Novel explanted human liver model to assess hepatic extraction, biliary excretion, and transporter function

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Novel Explanted Human Liver Model to Assess Hepatic Extraction, Biliary Excretion and Transporter Function

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Realistic models predicting hepatobiliary processes in health and disease are lacking. We therefore aimed to develop a physiologically relevant human liver model consisting of normothermic machine perfusion (NMP) of explanted diseased human livers that can assess hepatic extraction, clearance, biliary excretion, and drug–drug interaction (DDI). Eleven livers were included in the study, seven with a cirrhotic and four with a noncirrhotic disease background. After explantation of the diseased liver, NMP was initiated. After 120 minutes of perfusion, a drug cocktail (rosuvastatin, digoxin, metformin, and furosemide; OATP1B1/1B3, P-gp, BCRP, and OCT1 model compounds) was administered to the portal vein and 120 minutes later, a second bolus of the drug cocktail was co-administered with perpetrator drugs to study relevant DDIs. The explanted livers showed good viability and functionality during 360 minutes of NMP. Hepatic extraction ratios close to *in vivo* reported values were measured. Hepatic clearance of rosuvastatin and digoxin showed to be the most affected by cirrhosis with an increase in maximum plasma concentration (C_{max}) of 11.50 and 2.89 times, respectively, compared with noncirrhotic livers. No major differences were observed for metformin and furosemide. Interaction of rosuvastatin or digoxin with perpetrator drugs were more pronounced in noncirrhotic livers compared with cirrhotic livers. Our results demonstrated that NMP of human diseased explanted livers is an excellent model to assess hepatic extraction, clearance, biliary excretion, and DDI. Gaining insight into pharmacokinetic profiles of OATP1B1/1B3, P-gp, BCRP, and OCT1 model compounds is a first step toward studying transporter functions in diseased livers.

Study Highlights

WHAT IS THE CURRENT KNOWLEDGE ON THE TOPIC?

☑ It remains challenging to measure hepatic extraction, biliary clearance, and to assess transporter function in the current available preclinical models in healthy and especially in diseased states. More physiological models will result in better predictions of drug pharmacokinetics.

WHAT QUESTION DID THIS STUDY ADDRESS?

☑ To evaluate if normothermic perfusion of explanted diseased human livers can be applied to adequately measure hepatic extraction, clearance, and biliary excretion of rosuvastatin, digoxin, furosemide, and metformin in the absence and presence of perpetrator drugs.

WHAT DOES THIS STUDY ADD TO OUR KNOWLEDGE?

☑ The study presents the use and applicability of a novel dynamic hepatic model to measure hepatic extraction, biliary excretion, and transporter function in healthy and especially diseased livers.

HOW MIGHT THIS CHANGE CLINICAL PHARMACOLOGY OR TRANSLATIONAL SCIENCE?

☑ This model can be regarded as the bridge between *in vitro* and *in vivo* clinical studies. Studying drug pharmacokinetics using explanted human livers can serve as a basis to explore the differences in hepatic handling of drugs for patients with hepatic impairment and, in the future, can be applicable to other disease areas and/or organs.

Accurate prediction of drug disposition in patients with and without hepatic diseases remains difficult, as appropriate models are lacking. The liver plays an important role in drug handling and impairment or alteration of its function may greatly affect multiple processes. Upon first liver pass, after oral administration, drug bioavailability as well as drug clearance may be altered thereby

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affecting the drug's efficacy. Studies in liver cirrhosis have shown that increased bioavailability as well as reduced clearance lead to a higher prevalence of adverse drug reactions and drug–drug interactions (DDIs), which can result in safety issues and ultimately an increased risk for hospital admission.[1,2] Therefore, drug dosing should be tailored according to the varying degrees of liver dysfunction among patients with liver diseases. However, with the currently available preclinical and clinical models, it remains difficult to quantify the required tailoring of the dose related to the degree and type of liver dysfunction.[3]

Established *in vitro* and animal models are often used to study the pathology and pharmacological characteristics of drugs of varying diseases. However, translation of these findings to clinical practice remains challenging due to, among others, species differences in transporter expression and the difficulty to mimic dynamic liver processes.[4,5] Novel 3D models, like liver-on-a-chip and bile duct-on-a-chip models, have gained significant interest as a predictive platform to study liver processes due to the incorporation of hemodynamics.[6,7] Although these organ-on-a-chip models hold much promise, they are still in their infancy owing to the difficulty of mimicking (patho)physiological processes in the liver, such as portal and arterial blood flow and biliary excretion.[7] Normothermic machine perfusion (NMP) systems using human *ex vivo* whole organs overcome this problem because hepatic architecture is combined with (near) physiological hemodynamics. Thereby, use of human explanted liver whole organ enables to study hepatobiliary processes as well as liver disease-specific pharmacokinetics.[8–10]

In this study, we developed a novel hepatic model using diseased explanted human livers. Four model drugs (rosuvastatin, digoxin, furosemide, and metformin) with and without perpetrator drugs were used to study hepatic extraction, clearance, biliary excretion, and DDI. These model drugs are known substrates for different important hepatic uptake and efflux transporters and enabled comparison of the model to *in vivo* reported data.

MATERIALS AND METHODS

Human livers

Patients undergoing liver transplantation were included in this study. After providing informed consent, the patients approved the usage of the explanted liver for experimental study (**Figure 1**). The use of explanted liver tissue was approved by the medical ethical committee of the Leiden University Medical Center (B19.040). Patients with polycystic liver disease, with a transjugular intrahepatic portosystemic shunt, or waitlisted for recurrent-orthotopic liver transplantation were excluded from participation. Eleven human livers were included in the study. The underlying disease processes of these livers were primary biliary cholangitis ($n = 1$), nonalcoholic fatty liver disease ($n = 2$), alcoholic liver disease ($n = 3$), and hepatocellular carcinoma in the context of hepatitis B viral disease ($n = 2$). In addition, three discarded noncirrhotic livers, which were declined for transplantation, were included in this study. The reasons for being declined were steatosis ($n = 2$) and an occlusion of the right hepatic artery ($n = 1$). Immediately following explantation of the recipient diseased liver, a portal and arterial flush with cold Histidine-tryptophan-ketoglutarate

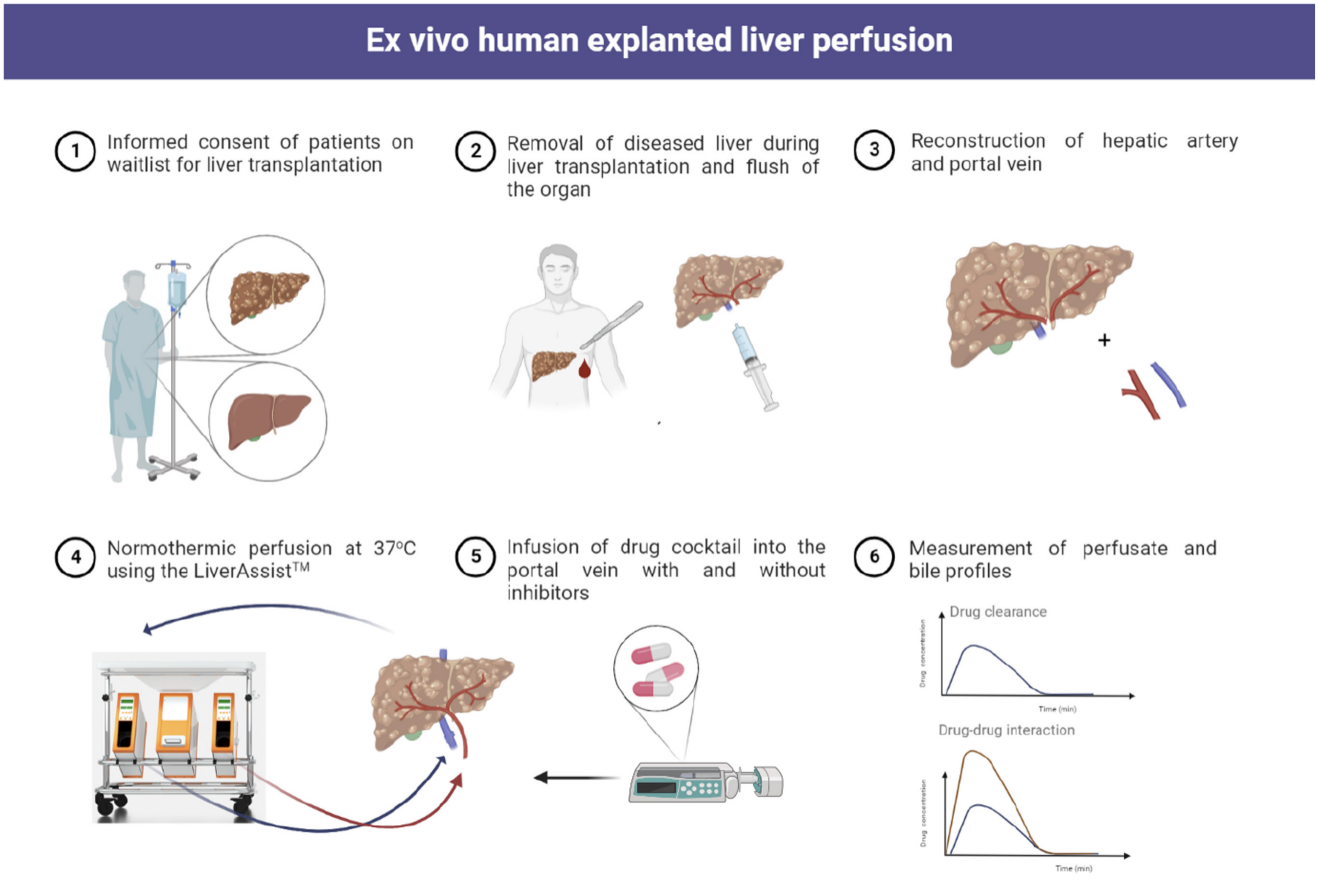


Figure 1 Schematic representation of normothermic explanted human liver perfusion setup.

(Carnamedica, Warsaw, Poland) preservation solution was performed. The period between explantation (i.e., clamping and transection of the portal and hepatic veins as the final step of the hepatectomy) and cold flush of the explanted liver (*ex vivo*), is described as the warm ischemia time. After a clean effluent flush, the liver was transported in cold preservation solution to the Organ Preservation and Regeneration room in the operating room complex. Here, under sterile conditions, a back table reconstruction of the right and left hepatic artery and portal vein was performed using surplus donor blood vessels, in order to facilitate cannulation (portal vein – 25 Fr cannula, hepatic artery – 12 Fr cannula) and connection to the machine perfusion device (Liver Assist device; XVIVO, Groningen, The Netherlands). Thereafter, the bile duct was cannulated. **Normothermic machine perfusion**

All human livers were perfused using the Liver Assist device. The machine consists of two centrifugal pumps, which provide a pulsatile flow to the hepatic artery and a continuous flow to the portal vein.[11] The system reservoir was filled with 2 L perfusion fluid containing 1:1 ratio of human red blood cells and fresh frozen plasma (Sanquin, Amsterdam, The Netherlands). Insulin, sodium taurocholate, heparin, and epoprostenol were provided as continuous infusion at a rate of 10 U/h, 1,041 U/h, 10 mL/h (2% w/v), and 8 µg/h, respectively, in order to maintain liver functioning and to facilitate bile flow. Additionally, nutrients (aminoplasmal 10E (B Braun Melsungen AG, Melsungen, Germany) and cernevit (Baxter BV, Utrecht, The Netherlands) were continuously provided (23 mL/hr) to keep the liver metabolically active (**Table S1**). Gas delivery to the Liver Assist consisted of 95% oxygen and 5% carbon dioxide at 1.5 L/min and the temperature was set at 37°C. The noncirrhotic livers were perfused with a portal pressure of 11 mmHg and the cirrhotic livers required perfusion at 14 mmHg to generate a sufficient portal flow. Mean arterial pressure was set at 50 mmHg. After 360 minutes of perfusion, the livers were submerged in formaldehyde, transported the pathology department, and were examined according to the institution's clinical guidelines dependent on the patient's underlying pathophysiology.

Drug administration during perfusion

Drug clearance in perfusate and bile of the drug cocktail (rosuvastatin, metformin, furosemide, and digoxin) were determined in the absence and presence of perpetrator drugs (quinidine, rifampicin, cimetidine, and probenecid; **Table S2**).[12] The doses applied to the system were based on clinically prescribed oral dosage and calculated as previously described in Stevens *et al*.[13] In short, portal doses of the drug cocktail compounds and inhibitors were calculated based on the fraction absorbed in the intestine to the portal vein, fraction of metabolism,[14] and circulating volume (**Table S2**). After 120 minutes of perfusion, a slow bolus for 10 minutes of the drug cocktail was administered via the portal vein at 1 mL/min to mimic oral absorption through the gut. Subsequently, perfusate and bile samples were taken for the following 120 minutes. Arterial samples were taken at $t = 120$, 122, 124, 126, 128, 130, 135, 140, 150, 160, 170, 180, 210, and 240 minutes. Additional portal samples were taken during the administration of the drug at $t = 126$ and $t = 130$ minutes to determine the hepatic extraction. Bile samples were collected in 10-minute fractions from 120 minutes onward. After 240 minutes, first, a slow bolus 10 minutes (1 mL/min) of perpetrator drugs (quinidine, cimetidine, rifampicin, and probenecid) was administered to the liver and after 5 minutes (at $t = 245$ minutes), again, a subsequent slow bolus of the drug cocktail was administered via the portal vein. The same sampling schedule for arterial samples and bile samples was followed. Biopsies were taken at the end of the first dosing ($t = 240$ minutes) and second dosing with inhibitors ($t = 360$ minutes). Perfusate and bile samples were immediately stored at ≤ −70°C until further processing.

Liver function assessment

Hepatic artery and portal vein flow were recorded from the Liver Assist machine. Perfusate samples and bile samples (collected under mineral oil to prevent bile exposure to ambient air[15]) were taken hourly to monitor liver viability (pH, glucose, lactate, etc.) using a RapidPoint 500 blood gas analyzer (Siemens, Germany). Alanine aminotransferase (ALT) concentration in the perfusate samples was measured by reflectance photometry (Reflotron-Plus System; Roche Diagnostics, Almere, The Netherlands). Perfusate and bile parameters were compared with defined criteria used in clinical transplantation studies; perfusate ALT < 6,000 and lactate < 2.5 mmol/L after 120 minutes of perfusion, biliary pH > 7.5.[16–18]

Histological analysis

Pre-perfusion ($n = 2$) and post-perfusion ($n = 2$) biopsies were taken for each liver, fixed in 10% formalin, and subsequently embedded in paraffin. Slices of 4 µm were cut and stained with hematoxylin and eosin (H&E) for examination using light microscopy.

Bioanalysis

The concentration of the drug cocktail was quantified using liquid-chromatography tandem mass spectrometry (LC–MS/MS; Waters, Etten-Leur, The Netherlands). Perfusate and bile sample (10 µL) were deproteinized with 100 µL acetonitrile (ACN) with the addition of 10 µL the isotopically labeled internal standards (1 µg/mL). Thereafter samples were vortexed, centrifuged, and supernatant was transferred to a 96-well plate and dried under nitrogen. Samples were dissolved in 100 µL 10% ACN + 0.1% formic acid and injected into LC–MS/MS for quantification. Details of the LC–MS/MS conditions used are shown in **Tables S3** and **S4**.

Chemicals

Rosuvastatin, digoxin, furosemide, and quinidine were obtained from Sigma-Aldrich (Zwijndrecht, The Netherlands). Metformin and rifampicin and cimetidine were obtained from Bioconnect (Huissen, The Netherlands). Heparin, sodium taurocholate (Sigma-Aldrich, Zwijndrecht, The Netherlands), insulin (Novo Nordisk, Alphen aan den Rijn, The Netherlands), and epoprostenol (Flolan; GlaxoSmithKline, Mississauga, ON, Canada) were obtained as indicated.

Data analysis and statistics

Data obtained during the perfusion studies was analyzed using Graphpad version 8 (GraphPad Software Inc., La Jolla, CA). Values for the area under the concentration time curve 0–120 minutes (AUC_{0-tau}) were calculated using the linear trapezoidal method. The AUC ratio (AUCR) was determined by dividing the $AUC_{125-245\,min}$ (with inhibitors) by the $AUC_{0-120\,min}$ (without inhibitors). The hepatic extraction ratio was calculated during the 10 minutes dosing period as follows: concentration entering the liver (portal vein) – concentration leaving the liver/concentration entering the liver. Significance of differences between the cirrhotic and noncirrhotic livers was tested using the Mann–Whitney *U* test. Data are presented as median and interquartile range (IQR) for nonparametric distributed data. *P* values below 0.05 were considered significant.

RESULTS

Explanted livers showed good viability during perfusion

Both cirrhotic ($n = 7$, characteristics; **Table 1**) and noncirrhotic ($n = 4$, characteristics, **Table 1**) livers had a stable arterial flow with minimal variation during perfusion; 235 mL/min (IQR: 214.7–249) in cirrhotic livers vs. 230 mL/min (IQR: 21.3–239.5) in noncirrhotic livers (**Figure 2a**). A significant lower portal flow in cirrhotic livers was observed compared with noncirrhotic livers of 523 mL/min (IQR: 489–557) vs. 1,678 mL/min (IQR: 1596–1710), respectively, $P < 0.001$ (**Figure 2b**). **Figure 2c** demonstrates perfusate lactate, which is a marker of liver function. Lactate clearance was observed after 30 minutes of perfusion in the noncirrhotic liver group and remained low (1.39 mmol/L, IQR:

Table 1 Liver characteristics and ischemic times of cirrhotic and non-cirrhotic livers

	Cirrhotic livers *N* = 7	Noncirrhotic livers *N* = 4	*P* values
Underlying disease	ALD (*n* = 3) NAFLD (*n* = 2) HBV + HCC (*n* = 1) PBC (*n* = 1)	Discarded liver (*n* = 3) HBV + HCC (*n* = 1)	n.a.
Age, years	59 (54–69)	63 (30–67)	0.545
Gender			
Male	6	4	
Female	1	0	
BMI, kg/m^2	29.4 (23.8–31.4)	26.8 (26.0–28.8)	>0.99
WIT, minutes	5 (4–6)	12 (5–14)	0.067
CIT, minutes	80 (71–99)	270 (105–507)	0.070
Weight of the liver, g	1,507 (1,297–2,005)	1,975 (1,394–2,008)	0.648
MELD	11 (9–23)	6 (6–6)	0.006

Differences between groups were analyzed using the Mann–Whitney *U* test.
ALD, alcoholic liver disease; BMI, body mass index; CIT, cold ischemia time; HBV, hepatitis B virus; HCC, hepatocellular carcinoma; MELD, model of end stage liver disease; n.a., not applicable; NAFLD, nonalcoholic fatty liver disease; PBC, primary biliary cholangitis; WIT, warm ischemia time..

0.48–0.29), whereas cirrhotic livers showed higher levels of perfusate lactate (13.25 mmol/L, IQR: 3.20–22.91) after 360 minutes of NMP). As marker of hepatocellular injury, release of ALT was measured throughout the perfusion (**Figure 2d**). Levels of ALT reached a plateau after 60 minutes of perfusion and remained stable until 360 minutes of perfusion. ALT levels were significantly higher in the noncirrhotic livers compared with the cirrhotic livers ($P = 0.017$). All livers produced bile during perfusion, but significantly more bile was produced by the cirrhotic livers 55 mL (IQR: 37–61) vs. 28 mL (IQR: 22–60) $P = 0.034$ (**Figure 2e**). The pH of produced bile during perfusion showed to be > 7.5 in cirrhotic as well as noncirrhotic livers (**Figure 2f**), demonstrating good cholangiocyte viability and meeting the defined viability criteria (Method section). To investigate the effect of perfusion on the integrity of the livers, biopsies of the livers pre- and post-perfusion were stained with H&E. **Figure 2g** shows a representative example of a noncirrhotic liver and a cirrhotic liver, before perfusion and post-perfusion ($t = 360$ minutes). The histopathological analysis indicated that the perfusion did not have obvious detrimental morphological effects on the liver tissue. Additional markers of hepatocellular injury and function and cholangiocyte viability can be found in **Figure S1**. Gene expression data of housekeeping genes, transporters, and enzymes can be found in **Figure S2**.

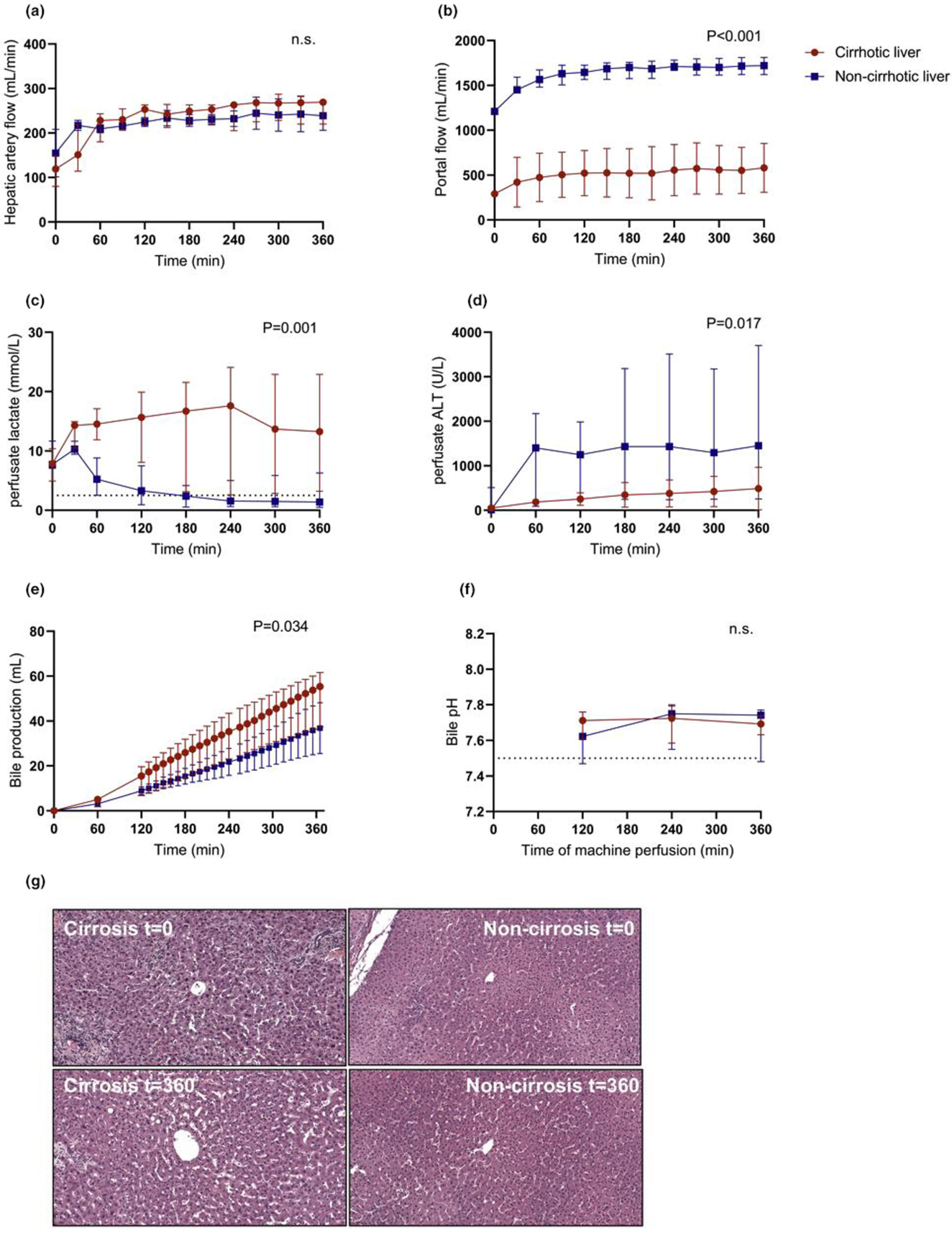
Hepatic clearance and biliary excretion of rosuvastatin and digoxin are affected by cirrhosis

To assess hepatic clearance and biliary excretion, a drug cocktail was infused to the portal vein (**Figure 3**, **Figure S2**, **Table S1**). Perfusate concentrations of rosuvastatin appeared to be the most affected by liver cirrhosis, with an approximate 11.5-fold increased maximum plasma concentration (C_{max}) in cirrhotic livers compared with the perfused noncirrhotic livers (463.3 ng/mL, IQR: 243.2–555.2) vs. 41.10 ng/mL (IQR: 7.01–71.02, $P = 0.024$) and 190-fold increased AUC_{0-tau} of 20.96 µg/mL (IQR: 11.61–29.98) vs. 0.11 µg/mL (IQR: 0.10–5.15, $P < 0.001$; **Figure 4a**). A comparable effect was observed for digoxin, with a perfusate C_{max} that was more than 3-fold higher in cirrhotic livers (10.03 ng/mL, IQR: 7.75–11.78) compared with noncirrhotic livers (3.46 ng/mL ng/mL, IQR: 2.33–7.80, $P = 0.038$) and an AUC_{0-tau} that was almost 3-fold higher; 629 ng/mL (IQR: 282–746) in cirrhotic livers vs. 222 ng/mL (IQR: 171–503) in noncirrhotic livers, $P = 0.003$ (**Figure 4d**). Biliary excretion of rosuvastatin and digoxin was higher in cirrhotic livers (66% and 51%, respectively) compared with noncirrhotic livers (47% and 17%, respectively), however, not significantly (**Figure 4b,e**). **Figure 4c,f** show lower intrahepatic levels of rosuvastatin and digoxin respectively in cirrhotic livers compared with noncirrhotic livers, which is in line with the biliary excretion. As can be observed in **Figure 4g–l**, cirrhosis had a minor effect on furosemide and metformin concentrations as C_{max} was 1.19 and 1.13 times higher in cirrhotic livers compared with noncirrhotic livers (not significantly). Metformin and furosemide were only minimally cleared through biliary excretion (in the range of 1–3%) which was not affected by the cirrhosis (**Figure 4h,k**) and also intrahepatic levels were comparable between cirrhotic and noncirrhotic livers (**Figure 4i,l**).

Hepatic extraction of rosuvastatin affected by cirrhosis

A unique application of the perfusion model is to sample from the portal vein (before the liver) and hepatic artery (after liver sample)

Figure 2 Liver functionality, viability, and injury markers measured in perfusate, bile, and tissue of normothermic perfused cirrhotic and noncirrhotic livers. (**a**) hepatic artery flow, (**b**) portal flow, (**c**) perfusate lactate, (**d**) perfusate ALT levels (**e**) bile production (**f**) biliary pH and of cirrhotic and noncirrhotic livers measured during 360 minutes of normothermic perfusion. (**g**) H&E staining of a cirrhotic liver and noncirrhotic liver, before perfusion ($t = 0$) and after perfusion (360 minutes; 200×). Data represent median and interquartile range in cirrhotic ($n = 7$) and noncirrhotic livers ($n = 4$). Differences between groups were analyzed using the Mann–Whitney *U* test; *P* value is presented in the right corner of each graph. ALT, alanine aminotransferase; H&E, hematoxylin and eosin; n.s., not significant.



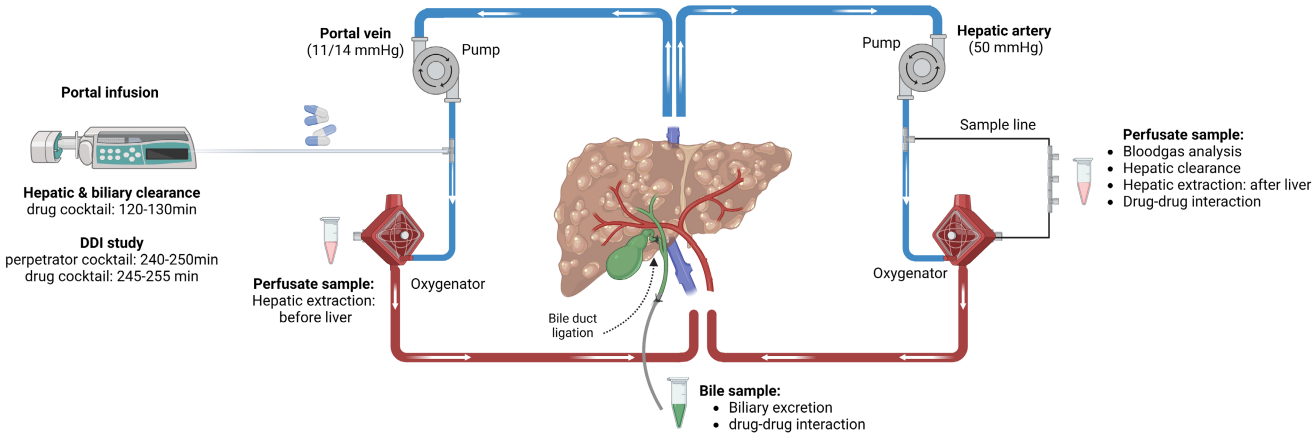


Figure 3 Schematic representation of normothermic machine perfusion setup of the liver to study drug hepatobiliary processes. DDI, drug-drug interaction.

during the dosing period (**Figure 5a**), enabling to determine the hepatic extraction ratio (**Figure 5b**). A high hepatic extraction by the noncirrhotic livers of rosuvastatin was measured; 0.70 (IQR: 0.69–0.83), which showed to be affected by cirrhosis (0.57, IQR: 0.42–0.67)). The hepatic extraction of digoxin, furosemide, and metformin, which are low hepatic extraction ratio drugs, did not show to be affected by cirrhosis.

Increased risk of DDI for rosuvastatin and digoxin

Figure 6 shows the results of the studies in which different drugs were used to inhibit the uptake and /or excretion of the drug cocktail from the previous section (**Figure 6a**). In both cirrhotic and noncirrhotic livers, rosuvastatin and digoxin AUC_{0-tau} and C_{max} were increased upon co-administration of a perpetrator cocktail (**Figure 6b,c**). However, the DDI, expressed as an increase in C_{max}, and increase in AUCR (i.e., ratio AUC of victim drug with and without inhibitors over 120 minutes) was more profound but not significant in the noncirrhotic livers than the cirrhotic livers. More specifically, the AUCR for rosuvastatin and digoxin was 5.6 (IQR: 3.1–13.3) and 8.1 (IQR: 4.6–20.5), respectively, in noncirrhotic livers compared with 1.4 (IQR: 0.9–1.9) and 2.2 (IQR: 1.3–3.5), respectively, in cirrhotic livers. No increase in AUC_{0-tau} and C_{max} was observed for the low-hepatic extraction ratio drugs furosemide and metformin. The inhibition of biliary excretion (expressed in AUCR) of rosuvastatin and digoxin, which are highly biliary excreted is shown in **Figure 6d**. The P-gp mediated biliary excretion of digoxin is shown to be inhibited as demonstrated by an AUCR of 0.28 (IQR: 0.11–0.52) in cirrhotic livers and 0.66 (IQR: 0.12–1.14) in noncirrhotic livers. Intrahepatic levels, demonstrated in **Figure 6f**, showed to be a 6-fold increase in cirrhotic livers and 1.6-fold increase in noncirrhotic livers upon co-administration of the inhibitors, which is in line with the inhibition of the biliary excretion. The BCRP mediated biliary excretion of rosuvastatin showed to be inhibited in the noncirrhotic livers (0.78, IQR: 0.75–2.20) whereas, on average, no inhibition of rosuvastatin in bile produced by cirrhotic livers was measured (0.98, IQR: 0.61–1.15). Co-administration of the inhibitor mix showed to mildly increase the intrahepatic accumulation by 1.6-fold and 2-fold in cirrhotic and noncirrhotic livers (**Figure 6e**). Metformin and furosemide showed to be minimally biliary excreted (range 1–3%), therefore no interaction in bile was presented here.

DISCUSSION

Here, we show for the first time the use of explanted human diseased livers as a model to assess hepatic extraction, biliary clearance, and transporter function. We successfully perfused 7 cirrhotic livers and 4 noncirrhotic livers for a period of 360 minutes, maintaining liver viability and functionality, as indicated by stable flow, bile production, proper histology pre- and post-perfusion, stable ALT values, and stable gene expression throughout the perfusion.

The use of NMP has proven to be beneficial for organ transplantation[11,19] and NMP has become a widely accepted method to assess viability of the donor liver prior to transplantation.[20,21] Many criteria of hepatocellular and cholangiocellular function have been described (e.g., lactate clearance, perfusate ALT, and biliary pH) to establish liver viability based on perfusion results and post-transplantation outcomes, demonstrating the robustness of the model in perfusion research.[15–19] The explanted cirrhotic livers perfused in this study met most of the criteria for hepatocellular function, except for lactate clearance and portal flow, whereas other hemodynamic parameters did not show significant changes. As expected, portal flow was lower in cirrhotic livers compared with noncirrhotic livers as a result of portal hypertension.[22]

Unique advantages of the whole organ perfusion model is [1] the dynamic environment, enabling to measure the hepatic extraction, and [2] the preservation of the biliary excretion route, thus allowing for the assessment of biliary excretion. The noncirrhotic livers showed to rapidly take up rosuvastatin and digoxin from the perfusate with a hepatic extraction ratio of 0.70 and 0.42, respectively, which are close to *in vivo* reported measures of 0.63 and 0.3, respectively.[23,24] In this study, hepatic extraction and clearance of rosuvastatin showed to be the most affected by cirrhosis as hepatic extraction decreased to 0.57 and a 190-fold AUC difference was observed. Rane *et al.*[25] reported that the clearance of hepatically cleared drugs with a high extraction ratio are related to blood flow

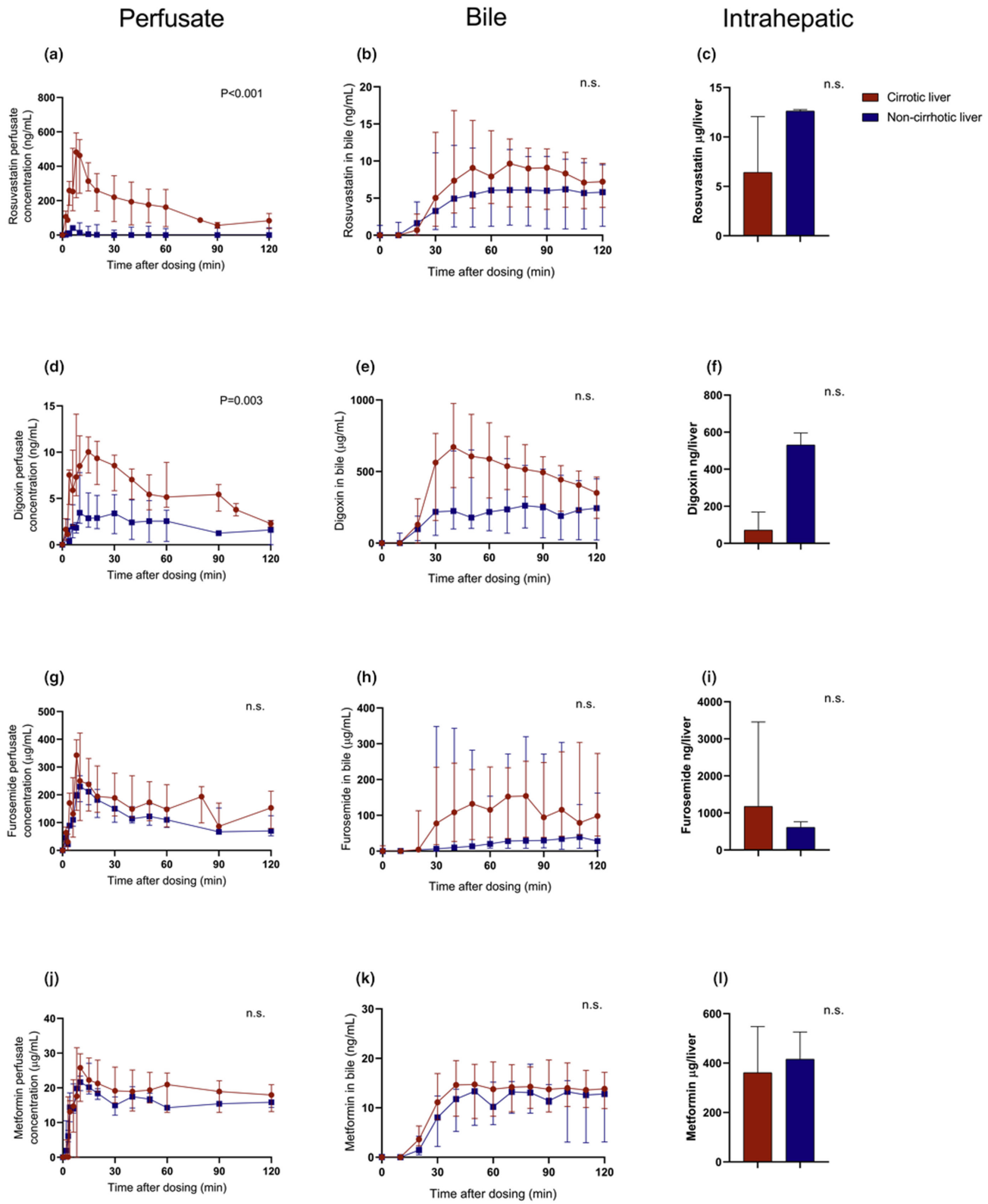


Figure 4 Pharmacokinetic profiles of rosuvastatin, digoxin, metformin, and furosemide in cirrhotic and noncirrhotic perfused livers. Rosuvastatin (applied dose of 1.80 mg) (**a**) perfusate levels, (**b**) biliary excretion of rosuvastatin, and (**c**) intrahepatic rosuvastatin levels. Digoxin (applied dose of 0.11 mg) (**d**) perfusate levels, (**e**) biliary excretion of digoxin, and (**f**) intrahepatic digoxin levels. Furosemide (applied dose of 0.77 mg) (**g**) perfusate levels, (**h**) biliary excretion of furosemide, and (**i**) intrahepatic furosemide levels. Metformin (applied dose of 74.40 mg) (**j**) perfusate levels, (**k**) biliary metformin excretion, and (**l**) intrahepatic metformin levels. Data represent median and interquartile range in cirrhotic ($n = 7$) and noncirrhotic livers ($n = 4$) for perfusate and bile. There were five in the cirrhotic and three in noncirrhotic livers for intrahepatic data. Differences in AUC between groups were analyzed using the Mann–Whitney *U* test; *P* value is presented in the right corner of each graph. AUC, area under the concentration time curve; n.s., not significant.

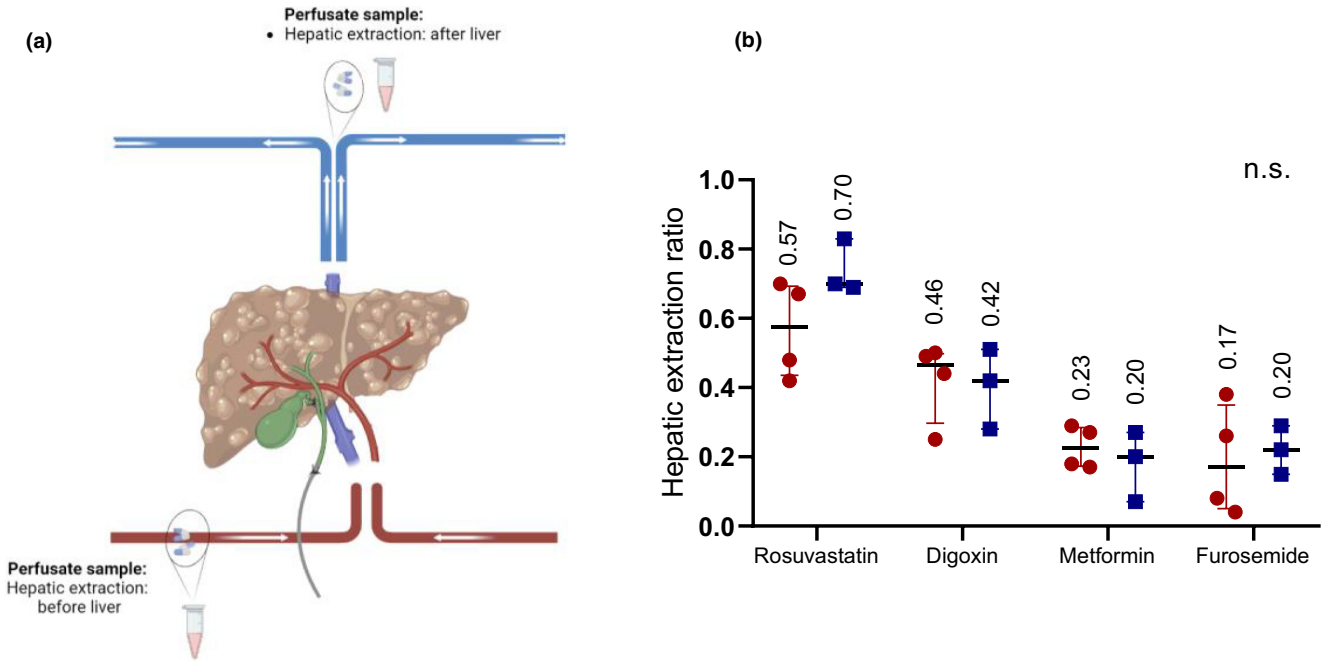


Figure 5 Hepatic extraction of drug cocktail compounds. (**a**) Schematic representation of sample points before and after liver (**b**) hepatic extraction ratio of rosuvastatin, digoxin, metformin, and furosemide (n = 3 noncirrhotic livers and n = 4 cirrhotic livers). Differences between groups were analyzed using the Mann–Whitney U test; P value is presented in the right corner of each graph. n.s., not significant.

and thus a major decrease in portal flow, as in cirrhosis, can dramatically affect the first passage across the liver.[26,27] However, changes in portal flow alone do not explain the 190-fold difference. Reduced uptake (C_{max}: 11.5-fold higher), as well as delayed elimination was observed. We hypothesize that decreased transporter abundance of OATP1B1/1B3, as reported in literature, contributed to the delayed elimination as we observed in the cirrhotic livers.[28–30] Hardly any studies are known regarding the effect of liver cirrhosis on, for instance, rosuvastatin pharmacokinetics. Only Simonson *et al.*[31] reported the effect of advanced liver cirrhosis on C_{max} and AUC values for two patients. This is substantiating the need for more knowledge regarding the role of cirrhosis in hepatic handling of drugs. *In vivo* studies demonstrated a high biliary excretion of rosuvastatin of ~ 76.8%, as measured by fecal excretion.[32] The *ex vivo* noncirrhotic livers showed a biliary excretion of 37% in 120 minutes, extrapolation of the data resulted in 77% total excretion of rosuvastatin, which is in line with *in vivo* data. Interestingly, digoxin showed a relatively high biliary clearance in cirrhotic (51%) and noncirrhotic (17%) livers during 120 minutes of perfusion. *In vivo* studies have shown that digoxin is extensively renally eliminated (75%).[33] However, multiple studies demonstrated that digoxin is highly involved in the enterohepatic circulation, thereby decreasing the *in vivo* fecal excretion of digoxin.[34,35] The two other compounds used in this study, furosemide and metformin, which are mainly renally cleared, showed a low hepatic extraction ratio and minor biliary excretion (≤ 3%) in both cirrhotic and noncirrhotic livers, which is in line with human *in vivo* data which showed that biliary eliminated was limited.[36,37] By showing the biliary excretion of two compounds, which are mainly biliary excreted and two that are not/minorly excreted via the biliary route, we demonstrated that the perfused liver retained its function while in the *ex vivo* environment. Interestingly, the percentage of biliary clearance was higher, for all compounds, in the cirrhotic perfused livers and lower intrahepatic levels of digoxin and rosuvastatin were measured. This might be due to an elevated bile flow which has been observed in patients with cirrhosis and which is confirmed in our model, resulting in a more efficiency biliary clearance.[38] In addition, it has been reported for digoxin that P-gp levels are significantly elevated in cirrhosis, which can contribute to a more efficient biliary clearance of digoxin.

The effect of DDIs in cirrhotic and noncirrhotic livers was subsequently determined by using a cocktail of perpetrator drugs. The noncirrhotic livers showed an increased AUCR for a DDI with rosuvastatin (3.52), whereas values between 2.48 and 5.38 for rosuvastatin with rifampicin as inhibitor have been observed,[39–41] showing good agreement with clinical data. Although it is observed that rifampicin can inhibit MRP2 and thereby limiting biliary excretion of rosuvastatin,[42] we observed minimal inhibition at the biliary level, and a minimal increase in the intrahepatic level. It is possible that the observed results are a consequence of inadequate portal dosing of rifampicin, as the perfusate concentration likely did not reach the desired levels. In our porcine perfusion, we have measured C_{max} levels of 7.3 µM (n = 10),[13] which is lower than 20 µM plasma levels in other clinical studies.[39] Digoxin showed a high increase in AUCR upon dosing with inhibitors, which is mainly the result of inhibiting uptake via OATP (rifampicin as inhibitor), as described by Lau *et al.*[43] Additionally, in cirrhotic as well as noncirrhotic livers, a decrease in the percentage of biliary excretion was observed and higher intrahepatic levels of digoxin were measured upon co-administration with inhibitors.

A decrease in P-gp-mediated biliary excretion was observed to an average inhibition of 0.64 in noncirrhotic livers. This is the same

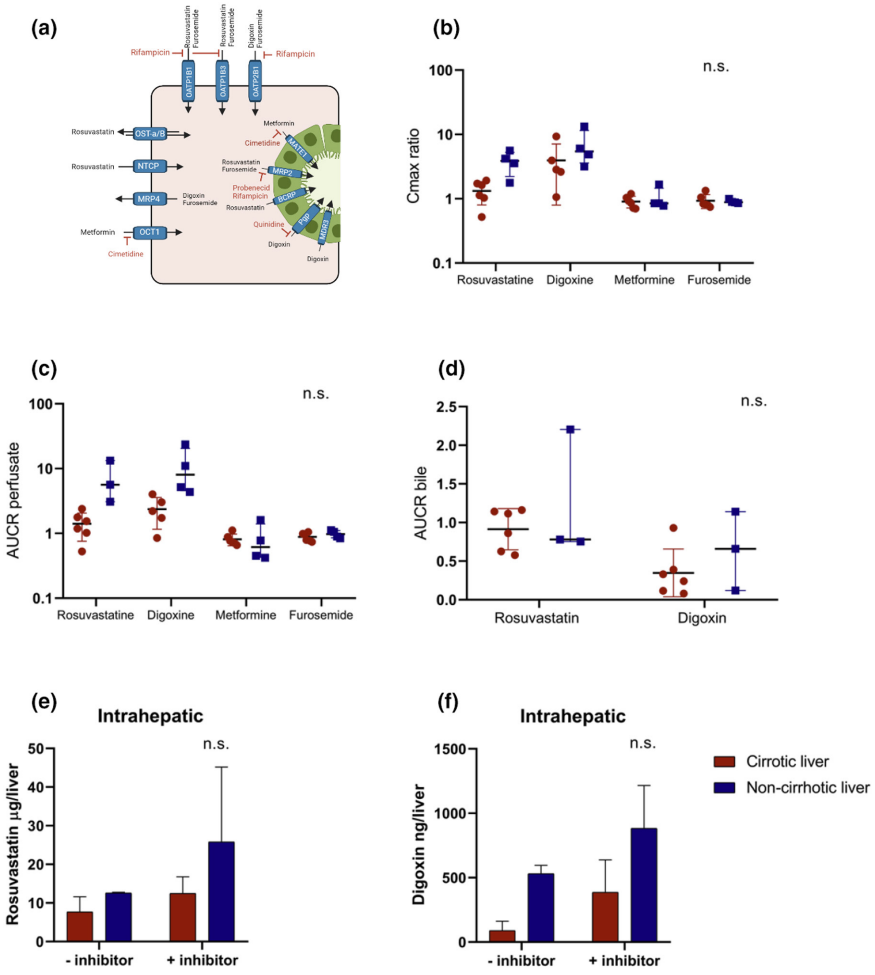


Figure 6 Effect of drug inhibitor mix on hepatic clearance of rosuvastatin, digoxin, metformin, and furosemide. (**a**) Graphical representation of relevant hepatic drug transporters for the victim drugs (rosuvastatin, digoxin, metformin, and furosemide) and the applied perpetrators (quinidine, rifampicin, cimetidine, and probenecid). (**b**) Ratio of perfusate C_{max} and (**c**) perfusate AUCR with and without applied perpetrator drugs for the cirrhotic and noncirrhotic livers. (**d**) Bile AUCR of digoxin and rosuvastatin with and without applied perpetrator drugs for the cirrhotic and non-cirrhotic livers. (**e**) Intrahepatic levels upon dosing inhibitor mix on rosuvastatin and (**f**) intrahepatic digoxin levels. Data represent median and interquartile range in cirrhotic ($n = 7$) and noncirrhotic livers ($n = 4$) for perfusate and bile, five in cirrhotic and three in noncirrhotic livers for intrahepatic data. Differences between groups were analyzed using the Mann–Whitney *U* test; *P* value is presented in the right corner of each graph. AUCR, area under the concentration time curve ratio; C_{max}, maximum plasma concentration; n.s., not significant.

inhibition we have observed in our porcine experiments. Because there is some variation between the human livers, we do recommend more replicates for future experiments. It remains difficult to compare to *in vivo* DDI observations because a major part of the DDI takes place at the intestinal level, when orally absorbed, thereby affecting the portal vein concentration. Still, the observations from this study showed that we could mimic a DDI with digoxin in this perfusion model leading to an increased C_{max} and AUCR.

Explanted livers obtained during orthotopic liver transplantation are currently only used for pathological assessment and subsequently discarded. Although many preclinical and laboratory animal models try to mimic liver diseases as best as possible, many models fail due to a lack of translation to the human situation. This model can be widely applied in a variety of research settings, however, implementation will only be feasible in a limited number of centers where liver transplantations are regularly performed. Considering the scarcity of explanted human livers for research purposes the utilization of porcine livers in early stages of drug development can prove to be a valuable approach, as we have previously shown.[13] Although we have used a limited time-window perfusion, recent studies have shown the possibility to prolong organ perfusion duration even up to 7 days[20] which will broaden the applicability of liver perfusion. Liver disease can affect the abundance of transporter proteins and/or metabolic enzymes. In fact, multiple studies have analyzed liver biopsies from patients with liver disease showing alterations in expression of specific proteins relevant for pharmacokinetics.[28–30,44] For instance, Drozdzik *et al*., showed an increase in P-gp and MRP4 and decreases in NTCP, OCT1, and OATP1B1 in patients with severe liver disease.[28] Although these studies already provided some hints toward altered pharmacokinetics and metabolism of drugs in patients with liver diseases, *ex vivo* perfusion of diseased livers offers a unique opportunity to directly study the effect of altered expression levels of transporter proteins and metabolizing

enzymes. In this study, we used known drug substrates for different important hepatic uptake and efflux transporters. Gaining insight into pharmacokinetic profiles of OATP1B1/1B3, P-gp, BCRP, and OCT1 model compounds is a first step toward studying transporter functions in diseased livers. Additionally, for many drugs, dosing advice is currently incomplete for patients with cirrhosis because of lacking evidence or showing major inter-individual differences. Studying drug pharmacokinetics using explanted human livers can serve as a basis to explore the differences in hepatic handling of drugs for patients with hepatic impairment even though to date it is yet too early to know what the exact place of this model is for clinical practice or drug development.[45]

In conclusion, we demonstrated for the first time NMP of diseased human livers explanted during liver transplantation and discarded donor livers to study hepatic extraction, clearance, biliary excretion, and DDIs. The ability to sample perfusate, bile, and tissue during and after dosing is a unique approach to gain insights into hepato-biliary processes, transporter function, and transporter abundance.

SUPPORTING INFORMATION

Supplementary information accompanies this paper on the *Clinical Pharmacology & Therapeutics* website (www.cpt-journal.com).

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CONFLICT OF INTEREST

The authors declared no competing interests for this work.

AUTHOR CONTRIBUTIONS

All authors wrote the manuscript. L.J.S., J.D., J.B.D., B.Vh., J.M.D., C.A.J.K., E.vdS., and I.P.J.A. designed the research. L.J.S., J.D., J.B.D., and I.P.J.A. performed the research. L.J.S., J.D., I.P.J.A., A.A., A.dV., and E.vdS. analyzed the data.

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