Lab on a Chip

CRITICAL REVIEW

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Bridging the gap between tumor-on-chip and clinics: a systematic review of 15 years of studies†

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Over the past 15 years, the field of oncology research has witnessed significant progress in the development of new cell culture models, such as tumor-on-chip (ToC) systems. In this comprehensive overview, we present a multidisciplinary perspective by bringing together physicists, biologists, clinicians, and experts from pharmaceutical companies to highlight the current state of ToC research, its unique features, and the challenges it faces. To offer readers a clear and quantitative understanding of the ToC field, we conducted an extensive systematic analysis of more than 300 publications related to ToC from 2005 to 2022. ToC offer key advantages over other *in vitro* models by enabling precise control over various parameters. These parameters include the properties of the extracellular matrix, mechanical forces exerted on cells, the physico-chemical environment, cell composition, and the architecture of the tumor microenvironment. Such fine control allows ToC to closely replicate the complex microenvironment and interactions within tumors, facilitating the study of cancer progression and therapeutic responses in a highly representative manner. Importantly, by incorporating patient-derived cells or tumor xenografts, ToC models have demonstrated promising results in terms of clinical validation. We also examined the potential of ToC for pharmaceutical industries in which ToC adoption is expected to occur gradually. Looking ahead, given the high failure rate of clinical trials and the increasing emphasis on the 3Rs principles (replacement, reduction, refinement of animal experimentation), ToC models hold immense potential for cancer research. In the next decade, data generated from ToC models could potentially be employed for discovering new therapeutic targets, contributing to regulatory purposes, refining preclinical drug testing and reducing reliance on animal models.

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1. Introduction

For almost one century, scientists have been developing and improving cell culture models to increase their resemblance to human *in vivo* conditions and relevance for clinical transition. In a similar vein, novel tumor-on-a-chip (ToC) technology has developed tremendously over the past decade and holds great promise for clinical applications in oncology. The present review focuses on the quantitative analysis of the tumor on chip field up to date and offers a clear outlook of the subject in terms of both, engineering and application approaches. We have brought together academic researchers (physicists and biologists), clinicians as well as pharmaceutical companies' research experts to offer a multifaceted point of view that reflects the diversity of the actors of the field.

Nowadays in Europe, the yearly number of newly diagnosed people with cancer is about 3.5 million. In 2021, the EU Cancer Mission outlined that if no further action is taken, this number will dramatically increase to more than 4.3 million by 2035. In the fight against cancer, the better understanding of cancer mechanisms and the development of more effective anti-cancer drugs still remain highly challenging. Although conventional and well-established

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treatments, such as chemotherapies, face many failures, the development of new therapeutic strategies, such as immunotherapies and targeted therapies, raises new hopes. Since 2015, the U.S. Food and Drug Administration (FDA) has approved more than 80 novel cancer drugs, illustrating a historically high level of successful clinical trials in oncology.[1] However, it is also worth mentioning that only 4 to 7% of potential anticancer drugs obtain final clinical approval[2] compared to 10 to 15% for other diseases.[3] Such low success rates call into question the preclinical studies whose aim is to predict the effect of therapeutic agents in terms of efficacy, safety and dosage. The analysis of clinical trial data (from 2010 to 2017) has shown that the two main reasons for these failures are the unmanageable toxicity and lack of clinical efficacy.[4] Preclinical toxicity studies are mainly assessed by animal testing. However, it is now established that animals are not always suitable models for human toxicity prediction as shown in the meta-analysis of Atkins *et al.*[5] that compares preclinical and clinical toxicity profiles of 108 anti-cancer drugs in animal models and humans. They highlighted that the main unpredictable toxicities are of neurologic/psychiatric, cutaneous, respiratory, and cardiovascular nature. Another reason for the low success rates of anti-cancer drug development is related to intrinsic drug efficacy. Recent studies have also shown that current *in vitro* and *in vivo* models are poor predictors of drug efficacy.[6] The dramatic failure rate of clinical trials not only challenges our ability to design and develop new drugs, but also the use of animal models for basic research. Common *in vivo* models include patient-derived xenografts in mice (PDX), which share several important characteristics with human tumors (*i.e.*, vascularization, 3D structure and metabolism). However, PDX models also lack some crucial features such as human stroma and pharmacokinetics as well as an intact autologous immune system since such models often consist of immune deficient mice to allow efficient engrafting.[7] It is also worth mentioning that syngeneic animal models will be even less predictive for novel anti-cancer therapies such as biologics, gene- and cell-based therapies, since these drugs are either mainly specific to human targeting molecular sequences or involved the human immune system.[8] Very recently, the FDA has removed the requirement of animal testing before human trials ("Modernization Act 2.0"). This FDA statement is an excellent opportunity as well as a strong responsibility for the scientific community to develop and share innovative *in vitro* models that would faithfully reproduce disease mechanisms while improving predictive power.

Within the past decades, researchers have developed more adequate 3D *in vitro* models such as spheroids and organoids. They can incorporate different cell types to better mimic the complex tumor microenvironment (TME) compared to conventional 2D cell cultures. While the use of spheroids for *in vitro* drug testing is popular due to their relative ease of handling, they still exhibit a low degree of structural complexity.[9] Organoids, cultured from embryonic, adult stem cells or induced pluripotent stem cells are better in mimicking the TME[10,11] and several studies have evidenced that they are relatively good predictors of chemotherapy response in patients.[12] However, the establishment of patient-derived organoids generally requires 1 to 3 months,[10] and thereby limits their application in clinics as a diagnostic tool to support the choice of a specific treatment. Organoids and spheroid cultures are also associated with some major limitations: (a) they are static models which can lead to the accumulation of biochemical waste within the cell aggregate, (b) they do not fully reproduce the immune response, presence of fibroblasts and vascularization, (c) they do not reproduce the mechanical properties of tumors which can influence drug response.[13]

New *in vitro* models for oncology research are required for both basic and preclinical research. These models should especially consider that tumors are complex ecosystems dynamically evolving over time. Indeed, the TME not only contains cancer cells, the surrounding extracellular matrix (ECM) with varying mechanical and physico-chemical properties, but also a variety of other cell types such as immune lymphoid and myeloid cells, cancer-associated fibroblasts (CAFs), pericytes and endothelial cells with specific spatial organization. All before mentioned TME components have been shown to play a significant role in tumor progression, metastasis development, and resistance to treatment.[14] Microfluidic technologies, and organ-on-chip approaches in particular have an enormous potential for the development of a new generation of 3D *in vitro* tumor models. ToC contains several features which make them highly attractive for both basic and translational research: (a) the capacity to control the cellular, mechanical and physicochemical conditions on-chip, (b) the compatibility with a wide range of analytical methods including transcriptomic analysis and live imaging, (c) the possibility to produce human and/or immunocompetent models, and (d) the relatively short experimental time of several days as compared to other 3D cancer models such as PDX or organoids, allowing clinical decision.

In this review, we conducted a systematic analysis of the publications related to ToC between 2005 and 2022 to provide the reader with a clear and quantitative vision of the emerging ToC field. Altogether, over 300 publications were identified using PubMed, Google Scholar and looking directly into key journals as shown Fig. S1.† In this systematic review, we identify crucial subject parameters and extracted percentages of each occurrence. We discuss the added values of ToC as well as the scope of possible applications: drug screening, cellular mechanisms understanding and personalized- and nano-medicine. The success of ToC for clinical applications will be determined by its ability to detect and validate new therapeutic targets as well as to guide the definition of the delicate balance between clinical dose, efficacy and safety.

2. Material and methods

We used several databases (such as Pubmed, Google Scholar) and searched for combinations of the following key words in the title and abstract (Fig. S1†): "tumor", "cancer", "microfluidic", "chip". We also searched directly for relevant publications in specific journals such as Lab On Chip. Publications with only computational approaches, as well as reviews were excluded during the first screen (titles and abstract). We screened references of more than 10 reviews to list all the ToC publications. During the second step, a qualitative analysis of all the publications results was performed and only studies including cell culture on chip were included (for example CTC sorting methods were excluded). For the purpose of the analysis, we created a table with information related to the most relevant aspects of OoC devices: a) cellular components: type (cell line, primary cells, freshly resected tumor, IPS cells, organoids), organ (breast, lung, pancreas, prostate, ovary/uterus, colorectal/intestine, liver, brain), spheroids (yes/no); b) physico-chemical control: flow, oxygen control, extracellular matrix; (c) applications: anti-cancer drug, readouts. Excel was used to count occurrence of these key points in all the publications. Over 300 publications were analysed.

3. Tumor-on-chip allows to control the reconstituted tumor micro-environment

Cancer is not defined solely by cancer cells but by the whole TME, including cellular and molecular components, ECM, as well as their complex interplay. Tumors have many biomechanical abnormalities such as elevated solid stress, interstitial pressure, and stiffness.[13] One beauty of the ToC approach is the possibility to finely control and thus dissect the role of each parameter of the reconstituted TME. Particularly, this includes the different cell types and their spatial organization, the ECM properties, the on-chip generation of biochemical gradients, the control of the gaseous environment or even the different mechanical forces at play.

3.1. Controlling physico-chemical properties of the TME

A. Features of the tumor extracellular matrix. The ECM is a complex compartment composed of matrix proteins (*e.g.* collagens and elastin), glycoproteins (*e.g.* fibronectin), glycosaminoglycans (*e.g.* heparan sulfate, hyaluronic acid), proteoglycans (*e.g.* perlecan, syndecan) as well as ECM-sequestered growth factors such as transforming growth factor-β, vascular endothelial growth factor or platelet-derived growth factor and other secreted proteins (*e.g.* proteolytic enzymes and protease inhibitors).[15] The ECM composition varies according to the organ nature and state[16,17] and confers the tissue certain porosity, permeability, stiffness and elasticity. These ECM properties are often altered during pathological conditions such as tumor growth.[18,19] Those dynamic changes affect cell differentiation, proliferation, survival, migration, and adhesion.[15] ECM composition along with its physical properties can also influence cell response to drugs by either enhancing drug efficacy or promoting drug resistance.[20] More precisely, changes in the ECM composition may influence drug response by modifying the expression of drug targets, or by changing the cellular defence mechanisms such as DNA repair upon nucleic acids damage or cell apoptosis.[15] For instance, it has been shown that adhesion to the ECM may contribute to drug resistance by activating several pro-survival pathways such as PI3K/Akt, p53 or ERK/MAPK cascade.[21] Cells can be affected not only by the biochemical composition of the ECM but also by its mechanical properties. Among the different mechanical characteristics, ECM stiffness has proven to be an important parameter, influencing cell division, signalling, migration, gene expression, ultimately leading to cell-mediated ECM remodelling.[15] Tumors are usually stiffer than the surrounding healthy tissue due to the ECM production by stromal cancer-associated fibroblasts, the increase of interstitial pressure and the unorganized microvasculature.[21]

Given the complexity and dynamic alterations of the ECM, one of the key points of ToC is the ability to properly select and design biomaterials to reconstitute the extracellular matrix *in vitro*. Here, we focused on ToC with extracellular matrix, but excluded articles dealing with hydrogel coating for 2D cell monolayers. Collagen I is the main ECM component *in vivo* and is by far the most used hydrogel in ToC being used in half of the ToC publications) (Fig. 1). Collagen I contains the tripeptide RGD (Arg–Gly–Asp) which is a very common motif in humans and animals responsible for cell adhesion.[22] Another important advantage of collagen is its stiffness, which can be adjusted easily through its concentration or by covalent crosslinking *via* non-enzymatic glycation.[23] The second most common ToC hydrogel is Matrigel which is a solubilized basement membrane matrix secreted by Engelbreth–Holm–Swarm mouse sarcoma cells. Nevertheless, it has been suggested that results based on Matrigel-cultured cells should be interpreted with caution[24] due to its influence on gene expression[25] as well as the lack of some human peptide motifs.[22] It is worth noticing that both collagen and Matrigel can vary highly between different batches or manufacturers which can affect ToC reproducibility. Besides Matrigel and collagen, there is a wide range of hydrogels available for ToC development such as fibrin, gelatin and agarose (Fig. 1). Another less common approach relies on *in vivo* extracted matrix. Romero-López *et al.* used a decellularized ECM extracted from both healthy and cancerous colon tissues and prepared hydrogels through enzymatic digestion.[26] However, variability in ECM extraction protocols could introduce further alterations in hydrogels, which may lead to inconsistent results.

Hybrid hydrogels are made up of building blocks that include biologically active peptides, proteins or synthetic structures. Hybrid hydrogels can be used to obtain desirable

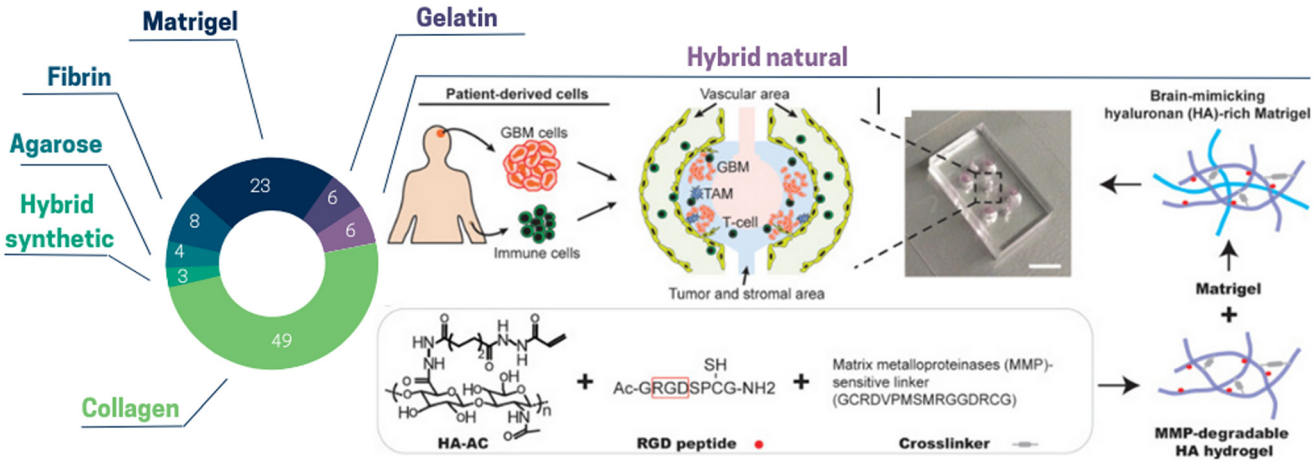

Fig. 1 Pie chart illustrating the proportion of the different extracellular matrix types used in ToC: collagen 49%, Matrigel 23%, fibrin 8%, gelatin 6%, hybrid natural 6%, agarose 4%, hybrid synthetic 3%. (Right panel) Hybrid natural: hydrogels can be created by interpenetrating growth-factor-reduced Matrigel matrix with MMP-degradable ha hydrogel for brain tissue-mimicking extracellular matrix. RGD peptides are conjugated onto acrylated hyaluronic acid (HA–AC) and crosslinked with MMP-degradable crosslinker.[27]

mechanical and biochemical characteristics *via* their functionalization with defined proteolytic sites and encapsulation of growth factors. Nevertheless, given that the ECM alone comprises more than 300 biochemical constituents,[28] this remains a daunting task. Among hybrid hydrogels in ToC, natural ones comprise alginate, fibrinogen, hyaluronan (HA), chitosan, and synthetic ones include poly-ethylene glycol (PEG), poly-caprolactone (PCL) and poly(lactic-*co*-glycolyc) acid (PLGA). Currently, these hydrogels have not been widely adopted. Cui *et al.*[27] used a hybrid brain tissue-mimicking hydrogel with RGD peptides conjugated onto acrylated hyaluronic acid (HA–AC) and crosslinked with MMP-degradable crosslinker (Fig. 1). With the combination of ToC and hybrid hydrogels, it is possible to not only control the ECM chemical composition and stiffness, but also to choose their spatial location. In a breast cancer model, Peela *et al.*[29] proposed a two-step photolithography approach to create an array of cells embedded in circular constructs, with a high stiffness matrix center surrounded by low stiffness matrix. They encapsulated three cell types separately to investigate cell migratory behavior, viability, and morphology. Importantly, cells migrating through the high stiffness circular constructs exhibited different invasive behaviors compared to those migrating through the surrounding matrix. They formed morphologically accurate structures without the addition of any biochemical stimuli, illustrating the versatility of ToC in creating a biomimetic tumor microenvironment.

There are still many challenges to define and improve ECM in order to mimic accurately *in vivo* conditions.[28] Due to the high heterogeneity between different cancer sub-types and even within the same TME, one single type of hydrogel cannot accurately recapitulate the 3D environment experienced by cells *in vivo*. A major challenge is still to synthesize hydrogel matrices that closely mimic the properties of the ECM components specific to each cancer subtype with properties controllable spatially and temporally. It should also be mentioned that fibroblasts and perivascular cells will be key in the future development of ToC as they also contribute to ECM production.

B. Mechanical forces at play. Another important parameter of the TME is the mechanical forces cells experience and convert into mechanical transduction pathways.[30] Among those forces, compressive stress and interstitial fluid pressure[31] are major players. Compressive stress results from the expansion of the solid tumor, but also from changes of the matrix stiffness. This can alter cancer cell properties such as cell cycle regulation[32] and drug resistance.[33] Leaky blood vessels and badly functioning lymphatic vessels can give rise to interstitial fluid pressure.[31] This drives interstitial flow in the tumor, exposing cells to shear stress, which in turn can strongly affect cancer cell migration, endothelial sprouting and even fibroblasts activation.[13] Apart from the mechanical forces at play in the tumor, stretching forces due to the tissue's function such as in uterus, intestine or lung can also impact the tumor development.[30]

Recent tools have been implemented to reproduce these tumor mechanical forces in ToC. To mimic compressive stress, Onal *et al.*[34] developed a chip with an integrated gas pressure micro-piston, applying dynamic compression on ovarian cancer cells. They studied the impact of cyclic stress on the cell nucleus, which is a mechanosensitive organelle, and demonstrated that the circularity of the cell nuclei was significantly less in compressed cells than in control. So far only a few ToC studies integrate controllable compressive stress, but this work highlighted that ToC constitutes a promising tool for studies of cell-mechanical force interaction.

In order to reproduce the interstitial fluid pressure and shear stress, fluidic control in ToC is made possible thanks to conventional fluid controllers also used for seeding cells, refreshing and controlling the cell culture media composition

over time. This fluidic control is mostly performed with syringe pumps[35–37] (half of ToC publications), peristaltic pumps[38–44] or tilted rocking platform[45] while the second half of the ToC studies does not report any flow control (Fig. S2†). Kocal *et al.*[35] investigated how shear stress affects the epithelial to mesenchymal transition of oesophageal cancer cells in a 2D ToC. From the third day of culture under flow, cancer cells experienced a phenotypic switch with a significant decrease in cell–cell adhesion (decrease in E-cadherin expression) as well as an increase of transendothelial migration capacity (increased expression of N-cadherin). Moreover, some ToC studies showed that shear stress can also affect cancer stem cell states. Ip *et al.*[46] found that spheroids grown under shear stress exhibited higher expression of stem cell markers Oct-4, c-Kit (CD117), efflux pumps ABCG2 and P-gp in contrast to static conditions. In clinical settings, a poor prognosis is associated with these factors, highlighting the link between shear stress applied on cancer cells and chemoresistance.[47]

Apart from cancer cells, shear stress can also have a high impact on normal endothelial cells. Several ToC studies showed that shear stress induced an elongated endothelial cell morphology as well as modified junctional protein expression[48] which can facilitate the extravasation of cancer cells. Kim *et al.*[49] demonstrated that the interstitial flow direction can also regulate the direction of capillary sprouting, suggesting angiogenesis occurs in the opposite direction of flow.

Among the mechanical forces experienced by cells *in vivo* is also peristalsis, which is the progression of coordinated muscle contractions. These forces are at play in the gut, oesophagi, uterus and many other organs. Only a few ToC devices have implemented physiological mechanical tissue deformation. Fang *et al.*[50] developed a microfluidic chip allowing high-throughput culture under peristalsis of human colon tumoroids to screen nanomedicines. They observed an increase of stem cell markers (Lgr5) and proliferation markers (Ki67) which could be linked to a peristalsis-induced high interstitial fluid pressure and suggested that peristalsis is also involved in the reduced nanoparticle internalization *via* clathrin-dependent endocytosis. In a recent study, Strelez *et al.* studied colon metastatic spreading in a peristalsis-tunable chip using colorectal cancer (CRC) cells from patients and showed a peristalsis-induced increase of tumor cell invasion.[51] Ao *et al.*[52] evidenced that mechanical stretching of prostatic normal tissue-associated fibroblasts (NAFs) alters the structure of secreted fibronectin. They suggested that mechanical stress is one of the critical factors in NAF activation into cancer associated fibroblasts (CAFs).

There is growing evidence that a wide range of mechanical stresses can alter tumor and stromal cells behavior and ToC appears to be a powerful and innovative technological tool to decipher the role of these various mechanical forces.

C. Tumor oxygen environment. In tumors, leaky and unorganized vascular networks contribute to an unstable oxygenation of microregions.[14] Low oxygen concentration areas named hypoxic zones, develop approximately 100 μm away from blood vessels range from 70 to 200 μm.[53] This is due to the combination of oxygen diffusion limitations and rapid consumption by proliferative cells. These hypoxic micro-regions are heterogeneously distributed within the tumor and lead to oxygen gradients. Hypoxia emerges as a pivotal factor in tumor progression and treatment resistance, through a response mainly ascribed to hypoxia-inducible factors (HIFs)[54] HIF activation in tumors can have several significant consequences: promoting the formation of new blood vessels, metabolic adaptation of cancer cells, enhancing invasiveness and metastasis.[54] Workstation and hypoxic chamber culture systems are the most common methods to create a hypoxic environment. However, they present major limitations: (a) they do not provide oxygen gradients as found *in vivo*, (b) they equilibrate slowly, (c) they only provide gaseous control without any control of dissolved oxygen, (d) they are not easily compatible with live-cell imaging. Several reviews detailed the various methods to control oxygen on-chip[55–57] but studies containing oxygen control in ToC are still rare. The main methods to control oxygen on chip are (Fig. 2): hypoxic incubator, inclusion of a side gas-channel, chemical induction and cell consumption in a low-gas permeable chip. The "gaseous channel" method relies on O_2 diffusion from a gaseous source channel to a channel containing cells across a thin, gas-permeable PDMS membrane. Koens *et al.*[58] described a double-layer microfluidic device (Fig. 2 bottom panel), where two parallel gas channels were located above the cell culture media and gel channels to enhance gas exchange. In addition, a gas-impermeable polycarbonate film was embedded in the device to prevent the diffusion of atmospheric oxygen. With this configuration, an oxygen gradient was established by supplying gas with 0% and 21% O_2 on each side of the gel channel (gradient from 3% to 17% O_2 across the gel). 3D migration trajectories of breast cancer cells inside the gel channel were assessed, and demonstrated that a lower oxygen concentration increases cell migration speed. Despite interesting performances, the main disadvantage of the "gas channel" approach remains that low O_2 concentrations (~0–1%) remain difficult to achieve. It also requires complex microfabrication and the evaporation of culture media can be accelerated by the flow of dry gas through the diffusion membrane. For ToC studies using "chemical induction", oxygen is modulated by on-chip chemical reactions that either generate or consume oxygen. Chang *et al.*[59] exploited the reduction of pyrogallol as oxygen scavenger. The device contains 4 sets of parallel-arranged serpentine channels with identical geometries to generate 6 different concentrations across the width of the channel. The oxygen level was decreased to 1% at the inlet and gradually increased to approximately 16% at the outlet. The device was exploited to perform drug testing on lung cancer cells with varying drug concentrations and under different oxygen pressures. Drug-induced death was assessed on a gradient of tirapazamine (TPZ), which is an original drug metabolized to a toxic radical (only at very low levels of oxygen), where it can exert an anti-

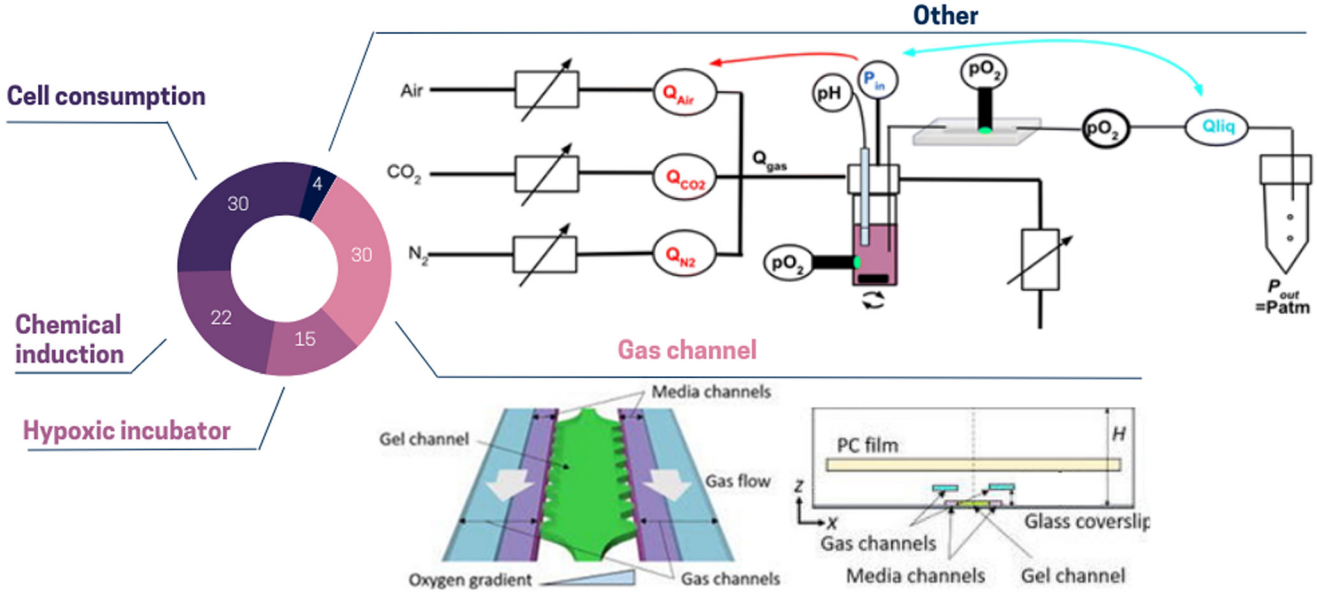

Fig. 2 Pie chart illustrating the proportion of different oxygen control strategies used in ToC: cell consumption 30%, gas channel 30%, chemical induction 22%, hypoxic incubator 15%, other 4%. (Top panel) Other: the dissolved oxygen level can be regulated by modulating pneumatic valves opening. This method simultaneously changes the gas composition and the pressure-driven flow.[62] (Bottom panel) Gas channel: to control dissolved oxygen levels in the media and gel channels, gas mixtures can be supplied to the two separated gas channels. To prevent from atmospheric oxygen diffusion, a polycarbonate film is embedded on top of the gas channels.[58]

cancer effect. This method has the advantage of eliminating the need for pressurized gas tanks, although it generally requires syringe pumps to continuously deliver the reagents. One third of the ToC studies reported oxygen concentration control by simple cell consumption of oxygen in a static chip fabricated with low gas permeable materials.[60] Oxygen consumption by cells can also be combined with constant flow of fresh media to modulate oxygen concentration on chip.[61] Despite simple implementation, this method suffers from major drawbacks, particularly its strong dependency on cell density and metabolic rate. We recently proposed an innovative method for on-chip oxygen control (Fig. 2 top panel)[62] called Oxalis (OXygen ALImentation System) that overcomes most of the cited limitations of current methods. Oxalis regulates simultaneously the gas composition and inlet reservoir pressure by modulating a pneumatic valve opening. Using this dual regulation, both the pressure-driven liquid flow-rate and the level of oxygen dissolved in the chip can be independently controlled. Oxalis offers unprecedented features such as an oxygen equilibration time lower than 3 minutes and accuracy of 3 mmHg. These performances can be reached for chip perfusion flow as low as 1 μL min^{-1}. This approach could thus be used in the future to generate oxygen gradients in ToC.

Reproducing the intricate gaseous environment of the tumor *in vitro* presents considerable challenges, but it holds immense importance as it offers the opportunity to capture spatial metabolic and phenotypic heterogeneity. Significantly, Ayuso *et al.*[63] demonstrated that cells situated farther from the lumen, where nutrients and oxygen originate, displayed upregulated genes associated with apoptosis resistance (*e.g.*, BIRC3), DNA damage induced by starvation (*e.g.*, GADD45G), and stress response (*e.g.*, ADM).

3.2. Controlling the cellular complexity on ToC

A. Cellular composition of the tumor microenvironment. The cellular composition of the TME varies between tumor subtypes. However, besides cancer cells the main cell types found in tumors include endothelial cells, immune cells and cancer associated fibroblasts (CAFs).[64] Tumor blood vessels are disorganized and are bigger in diameter (up to 225 μm) in contrast to physiological ones (up to 100 μm). Tumor blood vessels present an abnormal basement membrane, altered pericyte/endothelial cells ratios, and are highly permeable.[65] The TME contains a variety of immune cells including: different lymphocytes subtypes, tumor-associated macrophages (TAM), natural killer cells, dendritic cells, cancer associated neutrophils. Altogether, they play a major role in response to anti-cancer treatment. CAFs are a key component of the TME, with diverse functions such as matrix deposition and remodeling.[14] Moreover, CAFs are a highly heterogeneous cell population in primary tumors. Costa *et al.*[66] and Pelon *et al.*[67] identified four CAF subpopulations (CAF-S1-S4) in different breast cancer subtypes and metastatic lymph nodes (LN). In particular, the CAF-S1 are linked to an immunosuppressive environment, while both CAF-S1 and CAF-S4 promote cancer invasion and metastasis but with different mechanisms: CAF-S1 stimulate cancer cell

migration and initiate epithelial-to-mesenchymal transition through CXCL12 and TGFβ secretion, while highly contractile CAF-S4 induce cancer cell invasion *via* NOTCH signaling.

Cancer cell lines remain the primary tool for studying biological processes since decades due to their cost-effectiveness, ease of use, and ability to provide an unlimited number of consistent samples. As shown in Fig. 3, about 70% of ToC studies use predominantly cell lines. Most of these cell lines are part of the US National Cancer Institute (NCI) 60 human tumor cell lines anticancer drug screen (NCI60), which have been extensively characterized on a molecular level: exome sequence, DNA methylation, mRNA expression, protein levels and modifications, enzyme activity, and metabolomic profiling.[68] These cell lines theoretically allow an easy comparison between the results obtained inside ToC *versus* other *in vitro* models. However, it is also well-known and accepted that cell lines do not completely represent relevant models of *in vivo* tumors, since indeterminate transcriptomic, epigenetic, genetic and phenotype changes may occur during cell immortalization. Moreover, cells that have been cultured for several years and across different laboratories can present major differences as compared to primary cells and even to the initial source of such cell lines.[69] The use of cell lines therefore raises important questions. Is it essential for the scientific community to agree on the use of specific cell lines for every clinical cancer subtype? Which specific and standardized characterization methods should be performed to confirm genomic or phenotypic drifts or cell–cell contaminations? And at last, to which extent should cell-based models represent the *in vivo* pathology in terms of underlying biological mechanisms and response to treatment?

A more faithful approach to reconstitute the *in vivo* TME includes the use of primary cells directly extracted from fresh tissues or fluids with subsequent *ex vivo* culture. Since primary cells are only cultivated for a low number of passages, they are thought to display most of the differentiated properties of their tissue of origin.[69] Only one third of ToC incorporate primary cells mainly human peripheral blood mononuclear cells (PBMC), CAFs or endothelial cells (*e.g.* HUVECs). PBMCs include lymphocytes mostly T-cells, B cells including rare plasmocytes, NK cells, monocytes, rare dendritic cells and basophils. Numerous ToC studies use PBMCs, such as Boussomier-Calleja *et al.*[70] who studied the effect of monocytes on cancer cell extravasation at different stages of their life cycle. However, since PBMCs are extracted from healthy donors, they cannot be fully considered to perfectly mimic tumor immune cell infiltration. Here, the use of autologous cells offers the possibility to avoid recognition of non-self cells. This is especially crucial for testing immuno-oncology drugs, such as immune checkpoint inhibitors (ICI) that function by unleashing the cytotoxic activity of T-cells.

Among the endothelial cells, HUVECs were by far the most well-represented source of ECs in vascularized ToC models. Only a few studies used micro-vascular ECs from specialized tissues in ToC.[71,72] One major limitation of HUVECs and of most ECs is their non-tumoral origin. On the other hand, tumoral endothelial cells (TECs) are constrained by the isolation, availability, and viability challenges. Matsuda *et al.*[48] estimated that TECs represent around 2% of the overall cells in the tumor. As such, novel and more efficient isolation protocols need to be established to design fully patient-derived vascularized ToC models. Isolation of ECs from peri-tumor areas could consist of a reasonable compromise allowing isolation of tumor cells, immune cells, fibroblasts, and ECs from the same patient. Notably, some ToC integrate human induced pluripotent stem cell-derived endothelial cells (iPSC-EC).[73] In contrast to cell lines, iPSCs achieve immortality by inducing pluripotency rather than by transformation. For ToC development, iPSCs offer interesting advantages due to their ability to be reprogrammed into different types of tissues but their use in ToC remains scarce (only 1% of ToC publications). Lee *et al.*[74] integrated iPSC-derived cardiac cells in a heart-breast ToC. Fibrotic stages of

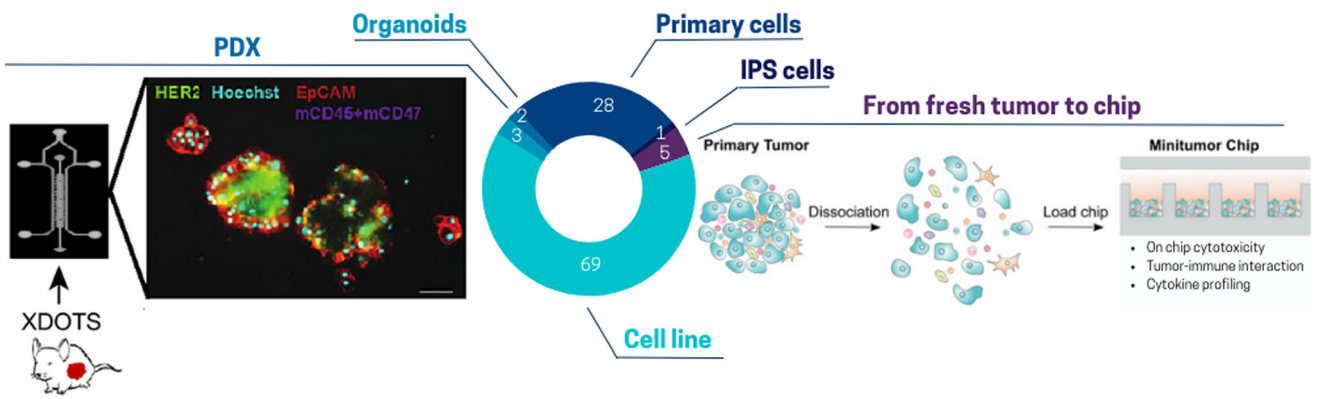

Fig. 3 Pie chart illustrating the proportion of different cell sources used for ToC: cell line 69%, primary cells (HUVECs, PBMCs, primary fibroblasts) 28%, PDX 3%, from fresh tumor to chip (freshly resected tumor, CTCs), IPS cells 1%, organoids 2%. (Left panel) Tumors are harvested from PDXs. After dissociation and filtration isolation, spheroids are produced in ultra-low attachment plate and seeded in the toc.[85] (Right panel) Freshly resected primary tumors can be subjected to tumor digestion using a human primary tumor dissociation kit and then seeded in a micro-well array ToC.[89]

iPSC-derived cardiac tissues have been promoted to assess the differential functionality in healthy and fibrotic cardiac tissues after treatment with chemotherapy. iPSCs also offer the possibility to design multi-organ platforms composed of various tissues from the same donor. For example, cardiomyocytes derived from iPSCs from patients with breast cancer were shown to model at the cellular level the doxorubicin-induced cardiotoxicity observed in some patients.[75]

Other studies used more complex cellular models (although it is still a minority among ToC publications) such as organoids,[76–79] PDX[80–88] or fresh surgical tumor sample.[82–87,90–96] The category "fresh surgical tumor sample" encompasses various form since there is diversity in patient tumor samples including slices, micro-dissected tumor tissues and even single cells. For instance, organotypic slices of PDX breast and prostate tumors were successfully cultured in chips with 6-well plate design for up to 14 days with an accurate prediction of cell death with conventional treatments.[86] Ivanova *et al.*[85] established a PDX ToC system for drug screening (Fig. 3 left panel). Several studies have also demonstrated the feasibility of growing circulating tumor cells (CTCs) isolated from blood samples.[96–98] Often proliferation of primary cells was limited to a few days or weeks but was sufficient for drug screenings.[96] ToC also indicated promising results for the maintenance of fresh surgical tumor samples in culture. Dorrigiv *et al.*[82] demonstrated that oxygen-permeability of microfluidic devices reduces the extent of hypoxia in tissue slices in comparison to 96-well plates. In addition, Chakrabarty *et al.*[86] reported maintaining proliferation in tissue slices for 14 days in ToC *versus* only 7 days with standard plates. Apart from tissue slices, diverse solid tumor types can be included on chip such as primary lesions, lymphadenectomy specimens, pleural effusions, ascites fluid, or resected metastases. Parsian *et al.*[95] fabricated a device with three PDMS layers, where the middle layer accommodates the tissue slices, and the top/bottom layers are perfused with media. Similarly, a study by Ao *et al.*[89] highlights the feasibility of integrating fresh tumor cells directly into microfluidic chips (Fig. 3 right panel). However, this model still lacks several features such as tumor-matching extracellular matrix, which has been demonstrated to affect several key processes, including tumor growth, immune infiltration and drug responses.[15] Alternatively, it is also possible to include micro-dissections of tumors.[81] Growing fresh tumor samples in 3D on ToC including its ECM could pave the way to a closer reflection of the *in vivo* tumor therapeutic response. However, standardized procedures still need to be developed to integrate cell populations isolated from fresh tumors.

B. Tumor cellular architecture. *In vivo*, drug response is influenced by different cell types of the TME through cell–cell interactions or biochemical and cytokines secretions. ToC can reproduce the cellular architecture complexity with great flexibility (varying geometries, positioning of each cell type, *etc.*). During the last 10 years, ToC have integrated cells in mono-, bi-, tri-culture or even more (Fig. 9A). Every cell type has *in vivo*-like organization within the tumor: endothelial cells form perfusable networks, fibroblasts and cancer cells are mixed in a 3D scaffold. About two third of ToC studies included isolated cells while one third used 3D cell aggregates such as spheroids. Spheroids have an organized cellular architecture and present more physiological cell–cell and cell-ECM interactions as compared to 2D cell culture.[99] Furthermore, they exhibit intrinsic metabolic gradients (nutrients, O_2, CO_2) that result in the formation of a multilayer structure: an external layer of proliferative cells, an intermediate layer of quiescent T-cells, and an inner layer consisting of necrotic cells. Despite these convincing features, spheroids generally still lack vascularization that provides cells with essential nutrients and growth factors.

In ToC, cellular architecture can be controlled using various microfabrication approaches. We classified ToC designs, inspired by Sleeboom *et al.*[100] according to five categories (Fig. 4): (a) compartmentalized chip, (b) micro-wells array, (c) 2D chip, (d) membrane chip, (e) lumen chip, and (f) others. Such classification does not cover the whole spectrum of published studies, but highlights the predominant strategies currently employed in the field of tumor-on-chip research. In turn, this classification does not completely capture the diversity and complexity of ToC approaches and some configurations can be a combination of several before mentioned categories.

Almost half of the ToC belong to the "compartmentalized chips" category.[76,101–109] Compartmentalization of cells embedded in hydrogels can be achieved by various physical means. Several studies use co-flow patterning flowing side by side to two different hydrogel solutions. Using this method, Jeong *et al.*[110] co-cultured mammary epithelial cells with human mammary fibroblasts and studied *in vitro* the transition from ductal carcinoma to invasive ductal carcinoma. Compartmentalization can also be created in a more reproducible way by playing with capillary forces.[108] Properly designed pillars induce surface tension to hold the hydrogels in a given area of the chip, while nutrients are supplied through media in adjacent channels. Adjei-Sowah *et al.*[109] (Fig. 4 top left panel) developed a tri-culture of endothelial cells, astrocytes and glioma stem cells utilizing a pillar compartmentalized chip and focused on the identification of ligand-receptor pairs. Compartmentalized chips are very versatile as different types of hydrogel can be arranged in a controlled manner. However, some applications require a fully free interface, while pillars can hinder homogeneous diffusion of the solute. Venzac *et al.* described a new method of compartmentalized chip, where rigid or semi-rigid structures – sliding walls – were inserted into a guiding channel open in the PDMS microfluidic chip sides.[111] The advantage of this method is the possibility to create channels of any size, independently of the hydrogel solutions properties.

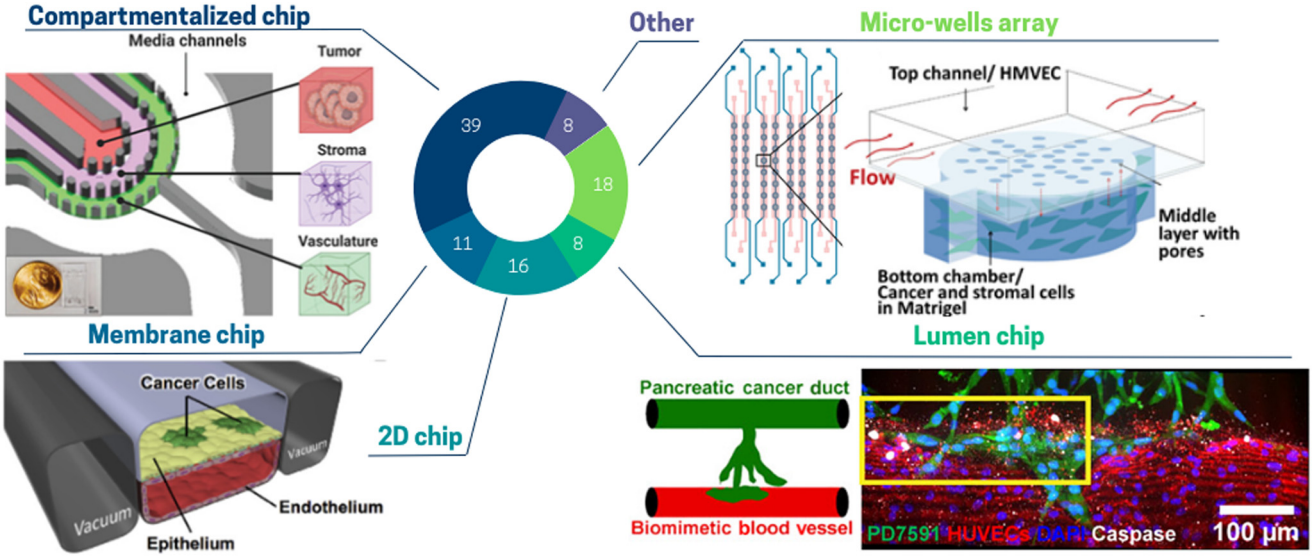

Fig. 4 Pie chart illustrating the proportion of different toc designs used to mimic cell architecture: compartmentalized chip 39%, micro-wells array 18%, 2D chip 16%, membrane chip 11%, lumen chip 8%, other 8%. (Top left panel) The glioblastoma toc model is a compartmentalized chip with three concentric cell culture regions separated by pillars: namely, the vasculature, stroma, and tumor regions surrounded by media channels.[109] (Top right) In this micro-well array ToC, cancer and stromal cells are encapsulated in Matrigel and seeded into the bottom layer, while flow is applied in the top layer.[116] (Bottom left panel) The 2-channel membrane toc contains human lung epithelial cells and a low density of lung cancer cells cultured on the upper surface of a porous ECM-coated membrane. Human lung microvascular endothelial cells are cultured on all four walls of the lower channel, forming a hollow vascular lumen.[71] (Bottom right panel) The lumen ToC is composed of two cylindrical channels embedded within a 3D collagen matrix. One channel is covered with endothelial cells to form a perfusable biomimetic blood vessel, and the other channel is covered with pancreatic cancer cells to form a pancreatic cancer duct.[129]

Another category is the "micro-wells array" which allows studying multiple and/or replicated conditions but often at the price of a limited cell complexity. This category addresses the need for multiplexing ToC studies.[89,112–115] Chi *et al.* (Fig. 4 top right panel) achieved an elevated reconstruction of the *in vivo* TME combined with high throughput screening.[116] They developed a three-layered ToC containing a tumor microvasculature and tumor–stromal microenvironment, along with high throughput screening capability (8 lines of 8 wells). This platform allowed studying the function of the endothelial barrier in drug response and resistance mediated by CAFs.

The "2D chip" approach includes all different designs containing cell cultures as monolayers These 2D models do not display *in vivo* stromal characteristics because their key point is to focus on technological advances, for instance, automation,[117] control of oxygen[62] or shear stress.[35] Kamei *et al.*[117] developed an integrated heart ToC containing three sets of artificial blood circulation loops thanks to the integration of pneumatic valves and a peristaltic pump. This chip allows to automatically perform the following sequence: Matrigel coating, cell introduction, flow circulation and cell staining. 2D chips can also be used to study cell migration. Agliari *et al.*[118] reproduced the interactions between cancer and immune cells and investigated the motility of spleen cells.

"Membrane chips", are composed of microchannels separated by a porous membrane.[41,52,71,79,86,119–121] Although cells are cultured as a monolayer on the membrane, such chips are not to be categorized as 2D chips as cells can migrate through the membrane pores. Membrane chips were originally developed by Huh *et al.*[121] A key feature of this device is the possibility to obtain an air–liquid interface (allowing respiratory epithelial cell differentiation) as well as to apply both controlled peristalsis and shear stress (respiratory lung motion). Although membrane chips were not often used for cancer studies, they are extensively in use for other physiological and pathological models (*e.g.* infection-induced recruitment of immune cells, breathing-induced absorption of nanoparticles).[122] In 2017, Hassell *et al.*[71] (Fig. 4 bottom left panel) created a ToC such membrane-based device. They seeded human lung cancer cells on a monolayer of primary alveolar cells on the same membrane chip, thus recapitulating a lung adenocarcinoma growth.

The category "lumen chip" consists of ToC in which a critical element is used to form lumen in hydrogels.[36,39,49,73,94,123–132] This design is typically used to model blood vessels in tumors or to tightly pack cells in a cylindrical compartment. Nguyen *et al.*[129] (Fig. 4 bottom right panel) describes a chip in which a biomimetic ductal channel containing pancreatic cancer cells is juxtaposed to a blood vessel consisting of an endothelialized perfused lumen. To build these lumen channels, acupuncture needles were withdrawn after collagen polymerization and endothelial cells or pancreatic cancer cells were seeded into each channel. They observed that pancreatic tumor cells invaded and occupied the lumen of the biomimetic blood vessel, resulting in apoptotic

endothelial cells in proximity to cancer cells. On the contrary, endothelial cells in the biomimetic blood vessels in absence of tumor cells invasion did not exhibit apoptotic activity. These data supported the notion that the "lumen chip" model, although being rather simple, allows for complex phenomena to be modelled and studied.

The incorporation of these diverse spatial TME architectures on chip has already provided valuable opportunities to investigate various tumoral mechanisms. Nevertheless, considering the advancements of biological technologies and knowledge, it is obvious that our technologies must evolve accordingly. The emergence of spatial transcriptomics has offered new and insightful perspectives into the architecture and functions of the TME. Consequently, there arises a necessity to further enhance the reconstituted TME on chip to mimic the *in vivo* spatial organization more faithfully. Compared to other cell types found in the TME, the 3D architecture of endothelial cells has been more deeply reproduced in ToC due to its specific organization of perfusable networks. Several *in vitro* approaches exist to replicate the vascular compartment, offering various levels of complexity of ECs arrangement (Fig. 5): patterned microchannel, sacrificial mold and self-assembling. The most straightforward method involves creating a 2D patterned microchannel lined with a monolayer of endothelial cells. This can be achieved by utilizing a membrane configuration, wherein the endothelial cells are seeded onto a porous membrane. The membrane serves to separate the main chamber into two compartments, while additional side channels allow for cyclic stretching of the porous membrane. This configuration is very well suited to study drug penetration of cancer cells extravasation under mechanical stresses. This configuration has been exploited by, Hassell *et al.*[71] to generate a lung ToC model with an air/liquid interface using a 3D endothelial vessel in the basal compartment seeded with lung microvascular ECs. Despite interesting features especially regarding the application of mechanical forces, this approach remains a simplified model of vascularization: geometries are square-shaped and sizes are highly different from tumor vessel networks *in vivo*. 3D blood vessel models with tubular structure have been created *via* several approaches such as bioprinting or guiding needles, called here as "sacrificial molds" and associated with the category of "lumen chips". In this configuration, the endothelial channel can be made in a wide range of hydrogels.[133,134] Miller *et al.*[128] used a commercial chip to incorporate patient derived tumor clusters into a 3D matrix crossed by a vessel mimicking lumen channel (Fig. 5 left panel). Key features include a controlled rate of directional media flow through the cellularized lumen. Although this strategy does not always produce complex geometries, the advantage lies in its full tunability in terms of cell input, perfusion, and 3D matrix. Finally, the most complex ECs organization consists of perfusable and self-assembling microvascular networks (MVNs),[101,135,136] which *in vivo* require interactions with pericytes or fibroblasts. Chen *et al.*[138] studied self-organizing perfusable human microvascular networks (Fig. 5 right panel). Self-assembly is usually restricted to the use of fibrin hydrogel, the only matrix allowing endothelial vessel sprouting, ramification and self-organization.[138] Recently this approach has been exploited by the group of R. Kamm.[38,108,137,139–141] They sequentially added fibroblasts to preform tumor spheroid.[38] They showed that this sequential approach could enhance vascularization and that the vessels close to the tumor spheroid surrounded by fibroblasts made are more perfusable.[38] All these methods have already allowed the study of several important tumoral or therapeutic mechanisms. However, other approaches are currently highly promising; especially photoablation, which could enable the generation of complex vascular networks while avoiding the use of fibrin.[142,143] Besides it is also worth mentioning that there are also room for improvement on the biological aspects of the reconstituted vascular compartment of the TME. *In vitro* replication of the vessel structure remains limited as tumor vascularization is known to be unorganized

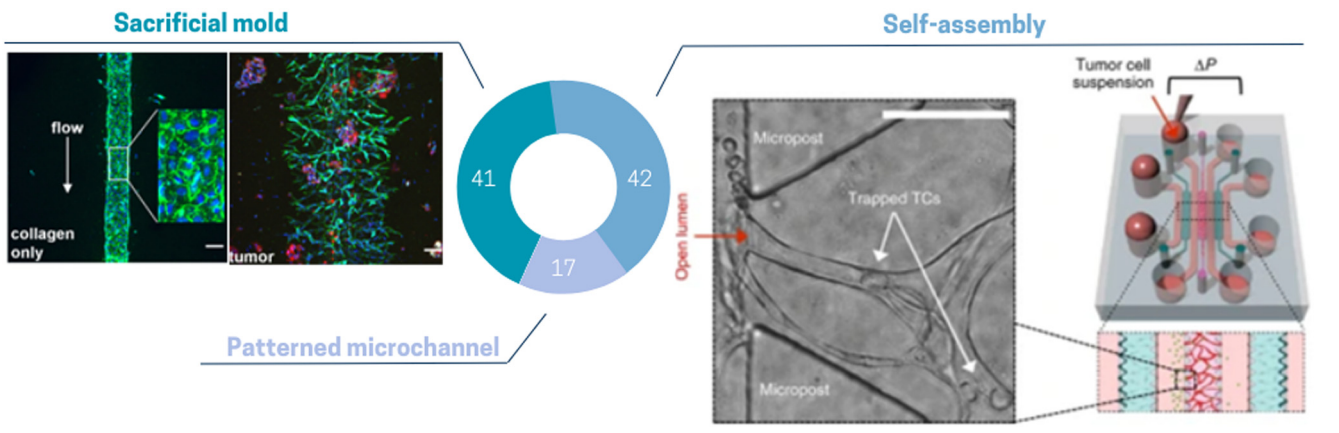

Fig. 5 Pie chart illustrating the proportion of different vascularization methods used in ToC: self-assembly 42%, sacrificial mold 41%, patterned microchannel 17%. (Left panel) Lumen can be formed by removing a retaining rod from collagen and further cellularizing with endothelial cells.[128] (Right panel) Endothelial cells elongate, form vacuoles and subsequently self-assemble into interconnected perfusable vascular networks.[137]

and tortuous, with a lack of pericyte coverage and fenestrated ECs, while most current models mainly focus on accurate perfusion and structural stability. Thus, the development of more accurate *in vivo* mimicking vascularized TME on a chip model remains a challenge.

Tumor-on-chip platforms offer a promising approach to replicate the complexity of tumors *in vivo* within controlled *in vitro* settings. These models provide a unique opportunity to mimic the intricate tumor microenvironment, encompassing factors like extracellular matrix, oxygen gradients, nutrient supply, cell architecture and cell–cell interactions.

4. Applications of ToC for pre-clinical studies for cancer treatment

4.1. A wealth of new information can be extracted from tumor-on-chip

There is a large variety of information which can be extracted from ToC studies. Considering ToC studies of the past 15 years, the most common readouts used in ToC are drug-induced cell death (52% of ToC publications), followed by proteomic (23%), transcriptomic (14%) and secretome (8%) analysis (Fig. 6). Additionally, more readouts such as imaging of hydrogel fibers, diffusion of a compound through the matrix, vessel permeability,[141] cell migration can also be obtained from ToC studies. This classification does not cover the whole spectrum but focuses on the most represented analytical readouts used on ToC; the analytical potential of ToC being continuously expanding.

The high proportion of ToC studies measuring drug-induced death emphasizes the potential of on-chip applications for drug screening. Very recently, Jun Ye Ong *et al.*, proposed an array device to culture PDX-derived spheroids combined with a drug concentration gradient to generate high throughput dose–response.[80] This work was the first to demonstrate a quantitative correlation between drug efficacies estimated on ToC with *in vivo* data. Protein-based studies are conducted in about one fourth of ToC studies and mostly rely on flow cytometry, western blot and immunostaining as for example the work Boussomier-Calleja *et al.*, which analyzed in ToC the expression of macrophage-like markers to characterize monocyte differentiation.[70] Transcriptomics performed on ToC have been used to decipher the impact of several TME parameters, such as oxygen levels, matrix stiffness, cell subsets identity or gene expression. The majority of these studies used RT-qPCR, although some studies performed bulk RNA-seq. Ayuso *et al.*, analyzed a panel of selected immunity-related genes to study stress-induced exhaustion of NK cells in co-culture with breast cancer spheroids.[123] They demonstrated that NK cells in bi-culture exhibited a different transcriptomic profile, dominated by exhaustion markers, as compared to naïve NK cells. Recent studies emphasized the feasibility of single cell RNA-sequencing (scRNA-seq) of ToC derived cells. In the work of Shirure *et al.*, several cell types (cancer cells, endothelial cells, fibroblasts, macrophages) were successfully recovered from chip devices and analyzed by scRNA-seq.[104] The authors identified macrophages to dramatically impact gene expression in both endothelial cells and fibroblasts. Importantly, they were able to identify two distinct endothelial cell phenotypes as a consequence of M1/M2 macrophages and tumor cells co-culture. Several clinical applications could emerge from integrating fresh patient derived tumors on chip in combination with scRNA-seq. For example, this approach can enable the detection of rare malignant and/or chemo-resistant cancer cells, and provide supporting evidence for further suitable treatment approaches. Analysis of secreted molecules by cells – secretome – can also offer crucial information about the functional responses of the tumor ecosystem. Ligand binding assays (LBA), such as ELISA or Meso Scale Discovery (MSD) platforms can be used to measure such secreted molecules. In Jenkins *et al.*, profiles of secreted cytokines from 3D microfluidic cultures of tumor spheroids were used to screen for the response of murine models and patient tumor samples to immune checkpoint blockade therapy (anti PD-1).[144] Although cytokine analysis is fairly simple and very classical in conventional *in vitro* models, it is still not often used in ToC experiments.[145] Recently, metabolic alterations have been shown to play a role in the sensitivity of cancer cells to drug and rewired metabolism is linked to rapid

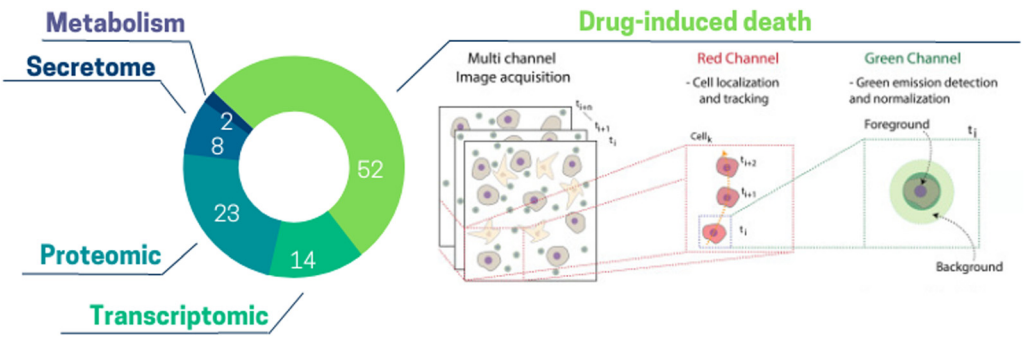

Fig. 6 Pie chart illustrating the proportion of different information extracted from ToC: drug-induced death 52%, proteomic 23%, transcriptomic 14%, secretome 8%, metabolism 2%. (Right panel) Drug-induced death: open-source computational method can be used to extract the temporal kinetics and the spatial maps of cancer death.[150]

tumor growth and proliferation.[146] Only a few ToC studies (2%) focused on cancer cells metabolism highlighting that this field remains to be further explored. Hou *et al.*[147] used ultra-performance liquid chromatography coupled with mass spectrometry (UPLC-MS) to detect metabolites such as 5′-deoxy-5-fluorocytidine or 5-fluorouridine, to study metabolism-induced anticancer bioactivity. Additionally, only a low number of studies apply live markers of metabolism such as pH, hypoxia or ROS, providing both temporal and spatial information[61] which are highly complementary to metabolic endpoint analysis. Among the new analyses that could be performed on ToC, it is worth citing the Assay-Guidance Manual published by the NIH Chemical Genomics Center.[148] It describes guidelines for robust assay development and recommends cell proliferation and cytotoxicity assays including[149] tetrazolium reduction, resazurin reduction, protease markers, and ATP detection, which are not commonly used on ToC up to date. Efforts could also be made to improve the compatibility of ToC with conventional analytical formats (*e.g.*, the plate reader) to be able to use conventional kits and standardized assessment protocols.

To our best knowledge, ToC studies pursuing genomic analysis are rare (only 1 study was identified among the over 300 reviewed in the current article). Zhang *et al.*[98] expanded circulating tumor cells (CTC) isolated from early-stage lung cancer patients with fibroblasts on a co-culture chip. Next-generation sequencing of 124 cancer-related genes revealed matched mutations between the primary tumor and cultured CTCs in 3 out of 8 paired CTC-tumor samples (CASP8, APC, TP53 and ERBB4 genes). Such studies suggest that the combination of ToC with next-generation sequencing could help revealing the genetic and epigenetic underpinnings of cancer, as well as help to redefine treatment paradigms.

In addition to the aforementioned readouts, the compatibility of ToC devices with high resolution imaging is also a major asset. ToC can be continuously imaged with automated video-microscopes which can additionally provide information about cell dynamics (*e.g.*, motility, cell–cell interactions, *etc.*) and cellular activities (*e.g.* death, division, pathway activation, *etc.*), at single-cell resolution. Novel types of information can be deciphered by looking at the behaviors of dynamic 3D microenvironments. However, a major bottleneck is the conception and development of appropriate computer tools to process and extract the richness of biological information encrypted in on-chip images and videos. New findings can be obtained by spying tumor ecosystem dynamics within ToC in the framework of interdisciplinary collaborations between biologists and computer scientists, taking advantage also of the great power of artificial intelligence (AI) tools. For instance, a computational method, named SpatioTemporal Apoptosis MaPper (STAMP), has been developed to extract the temporal kinetics and spatial maps of cancer cell death from ToC[150] (Fig. 6 right panel). STAMP revealed that cancer cells death induced by chemotherapy is transmissible, meaning dying cells promote apoptosis of cancer cells in proximity. Recently, ToC devices are also exploited to track immune cells activity around many individual cancer spheroids simultaneously at high spatiotemporal resolution. Ronteix *et al.*[151] combined parallel imaging of T-cells behavior with probabilistic modelling to investigate the rules governing T-cells recruitment. Additionally, imaging techniques may also be used to visualize intravasation and extravasation processes, which remains a challenge for *in vitro* models. ToC allows studying metastatic processes in a stepwise manner.[152] It enables investigating the impact of each parameter such as the presence of a specific cell type on cancer cell migration, intravasation or extravasation. ToC has been used to study the effect of monocytes, at different stages of their life cycle, on cancer cell extravasation in a 3D vascularized microfluidic model.[70] Boussommier-Calleja *et al.* showed that breast cancer cells are less prone to extravasation in the presence of monocytes highlighting the role of monocytes in metastatic progression.[70]

The next step will be to apply these analysis tools for evaluation of patient-derived ToC for clinical application such as patient stratification and drug response prediction. The use of 'personalized' ToC composed of primary cells isolated from fresh tumors will open the possibility to conceive novel diagnostic tools that exploit AI-based analysis on *ex vivo* live-cell biomarkers. Some recent proof-of-concept studies support this concept. For example, morphodynamic biomarkers of primary cancer cells cultured on chips were used to predict post-surgical risks of relapse in operated breast and prostate cancer patients with high accuracy using machine-learning algorithms.[153] Another study showed that a deep learning approach was able to correctly identify responding breast ToC cancer-immune co-cultures treated with a targeted therapy drug (trastuzumab) by using an atlas of immune cell trajectories.[154] This suggested the feasibility of the methodology to predict drug responses using the information hidden in cell ecosystem dynamics. In the future, it will be essential to intensively pursue and to greatly expand the interdisciplinary collaboration efforts between cancer biologists, clinicians and computer scientists in order to fully take advantage of a large number of hidden information that could be extracted from ToC experiments. The combination of ToC live imaging and advanced computational image analysis, involving AI, thus provide immense opportunities to characterize the behaviors governing tumor ecosystems and to understand their responses to anti-cancer treatments.

4.2. Testing the efficacy of chemotherapies

The TME composition has a profound effect on drug efficacy and deciphering the effect this complex environment is critical.[155] Since ToC allows mimicking the TME, it is a powerful platform to study drug effect as well as the dynamics of cell death in presence of drugs (Fig. 6). According to our analysis, the main anti-cancer treatments

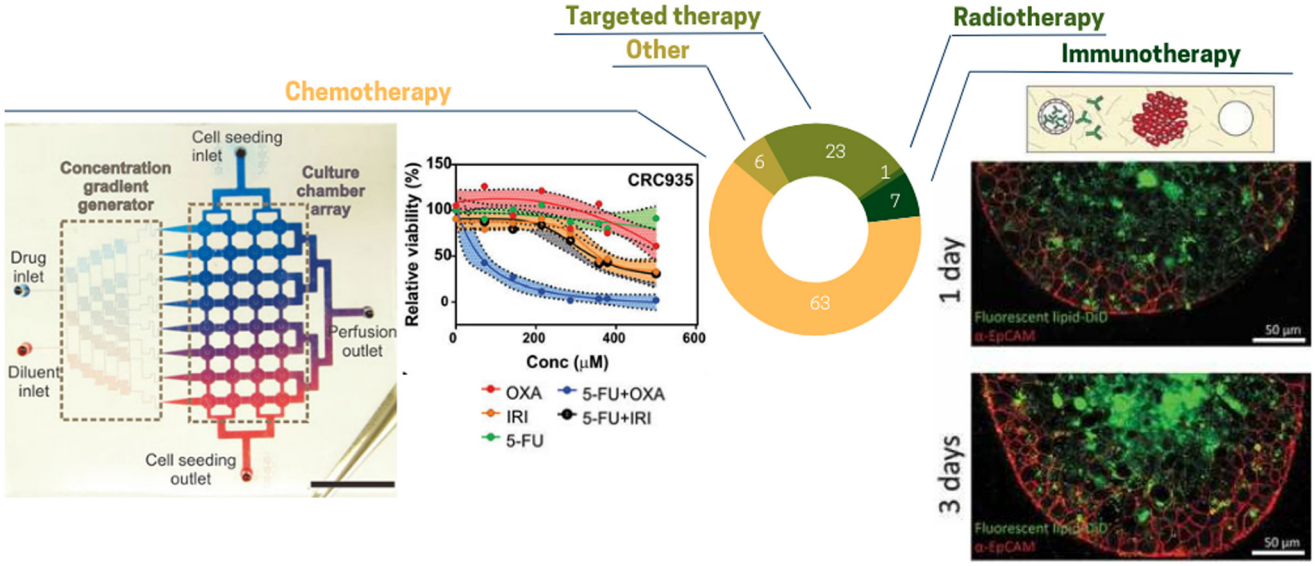

Fig. 7 Pie chart illustrating the proportion of different treatments tested in ToC: chemotherapy 63%, targeted therapy 23%, immunotherapy 7%, other 6%, radiotherapy 1% (right panel) concentration gradient generator to test different drug combinations.[80] (Left panel) Diffusion of antibody inside MCF7 spheroid.[124]

studied in ToC were chemotherapy (63% of ToC publications), targeted therapy (23%) and immunotherapy (7%) (Fig. 7).

Chemotherapy remains the frontline treatment for advanced-stage or metastatic malignancies for which loco-regional treatments, *i.e.* surgery and/or radiation therapy are not useful due to the dissemination of cancer cells in the whole body.[156] Chemotherapies tested in ToC are mostly taxol, doxorubicin, carboplatin or cisplatin,[35,77,106,110,116,117,120,145,157–160] in agreement with clinical practice. These treatments have been mostly applied to common cell lines such as lung cancer A549, breast cancer MCF7 or MDA-MB231. Using an on-chip co-culture of cell lines (cancer cells, CAFs, stem cells and an endothelial monolayer) Chi *et al.*[116] showed that the co-culture with CAF delayed the response of the TME to doxorubicin in comparison to the co-culture with normal fibroblasts. Apart from cell lines, ToC also enables the *in vitro* culture of patient samples for chemotherapy testing.[160] Chakrabarty *et al.*[86] tested the response to cisplatin using cisplatin-sensitive and cisplatin-resistant breast cancer PDX tumors. Interestingly, breast PDX cultured in the ToC platform showed a stronger response to cisplatin treatment than the conventional *ex vivo* culture method (6-well standard plates on an orbital shaker), suggesting that the ToC platform provides with a more optimal drug delivery into the tumor slices than the *ex vivo* culture method. Haque *et al.*[79] developed a ToC incorporating patient derived organoids (PDO) and stromal cells (pancreatic stellate cells and macrophages). They showed that targeting stroma cells (stellate cells by all-*trans* retinoic acid or macrophages by Clodrosome®) improved therapeutic effect of gemcitabine on cancer cells. Conversely, in a ToC model of breast cancer metastasis to bone,[161] including osteoblast-like cells seeded on a 3D-printed biomimetic bone scaffold, the sensitivity to cisplatin of PDX-derived triple-negative breast cancer (TNBC) cells was reduced with respect to conventional 3D cultures in Matrigel, which is consistent with the clinical observations that chemotherapy often failed to completely eliminate TNBC cells colonizing the bone.

Tumor chemosensitivity assays (TCAs) with conventional methods have been gaining attention over the past few decades. They provide a satisfactory negative predictive value (showing the possibility of drug resistance), however they only have a moderate positive predictive value (showing the possibility of drug sensitivity).[156] In this context, ToC appears as a future powerful tool for TCAs with the possibility to evaluate multiple drugs and multiple doses.[156]

4.3. Targeted therapy developments

Over the past two decades, there has been a tremendous shift in cancer treatment from chemo- to targeted therapy in cancer patients' subsets.[156] Targeted therapies often aim to deliver drugs to cells with molecular genetic alterations specific to cancer cells (gain of function, oncogene mutation or gene amplification).[162] Most of these targeted drugs have a much higher affinity for altered proteins in cancer cells than their normal counterparts in normal cells, which explains their lower toxicity (favorable therapeutic index). Targeted therapies can be roughly classified into two categories: small molecules (0.1–1 kDa) and macromolecules (greater than 1 kDa, also called biologics). Biologics are relatively complex molecules derived from living cells or through biological processes, such as monoclonal antibodies or antibody–drug conjugates.[163] Despite the recent interest and success of biologics, small molecules, which are made *via* chemical synthesis, are still in the picture of innovative drug research and development.

About one fourth of drug testing in ToC focused on targeted therapy. Targeted therapies exploring a wide range of targets have been tested on chip, among them kinase inhibitors (Tarceva, erlotinib),[71,164] HER2 inhibitors (trastuzumab),[165] CXC chemokine receptor inhibitors,[73] EGFR inhibitors (cetuximab).[165]

Nguyen *et al.* studied the effect of trastuzumab, a monoclonal antibody directed against the HER2 receptor, on breast cancer cells presenting HER2 amplification.[166] Trastuzumab alone or immune cells (PBMC) alone had mild effects on HER2+ cancer cells, but their combination induced a massive cancer cell apoptosis, exquisitely recapitulating on ToC a complex immune behavior, namely an anti-tumoral antibody-dependent cell cytotoxicity (ADCC). Live imaging combined with advanced cell tracking algorithms allowed to measure the number and the duration time of interactions between cancer and immune cells. In this HER2+ breast ToC, addition of trastuzumab specifically promotes long cancer–immune interactions (>50 min). Moreover, the presence of CAF (CAF-S1 sub-type) abolished this trastuzumab-dependent stimulation of cancer–immune interactions, suggesting that CAF-S1 cells may contribute to trastuzumab resistance by participating in immunomodulation.[166] Other targeted therapies such as anti-angiogenic drugs have been tested in vascularized patient-derived ToC vessels.[94] For different drug concentrations and different targets, the authors quantified vessel permeability as well as vessel sprouting and confluency.

Targeted therapies have already demonstrated their potential in clinics for different cancer subtypes and still holds great promise in particular with the recent emergence of the ADC drugs coupling targeting and chemotherapy precision delivery. However, small-molecule targeted anti-cancer drugs, despite high response rates and rare primary resistance, still face many challenges with the emergence of resistant clones.[162] Drug resistance could be linked to several mechanisms, including gene mutation or amplification, leading to parallel signalling pathway activation, apoptosis or autophagy dysregulation, *etc.*[167] We envision that the ability of ToC to recapitulate the cellular complexity of the tumor will be a strong asset for testing new targeted therapy testing, and we anticipate an increase of the anti-cancer drug application in ToC.

4.4. Pre-clinical studies on tumor-on-chip for immunotherapies

As ToC can include diverse immune cell types, it is emerging as a powerful model for immunotherapy testing. Immune system within the tumor microenvironment consists of adaptive and innate components[168] that are interdependent. The innate system is the first defense mechanism against tumor-specific or tumor-associated antigens, and it generates short-lived responses of antigen-specific immune cells such as monocytes, macrophages, dendritic cells and natural killer (NK) cells. On the other hand, the adaptive system can generate immune memory producing long-lasting responses through T-cells and B cells.[169] In turn, it has been shown that the presence of immune cells in the TME affects tumor progression, explaining why some of the on-going and very promising ToC strategies are focused on immunotherapy development.

Immunotherapies are innovative anti-cancer treatments that have revolutionized anticancer therapy since the early 2000s through unleashing the immune anti-cancer response.[170] Immune checkpoints are receptors expressed by immune cells that enable dynamic regulation of immune homeostasis and are particularly relevant to T-cell functionality.[171] Most immune check-point inhibitors (ICI) have been focusing on reinvigorating CD8+ T-cells to target cancer cells. While their success was originally thought to be dependent on local T-cell abundance, or on the cancer cell abundance of immune checkpoint targets, favorable responses to these therapies are still largely variable, particularly in solid tumors, where T-cell infiltration is highly variable and does not necessarily correlate with therapeutic efficacy.[172] Recent evidence suggested that there is more to be understood about the immune cell component within the TME and how to exploit them for therapeutic purposes. A major challenge to study tumor–immune interactions and develop therapies is the lack of effective and representative models. It is well established that the innate and adaptive immune systems of animal models, like rodents, are different from those of humans.[173] Consequently, syngeneic mouse tumor models, allowing for the participation of the native rodent immune system, rarely mimic human cancer behaviors and their applicability remains limited. PDX models are by nature immune-deficient models and are not suitable for studying immune checkpoint inhibitors targeting human T-cells. Humanized mice models could be used but such mouse humanization is still a highly variable, costly and time-consuming technology. Moreover, the approaches to investigate cancer cell escape from immune surveillance are limited to intravital microscopy in mouse models or observations of tissue slices from human tumor samples. This has motivated the development of ToC as a unique technological approach to reproduce the multiple layers of complexity of cancer–immune system crosstalk.[174–176]

Less than one tenth of the ToC studies focused on immunotherapies[27,38,83,89,123,177–180] but interestingly, the types of immunotherapy (alone or in combination) tested are broad, including immune checkpoint inhibitors (ICI), oncolytic viruses, and T-cell therapies. Parlato *et al.* reported that IFN-α-conditioned dendritic cells (DCs), grown in co-cultured ToC, exhibited remarkable migration and phagocytotic activity against colorectal cancer cells pre-treated with IFN-α and romidepsin, a histone deacetylase (HDAC) inhibitor.[177] In this model, DCs were cultured in the central chamber while both untreated and treated cancer cells were embedded in collagen I gel in the two adjacent chambers to evaluate DCs behavior in the extracellular matrix. An increase in DC migration toward pretreated colorectal cancer cells in this model was driven by

the CXCR4/CCL12 signaling axis, consistent with the DC responses *in vivo*. Most interestingly, by labeling DCs and SW620 with fluorescent dyes, cancer phagocytotic activity was captured in real-time using confocal microscopy, making this model more advantageous than *in vivo* models, where real-time monitoring of DC phagocytotic activity is not possible. Ayuso *et al.* developed a 3D microfluidic model that incorporated NK cells, endothelial channels (HUVECs), and MCF7 spheroids (Fig. 7 right panel). The authors studied antibody penetration into the spheroids, NK cell migration and antibody-dependent cell cytotoxicity (ADCC).[124] The dynamics of ADCC shown in this model *via* live imaging was remarkable. NK cells were able to directly penetrate deep into the spheroid core and destroy the cancer cells in a matter of hours, without first killing the outer layer of the tumor. Such studies highlight the power of ToC to pave the way for new studies which were not feasible with classical cell culture models. Indeed, ToC could enable researchers to focus on antibody dynamics and study the impact of various parameters separately such as endothelial permeability, tumor penetration or antibody clearance by tumor cells. A very recent study from Bi *et al.* interrogated the role of macrophages in tumor progression using a ToC device and downstream single cell RNA-seq. They introduced M1 or M2 macrophages into a 3D tumor-on-chip model to investigate tumor behaviors in response to these macrophage subsets.[104] In this model, M1 macrophages exhibited anti-tumor properties, whereas M2 macrophages showed significant pro-tumor effects, which is consistent with the current understanding of tumor-associated macrophages. However, to date, most ToC that incorporate immune cells do not allow for high-throughput testing. To address this limitation, Ronteix *et al.* recently introduced a platform for the parallel formation, manipulation and multiplexed observation of hundreds of tumor spheroids within stationary microfluidic droplets, in the presence of antigen-specific cytotoxic T lymphocytes.[151] Exploiting mathematical probabilistic models, the quantity and quality of spatiotemporally resolved data allowed to establish that the first recruited T-cells initiate a positive feedback loop to accelerate further recruitment to the spheroid, confirming the cooperation between T-cells in the tumor killing process.[151]

Injecting oncolytic vaccinia viruses (OVV) directly into the tumor microenvironment is an alternative to improve tumor antigen recognition and to strengthen T cell responses.[181] In a lung ToC model, infection by OVV was shown to increase cancer–immune interaction times leading to cooperative antitumoral activity of immune cells and OVV.[154] Proof-of-concept studies also illustrate the feasibility to exploit ToC models for innovative immunotherapies such as Chimeric Antigen Receptor (CAR)-T-cells. In the model developed by Pavesi *et al.*, human T-cells engineered to express tumor-specific T cell receptors (TCR-T-cells) were added into the adjacent channels to investigate the ability of the modified immune cells to migrate and kill the tumor target and their secreted soluble factors.[182] A recent work by Wan *et al.*[38] presented a vascularized *in vitro* model which can be used to evaluate CAR-T cell recruitment, killing capacity, and inflammatory response. After 96 h of perfusion, higher densities of both T-cells and dead cells were found in the CAR-T cell containing ToCs as compared to the control T-cell containing ToC in both co-mixed and sequential tumor spheroids.

Although ToC models offer a powerful experimental setting to quickly test immunotherapies, the vast majority of the immune-competent ToC models are so far based on cell lines and allogeneic immune cells, *i.e.* immune cells not coming from the same individual (mainly PBMC), exhibiting the obstacle of human leukocyte antigen (HLA) incompatibility. Immunotherapy testing requires the use of autologous cytotoxic T-cells to avoid the risk of allogeneic reactions, which, however, would require longer time than the few days needed for ToC experiences. We envision that one of the upcoming challenges in the field will be the development of patient-derived ToC for immunotherapy testing. Some latest works have shown the feasibility of such an approach. For example, a recent work reported the use of a glioblastoma-on-a-chip model to dissect a reconstituted immunosuppressive tumor microenvironment (composed of tumor-associated macrophages (TAM) and cytotoxic CD8+ T-cells) and its response to programmed cell death protein-1 (PD-1) checkpoint blockade. Interestingly, different glioblastoma subtypes displayed distinct CD8+ T-cells behaviors (extravasation, tissue infiltration, cytotoxic activities) as well as cytokine profiles. Moreover, co-targeting of PD-1 immune checkpoint and TAM-associated CSF-1R signaling improved therapeutic efficacy on-chip. However, again, human CD8+ T-cells were allogeneic, sorted from PBMCs, limiting the clinical significance.[27]

Even though immunotherapies can produce impressive and long-term responses in some cancer patients (*e.g.*, 20 to 40% of lung cancer patients), their clinical benefits remain unsatisfactory because the majority of cancer patients are non-responder.[170] What remains to be explored in these tumor–immune models is the incorporation of the adaptive immune cell component, in particular, autologous T-cells, as well as the integration of an *in vitro* immune organ, namely bone marrow- or lymph node-on-chip, to further understand tumor immunity and develop new therapeutics. Most models utilize a variety of cell sources, both immortal and primary cells in combination, making it difficult to study the adaptive immune response. A good metric of ToC would be to evaluate how deeply they are able to reproduce the *in vivo* functional readouts such as cytokine release, phagocytosis, IgM/IgG class switching.

Finally, immunotherapy is a fast-evolving field, and one of the further challenges in immunotherapy is the development of bispecific antibodies (BsAbs) that can directly target two different antigens on immune cells and/or tumors (tumor-associated antigens), synergistically engaging T-cells onto cancer cells, thereby increasing cytotoxic activity.[170] We anticipate that in the near future ToC technology will be able,

by reproducing the complexity of cancer–immune system crosstalk, to help the development of novel therapy exhibiting multiple cell interactions such as bispecific antibodies.

4.5. Drug combination in ToC

Over the years, the concept of combination therapy, which relies on combining two or more therapeutic agents, with different modes of action, has been introduced to overcome cancer treatment resistance. Such combination is either synergistic or additive, and therefore, a lower therapeutic dosage of each individual drug can be required, which also spares the cumulative toxicity.[183] Combination therapy exhibits numerous benefits, such as the ability to target multiple oncogenic pathways, to improve and prolong therapeutic responses while reducing the likelihood of therapeutic resistance.[184] This can include the combination of chemo- and radiotherapy, chemo- and immunotherapy, or chemotherapy and targeted agents.

In contrast to conventional cell culture, fluidic control in ToC allows for creating precise drug mixing and variations over time. Ong *et al.*[80] introduced a ToC with a concentration gradient generator (Fig. 7 left panel). This configuration allowed assessment of 8 drug concentrations and 5 different drug combinations. They showed that tumors did not respond to single-agent Oxaliplatin (OXA) and 5-fluorouracil (5-FU) treatments, but conversely had improved sensitivities when both drugs were combined (5-FU + OXA). This work represents a great illustration of the ToC potential for drug testing. Recent publications highlighted the possibility to test combined therapies on fresh surgical tumor[81] or PDX.[85] For example, Ivanova *et al.*[85] tested drug combinations in breast cancer patient xenograft-derived organotypic spheroids. They identified that neratinib and trastuzumab combination was more effective compared to each agent alone, and was associated with more robust inhibition of HER2. Importantly, Eduati *et al.*[91] presented a plug-based microfluidics platform for functional screening of drug combinations (56 different conditions with at least 20 replicates each). They suggested a novel drug combination for pancreatic cancer cell lines: PHT-427 and MK-2206, which consist of two serine/threonine kinase AKT inhibitors acting through different sites. This study highlights that the best drug combination can be different for each patient and that high throughput ToC holds a great potential for personalized medicine.

Finally, drug combinations can also include immunotherapies. With a vascularized ToC, Humayun *et al.*[73] demonstrated that by combining immunotherapies inhibiting IL-6, IL-8 and MMP-3, the extravasation events can be reduced. In the near future, ToC could be pivotal to decipher the intricate interplay between ADC and ICI. ADCs can selectively induce death of target-expressing tumor cells and activate tumor-specific adaptive immunity through increase of T-cells infiltration in the tumor microenvironment, whereas ICI reinvigorates exhausted T-cells, enhancing antitumor immune responses.[185]

4.6. Support for nanomedicine innovation

Besides these conventional anti-cancer treatments, recent advancements in nanotechnology have opened new windows for the discovery and development of anti-cancer nanomedicine strategies. Having gone through several generations of nanomedicine drug development, nowadays we are at the verge of multifunctional nanomedicine therapeutics, allowing for targeted drug delivery and stimuli-triggered nanosystems, as well as their combinations. Nanocarriers appear as powerful technologies to release anticancer drugs in a stable and controlled manner. Moreover, the use of stimuli can assist in the controlled release of the drug to ensure specific toxicity to the tumor tissue, while sparing the healthy tissue.[186] A variety of nanotechnology-based treatments have already gone through clinical trials, are marketed and are being currently used for clinical cancer therapy applications.[187] However, the prevailing majority of research on nanomedicine therapies for cancer care is still at the stage of preclinical studies.[188] Nowadays, conventional *in vitro* models for nanomedicine screening as well as *in vivo* animal models are unable to closely replicate human *in vivo* tissue environment which, in turn, significantly impedes adequate nanomedicine development and evaluation. Crucial parameters such as nanoparticles' toxicity, diffusion, internalization, accumulation oftentimes cannot be adequately assessed with conventional models. In regard to these limitations, ToC are gaining interest for the development and evaluation of nano-based therapies in tumors.[189] ToC may allow to finely evaluate these pivotal criteria of evolving nanotherapeutic approaches through precise control of microenvironment, phenotype, dynamic fluid flows and physiological gradients among the others – all of which affect nanomedicine's suitability and progress of preclinical studies.[190,191]

While multiple works in ToC have been focused on the various key aspects of nanomedicine evaluation for clinical applications, such as nanoparticles (NP) toxicity,[192,193] transport,[194,195] uptake,[193,196] accumulation,[197,198] they only represent 13% of reviewed ToC-related studies. For the purposes of this review, we will focus on the investigation of the therapeutic impact of NP-based therapies on tumor tissues, their efficacy and cellular effect readouts in ToC models. Broadly, nanomedicine therapies can be subclassified into drug nanocarriers and stimuli-responsive nano objects that function as a treatment itself upon activation.[187] The former has been mostly explored up to date as they allow to increase loaded conventional drug's stability, solubility, blood circulation time as well as to provide controlled drug delivery to the site of interest.[199] In the scope of ToC, multiple studies have been carried out to demonstrate effective tumor cell death following the nanocarrier drug exposure. Thereby, Liu *et al.* have reported a ToC model of human glioma to study tumor targeting with nanomedicine. They have demonstrated remarkable tumor reduction post-treatment with paclitaxel-loaded folate-

decorated NPs as well as significant cell death due to both apoptosis and necrosis.[200]

However, following the concerns of the high NP concentrations required and high systemic drug toxicity, a new line of research is dedicated to the stimuli-responsive nano objects that can be activated by endogenous or exogenous sources at the site of interest and operate as therapeutic agents themselves. Some of the most common non-invasive, stimulating therapies are exogenous triggering mechanisms such as photodynamic therapy (PDT) and hyperthermia-based therapies (*e.g.*, magnetic hyperthermia, photothermal therapy (PTT), ultrasound, among others).[201] PDT is based on the phototoxic reactions that stem from the photosensitizer activation by light within tumor cells.[201,202] Recently, Flont *et al.* designed a ToC model of ovarian cancer to evaluate the effect of PDT through free *vs.* nanoencapsulated photosensitizer on cancer cells.[203] Their results have demonstrated remarkably higher cytotoxicity of nanoencapsulated photosensitizer as compared to the free one, which was further boosted by the PDT. They also demonstrated that PDT induced ROS generation in cancer cells while having little effect on non-malignant cells. Another ToC study on externally triggered nanoparticles was conducted by Lee *et al.* on a co-culture of breast and glioblastoma cancer cells *via* gold nanorod-mediated PTT.[204] They have demonstrated drastic reduction in cell viability upon PTT exposure as compared to separate conditions with either nanorods presence or PTT, indeed indicating an advantage for the stimuli-induced effect.

Stemming from the beneficial characteristics and therapeutic outcomes presented by the stimuli-responsive nanoobjects, some of the recent strategies in nanomedicine therapies development have focused on the combination of nanocarrier and stimuli-responsive nanosystems, which allow for the dual action with triggered drug release, thus limiting the systemic exposure to the toxic chemotherapy. Agarwal *et al.* using a vascularized ToC model of breast cancer, have shown that nano-encapsulated doxorubicin is significantly more effective for cell death induction than free doxorubicin.[205] Moreover, the Dox release can be controlled *via* NIR irradiation thanks to the photothermal and photodynamic sensitivity of the NPs.[205] Another study involving stimuli-responsive nanocarrier drug delivery was conducted by Zervantonakis *et al.* in which they demonstrated in a ToC model of rat glioblastoma that Dox-loaded thermosensitive liposomes, when heated induce the greatest effect on tumor cell death and proliferation inhibition as compared to the single application of either drug-loaded NPs or heating.[206]

These different examples evidenced that ToC modeling would allow us to better investigate the effect of the new generation of nanomedicine therapies by being able to finely assess their influence on the tumor microenvironment in a human-mimicking tissue, therefore facilitating the transition towards the clinical studies.

5. ToC paths towards pharmaceutical and clinical applications

Classically, from the conception of a new drug to the launch of the product, the drug development process can take up to 15 years (although shorter times have been recently shown for targeted therapies), and cost upwards of $1 billion.[207] To date, the success rate of new anticancer drugs development is very low for two main reasons: the lack of clinical efficacy and the unmanageable toxicity of the drug.[4] One third of drugs fail in clinical phases due to an insufficient therapeutic index, meaning that the efficacious dose and the toxic dose are too close.[208] This number evidences the shortcomings of current preclinical models to accurately predict the right target at the right dose. In order to reduce such attrition between preclinical and clinical research, the FDA has recently requested 5 million US$ to develop a comprehensive strategy on alternative testing methods.

Among the different *in vitro* preclinical models, patient-derived cancer organoids (PDO) appear as a powerful new model. A recent review, including 60 studies, indicated that organoid cultures are faithful predictors of patient response to chemotherapy for different types of cancer.[12] However, the establishment of PDO is not equally effective for all solid cancers and cancer organoids still lack some cells component of the TME, including stromal cells, immune cells, and endothelial cells. Moreover, a big challenge of organoids is to align the timescales of model establishment (1–6 months) to those of clinical decision-making. ToC by combining a short establishment time and a high control of several TME features are expected to better predict patient's treatment response.[12] ToC has the potential to complement conventional preclinical models and the global ToC market size is projected to reach 110 million dollars by 2030.[209]

To make ToC seamlessly integrated into the drug discovery and development process, three main aspects must be considered: standardization, reproducibility, and throughput. Since ToC are still in the early-development stage, consistency and standardization are still lacking, and there is often variability in materials, device fabrication and operating conditions.[210] The US IQ MPS workshop 2022 and the European ORCHID project have clearly identified standardization as a fundamental pillar of the advancement of MPS technologies. However, until now, there are currently no specific standards that have been agreed upon. To meet these standards, ToC will have to be independently validated by third-party testing centers. In line with these requirements, the NIH/NCATS and its Tissue Chip Consortium has been working on the establishment of the Tissue Chip Testing Center program dedicated to organ on chip validation. This independent validation is a stepping stone for the MPS field to provide more confidence for industry to onboard this new technology. Beyond standardization, reproducibility is also

an important challenge from industry perspective; future studies would also be needed to properly assess the ToC reproducibility.

Another important consideration is related to the ability of this new technology to achieve the level of multiplexing that would allow rapid and efficient drug discovery at a large scale. So far, only few ToC studies were able to process samples at high throughput (*i.e.* custom micro-well arrays). High-throughput ToC would be a real breakthrough as it would allow for testing multiple compounds, and multiple concentrations in a complex and controlled reconstituted TME. Some studies recently showed the feasibility to automate ToC operations, such as media changes, pH testing, compounds monitoring (oxygen, lactate, glucose), or drug injection.[91] On chip flow control can be used to refresh cell culture medium preventing medium acidification and O_2 decrease.[211] Flow control of ToC should include a partial and controlled recirculation of cell medium to balance nutrients refreshing and cell secretions dilution. The automation of ToC models would considerably improve their ease of use and therefore their reproducibility then supporting regulatory and quality standards.[212] We believe a final goal would be to have automated high-throughput ToC with monitoring and feedback loop for a variety of key parameters (such as pH, oxygen and key functional metabolites).

Some pharmaceutical industries have already taken the step towards ToC tests in their pre-clinical studies. A recent review reported[213] collaborations between several MPS manufacturers (Mimetas, TissUse, Emulate) and pharmaceutical industries (Astrazeneca, Galapagos, Janssen, Novo Nordisk, Bayer and Roche) at different steps of drug discovery: target identification and validation, discovery, pharmacokinetics and pharmacodynamics, preclinical safety and clinical developments. These early adopter studies will provide a better understanding of how the technology is deployed. Gaining the confidence of more Research Contract Organizations (CROs) could also help increase ToC use at the target identification and validation steps.[214] In this section, we aim at providing insights on how ToC can bring added values for companies in their anti-cancer drug development process. We focus on three main aspects of this process:[215] (i) "trust in target" which identifies the right biological target and understand its role, (ii) "trust in targeted patient population" that defines the right patient population with any needed stratification strategies and (iii) "trust in therapeutic index" which identifies the right molecule that delivers the right exposure at the target site of action without compromising patient safety.

5.1. Trust in target

The development of a new drug starts with target identification and validation.[207] During this pre-discovery phase, high-throughput screening (HTS) campaigns are initiated to screen 10^3 to 10^6 potential drugs (called "hit") on cell lines. After screening, the "hits" identified are further evaluated through different methods (*e.g.* dose–response curves). Then, during the hit-to-lead stage, "lead" development candidates are synthesized and their efficacy is estimated. An increasing amount of evidence has established that while extremely informative, screens performed with 2D cell culture often do not recapitulate key information such as drug response and sensitivity. As a result, many targets have been likely missed by hit identification experiments. In this context, ToC offers a great advantage because of both increased complexity and versatility, to allow fit-for-purpose implementations for each disease. ToC can be set up with various degrees of complexity selected based on the specific need to be addressed. Moreover, ToC are compatible with a wide range of readouts such as genomics, proteomics and transcriptomics, which could allow the identification of new targets in presence of the TME. The combination of ToC with *in vivo* imaging technology would be tremendously useful for target identification and validation as it would allow to identify molecules capable of eliciting the desired effect with phenotypic live monitoring. Once ToC reaches higher throughput level, it will appear as a new high-content screening (HCS) tool allowing for multiparametric phenotypic outputs that can be used for more comprehensive drug screens.

5.2. Trust in targeted population

Personalized medicine aims to move apart for the "one-size fits all approach", by providing the most suitable medicine, at the right dose, for the right person, at the right time, at a reasonable cost.[216] This definition encompasses two ideas: (a) finding the most effective drug for an individual patient by testing different drugs on his/her tumor sample in the lab, (b) defining subpopulations of patients based on biomarkers to anticipate the efficient drug which should be chosen for an individual patient. Several prognostic and predictive markers have been identified (*e.g.*, somatic or germline mutations) to determine individual profile of each patient and tumor.[217] ToC could be a powerful tool in this field by combining functional testing on patient sample and biomarkers discovery during pre-clinical phases.[218]

Given the heterogeneity of primary tumor tissues, these ToC could be employed to assess the degree of heterogeneity in drug response according to various clinical characteristics, or genetically similar subgroups. The combination of ToC personalized medicine with artificial intelligence paves the way to digital/*in vitro* twins for the development of predictive responders and non-responders models.[219] The combination of patient ToC functional cytotoxicity assays with specific mathematical models could allow to predict the clinical response. For example, using conventional cell culture methods, Silva *et al.* developed a novel tool capable of predicting within 5 days the clinical response of multiple myeloma patients to 31 drugs over months, using fresh bone marrow aspirates.[220] They implemented a parametric model in which the likelihood of cell death depends on drug

concentration and exposure time. Importantly, the algorithm output is not only limited to a dichotomized response/no-response or depth of response, but can also predict the trajectories of clinical response during the first 3 months of treatment. The combination of mathematical algorithms with ToC could provide precise clinical insights on treatment efficacy in a timely manner and assist oncologists in practicing truly personalized management. The current challenge is to provide robust clinical validations of ToC-based predictions by performing rigorous correlations between *ex vivo* ToC responses and *in vivo* patient responses. This requires close collaborations with clinicians and establishment of appropriate patient cohorts.

5.3. Trust in therapeutic index

One of the main translational goals in drug development is the prediction of adverse events. Current toxicity regulations require testing first on a rodent and then on a larger mammal (pig, rabbit, dog, monkey) in order to identify the human dose prediction (HDP). However, very often animals tolerate higher dose than humans. For example, Chou *et al.*[221] used Emulate chips to create an *in vitro* preclinical model of human hematopoiesis that recapitulates many clinically relevant features of bone marrow pathophysiology in response to drugs and ionizing radiation. The bone-marrow-on-a-chip may serve as a human-specific alternative to animal testing for the study of bone-marrow pathophysiology.

A major drawback of conventional *in vitro* cancer models is that drugs are commonly delivered in fixed concentrations. However, this does not accurately simulate the pharmacokinetic (PK) concentration changes experienced *in vivo* due to drug metabolism and clearance mechanisms, and therefore reduces the predictive value of the data.

To solve this problem perfusion in ToC can be employed to simulate the liver's metabolism by changing the concentration over time, *i.e.*, a rise in blood concentration followed by a decrease. Recent work[222] reproduced *in vivo* PK profiles of different anti-cancer drugs over multi-week experiments, both as single-agent therapy or drug combinations. Interestingly, the authors compared the effects on tumor cell efficacy *in vitro* with efficacy seen in *in vivo* xenograft models. They reported that the incorporation of PK into ToC could improve *in vitro–in vivo* translation understanding for early therapeutic insight. Petreus *et al.*[223] designed a pump-driven microfluidic platform capable of mimicking *in vivo* anti-cancer drug pharmacokinetic profiles in ToC. Tumor xenografts were subcutaneously implanted in mice and subjected to the same treatment schedules as the spheroids in the chip and demonstrated that such microfluidic ToC platforms can successfully predict the efficacy. Incorporating flow into ToC could thus assist with the understanding of how different sequencing (continuous, intermittent, gapped schedules) of the drugs affect their efficacy, thereby guiding the design of confirmatory *in vivo* studies.

Physiologically based pharmacokinetic and pharmacodynamic (PK/PD) models could also be proceeded with multi-organ co-culture. Particularly, as liver and kidney play a big role in drug metabolism and clearance, there is a need to connect liver/kidney models to cancer tissue for the study of small molecules. In the case of biologics larger than 45 kDa, there is less interest in such systems because they are cleared from the system mainly by endocytosis.[224] McAleer *et al.*[225] described a multi-organ device with a PK/PD profiling of tamoxifen on MCF-7 breast cancer cells in the presence/absence of liver cells. They also simultaneously monitored the effect of tamoxifen on cardiac function. The efficacy of multi-organ MPS to predict human PK/PD was also demonstrated in other studies: for capecitabine, and its 5-fluorouracil metabolite, in a four-organ system, composed

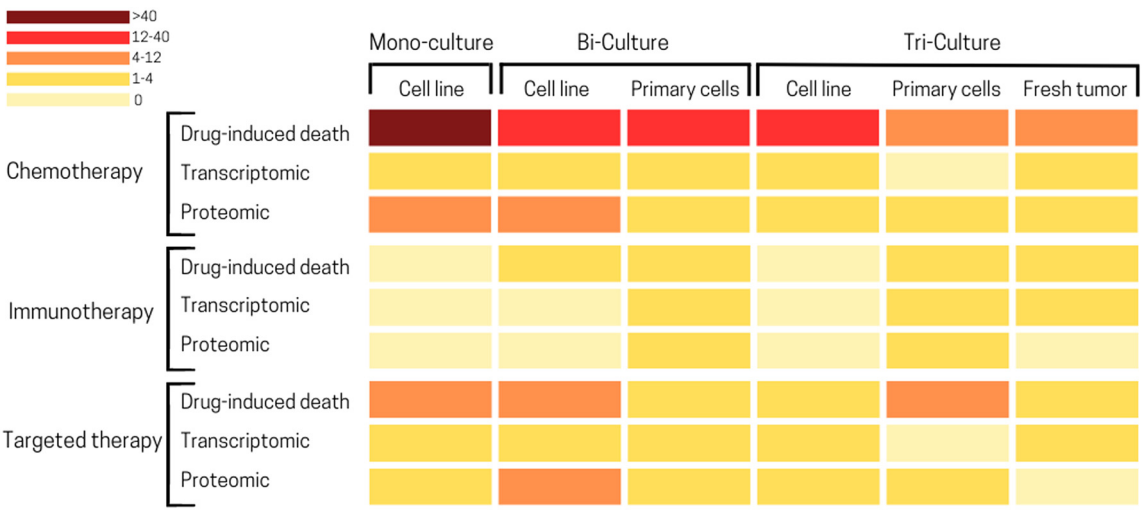

Fig. 8 Heatmap representing the number of publications according to the cellular complexity (mono-culture, bi-culture, tri-culture) with different cell sources (cell line, primary cells or fresh tumor) for different drug treatments (chemotherapy, immunotherapy, targeted therapy) and information extracted (drug-induced death, transcriptomic, proteomic).

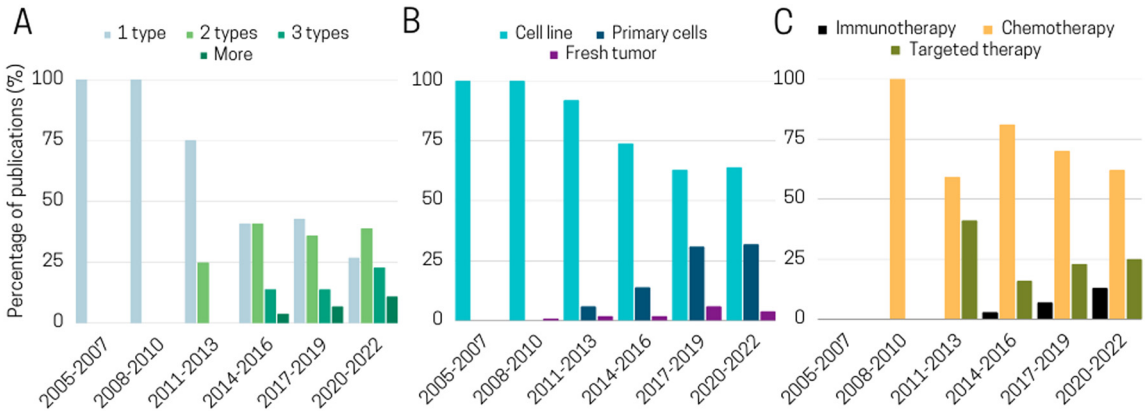

Fig. 9 Evolution of cellular complexity and drug tested over time during the last 15 years of ToC development. Histograms illustrating evolution of A. cell complexity, B. cell source and C. tested therapy in publications.

of intestine, liver, cancer, and connective tissue models;[226] for cisplatin using coupled bone marrow, liver and kidney chips.[227]

6. Conclusion

The past 15 years have witnessed significant advancements in the field of ToC. The microfluidics community in close collaboration with cell biologists and clinicians has actively engaged in this field and achieved impressive milestones. These efforts resulted over the years in an increasing level of complexity on-chip and, consequently, in a better biomimicry of these devices (Fig. 8 and S3†). In this review, our objective was to provide a comprehensive overview of the current state of ToC research and to identify the potential next steps and challenges. To achieve this, we conducted an extensive quantitative analysis of the literature, dissecting the diversity of cell models, drug treatments and extracted information (Fig. 9). Moreover, we highlighted the current promising results of ToC clinical validation.

In this review, we first focused into what sets ToC apart from other *in vitro* models, specifically explaining the various parameters that can be finely controlled in ToC systems. These parameters include the properties of the extracellular matrix, mechanical forces exerted on cells, the physico-chemical environment, cell composition, and the architecture of the tumor microenvironment. ToC ability to precisely and individually manipulate TME parameters enables researchers to dissect the influence of these TME parameters on tumor development, growth, invasion, and response to drugs.

Nevertheless, beyond their interest for basic research, ToC have also the potential to become a transformative technology for clinical and preclinical studies. This potential relies on several factors, notably their compatibility with a wide range of analytical techniques, both conventional and state-of-the-art, such as single-cell-based technologies. Moreover, we believe that the information that can be extracted from ToC remains largely untapped, presenting an avenue for future exploration. Particularly, there is a need to exploit the hidden insights within ToC-generated data, leveraging novel approaches in data analysis. By doing so, as a community, we could be able to unlock a wealth of valuable knowledge and further enhance the capabilities of ToC platforms.

When considering ToC implementation in clinical or preclinical studies, it is also necessary to mention the demonstrated potential and compatibility of ToC devices with a wide range of therapeutic modalities. These modalities encompass chemotherapy, targeted therapy, and more recently, immunotherapy (Fig. 9C). In the field of immunotherapy, ToC hold tremendous importance due to their ability to easily include immune cells, thus, to quite faithfully recapitulate the cell composition of the tumor microenvironment. While promising results have been achieved, significant challenges remain in working with autologous patient cells or incorporating adaptive immunity mechanisms into ToC models.

We also examined the potential of ToC for pharmaceutical industries. At this stage, of course ToC cannot replace all existing *in vitro* and *in vivo* models, and the future landscape will likely involve a combination of animals, organoids, and organ-on-chip platforms. Nevertheless, considering the high failure rate of clinical trials and the increasing emphasis on the 3Rs (replacement, reduction, refinement) principles by regulatory agencies worldwide, we envision that data generated through ToC models could be employed for regulatory purposes within the next decade. While ToC models are generally more costly than conventional cultures, their adoption is expected to occur gradually by identifying applications that will overcome existing barriers in the pharmaceutical industry. A survey conducted among pharmaceutical companies estimated that microphysiological systems could potentially save the industry up to approximately 25% in research and development expenses.[8]

Hence, ToC has the potential to significantly enhance the efficiency and cost-effectiveness of the drug discovery process especially by defining the delicate balance between clinical

dose, efficacy and safety. And the success of ToC for clinical applications will be determined by its ability to detect and validate new therapeutic targets as well as to help the clinicians to define the best therapeutic choices and sequences for patients.

Author contributions

This review was written by a consortium of engineers (CB, AD, SD, CC, CW), biologists (MB, IH, SL, MCP, FMG), clinicians (LC, GZ) and pharmaceutical industries members (DP, DM). Analysis of >300 publications were proceeded by CB and AD. Interviews of pharmaceutical industries and contract research organization were proceeded by CB.

Conflicts of interest

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