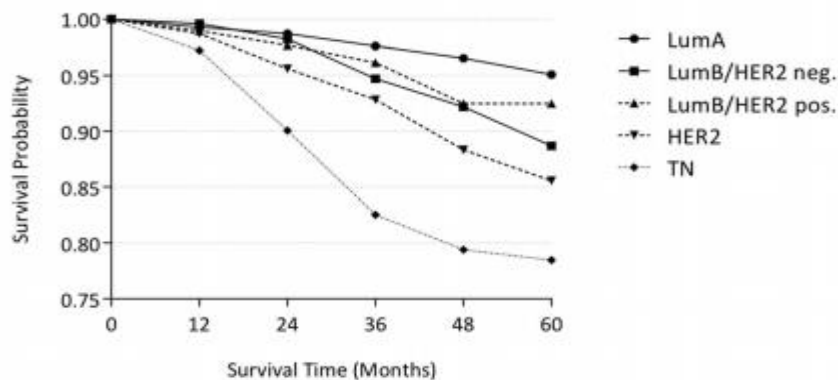


Figure 2 – Kaplan-meier plot of Overall Survival of local, unilateral non-metastatic breast cancer subtypes according to their molecular portrait and the corresponding survival time, measured in months after a lumpectomy or a mastectomy. Adapted from Hennings A, Riedel F, Gondos A, Sinn P, Schirmacher P, Marmé F, et al. Prognosis of breast cancer molecular subtypes in routine clinical care: A large prospective cohort study.

Observing the first image (Fig.2), a Kaplan-Meier plot compares the survival probability to the intrinsic subtype of BC in a two and half year period, after a lumpectomy or a mastectomy. As the results show, Luminal A has the best survival probability score and triple-negative has the lowest one. Since the results are from a prospective cohort study, with defined endpoints, the data does not show evidence of the effects of chemotherapy on survival probability (50).

Table 2 - Multivariable Cox proportional hazards analyses for 5-year post-recurrence on breast cancer mortality. Adapted from Kimbung S, Kovács A, Danielsson A, Bendahl P-O, Lovgren K, Stolt MF, et al. Contrasting breast cancer molecular subtypes across serial tumor progression stages: biological and prognostic implications.

<b>Biomarker status of primary tumours</b>	<b>N</b>	<b>Relative hazard</b>
<b>ER</b>	275	
Positive (reference)	210	1.0
Negative	65	2.2
<b>PR</b>	259	
Positive (Reference)	149	1.0
Negative	110	1.5
<b>Intrinsic Subtype</b>	175	
Luminal A (reference)	64	1.0
Luminal B vs Luminal A	81	1.3
HER2-enriched vs Luminal A	8	2.5
Triple Negative vs Luminal A	23	3.1

The table 2 represents the mortality in a five-year post-recurrence of BC according to gene expression subtype and intrinsic molecular subtype, defining the value as hazard ratios. As shown, patients with both ER and PR negative values were more likely to die. Also, the intrinsic subtypes were rated in accordance with the previous image, showing again, that the triple-negative phenotype has the worst prognosis. These values were adjusted for adjuvant endocrine or chemotherapy treatment, node status and age (51).

The above subject referred only to the prognostic value concerning primary tumours. It's important to understand that cancer can develop metastasis that will change the prognostic value and the therapy selected to the patient. Due to its relevance in patient outcome, it is necessary to discuss how metastasis develops and what are the consequences for the patients.

### **Breast Cancer Metastasis**

In every cancer, that is not removed or treated, there's always a chance that cells from a primary local tumour will migrate, through blood or lymph vessels, creating new tumour sites that will consume nearby tissues and organs.



This process is called metastasis. Due to the metastasis brutal invasive characteristic, current treatment isn't able to cure it. As for now, it can only stop its expansion, possibly leading to its elimination for a short period of time. Since recurrence probability stays high after tumour elimination, pharmaceutical companies are trying to develop better treatments that can lead to an increased length of time without new recurrences.

As the primary tumour gets access to the blood vessels, more possible sites become available within the body for new tumour cells to adhere. It's not yet explained why, for each type of tumour, there are some preferable sites than others. In breast cancer, a study recorded which sites were more targeted by metastasis in a small population sample (52). According to the figure 3, the most prevalent sites for BC metastasis are bone, lung and liver. It's important to notice that the image does not represent the first metastatic site and does not adjust the value for how many metastasis sites where before a new one shows up, it only shows the frequency on certain body organs. So how does the tumour forms metastasis?

Briefly, after the first appearance of a single cancer cell, due to its expression of molecules promoting proliferation and differentiation, it starts growing and multiplying faster than the surrounding healthy cells. Since this new tissue expands faster, it eventually reaches blood or lymphatic vessels and intravasates into them. On the blood stream, after evading the immune system they will adhere to capillary beds, followed by the extravasation into target tissue, and start to proliferate and induce angiogenesis. In this new site, just like the

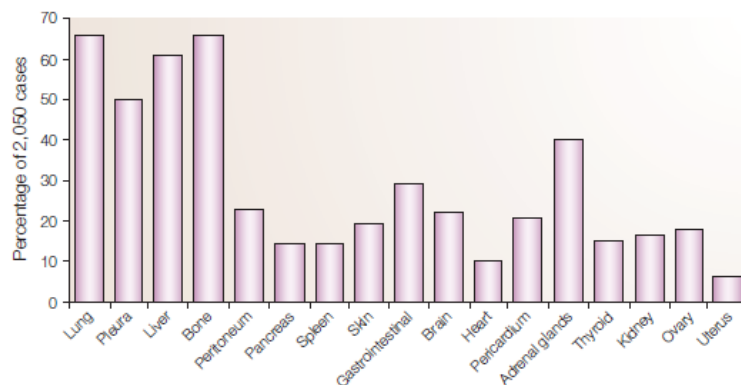


Figure 3 – The most common organs for a metastasis of breast cancer. Adapted from Weigelt B, Peterse Johannes L. Veer Laura J. Breast Cancer Metastasis: markers and models.

other, can now repeat the process, originating a second source of the metastasis (53). The detailed mechanisms of metastasis are complex and involve: cell to extracellular matrix and cell-to-cell adherence proteins alterations, morphologic changes, from epithelial to mesenchymal to promote cell migration and expression of angiogenic proteins (52). These steps are not further described since they're not essential for this thesis, however, they are critical to the successfulness of tumour metastasis and could become future targets for cancer treatment and so are worth mention.

As the prognosis of a metastasis-state cancer deeply worsens comparing to a local confined primary tumour, it's important to monitor as earlier as possible the induction of metastasis in order to increase the successfulness of the therapy.

The detection of early markers can increase the therapy efficiency through quicker elimination of metastasis. Many markers detect cancer cells within the blood, which can be very helpful because the tumour cell possibly hasn't adhered to a tissue target and hasn't also started to produce more cells that can lead to secondary metastasis. In association with early detection, quicker and less patient-invasive methods could increase the monitoring of the disease, also contributing to resolve the pathology.

Several markers are used to identify BC blood disseminated cells, which have great sensibility but lack specificity. No marker is exclusive of breast cancer disease, because it was also found in healthy individuals. However, some markers have been reported to be more specific in detection of breast cancer disseminated cells than others:

SCGB2A2 - Also known as mammaglobin, may be involved in signalling the immune response. Because it's not expressed on a variety of breast cancer subtypes, this protein has a limited use. SCGB2A1 and SCGB1D2 are other isoforms also used in detection, that have equivalent expression levels to SCGB2A2 (54).

PIP – Prolactin-inducible protein or gross cystic fluid protein-15, is specific and sensitive to apocrine differentiation, and its levels are correlated with ER and PGR levels (55). Although in low levels, PIP was found in other tissues, making it limited use.

TFF1 and TFF3 – Trefoil factor 1 and 3 are small cysteine-rich proteins containing one trefoil domain with 6 cysteines, linked by 3 disulphide bridges. These proteins are involved in the protection and regeneration of the luminal mucosa, preventing inflammation and benefiting cancer development. Estrogens increases the expression of these proteins, meaning they're markers for ER-positive breast cancer. Despite being specific, TFF1 and 3 are present in 65% of all breast cancer, limiting its prediction value (54).

SPDEF – SAM Pointed Domain containing Ets transcription Factor is a protein belonging to the ETs-domain family, whose expression deregulation, is associated with certain types of cancer (56). It's exclusively found in high epithelial tissues such as breast and prostate.

These are several other markers (54) used in single or multi-markers assays to detect breast cancer cells on the blood with different specific and sensibility degrees.

### **Breast Cancer Metastasis Prognosis**

As previously mention, a metastatic cancer has a severely worse prognosis, due to the invasiveness and the corresponding tissue damage in the organs affected. Relapse-free period and the different metastatic sites are crucial factors for evaluating the prognosis. A cohort study (57) with 797 patients was conducted, showing the overall survival correlated with the gene expression subtypes. Triple-negative subtype shows, again, the worse scenario with a

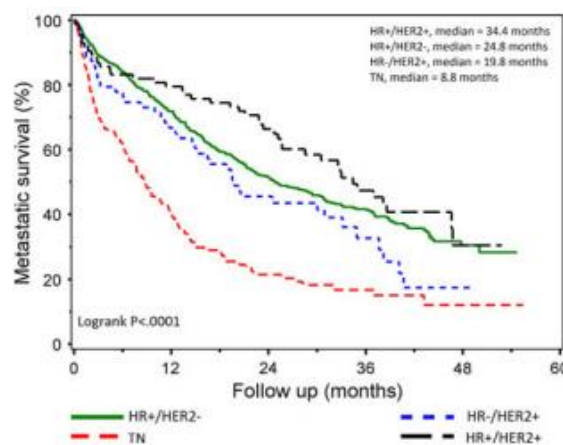


Figure 4 - Survival after diagnostic of a metastatic breast cancer according to the status of hormone receptor and HER2. Adapted from Lobbezoo DJA, van Kampen RJW, Voogd AC, Dercksen MW, van den Berkmortel F, Smilde TJ, et al. Prognosis of metastatic breast cancer subtypes: the hormone receptor/HER2-positive subtype is associated with the most favorable outcome.

medium survival value of 8.8 months, while HR+/HER2+ (Luminal B) shows the best prognosis, with a medium survival value of 34.4 months.

Compared to the primary tumour we can observe a marked decrease in the survival months after the diagnosis. This represents a bigger urgency in finding better treatments to target metastatic BC.

## **Treatment**

After a broad view of BC, it's important to understand the standard procedures currently used to treat it, in order to further discuss about targeted cancer therapies.

As mentioned, BC is very heterogenic, with different genes and proteins expressed on almost each new case, which makes it harder to have a unique and precise therapy that could overcome it. Besides, the treatment varies according the tumour stage (see Annexes).

On early stages, the patient submits to a lumpectomy, where the solid tumour plus the surrounding tissue will be removed, and might be followed up with radiotherapy, using x or  $\gamma$ -rays to damage the DNA of cancer cells, leading to apoptosis (58). Another option is to perform a mastectomy, removing all the affected breast.

On further stages, chemotherapy is added according to its phenotype (ER, PR and HER2 status), presence/absence of recurrences and/or metastasis. In chemotherapy, the approach to kill cancer cells is achieved with cytotoxic drugs that target: nucleic acids, DNA/RNA production, and cell cycle process. The general clinical use of chemotherapy is to shrink tumour size and to inhibit tumour growth so it can be further removed through surgery. In advance stages, chemotherapy will only sustain the patient's life for a short period of time.

These are some of the most used cytotoxic drugs in BC therapy. They can be single-used or mixed in combination, for increasing adjuvant results. Parameters like duration and amount, are deducted in each cancer case according to each patient response.

<b>Cytotoxic Drug</b>	<b>Mechanism of action</b>
Anastrozole/letrozole/exemestane	Inhibitor of aromatase enzyme.
Capecitabine/Fluorouracil	Inhibitor of thymine synthesis, breaking cell replication.
Carboplatin/Cyclophosphamide	Alkylates DNA forming adducts through crosslinks, blocking DNA replication or transcription.
Docetaxel/Paclitaxel	Binds to tubulin, perturbing the microtubule assembly and stopping cell cycle in G2/M phase.
Doxorubicin	Disturbs the DNA, inhibiting topoisomerase II.
Everolimus	mTOR inhibitor.
Lapatinib	Inhibitor of EGFR and of HER2 receptors
Methotrexate	Inhibitor of dihydrofolate reductase, stopping DNA synthesis.
Trastuzumab/Pertuzumab	Binds to the extracellular domain of the HER2 receptor, inhibiting cell- proliferation.

Most of the drugs lack BC selectivity. This leads to adverse effects manifested by the patients receiving the treatment. Besides the psychological burden, vomiting, nausea and hair loss are also a part of the therapy regime downside. Yet, after concluding the therapy, recurrences might appear, forcing the patient to do more procedures or, if the therapy fails, it will, ultimately, lead to death.

There's a worldwide need to find new markers and/or new drugs that can target BC cells exclusively, first suppressing cancer growth and then eliminating the malignant cells.

## **Objective**

Review and discuss Targeted Therapies in Breast Cancer and comparing with current therapy.

## **Materials and Methods**

To obtain information on targeted therapies in BC the following databases we're used: B-on, PubMed (on Best Match mode), Google Scholar, Nature, Cell and Clinical Oncology Journals.

To select the results, the following exclusion criteria were applied: older than 2007, exclusive detection studies, exclusive imaging applications. Inclusion criteria: perspective, novel, selective, in vivo. Only those who were accessible were used for discussion and only the top 25 matches were selected.

Concepts used: Novel/New, Targeted, Selective, Drugs, Breast Cancer.

## **Results**

By applying the above methods, a total of 40 articles were analysed for discussion.

## Discussion

From the above cytotoxic drugs currently used in BC therapy, a common characteristic stands out: none of these drugs are anti-BC designed. Although they have clinical value, they're only successful if the cancer is detected in an early stage, and it still must be further removed through a lumpectomy.

Some of the drugs act by inhibiting DNA synthesis, microtubule assembly and topoisomerase II. Even if it's administrated in a local-region, bypassing the blood circulation, the drug itself can't distinguish between malignant and normal cells, because these inhibitory mechanisms are also present in all cells. So, by killing healthy cells, besides failing the therapy purpose, adverse effects will likely occur.

Non-DNA targeted drugs aren't also BC specific. Lapatinib and trastuzumab act by binding to the HER2 receptor. HER2 (ErbB-2) receptor belongs to the human epidermal growth factor family, which is overexpressed in 20-30% of breast cancers (59). An overexpression is simply an abnormally high level of synthesis compared to the normal basal levels. This means that healthy cells also express this receptor, just not in large amounts as found in BC cells. Therefore, although these drugs will bind more often to cancer cells, they will also bind to normal cells, creating a lack in cancer cell selectivity. Lapatinib additionally binds to EGFR (ErbB-1) which is another receptor from the same family as HER2 and equally expressed in other organs, facing the same selective problem.

Everolimus acts by inhibiting the mammalian target of rapamycin (mTOR), which is a serine/threonine kinase belonging to the phosphoinositide kinase-related family of protein kinases (PIKK). It coordinates several cellular functions as survival, proliferation and differentiation (60). Mutations in the mTOR pathway have been found in increased levels in ER positive BC. These mutations allow an increment level of mTOR, which is associated with resistance to endocrine therapy. Everolimus can be combined with exemestane to increase clinical outcome (61). However, this approach lacks selectivity between normal and malignant cells.

Before analysing novel therapies in study, it's important to refer why some therapies fail to be more efficient on current clinical practices. Cancer cells can avoid being targeted by the human's immune response system, and by cytotoxic drugs. Regarding the immune response system, once T-cell recognises tumour cells, they release interferons through their T-cell receptor, which will boost the immune response by recruiting other leukocytes. One theory suggest that the malignant cell activates an interferon-inducible immune suppressive factors, limiting the immune response system and increasing tumour ability to survive. Another theory, only tested in mouse models, suggests that in the presence of T-cell pro-inflammatory cytokines, tumour cells changes its phenotype, reducing the expression of differentiated antigens, and assuming a less-differentiated state, thus avoiding the immune response system (62).

Cancer cells have different ways of resisting to a cytotoxic drug: 1) by changing the target from which the cytotoxic drug was designed. These changes usually involve mutations, lowering the binding between the drug and the target. This brings opportunity to develop further generations of cytotoxic drug that will target the altered phenotype. 2) upstream or downstream reactivation of target pathway. This resistance mechanism was found in anti-HER2 BC therapy with trastuzumab or lapatinib, through changes in the activation of the PI3K pathway, which, in turn, signals downstream of HER2. 3) drug-dependent survival. The malignant cell re-writes certain pathways signalling survival that become drug-dependent, meaning that they will only be active in the presence of the drug. In some cases, with the withdrawal of the cytotoxic drug, the pathway turns inactive, and the cell dies. [4] through cross-talk activation of similar pathways. When inhibiting, for example, the MAPK pathway, that leads to tumour survival, studies found that a similar pathway, PI3K, that also lead to tumour survival, was activated, suggesting a cross-talk between these signalling pathways upon internalization of cytotoxic drug.(63)

Understanding tumour-resistance development will help in developing novel generations of both resistance-inducible drug and therapies targeting the resistance acquisition.



Results found are grouped according to drug class, allowing a brief explanation of the pharmacokinetics proprieties and difficulties to overcome in target drug-delivery of each group.

### **Small-Molecules**

Administration of small-molecules may be the most difficult approach of developing efficient therapies. To design them, one must consider all the barriers facing drug delivery. The most important one, obligates the molecule to be stable in an aqueous solvent, around pH levels of 7.35-7.45, which are the physiological conditions, otherwise it will react and possibly lose its functional moiety. Other barriers such as drug absorption, distribution, metabolism, and excretion, may be avoided through an appropriate administration route choice.

Small-molecules are the generic drug type, and despite novel drug innovations, such as the creation of biosimilars, there's still an ongoing-search for novel small-molecules as a therapeutic agent.

A review (64), lists pharmaceuticals' small-molecules on clinical trials to be proven efficient in metastatic BC. These drugs target pathways that are quantitative altered in cancer cells. Nonetheless, these pathways, as previously discussed, are also present in normal cells, and so, these molecules fail to be a truly targeted therapy.

A study (65) showed a promising approach to TNBC androgen receptor (AR) positive subtype. It shows that administrating AR agonists on the malignant cells, revealed a 50% bigger growth inhibition effect compared to placebo. However, AR is not exclusively expressed in BC, it's also present in other tissues, such as: ovary, uterus and fallopian tubes (66), demonstrating the lack of selectivity in the current small-molecules targeting ability.

Many more studies and reviews(67–78), report promising small-molecules discovered, with different targeting strategies. However, there's always a lack of selectivity, because they only target an overexpressed pathway or receptor, not exclusive of BC.

A study stood out from the others, regarding selectivity in targeting malignant cells (79). It explains that carbonic anhydrase IX (CAIX) might be vital to the metastasis progression, and that it plays an important role in tumour survival on hypoxic conditions, through the regulation of the cell's pH. CAIX is a membrane-bound enzyme that reversibly catalyses the hydration of CO<sub>2</sub>, exclusively expressed on hypoxic tumours, including some forms of BC. This enzyme has been targeted with sulphonamide-based imaging probes and antibodies (80,81). Due to its selectivity, CAIX could also become an interesting target to the development of drug-delivery strategies. There are already some inhibitors, derivatives of acetazolamide, that show tumour growth inhibition but lack specificity for CAIX. Another inhibitor reported in a different paper (82) is CAI17, described as a highly selective sulphonamide-based inhibitor. CAI17 inhibitor acts as a prodrug and the strategy behind its development relies on the disulphide bond that will be further reduced due to the hypoxic conditions and the presence of thioredoxin 1 protein, forming a thiol group.

This discovery can also lead to novel tumour elimination strategies, as new drugs could locally force a hypoxic state in the malignant cells, allowing the endogenous expression of CAIX to be further targeted.

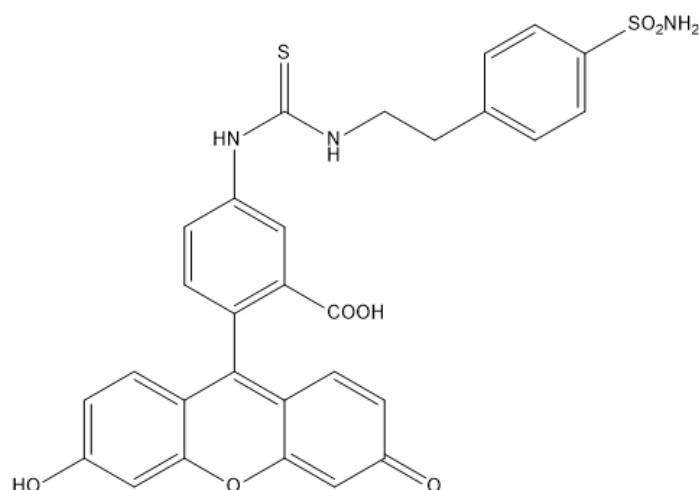


Figure 5 - Molecular structure of CAIX inhibitor CAI17. Adapted from Lou Y, McDonald PC, Oloumi A, Chia S, Ostlund C, Ahmadi A, et al. Targeting Tumour Hypoxia: Suppression of Breast Tumour Growth and Metastasis by Novel Carbonic Anhydrase IX Inhibitors.

Another inhibitor, EZN-2968, a synthetic locked nucleic acid antisense oligodeoxynucleotides is being developed to target (hypoxia-inducible factor) HIF-1 $\alpha$  (83). HIF-1 is a heterodimeric transcription factor activated in hypoxic conditions to maintain tumour survival and progression. It consists of two

subunits, HIF-1 $\alpha$  and HIF-1 $\beta$ . Only the HIF-1 $\alpha$  subunit expression is oxygen sensitive, while HIF-1 $\beta$  expression is uniquely concomitant. Despite also being expressed in normal cells, HIF-1 $\alpha$  undergoes a rapid degradation process, having a half-life of only 5 minutes. However, under hypoxic conditions, it can be stabilized through several pathways for longer periods. HIF-1 $\alpha$  also regulates CAIX expression levels (84). HIF-1 $\alpha$  represents a promising target regarding broad cancer selectivity (85).

As analysed, very few small-molecules have promising approaches to efficiently target BC cells. However, they have clinical potential as single targets, and can be proven useful when further conjugated with another molecule responsible for selectively delivering the drug.

### **Monoclonal Antibodies**

Monoclonal antibodies (mAb) are glycoproteins belonging to the immunoglobulin superfamily endogenously produced by B cells. They are  $\gamma$ -shaped, consisting of two chains: the heavy and the light chain. The heavy chain, has three constant regions and one variable region, while the light chain has one constant region and one variable region. Both variable regions form the antigen-binding fragment and when presented with an antigen they will bound exclusively to it, due to six peptide loops known as the complementarity-determining regions (CDR) (86,87). Manipulating the CDR has a great therapeutic value, since this region binds selectively with the complementary antigen, allowing novel drugs to be more efficient. This is the main reason why, after the release of the first mAb to the market, there's still an ongoing growth of its production (88).

mAb are high molecular weight proteins (usually about 150 kDa), and very water soluble. Such characteristics make them ideally stable in the vascular compartment, with very small percentage found in the extracellular fluid. Administration routes as sub-cutaneous or intra-muscular are not eligible due to their proprieties. Thanks to their high weight, filtration through the glomerulus does not occur, increasing its serum half-life, sometimes reaching 21 days. They reach the extracellular compartment due to extravasation, endocytosis or pinocytosis through the blood vessels. After reaching the extracellular fluid, they

bind through the CDR region and suffer cellular internalization followed by proteolysis, due to the lysosomal vesicles present within the cell (89).

These properties make antibodies a promising drug in targeting BC, despite failing at local-region administration.

Ganitumab, cixutumumab, dalotuzumab and OSI-906 are mAb, used in metastatic BC, designed to target insulin-like growth factor-1 receptor (IGF-1R). This receptor is implicated in cell growth pathways, of both normal and malignant cells. MEDI-573 and BI836845 act by binding to the IGF I and II, and not the receptor (64,90–92).

Other mAb such as bevacizumab were developed to target VEGF (93). Vascular endothelial growth factor, is a protein that increases blood vessels production. However, it's not its only role, it's also important in bone formation, wound healing and haematopoiesis, and so, it's not exclusive of malignant cells (94).

Yet again, despite the significant clinical value in BC, these antibodies fail to be a selective approach to treat BC, since they can't distinguish normal cells.

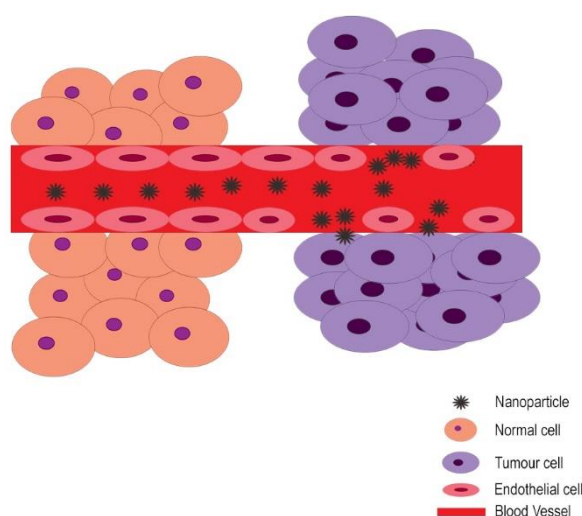
The pharmaceutical companies test their patent mAbs in different cancers to target cell-broad pathways, so they can additionally prove its value on another cancer type to increase their total drug market value. However, even with successful clinical value, these mAbs will not bring a targeted approach.

## **Nanoparticles**

Nanoparticles are compounds with an average range of 1 to 1000 nm ( $10^{-9}$  m). When used clinically they must achieve a closer range of 10 to 200 nm. This limitation is set due to two conditions. The smaller value accounts for the 5 nm renal filtration cut-off, increasing the drug's blood half-life and eventually increasing its efficiency. The bigger value accounts for the standard 220 nm filter used in theranostic applications. Theranostic is a term describing the diagnostic and therapeutic properties of the same drug, that advance nanoparticles present. Usually nanoparticle drugs may contain one or more of the following: liposomes, micelles, polymers, dendrimers and/or macromolecules (95,96). Nanoparticles are a great drug design option due to the enhanced permeability and retention

(EPR) effect on tumour cells, compared with other drug classes. The EPR effect is a phenomenon occurring in the vascular tissue around a solid tumour. Due to the tumour-induced angiogenesis surrounding blood vessels, there's a gap in the junction of the endothelial cells and, additionally, a lack of smooth muscle layers. This creates large fenestrations that allow drugs to leave the circulatory system and deposit on the tumour cells. Additionally, overexpression of vascular mediators such as nitric oxide, bradykinin, etc. enhances the permeability of these vessels (97). This effect grants additional selectivity compared with the previous discussed drug classes. To design nanoparticles, understanding its pharmacokinetics and pharmacodynamics is critical. Factors like PEGylation, size, composition, zeta potential, and shape, will determine its clearance due to its opsonization with macrophages on the liver and spleen (96).

The PEGylation steps consist of using polyethylene glycol (PEG) in the surface of these drugs to reduce serum protein binding through steric hindrance.



*Figure 6 - EPR effect. The endothelium cells in the blood vessels near the malignant cells have fenestrations that allow passage and deposition of nanoparticles.*

Although it has its relevance in drug deliver optimization, it also induces immune response and hypersensitivity, remaining its use unclear, and making necessary a benefit/risk analysis (96).

Another concept to keep in mind is ligand addition. A study compared the penetration ability of two micelles, one with ligands of the surface and another without them. Surprisingly, the micelles without the surface ligand showed better penetration. This effect is described as the binding site barrier, that difficults nanoparticle internalization (96).

Regarding BC targeting, one study (98) developed a curcumin lipid-base nanoparticle. It reports that curcumin, a yellow pigment, has anti-oxidant and anti-tumour properties, implicated in several cell mechanisms. However, a nanoparticle system had to be designed due to its poor stability on physiological conditions. To increase its targeting value, folate was added as a surface ligand, because many solid tumours have an increased expression of folate receptors. According to the results, this nanoparticle showed promising tumour growth inhibition, and reduced toxicity.

Folate receptors are reported to be great markers for targeting cancer in IV strategies, since they can be selective in targeting malignant cells. Normal cells express the folate receptor on the apical membrane surface of polarized epithelial cells, away from the blood circulation, preventing interaction with folate-mediated drugs. Upon malignant transformation, they lose its polarity and the folate receptor becomes available to the blood circulation, being ready to react with folate (99). However, a pregnant woman's breast cells also release folate to the milk in order to feed the embryo (100). Usage of folate to selective distinguish malignant cells requires a patient filtration to deliver an effective drug.

Other results found (101–106), share the selective targeting issue, due to the use of ligands that won't distinguish normal from malignant cells. However, their design strategies are different and interesting for discussion.

One group, developed a multifunctional magnetic polymer nanoparticle with "switchable On-Off" states. This nanoparticle acts accordingly to the environmental pH. On the blood the core stays intact, but when internalized to the cell, the acidic pH disintegrates the nanoparticle, allowing a release of the desirable drug. This effect was achievable through the use of hydrophobic polymer, D,L-lactic-co-glycolic acid (PLGA). This strategy largely increases drug stability in blood circulation (102).

Another nanoparticle design used mesoporous silica with fluorescein isothiocyanate (FITC) in the internal surface, conjugated with the HER2 mAb. The use of FITC-labelling antibodies allows imaging detection upon CDR binding to the antigen, through the emission of green fluorescence. The emission is granted by the structural changes suffered by the Fc portion of the antibody (107). This

mesoporous silica nanoparticles also allow a tailor design due to their controlled pore size and surface functionalization (103).

Another study (106), developed a slightly different nanoparticle. While the others had a core-like or porous-like structure, this study developed a biomimetic vector, with a rod-like structure. It contained: plasmid DNA, nuclear localisation signal, a DNA condensing motif, an endosomal disruptive motif, a cathepsin substrate and a cyclic targeting peptide, CPX (108). This fragmentation allows the condensation of the DNA to the nano-scale, protection from serum endonucleases, disruption of endosomal membranes and selective cell targeting. Interestingly, the targeted peptide showed selectivity to a specific BC cell line and not to a normal breast cell line. However, clinical practice has demonstrated that BC demands broader strategies to be developed to deliver an efficient therapy.

Two studies used gold nanoparticles (104,105). These compounds are widely explored in biomedicine applications. They have excellent compatibility with the physiological conditions, demanded by the cells, low toxicity, and can interact with a great variety of substances (109). One study found an interesting approach to treat cancer, using photothermal laser on the gold nanoparticles. Upon internalization of the gold nanoparticles, further near-infrared light is used to trigger the cells to enter hyperthermia, leading to cell death. The study showed that the amount of killed cells can be controlled by the exposure time and power density of the light. Additionally, cells that did not internalized gold nanoparticles, when submitted to the same treatment, maintained their integrity (104). This strategy can grant a precise and selective method to the treat BC, with the addition of a conjugated fluorescence probe and with real-time imaging technology, allowing the selection and destruction in real-time of each malignant cell.

As seen, the design of nanoparticles is complex with many different cores, layers and layouts able to integrate a variety of substances. Although nanoparticles are more complex drugs than small molecules or mAb, the selection of an appropriate selective target still is the critical step in delivering successful therapies. However, due to their EPR effect, they're additionally more selective than most of the drugs discussed so far.

## Bioconjugates

Bioconjugation is a branch in chemistry that studies the ability to link two functional molecules through a stable inert linker, that usually forms covalent bonds with the molecules. It can also have theranostics properties, depending on the nature and intent of the molecules used. As previously mentioned it can be used in the design of nanoparticles. However, in this drug class, only non-nanoparticle therapies will be discussed.

A group of conjugates that are gaining importance are antibody-drug conjugates (ADC). This drug class consists on the binding of a mAb to a cytotoxic drug via a stable linker. Through binding both molecules, the conjugate surpasses each ones' limitations while preserving their benefits. Another component, crucial to the design of ADC is the linker. The linker must be stable at physiological conditions, only releasing the drug when presented with a previous defined target, such as acidic pH or specific proteases. However, if the linker is not stable it can release the drug earlier than expected and increase drug toxicity (110).

One ADC design to BC is Trastuzumab-DM1 (111). DM1 is a maytansinoids derivative of the drug maytansine. It acts by binding with the microtubules inhibiting the mitosis process. The initial linker used consists of disulphide bridges, however, linker cleavage was reported as inefficient. When the linker changed to a thioether bridge, it showed increase efficiency. *In vivo* studies also confirmed the increased growth inhibition of the ADC compared with the mAb or the small drug alone, promising a better alternative to standard trastuzumab alone therapy.

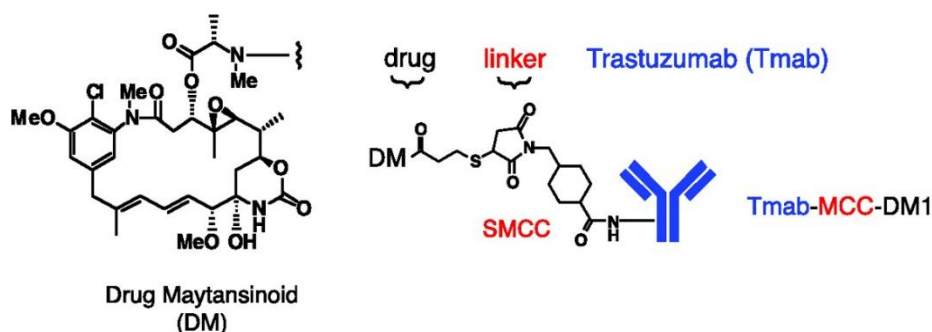


Figure 7 -Trastuzumab-DM1 structure scheme. Adapted from Lewis Phillip GD, Li G, Dugger DL, Crocker LM, Parsons KL, Mai E, et al. Targeting HER2-positive Breast Cancer with trastuzumab-DMA1, an Antibody-Cytotoxic Drug Conjugate.



Another ADC design used FITC-labelled trastuzumab-grafted G4 PAMAM [poly(amido)amine] dendrimers. Through the terminal amine of PAMAM, five FITC molecules were bonded. To link the mAb, a PEG-Maleimide spacer was used. After reacting a trastuzumab's amine with the Straut's reagent, the mAb was ready to be linked to the FITC-PAMAM-PEG-MAL structure, forming a complex conjugate. The dendrimers were previously loaded with docetaxel. *In vitro* fluorescent microscopy, showed a great growth inhibition effect of the conjugate, compared with dendrimer-loaded docetaxel without trastuzumab, and greater than docetaxel alone. Docetaxel plasma concentrations were also greatly increased with the use of the conjugated compared with docetaxel alone (112).

As the HER2 receptor is only overexpressed and not selectively expressed in BC, these strategies lack selective properties.

However, an interesting study designed a conjugate that can distinguish high HER2 expression levels from low levels (113). This study, aims to target STAT3 (signal transducer and activator of transcription 3), which is suspected to induce Erb2 transformation and tumour progression on Erb2 overexpressed BC cells. To selectively target these cells, some CPP (cell-penetrating peptides) showed promising results due to its effectiveness of crossing cell membranes and delivering bioactive cargos. One of those examples is the transduction and transactivation (TAT) protein of HIV. Using TAT derivatives conjugated with an anti-HER-2/*neu* peptide mimetic (AHNP) promises to be a great selective delivery system that will further incorporate STAT3BP, known to inhibit STAT3 signalling. The results, showed a selective internalization of the TAT-AHNP-STAT3BP to BC cell lines with an overexpression of HER-2, compared with cell lines with basal HER-2 expression levels. Additionally, it showed that *in vivo* results, where HER-2 overexpressed mouse had a greater apoptosis effect, with designed drug, compared with a HER-2 cell line with normal expression levels. This strategy is one of the effective BC target therapies due to its targeting ability described in this study. However, only 30% of the BC subtypes are HER-2 positive, demanding novel strategies to treat other BC subtypes.

Another study using CPP promises a novel strategy selectively targeting BC cells (114). The designed has: 1) SP90, a 12 amino-acid tumour homing peptide with selective properties to breast cancer cells, 2) C peptide, a 29 amino-

acid CPP derived from the heparin-binding domain of superoxide dismutase, 3) a Viral Protein R, a small apoptotic protein and 4) a green fluorescence protein. Although this study lacks *in vivo* report, it showed BC selectivity towards ER+/PR+/HER2- and triple-negative cell-lines, compared with normal breast cell and other cancer cell lines. Additionally, it showed that only the full drug presented cell internalization, while other incomplete parts failed to either be selective or internalized. Despite the targeting ability of SP90, there's no information about its molecular binding target.

These studies demonstrate the clinical value of drug designs containing CPP to selectively internalize drugs on BC cells, and can even be a promising approach to treat triple-negative cell line, which has the worst prognosis.

### **Future Perspectives**

As reviewed, BC is a dangerously disease, affecting mostly women world-wide. It's heterogenous, with many different phenotypes, being almost each case unique. Regarding current chemotherapy, it only acts as adjuvant, allowing a reduction of the growth and size of the tumour, so it could be further removed through a surgical procedure. Additionally, current therapy can't distinguish normal cells from malignant cells, causes adverse effects on the patient, leads to a painful therapy, causing some people to abandon it. In some advance forms of BC (metastasis), the drug therapy only allows an extension of the patient's life, usually few months.

There's a demand in finding a solution to treat BC. One without side effects and not invasive to the patient. To manage such achievement, as discussed, many researchers are developing novel approaches, to selectively target BC using drugs. However, only few prove to be an effective approach, because many did not have an unique target to deliver drugs selectively onto BC cells.

An interesting approach, is targeting either HIF-1 $\alpha$  or CAIX, since they're both selective markers expressed only when cells suffer hypoxia (84,115). Instead of neutralizing these proteins activity in cell survival, they should be kept expressed to design selective strategies. Regarding HIF-1 $\alpha$ , one hypothesis is to locally induce malignant cell expression. To do so, a study found out that under

47°C cells start to express HIF-1 $\alpha$ , through ERK and ARK pathways on lung cancer cell lines (116). Initially, BC cells would be exposed to the heat through a targeted approach, and next, an ADC could be designed, whereas the mAb would be developed to target the HIF-1 $\alpha$  and, linked to it, a drug reported to induce apoptosis on BC cells.

Another great strategy discussed was the use of folate as a selective ligand to target BC cells. Further investigating folate receptor expression, a study (117) compared the expression levels on both normal and malignant breast cell lines, as well as folate expression on other normal and malignant cells on different organs. As reported, larger expression was found in malignant breast cells, however, not in a selective amount from normal cells. Additionally, IV administration therapies would fail to be selective because lung and kidney normal cells lines express equal or bigger amounts of the folate receptor. The folate targeting strategy remains inconclusive, as different studies suggests different theories.

CPP also proved to be an efficient approach on selective internalizing onto BC, when attached with a homing protein. Their internalization is supposed to occur through the electrostatic affinity of the CPP positive charges to the negatively charged proteoglycans and phospholipids on cell surface. To design these peptides, it's important to attribute the right charge and hydrophobic proprieties, because it will decide if the peptide can enter the cell. This study (118) mentioned the importance of arginine residues on the CPP rather than lysine. This residue change is due to the interaction of arginine to the cell surface being greater than with the lysine group. The guanidinium group of arginine forms bidentate hydrogen bonds with the negatively charged phosphate, sulphate and carboxylate groups on cell surface, while lysine only forms one hydrogen bond.

Hydrophobicity also plays an important role as it is crucial to enter the cell lipid bilayer. Among hydrophobic residues, aromatic groups can evenly help both hydrophobic and charged proprieties of the CPP. Tryptophan was proved to be an efficient residue regarding cellular uptake. Plus, tryptophan, as arginine, can interact with sugar rings on cell surface. Additionally, tryptophan residues, when near to arginine residues, changes the later pKa of the guanidinium group to a more positively charged state, allowing a better cell internalization of the CPP.

Table 3 – Studied cell-penetrating peptides and their respective sequence's. Adapted from Bechara C, Sagan S. Cell-penetrating peptides: 20 years later, where do we stand?

<b>Peptide</b>	<b>Sequence</b>
<i>Protein derived</i>	
Penetratin	RQIKIWFQNRRMKWKK
Tat peptide	GRKKRRQRRRPPQ
pVEC	LLIILRRRIRKQAHASK
<i>Chimeric</i>	
Transportan	GWTLNSAGYLLGKINLKALAALAKKIL
MPG	GALFLGFLGAAGSTMGAWSQPKKKRKV
Pep-1	KETWWETWWTEWSQPKKKRKV
<i>Synthetic</i>	
Polyarginine	(R) <sub>n</sub> ; 6 < n < 12
MAP	KLALKLALKALKALKLA
R <sub>6</sub> W <sub>3</sub>	RRWWRRWRR

Ultimately conjugating CPP with the previously designed gold nanocages that, when targeted by near-infrared light causes cell death, seems an effective drug that can possibly lead to a strategic targeting method to treat BC cells.

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## Annexes

Table 4 – Breast Cancer Classification. Adapted from Breast Cancer Staging: TNM Classification for Breast Cancer

<b>Primary Tumour (T)</b>	
TX	Primary tumor cannot be assessed
T0	No evidence of primary tumor
Tis	Carcinoma in situ
Tis (DCIS)	Ductal carcinoma in situ
Tis (LCIS)	Lobular carcinoma in situ
Tis (Paget)	Paget disease of the nipple NOT associated with invasive carcinoma and/or carcinoma in situ (DCIS and/or LCIS) in the underlying breast parenchyma. Carcinomas in the breast parenchyma associated with Paget disease are categorized based on the size and characteristics of the parenchymal disease, although the presence of Paget disease should still be noted
T1	Tumor ≤ 20 mm in greatest dimension
T1mi	Tumor ≤ 1 mm in greatest dimension
T1a	Tumor > 1 mm but ≤ 5 mm in greatest dimension
T1b	Tumor > 5 mm but ≤ 10 mm in greatest dimension
T1c	Tumor > 10 mm but ≤ 20 mm in greatest dimension
T2	Tumor > 20 mm but ≤ 50 mm in greatest dimension
T3	Tumor > 50 mm in greatest dimension
T4	Tumor of any size with direct extension to the chest wall and/or to the skin (ulceration or skin nodules)
T4a	Extension to chest wall, not including only pectoralis muscle adherence/invasion
T4b	Ulceration and/or ipsilateral satellite nodules and/or edema (including peau d'orange) of the skin, which do not meet the criteria for inflammatory carcinoma
T4c	Both T4a and T4b
T4d	Inflammatory carcinoma
<b>Regional Lymph Nodes (N)</b>	
<b>Clinical</b>	
NX	Regional lymph nodes cannot be assessed (eg, previously removed)
N0	No regional lymph node metastasis

N1	Metastasis to movable ipsilateral level I, II axillary lymph node(s)
N2	Metastases in ipsilateral level I, II axillary lymph nodes that are clinically fixed or matted or in clinically detected* ipsilateral internal mammary nodes in the <i>absence</i> of clinically evident axillary lymph node metastasis
N2a	Metastases in ipsilateral level I, II axillary lymph nodes fixed to one another (matted) or to other structures
N2b	Metastases only in clinically detected* ipsilateral internal mammary nodes and in the <i>absence</i> of clinically evident level I, II axillary lymph node metastases
N3	Metastases in ipsilateral infraclavicular (level III axillary) lymph node(s), with or without level I, II axillary node involvement, or in clinically detected * ipsilateral internal mammary lymph node(s) and in the <i>presence</i> of clinically evident level I, II axillary lymph node metastasis; or metastasis in ipsilateral supraclavicular lymph node(s), with or without axillary or internal mammary lymph node involvement
N3a	Metastasis in ipsilateral infraclavicular lymph node(s)
N3b	Metastasis in ipsilateral internal mammary lymph node(s) and axillary lymph node(s)
N3c	Metastasis in ipsilateral supraclavicular lymph node(s)
<b>Pathologic (pN)</b>	
pNX	Regional lymph nodes cannot be assessed (for example, previously removed, or not removed for pathologic study)
pN0	No regional lymph node metastasis identified histologically. <i>Note:</i> Isolated tumor cell clusters (ITCs) are defined as small clusters of cells $\leq 0.2$ mm, or single tumor cells, or a cluster of $< 200$ cells in a single histologic cross-section; ITCs may be detected by routine histology or by immunohistochemical (IHC) methods; nodes containing only ITCs are excluded from the total positive node count for purposes of N classification but should be included in the total number of nodes evaluated
pN0(i-)	No regional lymph node metastases histologically, negative IHC
pN0(i+)	Malignant cells in regional lymph node(s) $\leq 0.2$ mm (detected by hematoxylin-eosin [H&E] stain or IHC, including ITC)
pN0(mol-)	No regional lymph node metastases histologically, negative molecular findings (reverse transcriptase



	polymerase chain reaction [RT-PCR])
pN0(mol+)	Positive molecular findings (RT-PCR) but no regional lymph node metastases detected by histology or IHC
pN1	Micrometastases; or metastases in 1-3 axillary lymph nodes and/or in internal mammary nodes, with metastases detected by sentinel lymph node biopsy but not clinically detected
pN1mi	Micrometastases (> 0.2 mm and/or > 200 cells, but none > 2.0 mm)
pN1a	Metastases in 1-3 axillary lymph nodes (at least 1 metastasis > 2.0 mm)
pN1b	Metastases in internal mammary nodes, with micrometastases or macrometastases detected by sentinel lymph node biopsy but not clinically detected
pN1c	Metastases in 1-3 axillary lymph nodes and in internal mammary lymph nodes, with micrometastases or macrometastases detected by sentinel lymph node biopsy but not clinically detected
pN2	Metastases in 4-9 axillary lymph nodes or in clinically detected internal mammary lymph nodes in the absence of axillary lymph node metastases
pN2a	Metastases in 4-9 axillary lymph nodes (at least 1 tumor deposit > 2.0 mm)
pN2b	Metastases in clinically detected internal mammary lymph nodes in the absence of axillary lymph node metastases
pN3	Metastases in $\geq 10$ axillary lymph nodes; or in infraclavicular (level III axillary) lymph nodes; or in clinically detected ipsilateral internal mammary lymph nodes in the presence of $\geq 1$ positive level I, II axillary lymph nodes; or in > 3 axillary lymph nodes and in internal mammary lymph nodes, with micrometastases or macrometastases detected by sentinel lymph node biopsy but not clinically detected; or in ipsilateral supraclavicular lymph nodes
pN3a	Metastases in $\geq 10$ axillary lymph nodes (at least 1 tumor deposit > 2.0 mm); or metastases to the infraclavicular (level III axillary lymph) nodes
pN3b	Metastases in clinically detected ipsilateral internal mammary lymph nodes in the presence of $\geq 1$ positive axillary lymph nodes; or in > 3 axillary lymph nodes and in internal mammary lymph nodes, with micrometastases or macrometastases detected by sentinel lymph node

	biopsy but not clinically detected
pN3c	Metastases in ipsilateral supraclavicular lymph nodes
<b>Distant Metastasis (M)</b>	
M0	No clinical or radiographic evidence of distant metastasis
cM0(i+)	No clinical or radiographic evidence of distant metastases, but deposits of molecularly or microscopically detected tumor cells in circulating blood, bone marrow, or other nonregional nodal tissue that are no larger than 0.2 mm in a patient without symptoms or signs of metastases
M1	Distant detectable metastases as determined by classic clinical and radiographic means and/or histologically proven > 0.2 mm

Table 5 – Breast Cancer Stages. Adapted from Breast Cancer Staging: TNM Classification for Breast Cancer

<b>STAGE</b>	<b>T</b>	<b>N</b>	<b>M</b>
0	Tis	N0	M0
IA	T1	N0	M0
IB	T0	N1mi	M0
	T1	N1mi	M0
IIA	T0	N1	M0
	T1	N1	M0
	T2	N0	M0
IIB	T2	N1	M0
	T3	N0	M0
	T0	N2	M0
IIIA	T1	N2	M0
	T2	N2	M0
	T3	N1	M0
	T3	N2	M0
IIIB	T4	N0	M0
	T4	N1	M0
	T4	N2	M0
IIIC	Any T	N3	M0
IV	Any T	Any N	M1

Table 6 - Amino Acid three letters and one letter codes.

<b>Amino Acid Code Designation</b>		
Amino acid	Three Letter Code	One Letter Code
Alanine	Ala	A
Arginine	Arg	R
Asparagine	Asn	N
Aspartic Acid	Asp	D
Cysteine	Cys	C
Glutamine	Gln	Q
Glutamic Acid	Glu	E
Glycine	Gly	G
Histidine	His	H
Isoleucine	Ile	I
Leucine	Leu	L
Lysine	Lys	K
Methionine	Met	M
Phenylalanine	Phe	F
Proline	Pro	P
Serine	Ser	S
Threonine	Thr	T
Tryptophan	Trp	W
Tyrosine	Tyr	Y
Valine	Val	V

## Conjugated gold nanoparticles synthesis scheme (119,120)

