REVIEW
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Microphysiological systems composed of human organoids in microfluidic devices: advances and challenges

Sistemas microfisiológicos compostos por organoides humanos em dispositivos microfluídicos: avanços e desafios

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ABSTRACT

Introduction: Models with higher predictive capacity and able to produce results at lower costs and in shorter times are needed for drug development. The microphysiological systems (MPS) that cultivate human tissues in three-dimensional histoarchitecture (3D) are promising alternatives for these objectives. **Objective:** This review work aims to address the state of the art of SMF development and illustrate the initial Brazilian experience with this technology. **Method:** The research and data collection covering the theme "Microphysiological Systems", and the subtopics "Microfluidic Devices" and "3D Culture of Human Cells", was based on electronic search in Capes Journals Portal, scientific databases Scopus, PubMed and Science Direct and with the Google Scholar search tool. **Results:** Among the existing microphysiological systems, those that are characterized by the culture of human tissues organized in three - dimensional histoarchitecture in microfluidic devices were recently introduced, as being the most promising ones. In addition, between the years 2000-2017, we recorded approximately increases of 12, 985 and 380 times in the number of academic publications related to the areas of Microfluidics, Organ-on-a-Chip and MPS respectively, illustrating the impact of this technology today. **Conclusions:** This relatively recent technology has high potential to overcome the limitations of current in vitro experimental models

KEYWORDS: Human-on-a-Chip; Organoids; Disease-on-a-chip; iPSC; Microphysiological Systems

RESUMO

Introdução: Modelos com maior capacidade preditiva e que produzam resultados a custos mais baixos e em prazos menores são necessários para o desenvolvimento de fármacos. Os sistemas microfisiológicos (SMF) que cultivam tecidos humanos em histoarquitetura tridimensional (3D) apresentam-se como alternativas promissoras para esses objetivos. **Objetivo:** Este trabalho de revisão tem por objetivo abordar o estado da arte mundial do desenvolvimento dos SMF e ilustrar a experiência brasileira inicial com essa tecnologia. **Métodos:** A pesquisa e coleta de dados abrangendo a temática "Sistemas Microfisiológicos", e os subtemas "Dispositivos Microfluídicos" e "Cultura 3D de Células Humanas", foi baseada em busca eletrônica no Portal de Periódicos Capes, nas bases de dados científicas Scopus, PubMed e *Science Direct* e utilizando a ferramenta de busca Google Scholar. **Resultados:** Dentre os sistemas microfisiológicos existentes, os que são caracterizados pelo cultivo de tecidos humanos organizados em histoarquitetura tridimensional em dispositivos microfluídicos foram recentemente introduzidos, como sendo os mais promissores. Além disso, entre os anos 2000-2017, registramos aumentos de aproximadamente 12, 985 e 380 vezes no número de publicações acadêmicas relacionadas às áreas de Microfluídica, *Organ-on-a-Chip* e SMF respectivamente, ilustrando o impacto dessa tecnologia atualmente. **Conclusões:** Essa tecnologia relativamente recente tem alto potencial para superar as limitações dos modelos experimentais *in vitro* atuais.

PALAVRAS-CHAVE: *Human-On-a-Chip*; Organoides; *Disease-on-a-Chip*; iPSC; Sistemas Microfisiológicos

INTRODUCTION

Preclinical tests in medical/biological research and drug development often do not accurately predict the responses observed in humans, which leads to high friction rates[1]. The US Food and Drug Administration (FDA) estimates that 92% of the medicines approved in animal testing fail in humans. Animal models provide access to systemic physiology, including distribution and metabolism in various tissues, immune system response, influence of microenvironments and organ-organ barriers and interactions, as well as behavioral responses. However, it has already been shown that the phylogenetic distance between humans and animals (for example, illustrated by proteomic changes or differences) decreases their predictive power[2,3,4,5,6]. Therefore, there is a clear need for better predictive models that can also reduce the time and cost of developing substances or products in various industries like pharmaceuticals, foodstuffs, cosmetics, sanitizers and agricultural products, to name a few.

Almost all of the safety tests required by drug regulatory authorities are currently done with animals. The few exceptions are not enough to comply with the required regulations[7,8]. Some *in silico* methods (computer simulations) are already available in Brazil with the support of the National Network of Alternative Methods[9] (RENAMA). These methods are useful for evaluating viability, but insufficient for the development and registration of innovative products, requiring experimental *in vivo* methods or the development of *in vitro* alternatives. Microfluidic devices or chips, which combine human tissues in a three-dimensional arrangement and stable conditions of homeostasis, may be the solution to this problem. Most of the times, human cells grown in two dimensions cannot adequately recap nor cover all functional aspects of tissues, tissue-tissue interfaces or the dynamics of human body organs[11,12,13,14,15]. Tissues modeled from human cells in microdevices can improve the predictive accuracy of preclinical efficacy and safety studies of medicines, cosmetics and other substances or products for human use[11,12,15,16].

The principle of the 3Rs (Replacement, Reduction and Refinement) was developed in 1959 by Russell and Burch[17] and promoted the consolidation of alternative methods. The term "alternative methods" may be defined as approaches having one or more of the following outcomes: 1) methods that induce the reduction of the number of experimental animals used in a given procedure or reduction to the minimum necessary; 2) refinement of the methodology that culminates in the significant reduction of the pain or discomfort suffered by the animals; 3) non-animal methods – full replacement of animals in a particular procedure or assessment[18].

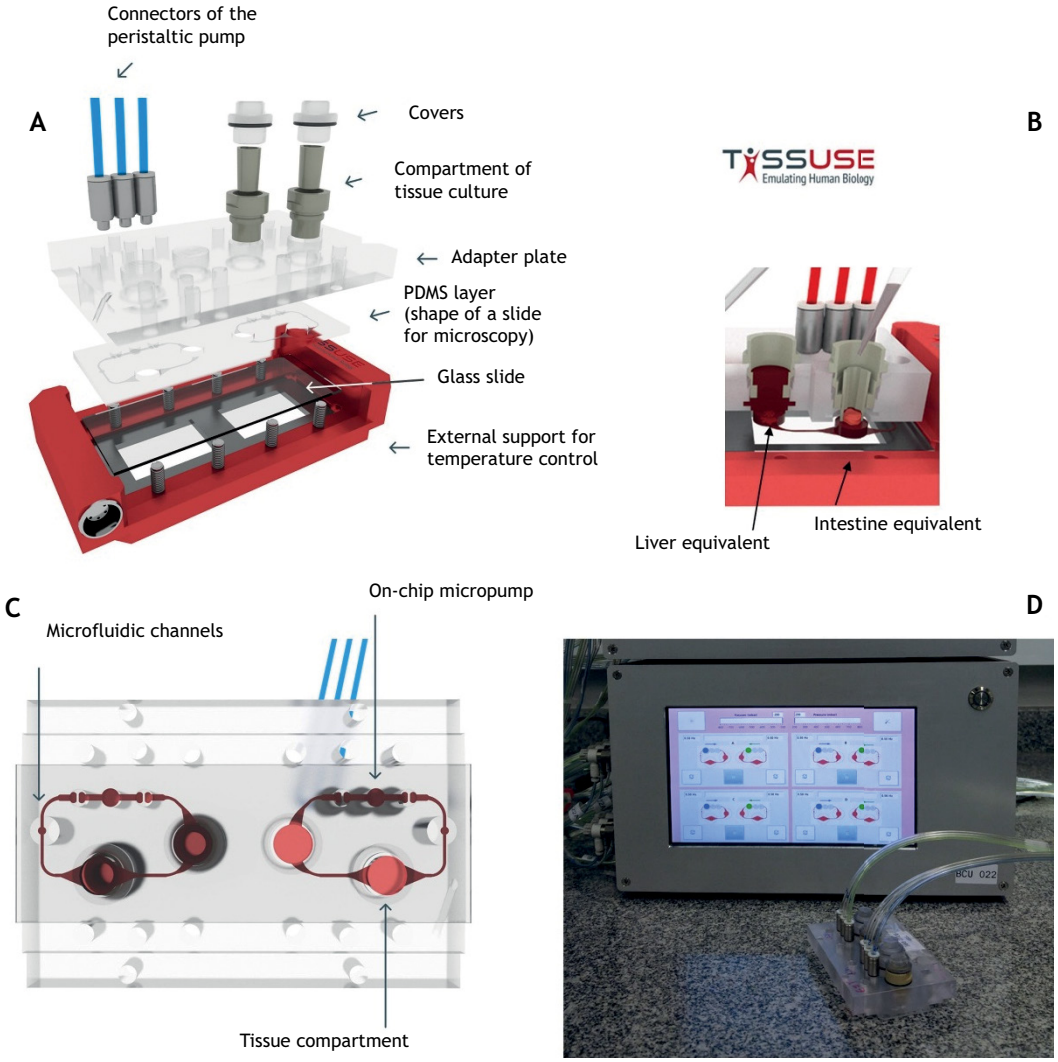
The increasing ethical and political pressure on the implementation of actions aimed at replacing the use of experimental animals led to the adoption in 2009 by the European Union of the regulatory requirement for the safety assessment of cosmetic ingredients through tests that do not use animals[19]. In 2013, the European Union officially banned the use of animals for cosmetic development research – ingredients and finished products – marketed in the EU[20].

These actions encouraged the development and adoption of alternative methods in Brazil and worldwide. Brazilian efforts and commitment to the promotion, implementation, development and validation of alternative methods to the use of animals led to the creation of RENAMA, in July 2012, by the Ministry of Science, Technology and Innovation (MCTI). In September 2012, the MCTI created the Brazilian Center for Validation of Alternative Methods (BraCVAM), a partnership between the National Sanitary Surveillance Agency (Anvisa) and the National Institute for Quality Control in Health of the Oswaldo Cruz Foundation (INCQS/Fiocruz). These were the first partnerships in Latin America to coordinate actions that could lead to the reduction, refinement or replacement of the use of experimental animals in Brazil[21]. In this sense, in 2015 the National Laboratory of Biosciences (LNBio), one of the three Central Laboratories of RENAMA (LNBio, National Institute of Metrology, Quality and Technology [Inmetro] and INCQs) started the *Human-on-a-Chip* project, aimed at the implementation and nationalization of technology based on microphysiological systems (MPS), with prospects of future developments that can comply with the 3Rs principle.

This review paper addresses the state of the art in the development of MPS, with emphasis on those that culture human organoids under microfluidics. It also illustrates the early Brazilian experience with this technology.

METHOD

The research and data collection that covered the topic of "Microphysiological Systems", as well as the subtopics of "Microfluidic Devices" and "3D Culture of Human Cells" (3D = three-dimensional), were carried out electronically on the Capes Portal, the scientific databases of Scopus, PubMed and Science Direct, with occasional assistance of the Google Scholar search engine. This paper aimed to critically review and gather the main pieces of information and updates on the aforementioned topics. The search and collection covered publications made from 2003 – the year in which development and studies in the field of tissue and microfluidic engineering became more robust – until the main studies recently published in the MPS area. The search also included the use of keywords or terms like alternative methods to animal testing in laboratory, predictive power of *in vitro* methods for substance tests, Human-on-a-Chip, Body-on-a-Chip, Organ-on-a Chip, iPSC (Induced Pluripotent Stem Cells), human organoids, Disease-on-a-Chip, microphysiological systems and microfluidics. Additionally, this review was complemented with a brief report aimed at illustrating the experience of LNBio to date in the development, proficiency and implementation of MPS technology in Brazil in partnership with the German company TissUse. The platform is operated by a controller unit coupled to a vacuum pump and combines a microfluidic channel system with two tissue culture compartments, each one of the size of the well of 96-well plates (liver) or 24-well plates (intestines), as shown in Figure 1. The constructive model of the TissUse 2-OC microfluidic device (Two-Organ-Chip) has two distinct



A) Schematic drawing of the constructive model of the TissUse microfluidic device. B) View of a 2-OC longitudinal section showing the culture compartments of intestine and liver equivalents. C) Bottom view of 2-OC with emphasis on the microfluidic channels that interconnect the tissue culture compartments. D) 2-OC platform installed and in operation at LNBio - Controller unit (peristaltic pump) connected to 2-OC.
Source: Adapted from https://www.tissuse.com/en/products/2-organ-chip/ and Maschmeyer et al. (2015).

Figure 1. Two-Organ-Chip platform by TissUse.

compartments per cell coculture circuit (Figure 1). The 2-OC were manufactured by applying standard mild lithography and molding of polydimethylsiloxane (PDMS) replicates (Sylgard 184, Dow Corning, Midland, MI, USA). This device consists of a PDMS slide containing the arrangements of the channels, the micropumps and the openings for the cell culture compartments, permanently attached to a glass slide (75 x 25 mm) for microscopy, by oxidation of low pressure plasma (Femto-Diener Electronic, Ebhausen, Germany). This permanent bond originates a single part that has microchannels with a height of 100 mm. The three on-a-chip peristaltic pumps (built in the PDMS slide) have a thickness of 500 mm. This microdevice for two organoids (2-OC) provides two features that improve its functionality: mechanical coupling and "humoral" communication between tissues[22]. The volume to culture two tissues (liver and intestines for example) is 900 µl per circuit. The total extracellular volume is about 1000 µl per circuit. The micropump provides a stable pulsatile flow of the fluid or culture medium. The upper and lower surfaces of the plates are transparent and allow visualization and morphological characterization of the organoids in real time. The direction of the perfusor flow within the chip compartments, as well as the flow rate, are determined according to type of tissue and experiment. In the case of 2-OC Intestines + Liver, the flow direction was from the intestines to the liver (emulating the intestinal absorption and direction of what was absorbed to the liver by the portal system). The established frequency was 0.8 Hz[22].

RESULTS AND DISCUSSION

Human organoids

The emulation of the *in vivo* histoarchitecture is essential for obtaining responses of physiological relevance of the *in vitro* human organ model. Three-dimensionality and intercellular

communication are fundamental characteristics for the full phenotypical and functional expression of most tissues[23]. No less important is the contact between different cell types. The three-dimensional complex microenvironment in which cells are organized *in vivo* enables interaction between different cell types and between cells and the extracellular matrix (ECM)[24,25,26].

Organoids are artificial structures that represent functional fragments of organs created for *in vitro* studies and capable of performing the fundamental *in vivo* functions of the equivalent organ[27]. Its conceptual conception starts from the morphofunctional units of the respective organs. For example, the hepatic lobes represent the morphofunctional units of the liver, which has about 1 million lobes. Each lobe contains about 1 million cells of 20 different types, mainly hepatocytes. The shape of the lobe is roughly hexagonal with the hepatic vein in the center and the hepatic triads (portal vein, hepatic artery and bile duct) at the vertices. A complex network of blood vessels (sinusoid) irrigates them[28]. Similar morphofunctional units can be identified in every organ of the human body[29].

Organoids are usually three-dimensional structures made up of different cell types organized in a given arrangement (microstructure). A very popular type of microstructure is the spheroid. The spheroidal organoid confection has several beneficial aspects that make it attractive: these models are easy to produce and handle, their size and composition can be relatively controlled (because the cells continue to proliferate after the formation of the sphere) and they do not require a mold to grow. This latter feature allows the cells to be arranged spontaneously during the aggregation process, increasing the chances of presenting an organotypic phenotype[30,31], in addition to the fact that they are free of the need to adhere to any type of non-physiological surface. Moreover, the possibility of using different cell types in the same spheroidal organoid enables the appearance of heterotypic intercellular contacts, imparting additional improvement to the aspects of tissue functionality and differentiation[23,27].

Other three-dimensional tissue models made *in vitro*, also very convenient and widely used, are the barrier organoids, including the intestinal barrier, renal barrier composed of epithelial cells of the proximal tubule or by glomerular podocytes, cornea, skin and blood-brain barrier.

Microfluidic devices

Although there is a great diversity of MPS, most of them are based on microfluidic devices that try to mimic the cellular environment of one or more human organs[32]. The term microfluidics refers to the flow of liquids in channels of micrometric dimensions, i.e. less than 1 mm in at least one dimension[33]. Microfluidic devices consist of interconnected microchannel assemblies that can be divided into two categories: 1) passive microfluidic system with flow determined by the force of gravity and 2) active microfluidic system with flow determined by the action of a pump that may or may not be part of a controlling unit. The microchannel network carved into the chip can be accessed through openings (inlets and outlets) connecting the interior to the exterior. It is through these orifices that the human tissues, substances and culture medium are integrated or withdrawn from the microfluidic device, with the use of tubes, syringe adapters, pipettes etc. Also through these openings the chips are connected to external active systems (pressure control, syringe or peristaltic pump) or passive forms (e.g. hydrostatic pressure).

When designing an MPS, establishing the flow regime of the microfluidic device is essential.

The choice of materials for the construction of microfluidic devices should take into account their possible impact on cell culture and also the properties of the substances to be tested. Various materials are currently used, such as: polymer (PDMS), silicone, ceramics, glass and metals. The construction of each involves specific processes: electronic deposition, corrosion, injection molding, embossing and smooth lithography in PDMS. More complex systems are usually made up of more than one type of material.

The PDMS polymer is probably the most widely used material for making the devices, since it is very convenient for cell culture. It is a transparent, gas permeable, biocompatible, low cost and easy to handle elastomer. However, the PDMS has the disadvantage of being able to adsorb and absorb small molecules with hydrophobic properties[34,35,36], a fact that significantly impacts on the accuracy of predictions involving the use of these substances. Alternatively, thermoplastic polymers such as polycarbonate (PC), polymethylmethacrylate (PMMA) and cyclic olefin copolymer (COC) which do not adsorb small molecules, can be used[23].

Microphysiological systems: Organ-on-a-Chip and Human-on-a-Chip (Body-on-a-Chip)

The Cellular Microfluidic Platforms or MPS are currently a technology that is in the development phase and still require significant progress in bioengineering. The advances made over the past decade in the creation of *in vitro* models of microtechnology-based biomimetic cultures have fostered a growing worldwide interest in the development of platforms combining human tissues with microfluidics, giving rise to the Organ-on-a-Chip term. In this case, the word chip comes from the English language and refers to a thin, small device (whether or not it contains electronic elements). Depending on the number of organs and the development of different cellular microenvironments, the Human-on-a-Chip or Body-on-a-Chip terms may also be used. With this technology, researchers expect to mimic *in vitro* the functionality of human organs *in vivo*, in order to better predict the effects of substances in the human body. The shear stimulus given by the flow is important for several functional aspects of the cultured tissues. Therefore, ensuring the physiological and appropriate flow in each case is critical for the establishment of MPS.

MPS projects should be focused on the optimization of the processes of preparation and culture of human tissues, so that the action of the microfluidic system or perfusion system provides mechanical shear stimulation at capillary and interstitial levels

within physiological parameters. In addition to allowing the removal of secreted substances or metabolites and allowing the interaction of cells located in different compartments or tissues, it promotes the creation of microenvironments with biomolecular gradients and the presence of controlled shear stress *in vitro*[23].

There is a great diversity of types of MPS (based on 2D or 3D culture, in parenchymal or barrier organoids, in passive or active flow, with or without electronic elements, such as sensors, electrodes etc.). Additionally, as mentioned before, the *in vivo* three-dimensional microenvironment in which the cells present relations with each other, with different tissues and with the ECM, directly affects the differentiation and function of each organ. With that in mind, we consider that MPS consisting of microfluidic devices for *in vitro* 3D tissue culture human in a controlled environment of mechanical and electrophysiological stimuli provide the best conditions of emulation possible[23].

Organ-on-a-Chip

Organs-on-Chips are biomimetic microenvironmental systems that contain microfluidic channels that carry culture medium with nutrients and extract catabolites from the tissues in culture. They can alternatively be defined as microscale models of human organs (from 10^{-6} to 10^{-4} in comparison with the original size)[37,38].

Organ-on-a-Chip systems seek to produce levels of tissue and/or organoid functionality that are not achieved by static cell culture, as well as to enable the real-time analysis of biochemical and metabolic parameters[15,29] like albumin, lactate dehydrogenase (LDH), glucose, O_2, glutathione (GSH) concentration, mitochondrial functional status, structure and morphology of the cell nucleus, redox status, ATP concentration etc. These parameters are monitored by means of internal or external biosensors, through microscopic images and analysis of the fluid collected from the system[39,40,41].

Organ-on-a-Chip models equipped with monitoring and detection (biosensor) accessories can provide important advantages related to time savings and improved reproducibility of the data produced. Furthermore, the possibility of continuous onsite monitoring of induced drug responses over a long time is crucial for adequate investigation of the pharmacological parameters and, consequently, to increase the predictive power of MPS. Emulate, Inc. of the Wyss Institute, located at Harvard University, has developed microdevices that have built-in electrodes for the measurement of Transepithelial Electrical Resistance (TEER), in order to noninvasively monitor the formation and integrity of several *in vitro* models of barrier organs like lungs or intestines[42].

Another example comes from the Center for Research and Innovation in Biomaterials from the Harvard Medical School, which developed a continuous and automated monitoring system in a modular, integrated platform equipped with multi-biosensors with biophysical and biochemical detection capabilities integrated into Organ-on-a-Chip modules. The system has a peristaltic pump, a microfluidic flow control module, modules containing biophysical sensors for O_2, pH, temperature and contraction of cardiac cells, module containing biochemical sensors for hepatic proteins (e.g. Glutathione S-Transferase alpha, albumin), cardiac proteins (creatine kinase), a mini-microscope for monitoring the morphology of organoids, in addition to a capture module of system bubbles[43].

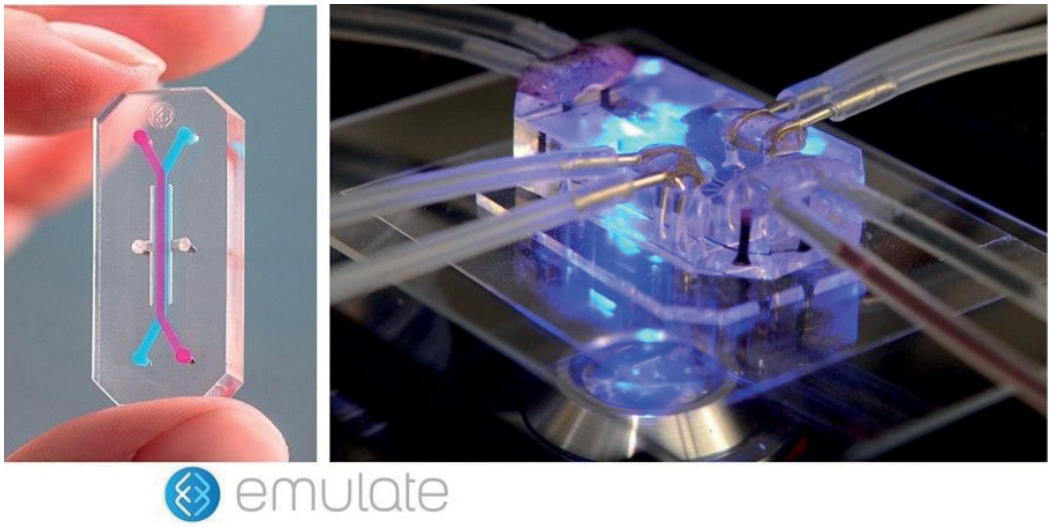
Thus, these MPS enable a more accurate emulation of internal environment conditions, including pressure and temperature conditions, nutrient diffusion (glucose, amino acids and lipids), trophic and growth humoral factors[10], as well as a more effective removal of catabolites. MPS have microfluidic diffusion pathways that permeate tissues similar to blood capillaries and allow three-dimensional culture with intercellular contact and shear stress, responsible in part for tissue morphology. These characteristics provide for the emulation of the *in vivo* conditions and purportedly lead to a preparation response pattern that is more similar to that of a living organism[15,44,45,46], as discussed above. With that, we conclude that the advent of the Organ-on-a-Chip technology is in line with the recognition that cellular morphogenesis, cell-cell interactions, and the biomechanical environment are as important as the cells themselves[47,48,49,50,51,52]. As examples we can cite the topographical orientation[53], spatial definition of culture[44,54,55,56], the shear stimulus and other types of mechanical stimulus (stretching, compression etc.)[57,58,59] and biochemical gradient[60,61].

This technology is an alternative to the use of animals in pharmaceutical, chemical and environmental applications[15].

Other important examples of Organ-on-a-Chip come again from the *Emulate* company. Human organoids cultured alone to date include: lungs (pulmonary microvascular endothelial cells interfaced with alveolar epithelial cells)[62], lower airways (primary human cells differentiated into mucociliary epithelium)[44,63], intestines (human CaCo2 cell lineage)[64], kidney (human proximal tubule epithelial cells)[65] and bone marrow (rat cells)[66]. Figure 2 illustrates the chip model by *Emulate*. In this technology, each organoid mimics the cellular interfaces of its respective organ, as well as some of its fundamental characteristics, for example, the peristaltic movement of the intestines.

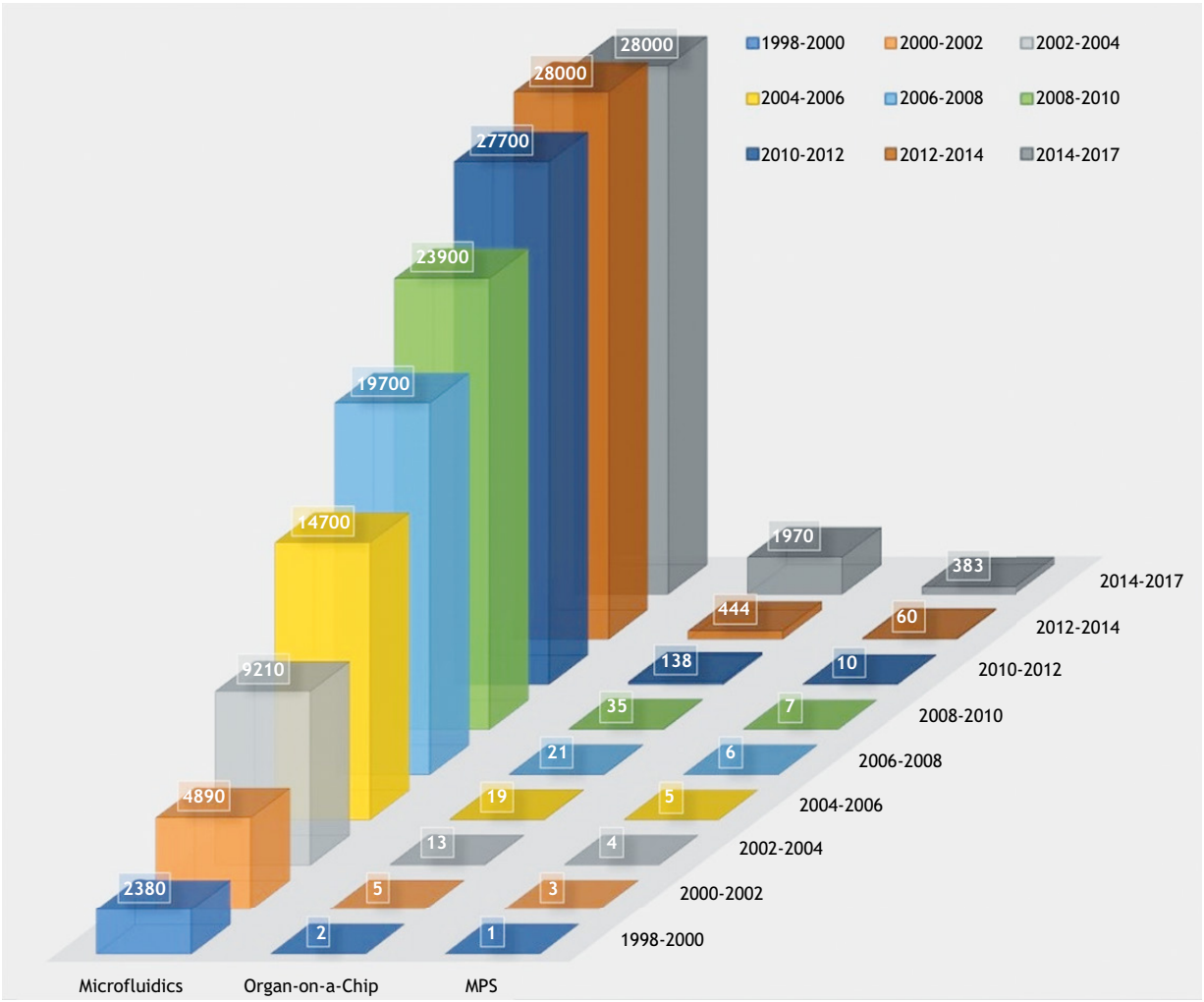
In 2016 the World Economic Forum specifically selected the Organ-on-a-Chip technology as one of the ten most promising emerging technologies in the world[67]. In this sense, the field of study related to microfluidic systems, Organ-on-a-Chip and MPS has experienced a great leap in the number of related academic publications over the last two decades. Between the years 2000-2014 there was an increase of approximately 12 times, 985 times and 380 times in the number of academic publications related to the areas of microfluidics, Organ-on-a-Chip and MPS, respectively (Chart). The increase in publications exemplifies the increasing activity and interest in this technology.

The following keywords: microfluidics, Organ-on-a-Chip and microphysiological systems were searched through Google Scholar. The numbers of publications found by this search for



Source: Adapted from Emulate (https://emulatebio.com/).

Figure 2. Example of Organ-on-a-Chip system by Emulate.



ECM: extracellular matrix; PDMS: polydimethylsiloxane; LDH: lactate dehydrogenase; TEER: transepithelial electrical resistance; iPSC: induced Pluripotent Stem Cells.

Chart. Numerical evolution of the number of publications.

each of the eight two-year intervals and the three-year interval are plotted from the year 1998 to the year 2017.

Human-on-a-Chip

The MPS based on the coculture of two or more interconnected organs in a microfluidic device are commonly called Multi-Organ-Chips. They are currently the precursor platforms for the future Human-on-a-Chip or Body-on-a-Chip that are under development and project over ten interconnected organs[68] already for the year 2018.

A good illustrative example of these types of MPS comes from the German company TissUse GmbH, a company derived from the Biotechnology Institute of the Technische University of Berlin.

The first prototypes consisted of devices for two interconnected organs under an adjustable flow generated by an external pump housed within a computerized controller unit (Figure 1). In these devices, called 2-Organ-Chip or 2-OC, studies were performed with organoid pairs, such as human skin biopsy and hepatic spheroids[22,69], human skin and hair follicle[70], *in vitro* reconstructed intestinal barrier and hepatic spheroids[22], neurospheres and hepatic spheroids[71].

More recently, culture of endothelial cells covering the luminal area of the microfluidic channels and the area of tissue culture wells have been incorporated and mimic the vascular interface between the tissues and the flow (circulation) inside the device[72]. A bone marrow model constructed on a ceramic template of hydroxyapatite coated with hydroxyapatite Sponceram® 3D (Zellwerk GmbH, Germany), which housed the coculture of stromal mesenchymal cells (MSC) and hematopoietic stem cells derived from blood of the umbilical cord (HSPC) viable for up to 28 days at 2-OC[73].

Subsequently, TissUse also developed devices for four organs (4-Organ-Chip or 4-OC) that gave rise to the publication that shows the culturing and maintenance of liver, intestine, skin and kidney equivalents interconnected in this device[74]. This new 4-OC device has two separate microfluidic compartments: one simulates blood circulation and another emulates the excretory circuit for the drainage of fluid (analogous to urine) secreted by the kidney equivalent.

MPS containing two or more organs have great potential for application in pharmacokinetic studies, which involve analyses of the absorption, distribution, metabolism, excretion and toxicity profiles (ADMETox) of a substance or drug, as well as being able to simulate human diseases *in vitro*. The study of the pharmacokinetic properties of a substance or drug candidate is one of the possible and promising applications of MPS and is a critical step in the drug discovery and development process. Traditional human cell models unmistakably reproduce the ADMETox properties observed *in vivo*, exhibiting a change in the level of exposure (when compared to humans) and impairing toxicological evaluation, since most cell responses evaluated depend directly and precisely on the level of exposure of the tissue to the tested drug[74,75,74,76,77,78,79,80,81].

Thus, an MPS that includes the integration of *in vitro* human intestinal and liver models, the two organs that are critical for bioavailability and systemic exposure responses, has significant relevance[82] and may represent an evolution in the predictive power in relation to static and non-integrated human cell culture models. An example of MPS for pharmacokinetic studies was developed by Murat Cirit's group of the Department of Biological Engineering of the Massachusetts Institute of Technology (MIT)[82]. The MPS consisted of a microfluidic platform (microdevice + infusion pump) in which integrated human intestinal and liver models were developed *in vitro*. These were maintained under continuous communication for the simultaneous investigation of pharmacokinetic parameters like absorption and after oral administration of a given compound (in the case of MPS containing intestine and liver, the emulation of oral administration is done through the application of the test substance in the intestinal compartment, whereas the mimicking of the intravenous administration is done through the application directly into the hepatic compartment). The authors demonstrated the possibility of obtaining intrinsic parameters such as permeability and hepatic clearance through the derivation of the data obtained in the MPS by mechanistic modeling. They also suggested in that study that the communication between the organs provided by MPS had a positive impact on the metabolic capacity of the liver model.

Another example of an MPS model optimized for pharmacokinetic investigations comes from the Laboratory of Biomechanics and Bioengineering at the Compiègne University of Technology, in Picardy, France. The group developed an MPS containing the human intestinal and liver models made *in vitro* and kept coupled under flow for the investigation of intestinal and hepatic first-pass metabolism of paracetamol[83]. This approach was also combined with a mathematical model aiming to estimate intrinsic *in vitro* parameters and to enable extrapolation for the *in vivo* processes. The study also showed the identification of metabolites such as paracetamol sulfate that was identified through the synergistic activity between the intestine and liver models that occurred in MPS. Both groups state in the aforementioned studies the importance and the great potential that MPS application may have in pharmacokinetic investigations, as well as the integration of the MPS-based *in vitro* and *in silico* approaches.

Disease-on-a-Chip

The *in vitro* Disease-on-a-Chip models are a variation or adaptation of the Multi-Organ-Chips intended for the emulation of pathological conditions. One of the illustrative examples is the model developed by the group of the Federal Institute of Technology of Zurich (ETH). The model in question used the culture of colorectal tumor tissue and murine hepatic tissue and enabled the evaluation of the efficacy of cyclophosphamide treatment with previous bioactivation in murine hepatic tissue in the treatment of colorectal tumor *in vitro*[84]. In addition to presenting a successful model of Disease-on-a-Chip, this group demonstrated the importance of simultaneous and integrated cultivation of more than one tissue provided by MPS. Inhibition of tumor growth was observed only in the experiments performed in microfluidic devices, that is, in the presence of flow. There was

no antitumor effect with cyclophosphamide in non-flow assays such as pipetting discontinuous transfer of supernatant from the static cultures of liver tissues treated with cyclophosphamide to cultures that are also stationary of colorectal tumor tissue[84].

In this work, the positive impact of the use of MPS on the response obtained in the test is explicit and was completely different from the response obtained in the test situation performed outside the MPS.

Another group cultured pancreatic ductal adenocarcinoma cells in 3D under flow and showed more physiological cisplatin response when compared to traditional *in vitro* culture models[85]. Tumor cells usually behave differently and exhibit distinct phenotype when grown outside the human body in 2D or even when cultured in 3D. The *in vitro* traditional 2D cell culture systems or under inserts of the Transwell® type, as well as spheroid models, for example, used to mimic the tumor microenvironment (TM), have shown limited predictive power of the therapeutic efficacy of various candidate drugs[86].

TM exerts a great influence on cellular behavior, especially in relation to survival, proliferation, invasiveness and sensitivity to treatment with compounds[87,88]. Typically, tumors are formed by cancerous and stromal cells (fibroblasts and immune cells) nourished by the vascular network. Understanding this intricate interrelationship of different cell types, as well as the interrelationship of cells with components of the extracellular matrix, is critical for the advancement of cancer treatment strategies[89]. In the case of traditional *in vitro* tumor models, i.e. non-MPS-based, the challenge is greater because of the absence of interaction between tumor cells and the extracellular matrix and differences in the intratumoral pH, oxygenation and nutrition gradient conditions found *in vivo*. In this sense, the application of MPS models in the investigation of the behavior and responses of tumor cells can be advantageous, insofar as the MPS can induce a tumor pattern in the tumor cells that is closer to the *in vivo* conditions by providing more approximate conditions of TM found in the human organism. Because of the advancement of microfabrication techniques, it is currently possible to develop microfluidic devices whose spatial organization through compartmentalization of cells and control over the diffusion of soluble factors critical to systemic homeostasis enable the reconstruction of complex cellular culture models that include the necessary integration between various cell types and between cells and components of the microenvironment[90].

The work of Albanese et al.[91], based on the tumor-on-a-chip model, demonstrated successful replication of the enhanced permeability and retention (EPR) effect *in vitro*. Several solid tumors have structural features that include hypervascularization, defective vascular architecture and impaired lymphatic drainage. This characterizes the known EPR effect described for specific molecules[92], typically macromolecular compounds, liposomes and nanoparticles, which tend to build up much more in tumor tissue than in normal tissue[93]. Albanese et al. have shown that the penetration and accumulation of nanoparticles in spheroids of tumor cells integrated into the microfluidic device were significantly influenced by the presence of flux and ECM in the environment. The study verified the importance of the interface region formed by the ECM, fluid and tissue surface. This interface region emulated in the MPS was shown to be necessary for *in vitro* replication of the EPR effect, behaving as a fluid-tissue interface reservoir, in which the nanoparticles build up and diffuse progressively into the tissue. This passive transport from the interface reservoir directly impacts the number of particles to be diffused into the tissue and appears to be able to predict the extent of nanoparticle accumulation within the tumor *in vivo*.

This work evidenced the importance of TM mimicking and the potential use of tumor-on-a-chip in the development and standardization of drug carriers, increasing the investigation of nanoparticle transport mechanisms through a tissue under physiological flow conditions and coupling with ECM.

There are also other examples of diseases emulated in MPS. The model based on endothelial cell-coated microchannels mimicking the intimate layer of blood vessels has also proved successful in mimicking pathological conditions *in vitro*[94]. In this, expression of the von Willebrand factor was observed in response to the stenosis of the microchannels, emulating the effect of the atheroma plaque (composed of fat, calcium and inflammatory cells, located in the artery wall). The application of MPS in disease research seems to be promising. There are already developments of MPS for neurodegenerative diseases, such as Parkinson's[95], infectious diseases or potential for infection of a certain tissue[96], vascular diseases such as thrombosis[63,97], airway diseases[62,63], inter alia.

The Brazilian Experience

The "*Human-on-a-Chip*" project is currently under development at LNBio, one of the four national laboratories allocated to the National Center for Energy and Materials Research (CNPEM). The first stage of the project was based on the development of human organoid models cultured in microfluidic devices manufactured and marketed by TissUse GmbH, a so-called Two-Organ-Chip or 2-OC device (Figure 2).

The microdevices manufactured by TissUse are composed of three types of materials: polycarbonate that makes up the adapter plate, PDMS in which are the microfluidic valves and channels and a glass slide that covers the PDMS (Figure 1A). The MPS model proposed by TissUse provides access to the cell culture wells, while keeping the microchannels isolated and protected, allowing the preparation and maturation of the organoids externally, their placement on chips and their subsequent withdrawal for histology. It also allows the use of a greater variety of human tissues, such as biopsy materials.

The LNBio is already able to successfully prepare and culture organoids of liver, heart and intestines in the 2-OC, in addition to the kidney, which is still under development.

Cells and tissues

Although primary cells from living donors or corpses have better functionality, they are complicated and uncertain to obtain

and may produce inconsistent results due to the fact that they originate from different donors in most cases. Depending on the origin of the tissue and the donor, they do not have the survival time that is necessary to perform longer tests.

Therefore, we chose cells from expandable lineages and/or iPSC cells. Their functionality is generally smaller, but we can control important parameters more effectively.

Equivalent human intestinal barrier

To produce organoids that emulate the intestinal barrier, we used cells from the CaCo2 lineage (ATCC HTB-37) (Figure 3A) associated with the HT29-MTX lineage (ATCC HTB-38) (Figure 3B). Both are epithelial lineages of adenocarcinoma of rectal colon that possess the majority of the morphologic and functional characteristics of absorption cells of the small intestine, including digestive enzymes and receptors. HT29-MTX cells also have the ability to secrete mucin and other compounds that form the intestinal mucus that assists the absorptive capacity[98,99,100]. The barrier was constructed for 21 days in the Transwell® insert whose microporous membrane was seeded with the coculture of the two intestinal lineages mentioned above (Figure 3C).

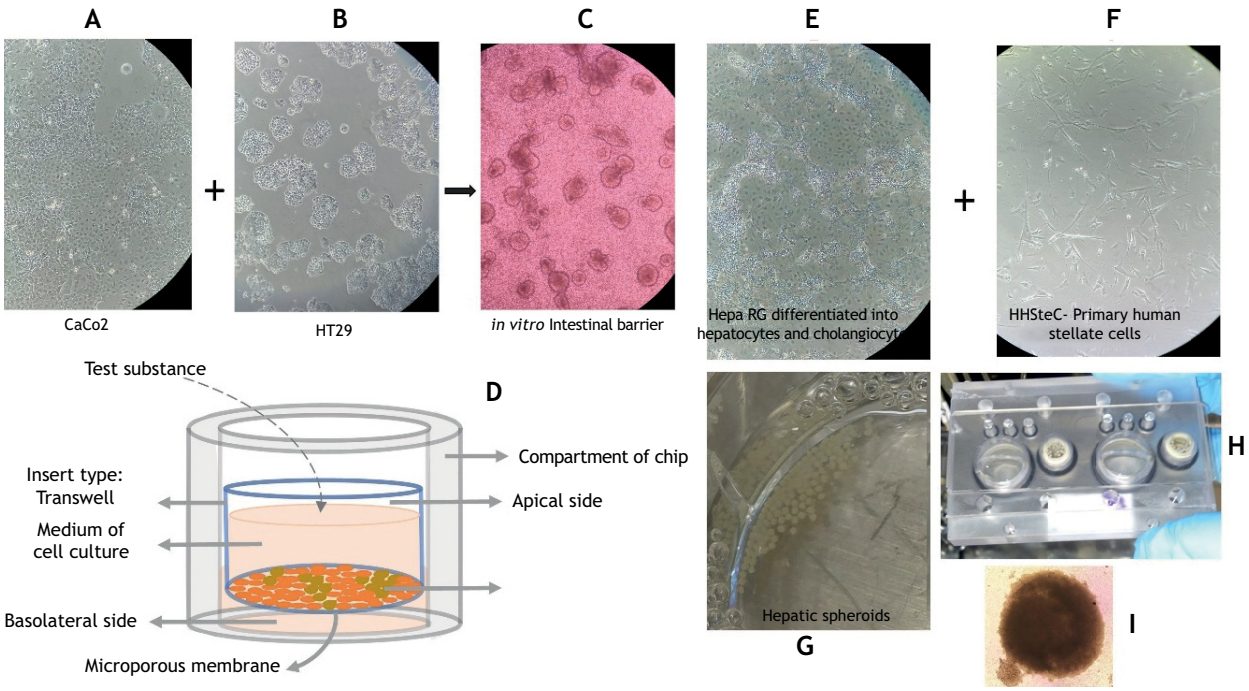
The insert, in addition to supporting the cell culture, also had the function of separating the organoid in two compartments or physically distinct sides mimicking the intraluminal (upper compartment or apical side) and interstitial/bloodstream (inferior compartment or basolateral side) intestinal regions (Figure 3D).

Equivalent human liver

For liver spheroids, we used HPR101 cell lineage differentiated into HepaRG® associated with the HHSteC lineage (Human Hepatic Stellate Cells #5300, Sciencell) (Figure 4). The first comes from a donor with hepatocellular carcinoma with concomitant infection with the Hepatitis C virus.

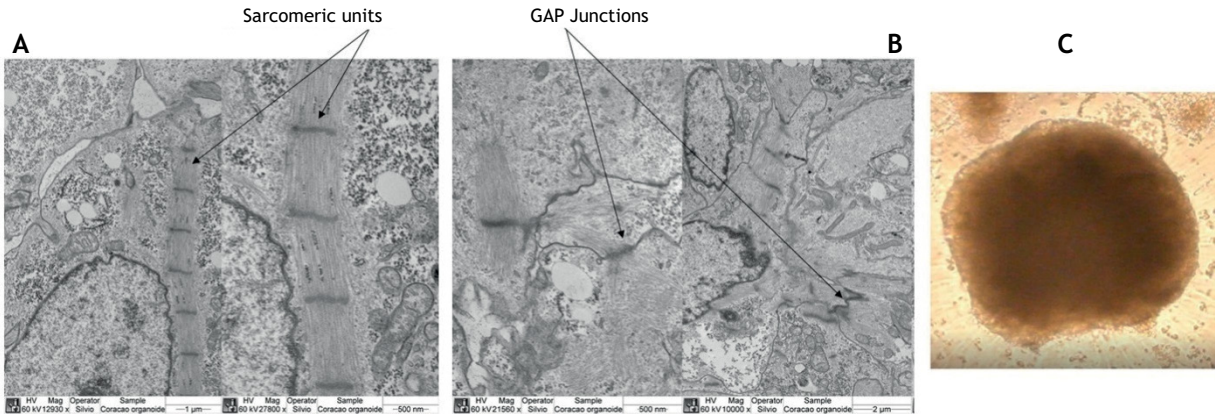
HepaRGs are progenitor liver cells capable of giving rise to fully differentiated adult hepatocytes and cholangiocytes. They have significant levels of hepatic functionality such as stable expression of CYP450 family enzymes and enzymes that act in drug conjugation (phase 2) and support the full replication cycle of hepatitis B virus[101]. The second produces cells of the intralobular connective tissue that present phenotype similar to that of myofibroblasts or lipocytes. These cells participate in the extracellular matrix homeostasis and liver repair, regeneration and fibrosis processes. The proliferation and migration of these cells, together with the expression of chemokines, are involved in the pathogenesis of hepatic inflammation and fibrogenesis.

In general, the culture of hepatocytes as spheroids has shown positive results with respect to hepatic function, due to the establishment of the homo and heterotypic cell-cell contacts and the presence of key components of ECM in and around the



A) Microscopic image of CaCo2 cells, with 10x magnification. B) Microscopic image of HT29-MTX cells, with 10x magnification. C) Coculture of CaCo2 and HT29-MTX cells, with 10x magnification. D) Schematic drawing of the equivalent model of intestinal barrier made in LNBio. E) Light microscopy image of differentiated 2D HepaRG cells, with 10x magnification. F) Light microscopy image of human star cells in 2D culture, with 10x magnification. G) Photograph of hepatic spheroids formed and collected. H) Hepatic spheroids integrated into the 2-OC - TissUse GmbH device. I) Light microscopy image of a hepatic spheroid, with 4x magnification.

Figure 3. Organoids of human intestines and liver - equivalent to the human intestinal barrier.



AB) Electron microscopy images of histological sections of cardiospheres or equivalent human heart. A) Highlights to the sequence of sarcomeric units, mitochondria and glycogen deposits in sarcoplasm. B) Highlight for GAP type junctions. C) Light microscopy image of a cardiosphere, with 4x magnification.

Figure 4. Human heart organoid - heart equivalent.

aggregates[69]. Figure 3 shows hepatic cells under 2D culture (Figure 3E and 3F), and the respective coculture in the spheroidal histoarchitecture (Figure 3G and 3I) integrated in the 2-OC (Figure 3H). The intestine and liver equivalents were kept under coculture at 2-OC for 14 days.

The human cardiomyocytes used for confection of the cardiosphere were generated from the reprogramming of blood cells (erythroblasts) obtained from a healthy 38-year-old male. The iPSC were differentiated into adult cardiomyocytes. The Brazilian company PluriCell Biotech provided the already differentiated cardiomyocytes, used for the confection of the organoids in the LNBio. When formed, the spheres (Figure 4C) were integrated into the 2-OC platform and cultured for conducting pilot experiments. Electron microscopy analysis of section transmission of cardiospheres allowed the identification of cellular structures compatible with a healthy cardiac cell, such as sarcomeric units, GAP junctions, intact mitochondria, glycogen stores, membranes and intact membranous compartments (Figure 4 A and B).

Interestingly, the work of Luni et al. has shown that the process of reprogramming human somatic cells into iPSC is highly influenced by the microfluidic environment (a 50-fold improvement over the most efficient reprogramming reported using human cells without genetic modifications), with direct differentiation into hepatocytes and functional cardiomyocytes in the same platform without an additional expansion step[102].

Prospects and challenges

Despite the great advances already made, human tissue engineering in three dimensions (3D) for the production of organ equivalents (at different scales and with different objectives), as well as the microfluidic devices, are in development phase. Advances in the management of embryonic stem cells and iPSC have already made great contributions and may expedite the progress of this field by providing access to several cell lineages for organoid production.

Vast prospects are also found in the field of personalized medicine with the possibility of "patient-specific" cell cultures[103,104]. The iPSCs are obtained from primary cells of the human body, such as blood cells (erythroblast), fibroblasts (obtained from skin), urinary tract epithelial cells (obtained from urine), which undergo dedifferentiation and in theory can be artificially redifferentiated into any cell type[105].

Cells of primary origin, although superior in functionality, are disadvantageous in aspects of logistic complexity, genetic variability (which may be beneficial only in some situations) and phenotypical/physiological instability *in vitro*. Cells from immortalized lineages have the advantage of simple access and handling, but their functionality at this time is smaller than that of primary cells. Despite the fact that adopting good laboratory practices is the most critical factor to ensure that cell cultures are free of various contaminants, lineage cells are at increased risk of contamination by *Mycoplasma*[106] and/or inter- and intra-species contamination and genetic instability[107,108].

Therefore, the availability of cell types, their adaptability and the most appropriate choice of cellular source for making increasingly realistic and stable organoids in MPS are some of the challenges for the establishment of the Human-on-a-Chip technology.

Furthermore, it is important to consider the need to develop a culture medium whose formulation/composition is optimized to meet the needs of the different cell types cocultured in multi-organ MPS. The choice of the medium is made according to each cell type, in order to improve adherence to the extracellular matrix, post-thaw viability, growth and replication. Situations where a cell type survives and grows perfectly in a medium in which another cell type does not go well are very common.

These factors make the formulation of a culture medium that is suitable for different tissues very challenging. The development of a culture medium formulation is one of the bottlenecks for the advancement of MPS technology. It is important for all types

of cell cultures (including 2D, no flow, no tissue coupling etc.) and it is often limiting to the integrated culture of two or more tissues. A study by Oleaga et al., published in 2016, opened a very encouraging prospect by demonstrating in MPS the simultaneous, successful 14-day culture of liver, heart, skeletal muscle, and neuronal tissue models in a circulating culture medium and free of FBS[75].

The variation occurring between batches of animal sera, especially fetal bovine serum (FBS), is critical and may interfere with drug development tests, for example[23]. Factors like the lack of knowledge on the exact composition of FBS, the occurrence of batch-to-batch seasonal and geographical variability[109] and unintended interaction with test substances[110,111] may lead to inconsistent results, concerns regarding laboratory staff safety in terms of health risk from accidental contact with endotoxins, mycoplasma and viral contaminants or prion proteins[112,113,114]; unexpected shortage in global availability[115,116], as well as ethical concerns about fetal distress[117]. In this sense, in favor of the replacement or elimination of FBS use, there are safety, scientific and ethical aspects, in other words, the search for the optimization of *in vitro* test systems, as well as the commitment to the 3Rs principles encourage the development and adoption of FBS-free culture medium formulations either chemically defined or with the use of replacement components[118]. Recently, human platelet lysates have been shown to be a promising alternative to FBS[116,119, 120, 121,122].

Another important aspect to be considered is that the pharmacokinetic profile study should precede toxicological and pharmacodynamic studies. This is because in all cases exposure to the effect should be related and the exposure will only be known through pharmacokinetic studies. For this, knowing the partition of drugs in the organs, distances of diffusion and metabolic rates is fundamental, with the intention of emulating the *in vivo* communication between the organs[123]. Therefore, a fundamental factor in achieving success is the proportional scaling of different organoids in order to reflect the relationship that occurs in the human body. The choice and application of an appropriate scaling method will ensure that a drug reaches the organs in concentrations similar to those expected in patients. Therefore, determining the principles and rules for sizing different MPS is one of the most critical steps for the development of MPS with great impact on future use in drug development studies[123,124]. Correct scaling will also ensure that paracrine factors reach other organs at physiological concentrations while maintaining the correct organ-organ coupling ratio in the MPS.

There are several types of scaling with different focuses or methods, of which we can cite: allometric scaling (the scale between the organoids and their human counterparts and each other), most commonly used[125,126], functional scaling[127] and multifunctional scaling[128] (which considers parameters like the compartmentalization of organoids in fluidic circuits, flow, routes of administration/excretion) and also the scaling based on organ volume and blood flow residence time[129]. The problem is that the most commonly used approaches to scaling such as direct miniaturization and allometric scaling are based on physical size only[128]. For further studies, there are reviews on scaling methods available in the Human-on-a-chip context[14,123,130,131,132,133].

To overcome the challenge of successful coculturing of different equivalents of *in vitro* interconnected human organs in an autonomous homeostasis situation, we must overcome other obstacles like the absence of synergistically integrated lymphatic, nervous, immune and vascular systems. Vascularization is particularly important. Usually, the cell volumetric density of the organoids in 3D does not correspond to that found in the *in vivo* organ due to pseudo-histoarchitecture and limitations of oxygen and nutrient delivery.

The integration of an endothelial lining into the MPS is a critical measure to improve their physiological performance. The presence of a network of vessels that is able to properly penetrate and perfuse a 300 µM spheroid, for example, will improve the supply of nutrients, oxygen and assist in the removal of metabolites. Other benefits include modulating the diffusion of hydrophilic molecules into the organoid and of the non-physiological strains that could more easily reach the cells[23]. In addition, endothelial cells have the ability to establish a vascular niche and provide *in vivo* and *in vitro* organogenesis.

The combination of robust emulation with the possibility of performing analyses and obtaining electrophysiological data through microelectrodes integrated into the MPS also opens up great prospects. Cardiac, musculoskeletal or neuronal tissues can be studied in this way[135,136]. Measurement of TEER in barrier integrity check on equivalent models of intestines[137], skin or cornea may also be another benefit of this functionality.

Additionally, 3D printers can be of great value in resolving the limitations of current MPS. By providing accurate, orderly and reproducible deposition of cell types and diverse materials, they open up the prospect of making organoids with complex histoarchitecture. In the case of the kidneys or spleen, the printing of human tissues in 3D is very convenient, since they cannot be fully recreated using the currently available techniques[138]. The Organovo company stood out in the area of human 3D tissue printing, offering models of liver (*ExVive™ 3D Bioprinted Human Liver Tissue Model*) and human kidney (*ExVive™ Human Kidney Tissue*)[139]. In addition, 3D printers open up the prospect of optimizing the development and manufacturing process of microfluidic devices, which is often laborious and intricate, requiring large investments and care (e.g. clean room and highly specialized human resources). Current 3D printing technology has advanced to a point where it allows the relatively inexpensive and rapid production of sophisticated microdevices, providing a promising alternative to the currently used protocols[140].

The flowchart of Figure 5 illustrates a proposed approach to the development of MPS in research centers, based on the data and information surveyed and interpreted here. It contemplates the reasons that warrant the investment in the implementation of MPS and the critical steps and aspects to be considered and weighted in this process.

CONCLUSIONS

Despite all the efforts and progress made toward developing experimental models that are as close as possible to the human physiological condition, this is not the most critical factor in the MPS vs. human body relationship. The results or data obtained from these platforms or MPS should not necessarily be realistically physiological. The most important thing is that they are comparable and transposable to the human being. In this sense, having and formalizing principles of extrapolation that enable us to accurately transpose the data obtained from these platforms is as much or more critical than the incessant search for the emulation of the physiology of the human organism *in vitro*.

The progress achieved so far in MPS projects suggests the high potential of this new approach to overcome the limitations of the experimental models used today. They will be able, in a shorter term, to reduce the use of laboratory animals and offer models with greater predictive potential applicable to pharmacology, drug development, disease emulation, personalized medicine as well as encourage the development of better tests that will contribute to the improvement of valuable products and substances in industries like food, cosmetics and agriculture.

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Conflict of Interest

Authors have no potential conflict of interest to declare, related to this study's political or financial peers and institutions.