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# Cholemic nephropathy causes acute kidney injury and is accompanied by loss of aquaporin 2 in collecting ducts

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**TITLE**

# Cholemic nephropathy causes acute kidney injury and is accompanied by loss of aquaporin 2 in collecting ducts

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**List of Abbreviations**

HRS – hepatorenal syndrome

AKI – acute kidney injury

APAP - acetaminophen

LTx – Liver transplantation

KTx – Kidney transplantation

CN – cholemic nephropathy

AST – aspartate aminotransferase

ALT – alanine aminotransferase

AP – alkaline phosphatase

GGT – gamma glutamyl transferase

CHE – cholinesterase

INR – international normalized ratio

CRP – C-reactive protein

PRBC – packed red blood cells

FFP – fresh frozen plasma

ULN – upper limit of normal

N/A – not applicable

ESRD – end-stage renal disease

RRT – renal replacement therapy

AQP2 – aquaporin 2

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## ABSTRACT

Impairment of renal function often occurs in patients with liver disease. Hepatorenal syndrome is a significant cause of acute kidney injury (AKI) in cirrhotic patients (HRS-AKI, type 1). Causes of non-HRS AKI include cholemic nephropathy (CN), a disease that is characterized by intratubular bile casts and tubular injury. As data on patients with CN is mostly obtained from case reports or autopsy studies, we aimed to investigate the frequency and clinical course of CN. We identified 149 patients who underwent kidney biopsy between 2000 to 2016 at the Department of Gastroenterology, Hepatology and Endocrinology. Of these, 79 had a history of liver disease and deterioration of renal function. When applying recent EASL criteria 45 of the 79 patients (57%) presented with AKI, whereas 34 patients (43%) had chronic kidney disease (CKD) (43%). Renal biopsy revealed the diagnosis of CN in 8 of the 45 patients with AKI (17.8%), whereas none of the patients with CKD was diagnosed with CN. Univariate analysis identified serum bilirubin, alkaline phosphatase and urinary bilirubin and urobilinogen as predictive factors for the diagnosis of CN. Histological analysis of AKI patients with normal bilirubin, elevated bilirubin and the diagnosis of CN revealed loss aquaporin 2 (AQP2) expression in collecting ducts in patients with elevated bilirubin and CN. Biopsy related complications requiring medical intervention occurred in four of 79 patients (5.1%).

In conclusion, CN is a common finding in patients with liver disease, AKI and highly elevated bilirubin. Loss of AQP2 in AKI patients with elevated bilirubin and CN might be the result of toxic effects of cholestasis and be in part responsible for the impairment of renal function.

## INTRODUCTION

Impairment of renal function is common in patients with acute and chronic liver disease and associated with an increased mortality (1). Recently, the European Association for the Study of the Liver (EASL) published a clinical practice guideline on the management of patients with decompensated cirrhosis including definitions of kidney disease. Depending on the dynamic and duration of renal impairment, three entities are considered: acute kidney injury (AKI), acute kidney disease (AKD) and chronic kidney disease (CKD) (2). Whereas the prevalence of CKD in patients with liver disease is not well defined (2), AKI is a common complication in patients with liver cirrhosis and hepatorenal syndrome as a cause of AKI (HRS-AKI) occurs in approximately 20% of hospitalized patients with decompensated liver cirrhosis (3).

Other causes of AKI (non-HRS-AKI) include inflammation, bacterial translocation, cardiac dysfunction and bile acids (4). In this context, a disease entity entitled cholemic nephropathy (CN) regained attention. Along with impaired renal function in the context of liver disease, these patients show characteristic histomorphological kidney alterations including intratubular casts and tubular injury (5). Even though CN was first described in the early 20$^{th}$ century, this disease entity has been neglected until recently. Moreover, most of the data on patients with CN was derived from case reports (6-15) or autopsy studies (16-18). The diagnostic work-up of impaired renal function in the context of liver cirrhosis usually includes non-invasive diagnosis and does not include kidney biopsy owing to coagulopathy with increased risk of bleeding (19). The aim of this study was to investigate the frequency, clinical course and histomorphological characteristics of CN in a tertiary care hospital over a period of more than 15 years (2000-2016).

## METHODS

### Patients

We identified 149 patients who underwent kidney biopsy between 2000 and 2016 at the Department of Gastroenterology, Hepatology, and Endocrinology at a tertiary care hospital (Hannover Medical School (MHH)). Of these patients, 50 had no history of liver disease and therefore were excluded from this study. Twenty of the remaining 99 patients with an existing liver disease were biopsied due to suspected renal malignancy, whereas 79 patients received a kidney biopsy due to deterioration of renal function and were further included in this retrospective study (Fig. 1). Core needle biopsies were performed in the Department of Nephrology at MHH by ultrasound guidance. All histological analyses of the human kidney tissues were performed at the Nephropathology Unit of the Institute of Pathology at MHH.

### Clinical and laboratory parameters

Clinical and laboratory patient data were assessed by retrospective chart review. The following clinical parameters were analyzed in this study: survival, underlying liver disease, liver cirrhosis defined by histological or ultrasonographic findings and bleeding complications. The following laboratory data were collected for further analysis: aspartate aminotransferase (AST), alanine aminotransferase (ALT), alkaline phosphatase (AP), gamma-glutamyl transferase (GGT), cholinesterase (CHE), bilirubin, albumin, International Normalized Ratio (INR), C-reactive protein (CRP) and complete blood count. Renal function was assessed by serum creatinine levels and urinary parameters. All laboratory parameters were taken at the time of the kidney biopsy. Additionally, the highest values for bilirubin and creatinine in the time course before the biopsy were included (maximum values).

**Definitions of kidney disease**

Patients were classified to the type of kidney disease based on the 2018 EASL clinical practice guideline on the management of patients with decompensated cirrhosis (2). Briefly, the diagnosis of AKI was based on an acute increase of serum creatinine (sCr) >26.5 µmol/l from baseline within 48 hours or an increase ≥50% from a recent sCr dating within the last three months before admission. Depending on the increase of sCr AKI was staged as follows:

- Stage 1: increase 26.5 µmol/l or 150-200% from baseline (1A <133µmol, 1B >133µmol)
- Stage 2: increase of 201-300%
- Stage 3: increase of >300% or initiation of renal replacement therapy.

CKD was defined as a GFR <60 ml/min/1.73m$^2$ for ≥ 3 months. Patients with preexisting CKD fulfilling the criteria of AKI were considered as "AKI overlapping CKD".

**Histology and immunohistochemistry**

Human tissue was fixed in 4% neutral buffered paraformaldehyde and embedded in paraffin according to standard routine procedures. For diagnostic purposes, sections of kidney tissue were stained by periodic acid-Schiff (PAS), hematoxylin and eosin (H&E), Prussian blue for iron deposits and Hall's bilirubin stain (20) according to routine protocols.

Immunostaining of serial sections was conducted with an automated platform (Ventana ULTRA; Ventana Medical Systems, Tucson, AZ, USA) using the following antibodies: monoclonal rabbit CD4 (clone SP35; Zytomed, Berlin, Germany), mouse CD8 (clone C8/144B, Dako, Glostrup, Denmark), mouse CD15 (clone MMA; BD

Biosciences, San Jose, CA, USA), mouse CD68 (clone PG-M1; Dako) and mouse CD163 (clone MRQ-26; Cell Marque, Rocklin, CA, USA). Mouse CD177 antibody (clone 4C4; Origene Technologies, Herford, Germany) incubation after heat induced epitope retrieval with T-EDTA buffer (pH 9.0; Zytomed) was followed by anti-mouse immunoglobulins conjugated with horseradish peroxidase (JIR-E, Cambridge, UK). Aquaporin 2 (AQP2; polyclonal rabbit; Sigma Aldrich, Darmstadt, Germany) and α/β tubulin (polyclonal rabbit; Cell Signaling, Danvers, MA, USA) stains were conducted using the ZytoChem Plus horseradish peroxidase Polymer system (mouse/rabbit; Zytomed) after peroxidase-blocking (3% H2O2, 10 min). For antigen retrieval, citrate buffer (pH 6.0; Zytomed) was used. Chromogen detection was accomplished by 3,3-diaminobenzidine (Zytomed) and nuclear counterstain by hemalum. Negative controls omitting primary antibody were included in all staining procedures.

**Histomorphological analysis**

Immune cell numbers were counted in five medium fields of view (20x) in the cortical tubulointerstitial area. AQP2 stains were evaluated by quantifiying the collecting ducts with normal and reduced positivity. Loss of stain is shown as percentages of collecting ducts with decreased positivity for AQP2. For α/β tubulins, the positively stained tubuli were displayed as percentages. Normal renal tissue was stained as an additional control and had 100% positivity for AQP2 in collecting ducts and 0% for α/β tubulins.

In order to further characterize histomorphological characteristics of CN we developed a questionnaire based on a recent study (21). We included 20 blinded acute tubular injury cases (7 CN, 7 cases with liver disease and elevated bilirubin without CN, 6 cases revealing pigment for other reasons (lipofuscin, iron, ochronosis/alcaptonuria, porphyria)) into this analysis. Histochemical stains (H&E,

PAS, Prussian blue for iron, Hall's stain) were digitalized using an Aperio scanner CS2 (Leica) at 40x magnification. Scans were evaluated in a blinded manner by 6 trained nephropathologists (>1 to >15 years of experience) with main emphasis on tubular damage and pigment scoring (Suppl. Table 1).

**Statistics**

Descriptive statistical analyses were performed using SPSS version 24 (SPSS Software Corp., Chicago, IL, USA) or Prism 7 (GraphPad, San Diego, CA) and R 3.4.1 (©2017, The R Foundation for Statistical Computing). Data was analyzed by an unpaired, two-sided t-test when two distinct groups were compared. When indicated, an additional sensitivity analysis using non-parametric Mann-Whitney-U test was performed. When analyzing more than two groups Kruskal-Wallis test was used. Two-sided Fisher´s exact test and chi-square test were used as indicated. Univariate cox regression analysis were performed for dependent variable time to outcome. In addition, Kaplan-Meier estimates were displayed to depict the different survival curves of CKD and AKI patients. Respective two-sided p-values of the log rank test were calculated. Univariate logistic regression analysis was used to examine the dependent variable cholemic nephropathy. As SPSS does not calculate odds ratios when populations contain a zero value, odds ratios for covariates bilirubin and urine bilirubin were calculated using the odds ratio calculator Medcalc®. A p-value of 0.05 or less was considered statistically significant. All data are expressed as means ± standard deviations. Questionnaire evaluation was performed using cross-tables and the agreement coefficients Fleiss' Kappa for the evaluation of all raters and Cohen's Kappa (22) in the subgroup analysis of the two expert raters (nephropathologists with more than 10 and more than 15 years of experience, respectively).

### Ethics

The study was approved by the local ethics committee (3525-2017, 8070_BO_K_2018).

## RESULTS

*Study cohort of patients with liver disease undergoing kidney biopsy*

As outlined in Fig. 1, over a period of 16 years a total of 79 patients with liver disease underwent kidney biopsy due to deteriorating renal function at our Department of Gastroenterology, Hepatology, and Endocrinology. The mean age of the patients was 52.2 ± 10.8 years and 71% were male patients (56 of 79). One quarter of the patients (20/79) had a history of organ transplantation (liver or kidney) and the average sCr at the time of biopsy was 256 ± 181 µmol/l.

When applying the recent EASL criteria (2) and analyzing the type of kidney disease, we observed that 45 of the 79 patients (57%) presented with AKI and 34 patients with CKD (43%). Of the 45 patients with AKI, 18 patients also had evidence of elevated sCr more than three months prior to kidney biopsy, thus presenting as “AKI overlapping CKD” (Fig. 1). Comparing one-year survival rates between the three different groups, we observed that both, patients with AKI or AKI overlapping CKD had a significantly higher mortality after one year (Fig. 2).

*Histopathologic diagnoses of patients with AKI and CKD*

The most common histopathologic diagnosis in our cohort of patients with AKI (including AKI overlapping CKD) and CKD included primary and secondary glomerular disease (including membranous glomerulonephritis (GN), MPGN, fibrillary glomerulopathy, post-/parainfectious-GN, Henoch-Schoenlein purpura and IgA nephropathy), noninflammatory tubulointerstitial disease and vascular disease

including hypertensive nephropathy (Fig. 3). Among the 25 patients with a history of organ transplantation, six (24.0%) showed signs of calcineurin inhibitor toxicity.

Of note, the diagnosis of CN was only observed in patients with AKI (8/45, 18%), whereas none of the patients with CKD was diagnosed with CN (0/34, p<0.01).

*Clinical characteristics of patients diagnosed with CN*

To further characterize the patients with CN (n=8), we compared them to the 37 patients with other causes of AKI (non-CN). As expected, both the mean bilirubin at the time of biopsy as well as the maximum bilirubin differed significantly between the two groups (Table 1) and 44.4% (8 of 18) of the patients with a bilirubin > 100 µmol/l in our cohort of patients with AKI were diagnosed with CN. Alkaline phosphatase, another marker of cholestasis, was also significantly higher in patients with CN compared to non-CN patients. There was no difference in liver synthesis as determined by INR. Importantly, 5 out of 8 patients with CN (62.5%) required renal replacement therapy versus only 6 of 37 patients (13.3%) of patients with other causes of AKI. Urine analysis revealed hematuria and proteinuria in both patient groups to the same extent of around 50%. Of note, all patients with CN were positive for bilirubin in the urine, while only 22% of non-CN patients had detectable urinary bilirubin. Similarly, urobilinogen, a degradation product of bilirubin was more often detected in the urine of CN patients than non-CN patients (Table 1).

Next, we were interested in factors that had an impact on survival in liver disease patients with AKI. We performed a cox regression analysis on time to death and identified dialysis and bilirubin as independent factors associated with mortality. Noteworthy, despite significantly higher MELD-score in the group of patients with CN (31 vs 22, p=0.001), the diagnosis of CN was not associated with an increased risk of death (Suppl. Fig. 1).

In order to identify factors associated with the diagnosis of CN we performed an additional univariate logistic regression analysis and identified bilirubin higher than five times the upper limit of normal (ULN), alkaline phosphatase higher than three times the ULN as well as detectable bilirubin and urobilinogen in the urine as independent risk factors (Table 3). Despite higher CRP in patients with CN at baseline, CRP was neither associated with an increased mortality in patients with AKI (Table 2) nor associated with the diagnosis of CN (Table 3).

*Clinical course of patients diagnosed with CN*

A total of eight patients were diagnosed with CN. While three patients recovered (#1-3), the remaining five patients required renal replacement therapy (RRT) (#4-8). Given this observation, we were interested, whether there are differences in the patient characteristics. All three patients that recovered (#1-3) had an acute episode of liver disease without significant comorbidities. Also, in all three cases a specific treatment was available: patient #1 was diagnosed with autoimmune hepatitis and received steroids, patient #2 was diagnosed with benign recurrent intrahepatic cholestasis (BRIC) and received rifampicin, patient #3 had an acute hepatitis B and received lamivudine and subsequently telbivudine. All three patients did not require RRT and kidney function recovered along with resolving cholestasis. The remaining five patients (#4-8) had advanced stages of various diseases: two patients had underlying liver cirrhosis (#4, #8), two patients were treated long-term on the ICU (#6, #7) and the remaining patient was liver transplanted with recurrent episodes of cholangitis (#5). All of these five patients underwent RRT; four out of five died within two years, the remaining patient underwent combined liver/kidney transplantation (#5). Detailed course of creatinine and bilirubin levels as well as liver related diagnoses are shown in the supplementary material (Suppl. Fig. 2, Suppl. Table 2).

*Histomorphological characteristics of patients diagnosed with CN*

The diagnosis of CN can only be verified by histopathological examination of kidney biopsies. In all eight patients diagnosed with CN, we observed bilirubin casts labelled by Hall's stain (20) within the tubular lumen or massive pigment inclusions in the tubular epithelial cells (Fig. 4, Suppl. Fig. 3). To rule out the deposition of iron pigment, we performed Prussian blue stain in serial sections and did not observe iron deposition in CN patients (Fig. 4). We were interested, whether CN can be distinguished from kidney biopsies with pigment inclusions other than bile cast and from kidneys of patients with various non-cholestatic liver diseases applying known histopathological criteria by six experienced nephropathologists from three different pathology departments in a questionnaire. Neither the evaluation of all six raters nor the subgroup analysis of the expert raters (nephropathologists with >10 and >15 years of experience) identified discriminating histopathological features between the chosen entities (Data not shown).

*Patients with elevated bilirubin and patients with CN show loss of AQP2 in collecting ducts*

In order to characterize the inflammatory infiltrate and characteristics of tubular injury in CN we performed a detailed immunohistochemical analysis. We investigated patients from three groups; patients with AKI and normal serum-bilirubin (n=5), patients with AKI and elevated serum-bilirubin (n=6) and patients with CN (n=6). No material was available from two patients diagnosed with CN. Detailed baseline characteristics of the patients included in this immunohistochemical analysis are presented in Suppl. Table 3. Baseline sCr was similar in all three groups. Mean serum bilirubin was 11, 134, and 525 µmol/l in the three patient groups, respectively. AQP2 expression in collecting ducts was significantly reduced in patients with high

bilirubin and patients with CN compared to patients with AKI but normal bilirubin. No differences between the three groups were observed in the expression of α/β tubulins which was in general high. No significant differences in cell infiltration of T cells (CD4, CD8), neutrophils (CD15) and macrophages (CD68) were revealed but CN patients had significantly lower numbers of M2 macrophages/monocytes (CD163) compared to patients with AKI and normal bilirubin (Fig. 5). CD177 positive neutrophil numbers were very low but higher in patients with AKI and normal bilirubin.

*Kidney biopsy related complications*

In line with the increased risk of bleeding in patients with advanced liver disease 25 patients (31.6%) had an INR > 2 or thrombocytes <100.000/µl. Biopsy related complications occurred in six of 79 patients (7.6%). Four patients required transfusion of packed red blood cells (PRBCs), two of those four underwent surgery or coiling, respectively, to control bleeding. Of the remaining two patients, one presented with a perirenal hematoma and the other one had an arteriovenous fistula, both did not require an intervention. No fatal complication occurred after kidney biopsy in our cohort.

## DISCUSSION

Clinical management of patients with liver disease and deteriorating renal function is challenging as a definite diagnosis of underlying renal cause can often only be made by kidney biopsy, which is seldom performed owing to increased risk of bleeding. Our study investigated the underlying cause of deteriorated renal function in a large cohort of patients with liver disease in a tertiary care hospital over a period of more than 15 years. We observed several important findings: (i) cholemic nephropathy was

diagnosed in 18% of patients with AKI, (ii) high serum bilirubin and alkaline phosphatase levels were associated with the diagnosis of CN, (iii) patients with AKI and elevated bilirubin as well as CN show loss of AQP2 in collecting ducts, (iv) bleeding complications were higher in our patient cohort compared to the published literature.

Various liver diseases are related with different forms of kidney alterations. It is well known, that HBV and HCV are associated with glomerular kidney disease (23, 24) and also patients with non-alcoholic fatty liver disease have a higher rate of CKD (25). In this context, an association of cholestasis with the impairment of renal function has already been described at the beginning of the last century (26, 27). Since then, most of the data on this disease entity, which is known as cholemic nephrosis, bile cast nephropathy or CN, has been derived from case reports (6-15) or autopsy studies (16-18). However, none of the studies systematically investigated the frequency of CN in a living cohort. Out of our 79 patients with liver disease who underwent renal biopsy 8 of the 45 patients with AKI were diagnosed with CN. Hepatorenal syndrome is a common cause of AKI in patients with liver cirrhosis (28). During 2000 and 2016, 935 treatment cases of “hepatorenal syndrome” were documented in our hospital. Given the low number of patients with AKI that underwent kidney biopsy (45 out of 935, 4.8%), CN is likely to be underdiagnosed.

In our cohort, we noted two types of CN. First, we observed a reversible form in patients with acute liver disease where a specific treatment was available (patients #1-3). All these patients developed AKI which resolved once bilirubin declined. In contrast, the five other patients (patients #4-8) suffered from disease conditions that are per se considered major risk factors for the development of severe AKI (stage 3) including liver cirrhosis or sepsis. Moreover, the underlying liver disease was chronic with no specific drug therapy available. CN in these patients did not resolve as

hyperbilirubinemia could not be improved by medical intervention. Also, CN in those patients was only one of many disease-defining factors and consecutively, all of these patients progressed to ESRD requiring RRT.

It is well accepted that dialysis is associated with increased mortality, which was also observed in our study. Another factor associated with mortality was serum bilirubin. Given that both bilirubin and creatinine are included in the Model for End-stage Liver Disease score (MELD-score), which predicts short-term mortality in patients undergoing transjugular intrahepatic portosystemic shunts (TIPS) (29) and suffering from end-stage liver disease (30), it is not surprising that CN patients who have both, a deteriorated renal function and an increased bilirubin, have significantly higher MELD-scores at baseline (31 vs 22, Table). However, one-year mortality was not different as compared to patients with non-CN AKI. At first sight, this might be contradictory, but this is reflected by the subset of patients that develops CN in an acute setting and has a benign course when hyperbilirubinemia resolves (patients #1-3).

We identified markers of cholestasis including bilirubin higher than five times the ULN and detection of bilirubin and urobilinogen in the urine as risk factor for the development of CN. It is not clear whether bilirubin has a direct toxic effect on the kidney, however it has been shown that bilirubin levels correlate well with levels of bile acids (31). In this context, a recent bile duct ligation study in mice identified bile acids as a major trigger of CN and the authors suggested that tubulotoxic injury caused a loss of AQP2 in collecting ducts (32). Loss of AQP2 expression in the collecting ducts is in line with other studies in rodents (33, 34) or human (35) kidneys which show an inverse correlation of AQP2 with toxicity and interstitial fibrosis. Of note, both in patients with AKI and elevated bilirubin as well as CN, we observed a loss of AQP2, whereas patients with AKI and normal bilirubin maintained AQP2

expression. Based on experimental evidence in a bile duct ligation model, it has been proposed that reduced renal AQP2 reflects an active escape mechanism from vasopressin-induced antidiuresis, providing a compensatory mechanism aimed at avoiding dilutional hyponatremia (36). Along these lines, bile acids may play a regulatory role as they can directly impact AQP2 expression through modulation of the farnesoid X receptor (FXR) and the G protein–coupled receptor (GPCR) (37). Additional studies will be needed to elucidate whether these vasopressin-independent pathways play a functional role in CN. Additionally, all groups showed high expression of α/β tubulin indicating severe kidney injury. Heterodimers of α- and β-tubulins assemble to tubulin and form microtubules which are major components of the cytoskeleton. Tubulins are also responsible for brush border formation (38). Kidney injury like ischemia increases tubulin expression as an adaptive response (39). The observed higher numbers of M2 macrophages identified by CD163 in AKI with normal bilirubin might mirror the well-known role of M2 in wound healing, which might be impaired in CN and AKI patients with elevated bilirubin leading to severe fibrosis as already described in mice with cholemic nephropathy (32). A subgroup of neutrophils (CD177-positive granulocytes) showed higher numbers in patients with normal compared to elevated bilirubin and CN. However, neutrophil cell numbers were so low that this difference is most likely random. Moreover, the data driven from the histomorphological questionnaire did not identify any specific features of renal damage in CN cases as compared to non-CN high bilirubin or other pigment associated cases, pointing out that 1) there are no established discriminating features of causes for tubular damage in general (21), 2) AKI patients with elevated bilirubin presented with the same toxic effect (especially tubular injury) (40) compared to CN but might have missed bile casts (sampling error), 3) Hall's stain is known to have a low sensitivity (18). A close cooperation between hepatologists,

nephrologists and nephropathologists including interdisciplinary case conferences may help to correctly diagnose CN.

One important question remaining is whether a diagnosis of CN has any therapeutic consequence. Extracorporal liver support systems such as the extracorporeal liver assist device (ELAD) may lower metabolites such as bilirubin without affecting creatinine. One case report has been published on the successful use of an extracorporeal albumin dialysis (ECAD) in a patient with cholestasis and AKI in whom kidney function quickly improved after the first session of ECAD (41). A randomized controlled study on the use of an extracorporeal cellular therapy (ELAD) in severe alcoholic hepatitis observed an effect on survival in patients with a MELD-score of <28, hyperbilirubinemia and no impairment of renal function (42). In contrast, there was no benefit for patients with a MELD-score higher than 28 owing to an increased creatinine in addition to hyperbilirubinemia. If one speculates that the extracorporeal elimination of hepatic metabolites including bilirubin has a beneficial effect on the renal deposition of bile cast, this prophylactic approach would be more efficient as opposed to a belated treatment once casts and cellular pigments are already deposited. Yet, given the enormous costs and logistic necessities, routine extracorporeal liver support systems are an unlikely therapeutic option. A pharmacological treatment approach would be more feasible. A study published recently showed that norursodeoxycholic acid (norUDCA) ameliorates cholemic nephropathy in bile duct ligated mice (43). Importantly, norUDCA did not positively affect liver function but might have a direct kidney specific therapeutic effect (43). Prospective studies in humans need to investigate a potential benefit for patients with highly elevated bilirubin and impaired renal function.

Finally, the question is whether it is justified to perform kidney biopsies in this patient population, where bleeding complications may occur not only owing to platelet dysfunction in uremia (44) but also due to concomitant liver dysfunction. Significant bleeding complications with the requirement of medical intervention (transfusion, coiling, surgery) occurred in 5.1 % of our patients (4 of 79). This is around five times higher than in a liver disease-independent study that only observed bleeding complications in 1.1% of patients (45). In a large systematic review of almost 10,000 patients, only 0.9% of patients who underwent kidney biopsy, required transfusion (46). Despite this apparently higher rate of bleeding complications, no fatal complications occurred and only two of the four patients underwent intervention (coiling, surgery). Based on these data, a kidney biopsy should only be performed in highly selected cases such as patients with HRS-AKI with non-response to standard treatment of HRS including albumin and vasopressors. Moreover, kidney biopsy might be required within clinical studies aiming to develop non-invasive diagnostics for the differentiation between HRS-AKI and CN, or in trials evaluating therapeutic agents such as norUDCA.

Our study has several limitations, including the small number of biopsy-proven CN patients and the retrospective, single center design. Moreover, follow up kidney biopsies to check whether bile casts are removed once kidney function improved were not available.

In conclusion, CN is a common finding in patients with liver disease, AKI and highly elevated bilirubin. Kidney biopsy should be considered at least in highly selected patients with HRS-AKI who do not respond to treatment and within clinical studies. Prospective studies are needed to define the real prevalence of CN in patients with liver disease and to investigate potential therapeutic interventions.

## FIGURE LEGENDS

**Figure 1:** Study population. Out of 149 kidney biopsies performed between 2000 and 2016, a total of 79 fulfilled criteria to enter the study.

**Figure 2:** Kaplan-Meier curves to display increased mortality of liver disease patients diagnosed with AKI compared to CKD. Patients with the diagnosis of AKI or AKI overlapping CKD have an increased one-year mortality as compared to patients with CKD ($p<0.001$ and $p=0.023$ of log rank test, respectively).

**Figure 3:** Distribution of histological diagnoses of kidney biopsy in a total of 79 patients with underlying liver disease, 45 of these patients presented with AKI, whereas 34 had CKD. Data are presented as percentages.

**Figure 4:** Representative photomicrographs of H&E (upper row), Hall's bilirubin stain (middle row) and Prussian blue iron reaction (lower row) in AKI patients with normal bilirubin (A, D, G), AKI patients with elevated bilirubin (B, E, H) and in patients with CN (C, F, I). Arrows point to bile casts, arrowheads indicate iron. Bar represents 100 µm, all micrographs at 40x.

**Figure 5:** Tubular integrity marker AQP2 and α/β tubulin expression (**A**) and counted immune cell infiltration (T cells: CD4, CD8; macrophages: CD68, CD163; neutrophils: CD15, CD177) (**B**). Marker expression was determined after immunohistochemical staining in samples from patients with AKI and normal bilirubin (NB, n=5), elevated bilirubin (EB, n=6) and cholemic nephropathy (CN, n=6). Bar represents 100 µm, all micrographs at 40x; arrows depict immunopositivity, asterisks show pigment. *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$.

## REFERENCE LIST

1. Fede G, D'Amico G, Arvaniti V, Tsochatzis E, Germani G, Georgiadis D, Morabito A, et al. Renal failure and cirrhosis: a systematic review of mortality and prognosis. J Hepatol 2012;56:810-818.

2. European Association for the Study of the Liver. Electronic address eee, European Association for the Study of the L. EASL Clinical Practice Guidelines for the management of patients with decompensated cirrhosis. J Hepatol 2018;69:406-460.

3. Garcia-Tsao G, Parikh CR, Viola A. Acute kidney injury in cirrhosis. Hepatology 2008;48:2064-2077.

4. Davenport A, Sheikh MF, Lamb E, Agarwal B, Jalan R. Acute kidney injury in acute-on-chronic liver failure: where does hepatorenal syndrome fit? Kidney Int 2017;92:1058-1070.

5. Krones E, Pollheimer MJ, Rosenkranz AR, Fickert P. Cholemic nephropathy - Historical notes and novel perspectives. Biochim Biophys Acta 2017.

6. Alnasrallah B, Collins JF, Zwi LJ. Bile Nephropathy in Flucloxacillin-Induced Cholestatic Liver Dysfunction. Case Rep Nephrol 2016;2016:4162674.

7. **Aniort J, Poyet A**, Kemeny JL, Philipponnet C, Heng AE. Bile Cast Nephropathy Caused by Obstructive Cholestasis. Am J Kidney Dis 2017;69:143-146.

8. Betjes MG, Bajema I. The pathology of jaundice-related renal insufficiency: cholemic nephrosis revisited. J Nephrol 2006;19:229-233.

9. Bredewold OW, de Fijter JW, Rabelink T. A case of mononucleosis infectiosa presenting with cholemic nephrosis. NDT Plus 2011;4:170-172.

10. Kiewe P, Korfel A, Loddenkemper C, Fischer L, Jahnke K, Notter M, Muhr-Wilkenshoff F, et al. Unusual sites of Hodgkin's lymphoma: CASE 3. Cholemic

nephrosis in Hodgkin's lymphoma with liver involvement. J Clin Oncol 2004;22:4230-4231.

11. Luciano RL, Castano E, Moeckel G, Perazella MA. Bile acid nephropathy in a bodybuilder abusing an anabolic androgenic steroid. Am J Kidney Dis 2014;64:473-476.

12. Patel J, Walayat S, Kalva N, Palmer-Hill S, Dhillon S. Bile cast nephropathy: A case report and review of the literature. World J Gastroenterol 2016;22:6328-6334.

13. Sequeira A, Gu X. Bile cast nephropathy: an often forgotten diagnosis. Hemodial Int 2015;19:132-135.

14. Tabatabaee SM, Elahi R, Savaj S. Bile cast nephropathy due to cholestatic jaundice after using stanozolol in 2 amateur bodybuilders. Iran J Kidney Dis 2015;9:331-334.

15. Werner CR, Wagner V, Sipos B, Buttner-Herold M, Amann K, Haap M, Malek NP, et al. [Acute kidney injury in liver failure]. Dtsch Med Wochenschr 2016;141:1559.

16. Mohapatra MK, Behera AK, Karua PC, Bariha PK, Rath A, Aggrawal KC, Nahak SR, et al. Urinary bile casts in bile cast nephropathy secondary to severe falciparum malaria. Clin Kidney J 2016;9:644-648.

17. Song J, Chang A. Jaundice-associated acute kidney injury. NDT Plus 2009;2:82-83.

18. van Slambrouck CM, Salem F, Meehan SM, Chang A. Bile cast nephropathy is a common pathologic finding for kidney injury associated with severe liver dysfunction. Kidney Int 2013;84:192-197.

19. Tripodi A, Mannucci PM. The coagulopathy of chronic liver disease. N Engl J Med 2011;365:147-156.

20. Hall MJ. A staining reaction for bilirubin in sections of tissue. Am J Clin Pathol 1960;34:313-316.

21. **Schumann-Bischoff A, Schmitz J**, Scheffner I, Schmitt R, Broecker V, Haller H, Brasen JH, et al. Distinct morphological features of acute tubular injury in renal allografts correlate with clinical outcome. Am J Physiol Renal Physiol 2018;315:F701-F710.

22. Fleiss JL, Levin B, Paik MC. Statistical methods for rates and proportions. 3rd ed. Hoboken, N.J.: J. Wiley, 2003: xxvii, 760 p.

23. Johnson RJ, Couser WG. Hepatitis B infection and renal disease: clinical, immunopathogenetic and therapeutic considerations. Kidney Int 1990;37:663-676.

24. Johnson RJ, Gretch DR, Yamabe H, Hart J, Bacchi CE, Hartwell P, Couser WG, et al. Membranoproliferative glomerulonephritis associated with hepatitis C virus infection. N Engl J Med 1993;328:465-470.

25. Musso G, Gambino R, Tabibian JH, Ekstedt M, Kechagias S, Hamaguchi M, Hultcrantz R, et al. Association of non-alcoholic fatty liver disease with chronic kidney disease: a systematic review and meta-analysis. PLoS Med 2014;11:e1001680.

26. Krones E, Wagner M, Eller K, Rosenkranz AR, Trauner M, Fickert P. Bile acid-induced cholemic nephropathy. Dig Dis 2015;33:367-375.

27. Clairmont P, Haberer Hv. Ueber die Anurie nach Gallensteinoperation. Mitt. Grenzgeb. Med. u. Chir. 1911;22.

28. Gines P, Guevara M, Arroyo V, Rodes J. Hepatorenal syndrome. Lancet 2003;362:1819-1827.

29. Malinchoc M, Kamath PS, Gordon FD, Peine CJ, Rank J, ter Borg PC. A model to predict poor survival in patients undergoing transjugular intrahepatic portosystemic shunts. Hepatology 2000;31:864-871.

30. Kamath PS, Wiesner RH, Malinchoc M, Kremers W, Therneau TM, Kosberg CL, D'Amico G, et al. A model to predict survival in patients with end-stage liver disease. Hepatology 2001;33:464-470.

31. Makino I, Nakagawa S, Mashimo K. Conjugated and unconjugated serum bile acid levels n patients with hepatobiliary diseases. Gastroenterology 1969;56:1033-1039.

32. **Fickert P, Krones E**, Pollheimer MJ, Thueringer A, Moustafa T, Silbert D, Halilbasic E, et al. Bile acids trigger cholemic nephropathy in common bile-duct-ligated mice. Hepatology 2013;58:2056-2069.

33. Kishore BK, Krane CM, Di Iulio D, Menon AG, Cacini W. Expression of renal aquaporins 1, 2, and 3 in a rat model of cisplatin-induced polyuria. Kidney Int 2000;58:701-711.

34. Kwon TH, Laursen UH, Marples D, Maunsbach AB, Knepper MA, Frokiaer J, Nielsen S. Altered expression of renal AQPs and Na(+) transporters in rats with lithium-induced NDI. Am J Physiol Renal Physiol 2000;279:F552-564.

35. Bedford JJ, Leader JP, Walker RJ. Aquaporin expression in normal human kidney and in renal disease. J Am Soc Nephrol 2003;14:2581-2587.

36. Brond L, Hadrup N, Salling N, Torp M, Graebe M, Christensen S, Nielsen S, et al. Uncoupling of vasopressin signaling in collecting ducts from rats with CBL-induced liver cirrhosis. Am J Physiol Renal Physiol 2004;287:F806-815.

37. Li S, Qiu M, Kong Y, Zhao X, Choi HJ, Reich M, Bunkelman BH, et al. Bile Acid G Protein-Coupled Membrane Receptor TGR5 Modulates Aquaporin 2-Mediated Water Homeostasis. J Am Soc Nephrol 2018;29:2658-2670.

38. Tonucci FM, Ferretti A, Almada E, Cribb P, Vena R, Hidalgo F, Favre C, et al. Microtubules regulate brush border formation. J Cell Physiol 2018;233:1468-1480.

39. Caron A, Desrosiers RR, Beliveau R. Kidney ischemia-reperfusion regulates expression and distribution of tubulin subunits, beta-actin and rho GTPases in proximal tubules. Arch Biochem Biophys 2004;431:31-46.

40. Wardle EN. Renal failure in obstructive jaundice--pathogenic factors. Postgrad Med J 1975;51:512-514.

41. Sens F, Bacchetta J, Rabeyrin M, Juillard L. Efficacy of extracorporeal albumin dialysis for acute kidney injury due to cholestatic jaundice nephrotoxicity. BMJ Case Rep 2016;2016.

42. Thompson J, Jones N, Al-Khafaji A, Malik S, Reich D, Munoz S, MacNicholas R, et al. Extracorporeal Cellular Therapy (ELAD) in Severe Alcoholic Hepatitis - A Multinational, Prospective, Controlled, Randomized Trial. Liver Transpl 2017.

43. Krones E, Eller K, Pollheimer MJ, Racedo S, Kirsch AH, Frauscher B, Wahlstrom A, et al. NorUrsodeoxycholic acid ameliorates cholemic nephropathy in bile duct ligated mice. J Hepatol 2017;67:110-119.

44. Kaw D, Malhotra D. Platelet dysfunction and end-stage renal disease. Semin Dial 2006;19:317-322.

45. Atwell TD, Spanbauer JC, McMenomy BP, Stockland AH, Hesley GK, Schleck CD, Harmsen WS, et al. The Timing and Presentation of Major Hemorrhage After 18,947 Image-Guided Percutaneous Biopsies. AJR Am J Roentgenol 2015;205:190-195.

46. Corapi KM, Chen JL, Balk EM, Gordon CE. Bleeding complications of native kidney biopsy: a systematic review and meta-analysis. Am J Kidney Dis 2012;60:62-73.

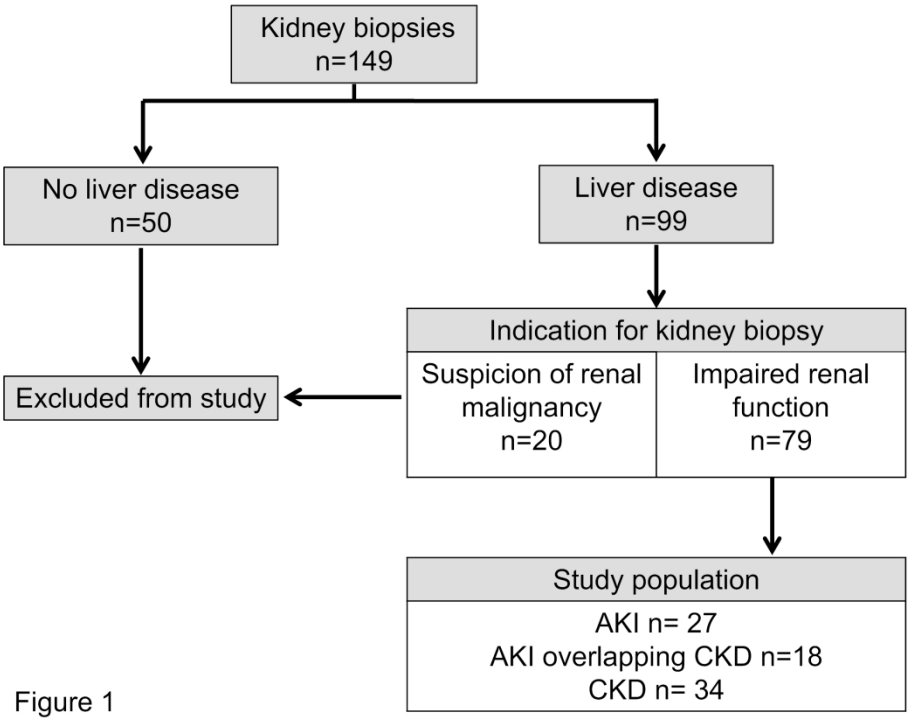


Figure 1

Figure 1

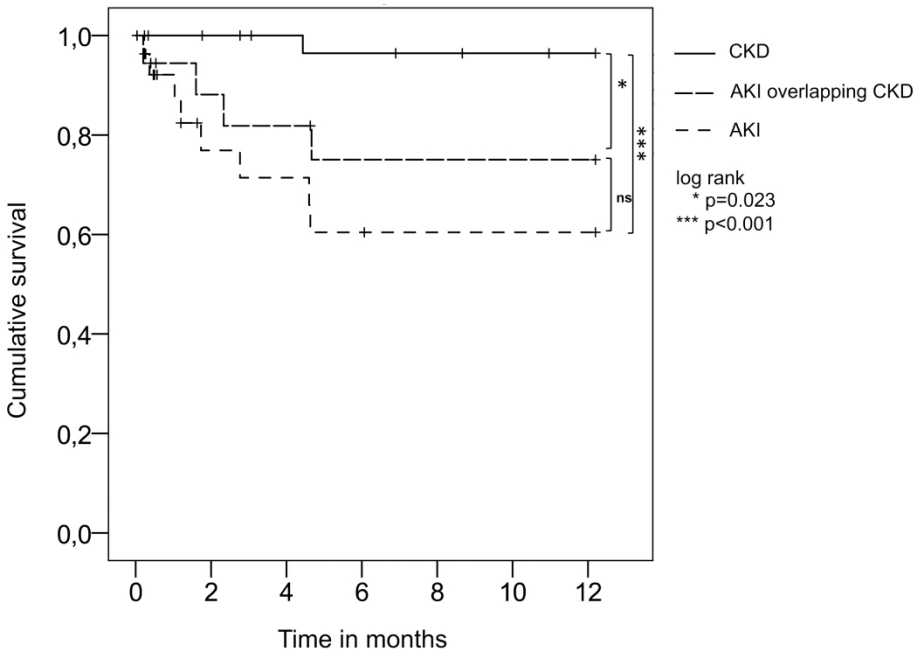


Figure 2

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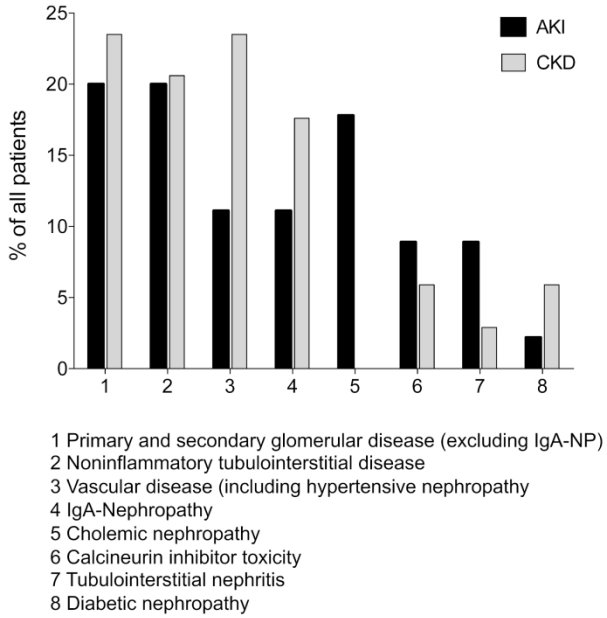


Figure 3

Figure 3

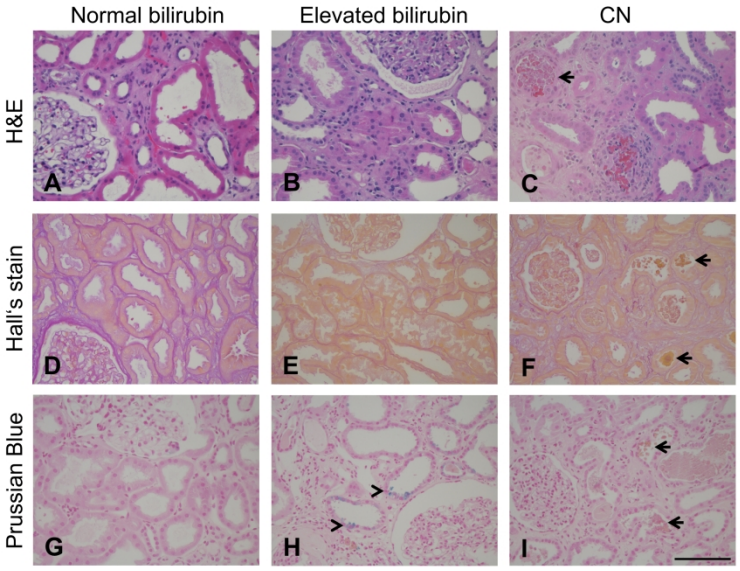


Figure 4

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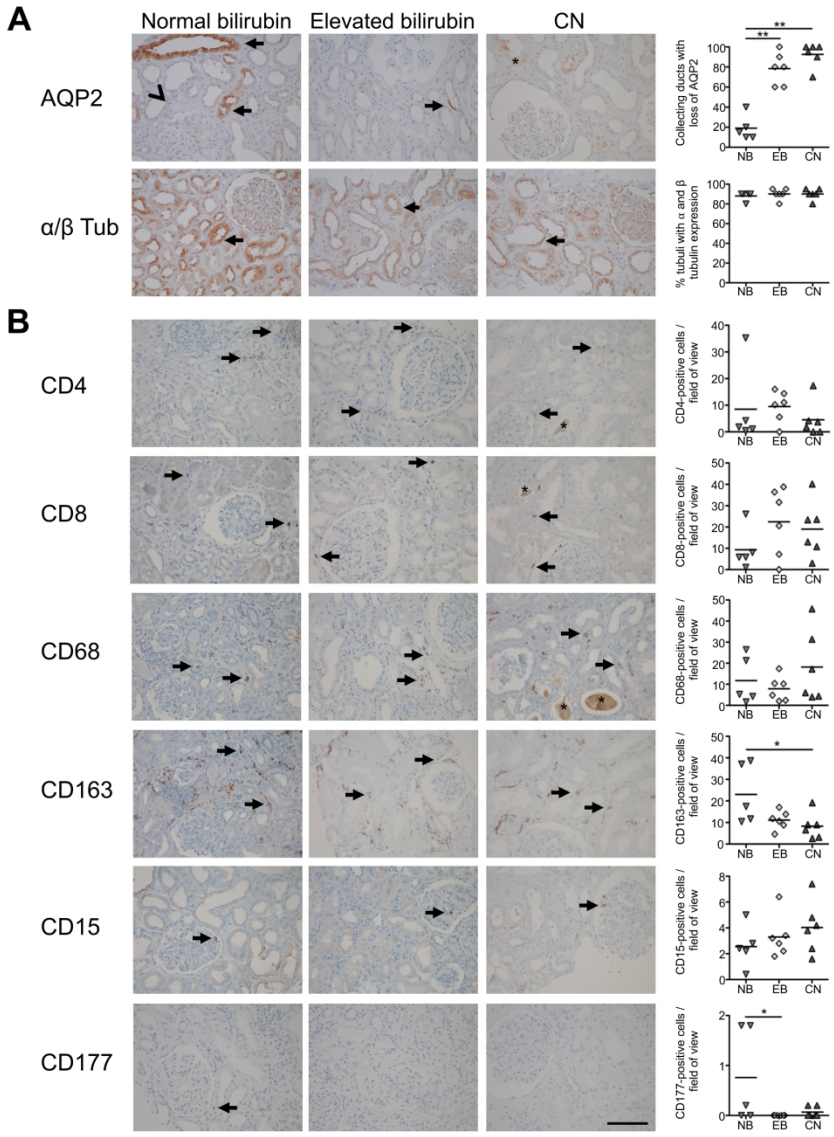


Figure 5

Figure 5

## Table 1: Patient characteristics

| | Cholemic Nephropathy | Other cause of AKI | p-value# |
|---|---|---|---|
| **Number of Patients** | 8 (17.8%) | 37 (82.2%) | |
| **Age (years)** | 52.6 ± 7.7 | 51.7 ±11.3 | |
| **Gender m/f** | 8 (100%)/0 | 27(73%)/10(27%) | 0.2 |
| **Cause of Liver Disease** | | | |
| **a) viral** | 3 (37.5%) | 8 (21.6%) | |
| **b) EtOH** | 0 | 9 (24.3%) | |
| **c) APAP** | 0 | 2 (5.4%) | |
| **d) NAFLD/NASH** | 0 | 2 (5.4%) | |
| **e) autoimmune** | 1 (12.5%) | 5 (13.5%) | |
| **f) others** | 4 (50%) | 11 (29.7%) | |
| **Liver cirrhosis** | 2 (25%) | 18 (56.3) | 0.2 |
| **Tx (LTx or KTx)** | 1 (12.5%) | 11 (29.7%) | 0.5 |
| **a) History of LTx** | 1 | 10 | |
| **b) History of KTx** | 0 | 0 | |
| **c) History of combined LTx and KTx** | 0 | 1 | |
| **INR** | 1.15 ± 0.15 | 1.27 ± 0.3 | 0.3 |
| **Bilirubin max [µmol/l]** | 779 ± 306 | 102 ± 139 | <0.001 |
| **Bilirubin [µmol/l]** | 466 ± 196 | 76 ± 118 | <0.001 |
| **Creatinine max [µmol/l]** | 418 ± 310 | 409 ± 136 | 0.9 |
| **Creatinine [µmol/l]** | 296 ± 245 | 325 ± 201 | 0.7 |
| **a) AKI Stage 1A** | 0 | 2 (5.4%) | |
| **b) AKI Stage 1B** | 1 (12.5%) | 7 (18.9%) | |
| **c) AKI Stage 2** | 1 (12.5%) | 8 (21.6%) | |
| **d) AKI Stage 3** | 6 (75%) | 20 (54.1%) | |
| **MELDscore** | 31 ± 5 | 22 ± 7 | 0.001 |
| | | | |
| **ALT [U/l]** | 120 ± 85 | 107 ± 263 | 0.9 |
| **AST [U/l]** | 129 ± 93 | 73 ± 69 | 0.06 |
| **AP [U/l]** | 761 ± 948 | 205 ± 218 | 0.002 |
| **GGT [U/l]** | 390 ± 538 | 198 ± 267 | 0.14 |
| | | | |
| **Albumin [g/l]** | 32 ± 14 | 27 ± 9 | 0.2 |
| **CHE [kU/l]** | 1.94 ± 1.13 | 3.47 ± 2.29 | 0.08 |
| | | | |
| **Leukocytes [$10^3$/µl]** | 10.6 ± 5.8 | 8.5 ± 4.4 | 0.3 |
| **Thrombocytes [$10^3$/µl]** | 192 ± 93 | 143 ± 73 | 0.1 |
| **C-reactive Protein [mg/l]** | 55 ± 35 | 29 ± 29 | 0.04 |
| **Hemoglobin [g/dl]** | 9.8 ± 1.4 | 9.9 ± 1.6 | 0.8 |
| | | | |
| **Urine-Bilirubin** | 8 (100%) | 12 (33.3%) | <0.001 |
| **Urine-Urobilinogen** | 4 (50%) | 5 (14%) | 0.042 |
| **Proteinuria** | 4 (50%) | 18 (51.4%) | 1.0 |
| **Hematuria** | 5 (62.5%) | 21 (58.3%) | 1.0 |
| **Dialysis** | 5 (62.5%) | 6 (13.3%) | 0.014 |

Displayed are means and standard deviations or relative and absolute frequencies.
#two-sided unpaired t-test or Chi-squared test, respectively

**Table 2:** Univariate cox regression analysis on time to death

| | Deceased n=17 | Alive n=28 | p-value | HR (95% CI)[#] |
|---|---|---|---|---|
| **Male** | 70.6% | 82.1% | 0.724 | 0.82 [0.29 – 2.35] |
| **Age >50** | 64.7% | 46.4% | 0.227 | 1.85 [0.68 - 5.03] |
| **Dialysis*** ** | 52.9% | 7.1% | <0.001 | 7.81 [2.65 – 23.06] |
| **Bilirubin > 2xULN*** | 94.1% | 46.4% | 0.027 | 9.74 [1.29 – 73.50] |
| **AP > 3xULN** | 52.9% | 10.7% | 0.092 | 2.29 [0.87 – 6.01] |
| **AST >3xULN** | 47.1% | 17.9% | 0.169 | 1.96 [0.75 – 5.08] |
| **ALT >3xULN** | 23.5% | 25.0% | 0.718 | 0.81 [0.26 – 2.51] |
| **CRP >50 mg/dl** | 23.5% | 25.0% | 0.848 | 0.89 [0.28 – 2.87] |
| **CHE <1.3 kU/l** | 29.4% | 21.4% | 0.302 | 1.75 [0.60 – 5.07] |
| **Leukocytes >10** | 35.3% | 25.0% | 0.131 | 2.19 [0.79 – 6.07] |
| **CN** | 29.4% | 10.7% | 0.432 | 1.52 [0.53 – 4.35] |
| **Cirrhosis** | 52.9% | 47.8% | 0.523 | 1.38 [0.51 – 3.72] |

[#]Hazard ratio and respective 95% confidence interval

**Table 3:** Univariate logistic regression analysis for the diagnosis of CN

| | CN | Non-CN | p-value | OR (95% CI) |
|---|---|---|---|---|
| **Bilirubin >5xULN*** | 100% | 27.0% | 0.011 | 44.52 [2.35 – 841.79]# |
| **AST >3ULN** | 50% | 24.3% | 0.158 | 3.11 [0.64 – 15.05] |
| **AP > 3xULN*** | 62.5% | 18.9% | 0.02 | 7.14 [1.37 – 37.22] |
| **CRP >50mg/dl** | 50% | 18.9% | 0.077 | 4.29 [0.85 – 21.48] |
| **U-Urobilinogen*** | 50% | 13% | 0.033 | 6.62 [1.16 – 33.17] |
| **U-Bilirubin*** | 100% | 33.3% | 0.019 | 33.32 [1.77 – 625.54]# |
| **CHE <1.3** | 50% | 18.9% | 0.077 | 4.29[0.85 – 21.47] |
| **ALT >3xULN** | 50% | 18.9% | 0.077 | 4.29[0.85 – 21.47] |
| **Cirrhosis** | 25% | 56.3% | 0.13 | 0.26 [0.05 – 1.49] |

# Odds Ratio (OR) calculated via Medcalc® (www. medcalc.org/calc/odds_ratio.php)

# Supplementary Material for

## Cholemic nephropathy causes acute kidney injury and is accompanied by loss of aquaporin 2 in collecting ducts

Jan Hinrich Bräsen*, Young-Seon Mederacke*, Jessica Schmitz, Kateryna Diahovets, Abedalrazag Khalifa, Björn Hartleben, Fermín Person, Thorsten Wiech, Eric Steenbergen, Annika Großhennig, Michael P. Manns, Roland Schmitt and Ingmar Mederacke#

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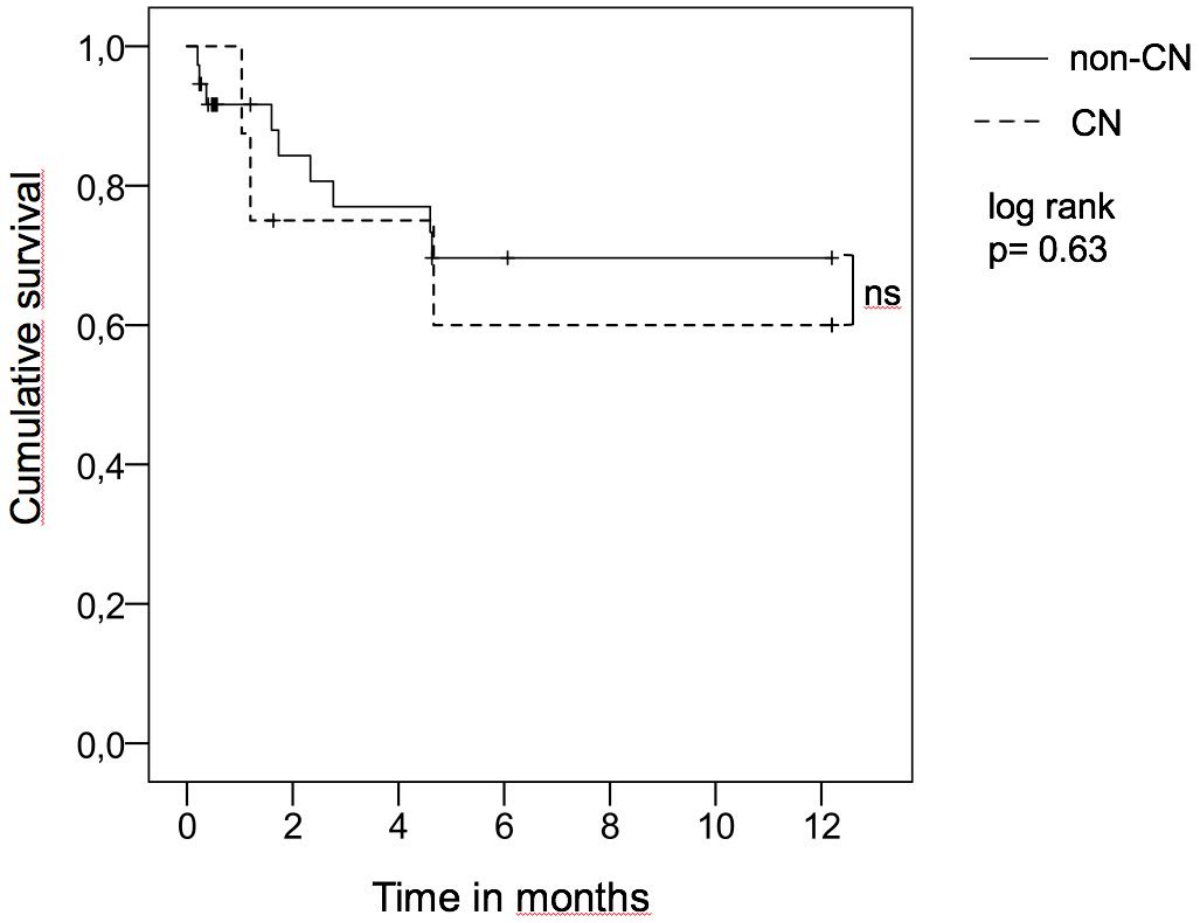
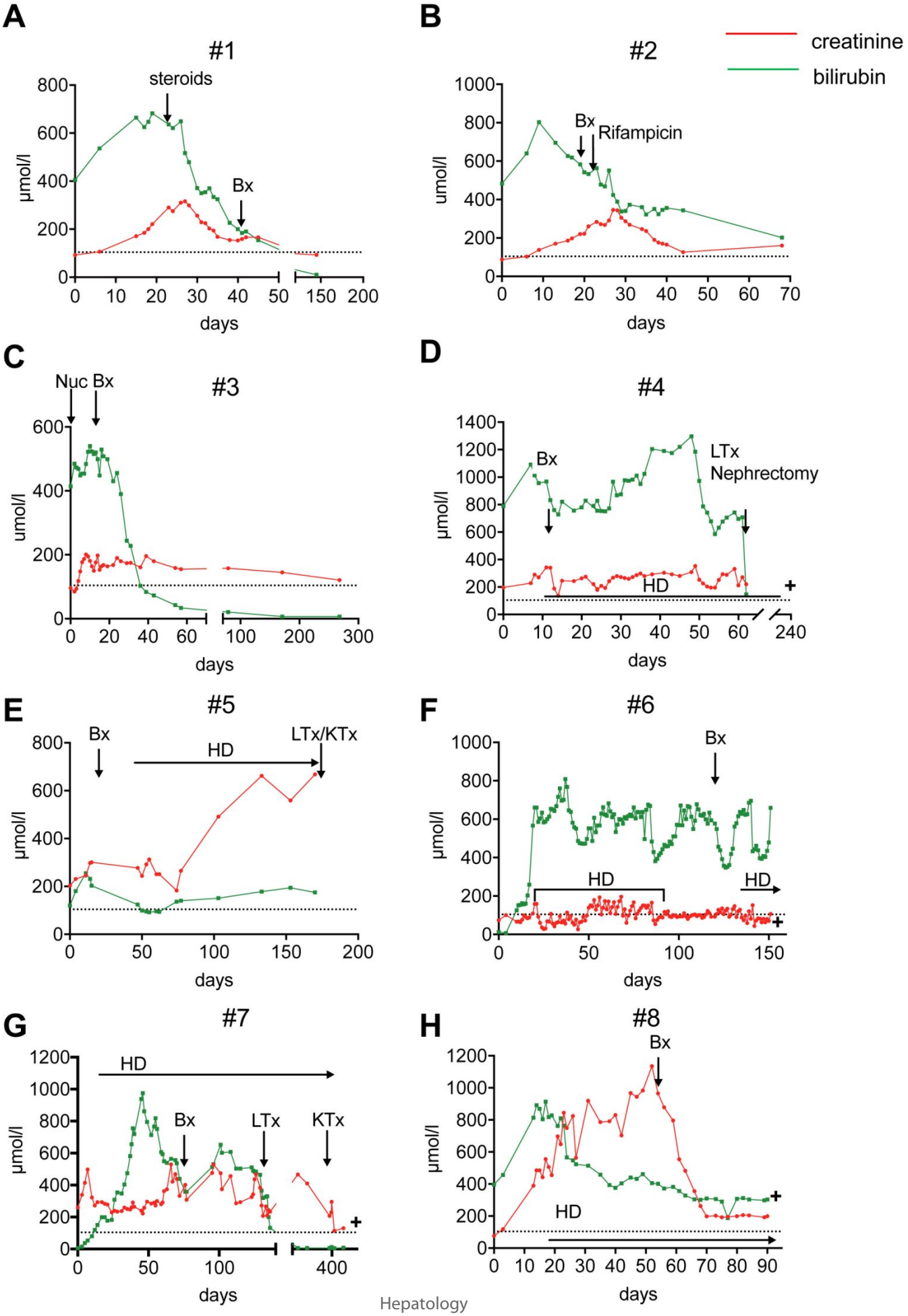



**Supplementary Figure 1:** Kaplan-Meier curves to display that CN (n=8) was not associated with an increased risk of death after one year when compared to patients with liver disease and other causes of AKI (n=37).


A
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steroids
Bx
µmol/l
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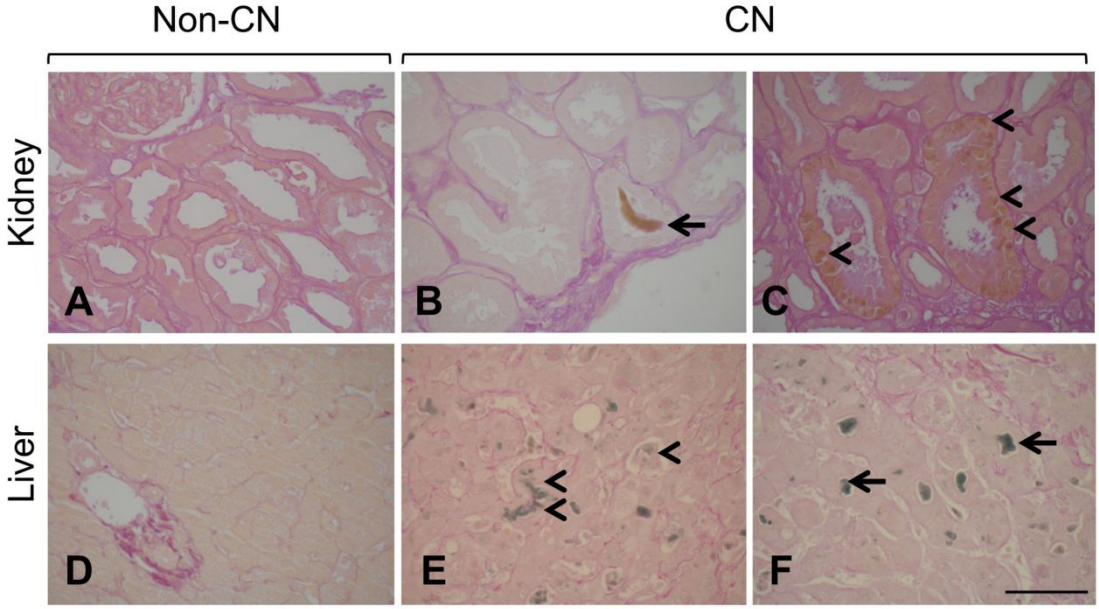
days
G
#7
HD
Bx
LTx
KTx
µmol/l
days
H
#8
Bx
HD
µmol/l
days

**Supplementary Figure 2:** Detailed course of creatinine and bilirubin in patients with the diagnosis of CN. Bx, kidney biopsy; HD, hemodialysis; Nuc, nucleoside analogue; KTx, kidney transplantation; LTx, liver transplantation; +, patient died; dotted line represents the upper limit of normal for creatinine.




**Supplementary Figure 3:** Hall's stains of human kidney in non-CN (A) as well as CN (B) cases, the latter revealing bile casts (arrows) and intracellular bile pigment (arrowheads). D shows normal liver, E and F cirrhotic liver with intracellular (arrowheads) and canalicular (arrows) bile casts. Bar represents 100 µm, all micrographs at 40x.

**Supplementary Table 1:** Histomorphological questionnaire

| | Scoring |
|---|---|
| 1. Glomerular injury (yes=1, no=0) | |
| 2. Typing and grading of tubulointerstitial injury (0-3+) | |
| • tubular cell hypertrophy | |
| • pyknosis | |
| • flattening | |
| • loss of brush border | |
| • cytoplasmic lucency | |
| • debris | |
| • tubular nuclei loss | |
| • vacuolization | |
| 3. luminal dilation (0-3+) | |
| 4. tubular casts (yes=1, no=0) | |
| • debris | |
| • pigmented | |
| • iron | |
| • Hall's | |
| • PAS | |
| 5. epithelial inclusions (yes=1, no=0) | |
| • cystic | |
| • pigment | |
| • iron | |
| • Hall's | |
| • PAS | |
| 6. interstitial edema (0-3+) | |
| 7. immune cell infiltrates (0-4)<br>(0= 0-5%, 1= 6-10%, 2=11-25%,3= 25-50%,4= >50%) | |
| 8. tubular atrophy (% of cortex) | |
| 9. interstitial fibrosis (% of cortex) | |
| 10. arteriosclerosis (0-3+) | |
| 11. arteriolosclerosis (0-3+) | |

**Supplementary Table 2:** Patients with the diagnosis of CN

| Patient | Age | Gender | Liver related diagnosis | CRP [mg/l] | Focus of infection |
|---|---|---|---|---|---|
| **1** | 62 | m | Acute autoimmune hepatitis | 93 | Erysipelas and urinary tract infection |
| **2** | 46 | m | Benign recurrent intrahepatic cholestasis | 8 | N/A |
| **3** | 64 | m | Acute hepatitis B | 35 | Acute hepatitis B |
| **4** | 52 | m | Cyptogenic liver cirrhosis | 44 | Spontaneous bacterial peritonitis |
| **5** | 55 | m | LTx due to chronic HBV infection 8 years earlier | 58 | Recurrent cholangitis, state after liver transplantation with biliodigestive anastomosis |
| **6** | 44 | m | Secondary sclerosing cholangitis (SSC) after sepsis due to pneumonia | 101 | Recurrent cholangitis, SSC |
| **7** | 45 | m | Drug-induced liver injury after long-term treatment on an ICU due to pneumonia, repeated exclusion of SSC | 17 | N/A |
| **8** | 50 | m | Liver cirrhosis due to chronic HCV infection | 84 | No focus identified (Exclusion of blood stream infection, spontaneous bacterial peritontits, urinary tract infection. Chest X-ray was not performed to exclude pulmonary infiltrate) |

**Supplementary Table 3:** Baseline characteristics of patients included in the detailed immunohistochemical analysis

| | AKI and normal serum-bilirubin | AKI and elevated serum-bilirubin | Cholemic nephropathy | p-value |
|---|---|---|---|---|
| **Number of patients** | 5 | 6 | 6 | |
| **Age (years)** | 49 ± 2 | 44 ± 12 | 53 ± 9 | 0.3 |
| **Gender male** | 3 | 5 | 6 | |
| **Cause of Liver Disease** | | | | |
| **a) viral** | 1 | 1 | 1 | |
| **b) EtOH** | 1 | 1 | 0 | |
| **c) APAP** | 1 | 0 | 0 | |
| **d) NAFLD/NASH** | 0 | 0 | 0 | |
| **e) autoimmune** | 0 | 2 | 1 | |
| **f) others** | 2 | 2 | 4 | |
| **Liver cirrhosis** | 2 | 2 | 1 | |
| **Tx (LTx or KTx)** | 1 | 3 | 0 | |
| **a) History of LTx** | 1 | 3 | 0 | |
| **b) History of KTx** | 0 | 0 | 0 | |
| **INR** | 1.19 ± 0.32 | 1.33 ± 0.47 | 1.15 ± 0.15 | 0.72 |
| **Bilirubin max [µmol/l]** | 14 ± 11 | 206 ± 140 | 851 ± 262 | <0.001[b] |
| **Bilirubin [µmol/l]** | 11 ± 7 | 134 ± 54 | 525 ± 184 | <0.001[b] |
| **Creatinine max [µmol/l]** | 318 ± 175 | 516 ± 213 | 318 ± 129 | 0.23 |
| **Creatinine [µmol/l]** | 298 ± 193 | 373 ± 132 | 199 ± 73 | 0.13 |
| **MELDscore** | 17 ± 3 | 29 ± 5 | 31 ± 5 | 0.006[a,b] |
| **ALT [U/l]** | 35 ± 13 | 116 ± 55 | 131 ± 101 | 0.19 |
| **AST [U/l]** | 59 ± 87 | 171 ± 260 | 126 ± 94 | 0.08 |
| **AP [U/l]** | 59 ± 26 | 317 ± 241 | 901 ± 1076 | 0.01[b] |
| **gGT [U/l]** | 66 ± 41 | 325 ± 240 | 461 ± 617 | 0.34 |
| | | | | |
| **Albumin [g/l]** | 28 ± 15 | 27 ± 12 | 28 ± 12 | 0.97 |
| **CHE [kU/l]** | 4.48 ± 2.39 | 3.53 ± 2.5 | 2.02 ± 1.24 | 0.22 |
| | | | | |
| **Leukocytes [$10^3$/µl]** | 7.8 ± 3.07 | 10.5 ± 6.4 | 11.7 ± 6.4 | 0.44 |
| **Thrombocytes [$10^3$/µl]** | 144 ± 55 | 126 ± 43 | 196 ± 94 | 0.44 |
| **C-reactive Protein [mg/l]** | 25 ± 40 | 36 ± 37 | 50 ± 39 | 0.29 |
| **Hemoglobin [g/dl]** | 11.2 ± 2.3 | 9.6 ± 2.2 | 10.2 ± 1.4 | 0.21 |
| | | | | |
| **Urine-Bilirubin** | 0 | 2 (33.3%) | 6 (100%) | 0.004 |
| **Urine-Urobilinogen** | 1 (20%) | 1 (16.6%) | 4 (66.6%) | 0.18 |
| **Proteinuria** | 3 (60%) | 4 (66.6%) | 3 (50%) | 0.59 |
| **Hematuria** | 3 (60%) | 3 (50%) | 4 (66%) | 0.96 |
| **Dialysis** | 0 | 1 (16.6%) | 3 (50%) | 0.13 |

Kruskal-Wallis test; a: normal bilirubin vs elevated bilirubin, b: normal bilirubin vs CN, c: elevated bilirubin vs CN

**TITLE**

# Cholemic nephropathy causes acute kidney injury and is accompanied by loss of aquaporin 2 in collecting ducts

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**Keywords**

liver disease, renal function, bile acids, hyperbilirubinemia, kidney biopsy, cholemic nephropathy, bile cast, aquaporin 2

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**List of Abbreviations**

HRS – hepatorenal syndrome

AKI – acute kidney injury

APAP - acetaminophen

LTx – Liver transplantation

KTx – Kidney transplantation

CN – cholemic nephropathy

AST – aspartate aminotransferase

ALT – alanine aminotransferase

AP – alkaline phosphatase

GGT – gamma glutamyl transferase

CHE – cholinesterase

INR – international normalized ratio

CRP – C-reactive protein

PRBC – packed red blood cells

FFP – fresh frozen plasma

ULN – upper limit of normal

N/A – not applicable

ESRD – end-stage renal disease

RRT – renal replacement therapy

AQP2 – aquaporin 2

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## ABSTRACT

Impairment of renal function often occurs in patients with liver disease. Hepatorenal syndrome is a significant cause of acute kidney injury (AKI) in cirrhotic patients (HRS-AKI, type 1). Causes of non-HRS AKI include cholemic nephropathy (CN), a disease that is characterized by intratubular bile casts and tubular injury. As data on patients with CN is mostly obtained from case reports or autopsy studies, we aimed to investigate the frequency and clinical course of CN. We identified 149 patients who underwent kidney biopsy between 2000 to 2016 at the Department of Gastroenterology, Hepatology and Endocrinology. Of these, 79 had a history of liver disease and deterioration of renal function. When applying recent EASL criteria 45 of the 79 patients (57%) presented with AKI, whereas 34 patients (43%) had chronic kidney disease (CKD) (43%). Renal biopsy revealed the diagnosis of CN in 8 of the 45 patients with AKI (17.8%), whereas none of the patients with CKD was diagnosed with CN. Univariate analysis identified serum bilirubin, alkaline phosphatase and urinary bilirubin and urobilinogen as predictive factors for the diagnosis of CN. Histological analysis of AKI patients with normal bilirubin, elevated bilirubin and the diagnosis of CN revealed loss aquaporin 2 (AQP2) expression in collecting ducts in patients with elevated bilirubin and CN. Biopsy related complications requiring medical intervention occurred in four of 79 patients (5.1%).

In conclusion, CN is a common finding in patients with liver disease, AKI and highly elevated bilirubin. Loss of AQP2 in AKI patients with elevated bilirubin and CN might be the result of toxic effects of cholestasis and be in part responsible for the impairment of renal function.

## INTRODUCTION

Impairment of renal function is common in patients with acute and chronic liver disease and associated with an increased mortality (1). Recently, the European Association for the Study of the Liver (EASL) published a clinical practice guideline on the management of patients with decompensated cirrhosis including definitions of kidney disease. Depending on the dynamic and duration of renal impairment, three entities are considered: acute kidney injury (AKI), acute kidney disease (AKD) and chronic kidney disease (CKD) (2). Whereas the prevalence of CKD in patients with liver disease is not well defined (2), AKI is a common complication in patients with liver cirrhosis and hepatorenal syndrome as a cause of AKI (HRS-AKI) occurs in approximately 20% of hospitalized patients with decompensated liver cirrhosis (3). Other causes of AKI (non-HRS-AKI) include inflammation, bacterial translocation, cardiac dysfunction and bile acids (4). In this context, a disease entity entitled cholemic nephropathy (CN) regained attention. Along with impaired renal function in the context of liver disease, these patients show characteristic histomorphological kidney alterations including intratubular casts and tubular injury (5). Even though CN was first described in the early 20$^{th}$ century, this disease entity has been neglected until recently. Moreover, most of the data on patients with CN was derived from case reports (6-15) or autopsy studies (16-18). The diagnostic work-up of impaired renal function in the context of liver cirrhosis usually includes non-invasive diagnosis and does not include kidney biopsy owing to coagulopathy with increased risk of bleeding (19). The aim of this study was to investigate the frequency, clinical course and histomorphological characteristics of CN in a tertiary care hospital over a period of more than 15 years (2000-2016).

## METHODS

### Patients

We identified 149 patients who underwent kidney biopsy between 2000 and 2016 at the Department of Gastroenterology, Hepatology, and Endocrinology at a tertiary care hospital (Hannover Medical School (MHH)). Of these patients, 50 had no history of liver disease and therefore were excluded from this study. Twenty of the remaining 99 patients with an existing liver disease were biopsied due to suspected renal malignancy, whereas 79 patients received a kidney biopsy due to deterioration of renal function and were further included in this retrospective study (Fig. 1). Core needle biopsies were performed in the Department of Nephrology at MHH by ultrasound guidance. All histological analyses of the human kidney tissues were performed at the Nephropathology Unit of the Institute of Pathology at MHH.

### Clinical and laboratory parameters

Clinical and laboratory patient data were assessed by retrospective chart review. The following clinical parameters were analyzed in this study: survival, underlying liver disease, liver cirrhosis defined by histological or ultrasonographic findings and bleeding complications. The following laboratory data were collected for further analysis: aspartate aminotransferase (AST), alanine aminotransferase (ALT), alkaline phosphatase (AP), gamma-glutamyl transferase (GGT), cholinesterase (CHE), bilirubin, albumin, International Normalized Ratio (INR), C-reactive protein (CRP) and complete blood count. Renal function was assessed by serum creatinine levels and urinary parameters. All laboratory parameters were taken at the time of the kidney biopsy. Additionally, the highest values for bilirubin and creatinine in the time course before the biopsy were included (maximum values).

**Definitions of kidney disease**

Patients were classified to the type of kidney disease based on the 2018 EASL clinical practice guideline on the management of patients with decompensated cirrhosis (2). Briefly, the diagnosis of AKI was based on an acute increase of serum creatinine (sCr) >26.5 µmol/l from baseline within 48 hours or an increase ≥50% from a recent sCr dating within the last three months before admission. Depending on the increase of sCr AKI was staged as follows:

- Stage 1: increase 26.5 µmol/l or 150-200% from baseline (1A <133µmol, 1B >133µmol)
- Stage 2: increase of 201-300%
- Stage 3: increase of >300% or initiation of renal replacement therapy.

CKD was defined as a GFR <60 ml/min/1.73m$^2$ for ≥ 3 months. Patients with preexisting CKD fulfilling the criteria of AKI were considered as "AKI overlapping CKD".

**Histology and immunohistochemistry**

Human tissue was fixed in 4% neutral buffered paraformaldehyde and embedded in paraffin according to standard routine procedures. For diagnostic purposes, sections of kidney tissue were stained by periodic acid-Schiff (PAS), hematoxylin and eosin (H&E), Prussian blue for iron deposits and Hall's bilirubin stain (20) according to routine protocols.

Immunostaining of serial sections was conducted with an automated platform (Ventana ULTRA; Ventana Medical Systems, Tucson, AZ, USA) using the following antibodies: monoclonal rabbit CD4 (clone SP35; Zytomed, Berlin, Germany), mouse CD8 (clone C8/144B, Dako, Glostrup, Denmark), mouse CD15 (clone MMA; BD

Biosciences, San Jose, CA, USA), mouse CD68 (clone PG-M1; Dako) and mouse CD163 (clone MRQ-26; Cell Marque, Rocklin, CA, USA). Mouse CD177 antibody (clone 4C4; Origene Technologies, Herford, Germany) incubation after heat induced epitope retrieval with T-EDTA buffer (pH 9.0; Zytomed) was followed by anti-mouse immunoglobulins conjugated with horseradish peroxidase (JIR-E, Cambridge, UK). Aquaporin 2 (AQP2; polyclonal rabbit; Sigma Aldrich, Darmstadt, Germany) and α/β tubulin (polyclonal rabbit; Cell Signaling, Danvers, MA, USA) stains were conducted using the ZytoChem Plus horseradish peroxidase Polymer system (mouse/rabbit; Zytomed) after peroxidase-blocking (3% H2O2, 10 min). For antigen retrieval, citrate buffer (pH 6.0; Zytomed) was used. Chromogen detection was accomplished by 3,3-diaminobenzidine (Zytomed) and nuclear counterstain by hemalum. Negative controls omitting primary antibody were included in all staining procedures.

**Histomorphological analysis**

Immune cell numbers were counted in five medium fields of view (20x) in the cortical tubulointerstitial area. AQP2 stains were evaluated by quantifiying the collecting ducts with normal and reduced positivity. Loss of stain is shown as percentages of collecting ducts with decreased positivity for AQP2. For α/β tubulins, the positively stained tubuli were displayed as percentages. Normal renal tissue was stained as an additional control and had 100% positivity for AQP2 in collecting ducts and 0% for α/β tubulins.

In order to further characterize histomorphological characteristics of CN we developed a questionnaire based on a recent study (21). We included 20 blinded acute tubular injury cases (7 CN, 7 cases with liver disease and elevated bilirubin without CN, 6 cases revealing pigment for other reasons (lipofuscin, iron, ochronosis/alcaptonuria, porphyria)) into this analysis. Histochemical stains (H&E,

PAS, Prussian blue for iron, Hall's stain) were digitalized using an Aperio scanner CS2 (Leica) at 40x magnification. Scans were evaluated in a blinded manner by 6 trained nephropathologists (>1 to >15 years of experience) with main emphasis on tubular damage and pigment scoring (Suppl. Table 1).

**Statistics**

Descriptive statistical analyses were performed using SPSS version 24 (SPSS Software Corp., Chicago, IL, USA) or Prism 7 (GraphPad, San Diego, CA) and R 3.4.1 (©2017, The R Foundation for Statistical Computing). Data was analyzed by an unpaired, two-sided t-test when two distinct groups were compared. When indicated, an additional sensitivity analysis using non-parametric Mann-Whitney-U test was performed. When analyzing more than two groups Kruskal-Wallis test was used. Two-sided Fisher´s exact test and chi-square test were used as indicated. Univariate cox regression analysis were performed for dependent variable time to outcome. In addition, Kaplan-Meier estimates were displayed to depict the different survival curves of CKD and AKI patients. Respective two-sided p-values of the log rank test were calculated. Univariate logistic regression analysis was used to examine the dependent variable cholemic nephropathy. As SPSS does not calculate odds ratios when populations contain a zero value, odds ratios for covariates bilirubin and urine bilirubin were calculated using the odds ratio calculator Medcalc®. A p-value of 0.05 or less was considered statistically significant. All data are expressed as means ± standard deviations. Questionnaire evaluation was performed using cross-tables and the agreement coefficients Fleiss' Kappa for the evaluation of all raters and Cohen's Kappa (22) in the subgroup analysis of the two expert raters (nephropathologists with more than 10 and more than 15 years of experience, respectively).

## Ethics

The study was approved by the local ethics committee (3525-2017, 8070_BO_K_2018).

## RESULTS

*Study cohort of patients with liver disease undergoing kidney biopsy*

As outlined in Fig. 1, over a period of 16 years a total of 79 patients with liver disease underwent kidney biopsy due to deteriorating renal function at our Department of Gastroenterology, Hepatology, and Endocrinology. The mean age of the patients was 52.2 ± 10.8 years and 71% were male patients (56 of 79). One quarter of the patients (20/79) had a history of organ transplantation (liver or kidney) and the average sCr at the time of biopsy was 256 ± 181 µmol/l.

When applying the recent EASL criteria (2) and analyzing the type of kidney disease, we observed that 45 of the 79 patients (57%) presented with AKI and 34 patients with CKD (43%). Of the 45 patients with AKI, 18 patients also had evidence of elevated sCr more than three months prior to kidney biopsy, thus presenting as "AKI overlapping CKD" (Fig. 1). Comparing one-year survival rates between the three different groups, we observed that both, patients with AKI or AKI overlapping CKD had a significantly higher mortality after one year (Fig. 2).

*Histopathologic diagnoses of patients with AKI and CKD*

The most common histopathologic diagnosis in our cohort of patients with AKI (including AKI overlapping CKD) and CKD included primary and secondary glomerular disease (including membranous glomerulonephritis (GN), MPGN, fibrillary glomerulopathy, post-/parainfectious-GN, Henoch-Schoenlein purpura and IgA nephropathy), noninflammatory tubulointerstitial disease and vascular disease

including hypertensive nephropathy (Fig. 3). Among the 25 patients with a history of organ transplantation, six (24.0%) showed signs of calcineurin inhibitor toxicity.

Of note, the diagnosis of CN was only observed in patients with AKI (8/45, 18%), whereas none of the patients with CKD was diagnosed with CN (0/34, p<0.01).

*Clinical characteristics of patients diagnosed with CN*

To further characterize the patients with CN (n=8), we compared them to the 37 patients with other causes of AKI (non-CN). As expected, both the mean bilirubin at the time of biopsy as well as the maximum bilirubin differed significantly between the two groups (Table 1) and 44.4% (8 of 18) of the patients with a bilirubin > 100 µmol/l in our cohort of patients with AKI were diagnosed with CN. Alkaline phosphatase, another marker of cholestasis, was also significantly higher in patients with CN compared to non-CN patients. There was no difference in liver synthesis as determined by INR. Importantly, 5 out of 8 patients with CN (62.5%) required renal replacement therapy versus only 6 of 37 patients (13.3%) of patients with other causes of AKI. Urine analysis revealed hematuria and proteinuria in both patient groups to the same extent of around 50%. Of note, all patients with CN were positive for bilirubin in the urine, while only 22% of non-CN patients had detectable urinary bilirubin. Similarly, urobilinogen, a degradation product of bilirubin was more often detected in the urine of CN patients than non-CN patients (Table 1).

Next, we were interested in factors that had an impact on survival in liver disease patients with AKI. We performed a cox regression analysis on time to death and identified dialysis and bilirubin as independent factors associated with mortality. Noteworthy, despite significantly higher MELD-score in the group of patients with CN (31 vs 22, p=0.001), the diagnosis of CN was not associated with an increased risk of death (Suppl. Fig. 1).

In order to identify factors associated with the diagnosis of CN we performed an additional univariate logistic regression analysis and identified bilirubin higher than five times the upper limit of normal (ULN), alkaline phosphatase higher than three times the ULN as well as detectable bilirubin and urobilinogen in the urine as independent risk factors (Table 3). Despite higher CRP in patients with CN at baseline, CRP was neither associated with an increased mortality in patients with AKI (Table 2) nor associated with the diagnosis of CN (Table 3).

*Clinical course of patients diagnosed with CN*

A total of eight patients were diagnosed with CN. While three patients recovered (#1-3), the remaining five patients required renal replacement therapy (RRT) (#4-8). Given this observation, we were interested, whether there are differences in the patient characteristics. All three patients that recovered (#1-3) had an acute episode of liver disease without significant comorbidities. Also, in all three cases a specific treatment was available: patient #1 was diagnosed with autoimmune hepatitis and received steroids, patient #2 was diagnosed with benign recurrent intrahepatic cholestasis (BRIC) and received rifampicin, patient #3 had an acute hepatitis B and received lamivudine and subsequently telbivudine. All three patients did not require RRT and kidney function recovered along with resolving cholestasis. The remaining five patients (#4-8) had advanced stages of various diseases: two patients had underlying liver cirrhosis (#4, #8), two patients were treated long-term on the ICU (#6, #7) and the remaining patient was liver transplanted with recurrent episodes of cholangitis (#5). All of these five patients underwent RRT; four out of five died within two years, the remaining patient underwent combined liver/kidney transplantation (#5). Detailed course of creatinine and bilirubin levels as well as liver related diagnoses are shown in the supplementary material (Suppl. Fig. 2, Suppl. Table 2).

*Histomorphological characteristics of patients diagnosed with CN*

The diagnosis of CN can only be verified by histopathological examination of kidney biopsies. In all eight patients diagnosed with CN, we observed bilirubin casts labelled by Hall's stain (20) within the tubular lumen or massive pigment inclusions in the tubular epithelial cells (Fig. 4, Suppl. Fig. 3). To rule out the deposition of iron pigment, we performed Prussian blue stain in serial sections and did not observe iron deposition in CN patients (Fig. 4). We were interested, whether CN can be distinguished from kidney biopsies with pigment inclusions other than bile cast and from kidneys of patients with various non-cholestatic liver diseases applying known histopathological criteria by six experienced nephropathologists from three different pathology departments in a questionnaire. Neither the evaluation of all six raters nor the subgroup analysis of the expert raters (nephropathologists with >10 and >15 years of experience) identified discriminating histopathological features between the chosen entities (Data not shown).

*Patients with elevated bilirubin and patients with CN show loss of AQP2 in collecting ducts*

In order to characterize the inflammatory infiltrate and characteristics of tubular injury in CN we performed a detailed immunohistochemical analysis. We investigated patients from three groups; patients with AKI and normal serum-bilirubin (n=5), patients with AKI and elevated serum-bilirubin (n=6) and patients with CN (n=6). No material was available from two patients diagnosed with CN. Detailed baseline characteristics of the patients included in this immunohistochemical analysis are presented in Suppl. Table 3. Baseline sCr was similar in all three groups. Mean serum bilirubin was 11, 134, and 525 µmol/l in the three patient groups, respectively. AQP2 expression in collecting ducts was significantly reduced in patients with high

bilirubin and patients with CN compared to patients with AKI but normal bilirubin. No differences between the three groups were observed in the expression of α/β tubulins which was in general high. No significant differences in cell infiltration of T cells (CD4, CD8), neutrophils (CD15) and macrophages (CD68) were revealed but CN patients had significantly lower numbers of M2 macrophages/monocytes (CD163) compared to patients with AKI and normal bilirubin (Fig. 5). CD177 positive neutrophil numbers were very low but higher in patients with AKI and normal bilirubin.

*Kidney biopsy related complications*

In line with the increased risk of bleeding in patients with advanced liver disease 25 patients (31.6%) had an INR > 2 or thrombocytes <100.000/µl. Biopsy related complications occurred in six of 79 patients (7.6%). Four patients required transfusion of packed red blood cells (PRBCs), two of those four underwent surgery or coiling, respectively, to control bleeding. Of the remaining two patients, one presented with a perirenal hematoma and the other one had an arteriovenous fistula, both did not require an intervention. No fatal complication occurred after kidney biopsy in our cohort.

## DISCUSSION

Clinical management of patients with liver disease and deteriorating renal function is challenging as a definite diagnosis of underlying renal cause can often only be made by kidney biopsy, which is seldom performed owing to increased risk of bleeding. Our study investigated the underlying cause of deteriorated renal function in a large cohort of patients with liver disease in a tertiary care hospital over a period of more than 15 years. We observed several important findings: (i) cholemic nephropathy was

diagnosed in 18% of patients with AKI, (ii) high serum bilirubin and alkaline phosphatase levels were associated with the diagnosis of CN, (iii) patients with AKI and elevated bilirubin as well as CN show loss of AQP in collecting ducts, (iv) bleeding complications were higher in our patient cohort compared to the published literature.

Various liver diseases are related with different forms of kidney alterations. It is well known, that HBV and HCV are associated with glomerular kidney disease (23, 24) and also patients with non-alcoholic fatty liver disease have a higher rate of CKD (25). In this context, an association of cholestasis with the impairment of renal function has already been described at the beginning of the last century (26, 27). Since then, most of the data on this disease entity, which is known as cholemic nephrosis, bile cast nephropathy or CN, has been derived from case reports (6-15) or autopsy studies (16-18). However, none of the studies systematically investigated the frequency of CN in a living cohort. Out of our 79 patients with liver disease who underwent renal biopsy 8 of the 45 patients with AKI were diagnosed with CN. Hepatorenal syndrome is a common cause of AKI in patients with liver cirrhosis (28). During 2000 and 2016, 935 treatment cases of "hepatorenal syndrome" were documented in our hospital. Given the low number of patients with AKI that underwent kidney biopsy (45out of 935, 4.8%), CN is likely to be underdiagnosed.

In our cohort, we noted two types of CN. First, we observed a reversible form in patients with acute liver disease where a specific treatment was available (patients #1-3). All these patients developed AKI which resolved once bilirubin declined. In contrast, the five other patients (patients #4-8) suffered from disease conditions that are per se considered major risk factors for the development of severe AKI (stage 3) including liver cirrhosis or sepsis. Moreover, the underlying liver disease was chronic with no specific drug therapy available. CN in these patients did not resolve as

hyperbilirubinemia could not be improved by medical intervention. Also, CN in those patients was only one of many disease-defining factors and consecutively, all of these patients progressed to ESRD requiring RRT.

It is well accepted that dialysis is associated with increased mortality, which was also observed in our study. Another factor associated with mortality was serum bilirubin. Given that both bilirubin and creatinine are included in the Model for End-stage Liver Disease score (MELD-score), which predicts short-term mortality in patients undergoing transjugular intrahepatic portosystemic shunts (TIPS) (29) and suffering from end-stage liver disease (30), it is not surprising that CN patients who have both, a deteriorated renal function and an increased bilirubin, have significantly higher MELD-scores at baseline (31 vs 22, Table). However, one-year mortality was not different as compared to patients with non-CN AKI. At first sight, this might be contradictory, but this is reflected by the subset of patients that develops CN in an acute setting and has a benign course when hyperbilirubinemia resolves (patients #1-3).

We identified markers of cholestasis including bilirubin higher than five times the ULN and detection of bilirubin and urobilinogen in the urine as risk factor for the development of CN. It is not clear whether bilirubin has a direct toxic effect on the kidney, however it has been shown that bilirubin levels correlate well with levels of bile acids (31). In this context, a recent bile duct ligation study in mice identified bile acids as a major trigger of CN and the authors suggested that tubulotoxic injury caused a loss of AQP2 in collecting ducts (32). Loss of AQP2 expression in the collecting ducts is in line with other studies in rodents (33, 34) or human (35) kidneys which show an inverse correlation of AQP2 with toxicity and interstitial fibrosis. Of note, both in patients with AKI and elevated bilirubin as well as CN, we observed a loss of AQP2, whereas patients with AKI and normal bilirubin maintained AQP2

expression. Based on experimental evidence in a bile duct ligation model, it has been proposed that reduced renal AQP2 reflects an active escape mechanism from vasopressin-induced antidiuresis, providing a compensatory mechanism aimed at avoiding dilutional hyponatremia (36). Along these lines, bile acids may play a regulatory role as they can directly impact AQP2 expression through modulation of the farnesoid X receptor (FXR) and the G protein–coupled receptor (GPCR) (37). Additional studies will be needed to elucidate whether these vasopressin-independent pathways play a functional role in CN. Additionally, all groups showed high expression of α/β tubulin indicating severe kidney injury. Heterodimers of α- and β-tubulins assemble to tubulin and form microtubules which are major components of the cytoskeleton. Tubulins are also responsible for brush border formation (38). Kidney injury like ischemia increases tubulin expression as an adaptive response (39). The observed higher numbers of M2 macrophages identified by CD163 in AKI with normal bilirubin might mirror the well-known role of M2 in wound healing, which might be impaired in CN and AKI patients with elevated bilirubin leading to severe fibrosis as already described in mice with cholemic nephropathy(32) . A subgroup of neutrophils (CD177-positive granulocytes) showed higher numbers in patients with normal compared to elevated bilirubin and CN. However, neutrophil cell numbers were so low that this difference is most likely random. Moreover, the data driven from the histomorphological questionnaire did not identify any specific features of renal damage in CN cases as compared to non-CN high bilirubin or other pigment associated cases, pointing out that 1) there are no established discriminating features of causes for tubular damage in general (21), 2) AKI patients with elevated bilirubin presented with the same toxic effect (especially tubular injury) (40) compared to CN but might have missed bile casts (sampling error), 3) Hall's stain is known to have a low sensitivity (18). A close cooperation between hepatologists,

nephrologists and nephropathologists including interdisciplinary case conferences may help to correctly diagnose CN.

One important question remaining is whether a diagnosis of CN has any therapeutic consequence. Extracorporal liver support systems such as the extracorporeal liver assist device (ELAD) may lower metabolites such as bilirubin without affecting creatinine. One case report has been published on the successful use of an extracorporeal albumin dialysis (ECAD) in a patient with cholestasis and AKI in whom kidney function quickly improved after the first session of ECAD (41). A randomized controlled study on the use of an extracorporeal cellular therapy (ELAD) in severe alcoholic hepatitis observed an effect on survival in patients with a MELD-score of <28, hyperbilirubinemia and no impairment of renal function (42). In contrast, there was no benefit for patients with a MELD-score higher than 28 owing to an increased creatinine in addition to hyperbilirubinemia. If one speculates that the extracorporeal elimination of hepatic metabolites including bilirubin has a beneficial effect on the renal deposition of bile cast, this prophylactic approach would be more efficient as opposed to a belated treatment once casts and cellular pigments are already deposited. Yet, given the enormous costs and logistic necessities, routine extracorporeal liver support systems are an unlikely therapeutic option. A pharmacological treatment approach would be more feasible. A study published recently showed that norursodeoxycholic acid (norUDCA) ameliorates cholemic nephropathy in bile duct ligated mice (43). Importantly, norUDCA did not positively affect liver function but might have a direct kidney specific therapeutic effect (43). Prospective studies in humans need to investigate a potential benefit for patients with highly elevated bilirubin and impaired renal function.

Finally, the question is whether it is justified to perform kidney biopsies in this patient population, where bleeding complications may occur not only owing to platelet dysfunction in uremia (44) but also due to concomitant liver dysfunction. Significant bleeding complications with the requirement of medical intervention (transfusion, coiling, surgery) occurred in 5.1 % of our patients (4 of 79). This is around five times higher than in a liver disease-independent study that only observed bleeding complications in 1.1% of patients (45) . In a large systematic review of almost 10,000 patients, only 0.9% of patients who underwent kidney biopsy, required transfusion (46) . Despite this apparently higher rate of bleeding complications, no fatal complications occurred and only two of the four patients underwent intervention (coiling, surgery). Based on these data, a kidney biopsy should only be performed in highly selected cases such as patients with HRS-AKI with non-response to standard treatment of HRS including albumin and vasopressors. Moreover, kidney biopsy might be required within clinical studies aiming to develop non-invasive diagnostics for the differentiation between HRS-AKI and CN, or in trials evaluating therapeutic agents such as norUDCA.

Our study has several limitations, including the small number of biopsy-proven CN patients and the retrospective, single center design. Moreover, follow up kidney biopsies to check whether bile casts are removed once kidney function improved were not available.

In conclusion, CN is a common finding in patients with liver disease, AKI and highly elevated bilirubin. Kidney biopsy should be considered at least in highly selected patients with HRS-AKI who do not respond to treatment and within clinical studies.

Prospective studies are needed to define the real prevalence of CN in patients with liver disease and to investigate potential therapeutic interventions.

## FIGURE LEGENDS

**Figure 1:** Study population. Out of 149 kidney biopsies performed between 2000 and 2016, a total of 79 fulfilled criteria to enter the study.

**Figure 2:** Kaplan-Meier curves to display increased mortality of liver disease patients diagnosed with AKI compared to CKD. Patients with the diagnosis of AKI or AKI overlapping CKD have an increased one-year mortality as compared to patients with CKD ($p<0.001$ and $p=0.023$ of log rank test, respectively).

**Figure 3:** Distribution of histological diagnoses of kidney biopsy in a total of 79 patients with underlying liver disease, 45 of these patients presented with AKI, whereas 34 had CKD. Data are presented as percentages.

**Figure 4:** Representative photomicrographs of H&E (upper row), Hall's bilirubin stain (middle row) and Prussian blue iron reaction (lower row) in AKI patients with normal bilirubin (A, D, G), AKI patients with elevated bilirubin (B, E, H) and in patients with CN (C, F, I). Arrows point to bile casts, arrowheads indicate iron. Bar represents 100 µm, all micrographs at 40x.

**Figure 5:** Tubular integrity marker AQP2 and α/β tubulin expression (**A**) and counted immune cell infiltration (T cells: CD4, CD8; macrophages: CD68, CD163; neutrophils: CD15, CD177) (**B**). Marker expression was determined after immunohistochemical staining in samples from patients with AKI and normal bilirubin (NB, n=5), elevated bilirubin (EB, n=6) and cholemic nephropathy (CN, n=6). Bar represents 100 µm, all micrographs at 40x; arrows depict immunopositivity, asterisks show pigment. *p < 0.05, **p < 0.01, ***p < 0.001.

## REFERENCE LIST

1. Fede G, D'Amico G, Arvaniti V, Tsochatzis E, Germani G, Georgiadis D, Morabito A, et al. Renal failure and cirrhosis: a systematic review of mortality and prognosis. J Hepatol 2012;56:810-818.

2. European Association for the Study of the Liver. Electronic address eee, European Association for the Study of the L. EASL Clinical Practice Guidelines for the management of patients with decompensated cirrhosis. J Hepatol 2018;69:406-460.

3. Garcia-Tsao G, Parikh CR, Viola A. Acute kidney injury in cirrhosis. Hepatology 2008;48:2064-2077.

4. Davenport A, Sheikh MF, Lamb E, Agarwal B, Jalan R. Acute kidney injury in acute-on-chronic liver failure: where does hepatorenal syndrome fit? Kidney Int 2017;92:1058-1070.

5. Krones E, Pollheimer MJ, Rosenkranz AR, Fickert P. Cholemic nephropathy - Historical notes and novel perspectives. Biochim Biophys Acta 2017.

6. Alnasrallah B, Collins JF, Zwi LJ. Bile Nephropathy in Flucloxacillin-Induced Cholestatic Liver Dysfunction. Case Rep Nephrol 2016;2016:4162674.

7. **Aniort J, Poyet A**, Kemeny JL, Philipponnet C, Heng AE. Bile Cast Nephropathy Caused by Obstructive Cholestasis. Am J Kidney Dis 2017;69:143-146.

8. Betjes MG, Bajema I. The pathology of jaundice-related renal insufficiency: cholemic nephrosis revisited. J Nephrol 2006;19:229-233.

9. Bredewold OW, de Fijter JW, Rabelink T. A case of mononucleosis infectiosa presenting with cholemic nephrosis. NDT Plus 2011;4:170-172.

10. Kiewe P, Korfel A, Loddenkemper C, Fischer L, Jahnke K, Notter M, Muhr-Wilkenshoff F, et al. Unusual sites of Hodgkin's lymphoma: CASE 3. Cholemic

nephrosis in Hodgkin's lymphoma with liver involvement. J Clin Oncol 2004;22:4230-4231.

11. Luciano RL, Castano E, Moeckel G, Perazella MA. Bile acid nephropathy in a bodybuilder abusing an anabolic androgenic steroid. Am J Kidney Dis 2014;64:473-476.

12. Patel J, Walayat S, Kalva N, Palmer-Hill S, Dhillon S. Bile cast nephropathy: A case report and review of the literature. World J Gastroenterol 2016;22:6328-6334.

13. Sequeira A, Gu X. Bile cast nephropathy: an often forgotten diagnosis. Hemodial Int 2015;19:132-135.

14. Tabatabaee SM, Elahi R, Savaj S. Bile cast nephropathy due to cholestatic jaundice after using stanozolol in 2 amateur bodybuilders. Iran J Kidney Dis 2015;9:331-334.

15. Werner CR, Wagner V, Sipos B, Buttner-Herold M, Amann K, Haap M, Malek NP, et al. [Acute kidney injury in liver failure]. Dtsch Med Wochenschr 2016;141:1559.

16. Mohapatra MK, Behera AK, Karua PC, Bariha PK, Rath A, Aggrawal KC, Nahak SR, et al. Urinary bile casts in bile cast nephropathy secondary to severe falciparum malaria. Clin Kidney J 2016;9:644-648.

17. Song J, Chang A. Jaundice-associated acute kidney injury. NDT Plus 2009;2:82-83.

18. van Slambrouck CM, Salem F, Meehan SM, Chang A. Bile cast nephropathy is a common pathologic finding for kidney injury associated with severe liver dysfunction. Kidney Int 2013;84:192-197.

19. Tripodi A, Mannucci PM. The coagulopathy of chronic liver disease. N Engl J Med 2011;365:147-156.

20. Hall MJ. A staining reaction for bilirubin in sections of tissue. Am J Clin Pathol 1960;34:313-316.

21. **Schumann-Bischoff A, Schmitz J**, Scheffner I, Schmitt R, Broecker V, Haller H, Brasen JH, et al. Distinct morphological features of acute tubular injury in renal allografts correlate with clinical outcome. Am J Physiol Renal Physiol 2018;315:F701-F710.

22. Fleiss JL, Levin B, Paik MC. Statistical methods for rates and proportions. 3rd ed. Hoboken, N.J.: J. Wiley, 2003: xxvii, 760 p.

23. Johnson RJ, Couser WG. Hepatitis B infection and renal disease: clinical, immunopathogenetic and therapeutic considerations. Kidney Int 1990;37:663-676.

24. Johnson RJ, Gretch DR, Yamabe H, Hart J, Bacchi CE, Hartwell P, Couser WG, et al. Membranoproliferative glomerulonephritis associated with hepatitis C virus infection. N Engl J Med 1993;328:465-470.

25. Musso G, Gambino R, Tabibian JH, Ekstedt M, Kechagias S, Hamaguchi M, Hultcrantz R, et al. Association of non-alcoholic fatty liver disease with chronic kidney disease: a systematic review and meta-analysis. PLoS Med 2014;11:e1001680.

26. Krones E, Wagner M, Eller K, Rosenkranz AR, Trauner M, Fickert P. Bile acid-induced cholemic nephropathy. Dig Dis 2015;33:367-375.

27. Clairmont P, Haberer Hv. Ueber die Anurie nach Gallensteinoperation. Mitt. Grenzgeb. Med. u. Chir. 1911;22.

28. Gines P, Guevara M, Arroyo V, Rodes J. Hepatorenal syndrome. Lancet 2003;362:1819-1827.

29. Malinchoc M, Kamath PS, Gordon FD, Peine CJ, Rank J, ter Borg PC. A model to predict poor survival in patients undergoing transjugular intrahepatic portosystemic shunts. Hepatology 2000;31:864-871.

30. Kamath PS, Wiesner RH, Malinchoc M, Kremers W, Therneau TM, Kosberg CL, D'Amico G, et al. A model to predict survival in patients with end-stage liver disease. Hepatology 2001;33:464-470.

31. Makino I, Nakagawa S, Mashimo K. Conjugated and unconjugated serum bile acid levels n patients with hepatobiliary diseases. Gastroenterology 1969;56:1033-1039.

32. **Fickert P, Krones E**, Pollheimer MJ, Thueringer A, Moustafa T, Silbert D, Halilbasic E, et al. Bile acids trigger cholemic nephropathy in common bile-duct-ligated mice. Hepatology 2013;58:2056-2069.

33. Kishore BK, Krane CM, Di Iulio D, Menon AG, Cacini W. Expression of renal aquaporins 1, 2, and 3 in a rat model of cisplatin-induced polyuria. Kidney Int 2000;58:701-711.

34. Kwon TH, Laursen UH, Marples D, Maunsbach AB, Knepper MA, Frokiaer J, Nielsen S. Altered expression of renal AQPs and Na(+) transporters in rats with lithium-induced NDI. Am J Physiol Renal Physiol 2000;279:F552-564.

35. Bedford JJ, Leader JP, Walker RJ. Aquaporin expression in normal human kidney and in renal disease. J Am Soc Nephrol 2003;14:2581-2587.

36. Brond L, Hadrup N, Salling N, Torp M, Graebe M, Christensen S, Nielsen S, et al. Uncoupling of vasopressin signaling in collecting ducts from rats with CBL-induced liver cirrhosis. Am J Physiol Renal Physiol 2004;287:F806-815.

37. Li S, Qiu M, Kong Y, Zhao X, Choi HJ, Reich M, Bunkelman BH, et al. Bile Acid G Protein-Coupled Membrane Receptor TGR5 Modulates Aquaporin 2-Mediated Water Homeostasis. J Am Soc Nephrol 2018;29:2658-2670.

38. Tonucci FM, Ferretti A, Almada E, Cribb P, Vena R, Hidalgo F, Favre C, et al. Microtubules regulate brush border formation. J Cell Physiol 2018;233:1468-1480.

39. Caron A, Desrosiers RR, Beliveau R. Kidney ischemia-reperfusion regulates expression and distribution of tubulin subunits, beta-actin and rho GTPases in proximal tubules. Arch Biochem Biophys 2004;431:31-46.

40. Wardle EN. Renal failure in obstructive jaundice--pathogenic factors. Postgrad Med J 1975;51:512-514.

41. Sens F, Bacchetta J, Rabeyrin M, Juillard L. Efficacy of extracorporeal albumin dialysis for acute kidney injury due to cholestatic jaundice nephrotoxicity. BMJ Case Rep 2016;2016.

42. Thompson J, Jones N, Al-Khafaji A, Malik S, Reich D, Munoz S, MacNicholas R, et al. Extracorporeal Cellular Therapy (ELAD) in Severe Alcoholic Hepatitis - A Multinational, Prospective, Controlled, Randomized Trial. Liver Transpl 2017.

43. Krones E, Eller K, Pollheimer MJ, Racedo S, Kirsch AH, Frauscher B, Wahlstrom A, et al. NorUrsodeoxycholic acid ameliorates cholemic nephropathy in bile duct ligated mice. J Hepatol 2017;67:110-119.

44. Kaw D, Malhotra D. Platelet dysfunction and end-stage renal disease. Semin Dial 2006;19:317-322.

45. Atwell TD, Spanbauer JC, McMenomy BP, Stockland AH, Hesley GK, Schleck CD, Harmsen WS, et al. The Timing and Presentation of Major Hemorrhage After 18,947 Image-Guided Percutaneous Biopsies. AJR Am J Roentgenol 2015;205:190-195.

46. Corapi KM, Chen JL, Balk EM, Gordon CE. Bleeding complications of native kidney biopsy: a systematic review and meta-analysis. Am J Kidney Dis 2012;60:62-73.