



Advanced Non-animal Models in Biomedical Research

Immuno-oncology



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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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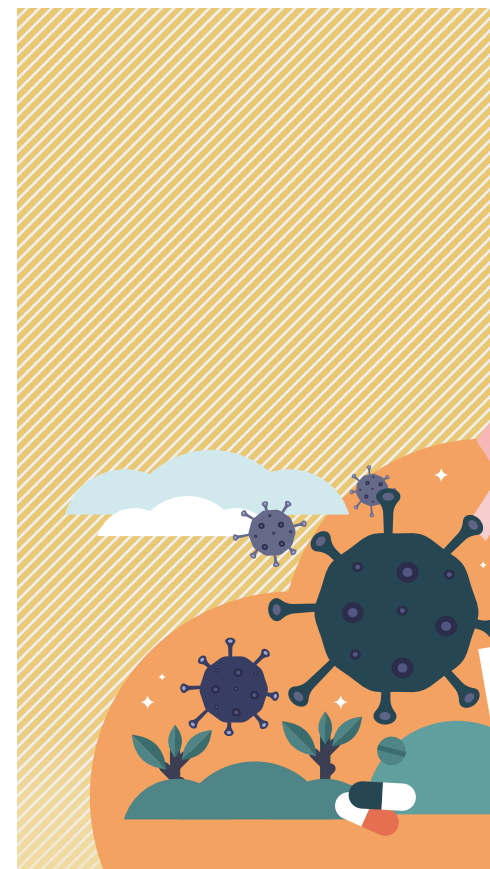
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Abstract

It is widely recognised that immuno-oncology has revolutionised cancer treatment. Nevertheless there are still major hurdles in addressing several key medical questions.

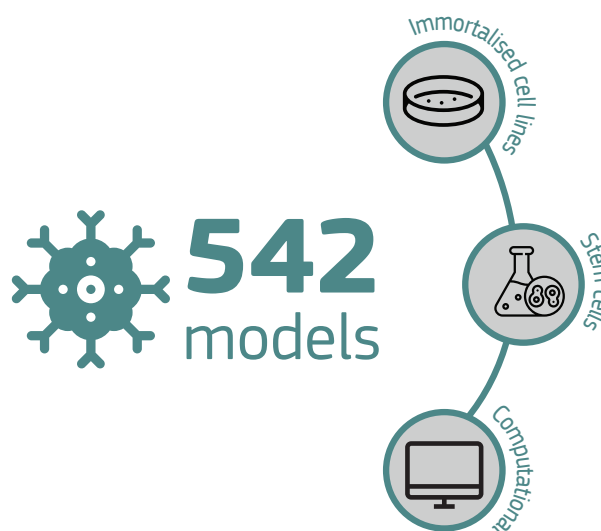
Among these, there is a lack of preclinical animal models capable of mimicking patient conditions and predicting responses to new therapies. Therefore, there is an ongoing need to develop more comprehensive, functional non-animal models.

The JRC's EU Reference Laboratory for alternatives to animal testing (EURL ECVAM) conducted a study to review the state-of-the-art of advanced non-animal models in use for immuno-oncology research.

In this study around 130,000 peer-reviewed publications on immuno-oncology were initially retrieved and screened for representative papers describing innovative and promising advanced non-animal models. The review identified 542 peer-reviewed articles as being the most relevant according to defined criteria.

The majority of these advanced models was focused on studying the biological mechanism responsible for cancer initiation and development and developing efficient and safe immunotherapies.

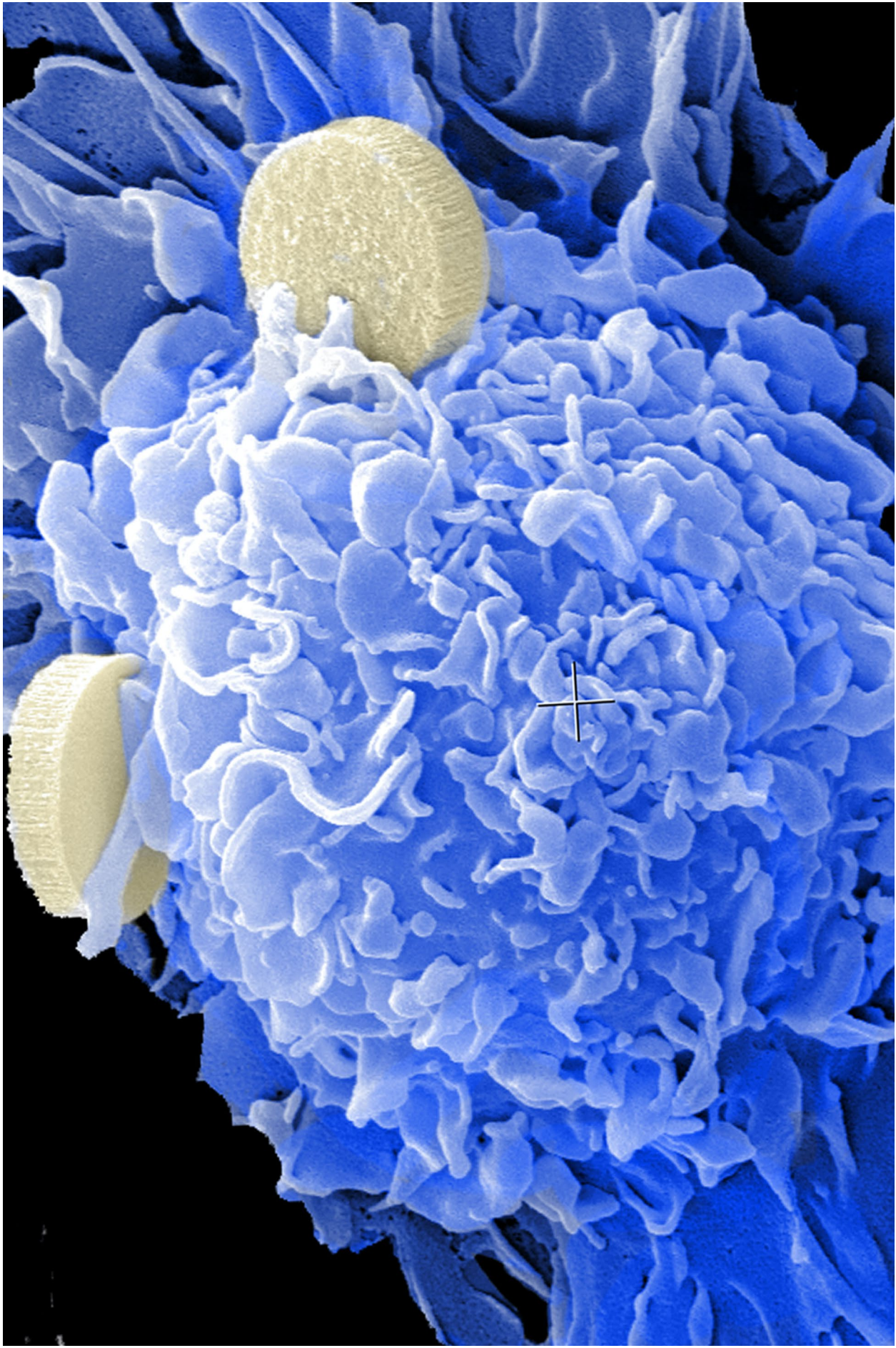
Approximately 10% of the analysed publications highlight the importance of novel and improved approaches to test the therapeutic strategies.



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1 Introduction

Cancer is a leading cause of death worldwide and the most important reason for premature mortality in countries with very high Human Development Index (which includes all 27 EU Member States), according to the International Agency for Research on Cancer (IARC) (Wild, *et al.* 2020). It is estimated that in 2020, there was approximately 2.7 million new cancer cases diagnosed in the EU-27¹. Thus the burden on healthcare systems and the society in general is enormous.

Cancer is a highly complex disease. It is now well-established that tumours consist not only of neoplastic cells, but also of other cell types, with immune cells having a prominent role. Our knowledge in the field has increased considerably in the last 20-30 years, and these scientific developments have led to profound advancements in the clinical therapeutic possibilities (e.g. with immunotherapy) (Galluzzi, *et al.* 2014). Still, we are far from fully elucidating the exact roles of the immune system in tumorigenesis and the optimal way to exploit the immune cells in therapies battling cancer.

The concept of immunosurveillance – i.e. the recognition and elimination of newly-formed tumour cells by the immune system – is not novel (Ribatti, 2017). Immunosurveillance of tumours is mainly exerted via control of antigens presented by the cells via the major histocompatibility complex (MHC) class I, where peptides derived from normal cellular (self) proteins are regularly ignored by CD8+ T cells, whereas those from mutated proteins trigger an adaptive immune response, through binding to the T cell receptor (TCR) (Fridman *et al.*, 2012; Coulie *et al.*, 2014).

Cancer development is often associated with

the lack of this specific and efficient immune system recognition. Loss of antigenicity can arise from the immune selection of cancer cells which lack or possess mutant immunogenic tumour antigens, as well as through the acquisition of defects or deficiencies in antigen presentation (e.g. loss of MHC expression), or dysregulation of antigen processing machinery (Seliger, 2008; Garrido *et al.*, 2016).

Another role of the MHC class I molecules in the innate immune system is to serve as ligands of inhibitory killer cell immunoglobulin-like receptors (KIRs) on natural killer (NK) cells. Under normal conditions, NK cells recognise as self-cells those expressing MHC class I molecules. On the other hand, acquisition of activating ligands in combination with reduced expression of MHC class I molecules on virus-infected and cancer cells, activates NK cell cytotoxicity (Raulet and Vance, 2006).

Several pieces of evidence have shown that tumours have evolved mechanisms enabling them to escape NK cell control. For example, some metastasising tumours show high MHC class I expression and loss or shedding of ligands for NK cell activating receptors. Furthermore, cancer cells may inhibit infiltrating cytotoxic T lymphocytes (CTLs) and NK cells by expressing or secreting immunosuppressive molecules. More subtle mechanisms operate through the recruitment of inflammatory cells that are actively immunosuppressive, including regulatory T cells (Tregs) and myeloid-derived suppressor cells (MDSCs) (Raulet and Vance, 2006).

1.1 Models in immuno-oncology

The idea that cancer is an immunological target, particularly under therapeutic pressure,

¹ <https://ecis.jrc.ec.europa.eu>

is not novel, but the exceedingly fast pace of molecular evolution started being appreciated only very recently, and is further accelerated by target therapies, with new aberrations appearing within days or weeks of treatment. Accumulating knowledge of biological mechanisms that determine the interaction between the immune system and cancer cells, have enabled the development of therapies restoring the capacity of the immune system to effectively target and annihilate tumour cells.

So far, it is widely recognised that immuno-oncology has revolutionised cancer treatment and targeting cancer cells by modulating the immune system has become an effective therapeutic option in many different malignancies. Nevertheless, major hurdles in addressing several key medical questions are the lack of adequate preclinical animal models, capable of mimicking patient conditions and predicting responders (and non-responders) to such new therapies.

Mouse models, including patient-derived xenograft (PDX) mice, are widely used to address questions in cancer research (Buque and Galluzzi, 2018). However, human cancer in the PDX mouse's biological environment leads to mouse-specific changes that invalidate the mouse as a descriptor of the human tumour or as a method for studying the responses that the cells may have to treatments (Sharpless and Depinho, 2006). These defects result in the misrepresentation of human tumour biology and limit the applicability of these kind of tools for translational research.

No currently available animal model can accurately predict off-target toxicity of immuno-oncology products. Furthermore, various other limitations of animal models might include questionable success rates of engraftment of a human tumour into - for example - a mouse, variable responses of tumours in mice versus humans, increased

stress and discomfort for the animals, and so on (Jackson and Thomas, 2017).

Therefore, there is an ongoing need to develop more comprehensive, functional and alternative non-animal models. Developing new immunotherapies could be greatly aided by human *in vitro* model systems that embody the diversity and interactions between tumor stromal immune populations (Boucherit *et al.*, 2020).

Conventional cell cultures fail to accurately predict drug responses in humans, as they do not properly mimic the complexity of the tumor microenvironment. Organoid methods are now widely used to culture cancer biopsies but typically only contain tumor cells and not immune components. The emerging technology of organ-on-chip (OoC), and specifically of tumor-on-chip (ToC), was born from the combination of cell biology, microfabrication, and microfluidics (Neal *et al.*, 2018). ToC platforms are generated by co-culturing tumor and stroma cells (immune cells, endothelial cells, fibroblasts) within 3D biomimetic matrices in microfluidics devices, also called "chip." They are immunocompetent, in that they recapitulate the interplay between immune and cancer cells. They can be personalised by introducing patient-derived autologous primary cells, and they can be treated with drugs and visualised in real time by video microscopy. ToC is a disruptive approach to investigate the drug-dependent plasticity of tumor ecosystems and the mechanisms underlying immunotherapy resistance. (Nguyen *et al.*, 2018).

In order to explore the trends of human-based *in vitro* and *in silico* models in the immuno-oncology research, EURL ECVAM organised a study based on a systematic literature review of 542 scientific peer-reviewed articles, published from January 2014 to March 2019, using non-animal models retrieved in [PubMed](#), [Scopus](#) and [Web of Science](#) databases.



2 Methodology

The review strategy that was used retrieved 136,084 candidate abstracts. After a selection based on titles and abstracts, 37,282 scientific articles were retrieved for the full-text selection.

The full-text analysis resulted in the selection of 542 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 immuno-oncology 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 algorithms, or mathematical / computational models, or 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 ancestor* 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

A total of 542 full-texts were eventually retrieved from where the data were extracted and analysed. However, in order to conclude on the selected full-texts, we applied five sequential strategies (Annex-Table 4), as illustrated in Figure 1.

Initially, a total of 136,084 scientific peer-reviewed journal articles were retrieved by applying Strategy A (Annex-Table 4). After the selection based on titles and abstracts applying Strategies B and C (Annex-Table 4), a remainder of 37,282 publications were finally sorted out for full text review.

During the analysis of the 37,282 publications, we observed a significant risk of redundancy for a few models, especially for studies in the application of immunotherapy where similar approaches and cell types were used. Such redundancy was partially overridden 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 following method-summary format including the following 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 Catalogue².

² <https://europa.eu/!6PXVf8>

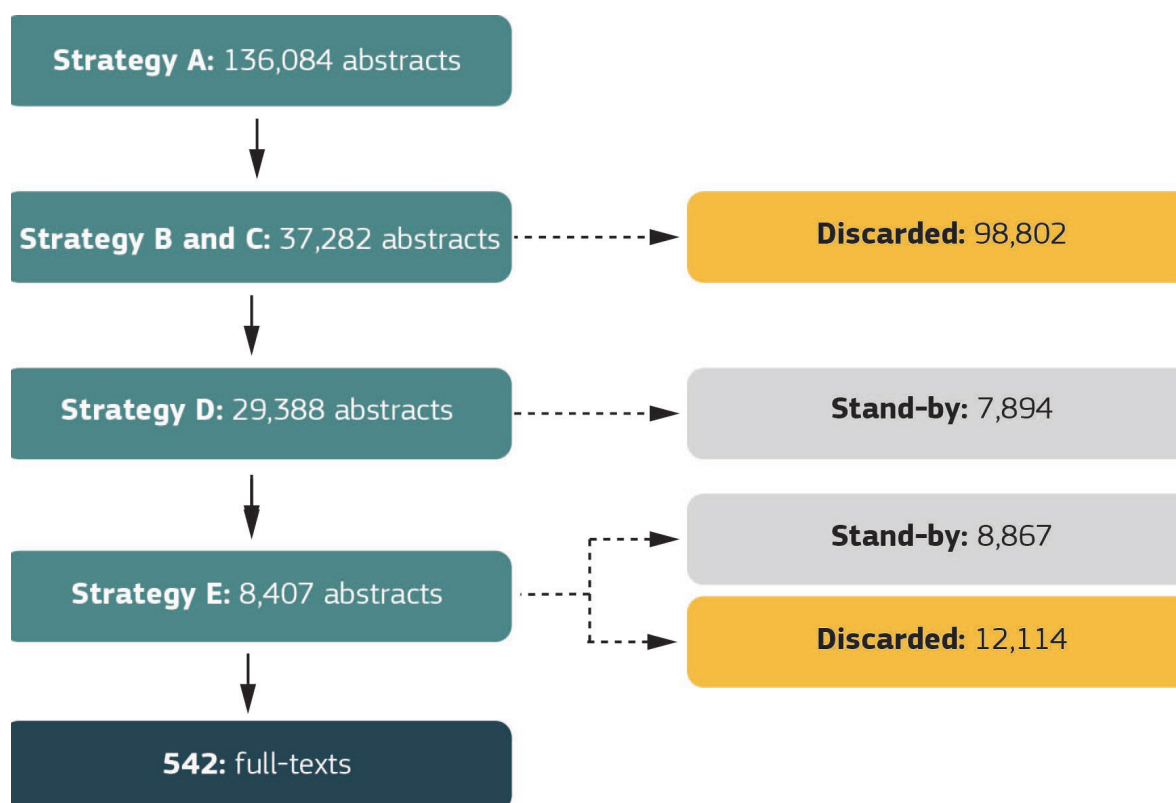
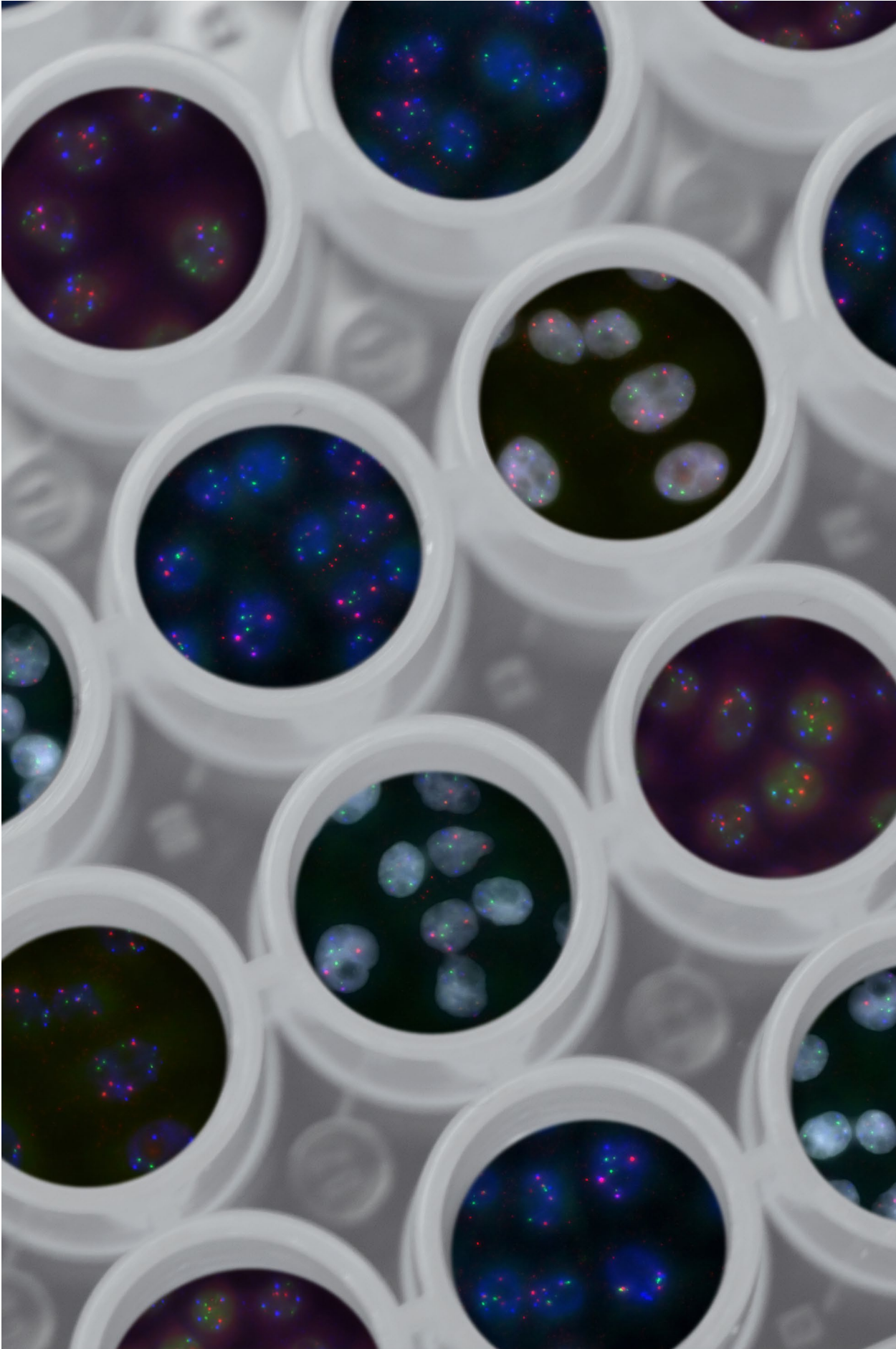


Figure 1: Selection process.



3 Results and discussion

3.1 Main applications of non-animal models in immuno-oncology

After retrieving a total of 136,804 abstracts following study criteria, 542 peer-reviewed articles published between January 2014 and March 2019 were selected and analysed.

Six types of cancers were the most frequently studied: colorectal, breast, melanoma, pancreatic, non-small cell lung and ovarian cancers (Figure 2). A total of 49.2% (n=267) of

retrieved publications were reporting studies on several other types or combinations of cancer models. On the other hand, 22.8% of retrieved publications were focused on general immune-cancer biology using different models as instrumental.

The number of articles published in scientific journals increased each year and, in 2018, 144 publications reported the use of non-animal models (Figure 2) representing a 105.7% increase within four years.

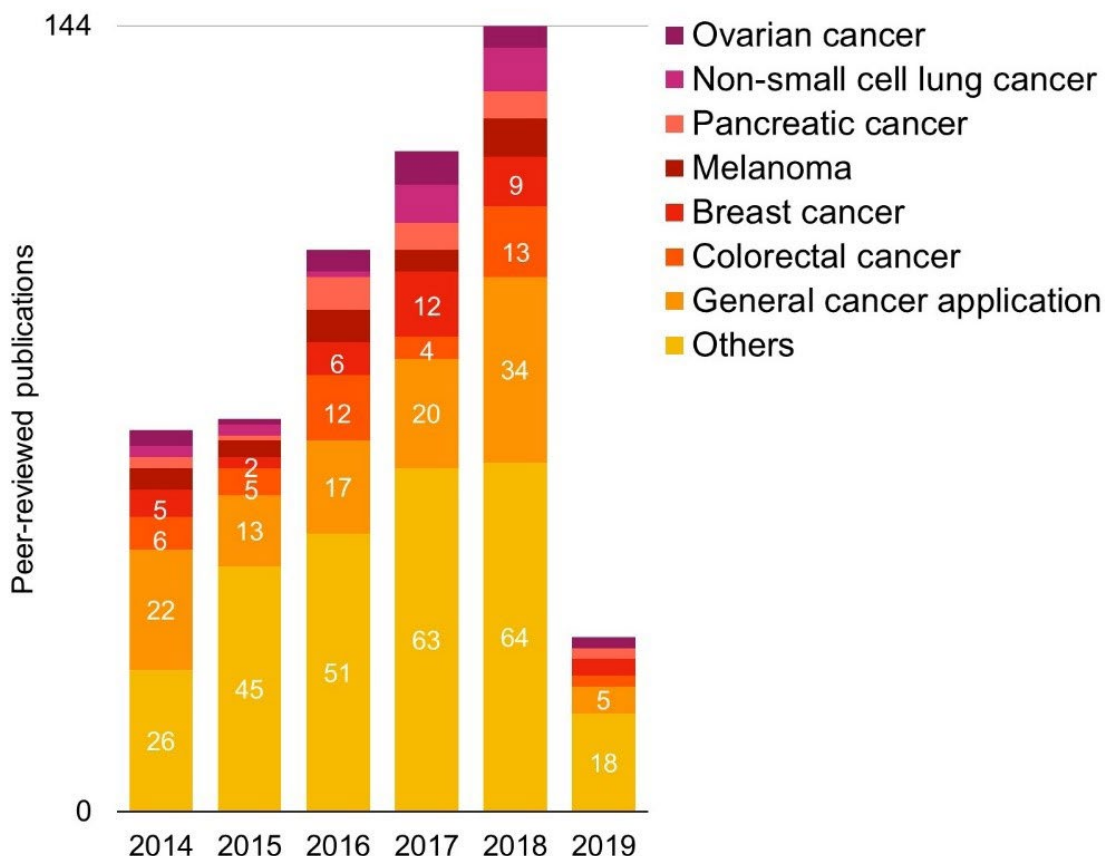


Figure 2: Distribution of peer-reviewed articles by year of publication from January 2014 to March 2019. Articles are classified by the main type of cancer their study focused on. The category “others” encloses all those types of cancers, where non-animal models were reported in less than 20 articles during the period of study. The category “general cancer application” includes studies exploring general immuno-oncology-related mechanisms or therapeutic strategies, which used any human based - cancer model in the methodology. The number of peer-reviewed publications are shown for the first four most abundant categories (others; general cancer application, colorectal cancer and breast cancer).

The majority of peer-reviewed articles were focused on developing immunotherapies (204 articles; Figure 3), spanning from a study developing a novel approach to generate tumour-specific polyclonal T cells for cancer immunotherapy (Saito *et al.*, 2016), to studies developing patient-derived organoids from clinical tissues (Braham *et al.*, 2018; Gao *et al.*, 2019). The second major use of non-animal models was to study cancer initiation and development (120 articles; Figure 3), such as the article from Neal and colleagues (Neal *et al.*, 2018) which focused on developing a new model for immuno-oncology studies, or the one from the Ribas group (Tumeh *et al.*, 2014), which studied the T cells driving a response to treatment in melanoma and how this cell population is regulated by the PD-1/PD-L1 axis,

or the impact of tumour purity in colon cancer (Mao *et al.*, 2018). Moreover, 92 studies used human-based models to explore anti-cancer therapies and other 46 research articles used these models to study immunomodulation of cancer physiology through compounds, to achieve immune- and chemotherapy-mediated cytotoxicity (Hamilton *et al.*, 2016), or potential effective strategies for enhancing the anti-tumour immune response (Abdellateif *et al.*, 2018).

Human-based models were also employed in 16 publications to determine molecular features which can represent biomarkers in specific cancer pathogenesis, either using *in silico* methods to identify neo-epitopes in cancer (Smart *et al.*, 2018), or comparing

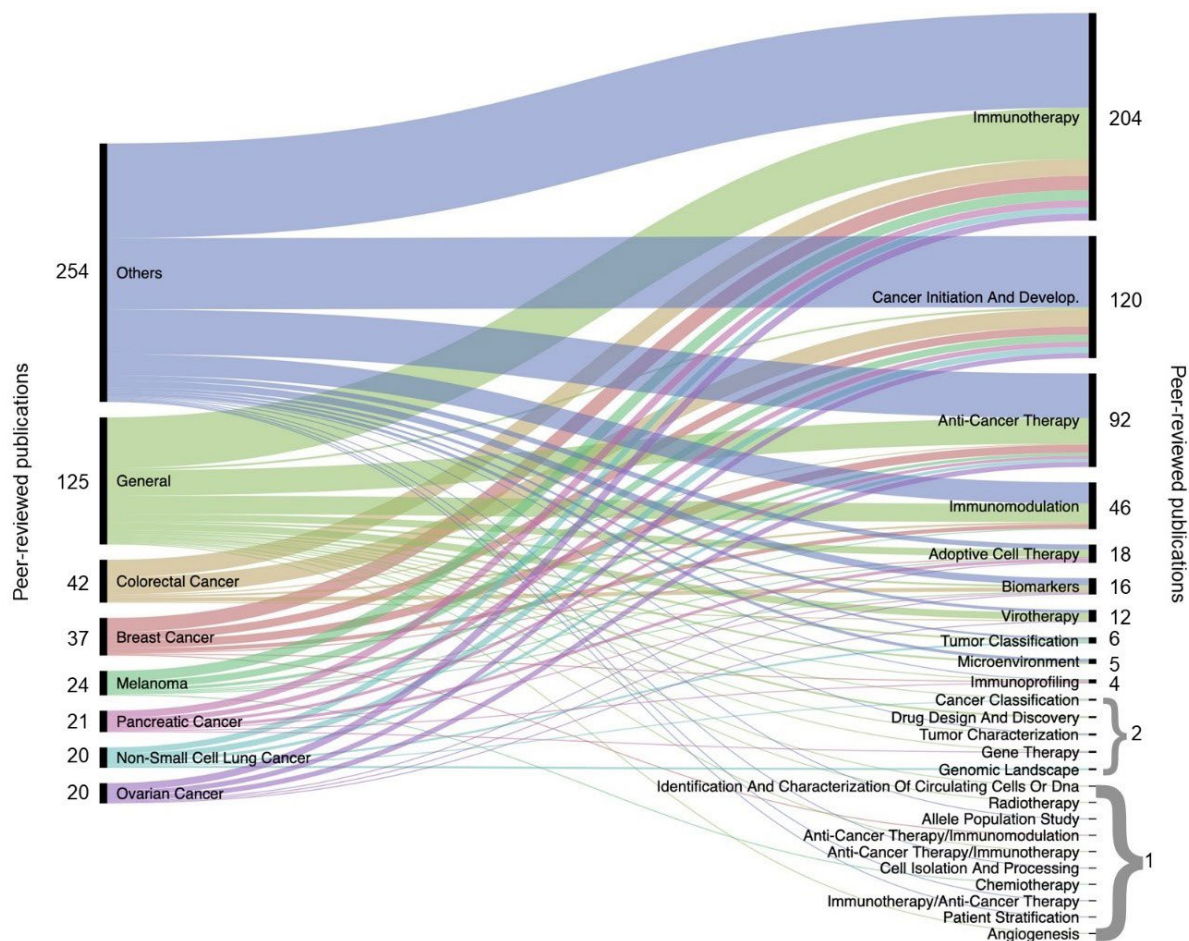


Figure 3: Human-based models are mainly used to study immunotherapies. Immunotherapies relate to a wide range of immune cell-based therapies, while anti-cancer therapies are defined as “therapeutic approaches based on drug candidates and/or physical properties that result in an immuno-related cell response”. Number of peer-reviewed publications focused on one or more immuno-oncology features is shown.

tumour antigens in cell line vs. primary cell line (Kloudová *et al.*, 2016).

Adoptive cell therapies and virotherapies were also explored by using human-based approaches in 30 studies, 18 and 12 research articles respectively. The remaining 35 studies employed non-animal models to dissect many other immune-related aspects of cancer pathogenesis, cancer stratification and therapeutic strategies (Figure 3), for example a study in neuroblastoma patients concluding that KIR3LD1 and HLA-B allele combinations can have a prognostic impact on patient survival after treatment (Forlenza *et al.*, 2016), or also patient stratification by assessing CCR7(+) mononuclear cells in the tumour microenvironment (TME), as a biomarker correlating to the progression of hepatocellular carcinoma (Shi *et al.*, 2016).

Six areas of applications of non-animal models in the immuno-oncology field were identified. In about 40% of retrieved publications, human-based models were applied to study or to model cancer molecular mechanisms

(Figure 4 A), with a gradual annual increase in the number of publications that reached 61 articles in 2018 (Figure 4 B).

A total of 35% of journal articles reported the use of these models in studies focusing on the development of therapies (Figure 4 A), including novel potential effective strategy for enhancing the antitumour immune response (Abdellateif *et al.*, 2018) or reducing the immune-mediated cytotoxicity (Hamilton *et al.*, 2016). During the five-year period under analysis, the interest in using human based approaches for development of therapies increased steadily. However, in 2018, a small reduction in absolute publication number was observed in comparison to 2017 (Figure 4 B).

Publications reporting their application in the development of new drugs or drug testing represented 14% of the total references (Figure 4 A). Moreover, this area of application experienced a clear increase from 2014 to 2018.

The development of new human-based models and methods represented 9.8% of all

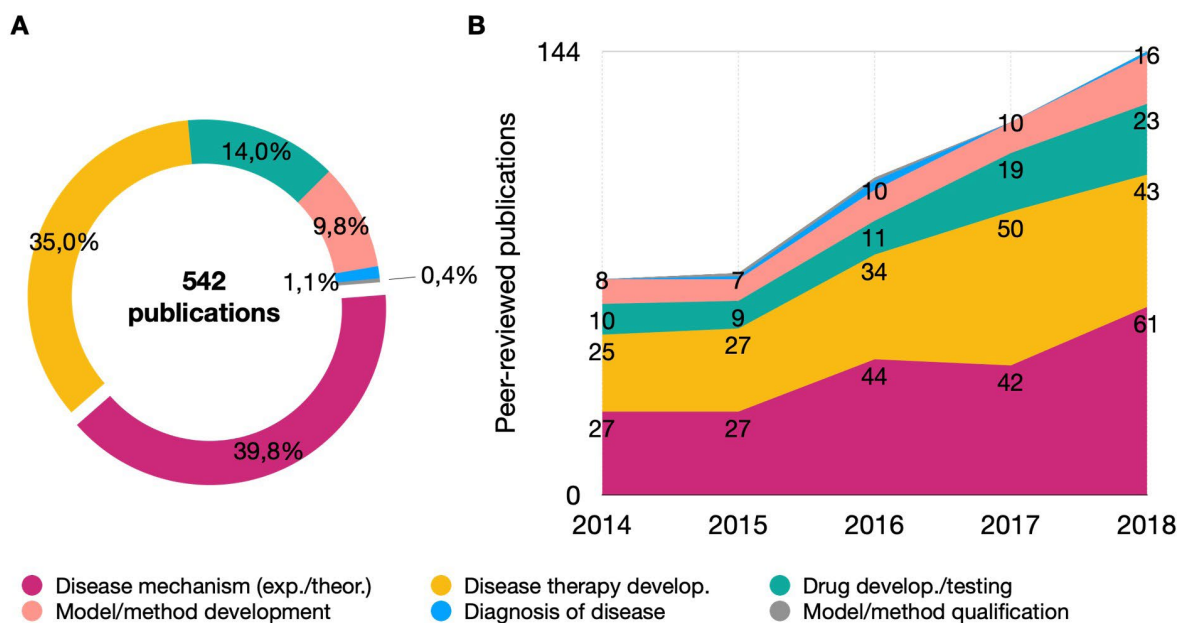


Figure 4: Six main applications for non-animal models in immuno-oncology research were identified. Panel A shows the percentage of each reported application in all retrieved articles, including publications from 2019. Panel B shows the distribution of articles by non-animal model application from January 2014 to December 2018 (2019 data are not included since only 3 months were analysed). The number of articles per year for the four major applications (disease mechanism, disease therapy development, drug development/testing, model/method development) are shown.

retrieved studies (Figure 4 A) and publications in this area increased from eight in 2014 to 16 in 2018 (Figure 4 B). Of interest is the development of new cell lines, e.g. cholangiocarcinoma (Thepmalee *et al.*, 2018) or colon cancer (Nagarsheth *et al.*, 2016), as well as a new *in vitro* model of MDSCs induction (Heine *et al.*, 2016).

A remaining 1.5% of studies (Figure 4 A) dealt with diagnosis of disease (1.1%; six articles) and model qualification (0.4%; two articles), both particularly relevant for the identification/validation of tumour antigens in cell line vs. primary cell line (Kloudová *et al.*, 2016) for

ovarian cancer, or biomarkers metastatic melanoma (Kotlan *et al.*, 2015).

3.2 Most represented categories of human-based approaches in immuno-oncology

The analysis of immuno-oncology scientific literature for non-animal models, showed that 88% of publications used *in vitro* models (Figure 5 A), with a clear trend increasing over time, from 62 publications in 2014 to 123 publications in 2018 (Figure 4 B). On the

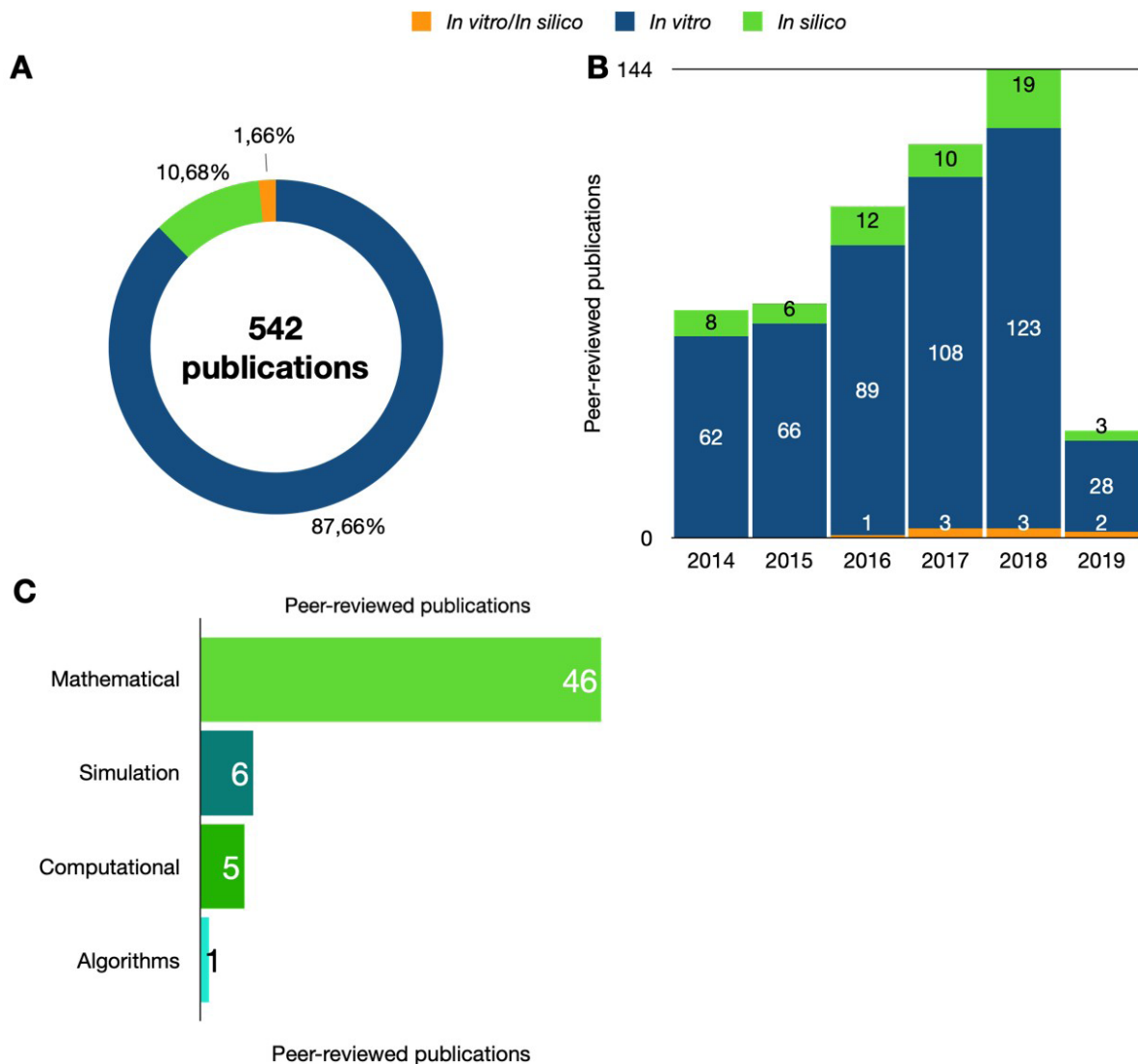


Figure 5: *In vitro* models were the most commonly used ones in immuno-oncology research. Panel A shows the percentage for each category in all 542 articles retrieved. Panel B shows the distribution of peer-reviewed articles by year of publication from January 2014 to March 2019. Panel C shows the number of peer-reviewed publications for each type of *in silico* models.

other hand, 11% were *in silico* models (Figure 5 A), mostly mathematical-based models (46 articles; Figure 4 C) and they increased from 8 studies in 2014 to 19 studies in 2018 (Figure 5 B). Up to 2% dealt with approaches integrating *in silico* and *in vitro* models (Figure 5 A).

Thirty out of 40 publications reporting the use of mathematical models were focused on modelling immunotherapies (Figure 6), mainly against cancer in general (Dawkins and Laverty, 2016; Lanzel *et al.*, 2016; Gong *et al.*, 2017) but also targeting bladder cancer (Starkov and Bunimovich-Mendrazitsky, 2016), melanoma (Jiang *et al.*, 2018), and prostate cancer (Nave and Elbaz, 2018) among others.

Eight publications described both *in vitro* and *in silico* models applied to the study of different aspects of immuno-oncology (Figure 6), such as immunotherapy (Valentini *et al.*, 2018), virotherapy (Liu and He, 2018), cancer initiation and development (Liu *et al.*, 2017), immunomodulation (Lanzel *et al.*, 2016) and tumour characterisation (Steele *et al.*, 2018).

The use of *in vitro* human-based models was significantly increasing in the period of study (Figure 7 A). It emerged that 65.4% of the total retrieved peer-reviewed publications (75% of *in vitro* reporting publications, Figure 7 B) used cell-based approaches to model many cancer features and interactions with the immune system (Figure 6). Patient biopsies were also used as *ex vivo* models in 113 articles (16% of total included studies) to investigate cancer initiation events and development, as well as immunotherapeutic approaches (Figure 6) (Guo *et al.*, 2017; Coscia *et al.*, 2018; Wei *et al.*, 2018), or tumour-array (Komdeur *et al.*, 2017). We found two publications using organ slices, both studying cancer initiation and development events focused on either breast or cervical cancer (Mani *et al.*, 2016; Komdeur *et al.*, 2017). We also retrieved 28 publications (6% of publications reporting *in vitro* models; Figure 7 B) using multiple approaches to model immuno-oncology features, by combining cell-based and *ex vivo* biopsy models (Figure 6). The remaining 3% of peer-reviewed publications reported the use of cell-free *in vitro* methods only, or in

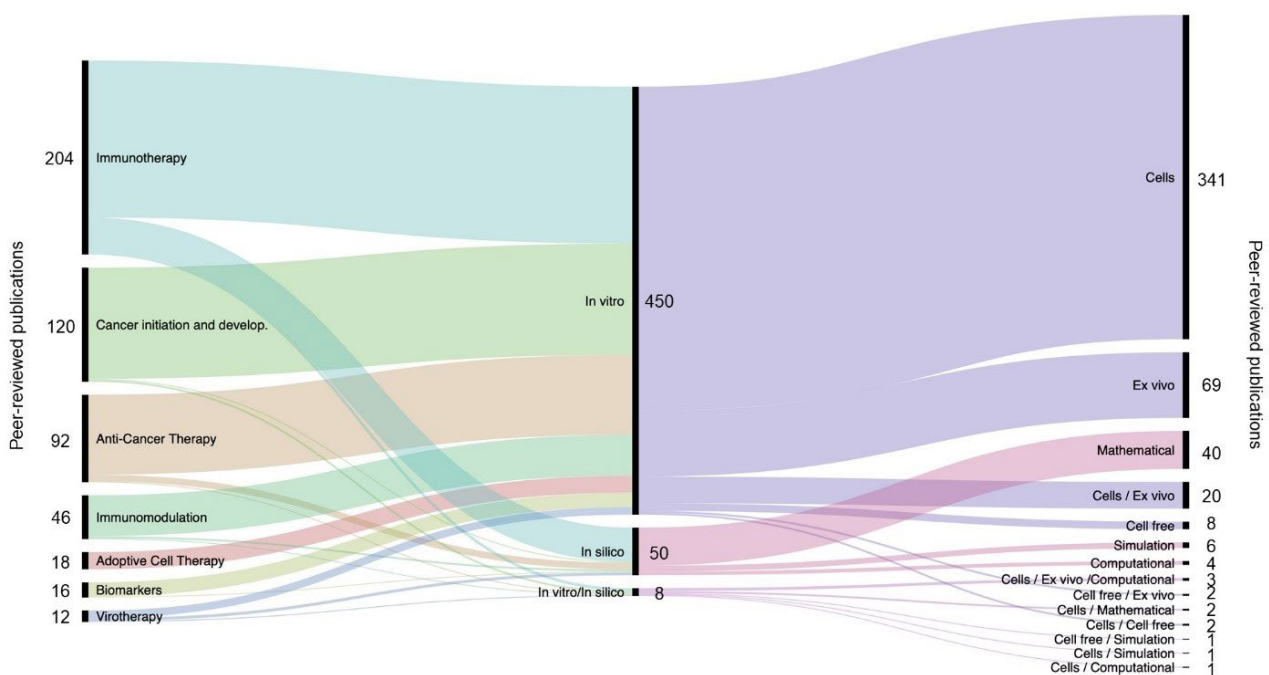


Figure 6: Distribution of the seven main research areas which resulted in a number of publications higher than 10 in immuno-oncology by type of human-based model used.

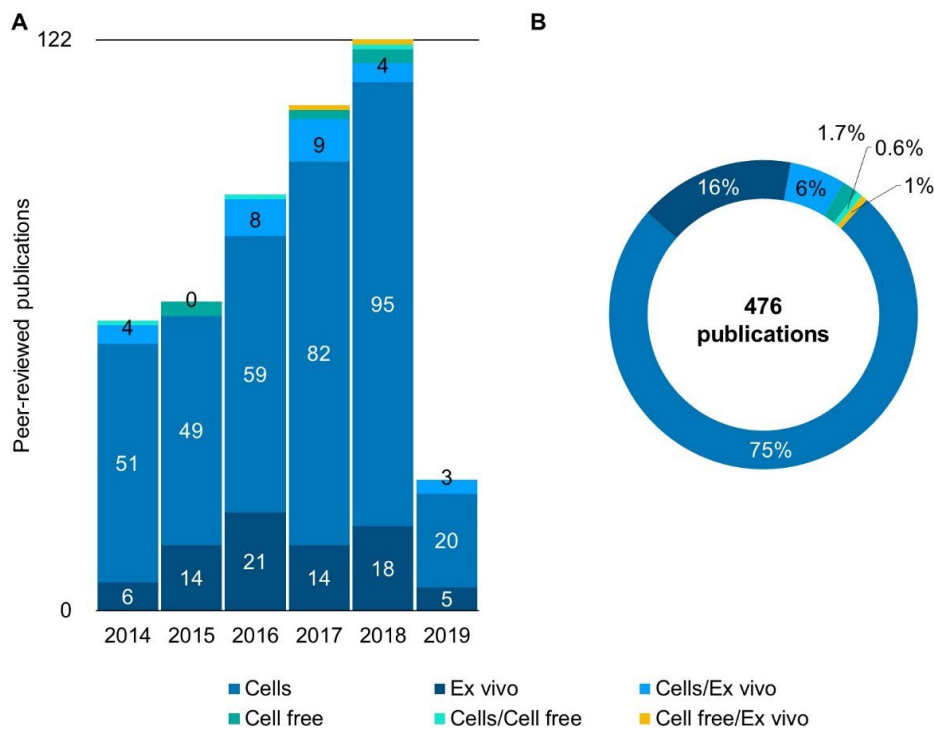


Figure 7: From January 2014 to March 2019 476 publications reported the use of *in vitro* non-animal models in immuno-oncology research. Panel A shows the distribution of peer-reviewed articles by year of publication from January 2014 to March 2019 and by type of *in vitro* model; values for the three most reported categories are shown per each year. Panel B shows the percentage for each category with respect to total *in vitro* non-animal models.

combination with cells or biopsies (Figure 7 B), such as liquid biopsies from patients with metastatic pancreatic cancer aimed to identify and to characterise circulating cells or nucleic acids (Sheng *et al.*, 2014).

A total of 392 studies out of 542 were identified dealing with cellular *in vitro* models (Figure 8). These publications reported the use of immortalised cell lines (38%), or primary cultures (32%), or a combination of both (29%; Figure 8 A). The number of publications for each category rose from 2016 onwards (Figure 8 B). Only one publication from 2019 used both immortalised cells and stem cells to study and target myeloid leukaemia (Guillaume *et al.*, 2019) and two publications from 2018 used both primary cultures, stem cells (Wenger *et al.*, 2018) and stem cell-like models (Chen *et al.*, 2018) to study glioblastoma multiforme and liver cancer, respectively (Figure 8 B).

Due to the importance of cell-based models, we disaggregated the data to provide a better

view of them (Figure 8): 134 publications described the use of biopsies as starting material for their *in vitro* models (Figure 8 C), and 30 of these studies performed primary cell culture from patient biopsies, mostly as monoculture, whereas in five studies they were cocultured in combination with immune cells to dissect immuno-oncology mechanisms, as in certain studies (Shiraishi *et al.*, 2016; Mo *et al.*, 2018) where biomarker profiles were assessed in patient-derived biopsies of gastric cancer and melanoma, in combination with a panel of immortalised cell lines. Furthermore, 5 studies were identified having performed coculture experiments with primary cultures of biopsies and immortalised cell lines to develop immunotherapeutic approaches and also to study cancer initiation events.

Nevertheless, the analysis revealed a great use of commercially available immortalised cell lines, such as breast cancer cell lines (MCF-7 and MDA-MB-231, 52 publications in total), lung carcinoma cell line (A549, 21 publications),

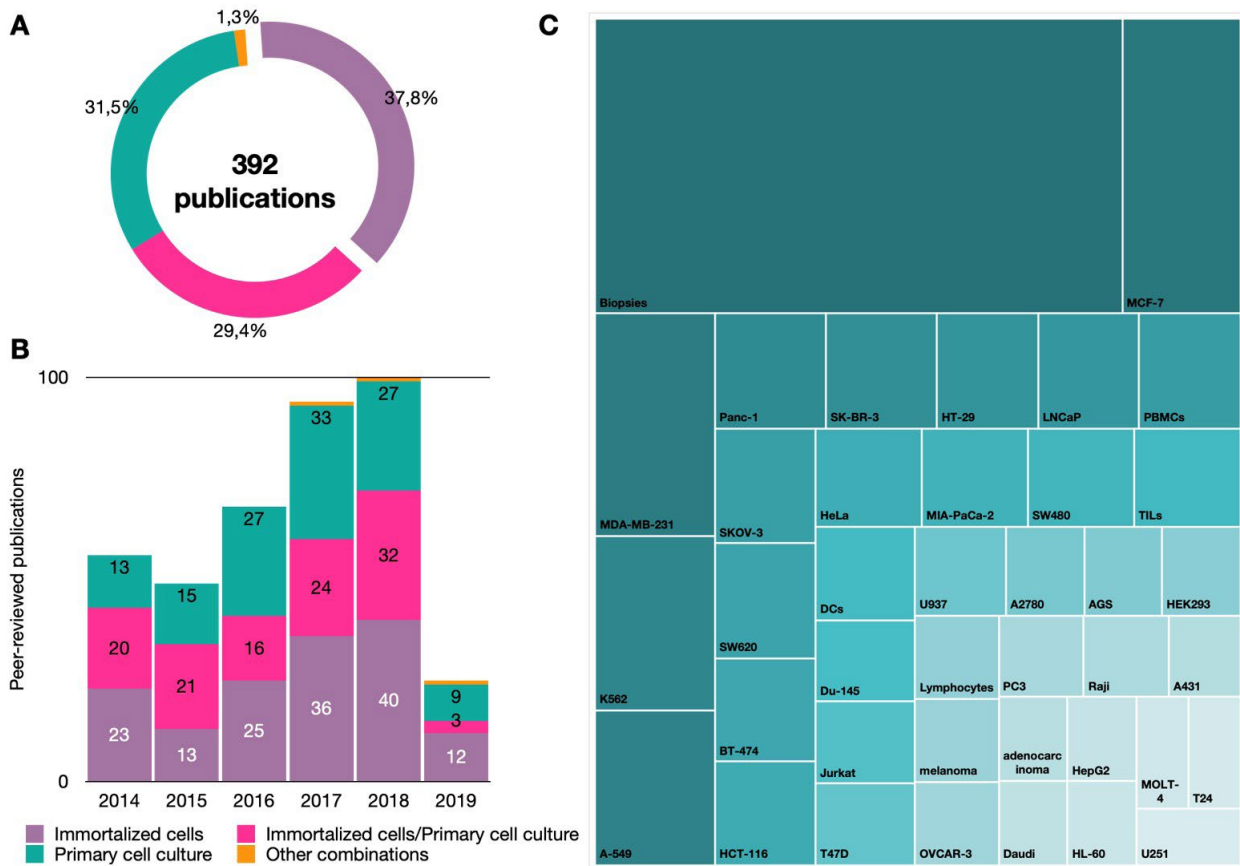


Figure 8: From January 2014 to March 2019, 392 publications reported various cell-based models in immuno-oncology research. Panel A shows the percentage for each type of human cell-based models category, with respect to total number of models. Panel B shows the distribution of peer-reviewed articles dealing with different types of human cell based-models by year of publication from January 2014 to March 2019; values for the three most commonly reported cell-based model categories are shown per each year. Only one publication dealing with “stem cells” (in yellow) was retrieved. Each square, in panel C, represents a human cell-based model. The area of each square is proportional to the number of identified publications, indicated as absolute number of articles.

bone marrow derived cells modelling chronic myelogenous leukaemia (K562, 17 publications; Figure 8 C). Other cell types, reported in more than five publications in the immuno-oncology field, are also shown in Figure 8 C.

3.3 Culturing conditions of human cell-based models

Acknowledging the importance of the culture conditions in better mimicking *in vivo* physiology, their different employment to use or develop *in vitro* cellular models has been analysed.

On one hand, 234 articles reported cell cultures of individual immortalised cell populations,

primary cells, stem cell-like and stem cells (Figure 9). On the other hand, 142 of the published studies employed co-culture systems, e.g. to model T cell induced cytotoxicity on lung cancer, cervical carcinoma, leukaemia and pancreatic cancer (Tal *et al.*, 2014).

Fourteen publications also employed human cells cultured into microphysiological systems (MPS) based on microfluidics (Figure 9) and six of these studies used 2D/3D or 3D cell cultures conditions (Figure 9) to model cell homing, immune invasion of tumour spheroids, and spheroid cytotoxicity in 2D/3D (Sherman *et al.*, 2018).

With respect to the dimension of the culturing conditions, 90.3% (363 articles) of all

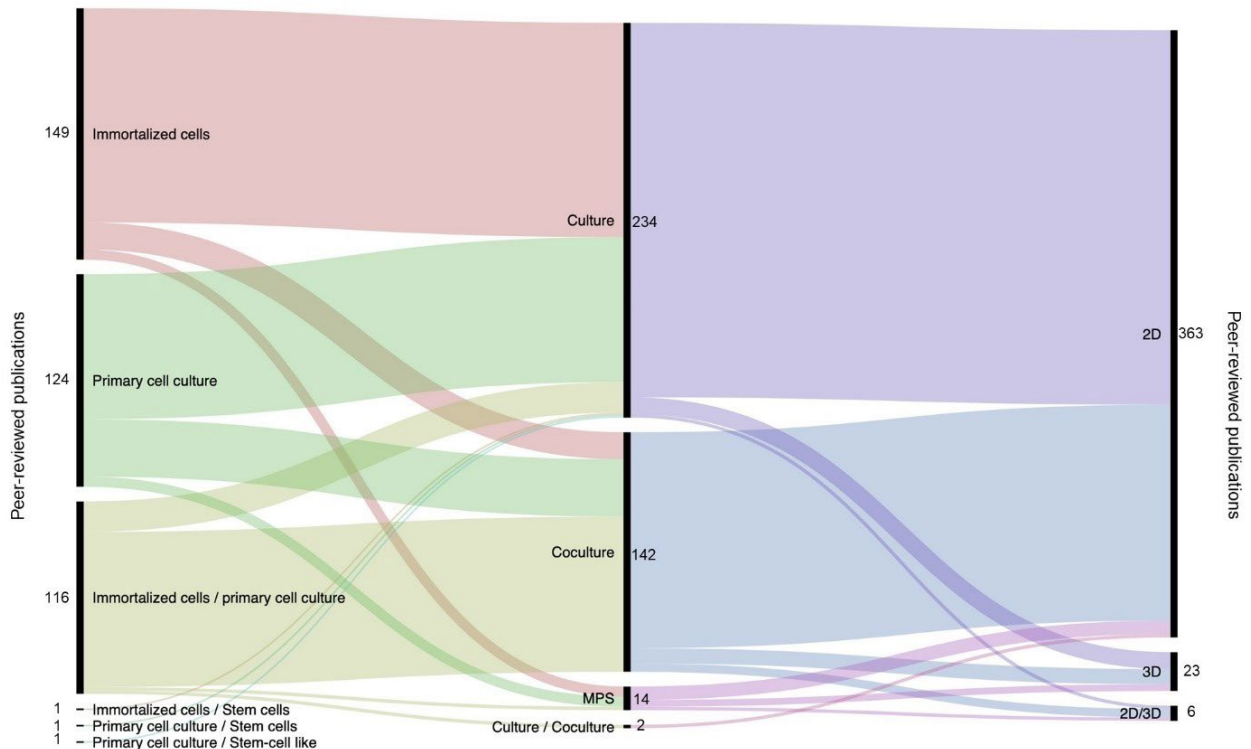


Figure 9: Types of human cell-based non-animal models by culturing conditions. Bars represent clusters of cell-based models (immortalised cells, primary cell cultures, stem cells and their combinations), clusters of cells culturing conditions (culture, co-culture, MPS or possible combinations) or spatial culture configurations (2D and/or 3D), and stream fields between the bars represent changes in the composition of these clusters over the analysed studies. The height of a bar represents the number of publications of the specific cluster and the height of a coloured stream area represents the number of the publications contained in both bars/clusters connected by the stream area. 2D cultures of immortalised cell lines and primary cultures are the most frequently reported in immunology research literature.

publications reporting *in vitro* models employed 2D culture techniques (Figure 9), including studies characterised by novel approaches to generate tumour-specific polyclonal T cells for cancer immunotherapy (Saito *et al.*, 2016) in melanoma, or a new *in vitro* model of MDSCs induction (Heine *et al.*, 2016).

Scaffolds, organoids or spheroid models (Poenick *et al.*, 2014; Zumwalde *et al.*, 2016; Sherman *et al.*, 2018) were used in combination with a 2D model to study immunotherapy or anti-cancer therapy assessing cytotoxicity, cell homing, and/or immune invasion in six publications. Only one study was focused on basic research on the TME by employing a 2D/3D model combination (Sethumadhavan *et al.*, 2017). While 23 studies reported culturing their cells in 3D (Figure 9 and Figure 10), by forming spheroids (nine publications)

and organoids (seven publications) but also through scaffolds (seven publications).

We observed a slight increase in the usage of 3D models over time, however this change followed the trend in the increased total number of publications, also observed regarding publications reporting 2D culturing conditions (Figure 10).

3.4 Trends of innovative studies in immunology research

Most of the studies retrieved were presenting proof of concept experiments and/or models (413 articles) and 29.8% (123 articles) of these were employing models already reproduced by other research teams (Figure 11), mostly

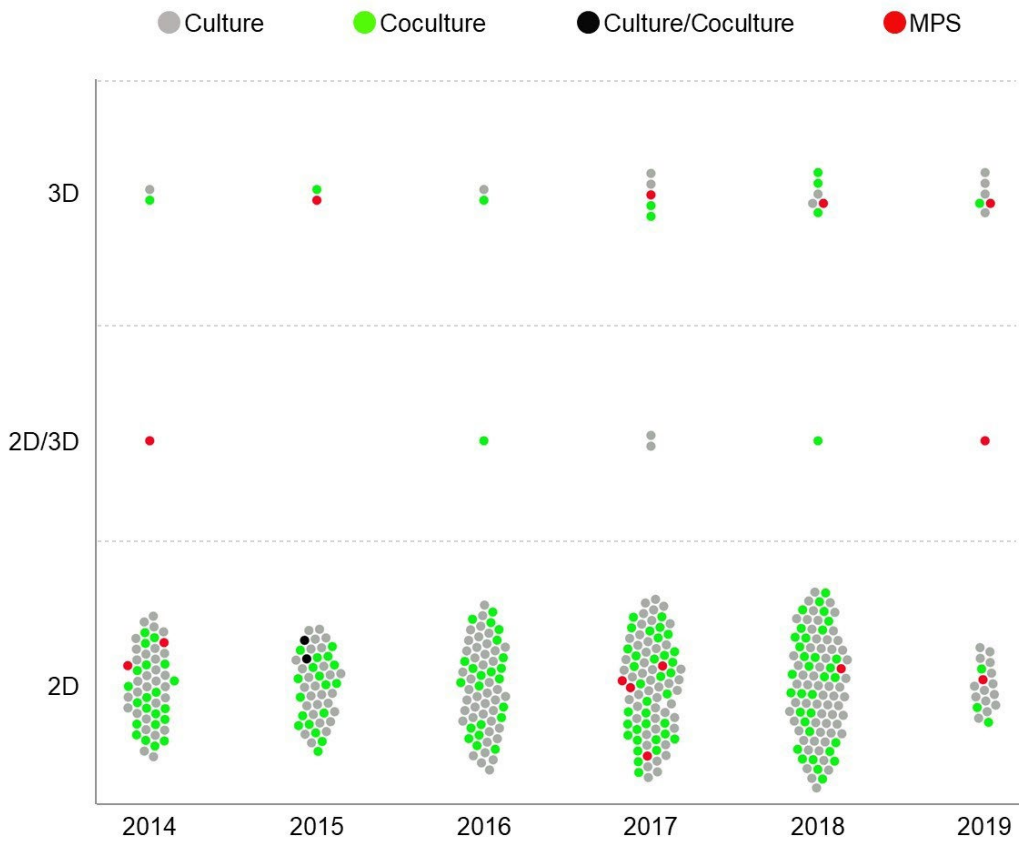


Figure 10: Distribution of articles by year of publication and by culture dimension, with colour coded culture conditions. Each dot represents a peer-reviewed publication.

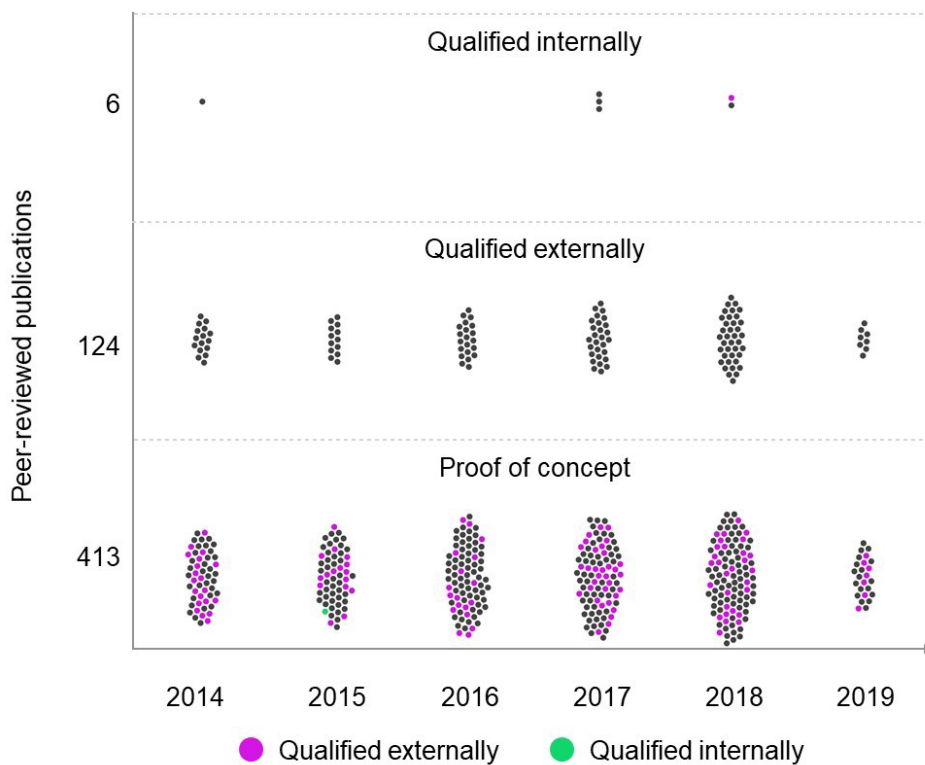


Figure 11: Distribution of peer-reviewed articles by status of models' use and year of publication. Each dot represents a peer-reviewed publication. Black dots represent publications reporting one model. Purple dots represent publications reporting multiple models, where one of the models has been already reproduced externally.

to develop therapeutic strategies (Figure 12). The other 70.2% were publications reporting proof-of-concept non-animal models studies or their application (Figure 10) on cancer mechanism, cancer therapeutic approaches and drug development and testing (Figure 12). Interestingly, all 58 publications employing *in silico* models were reporting proof-of-concept applications, highlighting the interest of *in silico* tools' applications to the complexity of immuno-oncology mechanisms looking for therapeutic clues.

A clear increase in the publication rate of proof-of-concept studies in the period of 2016 to 2018 was also observed (Figure 11).

Furthermore, 124 articles employed models reproduced by other research groups (Figure 11), mostly applied to dissect cancer mechanisms or in developing / testing drug candidates (Figure 12). Five articles used internally reproduced models and one article both internally and externally reproduced models (Figure 10), in particular reporting the

establishment of new cell lines (Gilabert-Oriol *et al.*, 2017; Li *et al.*, 2017; Thepmalee *et al.*, 2018). In addition, it is interesting to highlight a paper using “internally and externally reproduced ” cell lines, comparing the effect of DNA hypomethylating agents (DHAs) on human cutaneous melanoma cell lines that were generated from surgically removed metastatic lesions from melanoma patients and commercial human haematological cancer cell lines (Fazio *et al.*, 2018).

3.5 Throughput and information content of advanced models and methods

The 72.2% of all retrieved publications employed human immortalised cell lines and primary cultures (Figure 8 A and Figure 13) to mainly study immunotherapeutic strategies and cancer initiation and development (Figure 6). Since cell-based models were the most relevant non-animal models to be analysed

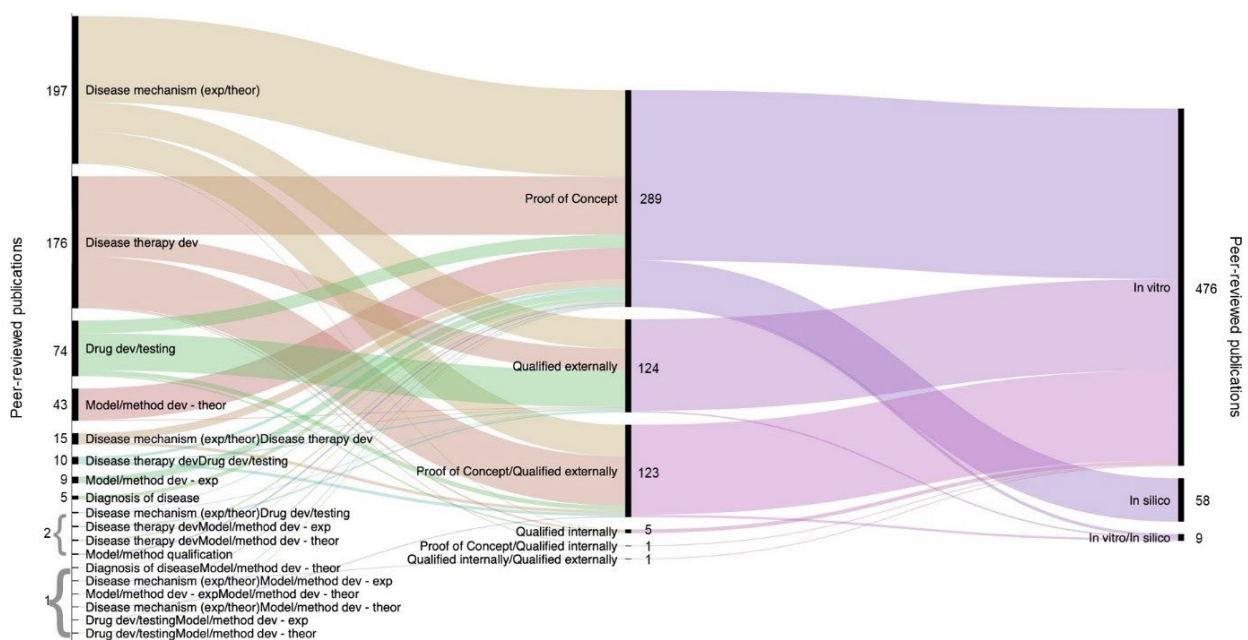


Figure 12: Number of publications by reported type of non-animal models, status and application. 24.7% of all retrieved publications dealt with cell-based models in proof-of-concept studies.

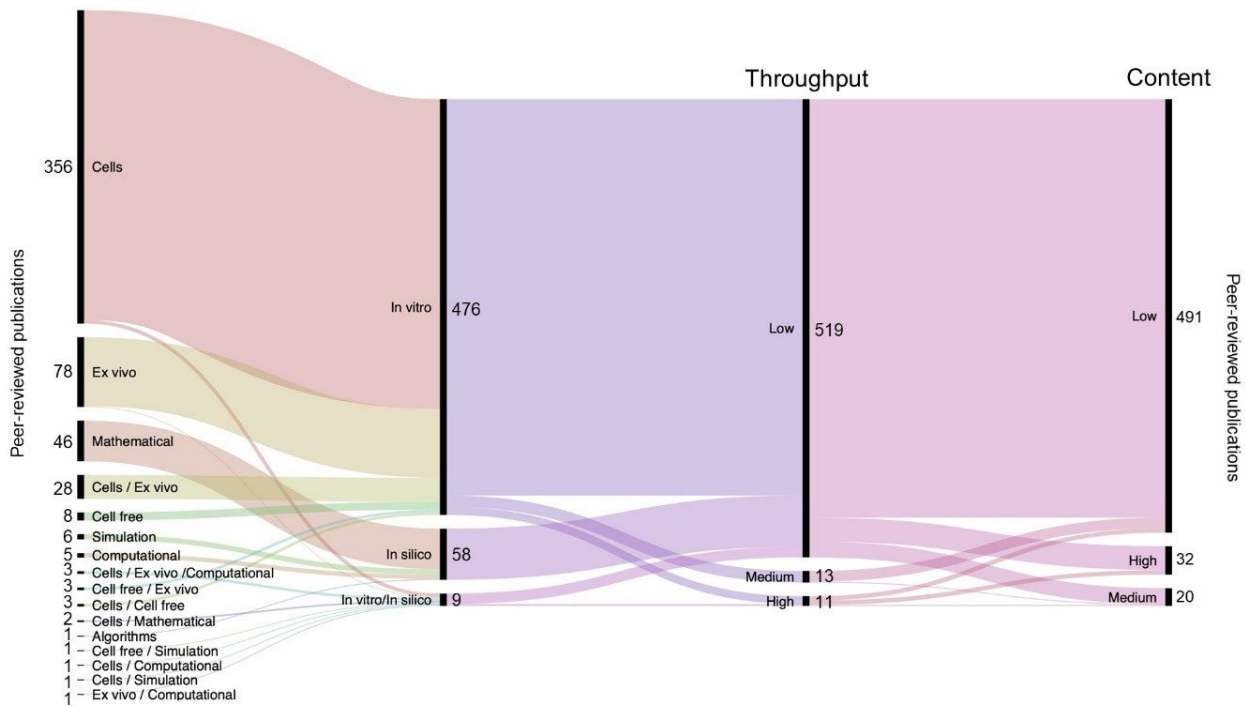


Figure 13: Number of articles by model and category, in conjunction with their throughput and analysis of content level. 87.3% (474 articles), of all peer-reviewed publications reported a low-throughput use of non-animal models coupled with a low content analysis

more in-depth, we determined the throughput³ (productivity or automation) and information content and analysis⁴ used to screen the biological endpoints or to retrieve phenotypic and molecular pathway information. We found 519 articles describing low-throughput use of non-animal models, 13 articles a medium-throughput level and 11 articles a high-throughput usage of non-animal models (Figure 13). From an information content perspective, 491 studies applied low content analysis methods, whereas 32 were reporting high-content analysis and 20 medium-content screening (Figure 13). This resulted in 87.3% of articles reporting both low-throughput use and low-content analysis of non-animal models. Interestingly, also *in silico* models were mostly used in low-throughput and low content manner (Figure 13).

We also analysed the relevance in the use of non-animal models to study a specific immuno-

oncology feature, also taking into consideration whether the model was used with a predictivity aim or not (Figure 14). Approximately a 50% distribution for the relevance of non-animal models in addressing the disease features was found. In fact, 275 articles (59.6%) were directly relevant, especially to study therapies and cancer physiology (Figure 14) (Lanzel *et al.*, 2016; Liu *et al.*, 2017) while, 268 peer-reviewed publications (49.4%) were using non-animal models as supportive model into their study for all the identified immuno-oncology features (Figure 14) (Valentini *et al.*, 2018).

Interestingly, only 91 articles reported a predictive use of non-animal models (Figure 14). However, most of the ones with predictive use (83.5%) had a direct relevance for the research targets under study (Figure 15) (Ma *et al.*, 2018; Mao *et al.*, 2018). Peer-reviewed articles reporting direct and supportive usage of human-based models were published with

³ Throughput is defined as the number of samples that can be processed in parallel.

⁴ Content is defined as the quantity of information retrieved by each sample with a single analysis or method.

similar rates along the period of time under consideration. The only exception was in 2015 where publications reporting a direct

application of non animal models were 45 compared to 27 publications using these models as supportive models (Figure 15).

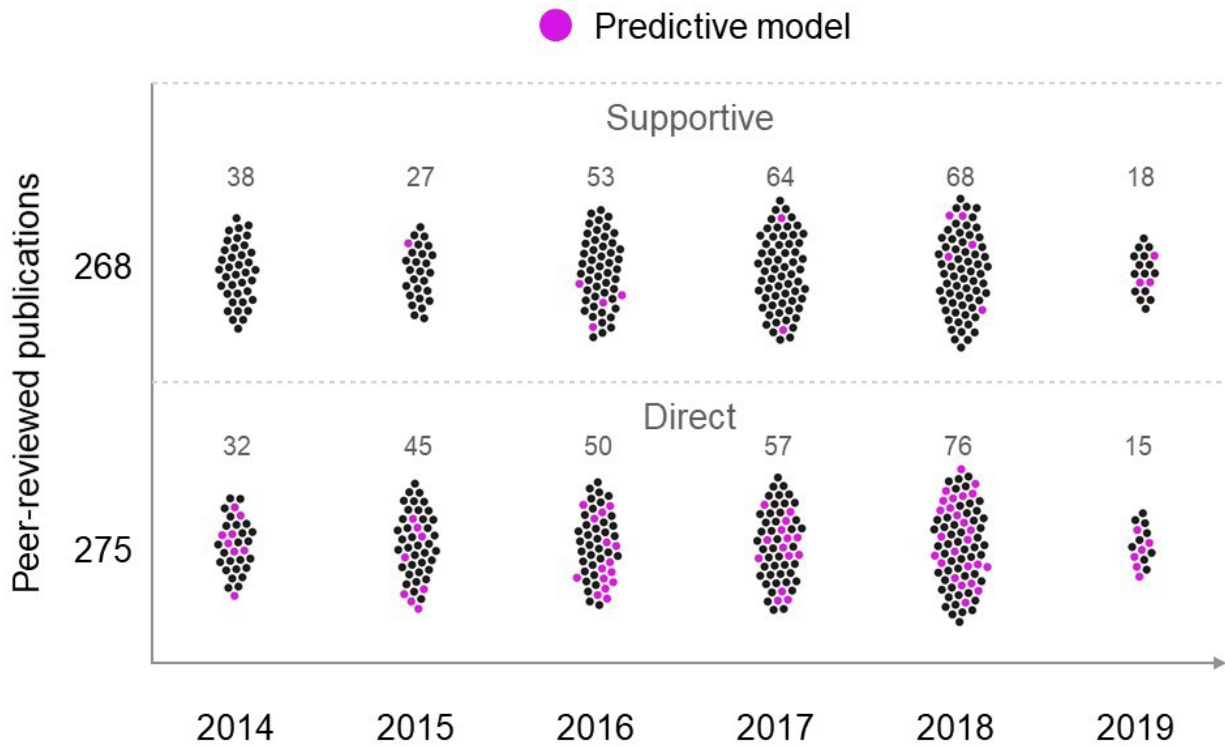


Figure 14: Number of articles and their distribution by their relevance and predictive use depending on the identified immuno-oncology research targets.

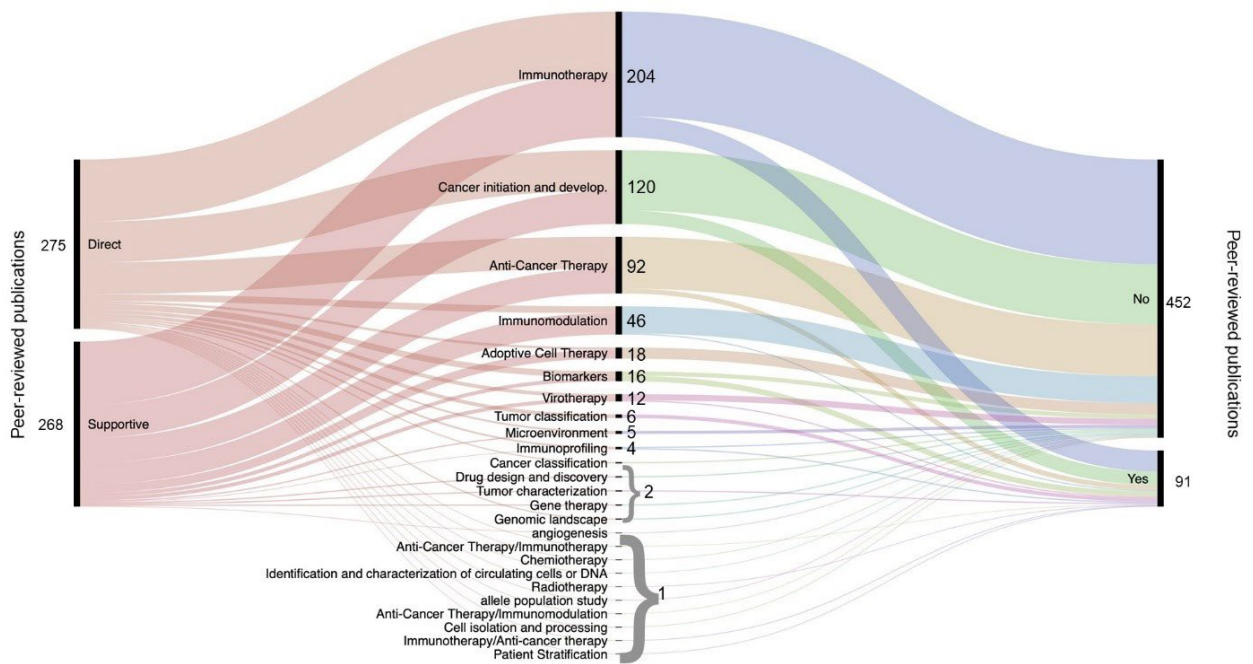
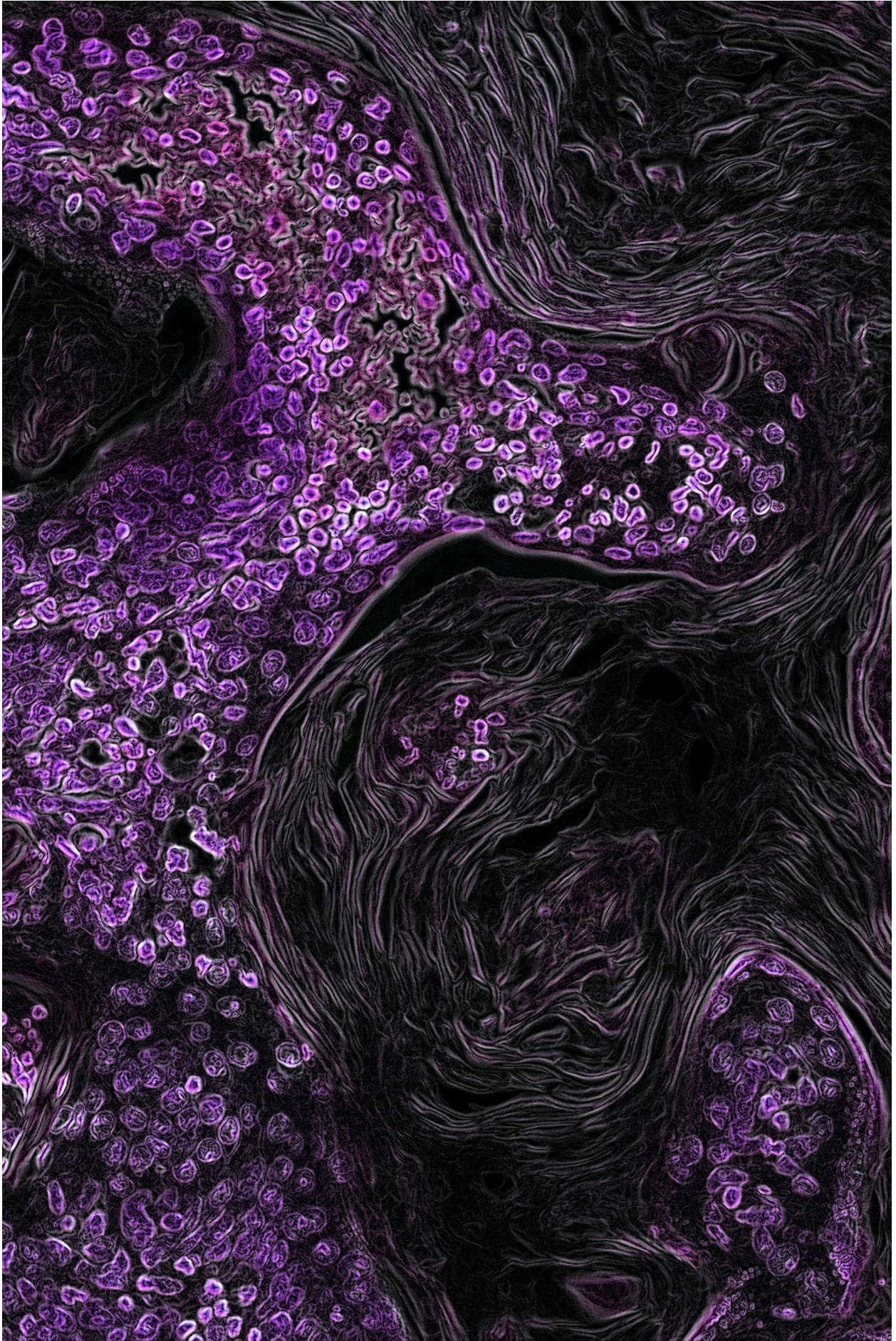


Figure 15: Number of articles and their temporal distribution by their relevance. Purple dots indicate a publication reporting a NAM with a predictive purpose in a specific immuno-oncology feature.



4 Conclusions

Recent advances in immuno-oncology research highlight the limitations of commonly used animal models in developing new approaches for cancer therapy. These models have failed to recapitulate the variable responses and potential toxicity seen in clinical settings.

For decades, cancer researchers have employed *in vitro* 2D cell cultures, and *in vivo* xenografts or genetically engineered animal models, but the high rate of ineffective compounds entering clinical testing indicates the need for more accurate models to predict efficacy, before subsequent clinical studies are effectively performed. In fact, 90% of the total biological immunotherapies entering clinical trials fail because of low efficacy and/or high toxicity, despite having undergone rigorous preclinical safety assessment (Arrowsmith, 2011; Begley and Ellis, 2012; Scannell *et al.*, 2012; Fernandez-Moure, 2016).

This is due to challenges and limitations related to the interspecies differences in key immune-biological aspects between humans and standard preclinical safety workhorses (Colucci *et al.*, 2002; Mestas and Hughes, 2004; Bailey *et al.*, 2013; Zschaler *et al.*, 2014; Haley, 2017). This high rate of failure is costly at both economic and social levels.

It is widely accepted that the spatial organisation and dynamic interplay of the complex cell-to-cell interactions in tumours are poorly mirrored by conventional *in vitro* and *in vivo* models. Tumours include both neoplastic cells and a diversity of non-neoplastic host components, collectively termed the TME (tumour micro-environment) which fosters carcinogenesis, tumour progression, and metastasis of malignant cells (Jin and Jin, 2020). This complex structure is regulated by the balance between cellular and humoral components, as well as several inflammatory mediators that support the growth of

neoplasms into an advanced tumour biomass, resulting in permanent alterations in cellular functions and response to immuno-oncology treatments.

In order to increase its reliability, a tumour model in the immuno-oncology field should reflect the heterogeneity of the tumour and contain components of the TME, in particular immune cells, the targets of immunotherapeutic drugs.

The most sophisticated animal models in use are the humanised immuno-oncology models, generated by the engraftment of PDXs into immunodeficient mice bearing human immune cells, but cost, time, throughput, and complete immune-compatibility, remain important challenges (Curran *et al.*, 2020). Even with timely improvement of these models, a developing HLA fully matched and personalised humanised mouse models will face other challenges, like the sub-optimal development of specific human immune cell types, due to lack of cross reaction of many cytokines and growth factors between mouse and human, or the residual mouse immune components, which mainly consist of macrophages and granulocytes that may also interfere with the responses in immuno-oncology studies and drug testing (DeNardo and Ruffell, 2019; Jaillon *et al.*, 2020).

Previous studies have investigated only defined aspects of the interaction of cells from adaptive and innate immune systems and tumour cell, highlighting the need for more comprehensive immuno-oncology models. *In vitro* screening models for targeted therapy, as a matter of fact, are an inevitable step, considering that the efficacy of immunotherapy is not uniform for all patients and/or cancer types.

To this aim, 3D *in vitro* models showed to be a better tool for addressing immune regulatory/modulatory questions for T cells, NK, and other

cell types of the immune system, compared to standard 2D cell culture techniques, which lack the necessary complexity to mimic *in vivo* heterogeneity, native histologic architecture, response to therapeutics and unravel multi-layered interplay overall.

It has to be considered that in 3D systems, the barriers that immune cells need to overcome are much greater than those in 2D. In order to mirror specific individual aspects — such as cellular migration or cancer immune evasion — immune cells not only need to migrate to the tumour site, but also to infiltrate a 3D structure, in order to attack the target cells.

Regardless of the advantages of the *in vitro* systems, a lack of tumour-specific 3D complex structure hampers the studies of crosstalk among cancer immunotherapy drugs, tumour cells, non-tumour cells and the TME, such as the presence of abnormal vascularisation and drug barrier. Moreover, it has been shown that phenotypic differences occur when tumour cells are cultured in 3D, allowing for higher resistance to cytotoxicity (Dangles-Marie *et al.*, 2003; Holmes *et al.*, 2011).

Hence, there is a clear need for new models that better mimic tumour biology and that can facilitate a more efficient and translatable drug discovery research. In this regard, we indeed found almost 10% of publications presenting new human-based models, highlighting the importance of novel and improved models to test the therapeutic strategies. This is also mirrored by the large number of proof-of-concept studies and the great use of patient biopsies to provide a closer look at human cellular and molecular environment.

This literature analysis demonstrated that the research community working in the immuno-oncology field is very much focused on the development and refinement of therapeutic strategies, reflecting the fact that cancer

represents one of the greatest targets for public health.

Most of the studies published until 2017 were focused on developing therapeutic approaches. However, in 2018 the aim of most studies changed into investigation of disease mechanisms. This possibly suggests a reorientation of the research community towards dissecting the molecular pathways, optimising the current strategies and creating new therapies.

A relevant limitation observed was the low use of high-content technologies such as omics, which can provide a broader and more in-depth view of the molecular dynamics, especially useful in dissecting the molecular interaction between cancer and immune cells.

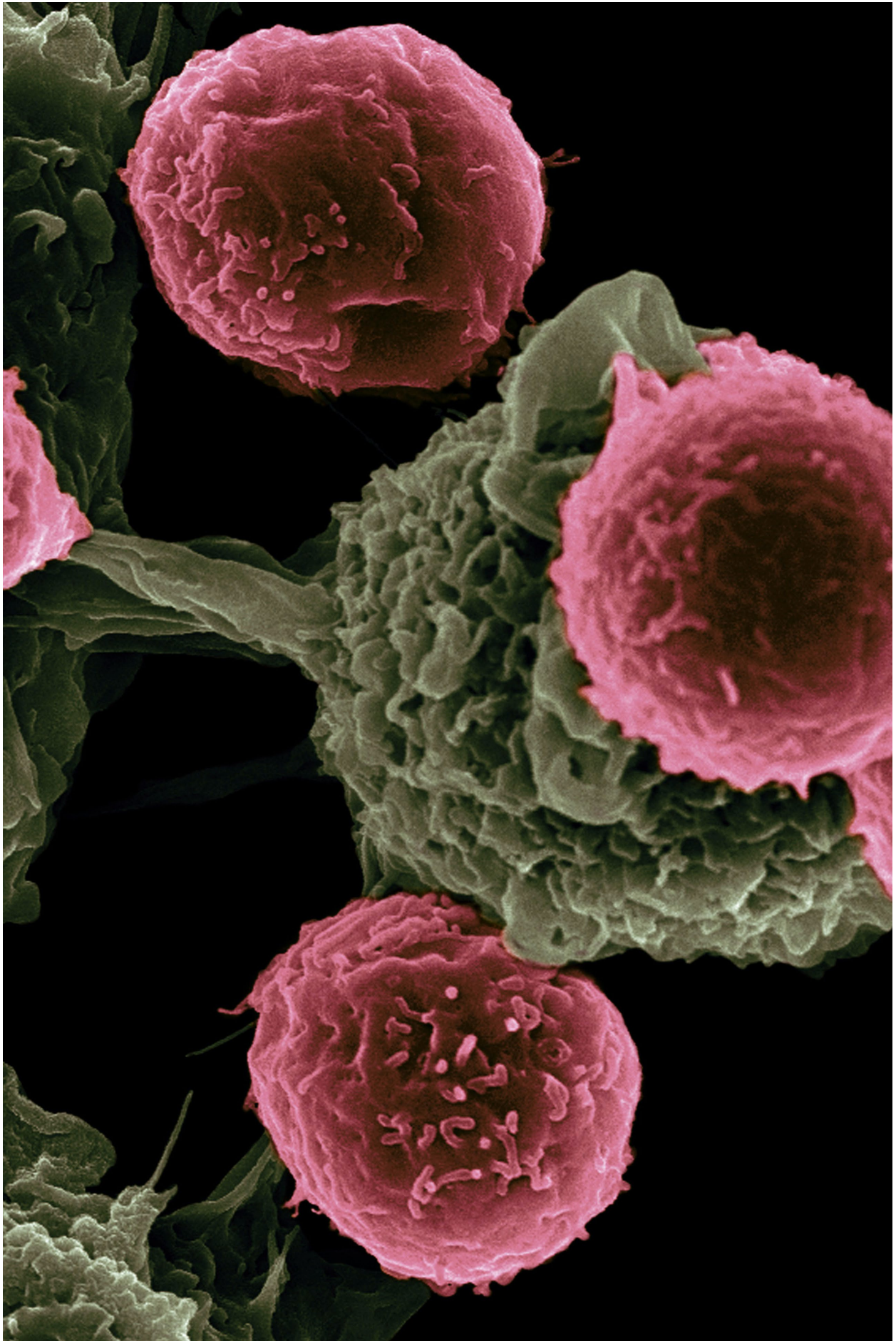
Considering the overall results of this systematic review, the main conclusions are the following:

1 The use of *in vitro* human-based models in immuno-oncology research is extensive; however, there is a clear need for more physiologically relevant models.

2 Non-animal models are mainly applied to test immunotherapies or other therapeutic strategies, but they are also employed to study the cancer initiation and development and its interaction(s) with the immune system.

3 *In vitro* cell-based models are the most frequently used, however they very often employ standard 2D cultures, under low-throughput conditions and involve analyses in a low-content mode.

4 Only half of the studies employed models as direct tools to study targeted aspects of immuno-oncology, and only one out of six as predictive models. Hence, there is certainly room for improvement in this regard.



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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
 - i. Explants
 - ii. Whole organ or organ slice
-

3. Cell-free assays

- a. Biochemical assays
-

4. Gene reporting assays

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

1. The study does not deal with immuno-oncology
2. Secondary literature (review, meeting abstract, etc.)
3. Duplicate
4. No <i>in vitro</i> or <i>in silico</i> model or method
5. In vivo study
6. Test method not able to measure endpoints
7. The study does not focus on development/characterisation 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. Non-English articles
13. Retracted publication
14. Published before 2014

Table 3: Specialised information sources on immuno-oncology research used for literature searches.

Societies	
European Society for Medical Oncology	https://www.esmo.org
Research institutions and programs	
Center for Immuno-Oncology – Dana-Farber Cancer Institute	https://www.dana-farber.org/center-for-immuno-oncology
Events	
Immuno-Oncology Summit Europe 2019	https://www.immuno-oncologyeurope.com/?gclid=EAIaIQobChMI_byt_9q44AIVx4jVCh05MAS4EAAYAiAAEgJmofD_BwE#
Immuno-oncology Summit	https://www.immuno-oncologysummit.com
Clinical Immuno-oncology symposium	https://immunosym.org
News platform	
Immuno-oncology News	https://immuno-oncologynews.com

Table 4: Definitions of the different strategies and their efficiency indicators (presence and inclusion rate).

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
Disease area	Type of cancer	For example: Breast cancer Colorectal cancer Leukaemia Lung cancer
Disease feature	The disease feature studied by the model	For example: Angiogenesis Cancer initiation and develop. Gene therapy Immunotherapy
Disease feature (1to 3)	Other disease feature studied by the model	See disease feature
Cellular input	Type of cellular input used to stimulate or treat the model	For example: Adaptive NK cells CAR T-cells
Model	Source of physical biological material or its registered properties in use	For example: A-549 Biopsy MCF-7
Category	The category of non-animal model assigned to the model	<i>In vitro</i> <i>In silico</i> <i>In vitro/in silico</i>
Type	More specifications of the model category	Cells Cell-free <i>Ex vivo</i> Computational Algorithm Simulation Mathematical
Cells	Biological material source, if any	Immortalised Primary Stem cells
Cell culture type	If the model employs cells, this field specifies the tpe of cell culture	Culture Co-culture MPS (Microphysiological systems)
Cel culture dimensions	If the model employs cells, this field specifies the dimensions of the cell culture	2D 2.5D 3D
3D	If the model uses 3D cell cultures, this field specifies the type of the 3D dimension	Scaffolds Spheroids Organoid
<i>Ex vivo</i>	If the model is based on <i>ex vivo</i> cells/ tissues, this field specifies the type of materials	Biopsies Liquid biopsies Organ slice Whole organ

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: Cell proliferation Cytotoxicity T-cell activation
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)
Status	Model developmental stage	Proof of concept (New method/model description) In research use (Method/model in use by research community but not widely deployed) Internally qualified (The research group is referencing its own previous article/s) Externally qualified (The research group is referencing previous article/s published by others and accepted by the research community) EMA or FDA approvals (Recognition by the European or USA regulatory agencies)
Content	Quantity of information retrieved	High Medium Low
Predictive		Yes (The model has a predictive purpose) No (The model has not a predictive purpose) N/A (Not specified)
Year	Publication year from 2014 to 2019	-
First author name	Name of the first author of the peer-reviewed article	-
Link to abstract (DOI)	Digital Object Identification number or link to retrieve the publication abstract.	-

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