CHAPTER 4

Biliary Bicarbonate, pH and Glucose are Suitable Biomarkers of Biliary Viability During Ex Situ Normothermic Machine Perfusion of Human Donor Livers

Alix P.M. Matton, Yvonne de Vries, Laura C. Burlage, Rianne van Rijn, Masato Fujiyoshi, Vincent E. de Meijer, Marieke T. de Boer, Ruben H.J. de Kleine, Henkjan J. Verkade, Annette S. H. Gouw, Ton Lisman, Robert J. Porte

Transplantation. 2019 Jul;103(7):1405-1413
Abstract

**Background:** *Ex situ* normothermic machine perfusion (NMP) can be used to assess viability of suboptimal donor livers prior to implantation. Our aim was to assess the diagnostic accuracy of bile biochemistry for the assessment of bile duct injury (BDI).

**Methods:** In a preclinical study, 23 human donor livers underwent 6 hours of end-ischemic NMP to determine biomarkers of BDI. Livers were divided into groups with low or high BDI, based on a clinically relevant histological grading system. During NMP, bile was analyzed biochemically and potential biomarkers were correlated with the degree of BDI. Receiver operating characteristics curves were generated to determine optimal cut-off values. For clinical validation, identified biomarkers were subsequently included as viability criteria in a clinical trial (n=6) to identify transplantable liver grafts with low BDI.

**Results:** Biliary bicarbonate and pH were significantly higher and biliary glucose was significantly lower in livers with low BDI, compared to high BDI. The following cut-off values were associated with low BDI: biliary bicarbonate >18 mmol/L (*P*=0.002), biliary pH >7.48 (*P*=0.019), biliary glucose <16 mmol/L (*P*=0.013), and bile/perfusate glucose ratio <0.67 (*P*=0.013). In the clinical trial, 4 out of 6 livers met these criteria and were transplanted, and none developed clinical evidence of post-transplant cholangiopathy.

**Conclusion:** Biliary bicarbonate, pH, and glucose during *ex situ* NMP of liver grafts are accurate biomarkers of BDI and can be easily determined point-of-care, making them suitable for the pre-transplant assessment of bile duct viability. This may improve graft selection and decrease the risk of post-transplant cholangiopathy.
Introduction

The gap between the demand and availability of donor livers for transplantation has stimulated the use of extended-criteria donor livers, including steatotic, elderly, and donation after circulatory death (DCD) liver grafts (1). These types of donor livers, however, have a higher risk of developing postoperative complications. Especially post-transplant cholangiopathy, or non-anastomotic biliary strictures (NAS), occur in 13 – 35% of DCD grafts, compared to 1 – 24% of livers donated after brain death (2-7). Although the pathogenesis of NAS is not fully understood, the degree of histological bile duct injury (BDI) at the time of transplantation has been identified as a strong predictor of the development of NAS after transplantation (8-10).

In an attempt to expand the donor liver pool, researchers are increasingly using ex situ machine perfusion of liver grafts prior to transplantation. When performed at 37°C, normothermic machine perfusion (NMP) renders the organ metabolically active, which allows for viability testing and the selection of potentially transplantable organs (11-14). Currently, criteria for the selection of suitable donor livers during NMP include bile production, lactate clearance, vascular flows, macroscopic appearance of the liver, transaminase concentration and pH buffering capacity (11-14). These parameters, however, mainly reflect hepatocellular function and injury, and do not provide information about cholangiocellular function or injury. Identification and determination of the diagnostic accuracy of biomarkers of BDI is clinically relevant as they may provide a missing tool in the selection of liver grafts with an anticipated low risk of NAS. Low biliary pH, bicarbonate and high biliary glucose during NMP have recently been associated with the development of NAS in three cases, and with histological bile duct stroma necrosis in five research livers (14). The authors reported that a biliary pH ≤7.4 during NMP was able to discriminate between these livers, but have provided no other cut-off values and have not reported on the implementation of these biliary parameters clinically.

Cholangiocytes lining the bile duct lumen and peribiliary glands actively contribute to the composition of bile by secretion of bicarbonate via cystic fibrosis transmembrane regulator (CFTR) and chloride-bicarbonate anion exchanger 2 (AE2) (15,16). As a result of this, bile in the extrahepatic bile duct has a pH ranging between 7.5 and 8.1 and bicarbonate concentration of 12 to 55 mmol/L (17,18). Moreover, cholangiocytes actively reabsorb glucose from bile via sodium-dependent glucose transporter, SGLT1, expressed on their apical plasma membrane domain, and another glucose transporter, GLUT1, on their basolateral domain (19,20). This results in very low biliary glucose concentrations under physiological conditions (21,22). These transporters are ATP-dependent, and studies have shown that ischemia leads to diminished function of SGLT1 in cholangiocytes, with subsequent decreased glucose reabsorption (23).

To determine the added value of specific biomarkers of biliary viability during NMP, we first examined whether histological BDI correlates with markers of hepatocellular injury. As this correlation was poor, we proceeded to identify biomarkers of BDI that can be easily assessed point-of-care. Our aim was to determine the diagnostic accuracy of biliary pH, bicarbonate, glucose and lactate dehydrogenase (LDH) concentration (24-27), to discriminate between livers with a high or low degree of BDI.
Materials and Methods

Donor Livers

Twenty-three human donor livers that were declined for transplantation nationwide and offered for research were used to determine suitable biomarkers of BDI during NMP in a preclinical study. All livers underwent six hours of NMP for viability testing after static cold storage, as described previously (28). In brief, livers underwent NMP at 37°C using a pressure-controlled perfusion device (Liver Assist, Groningen, the Netherlands), providing continuous portal flow and pulsatile arterial flow. For the preclinical study, informed consent was obtained from relatives of the donors and the study protocol was approved by the Medical Ethical Committee of the University Medical Center Groningen (METc, #2012.068) and by the Dutch Transplantation Foundation (NTS). For validation purposes, the identified cut-off values were prospectively applied to six human livers in an ongoing clinical trial as described below.

Histological Analysis

Two biopsies of the common or proper hepatic bile duct were collected: one during the back-table procedure prior to NMP and one at the end of NMP (proximally of the biliary catheter). All biopsies were fixed in formalin and paraffin-embedded. Slides were stained with hematoxylin and eosin (H&E) and assessed by light microscopy using an established, clinically relevant, histological BDI grading system (10) (Table 1). The presence of stroma necrosis, injury of the extramural peribiliary glands, and injury of the perivascular plexus in pre-transplant bile duct biopsies have been found to predict the development of NAS after transplantation (10). For this reason, we selected these three histological parameters for the present study. The range of the total BDI score was 0 – 7 (Table 1). Biopsies were independently scored in duplicate by two investigators (APMM and YdV) without knowledge of clinical or NMP data under supervision of an expert liver pathologist (ASHG), who resolved any discrepancies between the researchers’ scores.

Bile Sample Analyses

During NMP, bile samples were collected every 30 min under mineral oil (to prevent the exchange of CO₂ between the sample and ambient air, which would influence the pH and bicarbonate concentration) for immediate point-of-care determination of pH, bicarbonate and glucose concentration using an ABL90 FLEX analyzer (Radiometer, Brønhøj, Denmark). Additional bile samples were stored at -80°C and later analyzed for LDH using routine laboratory technique for standard patient care.
Table 1. Histological Bile Duct Injury Scoring System*.

| Grade | Bile Duct Wall Stroma Necrosis | Extramural Peribiliary Glands | Peribiliary Vascular Plexus |
|-------|--------------------------------|------------------------------|-----------------------------|
| 0     | No stroma necrosis             | No loss or injury of cells    | No vascular lesions         |
| 1     | ≤25% stroma necrotic           | ≤50% loss or injury of cells  | ≤50% of vessels necrotic    |
| 2     | >25% and ≤50% stroma necrotic  | >50% loss or injury of cells  | >50% of vessels necrotic or no longer visible |
| 3     | >50% stroma necrotic           |                              | -                           |

* Significant histological predictors of NAS were selected from op den Dries et al.10

Hepatocellular Injury and Function

Alanine aminotransferase (ALT) concentration, lactate clearance and bile production are commonly used parameters to assess hepatocellular injury and function during NMP (11-14). ALT and lactate concentrations in perfusate were analyzed as described previously (28). A peak ALT concentration <6,000 U/L was considered to reflect low hepatocellular injury. A lactate concentration of ≤2.5 mmol/L and cumulative bile production of ≥5 mL/kg liver within 2.5 h of NMP were considered to reflect good hepatocellular function.

Validation Cohort

Identified biomarkers of BDI were subsequently included as selection criteria, in addition to hepatocellular injury and function criteria, in a clinical trial of end-ischemic NMP for viability assessment of high-risk donor livers that were declined for transplantation nationwide based on perceived suboptimal quality (www.trialregister.nl; NTR5972). The study protocol was approved by the Medical Ethical Committee (METc #2017.281) and all patients gave written informed consent.

Statistics

Continuous variables were presented as median with interquartile range (IQR) and were compared between groups using the Mann-Whitney U test. Correlations were calculated using the Spearman’s rank correlation test. Receiver operating characteristics (ROC) curves were generated for biliary pH, bicarbonate, glucose, and LDH concentration, as well as for the bile/perfusate glucose concentration ratio and the delta between perfusate and biliary glucose concentration (perfusate minus biliary glucose) to illustrate diagnostic ability of the binary BDI-score. The first time point with an area under the ROC curve (AUC-ROC) >0.80 and a P <0.05 for each potential biomarker was selected to determine the cut-off value with the highest sensitivity and specificity to discriminate livers with low BDI from those with high BDI. Positive predictive value (PPV), negative predictive value (NPV), positive likelihood ratio (LR+) and negative likelihood ratio (LR-)
with corresponding 95% confidence interval (CI) were calculated using cross tabulation. Level of significance was set at $P$-value <0.05. All statistical analyses were performed using IBM SPSS version 23.0 (Chicago, IL, USA).

**Results**

**Donor Liver Characteristics**

In the pre-clinical study, 18 livers were from DCD donors and 5 livers were donated after brain death (DBD). The overall median (IQR) cold ischemia time was 8.1 (7.0 – 9.3) h and warm ischemia time (from withdrawal of life support until *in situ* cold flush) in DCD livers was 38 (33 – 42) min.

**Bile Duct Histology**

The degree of BDI per histological item in livers with low or high BDI is summarized in **Table 2**. Representative examples of H&E staining of bile ducts with low or high BDI are presented in **Figure 1**. The median BDI score before NMP was 4.0 (3.0 – 7.0) and 6.0 (6.0 – 7.0) after NMP, with 8 livers showing no change in the BDI score over time. The mean BDI score of the two biopsies ranged between 3 – 7, with 4.75 as the median of the range. This value was chosen to divide livers into a group with a low and a group with a high BDI score.

**Table 2. Comparison of Bile Duct Injury Score per Histological Item.**

|                  | Livers with a Low BDI Score (n=9) | Livers with a High BDI Score (n=14) |
|------------------|-----------------------------------|-------------------------------------|
| **Stroma Necrosis** (Grade 0 – 3) | 2.0 (1.5 - 2.0)                  | 3.0 (2.6 – 3.0)                      |
| **Extramural Peribiliary Glands** (Grade 0 – 2) | 1.0 (1.0 – 1.0)                  | 2.0 (1.5 – 2.0)                      |
| **Peribiliary Vascular Plexus** (Grade 0 – 2) | 1.0 (1.0 – 1.5)                  | 2.0 (2.0 – 2.0)                      |

Variables are presented as median and (interquartile range). Abbreviations: BDI, bile duct injury.
Figure 1. Representative histological H&E staining of an extrahepatic bile duct biopsy with a low histological bile duct injury (BDI) score (A) and a high BDI score (B). (A) Intact extramural peribiliary glands (encircled), intact vasculature (e.g. arrowhead pointing to vital arteriole in stroma) and no signs of stroma necrosis. Note that also the periluminal peribiliary glands are largely intact. (B) Severe injury to the extramural peribiliary glands with loss of cells (encircled), necrotic (arrowhead) or absent vessels and diffuse stroma necrosis. Note the denuded epithelial lining of the bile duct lumen (asterisk), as is the case in >90% of all donor bile ducts prior to transplantation.8-10
Hepatocellular Function and Injury Correlate Poorly with BDI

Overall, there was a weak correlation between peak ALT concentration in perfusate during NMP, a marker of hepatocellular injury, and the degree of BDI (Spearman r 0.424, P=0.044) (Figure 2A). In livers with extremely high ALT levels (>6,000 U/L), the BDI score was also high in 7 out of 8 cases (88%). However, when ALT levels were <6,000 U/L and livers could potentially be considered for transplantation based on an acceptable degree of hepatocellular injury (14), the correlation between ALT and BDI was very poor (Spearman r 0.192, P=0.493) with 7 out of 15 (47%) livers still having a high BDI score.

Similarly, 4 out of 9 (44%) livers with good lactate clearance also had high BDI (Figure 2B). There was no correlation between BDI and lactate concentration (Spearman r 0.349, P=0.103). Likewise, 10 out of 18 (56%) livers with high bile production also had high BDI. There was no correlation between BDI and cumulative bile production (Spearman r -0.291, P=0.178) (Figure 2C). Furthermore, there was no correlation between (cumulative) bile production and biliary pH, bicarbonate, LDH and the glucose and bile/perfusate glucose ratio (SDC, Figure S1). These findings indicate that markers of hepatocellular injury and function poorly predicted the degree of BDI, especially in cases that could potentially be considered for transplantation based on hepatocellular criteria. This supports the need for specific biomarkers of BDI during NMP.

Biliary pH and Bicarbonate Correlate Significantly with BDI

Biliary pH and bicarbonate concentration during NMP were significantly higher in livers with low BDI, compared to livers with high BDI (Figure 3A-B). As expected, biliary pH and bicarbonate were strongly correlated (Figure 3C). The line of best fit (R^2=0.7377) demonstrated that in the lower range of biliary bicarbonate small increases lead to a relatively large increase in pH, while at higher bicarbonate concentrations (>30 mmol/L) pH remained relatively stable.

Since bile production can be low or absent during the first 2 h of NMP, we only used bile samples collected after 2 h to determine optimal cut-off values to discriminate livers with
low BDI from those with high BDI (SDC, Table S1). Already after 2 h, a biliary bicarbonate concentration of 18 mmol/L discriminated between low and high BDI with an AUC-ROC of 0.91 (P=0.002) and a sensitivity, specificity, PPV, NPV >80% (Figure 3D-E).

The earliest time point at which biliary pH discriminated livers with low BDI from those with high BDI was 2.5 h (SDC, Table S1). At this time point, the optimal cut-off value for pH was 7.48, with an AUC-ROC of 0.81 (P=0.019) (Figure 3D-E).

**Biliary Glucose and Bile/Perfusate Glucose Ratio Correlate Significantly with BDI**

Glucose concentrations were lower in bile of livers with low BDI, compared to livers with high BDI score (Figure 4A). While biliary glucose gradually decreased in livers with low BDI, biliary glucose increased in livers with high BDI, generally remaining >20 mmol/L. In livers with high BDI, the median bile/glucose concentration ratio increased to 1 and remained stable throughout perfusion. In contrast, in livers with low BDI the median ratio was always ≤0.7 and gradually declined over time (Figure 4B-C). Another way to study the relation between biliary and perfusate glucose is by calculating the difference between perfusate and biliary glucose concentration (delta) (14). In livers with high BDI, the delta was lower compared to livers with low BDI, reaching significance at only 3 and 3.5 h NMP (Figure 4C). After several hours of NMP, negative delta values were reached.

The earliest time point at which biliary glucose and the bile/perfusate glucose ratio could discriminate livers with low BDI from those with high BDI was 2 h (SDC, Table S1). At this time point, the optimal cut-off values were 16 mmol/L and 0.67, respectively (both AUC 0.82; P=0.013) (Figure 4F-G). The delta between perfusate and biliary glucose did not result in an AUC > 0.80 at any time point, though at 3 h NMP an AUC of 0.79 resulted in a cut-off value of 2.3 mmol/L (P=0.030, Figure 4F-G, SDC, Table S1).
Figure 3. Biliary bicarbonate and pH produced during normothermic machine perfusion (NMP) correlated significantly with bile duct injury (BDI). Biliary bicarbonate concentration (A) and biliary pH (B) were significantly higher in livers with a low BDI score, compared to livers with a high BDI score at each time point during NMP. (C) Biliary pH and bicarbonate concentration were strongly correlated with each other. The line of best fit shows that in the lower range of biliary bicarbonate, small increases in biliary bicarbonate led to relatively large increases in biliary pH. At biliary bicarbonate >30 mmol/L, biliary pH remained relatively stable despite further increases in bicarbonate concentration. (D) ROC curves and (E) statistical analyses of biliary bicarbonate and pH used to discriminate high and low bile duct injury at the earliest time points. * p <0.05. More detailed results, including calculations for all time points of bile collection during 6 h of normothermic machine perfusion, are provided in SDC, Table S1. Abbreviations: AUC-ROC, area under the curve of the receiver operating characteristics curve; CI, confidence interval; LDH, lactate dehydrogenase; LR+, positive likelihood ratio; LR-, negative likelihood ratio; NPV, negative predictive value; PPV, positive predictive value; Sens, sensitivity; Spec, specificity.
Figure 4. Biliary glucose, bile/perfusate glucose ratio and biliary LDH correlated significantly with bile duct injury (BDI) during normothermic machine perfusion (NMP). (A) Biliary glucose concentration, inversely reflecting biliary epithelial cell function, was significantly lower in livers with low BDI, compared to livers with high BDI at nearly each time point during NMP. (B) In livers with high BDI, the bile/perfusate glucose concentration ratio was higher compared to livers with low BDI. (C) The delta between perfusate and biliary glucose concentration was lower in livers with high BDI, even reaching negative values at the end of NMP. (D) Livers with high BDI have relatively higher biliary glucose in relation to perfusate glucose concentrations, compared to livers with a low BDI. This resulted in a steeper slope in high BDI livers (slope 0.88), compared to low BDI livers (slope 0.62). (E) Biliary LDH concentration, a marker of biliary epithelial cell injury, was significantly higher at each time point in livers with a high BDI score compared to livers with a low BDI score. (F) ROC curves and (G) statistical analyses of biliary glucose, bile/perfusate glucose ratio, perfusate - biliary glucose delta and biliary LDH used to discriminate high and low bile duct injury at the earliest time points. * p <0.05. More detailed results, including calculations for all time points of bile collection during 6 h of normothermic machine perfusion, are provided in SDC, Table S1. Abbreviations: AUC-ROC, area under the curve of the receiver operating characteristics curve; CI, confidence interval; LDH, lactate dehydrogenase; LR+, positive likelihood ratio; LR-, negative likelihood ratio; NPV, negative predictive value; PPV, positive predictive value; Sens, sensitivity; Spec, specificity.

Biliary LDH Correlates Significantly with BDI

Biliary LDH concentration, a marker of biliary epithelial cell injury, was >2-fold higher in livers with high BDI, compared to livers with low BDI (Figure 4E). In both groups biliary LDH concentration declined gradually during NMP. The earliest time point with a significant AUC-ROC for biliary LDH was 2 h NMP (SDC, Table S1), with an optimal cut-off value of 3,689 U/L and an AUC of 0.83 (P=0.014) (Figure 4F-G).
Clinical Validation of Biliary Biomarkers during NMP

Based on the preclinical research data, we included biliary pH as one of the criteria for hepatobiliary viability assessment in a clinical trial of end-ischemic NMP of high-risk livers that were initially declined nationwide for transplantation. So far, 6 DCD livers (median Eurotransplant-Donor Risk Index (30) (ET-DRI): 2.9 (IQR 2.7 – 2.9)) have been included in this trial of which 4 (median ET-DRI: 2.9, (IQR 2.8 – 3.0); median United Kingdom-DCD risk score (31) 6.0 (IQR 5.8 – 7.5)) met all selection criteria for transplantation during NMP, including a biliary pH >7.48 within 2.5 h of NMP. In addition, biliary bicarbonate and glucose bile/perfusate ratio were within the ranges identified in the preclinical study (Figure 5). The recipients of these four liver grafts had an uneventful recovery and none of them developed clinical evidence of post-transplant cholangiopathy at a median follow-up of 8.3 months (IQR 7.6 – 10.1). Of the two livers that were declined for transplantation, the first fulfilled all hepatocellular criteria and was declined only on the basis of bile biochemistry (at 2.5 h NMP: cumulative bile production 45 mL (≥10 mL within 2.5 h NMP and ≥4 mL in preceding hour); perfusate lactate 0.3 mmol/L (<1.7 mmol/L); perfusate pH 7.38 (7.35 – 7.45)). The second liver was also declined based on hepatocellular criteria (cumulative bile production 57 mL; perfusate lactate 3.5 mmol/L; perfusate pH 7.25). At 2.5 h NMP, the perfusate ALT concentration was approximately 4,000 U/L and 6,000 U/L respectively. The retrospectively determined median BDI score for the transplanted livers was 3.2 (IQR 2.8 – 3.5) and 4.3 (3.0, 5.5) for the non-transplanted livers.
Figure 5. Clinical validation of biliary biomarkers during ex-situ normothermic machine perfusion (NMP) of transplanted and non-transplanted livers. Six livers that were declined for transplantation nationwide underwent end-ischemic NMP to assess their viability for transplantation in a clinical trial. According to protocol, viability assessment occurred within 2.5 h NMP. Four livers fulfilled the hepatobiliary criteria and were transplanted. There was no clinical evidence of post-transplant cholangiopathy at a median of 8.3 months (IQR 7.6 – 10.1) follow-up. Dotted lines indicate biliary biomarker cut-off values established in the preclinical study.
Discussion

In the present study, we have determined the diagnostic accuracy of the previously described biomarkers of biliary injury and viability, which can be easily assessed point-of-care during ex situ NMP of human donor livers. Biliary bicarbonate concentration >18 mmol/L, biliary pH >7.48, biliary glucose concentration <16 mmol/L, bile/perfusate glucose concentration ratio <0.67, and biliary LDH concentration <3,689 U/L within 2.5 h of NMP were strongly associated with low histological BDI. These findings have important clinical implications as the proposed biomarkers allow transplant teams to stratify livers grafts during ex situ NMP based on the risk of BDI, and potentially also that of post-transplant cholangiopathy. Biliary bicarbonate had the highest predictive value out of all of the studied biomarkers. This is likely because biliary bicarbonate is less influenced by other, non-biliary factors.

Ex situ NMP is increasingly being applied and explored as a tool to assess viability of liver grafts that were initially declined for transplantation based on a perceived high risk of early graft failure (11-14). In most centers, selection criteria are currently based on hepatocellular injury and function. The risk of post-transplant graft failure, however, is not only determined by the degree of hepatocellular injury, but also by the presence of biliary injury. Especially DCD livers have an increased risk of developing biliary complications (32). In a recent clinical study of 12 initially declined liver grafts that were identified as transplantable during end-ischemic NMP, 25% developed post-transplant cholangiopathy despite adequate hepatocellular function during pre-transplant ex situ NMP.13 These clinical data are in line with our observation that some livers with a low degree of hepatocellular injury can still have a high degree of BDI, which can be missed when no specific biliary viability criteria are used. This difference in hepatocellular and cholangiocellular injury can be explained by the greater susceptibility of cholangiocytes to ischemia-reperfusion injury and a slower post-ischemic recovery of intracellular ATP, compared to hepatocytes (23, 33).

The parameters we have selected as potential biomarkers of biliary viability were all based on the known physiological function of healthy bile duct epithelium. Two important physiological functions of biliary epithelium are active secretion of bicarbonate and reabsorption of glucose, leading to an alkalotic pH and very low biliary glucose concentrations at the level of the extrahepatic bile duct (17,18). We have previously suggested that these parameters could potentially serve as biomarkers of bile duct viability during machine perfusion (26,34,35). In the current study, we demonstrated that biliary pH, bicarbonate and glucose concentration indeed strongly correlate with histological BDI of liver grafts during NMP, confirming observations made earlier this year (14). In three independent clinical studies, the histological degree of BDI before liver transplantation has been identified as a significant predictor of the development of NAS after transplantation (8-10).

Our findings suggest that biliary epithelial cells of livers with high BDI are unable to secrete sufficient amounts of bicarbonate to raise the biliary pH. Increasing the biliary pH helps biliary epithelial cells to protect themselves against the toxic effects of hydrophobic bile salts, which is also known as the “biliary bicarbonate umbrella” (36). Low biliary pH and bicarbonate, therefore, not only reflect biliary injury/dysfunction, but may also contribute to additional biliary injury due to an absent bicarbonate umbrella (37). Biliary pH and bicarbonate were not linearly correlated, which is explained by the fact pH varies on a logarithmic scale, whereas bicarbonate does not. The pH values we observed in liver grafts with low BDI are within the normal range of biliary pH, which varies between 7.5 and 8.1 in the common bile duct (17). The same is true for biliary bicarbonate concentration, which normally ranges widely between 12 and 55 mmol/L (18). Interestingly, Watson et al. recently reported three patients that developed cholangiopathy after the transplantation of livers that were unable to produce bile with a pH >7.4 during NMP.14 This preliminary clinical observation is in line with the identified
biomarkers of BDI in the current study in preclinical livers as well as the clinical validation cohort.

While biliary glucose also correlated significantly with the degree of biliary injury, the interpretation of glucose values is slightly more complex. Post-ischemic reperfusion of a liver graft almost universally results in a pronounced increase of glucose levels in the perfusion fluid due to glycogenolysis (38). This contributes to a higher glucose concentration in the primary bile produced by hepatocytes, which affects the reabsorption of glucose from bile when it passes from the canaliculi to the common bile duct. Even when the biliary epithelium is intact, glucose reabsorption via SGLT1 and GLUT1 becomes insufficient when biliary glucose concentrations are too high, a phenomenon analogous to the renal threshold for glucose in kidney tubuli (22,39). In other words, high glucose concentration in the perfusion fluid (which is frequently seen during NMP) may affect the biliary reabsorption of glucose. Biliary glucose levels should therefore be viewed in relation to glucose levels in the perfusion fluid. To illustrate this, we have correlated glucose concentrations in bile with those in the perfusion fluid. The ratio between glucose in bile and perfusion fluid was almost 1 in livers with high BDI, while it was ≤0.7 in livers with low BDI. Others have reported the delta between perfusate and biliary glucose levels, though in our hands this did not result in a usable criterion. Our findings regarding biliary glucose are in line with an in vitro study with rat cholangiocytes, in which ATP-depleted cholangiocytes showed prolonged dysfunction of biliary glucose transporter SGLT1 and diminished glucose reabsorption (14,23). Similarly, in an experimental study in rabbits, higher glucose concentrations were found in bile of livers that had suffered from warm and cold ischemia, compared to livers that had not been ischemic (40).

The observations made for biliary bicarbonate, pH and glucose were supported by the data on biliary LDH concentrations. Based on animal experiments, biliary LDH has previously been proposed as a marker of biliary epithelial injury (24). In the current study, biliary LDH was indeed significantly higher in livers with high BDI, compared to livers with low BDI. In all livers, however, biliary LDH concentration declined gradually during NMP, which can be explained by an early washout of LDH from dead cholangiocytes when bile flow recurs. This is in line with previous research, where biliary LDH correlated with the length of cold ischemia in a rat liver model and previous studies that reported lower LDH concentrations in machine perfused livers compared to cold stored livers (25,27,41).

This study has some limitations. We took biopsies from the extrahepatic bile duct to assess the degree of histological injury, while the intrahepatic bile ducts can also be involved in post-transplant cholangiopathy. However, in a previous study, we have demonstrated that histological injury (in particular injury to the peribiliary vascular plexus, extramural peribiliary glands and stroma necrosis) assessed at the level of the extrahepatic bile duct also reflects the degree of injury in the intrahepatic bile ducts, as deep as the segmental bile ducts (42). Furthermore, there may have been some degree of sampling error in bile duct biopsies. To minimize this, the average BDI of two biopsies was taken. Furthermore, our preclinical data included 5 DBD livers, whereas the clinical livers were all DCD. However, when we excluded the DBD livers in the preclinical study, similar results were obtained. Lastly, some livers produce very little or no recorded bile during NMP. This raises the question as to whether or not such livers can be transplanted safely. We would advocate an approach tailored to the specific donor liver and recipient. For example, when assessing a liver with a high risk of developing NAS, production of good quality bile during NMP would be essential to allow cholangiocyte viability assessment prior to transplantation. On the other hand, for livers with a low risk of NAS, cholangiocyte viability assessment may not always be necessary, as has been reported in the literature (14).

Implementation of the identified biomarkers in larger clinical trials of NMP with longer follow-up, in which both DCD and DBD livers are included, is necessary to confirm their
utility in decreasing the incidence of post-transplant cholangiopathy. Machine perfusion has the potential to provide opportunities to ameliorate BDI, for instance through stem cell therapy. These cholangiocellular criteria could be applied to identify livers that require additional pharmacological interventions, and be used to assess the efficacy of such treatments. In conclusion, biliary bicarbonate, pH, glucose, and LDH during \textit{ex situ} NMP are accurate biomarkers of clinically relevant, histological biliary injury of livers grafts. These biomarkers can be easily determined point-of-care, making them suitable for the assessment of bile duct viability during NMP and the potential identification of donor livers with a low risk of developing post-transplant cholangiopathy.

**Acknowledgements**

The authors would like to thank all donors and donor relatives, as well as the Dutch Transplantation Society and its staff, for enabling the use of donor livers for research.
References

1. Jochmans I, van Rosmalen M, Pirenne J, Samuel U. Adult liver allocation in Eurotransplant. *Transplantation.* 2017;101:1542-1550.

2. de Vries Y, von Meijenfeldt FA, Porte RJ. Post-transplant cholangiopathy: Classification, pathogenesis, and preventive strategies. *Biochim Biophys Acta.* 2017.

3. Meurisse N, Vanden Bussche S, Jochmans I, et al. Outcomes of liver transplantations using donations after circulatory death: a single-center experience. *Transplant Proc.* 2012;44:2868-2873.

4. Jay CL, Lykensburg V, Ladner DP, et al. Ischemic cholangiopathy after controlled donation after cardiac death liver transplantation: a meta-analysis. *Ann Surg.* 2011;253:259-264.

5. Dubbeld J, Hoekstra H, Farid W, et al. Similar liver transplantation survival with selected cardiac death donors and brain death donors. *Br J Surg.* 2010;97:744-753.

6. Pine JK, Aldouri A, Young AL, et al. Liver transplantation following donation after cardiac death: an analysis using matched pairs. *Liver Transpl.* 2009;15:1072-1082.

7. Chan EY, Olson LC, Kisthard JA, et al. Ischemic cholangiopathy following liver transplantation from donation after cardiac death donors. *Liver Transpl.* 2008;14:604-610.

8. Hansen T, Hollemann D, Pitton MB, et al. Histological examination and evaluation of donor bile ducts received during orthotopic liver transplantation—a morphological clue to ischemic-type biliary lesion? *Virchows Arch.* 2012;461:41-48.

9. Brunner SM, Junger H, Ruemmele P, et al. Bile duct damage after cold storage of deceased donor livers predicts biliary complications after liver transplantation. *J Hepatol.* 2013;58:1133-1139.

10. op den Dries S, Westerkamp AC, Karimian N, et al. Injury to peribiliary glands and vascular plexus before liver transplantation predicts formation of non-anastomotic biliary strictures. *J Hepatol.* 2014;60:1172-1179.

11. Sutton ME, op den Dries S, Karimian N, et al. Criteria for viability assessment of discarded human donor livers during ex vivo normothermic machine perfusion. *Plos One.* 2014;9:e110642.

12. Mergental H, Perera MT, Laing RW, et al. Transplantation of Declined Liver Allografts Following Normothermic Ex-Situ Evaluation. *Am J Transplant.* 2016;16:3235-3245.

13. Watson CJE, Kosmoliaptsis V, Randle LV, et al. Normothermic Perfusion in the Assessment and Preservation of Declined Livers Before Transplantation: Hyperoxia and Vasoplegia-Important Lessons From the First 12 Cases. *Transplantation.* 2017;101:1084-1098.

14. Watson CJE, Kosmoliaptsis V, Pley C, et al. Observations on the ex situ perfusion of livers for transplantation. *Am J Transplant.* 2018.

15. Cohn JA, Strong TV, Picciotto MR, Nairn AC, Collins FS, Fitz JG. Localization of the cystic fibrosis transmembrane conductance regulator in human bile duct epithelial cells. *Gastroenterology.* 1993;105:1857-1864.

16. Martinez-Anso E, Castillo JE, Diez J, Medina JF, Prieto J. Immunohistochemical detection of chloride/bicarbonate anion exchangers in human liver. *Hepatology.* 1994;19:1400-1406.

17. Sutor DJ, Wilkie LI. Diurnal variations in the pH of pathological gallbladder bile. *Gut.* 1976;17:971-974.

18. Boyer JL. Bile formation and secretion. *Compr Physiol.* 2013;3:1035-1078.

19. Lazaridis KN, Pham L, Vroman B, de Groen PC, LaRusso NF. Kinetic and molecular identification of sodium-dependent glucose transporter in normal rat cholangiocytes. *Am J Physiol.* 1997;272:G1168-74.

20. Masyuk AI, Masyuk TV, Tietz PS, Splinter PL, LaRusso NF. Intrahepatic bile ducts transport water in response to absorbed glucose. *Am J Physiol Cell Physiol.* 2002;283:C785-91.

21. Schein CJ, Zumoff B, Kream J, Cassouto J, Hellman L. A blood-bile glucose barrier in man. *Gastroenterology.* 1968;54:1094-1097.

22. Guzelian P, Boyer JL. Glucose reabsorption from bile. Evidence for a biliohepatic circulation. *J Clin Invest.* 1974;53:526-535.

23. Doctor RB, Dahl RH, Salter KD, Fouassier L, Chen J, Fitz JG. ATP depletion in rat cholangiocytes leads to marked internalization of membrane proteins. *Hepatology.* 2000;31:1045-1054.

24. Vajdova K, Smrekova R, Kukan M, Lutterova M, Wsolova L. Bile analysis as a tool for assessing pathological gallbladder bile. *Hepatology.* 1976;17:971-974.

25. Wsolova L. Bile analysis as a tool for assessing pathological gallbladder bile. *Hepatology.* 1976;17:971-974.

26. Vajdova K, Smrekova R, Kukan M, Lutterova M, Wsolova L. Bile analysis as a tool for assessing pathological gallbladder bile. *Hepatology.* 1976;17:971-974.
27. Liu Q, Nassar A, Farias K, et al. Sanguineous normothermic machine perfusion improves hemodynamics and biliary epithelial regeneration in donation after cardiac death porcine livers. *Liver Transpl.* 2014;20:987-999.

28. Matton APM, Burlage LC, van Rijn R, et al. Normothermic machine perfusion of donor livers without the need for human blood products. *Liver Transpl.* 2018;24:528-538.

29. Laing RW, Mergental H, Yap C, et al. Viability testing and transplantation of marginal livers (VITTAL) using normothermic machine perfusion: study protocol for an open-label, non-randomised, prospective, single-arm trial. *BMJ Open.* 2017;7:e017733-2017-017733.

30. Braat AE, Blok JJ, Putter H, et al. The Eurotransplant donor risk index in liver transplantation: ET-DRI. *Am J Transplant.* 2012;12:2789-2796.

31. Schlegel A, Kalisvaart M, Scalera I, et al. The UK DCD Risk Score: A new proposal to define futility in donation-after-circulatory-death liver transplantation. *J Hepatol.* 2018;68:456-464.

32. Blok JJ, Detry O, Putter H, et al. Longterm results of liver transplantation from donation after circulatory death. *Liver Transpl.* 2016;22:1107-1114.

33. Noack K, Bronk SF, Kato A, Gores GJ. The greater vulnerability of bile duct cells to reoxygenation injury than to anoxia. Implications for the pathogenesis of biliary strictures after liver transplantation. *Transplantation.* 1993;56:495-500.

34. Bruinsma BG, Yeh H, Ozer S, et al. Subnormothermic machine perfusion for ex vivo preservation and recovery of the human liver for transplantation. *Am J Transplant.* 2014;14:1400-1409.

35. Weeder PD, van Rijn R, Porte RJ. Machine perfusion in liver transplantation as a tool to prevent non-anastomotic biliary strictures: Rationale, current evidence and future directions. *J Hepatol.* 2015;63:265-275.

36. Beuers U, Hohenester S, de Buy Wenniger LJ, Kremer AE, Jansen PL, Elferink RP. The biliary HCO\(_3\)(-) umbrella: a unifying hypothesis on pathogenetic and therapeutic aspects of fibrosing cholangiopathies. *Hepatology.* 2010;52:1489-1496.

37. Op den Dries S, Sutton ME, Lisman T, Porte RJ. Protection of bile ducts in liver transplantation: looking beyond ischemia. *Transplantation.* 2011;92:373-379.

38. Villalobos-Molina R, Saavedra-Molina A, Devlin TM. Effect of hypoxia and reoxygenation on metabolic pathways in rat hepatocytes. *Arch Med Res.* 1998;29:219-223.

39. Lawrence RD. Renal Threshold for Glucose: Normal and in Diabetics. *Br Med J.* 1940;1:766-768.
