Novel miRNA Profiling as a Biomarker to Predict Ischemic Cholangiopathy and Graft Loss in Donation after Circulatory Death (DCD) Liver Transplantation

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ABSTRACT

Research Question: What differences can be seen amongst donation after circulatory death (DCD) liver graft recipient whom develop ischemic cholangiopathy compared to DCD recipients who do not develop IC and donation after brain death recipients.

Background, significance, and rationale: The use of donation after circulatory death (DCD) liver grafts has emerged in the effort to address organ shortage through expanding criteria for donor selection. However, DCD liver transplantation has been associated with increased morbidity and graft loss vs. donation after brain death (DBD) liver grafts. Ischemic cholangiopathy (IC) is recognized as a major post-transplant complication that can occur following DCD liver transplantation, leading to graft dysfunction, potential graft loss, and in some cases, re-transplantation. Multiple mechanisms may contribute to cholangiocyte injury during DCD transplant, including ischemia and bile salt toxicity.

Materials and Methods: Our study investigated current literature surrounding the development and prevention of IC. Additionally, DCD with IC cohort was developed from the liver transplant recipient database. A cohort of DCD and DBD recipients who do not develop IC was used as controls. Clinical data was analyzed from the cohort groups. Lastly, a cohort of whether a change in RNA expression, including selective expression of circulating messenger RNAs (mRNA), associated with control of inflammatory markers and bile salt composition was evaluated between DCD recipients who develop IC compared with DCD and DBD recipients who did not develop IC.

Results: We first performed a literature review on the current knowledge surrounding the proposed mechanisms of cholangiocyte injury associated with IC and the clinical management of IC. We evaluated clinical data from our cohorts and found significant changes in creatinine and

ALT levels prior to transplant ($p<0.05$). Next, we evaluated miRNA from blood samples of recipients at specific time points before and after transplantation within a cohort of DCD recipients with established IC. Blood samples were undergoing evaluation by the time of this writing and will be evaluated in future studies. We anticipated that DCD recipients with IC will demonstrate an miRNA profile characterized by elevated concentrations of inflammatory markers and downregulation of bicarbonate and glucose transporters.

Conclusion: Our review paper provided understanding of risk factors contributing to the development of IC may play an important role in optimizing transplant outcomes, including patient and graft selection, preventing the development of IC, improving long-term liver graft function, avoiding re-transplantation, and improving morbidity within DCD liver graft recipients. Additionally, our clinical analysis further supported other current studies and provided understanding for potential biomarkers for detection of development of IC.

RESEARCH QUESTION

Is there a change in the miRNA profile immediately after liver transplantation related to inflammation and bile salt composition within DCD (donation after circulatory death) liver recipients who develop IC vs DCD and DBD (donation after brain death) recipients who do not develop IC? Is there a specific profile that may provide a risk assessment at early time points predictive of graft loss in the setting of DCD with IC?

INTRODUCTION, SIGNIFICANCE, RATIONALE

Chronic Liver Disease

Chronic liver disease has become a major global health burden as cirrhosis mortality rates have increased to 2% of total global deaths in 2010 [1] and liver disease is currently the 11th leading cause of death in the United States overall[2]. Cirrhosis results from chronic liver injury, inflammation, hepatocyte cell death, and progressive fibrosis. The development of cirrhosis drastically reduces liver function and can result in progressive liver failure and serious life-threatening complications associated with portal hypertension[3]. The most common causes of cirrhosis are alcohol-related liver disease, chronic hepatitis C, and nonalcoholic fatty liver disease in Western countries and chronic hepatitis B in Asia-Pacific regions[4].

The liver has many functions, including regulation of nutrients and protein synthesis. As a result, cirrhotic patients often suffer from malnutrition, cachexia, and protein synthesis dysfunction[5]. While the processes that lead to cirrhosis are complex and multifaceted, the loss of functional hepatocytes and increase in portal hypertension due to advancing fibrosis are the primary contributors to the clinical manifestations associated with end-stage liver disease. The loss of

functional hepatocytes leads to decreased metabolism of bilirubin and decreased synthesis of proteins such as albumin and clotting factors[3]. Meanwhile, increased fibrosis of the liver leads to portal hypertension, resulting in complications that define clinical decompensation, including variceal hemorrhage, hepatic encephalopathy, and ascites[3]. Patients with compensated cirrhosis may have little or no symptoms and a mean survival of ~6.5 years. In contrast, the mean survival for decompensated cirrhosis is ~2.5 years due to the risk of life threatening complications a patient is likely to experience during that time[3]. Patients with decompensated cirrhosis may be considered as candidates for liver transplantation.

Circulatory Death Liver

Historically, most organ donations for liver transplantation occur in the setting of donation after brain death (DBD). Donation after circulatory death (DCD) has been increasingly utilized in addition to DBD to address the discordance between organ availability and the number of patients on the liver transplant waitlist[6]. DCD liver grafts, by definition, have ischemic injury. Therefore careful selection of DCD donors and recipients is critical in order to minimize the potential for surgical complications, hemodynamic instability, and graft injury[6]. Although DBD and DCD recipients may have similar pre-transplant liver disease severity with no difference in median MELD score at transplantation and no reported difference in overall post-transplant survival, DCD liver transplantation is associated with an increased risk of graft loss[7], resulting in the potential need for repeat liver transplantation.

Graft loss associated with DCD liver transplantation has been mainly attributed to the increased incidence of biliary complications[6–8], in particular ischemic cholangiopathy (IC). The

incidence of IC has been reported in up to 10% of cases in established DCD LT programs[9]. Various mechanisms have been proposed in the pathophysiology of IC, primarily stemming from observed perioperative events involving the graft that characterize and differ between DBD and DCD liver transplantation, including differences in ischemic times, the presence of microthrombi, and changes in bile acid composition as a result of ischemia conditions. In this review, these proposed mechanisms will be summarized as well as management strategies to minimize the potential for IC and associated complications.

Mechanisms of Cholangiocyte Injury in Ischemic Cholangiopathy

In contrast with strictures occurring at the site of biliary anastomoses in association with factors including surgical technique, local ischemia, or bile leak,[10] IC is characterized by nonanastomotic biliary stricturing and can be found throughout the biliary tree. IC occurs more frequently in recipients of DCD liver transplants, with a reported incidence of up to 10-30% in DCD vs. 1-10% of DBD recipients,[9,11] and typically occurs within 3 to 6 months following liver transplantation[12,13]. Clinical features at the time of presentation are similar to anastomotic biliary strictures, including onset of jaundice, fever, or abdominal pain; however, some patients may remain asymptomatic[12]. Laboratory studies associated with IC typically demonstrate a pattern of abnormal liver function consistent with cholestasis. Ultimately, the diagnosis of IC is confirmed by the presence of intrahepatic strictures, dilatation or irregularity of the intra or extra hepatic bile ducts, either with or without biliary sludge formation, at sites other than the biliary anastomosis, which can be seen on magnetic resonance imaging, endoscopic retrograde cholangiography, or percutaneous transhepatic cholangiography[13,14].

Subtypes of IC have been identified based on imaging and location of stricturing disease, according to a study conducted by Croome et al., including diffuse necrosis, multifocal progressive, confluence dominant, and minor form[9]. These IC subtypes not only differ based on features seen on cholangiography, but may also have an impact on clinical course and long term outcomes, including rates of retransplantation[9]. Diffuse necrosis is severe abnormalities of the entire biliary tree, having the shortest amount of time of all the subtypes between transplant and diagnosis, suffering from frequent hospital admissions, and a nonexistent recovery as all patients were relisted for transplant[9]. Multifocal progressive has mild to moderate stenosis of the second-order peripheral ducts with progressive worsening over time, suffering from frequent hospital admissions, with ultimately 66% of patients being relisted for transplantation or were deceased by 5 years following initial transplant[9]. Confluence dominant is defined with strictures and casts confined to the biliary confluence that never expand beyond the confluence. Patients undergo multiple ERCP procedures in the first year following transplant, with ultimately 17% being relisted for transplant, being overall managed without retransplant[9]. Finally, minor form has mild radiologic abnormalities that ultimately resolve without developing more extensive strictures, with limited need for stent placement or repeat procedures, and with 100% graft survival up to year 3 after transplantation[9]. Management may involve cholangioplasty, stenting, drain placement, or revascularization[12,15]. In many cases IC is resistant to therapy and can result in long-term sequelae[15], including the need for retransplantion, which may be required in as many as 30% to 50% in some reports[16]. The source of cholangiocyte injury in IC appears to be multifactorial and may involve multiple mechanisms, including ischemia-related injury, immune-mediated injury, and cytotoxic injury induced by hydrophobic bile salts[15].

Ischemia

The utilization of DCD liver transplantation has given rise to the concept of donor warm ischemia time (DWIT). Classically, DBD liver transplants had only cold ischemia time (CIT), with cooling or chilling of the organ occurring simultaneously with removal of the organ's blood supply. CIT continues until the restoration of warm circulation after transplantation[17]. DCD donation is defined by standard circulatory arrest criteria with an additional period of DWIT,[18,19] which can be further divided into phases according to the National Conference on Donation After Cardiac death including a withdrawal phase and an acirculatory phase. Withdrawal phase is defined as the period from withdrawal of ventilatory support to cardiopulmonary cessation. Acirculatory phase is defined as the time from cardiopulmonary cessation until cold perfusion[17].

While there is a general consensus that prolonged DWIT negatively effects outcomes, there is no agreement on what length of DWIT is acceptable or how DWIT is defined[6]. According to the National Conference of Donation After Cardiac Death, warm ischemia time should not exceed 30 minutes for successful liver transplantation, as the potential risk of post-transplant biliary strictures increases after this timepoint[17]. Meanwhile, CIT should not exceed 8 hours[17]. Studies have demonstrated an association between longer DWIT length and the development of IC[13,14,20,21]. Moreover, both prolonged CIT and DWIT have been associated with early IC (within weeks to months) in contrast with later IC development (months to years)[13].

During transplantation, there is a both ischemia and reperfusion. The biliary system is more prone to ischemic injury compared to hepatic parenchyma. Unlike the hepatic parenchyma, which has dual blood supply from the hepatic artery and portal vein, the biliary system depends solely on the arterial supply[21]. In ischemic conditions, depletion of intracellular ATP results in the generation reactive oxygen species (ROS), resulting in intracellular calcium overload,

cytokine and caspase activation, and Bcl-2 gene production. The overall result is apoptosis of the cell[22]. Once reperfusion is obtained, inflammation can be further exacerbated due to introduction of immune cells, blood, and oxygen to the previously ischemic tissue. A further increase of ROS is seen within tissue as oxygen meets newly perfused tissue depleted of ATP stores[22–24]. Hepatocytes were seen to have more glutathione and produce less reactive oxygen species compared to bile duct cells, resulting in bile duct cells being more susceptible to reperfusion injury than hepatocytes[23]. Hepatocytes have a large regenerative capacity due to hepatic stellate and Kupffer cell regulation of cell proliferation, remodeling, fibrinogenesis after liver ischemic-reperfusion injury[25].Multiple mechanisms related to ischemic injury appear to be the cause of cholangiocyte injury at early time points during DCD liver transplantation. First is primary ischemia occurring during the process of transplantation involving DWIT and cold ischemia, which can result in diminished perfusion to the biliary tree. Secondary ischemia may occur after transplantation and is due to endothelial cell injury involving small vessels and capillaries. Some of the downstream effects of these ischemic events include the loss of peribiliary glands, leading to reduced cholangiocyte regeneration and alterations in bile flow and composition which can result in bile salt toxicitiy[26]..

Prior studies have evaluated biomarkers related to ischemia and inflammation in DCD and DBD in the setting of IC vs no IC. These studies using immunohistochemistry found only a modest correlation between inflammatory markers and the incidence of IC, overall finding that initial liver damage in the setting of DCD was similar to DBD[18].

Hemostasis and Thrombosis

Hemostatic changes within the transplanted organ may contribute to ischemic injury as a consequence of thrombotic events leading to vascular obstruction and altered perfusion; however, unlike ischemia associated with DWIT and CIT, the effects of vascular obstruction can persist after reperfusion of the organ. Thrombotic events which will be described in depth below in the setting of DCD liver transplantation include overt vascular obstruction such as hepatic artery thrombosis (HAT) as well as microthrombi resulting from endothelial injury.

Hepatic artery thrombosis (HAT) is strongly associated with the development of non-anastomotic strictures and IC as the hepatic artery is the only source of vascular supply to the donor biliary tree. The development of HAT can result in bile duct infarction, bile duct necrosis, bile peritonitis, and multifocal strictures of the intra and extrahepatic biliary tree[27]. The incidence of HAT in DCD recipients compared to other types of donation doubled[28]. HAT has been seen to have a 7.53 relative risk on the development of IC[29], and a meta-analysis found that HAT was the most identifiable risk factor for the development of IC[30]..

Microthrombotic events have also been proposed as a cause of ischemia occurring in DCD liver transplantation and a contributing factor to development of IC. When endothelial cells of the small arteries, capillaries, and veins experience injury due to ischemia or immune mediated processes during transplantation, the coagulation cascade is activated and forms microthrombi[26], exacerbating ischemic changes from peribiliary vasculature,[16] and resulting in necrosis. Injury to peribiliary glands and the vascular plexus prior to transplantation is strongly associated with the occurrence of biliary strictures after transplantation, suggesting that insufficient regeneration due to loss of peribiliary glands or impaired blood supply may explain the development of biliary strictures[31].

Uniquely, DCD donation varies from DBD by the administration of heparin, which may contribute to the increased risk of thrombotic events observed in DCD liver transplantation. Heparin administration to the DBD donor to prevent thrombus formation has been universally accepted. However, fewer DCD donors are administered heparin, with some local policies prohibiting such a practice[16].. In a study conducted by Narvaez et al., 5945 DCD donors were either administered premortem heparin or no heparin. Heparin was not associated with liver discard. No heparin was associated with an 18% higher hazard of overall graft failure compared to those who received heparin. Additionally, there was 81% increase in odds of primary nonfunction with DCD no heparin livers compared to heparin livers. Ischemic cholangiopathy was not an available endpoint for this study[32]. In another study, 22 participants were administered tissue plasminogen activator into the grafted vasculature after anastomoses was performed. In this study, there was a reduction of IC reports compared to average reports of IC across facilities (27% vs 50% during the time of this study). However, excessive bleeding was seen in 65% of recipients, not related to the dose of tpa administration[33].

Immune-Mediated Injury

Immunologic mechanisms may also contribute to the development of IC, whether autoimmune or as a result of host immune activity within the transplanted graft. Recurrence of an underlying autoimmune process such as primary sclerosing cholangitis[13,29] may increase the risk of IC. It should be noted that there are contraindicating reports as to whether autoimmune hepatitis could increase the risk of IC development[13,29]. Additionally, CMV infection prior to transplantation may also increase the relative risk of IC, possibly due to CMV-related vasculitis[21]. CMV inclusions have been observed histologically in arterioles adjacent to bile

ducts and in capillary endothelial cells of the gallbladder, suggesting the potential for direct CMV-mediated vasculitis within that supply the biliary tree[21].

The immunologic response at early stages following liver transplantation may also contribute to inflammation, apoptosis, and necrosis within the graft. Apoptosis of hepatocytes and biliary endothelial cells occurs during ischemia. After reperfusion of the organ, immune cells are introduced to the recently ischemic tissue[22]. Activation of the complement cascade occurs as intracellular components are introduced into the extracellular space. The activation of the complement cascade results in the local release of anaphylatoxins C3a and C5a as well as proinflammatory cytokines[22]. As proinflammatory cytokines and chemokines are released in response to both ischemic stress and activation of the innate immune system following reperfusion, neutrophil signaling and trafficking leads to further cell-mediated injury. Neutrophil activating proteins such as CXCL1, 2, and 5[34,35], and to a lesser extent, intercellular adhesion molecules (ICAMS), 1 and vascular adhesion molecules (VCAM) 1[24,36] play an essential role in early neutrophil aggregation and subsequent hepatocellular injury. Activated neutrophils then release myeloperoxidase and other proteases that cause direct injury to liver endothelial cells. Interestingly, the role of immune cells within the vasculature is also believed to cause platelet aggregation and thrombus formation, further perpetuating microthrombi and reperfusion injury as mentioned above[22].

Altered Bile Composition

Bile composition can identify the presence of cholangiocyte injury observed histologically, demonstrating that bile composition can serve as a marker to assess the presence of biliary duct injury, including IC in DCD liver transplant recipients[37,38]. In particular, increases

in bile glucose concentration correlate with the cholangiocyte injury[37]. Cholangiocytes lining the bile duct lumen and peribiliary glands actively contribute to the composition of bile[37]. Secretion of bicarbonate occurs via the cystic fibrosis transmembrane regulator (CFTR) and bicarbonate anion exchanger 2 (AE2)[39,40]. Additionally, cholangiocytes actively absorb glucose from bile using SGLT1 on the apical side of the membrane and GLUT1 transporters on the basolateral side[41], resulting in very low concentrations of glucose within bile. Due to these transporters being ATP-dependent, concentrations of glucose and pH within bile is indicative of cholangiocyte function and therefore degree of biliary disease[37,42], such that elevations in glucose and diminished pH correlated with a decline in cholangiocyte function coinciding with injury[37,38].

Bile acid toxicity resulting from alterations in bile composition may have a role in contributing to direct cholangiocyte injury. The detergent effects of bile acids, enhanced in the setting of bile acid toxicity, interfere with phospholipid integrity and can lead to induction of cholangiocyte apoptosis[15]. An alkaline environment deprotonates hydrophobic bile acids, making them less susceptible to cell membrane injury[15,43,44]. Cholangiocytes express bicarbonate-sodium exchangers on the apical membranes, allowing for the secretion of bicarbonate into bile acid, alkalizing the environment and being a protective factor for cholangiocyte against bile toxicity[38]. These exchangers are ATP dependent, and can become nonfunctional or damaged during ischemia or reperfusion[38]. Therefore, as pH decreases in the acidic environment associated with ischemic injury, bile acids have an increased ability to create direct injury. In support of these proposed mechanisms, studies have demonstrated that accumulation of bile salts to phospholipids in bile is associated with cholangiocyte injury in animal studies[45] and two prospective clinical studies[46,47]

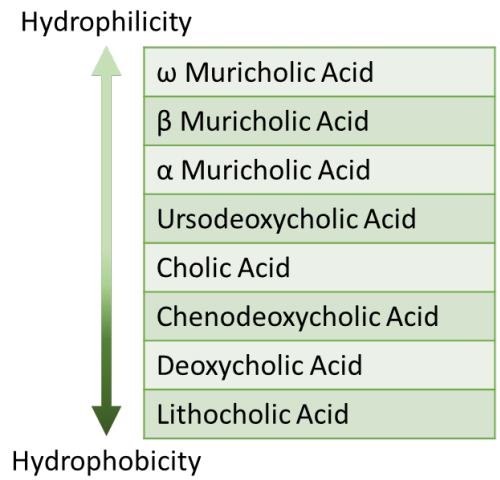

Figure 1

Mechanism of Measuring Cholangiocyte Injury

The field of measuring miRNA has been expanding. Circulating miRNA is quickly becoming a method of identifying biomarkers involved in the regulation of many disease processes[48]. Functional exosomal miRNA has been able to be extracted and purified from serum and cell culture systems. These miRNA cannot only detect particular disease processes, but have seen to forecast the progression of a disease as well. Particularly relevant to cholangiocyte injury and inflammation that may occur in the setting of IC following DCD liver transplantation, specific miRNA profiles have been associated with bile duct inflammation and impaired biliary bicarbonate secretion observed in primary sclerosing cholangitis (PSC) and primary biliary cholangitis (PBC).[48] Whether these miRNAs may also demonstrate altered expression in the setting of IC has yet to be determined.

MATERIALS AND METHODS

Establishing Review Paper

Research for the review was conducted using a literature search through PubMed, focusing on literature published in journals from the American Association for the Study of Liver Disease (AASLD) and American Society of Transplantation (AST). Additionally, attendance of the 7th Innovations in Transplantation Summit: Donation after Circulatory Death enhanced our updated understanding of current research within the field. We utilized multidisciplinary input from hepatologists and transplant surgeons who contributed to review, feedback, and authorship on the paper.

Clinical Information

The Baylor Scott & White Simmons Transplant Institute has performed more than 4,500 liver transplants, making it one of the largest transplant programs within the United States. This program has extensively recorded information about patients in a clinical database with stored specimens within a large biorepository. This information includes patient records, imaging, and frozen blood samples; known as the Liver Transplant Research Database System. This biorepository has been established with the intent on research regarding liver transplantation. We utilized this database during the first year of the project. These patients have graciously allowed for their demographic and clinical information as well as blood samples to be included within the database and biorepository for future research, and therefore permissions are obtained using the BSW protocols of information gathering in compliance and approval granted by the BSW Research Institute Institutional Review Board (see compliance plan).

Establishing patient groups

Three groups were generated for this study: DCD recipients who develop IC, DCD recipients who do not develop IC, and DBD donors who do not develop IC. Classification into each specific group was confirmed by review of medical records. Exclusion criteria include history of kidney transplantation, primary sclerosing cholangitis as the primary liver diagnosis, and presence of hepatic artery thrombosis. We developed a total cohort size of n=18, with 6 individuals per group.

Additionally, individuals with recurrent PSC after either DCD or DBD was used as controls compared to IC groups for evaluation of specific miRNA changes related purely to fibrosis formation.

Clinical outcomes

Clinical outcomes were obtained through access to patient medical records from the transplant database. Additionally, to confirm IC as opposed to other similarly presenting conditions (isolated biliary anastomotic structures, biliary strictures in the presence of hepatic artery thrombosis), a diagnosis of IC was confirmed by documented ERCP, PTC, surgically placed biliary catheter, or MRCP[9]. Key clinical data included for primary and secondary analyses including the incidence of graft loss, recipient clinical and demographic data, donor-related data, DWIT, and cold ischemia time (CIT). Specific surgical techniques including biliary tract flushing and solution and the type of machine perfusion (normothermic, hypothermic) will be analyzed as well[6,38].

mRNA profiling

We analyzed blood samples from the liver transplant biorepository at the following time points for patients in all groups (n=40): time points of 0, 30 days permitting) as IC typically presents within 12 months following liver transplantation[9] (Figure). Obtaining

blood samples: The Baylor Scott & White Liver Transplant Research Database has collected samples from transplant recipients. All samples were obtained retrospectively. From the protocols detailed by Lawrence et al. and Saravanan et al.[49], serum was required from each individual (75µL). Initial analysis using this volume was inadequate in quality, and therefore a second volume of 150µL was extracted, for a total of 225µL used.

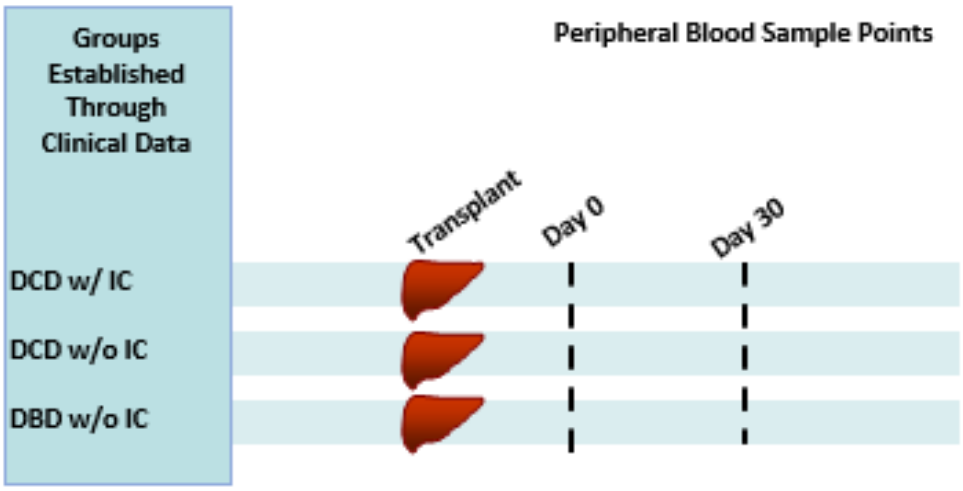

Figure 2

mRNA isolation from plasma: 150µL mRNA will be isolated using QIAGEN mRNA isolation kits. mRNA primers and Universal RT-PCR (Exiqon). These mRNA primers will be constructed using the mRNA transcriptome library and TruSeq RNA Library Prep Kit (Illumina, San Diego, CA, USA).[50] Target mRNA that we propose to evaluate are based on categories and include mRNA controlling the following mRNA inflammatory markers (Caspase-3 active, CCR5, CD44, CD90, COX-2, HIF1A, P21, TERT, and VEGF), mRNA controlling bile composition (ACOX2, AE2, AMACR, AQP1, AQP4, BSEP, CFTR, CYP7A1, GLUT1, MDR3, and SGLT1), and bile duct

pathology (Alkaline phosphatase, GGT, and LDH)[15,18,26,37,37–39,49,51–54] (Table 1). Laboratory work, including isolation and quantification will be performed at the BSW Research Institute in Dallas, Texas.

mRNA/NA	Fxn	Source
INFLAMMATORY		
COX-2	Inflammatory activation	Lopez-Lopez 2021[18]
CD90	Stem cell	Lopez-Lopez 2021
TERT	Senescence	Lopez-Lopez 2021
HIF1A	Hypoxia	Lopez-Lopez 2021, Lawrence 2019[49]
CD44	Cross-organ allograft rejection	Lopez-Lopez 2021
VEGF	Endothelial damage. 3-72 hours after	Lopez-Lopez 2021, Yi 2016[52]
P21	Early apoptosis	Lopez-Lopez 2021
Caspase-3 active	Cell damage	Lopez-Lopez 2021
CCR5 (chemokine receptor)	Bile duct injury immune related	Dries 2011[15]
MICROTHROMBI FORMATION		
TLR-4	Upregulated by neutrophils to activate platelets	Yazdani 2022[22]
Neutrophil elastase	Released by neutrophils during ischemic stress to form a clot	Yazdani 2022[22]
Cathepsin G	Released by neutrophils during ischemic stress to form a clot	Yazdani 2022[22]
BILE COMPOSITION		
CFTR	Cl- excretion	Cohn 1993[39], De Vries 2018[26]
AE2 (cholangiocyte exchanger)	Cl-HCO3- exchanger (buffer system)	De Vries 2018, Sutor and Wilkie 1976[53], Pisarello 2015[48]
BSEP (Bile-salt excretion pump)	Bile salt secretion	De Vries 2018
Multidrug resistant protein 3 (MDR3)	Bile salt, biliary, phospholipid secretion	De Vries 2018
GLUT 1	Bile glucose concentration	Gaurav 2020[38], Masyuk 2002[54], Matton 2019[37]

SGLT 1	Bile glucose concentration	Gaurav 2020, Masyuk 2002, Matton 2019
AQP1, AQP4	Bile glucose water exchanger	Gaurav 2020, Masyuk 2002
CYP7A1	Bile synthesis	Lawrence 2019
AMACR	Bile synthesis	Lawrence 2019
ACOX2	Bile synthesis	Lawrence 2019
BILE DUCT PATHOLOGY		
GGT		Lopez-Lopez 2021, Vajdova 2000[42]
Alkaline Phosphatase		Vajdova 2000
LDH	Biliary epithelium damage	Matton 2019, Vajdova 2000

Table 1-miRNA found in literature related to liver transplantation, inflammation, and bile acid composition

Bile Acid Phenotyping

Bile acid phenotyping was performed using the protocol as established through Biocrates® Bile Acids Kit. Bile acids that will be analyzed are demonstrated in table 2 below. 10µL of serum or plasma obtained from recipient samples of all cohorts on days 0, and 30 (or sample point approximated to said time points) for analysis.

Obtaining blood samples: The Baylor Scott & White Liver Transplant Research Database has collected samples from transplant recipients. All samples were obtained retrospectively.

No	Analyte	Name	Internal Standard	Human plasma/ serum	Mouse plasma	Calibration range (LLOQ – ULOQ in µM)
1	CA	Cholic acid	d5-CA	✓	✓	0.03 –75
2	CDCA	Chenodeoxycholic acid	d5-CDCA	✓	✓	0.02 – 30
3	DCA	Deoxycholic acid	d5-CDCA	✓	✓	0.02 – 10
4	GCA	Glycocholic acid	d5-GCA	✓	✓	0.03 – 75
5	GCDCA	Glycochenodeoxycholic acid	d4-GLCA	✓		0.02 – 20
6	GDCA	Glycodeoxycholic acid	d4-GLCA	✓	✓	0.01 – 10
7	GLCA	Glycolithocholic acid	d4-GLCA	✓	✓	0.01 – 5
8	GUDCA	Glycoursodeoxycholic acid	d4-GUDCA	✓	✓	0.01 – 10
9	HDCA	Hyodeoxycholic acid	d4-HDCA(b)		✓	0.01 – 5
10	LCA	Lithocholic acid	d4-LCA	✓	✓	0.01 – 5
11	MCA(a)	Alpha-Muricholic acid	d5-CA		✓	0.01 – 5
12	MCA(b)	Beta-Muricholic acid	d5-CA		✓	0.01 – 10
13	MCA(o)	Omega-Murichoclic acid	d5-CA		✓	0.01 – 5
14	TCA	Taurocholic acid	d5-TCA	✓	✓	0.02 – 50
15	TCDCA	Taurochenodeoxycholic acid	d5-TCDCA	✓	✓	0.01 – 20
16	TDCA	Taurodeoxycholic acid	d5-TCDCA	✓	✓	0.01 – 10
17	TLCA	Taurolithocholic acid	d4-GLCA	✓	✓	0.01 – 5
18	TMCA (a+b)	Tauromuricholic acid (sum of alpha and beta)	d5-TUDCA	✓	✓ **	0.01 – 10
19	TUDCA	Tauroursodeoxycholic acid	d5-TUDCA	✓	✓ *	0.01 – 15
20	UDCA	Ursodeoxycholic acid	d4-HDCA(b)	✓	✓	0.02 – 30

* Partial coelution with THDCA under UHPLC conditions, quantifiable only under HPLC conditions
** semi-quantitative for Waters Xevo™ TQ MS
✓ Generally present at very low concentrations (close to or < LLOQ) in healthy samples
✓ Generally present concentrations well above LLOQ in healthy samples

Table 2- Hydrophobic bile acids proposed for analysis using bile acid phenotyping

Statistical Analysis

A primary statistical analysis was performed evaluating miRNA levels and RNA gene expression at specified time points comparing DCD recipients with IC (DCD-IC) vs. DCD recipients without IC (DCD-nonIC) vs. DBD recipients, and a comparison of IC vs. nonIC groups. Selection of DCD-nonIC and DBD controls will be performed by propensity score matching based on baseline covariates. Pairwise comparisons between categorical variables was assessed using the chi-square and Fisher's exact tests. Continuous variables will be assessed for normal distribution and comparisons among variables with normal distribution was made using the two-sample T test (student's T test) or one-way analysis of variance (ANOVA) for multiple comparisons. Variables with nonparametric distribution will be made with the two-sample Wilcoxon rank-sum test or

Kruskal Wallis rank test for multiple comparisons. For correlation studies, Pearson's two-tailed correlation analysis will be performed. Multivariate logistic regression and receiver operator characteristic analysis with assessment of area under the curve (AUROC) was performed to evaluate predictors of IC and graft loss. A p-value of 0.05 will be considered statistically significant and all comparisons will be two-tailed.

RESULTS

Review Paper

A PubMed search elicited 58 results related to the topic at hand. Major topics on cholangiocyte injury included ischemic-reperfusion injury, thrombosis and micro thrombi, and immune mediated injury, and altered bile composition. Additionally, key preventative strategies include surgical strategies including duct-to-duct anastomosis. Preservation solutions were analyzed, finding various benefits and risks of the different solutions currently used. Additionally, normothermic regional perfusion, a newer method on organ preservation, was analyzed in conjunction to the development of IC.

Clinical Analysis

Development of Cohorts

Development of DCD cohorts is illustrated in figure 3. Individuals were evaluated from 2016 to 2019. An initial search of the biorepository yielded 149 DCD transplant recipients. Individuals were then evaluated for the development of IC, which yielded n = 12 for development of IC and n=137 for DCD recipients without development of IC. Exclusion criteria of history of kidney transplantation, primary sclerosing cholangitis as the primary liver diagnosis, and presence of early hepatic artery thrombosis (within 21 days of transplantation) was then applied, with n=10

DCD with IC without exclusion criteria and n=128 DCD without IC without exclusion criteria. When evaluating serum sample availability, n=6 for DCD with IC meeting previous criteria and n=86 for DCD without IC meeting previous criteria. The DCD without IC cohort was then controlled with DCD with IC cohort based on age, sex, and primary liver disease as seen in tables 3-5. Establishment of the DBD cohort (n=6) underwent a similar process as demonstrated above, and exclusion criteria and serum time points were applied directly into the biorepository database. DBD recipients were then matched and controlled with age, sex, primary liver disease to the other cohorts as seen in tables 3-5.

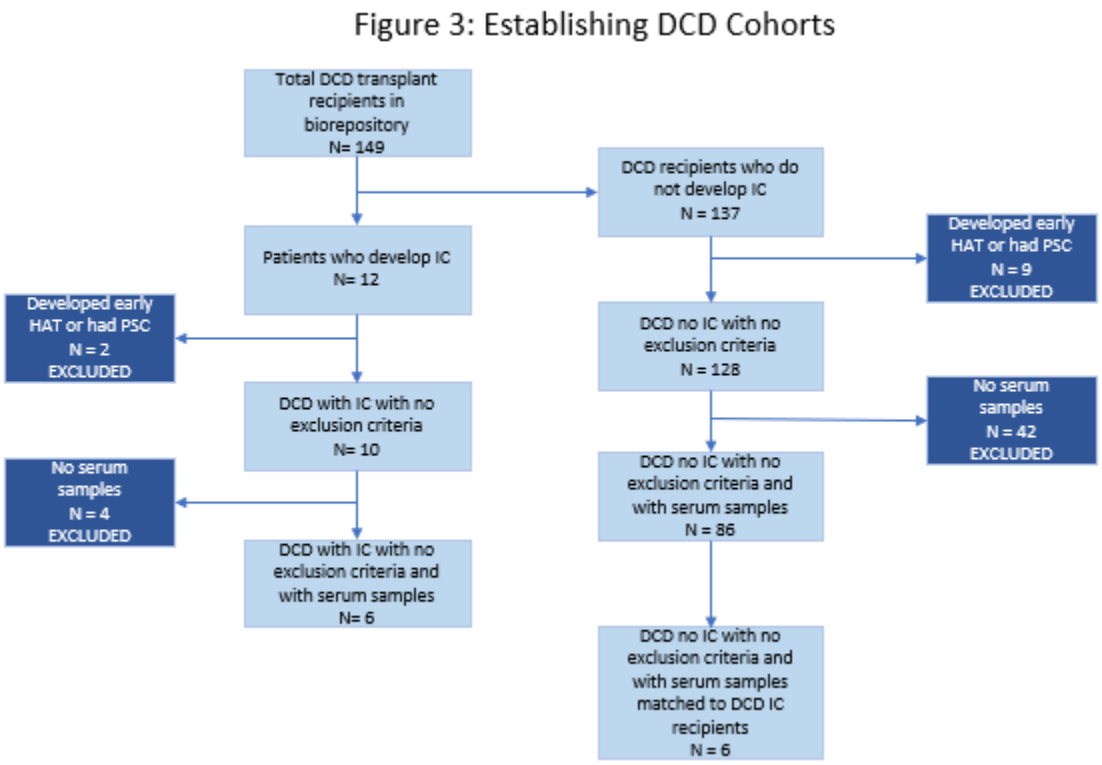

Figure 3. Establishing DCD Cohort

Age of Cohorts

Dependent Variable: Age

Tukey HSD

(I) Cohort	(J) Cohort	Mean Difference (I-J)	Std. Error	Sig.	95% Confidence Interval Lower Bound	95% Confidence Interval Upper Bound
DCD IC	DCD	1.833	4.922	.927	-10.95	14.62
	DBD	2.667	4.922	.852	-10.12	15.45
DCD	DCD IC	-1.833	4.922	.927	-14.62	10.95
	DBD	.833	4.922	.984	-11.95	13.62
DBD	DCD IC	-2.667	4.922	.852	-15.45	10.12
	DCD	-.833	4.922	.984	-13.62	11.95

Table 3. Age in Clinical Groups

Sex of Clinical Cohorts

		Sex Female	Sex Male	Total
Cohort	DCD IC	1	5	6
	DCD	2	4	6
	DBD	2	4	6
Total		5	13	18

Chi-Square Tests

	Value	df	Asymptotic Significance (2-sided)
Pearson Chi-Square	.554[a]	2	.758
Likelihood Ratio	.587	2	.746
N of Valid Cases	18		

a. 6 cells (100.0%) have expected count less than 5. The minimum expected count is 1.67.

Table 4. Sex controlled in clinical groups

Primary Liver Disease

		alpha 1 antitrypsin deficiency	autoimmune hepatitis	ETOH	HCC	hemachromatosis	HepC	NASH	polycystic liver	Total
Cohorts	DCD IC	0	0	1	1	1	3	2	0	8
	DCD	0	1	3	0	0	0	1	1	6
	DBD	1	0	3	2	0	1	2	0	9
Total		1	1	7	3	1	4	5	1	23

Chi-Square Tests

	Value	df	Asymptotic Significance (2-sided)
Pearson Chi-Square	15.307[a]	14	.358
Likelihood Ratio	16.984	14	.257
N of Valid Cases	23		

a. 24 cells (100.0%) have expected count less than 5. The minimum expected count is .26.

Table 5. Primary liver disease in clinical cohorts

NA-MELD

Na-MELD was calculated immediately prior to transplantation as demonstrated in table 6. No significance was seen in Na-MELD prior to transplantation between groups.

Na-Meld Prior to Transplantation

Dependent Variable: Na-MELD score prior to transplantation

Tukey HSD

(I) Cohort	(J) Cohort	Mean Difference (I-J)	Std. Error	Sig.	95% Confidence Interval Lower Bound	95% Confidence Interval Upper Bound
DCD IC	DCD	-9.93333	5.20528	.173	-23.5570	3.6903
	DBD	-.33333	4.96304	.998	-13.3230	12.6563
DCD	DCD IC	9.93333	5.20528	.173	-3.6903	23.5570
	DBD	9.60000	5.20528	.192	-4.0237	23.2237
DBD	DCD IC	.33333	4.96304	.998	-12.6563	13.3230
	DCD	-9.60000	5.20528	.192	-23.2237	4.0237

Table 6. Na-MELD in Clinical Cohorts

Immunosuppressive regimen

Immunosuppressive regimens immediately after transplant were evaluated. There were no significant difference in immunosuppressive regimens between cohorts as seen in table 7.

Immunosuppressive Regimen of Clinical Cohorts

		Cyclosporine	mycophenolate	prednisone	Sirolimus	tacrolimus	Total
	DCD IC	1	3	2	0	3	9
	DCD	0	4	2	1	4	11
	DBD	0	6	2	0	5	13
Total		1	13	6	1	12	33

Chi-Square Tests

	Value	df	Asymptotic Significance (2-sided)
Pearson Chi-Square	5.102[a]	8	.747
Likelihood Ratio	5.227	8	.733
N of Valid Cases	33		

a. 14 cells (93.3%) have expected count less than 5. The minimum expected count is .27.

Table 7. Immunosuppressive Regimen

Laboratory Analysis

Warm and Cold Ischemic Analysis

There was no significant difference in warm or cold ischemic times within or amongst all groups as seen in table 8.

Restrospective Laboratory Analysis

Laboratory testing immediately prior and after liver transplantation were evaluated in each cohort and compared. There was a significant increase in ALT levels prior to transplantation in DCD IC compared to DCD without development of IC (p=0.03, Mean difference 19.067, CI (1.79-36.35). Additionally there was a significant difference in alkaline phosphatase prior to transplantation when comparing DCD with IC and DCD without IC cohorts (p=0.02). Additionally, there was a significant difference in creatinine prior transplantation in DCD without IC and DBD. All of these results are demonstrated in table 8.

Restrospective Laboratory and Clinical Analysis

		Sum of Squares	df	Mean Square	F	Sig.
WIT (min)	Between Groups	29.400	1	29.400	.341	.575
	Within Groups	689.000	8	86.125		
	Total	718.400	9			
CIT (min)	Between Groups	21508.000	2	10754.000	1.663	.223
	Within Groups	96984.500	15	6465.633		
	Total	118492.500	17			
Hospital length of stay	Between Groups	.111	2	.056	.010	.990
	Within Groups	84.333	15	5.622		
	Total	84.444	17			
Na before transplant	Between Groups	10.111	2	5.056	.261	.774
	Within Groups	290.833	15	19.389		
	Total	300.944	17			
ALKPHOSBefore	Between Groups	15687.496	2	7843.748	3.237	.070
	Within Groups	33928.033	14	2423.431		
	Total	49615.529	16			
AST before transplant	Between Groups	434.778	2	217.389	.876	.437
	Within Groups	3720.833	15	248.056		
	Total	4155.611	17			
ALKPHOSAfter	Between Groups	5427.129	2	2713.564	2.803	.104
	Within Groups	10650.300	11	968.209		
	Total	16077.429	13			
AST immediately after transplant	Between Groups	2312652.333	2	1156326.167	.543	.593
	Within Groups	27664417.667	13	2128032.128		
	Total	29977070.000	15			
ALT prior to transplant	Between Groups	929.778	2	464.889	5.128	.020

	Within Groups	1359.833	15	90.656		
	Total	2289.611	17			
ALT immediately after transplant	Between Groups	1403548.833	2	701774.417	2.092	.163
	Within Groups	4360072.917	13	335390.224		
	Total	5763621.750	15			
Bilirubin before transplant	Between Groups	18.324	2	9.162	.605	.559
	Within Groups	227.085	15	15.139		
	Total	245.409	17			
Bilirubin immediately after transplant	Between Groups	20.838	2	10.419	1.108	.359
	Within Groups	122.202	13	9.400		
	Total	143.039	15			
INR prior to transplant	Between Groups	1.676	2	.838	.583	.570
	Within Groups	21.544	15	1.436		
	Total	23.220	17			
Cr prior to transplant	Between Groups	13.560	2	6.780	5.774	.014
	Within Groups	17.614	15	1.174		
	Total	31.174	17			

Table 8. Laboratory markers immediately prior and after transplantation

Survival of Graft After Transplant

Graft loss is indicated in table 9 in each clinical cohort. There was a 100% survival of patient survival 1 year after initial liver transplantation despite graft loss. There was significantly more graft loss in DCD IC recipients ($p<0.05$).

Graft Loss in Clinical Cohorts

Count

		graft loss		
		no	yes	Total
Cohort	DCD IC	3	3	6

	DCD	6	0	6
	DBD	6	0	6
Total		15	3	18

Chi-Square Tests

	Value	df	Asymptotic Significance (2-sided)
Pearson Chi-Square	7.200[a]	2	.027
Likelihood Ratio	7.902	2	.019
N of Valid Cases	18		

a. 3 cells (50.0%) have expected count less than 5. The minimum expected count is 1.00.

Table 9. Graft Loss in Clinical Cohorts

mRNA analysis

Initial mRNA isolation was performed with 50µL and sent for sequencing. Samples were sent to UT Southwestern for sequencing, however were deemed too low in quantity or quality for analysis. Isolation of mRNA was performed again with 150µL. mRNA primers were then constructed using mRNA transcriptome library and TruSeq RNA Library Prep Kit and sequenced at Baylor University Medical Center. mRNA is still undergoing sequencing.

DISCUSSIONS AND INNOVATION

Review Paper

The field surrounding ischemic cholangiopathy is rapidly changing due to new advances and increased focus on the disease amongst hepatology researchers. Review papers surrounding IC have already been published, although the latest was published in 2020[55]. The intent of the review paper was to coalesce the most up to date knowledge on IC. Additionally, this review paper attempted to discuss on multiple proposed theories on the developmental mechanisms of cholangiocyte injury occurring in IC, as well as discuss strategies and techniques during the transplant surgery that could prevent IC, including use of preservation fluid, surgical anastomosis

technique, and normothermic perfusion. Normothermic perfusion (NMP) in particular was noted as a preventative mechanism in order to optimize warm ischemia during transplantation and is considered a groundbreaking technique that could significantly reduce the development of IC.

The results of the review paper was a concise product intended for researchers studying ischemic cholangiopathy or physicians who care for patients undergoing DCD liver transplantation. In addition, this review paper will help stimulate discussions on prevention and detection measures, including ongoing research to identify potential biomarkers associated with the development of IC, and preventative measures including normothermic perfusion.

This paper also brought attention to areas requiring further study. For instance, HAT has been described as a risk factor for IC among DCD recipients, has been associated with a greater than 7-fold increase in risk of developing IC[29], and a meta-analysis found that HAT was the most identifiable risk factor for the development of IC[30]. However, the mechanism as to why IC develops within individuals who have hepatic artery stenosis is not well described. This review paper also investigated the field of measuring miRNA and mRNA as potential biomarkers. Overall, while the field has been expanding, there is still limited knowledge or biomarkers that can determine the risk of developing IC. This further justified including mRNA sequencing within this project to further expand the field of knowledge in this topic.

Clinical Analysis

The development of IC amongst DCD recipients was 8% within our cohort, which closely represents current reports of the incidence of IC of 10% in DCD LT programs[9]. Amongst the cohort groups, when controlled for age, sex, and primary liver disease, there was no

significant difference in Na-MELD prior to transplantation (table 6). Reported in literature, amongst DCD liver transplant (LT) programs, there is no difference in Na-MELD score at transplantation[7]. There was no significant difference in immunosuppressive regimen after transplantation (table 7). It should be noted that of the 18 total individuals used in this study, only one individual experienced acute cellular rejection without graft failure, and therefore graft rejection was not included in this analysis. All individuals survived one year after transplant. This is also consistent with current literature which finds that DCD liver transplantation is associated with no difference in overall post-transplantation survival[7].

As demonstrated in table 9, there was a significant loss of liver grafts in DCD IC compared with DCD or DBD cohorts ($p<0.05$). 50% of individuals in the DCD IC cohort had graft loss. Current literature suggests DCD liver transplantation is associated with higher graft loss[7]. Our cohort does suggest that compared to DBD, DCD does have a higher rate of graft loss. There was no graft loss amongst DCD without development of IC group, however, suggesting a high rate of graft loss in DCD was primarily when DCD recipients develop IC. This is consistent with current literature that supports graft loss associated with DCD liver transplantation has been mainly attributed to the increased incidence of biliary complications[6–8],

Ischemic times

There was no significant difference in cold ischemic time amongst cohorts (tables 7-8). There was no significant difference in DWIT amongst DCD without IC and DCD with IC (table 7). Prior studies have shown an association of DWIT with biliary stricture development[13,14,20,21]. The risk factor for the development of biliary strictures increases after 30 minutes[17]. One individual had DWIT greater than 30 minutes within the DCD IC cohort, all other transplants within this study had DWIT under 30 minutes. In prior studies, there was no significant graft failure loss for

DWIT 15-35 minutes[20]. There was no significant graft failure loss within this study with most DWIT within <30 minutes. These findings overall represent a program with controlled DWIT within The American Society of Transplant Surgeons recommendations for DWIT time limits[56]. It also indicates that while warm ischemic time can be a contributing factor, the development of IC is multifactorial.

When evaluating laboratory tests done prior and after liver transplantation, there was a significant difference ($p<0.05$) in ALT in DCD IC individuals prior to transplantation compared to DCD without IC development (table 8). From our knowledge, there have not been reports of elevated ALT in DCD recipients who develop IC in comparison to DCD without IC. Elevated ALT levels prior to transplantation could be due to several factors, including an increased inflammatory state prior to transplantation in individuals who develop IC. In theory, this increased inflammatory state could prime the immune system for a more robust immune response during and after the liver transplantation. However, it should also be acknowledged that levels of aminotransferases can fluctuate widely and correlate poorly with histopathologic activity. Additionally, the lack of significance for differences in aminotransferases after transplantation suggests that the levels of ALT prior to transplantation had no association with ALT after transplantation. Increased levels of serum alkaline phosphatase and bilirubin have predictive value in identifying risk of graft failure due to IC in DCD recipients[57]. This study did find a significant difference in alkaline phosphatase amongst DCD IC compared to DCD without IC, as demonstrated previously[57]. There was no significant difference in bilirubin seen in previous reports[57] (table 8).

mRNA analysis

Initial mRNA analysis was unable to be conducted due to a lack of quantity/quality of initial mRNA. All serum samples were obtained between 2016-2019. Serum samples were divided into multiple aliquots to prevent multiple thawing-refreezing cycles of the entire sample. It is unclear if the serum sample aliquots used for this analysis were previously thawed for other investigations. Second attempt at mRNA analysis resulted in 150µL of isolated mRNA that was able to be sequenced. Sequencing was occurring at the time of this writing and can be reported at a future date.

FUTURE DIRECTION

This project demonstrated common clinical findings within DCD recipients who develop IC. This study therefore helped solidify the understanding that factors such as age, sex, primary liver disease, immunosuppressive regimens, or Na-MELD were correlated with the development of ischemic cholangiopathy. This particular study saw an increase of ALT, while previous studies have seen an increase in bilirubin and alkaline phosphatase[57]. Future clinical studies could include further investigation into these common laboratory results to investigate if there is a repeatable correlation between ALT, alkaline phosphatase, and bilirubin in the development of IC. Further investigation could also include evaluation of inflammatory markers including WBC, CRP, or procalcitonin if taken to further investigate an increased inflammatory state within DCD recipients who develop IC compared to DCD without IC.

This was a retrospective study and was limited on quantity and timing of laboratory samples stored for study. A larger study would help increase power within the study. Additionally, a prospective study would allow for more accurate time points and more consistency in laboratory testing done prior and after transplantation as well as serum samples taken after transplantation.

mRNA was ultimately evaluated in this study in comparison to miRNA due to the quantity of mRNA in serum compared to miRNA and the stability of mRNA. miRNA, however, has been seen as potential biomarkers in the setting of primary sclerosing cholangitis (PSC) and primary biliary cholangitis (PBC)[22,48]. Therefore, specific investigation on miRNA, which helps to regulate mRNA, could be an important route of investigation for the establishment of a biomarker associated with IC and will be considered in a subsequent phase of this study.

CONCLUSION

Review Paper

When investigating the current literature on ischemic cholangiopathy, multiple mechanisms that could result in the development of IC were discovered, including ischemia-reperfusion, hemostasis and thrombosis, immune-mediated injury, and altered bile composition. Each of these mechanisms are being investigated in their respective right for the development of biomarkers for pathways associated with the disease, preventative strategies and management. In regards to preventative strategies, surgical techniques including organ perfusion solution[58] and type of surgical anastomosis have been investigated for the incidence of the development of IC and the prevention of IC[13,29]. In regards to biomarker development, miRNA profiles have been identified in other bile duct pathologies including primary sclerosing cholangitis and primary biliary cholangitis[22,48]. Further investigation is warranted for the development of biomarkers in IC specifically. Lastly, the immerging of normothermic machine perfusion (NMP) has been a novel field that was investigated. Multiple trials have been conducted for the utility of NMP in the prevention of IC in DCD recipients. Recent trials have seen reduction of IC, demonstrating the importance of maximally reducing ischemic times through portable NMP[59]. However, the

practical utility of NMP is a preventative factor for widespread utilization for many LT programs.

This paper aims to provide a general review of the most recent research and knowledge on the development, detection, and prevention of IC. It can be useful for providing general insight and management for the field of IC to a provider as this field continues to evolve.

Clinical Research

This clinical cohort demonstrated similar characteristics seen within literature today. Overall, there does not appear to be a difference in clinical characteristics including age, sex, Na-MELD, primary liver disease, or immunosuppressive regiment that results in significant increased incidence of IC amongst DCD recipients when compared to other DCD individuals and DBD recipients. It demonstrated a limited prevention strategy in regard to clinical risk stratification for individuals being evaluated for transplant. Instead, it appears as though events that surround the transplant surgery itself including warm and cold ischemia time, thrombotic disease, immune mediation, and bile acid composition are potential causes of IC development. Prior investigation has seen increases in alkaline phosphatase and bilirubin prior to transplant as indicators for the development of IC[57]. Alkaline phosphatase was seen nearly significantly different ($p=0.07$) but not found significant by this study's criteria. Bilirubin was not seen to be significantly different in this study. Additionally, this study found a significant difference in Cr prior to transplant amongst DCD with IC compared to DCD without IC ($p<0.05$). This was not found in other studies to our knowledge, and should be noted that none of the individuals within all of the cohorts qualified for liver-kidney transplant.

COMPLIANCE

Approval for research was obtained through the Baylor Simmons Transplant Research Committee at the Baylor Scott & White Annette C. and Harold C. Simmons Transplant Institute and the Baylor Scott & White Research Institute. IRB approval reference number: 384458

This was a retrospective study. Individuals within this study had already provided consent for use of biologic samples stored within the biorepository and to have their clinical information used for future research.

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