Amplifying the Impact of Kidney Microphysiological Systems: Predicting Renal Clearance Using Mechanistic Modelling Based on Reconstructed Drug Secretion

Pedro Caetano-Pinto1,2, Pär Nordell3, Tom Nieskens4, Katie Haughan1, Katherine S. Fenner1 and Simone H. Stahl1,4

1ADME Sciences, Clinical Pharmacology and Safety Sciences, R&D, AstraZeneca, Cambridge, United Kingdom; 2Department of Urology, University Medicine Greifswald, Greifswald, Germany; 3DMPK, Research and Early Development Cardiovascular, Renal and Metabolism (CVRM), BioPharmaceuticals R&D, AstraZeneca, Gothenburg, Sweden; 4CVRM Safety, Clinical Pharmacology and Safety Sciences, R&D, AstraZeneca, Gothenburg, Sweden

Abstract
Accurate prediction of pharmacokinetic parameters, such as renal clearance, is fundamental to the development of effective and safe new treatments for patients. However, conventional renal models have a limited ability to predict renal drug secretion, a process that is dependent on transporters in the proximal tubule. Improvements in microphysiological systems (MPS) have extended our in vitro capabilities to predict pharmacokinetic parameters. In this study a kidney-MPS model was developed that successfully recreated renal drug secretion. Human proximal tubule cells grown in the kidney-MPS, resembling an in vivo phenotype, actively secreted the organic cation drug metformin and organic anion drug cidofovir, in contrast to cells cultured in conventional culture formats. Metformin and cidofovir renal secretory clearance were predicted from kidney-MPS data within 3.3- and 1.3-fold, respectively, of clinically reported values by employing a semi-mechanistic drug distribution model using kidney-MPS drug transport parameters together with in vitro to in vivo extrapolation. This approach introduces an effective application of a kidney-MPS model coupled with pharmacokinetic modelling tools to evaluate and predict renal drug clearance in humans. Kidney-MPS renal clearance predictions can potentially complement pharmacokinetic animal studies and contribute to the reduction of pre-clinical species use during drug development.

1 Introduction
In the kidneys, secretion plays a major role in eliminating xenobiotics and metabolic by-products from the systemic circulation into the urine (Feng et al., 2010). The pharmacokinetic (PK) profile of renally cleared small molecule therapeutics is impacted by the activity of an array of both basolateral and apical transporters expressed in the proximal tubules (Fig. 1A). By actively removing a broad variety of substrates, these transmembrane proteins are determinants of drug distribution and efficacy (Yin and Wang, 2016; Moss et al., 2014). Drug-drug interactions leading to altered PK or nephrotoxicity (Sjögren et al., 2018) illustrate the significance of renal drug transporters and the importance of their functional evaluation during drug development (Zamek-Gliszczynski et al., 2018).

However, during pre-clinical drug development, the prediction of renal clearance has long relied on established in vitro and in vivo models. Cell lines are easily accessible and popular tools for characterizing drug uptake and PK parameters. However, a key challenge with the use of established cell lines is their limited ability to model and predict normal physiological conditions where transepithelial flux of molecules is mediated by a complex and coordinated system of transporters from multiple families. Immortalized proximal tubule cell lines (iRPTEC) that exhibit a more native phenotype are a valuable tool for nephrotoxicity assays. Nonetheless, their epithelial membrane polarity is limited...
as well as the expression of key uptake drug transporters (e.g., organic anion transporters, OATs) (Caetano-Pinto et al., 2016; Jenkins et al., 2012). Membrane polarity can be improved with the use of conventional transwell systems or capillary hollow fibers (Jansen et al., 2015). Growing cells on permeable porous membranes enables their basolateral and apical sides to face different compartments. However, in the hollow fibers characterized by Jansen et al. (2016), the basolateral membrane of the iRPTEC faces the lumen of the fibers, in contrast to the native physiology of the renal proximal tubules. iRPTEC can have limited monolayer integrity (Liang et al., 1999), which is crucial for transepithelial transport studies employed in renal clearance predictions. Nevertheless, the overexpression of otherwise absent drug transport machinery in iRPTEC expands their applications, as demonstrated by van der Made et al. (2019), where the OAT-mediated uptake clearance of uremic toxins was evaluated in vitro in a 3D kidney disease model based on micro-perfused hollow fibers. Unsurprisingly, human-derived primary renal proximal tubule epithelial cells (RPTEC) were shown to retain uptake and efflux drug transport activity and form tight monolayers (Brown et al.,

Fig. 1: Immunofluorescent characterization of RPTEC in 2D-plastic and kidney-MPS
(A) Schematic representation of key basolateral and apical drug transporter proteins expressed in human renal proximal tubule epithelial cells. Representation of the membrane localization of drug transporters in 2D-plastic (B) and kidney-MPS (C). Images were taken at 20x magnification, with 60x magnification detailed in the inset for selected panels. 2D-plastic: bright-field (D), f-actin (E), zonula occludens 1 (ZO1; F), acetylated tubulin (arrows highlight individual cilia in the inset) (G), organic anion transporter 1 (OAT1; H), organic cation transporter 2 (OCT2; I), P-glycoprotein (P-gp; J), sodium/potassium-ATPase (Na$^{+}$K$^{+}$-ATPase; K). Kidney-MPS: bright-field (L), f-actin (M), ZO-1 (N), acetylated tubulin (O), OAT1 (P), OCT2 (Q), P-gp (R), Na$^{+}$K$^{+}$-ATPase (S). Hoechst 33342 was used as a nuclear counterstain in all conditions. The f-actin panels (E, M) include a 100 µm scale bar to provide perspective on the cell dimensions in 2D and in kidney-MPS. Panels N and O represent a composite image of half of the kidney-MPS tube comprising approximately 20-30 individual sections stacked together. All other kidney-MPS panels represent the mid-section of the tube, where the membrane polarization features of the RPTEC are best distinguishable.
2008; Verhulst et al., 2008), offering an in vivo-like phenotype for in vitro PK applications.

On the other hand, pre-clinical animal models offer precise species-specific PK predictions. However, cross-species differences, namely the physiological disparities between the drug transport machinery of humans and commonly used rat and dog models, often lead to poorly representative renal clearance assessments (Davies and Morris, 1993; Jansen et al., 2020). Therefore, non-human primates are the most accurate species to evaluate PK parameters (Jansen et al., 2020; Shen et al., 2016). The challenge surrounding the prediction of drug secretion both in vitro and in vivo is one of the reasons behind the use of multiple cell models and animal species, such as rats, dogs and primates, with the aim of improving human translation.

Microphysiological systems (MPS) provide a dynamic culture platform that, when applied in kidney models, better mimics the renal proximal tubule microenvironment by enabling continuous luminal flow (Caetano-Pinto and Stahl, 2018). RPTEC grown in these models showed enhanced gene expression of drug transporters and functional gains when compared to cells cultured under conventional static conditions (e.g., multivell plates, transwells) (Jang et al., 2013; Maunsbach et al., 1987; Weber et al., 2016; Fried et al., 2018; Van Ness et al., 2021; Imaoka et al., 2021). Continuous luminal perfusion potentiates RPTEC differentiation (Duan et al., 2008), which, in combination with a tubular architecture, enables physiological cellular polarization. MPS chip designs usually only allow the incorporation of a limited number of cell types, enabling MPS models to capture functional tissue units but not the whole organ physiology (Caetano-Pinto and Stahl, 2018). This study relies on a so-called kidney-MPS that recapitulates human renal proximal tubule physiology responsible for active drug secretion in the kidney.

MPS models are increasingly being developed and employed for drug development applications, and effective examples of drug secretion are emerging, although still scarce (Weber et al., 2016; Chapron et al., 2020; Sakolish et al., 2020; Van der Made et al., 2019; Van Ness et al., 2021; Imaoka et al., 2021). Huang et al. (2018) recently outlined the lack of MPS approaches to predict renal drug clearance (Isoherranen et al., 2019; Wang et al., 2021). Often, accurate predictions of this PK parameter are hampered by inadequate and poorly representative systems. The derivation of appropriate parameters that are comparable to clinical endpoints is an important consideration in MPS study design (Cirit and Stahl, 2018; Ewart and Roth, 2021). Given the complexity of MPS, drug development applications can only be fulfilled with comprehensive in vitro-in vivo (MPS-to-human) translation approaches, as recently demonstrated by Imaoka et al. (2021).

Validated kidney-MPS models can improve in vitro renal clearance predictions. Pre-clinically improving human PK predictions with advanced in vitro tools can contribute to reducing the use of pre-clinical species in the short term. Further developments in MPS technologies may render extensive PK evaluation in different animals inefficient by delivering accurate renal clearance predictions. Therefore, in the long term, these systems may be able to eventually replace the use of animal models in dedicated PK applications (Ramsden, 2021).

In this study, a kidney-MPS was developed and applied to investigate transporter activity and predict renal secretion clearance ($CL_{sec}$). The biguanide drug metformin was used to investigate the cation excretion pathway, mediated by organic cation transporter 2 (OCT2, uptake), multidrug and toxin extrusion (MATE)1, and MATE2-K (efflux) (Deutsch et al., 2019; Nigam et al., 2015; Dunn and Peters, 1995). The anion excretion pathway, mediated by OAT1/3 (uptake) and multidrug-resistance-associated protein (MRP)2/4 (efflux), was evaluated using the antiviral cidofovir, reported to be an exclusive OAT1 (uptake) substrate (Cihlar et al., 1999; Van Ness et al., 2017; Plosker and Noble, 1999). Together, these pathways are responsible for handling the majority of renally secreted drugs (Nigam et al., 2015). The data obtained was used to build and calibrate a semi-mechanistic mathematical model of drug distribution within the kidney-MPS. The modelling framework enabled the quantification of renal drug secretion in the MPS and, subsequently, the prediction of human renal clearance in vivo by employing an in vivo to in vitro extrapolation (IVIVE) method. This approach expands the applications of kidney-MPS in drug absorption, distribution, and excretion studies and illustrates how to maximize their use during drug development.

2 Methods

Cell culture

Cryopreserved human RPTEC were acquired from Biopredic (cell donor: RPT101029; Tab. S1). Cells were cultured in flasks, microplates or transwells coated with 25 μg/mL human collagen IV (Sigma), using DMEM:F-12 GlutaMAX II medium (Invitrogen) supplemented with 1% penicillin/streptomycin (10,000 U/mL), 1% FCS, 10 ng/mL human epidermal growth factor, 32 ng/mL hydrocortisone, 4 μg/mL triiodothyronine, and ITS (insulin: 10 μg/mL, transferrin: 10 μg/mL, sodium selenite: 10 ng/mL). After thawing, cells were expanded once, harvested with trypsin-EDTA, filtered through a 100 μm pore size cell strainer (Corning), and plated for assays at a density of 75,000 cells/cm² in 96-well microplates (Coe Parmer) (2D-plastic) or 230,000 cells/cm² in 96-well transwell microplates – pore size: 0.4 μm (Millipore) (2D-transwell). In 96-well microplates, cells were grown until confluency (~5 days) before assays were performed, with medium changes every 48-72 h. In 96-well transwell microplates, medium was changed every 48-72 h, and transepithelial electrical resistance (TEER) of the cell monolayer was monitored from day 5 until values of at least 165 Ω·cm² were reached (typically at day 7-8). Monolayer leakage (both A2B and B2A) was assessed using lucifer yellow (LY: 40 μM; 60 min), and transport assays were performed when LY leakage was < 1% (supplementary file, Section 4).
We acknowledge that using animal-derived consumables such as fetal bovine serum, antibodies, and collagen matrix (see below) perpetuates the use of animal-based research products. Nonetheless, the percentage of serum used in our cultures was limited to 1% (v/v). The sourcing of alternative antibodies for the tubular markers selected for this study, the validation of their specificity, and optimization of experimental conditions was outside of our scope. The material used in our study had been previously validated and the target specificity of the antibodies used well documented in the literature, which facilitated our assay design. The same holds true for the use of rat tail collagen I gel. Although synthetic gels are available, implementing a new matrix would require extensive optimization, which was outside of our scope.

**MPS chip preparation**

The Nortis ParVivo® dual-channel chip, comprising two microfluidic circuits leading to a matrix chamber, were used as the MPS renal tubule model. The design incorporates two parallel micro-channels with a cross section of 125 µm and a length of 5800 µm, each formed by a glass micro-pin. One micro-channel corresponds to the renal tubule with perfusion driven through the lumen. The opposite channel remained cell-free and enabled perfusion through the extracellular matrix during experiments. MPS chips consist of a plastic frame and a PDMS body. They were coupled to a Nortis perfusion platform that pressurizes the chips and drives flow through the microfluidic channels. Chips were prepared according to manufacturer specifications, as previously reported (Van Ness et al., 2017).

Briefly, the extracellular matrix consisting of rat tail collagen I (5 mg/mL; Corning) was crosslinked with 200 µM genipin (Sigma-Aldrich) at pH 8-9. The addition of genipin improves collagen homogeneity and facilitates perfusion through the extracellular matrix, preventing erratic flows through the MPS-chip. Matrix was injected into the chips and left to polymerize overnight in a humidified incubator at 37°C. Subsequently, the glass micro-pins, now embedded in the collagen matrix, were removed to create the two parallel micro-channels. Chips were coupled to the Nortis perfusion platforms, reservoirs were filled with RPTEC culture medium, and chips were perfused overnight to wash and prime the extracellular matrix. The left channel was coated with 5 µg/mL human collagen IV (Invitrogen) for 10 min at 37°C, and 5 µL of a RPTEC suspension (10^5 cells/mL) was injected using a 5 µL Hamilton micro-syringe via the upstream injection port. To ensure RPTEC adhesion, chips were kept at 37°C in a humidified incubator for 24 h before flow was initiated through the renal tubule at a rate of 0.5 µL/min. Cells were cultured under flow for 7 or 8 days in a humidified incubator at 37°C before assays were performed. RPTEC in the renal tubule are estimated to experience a shear stress of 0.3-0.7 dyne/cm² during the culture period (Tab. S7). Tubule formation was monitored using a transmitted-light microscope. The successful perfusion of the chips was determined by measuring the effluent volume in the waste reservoirs (approximately 5 mL over a 7-day period). Each chip was monitored under a brightfield microscope to confirm that the ECM matrix was intact and the renal tubule properly connected to the microfluidic circuits. Chips that did not meet these criteria were discontinued and not used in the assays.

**Fluorescent functional and permeability assays**

Fluorescent probes were used to evaluate the activity of RPTEC cultured in both microplates and MPS, as well as the permeability of the kidney-MPS renal tubule. P-glycoprotein (P-gp) and MRP activity was determined using calcine-ace-toxyethyl (calcein-AM) and carboxyfluorescein diacetate succinimidy l ester (CFDA-SE), respectively. Both molecules diffuse through the cell membrane and are subsequently metabolized to their fluorescent forms (calcein and CFDA). Efflux activity was determined by blocking P-gp and MRPs with selective inhibitors valspodar and MK571, respectively, resulting in increased intracellular fluorescence. Assays were performed by incubating cells with 1 µM calcein-AM ± 5 µM valspodar or 2 µM CFDA-SE ± 50 µM MK571. In 2D assays, cells were incubated for 30 min in HBSS at 37°C and cellular fluorescence intensities were subsequently quantified using a Polar-star Optima microplate reader (BMG Labtech). In the kidney-MPS, the substrates (+ inhibitors) were perfused via the cell-free channel for 2 h, and fluorescence accumulation in the RPTECs or matrix was determined using a CV7000 (Yokogawa) automated high-content confocal microscope. To determine monolayer integrity in the kidney-MPS, 25 mg/mL inulin-FITC in RPTEC medium was perfused through the cell-free channel. Renal tubule perfusate was collected daily, for 7 days, and fluorescence quantified using a Polar-star Optima microplate reader. Furthermore, inulin-FITC (25 mg/mL in HBSS) was perfused either through an empty kidney-MPS-chip or the lumen of the renal tubule for 3 h. Fluorescence in renal tubule or extracellular matrix was evaluated using an Evos™ FL microscope (Invitrogen).

**Immunofluorescence**

RPTEC cultured in microplates were washed with HBSS, fixed with 4% formaldehyde for 10 min at room temperature (RT) and permeabilized using 0.1% Triton X (v/v)/2% BSA (w/v) in HBSS (permeabilization buffer) for 1 h. Kidney-MPS chips were washed with HBSS under continuous perfusion (flow rate: 1 µL/min) for a minimum of 2 h and subsequently fixed with 4% formaldehyde for 30 min at RT (flow rate: 2.5 µL/min). Following an additional HBSS wash, cells were permeabilized with permeabilization buffer for 1 h (flow rate: 2.5 µL/min). Incubation with the primary antibodies in both microplates and MPS chips was performed in static conditions (no perfusion) overnight at 4°C in permeabilization buffer. Subsequently, fluorescently conjugated secondary antibodies (Invitrogen) were incubated for 1 h, together with Hoechst 33342 (1:1000, Invitrogen) and Phalloidin-488 (1:200, Thermofisher). Figure S1 shows the specificity of the OAT1, OCT2, and P-gp antibodies used, and Table S2 lists antibodies and dilutions used. Image acquisition was carried out on a CV7000 automated confocal microscope (Yokogawa) or an LSM 700 confocal microscope (Zeiss).
Immunofluorescent image analysis
Acquired images were processed and analyzed using the open-source imaging software Fiji (Schindelin et al., 2012). The number of cells in the kidney-MPS renal tubule was estimated by counting Hoechst-stained nuclei in three selected tubule sections, reconstructed from z-stacks comprising 25 images. Cilium length was estimated from z-stack images by counting positive images for acetylated tubulin. Cell height and tubule diameters were estimated from the pixel/µm ratio using images representative of the renal tubule midsection. Fluorescence intensity was measured in sets of 3 individual images per replicate to estimate calcein and CFDA retention levels.

Gene expression analysis
Expression of pivotal proximal tubule markers was analyzed using custom-designed TaqMan™ gene expression array cards (Thermo Fisher, Waltham, USA; Tab. S3). RPTEC from a single donor were cultured in kidney-MPS or in plastic transwell microplates (pore size 0.4 µm; Millipore); three biological replicates, corresponding to different RPTEC aliquots were used. 2D-plastic and 2D-transwell cultured cells were harvested after DPBS washing (37°C), followed by directly adding RLT lysis buffer (RNaseasy® kit, Qiagen, Venlo, The Netherlands). Cells in MPS chips were perfused with HBSS (37°C) at 1 µL/min for 2 h, followed by perfusion with RLT lysis buffer at 5 µL/min for 20 min, collecting lysate at the renal channel. After 30 min incubation at RT, kidney-MPS chips were perfused with additional RLT lysis buffer at 5 µL/min for 30 min to collect additional cell lysates.

Two frozen human kidney cortex samples were obtained (BioIVT, Burgess Hill, UK). One fresh tissue kidney sample was obtained from a nephrectomy (Sahlgrenska University Hospital, Gothenburg, Sweden) for which collection complied with the Swedish Biobanks in Medical Care Act. Tissue samples (3x3x3 mm) were transferred to microcentrifuge tubes containing RLT lysis buffer (600 µL) and lysed for 2 min at 25 Hz using a TissueLyser (Qiagen).

Total RNA of all samples was isolated using the RNaseasy® kit (Qiagen), and cDNA was generated using the High Capacity Reverse Transcriptase kit (Applied Biosystems) in an Eppendorf Master-cycler. Taqman Array Cards were loaded with 300 ng cDNA using TaqMan Fast Advanced Master Mix (Qiagen) according to the manufacturer’s instructions. Quantitative polymerase chain reaction was performed on a Quantstudio 7 Flex thermocycler (Applied Biosystems). Data was analyzed by the 2-ΔCt and 2-ΔΔCt methods (Livak and Schmittgen, 2001) to compare absolute and relative gene expression, respectively, using hypoxanthin-guanin-phosphoribosyl-transferase 1 (HPRT1) as the housekeeping reference gene.

Statistical analysis of gene expression data
Gene expression data was analyzed using GraphPad Prism 8.0 (GraphPad Software, La Jolla California USA). An unpaired t-test corrected for multiple comparisons using the Holm-Sidak method with statistical significance of 0.05 was used to compare the different experimental groups. The 2D-plastic samples were used as the reference group, and analysis was performed based on the ΔCt values.

Perfusion and drug transport assays
For each drug transport assay, up to four chips were coupled to 1 mL syringes (BD) mounted on a multi-lane syringe pump (Perkin Elmer). Temperature was maintained at 37°C using a heating mat. Both microfluidic circuits were perfused at a flow rate of 1 µL/min, with flow initiated simultaneously upstream of the collagen chamber (Fig. 4A2-A3). The shear stress experienced by cells lining the tubule under assay conditions is estimated at ~0.7 dyne/cm² (Tab. S7). Perfusate samples from both channels were collected from the chip outlets in 30 min intervals over a period of 360 min. Perfusate volume was permanently monitored and chips excluded from the analysis if the perfusion became inconsistent. Drug transport assays were performed in HBSS/1% FCS/10 mM HEPES with different pH (basolateral: 7.4, apical: 6.5). [14C]-metformin and [3H]-cidofovir (Perkin Elmer) were prepared in solution with a ratio of 1:100 for radioactive:non-radioactive, each with a total concentration of 10 µM. This concentration was chosen to be below the Km values reported for both drugs for their primary uptake transporters (Morrissey et al., 2012). Drug exposure was achieved by perfusing the radiolabeled solutions via the cell-free channel (loading channel – basolateral compartment) and buffer via the renal channel (apical compartment). Assays were performed in the presence or absence of selective drug transport inhibitors imipramine (500 µM) or probenecid (1 mM), for metformin and cidofovir, respectively. These concentrations were selected to be substantially higher than the reported IC50 values for OCT1 and OCT2 (Morrissey et al., 2012) to maximize the inhibitory potential. Inhibitors were used both apically and basolaterally to achieve maximal inhibition. 5 µL of each perfusate sample was added to 5 mL of Ultima Gold scintillator (Perkin Elmer) and radioactivity quantified in a Tri-Carb 2100TR (Perkin Elmer). To determine transepithelial transport in static conditions, transwell microplates (Millipore) were used to perform assays under the same experimental conditions mentioned, but with an incubation period of 60 min instead. Determination of drug flux across the tubule and in transwell cultures is detailed in the supplementary information, Section 4 (Tab. S4, Fig. S3).

Mathematical modelling and in vitro to in vivo extrapolation
A semi-mechanistic mathematical model of drug disposition in the kidney-MPS platform was developed and implemented in MATLAB (Release 2020b, The MathWorks, Inc., Natick, MA, USA). Briefly, the model considers the net dispersion of drug across the physical extracellular matrix separating the loading and renal microfluidic channels, and in addition, the basolateral-to-apical transport into the renal channel (Fig. 6A; Tab. S8). Fitting the model to the outlet concentration-time data allows estimation of the key in vitro kinetic parameters Qm, PSp, and PSh, representing the first-order rate constant for flux through the ECM, and passive and active permeability-surface area product, respectively. Human renal clearance was predicted from PSp and PSh by accounting for derived surface area in vivo (Tab. S9) and...
Fig. 2: Efflux transporter activity and paracellular permeability in the kidney-MPS

Substrates calcein-acetoxyethyl (Calcein-AM) and carboxyfluorescein diacetate-succinimidyl ester (CFDA-SE) permeate the cell membrane passively and acquire fluorescence upon enzymatic cleavage by esterases to calcein and CFDA, respectively (A1). Although diffusing into the cells, calcein-AM efflux requires the activity of P-glycoprotein (P-gp). CFDA is impermeable and its efflux requires the activity of multidrug resistance proteins (MRPs). The use of the selective P-gp inhibitor valspodar prevents calcein-AM efflux and leads to the accumulation of calcein and, consequently, the increase in intracellular fluorescence. Likewise, inhibition of MRP activity by MK571 leads to the accumulation of CFDA, resulting in increased intracellular fluorescence (B1). Efflux drug transport activity is evident in the kidney-MPS from the accumulation of calcein and CFDA in the presence of selective inhibitors valspodar (B2) and MK571 (B3) relative to the uninhibited conditions (A2-3), respectively. Quantitative analysis demonstrates that cells cultured in 2D-plastic (n = 2) (C) and kidney-MPS (n = 3) (D) show similar calcein and CFDA accumulation (individual measurements represented). The paracellular permeability of the renal tubule in the kidney-MPS substantially decreases within the first few days of culture. When inulin-FITC is applied at the basolateral side of the renal tubule (extracellular), the fluorescence recovered in the apical side (luminal) decreases over time, showing a reduction in inulin-FITC leakage into the lumen consistent with increased tightness of the renal tubule (n = 2) (E). When perfused from the apical side, inulin-FITC diffuses throughout the extracellular matrix in a cell-free MPS-chip (E1), while in the kidney-MPS the fluorescence is contained within the lumen of the renal tubule, which prevents inulin-FITC from reaching the extracellular matrix (E2).
expressing kidney organ clearance as the net result of glomerular filtration, tubular secretion, and fractional tubular reabsorption following the method established by Kunze et al. (2014). Predictability was assessed by comparing estimates to reported clinical values before and after correction for filtered fraction. Since secretory clearance is not directly given from observations, calculation of this component was based on assuming a fraction reabsorbed estimated from MPS (Eq. S9\(^1\)) and a filtration clearance equal to \(f_u \times \text{GFR}\) (Eq. S10\(^1\)). A comprehensive description of the modelling and in vitro to in vivo extrapolation strategy, including parameters, assumptions, and equations is provided in the supplementary information\(^1\).

### 3 Results

#### 3.1 Kidney-MPS recreates renal proximal tubule morphology

A comparative immunofluorescence analysis of RPTEC grown in conventional two-dimensional culture (2D-plastic) and the kidney-MPS revealed that culture under microfluidic conditions enhanced crucial renal proximal tubule cell features, summarized in Figure 1.

In 2D-plastic, RPTEC maintained dense tight junctions (zonula occludens 1: ZO1) present between cells, clearly highlighting cellular boundaries. Cells presented a single primary cilium (acetylated tubulin) \(\sim10\ \mu\text{m}\) in length. Cytoskeleton filaments were concentrated in the cell periphery (f-actin) and co-localized with ZO1. The two major renal uptake transporters OAT1 and OCT2 were identified intracellularly in the perinuclear space and were not observed in the cell membrane (Fig. S2A\(^1\)). The efflux transporter P-gp was detected both in the cell membrane and intracellularly (Fig. S2B\(^1\)). These observations demonstrate that, despite retaining some key features, RPTEC polarity is reduced in conventional 2D culture.

In the kidney-MPS, RPTEC populate a channel \(\sim125\ \mu\text{m}\) in diameter and form a highly polarized, monolayered tubule. This 3D renal RPTEC tubule comprises approximately 10,000 cells (Fig. S4\(^1\)) with a height of about 20 \(\mu\text{m}\) and with well-defined basolateral and apical membranes. The renal tubule experiences shear stress between 0.3 and 0.7 dyne/cm\(^2\) (Tab. S7\(^1\)), which is within the previously reported physiological range (Vedula et al., 2017). Automated confocal microscopy image acquisition (Peel et al., 2019) determined that the tight junction marker ZO1 was homogenously expressed in all cells along the tubule and predominantly localized in the apical membrane. F-actin was mainly expressed at the cell periphery, and cells displayed a single primary cilium extending into the lumen, with a length of \(\sim20\ \mu\text{m}\). The drug uptake transporters OAT1 and OCT2 were consistently observed in the basolateral membrane whilst the efflux pump P-gp was localized in the apical membrane. Furthermore, the ion channel sodium-potassium ATPase (Na\(^+\)/K\(^-\)ATPase) was also localized in the basolateral membrane. From these findings it is evident that RPTEC exhibited distinct epithelial polarization when arranged in a tubular configuration and grown under continuous flow. Moreover, the evaluated drug transporters were correctly localized in the membrane, a characteristic that was absent in conventional 2D culture.

#### 3.2 Kidney-MPS drug efflux transport activity and permeability

The functional activity of RPTEC efflux transporters was demonstrated using fluorescent substrates in combination with prototypical inhibitors, in both 2D-plastic and the kidney-MPS (Fig. 2A1-B1). Activity of MRPs and P-gp was evaluated using the substrate CFDA-SE combined with the inhibitor MK571 or calcein-AM combined with valspodar, respectively. In 2D-plastic, the selective inhibition of MRPs and P-gp led to a 5.7 \(\pm\) 0.4 and 3.5 \(\pm\) 0.3-fold increase in fluorescent substrate retention in the cells, respectively (Fig. 2C). In the kidney-MPS, CFDA efflux under control conditions resulted in minimal intracellular retention, which could not be quantified. Therefore, MRP efflux activity was defined as CFDA extracellular fluorescence accumulation, for which an 8.1 \(\pm\) 0.1-fold increase was observed in the absence of inhibitor. P-gp inhibition led to a 5.4 \(\pm\) 0.1-fold increase in fluorescence retention in the renal tubule (Fig. 2D).

Inulin-FITC diffusion takes place solely paracellularly and is independent of active transport. Inulin transfer from the basolateral to the apical side of the renal tubule decreased substantially over the initial culture period. The trace levels of inulin-FITC fluorescence detected in the luminal effluent after day 3 reflect the presence of dense tight junctions in the kidney-MPS and a highly impermeable epithelial barrier. Conversely, when perfused apically inulin-FITC was contained inside the renal tubule, while in a cell-free chip (therefore without barrier function) extensive inulin-FITC fluorescence was observed throughout the extracellular matrix. In the kidney-MPS, RPTECs grown into a self-assembled renal tubule showed substantial apical drug transporter activity while forming an impervious monolayer. These results show that the key traits crucial for the study of drug transport across renal proximal tubule epithelium are present in this kidney-MPS.

#### 3.3 MPS platform enhances and potentiates the expression of renal drug transporters

To further compare the kidney-MPS to 2D culture conditions, the gene expression profile of key proximal tubule genes, including those encoding drug transporters, membrane channels, and RPTEC markers, was assessed across the culture formats. HPRT1 was used as the housekeeping reference gene for data normalization (Tab. S5\(^1\)). The analysis was performed using RPTEC from a single donor, grown in 2D-plastic, 2D-transwell or in the kidney-MPS for 2 or 7 days (MPS day 2 and MPS day 7). Human renal kidney cortex samples obtained from 3 donors were used as an in vivo reference.

The analysis revealed significant differences in mRNA expression levels between 2D-plastic, 2D-transwell and kidney-MPS for the analyzed genes (Tab. S4\(^1\)), as summarized in Figure 3. For drug uptake transporters, expression of OCT2 was similar in MPS and 2D-transwell but slightly lower compared to 2D-plastic. OAT3 expression was undetectable in the 2D culture formats as well as the kidney-MPS. OAT1 expression showed
the starkest difference between culture conditions: Completely absent in 2D, OAT1 was expressed in the kidney-MPS. For drug efflux transporters, MRP4 and P-gp showed the highest absolute expression levels of all drug transporters tested. MRP4 expression was similar in 2D-plastic, 2D-transwell, and kidney-MPS, while P-gp expression was lower in the kidney-MPS compared to 2D-plastic and 2D-transwell. Relative expression of MATE1 and MATE2-K was strongly increased in kidney-MPS day 2 and day 7 over 2D-plastic (13.7 ± 4.6-fold and 86.8 ± 48.4-fold, respectively).

In addition, the expression of the key renal proximal tubule markers aquaporin 1 (AQP1), sodium-glucose transport protein 2 (SGLT2), sodium-dependent phosphate co-transporter (NaPi), megalin (LRP2), and cubilin (CUBN) was confirmed in the MPS-cultured cells. Interestingly, NaPi was expressed in kidney-MPS and 2D-transwell but not in 2D-plastic. AQP1 showed a high expression in 2D and was further upregulated in the kidney-MPS. Hepatocyte nuclear factor 4 alpha (HNF4A), which regulates the expression of renal drug transporters, was also substantially increased in kidney-MPS day 7 (47.9 ± 24.0-fold relative to 2D-plastic). These results show that the expression of specific proximal tubule drug transporters and membrane proteins is maintained, enhanced, or even recovered in kidney-MPS, with the exception of P-gp, which is downregulated.
3.4 Kidney-MPS enables drug secretion through active transepithelial transport

Activity of drug transporters is fundamental for renal secretion. To determine transepithelial transport – movement of compounds across the cellular barrier – the secretion of prototypical renally cleared drugs was assessed in both 2D-transwell and kidney-MPS cultures (Fig. 5A; Tab. S6\(^1\)). All assays were performed using RPTEC from a single donor. For every experiment, a different batch of cells (aliquots) was expanded once after thawing. Each experimental condition was performed independently, using a total of 3 to 4 kidney-MPS chip replicates.

Gene expression levels of all genes investigated were evidently lower in the kidney-MPS compared to human renal cortex (Tab. S5\(^3\)). This observation supports previous reports describing a reduction in mRNA transcripts when RPTECs are isolated and cultured in vitro (Verhulst et al., 2008). Nevertheless, the kidney-MPS expressed all drug transporter genes analyzed except OAT3, MRP4, P-gp, and AQP1 maintained high expression levels, while the most pronounced reduction was observed for OAT1. Overall, these gene expression results demonstrate that, despite lower expression relative to renal tissue, RPTECs cultured as kidney-MPS demonstrate higher gene expression of drug transporters compared to as 2D cultures.
In 2D-transwell culture, RPTECs did not demonstrate any drug secretion capability (Fig. 5C1,C2). Transepithelial transport was assessed by exposing RPTECs grown on semi-permeable transwell inserts to metformin or cidofovir. Limited drug flux was observed from the basolateral to the apical compartment (B2A), which models the movement of drug from blood to urine. Further, no differences were observed in the apparent permeability coefficients (P_{app}; Tab. S6) for both drugs from B2A and vice versa (A2B), either in the presence or absence of inhibitors. Active drug secretion would result in an increased (and inhibitable) permeability from B2A compared to A2B.

In contrast to 2D setups, the compartments adjacent to the basolateral and apical cell surfaces in the MPS are not directly accessible for drug exposure or sampling in kidney-MPS. Instead, drugs are dispensed via inlet ports into the chips and perfusate is collected from the outlet ports downstream of the extracellular matrix chamber, where exchange of constituents between the drug donating (loading) and the receiving (renal) channels occurs (Fig. 4A3, 5B). In a cell-free chip configuration with no barrier function, metformin and cidofovir perfusion displayed overlapping concentration-time profiles at the renal outlet, reaching detectable levels from approx. 1.5 h and an apparent steady-state plateau within approx. 4 h of perfusion (Fig. 4B,C), indicating that in the absence of an RPTEC barrier drug dispersion in the MPS is governed primarily by the MPS-chip flow rate.
RPTECs lining the renal channel form a selective barrier to enable the translocation of drug into the renal lumen. Co-perfusion of selective inhibitors of OCT2 (imipramine) or OATs (probenecid) were used to evaluate the contribution of active drug secretion to the overall transepithelial transport of metformin and cidofovir (Wang et al., 2014; Takeda et al., 2001). The levels of both substrates in the renal perfusate were reduced in the presence of the renal tubule in relation to the cell-free setup (Fig. 5D1, 5D2), owing to the barrier function of RPTEC, and were further diminished in the presence of the inhibitor, corresponding to approx. 13% and 21% of the steady-state concentration reached after perfusion of metformin and cidofovir alone (Fig. 6B1,C1).

3.5 Semi-mechanistic modelling of kidney-MPS drug transport combined with in vitro to in vivo extrapolation can correctly predict renal drug clearance

While the empirical data clearly displayed the activity of key renal transporters in the kidney MPS, inferring a quantitative meaning to observations requires deconvolution of outlet drug concentration-time profiles. To this end, a bespoke mathematical modelling framework encompassing the temporal aspects of drug distribution along with the fundamental pathways governing transepithelial renal transport was developed (supplementary information, Sections 8, 9, and 10). Sequential fit of the semi-mechanistic model to kidney-MPS data from the cell-free channel and renal tubule in the absence and presence of specif-
Current in vitro systems, mostly 2D-cultured cells (Scotcher et al., 2016). Due to their ability to partially mimic in vivo physiology, MPS models have received interest from the biotech and pharmaceutical sectors, with the goal to enhance in vitro cultures and ultimately improve in vitro to in vivo translation of transporter-mediated drug clearance predictions (Cirit and Stokes, 2018; Ewart et al., 2017; Neuhoff et al., 2013; Huang and Isoherranen, 2018). In this study, a kidney-MPS model that demonstrates a tight and polarized human renal proximal tubule epithelium expressing cationic and anionic drug transport pathways enables robust evaluation of transepithelial flux. When transepithelial flux measurements were combined with a bespoke mathematical modelling strategy, transporter-mediated renal clearance of metformin and cidofovir was accurately predicted.

Conventional renal proximal tubule cultures demonstrate poor epithelial polarization (Fig. 1B) and limited secretion capacity of cationic drugs, such as metformin and cisplatin (Motohashi and Inui, 2013; Elsby et al., 2017). By incorporating continuous luminal shear stress through perfusion and a tubular architecture, the kidney-MPS enables cells to polarize with spatially correct localization of drug transporters in the basolateral and apical cell membranes (Fig. 1C). These features likely enable the substantial improvement in renal drug transporter activity (Caetano-Pinto and Stahl, 2018) responsible for the transepithelial transport of clinically relevant drugs in our kidney-MPS model. Shear stress was implicated in facilitating the transport of organic cations, demonstrated by increased OCT2 and MATE1 transport activity in transfected MDCK cell lines in dynamic culture (Jayagopal et al., 2019) as well as enhanced MATE2-K-mediated transport in primary RPTEC

<table>
<thead>
<tr>
<th>System</th>
<th>Parameter</th>
<th>Unit</th>
<th>Metformin estimate (95% CI)</th>
<th>Cidofovir estimate (95% CI)</th>
</tr>
</thead>
<tbody>
<tr>
<td>MPS model</td>
<td>$Q_{tr}$</td>
<td>$\mu$L/min</td>
<td>0.14 (0.13-0.15)</td>
<td>0.16 (0.14-0.18)</td>
</tr>
<tr>
<td></td>
<td>$PS_a$</td>
<td>$\mu$L/min</td>
<td>0.10 (0.081-0.13)</td>
<td>0.031 (0.019-0.043)</td>
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<tr>
<td></td>
<td>$PS_p$</td>
<td>$\mu$L/min</td>
<td>0.016 (0-0.034)</td>
<td>0.013 (0.0036-0.021)</td>
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<tr>
<td>In vivo scaling</td>
<td>$f_{ub}$</td>
<td>-</td>
<td>1</td>
<td>1</td>
</tr>
<tr>
<td></td>
<td>$CL_{r,fil}$</td>
<td>ml/min</td>
<td>120</td>
<td>120</td>
</tr>
<tr>
<td></td>
<td>$CL_{r,sec}$</td>
<td>ml/min</td>
<td>147 (102-190)</td>
<td>55 (40-75)</td>
</tr>
<tr>
<td></td>
<td>$f_{reab}$</td>
<td>-</td>
<td>0.16</td>
<td>0.13</td>
</tr>
<tr>
<td></td>
<td>$CL_{r}$</td>
<td>ml/min</td>
<td>227 (190-272)</td>
<td>153 (140-170)</td>
</tr>
<tr>
<td>Observed</td>
<td>Observed $CL_{r}$ (Graham et al., 2011)</td>
<td>ml/min</td>
<td>505 (252-758)</td>
<td>165 (60-270)</td>
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<tr>
<td></td>
<td>Obs. / pred.</td>
<td>-</td>
<td>2.2 (1.9-2.7)</td>
<td>1.1 (1.0-1.2)</td>
</tr>
<tr>
<td></td>
<td>Estimated $CL_{r,sec}$ (Cundy et al., 1995)</td>
<td>ml/min</td>
<td>481 (180-782)</td>
<td>69 (0-189)</td>
</tr>
<tr>
<td></td>
<td>Obs. / pred.</td>
<td>-</td>
<td>3.3 (2.4-4.5)</td>
<td>1.3 (0.9-1.7)</td>
</tr>
</tbody>
</table>

Table 1: Estimated model parameters and scaled renal clearance
Calculated assuming physiological parameters as specified in the supplementary file. Clinical pharmacokinetics as reported for metformin and cidofovir by Graham et al. (2011) and Cundy et al. (1995), respectively. Secretion clearance calculated from total renal clearance assuming the fraction reabsorbed estimated from MPS and a filtration clearance = $f_{u} \times GFR$. 4 Discussion

Accurate prediction of clearance by the kidneys is essential for drugs in development. Although the renal clearance of compounds that are mainly eliminated by glomerular filtration is reasonably estimated by established approaches, clearance of low-permeability candidate drugs that undergo active tubular secretion is poorly predicted (Mathialagan et al., 2017). The main reason for this is the limited active drug secretion capacity of current in vitro systems, mostly 2D-cultured cells (Scotcher et al., 2016). Due to their ability to partially mimic in vivo physiology, MPS models have received interest from the biotech and pharmaceutical sectors, with the goal to enhance in vitro cultures and ultimately improve in vitro to in vivo translation of transporter-mediated drug clearance predictions (Cirit and Stokes, 2018; Ewart et al., 2017; Neuhoff et al., 2013; Huang and Isoherranen, 2018). In this study, a kidney-MPS model that demonstrates a tight and polarized human renal proximal tubule epithelium expressing cationic and anionic drug transport pathways enables robust evaluation of transepithelial flux. When transepithelial flux measurements were combined with a bespoke mathematical modelling strategy, transporter-mediated renal clearance of metformin and cidofovir was accurately predicted.

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under flow (Fukuda et al., 2017). Recently, cisplatin nephrotoxicity was observed only after basolateral but not apical exposure in a similar kidney-on-a-chip, an indirect indication of cationic uptake activity that validates the physiological polarization of the kidney-MPS model (Nieskens et al., 2020). The enhanced metformin secretion observed in our kidney-MPS model is postulated to be the result of OCT2 localization to the basolateral membrane and the increased activity of the OCT2 - MATE1 - MATE2-K cation transport pathway. The upregulation of both MATE1 and MATE2-K at the gene level observed in the kidney-MPS further validates the impact of fluidic conditions on the expression of renal drug transporters but does not represent a functional gain in itself. Previously, transport of the anionic substrate para-amino hippuric acid (PAH) was demonstrated in a similar kidney-MPS model using freshly isolated renal proximal tubule cells (Weber et al., 2016). Moreover, the expression of morphine and its active metabolite morphine-6-glucuronide was also recently reported (Imaoka et al., 2021). With the determination of transcellular metformin and cidofovir flux, we confirm both anion and cation secretion activity in this model.

This shows that endogenous function of this key renal uptake transporter can be measured in vitro under physiological conditions, without depending on overexpression systems. Freshly isolated renal tubule cells initially demonstrate OAT1 and OAT3 expression and activity (Brown et al., 2008; Van der Hauwaert et al., 2014; Lash et al., 2006). However, cells de-differentiate when expanded and cryopreserved, losing or substantially reducing the expression of key tubular features (Sakolish et al., 2018). This change is evident in the cryopreserved RPTEC used (Fig. 3A). HNF4A activity is involved in the expression of solute carrier (SLC) transporters during renal development (Martovetsky et al., 2013, 2016). Its upregulation in the kidney-MPS (Fig. 3H) may suggest that this nuclear transcription factor plays a role in the recovery of OAT1 expression, indicating that cryopreserved RPTEC, under dynamic 3D culture, can re-differentiate, following pathways similar to those of kidney maturation (Martovetsky et al., 2013).

Applications of modelling-based methodologies to translate MPS in vitro data to in vivo renal parameters predictive of either renal clearance or drug-induced toxicity were recently demonstrated (Imaoka et al., 2021; Maass et al., 2019). These approaches maximize the impact of using MPS models in translational research and follow initial studies that explored the use of multi-organ MPS platforms encompassing liver and gut models to predict drug metabolism and absorption (Maass et al., 2017). More complex designs incorporating interconnected models were used to predict the pharmacokinetic profiles of different drugs (Maass et al., 2017; Edington et al., 2018). While these studies factor in different parameters towards a “physiome-on-a-chip” (Edington et al., 2018), our approach is directed at drug transport and renal secretion as a means to predict renal clearance. The kidneys are responsible for the elimination of a considerable number of drugs, and a fifth of all new compounds are estimated to fail in late drug development due to renal at-
where the drug permeability (\(\mu\text{L/min}\)) is higher than the drug flux (\(\mu\text{L/min}\)) through the matrix (PS > \(Q_\mu\)). In our approach, perfusion data from chips without cells were important to understand the drug distribution profile in the MPS and determine this characteristic of the system, which may be behind the apparent underprediction of metformin CL\(_r\). Drug concentrations at the loading outlets of the MPS-chip overlap (with and without inhibition), showing that the cell monolayer forms a rate-limiting barrier to drug translocation. With increasing efficiency of transporter-mediated transport, drug supply will, eventually, become a limiting factor, and transporter kinetic parameters will not be identifiable. This is a practical limitation applicable to any dynamic system of this nature.

Kidney-MPS recreates only the proximal tubule secretion and reabsorption; the other central CL\(_r\) parameter for IVVIE – glomerular filtration – depends on previously reported values. In order to maintain the simplicity of our approach, only CL\(_r\) modelling was attempted. A PBPK modelling strategy would require extensive use of non-MPS-derived parameters.

This study focused on well-described reference drugs, essentially with no protein binding (Scheen, 1996; Cundy et al., 1995) and expected to have minimal binding to the chip’s PDMS body (van Meer et al., 2017), an event that would hamper precise transepithelial flux determinations and conflict with the modelling strategy employed. Further testing should be undertaken to include compounds secreted by different transport pathways (e.g., OATP4C1-P-gp) and with different physicochemical properties (e.g., high lipophilicity, protein binding). The addition of an adjacent endothelial tubule can be used to study permeability across the renal vasculature and the proximal tubules, as recently demonstrated (Chapron et al., 2020). The MPS chip model used here incorporates a single renal tubule, which can be a limiting factor when it comes to the number of experimental replicates and data variability. In our study, the passive permeability data retrieved showed high variability; however, this has a lower impact on the overall prediction accuracies since the active secretion component is the main contributor to drug clearance of the tested drugs. In future pre-clinical studies, data variability could be mitigated by expanding the experimental replicates and employing cells derived from multiple donors. Prospective kidney-MPS designs could incorporate multiple renal tubules per chip, thus increasing the throughput and minimizing these issues.

Further, scaling strategies employing proteomics-based relative expression factors (REFs) could improve IVIVE predictions since they can incorporate the abundance of drug transporters at the protein level relative to surface area or cell number. However, the use of this approach is still limited by the biological specimen sizes in this MPS. A multi-segment chip recreating also glomerular and distal tubule components could capture filtration and reabsorption and enable all renal clearance endpoints to be derived from the same model. Apart from the proximal tubules, advances have been made towards developing a glomerulus-on-a-chip (Petrosyan et al., 2019; Doi et al., 2022); nonetheless, a multi-segment kidney-MPS is still in the future. With segments recreated on different MPS platforms with no interconnectivity, a modelling strategy such as ours offers a powerful tool to integrate PK parameters derived from different models and different sources.

A potential strategy to increase the experimental throughput of this kidney-MPS is to use a version of the semi-mechanistic micro-perfusion model where the parameters are derived from the MPS data at steady-state (Tab. S10) when the drug concentration in the loading channel outlet reaches the levels of the input. This approach estimates permeability within 25% of the perfusion-derived parameters and simplifies the experimental procedure, requiring far fewer samples to be collected and analyzed.

With this study we demonstrate that renal total and secretory clearance can be predicted within an acceptable 5-fold range, an industry standard commonly employed in pre-clinical development (Davies et al., 2020), using a model-based approach applied to a continuously perfused kidney-MPS that recapitulates endogenous drug transport activity. As MPS applications progress and a substantial body of evidence validates their usage, kidney-MPS can bridge the gaps of conventional in vitro systems in renal clearance predictions and play a major role in the reduction and potential future replacement of animals in PK studies.

References


Conflict of interest
All authors are currently AstraZeneca employees or were AstraZeneca employees during the execution of the experimental work presented in this manuscript and may hold AstraZeneca shares.

Data and code availability
Experimental data and the MATLAB code for the mechanistic model are available on request from the authors.

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Author contributions
Study concept: P.C-P., S.H.S., K.S.F.; study design and optimization: P.C-P., P.N., T.N., S.H.S.; experiment preparation, data generation, sample analysis: P.C-P., K.H., T.N.; T.N. conducted the gene expression analysis; P.N. developed the mathematical models and conducted the scaling; data analysis and interpretation: P.C-P., P.N., T.N., S.H.S.; manuscript preparation: P.C-P., P.N., T.N., S.H.S.; K.S.F. oversaw the research. All authors contributed to the manuscript revision.

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