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REVIEW ARTICLE

Towards human *ex vivo* organ perfusion models to elucidate drug pharmacokinetics in health and disease

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ABSTRACT

To predict the absorption, distribution, metabolism and excretion (ADME) profile of candidate drugs a variety of preclinical models can be applied. The ADME and toxicological behavior of newly developed drugs are often investigated prior to assessment in humans, which is associated with long time-lines and high costs. Therefore, good predictions of ADME profiles earlier in the drug development process are very valuable. Good prediction of intestinal absorption and renal and biliary excretion remain especially difficult, as there is an interplay of active transport and metabolism involved. To study these processes, including enterohepatic circulation, *ex vivo* tissue models are highly relevant and can be regarded as the bridge between *in vitro* and *in vivo* models. In this review the current *in vitro, in vivo* and in more detail *ex vivo* models for studying pharmacokinetics in health and disease are discussed. Additionally, we propose novel models, i.e., perfused whole-organs, which we envision will generate valuable pharmacokinetic information in the future due to improved translation to the *in vivo* situation. These machine-perfused organ models will be particularly interesting in combination with biomarkers for assessing the functionality of transporter and CYP450 proteins.

Abbreviations: ADME: absorption distribution metabolism excretion; DMPK: drug metabolism and pharmacokinetics; DDI: drug drug interactions; EHC: enterohepatic cyclus; PK: pharmacokinetics; PBPK: physiologically based pharmacokinetic modeling; CYP450: Cytochrome P450; OOC: organ-on-a-chip; PCS: precision cut slices; PCIS: precision cut intestinal slices; PCLS: precision cut liver slices; IPL: isolated perfused liver; IPK: isolated perfused kidney; NMP: Normothermic machine perfusion; OATP: organic anion transporting protein; PgP: P-glycoprotein; MRP: multi-drug resistance-associated protein)

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1. Introduction

Upon oral intake of a drug, intestinal absorption is the first barrier that affects the final drug concentration in the circulating blood (Shitara et al. 2005). Following intestinal absorption, drugs reach the liver via the portal vein, where they can be absorbed, metabolized and/or excreted into the bile. The liver is responsible for the biotransformation of many endogenous and exogenous compounds and is involved in the storage and biliary excretion of these compounds and their metabolites. In addition to the liver, extrahepatic tissues such as the GI tract and the kidneys significantly contribute to the clearance of drugs, as a diverse range of transporters and metabolizing enzymes also have been characterized in these organs (Krishna and Klotz 1994; Chan et al. 2004; Shitara et al. 2005; Müller and Fromm 2011; van de Steeg et al. 2012). Influx and efflux transporters (expressed on apical or basolateral membranes of these organs), and cytochrome P450 (CYP450) enzymes regulate the absorption of drugs across the intestinal wall into the portal blood, the clearance of drugs in the liver (and kidneys) and excretion by the biliary and renal pathways (Kusuhara and Sugiyama 2002). To determine how transporters and enzymes are involved in these processes is challenging as conventional research models are (too) simplified and do not properly reflect the conditions *in vivo*. For example, several studies have characterized the interplay between the efflux transporter P-glycoprotein (Pgp) and CYP3A4 (Benet et al. 2004; Lau et al. 2004). However, the role and contribution of transporters in biliary and renal clearance

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processes, the involvement of the enterohepatic cycle, and drug-drug interactions (DDI) in healthy and diseased tissues are still poorly understood.

Over the past decade, a variety of *in vitro*, *ex vivo, in vivo* and *in silico* preclinical models have been developed to characterize and predict absorption, distribution, metabolism and excretion (ADME) processes in the human situation. The ability to predict the ADME profile of a candidate drug in the preclinical phase is important to select drugs that are safe, effective and can advance to phase I clinical trials. Furthermore, the potential of the drug to interact with the kinetics of other drugs needs to be taken into account as predicting potential DDI is important for the patients' health. Concomitant administration of drugs can affect the pharmacokinetic and pharmacodynamic profile of one or both compounds. This can lead to a change in the systemic exposure or site-specific concentration of the drug, thereby altering the therapeutic outcome or increasing the risk of serious adverse events (Rowland et al. 1973; Singh 2006; Fagerholm 2008). Extensive research regarding the potential interactions between drugs is needed before later-stage clinical trials, as significant interactions can result in withdrawal of the drug from the market (Palatini and De Martin 2016). The impact of DDI on the development of new drugs was shown in a systemic review by the FDA showing that almost all new molecular entities were found to be perpetrators of metabolic interactions based on *in vitro* tests (Yu et al. 2014; Palatini and De Martin 2016). Therefore, it is important to study the functionality of transporters and enzymes, and their interplay in the liver, kidneys and GI tract as the most dominant organs involved in drug uptake, metabolism and excretion using proper preclinical models (Zhu 2020). This review describes the current models and novel developments using *ex vivo* tissues to improve the predictions of human ADME profiles and DDIs.

2. In vitro and in vivo models for the prediction of pharmacokinetics

2.1. In vitro *DMPK*

Multiple preclinical *in vitro* models have been developed to predict the pharmacokinetic profile and metabolic clearance of drugs. These models are mainly based on cell lines (e.g., caco-2, HEK293, MDCKII, HePaRG cells), primary cell cultures (e.g., hepatocytes), and isolated microsomes and vesicles to study organ function, drug metabolism, evaluation of transporter function and drug-induced organ toxicity (Li AP 2007). Genetically modified cell lines overexpressing a specific uptake or efflux transporter allow specific assessment of the interaction between drugs and transporter proteins. A major advantage of *in vitro* models is the possibility to study isolated processes such as phase I and II metabolism or to compare metabolism and transporter affinity across species (Lipscomb and Poet 2008). The medium- to high throughput nature of *in vitro* models makes them highly attractive in drug development screening processes. However, complex processes such as transporter-enzyme interplay are difficult to evaluate in *in vitro* models, as transport directionality and transporter-enzyme interactions at the organ level often cannot be established (Lau et al. 2004). Additionally, cell-based models frequently lack specific aspects of the organ, such as the presence of and interaction between different cell types or the ability to excrete bile or urine (Fabre et al. 1990; Guillouzo 1998). An example of an *in vitro* model that overcomes the problem of lacking bile excretion is the sandwich-cultured hepatocyte model. In this model, hepatocytes that are cultured between two layers of collagen form canalicular networks so that hepatobiliary disposition of compounds can be studied (Swift et al. 2010). A limitation of this model is the use of non-human hepatocytes leading to results that remain difficult to translate to the human *in vivo* situation due to differences in transporter expression between species (Swift et al. 2010).

2.2. *New developments in* in vitro *DMPK*

Currently, the development of new predictive preclinical *in vitro* models is a field that attracts a lot of attention. The discovery of organoids, a stem-cell-based 3D model derived from patient biopsy material, is generating serious attention especially in the field of disease development modeling (Sato and Clevers 2013). Furthermore, major improvements have been established lately in conventional cell models using a combination of 3D cell models and microfluidic technology, commonly referred to as 'organ-on-a-chip' (OOC). These models incorporate microfluidics and the flow thereby generated induces shear stress and mechanical strain to the cells, which has a major effect on cell adhesion, growth rate and differentiation processes (Yum et al. 2014). The OOC models can better mimic the complexity of *in vivo* organs and it is expected that they will be more useful than conventional *in vitro* cell lines (Bhise et al. 2014; Fowler et al. 2020; Zhu 2020). Kim et al. (2012), for example, reported a gut-on-a-chip model with the incorporation of a cyclic strain to mimic peristaltic motions, which is important for intestinal absorption processes and the ability to co-culture with

intestinal bacteria. An advantage of OOC models is the ability to connect multiple organs cultured on a chip using microfluids which allows study of the interconnection between metabolizing organs. Tsamandouras et al. (2017) developed a coupled gut-liver-chip model to study PK of multiple organs and the interconnection between the organs. Diclofenac and hydrocortisone were used as model compounds. A minor but significant difference was the formation of the CYP2C diclofenac metabolite 4-OH-DCF in gut-liver chip compared to the Liver-chip alone. Maschmeyer et al. (2015) reported a four-organ-chip including the intestine, liver, kidney and skin which can be used to study ADME processes. Although these systems are more complex than the conventional *in vitro* models, these systems remain cell based and as such limitations remain.

2.3. In vivo *animal DMPK models*

To study *in vivo* processes in the preclinical phase, a variety of laboratory animal models are used. Laboratory animals are useful in different stages of drug discovery and development and are systematically used to find bioavailable compounds, compare pharmacokinetics (PK) across species, determine the no-observed-adverse-effect-level (NOAEL), test DDI potential and ensure safety (Zhang et al. 2012). To study the contribution of specific transporters in absorption or clearance processes of a drug, knock-in or knock-out mouse models are often used (van de Steeg et al. 2010). However, most results obtained in pre-clinical studies using experimental animals do not translate well to the human situation (Shanks et al. 2009). An important contributor to this loss-in-translation is that PK processes are very different in animals and humans; i.e., the amount of drug that enters the circulation in animals shows little correlation to the relative amount measured in the blood of humans (Shanks et al. 2009). This is not only a result of differences in physiology between animals and humans, but also due to differences in transporter expression between species (Wendler and Wehling 2010). Humanized mouse models are a step toward human *in vivo* DMPK (Ito R et al. 2012). Katoh et al. (2007) showed the metabolism of CYP2D6 substrate debrisoquin in a humanized liver mouse model and showed the presence of human albumin in the blood of the chimeric mice indicating the presence of human hepatocytes. However, the prediction of intestinal absorption (or bioavailability) and renal and biliary excretion remain difficult, as there is an interplay of active transport and metabolism involved. Prediction of biliary excretion based on bile cannulation in rats or dogs often fail due to species differences (Guillouzo 1998; Ito K et al. 1998; Lentz 2008; Musther et al. 2014). The enterohepatic circulation (EHC) adds complexity to predicting biliary excretion. An increasing number of compounds is subjective to EHC, making it difficult to predict plasma profiles after oral or intravenous (iv) administration.

2.4. In vivo *human DMPK*

There are limited methods available to study clinical ADME processes in humans early in the drug development phase. One of these methods includes early-stage mass balance and metabolite profiling studies using a microtracer of the newly developed pharmaceutical compound labeled with [^{14}C] radiolabel to characterize the PK of the drug using highly sensitive accelerator mass spectrometry technology (Lappin et al. 2013; Swart et al. 2016; Mooij et al. 2017; van Groen et al. 2019). There are also some unique examples of studies using human subjects undergoing surgery where it is possible to measure intestinal permeability or biliary clearance. For example, *in vivo* intestinal permeability can be assessed using a Loc-I-Gut perfusion system, placed in the jejunum where during a single-pass perfusion, the permeability of a certain compound can be studied (Lennernäs 2007). To date, studying biliary excretion in humans is, only possible in postsurgical patients suffering from hepatobiliary diseases and remains very challenging (Ghibellini et al. 2006).

3. *Ex vivo* models

By using whole or partial organs and tissues, *ex vivo* models are recognized as the bridge between *in vitro* and *in vivo* models. When studying PK and DDI in health and disease, the complexity of the organ must be represented in the preclinical model. It is expected that this will lead to better predictions of DMPK than predictions from more simplified, single cell type *in vitro* systems (Benet et al. 2003; Vickers and Fisher 2005). In *ex vivo* models, the tissue morphology is maintained including cell-cell interactions, cell-matrix interactions, the complex efflux/uptake transporters and CYP enzyme expression. In the upcoming section the importance, advantages and limitations of existing *ex vivo* models for applications in preclinical DMPK research will be discussed (Table 1; Figure 1).

3.1. *Precision cut organ slices*

Precision cut organ slices (PCS) are widely used in the field of pharmacology and toxicology research (Vickers

Table 1. Overview of the discussed *ex vivo* models with their advantages and disadvantages.

Model				References	Advantages	Disadvantages
Precision cut slices	Intestine (PCIS)			• (van de Kerkhof et al. 2008) • (Possidente et al. 2011)	• High throughput model • Suitable for toxicity assessment	• One-compartmental model; No luminal and serosal compartment
	Liver (PCLS)			• (Renwick et al. 2000) • (De Graaf et al. 2010) • (Berginc et al. 2010)	• Suitable for human and animal derived tissue	• No biliary and renal excretion • Static model
	Kidney (PCKS)			• (Arakawa et al. 2017) • (Arakawa et al. 2019)		
Intestinal Two-compartmental models	Ussing Chamber			• (Sjöberg et al. 2013) • (Rozehnal et al. 2012) • (Haslam et al. 2011)	• Intestinal transport from A > B and B < A can be assessed	• Static model • Limited viability of the model, up to a maximum of 6h of incubation
	InTESTine			• (Westerhout et al. 2014) • (Stevens et al. 2019)	• Assessment of efflux transporters using inhibition studies	
	Everted gut sac model			• (Alam et al. 2012) • (Barthe et al. 1998)	• Multiple intestinal areas can be applied	
Perfusion models	Intestine			• (Lindahl et al. 1998) • (Gandia et al. 2004) • (Burks and Long 1966) • (De Vries MH et al. 1989) • (Midwoud 2010) • (Dawson et al. 2016)	• Relevant physiological morphology • Able to study biliary and renal excretion	• Difficult laboratory skills involved • Low throughput model • Viable for 3–4 h of incubation
	Liver (IPL)	Lobe	Human	• (Melgert et al. 2001) • (Schreiter et al. 2012) • (Schreiter et al. 20160		
		Whole liver		• (Villeneue et al. 1996)		
			Animal	• (Jablonski et al. 1971) • (Gores et al. 1986)		
	Kidney (IPK)			• (Nishiitsutsuji-Uwo et al. 1967) • (Van Crugten et al. 1991) • (Hori et al. 1993)		
Machine perfusion models	Whole Liver			• (Boehnert et al. 2013) • (De Vries et al. 2019) • (Watson et al. 2018) • (Eshmuminov et al. 2020)	• Suitable for (diseased and discarded) human organs • study biliary and renal excretion • Highly translatable to *in vivo* condition • Suitable for human (endogenous) biomarker research	• Complex chirurgical skills involved • Low throughput model • Limited to discarded and/or diseased human organs
	Kidney			• (Hosgood S et al. 2015) • (Nicholson and Hosgood 2013)		

and Fisher 2005; Baverel et al. 2013; Troth et al. 2019). It is an easy and medium-throughput technique where drug metabolism can be studied in an experimental and controlled situation with the main advantage that the tissue organization and cell-cell and cell-matrix interaction are maintained (Parrish et al. 1995; Guillouzo 1998; De Graaf et al. 2010). Multiple studies show the applicability of precision-cut slices for metabolic capacity assessment, drug induction, inhibition and drug transport to determine phase I and phase II metabolism and drug uptake by measuring the intracellular concentration in the slices at different time intervals (Renwick et al. 2000; van de Kerkhof et al. 2008; Possidente et al. 2011; Li M et al. 2016). The intestines, liver and kidneys are heterogenous organs with regional expression of transporters and enzymes in the intestine, metabolic zonation in the liver and cellular heterogeneity and structural complexity of the kidney. Therefore the PCS technique can be very useful for understanding the heterogeneity of the organ, an advantage over cellular *in vitro* systems (Parrish et al. 1995; Kanter et al. 2002). Li et al., for example used the precision cut intestinal slices (PCIS) technique to study regional expression of Pgp and the potency of Pgp inhibitors (Li M et al. 2016). Recently, Arakawa et al., studied azasetron uptake as substrate drug of efflux transporter mdr1a in rat kidney slices (Arakawa et al. 2019). Additionally, increased accumulation of azasetron was measured in the presence of the mdra1 inhibitor zusuquidar showing the ability to use organ slices for DDI studies. However, whether DDI leads to a higher systemic exposure or alters biliary or renal clearance cannot be fully mimicked since the tissue is freely floating in culture medium with direct communication of intraluminal and extraluminal compartments.

Organ slices are widely used and are still a relevant technique in the field of DMPK. Researchers use PCS to study nanoparticle formulations (Bartucci et al. 2020; Daga et al. 2020), anticancer drugs or molecular transport mechanisms (Estrada-Ortiz et al. 2017;

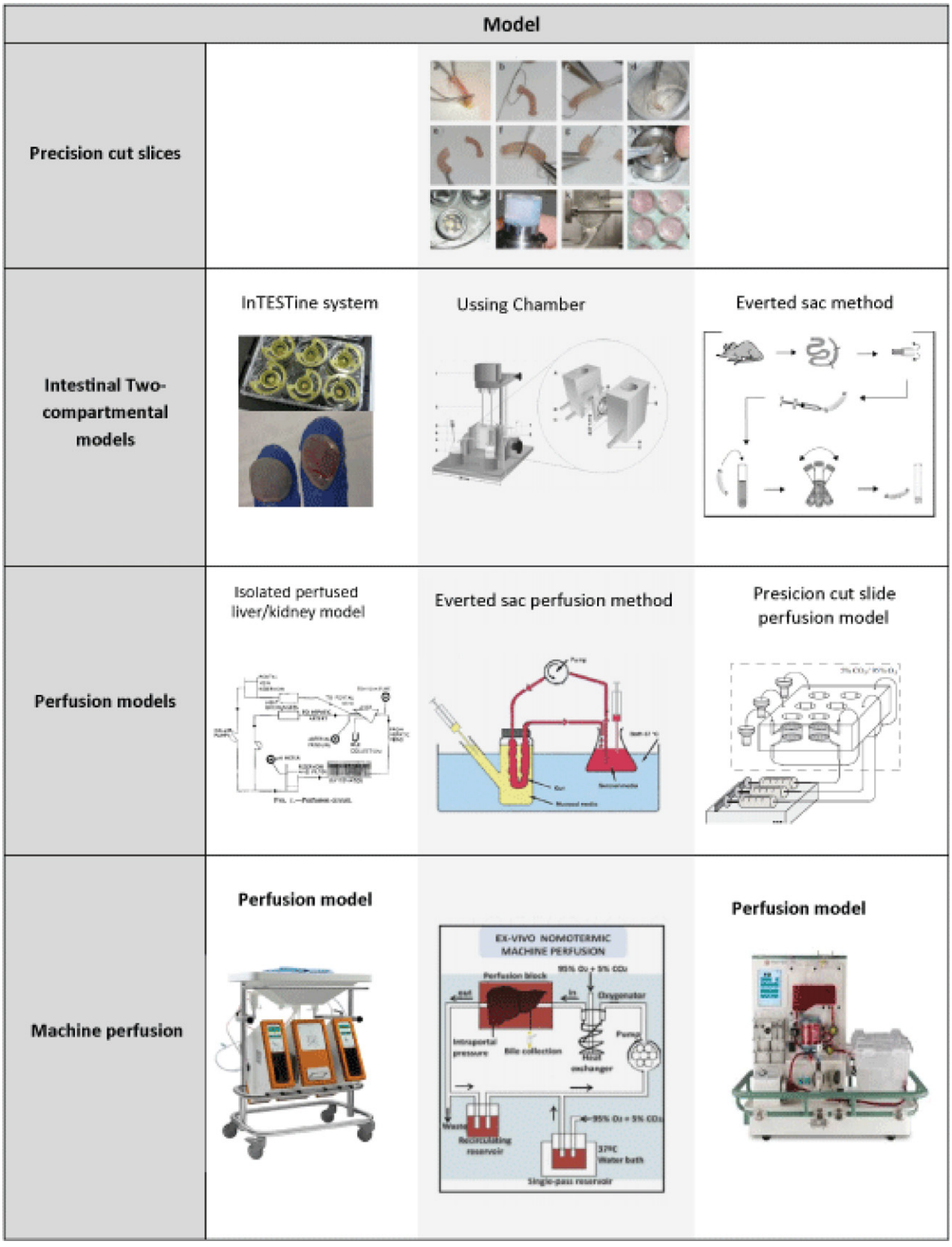

Figure 1. Schematic overview of the discussed *ex vivo* models. Prescision cut slices (De Graaf et al. 2010), InTESTine system (Westerhout et al. 2014; Stevens et al. 2019), Ussing Chamber (Sjöberg et al. 2013), Everted sac method (Santos et al., 1999), Isolated perfused liver/kidney model (Jablonski et al. 1971), Everted sac perfusion method (Gandia et al. 2004), Presicion cut slice perfusion model (Midwoud 2010) Liver perfusion models (Jiménez-Castro et al. 2013 OrganAssist; Organox).

Spreckelmeyer et al. 2017). Additionally, the PCS technology and microfluidics can also be combined (Meekel et al. 2018; Rodriguez et al. 2020). However, the main limitation of the model is that it is qualitative rather than quantitative as basic pharmacokinetic questions such as prediction of intestinal absorption, biliary and urinary excretion, enterohepatic circulation and the effect of DDI on these processes cannot be studied.

3.2. Intestinal two-compartment models

As described in Section 3.1.1., PCIS are freely floating in culture medium, so that there is no intestinal barrier between the luminal and the serosal compartment. However, to study intestinal absorption it is important to have two separate compartments. Therefore, several dual compartment *ex vivo* methods have been developed that allow study of intestinal absorption of drugs and nutrients while maintaining barrier integrity.

3.2.1. The Ussing system

The Ussing chamber is a model in which excised mucosal intestinal tissue is mounted vertically in the system under continuously oxygenated conditions. Subsequently, vectorial transport and intestinal wall metabolism of drugs can be studied. Several studies have shown the applicability of the Ussing system in determination of transporter expression and the transport of different drugs including substrates for efflux transporters (Haslam et al. 2011; Rozehnal et al. 2012; Sjöberg et al. 2013). For instance, the presence and activity of the efflux transporter Pgp was shown by incubating the Ussing system with the Pgp substrates digoxin, cimetidine, vinblastine, quinidine and verapamil, and a clear efflux effect was shown for the first 4 of the 5 compounds (Sjöberg et al. 2013). Hence, the Ussing system is a useful method to predict whether newly developed drugs can affect the intestinal absorption of another compound when dosed at the same time, or if they are a substrate for efflux transporters located in the gut. Unfortunately, the model is limited by its low throughput and the difficult laboratory preparation work involved.

3.2.2. Intestine system

Based on the Ussing system, Westerhout et al. (2014) developed a new intestinal two-compartment model called the InTESTine system, with easier handling and higher throughput. In this system, segments of the mucosal intestinal layer can be mounted horizontally in a 6-well or a 24-well plate device. Smaller tissue segments and reduced apical and basolateral volumes can be used in comparison with the Ussing system (Westerhout et al. 2014; Vaessen et al. 2017; Stevens et al. 2019). Using this model, CYP3A-mediated testosterone metabolism as well as the intestinal permeability of a subset of compounds was determined. Additionally, the suitability of the two-compartment model to study DDI with PhIP as a BCRP substrate and elacridar as inhibitor was shown (Westerhout et al. 2014). Recently, the same group showed the applicability of the InTESTine system with human intestinal tissue to predict the fraction absorbed (F_a) in humans (Stevens et al. 2019). Besides determining the transport of a subset of compounds across the intestinal wall, regional differences in transporter activity were assessed following the regional variability in Pgp expression in ileum and mid-colon tissue. This shows the additional value of a two-compartment intestinal model where drugs can be applied at both sides to study the interaction of the compounds at the transporter levels as well as the differential expression of transporters along the GI tract (Stevens et al. 2019).

3.2.3. Everted sac method

In contrast with the Ussing and InTESTine model, the everted sac method uses whole enclosed small parts of the intestines (Wilson and Wiseman 1954; Levine et al. 1970; Bridges 1980). In this model, the excised intestines are inverted, closed at both ends and placed in an oxygenated buffer. Drug metabolism, transport and the contribution of transporters to drug absorption can subsequently be studied (Roeselers et al. 2013). Several studies showed the use of the everted sac method to determine Pgp activity and to test compounds that are potential Pgp modifiers (Alam et al. 2012). For example, Barte et al., assessed the activity of the Pgp transporter by showing an increase in digoxin transport upon co-incubation with the Pgp inhibitors verapamil and quinidine (Barthe et al. 1998). Limitations of this model are that it is restricted to the use of animal tissue, that it uses a relatively large surface area and the presence of the muscularis mucosa layer which can result in an underestimation of drug transport (Alam et al. 2012). While the model is very suitable for different animal species, the model is rather low throughput with difficult set-up and preparation work involved and therefore not widely applied.

3.4. Perfusion models

The *ex vivo* tissue models discussed thus far study the fate of a drug in a static environment. However, *in vivo* blood flow continuously stimulates cells with chemical,

electrical and mechanical cues which can alter the behavior of a drug (Bhadriraju and Chen 2002). *Ex vivo* perfusion models make use of hemodynamics and therefore these models are suitable to determine specific functions of the whole organ in an isolated environment in the absence of other systemic effects (Roeselers et al. 2013). In the next section we focus on whole organ (liver and kidney) that have been developed to study biliary and renal excretion, the two most important excretion routes of drugs and tissue (intestine) perfusion models.

3.4.1. Intestinal perfusion models

Multiple intestinal perfusion methods have been developed. The incorporation of fluidics into conventional preclinical models using tissue explants has been reported by several research groups (Burks and Long 1966; De Vries MH et al. 1989; Gandia et al. 2004). For example, the everted sac model was developed into a perfusion model by Gandia et al. (2004). An advantage of using perfusion in contrast to the static everted gut sac model is that the serosal side is continuously perfused which can affect the kinetics of rapidly absorbed drugs during long term incubations. Additionally, the blood supply, intestinal tissue, innervation and clearance capabilities remain intact (Barthe et al. 1998; Stappaerts et al. 2015). The isolated perfused intestine is often used to assess the effect of flow on intestinal permeability and to determine the involvement of transporters and enzymes on drug absorption (Schanker et al. 1958; Prasad and Bhasker 2012). Many researchers have used the isolated perfused intestine technique to study CYP3A4-Pgp interplay or the specific contribution of drug transporters by using intestinal tissue from knockout animals (Stappaerts et al. 2015). A minor limitation of the perfusion technique involves the difficulty to properly measure the disappearance of a compound from the perfusion solution, especially for low permeable compounds (Stappaerts et al. 2015). Moreover, although the isolated perfused intestine is very useful for mechanistic evaluation, the limited viability of the tissue restricts the assessment of processes aside from drug transport or the assessment of transporter function (Maeng et al. 2011), such as host-microbe interactions and immune responses which play a significant role in gut health and barrier function but also the absorption and metabolism of drugs (van de Steeg et al. 2018). Extending the viability of the tissue would enable study of long-term exposure to drugs, intestinal absorption of low permeable drugs and the potential effects of drugs on the inflammatory state of the tissue. Here lies potential for OOC models that use fluidics to create a more physiologically relevant environment for the tissues. Although many studies show gut-on-a-chip perfusion models using single-cell lines (e.g., Caco-2, HT-29 cells) or using intestinal organoids, limited gut-on-a-chip models are known using the perfusion of tissue segments. Dawson et al., showed perfusion of inflammatory bowel disease (IBD) tissue segments in a dual-perfusion 'gut-on-a-chip-model' up to 72 h of incubation (Dawson et al. 2016). Proper viability was shown by the presence of the crypts and goblet cells and also a Ki67 staining showed the proliferative capacity of the tissue after incubation. However, barrier function and drug absorption were not studied. Together, these studies show the beneficial effect of fluidics on tissue viability enabling study of prolonged exposure of compounds on intestinal tissue.

3.4.2. Liver perfusion models

Liver perfusion models have attracted far greater attention than intestinal perfusion models. Using PCLS, various perfusion techniques have been developed; e.g., intra-tissue microneedle flow models, perfusion of the top and bottom of liver slices and flow through models (Khong et al. 2007; Schumacher et al. 2007; Midwoud 2010). The isolated perfused liver (IPL) model, a preclinical tool used to study the function of the whole liver and hepatobiliary disposition of drugs, uses perfusion of both the portal vein and the hepatic artery. Furthermore, IPL can be used to evaluate the physiology and pathophysiology of the liver and to study treatment of acute liver failure (Jablonski et al. 1971). A unique aspect of the model is the close representation of *in vivo* morphology since the 3D architecture of the tissue is maintained. The control over physiological conditions, and determination of specific functions of the whole organ in an isolated environment in the absence of other systemic effects are major advantages over *in vitro* and *in vivo* studies. For example, assessment of the effect of albumin concentration on clearance processes is widely studied using the IPL model. Tsao et al. (1988) studied warfarin uptake in the IPL in absence and presence of bovine serum albumin (BSA) to clarify the albumin-mediated uptake of warfarin (Stollman et al. 1983). Schary and Rowland (1983) found that the unbound fraction of tolbutamide varied in the perfusate upon varying concentrations of albumin or when using albumin from different species. Shand et al. (1976) clearly showed a reduction in hepatic extraction of phenytoin upon increasing the albumin concentration form 0.5 g/dl to 5.0 g/dL. Another physiological condition that can be controlled during perfusion is the applied flow rate. This is especially interesting since the

overall hypothesis is that the clearance of drugs is related to the organ perfusion flow and the extraction ratio. Lidocaine, a high hepatic extraction ratio drug is often used as a model drug in these studies showing flow-dependent extraction, in comparison with the low extraction ratio drug antipyrine which is not affected by hepatic flow (Pang and Rowland 1977). Additionally, the same researchers show in a different study that the metabolite of lidocaine, MEGX, appears to be flow dependent, thus showing that the parent drug and metabolites are in equilibrium with the concentration of the drug in the liver showing that the well-stirred model describes the hepatic drug clearance (Pang and Rowland 1977). To study the physiology of the liver and to understand the metabolic zonation of the liver, experiments have been performed using antegrade and retrograde perfusion directions (Pang et al. 1983; O'Sullivan et al. 1998). Livers from different species can be assessed; the most studied species in this context are pig and rat. Furthermore, an advantage of the IPL compared to the *in vivo* situation is the ability to study biliary secretion which is also an important improvement over the sandwich-cultured hepatocyte model (Guillouzo 1998). As the interaction between two drugs may affect the biliary clearance of a drug, the IPL is an excellent model to study the effect on hepatobiliary clearance and the involvement of uptake and efflux transporters. Pfeifer et al. used the IPL to show the biliary clearance of rosuvastatin, studying the involvement of the basolateral efflux transporters MRP2 and BCRP (Pfeifer et al. 2013). Using perfused livers from MRP2-deficient mice in the absence and presence of the BCRP inhibitor elacridar the researchers showed a strong reduction in rosuvastatin excretion in MRP2 deficient tissue in the presence of elacridar (Pfeifer et al. 2013). Booth et al. studied the concentration of the Pgp substrate doxorubicin in the presence and absence of the Pgp inhibitor quinidine. The biliary clearance was significantly reduced upon inhibition of Pgp, while perfusate and liver concentrations were not altered. The researchers also showed an increased intracellular concentration of the metabolite doxorubicin thus showing the added value of using the IPL model since the perfusate, liver and biliary secretion can be studied at the same time (Booth et al. 1998). The effect of enzyme-transporter interplay using the IPL was shown by Lau et al., who studied the disposition of digoxin and the effect on the systemic concentration when digoxin was dosed together with the organic anion transporting protein 1B1/1B3 (OATP1B1/OATP1B3) inhibitor rifampicin and the Pgp inhibitor quinidine (Lau et al. 2004). Although the IPL is a standardized and validated model, it is mainly applicable to rodents when studying drug pharmacokinetics. Furthermore, it is labor intensive, has a low throughput and the functional integrity is limited to up to a few hours (Bessems et al. 2006). The model is still used to study liver diseases as the model includes immune cells which are involved in many liver diseases. However, it remains difficult to recapitulate the metabolic and excretory function of the liver in a chip model, therefore the IPL is still the best model (Fowler et al. 2020).

3.4.3. Isolated perfused kidney (IPK)

Most drugs, in particular water-soluble drugs, are eliminated by the kidneys and excreted by the urine (Bekersky 1983). Renal secretion involves several processes such as glomerular filtration, tubular secretion and reabsorption (Van Ginneken and Russel 1989). The current main preclinical kidney models (based on primary cells or cell lines) primarily focus on the function of proximal tubuli cells. Therefore, *ex vivo* models where all main renal processes can be studied, including urinary excretion, are very valuable. Although isolated perfused kidney (IPK) is an interesting method to predict renal metabolism as well as renal clearance, only a handful of studies used the IPK model to study renal metabolism and excretion. Using IPK, Nishiitsutsuji-Uwo et al. (1967) showed the clearance of creatinine in the perfused rat kidney showing the function of the multidrug and toxin extrusion protein transporter 1/2 K (MATE 1/2 K) and the organic anion transporter (OCT). Although perfusion and the urinary flow remained constant, a decrease in clearance function over time was observed. Van Crugten et al. (1991) used the IPK technique to elucidate the renal mechanism involved in morphine clearance. They showed the additive value of a complex whole-organ model since glomerular filtration, active tubular secretion and possibly active reabsorption were processes involved in the metabolism and excretion of morphine. Besides elucidation of clearance mechanisms, DDI can also be predicted using the IPK technique. For instance, the renal tubular secretion of the Pgp substrate digoxin was shown in a study by Hori et al., where the researchers showed the dose-dependent inhibition of urinary secretion of digoxin upon co-incubation with Pgp the inhibitor quinidine (Hori et al. 1993).

4. Novel *ex vivo* perfusion models

Although substantial relevant information regarding PK using the isolated perfused organ method has been obtained, a major limitation of the model is the short

viability of 3–4 h, its animal origin, and the decline in integrity of the perfused organs (Gores et al. 1986; Guillouzo 1998). At present, there is a lot of development in the field of organ transplantation regarding organ preservation techniques using machine perfusion. Clearly, organ preservation is of main importance, but there is also an increasing interest in the viability and functional assessment of discarded donor organs which, after machine perfusion, might be used for transplantation when found to be of sufficient quality (Vogel et al. 2010; Watson et al. 2018). The use of pressure-driven perfusion pumps allows study of the function of a whole organ under conditions that are as close as possible to the *in vivo* situation. Many studies have shown viability and functionality of porcine and human livers during machine perfusion under normothermic conditions (37 °C) using a red blood cell-based perfusate (Borie et al. 2001; Boehnert et al. 2013; Watson et al. 2018; de Vries Y et al. 2019) even up to 7 days (Eshmuminov et al. 2020) Similar to normothermic machine perfusion (NMP) of the liver, NMP of the kidney is a method for quality assessment of extended criteria donor kidneys (Hosgood S et al. 2015). Porcine organs are often used for method validation and device development and it has been shown that normothermic machine perfusion (NMP) of organs is an excellent platform to study hepatic and renal processes (Butler et al. 2002; Jamieson et al. 2011; Hosgood SA et al. 2015; Vogel et al. 2017; Yoshikawa et al. 2018).

4.1. Normothermic machine perfusion for DMPK research

Ex vivo machine-perfused whole organs might be an interesting new platform to study pharmacokinetics and DDI in the liver and kidney. The commercially available pressure-driven perfusion machines for kidney and liver can manually apply and adjust pressure to theartery and portal vein (OrganAssist n.d.; Organox n.d.). Adjusting the pressure will result in an altered flow through the organ and this opens up the possibility to study for instance flow-dependent clearance of compounds. Additionally, researchers are able to take samples from multiple locations in the circulation (e.g., portal vein and vena cava inferior) to study the hepatic first pass effect, or to study intravenous versus portal dosing and the effect of DDI. Compared to isolated perfused-organ systems using rat organs or cannulated animal studies, the human or porcine machine perfusion models have relatively high circulating perfused volume (approximately 2 L) and bile and urine output (approximately >10mL). This has several advantages. Collection of perfusate and urine and bile samples over time and collection of tissue biopsies to study intracellular accumulation is easy which makes these models ideal for studying drug pharmacokinetics, metabolism, excretion and potential DDIs in a controlled setting. The applicability of machine perfusion for whole intestines has not (yet) been developed, probably because intestinal transplantations are not widely performed.

4.2. Opportunities involved in the perfusion of whole organs for DMPK

The major advantage of using whole-organ perfusion models which are perfused with a red blood cell-based perfusate offers a unique opportunity to study renal and biliary excretion pathways, allowing study of more complex pharmacokinetic processes which leads to a better understanding of *in vivo* processes. Currently, research is focused on finding novel endogenous biomarkers in bile, urine and blood which reflect the function and the status of organ-specific drug-metabolizing enzymes and drug transporters (Fromm 2012; Rodrigues et al. 2018).

An example of such an endogenous biomarker is bilirubin, a degradation product of heme, which is transported by the hepatic Organic Anion Transporting Protein 1B1 (OATP1B1) and OATP1B3 into the liver. In the liver, bilirubin is conjugated to bilirubin glucuronide by UGT1A1 and subsequently excreted by MRP2 in the bile or by MRP3 in the blood. This means that potential interaction of drugs with OATP1B1 and/or -1B3 transporter can be assessed by measuring the unconjugated bilirubin concentration in the blood. As OATP1B1 and OATP1B3 are involved in the clearance of a variety of drugs by the liver, applying an endogenous biomarker reflecting the function of these transporters is of major value (Chu et al. 2017). The conjugated bilirubin concentration in the bile reflects the involvement of MRP2 while conjugated bilirubin in the blood is a biomarker for MrRP3. In contrast to the IPL where often radiolabeled [^{3}H] bilirubin is used to measure bilirubin in a limited volume, a recent study by Eshmuminov et al. (2020) showed the bilirubin levels in the plasma and bile of perfused livers without using [^{3}H] bilirubin, thereby showing proper OATP1B1/1B3 function during perfusion of the livers. Additionally, coproporphyrin I (CPI) and III (CPIII) (byproduct of heme synthesis), dehydroepiandrosterone sulfate, conjugated and unconjugated bile acids and fatty acid dicarboxylates can also function as a biomarker of OATP1B1 and -1B3 function (Shen et al. 2016; Chu et al. 2017; Rodrigues et al. 2018). The application of endogenous biomarkers as a useful

tool to study transporter involvement in clinical trials was shown by Jones et al. (2020) The researchers measured the endogenous biomarkers CPI and CPIII in a clinical study to elucidate the involvement of OATP1 transporters for DDI of fenebrutinib with midazolam, simvastatin and rosuvastatin (Jones et al. 2020). As CPI and CPIII concentrations remained unchanged upon fenebrutinib administration, the researchers concluded that the observed DDI was due to involvement of CYP3A and BCRP rather than the OATP1B transporters. Likewise, different endogenous biomarkers for transporter functionality have been established for the kidney: hippurate and taurine (for transporter OAT1), 6B-hydroxycortisol (OAT3), thiamin (OCT/MATE1/2K), tryptophan (OCT2) and creatinine (MATE1/2K and OCT2). Besides endogenous biomarkers for transporter function it has been reported that 4B-hydroxycholesterol (4βHC) is an endogenous metabolite formed by the major hepatic metabolizing enzyme CYP3A4, thus functioning as a marker for CYP3A4 functioning (Chu et al. 2017). In a clinical study by Kasichayanula et al., it was shown that upon administration of ketoconazole, an inhibitor of CYP3A4, 4βHC decreased. Subsequently upon administration of rifampicin, increased 4βHC was measured (Kasichayanula et al. 2014). Endogenous biomarkers can play a significant role in organ perfusion as the biomarkers reflect the condition of the organ during perfusion. The ability to administer victim and perpetrator drugs in a controlled setting and the easy access to plasma, bile and urine can generate valuable pharmacokinetic and DDI knowledge. On the contrary, organ perfusion models can contribute to the validation of endogenous biomarkers e.g., the inhibition of specific transporters and studying the effect of a compound on the biomarker profile. Additionally, perfusion models can play a major role by the identification of new endogenous biomarkers.

Moreover, novel perfusion machines with incorporated pressure, temperature and flow sensors can also be a major advantage for the field of advanced physiologically-based pharmacokinetic (PBPK) modeling. PBPK models are mathematical multicompartmental models where assumptions based on data generated in *in vitro* and *ex vivo* models regarding transporter abundance, metabolizing enzymes and biliary and urinary excretion are made to predict the clearance of a drug (Fleishaker and Smith 1987; Jones and Rowland-Yeo 2013). Using these assumptions, simulated profiles are generated and subsequently validated with *in vivo* animal studies to see whether PBPK models match the *in vivo* data. The incorporation of experimental data derived from perfused organs into advanced PBPK models can be highly interesting. Clearance, excretion processes, intracellular concentrations, CYP enzymes and transporter expression can all be determined. At the same time, flow settings can be controlled. Of special interest for PBPK modeling are diseased organs since it is known that in liver disease for instance, different processes as the hepatic blood flow, CYP enzymes and transporter expression are altered as well as changes in liver and renal function (Jones and Rowland-Yeo 2013).

5. Application of *ex vivo* models in disease

Accurate predictions in diseased patients remain difficult as proper preclinical models for human diseases are often lacking. It would be a major advantage if diseased human tissues could be used earlier in the drug development process to assess disease-specific drug efficacy and safety. The challenge of using human tissue is the donor-to-donor variability and differences in disease status. However, this can be seen as an advantage since the use of *ex vivo* models is a step closer to the *in vivo* situation and thus may be a better prediction of *in vivo* DMPK processes. To measure the disease status or quality of the organs, endogenous biomarkers can play a role. This is currently widely applied in the field of organ transplantation using biomarkers in bile, urine and blood as quality assessment of perfused organs (Watson et al. 2018; de Vries Y et al. 2019).

5.1. Ex vivo *models in intestinal disease*

IBD, which includes ulcerative colitis and Crohn's disease, is a group of intestinal diseases with increasing prevalence (Bilski et al. 2019). It has been reported that the expression of certain transporters is altered in the inflamed intestinal regions of patients suffering from IBD (Blokzijl et al. 2007; Blokzijl et al. 2008). For example, it was shown that in mice with intestinal inflammation, Pgp transporter expression and activity was reduced (Buyse et al. 2005). Interestingly, in the non-inflamed regions an increased activity of Pgp was detected, showing a possible compensatory mechanism in the intestines. Using inflammatory and non-inflammatory regions from IBD patients, the application of PCIS or tissue segments mounted in a two-compartment setting can be helpful to determine the F_a of compounds, the effect of drug substrates for certain (efflux) transporters, DDI and the potential altered effect on first-pass metabolism. On the contrary, when specific drugs in the treatment for IBD need to be tested, an intestinal perfusion model using dual fluidics would be crucial to assess the effect of immune

modulating drugs on the inflamed intestinal tissue as a longer incubation period is needed and thus extended viability of the tissue needs to be guaranteed.

5.2. Ex vivo *models in liver disease*

Altered hepatobiliary transporter expression as well as affected CYP expression are found in patients suffering from liver diseases such as cirrhosis, nonalcoholic steatohepatitis and primary sclerosing cholangitis (George et al. 1995; Zollner et al. 2003; Czerwinski et al. 2019). This may drastically affect drug treatment and drug efficacy in these patients. The use of PCLS of diseased human liver tissue generated useful information regarding the altered metabolic capacity of the diseased liver (Westra 2014). Additionally, using human fibrotic tissue in PCLS has proven to be beneficial when studying potential antifibrotic drugs, DDI and drug induced liver injury (DILI) in fibrotic liver tissue (Westra 2014). Despite the fact that perfusion models can be very informative and unravel underlying mechanisms in liver disease, the perfusion of the isolated liver is only limitedly used for studying liver diseases using rodent livers as liver diseases need to be induced. Promising models include diet-induced (genetically modified) murine models of nonalcoholic steatohepatitis, reflecting the key inflammatory and cirrhotic processes involved (Morrison et al. 2018; Abe et al. 2019).

Machine perfusion of redundant whole human organs would be the holy grail, but obviously the use of this technology for pharmacokinetic application is hampered by the availability of healthy human donor organs. There are however opportunities when applying this technology (or machine perfusion) to 'diseased' organs which, following a pathological assessment are currently discarded after transplantation. Studying the pathophysiology of diseased livers using human orthotopic tissue is particularly helpful as animal models and human disease remain difficult to compare. However limited studies have evaluated PK processes of these redundant human organs. Villeneuve et al. were on of the first to describe the use of perfused whole human cirrhotic livers to study drug disposition and metabolism in diseased livers (Villeneuve et al. 1990, 1996). Their research showed that the application of diseased liver in a perfusion setting is very useful for studying the clearance function of the liver in the healthy and diseased state (Villeneuve JP et al. 1990). As very little is known regarding the uptake of drugs in humans and especially in cirrhotic patients, studying site-specific delivery of drugs in an isolated environment of the other organs is of additional value (Melgert et al. 2001). Melgert et al. and Schreiter et al. studied the functionality of a liver lobe and resected tissue of cirrhotic and non-cirrhotic livers that were being perfused under normothermic conditions. Melgert et al. showed phase I and phase II metabolism of lidocaine and 7-hydroxycoumarin in the liver lobe perfusion model (Melgert et al. 2001), while Schreiter et al. compared the metabolic activity of resected tissue from cirrhotic livers to non-cirrhotic livers (Schreiter et al. 2012). The metabolism of paracetamol, midazolam and diclofenac, metabolized by CYP1A1, CYP3A4 or CYP2C9, respectively, was studied and it was shown that cirrhotic resected tissue had diminished and slower phase I and phase II transformation of those drugs compared to non-cirrhotic tissue (Schreiter et al. 2012). The same researchers showed acetaminophen-induced liver injury of resected liver tissue as they were able to keep the tissue viable for up to 30 h by making use of NMP. Thereby they show the potential of using diseased perfused livers for the pharmacokinetic application and prolongation of the viability of resected tissue (Schreiter et al. 2016).

5.3. Ex vivo *models in kidney disease*

A kidney disease that affects kidney function is polycystic kidney disease (PKD). Patients develop fluid-filled cysts in their kidneys which can affect the PK of drugs. Besides altered kidney function, PKD can also affect other organs like the liver and thereby alter liver transporter expression and function (Bezençon et al. 2019). A case report from 1988 described the association of liver cysts with polycystic kidney disease (PKD) and the relation to MRP2 function (Salam and Keeffe 1989). Although the use of human tissue is preferred for the prediction of drug metabolism, the use of animal models can be useful in understanding the development of disease processes or the effect of diseases on the function of other metabolic organs. Bezençon et al. (2019) used the IPL model using PKD induced rats to determine the effect of PKD on hepatic transporter function and biliary excretion which in this case showed to be a very suitable model for this disease. On the contrary, in patients with liver disease compensatory mechanisms in extrahepatic tissues were observed as a result of the reduced hepatic elimination of drugs. For example, it was studied in humans *in vivo* as well as in rat models that with end-stage liver disease the transporters OATP1 and OATP2 were upregulated in the kidney (Wang et al. 2017). This renal compensation mechanism was also found for the clearance of *N*-methylnicotinamide, which was related to the severity of liver cirrhosis (Orlando et al. 2000). As a consequence, increased renal

exposure can result in a higher risk for renal DDI and drug induced injury showing the difficulty *in vivo* to study DMPK in diseased tissues. Another effect of kidney disease is that it might affect the plasma protein concentration and the ability of plasma proteins to bind to drugs, resulting in a higher unbound fraction of the circulating drug (Reidenberg et al. 1971). Kidney machine-perfusion models do not only create the possibility to assess the functionality of the organ and study the involvement of transporters, they also allow testing of different perfusate compositions and subsequently the effect on plasma protein binding can be assessed in an experimental environment.

6. Conclusion

This review provides an overview of available experimental predictive models to study drug absorption, metabolism and excretion processes, as well as DDI, in health and disease. The use of *ex vivo* models to predict pharmacokinetics has shown to be very useful and valuable considering the intact morphology and presence of metabolizing enzymes and transporters in the tissue. A diverse range of *ex vivo* models is available nowadays, each with their own advantages and limitations. Maintenance of the viability and integrity of the tissue is a major challenge observed for every model and tissue type. With increasing complexity more complicated problems and challenges also arise as more factors and unknown processes are involved. However, these complex models are expected to provide better translatability to the *in vivo* situation. The multifactorial and complex development of disease has often not fully been characterized and therefore makes it a huge challenge to properly predict pharmacokinetic processes in diseased patients. This indicates that improved preclinical models remain necessary to generate reliable translational results for both healthy and diseased patients.

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