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The potential of multi-organ-on-chip models for assessment of drug disposition as alternative to animal testing

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Abstract

The development of new medicines suffers from attrition, especially in the development pipeline. Eight out of nine drug candidates entering the clinical testing phase fail, mostly due to poor safety and efficacy. The low predictive value of animal models, used in earlier phases of drug development, for effects in humans poses a major problem. In particular, drug disposition can markedly differentiate in experimental animals versus humans. Meanwhile, classic *in vitro* methods can be used but these models lack the complexity to mimic holistic physiological processes occurring in the human body, especially organ–organ interactions. Therefore, better predictive methods to investigate drug disposition in the preclinical phase are needed, for which recent developments in multiorgan-on-chip methods are very promising. To be able to capture human physiology as good as possible, multiorgan-on-chips should feature 1) human cells endogenously expressing main transporters and metabolizing enzymes; 2) organ models relevant for exposure route; 3) individual organs-on-chip connected in a physiologically relevant manner; 4) a tight cellular barrier between the compartments; 5) organ models properly polarized in 3D; 6) allow for sampling in all major compartments; 7) constructed from materials that do not absorb or adsorb the compound of interest; 8) cells should grow in absence of fetal calf serum and Matrigel; 9) validated with a panel of compounds with known characteristics in humans; 10) an integrated computer model translating concentrations to the human situation. Here, an overview of available systems is presented and the difficult route towards a fully validated system is discussed.

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Efficacy, safety, and drug disposition in humans: lost in translation

For many severe, fatal, and debilitating diseases, treatment options are scarce, insufficient, or do not exist at all. Unfortunately, the drug development process is slow and extremely expensive. Regulatory authorities such as the United States Food and Drug Administration and the European Medicines Agency require the use of experimental animals to test absorption, distribution, metabolism, and excretion (ADME), efficacy and safety. It takes around 12 years to bring a drug to the market with costs estimated to amount to at least hundreds of millions of dollars up to $ 2.6 billion per drug that reaches the market [1]. The main reason for costs in terms of time and funds is the high attrition rate [2]. Currently, only one out of nine drug candidates (11%) that enters the clinical study phase is successfully introduced into the market. The main factor causing attrition is the lack of efficacy and safety in humans, even though available results from animal tests predict otherwise. The result of this is that effective medication for many severe and debilitating human diseases is currently not available.

It is commonly known that effects in experimental animals exposed to a compound of interest can be very different from effects observed in humans. This has been shown for pharmaceuticals [2–5] and for chemicals, especially for their carcinogenicity [6–9]. One of the main causative factors for the low predictivity observed for safety and efficacy when comparing experimental animals and humans is that drug disposition reflected by ADME processes is very different in experimental animals and humans. In other words, ADME of drugs determines their safety and efficacy profile, therefore predicting these processes (and drug metabolism and pharmacokinetics (DMPK), a related,

highly similar concept used for pharmaceuticals) in the preclinical phase is of the utmost importance. In a review by Shanks et al., the concentration of a specific compound that reaches the blood upon oral exposure is compared between several commonly used experimental animal species and humans. The plots show a shotgun pattern when human data were plotted against animal data [10]; the correlation between animal and human data was very low. A recent systematic review comparing human and animal renal clearance measured for 20 renally excreted drugs showed that rats significantly overestimate human renal clearance [11]. Clearly, results in animals do not always translate well to humans.

Despite this loss in translation, animal tests are obligatory for both pharmaceuticals and chemicals marketing. For instance, pharmacokinetic (PK) studies are routinely performed in animals in the drug discovery phase. Typically, these studies involve intravenous and oral administration of a clinically relevant formulation in rats followed by a thorough study of the disposition of a potentially new drug within the organism [12].

Another factor, that is quickly becoming more relevant, is the societal pressure to develop alternatives to animal tests because of ethical considerations. The most recent manifestation is that the US EPA has published a directive in September 2019 in which is stated that funding for animal testing will be reduced by 30% in 2025 and will be abolished completely by 2035.

Classic cell culture usually involves the culture of a commercially available cell line as a monolayer on a 2D surface (culture dish, flask, or wells plate). Medium is applied statically on top of the cells so that they receive nutrients, and periodic replacement is needed to provide fresh nutrients when the medium has been exhausted. Cells and immortalized cell lines are often derived from animal sources (e.g. MDCKII, LLC-PK1 cells) or human tumors (e.g. Caco-2, HT-29 cells). A notable shortcoming of such cell lines for investigation of ADME-DMPK is that they often lack metabolic competence; this means that toxicity can be both overestimated and underestimated because potentially toxic metabolites might not be formed, while a toxic parent compound may not be metabolized into nontoxic metabolites (e.g., Ref. [13]). Such simple systems do provide relevant information about basic features of the PK profile (e.g. intestinal absorption, hepatic metabolic rate, or transporter interactions) or *in vitro* toxicity profile of parent compounds, but not much more than that. Clearly, if we want to investigate more complex processes in humans, we need a higher level of complexity.

At the moment, fully validated alternative *in vitro* models to test ADME-DMPK are not yet available or at least not yet ready for implementation. But there have been exciting developments in the *in vitro* field in the last decade. Landmark studies on organoid- and organ-on-chip technology have shown the great potential of these technologies to replace the animal as the preferred model to predict the effects on humans. Examples include 1) the improved prediction of treatment efficacy for cystic fibrosis patients is made possible by culturing intestinal organoids [14], 2) the development of organ-on-chip systems in which cells are grown on tubular structures, which improves maturation and differentiation into multiple cell types, allowing for the investigation of the transport of substances across epithelial cell layers from a microfluidic flow mimicking vascular flow [15–17] and 3) the development of hepatic spheroids that express bile acid transporters [18]. Moreover, connecting multiple individual organ-on-chip systems can generate a system with which ADME-DMPK of pharmaceuticals, chemicals, or nutrients can be assessed.

Using multiorgan-on-chip technology to assess ADME-DMPK *in vitro*

Organ-on-chip revolves around the culture of one or more cell systems in a small chamber or bioreactor with a laminar flow, providing nutrients and appropriate levels of shear stress. An organ-on-chip system constitutes the following elements: 1) microfluidic chip, a credit card size device containing microchannels for medium flow and microchambers for cell culturing; 2) compartment to culture cells (commercial cell line, primary cells, stem cell–derived cells or organoid) or tissue segments/slices; 3) microfluidic flow through one or multiple microchannels/tubing providing culture medium and a test compound of interest and may additionally constitute: 4) optionally: scaffold or 3D gel on which cells are grown to simulate physiological structures or create barrier models; 5) Optionally: biosensors to measure endpoints such as shear stress, metabolism, barrier integrity and/or viability, or bio-actuators that stimulate relevant physical stimuli.

Using organ-on-chip, cells can be kept in culture for much longer before they dedifferentiate, undergo apoptosis or become senescent, opening the door to *in vitro* investigation of long-term exposures, in contrast to the acute exposures that are investigated with classic *in vitro* methods. Also because microfluidic flows are laminar, they only mix by diffusion, which means that biochemical gradients can be achieved and controlled more accurately permitting better control of the tissue microenvironment. Another advantage of shear stress induced by microfluidic flow on the cells is the fact that increased cellular maturation as well as differentiation into multiple cell types (especially for adult stem cells and induced pluripotent cell types) present in the organ of origin can be achieved, for example, for kidney [19] and intestine [16,20]. For ADME-DMPK specifically, a very relevant characteristic of organ-on-chip technology

is that multiple organ models in one or more chips can be interconnected. When each of those chips features a barrier system, the uptake, transport, and metabolism of a compound through the system can be monitored. Such a system can be based on a 2D membrane or, depending on the physiological structure of organs, on a hollow fiber membrane mimicking functional parts of organs such as the kidney (tubules [21]), gut (intestinal tube [16]), or liver (bile duct [15]). Different components would be present on either side of the membrane/hollow fiber, and transepithelial transport of compounds can be investigated by this approach.

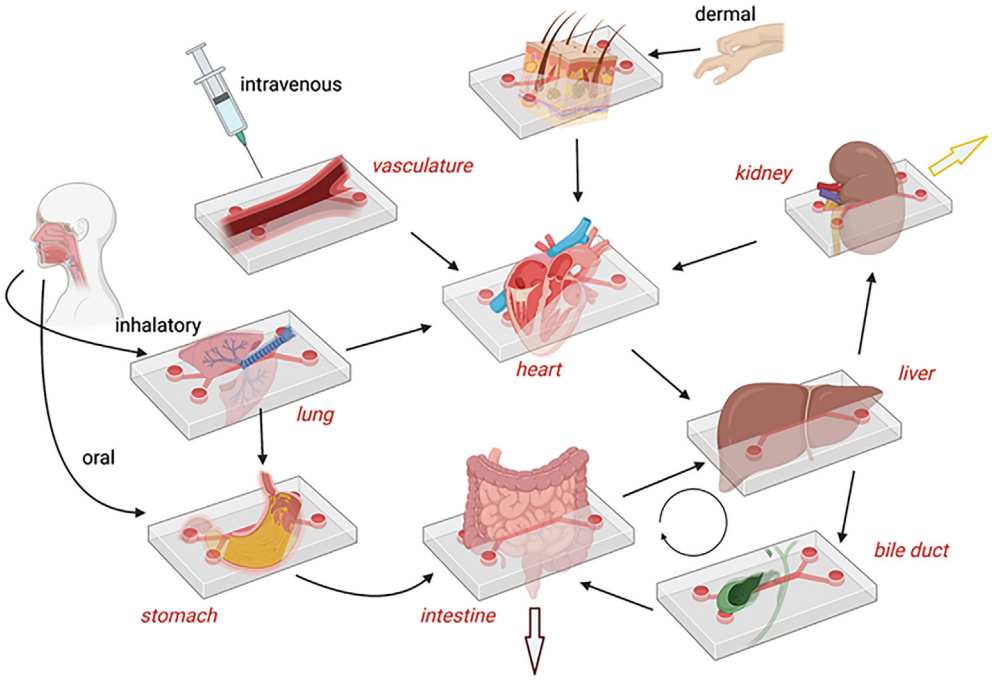
Of course, the selection of the appropriate individual organ modules in multiorgan-on-chip models highly depends on the mimicked exposure route. Examples of individual organ-on-chip systems that could be integrated in a multiorgan-on-a-chip system for ADME-DMPK assessment are summarized in Table 1. Figure 1 shows how such individual organ-on-chip modules could be connected to physiologically mimic the oral, intravenous, dermal, and inhalatory exposure routes. Here, it should be noted that different setups are possible and can have their value for specific purposes. To be able to completely replace the animal test for ADME-DMPK assessment of a novel compound, it will be necessary to integrate organ-on-chip modules for most relevant organs (or even multiple modules per organ for different organ parts, for example, the proximal tubule and the glomerulus for the kidney or the alveolar and bronchial regions for the lung). Sometimes very specific questions related to ADME-DMPK need to be answered, for which a more limited number of organ-on-chip modules would be appropriate. It can be important to investigate the interaction between two organ systems, for instance. Importantly, an ADME-OoC system should only be as complex as necessary because, inevitable, there are drawbacks to a more complex system (it certainly is more technologically challenging, but also problematic for validation of such a system, for achieving sufficient cellular function and viability in all integrated cell systems and so forth).

Table 1 Examples of single organ-on-chip models needed to create an ADME-DMPK-OoC model for each of the exposure routes.

Exposure route	OoC systems needed	Examples
Dermal	Skin-OoC	[34,35];
	Heart-OoC	[36,37];
	Liver-OoC	[38,39];
	Kidney-OoC	[40,41];
	Bile duct-OoC	[42];
	Intestine-OoC	[16,43];
Intravenous	Heart-OoC	See dermal exposure route
	Liver-OoC	See dermal exposure route
	Kidney-OoC	See dermal exposure route
	Bile duct-OoC	See dermal exposure route
	Intestine-OoC	See dermal exposure route
Oral	Stomach-OoC	[44];
	Intestine-OoC	See dermal exposure route
	Liver-OoC	See dermal exposure route
	Kidney-OoC	See dermal exposure route
	Bile duct-OoC	See dermal exposure route
Inhalatory	Lung-OoC	[45–47];
	Heart-OoC	See dermal exposure route
	Liver-OoC	See dermal exposure route
	Kidney-OoC	See dermal exposure route
	Bile duct-OoC	See dermal exposure route
	Intestine-OoC	See dermal exposure route

From a technical point of view, research groups that work on multiorgan chips have adopted three strategies to connect individual organ models in one system, as shown in Table 2. The first is to design one chip with different organs that are connected to each other by a simple gravity-driven flow. This is to decrease the complexity of the operation and, therefore, allow increasing the throughput of the experiments. Because of the simpler layout, these systems often benefit from more inert materials to pharmaceutical compounds, avoiding drug absorption and adsorption. The drawback of such systems is that the simplicity of the design reduces the complexity of the microenvironment in the integrated organs. Prof. Michael Shuler and Prof. James Hickman (co-founders Hesperos) are among the pioneers of pumpless multiorgan chips [22]. The second strategy keeps the different organ models in one chip but adds on-chip, e.g. TissUse [23] and CN BIO [24], or off-chip, e.g. Prof. Lecler's lab [25], peristaltic pumps to the system. This method enables researchers to use more physiologically relevant models, especially barrier models, in their multiorgan chips. This, consequently, comes with a price of more difficult operation and lower throughput than the first strategy. These two first strategies give us the opportunity to use standard static organ models, such as Transwell inserts, in a dynamic microenvironment of a microfluidic system. Although limited, sensors can also be integrated in these systems. The third strategy goes one step further in technical aspects and adds more physiological relevance and sensor technologies. The idea here is to model each organ in separate chips and connect them with tubing to each other to create a modular and independent multiorgan model [26]. This physiological complexity creates technical complexities which makes the operation of such systems more difficult and thus their throughput lower. Up to now, the physiological complexity of the multiorgan chips seems to be proportional to their technical complexity and inversely proportional to the throughput of these systems. More automation, standardization, and high-throughput readout technologies can help improve the relationship between complexity and throughput [27,28].

Figure 1



Main exposure routes (black text) for pharmaceuticals and chemicals and their disposition in relevant organ systems (red text) to be evaluated by a potential multiorgan-on-chip system; created with BioRender.com.

The road ahead for drug disposition on a chip

Multiorgan-on-chip technology is still in its infancy, and many challenges need to be overcome before a mature, fully validated ADME-chip system is ready to be implemented for regulatory testing. A number of technical challenges that need to be overcome include the following:

- Connection of the different individual chip modules is a challenge; this should be done in a physiologically relevant manner and the individual systems should receive the appropriate amount of shear stress in order for the cells to properly differentiate and mature.
- It can be difficult to keep such a complex system bubble-free and sterile to prevent infection with pathogens when handling it; for some organ-on-chip systems, this known to occur when changing medium or treating cells. Automation/robotization of the system can be a great asset in this regard as well as the integration of microsensors.
- Development of a universal medium that does not only keep all different cells alive, but also keeps them functional can be challenging. Due to the laminar flow in a microfluidic system, it might be possible to let various medium types flow through the system; otherwise, separated and isolated medium flows should be integrated into the system. When using organoid-based models, general organoid medium can work quite well (e.g., Refs. [29,30]).
- Also, to be a truly animal-free test method, it will be necessary to avoid the use of FCS and Matrigel.
- The choice of material for 3D printing of the chips and support structures is important: while PDMS is convenient and practical to work with, it is also known to be able to bind compounds and can release them in an unpredictable manner, which greatly limits the control one has over the intended compound concentration at a cellular site.

Clearly, the more complex an *in vitro* system is, the more difficult and time-consuming it will be to validate it [31,32]. Standardization of all the different components (cell system, 3D-printed chip, scaffold, medium, microfluidic flows, endpoints measured via biosensors etc.) is needed; each individual cell system should be tested for viability, functionality, and barrier function. Moreover, each individual system should still function properly after connecting them to form a predictive drug disposition system allowing study of ADME processes. Standardization of such a system, which is needed to ensure that reproducible results can be achieved, requires integration of microsensors to monitor relevant model parameters and could benefit greatly from extensive automatization/robotization.

Table 2 Multi-OoC systems in the scientific literature applicable for ADME-DMPK assessment.

	Group	Organs	Standardization	Technical complexity	Throughput/ automation	Material	Complexity of microenvironment	Sensors
ultiorgan system in a single chip with gravity-driven flow	Shuler and Hickman lab (Hesperos)	- Liver and intestine [48,49] - Liver and bone marrow [22,50] - Liver, tumor and marrow [50] - Liver and heart [51] - Liver, heart, muscle and neurons [52] - Liver, fat, kidney, bone marrow and intestine [53]	++	+	+++	++	+	++
	Sung lab (Hongik University)	- Liver, intestine [54–57] - Liver, tumor [58]	+	++	++	+	+	+
	Insphero (Akura™ Flow)	- Liver and tumor [59–61]	++	++	+++	+++	+	+
Multiorgan system in a single chip with peristaltic flow	TissUse (Humimic)	- Liver and neurospheres [62] - Liver and skin [63–65] - Liver and intestine [63] - Liver and lung [66] - Liver and testis [67] - Liver and kidney [68] - Tumor and skin [65]	++	++	++	++	++	+
	Griffith lab (MIT & CN BIO)	- Liver and intestine [24,69,70] - Liver, intestine, lung, heart muscle, brain, pancreas, skin and kidney [71]	++	++	++	+++	++	+
	Leclerc lab (CNRS Paris)	- Liver, intestine [25,72] - Liver and pancreas [73] - Liver and kidney [74]	++	++	++	+	+	++
Multiorgan system with multiple chips	Ingber lab (Wyss Institute)	- Gut, liver, kidney, heart, lung, skin, brain, vasculature [26]	+	+	+	+	+++	+
	Shrike (Harvard) & Khademhosseini (UCLA) lab	- Liver and heart [75] - Liver, lung and heart [76]	+	+	+	+	++	+++

A novel *in vitro* model is validated using a fit-for-purpose approach (i.e. the model works for a specific purpose intended). When an ADME-chip is to be used to assess pharmaceuticals in a contract research environment, it should usually comply with the good laboratory practice (GLP) standard which has significant implications; validated and highly standardized protocols are required for cell culture and all used equipment. Implementation in the R&D lab of a specific pharmaceutical company depends on the needs and preferences of that specific company, in terms of regulations little is imposed by the authorities. For regulatory testing of chemicals and food components however, strict regulations exist: an *in vitro* assay should be submitted to the European Union Reference Laboratory for alternatives to animal testing (EURL-ECVAM) so it can be validated by a test laboratory accredited by the European Union Network of Laboratories for the Validation of Alternative Methods (EU-NETVAL). Extensive guidance on how to develop a novel *in vitro* assay is given in the guidance document on Good In vitro Method Practices (GIVIMP), which has been drafted by the OECD [33]. Validation by EURL-ECVAM can be a lengthy procedure and it is important that a system is finalized at some point, because the introduction of small changes may implicate that the system needs to be re-validated. This is highly challenging in a field that is evolving so rapidly; the decision when to start a laborious and lengthy validation of a multiorgan-on-chip system is a tough call to make.

To test predictivity of drug disposition in a multi-organ-on-chip system, its performance should be validated against both human (gold standard, but availability can be limited) and animal (when human data are not available or not useful) ADME-PK data. Measurement of the concentration of parent compound and their metabolites in each organ compartment (e.g. blood and urine for the kidney, blood and bile duct for the liver, blood and gut lumen for the gut) using quantitative methods such as liquid chromatography-tandem mass spectrometry would be recommended. An integrated physiologically based PK model should be developed to translate the concentrations and volumes in the different compartments of the multiorgan-on-chip (e.g. bile, blood, urine, feces) to their human equivalent. Preferably, compounds should be included in the test battery that are well predicted by current methods (*in vitro a*nd *in vivo*) as well as compounds for which current systems fail to predict the human ADME properties. Examples of such compounds are well-known drugs such as antipyrine and verapamil (high permeability), theophylline and atenolol (low permeability), high organ extraction compounds such as propanol and morphine), and substances with more complex ADME characteristics include substrates for drug transporter proteins and/or metabolizing enzymes (diclofenac, digoxin, atorvastatin).

This will facilitate thorough assessment of the performance of the model in relation to animal experimentation. Important to note is that human data are the gold standard here, not animal data; a model that would excellently reproduce data obtained in experimental animals would still be of limited value when attempting to predict effects in humans.

Box 1. Recommendations/requirements for an *in vitro* system designed to mimic drug disposition/ADME.

1) Cells should be of human origin and should express functional transporter systems and metabolizing enzymes at physiological levels;
2) Selected chip modules mimicking specific organ functionalities models should be incorporated for the organs relevant for the exposure route of interest;
3) These models should be connected in a physiologically relevant way;
4) Cells should form a tight barrier between the different compartments relevant for ADME/PK that are mimicked by the system, such as blood, urine, feces, bile etc.
5) Organ systems should be properly 3D-polarized (*i.e.* transport should occur from apex to base or the other way around, just like in the actual organ);
6) Preferably, the system should allow for longer term cell culture, so ADME might be investigated upon a repeated dose regimen;
7) The system should allow for sampling in all the major compartments that are mimicked (e.g. blood, urine, bile, airway lumen or feces depending on the route of exposure), allowing for monitoring of transport through the system and metabolism of the compound of interest;
8) The system should be constructed from materials that do not absorb/adsorb the compound of interest;
9) Cells should grow in absence of FCS and Matrigel for the system to be truly animal-free and thus a real alternative to the animal test;
10) The system should be validated for a carefully selected panel of compounds with known ADME-DMPK in humans, using appropriate positive and negative controls;
11) Integrated physiologically based pharmacokinetic (PBPK) computer model translating *in vitro* findings to the human situation, that is, by upscaling the volumes in the individual organ-on-chip model compartments (blood, urine, bile feces etc.) to the volumes of respective compartments in people so that concentrations in humans can be predicted;
12) The system should incorporate a number of microsensors so that model parameters can be closely monitored; preferably, it should be automated/robotized as much as possible to facilitate standardization, which is difficult for such a complex system.

Conclusions and future perspective

Groundbreaking work has been performed in which steps have been taken towards the creation of multi-organ-on-chip models. In Box 1, the main characteristics for a multiorgan-on-chip system to provide a true alternative to an animal ADME-DMPK study are summarized. At present, none of the created models fulfill all of the criteria mentioned. When striving to create a model capable of improving on (and replacing) animal tests, close collaboration is needed between a great number of disciplines, that is, experts in cell biology, microfluidics, 3D printing, microelectronics, physiologically based PK modeling, artificial intelligence/machine learning and robotization should or could be linked up. Moreover, connections to R&D labs from the pharmaceutical, chemical and food industry as well as regulating agencies will be crucial to ensure that a system can be readily implemented and complies with regulatory, quality-related and practical requirements.

The complexity of such collaboration between stakeholders from diverse backgrounds requires setting up connective structures (*i.e.* hubs) from where stakeholder communication and project management is coordinated and where a database is present with information on experts in relevant fields. Independence of such structures/hubs would positively affect collaboration.

It is clear that the road ahead is long and is filled with plenty of obstacles, but the rewards upon achieving the goal (a fully developed and validated multiorgan-on-chip model) are great: faster and more cost-effective drug development resulting in better availability of treatment for all kinds of diseases, better testing of chemicals and food components and replacement of ethically sensitive animal tests by *in vitro* methods.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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In this review, the authors show that the bioavailability of a drug (i.e., the concentration that reaches the blood) is very different in humans compared to the most commonly used species in pre-clinical animal experiments, i.e. dogs, primates and rodents.

11. ** Jansen K, Pou Casellas C, Groenink L, Wever KE, Masereeuw R: **Humans are animals, but are animals human enough? A systematic review and meta-analysis on interspecies differences in renal drug clearance**. *Drug Discov Today* 2020, **25**. https://doi.org/10.1016/j.drudis.2020.01.018.
Species differences in transporter expression or substrate affinity might lead to prediction errors and limit the translatability of animal data. In an attempt to quantify differences in renal drug clearance between animals and humans, a systematic review and meta-analysis was conducted using published clearance data for a diverse set of renally excreted drugs. Identified differences were related to the drug excretion profile (filtration vs active secretion) and physicochemical drug properties (e.g., physiological charge and molecular weight). The authors demonstrated that rats, despite being the most commonly used model, are an inadequate species for preclinical renal drug clearance testing.

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The authors show that the predictive potential of advanced in vitro methods can be superior to animal tests. By studying patient-specific mutations in the cystic fibrosis transmembrane conductance regulator the authors show that the drug responses observed in mini-guts or rectal organoids can be used to predict which patients may be potential responders to the drug. Such organoids can be implemented in multi-

organ-on-chip systems, allowing for standardized and high-throughput analysis.

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A state-of-the-art bile duct model is described in this paper. Liver stem cells from mice were differentiated into cholangiocyte-like cells that were grown on hollow fiber membranes. This model can be integrated in a multi-organ-on-chip approach used to assess ADME-DMPK.

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The well-known and well characterized Caco-2 cell line was grown on an ECM-coated hollow fiber membrane under physiological shear stress. This approach resulted in improved phenotype, i.e. a more varied cellular population closer to the cellular variety observed in the human intestine.

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