

Review

Experimental Models as Refined Translational Tools for Breast Cancer Research

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Abstract: Breast cancer is one of the most common cancers worldwide, which makes it a very impactful malignancy in the society. Breast cancers can be classified through different systems based on the main tumor features and gene, protein, and cell receptors expression, which will determine the most advisable therapeutic course and expected outcomes. Multiple therapeutic options have already been proposed and implemented for breast cancer treatment. Nonetheless, their use and efficacy still greatly depend on the tumor classification, and treatments are commonly associated with invasiveness, pain, discomfort, severe side effects, and poor specificity. This has demanded an investment in the research of the mechanisms behind the disease progression, evolution, and associated risk factors, and on novel diagnostic and therapeutic techniques. However, advances in the understanding and assessment of breast cancer are dependent on the ability to mimic the properties and microenvironment of tumors *in vivo*, which can be achieved through experimentation on animal models. This review covers an overview of the main animal models used in breast cancer research, namely *in vitro* models, *in vivo* models, *in silico* models, and other models. For each model, the main characteristics, advantages, and challenges associated to their use are highlighted.

Keywords: oncology; breast cancer; experimental models; animal models; *in vitro*; *in silico*; pharmacological testing; strategies of drug development

1. Introduction

Over the last few decades, breast cancer has been the target of several studies regarding its molecular status, epidemiology, diagnosis, and treatment. Breast cancer is a major concern in women's health due to its high incidence and mortality rate. However, it must be noted that it also affects men, although with much less frequency. After analyzing breast cancer's trends and predictions for 2020 [1],

the overall decline in breast cancer mortality rate over the last decades stands out, and it appears that it will continue decreasing. In the European Union, breast cancer mortality rate declined 15.3% between 2002 and 2012, and it is predicted to fall around 10% until 2020. The same tendency applies for the USA. It is also important to mention that although Western lifestyle appears to be a major risk factor for breast cancer, the increased awareness of the population and the development of novel improved diagnostic and treatment strategies are major contributors to this decline. In addition, it is impossible to ignore the importance of the evolution in the research field when analyzing those numbers.

Breast cancers are differentiated in several types based on their features, allowing the best treatment course selection and better prediction of the expected outcomes [2]. Breast cancers can be distinguished based on histopathological types, tumor grade, tumor stage, and molecular subtypes, as schematically represented on Figure 1.

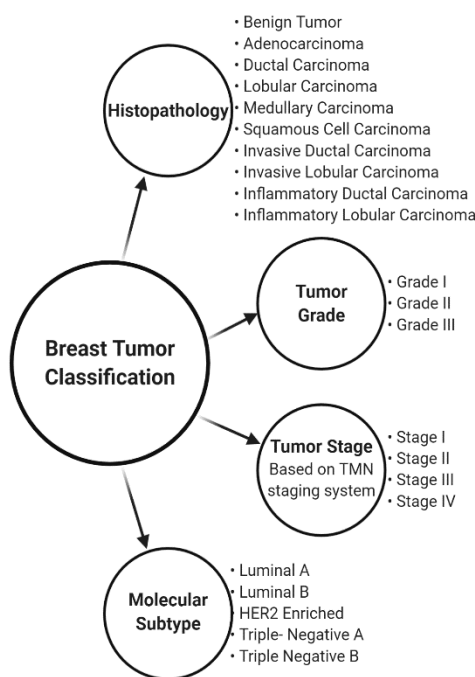


Figure 1. Schematic representation of the breast cancer classification depending on histopathological characteristics, tumor grade, tumor stage, and molecular subtype.

Histopathology characterizes the tumors regarding its site and morphology based on a microscopic evaluation of the cells. It gives information about tumor size, description (presence or absence of masses or margins), texture, and histological and architectural type. Based on the location of the tumors, they can be classified as lobular or ductal if they begin in the lobules or in the ducts, respectively. Moreover, depending on the morphology of the cells, tumors can be divided into non-invasive or benign, and invasive or malign. Non-invasive tumors, also known as carcinomas *in situ*, are limited to the area where they are original set and have typically better prognosis, although they can also evolve to invasive cancers.

The tumor grade classification, also known as the Elston and Ellis modified Scarff–Bloom–Richardson (SBR) is based on a numeric score attributed to a tumor [3]. It assesses how closely cancer cells resemble normal cells depending on three parameters: the tubule formation, nuclear pleomorphism, and mitotic rate.

The most used tumor stage classification system is based on the TNM staging system developed by the American Joint Committee on Cancer [4,5]. TNM is a two-step procedure, and it stands for Tumor, Nodes, and Metastasis [4,5]. Firstly, the cancer is classified based on its size and extension, on the spreading or not to lymphatic nodes, and on the presence or absence of metastasis. Then, the combination of the previous factors will be translated in an overall TMN score.

The molecular subtyping of breast cancer is based on the expression of specific genes, proteins, and cell receptors [6–10], such as Estrogen Receptor (ER), Progesterone Receptor (PR), Human Epidermal Growth Factor Receptor 2 (HER2), Tyrosine Kinase Receptor, and Ki-67 protein. Additionally, over the last few years, Androgen Receptors (AR) have drawn attention as possible new markers for breast cancer classification, prognosis predictors, and therapeutic targets [11–13]; however, their use is still controversial. Based on the ER, PR, and HER2 receptors status and on other proteins, breast cancers can be classified as Luminal A (LA), Luminal B (LB), HER2-Enriched (H), Triple-Negative A or basal-like (TNA), or as Triple-Negative B or normal-like (TNB), as represented on Table 1. This subtyping reflects not only differences in the molecular expression of the cancers, but also on their incidence, prognosis, and sensitivity to specific treatments [6,8,14].

Table 1. Molecular classification of breast cancer based on Estrogen Receptor (ER), Progesterone Receptor (PR) and Human Epidermal Growth Factor Receptor 2 (HER2) status [10,15–19].

	ER	PR	HER2	Ki-67	Notes
Luminal A	+ +/-	+/- +	-	Low	<ul style="list-style-type: none"> • Low-grade cancer and with the best prognosis
Luminal B	+ +/-	+/- +	+ -	Any High	<ul style="list-style-type: none"> • Higher expression of proliferation-associated genes • Worse prognosis than Luminal A
HER2 Enriched	-	-	+	High	<ul style="list-style-type: none"> • Faster growth and worse prognosis than Luminal cancers • Successfully treated with HER2 targeted therapies
Triple-Negative A ≡ Basal-like	-	-	-	High	<ul style="list-style-type: none"> • More common in women with BRCA1 mutations • Enriched in cytokeratins and integrins • Associated with metastasis and poor prognosis
Triple-Negative B ≡ Normal-like	-	-	-	Low	<ul style="list-style-type: none"> • Claudin-low cancers, overexpressing genes associated with tumor invasiveness and aggressivity

Nowadays, there are several therapeutic options available for breast cancer, which are possibly divided into two main categories: local treatments and systemic treatments. Local treatments allow minimizing the effect over healthy tissues, and they include [20–23] tumor surgery (which is considered the primary treatment for early stage breast cancer) and radiation therapy [24–26]. Systemic treatments are those in which the administered drugs will travel through the blood stream, reaching cells all over the body [21]. Regarding breast cancer systemic therapeutic options, four major groups can be considered: chemotherapy [20–22], hormone therapy [21,22], targeted therapy [27–30], and immunotherapy [31–34].

Despite the multiple therapies available for breast cancer treatment, their use and efficacy greatly depend on the patient, tumor classification and analysis of the clinician. Moreover, there are still some concerns that need to be addressed, including extensive side effects, the need of using higher doses of treatments to surpass the lack of specific targeting, and the few therapeutics available for certain cancers, in particular triple-negative breast cancer. In addition, breast cancer resistance to treatments and recurrence deserves special attention and further research. In this line, Breast Cancer Stem Cells (BCSC) are considered to play an important role [35,36], and thus, several studies have already hypothesized the importance of studying these cells to improve breast cancer prognosis and treatment efficacy [35–37]. For these reasons, it is imperative to continue the search for novel therapeutic options, which not only requires the search for new drugs, formulations, and therapies, but also the search and development of reliable breast models, whether in vitro, in vivo, or in silico. Thus, advances in breast cancer therapeutic approaches greatly depend on the availability of experimental models accurately mimicking human breast cancer’ features and microenvironments, which by their turn, depend on the ability of accurately detecting and classifying breast cancers.

2. Animal Models in Breast Cancer

As aforementioned, breast cancer is still one of the most common cancers in humans, which has boosted multifield efforts either to understand the mechanisms behind the disease progression, evolution, and associated risk factors, both to discover and develop innovative diagnostic and therapeutic tools. Nonetheless, advances in the breast cancer field depend on the ability to mimic tumors' properties and microenvironment, which can be achieved using animal models experimentation.

Great advances in animal models experimentation have been done over the last years to meet the demands of biomedical, medical, and pharmaceutical research [38,39]. Currently, animal models can be gathered into different groups depending on their biological complexity and if there is a coupling to non-biological entities or not, as represented in Figure 2 and explored further in this article. The simpler models in terms of biological complexity are *in vitro* models [19,40,41], which consist in the controlled growth, maintenance, and testing of single isolated cell lines or a synergy of different cell lines. Then, with higher complexity, there are *in vivo* models [42,43], which correspond to animal experimentation, representing a closer proximity to what is seen in humans, since they embody a better mimicking of biological microenvironments, as well as immunological responses. Furthermore, there are two other classes of models, the *in silico* models [44,45] and other models. *In silico* models are computational models that use mathematical equations and high computational power to represent and simulate biological properties and mechanisms. Other models include Phantoms and Microfluidic three-dimensional models, which use non-biological entities alone or coupled with cells, respectively, to physically mimic biological features and actions.

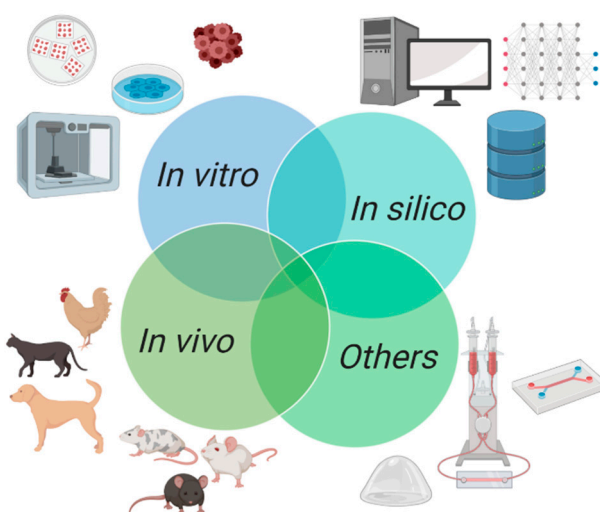


Figure 2. Schematic representation of experimental models currently available for breast cancer research, which are classified as *in vitro* models, *in vivo* models, *in silico* models, and others, including phantoms and 3D microfluidic chips.

The choice of the animal model that is most suitable for each project will greatly depend on several aspects, namely ethical concerns, the purpose and the severity of the procedures, the restrictions imposed on the models to use (genetic variability, immunity ...), financial and logistic resources, and the time available for the development of the project.

Currently, ethical aspects play an increasingly important role at the time of the decision of which animal model to use, because the well-being of the animals is becoming more and more valued [46–48]. In order to respect the animals' welfare and ensure the use of the strictly necessary number of animals, guidelines [49,50], included in the legislation, for the use of animals in scientific research were created. In Europe, the Federation of European Laboratory Animal Science Associations (FELASA) guidelines apply [50], which include the 3Rs Principles, which were firstly proposed by Russel and Burch in

1959 [51,52]. The use of animals for research purposes in Europe is regulated by the European Convention (ETS 123) for the Protection of Vertebrate Animals used for Experimental and Other Scientific Purposes [53], which was adopted on 18th March 1986 and by the Directive 2013/63/EU of the European Parliament and of the Council on the Protection of Animals Used for Scientific Purposes [54], adopted on 22ⁿ September 2010, both including the 3Rs Principles. Furthermore, in Europe, there is also the European Animal Research Association (EARA) [55], which is an organization whose main goals are to inform the community about animal experimentation in biomedical research and healthcare and to ensure transparency regarding the use of animals in research. Moreover, most countries, as well as each animal research center, create their own Animal Welfare and Ethics Body (ORBEA) to regulate and evaluate animal experimentation. The 3Rs stand for Replacement, Reduction, and Refinement, which compile a group of measures to encourage, among others, the reduction of the number of animals used by selecting other models. They also prompt the care of the animals to minimize their pain and distress as much as possible. Currently, a fourth R is included for responsibility, appealing to the sense of responsibility of the researchers to respect animals' welfare and to follow the animal experimentation guidelines. The application of the 4Rs implies not only ethical considerations, but also economical and common-sense considerations, because it is important to share the results of research projects, no matter good or bad, in order to avoid multiple experiments on the same subject leading to the sacrifice of more animals.

2.1. *In Vitro* Models in Breast Cancer

In vitro experimentation is based on the growth of single or multiple isolated cell lines in culture media. The cells can be grown on flat surfaces, on scaffold-based structures, or on an extracellular matrix. Cell culture can here be interpreted as the maintenance of animal or human cells outside of the donor in a viable state for long periods of time, leading to the creation of new generations of cells [56]. The *in vitro* models can use the culture of cellular adherent monolayers or suspensions of cells, resulting in two-dimensional models (2D models) or three-dimensional models (3D models).

In vitro models are a good alternative to implement the 3Rs Principles, since they may reduce the extension of the animal experimentation used, although *in vitro* experimentation also uses components from animal sources.

In vitro experiments allow studying several intracellular and intercellular mechanisms under controllable environmental conditions, such as cellular signaling pathways, metabolism, the absorption and excretion of substances, metastasis, and cellular proliferation. This type of experiments is frequently used because it allows the study of various cellular mechanisms and also because there is a vast list of cell lines available. Moreover, this method is considered to be easy and cheap to maintain compared to others. Nonetheless, it also presents some disadvantages such as the difficult mimicking of the conditions surrounding the cells, the lack of contextualization regarding the microenvironment and immunological response, and the different genetic expression and cellular markers of the cells, since those are the specific features of each individual.

2.1.1. 2D Models

Cell line cultures are one of the tools that researchers use the most in laboratory research in *in vitro* models. Apart from their advantages of unlimited self-replication and their ease of being handled and preserved, there are already multiple cell lines catalogued into different breast cancer subtypes, as summarized on Table 2, which make them meaningful models. Nonetheless, cell lines are known to accumulate genetic changes and mutations over time, which presents a big disadvantage of cell culture models. This phenomenon led researchers to debate whether or not cell lines are capable of maintaining their characteristics [40], because DNA alterations appear to be two times more common in breast cancer cell lines than in original tumors [15], which can cause phenotypical changes in the cells. Nonetheless, Comparative Genomic Hybridization (CGH) analysis hinted that cell lines are still representative of tumors, and that the same mutations happen in both the tumor and cell

lines [57]. Historically, the first breast cancer cell model that was discovered, established, and used was BT-20, in 1958 [58]. This cell line was derived from a 74-year-old female with a triple-negative invasive ductal carcinoma, and it was mainly used for pre-clinical studies. Later, in 1973, the Michigan Cancer Foundation discovered the MCF-7 cells [59], which is the most commonly used cell line in breast cancer research [41]. The popularity of MCF-7 is largely due to its exquisite hormone sensitivity through the expression of ER. This fact makes it an ideal model to study hormone response in breast cancer. Afterwards, other cell lines were discovered and categorized in tumor subtypes. The second most used breast cancer cell line is MDA-MB-231, followed by the T47D cell line [41].

The cell line nomenclature [15,60] is based not on the phenotypical characteristics of the cells, but on their origin: if they were isolated at the same laboratory, if they were from the same patient, or if they were isolated by serial subculture from the same initial population. For instance, the MCF series means that the cells were isolated at the Michigan Cancer Foundation [59]; the MDA series of cells were established at the M. D. Anderson Hospital and Tumor Institute [61]; 21 series were established from a patient diagnosed as having an infiltrating and intra-ductal mammary carcinoma (regardless their phenotypes or genotypes) [62]; and the SUM series of cells were isolated from different tumor species but established using the same selective media [63].

Molecular classification of the cell lines is very important, since it will indicate which cell line to choose, preferably depending on the end goal and the characteristics of the study [15,19,41]. Based on gene expression profiling, the cells in Table 2 are characterized by the status of four receptors normally used in breast cancer subtyping: ER, PR, AR and HER2. The nomenclature adopted to characterize the tumors depends on the receptors they express—Luminal A, Luminal B, HER2-Enriched and Triple-Negative—and the tumor type: adenocarcinoma (AC), benign tumor (B), carcinoma (C), ductal carcinoma (DC), invasive ductal carcinoma (IDC), invasive lobular carcinoma (ILC), inflammatory ductal carcinoma (InfDC), medullary carcinoma (MC), squamous carcinoma (SqC) or non-available information (NA).

The culture medium, or basic medium, is also an important factor to take into consideration regarding cells' needs, once each cell line has specific conditions to maintain its physiological and molecular features. In order to make the culture medium as similar to tumor microenvironments as possible, some refinement methods can be adopted, which can include the addition of elements such as glucose, amino acids, vitamins, inorganic salts, and even serums to provide attachment factors, hormones, and growth factors [64,65]. The main basic media utilized are [65] Ham's F12 liquid medium, Dulbecco's Modified Eagle's Medium (DMEM), and the Roswell Park Memorial Institute (RPMI) medium. The most commonly used culture media for each cell line are also presented in Table 2.

To conclude, despite all the advantages that cell lines present, they are still viewed as basic models that are unable to capture all the features and heterogeneity of cancer, so more additional advanced models must be addressed.

2.1.2. 3D Models

Biological systems are characterized by constant dynamic interactions between multiple elements, which complicate the understanding, mimicking, and knowledge of biological mechanisms, either in normal physiological conditions or altered states. By altered states, it is meant in the presence of pathology or in response to different environmental conditions, such as contact with chemicals, exposure to radiation, lack of nutrients, etc. Tumors present highly differentiated and heterogeneous structures [78], whose growth and proliferation are based on complex interactions between multiple cell types and the surrounding environment. This fact hinders the understanding of biological mechanisms and the discovery of more efficient treatments, since it requires the creation and use of more complex models that are able to successfully mimic complex tumor microenvironments.

Table 2. Characterization of breast cancer cell lines based on the receptors they express: Estrogen Receptor (ER), Progesterone Receptor (PR), Androgen Receptor (AR), and Human Epidermal Growth Factor Receptor 2 (HER2); and classification of the tumor they origin, into breast cancer subtypes depending on the receptors they express: Luminal A (LA), Luminal B (LB), HER2 Enriched (H), Triple-Negative A or basal-like (TNA), and Triple-Negative B or normal-like (TNB); and on their histopathological features: benign tumor (B), adenocarcinoma (AC), ductal carcinoma (DC), invasive ductal carcinoma (IDC), invasive lobular carcinoma (ILC), inflammatory carcinoma (InfC), inflammatory ductal carcinoma (InfDC), medullary carcinomas (MC), or squamous carcinoma (SqC). Additional information on the culture medias more commonly used for each cell line: Ham’s F12 liquid medium (HAM’s F12), Dulbecco’s Modified Eagle’s Medium (DMEM), Roswell Park Memorial Institute (RPMI) medium and Minimal Essential Medium Eagle-alpha modified with nucleosides and DFC1 medium (α -MEM/DFC1) [11,15,16,18,66–73].

Cell Line	ER	PR	AR	HER2	Tumor Classification	Notes	Ref.
BT-483	+	+/-	+	-	LA	IDC Medium: RPMI	[15,16,68–73]
HCC-712	+	+/-	NA	-		DC Medium: RPMI	[15,71]
KPL-1	+	-	NA	-		IDC Medium: RPMI	[15,72,74]
MCF-7	+	+	+	-		IDC Medium: RPMI, DMEM Ki67 low	[11,15,16,19,68–71,73]
MDA-MB-415	+	+/-	+	-	AC	Medium: DMEM	[15,68–70,73]
T-47D	+	+	+	-	IDC	Medium: RPMI Ki67 low	[11,15,19,68–73]
BT-474	+/-	+	+	+	LB	IDC Medium: RPMI Ki67 high	[15,16,19,68–73]
EFM-192A	+	+	NA	+		AC Medium: RPMI	[15,71]
IBEP-1	-	+	NA	+		IDC Medium: DMEM	[15,72]
MDA-MB-330	+/-	-	NA	+		ILC Medium: RPMI	[15,68,70,72]
UACC-812	+/-	-	+	+	IDC	Medium: RPMI, DMEM	[15,68–73]
ZR-75-30	+	-	NA	+	IDC	Medium: RPMI	[15,68–72]
21-PT	-	+/-	NA	+	H	IDC Medium: α -MEM/DFC1	[15,62]
HCC-1569	-	-	-	+		MC Medium: RPMI	[15,68,69,71,73]
MDA-MB-453	-	-	+	+		AC Medium: RPMI, DMEM Ki67 high	[11,15,16,19,66–73]
SK-BR-3	-	-	+	+		AC Medium: RPMI, McCoys Ki67 high	[15,19,68–73]
SUM-190PT	-	-	NA	+	InfC	Medium: Ham’s F12	[15,68–71]
SUM-225CWN	-	-	NA	+	IDC	Medium: Ham’s F12	[15,68–70]
DU-4475	-	-	NA	-	TNA	IDC Medium: RPMI	[15,68,70,72]
HCC-1806	-	-	+/-	-		SqC Medium: RPMI	[15,67,68,71,73]
HCC-70	-	-	+/-	-		DC Medium: RPMI	[15,68,69,71,73]
HMT-3522	-	-	NA	-		B Medium: DMEM, Ham’s F12	[15,75]
MA-11	-	-	NA	-	ILC	Medium: DMEM	[15,72,76,77]
MDA-MB-157	-	-	-	-	TNB	MC Medium: RPMI, DMEM	[15,68–73]
MDA-MB-231	-	-	+	-		AC Medium: RPMI, DMEM Ki67, E-cadherin, claudin-3, claudinin-4 and claudinin-7 low	[11,15,16,19,66–73]
SUM-149PT	-	-	NA	-		InfDC Medium: Ham’s F12	[15,68–71]
SUM-159PT	-	-	+	-	AC	Medium: Ham’s F12	[15,67–70]

The receptors are considered present in the cells (+), absent (-), information still controversial (+/-), or there is still non-available information (NA). Information about the main used media for each cell line is also presented, although it must be noticed that only the basic medias are mentioned, being those, in practice, frequently supplemented according to the cells’ maintenance needs, which are usually advised by the cell line suppliers. Popular supplements include fetal bovine serum (FBS), penicillin/streptomycin, insulin, L-glutamine, and non-essential amino acids.

It was in response to the need of developing more complex models that 3D in vitro models appeared. 3D in vitro models can be developed from previously well-known, isolated, and commercialized cell lines or from patient-derived samples collected through biopsies. They allow studying cell–cell interactions and cell–extracellular matrix (ECM) interactions. Moreover, 3D in vitro models make it

possible to analyze tumor formation, progression, and metastasis. This class of models includes tissue slice models, organoids, spheroids, and scaffold-based models.

Tissue Slice Models

Tissue slice models consist of thin slices of tissues collected from patients through biopsies, retaining their native state and preserving cell–cell and cell–ECM connections [79]. These models are very delicate to maintain and are subjected to higher ethical concerns once they use patient samples.

The preservation of the interactions between cells and cells and ECM makes the diffusion of the medium through the tissues possible [79], allowing the application of these models to study drug response [80] and the use of adenoviral vector strategies to develop novel gene therapies and virotherapies [81–83].

Organoids

Organoids are 3D assemblies of cells of one or more types with histologic arrangements at the micron scale similar to *in vivo* tumors [79,84]. More commonly, organoids are composed of malignant cells and supporting cells from the tumor microenvironment, such as fibroblasts, leucocytes, and endothelial cells, among others. Frequently, the cells used to create organoids are derived from patient samples obtained through biopsies, which leads to the formation of 3D structures that are able to mimic the tissue from where they were derived, representing a more realistic model. When patient samples are used, one of the techniques proposed to produce organoids is based on the disaggregation of the sample tissue followed by the reaggregation of the cells, allowing analysis of the ability of the cells to self-organize.

The use of organoids in breast cancer research has been proposed to study the interaction between fibroblasts and epithelial breast tumor cells [85], to study tumor progression [86], and to predict a drug response [87]. Nevertheless, these structures also present some drawbacks, namely the lack of blood flow. Some organoids are able to develop a vascular system, although it requires very complex production techniques, and the results are very variable [84].

Spheroids

Three-dimensional 3D spheroid models include sophisticated structures composed by one or more types of cells which, when grown enough, present a necrotic core surrounded by actively proliferating layers of cells [79,88]. The complex formed also includes an extracellular matrix (ECM) formed of proteins produced by the cells. These models are very similar to *in vivo* tumors, since they present similar genetic expression, growth kinetics, and cellular heterogeneity [88]. Moreover, the presence of a natural ECM allows mimicking a natural barrier to study the drug penetration, while the internal structure of the spheroids represents a great mimicking of *in vivo* tumors, where the internal region appears necrotic due to the deprivation of nutrients and oxygen.

Several methodologies were already proposed to produce 3D spheroids [79,88], such as the spinner flask technique, in which a suspension of cells is continuously stirred, encouraging the formation of cell aggregates; the liquid overlay technique [89,90], where the cells are cultured in non-adherent surfaces, supporting the formation of aggregates rather than adherence to the flask surface; the hanging drop technique [91], in which cells are cultured as droplets of medium on the lid of a petri dish, and the lid is turned over and placed on the bottom petri dish, followed by the culture of the cells until the spheroids reach the desired size; and magnetic levitation [92,93], where the cells aggregate in response to the application of magnetic fields. The methodology used will determine the production cost, since some techniques imply the use of specific materials and/or equipment. Moreover, the chosen production technique will influence the homogeneity of the spheroids obtained, which despite the efforts made so far is still a major concern.

Due to their potential as similar models to in vivo tumors, 3D spheroids have been increasingly explored [88,94]. They have already been used to study breast cancer cells invasiveness [91] and in drug delivery systems efficacy and distribution studies [89,90].

Scaffold-Based Models

Three-dimensional scaffold-based models rely on the growth of cells in 3D structures that will, in a certain way, mimic the extracellular matrix. The structures used in the models can be made of natural (e.g., collagen), semi-synthetic (e.g., chitosan), or synthetic (e.g., polycaprolactone) biomaterials [88]. Moreover, the cells can be cultured on previously fabricated/synthesized structures [95], or they can be 3D bio-printed [96]. The use of structural entities allows modeling small complex shapes [79], while simultaneously, when simulating the ECM, it creates some resistance to drug penetration, which is similar to what happens in in vivo tumors. Moreover, these models are usually characterized by spontaneous cell organization, possible heterogeneity, gene expression, and cellular phenotype, similar to in vivo tumors [88]. Despite the undeniable advantages of these models, they present some hindrances as well, such as the use of artificial ECM structures. Furthermore, it is required to use structural materials with high biocompatibility that are able to support the cells, which in the case of bio-printed structures must also be efficiently printable.

These models have been reported in projects involving the study of breast cancer' initiation, its hypoxia-driven cancer progression [97], and its response to treatments [95,98]. Additionally, they have been studied to replicate the tumor microenvironment and to be used in drug screening assays [99].

2.2. In Vivo Models in Breast Cancer

As mentioned above, the understanding of the mechanisms underlying breast cancer pathology and the development of new diagnostic and therapeutic options depend on the ability of animal models to mimic the tumors' properties and environment. When considering in vivo models, there are different animal species already reported, such as cats, dogs, rats, and mice [100,101].

2.2.1. Canine and Feline Models

Cats and dogs are natural models to study mammary tumors because they develop spontaneous malignant tumors with similar characteristics to human tumors, namely histopathological features [102, 103]. They have genetic variability comparable to humans and might be exposed to several carcinogens, similar to the ones to which humans [104] are prone.

To study the basis of age onset, incidence, and pattern of metastasis, cats are good models [103,105], presenting histological characteristics more similar to human breast cancer than murine and canine models [103,106]. Some feline mammary carcinomas present particular characteristics, such as overexpression of the HER2 receptor [107], tyrosine kinase receptor [105], and cyclin A [103], and the nuclear accumulation of the p53 [103] gene, making them suitable natural models of hormone-independent, HER2 overexpressing, and aggressive human breast cancer [38].

Canine models of mammary tumors have also similar characteristics to human breast cancer, namely the biological behavior and histological type of tumors [108]. Moreover, canine mammary tumors have comparable epidemiological factors, such as age-related incidence rate and the protective effect of early pregnancy [108,109]. HER2 overexpression and the deregulation of BRCA1 and BRCA2 genes, which are related to development and progression of breast tumors in humans, are also found in dogs' mammary tumors [109–111]. Furthermore, genetic alterations in the p53 gene in dogs can be associated with the development of canine mammary carcinomas and their increased malignancy [107,112].

2.2.2. Murine Models

In the European Union, the species more frequently used in experimental research are rats, *Mus musculus*, and mice, *Rattus norvegicus* [101,113]. Rats and mice present multiple advantages when

compared with other animal species, such as they are small and relatively cheap animals; they are easy to accommodate and manipulate; and they have many similarities with humans, namely a 98% genetic homology [114], which is one of the most important characteristics [100,101,113,115–118]. Two of the main differences between these two rodents species are size and weight, with mice possibly weighting 10 times less than rats [119,120].

In breast cancer-induced models, rat models, similar to what is seen in human breast cancer, present a high frequency of hormone dependence and show a tumor progression from ductal hyperplasia and ductal carcinoma in situ [121–123]. Those characteristics make them good models to study the development of breast cancer. Contrary to rats, mice models when compared with humans have a low frequency of hormone dependence and show a tumor progression from alveolar hyperplasia [121–123]. Regardless, they are good models to study the effect of genetic manipulations.

Within each species, the choice of the strain is a very important factor, since some features, such as the genetic variability, might affect the experimental design and the reproducibility of the models [124]. Depending on the genetic uniformity of laboratory rodents' colonies, they can be divided into inbred and outbred. Inbred animals are characterized by genetic uniformity, as a result of more than 20 generations of brother–sister mating or the equivalent. This class of animals is very useful to reduce the number of animals used in studies, to increase statistical power, and to improve experimental reproducibility, which represents major advantages when compared to the use of outbred colonies. There are different inbred colonies of rats—namely Fischer, F-344, and Lewis—and of mice, such as BALB/c and C57BL/6 [124–127]. In contrast, outbred colonies have no genetic uniformity, which is important to maintain the individual diversity, a characteristic that seems to be more similar to what happens in the human population. Moreover, outbred colonies allow increasing the fecundity and reducing costs [126,128]. There are different outbred colonies of rats, such as Sprague–Dawley, Wistar, and Long-Evans; and of mice, such as Black Swiss and CD-1 [124–127].

Researchers look for animal models that focus on particular features that better replicate the human breast cancer. However, this is a multifactorial and polygenic disease with significant morphological, histopathological, and molecular variation, as mentioned above. For this reason, it's difficult to find a single model representative of all human breast cancers. Nonetheless, a set of models can be used, with each one of them representing a particular subtype of tumor or characteristics of the disease [129,130].

There are numerous animal models reported to simulate different human cancers [131], specially breast cancer-induced models, which can include environmental models with chemical induction, transplanted tumors, and genetically engineered models [131].

Chemically-Induced Models

Environmental models consist in the induction of tumors through contact with chemical compounds, namely, compounds to which people can be exposed daily, through different pathways. Currently, there are only two chemical carcinogenic agents (7,12-dimethylbenzanthracene (DMBA) and N-methyl-N-nitrosourea (NMU)) that are able to induce mammary tumors in rats that exhibit histopathological characteristics and genetic alterations similar to those described in humans [101,132]. Both compounds are complete carcinogens that are able to induce cancer without the additional functions of tumor-promoting agents, and, in rats, they are capable of inducing hormone-dependent tumors [131,133–135]. In contrast, in mice, the tumors induced by the mentioned carcinogens tend to be hormone-independent [136]. DMBA is a compound that is present in cigarette smoke, coal, petrol, diesel engines, meat grilling, and roasting, that can be absorbed by the skin, respiratory, and gastrointestinal tracts. The inhalation of cigarette smoke can be one of the main examples of the human exposure to DMBA [131,137–142]. DMBA is a classical polycyclic aromatic hydrocarbon (PAH), an indirect-action carcinogen, that needs to be previously bioactivated by cytochrome P-450 located in the liver, and that can form adducts with DNA [143–146]. On the other hand, MNU is a direct alkylating agent by the methylation of guanine nucleosides that does not need previous bioactivation [101,147,148]. A single dose of both compounds can induce mammary tumors in Sprague–Dawley rats [149–152].

DMBA, administered at 50–56 days of age by gavage, in a single dose from 50 to 100 mg/kg can induce a high number of mammary tumors with 100% incidence in Sprague–Dawley rats [144,153]. Wistar rats can also be used, but recent studies showed that the number of mammary tumors induced in Wistar rats is lower when comparing with Sprague–Dawley rats [101,154]. A single intraperitoneal injection of MNU, at 50 days of age, with a dose of 50 mg/kg, is also capable of inducing mammary tumors with 100% incidence in Sprague–Dawley rats [101,117,148,155,156]. MNU can also be administered by other routes such as subcutaneously, intravenously, and by gavage, but the number of tumors induced is higher by intraperitoneal administration [101,157]. Comparing the mammary tumors induced with MNU and DMBA, MNU-induced mammary tumors tend to be locally more aggressive and spontaneously metastasized [158], and they can be used to induce other types of cancer models such as gastric, colorectal, and esophageal cancers [159–161]. Meanwhile, DMBA-induced tumors tend to be less aggressive, lacking local invasion and metastasis to distant organs [158,162].

Tumor latency is inversely related to carcinogen dose, whereas tumor incidence and the number of tumors per animal is related to the age of animals at the time of the carcinogen administration [163,164]. The age of the animals at the carcinogen administration time is considered to be the crucial point for carcinogenesis initiation [132,163,165]. The optimal age for carcinogen administration occurs between 45 and 60 days age, corresponding to the age of rat puberty, which is the stage where a faster expansion of the mammary gland epithelium occurs. At this age, the terminal end buds (TEBs) of mammary glands are numerous and differentiating into alveolar buds and terminal ducts, which maximizes the number of foci of tumoral initiation [132,163]. After this time, there is a decrease in the number of undifferentiated structures and the susceptibility of mammary glands to carcinogenesis decreases [166]. Similarly to what happens in rats, in human breast cancer during early adulthood, the cell replication rate is maximal, which is a phenomenon that tends to decrease considerably with age. This indicates that at the puberty age, the cellular turnover is faster, and consequently, there are increased chances for neoplastic processes to be initiated [166]. All this highlights the importance of the carcinogenesis initiation at the early adulthood stage.

In summary, chemically induced models have been widely studied and allow the investigation of certain compounds' effects on the initiation, promotion, and progression of breast cancer. Thus, they represent very useful tools to understand the impact of environmental factors. However, these models are time-consuming, and the tumors generated are still variable regarding their size and location.

Transplanted Tumors Models

Transplanted tumors models consist in the transplantation of living cancer cells in suspension or solid tumors obtained from a donor. They can be classified in cell-derived xenografts (CDX), patient-derived xenografts (PDX), or syngeneic models (allograft models) [167]. When the tumor donor and host are from the same species, the models are classified as allograft. Contrarily, when the tumor donor and host are from different species, they are classified as xenograft models. In the case of xenografts, the host animals must be immunocompromised to achieve greater tolerance to tumor transplantation [131,168]. In both models, if the tumor cells are implanted in the tumor site of origin, they are classified as orthotopic models, the transplantation of mammary tumor cells to the mammary fat pad as an example. On the other hand, when the tumor cells are implanted in a different place from their origin, they are classified as heterotopic models, the transplantation of tumor cells intraperitoneally or intramuscular being examples [101,133,169]. There are different tumor transplantation models, as described in Table 3.

Table 3. Summary of breast cancer cell-derived xenograft (CDX), patient-derived xenograft (PDX), and syngeneic mouse models [167].

Model	Implantation Site	Mice Strain	Cell Line	Tumor Classification
CDX	Subcutaneous (Heterotopic model)	BALB/c, Nude	MDA-MB-231 MDA-MB-435 BT474	TN TN LB
	Mammary fat pad (Orthotopic model)	NOD/SCID	MDA-MB-231 MDA-MB-4-35 SUM1315 MCF7 T47D	TN TN TN LA LA
	Tail vein (Metastatic model)	NOD/SCID	MDA-MB-231 SUM149	TN TN
PDX	Subcutaneous	BALB/c, Nude	/	/
	Mammary fat pad (Orthotopic model)	NOD/SCID NSG	/	/
	Humanized Mammary fat pad (Orthotopic model)	NOD/SCID	/	/
Syngeneic	Mammary fat pad	BALB/c	4T1	/

LA, Luminal A; LB, Luminal B and TN, Triple-negative.

Transplanted tumors models are well-established models that were mainly used to study new drugs' toxicity and therapeutic value [167]. CDX models are mostly useful to analyze tumor initiation and growth. They can also be used to study the metastatic process, although their clinical predictability is still limited. PDX models are based on the implementation of tumor fragments in murine animals, which leads to the development of tumors with the same features as the original tumor. Those features make these models especially advantageous to explore personalized treatments. Both CDX and PDX models might require the use of immunocompromised animals, which does not mimic what really happens in humans and increases the cost of the models. Syngeneic models present metastatic responses more alike to what is seen in human tumors, making them profitable resources to study the metastatic process and to test new anti-cancer and anti-metastatic drugs. Moreover, they do not require the use of immunocompromised animals, representing a better mimicking of real situations, although expressing genetic and histological properties possibly different from the humans. Additionally, they potentiate the study of novel immunotherapies.

Genetically Engineered Models

Genetically engineered models (GEM) correspond to animal strains with manipulated genetic alterations that can be classified as transgenic, knock-in, or knock-out, depending on if the addition, modification, or removal of DNA sequences occurred, respectively [101,170]. The main alterations involve the overexpression of oncogenes or loss of tumor-suppressor genes [171]. Comparing the number of genetically modified rats and mice, there are more genetically modified mice [172]. However, genetically modified rats can be good models to evaluate the role of Ras, BRCA1, and BRCA2 genes in the progression of malignant mammary tumors [151,152,173,174]. Genetically modified mice models of breast tumors can include the application of growth factors and receptors (TGF-alpha, ErbB2, and IGF-II), nuclear oncogenes (c-myc), viral oncogenes (polyoma middle T and SV40 large T), Ras genes (Ha-Ras and N-Ras), INT genes (Wnt family, INT family, FGF family), growth suppressor genes (p53, TGF-beta, BRCA1, and BRCA2), and genes affecting the cell cycle (cyclin-D1) [43].

Radiation Models

Ionizing radiation can induce tumors in the case of radiation overdose and overtime exposure, and mammary tissue is one of the most sensitive tissues to this kind of radiation [175]. The risk of developing breast cancer in cases of radiation exposure is related to the age at which exposure occurs, the risk being augmented in women under 20 years of age and more than 50 years old [176]. There are

different types of radiations with a potential carcinogenic effect, such as atomic radiation (alpha, beta, gamma, and neutrons), X-rays, and ultraviolet radiation [177]. The first report of X-ray irradiation and the induction of mammary cancer was in Sprague–Dawley rats [178]. More recent studies also used other rat strains such as F-344 [179,180], Wistar Furth, WAG, WM, and Lewis [181–184], but the Sprague–Dawley strain is considered the most likely to develop mammary cancer through radiation exposure [180,185,186]. Mice models can also be used to study radiation carcinogenesis, such as BALB/c [187]. Moreover, different mutant strains such as BALB/c with mutation in tumor suppressor gene p53 [188,189] and mice with double mutation in BRCA1 and p53 (strain unspecified) [190] can also be used. This type of models shows potential for studying the effect of the radiation exposure over the mammary tissue regarding tumor induction. Nonetheless, the data published so far on these models is still controversial, and consequently, more studies are required to prove their value and usefulness.

Despite the variety of in vivo models described in the literature, the choice of the most suitable model for each project is not obvious, so to make this task easier, Table 4 presents a summarized comparison of the main advantages and disadvantages identified by the authors (based on personal opinion) for each type of in vivo model here enumerated.

Table 4. Summary table exposing the main advantages and disadvantages identified by the authors for each breast cancer in vivo model mentioned in this review article.

Models		Pros	Cons	
Xenograft	CDX	Subcutaneous administration	<ul style="list-style-type: none"> Show primary tumor growth Easier control/monitoring of tumor growth 	<ul style="list-style-type: none"> Generally, the tumors do not metastasize
		Orthotopic administration	<ul style="list-style-type: none"> Allow analyzing the metastasis 	<ul style="list-style-type: none"> Costly and time-consuming (all xenografts)
	PDX	<ul style="list-style-type: none"> Tumors with similar features (histology, genomic signature, and heterogeneity) as the cells from the donor 	<ul style="list-style-type: none"> Require the use of immune-deficient animals, which can result in the lack of the immune system response 	
Syngeneic models (allograft)		<ul style="list-style-type: none"> More efficient metastasis with similar characteristics to the donated tumor sample 	<ul style="list-style-type: none"> The genetics and histology of tumors may not reflect the humane situation 	
GEM		<ul style="list-style-type: none"> Specific for studying molecular and pathophysiological pathways of breast cancer Specific reproduction of tumor formation and progression Specific mutations can be induced 	<ul style="list-style-type: none"> The mutation is “induced” in all cells Different inflammatory and desmoplastic response Different metastatic pattern compared to the one from humans Harder to mimic the period of tumor incubation 	
Tumor-induced by	Chemicals and Hormones	<ul style="list-style-type: none"> Useful to analyze the initiation, promotion, and progression of mammary carcinogenesis, and also to study the relation of environmental factors and breast cancer development Well described and tested 	<ul style="list-style-type: none"> Longer time to obtain the “sick” animal Harder to control the tumor size and location (which organs/tissues are affected) Do not show a clear evidence of invasiveness 	
	Radiation	<ul style="list-style-type: none"> Useful to study the effect of radiation and of fractionated dose 	<ul style="list-style-type: none"> Require more studies to clarify the different models 	

2.3. In Silico Models in Breast Cancer

Some of the main challenges and disadvantages of using in vitro and in vivo models are related to the difficulties intrinsic to the maintenance of the models, the high biological variability, and the ethical concerns inherent to the use of biological species. To surpass the mentioned disadvantages, in silico models were created to perform biological studies without using biological entities, allowing higher control over the experiments, to increase the number of parameter/variabilities to test and eliminating all ethical apprehensions [44,45].

In silico models combine complex mathematical equations with potent computational resources to simulate and investigate biological mechanisms. These models can be designed by gathering and analyzing the data obtained from previous in vitro and in vivo experiments [45,191], and/or by applying predictive and modeling techniques. They allow obtaining important information regarding chemical, physiological, and pathophysiological features, but at the moment, their validation is still dependent on in vitro and in vivo experiments.

The applications extent of in silico models is far-reaching. There are examples of models participating in different research fronts, going from the identification and understanding of the disease's cancer-driver factors [192–196], to its assessment regarding metabolism [197,198] and progression [199,200], to its diagnostic [201–204] and up to its treatment [205–210].

As aforementioned, the majority of the data analyzed for the design and development of in silico models comes from previous in vitro and in vivo experiments, which can be obtained from people belonging to the same group, from already published works [201], or from open sources such as libraries and databases. There are already well-organized and big databases gathering information about several cancers, especially regarding the cancer genome [211,212] and cancer proteome [213]. Moreover, researchers created a more advanced database with a million profiles, collecting information on genes, drugs, and disease states, which led to the creation of the so-called connectivity maps [214], potentiating the development of novel cancer-targeted therapies. Furthermore, some of the data used for creating in silico models can also be obtained from patients' medical exams [209,215] acquired prior their diagnostic, during the course of their treatments, or afterwards.

Genomic and proteomic data allow the development of computational methods not only to identify cancer-driver mutations and pathways, but also to discover novel drugs and more targeted therapies. The methods used to identify cancer-driver mutations and pathways can rely on [44,192]:

- using known pathways from public databases [194,216,217], which consists of the comparison of genetic modifications related to cancer with pathways already reported in the literature through the application of statistical and machine learning methods;
- network-based methods [193,195,218], identifying cancer-associated genes and pathways related with interactions at the cellular and molecular level in biological networks;
- learning cancer pathways de novo [196,219], making deductions on cancer genes and pathways based on the identification of co-occurrence patterns or mutual exclusivity between genetic aberrations, without replicating state-of-the-art knowledge.

The data extracted from databases and from work already published allow creating simulations of breast cancer fundamental aspects such as tumor growth [199,200], metabolism [197], and solute transport in tumor tissues [198]. Moreover, researchers developed computational methods to evaluate the efficacy of new targeted drugs. The use of machine learning to predict potential targeting sites was also described based on multiple data types, foreseeing, simultaneously, new drug repositioning opportunities and identifying similarities between drug classes [206]. It allowed making assumptions regarding previously unexplained clinical observations. There are also reports of the use of in silico methods combined with in vitro methods to assess the potential of new drugs as anti-cancer agents [207]. Tumors response to a single drug or a combination of drugs was already simulated [205,220], providing a very useful tool for developing more targeted and efficient drugs and for comparing the efficacy of different medicines.

Another sector in which in silico methods present high potentiality is in computer-aided decision making [215] which, likely, allows validating medical assessments regarding diagnosis and treatment options, leading to a reduction of the probability of human error occurrence. The majority of the computer-aided decision methods are related with the classification of the tumor types based on medical exams' data, such as mammograms [201,202] and infrared spectroscopy [203,204].

Additionally, in silico models can be applied to create virtual tissue models, also known as virtual phantoms, for assessing and predicting different tissues responses to different stimuli. This kind of

model can be designed based on medical data or can result from mathematical simulations. When considering the virtual models constructed based on medical data, there are projects reporting the use of computerized tomography data [221,222] and others reporting the use of 3D histopathological reconstructions [223,224]. Other types of models are designed by applying mathematical models, which are used for instance to optimize the acquisition of medical images [225] or by using finite element simulations to estimate the viscoelastic profile of breast tissues [226,227], as an example.

As discussed in this section, *in silico* models are valuable resources for doing a deeper and more complete analysis of the data obtained from *in vitro* and *in vivo* experiments. Moreover, computational simulations of biological systems, structures, and environments offer more controllable and flexible tools with enhanced easiness to evaluate multiple parameters in the same study than *in vitro* and *in vivo* systems. Nonetheless, it must be kept in mind that the greater the complexity of the system to study, the more computational power needed, and the extra technical and computational skills required.

2.4. Other Models Helpful in Breast Cancer

In addition to the models already mentioned, there are two different ones worth mentioning: the physical phantoms and 3D microfluidic models.

Physical phantoms are physical structures designed and produced to mimic biological structures' properties. Phantoms are frequently used to calibrate and optimize diagnostic techniques, such as X-ray-based imaging systems [228,229], microwave breast imaging systems [230,231], and terahertz spectroscopy systems [232]. Another common application of phantoms is to assess radiation doses reaching the tissues upon exposure to a radiation source. They are particularly important to determine the radiation dose reaching the tissues surrounding the irradiated area at radiotherapy sessions [233,234], since it will allow better treatment planning by adjusting the intensity, exposure time, and directionality of the radiation needed. Furthermore, the use of phantoms was already reported to mimic the dielectric properties of healthy and tumor tissues for further applications in novel diagnostic tools using millimeter-wave imaging [235].

3D Microfluidic models, also called on-chip 3D models, are particular models that combine in the same system micron-sized fluidic channels with tubing pumping peripherals, fluids, and cells. Once these models have cells, they could be considered as *in vitro* models, but since they use a microfluidic system, they will be here considered as a different class of models. The combination of cell culture and microfluidic systems bring multiple advantages for a closer approach to *in vivo* systems. The fact of using microchannels limits the volume of reagents needed, which makes cost reduction possible. Moreover, the use of circulating fluid allows maintaining a continuous perfusion of the cells and removing the degradation products resultant from the cellular metabolism, which is similar to what happens *in vivo*, making it possible to keep longer and more stable cell cultures. Furthermore, the fluid is pumped with a laminar flow, which is highly studied, allowing a controllable and predictable behavior, applied not only to the fluid but also to the entities flowing in the fluid, such as particles or cells. In summary, these systems demonstrate a terrific potential to better mimic *in vivo* tumor microenvironments, which make them ideal for studies of drug screening and disease modeling. On-chip 3D models are frequently used for drug screening assays, existing reports on the creation and characterization of these models [236], and reports already demonstrating the application of the models for drug screening tests [237], such as Carnosic Acid and Doxorubicin [238]. These models are also commonly used for therapy assessment, including the mimicking of magnetic particles guidance through narrow mammary ducts models [239] for developing possible future treatments for the evaluation of photodynamic [240] and photothermal [241] therapy systems. Moreover, these models are ideal candidates for modeling tumors' invasion mechanisms [242–244], tumors' microenvironments [245] and tumors' metastatic behavior [246,247]. Despite the undeniable potential and advantages of these models, they also present their weaknesses, such as the complexity to design, produce, and handle the systems' hardware. Moreover, the use of

cells, although presenting less ethical concerns than the use of animals, still implies the use of resources extracted from biological sources.

Overall, a brief comparison of the main advantages and disadvantages identified by the authors (based on personal opinion) for each type of model is summarized in Table 5.

Table 5. Summary table exposing the main advantages and disadvantages identified by the authors for each breast cancer model mentioned in this review article.

Models	Pros	Cons
In vitro	<ul style="list-style-type: none"> Cheap, relatively easy, and fast maintenance Wide range of cell lines available, depending on the properties to test Highly controlled experiments Ability to mimic intra and intercellular mechanisms Less ethical containments 	<ul style="list-style-type: none"> Lack of contextualization regarding the tumor microenvironment and immunological response, namely lack of blood flow Different expression of genes and markers from humans Less sensitivity to the same environmental features than humans Inevitable mutations of certain cellular features caused by the successive new generations
In vivo	<ul style="list-style-type: none"> Better mimicking of tumor microenvironment (angiogenesis, potential of replication, tissue invasion, apoptosis) and immunological response Closer genetic expression to humans Sensitivity to environmental conditions similar to the ones of humans 	<ul style="list-style-type: none"> Ethical concerns (mechanism of diseases' induction, type of treatments used, and number of animals sacrificed) Long experiments to induce the desired pathology in the animals More expensive (acquisition of the animals, maintenance of the animals, and method to induce the disease)
In silico	<ul style="list-style-type: none"> Absence of ethical concerns because there are no animals sacrificed Higher control over the experiments Ability to increase the parameters under study Less prone to factors external to the models Possibility to be used as validation method of other models 	<ul style="list-style-type: none"> Lack of complete biological mimicking of the tumor microenvironment and immunological response, although it can be mathematically simulated Need of using in vitro or in vivo data either to develop either predictive or modeling tools to validate the models Need of expertise computing skills and high computational power
Physical Phantoms	<ul style="list-style-type: none"> Absence of ethical concerns Ability to simulate biological structures and properties both in healthy and pathological states without using any biological entity 	<ul style="list-style-type: none"> Difficulty of designing and constructing biological structures with rigor, and mostly, accurately mimicking their properties
3D Microfluidic Models	<ul style="list-style-type: none"> Less ethical concerns than for <i>in vivo</i>, although they still use cells Better mimicking of tumor microenvironment (e.g., continuous cellular perfusion allowing also the removal of cellular metabolites) and immunological response Higher control and predictability over the fluid flow Need of smaller volumes of reagents due to the reduced size of the microfluidic channels, which can be related to lower expenses 	<ul style="list-style-type: none"> Complex design, production, and handling of microfluidic devices Requirements of specific microfluidic equipment (pump systems, tubing ...)

3. Conclusions

Breast cancer was always considered a complex disease, but with the evolution of the research, its complexity was proven and there is still a lot to discover. Research evolution and society pressure has demanded more and more improvements in what is known about cancer fundamentals, diagnostics, and treatments, which boosts novel and innovative projects on breast cancer. These current demands have already led to important and impactful discoveries, which while providing useful answers also demand the development of more advanced tools to fulfill the research needs. Some of those demands include the development of more sophisticated models to allow performing cutting edge and more accurate studies.

This work presents an extensive review on the main models used in breast cancer research, highlighting their main advantages, challenges, disadvantages, and potential depending on the applications. In the authors' opinion, *in vivo* models and 3D microfluidic models are overall the most informative and complete models when considering that they do a better job of mimicking live organisms systems. Nonetheless, they present strong disadvantages such as the ethical concerns inherent to the use of animals and the complexity of the microfluidic devices, respectively.

Among the *in vivo* models, there are several types of models already being used but there is no an ideal one, since it will greatly depend on the focus of the study. In CDX, PDX, and Syngeneic models, the development of tumors is more controlled, and their characteristics are more homogeneous and better defined. However, they use immunocompromised animals, which does not mimic what happens in humans and results in a lack of immune system response. In contrast, chemically induced models in addition to developing more heterogeneous tumors with longer latency do a better job of mimicking what may be underlined in the development of breast cancer in humans. These models might allow the analysis of different types of tumors, their development (carcinogenesis), and the impact of constant exposure to environmental toxic agents. Moreover, this type of model offers higher cost-effectiveness and greater ease of application.

To conclude, the authors believe that there is no single optimal model that is ideal for all the applications or studies, but a combination of multiple models may lead to exquisite results and discoveries and will surpass the main disadvantages inherent to individual models. The choice of the most suitable model will certainly depend on the research topic, the resources available, the preferences of the researcher, and on ethical concerns related to the use of biological resources, so there is no standard model or group of models to be used; instead, it will be tailored to each project.

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