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Microphysiological systems for ADME-related applications: current status and recommendations for system development and characterization

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Over the last decade, progress has been made on the development of microphysiological systems (MPS) for absorption, distribution, metabolism, and excretion (ADME) applications. Central to this progress has been proof of concept data generated by academic and industrial institutions followed by broader characterization studies, which provide evidence for scalability and applicability to drug discovery and development. In this review, we describe some of the advances made for specific tissue MPS and outline the desired functionality for such systems, which are likely to make them applicable for practical use in the pharmaceutical industry. Single organ MPS platforms will be valuable for modelling tissue-specific functions. However, dynamic organ crosstalk, especially in the context of disease or toxicity, can only be obtained with the use of inter-linked MPS models which will enable scientists to address questions at the intersection of pharmacokinetics (PK) and efficacy, or PK and toxicity. In the future, successful application of MPS platforms that closely mimic human physiology may ultimately reduce the need for animal models to predict ADME outcomes and decrease the overall risk and cost associated with drug development.

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Introduction

Opportunities for MPS in ADME sciences

In pharmaceutical drug discovery and development, early ADME screening assays optimized for low cost/high throughput enable the identification of the most promising lead compounds. In contrast, detailed mechanistic investigations are made for a smaller number of potential drug candidates and used to support new drug applications. A well-developed battery of assays to predict ADME endpoints and human PK using human-specific reagents now exists. Yet gaps remain, and the field will benefit from advances in cell culture techniques such as those provided by microphysiological system (MPS) technologies.[1] Opportunities include the ability to evaluate ADME or toxicology endpoints for a longer duration of time under more physiologically or pathophysiologically relevant conditions.

There are many definitions of MPS in the literature and there is no consensus on what constitutes an MPS. As such, we define MPS as going beyond traditional 2D culture and include several of the following design aspects: a multi-cellular environment within biopolymer or tissue-derived matrix, a 3D structure, mechanical factors such as stretch or perfusion (*e.g.* breathing, gut peristalsis, flow), incorporating primary or stem cell derived cells, and/or inclusion of immune system components. Some MPS platforms may be utilized as fluidically isolated single systems or connected through fluidic circuits to model the function of interconnecting tissues.[2]

One of the most critical parameters determined in ADME assays is metabolic clearance, which guides human dose projection. Both the decision to move a compound to the clinic and the early clinical development plans rely heavily on the projected dose. Primary cells such as hepatocytes are considered the gold standard for studies such as metabolism-based clearance. However, a limitation to their use is the loss of metabolic function over time in culture.[3] This makes accurate prediction of low clearance compounds very challenging. Recently, relay incubation methods and longer term hepatocyte culture systems such as HepatoPac™

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and Hurel, have provided some success in filling these gaps.[4–6] However, such solutions are resource intensive and/or have limitations in the accuracy of their predictions.[7,8] Similarly, enzyme induction and time-dependent inhibition (TDI) testing of cytochrome P450 (CYP) would greatly benefit from long-term measurements in a more stable test system since the enzyme expression and activity readouts can be confounded by loss of metabolic function with time in conventional models. Long-term culture is expected to be more stable, recover lost enzyme (*e.g.* with TDI) and may be more sensitive to effects from secondary metabolism.[9] Additionally, our success in improving metabolic stability in the design of molecules has resulted in a shift towards transporter mediated clearance. However, the ability to measure and extrapolate transport mediated clearance is currently limited. An MPS model capable of both metabolism and bile flow, which could be sampled for LC-MS/MS analysis would be particularly attractive.

In vivo predictions are based on assay validation through *in vitro* to *in vivo* extrapolations (IVIVE). These are typically constructed by physiological scaling of the *in vitro* data and comparison with the *in vivo* results using a diverse set of compounds.[10] In addition to clearance, IVIVEs have been reported for other endpoints such as induction and drug–drug interactions.[11–13] Mathematical treatment of the data beyond physiological scaling is sometimes required to address prediction bias (*e.g.* under-prediction of clearance).[14] The need for such empirical scaling factors is of concern to ADME scientists as it indicates that either some element is missing from the *in vitro* system (*e.g.* low enzyme or transporter expression/metabolism by another tissue) or the method for scaling the data to the *in vivo* situation is deficient in some way. An optimal MPS model, which by design should be more physiological, should only employ scalars that can be explained mechanistically.

Another MPS opportunity would be to study ADME endpoints in models of special populations such as genetic variants or disease. Such models could have utility in predicting or understanding human pharmacokinetic variability. In the case of the liver, certain functions and pathobiology derive from the complex interactions supported by the architecture and heterogeneity found in the intact organ. Reproducing the effects of disease states such as hepatitis C or non-alcoholic steatohepatitis on ADME endpoints may also benefit from MPS models.

Finally, due to the ability to form linked organ microstructures, MPS should be expected to augment or replace simpler ADME assays in reproducing aspects requiring multi-organ function. There might be an opportunity for understanding complicated pharmacokinetic phenomena such as enterohepatic recirculation by linking a functioning model of biliary excretion to a model of gut stability and absorption. Indeed a future whole-body pharmacokinetic and pharmacodynamic simulations or "physiome-on-a-chip" can be envisioned.[15] However, it should be emphasized that such models are some distance from commercial realisation. For such complex linked models to be successful, it is likely that the organotypic functions will first need to be individually optimized and validated. A vision of the potential implementation of MPS in the ADME discipline is represented in Fig. 1. Well-characterized and validated organotypic models could be used in specific context to derive organ level PK parameters such as intrinsic clearance due to metabolism and transport. These endpoints could be scaled with mechanistic or physiological models to estimate whole body PK parameters. Finally one can envision a highly evolved multi-organ chip model which could be used to generate whole body PK parameters with the ability to produce organ–organ interactions and to study PK/PD relationships.

MPS platform requirements for ADME studies

ADME studies involve quantitative measurements of the test compounds (and metabolites) which are required for drug–drug interaction (DDI) and PK parameter estimates to allow for IVIVE as previously discussed. As a consequence:

The authors are actively involved in facilitating the development of MPS for ADME applications within their various organizations, which spans 9 leading pharmaceutical research and development companies. They have come together through the IQ consortium MPS affiliate to share their interest in the evolution and application of MPS systems. In the current article, experience of challenges faced in the discovery and development of new drug molecules is combined knowledge of, and aspirations for, MPS systems. The result is an overview of the current state of the art, which also aims to give some guidance to system developers of where MPS can add most value and suggestions of validation work which could demonstrate this. (Top row: Anshul Gupta, Niresh Hariparsad, Jonathan Phillips, Jane R. Kenny, Wen Li Kelly Chen. Bottom row: David B. Duignan, Jennifer Liras, W. George Lai, Stephen Fowler and Jinping Gan).

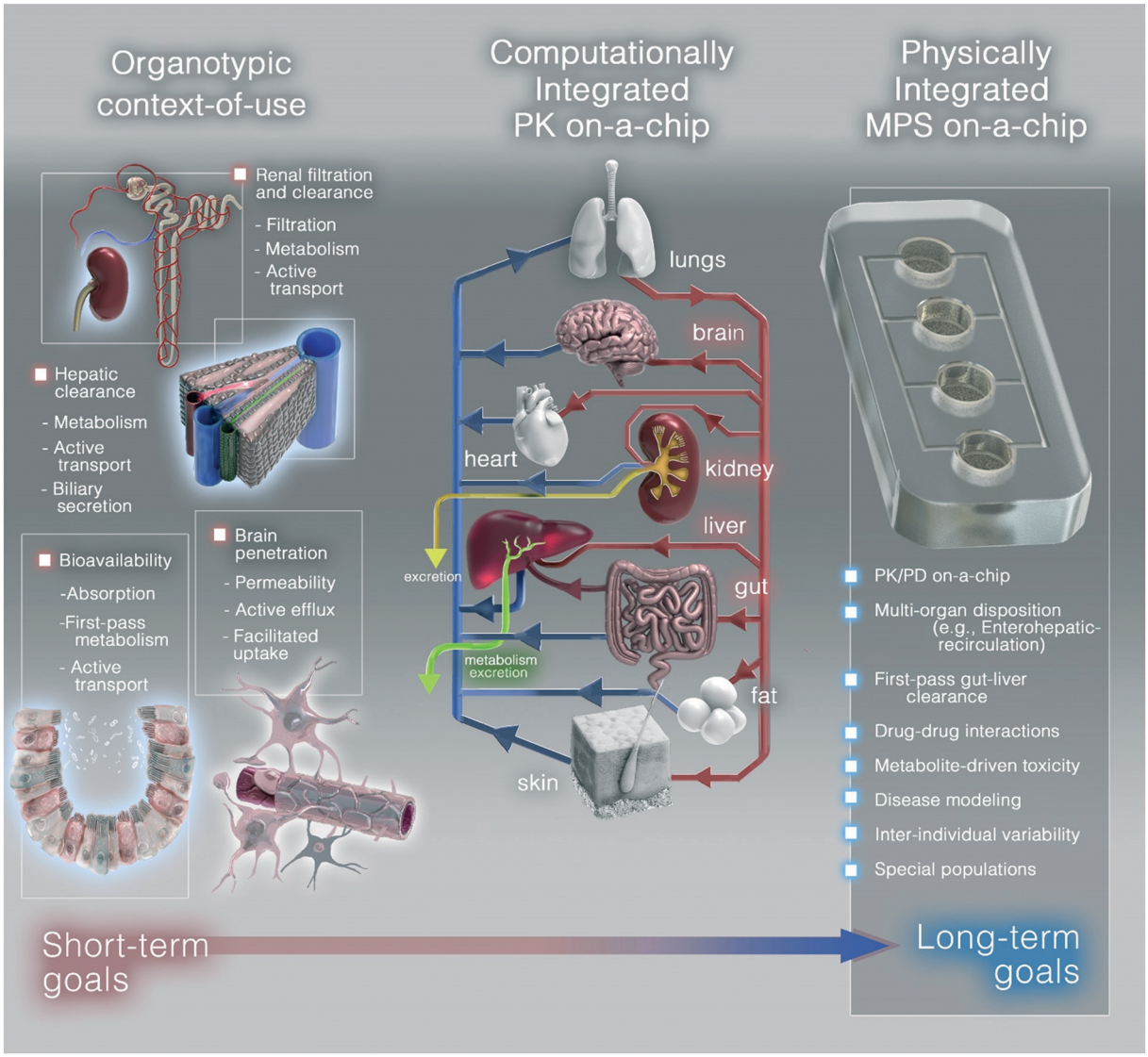

Fig. 1 A schematic to highlight potential applications of MPS systems in the ADME discipline. In the short-term, the focus should be to develop well characterized and validated organotypic models to recapitulate underlying processes (*e.g.* metabolism, transport) that define intrinsic clearance of drugs in organs of distribution and elimination. Such endpoints obtained from organotypic models could then be computationally integrated using physiologic parameters and mechanistic models to determine whole body PK. Finally, the long-term vision of such efforts would be to develop a highly evolved multi-organ chip model by establishing physiologic flow between organs to produce organ–organ interactions which will allow for the study of inter-dependent PK, PK/PD and TK/TD relationships *in vitro*. Illustration by Victor O. Leshyk.

- MPS fabrication material ideally should exhibit low non-specific binding to minimize loss of test article to the material.
- Sterile sampling needs to be possible and practical.
  - System media volumes need to be sufficient such that multiple sampling does not significantly deplete media.
  - Traditional culture media exchange cannot be performed for many ADME studies where drug depletion is measured. This limits cellular lifetime to a few days and accentuates any evaporation issues.
- Sufficient cellular mass is needed in the system to produce measurable metabolism or transport effect.
- Sufficient throughput is needed in an MPS platform to allow triplicate determinations for multiple test compound concentrations and prerequisite positive and negative controls.
- Media recirculation is preferred to allow for adequate metabolic turnover of compounds.

These considerations are important for any organ used for ADME applications and form a starting point in the design of the MPS platforms. Table 1 summarizes design attributes of new MPS models seen as minimum requirements for an ADME setting as well as features desirable to enable additional applications in the future. This table highlights pharmaceutical industry needs to system developers. Table 2 presents basic expectations for system characterization. The goal is to provide guidance for relevant, standardised and straightforward characterization.

Table 1 MPS system design considerations for ADME-related applications

	Intestine	Liver	Brain	Kidney	Linked systems
General system requirements for ADME applications	• Low (<5%) non-specific binding of lipophilic drugs (cyclosporin A, ritonavir, chlorpromazine, saquinavir) in cell-free system • Ability to collect multiple timepoint samples for LC-MS/MS analysis (*e.g.* 20μL) without significant media volume change (<10%) • Low evaporation (*e.g.* <20% over 1 week with no media replenishment) • Highly reproducible system manufacturing (low test-to-test variability; CV < 5% on system assessments (cell-free)) • Direct fluid sampling from all system compartments (especially barrier tissues and multi-organ systems) • Ability to access cell compartment for mRNA and cellular uptake measurements at end of study • Live and post-experiment imaging of microtissues possible • Continuity of supply – cells and/or system consumables available on demand • Usability – high quality data can be generated successfully in multiple labs				
Future development considerations for system design	• Recirculating systems preferred to allow multiple passes of compound past the cells • On-board sensors for continuous monitoring of oxygen, pH, TEER • Compatible with live-imaging • Automated media change, sampling, or drug dosing and unattended operation • Ability to control flow rate to different levels • Can be applied in linked organ systems				
Organ-specific system requirements for ADME applications[a]	- Flow of media to simulate GI flow and enteric blood flow - Demonstrable barrier function - Ability to adjust pH	- Media flow through liver microtissue for scaling to physiologically-based pharmacokinetics (PBPK)	- Demonstrable barrier function - Ease of noninvasive imaging	- Qualitative demonstration of kidney-relevant transporter and/or metabolic activities - Ability to adjust pH	
Desirable for extended applications	- Differentiation to multiple cell types - Multiple regions of the intestine represented - Peristalsis - Modulation of oxygen tension - Accommodation of microbiome	- Multiple cell types to support hepatocyte activities and enable hepatocyte gene expression modulation - Bile canaliculi which can be sampled for biliary clearance and future entero-hepatic recirculation models	- CSF barrier model	- Multiple cell types or regions of the kidney represented	- Physiologically scalable organ composition and fluid flow - Delivery of media from liver to gut to simulate enterohepatic recirculation - Ability to circulate immune cells - Modular platform to accommodate different MPS (gut–liver or liver–kidney)

[a] System requirements: sample volume, flow, recirculation/single pass. See Table 2 for ADME needs.

This manuscript aims to bring a pharmaceutical industry perspective to ADME applications of complex *in vitro* models or MPS platforms. We start with absorption in the intestine and move on to metabolism by the intestine and liver followed by distribution to key tissues such as the brain before concluding with excretion by the kidney and consideration of linked systems. We will set out the current status of adopted technologies, indicate unmet ADME needs and propose MPS platform characterization to enhance system comparability and enable more rapid acceptance and uptake by the pharmaceutical industry. Whilst this manuscript focuses largely on small molecule ADME endpoints, which have been investigated first, the opportunity to develop new, long-term systems to study the distribution and disposition of biologics over a period of many days may prove to be an area where MPS bring significant impact to ADME in the future.

Intestinal models for absorption and first-pass metabolism

Oral delivery is the preferred route for administration of most drugs and attaining adequate oral exposure is of vital importance. The systemic bioavailability of an orally dosed drug is determined by the product of its intestinal absorption (fraction absorbed, Fa), and first-pass metabolism in the intestine (intestinal availability, Fg) and liver (hepatic availability, Fh). Therefore, in addition to minimising hepatic clearance, attaining high intestinal absorption and low intestinal metabolism are key outcomes in drug discovery.

Intestinal absorption is a complex process which occurs mainly in the upper GI tract (primarily the jejunum). The fraction absorbed is dependent on the physiochemical properties of the drug, its dissolution rate, its solubility and

Table 2 Unmet ADME needs and recommendations for baseline MPS characterization for ADME-related applications

	Intestine	Liver	Brain	Kidney (proximal tube)	Linked systems
Unmet ADME needs[a]	- First-pass metabolism on transit through tissue layer for CES2, CYP3A4, UGT substrates	- Measurable biliary and sinusoidal efflux	- Small molecule: quantitative prediction of unbound brain/plasma ratio	- Apical–basolateral transport	- Predict bioavailability of drugs with gut and liver metabolism
	- Can be adapted to gut–liver system	- Scalable uptake clearance	- Large molecule: predict receptor-mediated transcytosis	- Kidney-relevant metabolism	- Effect of induction and inhibition on bioavailability
		- Improved clearance IVIVE for AO, CYP and UGT-metabolised drugs including metabolically stable compounds		- Multi-drug interaction on transport/metabolism	- Distribution tissue (*e.g.* adipose) for closer mimicking of *in vivo* PK situation
		- Correct steady-state prediction of induction and time-dependent inhibition			- Evaluation of disposition pathway of chemicals by a link of liver model and kidney model
Recommended baseline characterisation	- Scalable gut first pass metabolism lovastatin, midazolam & repaglinide (CYP3A4 exhibiting low, moderate and high Fg[177]) raloxifene (UGT) irinotecan (CES2)	- Active and stable CYP, UGT, AO activities. Midazolam metabolism (CYP3A short-term incubation marker), tolbutamide clearance (CYP2C9 long-term incubation marker)[6,62]	- TEER value (>300 Ω cm^2)	- Transepithelial barrier function	- Relative contribution of intestinal and hepatic CYP3A4 metabolism of docetaxel,[180] midazolam, cyclosporine
	- Scalable P-gp and BCRP efflux activities. Markers: digoxin (P-gp); rosuvastatin or topotecan (BCRP)	- Rifampicin and IL6 (CYP3A4 induction/induction suppression of mRNA and enzyme activity) rosuvastatin (OATP uptake)[69]	- Demonstration of P-gp function (efflux ratio of digoxin >2)	- Polymyxin B,[178] gentamicin (megalin, PepT2)	- Bioavailability of drugs (*e.g.*, cyclosporine, midazolam) in the presence of CYP3A4 inducer (*e.g.*, rifampicin) or inhibitors (itraconazole)[181]
		- Canalicular efflux of d8-taurocholic acid	- Demonstration of MPP+ formation from MPTP	- Furosemide, cisplatin (OCT2/MATE1/2K)	- Demonstrate maintenance of tissue-specific function over time (at least 1 week, preferably >2 weeks)
		- Can establish steady state drug exposure for predicting complex DDI	- Demonstration of permeability rank ordering of sucrose, mannitol, morphine, propranolol - Demonstration of uptake transporter activity - Change in verapamil brain exposure ± cyclosporin A - Demonstration of transcytosis of transferrin or insulin	- Tenofovir[179] (OAT1/3, MRP4)	- Controls to demonstrate the contribution of individual MPS to the multi-MPS interactome

Table 2 *(continued)*

	Intestine	Liver	Brain	Kidney (proximal tube)	Linked systems
Current standard(s)	- Caco-2 or MDCK for permeability	- Suspension pooled cryopreserved hepatocytes;	- Non-endothelial cell culture systems (MDCK, Caco-2)	- Kidney microsomes	
	- Stably transfected cell lines for transport[182]	- Cultured (monolayer or sandwich) cryopreserved hepatocytes;	- Brain endothelial cultures in transwells: primary or immortalized cell lines, with or without astrocytes and pericytes	- Isolated primary rat and human proximal tubule cells[183]	
	- S9, intestinal microsomes and cytosol for metabolism	- Long-term hepatocyte coculture systems[6,62,65,68,69]	- Live animal models	- Stably transfected cell lines	
	- Literature Fg values[177]		- Change in verapamil brain exposure ± cyclosporin A	- Kidney tissue slices - Perfused, intact kidney - Live animal models	

[a] ADME needs: superiority to existing system, unique opportunity that is not possible with traditional technologies, disease models/disposition in disease populations.

its membrane permeability. Moreover, permeability and absorption are also affected by the physiology of the lumen environment – pH, mucus layer and microbiome, to name a few. There are multiple mechanisms for drug absorption – passive diffusion, active transport, paracellular and lymphatic uptake. In addition, efflux transporters such as P-glycoprotein (P-gp) and breast cancer resistance protein (BCRP) can limit absorption for poorly permeable drugs. An extensive body of literature exists describing the absorption process in detail.[16–18]

Fg contributes to overall bioavailability for highly metabolized drugs, sometimes quite significantly for CYP3A4 substrates such as midazolam and nifedipine.[19,20] In addition to CYP3A4 (~80% of total CYP content), human intestinal epithelial cells express other CYPs including CYP2C9 and CYP2J2. Besides CYPs, enterocytes express other important phase I and phase II enzymes such as UDP glucuronosyltransferase (UGT), sulfotransferase (SULT), alkaline phosphatase (ALP) and carboxyl esterase 2 (CES2).[21–23] Active transport by enterocytes is also an important factor for the movement of chemicals across the intestinal barrier. On the luminal (apical side), multiple uptake and efflux transporters are expressed. Of these, most attention has been focused on P-gp and BCRP due to their association with multi-drug resistance of chemotherapeutics. There are also transporters expressed on the basolateral side of enterocytes that are different from those expressed apically, and the apical and basolateral transporters work in concert to shuttle drugs into and out of the intestine.[24]

Two common cell-based systems for permeability measurement are Caco-2 and MDCK (Madin–Darby canine kidney). Caco-2 cells (derived from a human colorectal carcinoma) form a polarized monolayer which are used to assess cellular permeability and active *vs.* passive uptake mechanisms. Caco-2 cells can exhibit higher trans-epithelial electrical resistance (TEER), a measure of barrier integrity, relative to human gut, and they express low levels of CYP3A4 and efflux transporters.[25,26] Nevertheless, Caco-2 permeability generally shows a good relationship with human absorption.[27] MDCK cells, with or without transfection with one or more efflux transporters, is a more common cellular model since it does not require long culture times. Permeability assays using either Caco-2 or MDCK cells are useful models to aid in the prediction of human absorption. However, neither model possesses representative *in vivo* CYP and transporter activity.[28] Moreover, these are static 2D systems that do not possess many of the physiologic features seen *in vivo* such as mucus secretion (goblet cells), microbiome and peristalsis.

Desired functionality of intestine MPS

Intestinal MPS models have the potential to recapitulate *in vivo* complexity and function in a 3D environment with physiologically relevant fluid flows and fluid–cell interactions. The aim for intestinal MPS in ADME applications is to capture transport and metabolism processes, which cannot currently be assessed, as exemplified in Table 2. To this end, the desired near-term functionalities necessary for intestine MPS includes:

- An appropriate barrier function – polarized monolayers with tight junctions reflective of those *in vivo* (*i.e.* TEER values in the range of 50–100 Ω cm^2).[25]
- Maintenance of cell viability and architecture for up to a week.
- The ability to sample easily from both the apical and basolateral sides.
- Verified asymmetric distribution of key transporter expression similar to *in vivo*.
- Functional expression of relevant drug uptake transporters such as peptide transporter 1 (PEPT1), organic-anion-transporting polypeptide 2B1 (OATP2B1), and efflux transporters (P-gp and BCRP).
- Drug metabolizing activity (*i.e.* CYP3A4, CES2, UGT).
- The ability to reproduce inhibition and/or induction of metabolic enzymes and transporters such as occur in natural product drug–drug interactions (*e.g.* with grapefruit juice).[29]

• Recapitulate dynamic nature of intestinal lumen (by introduction of flow, which is shown to enhance barrier functionality).

Progress in intestinal MPS model development

Much progress has been made in recent years with respect to intestinal MPS chip models. Early intestine chip models were simple models with microfluidic laminar flow that focused on culturing Caco-2 cell monolayers on a porous membrane, thereby mimicking both an apical and basolateral compartment.[30,31] It was quickly recognized that additional cell types were needed in an intestinal chip to better mimic *in vivo* conditions and increase tissue functionality. In this regard, Shuler *et al.* co-cultured Caco-2 cells with the goblet-like cells HT29-MTX.[32] This early gut chip exhibited barrier function and it was used to show that acetaminophen is absorbed and metabolized in intestinal cells. A major advancement in gastrointestinal and organ chip models was development of a pumpless microfluidics system whereby fluid flow across cells or compartments is determined by microfabrication of the MPS and gravity flow in a rocking platform.[33] A further improvement in intestine MPS has been the development of 3D models that allow cell monolayers to form on polymeric scaffolds resulting in villi-like structures.[34] Another advance was the ability to co-culture Caco-2 cells with anaerobic bacteria, which is a key feature to better reflect *in vivo* conditions. However, a limitation was the short duration of incubation (<24 h).[35]

Ingber and colleagues have shown the ability to culture multiple cells types along with mimicking peristalsis.[36] In this "dynamic" gut chip, Caco-2 cells are grown on the upper chamber while endothelial cells are cultured on the lower chamber and cyclic suction within the device mimics the mechanical deformations that occur *in vivo*. Under these conditions, the villi contain all 4 cell types (epithelial, goblet, enteroendocrine and Paneth) and they maintain a columnar architecture.[37] Other features of this model are the continuous flow of media and mucus production, enabling co-culturing with commensal bacteria with direct contact with epithelial cells. Epithelial cells in this model can be cultured for several weeks. These chips were also shown to exhibit complex immune–microbiome inflammatory interactions relevant to inflammatory bowel disease (IBD).[36] Introduction of lipopolysaccharide (LPS) resulted in secretion of pro-inflammatory cytokines into the basolateral side of the microfluidic channel.

Investigations are also ongoing to develop intestine MPS that use primary human intestinal cells or organoids, instead of Caco-2 cells, as these models should better reflect *in vivo* functionality. Chen *et al.* have developed a gut chip that incorporates human intestinal epithelial cells that form organoids.[38] These cells are isolated from human colon biopsies and maintain tight junctions, GI barrier functionality (measuring permeability of various drugs) and CYP and P-gp expression at higher levels than that seen in Caco-2 cells. This system has been combined with liver to form a 2-organ system. Other investigators have developed the intestine chip which also utilizes intestine organoids. Kasendra *et al.* have utilized organoids derived from biopsies or tissue resections to isolate epithelial cells and have shown differentiation into the 4 main cells types and functionality that exceeds that shown in the gut chip.[39] Workman *et al.* have also developed an intestine chip that uses epithelial cells from intestinal organoids derived from pluripotent stem cells.[40] This model showed cell differentiation, enhanced functionality as well as a physiologic cellular response to interferon gamma stimulation. Advantages of using induced pluripotent stem cells (iPSC)-derived organoids are that they contain mesenchymal cells, which should lead to a more *in vivo*-like architecture of crypts and micro-villi and they will more easily allow for investigations of individual responses and personalized medicine.[39,40] A limitation of intact organoids is the orientation of cells with the apical side buried inside the sphere which provides a challenge in administration of compounds to the apical side.

To address this, a recent report described a culturing methodology of small intestinal organoid which flips the apical side out.[41] This type of organoid may enable administration of compounds by simple addition to culture media.

Perspectives for intestinal MPS development

Investigators recognized early on the need to have this model better reflect the complex physiological conditions *in vivo* – mucous production, metabolically competent epithelial cells, mixed cell populations, peristalsis and the presence of a microbiome. ADME opportunities for intestinal MPS models lie beyond permeability and barrier function, which are adequately served with basic transwell models. As described in this section, intestinal MPS can add value where:

• Key drug transport mechanisms are active (P-gp and BCRP).

• First-pass gut metabolism is active and scalable to the *in vivo* setting.

• Adaptive responses of multiple cell types in the model due to drug treatment and disease may be assessed.

The source of the epithelial cells used in intestinal MPS is critical. Much of the system development with intestinal MPS has utilized Caco-2 cells. With the physiologic conditions achieved in the most advanced MPS, this cell line forms micro-villi and produces mucus. Reports suggest they can exhibit CYP3A4/5 metabolism close to that observed in human jejunum epithelial cells.[37] Nevertheless, additional progress is needed to ensure the metabolic and transporter activity of the epithelial cell line is similar to that observed *in vivo*. Since it has been shown that primary human intestinal organoids exhibit higher CYP and transporter function compared to Caco-2,[36] incorporation of primary organoids into intestine MPS would potentially lead to more *in vivo*-like function, but such an MPS would require a ready supply of high quality primary cells or organoids. To aid

characterization, cryopreserved human enterocytes and intestinal mucosa are now commercially available and may be used for comparison of mRNA levels and enzyme activities in the absence of fresh human tissue samples.[42]

For intestinal MPS, there is also the potential to develop chips to reflect disease states (*e.g.* IBD), to test potential mechanisms of diseases and to assess the efficacy of drug candidates. For example, Shin and Kim have developed a human gut "inflammation-on-a-chip", in which they reproduced dextran sodium sulfate-induced inflammation observed in a mouse *in vivo* model in order to create a modular model of human gut inflammation.[43] They were able to individually uncouple then recouple factors important for intercellular host-microbiome cross-talk associated with inflammation. As a result, they showed that barrier dysfunction is a very critical trigger of inflammation onset.

Another important aspect of an intestine MPS platform is the potential ability to study regional absorption of drugs by incorporating epithelial cells or organoids from the duodenum, jejunum, ileum and/or colon.[44–46] Representation of the ileum would also allow for incorporation of enterohepatic recirculation of bile acids. In addition, the presence of colon-derived epithelial cells would not only allow for the study of colonic absorption, but would also allow for testing of colon-targeted prodrugs, which is another specific ADME application.[47,48] To best simulate *in vivo* conditions, an MPS with colon epithelial cells will need to incorporate commensal anaerobic bacteria to reflect the microbiome. The microbiome is often considered an organ by itself, and it is now recognized as important for metabolism, pharmacology and a healthy symbiosis with the host.[49] The ability to maintain anaerobic conditions and the culturing of anaerobic bacteria in contact with human intestinal epithelial cells has recently been demonstrated for at least 5 days.[50]

Liver models for clearance, metabolite identification, induction and transport

Liver is the main organ of clearance for many drugs due to its high metabolic enzyme capacity, physiological positioning as a barrier to systemic exposure of orally administered drugs and biliary excretion functionality. The liver has a complex architecture comprised mainly of hepatocytes, endothelial cells, resident macrophages (Kupffer cells) and stellate cells.[1,51] A significant difference in oxygenation of the periportal and perivenous regions exists, with resulting differential drug metabolising gene expression. Hepatocytes *in vivo* therefore have sub-populations with different drug metabolizing capabilities and metabolism-dependent toxicity sensitivities.[1,51,52] In contrast to this *in vivo* complexity, *in vitro* investigations of drug metabolism typically use highly simplified cultures of primary hepatocytes in either short-term suspension or 2D plated cultures.[53] Data generated may then be used to try to predict a particular aspect of the *in vivo* situation such as metabolism, transporter-mediated clearance or induction. Cryopreserved primary hepatocyte suspension cultures meet availability, handling convenience and high metabolic enzyme activity requirements which, in combination with high cell concentration, make them effective drug metabolism screens. One aim of microphysiological liver systems is to capture more of the *in vivo* complexity by creating a more physiologically relevant environment. Such systems may provide better cell differentiation and cell–cell signalling can be promoted, enabling experiments more suited to *in vivo* drug metabolism, drug toxicity and disease state characterization to be performed. Highly recommended reviews include the wide-ranging overview presented by Godoy *et al.* as well as more recent reviews.[1,54–59]

Over the last few years, advanced 2D hepatocyte (co-culture) systems have been validated for drug metabolism assessment, especially for compounds with very high metabolic stability.[9,60] The systems have rapidly found application in late-stage pharmaceutical discovery and development because the drug-metabolising enzyme and transporter activities are retained at a similar level as those found in suspension cultures of cryopreserved human hepatocytes. The systems enabled prediction of human clearance for very metabolically stable drugs, for which *in vitro* intrinsic clearance measurement was not previously possible.[6,61] The use of micro-patterning allowed Khetani and Bhatia to develop a plate-based system containing islands of primary human hepatocytes surrounded by mouse 3T3 fibroblast cells and to demonstrate the viability and activity of the hepatocytes over culture periods of more than 4 weeks.[4] Chan *et al.* and Lin *et al.* established the utility of these micro-patterned hepatocyte co-cultures for the prediction of human metabolic clearance.[8,62] Bonn *et al.* and Hultman *et al.* also reported on prediction of hepatic clearance using an alternative co-culture system.[61,63] The degree of clearance predictivity compared well with short-term suspension culture systems, but with measurement down to ~0.2 μL min^{-1} per million cells reported, ~10-fold lower than attained in classical suspension culture experiments.[6,64] Metabolite identification and enzyme induction have been demonstrated and new experimental opportunities explored.[65–67] For instance, Moore *et al.* combined short-term uptake experiments with long-term enzyme induction assessments and reported on how active uptake affected the apparent induction potency of rifampicin.[68] Other case studies have also shown that multiple short-term and long-term endpoints can be combined to allow the interaction of transport, metabolism and induction processes to be better understood.[69,70] Further applications of the co-cultured hepatocyte systems show how elements of drug metabolism, disease modelling and *in vitro* pharmacology may also be addressed. March *et al.* and Ploss *et al.* have shown that hepatocytes in long-term co-culture systems can support infection by hepatitis C and hepatitis B

viruses, respectively.[71,72] Kratochwil *et al.* further showed the effect of hepatitis B infection and examined the effect on drug-metabolising enzyme expression, indicating how the long-term hepatocyte culture technology now allows more interdisciplinary approaches to be taken.[69]

Prediction of biliary secretion remains highly challenging.[73–75] Indeed, the contribution of biliary secretion to human drug clearance is often inferred from human mass balance studies relatively late in drug development and may not easily be defined in the absence of an intravenous administration arm in the study.[76] Hepatocytes either in co-culture or sandwich culture generate 'bile pockets', similar to the bile canaliculi found *in vivo*, into which drugs and metabolites may be secreted. As treatment with media lacking calcium opens up these pockets, it is possible to make assessments of drug levels in cell culture media with or without calcium to assess biliary clearance.[77] This methodology requires excellent reproducibility of experimental handling, a strong active biliary secretion activity and sensitive analytics to enable a small difference in drug concentration to be measured with confidence. In addition, it is challenging to maintain supplies of transport-active batches of primary hepatocytes. Alternative culture or MPS models in which the bile could be directly sampled are therefore of extremely high interest for ADME scientists and are currently a major unmet need.

Tissue spheroids are a more complex system, with 3D structure and cell–cell interactions in a format also allowing drug screening activities to be performed. Various authors have reported advantages of 3D systems in metabolism or toxicity testing applications.[78,79] Frey *et al.* showed that hanging drops containing spheroids could be connected, allowing transfer of media between spheroids of different types, creating a simple multiple tissue system.[80] In this way, hepatocyte spheroids were able to bioactivate cyclophosphamide, inhibiting the growth of tumour spheroids in another droplet. Although spheroids may be cultured for extended periods and retain enzyme activities, the low number of cells make many ADME measurements challenging. Methods to increase the number of spheroids in an individual experiment, and therefore enhance system suitability for ADME applications include bioprinting, permeable gel encapsulation and use of multiple microcavities within the same microtiter plate well.[81–83] To date, these approaches still need to be characterised and their applicability to ADME applications established.

Desired functionality of liver MPS platforms

Microphysiological liver culture systems are eagerly anticipated by the ADME community due to their potential to enable new mechanistic investigations to be performed with enhanced translational capacity. To date, issues of availability, characterization, reproducibility, throughput and cost mean that MPS models have yet to be routinely applied.

Basic liver MPS characterization for ADME applications should include:

- Demonstration of long-term system suitability (cell survival and hepatocyte-like activity retention, lack of media evaporation and non-specific binding).
- Metabolism of drugs to known products and intrinsic clearance determination using relevant drug compounds under both short- and long-term incubation conditions (*e.g.* midazolam and tolbutamide).
- Active uptake and efflux of drug transport substrates (*e.g.* rosuvastatin).
- Biliary efflux of drugs, drug metabolites and bile acids (*e.g.* taurocholic acid), ideally with bile duct-like structures to allow direct sampling.
- Interplay of uptake, metabolism and subsequent biliary excretion/sinusoidal excretion.[84]
- Enzyme induction capability (mRNA, enzyme activity) demonstrated using strong inducers such as rifampicin.

Perspective for liver MPS

Early experiments showed the importance of cell co-culture and the effect of media flow and potentially of shear stress on drug metabolizing enzyme expression[85] and the absolute need to prevent non-specific binding of drug to the apparatus.[86] The focus of early MPS model development was on cell survival and hepatocyte-like activity, with albumin generation, urea secretion and cell survival assays used as the main test system readouts. As the field has evolved, researchers have shown more ADME-relevant activities, for example demonstration of CYP activities and the inducibility of CYP enzymes.[87–90] Unfortunately, the test systems used (*e.g.* pro-fluorescent or pro-luminescent assays) and comparator cells selected have not allowed ADME-relevant characterization or system comparisons to be made. Table 2 summarizes example drugs which may be used to characterise liver MPS systems in a manner more relevant to their end use. Some more recent studies have shown ADME-relevant characterization: Kratochwil *et al.* provided an example of how cryopreserved hepatocytes can be used to benchmark different system activities.[6] Tsamandouras *et al.* examined the *in vitro* pharmacokinetics of 6 different drug compounds in a microphysiological liver system using cryopreserved hepatocytes.[91] Sarkar *et al.* reported on an immunocompetent MPS model where Kupffer cells and hepatocytes were co-cultured to study diclofenac metabolism.[92] Chen *et al.* showed quantitative clearance measurement and pharmacokinetic modelling from an intestine–liver system incorporating hepatocytes and Kupffer cells in the liver compartment and with enterocytes, goblet cells and dendritic cells in the intestinal compartment.[93] The implementation of hepatocyte–Kupffer cell co-cultures is a likely next step in the development of long-term liver MPS systems. Co-cultures have been shown to be viable and to enable interleukin-mediated effects on hepatocyte activity and hepatotoxicity to be assessed.[93–95] This could lead to

development of ADME and toxicity test systems performing under inflammatory response conditions, coming closer to the *in vivo* situation in inflammatory disease states. Future co-cultured MPS platforms may also be used to interrogate the mechanisms by which human hepatocytes produce damage-associated molecular patterns (DAMPs) that can further activate inflammasomes in macrophages.[96]

Quantitative validation and characterization of MPS are key to use and acceptance by industry and regulatory agencies. During system development rapid and low-cost characterization methods such as mRNA profiling can be used in place of drug metabolism studies. However, for final applicability demonstration of uptake, efflux, metabolism, induction and inhibition properties of real drug compounds need to be demonstrated. Although the main focus of MPS development is the generation of human systems, analogous MPS using cells from preclinical species such as rat, mouse or monkey would enable IVIVE establishment for processes (*e.g.* biliary secretion) for which human *in vivo* data are missing. A close collaboration between system designers, experimental ADME scientists and modelling and simulation experts would be beneficial to ensure that relevant characterization is performed to enable recognition and acceptance by the wider scientific community and regulatory authorities.[97]

CNS models for brain exposure predictions

There is tremendous unmet medical need in serious diseases of the CNS, such as Alzheimer's disease, Parkinson's disease, and brain cancers. A key challenge in the discovery and development of CNS therapeutics is the inability to adequately predict brain penetration in humans.

The blood–brain barrier (BBB) is a collective term to describe layers of cells at the interface between the brain and the systemic circulation.[98] The BBB is comprised of endothelial cells, basal lamina, end feet of astrocytes, and pericytes. The endothelial cells are connected by a complex network of tight junctions and adherence junctions.[99] The cellular context of the BBB and many *in vitro* models that aim at mimicking it has been extensively reviewed recently.[99–101] The often modelled features of BBB are the endothelial barriers between circulating blood and CNS, which allow entry of essential nutrients into the brain but prevents the entry of toxic compounds and pathogens while effluxing waste back into circulation.[99,100,102] The less modelled barriers are the blood–cerebrospinal fluid (CSF) barrier and the CSF–brain barrier.[103]

Similar to all other endothelial barriers, the primary drivers for drug penetration are membrane permeability and active uptake. Because the uniquely structured network of junction proteins makes it impossible for paracellular permeation of small molecules, the permeation of hydrophilic molecules into the CNS relies upon specific uptake transporters, such as glucose transporter 1 (GLUT1) for glucose transport and L-type amino acid transporter 1 (LAT1) for essential amino acid transport. On the other hand, efflux transporters (P-gp, BCRP, MRP4) are expressed on the apical side to effectively prevent the entry of lipophilic molecules, and this is the primary mechanism behind the failure of many anti-cancer compounds in the treatment of primary or metastatic brain tumours. The expression and function of transporters at the BBB are comprehensively reviewed by Stieger and Gao,[104] and the International Transporter Consortium has proposed the following transporters as important in drug development: P-gp, BCRP, MRP4, MRP5, OATP1A2, OATP2B1, ENT1 (equilibrative nucleoside transporter 1, SLC29A1) and ENT2 (SLC29A2). In addition to passive permeation and active transport, there are also drug metabolizing enzymes in the brain than can impact efficacy or generate toxic metabolites.[105,106] Human brain endothelial cells express multiple CYP enzymes such as CYP3A4, 2C9, 2C19, 2A6, 2E1, and 2J2. On the other hand, CYP2B6, 2D6, and 3A43 were reported to be expressed in neuronal cells. In addition, other enzymes for neuronal function include acetylcholinesterase, alkaline phosphatase, γ-glutamyl transpeptidase, monoamine oxidases.[107] Another important function of the BBB is the transcytosis of endogenous proteins through their respectively specific receptors such as transferrin receptor (TfR1), insulin receptor (IR), and low density receptor related protein-receptor (LRP-1). Exploiting these receptors could allow access to the CNS of biotherapeutics that could not normally cross the BBB,[108–110] for instance antibody fusion proteins and siRNAs.[111,112]

Current methods of predicting human brain exposure typically involve a comprehensive set of *in vitro* and *in vivo* testing, including *in vitro* permeability assessment, *in vitro* transporter substrate evaluations including P-gp and BCRP, *in vivo* brain to plasma ratio determination typically in a rodent species, and transwell BBB models.[103,113] The totality of these data is then integrated to predict the human brain exposure. A recent publication utilizing *in vitro* transport assays with a physiologic model has demonstrated high concordance between predicted and observed brain penetration.[114]

MPS models of BBB

A typical MPS model of BBB consists of microfluidic channels separated by a porous membrane upon which are microvascular endothelial cells on one side and astrocytes/pericytes on the other.[101,115–119] The flow of medium and the interaction with pericytes/astrocytes promote tight junction formation and polarization of the endothelium.[120,121] The systems are also fabricated with embedded electrodes for TEER (trans-epithelial electrical resistance) measurement and with transparent material for imaging.[122,123] Some models add neurons on top of the astrocyte layer to mimic the brain compartment,[115] which allows for direct functional measurement of neuronal activity and therefore enables

pharmacokinetics/pharmacodynamics (PK/PD) relationship analysis. Nevertheless, most models are chimeric with cells isolated from primary animal sources or immortalized brain endothelial cells.

Despite the promise of increased tight junction protein expression of a flow based system as compared with a static one, the currently available BBB chips typically exhibit TEER values of ~200 Ohm cm^2 (target >300 Ohm cm^2) although very high TEER values were reported by the Shuler lab.[124–126] In addition to TEER value, the permeability of different sized molecules such as mannitol, Lucifer yellow, and FITC-dextran is often used to assess the tight junction formation. The shear stress introduced by media flow not only promotes tight junction formation but also induces the expression of CYP enzymes that may be important in the context of explaining CNS active drug resistance.[127]

The use of primary cells to develop BBB models has its limitations. Isolations of primary human brain endothelial cells and astrocytes are technically challenging. Purity issues in isolation may be the root cause of variability. Moreover, cryopreservation and time in culture cause neural cell types to lose phenotype quickly.[128] Species differences in transporter abundance can also limit predictive power.[129] An emerging new approach to establish functional BBB model is to prepare human iPSC derived neurovascular unit.[124,130,131] This transformative approach may offer a scalable renewable resource that can create physiological relevant models to study permeation, transport, metabolism, toxicity, and pharmacology of novel therapeutics. Current efforts in this area focus on the acceleration methodologies in the maturation of differentiated cell types.[125]

Desired functionality of CNS MPS models for ADME

A successful BBB model needs to demonstrate the functional effect of efflux transporters and drug metabolizing enzymes.

- The ability to discriminate the permeability of low, medium, and high brain penetrating drugs should be demonstrated. A test set of compounds[132] can include mannitol (low), indomethacin (medium), and propranolol (high). Prediction accuracy needs to exceed that of other cell monolayer systems which could otherwise be used as proxies.
- For efflux transporters, the functional expression of P-gp and BCRP needs to be demonstrated using relevant drug molecules. Both of these transporters are expected to be expressed on the apical membrane of the polarized endothelial cells. The functional efflux of ABCB1 substrates such as digoxin is required, typically expressed as permeability ratios of basolateral to apical over apical to basolateral.
- For influx transporter activity, glucose uptake by GLUT1 needs to be demonstrated.
- Functional assessment of CYP activity can be done by monitoring the disappearance of carbamazepine, a CYP3A4 substrate.[133]
- The system should allow for penetration of large molecules through receptor-mediated transcytosis *via* functional expression of TfR1, IR, and LRP-1.
- Sustained barrier function as indicated by TEER values over time of testing, usually up to a week.
- IVIVE in preclinical development of CNS-targeting biotherapeutics.

Perspective for brain MPS

Although the addition of shear stress with media flow has been shown to increase the expression of tight junction proteins, the current models of BBB chips are still not consistently demonstrating TEER values close to the *in vivo* situation, and further work in the control of cell sources and improved engineering may be key for performance improvement. Furthermore, there is reasonable qualitative concordance between the predicted brain penetration and *in vivo* values of small molecule drugs as measured by CSF and/or PET imaging with static BBB cultures and particularly with specific transporter assays such as P-gp transfected cell lines.[134,135] Therefore, a MPS BBB model in this setting faces a high bar in differentiating from these simpler models, and it is reasonable to expect more quantitative prediction of free brain/plasma ratio for it to be adopted in this setting. In addition to transwell models, there is much interest in the development of brain spheroid models. The spheroids are self-assembling multicellular system encased by brain endothelial cells.[136] Spheroid models are challenging for ADME studies of small molecules due to the difficulty to sample the interior of the spheroid. On the other hand, penetration of fluorescently-tagged large molecules and peptides can be assessed using sensitive and non-invasive imaging methodologies.[136] Therefore, for these drug modalities brain spheroid models can initially be applied in an ADME setting, whilst the challenges of compound measurement from spheroid cores is addressed.

The inclusion of functional neurons in a brain MPS model, which couples BBB penetration with efficacy readout, allows PK/PD studies within a chip.[137] In addition, if the brain microtissue is part of a linked organ system, which provides drug metabolizing organ such as liver, one could assess the impact of circulating metabolites in neuronal pharmacological activity or toxicity. In addition, substantial opportunities exist to take advantage of endogenous receptors and transporter at the BBB to facilitate the uptake of large molecule biotherapeutics and gene-therapies for serious CNS diseases.[110] Finally, advanced BBB MPS platforms such as CNS disease-on-a-chip are envisioned to study the effect of age and diseases such as brain metastasis and cerebral inflammation on brain penetration of therapeutics.

In summary, clear advantages of a brain MPS model need to be demonstrated over current practices in ADME applications before wide adoption of these systems in the industry.

Kidney MPS for drug metabolism and excretion

The kidney is an organ which plays an essential role in the clearance of many drugs and yet ADME tools available for probing kidney function remain largely underdeveloped. A significant proportion of drugs and circulating drug metabolites are excreted *via* the kidney. Although cellular tools are starting to emerge, it is likely to be many years before a functional model of a renal tubule/nephron containing the *in vivo* cell types and morphology is developed. While a nephron is composed of various parts, *e.g.* glomerulus, proximal convoluted tubule (PCT), distal convoluted tubule (DCT); the most critical gap in IVIVE comes from PCT due to high levels of metabolic enzymes and transporters expressed in this region of nephron. Hence, a model which can demonstrate functionality of PCT, would be of highest interest and priority at this point in time. In this section we highlight the ADME aims for a kidney MPS model and key characterization criteria which will indicate potential value to the pharmaceutical industry.

The kidney has many important functions but is specialized in the elimination of undesirable substances from the circulation. In addition to passive filtration, transmembrane proteins expressed primarily in the epithelial cells of PCTs are directly responsible for the tubular secretion and tubular reabsorption of drugs and many other exogenous and endogenous substances. These transporters are expressed either at the basolateral or apical (luminal) membrane of proximal tubular epithelial cells. Basolateral transporters are responsible for the cellular uptake of substances from the blood, while apical transporters ensure their efflux into the tubular lumen. Thus, having a thorough understanding of the mechanisms involved in the elimination of drugs can provide important information related to their clearance, the potential for the occurrence of renal or other organ toxicity, the effect of elimination of a test compound in patients with compromised renal function and drug–drug interactions.

Understanding anatomical differences in metabolic enzyme and transporter expression helps to shape approaches for MPS platforms. The regional distribution of metabolic enzymes and transporters are disproportionally concentrated in the cortical layer of the kidney but very little, if any, drug metabolism or transporter activity occurs in the glomerulus. In contrast to the glomerulus, the proximal tubule segments contain the greatest amount of metabolic and transporter activity in the kidney. Considering that the proximal tubule is a commonly targeted kidney region for nephrotoxicants, there have been efforts to develop MPS models to recapitulate proximal tubule function.[138] These models show promise for toxicity assessment; and should be further characterized for drug metabolism and transport capacity; enabling mechanistic understanding of exposure related toxicological outcome.

Clearance by the kidney

Renal clearance typically refers to the proportion of total drug that is eliminated by the kidney unchanged. The total amount excreted is the net result of glomerular filtration, tubular secretion and reabsorption. When glomerular filtration constitutes the major portion of renally excreted drug clearance, predictions based on preclinical animal models work reasonably well. However, when active transport mediated secretion and/or reabsorption contributes significantly to total renal clearance, preclinical models are not sufficient for human renal clearance prediction.[139,140] Significant knowledge gaps continue to exist in our understanding of species differences of renal transporters, which results in reduced confidence in human clearance predictions and dose projections. Overall, improving our understanding of mechanisms involved in renal clearance will allow us to improve clearance and drug–drug interaction predictions during drug discovery.

Tubular secretion plays a major role in the elimination of numerous drugs, such as β-lactams and antivirals. Transport proteins participating in tubular drug secretion or reabsorption includes organic anion transporters (OATs), organic cation transporters (OCTs), OATPs, urate transporter 1 (URAT1), multidrug and toxic compound extrusion (MATE) proteins, nucleoside transporters and others. They mediate transport and molecular exchanges along chemical gradients. Reviews about this transporter superfamily have recently been published.[139,140] The ATP-dependent efflux transporters belong to the ABC family and perform active, energy-consuming molecular transport. Those relevant for renal tubular secretion include MRP2 and 4,[141–143] P-gp,[144–147] and BCRP.[144–146]

Glucuronidation is a key metabolic function of the proximal tubule. Drugs such as acetaminophen, morphine, and furosemide have been described as having kidney glucuronidation activity.[148–150] Induction of glucuronidation has also been demonstrated. Compounds such as phenobarbital and β-naphthoflavone represent established models of UGT induction in the kidney.[151,152] *O*-Aminophenol and *p*-nitrophenol are examples of generic UGT substrates, which have been used to verify the endogenous and induced glucuronidation activity of kidney MPS models. These compounds can be used as tools to aid development of kidney MPS to characterize glucuronidation activity under various model conditions prior to studies using more relevant drug molecules.

The variety and relative abundance of metabolic activity tends to diminish outside-in, from the cortex to the outer medulla, with some types of reductase activity relegated to the inner medulla. This feature of the kidney helps emphasize the importance of richly characterized proximal tubule models. While the proximal tubule captures the majority of renal metabolic activity, some specific activities are unique to more distal nephron segments. Certain steroid hydroxylases are expressed in the distal tubule and collecting

duct. Aldose reductase activity is concentrated in the inner medullary regions where the loop of Henle and collecting duct are located. MPS models of these downstream nephron regions should consider how to verify retention of those specific metabolic functions in order to accurately recapitulate the physiology in those areas.

MPS models can also be evaluated for their suitability to study tissue exposure/accumulation questions in relation to renal toxicity. For example, colistin and polymyxin B are known to accumulate in renal proximal tubules resulting in dose-limiting nephrotoxicity in patients. Colistin/polymyxin B mediated toxicity has been shown to be related to significantly higher exposure or accumulation of the parent drug and/or its metabolite in the kidney and could only be reproduced in a rat model under specific dosing requirements.[145] Despite some success in the field with the rat model, there are significant gaps that exist in human translation of such data and relating it to clinical outcome. *In vitro* systems that would mimic renal cortex or proximal convoluted tubular cells that can preserve the physiology, metabolic and transporter expression, would be an ideal tool to provide insights into tissue distribution or exposure queries.

Existing kidney systems for ADME studies

Kidney microsomes can be used as a benchmark for metabolic enzyme activities, including CYPs, flavin-containing monooxygenase, epoxide hydrolases, ketone reductases, SULTs and cysteine conjugate beta-lyases. Taub *et al.* used kidney microsomes to demonstrate a sex-, species- and kidney-specific metabolite was likely the mechanism for increased incidence of mouse renal adenocarcinomas related to lifetime exposure to empagliflozin.[153]

Cell lines over-expressing a single renal transporter such as OAT1, OAT3 or OCT2 are increasingly being used to evaluate transporter-mediated renal elimination. However, due to the concern of expression differences between *in vitro* models and *in vivo*, a relative activity factor approach has been employed to improve renal clearance predictions.[154] Primary human renal proximal tubular (HRPT) cells have also been occasionally utilized, although their use has been limited to complex situations where ADME properties can explain certain toxicities. Supply of consistently performing, good quality HRPTs remains a significant challenge and hinders their use.

Therefore, kidney MPS could offer a more stable enzymatic and transporter expression, and desired tissue architecture allowing greater opportunity for quantitative renal clearance or metabolism evaluations. In addition, control of fluidic rates in MPS models can allow for more accurate simulation of kinetic/physiologic conditions in the body. For approximating clearance, these features would provide greater potential for accurate prediction, as compared to currently available *in vitro*/*ex vivo* models.

Desired functionality of kidney MPS models

Currently, there are no gold standards for translational prediction of human renal drug clearance outside of live animal models. The most useful MPS models will behave similarly to the intact nephron, where kinetic assessment of drug clearance can be reliably measured.

Recapitulating the nephron's sorting function, at the microenvironment level, is accomplished through a set of diverse, specialized functional units comprised of different zones, cell types, basement membrane compositions and soluble factors. A number of these physiological kidney features may be perturbed by drug treatment, leading to drug–drug interactions and/or adverse effects. Ideally, all of these functions would be present and measurable in a kidney MPS. Limiting the MPS to a specific nephron component could allow focus on accurate representation of key endpoints such as: filtration rate, blood flow/pressure, transport, and excretion levels of urinary components (electrolytes, protein and metabolites). While recent publications indicate that an array of transporters are expressed in the kidney,[155,156] any MPS model that is developed to understand renal disposition, nephrotoxicity or DDI risk should at minimum have functional activity of OAT1/3 and OCT2 on the basolateral side and P-gp, BCRP, MRP2 and MATE on the apical side of the proximal tubule.

For ADME applications, kidney MPS models should ideally demonstrate:

- Barrier function between representative blood and urinary compartments.
- Appropriate reuptake transport from intraluminal space into the blood compartment (apical to basolateral).
- Kidney-relevant metabolism function (*e.g.*, vitamin D).
- Capacity to probe known drug–drug interaction(s) influencing transport and/or metabolism especially with respect to potential tissue accumulation.

Perspective for kidney MPS

Most of the efforts that have gone into the development of 'kidney-on-a-chip' to date are focused on creating a model to evaluate renal toxicity. While studying toxicity in such models may seem to be the most important endpoint, if the underlying mechanism/s cannot be recapitulated, we may only partially accomplish the goals of such an exercise. It is critical to understand if nephrotoxicity is drug exposure related. Establishing such findings early in a program can enable a strategy to reduce drug exposure in a discovery program. Efforts have been initiated in the right direction as highlighted by Chang *et al.* but demonstration of expression and activity of key enzymes and transporters in this system would be the next most logical step.[157] Weber *et al.* have demonstrated expression of metabolic enzymes and transporters including some data on function in a kidney MPS model.[158] Such advances can tremendously help in moving the field when translatability to the *in vivo* situation has been demonstrated.

Linked organ MPS

The purpose of ADME studies is to enable the understanding of bio-distribution and exposure. At present, these parameters are largely either measured in animal studies or predicted for man. Although isolated MPS are valuable for modelling certain tissue-specific functions, there exists non-linear, dynamic organ crosstalk, especially in the context of disease or toxicity, that cannot be captured by studying these processes in isolation. The value-added applications for inter-linked MPS, such as the one depicted in Fig. 1, should address specific questions at the intersection of PK and efficacy, or PK and toxicity, which were previously reserved to the realm of animal studies. Bioengineered platforms capable of inter-connecting up to 10 MPS of varying complexity have been reported;[15] however, such systems are impractical for industrial implementation. In the near-term, lower-order (2–4 tissues) inter-linked MPS are more tractable and can be used to examine the secondary effects of drugs on (un)intended target tissues. The ability to model and measure the temporal dynamics of organ-specific drug and metabolite profiles is essential to predicting both on-target and off-target activities, because systemic drug exposure can be a poor predictor of target coverage and the pharmacodynamic effects at the site of action. To this end, a multi-compartment *in vitro* system amenable to interrogation and measurement can help uncover mechanisms underlying complex drug actions at the local and systemic level.

To date, most of the multi-MPS studies have been liver-centric,[159] designed to interrogate the metabolic interplays between the liver and various tissue types (gut, kidney, pancreas, heart, tumour).[15,30,38,93,159–169] These proof-of-concept applications include first-pass metabolism,[30,161,162,168] metabolic activation or inactivation of parent compounds and their effects on downstream tissues[160,165,166,169] and tissue-crosstalk in disease modelling.[93,163,164]

Modelling integrated drug disposition

Determining the first-pass metabolism of oral drugs is important for the prediction of bioavailability in humans. To this end, early multi-MPS efforts attempted to model intestinal absorption and hepatic metabolism in gut–liver systems.[38,161,162,168] However, as indicated in the Introduction and shown in Table 1, both cell culture and ADME study requirements need to be met. All these studies have at least one or more of the following limitations that undermine their utility in PK applications;[161] 1) the material of device construction (*e.g.*, polydimethylsiloxane, PDMS) can non-specifically adsorb and also absorb hydrophobic compounds, which limits cellular exposure to drug and the generation of metabolites; 2) the use of low cell number and media volume in some microfluidic systems can pose a challenge for analytical detection; 3) the inability to access all tissue compartments for organ-specific drug/metabolite profiling over time; 4) the lack of a mathematical methodology for extracting PK parameters necessary for IVIVE; 5) the use of cell lines, which lack the relevant enzymes and transporters for PK studies.

A recent paper by Tsamandouras *et al.* reported the use of a non-PDMS, mesofluidic gut–liver system along with mechanistic model-based analysis for quantitative PK investigation of diclofenac, with the goal to address some of the limitations outlined above.[161] Although the work was proof-of-principle in nature, it offered a framework on how one can design, operate, and interpret multi-MPS experiments for PK investigations. One can dissect the organ-specific contribution to first-pass metabolism by monitoring the parent drug and metabolites profile over time in disconnected and inter-connected gut and liver MPS following different routes of administration (oral: apical gut; I.V.: systemic). When used in conjunction with specific inducers or inhibitors of known transporters and metabolic enzymes, MPS can provide a versatile platform to examine the complex temporal interplay of transport and metabolism within and across organ systems. Future work should include the evaluation of compounds across a diverse chemical space for which human PK is known in order to validate use of a gut–liver system as a predictive model. Compounds (Table 2) with clinically estimated intestinal and hepatic clearance should be included in this data set.

We foresee that a gut–liver system consisting of entirely primary cells should be possible in the near future given the recent advances in intestinal organoid technology and the availability of primary hepatocytes. Such a system can significantly improve the physiological relevance of the gut–liver model. Ultimately, a transformative goal would be to incorporate enterohepatic circulation with separate bile flow connecting to the luminal side of the gut. This will support numerous applications in both PK/PD and toxicity assessment.

An additional area of opportunity to consider for linked organ MPS models is to explore their utility for oligonucleotide drugs. It is well established that GalNAc-conjugated oligonucleotide drugs distribute extensively in the liver by ASGR1 mediated endocytosis, followed by kidney. The mechanism that enables this class of drugs to distribute to kidney is unknown. The technology preferentially targets the liver, although with an increasing dose, the distribution shifts to kidney, highlighting a capacity limit to liver dosing. Prediction of exposure, extent and duration of response in human proves challenging. MPS may serve as new tools to study oligonucleotide distribution between liver and kidney and help establish the exposure-response relationship for dose selection.

Modelling PK/PD and toxicokinetics/toxicodynamics (TK/TD)

One of the most powerful applications of a multi-MPS model is to capture not only the direct effect of the drug on the target but also the effects of metabolites and downstream pharmacodynamics of the drug as it undergoes sequential modifications throughout different parts of the body. In a proof-of-concept study, Chang *et al.* joined a liver and a

kidney chip to establish the causal link between bioactivation and transport of aristolochic acid (AA-I) and resulting kidney toxicity.[87] They demonstrated hepatic metabolism *via* nitro-reduction and subsequent sulfonation of the parent drug. Moreover, it was shown that the toxic AA-I metabolite could be transported out of hepatocytes by MRP3/4 and was taken up into the kidney tubule cells *via* OAT transporters, as inhibition of OAT rescued cytotoxicity. This study demonstrated the utility of inter-linked MPS for mechanistic dissection of a dynamic, multi-faceted phenomenon involving phase I and phase II metabolism and transport interacting across organ systems. The ability to isolate and connect MPS to address specific questions can help ascertain the sequential, organ-specific contributions to a given phenotype of interest.

Similarly, others have studied the impact of hepatic transformation of parent drugs on other downstream organs, such as the heart, using a multi-MPS platform. Skardal *et al.* reported an interacting liver and cardiac spheroid system, where hepatic clearance of propranolol de-suppressed the inhibitory effect of propranolol on epinephrine-induced increase in cardiac beat rate.[166] Zhang *et al.* applied a different heart–liver device to show that capecitabine elicited a cardiotoxic effect only in the presence of hepatocytes, presumably due to metabolic conversion to 5-fluorouracil.[167] Oleaga *et al.* used a coupled liver–heart system to examine the effects of two drugs with known metabolic consequences. Cyclophosphamide, which is known to produce cardiotoxic metabolites following hepatic transformation, was shown to induce a more severe cardiomyocyte dysfunction *in vitro* in the presence of hepatocytes.[160]

While these studies showed promise, they are mostly descriptive in nature and lack the quantitative rigor required for translational applications (for instance, use of physiologically relevant drug and metabolite concentrations). Thus far, quantitative PK/PD or TK/TD analysis in tissue compartments of interlinked MPS has been limited. Drug and metabolite sequestration and unpredictable release from the device can result in non-physiological dose selection and confound biological interpretation. A more thorough accounting for drug binding to media components, biological matrices and device components is necessary before these systems can be applied effectively in an industry setting.

The PK/PD and TK/TD properties of a drug depend on the physiological context of the system, where the underlying biology can influence the activities of key proteins involved in biotransformation as well as target (off-target) abundance. Linked MPS provide new opportunities to study the interactions of pharmacology and patient factors (*e.g.*, genetics and disease states) by improving our ability to model complex biology.

Chronic multifactorial human diseases with diverse clinical phenotypes are difficult to capture in animal models. This is especially pertinent in diseases associated with metabolic and immune dysregulation, where disease manifests at both the local and systemic level and co-morbidity across organ systems is common. An example of this is non-alcoholic fatty liver disease (NAFLD), which afflicts approximately 25% of the global population, and is commonly associated with type 2 diabetes and other metabolic and immune dysfunction.[170] Dysregulation across multiple organ systems, including gut/microbiome, liver and the adipose tissue, has been implicated in driving disease progression. Multi-organ interplay has also been reported to play a role in other inflammatory conditions, such as IBD and sepsis.[171–173]

Recent proof-of-principle multi-MPS studies have demonstrated that aspects of metabolic and immune crosstalk across organ systems can be captured *in vitro*, albeit at a simple level. Lee *et al.* reported a gut–liver system, where they studied intestinal lipid absorption and hepatic lipid accumulation to model processes involved in hepatic steatosis.[163] Bauer *et al.* reported a functional coupling of pancreatic islets and liver spheroids, where insulin release in response to a glucose stimulus promoted hepatic glucose uptake and maintenance of glucose homeostasis.[164] Chen *et al.* reported an integrated gut–liver system, where modulation of bile acid signalling was observed in baseline gut–liver interactions as evidenced by gut-derived FGF19 production and the associated downregulation of hepatic CYP7A1 gene expression.[93] Moreover, highly non-linear regulation of cytokine signalling, particularly CXCR3 ligand (*e.g.*, CXCL9, 10, 11) production, was observed in the gut–liver interactome under inflammatory conditions. Inflammatory gut–liver crosstalk was also accompanied by concomitant modulation to hepatic metabolic processes, such as down-regulation of xenobiotic- and lipid metabolism-related gene expression. Together, these data suggest that inter-linked MPS have the potential to model immune and metabolic interplay in complex (patho)physiology. This is exemplified in a study by Long *et al.*,[174] in which metabolism and immune interplay was demonstrated to influence drug–drug interaction in an immune-competent liver MPS, whereby tocilizumab, an anti-IL6-R monoclonal antibody de-suppressed CYP3A4 activity under IL-6-induced inflammation to reduce the half-life of CYP3A4 substrate simvastatin hydroxy acid. Given that the intestine also contributes significantly to first-pass metabolism, an immune-competent gut–liver system, ideally containing a microbiome component, would be relevant to interrogate potential drug–drug interactions across organ systems under normal and perturbed contexts, thereby enabling the assessment of PK and safety margins in different patient populations.

The ability to add/subtract specific cells/tissues from the *in vitro* platform in a modular manner and to control precisely the spatial and temporal dosing strategy can help elucidate cell or tissue-specific drivers to integrated systems response. These systems may enable the discovery and characterization of tissue-specific efficacy and safety biomarkers. Ultimately, the goal is to derive a detailed understanding of drug effects in the integrated physiome (from molecules → cells → multi-cellular tissues → interacting organ systems).

Ongoing challenges and development needs

Although there are essential criteria for an MPS to be characterized for PK, efficacy and toxicity applications are often unique and MPS platforms need to have flexibility to allow end users to customize towards specific applications. Therefore, a one-size-fits-all model is insufficient to meet all the emerging applications, as the classes of drugs under development continues to evolve beyond small molecules to include other modalities such as antibodies, growth factors, antibody–drug conjugates, oligonucleotides, gene and cell therapies. This presents a large gap in existing capabilities and therefore an opportunity where MPS technology can make a large impact in preclinical drug testing.

A defined media should link multiple tissues without adversely impacting tissue functions. This requires understanding the minimally essential components for supporting organ-specific functions. In the case where a supplement is necessary for one tissue but detrimental to another, local administration and/or controlled release strategies can be used for local delivery of essential components. For example, selected media component can be added to specific tissue compartment in a temporal fashion such that the rate of addition roughly matches the rate of consumption to minimize systemic distribution. Alternatively, a more sophisticated approach may involve engineering synthetic matrices with cleavable growth factors to provide tissue-specific cues while minimizing systemic effects. Ultimately, the ideal integration should occur *via* vascular coupling (*i.e.*, tissue-specific vasculature) such that individual MPS can sustain their respective functions in tissue-specific microenvironments and can communicate *via* the vascular channel that interconnects all MPS in a systemic circulatory loop. Such a system is particularly pertinent to the study of vascular penetration and tissue distribution of large molecules and circulating cells.

Conclusions

Accurate prediction of ADME and toxicological properties in the clinic has been a continual challenge for drug development scientists. In contrast to traditional *in vitro* systems that have the intrinsic limitation of being static, short-lived and discrete, dynamic organotypic MPSs are more physiologically relevant models that have the potential to deliver a more reliable prediction of human responses. Over the past decade, significant progress has been made in the optimization of both 3D co-culture systems and flow-based dynamic systems, resulting in success of the technology in several areas, mostly related to demonstration of efficacy and safety end-points. However, the progress in the area of ADME is limited. One potential reason for this slow development is lack of demonstration of clear advantages of organotypic MPS over the traditional approaches. Although, safety and efficacy endpoints should be evaluated in relation to exposure in the relevant organ/tissue. Additional reasons include high cost of running MPS model over traditional systems. For more extensive industrial application and regulatory acceptance in the future, it would be crucial for the new *in vitro* technologies to be qualified against clinical data to demonstrate improved predictive power over existing technologies. Although undisputed reports are still lacking, many directions are promising for such improvements. This review highlights the significant progress to date, pinpoints the unmet needs, describes the model characterization requirement for industry adoption and proposes short and long-term utility of the model.

Overall, there is hope that MPSs will be able to fill the gaps in ADME field that traditional systems are unable to overcome. To accomplish these goals, it would be beneficial if the development of these models is conducted in a systematic manner to maximize efficiency and provide clear and interpretable study outcomes. This should include profiling of the various available MPS models. Partnership of bioengineers with ADME scientists in an industry wide collaborative effort across organizations might be the fastest and most efficient way to accomplish such goals and make the systems affordable for wider application. It is also critical that short-term and long-term goals (Fig. 1) are kept in mind to help focus the efforts:

- Short term objectives would include developing MPS for individual organ systems (*e.g.* liver, kidney, lung *etc.*), characterization of such systems for expression of ADME genes and evaluating their performance for functional activity and their utility for PK/PD, drug disposition and DDI.
- Long term objectives should focus on linking these individual organs together to fill more challenging gaps such as entero-hepatic recirculation, with the ultimate goal of creating "human-on-a-chip". If successful, the fully integrated system should be able to offer significant advantages over traditional systems; for example, the ability to determine effect of a metabolite formed in one organ over a different organ.

One of the most critical next steps is for MPS developers to modify the design of MPS models to allow the ability to collect sufficient samples over time for temporal drug quantification, and routinely make use of material that is low binding to avoid loss of test article to the apparatus. Future improvement of MPS systems can focus on recapitulating organ-specific vasculature and cellular heterogeneity of the complex organs (*e.g.* liver, intestine, kidney, brain). Promising approaches have already been reported to reproduce functional microvascular structures of MPS models.[175,176] Additionally, it will be useful to also expand the current co-culture systems to include relevant immune cells (*e.g.* macrophages, individual T cells), to assess the contribution of immune activation to drug toxicity. Building these elements in the MPS model can, perhaps allow the conduct of *in vitro* PK/PD studies and help triage molecules more efficiently, potentially reducing animal studies. While compatibility of genetic background is an important consideration in constructing functional and meaningful co-culture and organ-linked systems for applications involving

immune response, it is highly challenging to rely on only the donor-based system to achieve sufficient genetic compatibility of increasingly complex MPS systems. However, for clinical predictions, the value of controlling genetic background may be more in the ability to characterize the impact of pharmacogenomics. Therefore, the advancement of iPSC technology can have a game-changing impact on the production of MPS systems that truly represent individual physiology for ADME prediction with personalized precision. To achieve such objectives, the current iPSC systems will have to benchmark adequately against the primary human cells for matured ADME functions. The co-culture MPS system, on the other hand, may provide an *in vitro* platform to boost the maturation of iPSC cells. Furthermore, the emerging genome editing technologies, such as CRISPR-Cas9, may help edit in desired genetic background for ADME applications.

With the parallel advancement in other technologies, such as iPSC, the future organotypic MPS have the capability of producing *in vitro* systems that are truly individualized and representative of specific populations. In the future, successful application of organotypic MPS in ADME studies may improve the human prediction, reduce current dependence on preclinical animal models and reduce the overall risk and cost in drug development.

Abbreviations

ABC ATP binding cassette
ADME Adsorption, distribution, metabolism and excretion
BBB Blood–brain barrier
BCPR Breast cancer resistance protein
CES Carboxylesterase
CNS Central nervous system
CSF Cerebrospinal fluid
CYP Cytochrome P450
DCT Distal convoluted tubule
ENT Equilibrative nucleoside transporter
HRPT Human proximal convoluted tubule
IBD Inflammatory bowel disease
iPSC Induced pluripotent stem cell
IVIVE *In vitro–in vivo* extrapolation
LC-MS/MS Liquid chromatography – tandem mass spectrometry
LPS Lipopolysaccharide
MATE Multidrug and toxic compound extrusion protein
MDCK Madin–Darby canine kidney
MPS Microphysiological system
MRP Multidrug resistance protein
OAT Organic anion transporter
OATP Organic anion transporting polypeptide
OCT Organic cation transporter
Papp Apparent permeability
PCT Proximal convoluted tubule
PD Pharmacodynamic
PDMS Polydimethylsiloxane
PEPT Peptide transporter
PET Positron emission tomography
P-gp P-Glycoprotein
PK Pharmacokinetic
SULT Sulfotransferase
TD Toxicodynamics
TDI Time-dependent inhibition
TEER Trans-epithelial electrical resistance
TK Toxicokinetics
UGT UDPGA-glucuronosyl transferase

Authorship statement

All authors contributed to the writing, reviewing and editing of this manuscript.

Conflicts of interest

There are no conflicts of interest to declare.

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