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Microphysiological systems to study colorectal cancer: State-of-the-art

To cite this article before publication: Pedro Ramos *et al* 2023 *Biofabrication* in press https://doi.org/10.1088/1758-5090/acc279

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Microphysiological systems to study colorectal cancer: State-of-the-art

Abstract

Basic pre-clinical research based on 2D cultures have been very valuable in colorectal cancer (CRC) research but still have failed to improve patient prognostic outcomes. This is because they simply do not replicate what happens *in vivo, i.e.* 2D cultured cells system cannot replicate the diffusion constraints usually found in the body. Importantly, they also do not mimic the dimensionality of the human body and of a CRC tumour (3D). Moreover, 2D cultures lack the cellular heterogeneity and the tumour microenvironment such as stromal components, blood vessels, fibroblasts, and cells of the immune system. Cells behave differently whether in 2D and 3D, in particular their different genetic and protein expression panels are very different and therefore we cannot fully rely on drug tests done in 2D. A growing field of research based on microphysiological systems involving organoids/spheroids or patient-derived tumour cells has become a solid base for a better understanding of the tumour microenvironment and as a result is a step towards personalized medicine. Furthermore, microfluidic approaches have also started to open possibilities of research, with tumour-on-chips and body-on-chips being used in order to decipher complex inter-organ signalling and the prevalence of metastasis, as well as CRC early-diagnosis through liquid biopsies. Herein, we focus on the state-of-the-art of CRC research with emphasis on 3D microfluidic *in vitro* cultures - organoids, spheroids – drug resistance, circulating tumour cells and microbiome-on-a-chip technology.

Keywords: colorectal cancer, spheroids, patient-derived organoids, tumour microenvironment, microbiome, microfluidics

1. Introduction

Mortality rates due to colorectal cancer (CRC) reached its peak around 1990, where number of countries have introduced population-based colorectal cancer screening programs, targeting mostly people in their 50s and 60s [1]. Advances in diagnosis and therapy allowed the numbers to decrease. On the other hand, as socio-economic transition occurs globally, CRC incidence and mortality rates increases again. Latest data on CRC shows there were more than 1.9 million new cases in 2020 in the world and 935.173 deaths, being the second most common cancer in woman [2]. Microphysiological systems (MPS) are organ-specific cultures that replicate the functions of human organs or tissues in a limited space to create miniaturized human systems, thereby with the possibility of emulating the interconnection of organs – "body-on-a-chip" [3]. On the other hand, more common "organs-on-a-chip" are cell culture microfluidic devices that provide mechanical cues, tissue-tissue interactions, and vascular perfusion to recapitulate the local microenvironment of native organ-specific cellular organizations [4]. MPS have useful applications in: 1) pathobiology elucidation, 2) drug screening and drug safety testing 3) disease modelling and 4) circulating tumour cells (CTCs) entrapment. Such devices have been utilized to study key functional and mechanical properties of several human organs, in a multidisciplinary effort involving bioengineers and biologists, including lung alveoli [5] and bronchioles [6], kidney tubules [7] and glomeruli [8], small intestine [9], liver [10], bone marrow [11], and the blood brain barrier [12], among others [13].

For several decades, cancer research has been carried out in 2D flat monolayer plastic flasks, and with the advent of MPS or organs-on-chip many avenues have opened that revolutionized the field of cancer preclinical models, including CRC research [14]. 2D models fail to provide insights into the immune infiltrate, vasculature recruitment and stroma compartment interactions. New 3D models such as organs-on-chip, body-on-chip and/or organoids better recapitulate the microenvironment of tumours and offer possibilities of studying these with higher precision and focus on the key steps of the cancer cascade: tumour growth and progression [15], angiogenesis [16], metastasis [17]; and improve drug development efforts [18].

Furthermore, the synergistic combination of organoids (see BOX1) with organs-on-chips have come as a leap in cancer research [19]. Tumour organoids, in comparison to cell lines, better emulate the genetic and phenotypic make-up of patient tumours, thereby paving the way to oncological personalized medicine [20]. On the other side of the synergistical relationship, microfluidic organs-on-chips may present several advantages such as requiring smaller amounts of sample when comparing with other systems (2D, 3D), enhanced imaging/quantification, enhanced throughput, increased controllability, and they are an inexpensive tool. As a disadvantage: it does require extensive training for use, a cleanroom fully equipped to prepare the devices, and some optical difficulties and reproducibility issues may arise, as well as reduced optical transparency due to PDMS [21].

The current developments involving MPS or tumour-on-chips that use spheroids and organoids dedicated to the study of CRC are overviewed herein. Liquid biopsies with a particular focus on CTCs microfluidic platforms will be discussed. Also, the human microbiome has gained renewed interest as the advent of genomic sequencing technology allowed a deeper understanding of its structural diversity and functionality [22]. Microfluidic approaches involving a gut/microbiome interface have moved forward the understanding of the cell signalling involved in the link of the microbiome and the onset of CRC [23][24]. Therefore, it is within the scope of this review to analyse the key aspects of tumour in recent years [25].

BOX 1

Different concepts: Spheroids *vs.* Organoids

Spheroids

Spheroids are multicellular self-assembly structures that can be generated according to four main categories: suspension culture, non-adherent surface method, hanging drop and scaffold-based culture. Suspension cultures can be obtained in a spinner flask bioreactor or a rotary cell culture system. The non-adherent surface method can be used by utilizing non-adherent surface coatings such as agarose, poly-HEMA and pluronic acid. Hanging drop forms spheroid cultures in small hanging droplets and scaffold-based culture can be achieved with natural hydrogels (chitosan, alginate, silk fibroin) or synthetic hydrogels (polyethylene glycol (PEG), polylactic acid (PLA) and polyglycolic acid (PGA)) [26] (Figure 1).

Organoids

Organoids are entirely different from spheroids. They are complex clusters of organ-specific cells, such as those from the stomach, liver, or bladder. They're made of stem cells or progenitor cells and self-assemble when given a scaffolding extracellular environment. When that happens, they grow into microscopic versions of parent organs." (see BOX 2 for different types of organoids) [27].

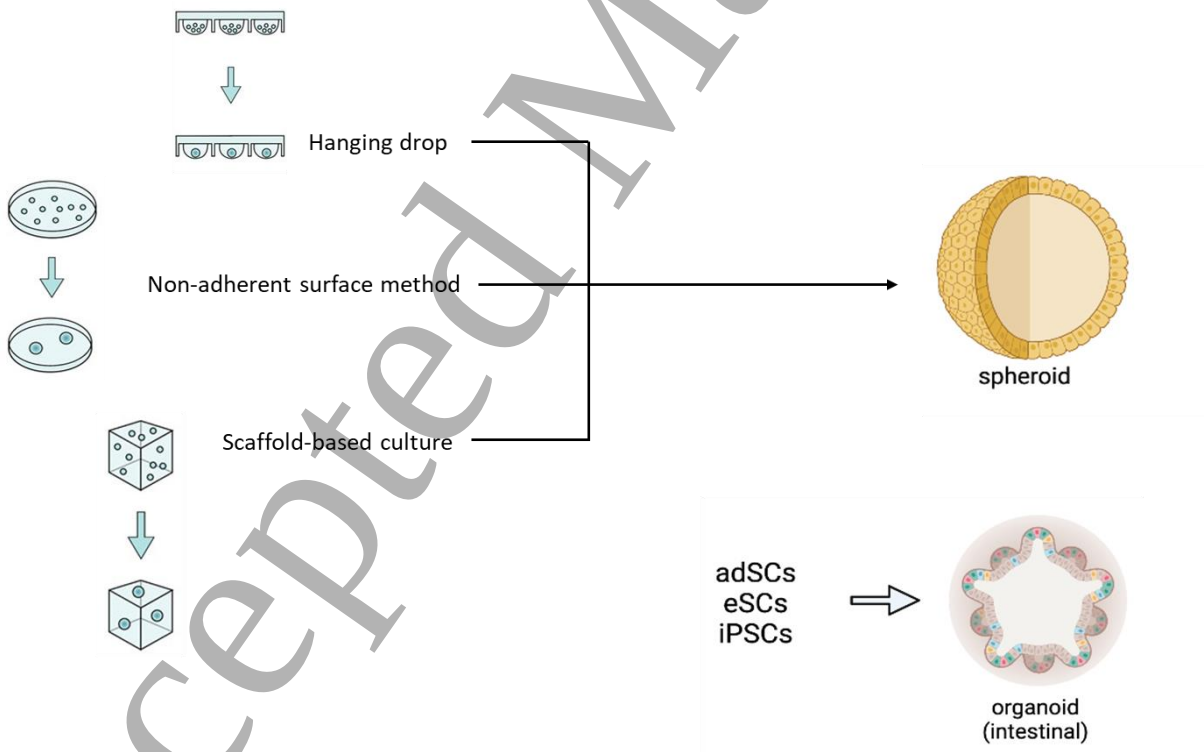

Figure 1 – Schematics as a visual help to understand the differences between spheroids and organoids. Created with BioRender.com

BOX 2

Tumour organoids: Beyond the cell lines

AdSCs (adult stem cells) organoids

The method for the development of CRC organoids was first devised by Sato *et al.*[28] by working on the established long-term culture conditions under which single crypts or leucine-rich repeat-containing G protein-coupled receptor ($LGR5^{+}$) intestinal stem cells, derived from mouse small intestine, expand over long periods. An adaptation of the cocktail of growth factors that mimic the *in vivo* stem cell niche (including WNT stimulators, such as Wnt3A and R-spondin-1, and activators of tyrosine kinase receptor signalling), and embedment in Matrigel® allowed the cells to expand indefinitely [28]. As a result, cells derived from this single stem cell grew out as organotypic and as highly polarized epithelial structures with proliferative crypt and differentiated villus compartments [29].

This same protocol has been used not only for healthy tissues of different organs including, colon [28], liver [30], pancreas [31], prostate [32], but also for malignant tissues [27], whereby patient-derived tumorigenic mutations can be further investigated in *in vitro* cultures. Hence, these long-term organoid cultures from patient-derived tumour tissues resemble the tumour epithelium they were derived from – both phenotypically and genetically. This characteristic of organoids is important since tumour-specific heterogeneity of cancer cell lines is gradually lost through epigenetic and genetic drift in the long-term culture [33]. Besides, other limitations of cell lines include the absence of normal tissue-derived control cell lines as references and lack of interaction with other stroma and immune cells [27]. This can have an effect on the drug response of targeted therapy agents [34]. Another method to generate tumour organoids is to use CRISPR/Cas9 genome editing to cause the desired tumourigenic mutations in normal healthy adult stem cell patient-derived tissue or iPSCs [35].

iPSC (induced pluripotent stem cells) organoids

Tumour organoids can also be established from iPSCs, where, in principle, it is possible to reprogram epithelial tumour stem cells to become pluripotent. However, generating epithelial tumour organoids from these iPSCs would be challenging, as cancer genetic and epigenetic abnormalities might interfere with the differentiation trajectories [36]. According to Clevers *et al.* [27], it is more practical to generate tumour organoids directly from cancers rather than to involve an intermediate iPSCs step.

CRC organoid biobanks

The possibility to cryopreserve organoids led to the development of living organoid biobanks of healthy and diseased tissues. An organoid biobank for CRC patients was established by van de Wetering *et al.* [37]. The biobank is constituted of organoid cultures from 20 CRC patients, as well as organoids from adjacent normal tissue. This biobank allows for studies of the tumourigenic genetic diversity of CRC as well as it enables high-throughput drug screens permitting the detection of gene-drug associations. These aspects are important for patient-centered therapeutic development (Fig. 2).

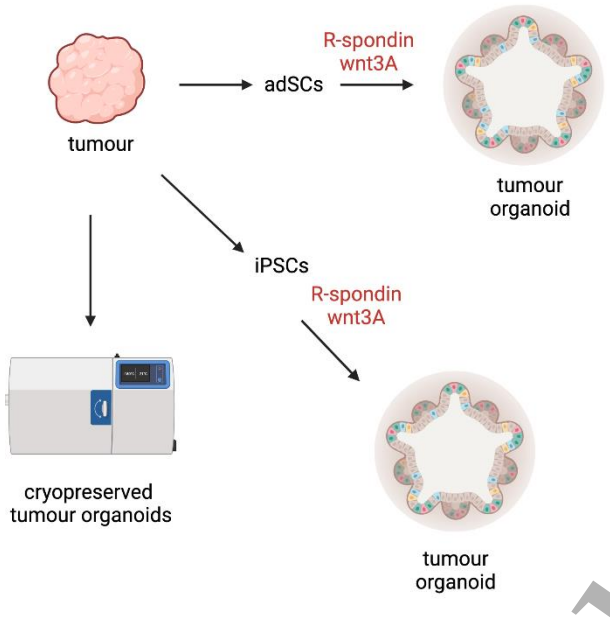

Figure 2 – Schematics of the cellular approaches to produce and cryopreserve tumour organoids. Created with BioRender.com.

2. The paradigm of colorectal cancer: Fundamentals and research directions

CRC, according to recent data (2020) of the IARC (International Agency for Research on Cancer), has the fourth highest incidence rate and the third highest mortality rate worldwide [38]. CRC is caused by both environmental factors such as excessive consumption of red meat, alcohol, smoking, and by genetic mutations that target oncogenes (*WNT*, *RAS/MAPK*, *PI3K*, *TGFB1*), tumour suppressor genes (*AR1D1A*, *CTNNB1*, *DCC*, *FAM123B*), proto-oncogenes (*BRAF*, *ERBB2*, *GNAS*) and genes related to DNA repair mechanisms [39]. These mutations structure the classification of CRC as sporadic (70%), familial (25%) or inherited (5%) [40]. Such a multi-hit target makes CRC, like many other cancers, not prone to a specific molecular therapy that could be effective for all patients.

The intestinal architecture is composed of epithelial folds to increase absorption surface area, that can be subdivided into two distinct areas: the villus at the top of the fold and the crypt at the bottom (Fig.1) [14]. It's in the crypt that self-replenishing processes occur involving the LGR5+ intestinal stem cells (ISCs) that maintain tissue self-renewal by undergoing 4 to 5 replication cycles and turning into progenitor stem cells, *i.e.* the transit amplifying (TA) cells [41]. These cells migrate upward through the villus and differentiate into: nutrient absorbing enterocytes, mucus producing goblet cells, and enteroendocrine cells that secrete various hormones and exert local regulatory effects [14]. On the contrary, differentiated paneth cells migrate downward and are responsible for stem cell niche maintenance secreting WNT3 and EGF signals [28]. Moreover, paneth cells play an important role in the intestinal homeostasis: they are the most highly secretory of the four lineages of epithelial cells that form the intestinal mucosa. Their main function is to produce, package and export a variety of antimicrobial proteins and peptides including α-defensins, angiogenin-4, lysozyme and secretory phospholipase A2 (regulation of microbiota) [42]. The continuous proliferation of cells in the crypt is compensated by cells shedding off at the tip of the villus through apoptotic

programmes. The regulatory signalling pathways responsible for maintaining tissue homeostasis are WNT, Notch, BMP, and Hedgehog [43].

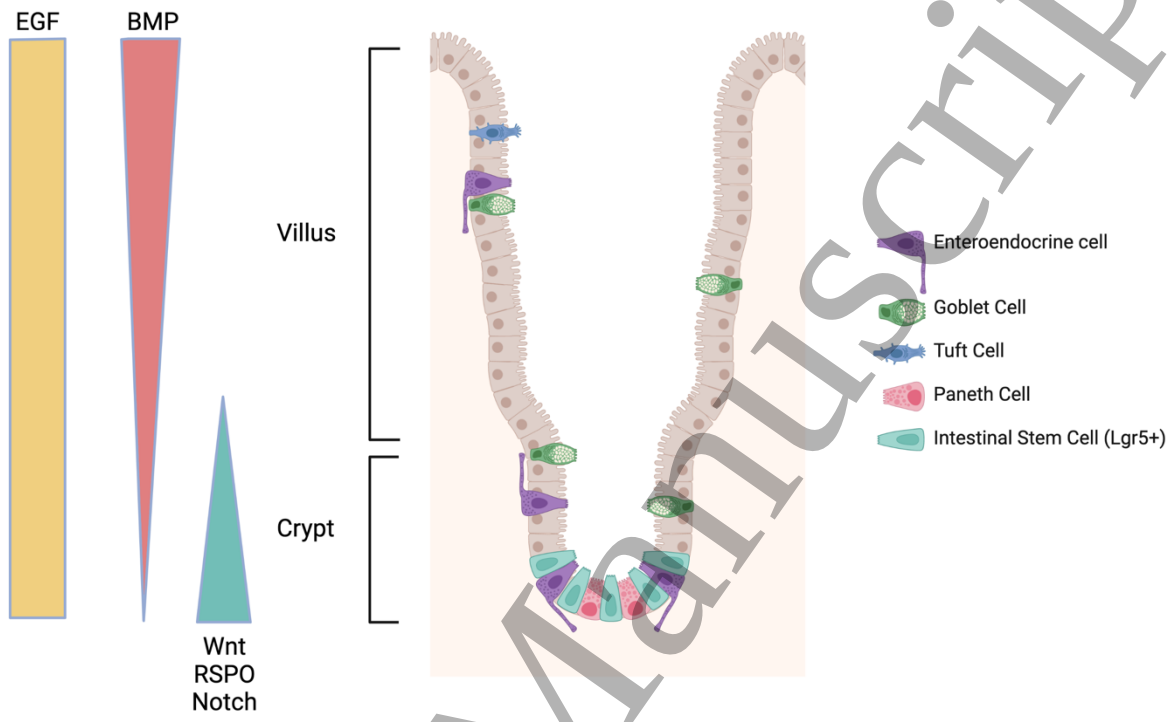

Figure 3. Intestinal epithelial structure with villus and crypt, composed of enteroendocrine cells, goblet cells, tuft cells, paneth cells and intestinal LGR5+ stem cells. EGF, BMP, Wnt, RSPO and Notch signalling pathways activity. Created with BioRender.com

The highly regenerative potential and the permanent contact with nutrients and microbiota makes the intestinal epithelium very prone to the onset of CRC. The development of CRC starts with a benign adenoma that transforms into a high-grade adenoma and eventually evolves into an invasive tumour with loss of epithelial intestinal structure [43]. In late stage carcinoma, intestinal epithelium may undergo the epithelial-mesenchymal-transition (EMT) which promotes invasiveness, migration and metastasis [44]. Significant advancements have been made in the knowledge of the mechanism of action of CRC but less has been done in the translation into the clinics. This is much due to a lack of *in vitro* models that resemble tissue architecture.

In respect to animal models, there are some convincing limitations to their use. Studies are being performed on whole, living and conscientious organisms, which can prove to be unethical if harm or distress is caused. It is a lot harder to control every variable in a living animal, so the results may not be reliable nor applicable to a wider population. Moreover, they are very expensive and time consuming [45][46]. Another limitation that frequently arises in heterotopic models of CRC is the absence of metastasis from the subcutaneous location made the cancer model inappropriate for study of the spontaneous metastatic process. Making these models using commercially available cell lines is also a pitfall because they are altered *per se*

[46]. However, with the advent of intestinal organoids developed by Sato *et al.* [47] (see BOX 2) and microfluidic organs-on-a-chip, new light has been shed.

The most common mutation in CRC is the *APC* mutation in ISCs. APC is responsible for regulating the WNT signalling and promoting the phosphorylation and degradation of β-catenin. *APC*-mutants cause a constitutive activation of the WNT pathway, leading to uncontrolled proliferation of intestinal stem cells (ISCs) and furthermore, *APC*-mutants inhibit the proliferation of surrounding wild-type ISCs crypt cells, in essence outcompeting them. Flanagan *et al.* [48] have devised a strategy to overcome this competitiveness at this very early stage of tumour initiation by looking at the transcriptomic analysis of *APC*-mutants and identifying genes differentially upregulated. The results identified NOTUM, a key mediator in *APC* mutation fixation and that could be targeted to restore wildtype competitiveness. NOTUM inhibitors are currently under development. NOTUM was identified as an extracellular carboxylesterase that removes the palmitoleate moiety from Wnt proteins, thereby rendering them inactive. In another approach, also exploiting the competitiveness of *APC* mutants over wild type ISCs, used intestinal organoids combined with analyses of *in vivo* clonal dynamics to find ways to restore the fitness of these wildtype ISCs and thereby limit the expansion of the pre-malignant clones [49]. That approach envisions the discovery of pharmaceutical targets capable of hampering CRC development in high-risk patients. The results showed that the use of lithium chloride that inhibits WNT antagonists secreted by *APC* mutants prevented the formation of adenomas by allowing the maintenance of the wildtype ISCs niche. The use of intestinal organoids served as a suitable disease model.

An increasingly important aspect of cancer research is the tumour microenvironment (TME), which in the case of CRC is characterized by a disorganized interstitial extracellular matrix including the presence of immune cells, mesenchymal stromal cells, cancer associated fibroblasts (CAFs) and tumour-associated endothelial cells (TECs). The TME acts as a natural barrier to the progression and invasiveness of cancer but as the genetic malignancy of the tumour evolves, the TME is remodelled in several ways [50]. One of the main changes related with the extracellular matrix (ECM) of the TME is the occurrence of fibrosis in the tumour tissues, which is explained by the secretion of collagen fibres by CAFs [51]. This causes an increase in tissue stiffness, which promotes tumourigenesis and treatment resistance [52]. Furthermore, the remodelling of the ECM is also induced by matrix metalloproteinases and promotes cancer cell migration and invasiveness. Li *et al.* [50] have verified the changes in expression of collagen type I and type IV and MMP-2 and -9 in different stages of CRC and reached the conclusion that there is an increase in the expression of these molecules as the tumour stage advances.

In another study, the tumour microenvironment was replicated in 3D tumour-stroma co-cultures *in vitro* and *in vivo*, thereby simulating the physiological properties that are found in native CRC tissue [53]. Important to retain is the knowledge that ECM architecture and tissue mechanical properties can modulate the behaviour of surrounding cells, including cancer cells, via mechanomodulation processes [54]. These types of experiments help bridge the gap between 2D *in vitro* models and animal models, and provide better platforms for drug development efforts. In fact engineering the ECM to recapitulate the tumour microenvironment is a growing field of research, where natural- or- synthetic- based ECMs [55] can be used and modified with protein treatments and peptide motifs in order to increase cell adhesion and motility, and thereby modulate tumourigenesis [56]. Teasing apart the roles of these players in the TME can lead to the discovery of new drug targets and therapeutic approaches.

Another state-of-the-art approach to model the TME of CRC is through 3D bioprinting [57]. Co-culture of CRC cells with TME-associated cells such as cancer-associated fibroblasts (CAFs) and tumour-associated endothelial cells (TECs) was attained by developing a conditioned culture methodology where these stroma cells (CAFs and TECs) became activated and pro-tumourigenic [58]. Then, the three cellular components (HCT-116, CAFs and TECs) were bioprinted together with a scaffold made of collagen-PCL (polycaprolactone). The expression of genes related with stroma activation, epithelial-to-mesenchymal transition and others were evaluated by quantitative RT-PCR. The results obtained showed overexpression of tumour-related genes and remodelling of the ECM, and metabolic profiles and malignant transformation were similar to *in vivo* counterparts. That 3D bioprinted *in vitro* model presents itself as suitable for further tumour progression studies.

A major attribute of the TME of CRC is the disorganized vascular elements crucial for the access to nutrients and oxygen as well as functioning as the gatekeepers for tumour metastasis [59]. These vascular elements include endothelial cells, smooth muscle cells, pericytes and progenitor endothelial cells. The so-called tumour-associated endothelial cells (TECs) are responsible for the secretion of the vascular endothelial growth factor (VEGF), and their family of receptors are the targets of angiogenesis anti-cancer therapy [60]. VEGF is responsible for recruiting new blood vessels and incorporating endothelial cells into the newly formed vascular tissue. VEGF overexpression is associated with a poor prognosis outcome in CRC patients [59].

3D *in vitro* tumour models of perfused vascularized networks are thus advantageous platforms to study the implications of a vascular bed in tumour progression, and this can have a tremendous effect in angiogenesis research by complementing the use of animal models. A research study by Sobrino *et al.* [61] has developed a tumour-on-a-chip loaded with HCT-116 cells and human endothelial colony forming cell-derived ECs (ECFC-ECs). The microfluidic device is composed of two microchannels separated by 3 diamond-shaped tissue chambers. In these chambers EC cells migrate outward and anamastose - forming a fully developed vascular bed. Anti-angiogenic drugs were tested and it was observed the efficacy of combined drugs that target VEGFr, PDGFR and TIE2 that do regress the vasculature. The metabolic profile of the vascular network was also studied and it was observed a higher glycolytic rate in the tumour, moderate glycolytic rate in the EC vascular bed and low glycolytic rate in stroma cells. These results are consistent with the deregulated cellular energetics expected to be found according to the hallmarks of cancer [62]. This vascular platform is not pre-patterned, is simple, it requires no external pumps and tubing, and presents itself suitable to model physiologic cues and pharmacology therapy.

Finally, immunotherapies had a major breakthrough in various haematological malignancies with the use of chimeric antigen receptor (CAR)-engineered lymphocytes, against leukaemia for example [63]. However, with solid tumours, such as CRC, efficient 3D *in vitro* models are needed to test CAR-T cytotoxicity. Schnalzger *et al.* [64] have developed a 3D model for CAR-mediated cytotoxicity using patient-derived CRC organoids. This study uses the technology developed by Sato *et al.* [28] in the field of organoids as well as established biobanks of normal and healthy tissue of CRC patients (see BOX 2). The addition of stromal cells allowed the reconstruction of the desired TME to study immune-tumour crosstalk between CAR-T cells and CRC organoids. The study of T-cell exhaustion showed that only a fraction of CRC tumours was affected, nevertheless displaying the potential of using organoid systems for the development of cancer immunotherapies.

Furthermore, interestingly, the metabolic profile found in the TME with high glycolytic rates and low OXPHOS (oxidative phosphorylation) is understood to impair the activity of anti-tumour immune cells such as the engineered CAR-T cells or normal T-cells, a process known as T-cell exhaustion [65]. Hence, tumour dysfunctional metabolism interferes with CAR-T cells therapy [66]. A metabolic rewiring of these cells needs to be done to handle better the metabolic dysfunction of tumours. Thus, a new era of further unravelling the complex interactions between immune, stromal and tumour cells is in the horizon. For that 3D *in vitro* models capable of replicating TME with immune infiltrate, appropriate nutrient supply and shear stress are essential to attain those goals. Some of these topics will be mentioned in the review.

3. Colorectal cancer (CRC) 3D models

2D cell culture models have proven to be unsuccessful when it comes to model the extracellular environment and other physiological relevant aspects such as stroma compartments, drug delivery conditions and vasculature. As a result, 3D technology has become increasingly used in many research applications, including disease modelling, drug screening and regenerative medicine [67]. It is important to take into account the importance of pre-clinical models since the drug selection that takes place in this stage has a financial impact in late-stage clinical trials, where about only 1 in 15 drugs entering phase I clinical trials is finally approved by the US Food and Drug Administration (FDA) [68]. The advantages and disadvantages of spheroids, organoids and microfluidics 3D *in vitro* models are illustrated in Fig. 4.

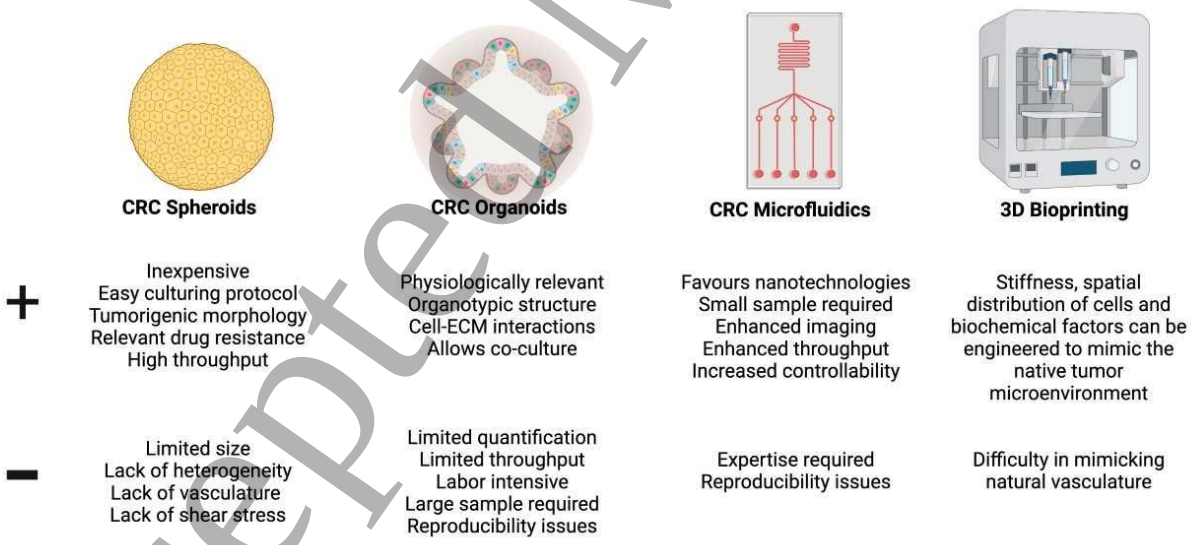

Figure 4. Main advantages (+) and disadvantages (-) of spheroids, organoids, microfluidics and 3D bioprinting as CRC 3D models. Created with BioRender.com

3.1. Spheroids, microfluidics and 3D printed devices

Spheroids are multicellular self-assembly structures that can be generated from cell suspensions and be typically used either in a scaffold-free or scaffold-based mode. Scaffold-free spheroids are user-friendly, relatively inexpensive, and rapid. Some of the disadvantages of spheroids models lay on the fact that they lack nutrient supply, shear stress, in and outlets, and mechanical cues, but when combined with microfluidics these challenges can be overcome. Presented below are studies using colon tumour spheroids (TS) combined with microfluidic devices or 3D printed platforms that enhance physiological properties observed in *in vivo* counterparts.

3.1.1. Tumour development studies

"Spheroids recapitulate the tumour physiology and architecture by recreating necrotic cores. This ability has been exploited by several research groups to better understand cancer processes such as cell growth, proliferation and migration, vasculature recruitment and stroma interactions as well as drug delivery studies." Zhao *et al.* [69] have optimized a 3D printed hanging drop dripper that can be directly mounted on a 96/384-well plate to perform several TS-based assays: drug resistance analysis, tumour spreading and inhibition analysis, 3D tumour metastasis analysis and a 3D tumour transendothelial migration study (Fig. 4A). One of the highlights of this approach is that it avoids the use of expensive time-consuming microfluidics, based on photolithography fabrication. It works by cultivating TS on a 3D printed hanging drop that allows easy retrieval of the cellular lump for downstream analysis. Results suggest that malignant breast cancer (MDA-MB-231) cell aggregates presents a more metastatic morphological phenotype than the non-malignant breast cancer (MCF-7) and colonial cancer (HCT-116) cell spheroid. It was also seen an up regulation of epithelial-mesenchymal transition (EMT) relevant genes. This malignancy feature was confirmed with the tumour transendothelial assay.

In another example of the relevance of the TME for preclinical studies, Jeong *et al.* [70] have co-cultured HT29 TS with fibroblasts and observed an increase in the diameter of spheroids in the presence of the co-culture when compared to the monoculture. This microfluidic chip contained four units, with each unit composed of seven channels for cell loading and culture medium fill. The middle channel was loaded with TS, and it was sided by culture medium channels and fibroblasts channels. The ratio of cancer cells to fibroblasts was of 1:1.2 as it was found in the literature to be the ratio comprising *in vivo* relevancy [71]. The ECM scaffold was composed of rat tail collagen type I, and fibroblast activation into cancer-associated fibroblasts (CAFs) was demonstrated by higher expression of α-SMA. The co-culture showed increased resistance to paclitaxel, a common standard of care CRC therapy, when compared with the mono-culture. This efficient co-culture microfluidic device showed physiological relevance and it could be used for further drug development efforts and insights into the mechanisms of the CRC TME [56] [72].

In a more recent study, Sargenti *et al.* [73] have explored the value of CRC spheroids as aggregations of epithelium cells undergoing metabolic, physical, and chemical modifications, and as a result emulating an *in vivo* tumour. The present work uses an improved software technique for measurement of weight, size and mass density of sphere-like samples to study variations to the system caused by the action of natural killer cells (NK). NK cells were used based on their prevalence in the native CRC patient tissue and broad capacity of recognizing cancer targets without the need for antigen presentation, unlike other lymphocytes

such as T-cells. The technology developed was based on a fluidic-core chip equipped with a bright-field imaging setup connected to a customized software capable of assigning a circular reference to each image and thereby extrapolating the radius of the analysed spheroids. The system was further equipped with a peristaltic pump, a temperature sensor and a flow-circuit with inlet and outlets. The results showed the efficacy of NK cells in targeting TS, demonstrated by measuring weight loss and diameter shrinkage. The infiltration rate of NK cells in TS was correlated with exposure time and type of CRC cell line. This study contributes to the standardization of TS-based assays and immunotherapy research. Overall, 3D TS research provides reference models for deepening insights into tumour development.

3.1.2. Drug response and drug screening analysis

A drug screening and drug response study using TS research was developed by Petreus *et al.* [74]. A microfluidic platform was constructed to evaluate drug pharmacokinetics and treatment response. It is argued that current microfluidic devices still lack the ability to serve as cell models that reproduce the real physiological drug exposure. In this study, a microfluidic platform was constructed to sequentially deliver up to eight concentration points on the PK profile for a specific drug or combination of drugs. In this case, a single channel microfluidic Ibidi chip was used and adapted to handle eight CRC cell line spheroids (SW620) encapsulated in Matrigel® droplets, that were treated with a topoisomerase-I (TOP1) inhibitor, irinotecan and its combination with an oral inhibitor of ATM kinase (AZD0156) (Fig. 5B). As a measure of cancer cell response, spheroid volume and viability were determined as well as changes in mechanistically relevant pharmacodynamic biomarkers (YH2AX, cleaved-caspase 3 and KI67). Furthermore, it was carried out a parallel experiment in a 2D plate format to explore the differences between a 2D and 3D microfluidic environment. The drug response predictability assessment of the platform was carried out by comparing its results with *in vivo* results found in the literature. This is a useful example of the combination of 3D spheroid microfluidics with the study of drug delivery conditions in CRC cell lines. This microfluidic chip overcomes the limitations of some commercial chips such as low throughput, high cost and lack of ability to reproduce physiological exposure of drugs. It became demonstrated that this platform can predict the efficacy of *in vivo* drug responses and guide drug dose and scheduling, thereby decreasing the need to use animal testing. This is a good example of what is happening in science overall, an urge to complement (replace in extreme cases), the animal studies for MPS, which can give us results such as the ones demonstrated by Petreus *et al.* [74].

Furthermore, Lim *et al.* [75] have developed a microfluidic spheroid culture device with a concentration gradient generator (Fig. 5C), composed of concave microwells with several serpentine micro-channels. HCT-116 cells were injected through the cell injection holes with approximately 110 cells per microwell being captured. Upon formation of spheroids, irinotecan was injected into the microfluidic platform and the responses of the spheroids were evaluated.

The results obtained showed that the number of spheroids, roundness, and cell viability, were affected by increasing concentrations of drug gradient, thereby making this platform a putative platform for screening the efficacy of cancer drugs [75].

In another approach, Thakuri *et al.* [76] have developed a high-throughput screening of 25 anti-cancer drugs targeted to colon cancer spheroids. In order to overcome the limitations of spheroids such as lack of homogenized spheroid size, the authors have devised a robotic-based technology using a 3D two phase aqueous system, with immiscible solutions of

polyethylene glycol (PEG) and dextran (DEX). The biomechanics and tensile strengths of the solutions allowed for the spontaneous formation of a viable spheroid, upon loading of HT-29 colon cancer cells into the system. Drug tests with 25 compounds such as paclitaxel, doxorubicin and oxaliplatin were carried out in a high-throughput fashion to quantitatively determine the effectiveness in decreasing TS viability. Furthermore, a combination of kinase inhibitors with drug compounds was also evaluated as putative therapy. This platform aims to provide a more user-friendly robotic-based approach to drug screening using TS, and ultimately attempts to curtail the high attrition rates of anti-cancer drug compounds in clinical studies.

For cell therapy, bioprinting has been used to improve T cell expansion and function using alginate and alginate–gelatin scaffolds to mimic lymph vessels, resulting in the differentiation of CD4+ cells into the central memory type and differentiation of CD8+ cells into effector memory type [77].

3.1.3. Drug delivery analysis

An interesting approach to improve drug delivery into solid tumours, that is still a major obstacle to overcome, uses microbubble mediated sonoporation with an ultrasound trigger to enhance the uptake of drugs into cancer cells [78]. For the study of this approach, a microfluidic platform was constructed that combined the use of CRC spheroids with the possibility of a dynamic flow and shear stress. Spheroid traps were built into a polydimethylsiloxane (PDMS) microfluidic device fabricated using standard photolithography and soft lithography techniques and were loaded with pre-grown CRC spheroids (Fig. 5D). Drug delivery studies were performed with doxorubicin, a common standard-of-care CRC therapy, and it was observed a reduced cancer cell viability with doxorubicin combined with microbubble mediated sonoporation than with doxorubicin alone, which highlights this technique as a potential tool to increase drug delivery efficacy.

Thao *et al.* [79] have used a HCT-116 TS model to test a delivery system for doxorubicin, and apoptotic inducer protein termed TRAIL (tumour necrosis factor-related apoptosis-inducing ligand). This system consisted of doxorubicin-bound albumin attached to TRAIL. The synergic combination of chemotherapeutic agent with apoptosis had been previously reported by the authors. In this case, albumin-bound Nanoparticle technology (nab™ [80]) was used, which exploits the leaky tumour blood vessels and affinity of tumour cells for albumin, for nutrient uptake. Hence, microspheres were loaded with doxorubicin and TRAIL, which were then bound to albumin, and its delivery efficacy and cytotoxicity was demonstrated by measurement of fluorescent-modified TRAIL protein and by MTT based-assay, FITC Annexin V, and TUNNEL assay in *in vitro* HCT-116 TS models. Furthermore, a high anti-tumour efficacy was observed in HCT-116 colon cancer-bearing mice. This approach demonstrates the added value of combining chemotherapeutics with anti-apoptotic adjuvants in loaded cancer-targeted nanoparticles, however ethical issues are raised with the use of animal models.

Sbirkov *et al*. used a CELLINK RGD bioink to print Caco-2 cells or primary CRC. After validation, the team tested three of the most commonly used chemotherapeutics in CRC—5-fluoruracil (5-FU), oxaliplatin, and irinotecan as single agents on both monolayers of Caco-2 cells and on 3D bioprints. Only oxaliplatin remained effective at similar concentrations in 2D and 3D cells, while the other two chemotherapeutics failed to reproduce similar IC50 values to the ones previously determined on 2D [81].

In another study using a multicellular TS model with increasing complexity, Bauleth-Ramos *et al.* [82] have attempted to meet the critical need for 3D models to be capable of

augmenting the clinical translation of nanoparticles (NPs) to CRC treatment. One of the issues with 2D models is the absence of a physical barrier such as the one provided by ECM. Hence, their 3D model composed of HCT-116 cells, human intestinal fibroblasts and monocytes developed a physiological tumour-like organization with a necrotic core, where the cells secreted ECM, and monocytes differentiated into M2 pro-tumour phenotypes. NPs were made of biomaterial spermine-modified acetylated dextran and loaded with chemotherapeutic Nutlin-3a (Nut3a) and immunotherapy adjuvant granulocyte-macrophage colony-stimulating factor (GM-CSF). The results showed that drug penetration was higher in control 2D models when compared to the proposed 3D model due to the presence of ECM, as predicted. It was also observed the polarization of macrophages into M1 anti-tumour phenotypes, and an anti-proliferative effect was observed in both 2D and 3D models. The authors suggest that these NPs could be a putative therapeutic approach to CRC treatment having proved to be successful in this 3D *in vitro* model.

Furthermore, Baye *et al.* [83] have carried out a study on using TS as 3D tumour models by evaluating the impact of necrosis of TS on nanoparticle penetration using a microfluidic device. The authors report how to manipulate the properties of TS, such as size and the presence of core necrosis, by changing the glucose concentration of the culture medium. Secondly, a PDMS microfluidic device was devised equipped with a microparticle imaging velocimetry. Rhodamine-B conjugated polystyrene beads were delivered through a flow channel in the direction of the immobilized spheroids at a flow rate controlled by a syringe pump. Fluorescence of nanoparticles was analysed, and the results showed a heterogenous accumulation of NPs at the tumour perimeter correlating with different fluid flow velocities. This study highlights the importance of understanding the way TS size and necrotic core affect nanoparticle distribution, since they have an implication in tumour drug resistance. Drug delivery analysis remains an important field of study to improve anti-cancer drug therapy. The main 3D spheroid models described thus far, its main research applications, advantages and disadvantages are summarized in Table 1.

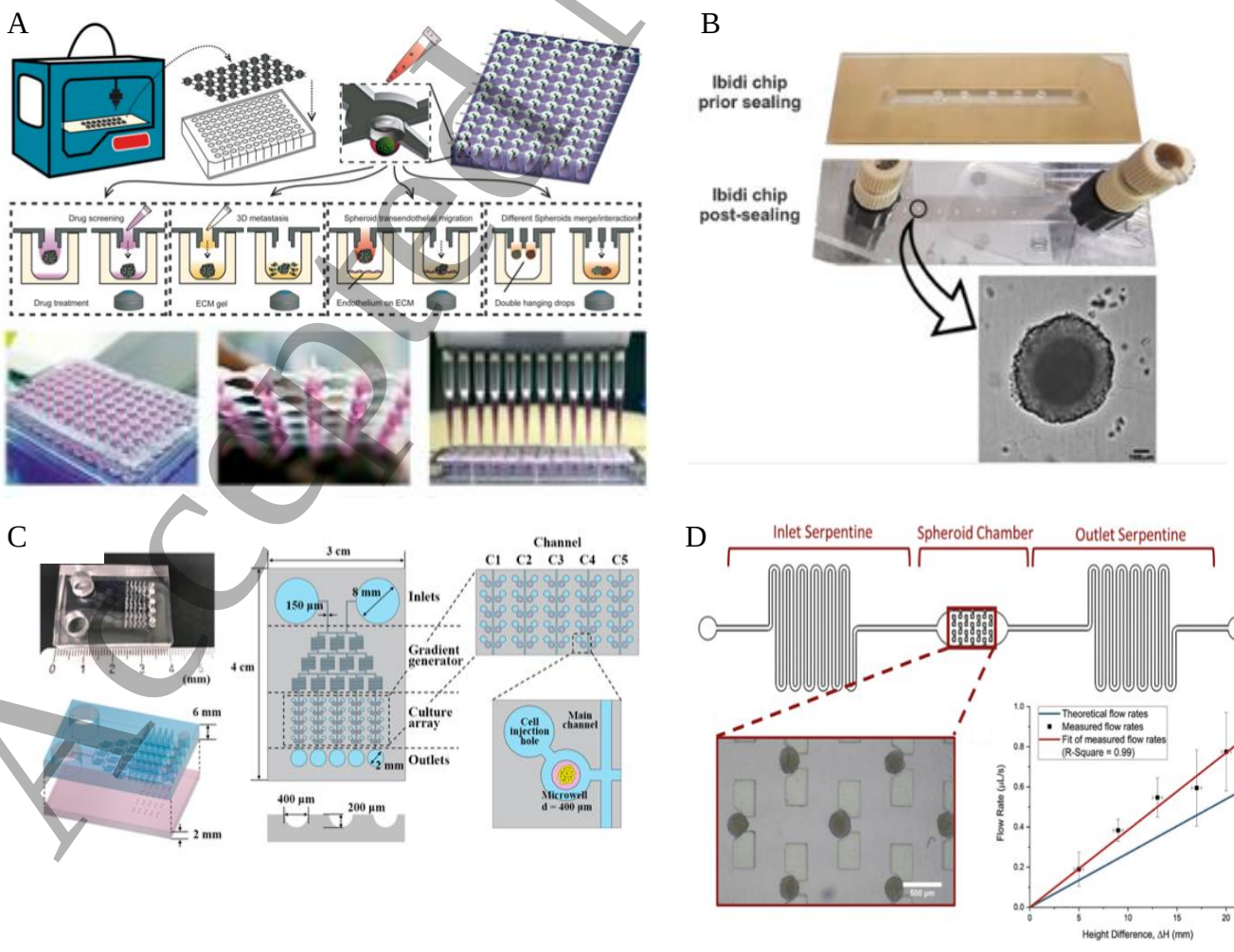

Figure 5. 3D microfluidic devices using CRC spheroids. (A) 3D printed hanging drop dripper that can be directly mounted on a 96/384-well plate to perform several TS-based assays. Reprinted with permission from [69] *Copyright © 2019 Springer Nature*. (B) Single channel microfluidic Ibidi chip used and adapted to handle eight CRC cell line spheroids (SW620) encapsulated in Matrigel® droplets. Reprinted with permission from [74] *Copyright © 2021 Springer Nature*. (C) Microfluidic spheroid culture device with a concentration gradient generator. Reprinted with permission from [75] © *2018 MDPI*. (D) Microfluidic platform with microbubble mediated sonoporation with an ultrasound trigger to enhance the uptake of drugs into cancer spheroid cells. Reprinted with permission from [78] *Copyright © 2020 Elsevier*.

Table 1 - Main research applications for 3D *in vitro* microfluidic colorectal cancer spheroids models.

Model	Applications	Model Cells	Characteristics	Advantages	Disadvantages	Reference
3D *in vitro* spheroid	Drug resistance analysis, tumour migration, metastasis	HCT-116/MDA-MB-231/MCF-7	3D printed hanging drop dripper	High throughput, Downstream analysis, Inexpensive	Limited size, Lack of ECM component, Lack of vasculature	[69]
3D *in vitro* spheroid co-culture	Drug resistance analysis, drug development	HT29 and Fibroblasts	Microfluidic chip with four units, seven channels each	ECM component (collagen), Physiological relevance	Limited size, Lack of shear stress, Lack of vasculature	[70]
3D *in vitro* spheroid / immune cells	Standardize TS assays, analyse immune response	HT29/HCT-15/SW620/DLD-1 and Neutrophils	Fluidic core chip linked to sensing software	High throughput, Reproducibility, Standardization	Limited size, Lack of Shear Stress, Lack of vasculature, Requires specialized software, Lack of ECM component	[73]
3D *in vitro* spheroid	Drug response and screening	SW620	Single channel microfluidic chip	ECM component (Matrigel®), High throughput, Inexpensive, Physiological drug exposure	Limited size, Lack of shear stress, Lack of vasculature	[74]
3D *in vitro* spheroid	Drug response and screening	HCT-116	Microfluidic chip with concentration gradient generator	Spheroid characterization, High throughput, Concentration gradient	Limited size, Lack of ECM component, Lack of shear stress, Lack of vasculature	[75]
3D *in vitro* spheroid / two phase aqueous system	Drug response and screening	HT-29	System of immiscible solutions of PEG and dextran (DEX) for spheroid trapping	High throughput, User-friendly, Preliminary screenings	Requires knowledge of robotics, Lack of ECM component, Lack of shear stress, Lack of vasculature	[76]
3D *in vitro* spheroid	Drug delivery analysis	HCT-116	Microfluidic device using microbubble mediated sonoporation	Dynamic flow, shear stress, Quantitative analysis, Physiological Relevance	Limited size, Lack of vasculature	[78]

3D *in vitro* spheroid	Drug delivery analysis	HCT-116	Delivery system for doxorubicin and apoptotic inducers loaded on nanoparticles	Synergistical combination of drugs	Limited size, Lack of ECM component, Lack of vasculature, Lack of shear stress	[79]
3D *in vitro* spheroid co-culture / immune cells	Drug delivery analysis	HCT-116, Fibroblasts and Monocytes	Delivery system for Nutlin-3a loaded nanoparticles	ECM component (fibronectin), Immune system component	Limited Size, Lack of vasculature, Lack of shear stress	[82]
3D *in vitro* spheroid	Drug delivery analysis	HCT-116, A549, U87MG	Microfluidic device equipped with microparticle imaging velocimetry	Fluid flow, Shear stress	Limited Size, Lack of ECM component, Lack of vasculature	[83]

3.2. Organoid models

Organoids are cellular organotypic structures, derived from AdSCs (as well as iPSCs or ESCs) that with the right cocktail of growth factors differentiate stochastically into microstructures resembling *in vivo* organization (see BOX2) (Fig. 6). Tumour organoids can be derived directly from CRC patients or generated with an intermediate step to introduce tumourigenic mutations with CRISP-Cas9 genome editing in iPSCs [35]. Furthermore, access to adjacent healthy tissues, in the case of tumour organoids derived from patients, allows the possibility of genotypical and phenotypical comparisons. This is a type of control sample that is not possible with tumour cells lines and it is argued that these commonly used cell lines can drift genetically and phenotypically with an increasing number of passages [33]. However, a multi-omics study carried out by Berg *et al.* [84] on 34 CRC cell lines revealed that cell lines are an accurate molecular model of primary carcinomas.

In general, organoids have revolutionized the field of cancer research by allowing a more physiologically relevant microenvironment, however some limitations remain to be resolved related with reproducibility issues that can be categorized into three main categories: non-standardized cancer-tissue sources, ill-defined medium formulations and heterogenous animal-derived 3D matrices [85]. This section exploits CRC organoid applications in drug screening and tumour heterogeneity analysis at the molecular level.

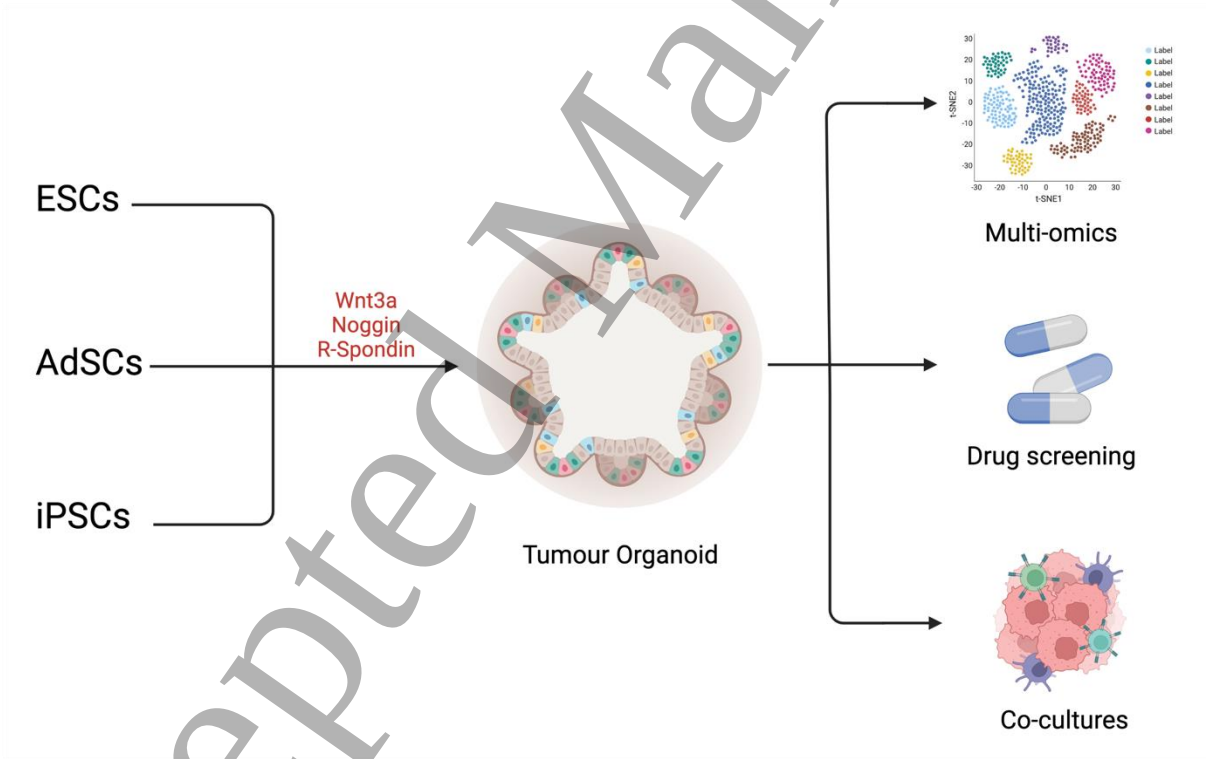

Figure 6 - Schematic representation of organoids derived from either ESCs, AdSCs or iPSCs with common research applications such as multi-omics, drug screenings and co-cultures. Created with BioRender.com

3.2.1. Drug screening

The organotypic features of organoids make them ideal platforms for drug testing. Crespo *et al.* [86] have developed a robust protocol for developing colonic organoids (COs). A control healthy organoid and a mutated organoid were generated for comparative studies. The first one was a CO derived from human ESCs and contained stem cells, transit amplifying cells, goblet cells and endocrine cells. The mutated version was derived from familial adenomatous polyposis (FAP) patients with APC mutations, thereby having enhanced WNT signalling activity and higher epithelial proliferation. The cancer cells were reprogrammed into iPSCs (FAP-iPSCs), and with the right cocktail of growth factors grown into COs (FAP-COs). RNA-seq analysis showed a strong upregulation of the WNT pathway in the FAP-COs as compared to wildtype COs. Furthermore, qRT-PCR and Ingenuity Pathway Analysis consistently showed upregulation of WNT pathway genes in the FAP-COs, indicating a genetic profile consistent with the expected molecular profile of CRC cells. COs were cultured in Matrigel®, that is an ill-defined animal-derived matrix and therefore could potentiate reproducibility issues. Two drug candidates were tested: XAV939 and rapamycin that caused lower proliferation in FAP-COs. However, wildtype COs also showed lower proliferation with these compounds, which indicates lack of specificity for the compounds, limiting their therapeutic use. However, another drug compound studied, geneticin, decreased WNT over-activation and stopped hyper-proliferation of FAP-COs without compromising wildtype COs. In essence, this study exemplified the use of COs as a drug screening platform with potential clinical applications [86].

Cho *et al.* [87] have also used patient-derived organoids (PDOs) to study WNT signalling and the relapse of cancer occurrence in patients treated with 5-fluorouracil (5-FU), a first line treatment for CRC patients. Although 5-FU treatment is commonly used in CRC patients, a poor prognosis is still associated with this drug regimen. The authors showed that treatment of tumour organoids with 5-FU caused the activation of the WNT signalling, inducing β-catenin activation and subsequent activation of cancer stem cells (CSCs). The enrichment of Lgr5+ cells – CSCs – seems to be responsible for the relapse of cancer, following 5-FU treatment. Combinatorial treatment of 5-FU with inhibitor LGK-974 caused an inhibition of cancer stem cell markers, and as a result reduction of tumour growth. Furthermore, it was shown the involvement of p53 in the transcription of WNT ligands (WNT3), responsible for promoting WNT signalling. Resistance to 5-FU is a common issue in CRC patients, and PDOs have allowed a better understanding of the molecular events mediating this resistance.

In another study [88], the MAPK signalling pathway dynamics and EGFR inhibitors were studied using CRC PDOs. PDOs were also cultured in Matrigel® and were selected for wild type or mutant MAPK signalling. Using an improved version of a FRET-based ERK biosensor termed EKAREN5, the results revealed extensive ERK activity oscillation in wild-type and mutant PDOs. These dynamics were studied, and autonomous autocrine/paracrine production of EGF-ligands was taken into consideration. In essence, it was found that pan-HER inhibition - that affects downstream MAPK signalling – combined with pan-RAF inhibition caused growth arrest, as opposed to single treatments. Inhibition studies of the complex dynamics of MAPK effectors in *KRAS* and *BRAF* mutants showed effective combinatorial treatments and provided mechanistic insights into these molecular pathways. PDOs proved to be potential efficient 3D preclinical models.

As an example of the synergistically combination of organoids with microfluidic devices, Pinho *et al.* [89] have developed a CRC organoid-on-a-chip termed Organoid Chip. The chip was fabricated using a high-speed milling machine, where microfluidic channels are constructed using rotating cutting tools. A CRC organoid line Iso-50 was obtained

commercially and cultured according to the manufacturer's instructions. The organoids were embedded in Matrigel® and transferred to the microfluidic chip. Viability assays were performed, and it was observed an increase in viability and proliferative activity on-chip. Further studies showed no significant differences in response to 5-FU treatment on-chip and on-plate, which indicates stability and accuracy of the model. Furthermore, the organoid culture within the chip showed an increased morphological-forming efficiency than the culture on a regular 24-well plate. Thus, the microenvironment provided by the Organoid Chip allows a better capacity for generation of organotypic structures and as a result conductance of drug development studies, disease modelling and personalised medicine studies, if PDOs are to be used.

3.2.2. Tumour heterogeneity analysis

Tumour organoids provide a useful platform to analyse and model tumour heterogeneity, since there is a realistic recapitulation of *in vivo* molecular profiles. Tumour heterogeneity can be considered as intra-tumoural or inter-tumoural, and is responsible for drug resistance in cancer patients [90]. For intra-tumoural heterogeneity, single cell resolution analysis provides a more accurate access to the intrinsic molecular profiles of tumour cells and as a result the development of more efficient drug treatments. For instance, Roerink *et al.* [91] have analysed single-cell genetic, epigenetic and transcriptomic profiles derived from patient-cancer cells. PDOs were generated and cultured in Matrigel®. Normal healthy colonic cells were also used to generate organoids for data comparison. The results showed that clonal expansion of organoids accumulated an increasing level of somatic mutations, contributing to the formation of cellular subpopulations within the same tumour. The mutation load was higher than in normal PDOs clones. Genetic mutations such as deletions, inversions, tandem duplications and translocations were observed, and mutational signatures were assigned to each clone in light of its phylogenetic tree. Single cell RNA-seq was performed to evaluate the transcriptomic profiles and to compare it with methylation profiles. Different clusters were observed for each PDO, and diversification of methylation was also observed – indicating high clonal dynamics related with elevated mutational rate. Drug treatments with 5-FU, doxorubicin, EGFR inhibitor, amongst other drugs, were carried out, and different drug responses were observed as expected, according to the mutational load of cells. Thus, intra-tumoural heterogeneity is an important aspect to take into consideration when elaborating drug regimens and is a cornerstone of personalized oncological medicine.

Furthermore, by analysing the epigenetic landscape of a library of PDOs, Chiara *et al.* [92] have unveiled a tumour maintenance regulatory mechanism based on the action of transcriptional co-activators YAP/TAZ. First, PDOs were selected according to RNA-seq results in order to obtain a panel representative of the intra-cellular heterogeneity found *in vivo.* PDOs were then tested for their deregulated microstructures recapitulating human CRC. Finally, RNA-seq was performed to compare adjacent normal tissue PDOs with CRC PDOs, for identification of differentially regulated genes, showing enrichment of tumour-related genes in CRC PDOs when comparing with wild type PDOs. With this established, epigenetic profiles were then carried out by performing *de novo* chromatin state discovery. Deconvoluting software was used (ChromHMM) to analyse large data sets, and motif discovery tools suggested a role for transcriptional coactivators YAP/TAZ, as putative regulators of the conserved CRC enhancer. Overall, PDOs provided a robust 3D model for the discovery of an intrinsic gene-regulatory mechanism of CRC and potentially of other malignancies.

In another study using organoids as a 3D model, a mass cytometry workflow was developed to overcome the difficulties of measuring post-translational modifications (PTMs) in CRC organoids [93]. PTMs regulate signalling networks underlying fundamental phenotypes and are frequently dysregulated. Wild type intestinal organoids were derived as described by Sato *et al.* [28] and CRC organoids were derived from murine models carrying oncogenic mutations. Organoids were cultured in Matrigel® and were pre-treated with protease and phosphatase inhibitors to preserve cell state and fixed with appropriate reagents before performing mass cytometry. The authors carried out a sequence of experiments enabling identification of cell-type and cell-state specific signalling measurements in organoids based on present PTMs. Thiol-reactive Organoid Barcoding *in situ* (TOB*is*) was then performed to compare wild type organoids with CRC organoids in the presence of stroma. Moreover, by comparing mono-cultures with co-cultures of CRC organoids with fibroblasts and macrophages, it was found that epithelial oncogenic mutations in CRC organoids mimic signalling networks otherwise induced by fibroblasts and macrophages. A large amount of research has been carried out about oncogenic driver CRC mutations but less is known about how microenvironmental cues from stromal and immune cells affect signalling networks linked to PTMs, which highlights the relevance of the TME for CRC progression.

Another way to better emulate the TME is by using more native-like ECMs such as collagen and hyaluronic acid hydrogels - as opposed to the common use of Matrigel®. For instance, Luo *et al.* [94] have co-cultured CRC PDOs with cancer-associated fibroblasts (CAFs) embedded in a hyaluronan gelatin hydrogel. RNA- and whole exome-sequencing showed that molecular profiles were similar to native CRC tissue which strengthens the logic of using well-defined biomimetic scaffolds. Furthermore, it was shown that intrinsic biological pathways were restored in the co-culture and not in the culture of PDOs alone, as in the previous case. In an interesting parallelism with pancreatic cancer, Ting *et al.* [95] have highlighted the relevance of the stroma architecture in shaping intratumoural heterogeneity of PDACs (pancreatic ductal adenocarcinoma). Co-cultures with CAFs identified relevant single-cell population shifts towards signature events assigned to EMT and PRO (proliferative) phenotypes linked with MAPK and STAT3 signalling. The authors then aimed to identify the CAF-secreted factors responsible for these phenotypic changes and identified TGF-β1 as an important regulator protein.

Tumour organoid libraries serve as a panel of heterogeneity diversification of CRC. Several CRC organoid libraries have been established with the purpose of analyzing this heterogeneity in 3D *in vitro* models. Perhaps the most recognized one is the organoid biobank developed by van de Wetering *et al*. [34] (see BOX 2), where the four CRC consensus molecular subtypes (CMS) are represented. The utility of the library was proven as a high-throughput drug screening platform, providing information on drug sensitivity data and gene-drug association data. As an example of the potential of these libraries: it was confirmed the activity of the anti-cancer drug cetuximab in a subset of KRAS wild-type organoids as seen in clinical examinations.

Nikolaev et al. [96] took advantage of tissue engineering and the intrinsic self-organization properties of stem cells to induce the formation of tube-shaped epithelia with an accessible lumen and a similar spatial arrangement of crypt- and villus-like domains to that found *in vivo*. To achieve such grand challenge, hydrogels such as a mixture of Matrigel® and Collagen type I were used in a perfusable platform to generate a hybrid microchip system. It consists of an elastomeric device with a central chamber for hydrogel loading and subsequent organoid culture, flanked by a pair of (inlet and outlet) reservoirs for cell loading and luminal perfusion [96]. That model exhibits exceptional cell-type diversity (macrophages, endothelial cells myofibroblasts), tissue architecture and function [96].”

Another aspect that has been under investigation is the role of peristalsis in colon. Fang *et al*. [97] presented an organoid-on-a-chip with peristalsis-like movements. Human colon tumour organoids growing in the microwell were cyclically contracted by a pressure channel, mimicking the *in vivo* mechano-stimulus by intestinal muscles. This model exhibits exceptional cell-type diversity (macrophages, endothelial cells myofibroblasts), tissue architecture and function [96]. When treated with ellipticine-loaded polymeric micelles (an anti-cancer drug), the organoids showed reduced uptake under peristalsis and resulted in compromised anti-tumour efficacy [97]. Therefore, conventional culture of the colon organoids that cannot replicate the peristalsis microenvironment, may possibly lead to a gap between organoid models and colon tissues *in vivo*.

Organoids present several technical challenges such as reproducibility issues and lack of standardization across laboratories. However, a recent study exploring the microtopography of hydrogels has showed the possibility of a deterministic patterning of organoids [98]. This would overcome the problems related with the stochastic differentiation and structural conformation of organoids and could lead to a standardization and more efficient high throughput data production across laboratories. In essence, the breakthrough of developing long term cultures of intestinal organoids derived from a single stem cell [47] allowed the possibility of working with a more physiological relevant 3D model when comparing with spheroids or common cell lines. Likewise organoid cultures could bridge the gap between animal models and clinical trials, thereby improving drug treatment and more effectively targeting drug resistance.

3.3. Drug resistance studies

Drug resistance in cancer is intrinsically connected with tumour heterogeneity, whether it is intra-tumoural – within the same tumour cell population – or inter-tumoural – between different patients [90]. This acquired heterogeneity can be caused by genomic instability but also by germ line mutations, somatic profiling differences and environmental factors. One proposed model also points out a Darwinian-like process where tumour cells are selected according to a growth advantage in the restricted environment of early tumour formation [99]. Thus, tumour cells can adapt and evolve in a dynamic process that can be used to resist to cancer therapy, presenting an enormous clinical challenge. With the advent of next-generation sequencing and genomic technology it is now possible to analyse this tumour heterogeneity – through single-cell sequencing and liquid biopsies – and provide better ways to circumvent tumour drug resistance.

One of the first line treatments for colorectal cancer is 5-FU that can be administered after tumour surgery removal and in advanced cancer stages such as metastatic tumours. 5-FU is metabolized by cells and its product metabolites are responsible for a cytotoxic effect that impairs DNA and RNA replication leading to cell death [100]. CRC cells are able to interfere with cellular mechanisms such as apoptosis, autophagy, oxidative stress, mitochondrial activity and EMT in order to circumvent the cytotoxic effects of 5-FU [101]. Other mechanisms include drug efflux, epigenetic alterations and miRNA dysregulation. The tumour microenvironment can also be responsible for acquired drug resistance, through stromal components like CAFs or immune cells, that can secrete growth factors and cytokines with inhibitory effects of 5-FU [101]. Importantly, cancer stem cells (CSCs), with an enhanced self-renewal capacity, are also regarded as a niche of resistance due to an upregulation of growth signalling pathways such as Wnt, Notch, Hedgehog and Hyppo/Yap [102]. These cells can quickly re-establish their number after the bulk tumour mass is removed.

Establishing 3D models, that respect the intra-tumoural architecture, is crucial to analyse and understand drug resistance mechanisms – using organoids or spheroids embedded in hydrogels, with the possibility of being incorporated in microfluidic platforms. This section focuses on microfluidic chips with increasing complexity that aim to understand and unravel the intricacies of CRC drug resistance.

3.3.1. Microfluidic approaches for CRC drug resistance

Velasco *et al.* [103] have developed a lab-on-a-chip platform that allows for a real time monitoring of cytotoxic effects of anti-cancer compounds by electrical cell characterization – using dielectrophoresis (DEP) technique – followed by electrical impedance analysis, which is a label-free approach that can replace expensive and large optical systems. This methodology has the advantage of being cost-effective and with high throughput, and is a suitable alternative for laboratories and clinical scenarios with financial restrictions. As a proof-of-concept experiment, HCT-116 colorectal cancer cells were treated with apoptotic inducer, topoisomerase I inhibitor, 7-ethyl-10-hydrocamptothecin (SN38). The electrical impedance characterization showed that the cells underwent apoptosis and necrosis – the results were confirmed by optical imaging. Moreover, in a different study with increasing complexity, it was developed a gravitational microfluidic platform (GMP) equipped with micro-electro-mechanical sensors (MEMS) integrated in a 3D fiber-inspired smart scaffold (FiSS) [104]. This system allowed measuring tumour growth and metabolism in a non-invasive touch-free manner. Gravitational flow was used instead of external pumps, which allowed high-throughput studies. The platform was loaded with HT-29 and HCT-116 colorectal cancer cell lines that were grown into 3D tumouroids, and after 48h were treated with 5-FU and oxaliplatin. Shear stress was optimized using numerical model to establish physiological flow rate distributions. Increased drug resistance to drug treatment was observed, making this system a more realistic model of the *in vivo* scenario, thereby increasing the standard level of efficacy for drugs to be approved for downstream clinical trials. Wang *et al.* [105] have downloaded the scRNA-seq data of nine CRC patients and by using data mining software have performed cell-cell communication analysis, pathway enrichment analysis, critical regulator identification and immune-cell abundance tests. The results tagged patients with consensus molecular subtypes (CMS), with special attention given to CMS4 that showed highest stromal infiltration and activation of epithelial-to-mesenchymal transition (EMT), making it more resistant to chemotherapy. Although the authors state that the number of patients in the study to infer clinical data, further studies will provide more details about chemotherapeutic resistance, paving the way to precision oncological therapy.

4. MPS for inter-organ signalling: Body-on-a-chip

MPS can be used to mimic the human body by interconnecting different organs-a-chip through microfluidic channels or tubing, as well as enhancing our understanding on cancer progression. For instance, La Valley *et al.* [106] have developed a body-on-a-chip comprising three different compartments: gut - HCT-116 colon cancer spheroids – liver - HepG2/C3A hepatocytes - and bone marrow - HL-60 promyeoloblasts to evaluate drug toxicity (Fig. 7A). The microfluidic device was composed of individual channels with inlet and outlet for fluid flow to each

chamber and it was made of poly(methylmethacrylate) (PMMA) etched by VersaLaser VLS3.60 cutting and CO_2 laser. Chambers were pre-coated with collagen type I and upon cell loading into the chip, cells were re-suspended in collagen gel solution, prior to hydrogel polymerization. The system in operation was subjected to the action of a pumpless rocker platform custom made to a ±15° tilt angle at every 1 min interval. The drug Tegafur, an oral prodrug version of the standard-of-care CRC treatment 5-FU, was administered to the system and the results showed cellular depletion in the gut construct, with increased resistance observed when aggregated cellular spheroids were formed - likely due to hampered drug penetration. It was also observed increased drug cytotoxicity in the presence of the liver construct, due to P450 activity – required to metabolize Tegafur into active metabolites. Furthermore, drug toxicity side effects were observed in the bone marrow construct, which shows the potential of this *in vitro* platform to be used in improved drug development efforts and drug toxicity studies. The platform allows for further physiological-based pharmacokinetic drug studies.

Aleman *et al.* [107] have developed a microfluidic chip synergistically combined with the use of four organoid systems: gut, liver, lung and endothelial barrier, termed multi-site metastasis-on-chip (MOC), in order to evaluate the metastatic preference of cancer cells (Fig. 7B). Basically, five chambers are connected by microfluidic flow channels. Cancer cells originating from CRC organoids disseminate into the circulation and evade a downstream chamber. The MOC was fabricated by photolithography and ECM-based HA/gelatin hydrogels were used as scaffolds for cell seeding of HCT-116 cell lines. The question addressed in this study was to test whether cancer metastases gave more preference to the proximity factor or to the tumour microenvironment factor of a metastatic site. The rationale behind the study is that CRC metastases are commonly found in the liver, but would this be because the liver is where the vascular/lymphatic drainage from the gut occurs or because the liver microenvironment is more prone to assimilate metastasis. Results provided have shown that HCT-116 CRC cells preferentially home to the liver and lung constructs, as expected. In brief, the authors state that this body-on-a-chip could help further understand the behaviour of metastatic cells and help identify novel therapeutic targets.

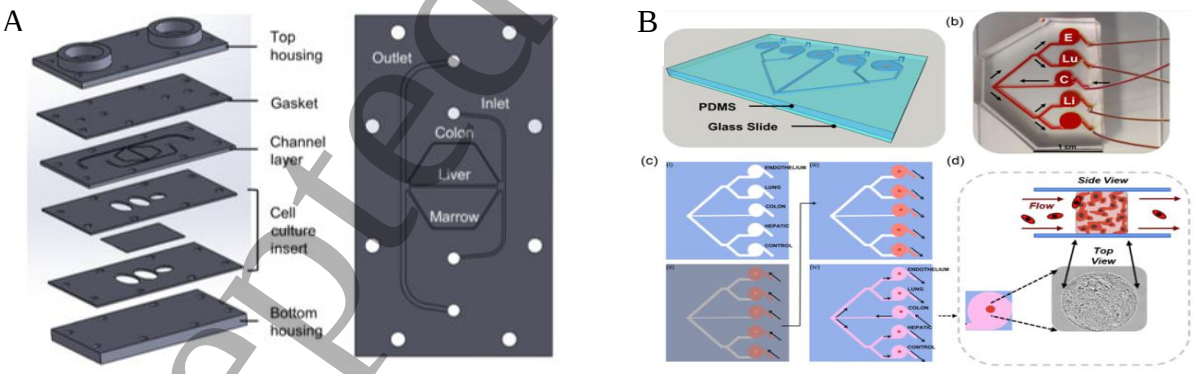

Figure 7. Body-on-a-chip platforms with different applications. (A) Body-on-a-chip with gut, liver and bone marrow constructs for drug toxicity studies. Reprinted with permission from [106] *Copyright © 2020 American Institute of Chemical Engineers*. (B) Body-on-chip platform to study the metastatic preference of HCT-116 cells in chip equipped with multi-organ chambers and fluid flow. Reprinted with permission from [107] *Copyright © 2018 Wiley Periodicals, Inc.*

5. Emerging tools for personalized oncological medicine: liquid biopsy

Colorectal cancer patients are not usually diagnosed in an early-stage of the disease, which severely affects their survival outcome. For distant metastasis there is a less than 20% of a five-year survival rate [108]. The standard clinical procedures for detection of CRC include endoscopic and radiological imaging, however these invasive techniques have poor patient compliance [109]. Other non-invasive methods exist such as faecal occult blood test (FOBT) [110] and monitoring biomarkers such as the carcinoembryonic antigen (CEA) and carbohydrate antigen 19-9 (CA19-9) [111], which have a relatively low sensitivity and specificity [112] [113].

The discovery that tumours shed part of their DNA into the blood stream traces back to 1948 by the scientists Mandel and Métais [114], however with the increasing capabilities of advanced next-generation genomics and transcriptomics, this seminal discovery opened up new possibilities for non-invasive early diagnosis, treatment monitoring and predictive and prognostic values in CRC [115] and other cancer types [116]. With a simple non-invasive liquid biopsy, circulating tumour DNA (ctDNA) can be detected and analysed in terms of their somatic mutations [117] and DNA methylation profiles [118], and give a "snapshot" of the dynamic real-time tumour burden [119] (Fig. 6). Other biomarker molecules have been utilized such as microRNAs [120], metabolomic markers [121] and microbial markers [122] [123]. CTCs also gives us a hint into the metastasis status of the cancer [124] and can provide important predictive biomarkers [125]. Thus, this novel analytical tool – liquid biopsy - has been advancing the field of cancer prevention, relapse recurrence and drug resistance, and some have viewed it as the "Rosetta stone of biomedicine", instrumental for personalized oncological medicine [126]. This section will discuss comprehensive studies using liquid biopsy for CRC clinical approaches, with a particular focus on CTCs microfluidic platforms.

5.1. Circulating tumour cells (CTCs)

CTCs are difficult to detect due to their scarcity (<10 CTCs/billions of blood cells) [127], and a cut-off measurement for metastatic CRC has been defined as ≥ 3 CTCs in 7.5 mL of peripheral blood, correlating with an unfavourable prognosis [128]. This allows for a stratification of patients with different prognosis and a tailoring of the most suited drug regimens. Currently the CTC - Cell Search System [129], designed for analytical accuracy and reproducibility, has been approved by the FDA for CTC enumeration in a clinical setting for mtCRC (metastatic CRC) patients, as well as breast and prostate cancer.

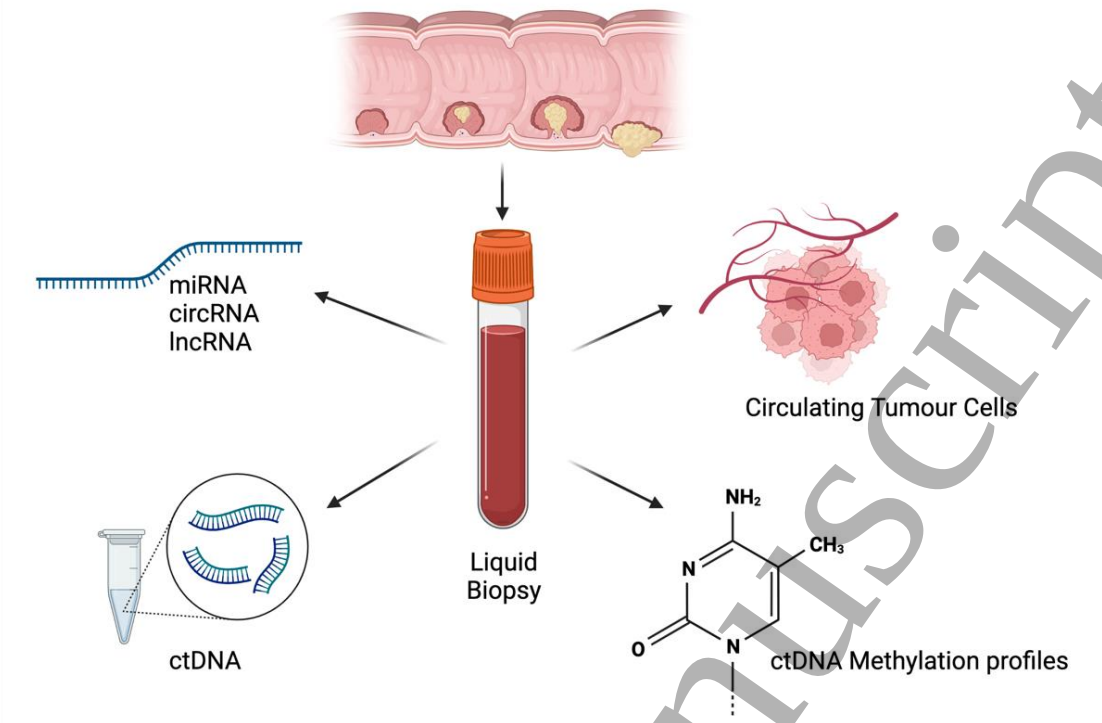

Figure 8. Schematic representation of a CRC patient liquid biopsy that can be analysed for CTCs, ctDNA methylation profiles, ctDNA somatic mutations and non-coding RNA (ncRNA): microRNA (miRNA), circular RNA (circRNA) and long non coding RNA (lncRNA). Created with BioRender.com

5.1.1. Microfluidic platforms for CTC isolation

Liquid biopsy can be used to obtain blood samples from CRC patients and several microfluidic platforms have been developed with the purpose of isolating CTCs. For instance, Ribeiro-Samy *et al.* [130] have developed a microfluidic chip that isolates CTCs from peripheral blood of CRC patients. This microfluidic device (Fig. 9A) was designed to split the blood equally in 4 different modules, whereby blood cells can deform and gently flow through, and other larger more rigid cells are retained in the filter. As such, the device so-called CROSS chip captures CTCs based on their size and deformability with an efficiency of 70%. The captured cells can be used to stratify patients according to their prognosis, as well as be further characterized by PCR. Another example of a microfluidic application for capturing CTCs of CRC patients is provided by Su *et al.* [131] whereby a microfluidic platform with four layers: the top layer – that contained microvalves and micropumps for gas control – two middle layers – fluidic control layers with microchannels – and the bottom layer – contained the microfeatures responsible for cell trapping (Fig. 9 B). As the previous example, the capture of CTCs was possible due to a system of trapping chambers (about 5600) that ensured that larger cancer cells got trapped while other blood cells with smaller size escaped. Similarly, it was possible to carry downstream molecular analysis (*e.g.*, PCR and FISH assays).

Ongoing research has been carried out to obtain CTCs information beyond cell number, including analyzing intrinsic biomarkers in specific clinical contexts. For instance, Raimondi

et al. [132] have analyzed the expression of programmed death-ligand 1 (PD-L1) in CTCs using the Cell Search System for a cohort of mCRC patients being treated with regorafenib as the third line of treatment. The aim of the study was to understand if PD-L1 expression in CTCs could be used as a predictive biomarker for response and clinical benefit in mtCRC patients treated with regorafenib. Results showed a better response rate to the drug in PD-L1+ CTCs and in those whose expression value, evaluated by fluorescence microscopy, decreased 4 and 8 weeks after initiation of treatment. In essence, longitudinal liquid biopsies and PD-L1 CTC analysis in mtCRC patients could be a useful clinical tool to distinguish between responders and non-responders to treatment with regorafenib, thereby optimizing patient treatment.

An important study in microfluidic capture of CTCs focused on the different types of ceilings in the platform design [133]. These devices work by antibody targeting of cancer cells that are captured by an incorporated microarray with a capture element. The authors describe the physical relevance of the ceiling conformations in the microfluidic device, which can affect the flow of cells through the platform. The herringbone structure (HBS) ceiling is described as the best suited to disrupt laminar flows and increase contact between tagged CTCs and reactive microarray. EpCAM capture method was used for comparison with established Cell Search System. In the study, comparative studies were made between different types of ceiling and the HBS ceiling outperformed the others resulting in a higher levels of tumour cell recovery from blood samples. Cell viability studies and feasibility of downstream genetic analysis showed the potential of this platform for clinical use.

Thus, CTCs analysis not only provide enumeration data that can give a metastatic status of the patient [130] but can also guide therapeutic decisions by analyzing protein expression and other common CRC genetic mutations such as *BRAF* and *PIK3CA* [134]. Furthermore, in an interesting approach De Angelis *et al.* [135] have exploited the invaluable biological tumour material provided by CTCs and expanded them into CTCs-derived organoids (CTCDOs) in a proof-of-concept experiment. The reasoning behind this approach relates with technical difficulties in expanding CTCs in culture (can take up to months) which hampers clinical decisions. Furthermore, CTCDOs have showed distinctive drug sensitivity as well as features of EMT profile and expression of stemness-associated proteins. The workflow initiated with obtaining tumour tissue from CRC patients and develop it to PDOs according to Sato *et al.* [28] methodology. These PDOs developed organotypic structures as expected, were transfected with bioluminescence molecules and then were inoculated orthotopically into the colon of immunocompromised mice. The CTCs generated by these mice were isolated and derived into CTCDOs for further analysis such as drug screenings and proteome profiling. As a control xenograft-derived organoids were also maintained to compare results and rule out the effects of cell passage in the mice. Results showed that CTCDOs could serve as faithful model of the tumour biology allowing the possibility of investigating features of metastatic CRC cells, identify new prognostic markers and prevent metastasis. CTCDOs are an important technical achievement in the prognosis and monitoring of tumour burden. In brief, CTCs are a less invasive way to obtain tumour tissues, which may benefit of increased patient compliance. However, with the emergence of next-generation sequencing combined with liquid biopsies there has been a shift towards ctDNA enquiries [124], which is beyond the scope of this review.

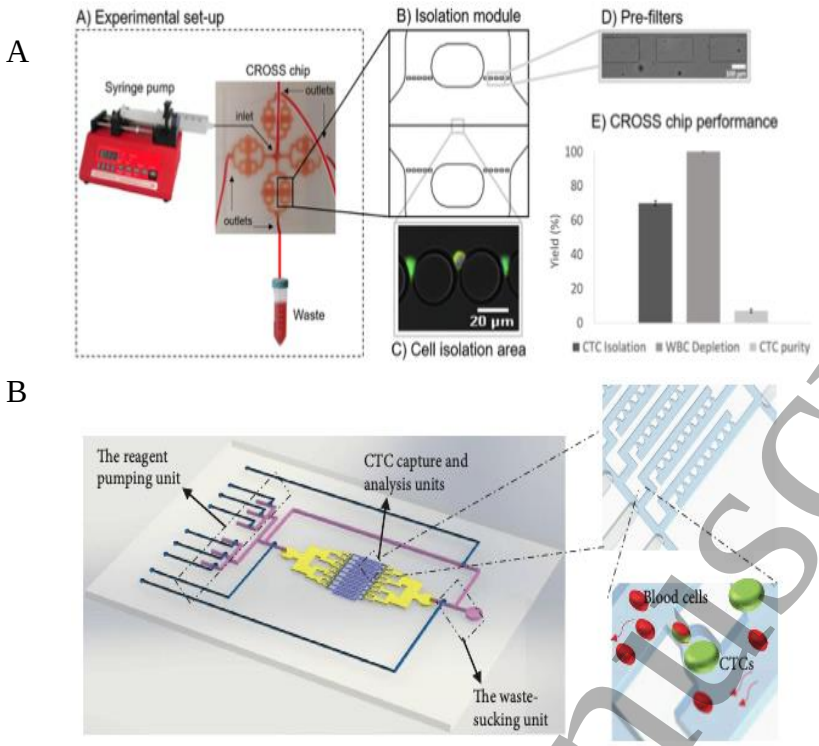

Figure 9. Microfluidic devices for CTC isolation and entrapment. (A) Microfluidic chip that isolates CTCs from peripheral blood of CRC patients. Reprinted with permission from [130] *Copyright © 2019 Springer Nature*. (B) Microfluidic chip that captures CTCs from blood of CRC patients with a system of trapping chambers (about 5600). Reprinted with permission from [131] *Copyright © 2019 Hindawi.*

6. Microbiome and gut-on-a-chip microfluidics

The integration of the effects of the human microbiome as responsible effectors of diseases such as CRC is complex and to individualize a single cause is a daunting task, given the immensity of the human microbiome [136]. The microbiome comprises archaea, eukaryotes, protozoa, and bacteria that live symbiotically inside the human body, being the intestine its densest population localization [137]. It is composed of about $3.8x10^{13}$ bacteria in the colon and the dominant bacteria are the Proteobacteria, Bacteroidetes and Actinobacteria at the phylum level [138][137]. The human microbiome plays an important role in human well-being by providing intestinal protection from pathogens, contributing to intestinal topography/architecture, food and drug metabolism and immune regulation [139]. Host-microbiome interactions have been difficult to study due to the poor technology when it comes to analysing non-cultivable microbes, however with the advent of high-throughput sequencing it has been possible to increase the understanding of the structural diversity and functionality of the human microbiome [140]. As such, mounting evidence has implied a disruption of the microbiome balance (dysbiosis) in the development of several diseases such as Inflammatory Bowel Disease (IBD) [141], Celiac Disease [142], Obesity [143], Autism Spectrum Disorder [144] and neurodegenerative disorders such as Alzheimer's [145] and Parkinson's Disease [146], and finally CRC [137] [24].

Although many of the mechanistic aspects of the causality that links dysbiosis and the onset of CRC remain to be elucidated, one of the main contributors for tumour progression in the colon is the *Fusobacterium nucleatum*, a non-colitogenic bacteria that is found in high concentrations in CRC patients [39] [148]. *Fusobacterium nucleatum* has been associated with microsatellite instability [149] and murine studies have suggested that *F. nucleatum* could promote proliferation and inflammation in E-cadherin expressing CRC cells [150]. *Pks+ E. coli* strains are also responsible for inducing CRC initiation as described below [151]. To study the microbiota, murine models and clinical studies have been carried out, however several limitations such as restricted access to the gut *E. coli* and reduced possibility of *in vivo* monitoring have hampered an in depth investigation [139]. However, some 3D *in vitro* models have been developed with increasing complexity over the years namely: gut-on-a-chip [152], gut-on-a-chip with microbiome [23], body-on-a-chip with gut/microbiome interface [153] and lastly intestinal organoids with microbiome interface [136][154].

For example, Biagini *et al.* [139] have biofabricated by electrospinning a 3D gelatin structure to serve as a scaffold for microbial growth. This 3D model was shown to have the appropriate mechanical properties to support microbial growth, measured by crystal violet assays. To evaluate the state of the natural diversity of the cultured microbiota, metagenomic analyses and RT-qPCRs were performed, and the results showed a biodiversity consistent with the one found in the original sample. The authors postulated that this model showed the potential to be synergistically combined with microfluidic platforms in order to increase the resemblance of *in vivo* environments, for example, in terms of modulation of metabolite production in response to different factors such as nutrients, drugs and probiotics.

In fact, with the advancement of microfabrication techniques such as soft photolithography and microfluidics, *in vitro* models such as the one mentioned before could potentially be made dynamic by incorporating fluid flow, mechanical cues, and tissue barriers as well as be monitored for changes in the biological responses of cell cultures within the microfluidic platform [155]. For instance, Kim *et al.* [156] have developed an *in vitro* model comprising a microfluidic platform – a human gut-on-a-chip – composed of two microchannels separated by an ECM layer and lined with Caco-2 cells (human intestinal epithelial cell line). The ECM layer contained rat type I collagen and Matrigel. To study human host cell-microbiome interactions, the bacteria *Lactobacillus rhamnosus* (LGG) was loaded to the apical surface of Caco-2 cells and its viability studied with ß-galactosidase catalytic activity assays. Mechanical strain was applied to the system by means of vacuum chambers on the sides of the microfluidic channels. The microfluidic model showed the potential to be used as a platform suited for transport, absorption and toxicity studies, as well as for drug development efforts.

In a more complex example, a research team developed an oxygen-sensitive gut-on-a-chip populated with aerobic and anaerobic microbes [157]. Oxygen sensors were integrated into a microfluidic device that also contained two microchannels separated by a porous membrane, with the top channel layered with Caco-2 cells, and the bottom channel layered with human intestinal microvascular endothelial cells (HIMECs). The establishment of a hypoxic microenvironment allowed the proliferation and support of a physiologically significant amount of microbial growth, resembling the native microbiota in terms of diversity and abundance. Through 16s RNA metagenomics analysis it was identified approximately 200 operational taxonomic units (OTUs), which is similar to the number of OTUs expected for the *in vivo* microenvironment – 280 OTUs – and within these results 11 genera were subcategorized including: *Eubacterium, Bacteroides and Enterococcus*. This proof-of-concept model successfully recapitulated the complex epithelial-microbe interactions and could potentially be used to discover new microbiome-related therapeutics and as a CRC research platform.

Similarly, Shah *et al.* [23] have developed the HuMiX chip with a human-microbiome crosstalk comprising three microchambers: perfusion microchamber, epithelial microchamber (with Caco-2 cells) and microbe microchamber (with LGG) (Fig. 10A). In this case, the chip was adapted in order to measure transepithelial electrical resistance (TEER) for the evaluation of Caco-2 cells differentiation. The results showed that this model enables microbial co-culture with intestinal cells and further transcriptomic, metabolomic and immunological analyses. Up-regulation and down-regulation of several genes was observed as a result of Caco-2 cells-microbiome interaction consistent with *in vivo* profiles under aerobic and anaerobic conditions. Furthermore, the co-culture with the microbial population showed a differential regulation of the expression of several cancer-related miRNAs. In brief, this chip showed transcriptomic results analogous with *in vivo* data and allowed a deeper understanding of the host-microbiome interactions at genetic and molecular level, making it a potential tool for drug screening, drug discovery and nutritional studies. Interestingly, the same research team integrated the HuMix chip [158] (loaded with Caco-2 cells and LGG) with -omics data (transcriptomics and metabolomics) and *in silico* simulations to gain knowledge on the basic signalling interplay between a high-fibre diet and CRC-epithelial cells (Caco-2 cells) (Fig. 10B). It was found that a symbiotic regimen (combination of prebiotics and probiotics) diminished the renewal capacity of CRC-derived cells. The extension of the microbiome population and its dynamic involvement in metabolism make it a rich field of study for metabolomics and this topic in regards to the microbiome and host-microbe interactions has been extensively reviewed here [140].

Pulschof *et al.* [151] have dedicated their lab to the intersection of organoids/organs-on-chip with the gut microbiome and have identified a strain of *E. coli* that causes a signature mutation in CRC. This genotoxic *E. Coli* harbours an operon termed *pks* responsible for the production of the toxin colibactin. Colibactin induces DNA damage such as interstrand crosslinks and double strand breaks. The authors investigated the extent of these genetic mutations at different levels. Firstly, intestinal organoids were co-cultured with *pks+ E. coli* and DNA damage was detected, induced by the *pks+* operon. A knockout strain of *pks* was used as a negative control. Secondly, whole genome sequencing (WGS) was performed in intestinal organoids under long-term exposure to *pks+ E. coli* proving that this mutational signature is a direct consequence of this genotoxic strain of *E. coli*. Thirdly, WGS data of a cohort of 496 solid CRC metastases was analysed and a strong prevalence of the two *pks* signatures was revealed. Furthermore, the oncogenic potential of these mutations was assessed and it was found that the *APC* mutant contained the higher number of mutations induced by the two signatures of *pks+ E. coli*: single bases substitutions (SBS-*pks*) and inserts and deletions (indels-*pks* or ID-*pks*). In summary, this study showed that the microbiome can play a role in the onset of CRC by exposure to the *pks+ E. coli.* Therefore, it may be possible to reduce the prevalence of CRC through detection and removal of *pks+ E. coli* contacts. With the advancement of microfluidic technologies, it could be feasible to co-culture the microbiome with gut cells, which offers the possibility to investigate the mechanisms and relationships behind gut dysbiosis and CRC development, leading to the discovery of potential new drug targets.

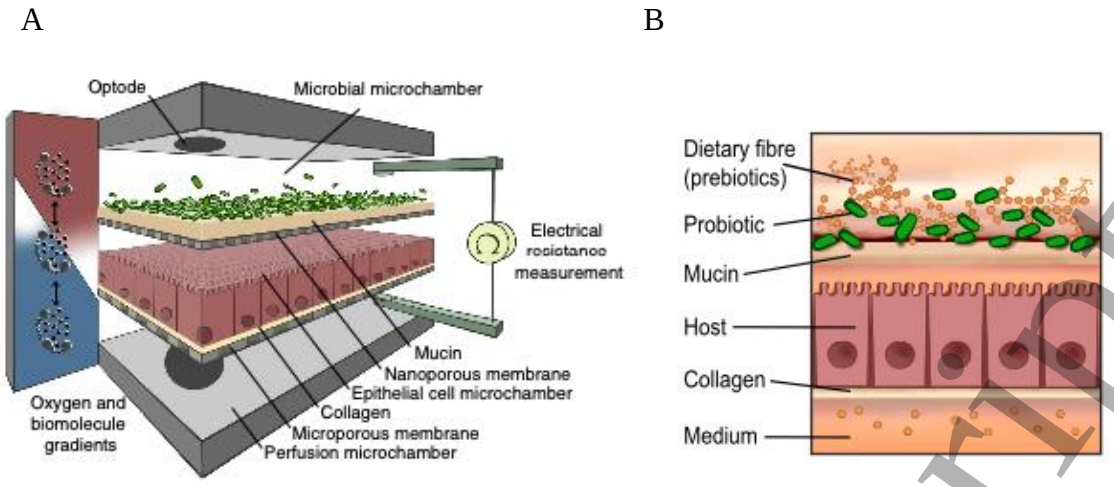

Figure 10. Several 3D microfluidic gut/microbiome-on-a-chip. (A) HuMiX chip equipped with TEER measurement device and, oxygen and biomolecule gradients. Reprinted with permission from [23] *Copyright © 2016 Springer Nature.* (B) HuMiX chip for the study of the impact of high-fibre diet (prebiotics and probiotic). Reprinted with permission from [158] *Copyright © 2019 Elsevier.*

7. Future perspectives

The initial motivation for creating MPS was to increase the speed, efficiency, and safety of pharmaceutical development and testing. Paying particular regard to the fact that neither monolayer monocultures of immortal or primary cell lines nor animal studies can adequately recapitulate the dynamics of drug-organ, drug-drug, and drug-organ-organ interactions in humans, other applications are already being studied besides cancer. MPS can be used to determine of the effect of environmental toxins on humans, identification, characterization, and neutralization of chemical and biological weapons, controlled studies of the microbiome and infectious disease that cannot be conducted in humans. Nevertheless, there are some technical challenges to be addressed, such as achieving a higher level of complexity, recreate the microenvironment having in consideration ECM composition, stiffness and typography and obtaining human samples. Most of all, the field is missing regulatory science for validating the MPS models. Nevertheless, from our perspective, it seems certain that progress will be made towards providing more physiologically realistic alternatives.

8. Conclusions

Cancer remains a heavy burden in our society and the occurrence of drug resistance and undesired side effects of current therapies, demand new approaches in the research field. 3D models come to bridge a gap between 2D models/murine models and clinical trials. This new pre-clinical tool that includes the use of spheroids/organoids with putative incorporation in microfluidic platforms can trail a way to better drug regimens, identification of novel drug targets and development of patient-centred therapies.

Herein, we described several spheroid-based assays using microfluidic platforms that evaluate not only malignancy potential through migration assays, but also the ability of immune cells to penetrate spheroids, high throughput drug screenings and analysis of drug delivery and

distribution. CRC PDOs have been described, including its applications as drug screening platforms and also as experimental models to recapitulate the intrinsic complexity and heterogeneity of a tumour. Insights into complex signalling pathways such as the MAPK and WNT signalling, as well as transcription factors YAP/TAZ and PTMs have been described. Co-cultures with fibroblasts and macrophages have also been exemplified using PDOs, and the importance of the tumour microenvironment for more realistic 3D *in vitro* models was highlighted. Finally, body-on-chips systems for drug toxicity studies and to recapitulate CRC metastasis were mentioned. Furthermore, MPS can be developed to capture CTCs and carry out further genetic analysis. The novelty of CTCDOs was discussed and it could be an important tool for more precise drug regimen. In general, the need for less invasive clinical approaches to CRC makes liquid biopsies a promising tool for the clinical practice.

The intersection between the microbiome and intestinal 3D models with increasing complexity was reviewed. The putative role of the microbiome on the onset of CRC makes this a growing field of cancer research. Intestinal organoids can also be used to study gut/microbiome interactions in the CRC microenvironment. Organoids are a great tool to mimic the *in vivo* differentiated cellular structures such as intestinal microvilli, however there are still some reproducibility issues related with the stochastic nature of organoid differentiation and the use of ill-defined matrices and non-standardized culture mediums.

In summary, MPS to study CRC are an important tool to understand and replicate the CRC pathobiology *in vitro* and as a result improve drug development efforts and pave the way to personalized oncological medicine.

Acknowledgments

The authors thank Project "HEALTH-UNORTE: Setting-up biobanks and regenerative medicine strategies to boost research in cardiovascular, musculoskeletal, neurological, oncological, immunological and infectious diseases", ref. NORTE-01-0145-FEDER-000039 funded under the program NORTE-45-2020-20 – Sistema de Apoio à Investigação Científica e Tecnológica – "Projetos Estruturados de I&D&I" UNorte. The authors also thank the funds provided under the IET A. F. Harvey Engineering Research Award 2018 (ENG ThE CANCER). The EU Framework Programme for Research and Innovation H2020 on FoReCaST (grant agreement no.668983) is also significantly acknowledged.

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