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Urinary Bile Acid Indices as Prognostic Biomarkers for the Complications of Liver Diseases

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URINARY BILE ACID INDICES AS PROGNOSTIC BIOMARKERS FOR THE COMPLICATIONS OF LIVER DISEASES

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URINARY BILE ACID INDICES AS PROGNOSTIC BIOMARKERS FOR THE COMPLICATIONS OF LIVER DISEASES

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Hepatobilary diseases cause the accumulation of toxic bile acids (BA) in the liver, blood, and other tissues, which may lead to an unfavorable prognosis. In this study, we compared the urinary BA profile in 257 patients with hepatobilary diseases during a 7-year follow-up period. We investigated the use of the urinary BA profile to develop logistic regression models to predict the prognosis of hepatobiliary diseases in terms of developing disease-related complications, especially for ascites. The urinary BA profile was characterized by calculating BA indices, which quantify the composition, metabolism, hydrophilicity, and toxicity of the BA profile. All patients had high total and individual BA concentrations. The percentages of primary BA (CDCA and LCA) were high, while the percentages of secondary BA (MDCA and DCA) were low in patients. BA indices had lower inter- and intra-individual variability than absolute total and individual BA concentrations. The changes of the BA indices were associated with the probability of developing ascites in the entire liver-patient population using logistic regression analysis. BA indices were proved as prognostic biomarkers for hepatobilary diseases.

We have developed and validated a prognosis model based on BA indices to predict the prognosis of ascites in the entire liver-patient population. Other models,

including non-BA, original MELD, and mixed BA and non-BA models, were also developed to compare their performance with our BA model. Overall, the mixed BA and non-BA model was the most accurate based on Akaike information criterion (AIC) and receiver operating characteristic (ROC) analyses. The mixed BA and non-BA had lower AIC values indicating a smaller error of distribution and a better trade-off between goodness of fit vs. degrees of freedom. Moreover, the mixed BA and non-BA model had highest area under the ROC curve (AUC) values indicating higher accuracy than other models. One application of the mixed BA and non-BA model could be used to predict the development of ascites in patients diagnosed with liver-disease at early stages of intervention, such as liver transplantation. This will assist in supply allocation and physician decisions when treating liver diseases.

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AIC: akaike information criterion

ALP: alkaline phosphatase

ALT: alanine transaminase

APRI: AST/ platelet ratio index

ASBT: Na+ dependent bile salt transporter

AST: aspartate transaminase

AUC: area under the ROC curve

B: regression coefficient

BA: bile acids

BA-CoA: bile acid coenzyme-A

BAT: bile acid-coenzyme A: amino acid N-acyltransferase

BSEP: bile salt export pump

C27-3β-HSD: C27-3β-hydroxylated dehydrogenase

CA: cholic acid

CDCA: chenodeoxycholic acid

CMC: critical micelles concentration

CTP: child-Turcotte-Pugh

CYP8B1: cytochrome p450 family 8 subfamily B member 1

DCA: deoxycholic acid

ESI: electrospray ionization

FXR: farsenoid-X-receptor

G: glycine

G-BA: glycine-amidated bile acids

GGT: glutamyl transferase

Gly-MCA: glycine-β-MCA

HCA: hyocholic acid

HDCA: hyodeoxycholic acid

HI: hydrophobicity index

HL: hosmer–lemeshow

INR: international normalized ratio

IS: internal standard

LCA: lithocholic acid

LC-MS/MS: liquid chromatography-tandem mass spectrometry

LT: liver transplantation

MCA: muricholic acid

MDCA: murideoxycholic acid

MDR: multidrug resistance protein

MELD: model for end-stage liver disease

MeOH: methanol

MRP: multidrug resistance–associated protein

NASH: nonalcoholic steatohepatitis

NPV: negative predictive value

NTCP: sodium-taurocholate co-transporting polypeptide

OATP: organic anion-transporting polypeptide

OATPs: organic anion transporting polypeptides

OH: hydroxyl group

OR: odds ratio

PBC: primary biliary cholangitis

PPV: positive predictive value

PSC: primary sclerosing cholangitis

ROC: receiver operating characteristics

RSD: relative standard deviation

SE: standard error

SEN: sensitivity

SPE: specificity

SULT2A1: sulfotransferase 2A1

T: taurine

T-BA: taurine-amidated bile acids

TGR: G-protein-coupled membrane receptor

TIPS: transjugular intrahepatic portosystemic shunt

U: unamidated

UDCA: ursodeoxycholic acid

UPLC: ultra-performance liquid chromatography

CHAPTER 1

INTRODUCTION

1.1 Bile acids (BA) synthesis, metabolism, and enterohepatic recirculation

Bile Acids (BA) are synthesized in the liver and excreted into bile, which flows to the small intestine through the bile duct [1]. BA synthesis takes place in liver cells through cytochrome P450-mediated oxidation of cholesterol in many steps [2]. The major pathway of BA synthesis is initiated by hydroxylation of cholesterol at the 7α position through the action of the CYPA1 enzyme [3]. The next step of BA synthesis is the oxidation of the 3β-OH and isomerization of the C5-C6 double bond by the microsomal C27-3β-hydroxylated dehydrogenase (C27-3β-HSD). The forming intermediates are either involved in hydroxylation at the 12α position through the action of the CYP8B1 enzyme or passed to the next step [4]. The intermediates with 12α hydroxylation produce CA, while intermediates that are not involved in hydroxylation produce CDCA and CA that belong to primary BA in humans. The next step of BA synthesis is the hydroxylation and oxidation of a carboxylic acid. This occurs at the C27 position through the action of the CYP27A1 enzyme followed by the bile acid coenzyme-A (BA-CoA) synthetase [5]. The side chain of these C27 intermediates is decreased to C24 BA through β oxidation. The final step of BA synthesis is involved in amidation of the BA-CoA with glycine(G) or taurine (T) via amino acid N acyltransferase (BAT) [5].

BA can also be synthesized by alternative pathways, which do not require the enzyme CYP7A1 to initiate their synthesis [6]. The alternative pathways of BA synthesis are initiated through the hydroxylation of cholesterol at side chains C24, C25,

or C27 by the action of the CYP7B1 and CYP39A1 enzymes [7]. Compared with the major pathway of BA synthesis, the alternative pathways produce more CDCA. Also, these pathways are linked to conditions with deficiency in CYP7A1 activity [7].

The enterohepatic recirculation of BA describes the cycle of BA absorbed from the intestine into the liver and then re-secreted into bile [1]. BA are excreted from liver into bile through efflux transporters, which include the bile salt export pump (BSEP), multidrug resistance protein 3 (MDR3), and multidrug resistance-associated protein 2 (MRP2) [8]. After meal ingestion, cholecystokinin secretion prompts the gallbladder to contract and empty its contents into the duodenum [9, 10].

Most amidated BA in the small intestine are absorbed in the ileum through the apical Na+-dependent bile salt transporter (ASBT) or organic anion-transporting polypeptides (OATPs) [9, 11]. These two transporters have higher affinity on amidated BA compared with unconjugated BA [12]. Therefore, unconjugated BA are passively absorbed via the intestinal tract due to low affinity on transporters and their unionized forms [9, 10]. Also, partial deamination occurs from the bacteria in the small intestine, and unconjugated BA are passively absorbed [13].

Unabsorbed BA are transferred from the small intestine to the large intestine [1]. BA undergo bacterial transformation of deamidation and dehydroxylation in the large intestine [1, 14]. Due to the dehydroxyaltion of primary BA at the 7α position, secondary BA are produced via bacterial transformation, such as DCA and LCA [1, 14]. Absorbed BA are extracted by the liver through active or passive diffusion. The

majority of BA are amidated in hepatocytes. Other metabolic pathways take place such as sulfation and hydroxylation [1]. BA are excreted in feces when they are not absorbed in the intestines [1, 14]. Finally, the enterohepatic cycle is completed when the newly synthesized and reabsorbed BA are re-excreted into bile.

1.2 BA structure, function, and toxicity

BA are amphipathic steroid molecules synthesized in the liver from cholesterol [3]. **Figure 1.1** indicates the chemical structure of the major BA, which includes cholic acid (CA), muricholic acid (MCA), hyocholic acid (HCA), chenodeoxycholic acid (CDCA), deoxycholic acid (DCA), ursodeoxycholic acid (UDCA), murideoxycholic acid (MDCA), hyodeoxycholic acid (HDCA), lithocholic acid (LCA), their glycine (G) and taurine (T) amidates, and sulfate conjugates. Based on their chemical structure, BA can be sorted into mono-OH BA (LCA), di-OH (MDCA, UDCA, HDCA, CDCA, and DCA), and tri-OH (CA, HCA, and MCA).

The physiological functions of BA include cholesterol absorption and elimination, fat absorption, and maintenance of a healthy microbiome [15]. Moreover, the absorption of lipids and fat-soluble vitamins by emulsification is also related to BA's physiological functions [16]. BA work as signalizing molecules by binding to numerous receptors, especially the surface G-protein-coupled membrane receptor (TGR5) and the nuclear farsenoid-X-receptor (FXR) [17]. Based on that, BA are involved in regulating gene expression on cholesterol, glucose metabolism, and

homeostasis. For example, one type of primary BA, ursodexoxycholic acid (UDCA), is associated with the treatment of cholestatic liver diseases[18].

BA also have cytotoxic and pathological effects at high concentrations. BA degrade cell membranes, have necrotic effects on mitochondria, detergent effects on biological membranes and promote cell mutations that produce cancer [19-21]. In more detail, BA bind to the lipid bilayer and increase solubility of plasma membrane components at high concentrations. At the intracellular level, BA decrease the mitochondria integrity, and lead to the influence of permeabilization of mitochondria membranes, such as depolarization of the organelle and mitochondrial swelling [22]. Based on that, BA cause mitochondrial collapse, release cytochrome c, and lead to apoptosis. Moreover, BA toxicity is associated with hydrophobicity [22]. The increasing of BA hydrophobicity is linked to the efficiency of BA to solubilize membrane lipids [22]. Therefore, BA toxicity increase when more hydrophobic BA are synthesized.

1.3 Differences among individual BA

Individual BA are different from each other through to their physicochemical properties, physiological, and pathological functions. One physicochemical property is the lipophilicity of BA, which is determined by the side chain structures and BA nucleus [23]. Amidation of BA side chain with G and T cause the reduction of lipophilicity by decreasing pka, and it led to the increased solubility. For example, the acidity of unconjugated BA is associated with G and T amidation. As amidation

increases, pka is decreased from unamidated BA to G and T amidated BA [24]. The number of hydroxyl groups (OH) on steroids nucleus shows an opposite relationship with BA lipophilicity. For example, tri-OH BA (CA and MCA) is less lipophilic than di-OH BA (CDCA and DCA), which in turn is less lipophilic than mono-OH BA(LCA). Moreover, the position and stereochemistry of OH groups are related to BA lipophilicity [24]. For example, di-OH BA (UDCA) is less lipophilic than tri-OH BA (CA). The completed ionization of BA at physiological PH causes decreasing in lipophilicity and increasing in solubility and leads to inactivation of membrane permeability.

BA are amphipathic molecules, and their anions self-associate to form micelles in water. The critical micelles concentration (CMC) is one of the important parameters for BA cytotoxicity. It shows the propensity of molecules to dissociate or aggregate in solutions and their level of toxicity [25]. CA has higher critical micellar concentrations than DCA and CDCA; therefore, it has less cytotoxicity at a given concentration [26]. Moreover, BA hydrophobicity is another critical parameter to determine BA toxicity. BA are planar molecules with two “faces”. The one face does not have OH groups, making it hydrophobic. The other face has OH groups, making it hydrophilic. Based on this, BA hydrophobicity also depends on the number, position and orientation of OH groups. The hydrophobic index (HI) is used to describe the balance of hydrophilic and hydrophobic of individual BA. HI of BA is calculated from the retention time and capacity factor on a C18 column [1]. The range of HI is from -0.94 for the hydrophilic BA (T-UDCA) to +1.46 for the hydrophobic BA(LCA).

The hydrophobicity of individual BA is linked to membrane damage [26]. The lower value of HI, the higher concentration of hydrophilic BA indicate the lower cytotoxicity of BA [27]. Therefore, the individual BA such as DCA, CA, and UDCA can be ranked based on their cytotoxicity, [26].

Affinity to various BA receptors can be influenced by the structural differences of Individual BA. The G protein-coupled receptor (TGR5) works as a cell-surface receptor responding to BA [28]. For instance, primary BA (CDCA and CA) are less potent TGR5 activators than secondary BA (LCA and DCA) [28]. Farnesoid X receptor(FXR) is one type of nuclear receptor of transcription factors that regulates BA metabolism [29, 30]. For example, primary BA such as CDCA is limited by FXR activation, while secondary BA such as DCA is not [31]. However, glycine-β-MCA (Gly-MCA) works as a FXR inhibitor in the intestine [30].

Individual BA are also differentiated by their pathological effects. For instance, hydrophobic BA such as LCA cause cholestasis in rats and mice. However, hydrophilic BA such as CA cause hypercholeresis [32]. CA is also less likely than LCA to cause red blood cell hemolysis [32]. T-amidates are less cytotoxic than G-amidates and cause less cell membrane lysis than the corresponding G-amidates [32, 33]. The amount and composition of the BA pool must be maintained to keep normal physiological levels. This also prevents toxicity from the accumulation of toxic BA.

1.4 Species Differences of BA

Major species differences in BA metabolism have been reported in previous studies [34-38]. The detoxification of BA mainly focuses on several pathways, such as conjugation (sulfation or glucuronidation), amidation (glycine or taurine), and hydroxylation by CYP3A [1]. Glycine amidation is less likely to increase BA hydrophilicity and decrease their toxicity than taurine amidation. Glycine amidation is mainly observed in humans[39, 40], rabbits[41], and minipigs[42], while taurine amidation is mainly observed in mice [6], rats [43], and dogs [44]. Hydroxylation at the 6-α,6-β, and 7-β positions, which is the major pathway to produce hydrophilic toxic BA, including MCA (mice), HCA (pigs) and UDCA(bears) [27]. BA sulfation are more observed in humans and chimpanzees, and less observed in rabbits, rats and mice [6]. BA glucuronidation are a minor pathway in numerous species such as rats, chimpanzees, mice and humans, while dogs show a high level of glucuronidation [45].

Major species differences are also reported in BA transport [17, 30, 32]. The contribution of efflux through multidrug resistance–associated protein (MRP) transporters to drug induced-toxicity are 5-fold lower in humans than rats [34]. The affinity of MRP3 transporters in humans is relativity less than in rodents [55]. Similarly, the uptake affinity of BA via NTCP (sodium-taurocholate co-transporting polypeptide) and OATP (organic anion-transporting polypeptide) transporters is higher in rats than in humans [35]. Also, OATP1 and OAPT3 are not effective in humans, dogs or rodents[36].

Moreover, major species differences in BA-induced toxicity have been reported in previous studies and explained by species difference in BA metabolism [34-38]. CDCA cause harmful hepatic toxicity in monkeys [46], rabbits [47] and dogs [48] because they lack BA sulfation capabilities. Sulfation is the major pathway of BA metabolism in humans and chimpanzees, therefore CDCA therapy is not linked to hepatic injury for these species [46, 49-52]. Also, LCA and DCA are both hepatotoxic in rabbits because of the lack of BA sulfation and hydroxylation [41, 47, 53]. Humans are less resistant to CDCA, LCA than mice [54] and rats [55] because of their BA are less hydrophilic due to hydroxylation and taurine amidation. Therefore, species difference to BA toxicity is mainly determined by their capability to efficiently metabolize BA.

There are some limitations when using animal models for studying BA toxicity in their metabolism. BA sulfation has been considered as a primary detoxification mechanism [1]. Amidation of BA with glycine and taurine amino acids enhance their solubility and decrease their toxicity [56]. The sulfation of BA is highest in humans and chimpanzees, while other species are very low across all BA in a vivo and in vitro study. Also, the amidation of BA is highest in humans and lowest in rats in the same study [45]. BA sulfation and amidation are important to understand the balance between physiological and pathological effects [39]. For example, the inhibition of BA sulfation and amidation decrease transporter-medicated vectorial transport and effect

the liver's ability for drug-induced adaptation [57]. Based on these limitations, using animal models are not as useful as human models for studying BA toxicity.

1.5 BA and Hepatobiliary diseases

Cholestatic liver diseases are a diverse group of hepatobiliary diseases [2]. The major cholestatic liver diseases include primary biliary cirrhosis (PBC) and primary sclerosing cholangitis (PSC) [58]. Patients with PSC are most likely to develop cirrhosis and end-stage liver disease [58]. Around 90 percent of patients with compensated cirrhosis develop into ascites [59]. In liver disease severity, there are decompensated and compensated liver diseases. Patients with decompensated liver diseases have severe complications, including liver damage and severe to the point where the liver can no longer function. These complications include ascites [59], bacterial peritonitis [60], encephalopathy [61], GI bleeding [62], hepatobiliary carcinoma [63], hepatorenal syndrome [64], jaundice [65], peripheral edema [66], and portal hypertension [67]. Patients with compensated liver disease do not have severe complications, which means the liver is scarred, but it can still perform most basic functions [68].

BA have deleterious effects on the liver which includes cholestasis, changes in liver structure, and hepatocyte ultrastructure [1, 69]. Cholestatic liver diseases are associated with bile flow reduction, which is caused by the impairment of bile flow into bile duct or defects in bile production [2]. Cholestatic liver diseases cause BA

accumulation in the liver, spread into the circular system, extrahepatic tissues, and urine. Many research studies report the changing of BA concentrations in the blood and urine at liver disease conditions [1, 70-72].

There are several human and animal studies illustrating the link between the accumulation of toxic BA in the liver, blood and extrahepatic tissues, and unfavorable liver disease prognosis [2, 39, 73, 74]. The accumulation of toxic BA in cholestasis leads to hepatoxicity and extrahepatic toxicity [75]. For instance, BA concentrations correlate to liver and bile duct damages in diseased rabbits, rats and humans [73, 76-78]. Also, patients with high concentrations of BA are more likely to have hepatobiliary complications after liver transplantation[73]. The intracellular accumulation of toxic BA influences the upregulation of proteins connected with hepatic bile secretion due to the imbalance of BA receptors such as FXR. After that, it inhibits the hepatocellular uptake of BA and BA synthesis [74]. Moreover, toxic individual BA are more associated with the damage inflicted on hepatocytes and cholangiocytes than total levels of BA [2]. Therefore, the evidence from animal and human studies supports the causal link between the accumulation of toxic BA and unfavorable prognosis of hepatobiliary diseases.

1.6 BA as biomarkers of liver diseases

In the US, ten percent of people diagnosed with cholestatic liver diseases led to end up with liver transplantation (LT) [79]. Even though liver transplantation is a well-known therapy for patients with cholesteric liver diseases, one of the major challenges

is a larger portion of the overall complications occur after LT [80]. For example, PBC and PSC relapse after liver LT, and affect graft outcomes during a long period. Moreover, Immunosuppression in LT with cholesteric liver disease is poorly understand because of the increased acute cellular rejection in patients with cholesteric liver diseases [79]. There are not enough data indicating a relationship between a immunosuppression regimen and the risk of relapsing for liver cholesteric liver diseases after patients undergo LT [79].

Aspartate transaminase (AST), alanine transaminase (ALT), alkaline phosphatase (ALP), glutamyl transferase (GGT), serum creatinine, protime, international normalized ratio (INR) are most commonly used as individual biomarkers for the prognosis of hepatobiliary diseases **(Table1.1)**. However, these biomarkers are not specific to bile duct injuries or the liver, and may more commonly be associated with non-hepatobiliary conditions [81]. For example, elevated level of serum ALT is linked to toxicity in other organs besides the liver. Using these biomarkers can lead to an under evaluation of the severity of the problem [82]. For example, ALT works as a poor indicator of disease severity for hepatobiliary diseases such as cholestasis [83]. In evaluating liver diseases, models with multiple parameters are preferred and show high accuracy compared with models using an individual parameter, such as the Child-Turcotte-Pugh (CTP) and the Mayo model for end-stage liver disease (MELD) score.

Many models, scores and criteria have been developed to predict the prognosis of hepatobiliary diseases (**Table1.2).** The CTP score, originally used to determine the risk of shunt surgery for liver disease severity [84]. The CTP score use three biological variables (serum albumin, serum bilirubin and prothrombin) and two clinical variables (ascites and encephalopathy). However, there are several limitations for the CTP score. Variables of ascites and encephalopathy are easily affected by extraneous factors [85]. Also, variables of bilirubin and creatinine make the end of the CTP scale inaccurate [86].

Another model for liver diseases is the Mayo model for end-stage liver disease (MELD). It is used to determine a patient's eligibility for liver transplantation in many countries [87]. MELD uses three parameters which are serum bilirubin, International normalized ratio (INR), and creatinine. The MELD score contains a metric using a continuous scale to predict the ranking of patients by disease severity [85]. These three variables are reproducible and easy to measure. Combined together, they give a high accuracy on how the liver is functioning than CTP. The level of creatinine is related to kidney function. The level of bilirubin shows how well the liver clears bile. INR reflects how well the liver makes factors needed for blood clots [85, 86]. When MELD was implemented, it decreased post-transplant mortality rates. MELD also led to accurate predictions of surgical outcomes with alcoholic hepatitis and cirrhosis patients [88].

Even though it is used globally, MELD still has several limitations. MELD calculation is based on three variables that are not specific to all hepatobiliary diseases [87]. For example, patients with a high level of serum creatinine are likely to have kidney disease. The changing status of serum bilirubin is linked to other conditions like hemolysis or sepsis [89]. Moreover, in several studies, patients with a low MELD score represent a high mortality rate and a less accurate MELD score [89, 90]. Based on these issues, using the MELD score to estimate liver disease severity needs to be reconsidered.

Further diagnosis and prognosis of liver disease is critical and depends on invasive procedures, endoscopic treatment and evaluation of liver biopsies [91]. Based on these, noninvasive biomarkers are needed to help on prognosis, diagnosis, and evaluation. For several decades, BA has been considered as potential biomarkers for many hepatobiliary diseases based on their accumulation and hepatoxicity in hepatobiliary diseases [1]. For example, PSC [92], PBC [92], alcoholic liver disease [92], nonalcoholic fatty liver disease [93], hepatitis intrahepatic cholestatic of pregnancy [94]. BA biomarkers are an accurate, noninvasive option that can improve the diagnosis and prognosis of liver diseases [95-97]. Not only are they being more accurate, but they are also a vital addition to treatment and evaluation of hepatobiliary diseases. They could improve the therapeutic outcomes for these diseases.

1.7 BA indices

Even though BA as biomarkers have been extensively used for hepatobiliary diseases, they have not been effectivity used in clinical studies due to several limitations. Individual BA concentrations are better correlated to the hepatobiliary liver condition than total BA concentrations due to the difference in the various BA's physiological and pathological properties [26, 72]. Moreover, total and individual BA concentrations reflect high inter- and intra-individual variability and make it hard to identify baseline ranges in the absence of liver diseases. BA have shown high inter-individual variability based on several factors, including gender, alcohol consumption, and obesity [97-100]. Also, serum and urinary BA levels show high intra-individual variability due to many factors, such as medication intake, and food ingestion [71, 101-103].

Based on these limitations, we have investigated the concept of "BA indices", which are ratios calculated from the absolute individual BA concentration and their metabolites [39]. These ratios are used to characterize BA profiles by quantifying BA composition, hydrophilicity, toxicity, formation of secondary BA, and metabolism [2, 39, 104, 105]. BA indices have numerous benefits compared to total and individual BA concentrations. BA indices have low inter- and intra-individual variability. For total and individual absolute BA concentrations in urine, the relative standard deviation (RSD) is from 66% to 256%, but it is from 10% to100% for BA indices in the same population of health subjects [39]. Serum BA level increases after food ingestion

because of cholecystokinin's release, which leads to gallbladder contraction resulting in increasing bile flow into intestine [39]. Therefore, feeding status has to be controlled before the use of serum BA as a reliable biomarker. Moreover, the absolute and most individual BA concentrations increase more than 2-fold one hour after a standard meal. However, BA indices only change 10% in the same individual BA after a standard meal [39]. Also, the same trend has shown in urine, urinary BA indices have smaller inter-and intra-variability than in serum. For instance, the percentage of RSD of overall BA was 8% and 47% in urine and serum [39]. Moreover, urinary BA indices are resistant to feeding status compared with absolute BA concentrations in the same population [39]. Therefore, noninvasive urinary BA indices are significantly better than absolute urine or serum BA concentrations for treating hepatobiliary diseases. In addition, urinary BA indices have better performance than serum liver enzymes such as ALT and AST or total BA concentrations in humans and in animal models for cholestatic liver disease diagnosis and prognosis [2].

1.8 Research objectives

In this study, we have extended the application of BA indices to predict liver disease prognosis by recruiting 257 patients with liver diseases over a period of seven years. The study focuses on developing prognostic models based on BA indices to predict the individual complication in the entire liver-patient population. In other words, it is used for indicating the prognosis of the complication from a grouped population of

liver disease subtypes, with an emphasis on the relationship between the BA indices and the severity of the complication. The various BA, non-BA, and MELD models were compared for their accuracy in predicting the prognosis of liver diseases via statistical tests.

CHAPTER 2

URINARY BILE ACID INDICES AS PROGNOSTIC BIOMARKERS FOR ASCITES ASSOCIATED WITH LIVER DISEASES

2.1 Introduction

Cholestatic liver diseases is a diverse group of hepatobiliary diseases associated with limitations in bile flow due to a failure of bile flow or an impairment in bile production [2]. Relatively common cholestatic liver diseases include primary biliary cirrhosis (PBC) [58], primary sclerosing cholangitis(PSC) [58], alcoholic liver disease [106], and nonalcoholic fatty liver disease [93].

Common complications associated with cholestatic liver diseases include ascites [59], bacterial peritonitis [60], encephalopathy [61], GI bleeding [62], hepatobiliary carcinoma [63], hepatorenal syndrome [64], jaundice [65], peripheral edema [66], and portal hypertension [67]. In particular, ascites is one of the most common complications associated with cirrhosis. The risk of developing ascites is around 60% if the cause of cirrhosis has not been treated [107]. Cirrhosis is an advanced-stage liver disease caused by fibrosis, which impedes the intrahepatic blood flow, increases portal blood pressure, and causes accumulation of fluids in the peritoneal cavity (ascites) [108]. The survival of cirrhosis patients decreases from 80% to 50% when these patients are diagnosed with ascites [109]. Cirrhosis patients with ascites experience several symptoms, such as nausea [110], abdominal distention [111], dyspnea [112], edema [113], and hepatorenal syndrome [114].

Aspartate transaminase (AST), alanine transaminase (ALT), alkaline phosphatase (ALP), glutamyl transferase (GGT), serum creatinine, protime, and INR (international normalized ratio) are commonly used biomarkers for the diagnosis and

prognosis of liver diseases [81-83]. However, these biomarkers are not specific to bile duct or liver injuries, and may be related to non-hepatobiliary conditions [81]. Therefore, models with multiple parameters/markers were developed to better predict the prognosis of liver diseases with higher accuracy than individual parameters [84, 86].

Models with multiple parameters have been used globally to predict survival of hepatobiliary disease-related complications such as the Child-Turcotte-Pugh (CTP) and the Mayo model for end-stage liver disease (MELD) scores [85, 109]. The CTP score was originally used to determine the risk of shunt surgery for severity of liver disease and its complications, such as GI bleeding and encephalopathy [115, 116]. The MELD score was originally used to estimate survival of liver patients undergoing the transjugular intrahepatic portosystemic shunt (TIPS) [85]. The MELD score is currently used to determine a patient's eligibility for liver transplantation [87]. In addition, the MELD score is used as a good predictor of outcome in liver disease complications, such as GI bleeding and portal hypertension [85, 115]. Even though the CTP and MELD scores have been used globally, they still have several limitations. Variables of ascites and encephalopathy are easily affected by extraneous factors in the CTP score [85]. And the MELD score has a poor evaluation for patients with cholestatic liver disease-related complications, such as ascites and encephalopathy [86].

More recently, bile acids (BA) have been considered as potential biomarkers for prognosis of hepatobiliary diseases [117, 118]. BA are synthesized in the liver and excreted into bile, which then flows to the small intestine via the bile duct [8]. BA have many physiological functions, such as fat absorption and cholesterol elimination [15, 17]. Compared to their physiological functions, BA also exhibit pathological effects at high BA concentrations. They are associated with necrotic effects on mitochondria, detergent effects on biological membranes, and cancer promoting effects [20, 21]. There are a plethora of human and animal studies illustrating the link between the accumulation of toxic BA in the liver, blood and extrahepatic tissues, and unfavorable liver disease prognosis [2, 39, 73, 74].

However, BA have not been widely used in the clinic as biomarkers for liver diseases due to several limitations. Individual BA concentrations are better correlated to hepatobiliary diseases than total BA concentrations due to the difference in the various BA's physiological and pathological properties [26, 72]. Both individual and total BA concentrations have high inter-and intra-variability under normal conditions due to several factors including weight, gender, and alcohol consumption, food ingestion, diurnal variation, and medication intake. Therefore, the normal baseline ranges are difficult to establish [71, 97-103].

To address these limitations, we have established the concept of “BA Indices.” BA indices are ratios calculated from the absolute individual BA concentration and their metabolites [2, 39, 104, 105]. BA indices have markedly low inter-and intra-individual

variability and are more resistant to the above-mentioned cofactors than absolute BA concentrations. For example, the absolute total and individual BA concentrations increased more than 2-fold in individuals one hour after a standardized meal, while BA indices changed less than 10% in the same individuals [39]. Furthermore, we have demonstrated that urinary BA indices outperformed the currently used blood liver enzymes as biomarkers for cholestatic liver diseases [2, 39]. In addition, we have recently developed a BA-based survival model (the BA score (BAS) model) to predict the prognosis of cholestatic liver diseases [119]. BAS had a higher true-positive and true-negative prediction of 5- and 3-year death and liver transplant than other non-BA models including MELD.

Multiple markers and models are used to predict the survival of cholestatic liver diseases [120, 121]. However, very few studies have addressed the prognosis of cholestatic liver disease-related complications. For example, the CTP score has widely been used in the prognosis of cirrhosis, but it does not provide clear guidance of prognosis for cirrhotic patients with complications [122]. Similarly, the MELD score has extensively been used to prioritize cirrhotic patients awaiting liver transplantation [123], but it still does not correlate with cirrhosis-related complications, including encephalopathy and bacterial peritonitis [124]. Therefore, there is a critical need for markers/models to particularly predict complications of liver diseases.

In this study, we have expanded the application of BA indices to predict complications, especially ascites, in patients with liver diseases. The study focuses

on developing prognostic models based on BA indices to predict the development of ascites in liver patients.

2.2 Materials and methods

2.2.1 Study participants

Patients with hepatobiliary conditions were diagnosed by University of Nebraska Medical Center's (UNMC) hepatology Clinic (Omaha, NE, USA). The institutional review board (IRB) approved this study at UNMC. Hepatobiliary conditions included Chronic Hepatitis C, Chronic hepatitis B, Alcoholic Liver disease, Primary biliary cholangitis (PBC), Primary Sclerosing Cholangitis (PSC), Autoimmune Hepatitis, Alpha-1-antitrypsin deficiency, Nonalcoholic Fatty Liver Disease, Nonalcoholic Steatohepatitis (NASH), Cryptogenic Cirrhosis and Nonalcoholic Steatohepatitis. The following complications were diagnosed and monitored by the hepatologists: Hepatobiliary Carcinoma, Gastrointestinal Bleeding, Portal Hypertension, Ascites, Peripheral edema, Encephalopathy, Jaundice, Bacterial Peritonitis, Hepatorenal Syndrome. Two-hundred fifty-seven patients with cholestatic liver diseases between the ages of 19 and 65 years (121 female and 136 male) were treated at the UNMC from November of 2011 to December of 2018 were recruited into the study. Thirty milliliters' urine samples were collected from patients on every visit to the hepatology Clinic. All urine samples were stored at -80℃ before BA analysis using liquid chromatography-tandem mass spectrometry (LC-MS/MS) until analyzed.

2.2.2 Non-BA parameters

The performance of potential biomarkers from the urinary BA profile has also been compared with the performance of existing markers of liver function including alanine transaminase (ALT), aspartate transaminase (AST), serum creatinine, albumin, bilirubin, international normalized ratio (INR), protime, AST/ ALT ratio, and AST/ platelet ratio index (APRI).

2.2.3 Bile acid (BA) quantification by liquid chromatography-tandem mass spectrometry (LC-MS/MS)

BA concentrations were quantified by LC-MS/MS, as described previously [2, 6, 39, 40, 104]. Briefly, a Waters ACQUITY ultra performance liquid chromatography (UPLC) system (Waters, Milford, MA, USA) coupled to an Applied Biosystem 4000 Q TRAP® quadrupole linear ion trap hybrid mass spectrometer with an electrospray ionization (ESI) source (Applied Biosystems, MDS Sciex, Foster City, CA, USA) was used to perform the LC-MS/MS analysis. All chromatographic separations were performed with an ACQUITY UPLC® BEH C18 column (2.1x 150 mm, 1.7 µm) equipped with an ACQUITY UPLC C18 guard column (Waters, Milford, MA, USA). The following MS source settings were used: temperature, 500°C; ion spray voltage, −4000 V; collision gas pressure, high; curtain gas, 20; gas-1, 35; gas-2 35 (arbitrary units); Q1/Q3 resolution, unit; and interface heater, on. Mobile phase consisted of 7.5 mM ammonium bicarbonate, have been adjusted to pH 9.0 by using ammonium

hydroxide (mobile phase A) and 30% acetonitrile in methanol (mobile phase B) at a total flow rate of 0.2 ml/min. The gradient profile was held at 52.5% mobile phase B for 12.75 minutes, increased linearly to 68% in 0.25 minutes, held at 68% for 8.75 minutes, increased linearly to 90% in 0.25 minutes, held at 90% for one minute and finally brought back to 52.5% in 0.25 minutes and then followed by 4.75 minutes re-equilibration (total run time of 28 minutes per sample).

2.2.4 Preparation of standard solutions and calibration curves

For the preparation of standard solutions and calibration curves, blank matrices were obtained by charcoal stripping as mentioned early [2, 6, 39, 104]. Stock solutions of individual unsulfated BA and the IS (2H4-G-CDCA) were prepared in methanol (MeOH) at a concentration of 10 mg/mL and stock solutions of individual sulfated BA were prepared in deionized water at a concentration of 1 mg/mL. Human urine was incubated with 100 mg/mL activated charcoal for two hours to remove endogenous BA from the matrix. The mixture was then centrifuged at 16000 x g for 10 min, and the supernatant was aspirated and filtered using a 0.22-µm nylon filter. The filtrate from the stripped urine matrix was used for preparing the calibration curve. Eleven-point calibration curve was prepared by spiking 10 µL of the appropriate standard solutions and 10 µL of the IS stock (2H4-G-CDCA) into 100 µL of the stripped urine matrices. The final concentration of IS was 500 ng/ml and the dynamic range

of the standard curves for the various unsulfated and sulfated BA analytes was 1-1000 ng/ml.

2.2.5 Sample preparation

Solid phase extraction was used to extract urine samples as mentioned previously [2, 6, 39, 40, 104]. 100 µL of urine samples were spiked with 10 µL of internal standard (IS), vortexed and loaded on to SupelcleanTM LC-18 SPE cartridges pre-conditioned with 4 mL MeOH, followed by 4 mL H_2O. Loaded cartridges were then washed with 3 mL H_2O and eluted with 4 mL MeOH. The eluates were evaporated under vacuum at room temperature and reconstituted in a 100 µL of 50 % MeOH solution. Ten microliters of reconstituted samples were injected for LC-MS/MS analysis.

2.2.6 Calculation of BA indices

The BA profile in urine was characterized using BA “indices”, as we have described previously [2, 39, 40, 45, 104]. **Table 2.1** shows a summary of the BA indices used in the current study. BA indices describe the composition, hydrophilicity, formation of 12α-OH BA by CYP8B1, metabolism, and formation of secondary BA by intestinal bacteria. The composition indices were calculated as the ratio of the concentration of individual BA in all their forms (unamidated, amidated, unsulfated and sulfated) to the total concentration of BA. Hydrophilicity indices include the

percentages of the BA pool exist as mono-, di-, or tri-OH BA as well as the hydrophobicity index (HI) of the BA pool. The percentages of mono-OH BA (LCA), di-OH BA (UDCA, MDCA, HDCA, DCA and CDCA) and tri-OH BA (CA, MCA, and HCA) were calculated as the ratio of the concentration of the sum of the respective BA in all their forms to the total concentration of BA. HI was calculated according to the Heuman index, which based on the relative contributions of the individual BA to the total BA pool and their HIs [125].

12α-OH BA are formed by CYP8B1 in the liver and include DCA, CA, Nor-DCA, and 3-dehydroCA. Therefore, CYP8B1 activity can be measured by the ratio of 12α-OH BA to the remaining of all other BA (non-12α-OH BA). Another marker for CYP8B1 is the ratio of CA to CDCA because CA is formed by the 12α hydroxylation of CDCA. In the same way, the ratio of 12α-OH (DCA, CA, Nor-DCA, and 3-dehydroCA in all their forms) to non-12α-OH (HDCA, CDCA, UDCA, LCA, MDCA, MCA, HCA, 12-oxo-CDCA, 6-oxo- LCA, 7-oxo-LCA, 12-oxo-LCA, isoLCA, isoDCA in all their forms) was calculated.

BA are primarily metabolized by sulfation, and glycine (G), and taurine (T) amidation in the liver. The percentage of sulfation of individual BA was calculated as the ratio of the concentration of sulfated BA, in both the unamidated and amidated forms, to the total concentration of individual BA in all their forms (unamidated, amidated, unsulfated, and sulfated). The percentage of amidation of individual BA was calculated as the ratio of the concentration of amidated BA, in both the unsulfated

and sulfated forms, to the total concentration of individual BA in all their forms (unamidated, amidated, unsulfated, and sulfated). In addition, percentages of amidation were divided into the percentages of BA existing as taurine (T) or as glycine (G) amidates.

Primary BA are synthesized in the liver and secreted into the intestine via bile, where they are metabolized by intestinal bacteria into secondary BA. The ratio of primary (CA, CDCA, MCA and HCA in all their forms) to secondary BA (DCA, LCA, UDCA, HDCA, MDCA, Nor-DCA, 12-oxo-CDCA, 3-dehydroCA, 6-oxo-LCA, 7-oxo-LCA, 12-oxo- LCA, isoLCA, and isoDCA in all their forms) was also calculated.

2.2.7 Model development

Logistic regression analysis was used to develop prognostic models to predict the prognosis of hepatobiliary diseases in terms of developing disease-related complications. Models were constructed to predict (i) various individual complications and (ii) all complications combined (pooled) in the entire liver-patient population as well as in the individual disease subtype-populations (patient groups with specific disease subtypes). All statistical analysis was conducted using the Statistical Product and Service Solutions (SPSS) software, version 26 (IBM corporation, Armonk, NY, USA).

We developed models that can be classified into six categories: (i) BA variables only, (ii) Non-BA variables only, (iii) Mixed BA and non-BA variables, (iv) Original Model

for end-stage liver disease (MELD), (v) MELD variable with coefficients from our data set, and (vi) Original MELD modified with BA and/or non-BA variables.

Individual BA and/or non-BA variables were analyzed as possible predictors in a univariate logistic regression analysis. Significant variables (P<0.05) were selected from the univariate analysis to include in the multivariate analysis. The backward elimination regression method was used to retain the most significant variables with retention criteria of P < 0.05 during the multivariate analysis.

The estimated odds ratio (OR) of developing complications by BA and/or non-BA variables was calculated from the final multivariate logistic regression model for all subjects.

$$\log\ (\mathrm{OR}) = \log\left[\frac{\widehat{\mathrm{P}}}{1-\widehat{\mathrm{P}}}\right] = \mathrm{a} + \mathrm{b}_1\mathrm{x}_1 + \cdots + \mathrm{b}_\mathrm{k}\mathrm{x}_\mathrm{k}$$

Where $\widehat{\mathrm{P}}$ is the probability of developing complications; a is the intercept; and b represents regression coefficients for the x variables [126].

The final multivariate logistic regression model describes the association between significant BA and/or non-BA variables and the odds of developing complications. Then, we rewrote the multivariate logistic regression model as a function of the predicted probability, which transforms the estimated probabilities of complications to a scale of 0 to 1 using the following equation:

$$\widehat{\mathrm{P}} = \frac{\exp(\log\ (\mathrm{OR}))}{1 + \exp(\log\ (\mathrm{OR}))}$$

2.2.8 Model goodness of fit, validation and performance

Goodness of fit was assessed by using the Hosmer–Lemeshow test for logistic regression models. This test compares the observed number of individuals to the expected number of individuals in each pattern, which shows how well the data fits into the model [126]. In general, the Hosmer–Lemeshow test indicates a poor fit if the value is less than 0.05.

We used Akaike information criterion (AIC) to estimate out-of-sample prediction error from multivariate logistic regression models [127]. AIC values were derived from the likelihood function of models and result in a maximum likelihood estimate in the same data set [127]. Therefore, AIC values were used to compare models with different error distribution. Minimizing AIC values represents a good trade-off between goodness of fit and degrees of freedom [128]. The AIC values were calculated by:

$$AIC = -2\ln(L) + 2K$$

Where L is the maximized likelihood function; K is the number of parameters in the different models [129].

Bootstrapping was used to validate the models. Bootstrapping is a resampling technique used to estimate statistics on a population by sampling a dataset with replacements [130]. The parameters included P-value, Bias, and Standard Error [131]. The bootstrapping estimate of bias indicated the difference between the estimates computed using the original sample and the mean of the bootstrap estimate.

The standard error represented the standard deviation of the estimator and reflects how far our sample estimate deviates from the actual parameters [132]. The range of regression coefficients (B) was defined as the 95% confidence interval of the bootstrap estimator. Acceptance criteria of P-values were set at 0.05.

We also performed receiver operating characteristic curve (ROC) on the scores from multivariate logistic regression models to determine their cut-off value in differentiating patients with or without ascites. The cut-off values with optimum specificity vs. sensitivity were selected and the areas under the ROC curve (AUC) values were calculated. AUC of 0.9 or greater is rarely seen, AUC between 0.8 and 0.9 indicates excellent diagnostic accuracy, and any AUC over 0.7 may be considered clinically useful [126].

The performance of the different models in predicting the occurrence of complications were compared using statistical outcomes from the Hosmer–Lemeshow test, AIC values, bootstrapping, and AUC values.

2.3 Results

2.3.1 Demographics

Table 2.2 shows a summary of patients who participated in this study. The demographic variables included age, BMI, gender, and race. During the 7-year follow-up period, there were 257 patients with cholestatic liver diseases. The

development of the following liver disease-related complications was monitored: ascites (62), bacterial peritonitis (2), encephalopathy (36), GI bleeding (18), hepatobiliary carcinoma (15), hepatorenal syndrome (1), jaundice (7), peripheral edema (63), and portal hypertension (106).

2.3.2 Univariate logistic regression analysis for ascites prediction in the entire liver-patient population

Table 2.3 shows the results of univariate logistic regression analyses for ascites prediction by BA indices in the entire liver-patient population. The odds ratio (OR) quantifies the magnitude of the risk of developing ascites per one unit as well as 10% and 20% change of the normal value changes in BA indices. We found correlation between the odds of developing ascites and many BA indices ($P < 0.05$). Positive regression coefficients (B) values indicate that odds of developing ascites increase with increasing the values of BA indices, while negative coefficients imply the odds of developing ascites increase with decreasing the value of BA indices. For example, for every 20% increase in the % CDCA, the odds of developing ascites increased 1.387-fold (OR: 1.387; $P < 0.05$). In contrast for every 20 % increase in %MDCA, the odds of developing ascites decreased 0.774-fold (OR: 0.774; $P<0.05$).

We performed the same univariate logistic regression analysis for demographics and non-BA parameters as well (**Table 2.4).** For demographics, only gender was significant ($p < 0.05$), with the odds of developing ascites being significantly 1.3-fold

higher in males than females. For non-BA parameters, increasing levels of creatinine, INR, protime, AST, bilirubin, AST/ALT, and MELD significantly increased the odds of developing ascites, whereas decreasing levels of albumin and ALT significantly increased the odds of developing ascites. For every 20 % increase in the INR, the odds of developing ascites increased 1.391-fold (OR: 1.391; $P < 0.05$). In contrast, for every 20 % increase in the albumin, the odds of developing ascites decreased 0.231-fold (OR: 0.231; $P < 0.05$).

2.3.3 Multivariate logistic regression analysis for ascites prediction in the entire liver-patient population

The BA Model

In multivariate logistic regression analysis, a backward elimination regression was used to retain the most significant BA variables from univariate analysis.

The only BA variables retained in the multivariate model were %MDCA and % Primary BA, which were independently predictive of developing ascites **(Table 2.5.a)**. The estimated odds ratio (OR) of developing ascites as a function of BA variables (BA-OR) for individual patients were calculated using this equation:

$$\text{BA score} = \text{Log (BA-OR)} = \text{-3.463-(2.452} \times \%\ \text{MDCA)} + (0.045 \times \%\ \text{PrimaryBA})$$

The predicted probability ($\widehat{P}$) of ascites as a function of BA (BA-$\widehat{P}$) variables is then calculated using this equation:

$$\text{BA}\ (\widehat{P}) = \frac{\exp\ (\text{Log (BA OR)})}{1 + \exp(\text{Log (BA OR)})}$$

Figure 2.1.a shows the probability of developing ascites (BA-$\widehat{\mathrm{P}}$) as predicted by the BA score.

For example, for a patient with a %MDCA of 1%, and %Primary BA of 30%, the estimated odds ratio (BA-OR) of developing ascites by BA variables:

BA score = Log (BA-OR) = -3.463-(2.452 ×1%) + (0.045 ×30%) = -4.564

Then, the predicted probability of developing ascites (BA-$\widehat{\mathrm{P}}$) by BA variables can be calculated as:

$$\mathrm{BA}\ \left(\widehat{\mathrm{P}}\right)\ = \frac{\exp\ (-4.565)}{1+\ \exp(-4.565)} = 0.01$$

We tested the effect of the significant demographic variables from univariate analysis, i.e., gender, on this BA multivariate model. Gender was retained in the multivariate analysis but with no-minimal improvement of model validation criteria, including the bootstrapping approach **(Appendix. Table A**). For example, %MDCA and %Primary BA variables did not show any improvement for their p-values when compared with the BA model without gender. The value of bias, standard error, and relative standard error was not decreased in the BA model with gender. Furthermore, gender was retained in the BA model with no-minimal improvement for model comparison, including akaike information criterion and area under the ROC curve (**Appendix. Table B)**. For example, the AIC and AUC value was 215.63 and 0.833 which resulted in relatively minimal improvement to both values from the BA model without gender (AIC:223.56; AUC:0.811). Therefore, we did not include gender in the multivariate logistic regression model.

The Non-BA model

Albumin level and MELD were the only significant predictive variables of developing ascites **(Table 2.1.b)**. The estimated odds ratio (OR) of developing ascites as a function of non-BA variables (non-BA-OR) for individual patients was calculated from this equation:

$$\text{non} - \text{BA score} = \text{Log}\ (\text{non BA OR}) = 0.947 - (1.205 \times \text{Albumin level}\ \left(\frac{\text{g}}{\text{dl}}\right)) + (0.189 \times \text{MELD})$$

The predicted probability ($\widehat{\text{P}}$) of developing ascites as a function of non-BA (non-BA-$\widehat{\text{P}}$) variables were calculated using this equation:

$$\text{Non BA}\ \left(\widehat{\text{P}}\right) = \frac{\exp(\text{Log}\ (\text{non BA OR}))}{1 + \exp(\text{Log}\ (\text{non BA OR}))}$$

Figure 2.1.b shows the probability of developing ascites as predicted by the non-BA score.

For example, for a patient with albumin level of 1 g/dl, and MELD of 5, the estimated odds ratio (non-BA-OR) of developing ascites by non-BA variables:

$$\text{non} - \text{BA score} = \text{Log}\ (\text{non BA OR}) = 0.947 - (1.205 \times 1\ \left(\frac{\text{g}}{\text{dl}}\right)) + (0.189 \times 5) = 0.687$$

Then, the predicted probability (non-BA-$\widehat{\text{P}}$) of developing ascites by non-BA variables can be calculated as:

$$\text{Non BA}\ \left(\widehat{\text{P}}\right) = \frac{\exp(0.687)}{1 + \exp(0.687)} = 0.67$$

We performed the same multivariate logistic regression analysis for demographics and non-BA parameters as well. The results of the demographic variable (gender) were the same as the BA model. Because of no-minimal improvement on model validation and comparison, we did not include it in the multivariate logistic regression for the non-BA model **(Appendix. Table A-B)**.

The Mixed BA and Non-BA model

The variables retained in the multivariate model were %CDCA, primary/secondary BA, albumin level, and MELD which were independently predictive of developing ascites **(Table 2.5.c)**. The estimated odds ratio (OR) of developing ascites by mixed BA and non-BA for individual patients was calculated from this equation:

$$\text{mixed BA and non} - \text{BA score} = \text{Log}\ (\text{mixed BA and non BA OR}) = -0.275$$
$$+ (0.029 \times \%\text{CDCA}) - \left(0.077\ \times \frac{\text{PrimaryBA}}{\text{SecondaryBA}}\right)$$
$$- \left(1.143\ \times \text{Albumin level}\left(\frac{\text{g}}{\text{dl}}\right)\right) + (0.189\ \times \text{MELD})$$

The predicted probability ($\widehat{\text{P}}$) of developing ascites as a function of mixed BA and non-BA (mixed BA and non-BA-$\widehat{\text{P}}$) variables were calculated using this equation:

$$\text{Mixed BA and non BA}\ \left(\widehat{\text{P}}\right) = \frac{\exp(\text{Log}\ (\text{mixed BA and non BA OR}))}{1 + \exp(\text{Log}\ (\text{mixed BA and non BA OR}))}$$

Figure 2.1.c the probability of developing ascites as predicted by the mixed BA and non-BA score.

For instance, for a patient with %CDCA of 15%, Primary/Secondary BA of 1, Albumin level of 1 g/dl, and MELD with 2, the estimated odds ratio (mixed BA and non-BA-OR) of developing ascites by mixed BA and non-BA variables:

$$\text{mixed BA and non} - \text{BA score} = \text{Log}\ (\text{mixed BA and non BA OR}) = -0.275$$
$$+ (0.029 \times 15) - (0.077\ \times 1) - \left(1.143\ \times 1\left(\frac{\text{g}}{\text{dl}}\right)\right) + (0.189\ \times 2)$$
$$= -0.682$$

Then, the predicted probability (mixed-BA and non-BA -$\widehat{\text{P}}$) of developing ascites by mixed BA and non-BA variables can be calculated as:

$$\text{Mixed BA and non BA}\ \left(\widehat{\text{P}}\right) = \frac{\exp(-0.682)}{1 + \exp(-0.682)} = 0.34$$

The demographic variable (gender) results for multivariate regression analysis in this model were the same as the previous models **(Appendix. Table A-B)**. Thus, we did not include gender in the multivariate logistic regression for the mixed BA and non-BA model.

The Original MELD model

We also performed the same multivariate logistic regression analysis for the MELD parameter **(Table 2.5.d)**. The estimated odds ratio (OR) of developing ascites as a function of original MELD variables for individual patients was calculated from this equation:

$$\text{original MELD score} = \text{Log}\ (\text{MELD} - \text{OR}) = -4.049 + (0.276\ \times \text{MELD})$$

The predicted probability ($\widehat{\text{P}}$) of developing ascites as a function of original MELD variables were calculated using this equation:

$$\text{MELD}\ (\widehat{\text{P}})\ = \frac{\exp(\ \text{Log}\ \ (\text{MELD}))}{1 + \ \exp(\ \text{Log}\ \ (\text{MELD}))}$$

Figure 2.1.d shows the probability of developing ascites as predicted by the original MELD score.

For example, for a patient with MELD of 1, the estimated odds ratio (MELD) of developing ascites by MELD variables:

$$\text{original MELD score} = \text{Log}\ \ (\text{MELD} - \text{OR}) = -\ 40.49 + (0.276\ \times 1) = -3.773$$

Then, the predicted probability (MELD-$\widehat{\text{P}}$) of developing ascites by MELD variables can be calculated as:

$$\text{MELD}\ (\widehat{\text{P}})\ = \frac{\exp(\ -\ 3.773)}{1 + \ \exp(\ -\ 3.773)} = 0.02$$

Similar to the BA model development, we did not include gender in this model **(Appendix. Table A-B)**.

Other Hybrid Models

In addition, we used the same methodology to develop other models **(Appendix. Table C**) including: (i) MELD variables with coefficients from our data set to create a model with the original MELD variables, but with model coefficients derived from our data set. In this model, creatinine and INR variables from the original MELD were not statistically significant. (ii) Original MELD modified with BA or non-BA variables at a time, to test if the performance of the original MELD could be improved by adding significant BA or non-BA parameters from the univariate analysis. Original MELD modified with BA variables only did not pass the Hosmer–Lemeshow test (P-value <0.05), while original MELD modified with non-BA variables only did improve the

performance of the original MELD variables. However, this model has poor performance because of the low AUC (0.865) and high AIC (171) values compared to the mixed BA and non-BA model. (iii) Original MELD was modified with both BA and non-BA variables, to test if the performance of the original MELD could be improved by adding both significant BA and non-BA parameters from the univariate analysis. This model did not result in any improvement compared to the mixed BA and non-BA model **(Table 2.5.c).** In this model's performance, AUC (0.875) and AIC (167) values were the same as the mixed BA and non-BA model. Since none of these models has improved the performance of our main models, we did not further evaluate any of these approaches.

Similar to the BA model development, gender was not included in other hybrid models **(Appendix. Table D)**.

2.3.4 Model goodness of fit, validation, and performance

The Hosmer–Lemeshow test was used as one criteria to evaluate goodness of fit for all logistic regression models. For the BA model, the p-value of the Hosmer–Lemeshow (HL) test was 0.168 ($p>0.05$), which means that the observed and expected results were not significantly different, indicating the logistic regression of the BA model fit the data well. Other models including the non-BA model ($p=0.228$), the mixed BA and non-BA model (p-value = 0.11) also had a p value > 0.05. For the original MELD

model, the p-value of the Hosmer–Lemeshow test was 0.029 ($p< 0.05$), indicating the logistic regression of the original MELD model did not fit the data well **(Table 2.6)**.

Table 2.6 also shows the Akaike information criterion (AIC) for ascites prediction. AIC values were used to compare models with different error distribution. The AIC values for the BA, non-BA, mixed BA and non-BA, and original MELD models were 223.56, 170.81, 167.3, and 180.45. The BA model had a larger AIC value than the non-BA, mixed BA and non-BA, and original MELD models, which means this model did not have a good trade-off between goodness of fit and degrees of freedom. This indicates that the logistic regression of the BA model demonstrated a large error distribution.

Table 2.7 describes the bootstrapping validation for ascites prediction. Bootstrapping validation results for all four models indicated that the regression coefficients (B-value) were in the range of the 95% confidence intervals and p-values were statistically significant for all covariates ($p\text{-value}<0.05$). Bias values were relatively small (-0.056 to 0.016), which means the estimates calculated using the original sample and the mean of the bootstrap estimate were not significantly different. In contrast, standard error (SE) and relative standard error (RSE) (0.02% to 296.3%) values of the bootstrapping analysis were relatively high, which may reflect our sample estimate derivates far from the actual parameter (**Appendix. Figure A)**.

Figure 2.2 shows the receiver operating characteristics (ROC) curves of all four models for ascites prediction. The area under the ROC curve (AUC) for the BA, non-

BA, mixed BA and non-BA, and original MELD were 0.81, 0.87, 0.88, and 0.86, respectively.

We also calculated the sensitivity (SEN), specificity (SPE), positive predictive value (PPV) and negative predicative values (NPV) from ROC analysis **(Table 2.6)**. For instance, in the BA model, the sensitivity and specificity were 33.90% and 88.30%, the positive and negative predictive values were 48.80% and 80.20%.

Potential cut-off values of all 4 model scores to best differentiate patients with vs. without ascites were selected based on the optimum sensitivity vs. specificity from ROC analysis. The ROC-optimum cut-off values for BA, non-BA, mixed BA and non-BA models, and original MELD models for ascites prediction were -0.99, -1.18, -1.06, and -1.09, respectively **(Table 2.6)**.

Moreover, we tested if patient populations with scores below vs. higher than these optimum cut-off values can be distinguished using ROC analysis. The p-value of AUCs were used to find statistically significant differences between the low- vs. high-score populations (**Figure 2.3 and Table 2.8).** The null hypothesis for p-value of AUCs were AUC=0.5.

2.3.5 Prediction for other complications

We also followed the same approach to predict other complications of liver diseases including bacterial peritonitis, encephalopathy, GI bleeding, hepatobiliary carcinoma, hepatorenal syndrome, jaundice, peripheral edema, and portal

hypertension. **Appendix. Table E** shows the ROC analyses, p-values of the bootstrapping, Hosmer-Lemeshow tests, and Akaike information criterion (AIC) tests for the BA models. **Appendix. Tables F-H** show similar results for non-BA, mixed BA and non-BA, and original MELD models.

2.4 Discussion

In this study, we have examined the ability of BA indices to predict complications in patients with liver diseases. Logistic regression analysis was used to develop models to predict the prognosis of hepatobiliary diseases in terms of developing disease-related complications. In addition to the BA model, we have developed (i) non-BA, (ii) mixed BA and non-BA variables to compare with the BA-only and non-BA only models. (iii) MELD variables with coefficients from our data set were used to create a model with the original MELD variables, but with model coefficients derived from our data set. (iv) Original MELD was modified with BA and/or non-BA variables, to test if the performance of original MELD can be improved by adding significant BA and non-BA parameters from the univariate analysis. First, individual BA and non-BA variables were analyzed as possible predictors of developing ascites in a univariate logistic regression analysis. Then multivariate models were built using backward elimination regression, where only the most significant variables from the univariate regression were retained.

The final multivariate logistic regression models were then validated using bootstrapping method. Goodness of fit criteria also included the Hosmer-Lemeshow test, the Akaike information criterion (AIC), and multiple parameters from the receiver operating characteristic (ROC) analyses.

From univariate logistic regression analysis, total UDCA, total CA, total MCA, %CDCA, %sulfation, total Mono-OH, % T-amidation, % tri-OH, % non-12α-OH, and % primary BA significantly increased the odds of having ascites, whereas total DCA, total HDCA, %LCA, % G-amidation, %mono-OH, and % secondary BA decreased the odds of having ascites (**Table 2.3)**.

For demographics, univariate logistic regression analysis showed that the odds of having ascites was significantly 1.3-fold higher in males than females. For non-BA parameters, creatinine, INR, protime AST, bilirubin, AST/ALT, and MELD increased the odds of having ascites, whereas albumin and ALT decreased the odds of having ascites (**Table 2.4)**.

Using multivariate logistic regression analysis, we have constructed these final models for ascites prediction:

(i) The BA variables (BA-OR) model for ascites prediction:

$$\text{BA score} = \text{Log}\ (\text{BA OR}) = -3.463 - (2.452 \times \%\text{MDCA}) + (0.045 \times \%\text{PrimaryBA})$$

(ii) The non-BA variables (non-BA-OR) model for ascites prediction:

$$\text{non} - \text{BA score} = \text{Log}\ (\text{non BA OR}) = 0.947 - (1.205 \times \text{Albumin level}\ \left(\frac{\text{g}}{\text{dl}}\right)) + (0.189 \times \text{MELD})$$

(iii) The original MELD variables (MELD-OR) model for ascites prediction:

$$\text{original MELD score} = \text{Log}\ (\text{MELD OR}) = -4.049 + (0.276 \times \text{MELD})$$

(iv) The mixed BA and non-BA variables (mixed BA and non-BA-OR) model for ascites prediction:

$$\text{mixed BA and non} - \text{BA score} = \text{Log}\ (\text{mixed BA and non} - \text{BA} - \text{OR}) = -0.275 + (0.029 \times \%\text{CDCA}) - \left(0.077 \times \frac{\text{PrimaryBA}}{\text{SecondaryBA}}\right) - \left(1.143 \times \text{Albumin}\left(\frac{\text{g}}{\text{dl}}\right)\right) + (0.189 \times \text{MELD})$$

Gender was the only significant demographic variable in univariate logistic regression analysis for all models. However, it was not included in these models because it resulted in but with no-minimal improvement of model validation criteria including bootstrapping, AIC, and ROC-AUC (**Appendix. Tables A-D)**. Therefore, we did not include gender in the multivariate logistic regression model.

Cholestatic diseases are associated with impaired bile flow to the intestine, which is expected to translate into reduced transformation of primary BA into secondary BA by intestinal bacteria. Therefore, an accumulation of primary and a decrease in secondary BA in the blood may indicate further impairment in bile flow and existing liver disease [2, 133-136]. This was in agreement with the BA model, where increasing % Primary BA and decreasing %MDCA (a secondary BA) were the final significant predictors of liver disease prognosis. Furthermore, we have previously demonstrated survival model development for death prediction using cox regression

analyses. The same results have shown in their BA model, where increased %CDCA and %Tri-OH BA (both are primary BA) were the significant predictors of liver disease prognosis into death.

As shown in **Figure 2.1**, the probability of developing ascites increased as a function of BA, non-BA, mixed BA and non-BA original MELD, and original MELD scores. In general, logistic regression analysis produces a S-shaped curve, when predicated probability is plotted against the explanatory score [137]. All four models produced such S-shaped curves except for the BA score. This is expected in the absence of extreme values of BA scores from our data set. However, with more subject enrollment in the future, more extreme BA score values; therefore, S-curve shape, are expected.

Hosmer–Lemeshow test was one of the criteria to evaluate the goodness of fit for logistic regression models. The Hosmer–Lemeshow test results supported the validity of the BA, non-BA, and mixed BA and non-BA models (P-value >0.05), but not the original MELD model **(Table 2.6).** The original MELD model was the only model with P-value < 0.05, which indicates the expected and observed results were significantly different.

We also used Akaike information criterion (AIC) to compare the estimated out-of-sample prediction error from multivariate logistic regression models. Minimizing AIC values represents a good trade-off between goodness of fit vs. degrees of freedom [128]. The AIC value of the BA, non-BA, and original MELD models were 233.56,

170.81, and 180.45, which were higher than the AIC value of the mixed BA and non-BA model (167.3) (**Table 2.6**).

Models were validated using the bootstrapping method **(Table 2.7)**. Bootstrapping is a resampling technique used to estimate statistics on a population by sampling a dataset with replacement [130]. Random samples were taken one at a time, with replacement from our data set to create a series of 1000 new data sets. Statistics were calculated by comparing these data sets. In the BA model, the relative standard error was relatively large because the model parameter (%MDCA) has a high relative standard error (**Appendix. Figure A**). This could be due to the fact that %MDCA was not normally distributed in the original data set and because the sample size was relatively small [138]. Despite the high relative standard error, the BA model could be considered to pass the bootstrapping validation given the relatively small sample size of our study. Overall, the bootstrapping validation results supported the validity of the BA, non-BA, mixed BA and non-BA, and original MELD models for ascites prediction.

ROC analysis was used to compare the models for their accuracy to predict liver patient prognosis into complications such as ascites. The higher the arear under the ROC curve (AUC), the greater the overall accuracy of the marker in distinguishing between groups. For prognostic models, AUC of 0.9 or greater is rarely seen. AUC between 0.8 and 0.9 indicates excellent accuracy. And any AUC over 0.7 may be

considered clinically useful [139-141]. Therefore, all four models show high accuracy for ascites prediction.

ROC analysis was also performed to test sensitivity, specificity, and positive and negative predictive values **(Table 2.6)**. The sensitivity is the proportion of true positive patients (patients who were predicted to have ascites and actually did have ascites) to the actual positive patient population (total number of patients who actually did have ascites). The specificity is the proportion of true negative patients (patients who were predicted not to have ascites and actually did not have ascites) to the actual negative patient population (total number of patients who actually did not have ascites). The positive predictive value is the proportion of true positive patients to the total number of predicted positive patients. The negative predictive value is the proportion of true negative patients to the total number of predicted negative patients. The high sensitivity and specificity correspond to the high positive and negative predictive values, vice versa. Predictive values are more commonly used than sensitivity and specificity in clinical studies [137]. The higher positive and negative predictive values are preferred comparing model performance. Based on that, we compared positive and negative predictive values for all four models. The non-BA model has higher positive and negative predictive values than other models. In addition, the mixed BA and non-BA model also has high predictive values closed to the non-BA model. Therefore, both non-BA and mixed BA and non-BA models show better model performance than others.

Moreover, ROC analysis was used to determine potential cut-off values which quantify the normal range of biomarkers. The selection of optimum cut-off values is a tradeoff between sensitivity vs. specificity, where lower cut-off values are associated with higher sensitivity but lower specificity, and vice versa. Scores for the BA, non-BA, mixed BA and non-BA, and original MELD models were identified as cut-off values with optimum sensitivity vs. specificity, which were -0.99, -1.18, -1.06, and -1.09 respectively **(Table 2.6)**. For example, a BA score of -0.99 was considered an optimum cut-off value in differentiating patients with vs. without ascites because it maintained a balance between sensitivity (74%) vs. specificity (74%).

These ROC optimum cut-off values were used to split the overall patient population into two populations for every model. One population contained patients with model scores higher than the cut-off score and the other contained patients with model scores lower than the cut-off score. The p-value of AUCs from the two populations for every model were then used to find statistically significant differences (**Figure 2.3 and Table 2.8**). The p-value of AUCs are smaller than 0.05 and lead to the rejection of the null hypothesis, indicating AUCs are above the reference line (AUC=0.5), and vice versa. Only ROC-optimum cut-offs for the BA score (-0.99) resulted in statistically significant different AUCs based on their p-values; therefore, they were able to distinguish high- vs. low-score patient populations.

In addition to ascites, we attempted to develop similar models for the prediction of other common liver disease complications including bacterial peritonitis,

encephalopathy, GI bleeding, hepatobiliary carcinoma, hepatorenal syndrome, jaundice, peripheral edema, and portal hypertension **(Appendix. Tables E-H)**. None of these complications were as accurately predicted as ascites by any of the BA and non-BA models. In general, models for the prediction of other complications had lower sensitivity, lower specificity, lower AUC values, and higher AIC values. This could be due to the fact that other complications were less common than ascites (except for portal hypertension and peripheral edema) in our study. Overall, improving prediction accuracy would require an increase in the study population to predict all these other complications.

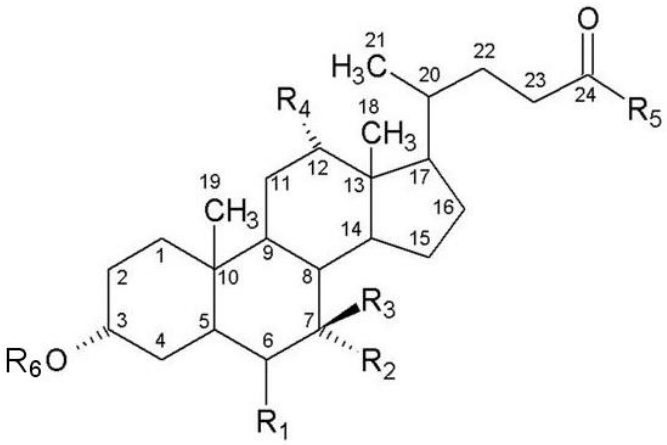
2.5 Conclusions

We have developed and validated a prognosis model based on BA indices to predict the development of liver disease complications such as ascites. Other models, including non-BA, mixed BA and non-BA, and original MELD models, were also developed to compare their performance with our BA model. Overall, the mixed BA and non-BA model was the most accurate based on AIC and ROC analyses. The mixed BA and non-BA had lower AIC values indicating a smaller error of distribution and a better trade-off between goodness of fit vs. degrees of freedom **(Table 2.6)**. Moreover, the mixed BA and non-BA model had the highest AUC values indicating higher accuracy than other models **(Figure 2.2).** Therefore, the mixed BA and non-BA model could be used to predict the development of ascites

in patients diagnosed with liver-disease at early stages of intervention, such as liver transplantation. This will assist in supply allocation and physician decisions when treating liver diseases.

2.6 Figures and Tables

Figure 1.1 The chemical structure of major BA and their glycine, taurine, and sulfate conjugates.



Bile acid	R_1	R_2	R_3	R_4
Tri-OH BA				
Cholic acid (CA)	H	OH	H	OH
α-Muricholic acid (α-MCA)	β-OH	OH	H	H
β-Muricholic acid (β-MCA)	β-OH	H	OH	H
ω-Muricholic acid (ω-MCA)	α-OH	H	OH	H
Hyocholic acid (HCA)	α-OH	OH	H	H
Di-OH BA				
Chenodeoxycholic acid (CDCA)	H	OH	H	H
Deoxycholic acid (DCA)	H	H	H	OH
Ursodeoxycholic acid (UDCA)	H	H	OH	H
Mono-OH BA				
Lithocholic acid (LCA)	H	H	H	H
R_5				
Unamidated BA	OH			
Glycine-amidated BA (G-BA)	NH_2CH_2COOH			
Taurine-amidated BA (T-BA)	$NH_2CH_2CH_2SO_3H$			
R_6				
Unsulfated BA	H			
Sulfated BA	SO_3H			

Figure 2.1 The relationship between the BA, non-BA, mixed BA and non-BA, and original MELD model scores and the probability of developing ascites.

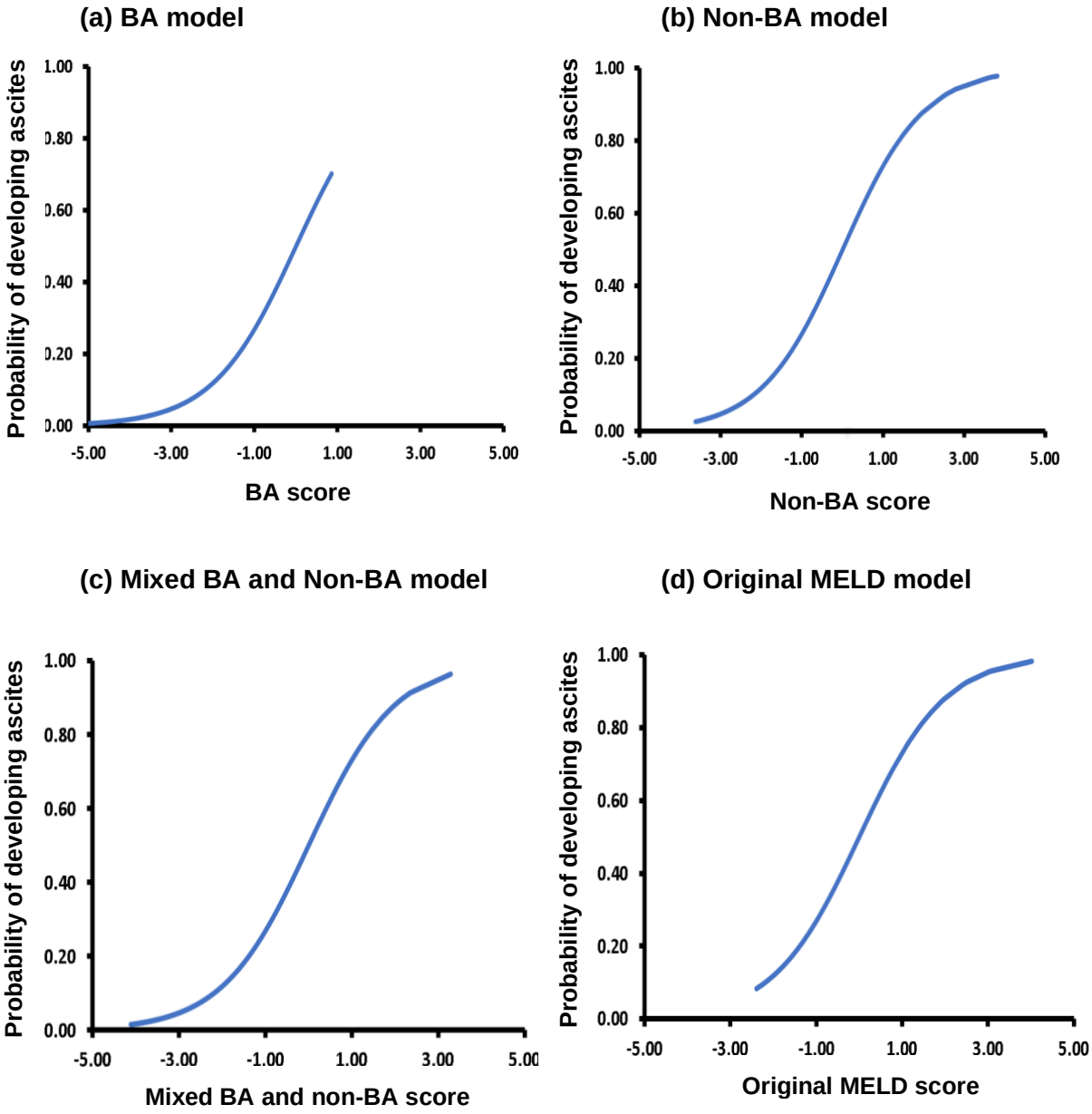


Figure 2.2 Receiver operating characteristics (ROC) curves of the BA, non-BA, mixed BA and non-BA, and original MELD models for ascites prediction. The area under the ROC curves (AUC) for (a) BA model, (b) non-BA model, (c) mixed BA and non-BA model, and (d) original MELD model for differentiating patients with ascites from patients without ascites.

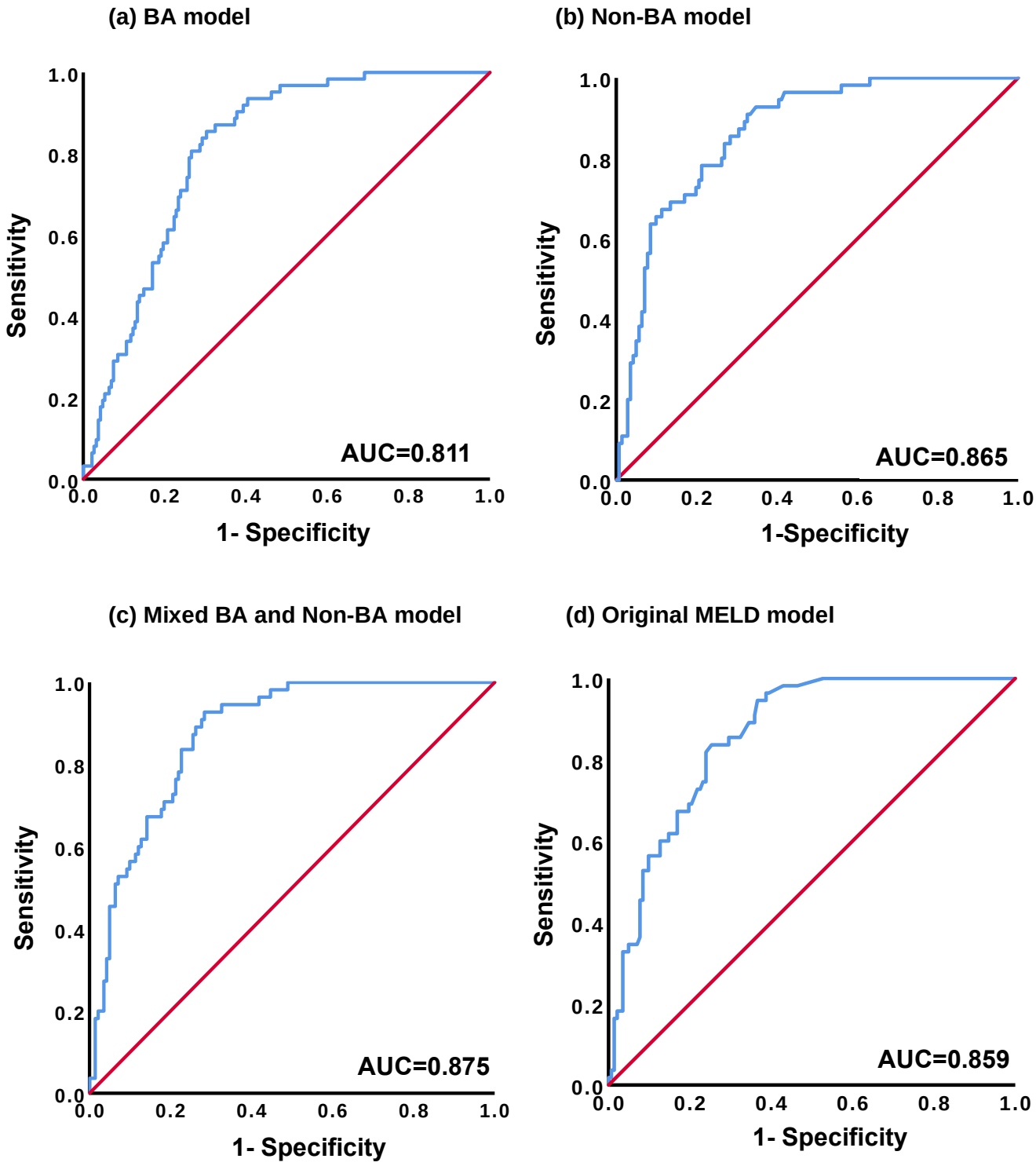
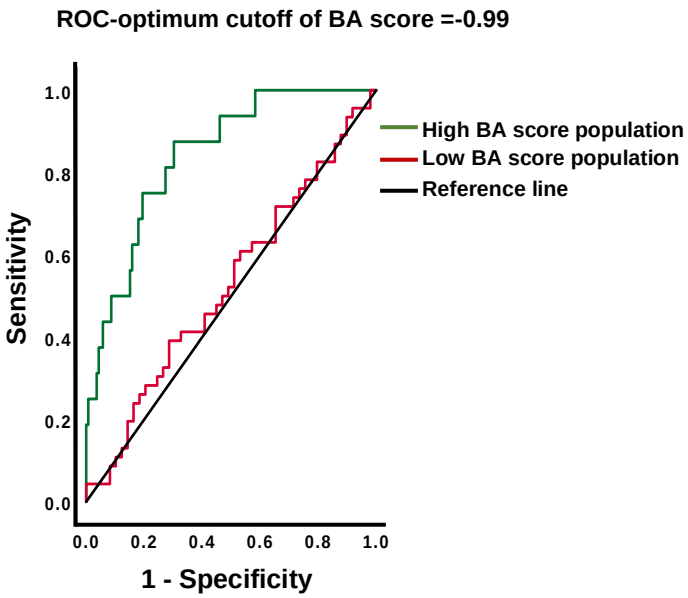
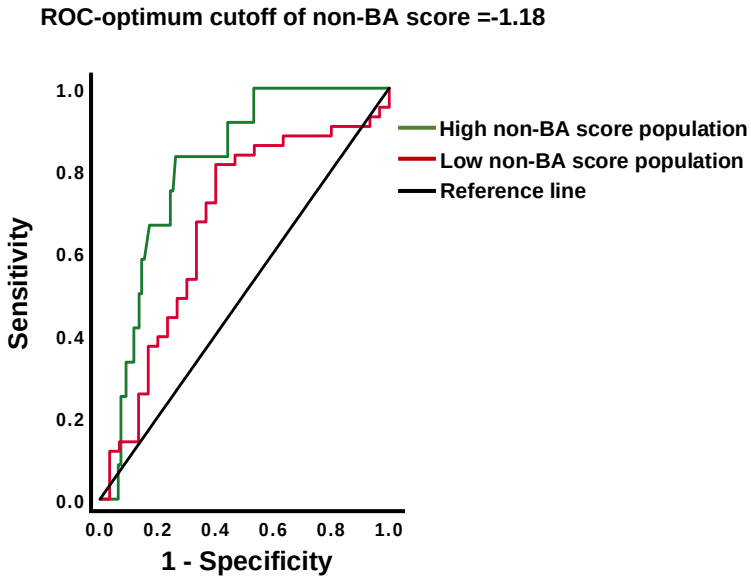


Figure 2.3 ROC analysis using optimum cut-off values in BA, non-BA, mixed BA and non-BA, and original MELD model scores.

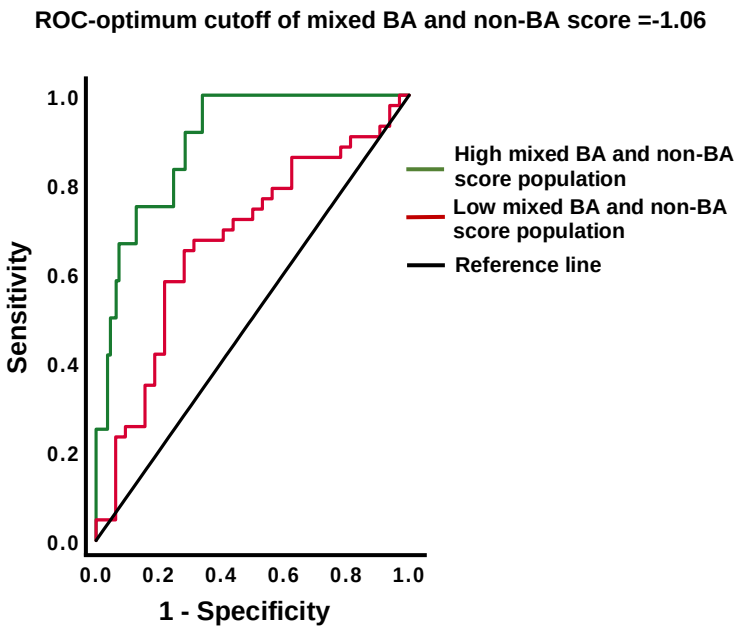
(a) BA model



(b) Non-BA model



(c) Mixed BA and non-BA model



(d) Original MELD model

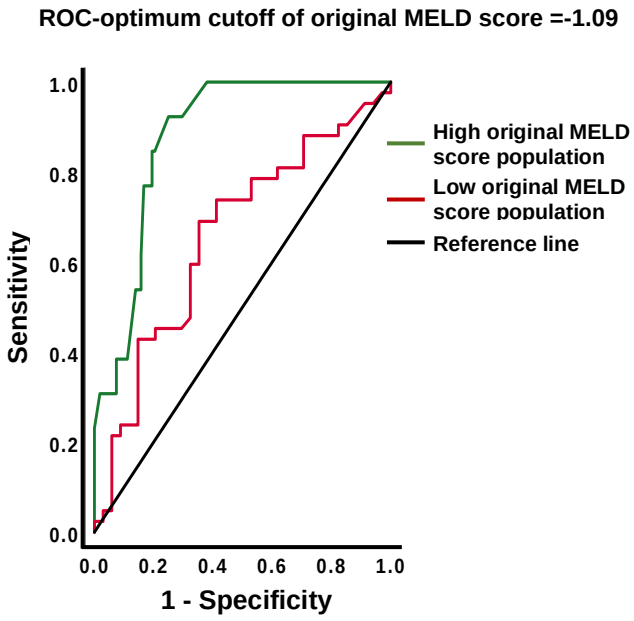


Table 1.1 Currently used biomarkers for hepatobiliary diseases.

Biomarker	Normal Range	Disease
Aspartate aminotransferase. (AST)	8IU/L- 48 IU/L	Hepatocellular injury with any cause[141] Nonalcoholic fatty liver disease[142] Primary biliary cirrhosis (PBC)[143]
Alanine aminotransferase (ALT)	7U/L- 55 U/L	Hepatocellular injury with any cause[141] Nonalcoholic fatty liver disease[142] Primary biliary cirrhosis (PBC)[143]
Gamma-glutamyl transferase. (GGT)	8U/L- 61 U/L	Biliary or pancreatic disease[141] Primary biliary cirrhosis (PBC)[143]
Alkaline phosphatase (ALP)	40U/L-129U/L	Cholestatic liver disease[141] Primary biliary cirrhosis (PBC)[143]
Albumin	3.5-5.0 g/dL	Nephrotic syndrome[141] Cirrhosis[144]
Total proteins	6.3-7.9 g/dL	Hepatitis C Alcoholic fatty liver disease[145]
Total bilirubin	0.3-1 mg/dL	Cirrhosis Nonalcoholic fatty liver disease[142]
Unconjugated bilirubin	0.2-0.8 mg/dL	Cirrhosis Nonalcoholic fatty liver disease[142]
Conjugated bilirubin	0.1-0.3 mg/dL	Cirrhosis Nonalcoholic fatty liver disease[142]
Lactate dehydrogenase (LD)	122-222 U/L	Hepatocellular carcinoma Acute liver failure (ALF)[146]
Prothrombin time (PT)	9.4-12.5 seconds	Prolonged in liver disease ,Pancreatic insufficiency[141];Cirrhosis[147]
International normalized ratio (INR)	~1.1	Cirrhosis[148]; Non-alcoholic fatty liver disease[149]
Serum creatinine	0.84-1.21 milligrams per deciliter	Nonalcoholic fatty liver disease[150] Hepatobiliary diseases [2]

Table1.2 Summary of models/scores/criteria for prediction of hepatobiliary disease prognosis

Models, Scores or Criteria	Disease	Outcomes	Study population	Normal range
Body composition-MELD (BC-MELD) = MELD score+3.59*skeletal muscle mass index (SM)I+5.42*high intramuscular adipose tissue content (IMAC)+2.06*high visceral-to-subcutaneous adipose tissue area ratio (VSR).[151]	Cirrhosis with liver transplantation	LTM	173 patients (male, 97; female 76) as acute liver failure as the indication for LT.	NA
Risk score=0.002*Carbohydrate Antigen 19-9 ((CA-199) +0.072*Age-6.612d[152]	Atypical bile duct hyperplasia	The model for predicting atypical hyperplasia in the intrahepatic bile duct	Total 375 patients. The atypical hyperplasia group 36 patients (man,15 and women,21). The non-atypical hyperplasia group 339 patients (93 males, 246 female)	NA
HBV-ACLF MELD (HAM) model= 0.174* MELD + 1.106 * hepatic encephalopathy (HE) -(0.003*alpha-fetoprotein(AFP))+ (0.237*white blood cell (WBC)) + (0.103 *Age) - 11.388[153]	Hepatitis B virus related acute-on-chronic liver failure (HBV-ACLF)	STM	A total of 530 HBV-ACLF patients. training cohort (300 patients) and validation cohort (230 patients)	NA
Risk score = 3.090 + 0.035 *Age (years) - 0.050 *PTA (%) + 0.005 * TBIL (mmol/L) + 0.044 *D/T (%) - 0.072 * Na (mmol/L) + 0.180 * HBV DNA (log10IU/mL) [154]	Acute deterioration (AD) of hepatitis B virus (HBV)-related chronic liver disease (CLD)	The verification and evaluation the new prediction model	754 patients with AD of HBV-related CLD, training cohort (580 patients) and a validation cohort (174 patients)	Score > -2.12 (higher survival rate) Score <-2.12 (lower survival rate)

ABIDE model= [2.003*INR+ 0.824*AST/ALT ratio + 0.821*(Type 2 diabetes:0 if absent, 1 if present) + 0.806*(esophageal varices: 0 if absent, 1 if present) + 0.332 *total bilirubin.[155]	Non-alcoholic fatty liver disease (NAFLD)	LTM related to liver cirrhosis in NAFLD patients	512 patients in derivation cohort, 299 patients with compensated cirrhosis 244 of 346 in validation cohorts	The high score ≥ 4.1 The low model score <4.1
Chronic Liver Failure Consortium (CLIF)-C Acute-on-chronic liver failure (ACLF) score= 10 x (0.33 *CLIF-C OF + 0.04 * age + 0.63 * Ln [leukocyte count] -2)[156]	Acute-on-chronic liver failure (ACLF)	STM	A total 177 patients with Acute-on-chronic liver failure (ACLF), Male (132) Female (45)	The score ≤ 39 with a higher survival rate. The score ≥ 51 with a lower survival rate.
The age-bilirubin-international normalized ratio-creatinine (ABIC) score = (age *0.1) + (serum bilirubin * 0.08) + (serum creatine * 0.3) +(INR * 0.8)[157]	Acute-on-chronic hepatitis B liver failure (HBV-ACLF)	STM	A 398 total patients diagnosed with HBV-ACLF, a training cohort of 305 patients and a validation cohort of 93 patients	The score > 9.44 With shorter survival time. The score ≤ 9.44 had longer survival
The Platelets- albumin-bilirubin (PALBI) score = (2.02*log10 bilirubin) +(-0.37*(log10 bilirubin)2) +(-0.04 *albumin) +(-3.48*log10 platelets) +(1.01* (log10 platelets)[158]	Cirrhosis	Prognostic indicator of mortality	A total 195 patients,127 male, 68 Female, median age 66 years	PALBI score: grade 1 (score ≤-2.53), grade 2 (>-2.52 to -2.09), and grade 3 (>-2.09)

The albumin- bilirubin (ALBI) score = 0:66 × log10 bilirubin (μmol/l) − 0.085 × albumin (g/l) [159]	Decompensated cirrhosis	STM	A total 456 patients with DeCi, The median age 53.5, Male(302), Female (154)	NA
ICGR15-MELD model= 0.117 × ICGR15 + 0.128 × MELD score − 3.446.[160]	Early allograft dysfunction (EAD) and early postoperative complications after LT	The accuracy of model	A total 87 consecutive liver transplant patients, a training cohort (n=61) and an internal validation cohort (n=26)	The score ≥0.098 (66.7% of EAD incidence). The score< 0.098 (6.5% of EAD incidence)
CLIF Consortium Acute Decompensation scores (CLIF-C ACLF) = 10×[0.03×Age(year) + 0.66×Ln (Creatinine(mg/dL)) + 1.71×Ln (INR) + 0.88×Ln (WBC (10^9 cells/L)) - 0.05 × Sodium(mmol/L)+8[161]	Chronic Liver Failure	STM	A total 209 patients with ACLF and 1245 patients without ACLF (Chronic Liver Failure)	NA
Lille Model and MELD Score= [2.4778 * (Lille model - 0.4114) + 0.0695 * (MELD – 24.6812)] * 0.9836 [162]	Alcoholic hepatitis	STM	A total of 712 patients. 67 patients from the derivation data set and 108 patients from the validation data set from 8 pooled cohort studies.	NA
Lille Model and Maddrey DF score= S = 2.5373 * (Lille model - 0.4195) + 0.0095 * (Maddrey's DF - 61.8519)] * 0.9850[162]	Alcoholic hepatitis	STM	A total of 712 patients. 67 patients from the derivation data set and 108 patients from the validation data set from 8	NA

			pooled cohort studies.	
Lille Model and ABIC Score= S = 2.3260 * (Lille model - 0.4114) + 0.2362 * (ABIC - 8.3882)] *0.980[162]	Alcoholic hepatitis	STM	A total of 712 patients. 67 patients from the derivation data set and 108 patients from the validation data set from 8 pooled cohort studies.	NA
Modified CTP score, Second modified CTP score, and creatinine-modified CTP core (Ascites, Encephalopathy, Serum bilirubin, Albumin and INR)[163]	Cirrhosis	The transplant-free survival in.	A total 30,897 cirrhotic patients with at least 5 years of follow-up, (72.3 %) male (97.2 %) cirrhotic patients	NA
King's College criteria (KCC) (The grade of hepatic encephalopathy, arterial blood pH, prothrombin time, and serum creatinine)[164]	Acute liver Failure	STM	100 consecutive patients with acetaminophen-induced ALF	NA
APACHE II score = acute physiology score + age points + chronic health points[116]	Acute-on-chronic liver failure	STM	100 patients were enrolled in the study, including 87 males and 13 females, with a median age of 49 years	NA
The sequential organ failure assessment (SOFA) score (PaO2/FiO2 (mmHg), Plateletsx 103/mm, Bilirubin (mg/dl) Glasgow Coma Sore. Creatinine, (mg/dl), hypotension (yes or no))[165]	Paracetamol-induced acute liver injury	Comparing prognostic accuracy on both modified MELD and SOFA score	A total of 138 patients (61 males, 77 female). 125 were classified as ‘non-paracetamol’ cases, and 123 patients had taken a staggered	NA

			paracetamol overdose.	
MESO=[MELD/Na (mmol/L)] +100 iMELD=MELD+[age(years)*0.3]-[0.7*Na(mmol/L] +100, UKELD=5*(1.5*ln(INR)+0.3*ln(creatinine(ulmol/L)+0.6*ln(bilirubin(lmol/L)-13*ln(mmol/L)+70 [165]	Paracetamol-induced acute liver injury	Comparing prognostic accuracy on both modified MELD and SOFA score	A total of 138 patients (61 males, 77 female). 125 were classified as 'non-paracetamol' cases, and 123 patients had taken a staggered paracetamol overdose.	NA
MDF (Maddrey's discriminant function) =4.6(prothrombin time -control time) + serum bilirubin [in µmol/L]/17.1[166]	Alcoholic hepatitis	STM	A total 66 patients with alcoholic hepatitis	NA
The Glasgow alcoholic hepatitis score (GAHS)[167]	Alcoholic hepatitis	STM	A total 241 patients with alcoholic hepatitis	The score<9 (high survival rate) The score ≥9(lower survival rate)
Beclere model = (0.0484 × [Age in Years] + 0.469 × [encephalopathy] + 0.537 × Loge [Bilirubin in µmol/L] - 0.052 × [Albumin in g/L] [168]	Alcoholic hepatitis	STM	A total 183 patients enrolled in the study	NA
The Alcoholic Hepatitis Histologic Score (AHHS) (Stage of fibrosis, Bilirubinostasis, Neutrophil infiltration, Megamitochondria)[169]	Alcoholic hepatitis	STM	A Total 121 patients admitted to the Liver Unit in Spain, and a total 205 patients from 5 academic centers in the	low morality (0-3 points) Moderate morality (4-5 points).

			United States and Europe	High morality (6-9 points)
TAP score = 100 × (exp [lr]/ 1 + exp [lr]; Note lr = −3.71 + (0.34*TMA) - (0.087*Pentane) [170]	Alcoholic Hepatitis	The severity of patients with Alcoholic Hepatitis	A total of 80 patients, 43 healthy subjects without liver disease	The score ≥36 with high mortality
The CLIF-SOFA score (Bilirubin, Cerebral failure, INR, mean arterial pressure, partial pressure of arterial oxygen/fraction of inspired oxygen)[171]	Acute-on-chronic liver failure	STM and LTM	A total 1349 patients with ACLF	NA
GLOBE score = 0.044378 * age at start of UDCA therapy + 0.93982 * LN(bilirubin times the upper limit of normal [ULN] at 1 year follow-up)+0.335648*LN(alkaline phosphatase times the ULN at 1 year follow-up) - 2.266708 * albumin level times the lower limit of normal (LLN) at 1 year follow-up)- 0.002581 * platelet count per 109/L at 1 year follow- up + 1.216865[172]	Primary biliary cirrhosis	The transplant-free survival for patients with PBC	4119 patients with PBC treated with ursodeoxycholic acid in European and North American countries	NA
UK-PBC Risk Scores [173]	Primary biliary cirrhosis	LTM	4,099 patients with PBC	NA
Rochester I Criteria: ALP 2* ULN (upper limit of normal)[174]	Primary Biliary Cholangitis	LTM	A total 180 patients, who we continue to follow with PBC.	NA
Paris I Criteria: ALP 3× ULN; AST 2× ULN; and TB 1 mg/dL [174]	Primary Biliary Cholangitis	LTM	A total 292 patients with PBC	NA
Rotterdam Criteria: TB <1× ULN and albumin >1× LLN[174]	Primary Biliary Cholangitis	LTM	A total 375 patients with PBC and median follow-up time was 9.7 years	NA
Toronto Criteria: ALP 1.67× ULN[174]	Primary Biliary Cholangitis	LTM	A total 69 Patients with PBC	NA
Paris II Criteria: ALP 1.5× ULN; AST 1.5× ULN; and TB 1 mg/dL[174]	Primary Biliary Cholangitis	LTM	A total165 patients with	NA

			early-stage PBC followed up for an average 7 years	
UDCA: Albumin(,38g/L) , Histologic stage (>3), Lack of biochemical response at 1 year.[175]	Primary Biliary Cholangitis	LTM	A total 192 patients with PBC	NA
Glasgow alcoholic hepatitis score (GAHS): Age, Leukocytes, Urea, PT (prothrombin) ratio, Bilirubin[176]	Alcoholic hepatitis	STM and LTM	A total 274 patients with alcoholic hepatitis	NA
Lille model= exp(R)/(1 + exp(-R); R= 3.19–0.101 * (age in years) + 0.147 * (albumin day 0 in g/L) + 0.0165 * (bilirubin day 0 - bilirubin day 7 (mmol/l)) - 0.206 * (renal insufficiency) - 0.0065 * (bilirubin day 0 in mmol/l)- 0.0096 (PT in seconds)[176]	Alcoholic hepatitis	STM	A total 274 patients with alcoholic hepatitis	NA
MELD-Na score= MELD + 1.59 * (135-Na), with maximum and minimum Na of 135 and 120 mEq/L [176]	Alcoholic hepatitis	STM	A total 274 patients with alcoholic hepatitis	NA
Mayo model = 0.871 log. (bilirubin in mg/dl) +-2.53 1og (albumin in gm/dl) +0.039* age in years +2.38 log. (prothrombin time in sec) +0.859 edema[177]	Primary Biliary Cirrhosis	LTM	A total 106 Mayo Clinic primary biliary cirrhosis patients	NA
The Nutritional Index (CONUT) [178]	End-Stage Liver Diseases (ELD)	LTM	A total 58 patients with end-stage liver diseases	NA
Prognostic nutritional indices (Onodera: PNI-O) = 10Albumin + 0.005(total lymphocyte count))[178]	End-Stage Liver Diseases (ELD)	LTM	A total 58 patients with end-stage liver diseases	NA
Actin-free Gc-globulin combine with King's college hospital criteria [179]	Acute liver failure (ALF)	STM	A total of 252 patients with varying etiologies from the U.S. ALF Study Group	NA

Clichy's Criteria (Factor V, age, and the presence of grade 3-4 encephalopathy)[180]	Fulminant hepatic failure (FHF)	STM	A total 120 consecutive patients with FHF, adults (n = 64) and children (n = 56)	NA
End-Stage Liver Disease (PELD) = 4.80*[Ln serum bilirubin (mg/dL)] + 18.57*[Ln INR] - 6.87*[Ln albumin (g/dL)] + 4.36*(year old) + 6.67*(growth failure)[180]	Fulminant hepatic failure (FHF)	STM	A total 120 consecutive patients with FHF, adults (n = 64) and children (n = 56)	NA
London Criteria (muscle fatigability or weakness presence of symptoms including the brain and centra nervous system, autonomic dysfunction, fluctuation of symptoms)[181]	Acute liver failure	STM	A total 61 patients had fulminant liver failure	NA
Hangzhou Criteria (Total tumor diameter less than or equal to 8 cm, Total tumor diameter more than 8 cm, with histopathologic grade I or II and preoperative AFP level less than or equal to 400 ng/mL)[182]	Hepatocellular Carcinoma	LTM	A total 195 patients with HCC were retrospectively analyzed and various clinical and pathological factors	NA
logit(P)=-4.595+0.824×fibrinogen concentration (g/L) + 0.641 × AFP score 1 for AFP<=20ng/ml, 2 for 20<AFP<=100ng/ml, 3 for100<AFP<=200ng/ml, 4 for 200<AFP<=400ng/ml, 5 for AFP>400ng/ml)[183]	Hepatocellular Carcinoma	LTM	A total of 119 patients receiving liver transplantation for 43hepatocellular carcinoma	The score < -0.85 with better outcome The score > -0.85 with less outcome
Milan Criteria (Single tumor less than 5 cm in size, no more than three tumors, all less than 3 cm in diameter)[184]	Hepatocellular Carcinoma	LTM	A total 195 patients with HCC were retrospectively analyzed and various clinical and pathological factors	NA

UCSF criteria (1 tumor ≤6.5 cm or ≤3 tumors with the largest tumor diameter ≤4.5 cm and total tumor diameter ≤8 cm)[185]	Liver transplantation (OLT) for patients with hepatocellular cancer (HCC)	LTM	A total of 3,434 patients underwent OLT for HCC during the study period	NA
Radiomics score=2.688195- 4.306105e-09× (Contrast_0) + 7.882485e-08× (Cluster Prominence_0) + 3.492191× (Information measure of correlation2_0) + 3.088437× (Inverse difference normalized (INN)-0)-2.511158× (Information measure of correlation2_2)-1.641851× (Energy_2.5)[186]	Solitary hepatocellular carcinoma (HCC)	LTM	A total of 319 solitary HCC patients	Rad score>4.32 with high mortality Rad score≤ 4.32 with low mortality
Barcelona Criteria (ALP)[174]	Primary Biliary Cholangitis	LTM	A total 292 patients with PBC	NA
MELD-XI score = 5.11 * ln (serum bilirubin) + 11.76 * ln (serum creatinine) + 9.44 [187]	End-Stage Liver Disease in pediatric patients undergoing orthotopic heart transplant	STM and LTM	A total 2,939 patients met the inclusion criteria	NA
Adam's score (Age, Presence of extrahepatic metastases; Major hepatic resection, R2 resection DFI, Primary tumor type)[188]	Non colorectal, non-neuroendocrine (NCNN) liver metastases	Prediction of survival rate	A total 78 consecutive patients with NCNN liver metastases	Low score (0-3) Medium score (4-6); High score (7-10)
ALFSG prediction model= Logit SS= 2.67 – 0.95(HE*)+1.56(Etiology*)-1.25(Vasopressor Use*) - 0.70 (ln bilirubin) - 1.35 (ln INR)[189]	Acute liver failure	LTM	A total 1974 patients who met criteria for ALF	NA
NAFLD fibrosis score = -1.675 + 0.037 * Age (yrs)+0.094*BMI(kg/m2)+1.13 * IFG/diabetes (Yes=1, No=0) + 0.99 * AST/ALT ratio-0.013 * Platelet (10^9/L) - 0.66 *Albumin (g/dl)[190]	Non-alcoholic fatty liver disease (NAFLD)	The liver-related mortality	A total 646 biopsy proven NAFLD patients	NAFLD score<-1.45 (Low) NAFLD score>0.67(High)

FIB-4 index= Age (yrs) * AST [U/L]/(Platelet [10^9/L] * (ALT [U/L]^1/2)[190]	Non-alcoholic fatty liver disease (NAFLD)	The liver-related mortality	A total 832 patients with NAFLD	FIB-4 index<1.3(Low); FIB-4 index>3.25(High)
BARD score (AST/ALT ratio> 0.8=2 points; BMI>28=1 point; Presence of diabetes= 1points; Score range from 0 to 4 points)[190]	Non-alcoholic fatty liver disease (NAFLD)	The liver-related mortality	A total 827 patients with NAFLD	NA
APRI score (AST to Platelet Ratio Index). (AST [IU/L])/(AST upper limit of normal [IU/L])/(Platelet [10^9/L])[190]	Non-alcoholic fatty liver disease (NAFLD)	The liver-related mortality	A total 236 patients fulfilled in this study	NA
Hepascore = exp [-4.185818 - (0.0249 * Age) + (0.7464* SEX) + (1.0039*a2 macroglobulin) + (0.0302*Hyaluronic acid)+(0.0691 Bilirubin) - (0.0012*GGT)][190]	Non-alcoholic fatty liver disease (NAFLD)	The liver-related mortality	A total 510 patients with hepatitis B or C and matched on fibrosis stage were included	NA
FORNs score=7.811 -3.131ln (platelet count) +0.781ln(GGT)+3.467ln(age)-0.014(cholesterol)[191]	Chronic Hepatitis C Patients Without Hepatic Fibrosis	The liver-related mortality	The cohort study included 502 consecutive patients with chronic hepatitis C.	FORNs score <4.21 and >6.9with significant fibrosis.
BARDI score (improved BRAD score by adding INR)[192]	Advanced liver fibrosis in nonalcoholic fatty liver disease	The liver-related mortality	A total 107 patients with biopsy proven NAFLD were enrolled.	NA
Frailty index=(-0.33*gender- adjusted grip strength)+(-2.529*number of chair stands per second)+(-0.04* balance time)+6[193]	Cirrhosis	The liver-related mortality	A total 536 patients enrolled in the study	NA
The donor risk index (DRI) (Age, COD (cause of death), DCD (donation after cardiac death), Partial/Split, race, regional/ national share, height, CIT (cold ischemia time)[194]	Nonalcoholic fatty liver disease	The liver-related mortality	A total 20023 transplants, using livers from deceased donors	Donor risk index (≤1.1or >1.5)
The balance of risk (BAR) score (MELD score, cold ischemia time, recipient age, donor age, previous liver transplantation,	End-stage liver disease	The mortality and	A total 233 patients	BAR score>18 (higher

and life support at the time of transplant)[195]		posttransplant outcome		survival rates) BAR≤18 (lower survival rates).
ADOPT-LC score (score range from 0-8) (Age CTP class (A, B, C), Charleston comorbidity index, Duration of anesthesia (<180, 181-420, >420))[196]	Cirrhosis	The in-hospital mortality	A total 2197 patients are involved in this study.	NA
Model for Early Allograft Function Scoring (MEAF)[197]	Early allograft dysfunction	STM	A study including 1026 consecutive liver transplants patients was performed for MEAF score development	NA
ALF in-hospital mortality score (ALFIHMS) = 0.714 + 0.02 (total bilirubin) + 0.03 (APACHE II score) × 10[198]	Acute liver failure (ALF)	The in-hospital mortality	55 individuals with ALF were included in the study.	ALFIHMS score>15 with 50% higher in-hospital mortality.

Note: STM and LTM represents the short-term mortality and long-term mortality.

Table 2.1 List of BA indices.

Composition	**Hepatic Metabolism**	**Hydrophilicity**	**CYP8B1 Activity**	**Intestinal Contribution**
Concentration of individual BA	Total Sulfated	Total Mono-OH	Total 12α-OH	Total Primary
% of individual BA	Total G-amidated	Total Di-OH	Total non-12α-OH	Total Secondary
	Total T-amidated	Total Tri-OH	12α-OH/ non12α-OH	Primary/ Secondary
	% Sulfation	% Mono-OH	CA/ CDCA	% Primary
	% Amidation	% Di-OH	% 12α-OH	% Secondary
	% G-amidation	% Tri-OH	% non-12α-OH	
	% T-amidation	HI		

Table 2.2 Demographics.

Total Patients(N)	257
Gender	
Male	136
Female	121
Age (years)	
Mean ± SEM	52.2 ± 0.71
Body Mass Index (BMI)	
Mean ± SEM	30.7 ± 0.45
Race	
White	217
Black	11
Asian	7
Hispanic	4
Others	18
Liver disease complications	
Ascites	62
Bacterial peritonitis	2
Encephalopathy	36
GI bleeding	18
Hepatobiliary carcinoma	15
Hepatorenal syndrome	1
Jaundice	7
Peripheral edema	63
Portal hypertension	106

Table 2.3 Univariate logistic regression analyses for the prediction of developing ascites in the entire liver-patient population based on BA indices.

BA (μM) / BA indices	B-value (Regression Coefficient)	P-value	Odds ratio (OR): Exp (B)		
			1 unit	10%	20%
Total BA	0.002	0.059	1.002	1.010	1.020
Total LCA	0.024	0.275	1.024	1.007	1.013
Total UDCA	0.001	0.538	1.001	1.002	1.004
Total CDCA	0.009	0.002	1.009	1.017	1.034
Total DCA	-0.001	0.871	0.999	0.999	0.999
Total HDCA	-20.099	1.000	0.000	0.980	0.961
Total MDCA	-20.104	0.999	0.000	0.923	0.851
Total CA	0.052	0.007	1.053	1.013	1.027
Total MCA	0.008	0.528	1.008	1.002	1.005
Total HCA	0.407	0.012	1.502	1.007	1.015
% LCA	-0.071	0.004	0.931	0.936	0.877
% UDCA	-0.049	0.000	0.952	0.892	0.795
% CDCA	0.048	0.000	1.049	1.178	1.387
% DCA	-0.061	0.000	0.941	0.908	0.825
% HDCA	-6.66	0.108	0.001	0.980	0.960
% MDCA	-3.281	0.003	0.038	0.880	0.774
% CA	0.065	0.005	1.067	1.040	1.081
% MCA	-0.007	0.713	0.993	0.996	0.991
% HCA	-0.671	0.001	0.511	0.977	0.954
Total Unamidated	0.016	0.076	1.016	1.009	1.017
Total G-amidated	0.002	0.103	1.002	1.008	1.017
Total T-amidated	0.019	0.016	1.019	1.011	1.021
% Amidation	0.041	0.017	1.042	1.433	2.054
% G-amidation	-0.004	0.665	0.996	0.970	0.940
% T-amidation	0.037	0.002	1.038	1.039	1.080
Total Unsulfated	0.061	0.076	1.016	1.009	1.017
Total Sulfated	0.002	0.061	1.002	1.009	1.018
% Sulfation	0.012	0.338	1.012	1.106	1.224
Total Mono-OH	0.024	0.275	1.024	1.007	1.013
Total Di-OH	0.002	0.074	1.002	1.008	1.017
Total Tri-OH	0.018	0.029	1.018	1.010	1.021
% Mono-OH	-0.071	0.004	0.931	0.936	0.877
% Di-OH	0.018	0.095	1.018	1.142	1.304
% Tri-OH	0.021	0.108	1.021	1.027	1.055
Total 12α-OH	0.008	0.162	1.008	1.007	1.014
Total non-12α-OH	0.002	0.068	1.002	1.008	1.017
12α-OH/ non12α-	-0.787	0.114	0.455	0.974	0.948
CA/ CDCA	-0.997	0.159	0.369	0.974	0.949
% 12α-OH	-0.033	0.014	0.968	0.928	0.861
% non-12α-OH	0.033	0.014	1.034	1.291	1.666
Total Primary	0.007	0.003	1.007	1.017	1.034
Total Secondary	0.001	0.543	1.001	1.003	1.005
Primary/ Secondary	0.09	0.001	1.094	1.020	1.041
% Primary	0.049	0.000	1.050	1.258	1.582
% Secondary	-0.049	0.000	0.952	0.770	0.594
HI	0.074	0.012	1.077	0.999	0.998

BA concentrations are in (μM), while BA indices are in percentage. HI is hydrophobicity index.

Table 2.4 Univariate logistic regression analyses for the prediction of developing ascites in the entire liver-patient population based on demographics and non-BA parameters

Demographics and. Non-BA parameters	B-value	P-value	Odds ratio (OR): Exp (B)		
			1 unit	10%	20%
Age(year)	0.012	0.366	1.012	1.000	1.001
BMI	-0.008	0.685	0.992	1.000	0.999
Gender	1.291	0.000	3.636	NA	NA
Race	*	0.258	*	*	*
Creatinine (mg/dL)	0.048	0.601	1.049	1.005	1.010
Albumin (g/dL)	-1.980	0.000	0.138	0.481	0.231
INR	1.529	0.000	4.614	1.180	1.391
Protime (sec)	0.133	0.000	1.142	1.156	1.337
AST (U/L)	0.003	0.168	1.003	1.017	1.034
ALT (U/L)	-0.004	0.257	0.996	0.977	0.955
Bilirubin (mg/dL)	0.536	0.000	1.709	1.069	1.142
AST/ALT	1.895	0.000	6.653	1.246	1.552
MELD	0.276	0.000	1.318	1.281	1.642

B-value: regression coefficient. *Race is a categorical variable which contain five race groups. There are five values for B-value and OR, one for each race group, which are not shown, because was not statistically significant in univariate logistic regression analysis. NA: Not applicable.

Table 2.5 Multivariate logistic regression analyses for ascites in the entire liver-patient population.

(a) BA model

BA Parameters	B-value (Regression Coefficient)	Standard Error	P-value	Odds ratio (OR): Exp (B)		
				1-unit	10%	20%
Intercept	-3.463	-	0.000	0.031	-	-
% MDCA	-2.452	1.112%	0.027	0.086	0.909	0.826
% PrimaryBA	0.045	0.008%	0.000	1.046	1.234	1.524

Using the regression coefficients from this table, the estimated (OR) of developing ascites by the BA model is:

BA score=Log (BA-OR)= -3.463-(2.452 ×% MDCA) +(0.045 ×% Primary BA)

(b) Non-BA model

Non-BA parameters	B-value (Regression Coefficient)	Standard Error	P-value	Odds ratio (OR) : Exp (B)		
				1-unit	10%	20%
Intercept	0.947	-	0.560	2.577	-	-
MELD	0.189	0.050	0.000	1.208	1.185	1.404
Albumin level	-1.205	0.387	0.002	0.300	0.640	0.410

Using the regression coefficients from this table, the estimated (OR) of developing ascites by the Non-BA model is:

non-BA score=Log (Non-BA-OR) = 0.947+ (0.189 × MELD) -(1.205 × albumin level)

(c) Mixed BA and Non-BA model

Mixed BA and non-BA parameters	B-value (Regression	Standard Error	P-value	Odds ratio (OR): Exp (B)		
				1-unit	10%	20%
Intercept	-0.275	1.768	0.894	0.79	-	-
% CDCA	0.029	0.012%	0.014	1.029	1.104	1.218
PrimaryBA/SecondaryBA	-0.077	0.032	0.015	0.926	0.983	0.967
Albumin level	-1.143	0.407	0.004	0.319	0.655	0.429
MELD	0.189	0.053	0.000	1.208	1.185	1.404

Using the regression coefficients from this table, the estimated (OR) of developing ascites by the mixed BA and non-BA model is

mixed BA and non-BA score=Log (BA-OR) = -0.275+(0.029×%CDCA) –
(0.077×Primary BA/Secondary BA) - (1.143 × Albumin level) + (0.189 × MELD)

(d) Original MELD model

MELD Parameters	B-value (Regression Coefficient)	Standard Error	P-value	Odds ratio (OR): Exp (B)		
				1-unit	10%	20%
Intercept	-4.049	0.554	0.000	1.317	-	-
MELD	0.276	0.045	0.000	0.017	0.026	0.001

Using the regression coefficients from this table, the estimated (OR) of developing ascites by the original MELD model is:

original MELD score= Log (MELD-OR) = -4.049+ (0.276 × MELD)

Table 2.6 Model comparisons for ascites prediction.

(a) BA model

ROC Analysis						
SEN	SPE	PPV	NPV	Cutoff value (SEN, SPE)	HL(P-value)	AIC value
33.90%	88.30%	48.80%	80.20%	-0.99 (74%, 74%)	0.168	223.56

(b) Non-BA model

ROC Analysis						
SEN	SPE	PPV	NPV	Cutoff value (SEN, SPE)	HL(P-value)	AIC value
56.40%	91.50%	72.10%	84.30%	-1.18 (78%, 78%)	0.228	170.81

(c) Mixed BA and Non-BA model

ROC Analysis						
SEN	SPE	PPV	NPV	Cutoff value (SEN, SPE)	HL(P-value)	AIC value
54.50%	90.10%	68.2%	83.60%	-1.06 (78%, 78%)	0.11	167.3

(d) Original MELD model

ROC Analysis						
SEN	SPE	PPV	NPV	Cutoff value (SEN, SPE)	HL(P-value)	AIC value
45.50%	91.50%	67.60%	81.30%	-1.09 (76%, 76%)	0.029	180.45

SEN (sensitivity), SPE (specificity), PPV (positive predictive value), NPV (negative predictive value). P-value is for the Hosmer-Lemeshow test (HL). AIC is Akaike information criterion.

Table 2.7 Bootstrapping validation for ascites predication models.

Variables	B-value	Bias	SE	RSE	p-value	95% CI	
						Lower	Upper
BA model							
Intercept	-3.463	-0.049	0.548	-	0.001	-4.666	-2.445
% MDCA	-2.452	-0.192	0.948%	296.3%	0.002	-4.823	-1.148
% PrimaryBA	0.045	-0.049	0.008%	0.02%	0.001	0.032	0.061
Non-BA model							
Intercept	0.947	-0.056	1.702	-	0.554	-2.606	4.139
MELD	0.189	0.009	0.062	0.59%	0.001	0.086	0.325
Albumin_level	-1.205	-0.014	0.389	11.21%	0.001	-2.028	-0.490
Mixed BA and non-BA model							
Intercept	-0.236	-0.052	2.029	-	0.897	-4.572	3.484
% CDCA	0.029	-0.002	0.013%	0.03%	0.013	-0.001	0.052
Primary/Secondary	-0.077	0.012	0.055	1.58%	0.028	-0.164	0.053
Albumin (g/dL)	-1.158	-0.023	0.46	13.26%	0.005	-2.108	-0.219
MELD	0.189	0.016	0.066	0.63%	0.003	0.087	0.341
Original MELD model							
Intercept	-4.049	-0.098	0.658	-	0.001	0.183	0.411
MELD	0.276	0.007	0.061	0.59%	0.001	-5.573	-2.996

B-value (Regression Coefficient). SE (Standard Error). RSE (Relative standard Error). CI (Confidence Interval).

Table 2.8 ROC analysis using optimum cut-off values.

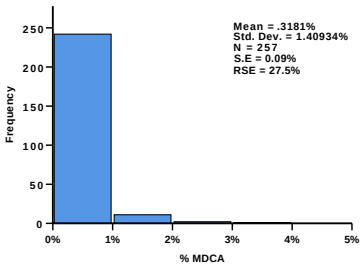
Cutoff	AUC	P-value	SE	95% CI	
				Lower	Upper
BA score					
High BA score<-0.99	0.842	0.00	0.05	0.752	0.932
Low BA score≥-0.99	0.527	0.65	0.06	0.41	0.644
Non-BA score					
High non-BA score<-1.18	0.806	0.00	0.05	0.707	0.905
Low non-BA score≥-1.18	0.670	0.01	0.07	0.538	0.801
Mixed BA and non-BA score					
High BA and non-BA score<-1.06	0.895	0.00	0.04	0.821	0.970
Low BA and non-BA score≥-1.06	0.672	0.01	0.06	0.546	0.797
Original MELD score					
High original MELD score<-1.09	0.879	0.00	0.04	0.809	0.949
Low original MELD score≥-1.09	0.657	0.01	0.06	0.532	0.782

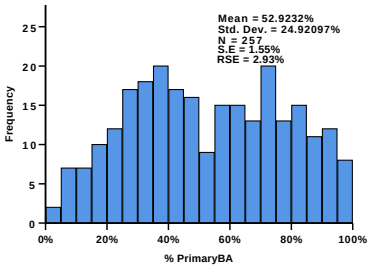
AUC is the area under the ROC curve. SE (Standard Error). CI (Confidence Interval).

Appendix

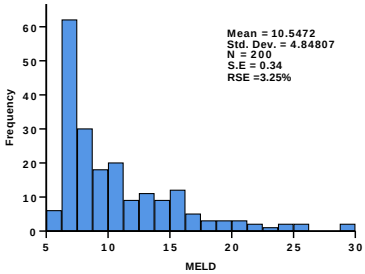
Figure A. Histograms for the BA, non-BA, mixed BA and non-BA, and original MELD model's variables.

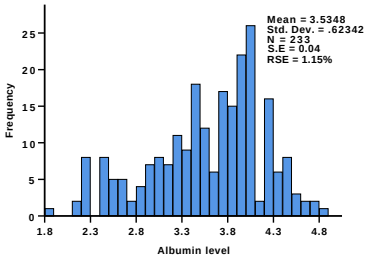
(a) BA model's variables



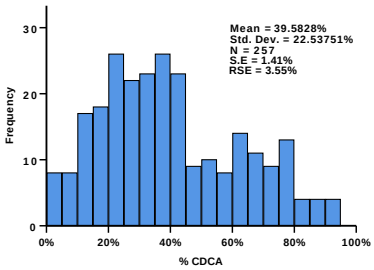


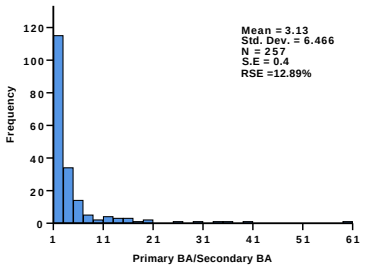
(b) Non-BA model's variables

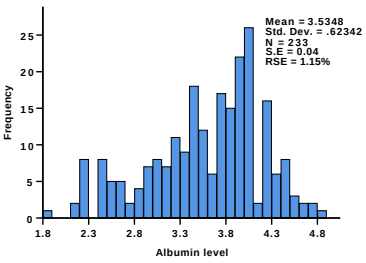


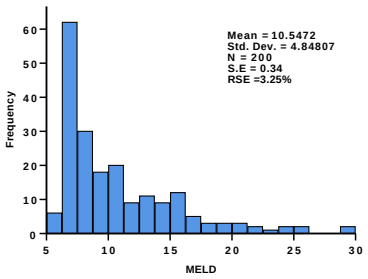


(c) Mixed BA and non-BA model's variables









(d) Original MELD model

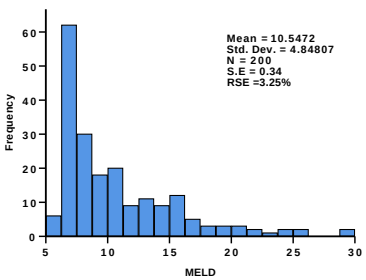


Table A. Bootstrapping validation for ascites predication models with gender.

Variables	B-value	Bias	SE	RSE	p-value	95% CI	
						Lower	Upper
BA model							
Intercept	-4.057	-	-	-	-	-	-
% MDCA	-2.568	-0.201	1.07%	334.4%	0.009	-5.309	-1.096
% PrimaryBA	0.044	0.001	0.008%	0.02%	0.001	0.03	0.062
Gender	1.121	0.023	0.404	76.2%	0.003	0.387	1.996
Non-BA model							
Intercept	0.385	-	-	-	-	-	-
MELD	0.180	0.016	0.065	0.62%	0.003	0.086	0.347
Albumin_level	-1.248	-0.023	0.409	11.57%	0.001	-2.131	-0.480
Gender	1.213	0.011	0.482	91.0%	0.004	0.368	2.263
Mixed BA and non-BA model							
Intercept	-0.54	-	-	-	-	-	-
% CDCA	0.025	-0.001	0.014%	0.04%	0.026	-0.003	0.052
Primary/Secondary	-0.068	0.006	0.054	1.7%	0.042	-0.159	0.059
Albumin (g/dL)	-1.230	-0.03	0.438	12.4%	0.002	-2.151	-0.438
MELD	0.181	0.019	0.064	0.61%	0.003	0.084	0.327
Gender	1.127	0.056	0.528	99.6%	0.01	0.224	2.322
Original MELD model							
Intercept	-4.696	-	-	-	-	-	-
MELD	0.270	0.014	0.064	0.61%	0.001	0.180	0.425
Gender	1.083	0.043	0.446	84.1%	0.011	0.294	2.070

B-value (Regression Coefficient). SE (Standard Error). RSE (Relative standard Error). CI (Confidence Interval).

Table B. Model comparison for ascites prediction with gender.

The BA model with gender	
AUC value	AIC value
0.833	215.63
The non-BA model with gender	
AUC value	AIC value
0.872	164.15
The mixed BA and non-BA model with gender	
AUC value	AIC value
0.878	160.8
The original MELD model with gender	
AUC value	AIC value
0.855	175.29

AUC is the area under the ROC curve. AIC is Akaike information criterion.

Table C. Other models for ascites prediction.

Other Models	Logistic(P)	Bootstrapping(P)	HL(P)	AUC	AIC value
MELD variables with coefficients from our data set					
Creatinine	0.739	NA	NA	NA	NA
INR	0.155	NA			
Bilirubin	0.000	NA			
Original MELD modified with BA variables					
MELD	0	0.002	0.037	0.859	171
%PrimaryBA	0.009	0.005			
Original MELD modified with non-BA variables					
MELD	0.000	0.001	0.228	0.865	171
Albumin level	0.002	0.001			
Original MELD modified with BA and non-BA variables					
%CDCA	0.014	0.013	0.11	0.875	167
Primary/SecondaryBA	0.015	0.028			
Albumin level	0.005	0.005			
MELD	0.000	0.003			

(P) is P-value. NA: Not applicable. Bootstrapping was not performed because P-values of model parameters were not significant (P-value > 0.05). HL is the Hosmer–Lemeshow test. AUC is the area under the ROC curve. AIC is Akaike information criterion.

Table D. Other models for ascites prediction with gender.

Other Models	Logistic(P)	Bootstrapping(P)	HL(P)	AUC	AIC value
MELD variables with coefficients from our data set					
Creatinine	0.537	NA	NA	NA	NA
INR	0.091	NA			
Bilirubin	0.000	NA			
Gender	0.002	NA			
Original MELD modified with BA variables					
MELD	0.000	0.001	0.043	0.862	171
%PrimaryBA	0.017	0.025			
Gender	0.02	0.021			
Original MELD modified with non-BA variables					
MELD	0.000	0.003	0.706	0.870	165
Albumin level	0.001	0.001			
Gender	0.006	0.008			
Original MELD modified with BA and non-BA variables					
%CDCA	0.031	0.026	0.145	0.878	161
Primary/SecondaryBA	0.032	0.042			
Albumin level	0.003	0.002			
MELD	0.001	0.003			
Gender	0.013	0.01			

(P) is P-value. NA: Not applicable. Bootstrapping was not performed because P-values of model parameters were not significant (P-value > 0.05). HL is the Hosmer–Lemeshow test. AUC is the area under the ROC curve. AIC is Akaike information criterion.

Table E. Prediction of other liver disease complications using BA models.

	ROC Analysis							
Other Complications	**SEN**	**SPE**	**PPV**	**NPV**	**AUC**	**B(P)**	**HL(P)**	**AIC value**
Bacterial peritonitis	0%	100%	0%	99.2%	0.952	0.001	0.967	22.39
Encephalopathy	2.8%	98.1%	20.0%	85.7%	0.777	0.001	0.744	177.75
GI bleeding	0%	100%	0%	92.8%	0.791	0.001	0.027	112.81
Hepatobiliary carcinoma	0%	100%	0%	94%	0.745	0.001	0.714	104.52
Hepatorenal syndrome	NA	NA	NA	NA	NA	NA	NA	NA
Jaundice	14.3%	100%	100%	97.6%	0.867	0.001	0.218	55.22
Peripheral edema	17.5%	98.80%	64.7%	77.7%	0.710	0.553	0.418	262.89
Portal hypertension	63.2%	82.6%	72.8%	75.3%	0.813	0.001	0.480	266.10

SEN (sensitivity), SPE (specificity), PPV (positive predictive value), NPV (negative predictive value). HL is the Hosmer–Lemeshow test. AUC is the area under the ROC curve. B(p) is the P value for Bootstrapping method. AIC is Akaike information criterion. NA: Not applicable.

Table F. Prediction of other liver disease complications using non-BA models.

	ROC Analysis							
Other Complications	**SEN**	**SPE**	**PPV**	**NPV**	**AUC**	**B(P)**	**HL(P)**	**AIC value**
Bacterial peritonitis	NA	NA	NA	NA	NA	NA	NA	NA
Encephalopathy	24.2%	97.0%	61.5%	86.4%	0.829	0.001	0.140	145.28
GI bleeding	0.0%	100.0%	0.0%	92.9%	0.762	0.001	0.588	105.72
Hepatobiliary carcinoma	NA	NA	NA	NA	NA	NA	NA	NA
Hepatorenal syndrome	NA	NA	NA	NA	NA	NA	NA	NA
Jaundice	25.0%	99.1%	50.0%	97.3%	0.961	0.001	0.967	43.63
Peripheral edema	38.6%	90.7%	59.5%	80.7%	0.839	0.003	0.225	193.34
Portal hypertension	67.4%	82.2%	78.0%	72.8%	0.818	0.005	0.251	213.17

SEN (sensitivity), SPE (specificity), PPV (positive predictive value), NPV (negative predictive value). HL is the Hosmer–Lemeshow test. AUC is the area under the ROC curve. B(p) is the P value for Bootstrapping method. AIC is Akaike information criterion. NA: Not applicable.

Table G. Prediction of other liver disease complications using mixed BA and non-BA models.

	ROC Analysis							
Other Complications	**SEN**	**SPE**	**PPV**	**NPV**	**AUC**	**B(P)**	**HL(P)**	**AIC value**
Bacterial peritonitis	0.0%	100.0%	0.0%	99.2%	0.952	0.004	0.967	22.39
Encephalopathy	24.2%	86.4%	61.5%	86.4%	0.829	0.001	0.14	145.28
GI bleeding	0.0%	100%	0.0%	92.8%	0.809	0.008	0.886	111.72
Hepatobiliary carcinoma	0.0%	100%	0.0%	94.0%	0.717	0.001	0.703	107.07
Hepatorenal syndrome	NA	NA	NA	NA	NA	NA	NA	NA
Jaundice	NA	NA	NA	NA	NA	NA	NA	NA
Peripheral edema	50.9%	91.9%	69.0%	84.1%	0.857	0.352	0.694	188.06
Portal hypertension	67.7%	87.4%	80.7%	77.6%	0.858	0.006	0.09	223.88

SEN (sensitivity), SPE (specificity), PPV (positive predictive value), NPV (negative predictive value). HL is the Hosmer–Lemeshow test. AUC is the area under the ROC curve. B(p) is the P value for Bootstrapping method. AIC is Akaike information criterion. NA: Not applicable.

Table H. Prediction of other liver disease complications using original MELD models.

	ROC Analysis							
Other Complications	**SEN**	**SPE**	**PPV**	**NPV**	**AUC**	**B(P)**	**HL(P)**	**AIC value**
Bacterial peritonitis	NA	NA	NA	NA	NA	NA	NA	NA
Encephalopathy	24.2%	97.0%	61.5%	86.4%	0.829	0.001	0.14	145.28
GI bleeding	27.1%	90.5%	75.9%	52.8%	0.684	0.001	0.72	108.07
Hepatobiliary carcinoma	NA	NA	NA	NA	NA	NA	NA	NA
Hepatorenal syndrome	NA	NA	NA	NA	NA	NA	NA	NA
Jaundice	90.9%	91.0%	75.9%	97.0%	0.939	0.001	0.799	43.9
Peripheral edema	28.1%	93.6%	64.0%	76.2%	0.778	0.001	0.279	207.81
Portal hypertension	63.2%	81.4%	75.9%	70.3%	0.818	0.001	0.022	221.49

SEN (sensitivity), SPE (specificity), PPV (positive predictive value), NPV (negative predictive value). HL is the Hosmer–Lemeshow test. AUC is the area under the ROC curve. B(p) is the P value for Bootstrapping method. AIC is Akaike information criterion. NA: Not applicable.

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