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# Normothermic perfusion of cirrhotic and non-cirrhotic explanted human livers: applicability to study drug pharmacokinetics

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**Abstract**

Background and Purpose: 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 be used to investigate hepatic first-pass, clearance, biliary excretion and drug-drug interactions. Experimental approach: Eleven livers were included in the study, seven with a cirrhotic and four with a non-cirrhotic disease background. After explantation of the diseased liver, the liver artery and portal vein were reconstructed followed by NMP. After 120 minutes of perfusion, a drug cocktail (rosuvastatin, digoxin, metformin and furosemide) was administered to the portal vein and 120 minutes later, a second bolus of the drug cocktail was co-administered with drug inhibitors to study relevant drug-drug interactions. Key results: The explanted livers showed good viability and functionality after explantation and 360 minutes of NMP. Hepatic first-pass and clearance of rosuvastatin and digoxin showed to be the most affected by cirrhosis with an increase in Cmax of 10.03 and 2.89 times, respectively, compared to non-cirrhotic livers. No major differences were observed for metformin and furosemide. Drug-drug interaction of rosuvastatin or digoxin with inhibitors were more pronounced in non-cirrhotic livers compared to cirrhotic livers. Conclusions and Implications: Our results demonstrated that explanted cirrhotic and non-cirrhotic livers were suitable for NMP and we demonstrated the applicability to study hepatic first pass, clearance, biliary excretion and drug-drug interaction. This model can be applied in a variety of research settings for hepatology, transplantation and pharmacology

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**Authors contribution:**

Concept and design: LJS, JD, JBD, BvH, JMD, CAJK, ES, IPJA.

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Acquiring data: LJS, JD, JBD, IPJA, AA, AdV, AM

Writing and review of article: : LJS, JD, JBD, BvH, AM, JMD, AA, AdV, WV, CAJK, ES, IPJA.

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**Background and Purpose:** 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 be used to investigate hepatic first-pass, clearance, biliary excretion and drug-drug interactions.

**Experimental approach** : Eleven livers were included in the study, seven with a cirrhotic and four with a non-cirrhotic disease background. After explantation of the diseased liver, the liver artery and portal vein were reconstructed followed by NMP. After 120 minutes of perfusion, a drug cocktail (rosuvastatin, digoxin, metformin and furosemide) was administered to the portal vein and 120 minutes later, a second bolus of the drug cocktail was co-administered with drug inhibitors to study relevant drug-drug interactions.

**Key results:** The explanted livers showed good viability and functionality after explantation and 360 minutes of NMP. Hepatic first-pass and clearance of rosuvastatin and digoxin showed to be the most affected by cirrhosis with an increase in Cmax of 10.03 and 2.89 times, respectively, compared to non-cirrhotic livers. No major differences were observed for metformin and furosemide. Drug-drug interaction of rosuvastatin or digoxin with inhibitors were more pronounced in non-cirrhotic livers compared to cirrhotic livers.

**Conclusions and Implications** : Our results demonstrated that explanted cirrhotic and non-cirrhotic livers were suitable for NMP and we demonstrated the applicability to study hepatic first pass, clearance, biliary excretion and drug-drug interaction. This model can be applied in a variety of research settings for hepatology, transplantation and pharmacology.

**Keywords:** liver, hepatic clearance, biliary excretion, transplantation

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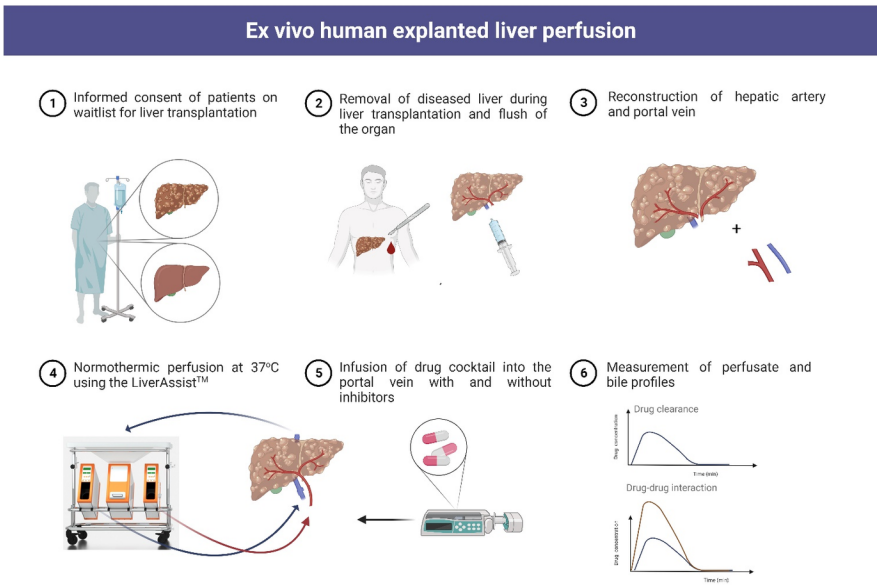


**Graphical abstract**

**List of abbreviations**

ALD Alcoholic liver disease

ALT Alanine aminotransferase

AST Aspartate aminotransferase

AUC Area under the curve

AUCR Area under the curve ratio

CIT Cold ischemia time

Cmax Maximum concentration

HA Hepatic artery

HTK Histidine-tryptophan-ketoglutarate

HBV Hepatitis B virus

HCC Hepatocellular carcinoma

MELD Model for end stage liver disease

NAFLD Non-alcoholic fatty liver disease

NMP Normothermic machine perfusion

OLTx Orthotopic liver transplantation

PBC Primary biliary cholangitis

PBPK Physiologically based pharmacokinetic modelling

PV Portal vein

WIT Warm ischemia time

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## Introduction

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 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 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 degree 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 haemodynamics (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 since 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 feasibility study we explored the applicability of using explanted human diseased livers for NMP research. Besides standard liver function assessment, we explored liver functionality by studying drug pharmacokinetics; hepatic first-pass, clearance and biliary excretion of four model compounds (rosuvastatin, digoxin, furosemide and metformin). These compounds are known substrates for different important hepatic uptake and efflux transporters. We also studied drug-drug interactions of the model drugs with a cocktail of perpetrator drugs.

## 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. 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 (TIPS), 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 (PBC, n=1), non-alcoholic fatty liver disease (NAFLD, n=2), alcoholic liver disease (ALD, n=3), hepatocellular carcinoma in the context of Hepatitis B viral disease (HBV+HCC, n=2). In addition, three discarded non-cirrhotic livers which were declined for transplantation were included in this study. The reasons for decline were; steatosis (n=2) and a occlusion of right hepatic artery (n=1). Immediately following explantation of the recipient diseased liver, a portal and arterial flush with cold Histidine-tryptophan-ketoglutarate (HTK) (Carnamedica, Warsaw, Poland) preservation solution was performed. The period between explantation (i.e. clamping and transection of the portal and hepatic veins as final step of the hepatectomy) and cold flush of the explanted liver (*ex vivo* ), is described as the warm ischemia time (WIT). After a clean effluent flush, the liver was transported in cold preservation solution to the Organ Preservation and Regeneration room in the OR 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- 25Fr cannula, hepatic artery – 12Fr cannula) and connection to the machine perfusion device

(LiverAssist$^{TM}$ device, XVIVO, Groningen, the Netherlands). Thereafter, the bile duct was cannulated in order to collect bile fractions during perfusion. Additional flush of the hepatic artery and portal vein was performed on the back table.

**Normothermic machine perfusion**

All human livers were perfused using the LiverAssist$^{TM}$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 2L perfusion fluid containing 1:1 ratio of 4x packs of human red blood cells and 4x packs of fresh frozen plasma (Sanquin, Amsterdam, the Netherlands). Insulin, sodium taurocholate, heparin and epoprostenol were provided as continuous infusion at a rate of 10U/h, 1041U/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 (23mL/hr) to keep the liver metabolically active (Supplemental table 1). Gas delivery to the LiverAssist$^{TM}$ consisted of 95% oxygen and 5% carbon dioxide at 1.5 L/min and the temperature was set at 37°C. The non-cirrhotic 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. Upon perfusion, additional boluses of sodium bicarbonate and glucose were given when necessary to maintain physiological pH (˜7.35-7.45) and glucose (>5mmol/L) levels. After 360 minutes of perfusion, the livers were submerged in formaldehyde and 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 drug inhibitors (quinidine, rifampicin, cimetidine and probenecid). The selection of the drug cocktail applied in this study was based since the cocktail is representative for different important hepatic and renal uptake and efflux transporters (12). The dosage applied to the system was based on the typical oral dosage and was corrected for the fraction absorbed in the intestine to the portal vein, fraction of metabolism and circulating volume which is shown in supplemental table 2. After 120 min. of perfusion, a slow bolus for 10 min of the drug cocktail was administered via the portal vein at 1mL/min to mimic oral absorption through the gut. Subsequently, perfusate and bile samples were taken for the following 120 min. Arterial samples were taken at t=120, 122, 124, 126, 128, 130, 135, 140, 150, 160, 170, 180, 210, and 240 min. Additional portal samples were taken during the administration of the drug at t=126 and t=130 min to determine the hepatic extraction. Bile samples were collected in 10 minute fractions from 120 min onwards. After 240 min., first a slow bolus 10 min (1mL/min) of inhibitor mix (quinidine, cimetidine, rifampicin and probenecid) was administered to the liver and after 5 min (at t=245 min.), 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. Perfusate and bile samples were immediately stored at [?]-70degC until further processing.

**Liver function assessment**

Hepatic artery and portal vein flow were recorded from the LiverAssist$^{TM}$ machine. Arterial and venous perfusate samples and bile samples (collected under mineral oil to prevent bile exposure to ambient air (13)) were taken hourly to monitor liver viability (pH, glucose, lactate etc.) using a RapidPoint 500 blood gas analyzer (Siemens, Germany). The total bilirubin, alanine aminotransferase (ALT) and aspartate aminotransferase (AST) concentration in the perfusate samples was measured by reflectance photometry (Reflotron-Plus system, Roche diagnostics, Almere, the Netherlands). Perfusate and bile parameters were compared to defined criteria used in clinical transplantation studies; perfusate ALT <6000 and lactate <2.5 mmol/L after 120 min of perfusion, biliary pH >7.5, Δ glucose perfusate – bile <10 mmol/L, Δ bicarbonate bile – perfusate >0 mmol/L (14-16).

**Histological analysis**



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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 4μm were cut and stained with hematoxylin & eosin (H&E) or Sirius red for examination using light microscopy.

**Bioanalysis**

The concentration of the drug cocktail was quantified using 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 of μL the isotopically labelled internal standards (1μg/mL). Thereafter samples were vortexed, centrifuged and supernatant was transferred to 96 well plate and dried under nitrogen. Thereafter, samples were dissolved in 100 μL 10% ACN + 0,1% formic acid and injected in to LC-MS/MS for quantification. Details of the LC-MS/MS conditions used are shown in supplemental table 3.

**Chemicals**

Rosuvastatin, digoxin, furosemide, 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 Inc, Mississauga, ON, Canada) were obtained as indicated.

**Data analysis and statistics**

Data obtained during the perfusion studies was analyzed using Prism version 8 (GraphPad, California, USA). Values for the area under the concentration time curve 0 -120 min ($AUC_{0\text{-}tau}$) were calculated using the linear trapezoidal method. The area under the concentration time curve ratio (AUCR) was determined by dividing the $AUC_{125\text{-}245\ min}$ (with inhibitors) by the $AUC_{0\text{-}120\ min}$ (without inhibitors). The hepatic extraction ratio was calculated during the 10 min dosing period as following: concentration entering the liver (portal vein) - concentration leaving the liver / concentration entering the liver. Significance of differences between the cirrhotic and non-cirrhotic livers was tested using the Mann-Whitney U test. Data is presented as median and inter-quartile range (IQR) for non-parametric distributed data. P-value below 0.05 was considered significant.

**Results**

**Altered portal flow in cirrhotic livers**

Both cirrhotic (n=7, characteristics Table 1) and non-cirrhotic (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 non-cirrhotic livers, Figure 1A). A significant lower portal flow in cirrhotic livers was observed compared to non-cirrhotic livers (523 mL/min (IQR:489-557) vs 1678 mL/min (IQR:1596-1710)), respectively, $p<0.001$ (Figure 1B). Arterial resistance remained stable and did not significantly differ between the cirrhotic and non-cirrhotic livers ($p=0.115$) (Fig 1C) whereas the portal resistance of the cirrhotic livers proved to be higher over the entire perfusion time compared to non-cirrhotic livers ($p<0.001$) (Fig 1D). 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 1E).

**Explanted livers showed good viability during perfusion**

As markers of hepatocellular injury, release of ALT and AST into the perfusate was measured throughout the perfusion and results are presented in figure 2 A and B. Levels of ALT and AST levels were significantly higher in the non-cirrhotic livers compared to the cirrhotic livers. Figure 2C-F demonstrates the liver function parameters measured in perfusate. Lactate clearance was observed after 30 min of perfusion in the non-cirrhotic liver group and lactate levels remained low (1.39 mmol/L (IQR:0.48-.29)) while cirrhotic livers showed higher levels of perfusate lactate (13.25 mmol/L (IQR:3.20-22.91) after 360 min of NMP). A correlation between perfusate lactate levels (lactate AUC) and the MELD score of the patients ($R^2=0.793$) was observed. All livers were able to maintain albumin perfusate levels within physiological serum levels.

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Perfusate total bilirubin levels were significantly higher in cirrhotic livers compared to non-cirrhotic livers (29.63 μmol/L (IQR:22.37-34.81) vs 10.30 μmol/L (IQR:9.67-14.27) respectively, p=0.026). Viability markers as measured in bile (figure 2G-J) demonstrated good cholangiocyte viability in all livers with a biliary pH >7.6, high bicarbonate excretion and stable bilirubin excretion into bile. Glucose resorption function was better preserved in cirrhotic livers (Δ glucose <10 mmol/L) than in non-cirrhotic livers, meeting the defined viability criteria (see method section).

**Histological characterization of cirrhotic and non-cirrhotic liver pre- and post-perfusion**

Biopsies of the livers pre-, and post-perfusion were stained with H&E and Sirius red to characterize morphology and collagen (Figure 3). In figure 3A, a Sirius red staining of a representative cirrhotic liver and a representative non-cirrhotic liver pre-perfusion is shown. Median percentage of Sirius red staining positive for cirrhotic livers was 36.25(IQR:23.13-64.38) versus 8.5(IQR:5.56-14.44) in the non-cirrhotic livers. In the cirrhotic livers, nodules of regenerating hepatocytes are visible, surrounded by thick bands of fibrous tissue. The extensive fibrosis is accompanied by biliary duct hyperplasia, angiogenesis and inflammation. In the non-cirrhotic livers, minimal to mild periportal and sinusoidal fibrosis was detected (Figure 3B). To investigate the effect of perfusion on the integrity of the livers, biopsies were taken pre- and post-perfusion. Figures 3 show examples of a non-cirrhotic liver and a cirrhotic liver, before perfusion (3C & 3D) and post-perfusion (3E & 3F). The histopathological analysis indicated that the perfusion did not have obvious detrimental morphological effects on the liver tissue.

**Hepatic first pass, clearance and biliary excretion of rosuvastatin and digoxin affected by cirrhosis**

To study the application of NMP to assess hepatic first pass, clearance and biliary excretion, a drug cocktail (rosuvastatin, digoxin, furosemide and metformin) was infused to the portal vein (schematic presentation of experimental set-up is shown in Figure 4). The drug cocktail is representative for different important hepatic uptake and efflux transporters (Supplemental table 1; e.g. organic anion transporter protein 1B1/1B3 (OATP1B1/1B3), P-glycoprotein (Pgp), organic cation transporter 1 (OCT1), breast cancer resistance protein (BCRP). Perfusate concentrations of rosuvastatin appeared to be the most affected by liver cirrhosis, with an approximate 34-fold increased Cmax in cirrhotic livers compared to the perfused non-cirrhotic livers (463.3 ng/mL (IQR: 243.2-555.2) vs 13.50 ng/mL (IQR: 7.01-71.02), p=0.024) and 20-fold increased $AUC_{0\text{-}tau}$ (20962 (IQR: 11617-29981) versus 107 ng/mL (IQR:100-5154, p<0.001) (Figure 5A). A comparable effect was observed for digoxin, with a perfusate Cmax that was more than 3-fold higher in cirrhotic livers (10.03 ng/mL (IQR:7.75-11.78)) compared to non-cirrhotic livers (3.46 ng/mL ng/mL (IQR:2.33-7.80, p=0.038)) and an $AUC_{0\text{-}tau}$ that was almost 3-fold higher (629 ng/mL (IQR:282-746) in cirrhotic livers versus 222 ng/mL (IQR:171-503) in non-cirrhotic livers, p=0.003) (Figure 5B). Biliary excretion of rosuvastatin and digoxin was higher in cirrhotic livers (66% and 51% respectively) compared to non-cirrhotic livers (47% and 17%, respectively), however not significant (Figure 5E-F). As can be observed in Figure 5C-D, cirrhosis had a minor effect on furosemide and metformin concentrations as Cmax was 1.19 and 1.13 times higher in cirrhotic livers compared to non-cirrhotic livers (not significant). Metformin and furosemide were only minimally cleared through biliary excretion (in the range of 1-3%) which was not affected by the cirrhosis (Figure 5G-H)). The hepatic extraction was determined by sampling from the portal vein and hepatic artery. Hepatic extraction ratio of rosuvastatin tended to be affected by cirrhosis (not significant) and was 0.48 (IQR:0.42-0.67) versus 0.70 (IQR:0.69-0.83) in non-cirrhotic livers (Figure 5I). Hepatic extraction of the other drugs was not affected by cirrhosis.

**Increased risk of drug-drug interaction for rosuvastatin and digoxin**

Figure 4 shows the application of NMP to assess drug-drug interactions in cirrhotic and non-cirrhotic livers. Figure 6A shows the results of these studies in which different drugs were used to inhibit the uptake and /or excretion of the drug cocktail from the previous section. In both cirrhotic and non-cirrhotic livers, rosuvastatin and digoxin $AUC_{0\text{-}tau}$ and Cmax was increased upon co-administration of a perpetrators cocktail (Figure 6B,C). However, the drug-drug interaction, expressed as an increase in Cmax, and increase in AUCR

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(i.e. ratio AUC of victim drug with and without inhibitors over 120 min) was more profound but not significant in the non-cirrhotic 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 non-cirrhotic livers compared to 1.4 (IQR: 0.9-1.9) and 2.2 (IQR: 1.3-3.5) respectively in cirrhotic livers. No increase in $AUC_{0\text{-tau}}$ and Cmax was observed for the low-hepatic extraction ratio drugs furosemide and metformin

**Discussion**

Here we demonstrate for the first time the use of NMP of explanted diseased human livers and the applicability for pharmacokinetic research. We successfully perfused 7 cirrhotic livers and 4 non-cirrhotic livers for a period of 360 min, maintaining liver viability and functionality, as indicated by stable flow, bile production, albumin synthesis, bilirubin excretion, proper histology pre- and post-perfusion and stable ALT and AST values. Additionally, the model showed to be useful to study hepatic first pass, clearance, biliary excretion and drug-drug interaction.

The use of NMP has shown to be of great benefit in the field of organ transplantation (11, 17). Over the past years, NMP has become a widely accepted method to assess viability of the donor liver prior to transplantation (18, 19). Many criteria of hepatocellular and cholangiocellular function have been described (e.g. lactate clearance, perfusate AST, perfusate glucose levels and biliary pH, bicarbonate and glucose levels) to establish liver viability based on perfusion results and post-transplantation outcomes, which demonstrates the robustness of the model in perfusion research (13-17). 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 to be significantly affected. As expected, portal flow was lower in cirrhotic livers compared to non-cirrhotic livers as a result of portal hypertension (20). Cholangiocellular function remained intact during 360 min of NMP as shown by the presence of glucose resorption and bicarbonate excretion.

We previously showed the application of NMP of porcine livers to predict drug-drug interaction on plasma exposure of commonly prescribed statin drugs (21). The degree of drug-drug interactions derived from the NMP model were in good agreement with clinical data showing no effect of ischemia-reperfusion injury on the pharmacokinetics of drugs. Literature is scarce regarding human models that predict drug pharmacokinetics especially in cirrhotic conditions. In the current study we demonstrate the application to study hepatic first pass, clearance and biliary excretion in a perfused explanted human liver model. This approach showed to give comparable results with published *in vivo* human data. In patients with liver cirrhosis, differences can arise in portal vein concentration after oral dosing due to differences in intestinal absorption and/or portal hypertension compared to individuals without liver cirrhosis. However, in this study we administered the same bolus to the portal vein to study differences in hepatic first pass and clearance between the cirrhotic and non-cirrhotic livers. The non-cirrhotic livers showed to rapidly take up rosuvastatin and digoxin from the perfusate with a hepatic extraction ratio of 0.74 and 0.40 respectively which are close to*in vivo* reported measures of 0.63 and 0.3 respectively (22, 23). In this study, hepatic first-pass and clearance of rosuvastatin and digoxin showed to be the most affected by cirrhosis. Rane et al. (24) reported that the clearance of hepatically cleared drugs with a high extraction ratio are related to blood flow and thus a major decrease in portal flow as in cirrhosis can dramatically affect the first passage of across the liver (25, 26). In addition, *in vivo* studies demonstrated a high biliary excretion of rosuvastatin of approximately 76.8% as measured by fecal excretion (27). The *ex vivo*non-cirrhotic livers showed a biliary excretion of 37% in 120 min, extrapolation of the data resulted in 77% total excretion of rosuvastatin which is in line with *in vivo* data. Interestingly, digoxin showed a relative high biliary clearance in cirrhotic (51%) and non-cirrhotic ( 17%) livers during 120 min of perfusion. *In vivo*studies have shown that digoxin is extensively renally eliminated (75%) (28). However, multiple studies demonstrated that digoxin is highly involved in the enterohepatic circulation, thereby decreasing the*in vivo* fecal excretion of digoxin (29, 30). 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 non-cirrhotic which is in line with human *in vivo* data which showed that biliary eliminated was limited (31, 32). Interestingly, the percentage of biliary

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clearance was higher, for all compounds, in the cirrhotic perfused livers. This might be due to an elevated bile flow which has been observed in patients cirrhosis and which is confirmed in our model, resulting in a more efficiency biliary clearance (33).

The effect of drug-drug interactions in cirrhotic and non-cirrhotic livers was subsequently determined by using a cocktail of inhibitors. The non-cirrhotic livers showed an increased AUCR for a drug-drug interaction with rosuvastatin (3.52) where values between 2.48 – 5.38 for rosuvastatin with rifampicin as inhibitor have been observed (34-36). Rosuvastatin is mainly inhibited at the hepatic level, since it is an OATP substrate, and therefore the perfused liver model is showing to properly predict the degree of drug-drug interaction for rosuvastatin. Digoxin, showed a high increase in AUCR upon dosing with inhibitors, which is potentially the result of inhibiting uptake via OATP2 (rifampicin as inhibitor) as well as biliary secretion via Pgp (quinidine as inhibitor). However, this is difficult to compare to*in vivo* observations since s major part of the drug-drug interaction 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 drug-drug interaction with digoxin in this perfusion model leading to an increased Cmax and AUCR.

Explanted livers obtained during orthotopic liver transplantation are currently only used for pathological assessment and subsequently discarded. While 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. Despite the advantages of utilizing explanted livers, such as specific disease characteristics, maintained hepatic architecture and presence of systemic inflammation and collagen content, this NMP explanted liver model is not widely adopted (or has not been reported) (4). Considering the frequency of orthotopic liver transplantation, and the ease of combining this with the relative simple procedure to prepare the liver for perfusion, this model can be widely applied in a variety of research settings (37). For the field of drug development, use of perfused explanted livers might not only contribute to the reduction in the use of laboratory animals, but more importantly enable better predictions on drug pharmacokinetics*in vivo* . It will increase our knowledge on hepatic blood flow, hepatic first pass effect and biliary clearance of drugs under normal and cirrhotic conditions. Besides changes in blood flow and collagen content, changes in pharmacokinetics of drugs in patients with liver disease might also be the result of alterations in expression levels 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 (38-41). For instance, Drozdzik et al., showed an increase in Pgp and multidrug resistance protein 4 (MRP4) and decreases in NTCP, OCT1 and OATP1B1 in patients with severe liver disease (39) . Although these studies already provided some hints towards 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 OATP1B1/1B2, Pgp, BCRP and OCT-1 model compounds is a first step towards studying transporter functions in diseased liver. This information can be easily extrapolated to other drugs which are substrates for one of the transporters assessed in this study. Additionally, for many drugs, dosing advice is currently incomplete for patients with cirrhosis because of lacking evidence or showing major interindividual differences. Studying drug pharmacokinetics using explanted human livers may substantiate dosing advice for this group of patients (42).

In addition to using cirrhotic liver perfusion to study drug pharmacokinetics, this model provides new possibilities to study specific liver functions under different pathological circumstances for multiple applications, e.g. disease specific biomarkers. Currently, there are several examples of using machine perfusion platforms to study organ reconditioning, pharmacological interventions to prevent ischemia-reperfusion injury, defatting steatotic livers or studying the effect of oncolytics (43-47). Recent studies have adapted machine perfusion to demonstrate the possibility to prolong organ perfusion duration (48-50), with perfusion of human and porcine livers for up to 7 days (18). This might lead to increased knowledge regarding disease pathology and enable the *ex vivo* treatment of discarded or explanted livers with medication or stem cells (51). Such future interventions would certainly increase the availability of suitable donor organs.

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In conclusion, we demonstrated for the first time stable NMP of diseased human livers explanted during liver transplantation and discarded donor livers to study hepatic first pass, clearance ,biliary excretion and drug-drug interactions. This approach provided comparable results to published *in vivo* human data supporting its applicability as a robust *ex vivo* drug handling model.

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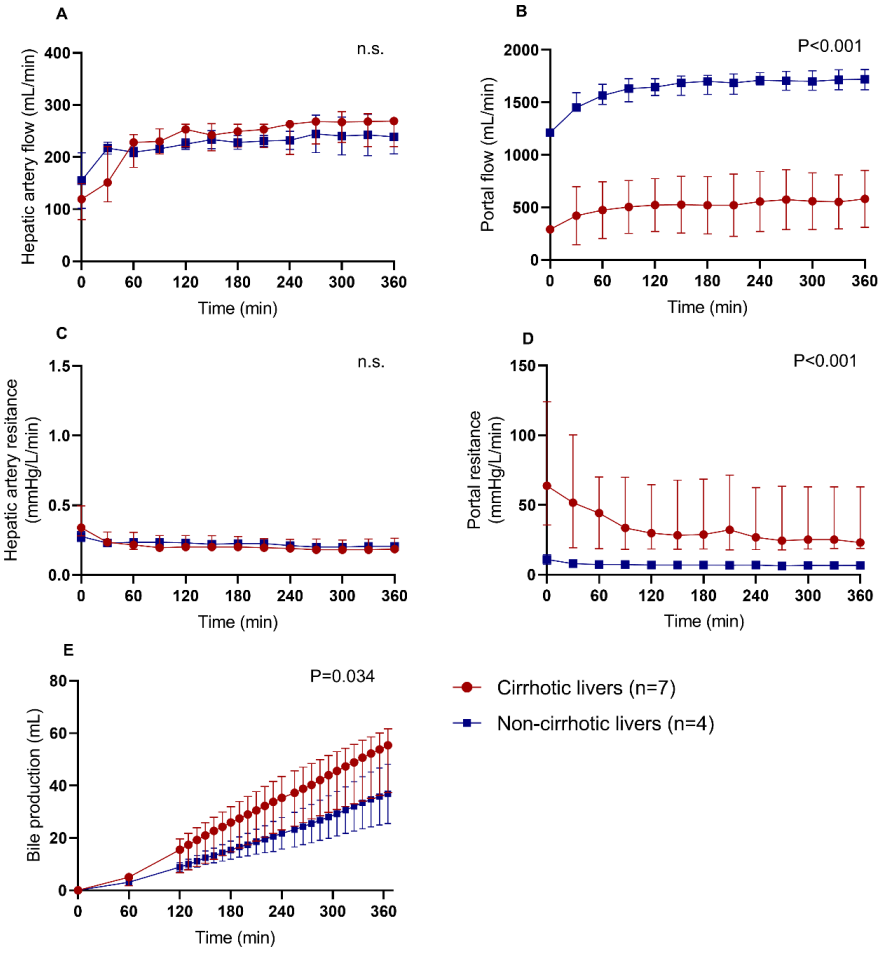
## Figures

### Figure 1



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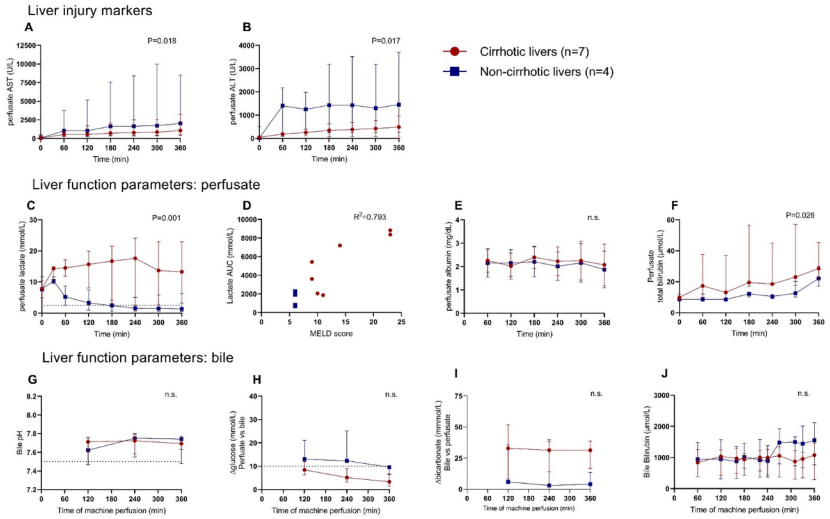


**Figure 2**

**Figure 3**

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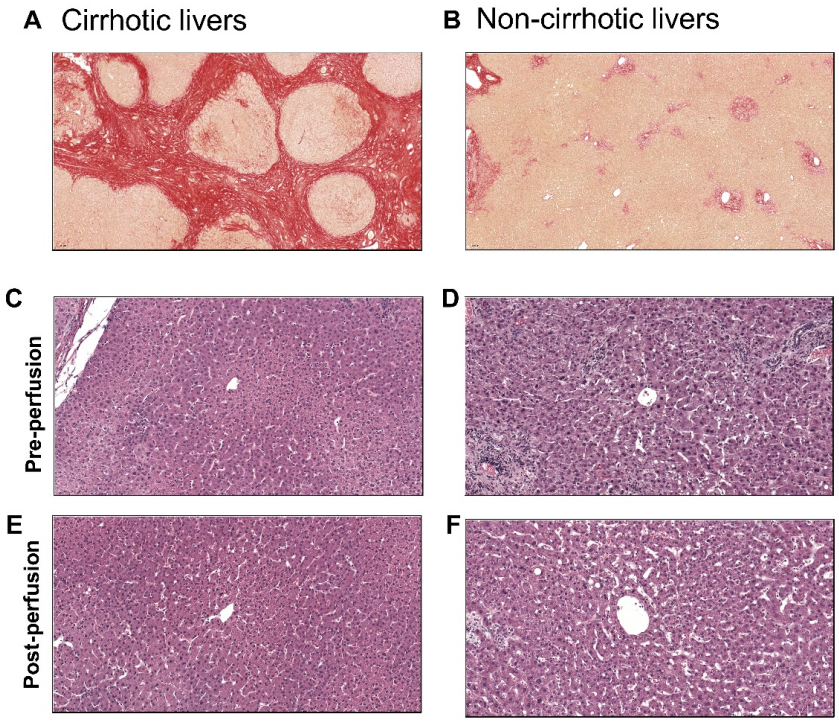
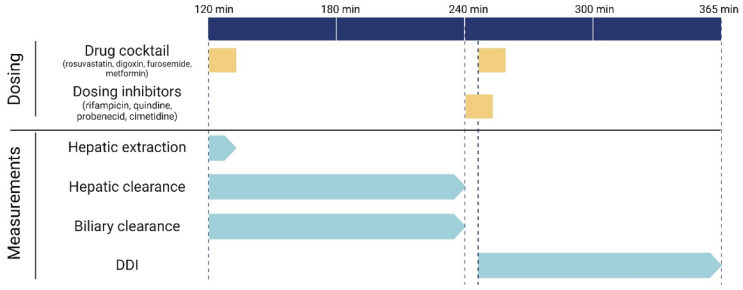



**Figure 4**



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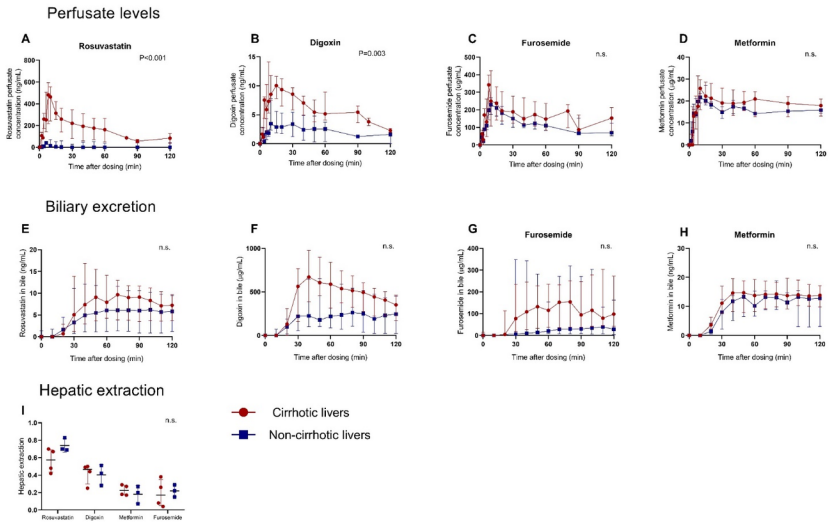


**Figure 5**

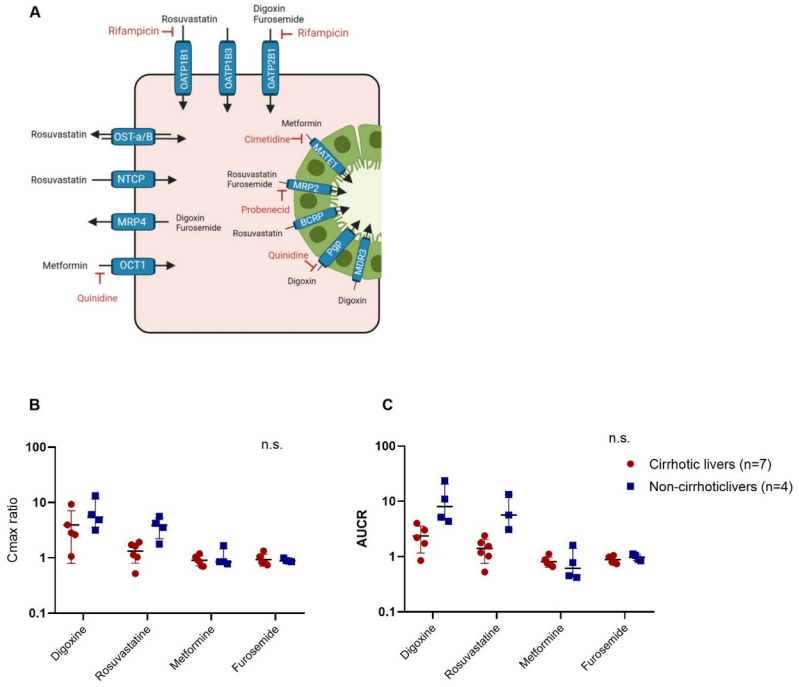


**Figure 6 Table 1** . Liver characteristics and ischemic times of cirrhotic and non-cirrhotic livers. Data represents median with interquartile range

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| | | **Cirrhotic livers** *N=7* | **Non-cirrhotic livers** *N=4* | **p** |
|---|---|---|---|---|
| **Underlying disease** | **Underlying disease** | ALD (n=3) NAFLD (n=2) HBV + HCC (n=1) PBC (n=1) | discarded liver (n=3) HBV + HCC (n=1) | na |
| **Age (years)** | **Age (years)** | 59 (54 - 69) | 63 (30 – 67) | 0.545 |
| **Gender** | **Male** | 6 | 4 | |
| | **Female** | 1 | 0 | |
| **BMI (kg/m²)** | **BMI (kg/m²)** | 29.4 (23.8 – 31.4) | 26.8 (26.0-28.8) | >0.99 |
| **WIT (min)** | **WIT (min)** | 5 (4 – 6) | 12 (5 - 14) | 0.067 |
| **CIT (min)** | **CIT (min)** | 80 (71 - 99) | 270 (105 - 507) | 0.070 |
| **Weight of the liver (g)** | **Weight of the liver (g)** | 1507 (1297 - 2005) | 1975 (1394-2008) | 0.648 |
| **MELD** | **MELD** | 11 (9 – 23) | 6 (6 – 6) | 0.006 |

PBC = primary biliary cholangitis, ALD = alcoholic liver disease, NAFLD = non-alcoholic fatty liver disease, HBV = Hepatitis B virus, HCC = hepatocellular carcinoma), na= not applicable; differences between groups were analyzed using the Mann-Whitney U test

**Table 2** Overview of the hepatic uptake and biliary clearance of rosuvastatin, digoxin, metformin and furosemide. Values are median with interquartile range. Differences between groups were analyzed using the Mann-Whitney U test

| | | **Perfusate** | **Perfusate** | **Perfusate** | **Bile** |
|---|---|---|---|---|---|
| | | **Cirrhosis** | **Non-cirrhosis** | **p** | **Cirrho** |
| **Rosuvastatin** | Cmax (µg/mL) | 0.46 (0.24-0.55) | 0.04 (0.01-0.07) | 0.024 | 10.23 ( |
| | $AUC_{0\text{-}tau}$ | 20.96 (11.61-29.98) | 0.11 (0.10-5.15) | <0.001 | 1194.10 |
| | Biliary excretion (% of dose) | - | - | - | 66.34 ( |
| **Digoxin** | Cmax (ng/mL) | 10.03 (7.75-11.78) | 3.46 (2.33-7.80) | 0.038 | 671.11 |
| | $AUC_{0\text{-}tau}$ | 629.70 (282.30-746.10) | 222.80 (171.00-503.40) | 0.003 | 62268 ( |
| | Biliary excretion (% of dose) | - | - | - | 51.89 ( |
| **Metformin** | Cmax (ug/mL) | 25.79 (21.79-33.75) | 22.84 (21.29-27.58) | 0.352 | 12.63 ( |
| | $AUC_{0\text{-}tau}$ | 2248.00 (1648.00-2807.00) | 1896 (1780.00-2061.00) | 0.187 | 2235.26 |
| | Biliary excretion (% of dose) | - | - | - | 3.02 (1. |
| **Furosemide** | Cmax (µg/mL) | 0.27 (0.15-0.42) | 0.23 (0.18-0.27) | 0.476 | 0.16 (0. |
| | $AUC_{0\text{-}tau}$ | 18.61 (11.65-28.62) | 14.32 (10.37-18.64) | 0.073 | 17.53 ( |
| | Biliary excretion (% of dose) | - | - | - | 2.27 (0. |

**Figure Legends**

**Figure 1 . Hemodynamics, vascular resistance and bile production of normothermic perfused cirrhotic and non-cirrhotic livers.**(A) hepatic artery flow, (B) portal flow, (C) hepatic artery resistance, (D) portal resistance and (E) bile production of cirrhotic and non-cirrhotic livers measured during 360 min of normothermic perfusion. Data represent median and interquartile range in cirrhotic (n=7) and non-cirrhotic livers (n=4). Differences between groups were analyzed using the Mann-Whitney U test

**Figure 2. Liver injury and liver function markers, measured in perfusate and bile during normothermic machine perfusion.**(A) Perfusate AST and (B) ALT. Liver function parameters measured in perfusate, including; (C) lactate, (D) relation of perfusate lactate AUC and MELD score, (E) albumin and (F) total bilirubin. Liver function parameters measured in bile; (G) pH, (H) Δglucose perfusate vs bile,

(I) Δbicarbonate bile vs perfusate and (J) bile bilirubin levels during 360 min of perfusion. Data represent median and interquartile range in cirrhotic (n=7). and non-cirrhotic livers (n=4). Differences in AUC between groups were analyzed using the Mann-Whitney U test; p value is presented in right corner of each graph

**Figure 3. Histological characterization of liver biopsies taken pre- and post-perfusion.** (A) Sirius red staining of a cirrhotic liver (50X) and (B) Sirius red staining of a non-cirrhotic liver (50X).(C) H&E staining of a -cirrhotic liver, before perfusion (200x), (D) non-cirrhotic liver pre-perfusion (200x), ( E) Cirrhotic liver after 6 hours of perfusion (200x) and (F) non-cirrhotic liver after 6 hours of perfusion (200x).

**Figure 4. Schematic representation of the experimental set-up for studying pharmacokinetics of a cocktail of drugs and the effects of drug-drug interactions.** Stable liver perfusion was maintained in the first 120 min of perfusion. Between 120-130 min, the drug cocktail was infused into the portal vein (1mL/min), during this time the hepatic extraction of the drugs was measured. Hepatic clearance as well as biliary clearance was measured for 120 min (120 -240 min). Thereafter, at t=240 min, a drug inhibitors were infused into the portal vein for 10 min (240 – 250). Between 245 – 255 the drug cocktail was infused into the portal vein to study drug-drug interactions. Perfusate and bile samples studying the extent of drug-drug interactions were taken between 245 and 365 min

**Figure 5. Pharmacokinetic profiles of rosuvastatin, digoxin, metformin and furosemide in cirrhotic and non-cirrhotic perfused livers** . Perfusate levels of (A) rosuvastatin (applied dose of 1.80 mg), (B) digoxin (applied dose of 0.11 mg), (C) metformin (applied dose of 74.40 mg), and (D) furosemide (applied dose of 0.77 mg) in cirrhotic and non-cirrhotic normothermic perfused livers. Biliary excretion profiles of the administered cocktail of drugs (E) rosuvastatin, (F) digoxin, (G) metformin and (H) furosemide. (I) hepatic extraction of drug cocktail compounds measured during dosing (n=3 non cirrhotic livers, n=4 cirrhotic livers). Data represent median and interquartile range in cirrhotic (n=7) and non-cirrhotic livers (n=4), unless indicated otherwise. Differences in AUC between groups were analyzed using the Mann-Whitney U test; p value is presented in right corner of each graph

**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, probenecid), (B) Ratio of perfusate Cmax and (C) AUCR with and without applied perpetrator drugs for the cirrhotic and non-cirrhotic livers. Data represent median and interquartile range in cirrhotic (n=7) and non-cirrhotic livers (n=4). Differences between groups were analyzed using the Mann-Whitney U test

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