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Advanced Non-animal Models in Biomedical Research

Breast Cancer

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This collaborative study was coordinated by Laura Gribaldo on behalf of the JRC's EU Reference Laboratory for alternatives to animal testing (*EURL ECVAM*).

The collection of non-animal models described in this report is publicly available from the *JRC Data Catalogue*.

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Abstract

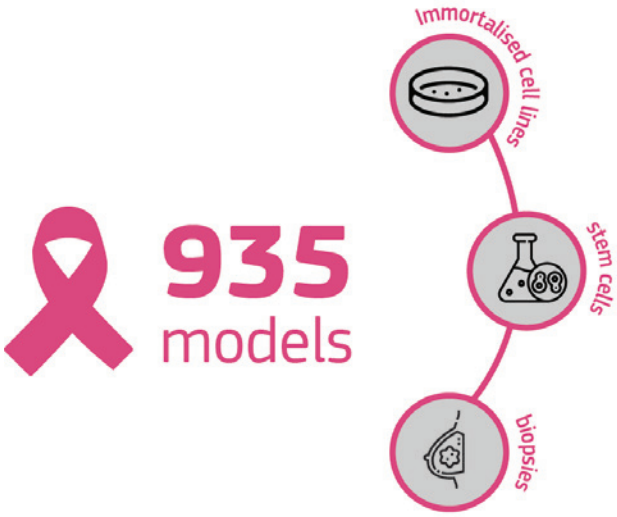
The European Cancer Information System (ECIS) indicates that in the EU over 355,000 women were diagnosed with breast cancer in 2020 (13.3% of all cancer diagnoses).

Despite advances in early detection and better understanding of the molecular bases of breast cancer biology, approximately 30% of all patients with early-stage breast cancer have recurrent disease, which is metastatic in most cases.

To offer better treatment with increased efficacy and low toxicity, selecting therapies based on the patient and the clinical and molecular characteristics of the tumour is necessary.

Although preclinical breast cancer research relies heavily on animal models, experimental approaches that more accurately recapitulate breast cancer pathogenesis are still missing. Furthermore, *in vivo* models, mostly rodents, present some disadvantages: high costs; high-variability (due to lack of standardisation across laboratories); and most importantly the physiological environment provided is not the human one.

Therefore, the JRC's EU Reference Laboratory for alternatives to animal testing (EURL ECVAM) launched a study to survey the state of the art of human-based models for breast cancer described in the scientific literature from January 2014 to March 2019, by retrieving specific information from 935 selected peer-reviewed publications.



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1 Introduction

Breast cancer is the most commonly diagnosed cancer among women worldwide. Although there have been several breakthroughs in the treatment of breast cancer in the past few decades, the high incidence of relapse and progression after conventional therapies is deeply concerning and indicates a great need for developing new therapeutics (Ju *et al.*, 2018).

1.1 Overview of breast cancer

Breast cancer is curable in about 70–80% of patients with early-stage, non-metastatic disease. Advanced breast cancer with distant organ metastases is considered incurable with currently available therapies.

On the molecular level, breast cancer is a heterogeneous disease; molecular features include activation of human epidermal growth factor receptor 2 (HER2, encoded by ERBB2), activation of hormone receptors (oestrogen receptor and progesterone receptor) and/or BRCA mutations. Treatment strategies differ according to molecular subtype.

> *Less than 10% of investigational drugs based on new molecules proceed beyond early development*

The impressive increase in knowledge in the field of molecular biology and immunology has helped to elucidate the molecular characteristics of cancer representing the basis for a plethora of upcoming drugs. However, although important improvements have been achieved in recent years in terms of metastatic breast cancer outcomes, more and better treatments are needed. Additionally, the mechanisms underlying tumour resistance and how to overcome it are main topics of ongoing research.

Knowing the driving pathway at every given moment will enable the correct determination of the optimal sequence of therapies, which currently is largely unknown for all advanced breast cancer subtypes.

All existing therapies hit less than 500 molecular targets (Drews, 2000) suggesting that there are many unexplored targets for drug discovery within the human interactome that comprises possibly 1 million proteins and over 1 trillion potential interconnections. Nonetheless, there are clearly other limitations in drug development. Less than 10% of investigational drugs based on new molecules proceed beyond early development (Kola and Landis, 2004); the approval rate for new oncology drugs is about 5% (Kinders *et al.*, 2007).

Perhaps the lack of significant progress partly reflects the drug development process in which preclinical animal models play a central role. The leading causes of attrition of new drugs are generally cited as being unpredictable toxicities and lack of efficacy, the early identification of which are primary goals for preclinical animal models.

Furthermore, as breast cancer is a highly heterogeneous disease, heterogeneity is often evident even within the same tumour. Cell line xenografts and genetically manipulated mouse models are more homogeneous. The relative homogeneity of these models may

render them overpredictive or underpredictive, depending on how prevalent their phenotype is in the human disease. A prediction of high sensitivity often leads to a drug being considered a strong target for human testing.

Overpredicted drugs would show limited activity/potency in human efficacy trials and so (after significant investment) experience a high attrition rate if the animal models were too sensitive. While current breast cancer models may well overpredict sensitivity relative to the human disease, it is difficult to assess underprediction because the lack of activity in preclinical animal models could lead them to be dropped early.

1.2 Recent development in breast cancer research

In recent years, great progress has been made in the molecular target therapy of breast cancer, representing the pioneering field of precision medicine. Trastuzumab is regarded as the cornerstone of targeted therapy in HER-2-positive breast cancer and shows considerable efficacy in both neoadjuvant and adjuvant therapy (Ross *et al.*, 2009; Ishii *et al.*, 2019; Zimmer and Denduluri, 2019).

CDK4/6 and mTOR inhibitors exhibit the ability to reverse the resistance to endocrine and targeted agents to some extent (Yardley *et al.*, 2013; Goel *et al.*, 2017). PARP inhibitors also show immense potential in the treatment of the BRCA1/2-mutated subgroup (McCabe *et al.*, 2006). Given that the hallmark of breast cancer is the great heterogeneity in which biomarkers are diverse not only between primary and metastatic tumours, but also within a single tumour or during tumour progression, in recent years surrogate intrinsic tumour phenotypes have also been used for treatment individualisation.

At research level, efforts continue for a better understanding of the biological heterogeneity of breast cancer, as well as of mechanisms of tumour resistance and biomarkers predictive of response to the different therapeutic options. The generation of primary cell lines from human tumour biopsy is highly informative to identify novel biomarkers for the development of personalised “antigen-specific antibody”.

This approach overcomes the use of animal models for xenograft injection of commercial tumour cell lines, which partially resemble the real expression of breast cancer biomarkers. In addition, xenografts using human cell lines to test drug responses do not often correlate with clinical activity in patients. This is due to immunological deficits such as loss of T- and B-cell responses, depending on the selected mouse models (Richmond and Su, 2008). Moreover, the selection of cancer stem cells for *in vitro* cell culture from tumour biopsy and their protein and genetic characterisation is an exhaustive method to characterise each tumour population with the aim to develop specific therapy (Jin *et al.*, 2017).

The generation of primary cell lines from human tumour biopsy is highly informative to identify novel biomarkers for the development of personalised “antigen-specific antibody”

Recent studies reported the role of inflammation in the development of breast cancer, supporting the evidence that tumorigenic signalling pathways are not sufficient for explaining a complete breast tumour progression. In this

contest also the role of the immune system is crucial. During the onset of breast cancer, immune system cells like lymphocytes, NK cells and macrophages produce inflammatory factors in the tumour microenvironment. Some of these factors are cytokines, like TNF-a, TGF-b, and interleukins, exerting a pivotal role in tumour progression and contributing to the induction of proliferation, angiogenesis and epithelial-mesenchymal transition (Bahiraee *et al.*, 2019).

Breast cancer research is mostly carried out using animal models, which has been instrumentally useful so far since they can provide a physiologically relevant microenvironment and an intact immune system. From this perspective, many animal models have been generated. We can classify the *in vivo* models either as Genetic ally Engineered Mouse Models (GEMM, either conventional or conditionals), as Xenograft based (Cell-line Derived Xenograft, CDX; and Patient-Derived Xenografts, PDX) and as Syngeneic-transplant based (Park *et al.*, 2018).

Although preclinical breast cancer research has relied until now on *in vivo* models, approaches that accurately recapitulate breast cancer pathogenesis are still missing (Manning *et al.*, 2016). Furthermore, *in vivo* models also present certain disadvantages. In fact, *in vivo* murine models have: high costs; high-variability, due to lack of *in vivo* models standardisation across laboratories; and most importantly the physiological environment provided is the mouse microenvironment (Holen *et al.*, 2017), not the human.

In order to explore the trends of human *in vitro* and *in silico*-based models in breast cancer research, we present here the results of the systematic literature review of 935 scientific peer-reviewed articles, published from January 2014 to March 2019, using non-animal methods in breast cancer research, retrieved in *PubMed*, *Scopus* and *Web of Science* databases.

2 Methodology

The review strategy employed retrieved 119,722 candidate abstracts. After a selection based on titles and abstracts, 48,327 scientific articles were retrieved for the full-text selection.

The full-text analysis resulted in a selection of 935 articles, from which all the identified data were extracted and analysed.

2.1 Selection criteria

The systematic search strategy considered any scientific article describing or dealing with *in vitro* human models or methods or assays or test systems in the field of breast cancer research, based on the dynamic classification shown in Annex - Table 1, as inclusion criteria.

In addition, it was considered as inclusion criteria any scientific article describing or dealing with any *in silico* model, such as an algorithm or mathematical or computational simulations.

The following initial set of flagged search terms was determined as inclusion search terms, for the publications retrieval based on title/abstract analysis:

model OR assay* OR "test* system*" OR "in vitro" OR "ex vivo" OR in-vitro OR ex-vivo OR organoid* OR spheroid* OR 3D OR coculture OR co-culture OR microfluidic* OR microphys* OR biops* OR explant* OR "cell culture" OR "stem cell*" OR stem-cell* OR "primary culture" OR simulation* OR algorithm* OR mathematic* OR computation* OR chip*

The search strategy proposed considered the exclusion criteria listed in Annex - Table 2 and the following initial set of flagged search terms were determined as exclusion search terms for the publications retrieval based on title/abstract analysis:

"mouse model" OR murine OR mice OR rat OR rats OR "Controlled Study" OR "Priority Journal" OR "Major Clinical Study" OR "Animal Experiment" OR "Animal Model" OR "Animal Tissue" OR "Prognosis" OR "Follow Up" OR "Follow-Up" OR "Retrospective Stud" OR "Prospective Study" OR "Case Control Study" OR "case stud*" OR "case-stud*" OR "Nude Mouse" OR "Psychology" OR review OR "Case Report" OR questionnaire* OR "Diagnostic Imaging" OR "Mammography" OR cross-sectional OR survey* OR "Meta-Analysis" OR "meta-analysis" OR hiv OR infection* OR aids OR hepatitis OR influenza OR "clinical trial*" OR xenotransplant* OR xenograft* OR papilloma* OR gvhd OR "qualitative study" OR workshop OR sympos* OR "conference* proceeding*" OR cohort OR descent OR ancestr* OR participant* OR population OR gwas OR "genome wide analysis" OR "methyl* analys*" OR polymorphism**

2.2 Information sources

To perform the systematic literature search, it was agreed to focus on human-based models published in the last five years (January 2014 up to March 2019). In order to generate the most inclusive datasets, multidisciplinary citation databases and indexing services (Web of Science and Scopus) and the specific biomedical sciences citation database, PubMed, were used. Furthermore, grey literature sources of information were monitored to retrieve news and/or highlights on non-animal methods in the field (Annex - Table 3).

2.3 Systematic search

We finally retrieved 935 full-texts from where the data were extracted and analysed. However, to end with the selected full-texts,

we applied five sequential strategies (Annex - Table 4) as illustrated in Figure 1.

We initially retrieved a total number of 119,722 scientific peer-reviewed journal articles by applying Strategy A. After the selection based on titles and abstracts applying Strategies B and C, we finally sorted out 48,327 publications for full text review. During the analysis of the 48,327 publications, we observed a significant risk of redundancy for few models, especially for immortalised cell lines. We managed to partially override such redundancy by designing a new strategy applied to abstracts (strategy D).

However, such high-represented models are still over represented in the repository due to lack of their specific description in the abstract texts. We therefore designed a new strategy (strategy E) to specifically retrieve peer-reviewed publications reporting new models, lowering redundancy of high literature represented models.

2.4 Method summary

The data from the scientific articles were extracted based on the method-summary format including fields that are reported in Annex - Table 5. The resulting collection of advanced non-animal models is publicly available from the EURL ECVAM collection in the *JRC Data Catalogue*[1].

1 *https://europa.eu/!bM83pv*

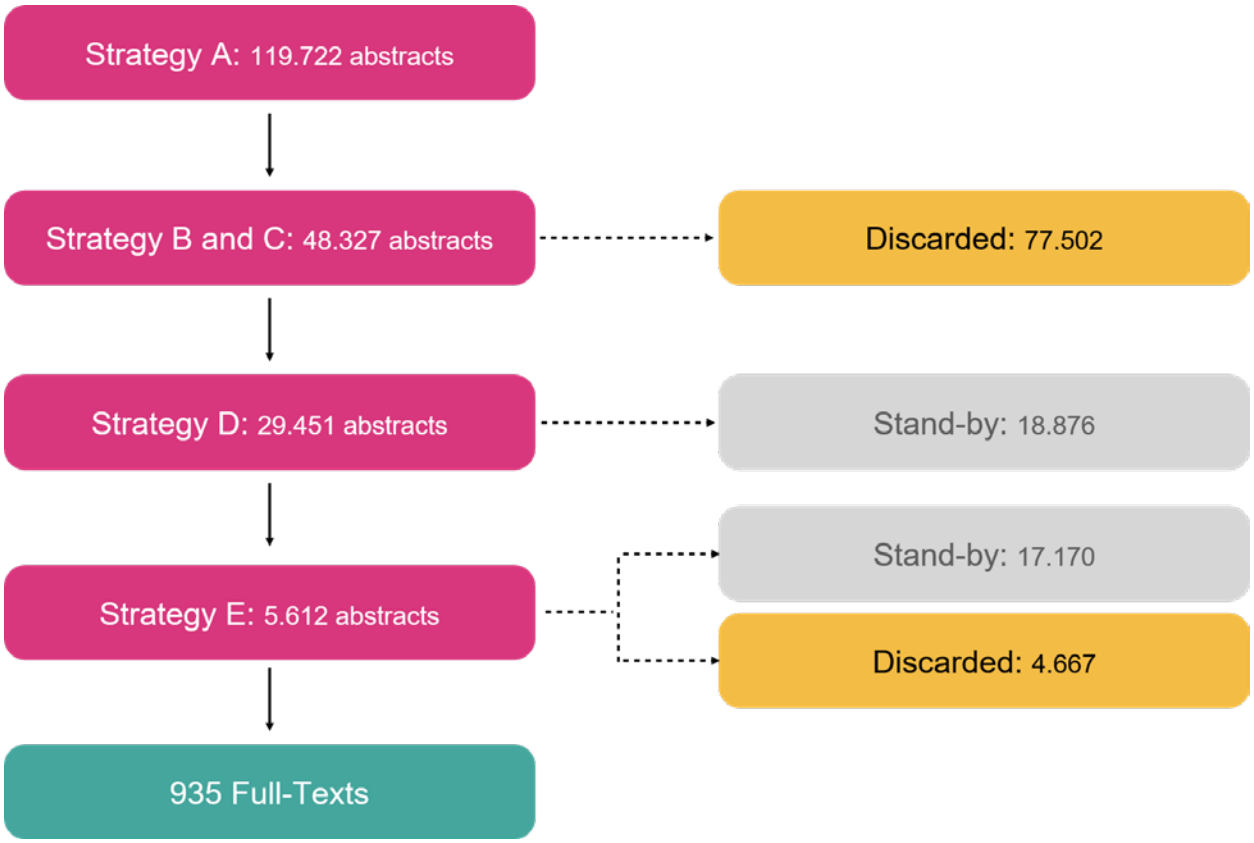

Figure 1: Selection process.

3 Results and discussion

3.1 Publications employing human-based models

After retrieving a total of 119,722 abstracts following our study criteria (see Section 1), we have selected and analysed 935 peer-reviewed articles published from January 2014 to March 2019. The vast majority of articles (924) dealt specifically with breast cancer and only 11 articles were focused on the role of inflammation in breast cancer research (Figure 2). The number of journal articles published increased each year and, in 2018, 214 publications reported the use of non-animal models compared to the 129 articles of 2014 (Figure 2) which is the 66% more within 4 years.

The majority of peer-reviewed articles focused on breast cancer initiation and development (384 articles; Figure 3), including paramount processes like epithelial–mesenchymal transition, cancer stem cell (CSC) formation, metastatic behaviour or mutation functional significance (Mylona *et al.*, 2014; Feng *et al.*, 2015; Avivar-Valderas *et al.*, 2018; Bocci *et*

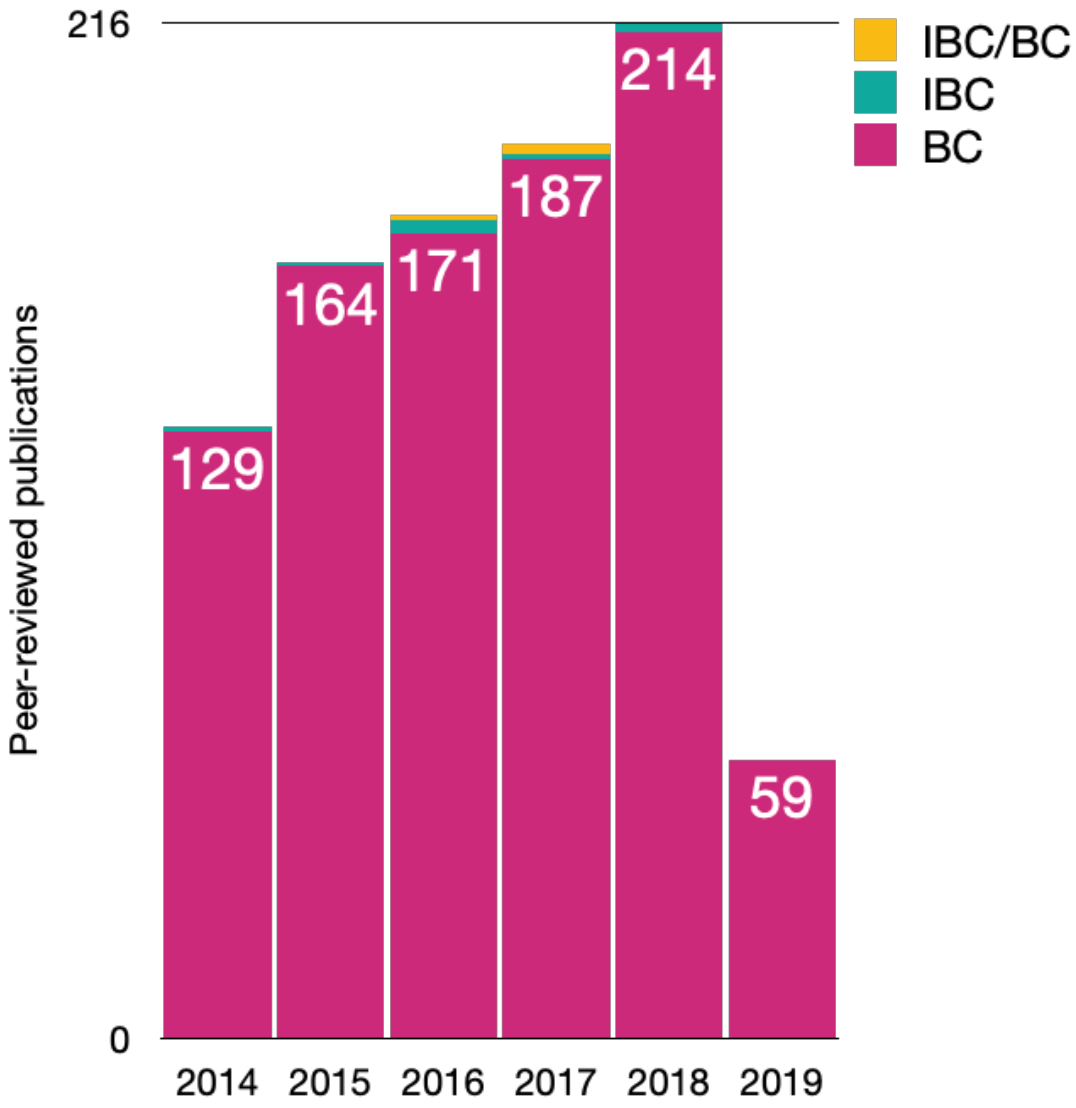

Figure 2: Distribution of peer-reviewed articles by year of publication from January 2014 to March 2019. Articles are classified for their main focus: BC, Breast cancer; IBC, inflammatory breast cancer; or both IBC/BC.

al., 2019). A total of 210 scientific articles employed non-animal models to develop and test pharmacological and/or physical treatment to stop or revert breast cancer pathogenesis (Figure 3), such as small molecules characterisation and testing and large-scale high-content screening based on advanced culture systems (Härmä *et al*., 2014; Li *et al*., 2016). Moreover, microenvironment-tumour interactions were studied in 75 publications (Figure 3), also using lab-on-chip technologies (Choi *et al*., 2015; 2016).

Forty-nine articles used non-animal models to study metastasis, reporting the development of new cell substrates able to prompt invasion and migration mimicking *in vivo* conditions (Cavo *et al*., 2018). A total of 36 articles focused on studying metastasis microenvironment, also presenting new models potentially scalable to high-throughput (Nagaraju *et al*., 2018) or describing specific players in the tumour-stroma interaction (Hohensee *et al*., 2017). Non-animal models were also employed in 28 publications to dissect breast cancer initiation and development with the aim of testing pharmacological and/or physical treatment (Figure 3), e.g. to evaluate photodynamic therapy (Yang *et al*., 2015), or chemical drug efficiency to inhibit stem/progenitor proliferation (Farnie *et al*., 2014).

Furthermore human-based models were used in 30 publications for tumour detection and classification for clinical stratification purposes, including emerging methods such as circulating tumour cells analysis (Hainsworth *et al*., 2016) or *in silico* prediction models

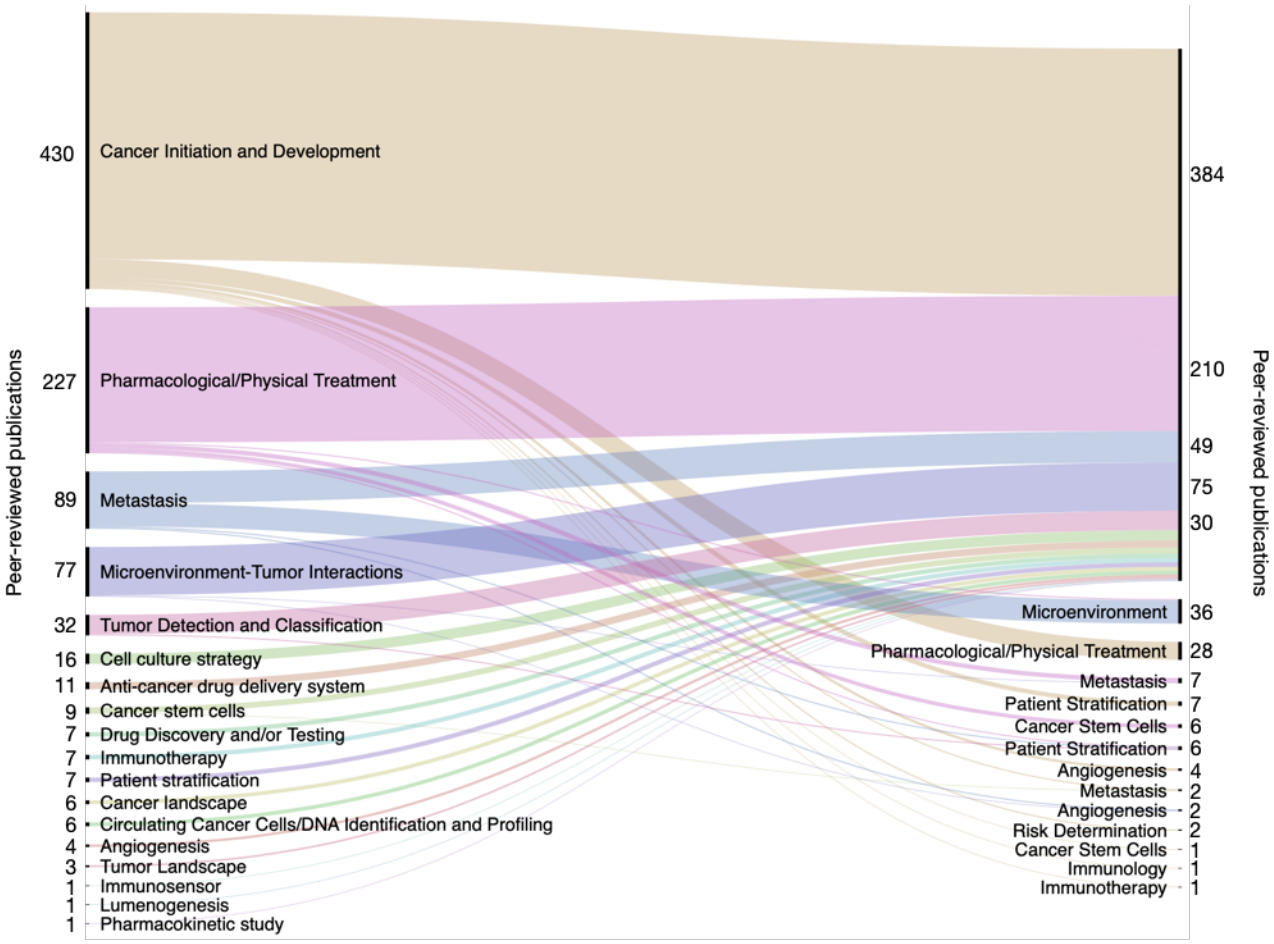

Figure 3: Number of peer-reviewed publications focused on one or more breast cancer disease features. E.g.: 384 publications out of 430 were focused on breast cancer initiation and development only, the remaining 46 publications were focused on breast cancer initiation and development plus other disease features.

(Karapanagiotis *et al.*, 2018). The remaining studies were tackling many other aspects of breast cancer pathogenesis and therapeutic strategies (Figure 3).

3.2 Fields of application of advanced models: from cancer pathogenesis to drug discovery

In our analysis we identified eight areas of application of non-animal models in breast cancer research. They are listed in Figure 4.

More than half of the publications focused on certain aspects of breast cancer pathogenesis to identify or to model specific disease mechanisms (51.2%; Figure 4A). This area showed an annual increase in the number of publications, which reached 108 articles in 2018 (Figure 4B). A total of 29.5% of journal articles reported the use of advanced models in studies on drug development and testing of their efficacy against breast cancer (Figure 4A), using chemical libraries and not-directed to a specific target (Chen *et al.*, 2018) or directed to specific cellular players (Patidar *et al.*, 2016).

During the 5-year period under analysis, the interest in using non-animal models for drug development in breast cancer increased overtime, almost doubling the number of publications (36 articles in 2014 vs. 70 in 2018; Figure 4B). Publications reporting the development in breast cancer research of new models, such as new cell lines (Ali *et al.*, 2017), or new techniques, represented 10.9% of all the retrieved articles (Figure 4A), with 14 articles published in 2018 and a median of 21 articles from 2015 to 2018 (Figure 4B).

A remaining 8.3% of publications dealt with the theoretical development of non-animal models including models aiming to predict cell survival to therapy (2.7%), both physical (Sung *et al.*, 2018) and chemical (Lucantoni *et al.*, 2018), breast cancer diagnostic applications

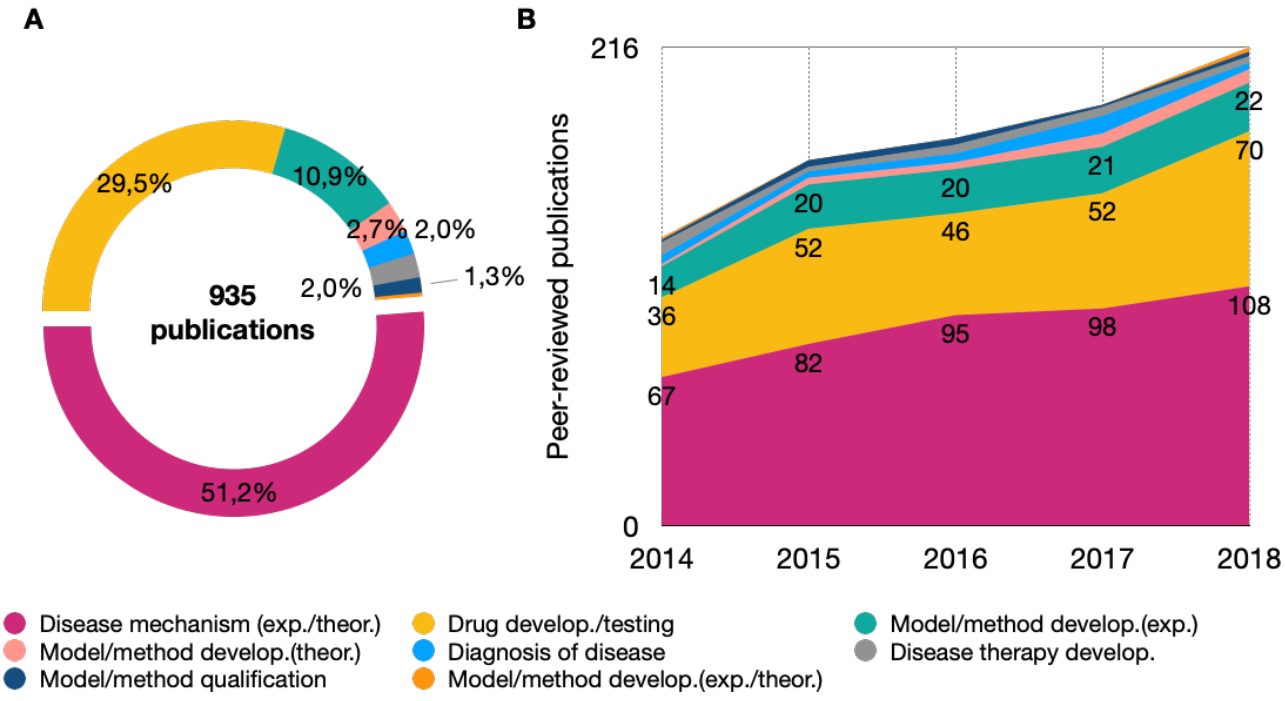

Figure 4: Eight applications for non-animal models in breast cancer research were identified. Panel A shows the percentage of each reported application in all retrieved articles, including publications from 2019. Panel B shows the distribution of articles by model application in breast cancer research from January 2014 to December 2018. The number of articles per year for the three major applications are shown.

(2.0%), development of disease therapies (2.0%), qualification for specific applications (1.3%) (Figure 4A). Finally, 3 articles (0.3%) described their development both theoretically and experimentally.

3.3 Distribution of models into categories

The analysis of the scientific literature found that on the one hand 91% out of 935 selected publications used *in vitro* models for breast cancer research (Figure 5A), with a clear trend increasing overtime (Figure 5B). On the other hand, only a 5% were *in silico* models (Figure 5A). Thirty-eight articles (4%) dealt with models integrating *in silico* and *in vitro* (Figure 5A). The publication of articles presenting such *in vitro*/*in silico* models increased from the average of 5 articles per year to 15 articles in 2018 (Figure 5B). Whereas the use of *in silico* models alone were reported in a maximum of 12 publications in 2017 to a minimum of 6 articles in 2018 (Figure 5B).

The *in silico* models reported in 49 articles were mainly computational models and algorithms for cancer initiation and development studies and for tumour detection and classification (Figure 5C) (Greenbaum *et al.*, 2014; Chapa *et al.*, 2016). Thirty-eight publications using both *in vitro* and *in silico* methods were mainly integrating breast cancer cell lines-based models in combination with an *in silico* model (31 articles, Figure 5D). These

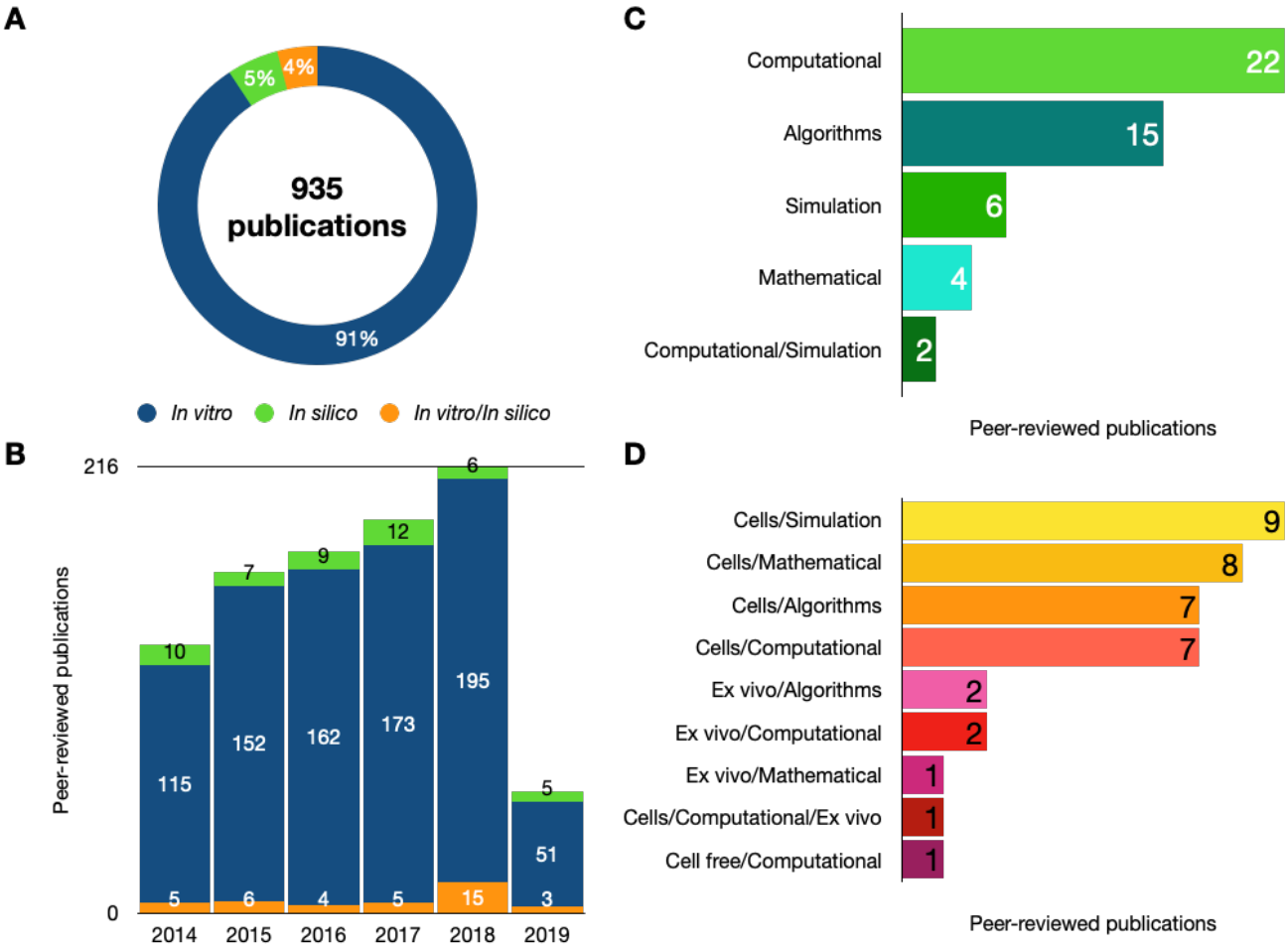

Figure 5: Panel A shows the percentage for each model category in all retrieved 935 articles. Panel B shows the distribution of peer-reviewed articles by year of publication from January 2014 to March 2019. Panel C shows the number of peer-reviewed publication for each type of *in silico* model/method. Panel D shows the number of peer-reviewed publications reporting the use of both *in vitro* and *in silico* human-based models.

publications were mainly focused on breast cancer initiation and development (17 articles) and also on pharmacological and physical treatment of breast cancer cells (14 articles). Those studies that combine machine learning and *in vitro* approaches to identify new marker for evaluation in risk assessment (Ren *et al.*, 2018) or those developing mathematical model to study tumour evolution (Reiter *et al.*, 2018) are of particular interest.

Focusing on breast cancer research, *in vitro* studies from 2014 to 2018 used mainly cell-based models with a constant increase year after year (Figure 6A), representing 84% of total retrieved articles (Figure 6B). The use of human *ex vivo* models was reported in 107 peer-reviewed scientific articles during the period under analysis (Figure 6A), which corresponds to 12% of the total *in vitro* models we analysed (Figure 6B). Most *ex vivo* models were patient biopsies (88 articles), mainly solid biopsies used for the identification of biomarkers (Vici *et al.*, 2014) or clinical applications, e.g. immunotherapy (Chen *et al.*, 2019), while liquid biopsies were less represented (Bingham *et al.*, 2017; Zhong *et al.*, 2018) (Figure 6C).

Organ slice, intended as explanted tumour cut to be used for *in vitro* studies (Carranza-Torres *et al.*, 2015), were reported in 15 publications (Figure 6C).

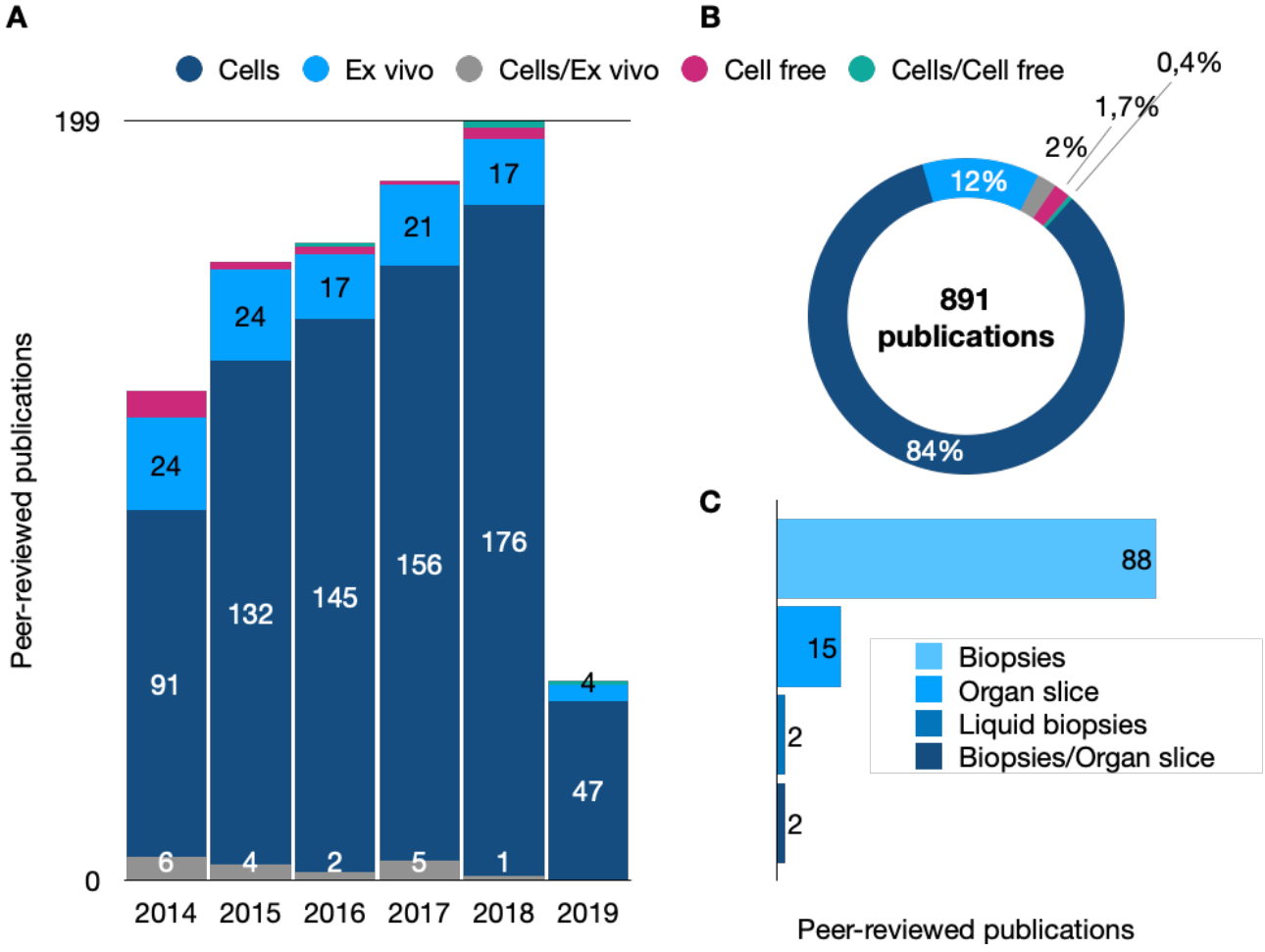

Figure 6: From January 2014 to March 2019, a total of 891 publications were reporting the using of *in vitro* models in breast cancer research. Panel A shows the distribution of peer-reviewed articles by year of publication from January 2014 to March 2019 and by type of *in vitro* model; values for the three most reported categories are shown per each year. Panel B shows the percentage for each category with respect to total *in vitro* models. Panel C shows the number of peer-reviewed articles per each type of *ex vivo* model.

The use of both cellular and *ex vivo* models in the same study represents another research approach (Chi *et al.*, 2017), with 2% of publications reporting this strategy to address their research hypothesis (Figure 6A and B). Cell free approaches alone or in combination with cells were used in few cases (2.1% in total), representing a small niche of *in vitro* models, but including a large spectrum of disease areas spanning from the mutational landscape (Kirkizlar *et al.*, 2015) to the development of immunosensors (Eletxigerra *et al.*, 2016) (Figure 6A and B).

A total of 801 out of 935 publications dealt with cellular *in vitro* models. Due the importance of this category, we disaggregated the data to provide a better view of cellular models in use. In 82% of the publications using human cell-based models, immortalised breast cancer cell lines were employed (Figure 7A). A total of 10% of the publications reported the use of immortalised cell lines and stem cell-like *in vitro* models and a 5% of research articles reported the use of human primary cultures (Figure 7A). Twenty-two articles (3%) reported the use in combination of immortalised cells and primary cell culture, while 8 articles (1%)

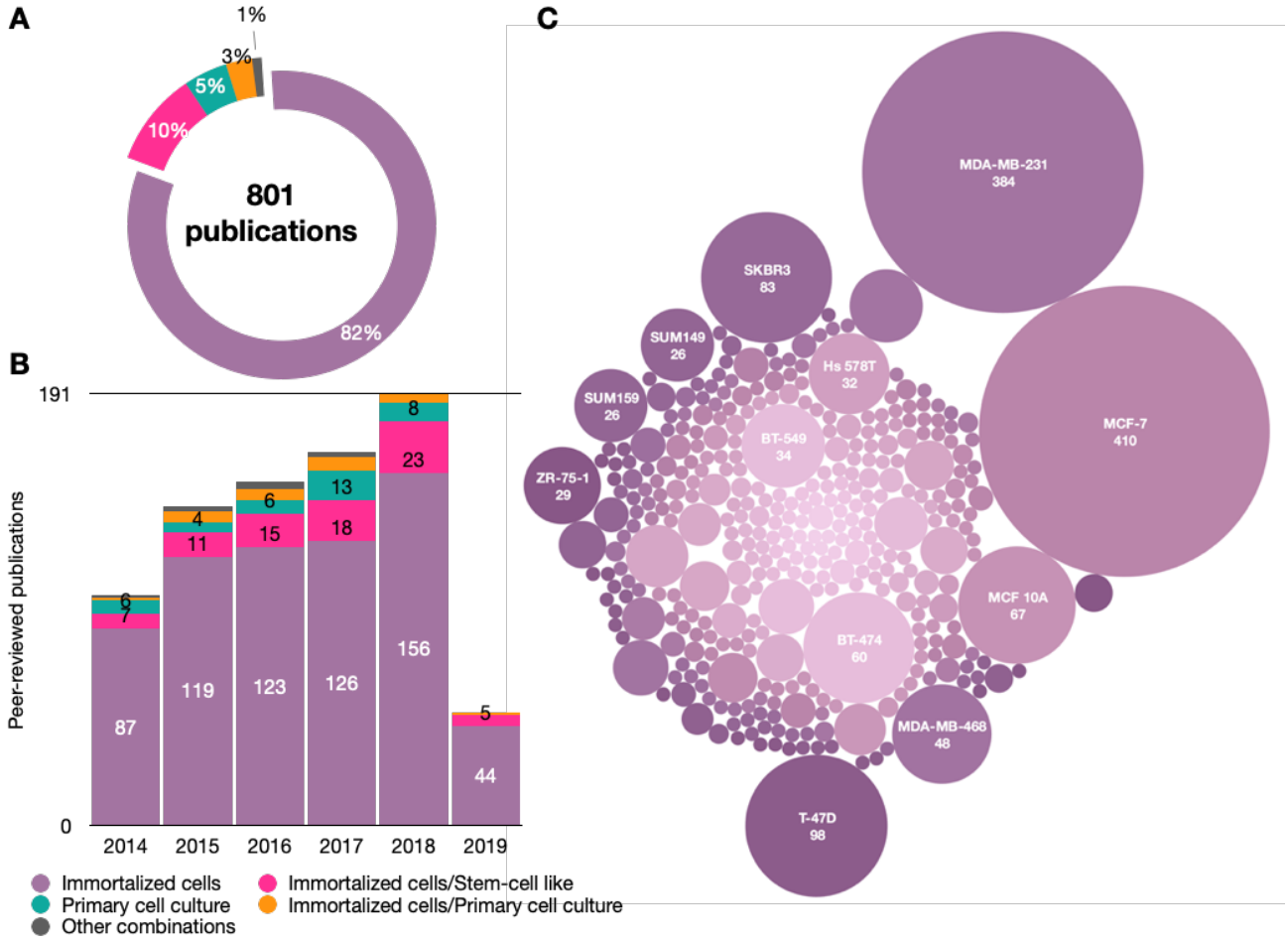

Figure 7: From January 2014 to March 2019, a total of 801 publications reported the use of immortalised cell lines in breast cancer research. Panel A shows the percentage for each type of human cell-based models category with respect to total number of cellular models. Panel B shows the distribution of peer-reviewed articles dealing with different type of human cell based-models by year of publication from January 2014 to March 2019; values for the three most reported cell-based models category are shown per each year. Each bubble, in panel C, represents a human immortalised cell line. The area of each bubble is proportional to the number of publications employing the specific immortalised cell line, the 12 most abundant human immortalised cell lines are indicated with the corresponding absolute number of references.

used as experimental paradigms multiple cellular models (Figure 7A).

Immortalised cell lines reporting in journal articles increased from 87 publications in 2014 to 156 in 2018 (Figure 7B). Whereas, the number of publications using immortalised cells and stem cell-like models increased, during the same period, more than three times from 7 to 23 research articles (Figure 7B). On the other hand, research articles reporting primary cell culture showed relatively constant rate of publications in our 5-year analysis (Figure 7B).

Regarding breast cancer immortalised cells, we observed hundreds of different cell lines employed in several experimental paradigms (Figure 7C). Breast cancer cells lines included cells derived by different sources and cells genetically modified from original cell lines with similar genomic background. The most common cell lines reported in peer-reviewed publications from January 2014 to March 2019 were in order of frequency of use: MCF-7; MDA-MB-231; T-47D; SKBR3; MCF 10A; BT-474; MDA-MB-468; BT-549; ZR-75-1; Hs 578T; MDA-MB453; SUM149; SUM159 (Figure 7C).

3.4 Increased use of 3D models

Culture conditions are very important to understand whether disease modelling is able to mimic as much as possible the physiological microenvironment of breast cancer. A better modelling of physiological conditions enhances the reliability of *in vitro* results.

A total of 83.2% (655 articles) of all publications employing cell-based models, reported cell cultures of individual immortalised cell populations and stem cell-like and primary cells (Figure 8). Only 9.6% (77 articles) of the published studies employed co-

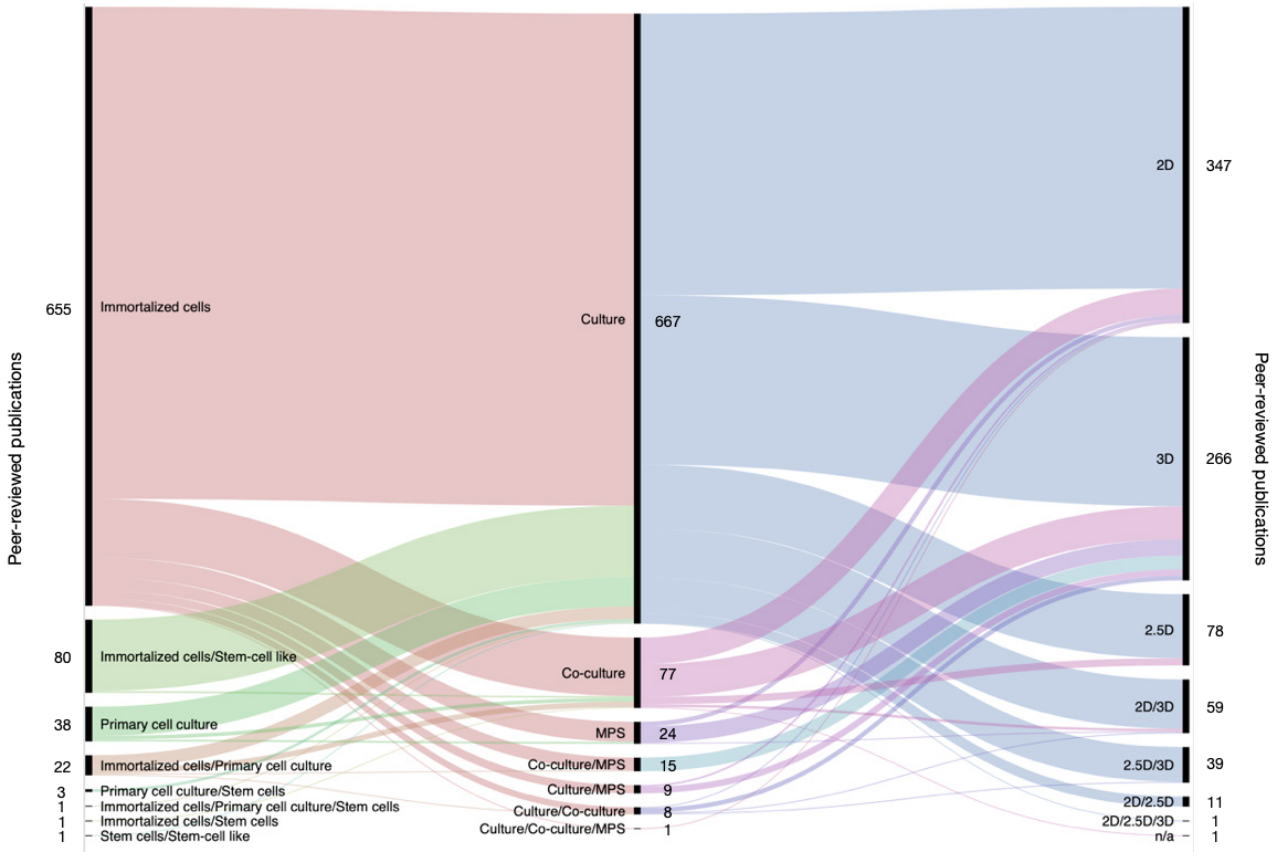

Figure 8: Type of human cell-based model by culturing conditions.

culture systems, e.g. to study chemotaxis of metastatic cells or to model the crosstalk with tumour microenvironment (Chung *et al.*, 2017; Daubriac *et al.*, 2018), and 3% (24 articles) of articles reported the use of microphysiological systems (MPS), mostly microfluidics culture models for 3D (Sabhachandani *et al.*, 2016) and 2D (Ren *et al.*, 2018) applications (Figure 8). The remaining 4.2% of studies (33 articles) employed several cell-based systems and culture conditions.

Less than half of the individual cell populations were cultivated in 2D (347 articles) and 33.2% were cultivated in 3D conditions (266 articles; Figure 8), where the use of scaffolds (63 articles) was the main approach, e.g. for the study of matrix topography (Riching *et al.*, 2014; Clay *et al.*, 2016) or microenvironment and metastasis (Eslami Amirabadi *et al.*, 2017), followed by organoids (15 articles), including patient-derived organoids (Shirure *et al.*, 2018) and spheroids (15 articles), also used in microfluidic systems (Xia *et al.*, 2017).

In 7.4% of studies it is reported the use of both 2D and 3D models (59 articles; Figure 8), eventually comparing the two models or to highlight the value of their complementary use (Breslin and O'Driscoll, 2016; Yildiz-Ozturk *et al.*, 2017). Moreover 9.7% of publications reported extracellular matrix-embedded conditions (2.5D) for individual cell cultures (78 articles; Figure 8). On the other hand, cellular co-culture models were almost equally used in 2D and 3D culture systems, while MPS were mainly used in 3D conditions and a minor fraction in 2D (Figure 8). A total of 39 articles (4.9%) reported the use of two individual cell populations cultivated both in 2.5D and 3D conditions (Figure 8).

The analysis of data collected showed that 3D models increased over time and, in 2018, they equaled the number of publications reporting 2D model implementation (Figure 9A). A small increase in the use of multiple models in the same research article has been observed, from 2014 to 2018, especially in 3D conditions (Figure 9A).

Spheroids, formed by single cell population or in co-culture with other cells, were the most employed 3D models (278 articles), followed by the use of scaffolds (Figure 9B). Of note, the use of spheroids and scaffolds in microfluidic systems was also reported in 41 peer-reviewed publications.

3.5 Relevance of advanced models for the disease features under analysis

Of all retrieved publications reporting non-animal models 22.4% focused on pharmacological and physical treatments of breast cancer and 41% studied the breast cancer initiation and development mechanisms (Figure 3).

To analyse these features, the throughput[2] (productivity or automatisation) and the content analysis are key to screen several treatments in many different models and to retrieve as much molecular signalling information as possible. Hence, we screened the articles to determine the level of throughput and content of analysis[3] and we found that 770 articles described low-throughput use of models, 112 articles a medium-throughput level and 50 articles a high-throughput usage (Figure 10).

From a content perspective, 835 studies applied low content analysis methods, whereas 83 were reporting high-content analysis (Figure 10). This resulted in 73.6% of articles reporting both low-throughput use and low-content analysis (Figure 10). On the one hand, 4.1% of the publications reported high-

2 Throughput is defined as the number of samples that can be processed in parallel.

3 Content is defined as the quantity of information retrieved by each sample with a single analysis or method.

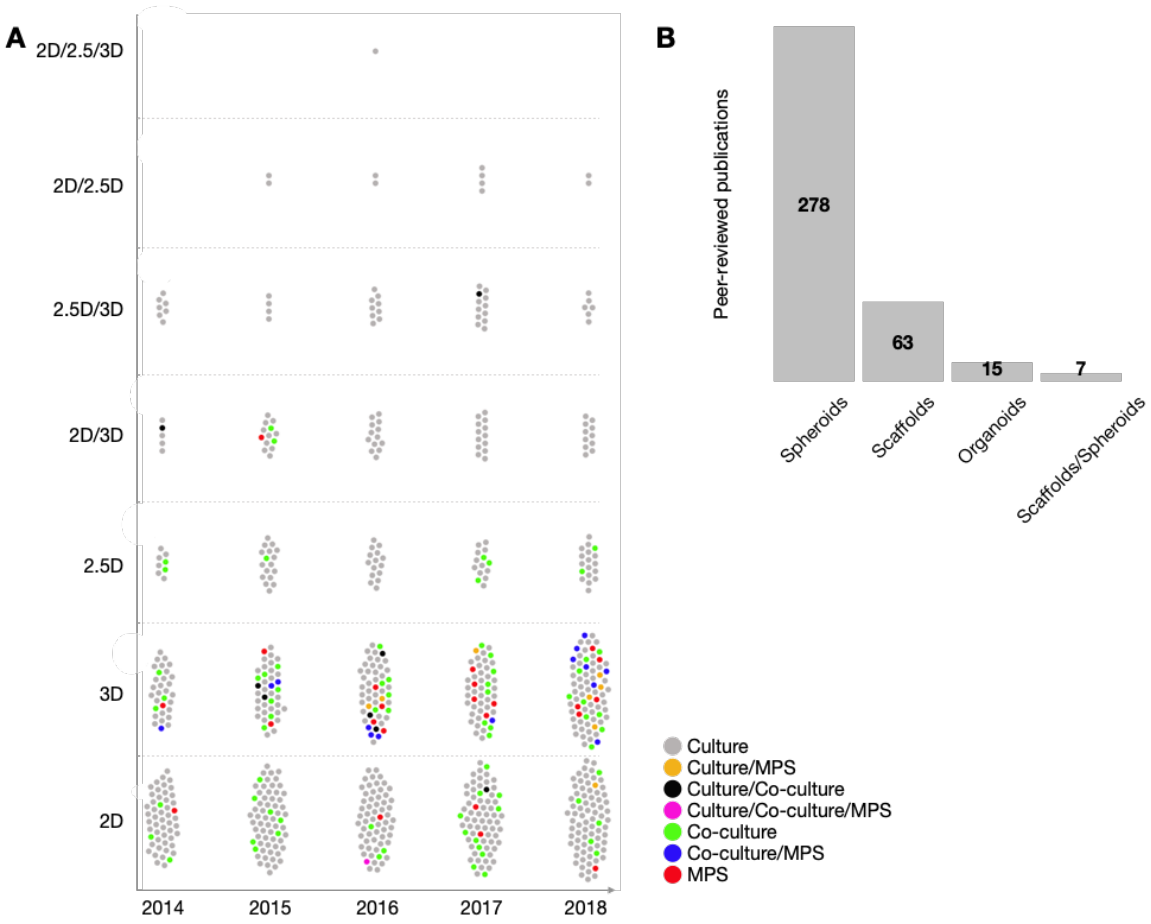

Figure 9: Distribution of articles by year of publication and by culture dimension. A total of 188 articles reported the use of multiple models with different culture dimensions. Each dot represents a peer-reviewed publication.

throughput use of non-animal models, but low content analysis. On the other hand, 7.3% of publications employed high content methods of analysis, but low throughput (Figure 10). Only 1.1% of the publications employed human-based models in high-throughput and applying high-content methods of analysis (Figure 10).

To understand whether non-animal models were relevant to study a specific disease feature, we analysed each article taking into consideration the type of human-based model used to address a disease feature (Figure 11). Seventy-nine percent of all retrieved publications implemented cellular models, where 36.6% were used to study breast cancer initiation and development and 21% were employed to test pharmacological and physical treatments (Figure 11). Cell-based models were also the main models to study metastatic processes and microenvironment-tumour interactions, representing 7.8% and 6.8% of all studies, respectively (Figure 11). In the 4.3% of all peer-reviewed publications, *ex vivo* models were employed to study breast cancer initiation and development (Figure 11).

Then, we analysed the relevance of each human-based model with respect to the target disease feature under consideration. We found 630 peer-reviewed publications where the models had direct relevance for the disease feature that was studied (Figure 12). On the one hand, direct relevance was observed in 28.3% of the articles studying breast cancer initiation and development, 17.3% of the articles dealt with pharmacological or physical treatment, 6,4% focused on metastasis and 6,4% focused on microenvironment-tumour interactions. 2.7% of total publications were having a direct relevance for their use in tumour detection and classification (Figure 12). On the other hand, we determined that 258 scientific articles

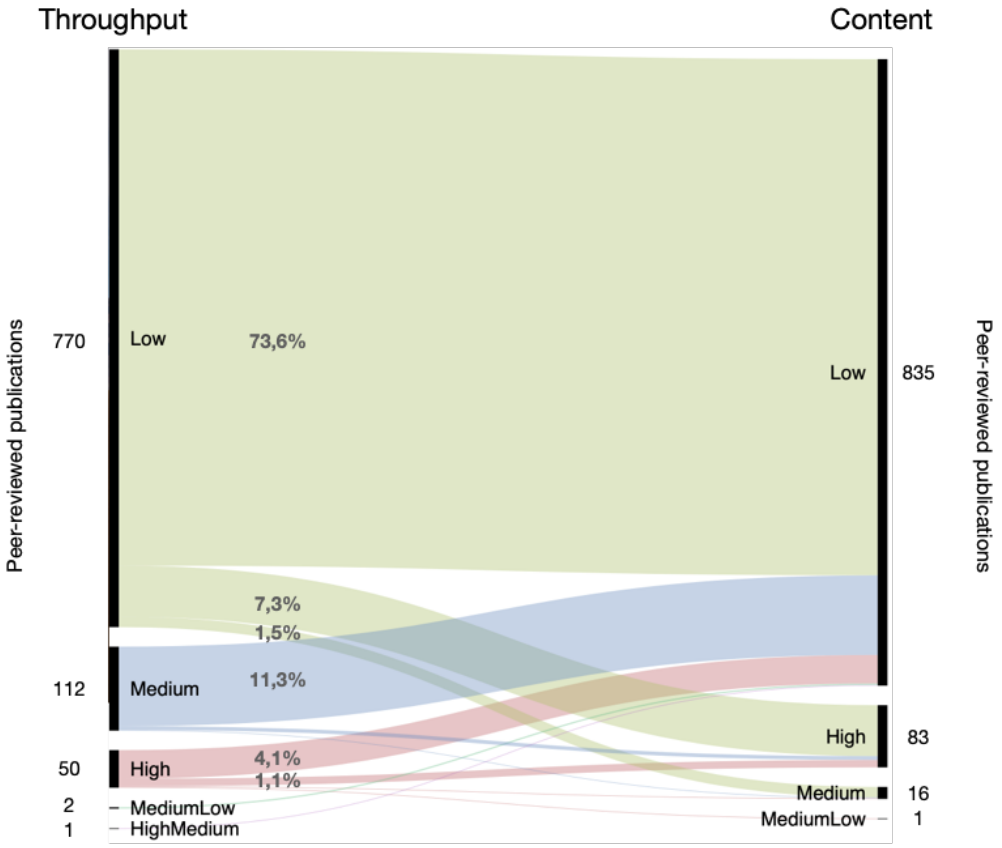

Figure 10: Number of articles by their throughput and analysis of content level.

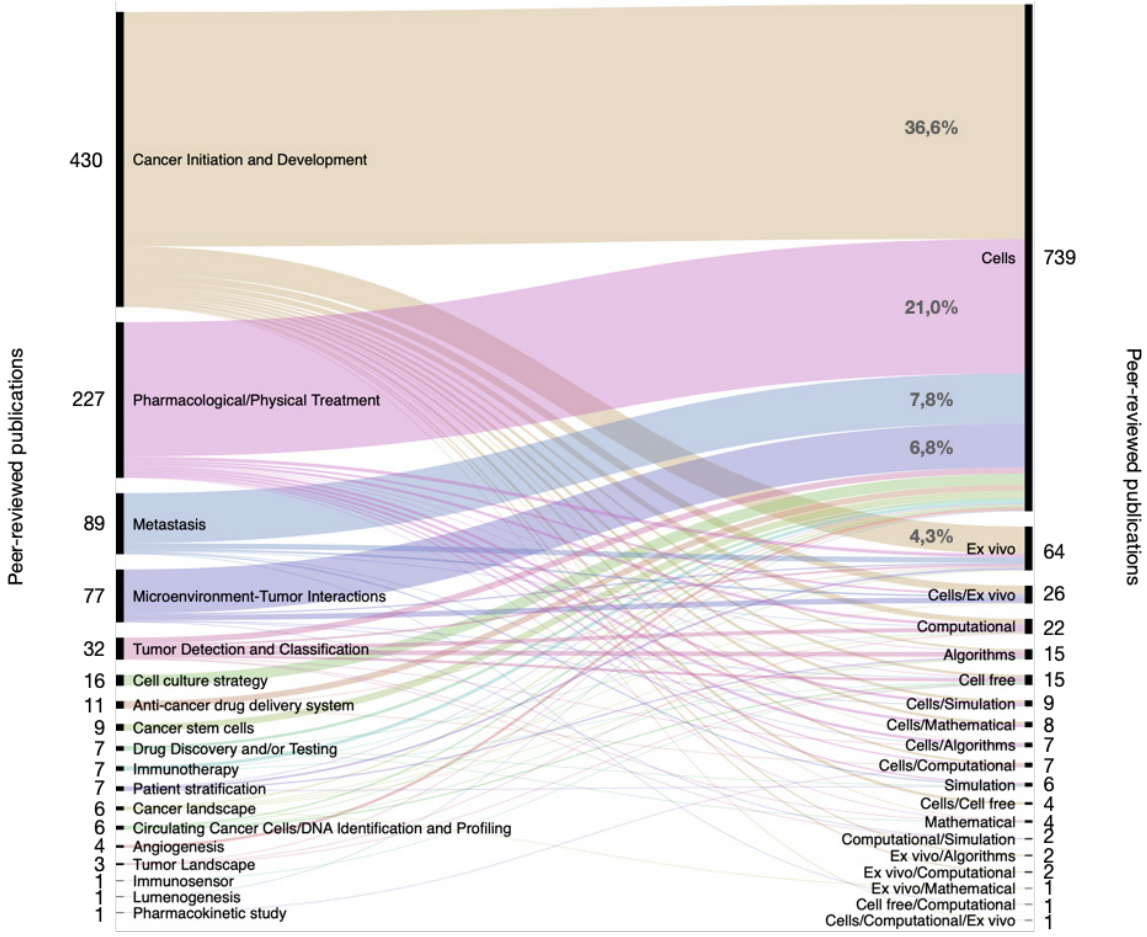

Figure 11: Number of articles and their distribution by the type of model used to address specific breast cancer features. Only percentage greater than 1% of the total retrieved peer-reviewed publications are shown.

employed non-animal models in a supportive manner for the specific disease feature they addressed (Figure 12). This was assessed in 14.4% of the total retrieved publications for studying breast cancer initiation and development, 6.8% of the articles to test pharmacological or physical treatments, while 4% of the publications dealt with metastasis and microenvironment-tumour interactions (2.4% and 1.6% respectively) (Figure 12). We were not able to determine the relevance of 47 publications, correspondong to 5% of the total publications, mostly dealing with cancer initiation and development (Figure 12).

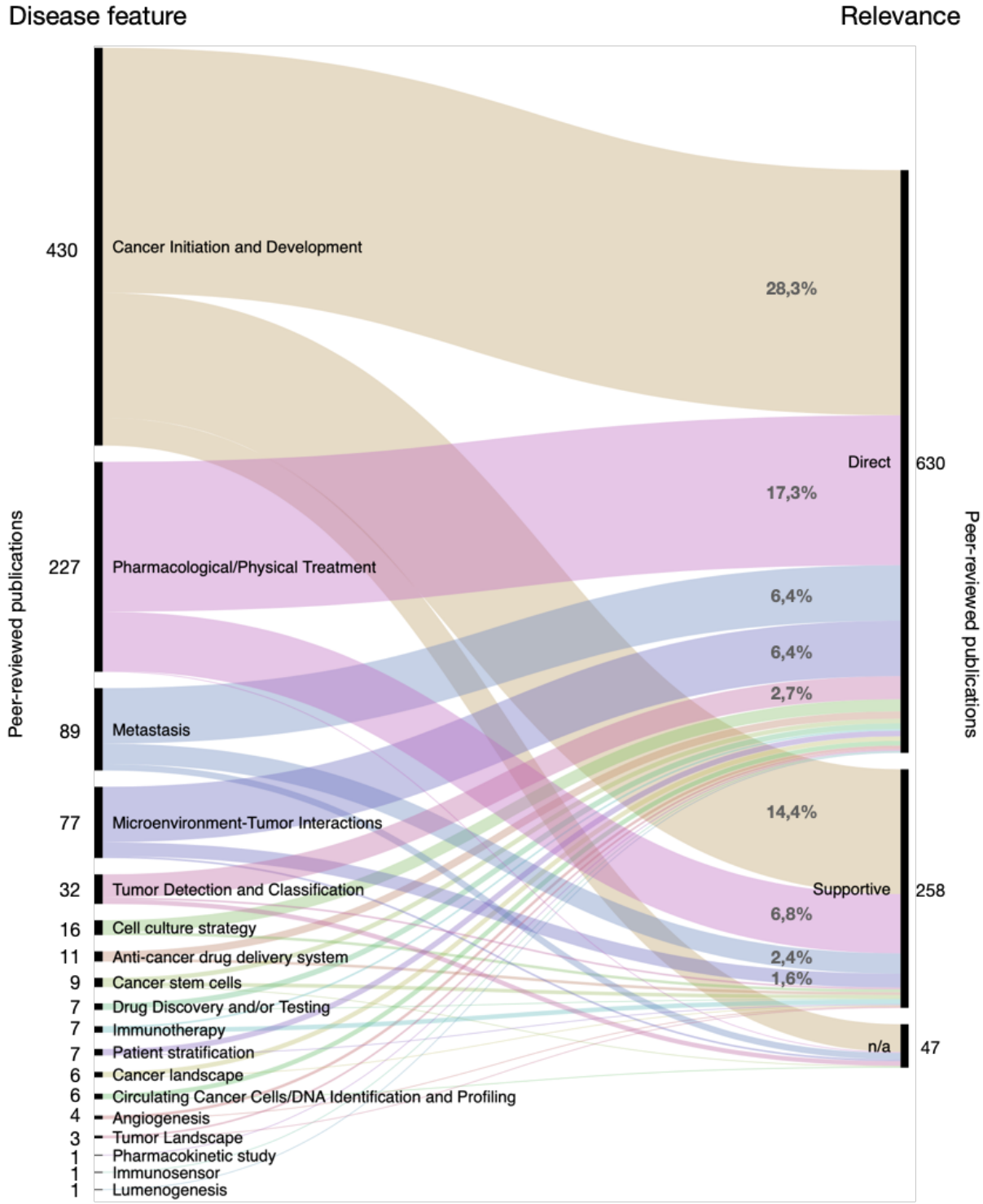

Figure 12: Number of articles and their distribution by the relevance for the use of non-animal modelNAMs in studying specific breast cancer features.

4 Conclusions

The European Cancer Information System (ECIS) indicates that in the EU over 355,000 women were diagnosed with breast cancer in 2020 (13.3% of all cancer diagnoses). Although 5% to 10% of breast cancer are likely hereditary, this high incidence highlights the lack of knowledge about the events triggering breast cancer initiation and whether it skips immunological recognition.

This systematic review of human-based models for breast cancer resulted in 935 articles using *in vitro* and/or *in silico* models to study 18 different aspects of breast cancer pathogenesis. The main disease features addressed were breast cancer initiation and development at cellular levels by using mainly immortalised cell lines in particular, and *ex vivo* models such as tumour biopsies. The main interest in breast cancer initiation and development among the research community mirror the great importance in discovering the molecular bases of the starting events.

The incidence of female breast cancer increased steadily from 1970 to 2016 (WHO 2020) and the biomedical research also has increased its efforts. Our 5 year-time analysis captured a similar trend with peer-reviewed publications, with the use of non-animal models increasing each year. In parallel we also observed an increase in their usage for drug development and testing, which indicates the importance of finding new and more effective treatments for breast cancer. On the other hand, the publication of new human-based models for breast cancer research was steady. However, the use of 3D models has increased suggesting a better approximation to breast cancer physiology, especially through the use of spheroids.

Our analysis showed that the implementation of human-based *in silico* models in breast cancer research is still a minor niche, but applied to all disease features. It is also worth pointing out the small group of publications reporting the use of both *in vitro* and *in silico* models, which has great potential.

Human breast cancer immortalised cells end up being the undisputable most used model reported in the systematic review. In fact, hundreds of cell lines have been found as reported in 747 peer-reviewed publications, including genetic engineered lines derived from commercially available or already qualified cell lines. They have been employed to study breast cancer initiation, treatments, metastatic process and the microenvironment-tumour interactions using several culturing conditions.

Regarding the selection of immortalised cell lines for modelling metastatic breast cancer an insightful evaluation has been made by an integrative analysis of genomic data (Liu *et al.*, 2019). However, as in previous studies, most publications reported a low-throughput and low-content analysis. This was interesting since most of the models were immortalised cell lines in relative standard culture conditions, which would have allowed process automation and the analysis through omics.

Considering the overall results of the review, the main conclusions are the following:

1 The use of non-animal models in human breast cancer research is extensive, especially in relation to cell-based *in vitro* models.

2 The human-based models are mostly applied to elucidate disease mechanisms and to test drug candidates. In particular, human-based models are focused on studying breast cancer initiation and development and on pharmacological or physical treatments.

3 Qualified and commercially available human breast cancer immortalised cell lines represent the most common models.

4 *In vitro* immortalised cells are often cultured in 3D conditions, such as spheroids, to better mimic breast cancer pathophysiology.

5 Two-thirds of publications employed non-animal model as a relevant model to study the disease features of interest in breast cancer research.

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6 Annex

Table 1: Inclusion criteria used to retrieve scientific articles from literature.

1. Cells cultures and/or co-cultures in 2D, 2.5D, 3D or Microphysiological Systems (MPS)
a. Primary cell cultures
b. Immortalised cell lines
c. Stem cells (SCs)
i. Pluripotent SCs
• Induced pluripotent SCs (iPSCs)
• Embryonic SCs (ESCs)
ii. Multipotent SCs
• Somatic SCs
• Fetal SCs
2. *Ex vivo* material
a. Biopsies
b. Organotypic cultures
c. Stem cells (SCs)
i. Explants
ii. Whole organ or organ slice
3. Cell-free assays
a. Biochemical assays
4. Gene reporting assays
5. *In silico*
a. Algorithm
b. Mathematical
c. Computational
d. Simulations

Table 2: Exclusion criteria used to retrieve scientific articles from literature.

1. The study does not deal with breast cancer
2. Secondary literature (review, meeting abstract, etc.)
3. Duplicate
4. No *in vitro* or *in silico* model or method
5. In vivo study
6. Test method not able to measure endpoints
7. The study does not focus on development/characterization of a valuable alternative test method/model
8. No information on applications
9. The study does not provide mechanistic/pathophysiological or biological relevance
10. No biomedical research application
11. No valuable non-animal model or method
12. Not English articles
13. Retracted Publication
14. Published before 2014

Table 3: Information sources used for literature searches.

Multidisciplinary citation databases and indexing services	
Web of Science	*http://webofknowledge.com/WOS*
Scopus	*http://www.scopus.com/*
Google Scholar	*http://scholar.google.com/*
Biomedical sciences citation databases	
PubMed	*http://www.ncbi.nlm.nih.gov/pubmed*
Foundations	
American Breast Cancer Foundation	*http://www.abcf.org/*
Breast Cancer Research Foundation	*http://www.bcrf.org/*
Breastcancer.org	*http://www.breastcancer.org/*
Societies	
Breast Cancer – American Cancer Society	*https://cancer.org/cancer/breast-cancer.html*
Eusoma	*http://www.Eusoma.org*
Research institutions and programs	
NIH – National Cancer Institute	*https://www.cancer.gov/types/breast/research*
California Breast Cancer Research Program	*http://cbcrp.org*
Three-dimensional breast cancer models for X-ray Imaging research – MaXIMA	*https://maxima-tuv.eu/*
Breast Cancer Research Program | Vanderbilt-Ingram Cancer Center	*https://www.vicc.org/research/programs/breast*
Westmead Breast Cancer Institute	*https://www.bci.org.au*
Fred Hutch	*https://www.fredhutch.org/en/diseases/breast-cancer.html*
Charities	
Breast Cancer Care	*https://www.breastcancercare.org.uk/*
Breast Cancer Now – the UK's largest breast cancer research charity	*https://breastcancernow.org*
Pink Ribbon Foundations	*www.pinkribbonfoundation.org.uk*
Events	
European Breast Cancer Conference (EBCC)	*www.ecco-org.eu/EBCC*
Advanced Breast Cancer - 5th ESO-ESMO International Consensus Conference	*http://www.abc-lisbon.org*
IABCR 2019 – 31st International Association for Breast Cancer Research Conference	*https://www.eacr.org/meeting/iabcr-2019-31st-international-association-for-breast-cancer-research-conference*
4th Breast Cancer Congress	*www.breastanbul.org*
10th Euro Breast Cancer Summit	*https://eurobreastcancer.cancersummit.org/2019*
Melbourne International Breast Congress	*https://melbournebreast2018.org*

Table 4: Organisations relevant to respiratory disease modelling and non-animal methods.

	Strategy	Definition	Presence rate[1]	Inclusion rate[2]	Pros & cons
A	**Bottom-Up**	Wide-range strategy without any search term exclusion. This strategy retrieves a large amount of publications.	It should guarantee the highest presence rate among the bottom-up strategies.	Inclusion rate could be low.	A large amount of publications must be screened.
B	**Bottom-Up + Scoring system**	Wide-range strategy followed by a ranking system based on search terms scores.	Top rank should have the higher presence rate than the intermediate and low ranks.	This strategy should concentrate the eligible publications into the top rank (score >200).	Higher amount of publications than Strategy A; however the absolute number of publications could be lower.
C	**Bottom-Up + Top-down + Scoring system**	Wide-range strategy followed by a ranking system based on search terms scores. The exclusion terms are tailored by analysing eligible publications specific for each lot.	This strategy concentrated the eligible publications into a top-ranking class (score >200).	Top rank should have higher inclusion rate than Strategy C.	Higher publications than in Strategy A.
D	**Stand-by**	The most represented redundant models are actively searched and shelved.	Not applicable	Not applicable	It avoids information dilution of new models, which are underrepresented. On the contrary, some applications of most represented models can be lost.
E	**Enrichment**	Specific search terms for new models' retrieval.	Not applicable	Not applicable	It enriches the search with underrepresented models.

1 Presence rate: Percentage of pre-selected eligible publications existing inbuilt dataset for each lot.

2 Inclusion rate: Percentage of eligible publications selected by title and abstract analysis.

Table 5: Agreed categories for data extraction.

Field	Definition	Drop-down option
Model number	Model of breast cancer which is described in a paper	NA
Breast cancer type	Type of breast cancer	BC (Breast Cancer) IBC (Inflammatory Breast Cancer) BC/IBC
Breast cancer subtype	Morphological, molecular and clinical characteristics investigated in the model	For example: BC (breast cancer) Claudin-low BC DCIS (Ductal Carcinoma in Situ) IBC (Inflammatory Breast Cancer) IDC (Invasive Ductal Carcinoma) IDC/ILC ((Invasive Ductal Carcinoma/ Invasive Lobular Carcinoma)
Disease features	The disease feature studied by the model	For example: Cancer initiation and development Angiogenesis Metastasis Cancer landscape Drug discovery and/or testing
Category	The category of non-animal model assigned to the model	*In vitro* *In silico* *In vitro/in silico*
Type	More specifications of the model category	Cells Cell-free *Ex vivo* Computational Algorithm Simulation Mathematical and their combinations
Cells	Biological material source, if any	Immortalised Primary Stem cells n/a
Source	Biological material source, if any	MCF-7 MDA-MB-231 fibroblasts and endothelial cells liquid biopsy
Cell culture type	If the model employs cells, this field specifies the tpe of cell culture	Culture Co-culture MPS (Microphysiological systems) and their combinations
Cel culture dimensions	If the model employs cells, this field specifies the dimensions of the cell culture	2D 2.5D 3D and their combinations
3D	If the model uses 3D cell cultures, this field specifies the type of the 3D dimension	Scaffolds Spheroids Organoid and their combinations

Ex vivo	If the model is based on *ex vivo* cells/ tissues, this field specifies the type of materials	Biopsies Liquid biopsies Organ slice Whole organ and their combinations
Applications	Main scientific aim or application of the model	For example: Diagnosis of diseases Model/method development Diseases mechanism Drug development/testing
Biological endpoints	List of potential biological endpoints used in a model system to describe the disease mechanism and/or study focus	For example: Cytotoxicity Cell proliferation Invasion Metabolic activity
Throughput	Regarding productivity/automatisation of the model	High Medium Low
Potential	Possible multiple model application in addressing disease features	Yes (The method/model has future potential for its breast cancer applications). No (The method/model has no future potential for its breast cancer applications). n/a (not specified)
Relevance	Biological relevance of the model for the disease feature in replacing animal models	Direct (The model is sufficient for the conclusions of the study). Supportive (The model is partially supporting the conclusions of the study). n/a (not specified)
DOI or link	Digital Object Identification number to retrieve the publication abstract. If not available, an alternative link is provided	-
First author name	Name of the first author of the peer-reviewed article	-
Year	Publication year from 2014 to 2019	-

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