Pumping new life into preclinical pharmacokinetics: exploring the pharmacokinetic application of ex vivo organ perfusion
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CHAPTER 05

Unraveling and enhancing the dynamics of hepatic bile acid and cholesterol metabolism during ex vivo normothermic machine perfusion; a path to improved liver function through conjugated bile acid infusion

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Abstract

Ex vivo liver normothermic machine perfusion (NMP) does not fully recapitulate physiological liver function due to the absence of the enterohepatic circulation as only infusion of the bile acid taurocholate (TCA) is applied in most protocols. In this study we characterized the *de novo* bile acid synthesis and cholesterol homeostasis during liver NMP. We hypothesized that addition of a more diverse pool of (conjugated)bile acids during liver NMP would decrease the metabolic burden of *de novo* synthesis and thereby improve liver function during NMP.

First, human and porcine livers were perfused for 360 min at 37°C and perfusate containing TCA. Next, the infusion of different conjugated bile acid mixes was assessed during porcine and human liver perfusion. Perfusate, bile and tissue samples were obtained to study liver viability, functionality, gene expression, cholesterol and bile acid levels.

During human and porcine perfusions with TCA infusion, composition of bile was comparable to literature however, synthesis rates were above physiological average and a decrease over time in cholesterol perfusate levels was observed. Additionally, over time a decreased expression of bile acid synthesis related genes, increased gene expression of cholesterol metabolism related genes and decreased expression in bile acid-dependent uptake and efflux transporters were detected. Upon infusion of a conjugated bile acid mix lower AST and ALT values and stable cholesterol homeostasis was observed after 720 min of perfusion. Perfused human livers showed appropriate function and good functioning livers showed rapid bile acid clearance from the perfusate into the bile.

In this study we reveal new insights that infusion of (un)conjugated bile acids in NMP alleviated the burden of the *de novo* bile acid synthesis and improved liver function.

Introduction

Ex vivo normothermic machine perfusion (NMP) of the liver is a well-known technique in the field of organ transplantation to assess metabolic processes and liver function[1,2]. Additionally, NMP can be used as a platform for drug intervention studies, drug pharmacokinetics and disease modelling[3-7]. During NMP, livers are perfused with a red-blood cell based perfusate through the hepatic artery and the portal vein under oxygenated conditions at 37°C. Many protocols depend on infusion of taurocholate (TCA) to induce bile flow[8,9] as current liver viability assessment is partly focused on bile production and bile composition as well as biliary pH, glucose and bicarbonate levels[10,11]. However, TCA is only one of many bile acids found in the bile acid pool which encompasses a variety of (conjugated) bile acids, each with a specific function and contribution to the overall bile metabolism[12-14].

Under physiological conditions the liver synthesizes bile acids from cholesterol through either the classic or alternative pathway, initiated by cholesterol 7α-hydroxylase (CYP7A1) or sterol 27-hydroxylase (CYP27A1), respectively[15]. The synthesized primary bile acids cholic acid (CA) and chenodeoxycholic acid (CDCA) are subsequently conjugated with glycine (G) or taurine (T). After synthesis, conjugated primary bile acids are excreted across the canalicular membrane and drained via the biliary tree into the gall bladder, from where the bile acids are subsequently secreted into the duodenum upon a postprandial signal[13]. In the intestinal tract, bile acids are transformed by intestinal bacteria into secondary bile acids such as deoxycholic acid (DCA), reabsorbed by the intestinal cells and are transported back to the liver via the portal vein[12,15,16]. In the portal vein, the bile acid composition predominantly comprises of GCA, GCDCA and GDCA[17]. This efficient recirculation of bile acids is known as the enterohepatic circulation, where approximately 95% of the total amount of bile acids recirculates between the intestine and the liver[18,19]. The loss of bile acids is mainly due to excretion of bile acids via the feces. As a consequence, the loss of bile acids is compensated by *de novo* synthesis in the liver to maintain homeostasis and a constant bile acid pool[13] in which bile acids regulate their own homeostasis by providing a negative feedback on bile acid biosynthesis genes such as CYP27A1, CYP7A1 and sterol 12α-hydroxylase (CYP8B1). This downregulation is mediated by activation of farnesoid X receptor (FXR) by bile acids, which upon activation also prevents toxic intracellular accumulation of bile acids by inhibiting bile acid uptake and stimulating bile acid export out of the liver[20,21]. Thus, FXR activation suppresses gene expression of hepatocyte bile acid influx transporters such as sodium taurocholate co-transporting polypeptide (NTCP), organic anion transporter 2 (OAT2), and the organic anion-transporting polypeptides (OATP) 1B1, 1B3 (humans) and 1B4 (pigs)[22]. Concomitantly, FXR activation increases gene expression of canalicular efflux transporters such as bile salt export pump (BSEP) and multidrug resistant-related protein (MRP) 2, which secrete divalently conjugated bile acids into bile and is bile acids are subsequently stored in the gallbladder[21]. Alternatively, bile acids can be exported basolaterally in the systemic circulation via MRP3, which is also upregulated upon FXR activation[23]. As this hepatobiliary transporter system is also responsible for the transport of other substances such as nutrients, endogenous compounds and drugs, is tightly regulated[24,25].

Current clinical and lab-based NMP protocols recreate physiological perfusate compositions in order to maintain and assess liver vitality and functionality thereby only including TCA as bile acid[26]. Here we hypothesize that this imposes a substantial burden on the liver during NMP as it is forced to engage in the *de novo* bile acid synthesis without the support of endogenous bile acids. The *de novo* bile acid synthesis is an energy consuming process using ATP[13]. However given the importance of maintaining optimal organ viability and functionality during *ex vivo* liver perfusion prior to transplantation, it is critical to prevent the depletion of ATP reserves which is essential for various other physiological processes[27]. In our study, we addressed this issue and aimed to characterize the *de novo* bile acid synthesis by profiling the biliary bile acid excretion, cholesterol homeostasis and transporter expression during *ex vivo* liver NMP. We hypothesized that introduction of a variety of bile acids during liver NMP would alleviate the strain of the *de novo* synthesis by the liver and improve the physiological resemblance.

Methods

Procurement, preservation and perfusion of porcine livers

Porcine livers were procured, preserved and perfused as previously published[28]. Standard perfusion protocol consisted of infusion with TCA at a fixed rate of 0.2 g/hr.

Porcine liver perfusion bile acid infusion studies

Porcine liver perfusions were performed using varying compositions of bile acid mixes, summarized in Table 5.1:

- Standard protocol
  The standard protocol consisted of infusion of TCA at 0.2 g/hr (0.39 mmol/hr), n=5 studies up to 360 min and n=2 up to 720 min of perfusion.
- Simple conjugated bile acid mix
  A bile acid mixture was continuously infused in the portal vein during porcine liver NMP at a rate of 0.15 g/hr (0.41 mmol/hr)[8] (n=2). The bile acid mixture consisted of GCDCA, 40%, GCA, 40%, CDCA 10% and CA 10%, representing the two most prevalent human bile acids supplemented with unconjugated primary bile acids to alleviate the strain of the *de novo* synthesis. Livers were perfused for a total time of 720 minutes.
- Complete conjugated bile acid mix
  To study the effect of a more completed conjugated bile acid mix was continuously infused in the portal vein at a rate of 0.15 g/h (0.35 mmol/hr) (n=3). This mixture consisted of GCA, 24%, GCDCA, 24%, GDCA, 24%, TCA, 11%, tCDCA, 11%, DCA, 5%, CA, 0.6% and CDCA, 0.6%, more closely resembling human *in vivo* conditions[17]. Additionally, 3 mL/hr of omegaven (Fresenius, 's Hertogenbosch, the Netherlands) was infused. Livers were perfused for a total time of 660 minutes.

Table 5.1 - Composition of bile acid mixes.

Standard protocol	Conjugated simple bile acid mix	Conjugated complete bile acid mix
TCA (100%)	GCDCA (40%)	GCA (24%)
	GCA (40%)	GCDCA (24%)
	CDCA (10%)	GDCA (24%)
	CA (10%)	TCA (11%)
		TCDCA (11%)
		DCA (5%)
		CA (0.6%)
		CDCA (0.6%)

Human liver perfusion

Human explanted and discarded livers were perfused as previously published[6]. Standard perfusion protocol consisted of infusion with TCA at a fixed rate of 0.2 g/hr. The samples (perfusate, bile and tissue) used in this research with TCA infusion were derived from a study which was previously published[6].

Human liver perfusion bile acid infusion studies

Livers were perfused with the simple conjugated bile acid mix consisting of GCDCA, (40%), GCA, (40%), CDCA (10%) and CA (10%). An overview of the condition and characteristics of the liver is shown in Table 5.2. Continuous bile acid infusion was applied at 0.15 g/hr.

Table 5.2 - Donor characteristics and ischemic times of human and porcine livers.

	Liver #1	Liver #2	Liver #3	Porcine livers
Reason for decline	Declined after 13h of NMP Necrotic artery patch	Alcohol and smoking history	Choledocholithiasis	-
DBD/DCD	DCD	DBD	DCD	DCD
Age (years)	51	63	51	~ 6 months
Gender	Female	Female	Male	Male
BMI (kg/m^2)	19.9	28	23	-
WIT (min)	16	0	14	9 - 15
CIT (min)	387	226	318	180-210
Weight of the liver (g)	2025	1877	2177	2100 - 2500

Liver #1: Liver was perfused using the OrganOx machine (OrganOx, Oxford, UK), with a perfusate of red blood cells and gelofusine. Continuous bile acid infusion was given from t=15 hours till t=20 hours at 0.15 g/hr. To study clearance capacity, indocyanine green (ICG) was added to the reservoir at t=19h and subsequently, perfusate and bile sample were taken. Infusion speed was increased after 20 hours to 0.3g/hr. An additional bolus (0.2 g) was given at 20.5 hours to study the effect of high bile acids concentrations on liver function.

Liver #2 - #3: Livers were perfused using the Liver Assist device, with a perfusate of red blood cells and plasma. Continuous bile acid infusion was given from t=0 hours till t=12 hours at 0.15 g/hr. ICG was dosed to the reservoir at t=120 min to study liver clearance capacity. During perfusion of liver #2 a bile acid bolus of 0.2 g was given after 6 hours of perfusion to study the effect of high bile acids concentrations on liver function.

Liver function assessment

Several parameters were measured to assess liver viability and functionality. Aspartate transaminase (AST) and alanine transaminase (ALT) and ICG concentrations were measured as previously published. Data of ALT and AST was expressed as a percentage of AST (U/L) and ALT (U/L), where the highest level of the control study was set at 100%.

Bile acid analysis

Bile acid composition was measured by reverse-phase high performance liquid chromatography (HPLC) as described previously[29]. Total bile acids levels in perfusate and bile were measured by the Total Bile Acid Assay kit (Diazyme Laboratories, Poway, USA) using a microplate reader set at 405 nm.

Cholesterol assay

Cholesterol levels were quantified using the enzymatic CHOD-PAP assay (Roche Diagnostics, Basel, Switzerland). Perfusate total cholesterol was measured spectrophotometrically using an enzymatic assay (Roche diagnostics, Almere, the Netherlands) according to the manufacturer's instructions.

RT-qPCR analysis

RNA was extracted from liver tissue using the RNeasy Mini Kit (Qiagen) according to manufacturer's instructions. cDNA was synthesized from RNA using the iScript™ Reverse Transcription Supermix (Bio-Rad, Hercules, California, USA) according to manufacturer's description. Forward and reverse primers, of which details can be found in Supplementary Table S5.1 and S5.2, were designed for both human and porcine tissue. An Applied Biosystems™ 7500 Fast Real-Time PCR System (Fisher Scientific) was used to run the RT-qPCR using SYBR green (Bio-Rad, Hercules, California, USA).

RNA sequencing

After RNA isolation, RNA quality was evaluated using a Fragment Analyzer (tot.RNA conc. & RQN). Total RNA was processed into tagged random sequence libraries (NEBNext Ultra II Directional RNA Library Prep Kit for Illumina, NEB #E7760S/L, Biolabs) and sample quality was checked for proper size distribution (300-500 bp peak, Fragment Analyzer). The mixed (multiplex) sample libraries were sequenced on an Illumina NovaSeq6000 sequencer with a paired-read 150-cycle sequencing protocol at GenomeScan BV (Leiden, the Netherlands), resulting in ~18-26 million read counts per sample. Clustering and DNA sequencing using the NovaSeq6000 was performed according to manufacturer's protocols. A concentration of 1.1 nM of DNA was used yielding paired end reads (2x 150bp). NovaSeq control software NCS v1.7 was used. Image analysis, base calling, and quality check was performed with the Illumina data analysis pipeline RTA3.4.4 and Bcl2fastq v2.20. Trimmed Fastq files (Trimmomatic software) were merged (in case of Paired-end reads) and aligned to the reference genomes (Human: Homo_sapiens.GRCh38.gencode.v29; Pig: GCF_000003025.6_Sscrofa11.1_genomic) using the STAR 2.5 algorithm with default settings (https://github.com/alexdobin/STAR). Based on the mapped read locations and the gene annotation, Htseq-count 0.6.1p1 was used to count the read mapping frequency/gene (transcript region) resulting in read mapping frequency per gene. Expression was normalized based on total counts per sample and corrected for GAPDH expression.

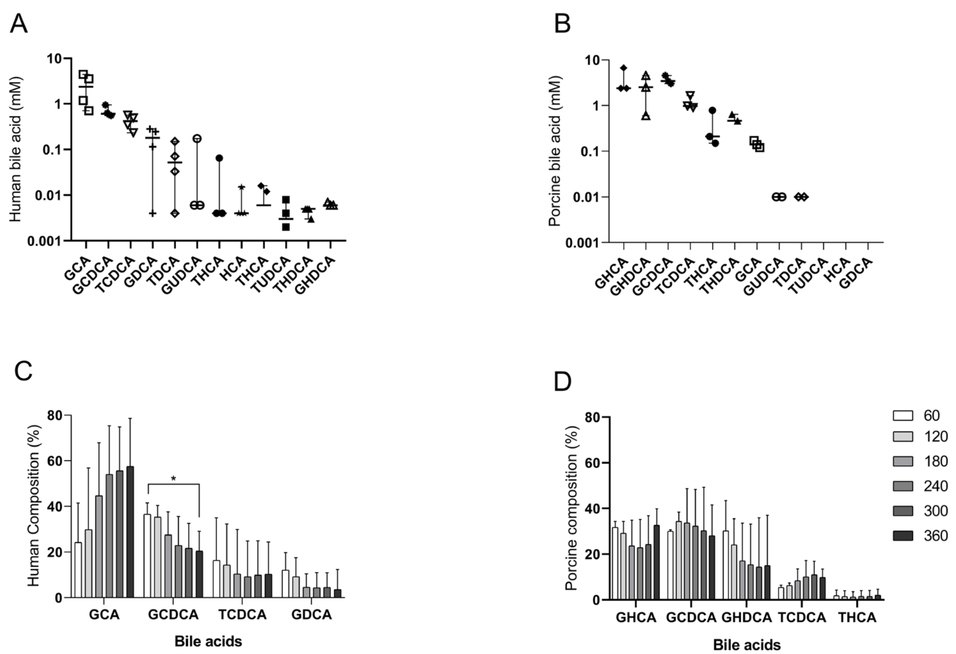
Data analysis

Data was analyzed and visualized using GraphPad Prism 8.0.1 (GraphPad Inc., La Jolla, California, USA). Statistics were performed as indicated in figure legends. Values for the area under the concentration time curve (AUC) were calculated using the linear trapezoidal method. Significance of differences between the intervention and standard protocol was tested using the Mann-Whitney U test. Data is presented as median and range for non-parametric distributed data. P-values below 0.05 were considered significant.

Results

Characterization of the *de novo* bile acid synthesis

To study the *de novo* bile acid synthesis in the standard protocol with the infusion of TCA, the produced bile during NMP of human and porcine livers was characterized. TCA was not presented in the graphs since TCA was administrated and the measured output was in line with the administration. In human bile, GCA, GCDCA and TCDCA were the three most abundant bile acids, followed by tauroursodeoxycholic acid (UDCA), taurohyodeoxycholic acid (THDCA) and glycohyodeoxycholic acid (GHDCA) the least (Figure 5.1A). Porcine bile showed the highest abundance of GHC, gHDC and GCDCA but with minimal detection of TUDCA, HCA and GDCA (Figure 5.1B). Figure 5.1C-D show the bile composition of hourly fractions from human and porcine bile. For human bile, the bile acid GCDCA decreased significantly over time (36% at t=60 min, to 20% at t=360 min compared to t=0). Porcine bile composition showed no significant changes and remained stable over time. The total bile acid excretion was calculated and expressed as cumulative bile acid excretion in grams over time to illustrate the total synthesis of bile acids during NMP (Figure 5.1E). The total bile acid excretion showed to be higher in porcine bile compared to in human bile during 360 minutes of perfusion with values of 0.67±0.22g in porcine bile versus 0.14+0.04 in human bile. Figure 5.1F shows the biliary excretion of TCA which is a marker for NTCP (uptake) and BSEP (excretion) function. The dotted line shows the administrated TCA dose during *ex vivo* liver perfusion (0.2g/hr). In human as well as porcine livers, the excretion of TCA was linear and constant in time indicating proper NTCP as well as BSEP function. We also investigated bile acid conjugation to glycine as main conjugated product (Figure 5.1G). Human bile conjugation to glycine increased during perfusion from 76±11% to 82±7% after 240 minutes of perfusion, whereafter it remained stable thereafter until 360 minutes of perfusion. In contrast, glycine conjugation in porcine livers was stable over time, with 88±3% conjugation at t=60 min versus 84±1% conjugation after 360 min of perfusion, showing no significant change. Lastly, the perfusate cholesterol levels were quantified as cholesterol is the precursor of bile acid synthesis. During human liver perfusion, perfusate cholesterol levels remained stable until 240 minutes after perfusion, whereafter a decreasing trend was observed (Figure 5.1H) whereas in pigs, perfusate levels of cholesterol decreased throughout the perfusion, starting at 80 mg/dL at t=60 min and with a final concentration of 50 mg/dL after 360 min of perfusion.



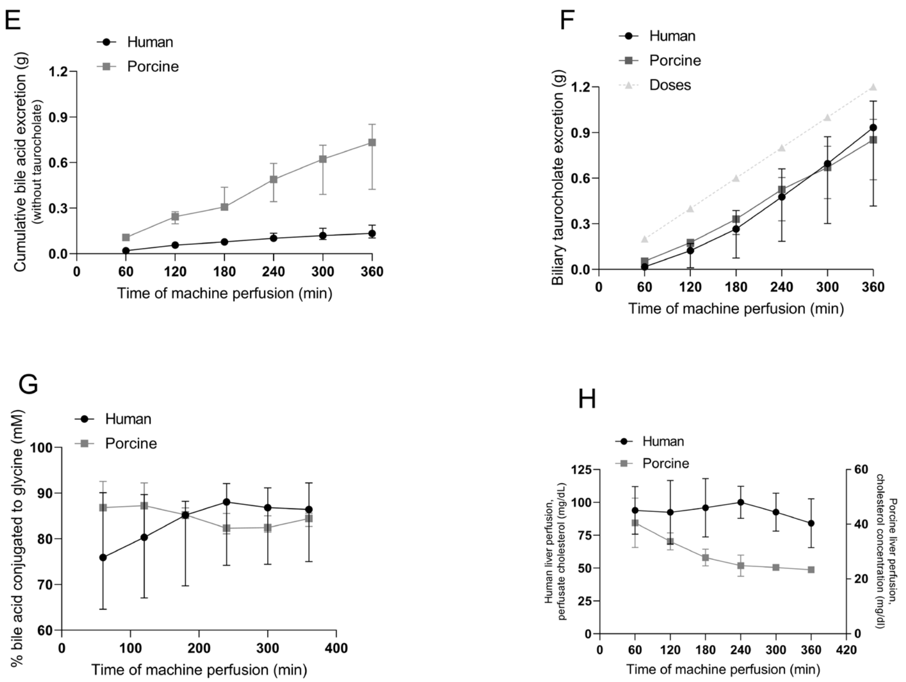


Figure 5.1 - Characterization of human and porcine de novo bile acid synthesis and cholesterol metabolism (A) average human bile acid composition (t=60-360min), (B) average porcine bile acid composition (t=60-360min), (C) human bile acid composition over time and (D) porcine bile acid composition over time, (E) Cumulative bile acid excretion in grams during NMP for human and porcine livers, (F) biliary excretion of taurocholate in human and porcine livers versus the administered taurocholate dose, (G) % bile acids conjugated to glycine in human and porcine bile and (H) perfusate cholesterol concentration during 360 min of perfusion. Data represents median ± range of n=4 human livers and n=3 porcine livers. Differences between groups were analyzed using the Mann-Whitney U test

Dysregulated cholesterol- and bile acid gene expression during NMP with TCA

RNA sequencing was performed on tissue biopsies taken at t=0 min and t=360 min of perfusions using the standard protocol with TCA. Genes involved in cholesterol synthesis, cholesterol import and export, bile acid synthesis, bile acid conjugation, and bile acid transporter expression were analyzed since bile acid transporter gene expression is regulated through bile acid signaling[30] (Figure 5.2). The genes LDL-r, HMG-CoA and NPC1, responsible for cholesterol uptake, cholesterol synthesis and intracellular transport, respectively, exhibited increased expression in human liver biopsies at t=360 min. Notably, NPC1 demonstrated a significant 1.8-fold upregulation ($p<0.05$) (Figure 5.2A), indicating the intracellular need for cholesterol. The genes ABCA1 and ABCG8, involved in cholesterol efflux, showed a 0.8- and 0.3-fold decrease, however not significant. In pig liver tissues the genes involved in cholesterol uptake and synthesis showed a strong upregulation of which LDL-r (fold change of 3.4, $p<0.05$), NPC1L1 (fold change of 4.5, $p<0.05$) and NPC1 (fold change of 4.5 $p<0.01$) (Figure 5.2B). Additionally, ABCG8 strongly decreased to 0.3 fold change ($p<0.01$). Regarding cholesterol metabolism, the effect on gene expression were comparable, however, it was more pronounced in pig liver tissues. Figure 2C-D demonstrates the expression of bile acid synthesizing enzymes in human and pig liver biopsies. In human liver biopsies, CYP27A1 and CYP8B1, and bile acid conjugating enzymes (bile acid:amino acid transferase, BAAT [human] and bile acid:CoA synthase, BACS) in general had a decreased gene expression following 360 min liver NMP, though only CYP27A1 ($p<0.05$) and CYP8B1 ($p<0.05$) were statistically significant in human liver tissues. In pig liver biopsies, CYP27A1 ($P<0.05$) and CYP7A1 ($P<0.05$) were significantly decreased after 360 min of liver NMP and CYP8B1 and BACS showed decreasing trend over time. Figure 5.2E and 5.2F present data on bile acid uptake and efflux transporter expression showing that a decrease for all measured bile acid transporters was observed over time. For example, in human liver biopsies, OAT2 (fold change of 0.43, $p<0.05$) and BSEP (fold change of 0.45, $p<0.05$) significantly decreased and in porcine liver tissue, OATP1B4 (fold change of 0.41, $p<0.01$) and BSEP (fold change of 0.32, $p<0.05$) showed a significant decrease. Together, similar trends in gene expression were observed between porcine and human livers, however, effects on cholesterol uptake, intracellular transport and synthesis were more uniform in pig liver biopsies. These results combined with the bile acid synthesis and cholesterol data strongly suggest that bile acid homeostasis is dysregulated during liver NMP using current standard protocol of TCA infusion.

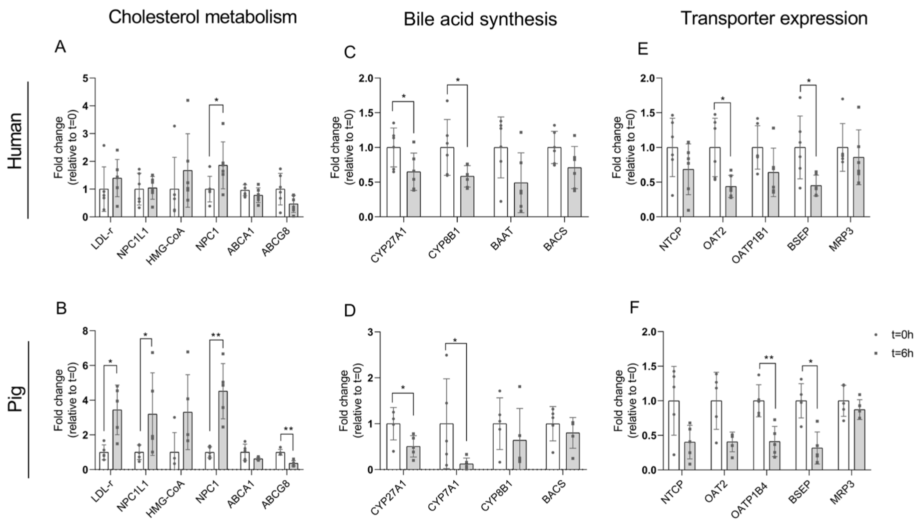


Figure 5.2 - Gene expression pattern before and after 360 min of human and porcine liver NMP (A) gene expression pattern of various genes in cholesterol metabolism such as influx transporters (LDL-r, NPC1L1), cholesterol biosynthetic enzyme (HMG-CoA reductase), intracellular cholesterol transporter (NPC1) and efflux transporters (ABCA1, ABCG8) before and after 360 minutes of NMP in human and (B) pig livers. (C) Gene expression pattern of enzymes involved in bile acid biosynthesis (CYP27A1, CYP7A1, CYP8B1) and conjugation (BACS, BAAT) before and after 360 minutes of human and (D) pig livers. (E) gene expression pattern of uptake and efflux transporters involved in bile acid uptake and excretion before and after 360 min of NMP in human and (F) pig livers. Data was expressed as FC relative to expression at t=0. Bars represent the mean of n=5 (porcine) or n=6 (human) independent experiments whereas lines represent individual livers. Differences between groups were analyzed using the Mann-Whitney U test

Infusion of conjugated bile acid mix enhances liver viability and function during NMP

After the observed increase in cholesterol-related gene expression and decrease in bile acid transporter gene expression in livers during NMP, we aimed to study the effect and addition of unprocessed bile on cholesterol and bile acid transporter gene expression during NMP of the liver. Case studies were performed by infusing unprocessed bile (10 mL/hr) during NMP, showing stimulated expression of several genes (Supplemental Figure S5.1). Since the use of unprocessed bile will never be applicable in the clinical setting, we aimed to investigate whether infusion of a variety of specific bile acids during liver NMP would alleviate the burden of the *de novo* synthesis by the liver and improve organ physiology. Various combinations of bile acids, both conjugated and unconjugated, were tested and compared to the standard protocol with TCA only. First attempts to perfuse porcine livers with primary bile acids only

(CDCA +CA) resulted in elevated ALT levels by potentially direct toxic effects of these primary bile acids (Supplemental Figure S5.2). Thus different strategies were explored. Figure 5.3 shows the liver viability and functionality parameters of upon infusion of a simple conjugated bile acid mix and a complete conjugated bile acid mix with Figure 5.3A showing the cumulative bile production during 720 minutes of perfusion. All livers produced consistent amounts of bile during 720 min of perfusion and no significant differences were observed between the groups. Perfusate ALT and AST were measured to study liver injury (Figure 5.3B-C). Figure 5.3B shows the AST release during 720 minutes of perfusion. Compared to the standard protocol using TCA, both the simple conjugated- and complete conjugated bile acid mix tended to show lower AST (average 56.3±9.9% and 63.5±10.8% respectively) and ALT levels (average 57.1±10.1% and 84.7±17.5% respectively). To study liver clearance capacity, a bolus of ICG was administered after 120 minutes of perfusion, as ICG is cleared through transporter-mediated mechanisms (OATP, NTCP, MRP2)[31] (Figure 5.3D-E). Efficient ICG clearance from the perfusate was observed in all livers, which was comparable to standard perfusions. The biliary excretion on ICG into bile tended to be faster for the simple conjugated bile acid mix, showing a steeper elimination curve compared to the standard and complete conjugated bile acid mix, however this difference was not significant (Figure 5.3E).

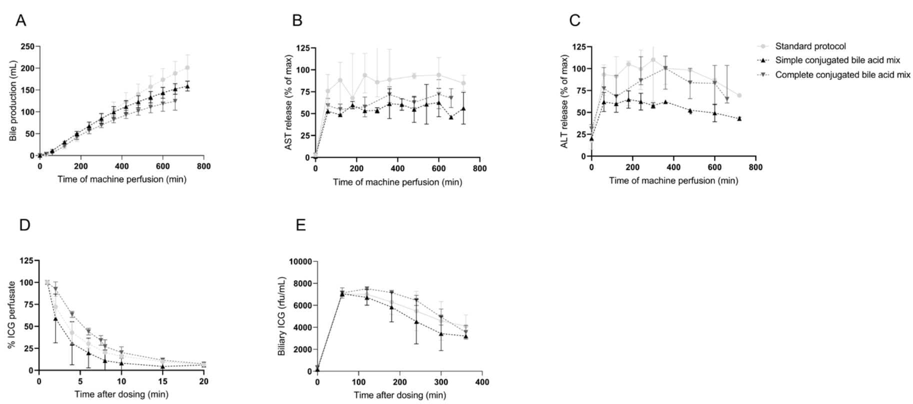


Figure 5.3 - Liver functionality during porcine liver NMP comparing the standard protocol versus continuous infusion with a simple conjugated bile acid mix versus continuous infusion with a complete conjugated bile acid mix. (A) Bile production during 720 min of perfusion, (B) AST release in the perfusate and expressed as % of highest AST release observed in the standard protocol, (C) ALT release in the perfusate and expressed as % of highest AST release observed in the standard protocol, (D) ICG elimination from the perfusate after a single bolus administration expressed as % of highest observed concentration (Cmax) and (E) biliary excretion of ICG studies over 360 min after administration. Data represents median ± range of standard protocol n=2, conjugated simple bile acid mix n=2, conjugated complete bile acid mix n=3.

Conjugated bile acid mix moderates *de novo* bile acid synthesis in porcine livers

Next, bile acid concentration in the perfusate and bile were determined of the perfusion with simple-, complete conjugated bile acid and TCA infusion. Figure 5.4A demonstrates the perfusate total bile acid concentration. In the standard protocol (TCA infusion), an increase in perfusate bile acid concentration was observed at t=60 min of perfusion whereas the simple conjugated- as well as complete conjugated bile acid mix showed lower perfusate bile acid levels indicating the ability of the perfused liver to efficiently clear the infused bile acids from the perfusate. Figure 5.4B shows the excreted bile acid concentration in bile over time demonstrating no differences in the excretion of total bile acids between protocols. The cumulative total bile acid output, visualized in Figure 5.4C, also showed no significant differences among the protocols. The total bile acid output was 4.18 mmol in 12h (4.8 mmol was infused in 12h) for standard protocol, 3.9 mmol in 12h (4.9 mmol was infused in 12h) for the conjugated simple bile acid mix and 3.0 in 11h (3.9 mmol was infused in 11hr). Cholesterol, which is the precursor of bile acids, remained at higher concentrations in the perfusate throughout the perfusions when bile acid mixes were supplemented, suggesting a reduced need for the intrahepatic conversion of cholesterol into bile acids. This is visualized in Figure 5.4D by the delta cholesterol (t=end - t=60 min of the perfusion), showing a drop of 25.9±4.4 mg/dL in perfusate levels of cholesterol in the standard protocol group. That result likely indicates a high consumption of cholesterol by the liver, needed for bile acid synthesis. The simple conjugated bile acid mix showed a subtle increase in cholesterol perfusate (Δ 5.3±1.3 mg/dL, $p<0.05$) and the complete conjugated bile acid mix demonstrated a small (not significant) reduction in cholesterol consumption compared to the standard protocol (Δ 12.8±15.2 mg/dL). Biopsies of the liver were taken at several time points during perfusion to study gene expression (Figure 5.4E-J). The simple conjugated bile acid mix increased the FXR gene expression to some extent at 360 and 540 min of perfusion (fold change 1.5), thereafter FXR expression returned to baseline (Figure 5.4E). In the complete conjugated bile acid mix FXR expression was stable over the length of the perfusion. HMG Co-A reductase as well as LDL-r gene expression increased upon the infusion of the different bile acid mixtures, in a similar manner compared to the standard protocol (Figure 5.4F-G). The expression of OATP1B4 showed a delayed decrease (after 360 min of perfusion) compared to the standard protocol showing a decrease after 240 min (Figure 5.4H). BSEP and NTCP (Figure 5.4I-J) both showed a fluctuating gene expression profile, however after 720 min of perfusion, a decrease was observed in all protocols.

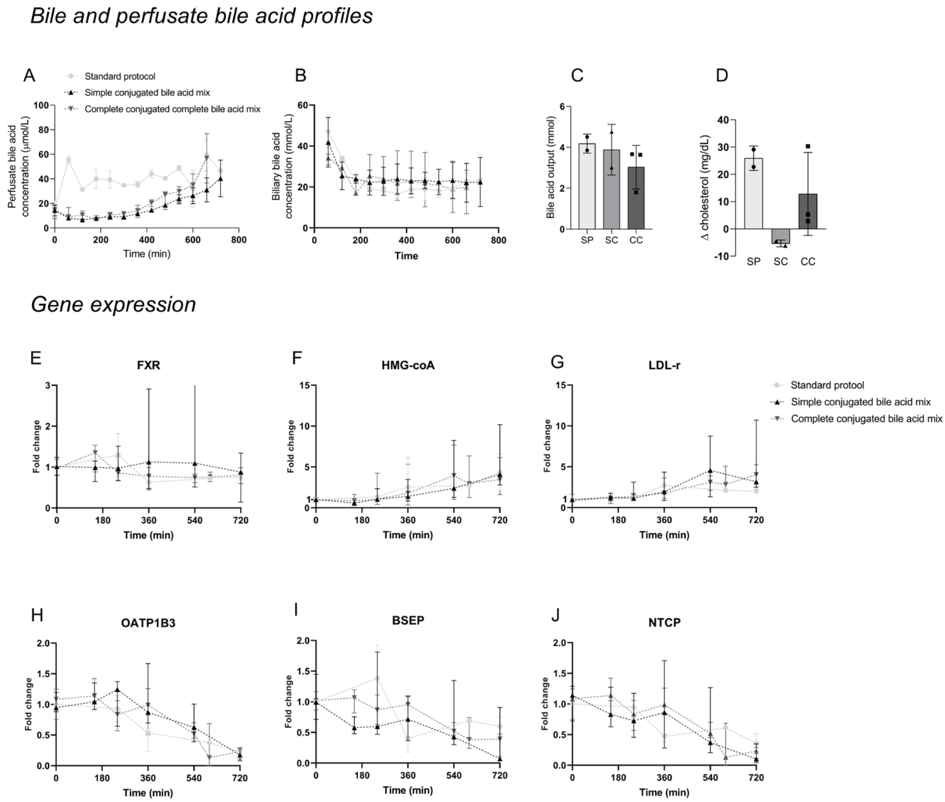


Figure 5.4 - Bile and perfusate bile acid profiles and corresponding gene expression characterization during 720 min of perfusion in the standard protocol versus continuous infusion with a conjugates simple bile acid mix versus continuous infusion with a conjugated complete bile acid mix. (A) perfusate bile acid concentration during NMP, (B) biliary bile acid concentration during NMP, (C) total bile acid output during NMP as corrected for total bile flow, SP=standard protocol, CS=conjugated simple bile acid mix, CC=conjugated complete bile acid mix, (D) delta cholesterol: end of perfusate – t=60 min. Gene expression profile during porcine liver NMP of (E) FXR, (F) HMG-CoA, (G) LDL-r, (H) OATP1B4, (I) BSEP and (J) NTCP. Data represents median ± range of standard protocol n=2 (n=8: 0-360 min, n=2: 360-720 min), conjugated simple bile acid mix n=2, conjugated complete bile acid mix n=3.

Bile acid challenge as hepatobiliary function assessment in human livers

The simple conjugated bile acid mix was chose to be applied during 3 independent human liver perfusions (referred to as liver #1, #2 and #3) because of its positive effects on ALT AST release and ICG clearance observed in the porcine liver experiments. Donor characteristics are presented in Table 5.2 and set-up of the liver perfusion is illustrated in Figure 5.5A. Human liver perfusion demonstrated a proper bile flow during the infusion of the simple conjugated bile acid mix in all three livers (Figure 5.5B). Human livers were able to rapidly clear lactate, of which the subsequent levels remained stable during the perfusion duration demonstrating appropriate hepatocellular function (Figure 5.5C). ALT and AST levels in liver #1 and #2 remained low and stable during the perfusion whereas liver #3 had relatively high ALT levels (1800 U/L) from the start of the perfusion, likely relating to the presence of a pre-existing pathological condition (including choledocholithiasis). Bile pH was alkalotic (>7.5) from all three livers and remained stable throughout the perfusion (Figure 5.5E). The plasma cholesterol levels were stable over time (Figure 5.5F). The functionality of the livers was assessed by studying ICG clearance from the perfusate after 4h and 2h for liver #1 and #2-#3 respectively (Figure 5.5G). Overall, rapid elimination of ICG from the perfusate was observed with subsequent biliary elimination detected 20 min after administration. Additionally during perfusion, a bile acid challenge was performed to liver #1 and #2 by providing a bolus of simple conjugated bile acids (0.2g) to the perfusate and assessing clearance of these bile acids as a functional parameter. Prior to the bile acid bolus in liver #1 and #2, perfusate and biliary bile acid concentrations remained stable over time (5.5±1.4 μmol/L in perfusate, 15.3±2.1 mmol/L in bile in #1, 4.3±2.2 μmol/L in perfusate, 16.3.7±1.5 mmol/L in bile in #2). Upon administration of a bile acid bolus, the perfusate levels showed to peak and rapidly decline indicating the ability to efficiently clear bile acids from the circulation. This was also illustrated by an increase in bile acid concentration in bile. Liver #3 suffered from choledocholithiasis as is visualized by the limited bile acid output and decreased bile acid absorption from the perfusate (Figure 5.5I). The data shows that the simple conjugated bile acid mix supports proper liver function and is safe to use however did not have pronounced effects on bile acid-regulated genes (Supplemental Figure S5.3, supplemental Figure S5.4).

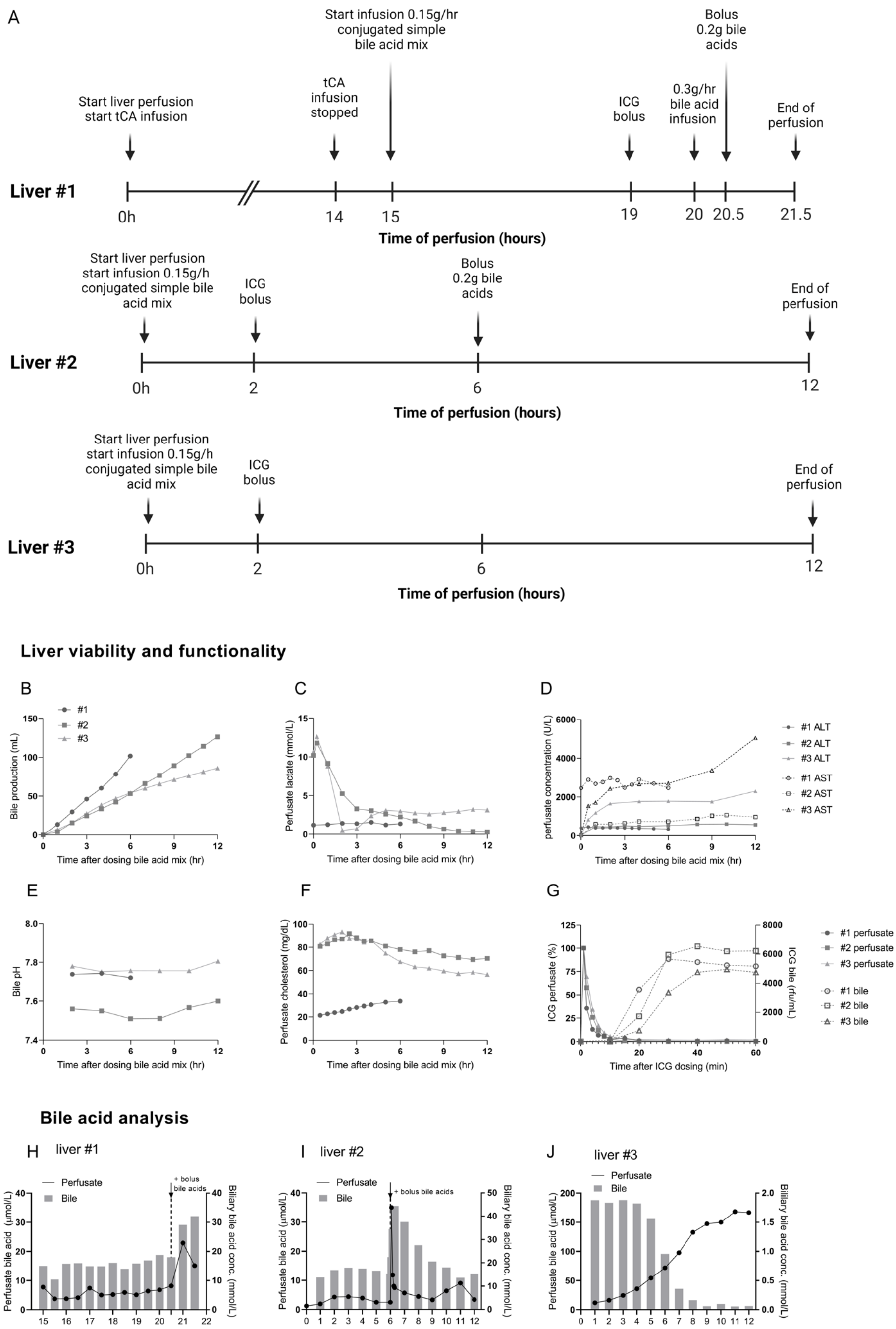


Figure 5.5 - Human liver perfusion with administration of conjugated simple bile acid mix. (A) schematic overview of the study set-up (B) Cumulative bile production, (C) perfusate lactate

concentration, (D) perfusate ALT and AST concentration, (E) Bile pH, (F) cholesterol perfusate concentration, (G) ICG perfusate and ICG biliary excretion measured after 4 hours of perfusion liver #1, 2 hours of perfusion liver #2 and #3, (H) perfusate bile acid concentration during 15-20 hours and 20 – 21,5 with increased bile acid infusion + additional bolus liver #1, (I) perfusate and biliary bile acid concentration in liver #2 with a bile acid bolus at t=360 min and (J) perfusate and biliary bile acid concentration in liver #3 min. Data represents mean ± SD n=3 individual experiments.

Discussion

Bile acid metabolism, is a complex phenomenon in our body and often not taken into account during *ex vivo* liver perfusion as standard protocols only apply TCA. In the first part of this study, applying the standard protocol with TCA only, we showed that during *ex vivo* liver NMP bile acid and cholesterol homeostasis was dysregulated. In the second part of this study, we described that infusion of a (un)conjugated bile acid mixture alleviated the burden of the *de novo* bile acid synthesis in the liver by showing decreased ALT and AST levels while retaining appropriate liver functions. These finding provide valuable new insights for improving the physiological resemblance of bile acids (and cholesterol) metabolism during NMP and thereby improving organ viability and functionality.

The human body is a complex biological system with continuous feedback systems and a dynamic interplay between organs, which is essential for maintaining homeostatic processes. Interestingly, the perfused livers in the first part of the study with TCA infusion appeared to be comparable to biliary drainage models where the EHC is interrupted[32-34]. During NMP, a decrease in perfusate cholesterol was observed accompanied by an increase in HMG-CoA reductase expression and altered cholesterol gene expression profile, similar to biliary drainage models. Kuipers et al.[32] and Smit et al.[34] demonstrated in rats that interruption of the EHC rapidly reduced plasma cholesterol and biliary bile acid output, and interestingly, increased food intake[32]. Additionally, a rapid decrease in bile flow was observed in the first hours after cannulation[32-34]. In humans, a similar phenomenon was observed, with Shoda et al.[35] reporting the low biliary secretion of bile acids just after biliary drainage. These findings indicate that the absence of EHC *in vivo* profoundly impacts bile acid and cholesterol homeostasis, suggesting that incorporating EHC during liver perfusion could enhance liver function and homeostasis.

The intervention studies with (un)conjugated bile acids indicated that cholesterol consumption in the liver was equivalent to cholesterol synthesis, since cholesterol perfusate levels remained more stable compared to control perfusions with TCA only. Supplementation of an (un)conjugated bile acid mixture reduced the requirement for *de novo* bile acid synthesis, demonstrated by the reduced cholesterol consumption while retaining similar bile acid output. Furthermore, peak bile acid concentration was observed in the standard protocol at t=60 min, which was absent during the perfusions with conjugated bile acid infusion. Higher levels of ALT and AST at t=60 were observed for the standard protocol compared to the intervention protocols possibly attributed to the elevated perfusate bile acid levels[13]. After static cold storage, the liver is subjected to a sudden increase in temperature when connected to the perfusion device resulting in a rapid increase in metabolic rate. The (un)conjugated bile acid mix may support this recuperation phase as evidenced by efficient bile acid clearance during the first hours of perfusion. However, more research is needed to study the optimal bile acid infusion composition especially given the potential for prolonged *ex vivo* liver perfusion. Current studies show prolonged *ex vivo* liver perfusion duration up to 7 days or longer[8,36] and thus the need for bile acid supplementation will become more substantial. Eshmuminov highlighted the utilization of UCDCA in-stead of TCA, based on UCDCA's epithelial protection properties[11]. Yet, using a single bile acid during perfusion does not replicate the diverse pool normally synthesized and excreted by the liver. Infusing a pool of (un)conjugated bile acid could efficiently extend perfusion duration by reducing the necessity for *de novo* synthesis, thus resulting in reduced energy requirement as the *de novo* synthesis of bile acids is an energy demanding process[12,13].

In the human liver perfusions using supplementation of the simple conjugated bile acid mix, stable cholesterol perfusate levels were observed, indicating reduced use of cholesterol for the conversion to bile acids. Good liver function and safe use of the simple conjugated bile acid mix was observed by showing lactate clearance, alkalotic bile pH and ICG clearance. In 2 out of 3 human livers efficient removal of the bile acid bolus from the perfusate was observed and subsequent increased excretion of total bile acids was measured as well as an increase in bile production. This indicates presence and appropriate function of the transporters NTCP and BSEP, highlighting the potential utility of a bile acid challenge as a tool to assess viability and/or quality during NMP. Notable, one of the livers (liver #3) from a patient with underlying pathology of gall stones, showed diminished clearance of bile acids, potentially explained by the lower abundance of the transporters NTCP and BSEP in this donor[37].

In this study it was hypothesized that bile acid signaling would be one of the main mechanism in regulating stable (ADME) gene expression during NMP. However, the gene expression profile in pigs following intervention with the (un)conjugated bile acid pools continued to exhibit effects on bile acid-regulated genes. It is important to acknowledge that the porcine bile acid composition differs from that of humans, showing higher presence of hyocholic acid and its conjugates. The most optimal scenario would have been to mimic porcine bile acids however obtaining hyocholic acids in mg scale was economically unfeasible[38]. Although to a lesser and more delayed extent, also a decreasing trend in relevant gene expression was observed during the human liver perfusion. It could be that other mechanisms such as circadian rhythm overpowered gene regulation. It is known that genes involved in bile acid metabolism are regulated by central and hepatic circadian clock genes in addition to the timing of food intake, which was not taken into account in this study[18,39]. Therefore, longer term perfusion of the livers (>24 hours) is needed to study potential fluctuation in gene expression regulated by circadian clock genes.

Conclusion

In conclusion, replacing standard TCA infusion during liver NMP with a more physiologically representative bile acid pool to stimulate the enterohepatic recirculation of bile acids has yielded promising results. The infusion of a more physiologically relevant bile acid mixture containing a variety of (un)conjugated bile acids showed a decreased release of hepatic injury markers and better maintenance of stable cholesterol levels in the perfusate, compared to standard NMP. Moreover, the results demonstrated that the infusion of (un)conjugated bile acids may enhance liver function during NMP, thereby pointing towards potential advancements in liver preservation during the and transplantation process.

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Supplementary materials

Table S5.1 - Human primer sequences.

Species	Gene/protein	Primer sequences forward (FW) and reverse (REV) '5-3'
Human	*GAPDH*/GAPDH	FW: ATGGAAATCCCATCACCATCTT
		REV: CGCCCCACTTGATTTTGG
	ACTB/ACTB	FW: GCTGCCCTGAGGCACTCTT
		REV: GGATGCCACAGGACTCCATG
	ABCB11/BSEP	FW: AAAGCACTCATTTGCCCCTG
		REV: TCTTGTAGATTCCTGCCGCC
	NR1H4/FXR	FW: TGTGAGGGGTGTAAAGGTTTCT
		REV: GCCAACATTCCCATCTCTTTGC
	HMGCR/HMG-CoA reductase	FW: TACCATGTCAGGGGTAC
		REV: CAAGCCTAGAGACATAAT
	LDLR/LDLR	FW: GTGCTCCTCGTCTTCCTTTG
		REV: GCAAATGTGGACCTCATCCT
	SLC10A1/NTCP	FW: CTTCTGCCTCAATGGACGGT
		REV: AGGCCACATTGAGGATGGTG
	SLCO1B3/OATP1B3	FW: GGGTGAATGCCCAAGAGATA
		REV: ATTGACTGGAAACCCATTGC

Table S5.2 - Porcine primer sequences.

Species	Gene/protein	Primer sequences forward (FW) and reverse (REV) '5-3'
Porcine	*GAPDH*/GAPDH	FW: ATCGTCAGCAATGCCTCCTG
		REV: ACCGTGGTCATGAGTCCCTC
	ABCB11/BSEP	FW: GGATTCATGTGGTGTCTCATCTTT
		REV: ACAAGGGTTCCTGCTGTGTATTC
	ABCG8/ABCG8	FW: AGACGCAATCCTCAATGCCA
		REV: GCAGGAGTCTGGGCTTGAAT
	CYP27A1/CYP27A1	FW: TTGAGAAACGCATTGGCTGC
		REV:ATCCAGGTATCGCCTCCAGT
	NR1H4/FXR	FW: ATGGGAATGTTGGCTGAATGT
		REV:TGTTGAGGTCACTTGTCGCA
	HMGCR/HMG-CoA reductase	FW: GACTCCGTTGACTGGAGACG
		REV: AAAGAGGCCATGCATTCGGA
	ABCC2/MRP2	FW: TCACCTGCAACTTGGGTTGT
		REV: GTCTCTAGATCCACCGCAGC
	SLC10A1/NTCP	FW: TTACCCCCAAAAGCCTCACC
		REV: TTTTATGCCTGTGGGGCACT
	SLC22A7/OAT2	FW: CTGGGAATACGACCACTCGG
		REV: GGCTCTGTTCAGGCCTTTCT
	SLCO1B4/OATP1B4	FW: CCTCTTCATTGGGAACCATCTC
		REV: GAATTCCTCCTAGCGTTCGAATAGT
	ABCB1/P-gp	FW: AAATTGTGTGAATTGCCCAGATAAC
		REV: GCCACAGTAATAATAGGCGTACACTG

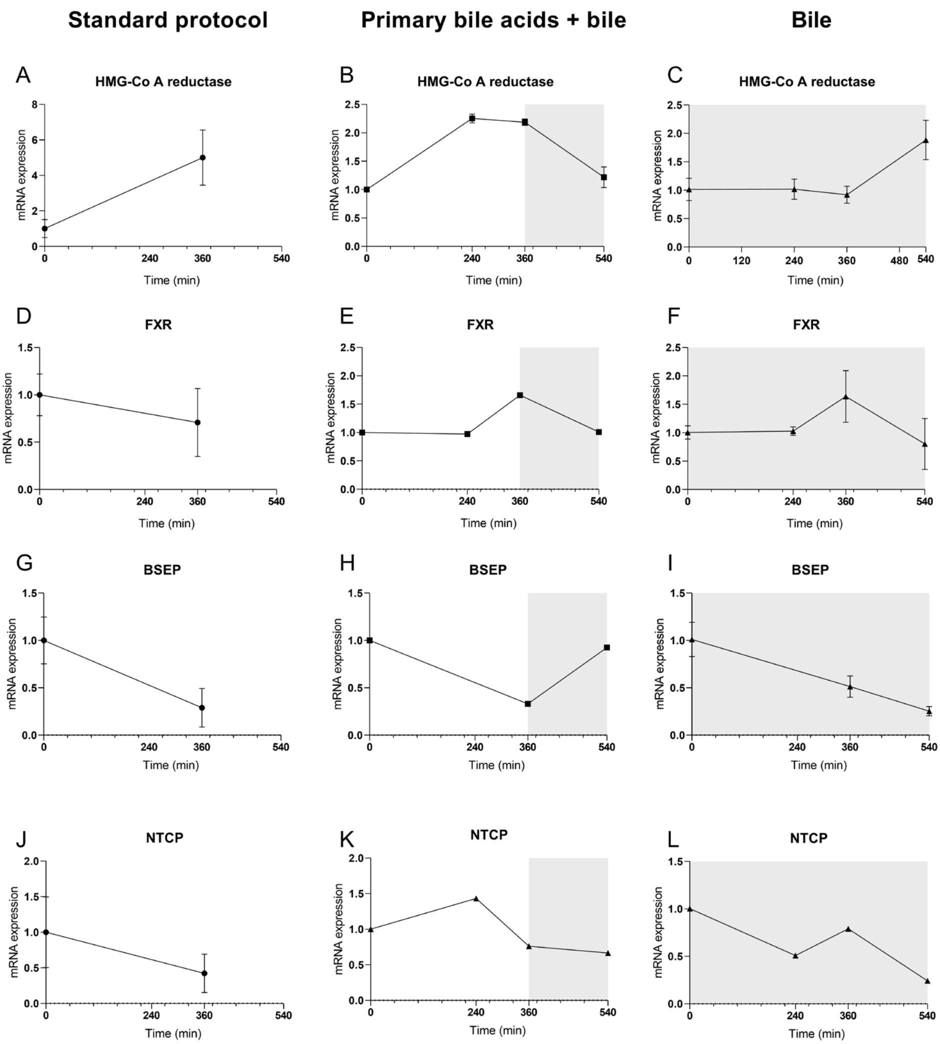

Figure S5.1 - Case studies showing gene expression profile upon standard protocol, infusion with primary bile acids + unprocessed bile or continuous infusion of unprocessed bile during NMP in porcine livers. (A-C) Gene expression of HMG-CoA, (D-F) FXR, (G-I) BSEP and (J-L) NTCP. Data represents median ± + IQR, n=5 for the standard protocol and n=1 for primary bile acids + bile and n=1 for bile condition. Primary bile acid infusion: To study the effect of primary bile acids and porcine bile, tCA was replaced by the primary bile acids CDCA and CA (total rate of 0.2g/hr (0.37mmol/hr)) (n=1). Liver was perfused for 360 minutes, thereafter bile from the gall bladder was infused at a rate of 10mL/hr from 360 minutes until 540 minutes. Continuous bile infusion: To study the effect of continuous bile infusion, tCA was replaced by porcine bile (pooled from n=5 pig gall bladders) (n=1). Bile was infused at a rate of 10 mL/hr. Livers were perfused for a total time of 540 minutes.

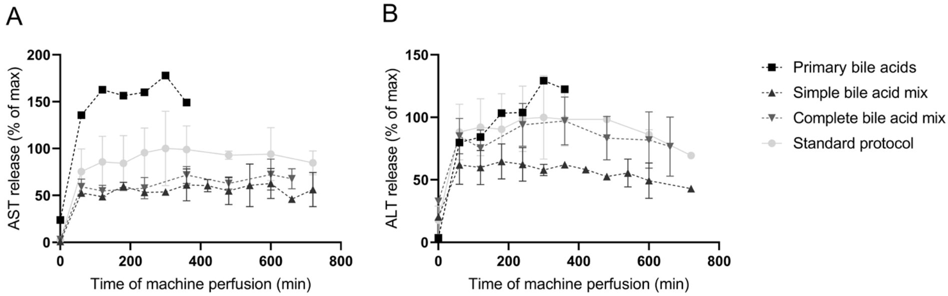

Figure S5.2 - AST and ALT levels during infusion of different bile acid mixes. Livers were perfused and continuous infusion of primary bile acids (CDCA+CA), conjugated simple bile acid mix, conjugated complete bile acid mix or with tCA (standard protocol). (A) perfusate AST and (B) perfusate ALT release. Data represents mean with range (min-max).

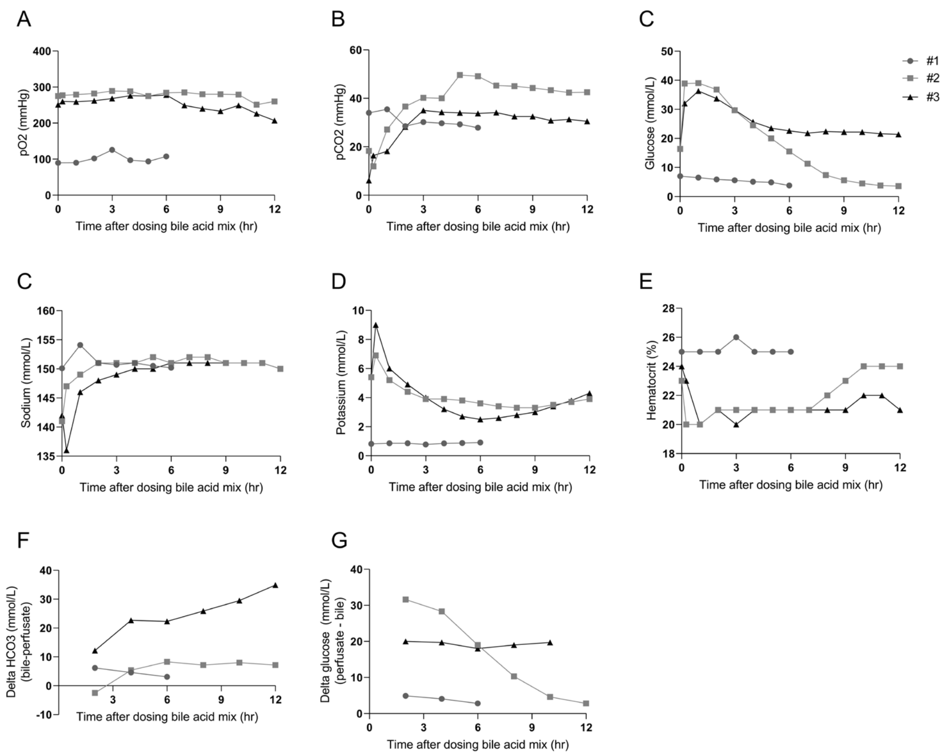

Figure S5.3 - Bloodgas analysis data of human liver perfusions with continuous infusion of conjugated simple bile acid mix. (A) pO2, (B) pCO2, (C) perfusate glucose, (D) perfusate sodium, (E) perfusate potassium, (F) perfusate hematocrit level (G) delta HCO3 (bile-perfusate) and (I) delta glucose (perfusate-bile). Data represents individual data of n=3 experiments.

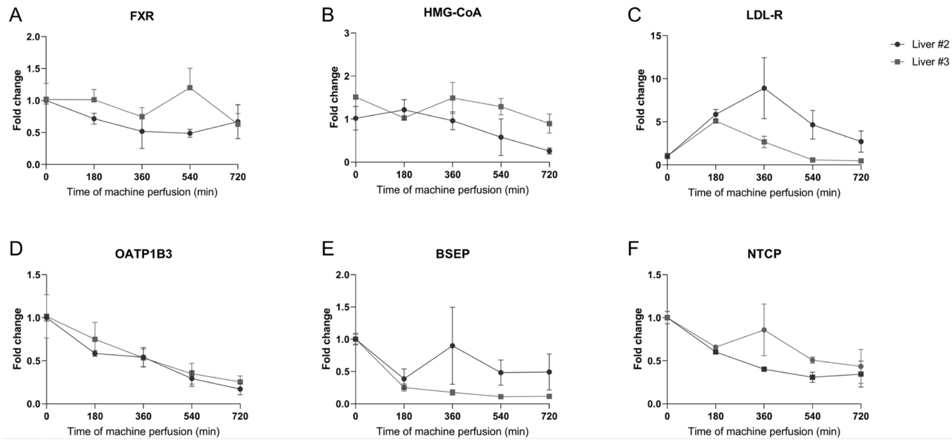

Figure S5.4 - Gene expression profile of human liver #2 and #3 during 720 min of perfusion. Gene expression profile during human liver NMP of (A) FXR, (B) HMG-CoA, (C) LDL-r, (D) OATP1B3, (E) BSEP and (F) NTCP. Data represents n=2. No biopsies were taken from liver #1 which perfused using the OrganOx system due to inability of the closed system