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Quang Trung Tran
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Dekan: Prof. Dr. Karlhans Endlich

1. Gutachter(in): Prof. Dr. Markus M. Lerch

2. Gutachter(in): Prof. Dr. Julia Mayerle

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Die in dieser Dissertation vorgestellten Arbeiten wurden ausgeführt unter der Betreuung des Dissertationskomitees:

Prof. Dr. Markus M. Lerch, Universitätsmedizin Greifswald und Ludwig-Maximilians-Universität München

Prof. Dr. Ali A. Aghdassi, Universitätsmedizin Greifswald

Prof. Dr. Uwe T. Bornscheuer, Universität Greifswald

und Prof. Dr. Michael Lalk, Universität Greifswald

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List of abbreviations

ANC	: Acute necrotic collection
AP	: Acute pancreatitis
APACHE	: Acute physiology and chronic health evaluation
AUC	: Area under the curve
BAs	: Bile acids
BPDL	: Bile and pancreatic duct ligations
CCK	: Cholecystokinin
ERCP	: Endoscopic retrograde cholangiopancreatography
LAMS	: Lumen-apposing metal stent
LCA	: Lithocholic acid
LDH	: Lactate dehydrogenase
NAC	: *N*-acetyl cysteine
PDL	: Pancreatic duct ligation
PEP	: Post ERCP pancreatitis
PI	: Propidium-iodide
RCT	: Randomized controlled trial
ROC	: Receiver operating characteristic
TCDC	: Taurochenodeoxycholic acid
TLCS	: Taurolithocholic acid 3-sulfate;
TUDCA	: Tauroursodeoxycholic acid
WON	: Walled-off necrosis

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Abstract

Acute pancreatitis (AP) is one of the most common and widely increasing gastrointestinal diseases leading to hospitalization without specifically available therapy. Among various etiologies, biliary origin is the most common cause. However, the effects of BAs, given systemically, on AP remains elusive. A detailed characterization of the mechanisms through which BAs contribute to the pathogenesis and severity of AP will greatly improve our understanding of the underlying pathophysiology and may facilitate the development of treatment, early identification of complications, and prevention for AP. In this view, the roles of different circulating BAs using *in vitro*-to-*in vivo* models were investigated and the underlying mechanisms through which BAs modulate the severity of AP were addressed. The impact of hydrophobic and hydrophilic BAs on both, isolated acinar cells and different animal models induced by repetitive injections of caerulein or L-arginine, ligation of the pancreatic duct (PDL) or combined bile and pancreatic duct ligation (BPDL), were tested. Disease severity was assessed by biochemical and histological parameters. Serum CCK concentrations were determined by enzyme immunoassay. The binding of CCK1 receptor was measured using fluorescent-labeled CCK. Human BA profiles in AP patients were quantified and that were correlated with etiology as well as clinical course. In acinar cells, hydrophobic BAs mitigated the damaging effects of CCK. The same BAs further enhanced pancreatitis in L-arginine and PDL-based pancreatitis whereas they ameliorated pancreatic damage in the caerulein and BPDL models, in which CCK was involved. The chemical effect of BAs on protease trypsin was also observed, however, it was similar between hydrophobic and hydrophilic compounds. Mechanistically, the binding affinity of the CCK1 receptor was significantly reduced by hydrophobic BAs. In patients, the sum of hydrophobic but not hydrophilic BAs correlated with the etiology and severity of AP.

Post-endoscopic retrograde cholangiopancreatography (ERCP) pancreatitis (PEP) was reported to be related with CCK and several pharmaceutical agents have been used to prevent this most common and potentially severe complication, but those are of limited benefit. In this regard, our multicenter multinational randomized control trial was designed to compare the efficacy of indomethacin and *N*-acetylcysteine (NAC) for the prevention of PEP. A total of 432 ERCP patients from 6 countries were recruited and randomly assigned to receive either NAC (group A, 84 cases), indomethacin (group B, 138 cases), NAC + indomethacin (group C, 115 cases) or placebo (group D, 95 cases) two hours before procedure. The rate of PEP in groups A, B and C in comparison with placebo were 10.7%, 17.4%, 7.8% vs 20% ($p = 0.08$, 0.614 & 0.01, respectively).

Among complications of AP, infection of pancreatic necrosis is one of the most severe consequence that mostly necessitates interventional therapy. A model to identify parameters that are useful for the prediction of infected necrosis at an early stage was developed. A retrospective analysis was conducted in 705 AP patients, who underwent contrast-enhanced

computed tomography (CT scan). Both laboratory and clinical parameters were analyzed for an association with infected pancreatic necrosis, which was microbiologically confirmed. A logistic regression analysis with stepwise inclusion of significant variables was used to develop a prediction model. We tested the model quality by receiver operating characteristics analysis. We found a significant association between 11 parameters with an infection including albumin, creatinine, C-reactive protein (CRP), and alcoholic etiology, which were independent variables in the final predictive model with an area under the curve of 0.819.

In the same cohort in which we developed the prediction model above, 89 AP cases with necrotic complications diagnosed by CT scan were identified. These complications with high morbidity and mortality required endoscopic drainage, which possibly accompanies severe adverse events. All complications which occurred in patients who underwent those procedures and their associated features were retrospectively analyzed. Positive necrosis cultures and a larger diameter of the intervened necroses were significant factors associated with the occurrence of adverse events, in which the former was the most significant predictor with Odds Ratio of 6.1.

The entire work demonstrated that hydrophobicity of BAs and the involvement of CCK are relevant for the clinical course of AP. Systemic BAs may affect the severity of AP by interfering with the binding of the CCK1 receptor. Oral NAC is effective for prevention of PEP and the combination of NAC plus indomethacin resulted in the lowest PEP rate. A model using albumin, creatinine, CRP, and alcoholic etiology can differentiate infected and sterile pancreatic necrosis and control of infection is crucial for successful endoscopic drainage therapy in complicated AP. The exact pathophysiologic mechanisms, especially in CCK-related pathways, and the potential impact of BAs in human AP, in particular in preventing PEP, need to be investigated in further studies.

Key words: Acute pancreatitis, bile acids, CCK, CCK1 receptor, infected pancreatic necrosis, endoscopy intervention, RCT

Zusammenfassung

Die akute Pankreatitis (AP) ist eine der häufigsten gastrointestinalen Erkrankungen, die eine stationäre Behandlung erfordern, und weist eine weltweit steigende Inzidenz auf. Eine spezifische Therapie ist gegenwärtig nicht verfügbar. Global betrachtet liegt den meisten Fällen eine biliäre Ätiologie zugrunde. Die Auswirkungen von systemisch verabreichten Gallensäuren (GS) auf die AP sind bislang jedoch nicht vollständig aufgeklärt. Eine detailliertere Charakterisierung der Mechanismen, durch die GS zur Pathogenese und zum Schweregrad der AP beitragen, könnte das Verständnis der zugrundeliegenden Pathophysiologie verbessern und die Entwicklung von Behandlungen, die frühzeitige Erkennung von Komplikationen und die Prävention der AP ermöglichen. Als Hauptteil der vorliegenden Arbeit wurde daher die Rolle verschiedener zirkulierender GS anhand von in *in-vivo*- sowie *in vitro*-Modellen untersucht und die Mechanismen, durch die GS den Schweregrad der AP modulieren, adressiert. Hydrophobe und hydrophile GS wurden sowohl an isolierten Azinuszellen als auch in verschiedenen Mausmodellen getestet, die auf wiederholten Injektionen von Caerulein oder L-Arginin, Ligatur des Pankreasgangs (pancreatic duct ligation, PDL) oder Ligatur von Gallen und Pankreasgangs (bile and pancreatic duct ligation, BPDL) basierten. Der Schweregrad der Erkrankung wurde anhand biochemischer und histologischer Parameter beurteilt. Die CCK-Konzentrationen im Serum wurden mittels Enzymimmunoassay quantifiziert. Die Bindung des CCK1-Rezeptors wurde mit fluoreszenzmarkiertem CCK gemessen. Die GS-Profile bei Patienten mit AP wurden bestimmt und hinsichtlich Assoziationen mit der Ätiologie sowie dem klinischen Verlauf korreliert. In den Azinuszellen schwächten hydrophobe GS den schädlichen Effekt von CCK ab. Zudem verstärkten hydrophobe GS die Pankreatitis in der L-Arginin- und PDL-basierten AP, während sie die Pankreasschädigung in den Caerulein- und BPDL-Modellen, bei denen CCK beteiligt ist, verringerten. Die chemische Wirkung der GS auf Trypsin wurde ebenfalls betrachtet, war jedoch bei hydrophoben und hydrophilen Verbindungen ähnlich. Mechanistisch gesehen wurde die Bindungsaffinität des CCK1-Rezeptors durch hydrophobe BAs deutlich verringert. Bei den Patienten korrelierte die Summe der hydrophoben, nicht aber der hydrophilen GS, mit Ätiologie und Schweregrad der AP.

Es gibt Hinweise, dass die post-endoskopische retrograde cholangiopankreatikographie (ERCP) Pankreatitis (PEP) mit CCK in Verbindung steht. Mehrere pharmazeutische Mittel wurden zur Verhinderung dieser häufigsten und potenziell schwerwiegenden Komplikation eingesetzt, wobei der Nutzen aber begrenzt blieb. Vor diesem Hintergrund wurde eine multizentrische, multinationale, randomisierte Kontrollstudie konzipiert, um die Wirksamkeit von Indomethacin und *N*-Acetylcystein (NAC) zur Prävention von PEP zu vergleichen. Insgesamt wurden 432 ERCP-Patienten aus 6 Ländern rekrutiert, die zwei Stunden vor dem Eingriff nach dem Zufallsprinzip entweder NAC (Gruppe A, 84 Fälle), Indomethacin (Gruppe B, 138 Fälle), NAC + Indomethacin (Gruppe C, 115 Fälle) oder Placebo (Gruppe D, 95 Fälle)

erhielten. Die PEP-Rate in den Gruppen A, B und C betrug im Vergleich zur Placebo-Behandlung 10,7 %, 17,4 %, 7,8 % bzw. 20 % (p=0,08; 0,614 bzw. 0,01).

In Bezug auf Komplikationen der AP stellt eine infizierte Pankreasnekrose ein schwerwiegendes unerwünschtes Ereignis dar, welches in der Regel eine interventionelle Therapie erfordert. In einer weiteren Studie wurde daher ein Modell zur frühzeitigen Vorhersage einer infizierten Nekrose entwickelt. Hierzu erfolgte eine retrospektive Analyse von 705 Patienten mit AP, bei denen eine kontrastmittelverstärkte Computertomographie (CT) durchgeführt wurde. Sowohl Labor- als auch klinische Parameter wurden auf einen Zusammenhang mit einer mikrobiologisch bestätigten, infizierten Pankreasnekrosen getestet. Eine logistische Regressionsanalyse mit stufenweiser Einführung signifikanter Variablen wurde zur Entwicklung eines Vorhersagemodells verwendet. Die Qualität des Modells wurde mittels Receiver-Operating-Characteristics-Analyse geprüft. Elf Paramater waren signifikant mit einer infizierten Pankreasnekrose assoziiert; darunter Albumin, Kreatinin, C-reaktives Protein (CRP) sowie eine alkoholische Ätiologie, die jeweils als unabhängige Variablen in das finale Vorhersage-modell einflossen. Dieses wies eine Fläche unter der Kurve (AUC) von 0,819 auf.

In derselben Kohorte, für die das obige Vorhersagemodell entwickelt wurde, wurden 89 AP-Fälle mit nekrotischen Komplikationen identifiziert, die mittels CT diagnostiziert wurden. Diese Komplikationen bedingen eine hohe Morbidität und Mortalität erforderten eine endoskopische Drainage, die mit schweren unerwünschten Ereignissen einhergehen kann. Alle Komplikationen und die damit verbundenen Merkmale, die bei Patienten auftraten, die sich diesem Verfahren unterzogen, wurden retrospektiv analysiert. Positive Nekrosekulturen und ein größerer Durchmesser der behandelten Läsionen waren signifikante Faktoren, die mit dem Auftreten von unerwünschten Ereignissen assoziiert waren, wobei Ersteres mit einem Odds Ratio von 6,1 den stärksten Prädiktor darstellte.

Zusammenfassend zeigt die vorliegende Arbeit, dass die Hydrophobizität von GS und die Beteiligung von CCK für den klinischen Verlauf der AP relevant sind. Systemische GS können den Schweregrad der AP beeinflussen, indem sie die Bindung des CCK1-Rezeptors beeinträchtigen. Die Gabe von NAC ist wirksam zur Vorbeugung einer PEP und die Kombination von NAC und Indomethacin führte zu der geringsten Ereignisrate. Ein Modell, das Albumin, Kreatinin, CRP und alkoholische Ätiologie berücksichtigt, kann zwischen infizierter und steriler Pankreasnekrose unterscheiden. Bei Vorliegen einer infizierten Nekrose ist die Kontrolle der Infektion dabei entscheidend für eine erfolgreiche endoskopische Drainagetherapie. Die genauen pathophysiologischen Mechanismen, insbesondere in den CCK-bezogenen Signalwegen, und das Potenzial von GS bei der AP im Menschen, insbesondere zur Prävention der PEP, müssen in zukünftigen Studien allerdings weiter untersucht werden, um einen tatsächlichen therapeutischen Nutzen hervorzubringen.

1. Introduction

Acute pancreatitis (AP) is one of the most common gastroenterological diseases that requires hospital admission with a steadily and globally increasing incidence over the last five decades [1]. Despite one fifth of patients may suffer from a severe course with (multi-)organ failure and high mortality, the specific treatment of AP is not yet available [2]. Pathogenically, AP starts in the pancreatic acinar cells, the exocrine units which are vulnerable to extracellular stimuli [3], [4]. In these cells, digestive proteases, initiating with trypsin [5], are prematurely activated after co-localization with cathepsin B, a lysosomal hydrolase. An excessive activation without balance in the degradation of these enzymes may damage pancreatic acini [6], [7].

Among various causes, biliary origin is the most common etiology which accounts for up to half of all cases [8]. Bile acids (BAs), the most predominant component of bile juice, are synthesized from cholesterol and are conjugated with glycine or taurine in the liver. The first BA called cholic acid was discovered in 1848 and others were subsequently revealed as described by Wieland, a German chemist, in his Nobel lecture [9]. After being secreted into the intestine, the primary BAs are metabolized to secondary BAs by gut microbiome. They are mainly reabsorbed through ileum and recycled via the enterohepatic circulation [10], [11], [12]. BAs can be classified according to their chemical properties based on their hydrophobicity index with different biological effects [13,14]. BAs are not only essential for lipid digestion but also play the important role in many diseases and have been recently gaining great attention in both rodents and humans from the pathogenesis to the treatment [15,16], [17], [18].

In AP, the known knowledge about the role of BAs is limited and so far, mainly based on retrograde ductal infusion models [4,19,20]. Data on the modulation of AP severity in cholestatic disorders with increased serum concentration of BAs is insufficient. The mechanisms of how BAs affect acinar cells in AP, when given systemically, have not been elucidated. Further elucidation of the underlying mechanism through which bile stones induce AP and how BAs play their roles in this progress would remarkably enhance our knowledge about pathophysiology of the disease and allow us to develop therapeutic and preventive strategies for biliary pancreatitis [21].

Among the pancreas-related endoscopic interventions, endoscopic retrograde cholangiopancreatography (ERCP) is one of the most common procedures and is increasingly indicated [22,23]. The major complication of ERCP is AP, with a prevalence of 2.1% to 24.4%, overall 9.7% [24-26], called post ERCP pancreatitis (PEP). Various medications and interventions have been used to mitigate its severity and prevent PEP, but are of limited benefit [27-29]. In this regard, *N*-acetyl cysteine (NAC), a safe and inexpensive medication with a broad anti-oxidant effect may inhibit inflammatory intermediates and oxidative stress and potentially prevent PEP [30,31].

As mentioned above, AP may progress to a more severe condition and approximately 5 to 20% of patients develop necrotizing AP with long hospital stays and a high death rate, which requires intensive treatment [32]. Early prediction of infected necrosis for timely intervention is therefore essential to improve the outcome. Several multiparameter scores [33], [34], [35] have

been used for grading AP severity and predicting mortality. However, they require a large number of parameters at different time points while the accuracy of prediction for infected necrosis is not convincing.

When necroses are established in AP, these lesions may accompany infection, severe pain, or continuous enlargement, causing obstruction of the gastric outlet or biliary tract, which indicate drainage and necrosectomy [36,37]. These procedures have been shifting remarkably during the last decade from surgery to endoscopy with many advantages [38,39]. The methods of endoscopic interventions include the creation of an orifice, which connects the lesion with stomach or duodenum. Normally, a lumen-apposing metal stent (LAMS) or (multiple) plastic stent(s) are placed into the orifice for continuous drainage and repeated necrosectomies [40], [41]. Common adverse events during endoscopic treatment can be minor such as stent obstruction, stent dislocation, residual lesions, but may also be life-threatening, including severe systemic infection and bleeding [42,43]. The concrete risk factors that trigger the aforementioned complications are not yet fully understood.

Considering all of above, the work presented in this dissertation aims to:

- Investigate the impact of hydrophobic and hydrophilic BAs using *in vitro*-to-*in vivo* models and proposed the underlying mechanisms how BAs modulate AP severity.
- Evaluate the efficacy of oral NAC in comparison with rectal indomethacin and placebo in preventing of PEP.
- Establish a prediction model for identification of infected necrosis in AP.
- Identify the risk factor of adverse events during endoscopic drainage therapy in AP patients with pancreatic necrosis.

The main methods, key results including unpublished data and general discussion together with the appendix of papers published from our work are presented in this cumulative dissertation.

2. Main methods

2.1. Experimental work:

Information is provided about chemicals and materials as well as detailed methods concerning: Isolation and stimulation of mouse pancreatic acinar cells, CCK1 receptor-binding assay, quantification of CCK concentration, protease activation assays, quantification of intracellular calcium mobilization in living acinar cells, amylase and lipase measurement, measurement of propidium iodide exclusion and release of lactate dehydrogenase (LDH), myeloperoxidase measurement, quantification of total bile acids, examinations of histopathological changes, and animal models induced AP were presented in the paper [44] attached below.

Beside investigation of the biological effects of BAs in AP, we have also tested the chemical interaction between BAs and trypsin: taurolithocholic acid 3-sulfate (TLCS) and tauroursodeoxycholic acid (TUDCA) at different concentrations from 0-500 µM that were mixed with trypsin (T6567, Sigma-Aldrich), which had the best concentration curve at 0.2 µM and/or the Rhodamin 110 fluorescent substrate only (13558-31-1, Sigma-Aldrich). The

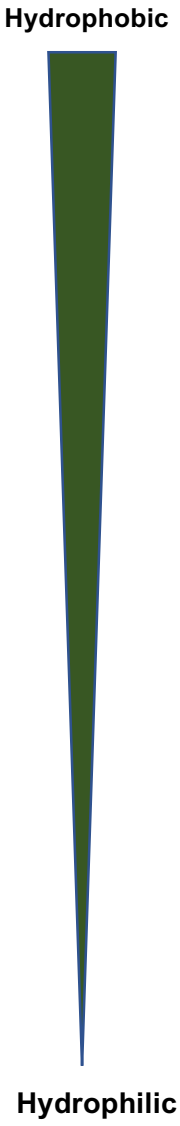
fluorescent substrate of trypsin was R-110 BZIPAR (Bio Trend, Pambio-Noranco, Switzerland, 10208) with the final concentration of 10 µM. The trypsin activity was measured via fluorescent intensity using a FLUOStar Omega fluorometer (BMG Labtech GmbH, Ortenberg, Germany) at the excitation and emission wavelengths of 485 nm and 520 nm, respectively.

2.2. Clinical work:

With regard to the part of unpublished data about BAs profile in patients, we have performed sample collection and quantification of human BAs. The study was approved by the institutional review board (registration no. III UV 91/03). Serum from patients with AP was collected within 24 hours after hospital admission. Quantification of BAs was performed by liquid chromatography tandem mass spectrometry-based assay using the AbsoluteIDQ® BAs kit (Biocrates Life Sciences, Innsbruck, Austria) according to the manufacturer's protocol. The measured BAs are listed in *table 1*. The workflow was managed by MetIDQ and the data was analyzed by Oracle database XE software.

Table 1. List of bile acids profile classified by their hydrophobicity index measured by AbsoluteIDQ® BAs kit [14,45]

Nr.	Abbreviation	Bile acids full name	Hydrophobicity index
1	LCA	Lithocholic acid	+1.23
2	GLCA	Glycolithocholic acid	+1.05
3	TLCA	Taurolithocholic acid	+1.00
4	DCA	Deoxycholic acid	+0.72
5	GDCA	Glycodeoxycholic acid	+0.65
6	TDCA	Taurodeoxycholic acid	+0.60
7	CDCA	Chenodeoxycholic acid	+0.59
8	GCDCA	Glycochenodeoxycholic acid	+0.51
9	TCDCA	Taurochenodeoxycholic acid	+0.46
10	CA	Cholic acid	+0.13
11	GCA	Glycocholic acid	+0.07
12	TCA	Taurocholic acid	0.00
13	HDCA	Hyodeoxycholic acid	-0.31
14	UDCA	Ursodeoxycholic acid	-0.35
15	GUDCA	Glycoursodeoxycholic acid	-0.43
16	TUDCA	Tauroursodeoxycholic acid	-0.47
17	TMCA(a+b)	Tauromuricholic acid alpha and beta	> -0.84 & < -0.78
18	MCA(b)	Muricholic acid, beta	-0.78
19	MCA(a)	Muricholic acid, alpha	-0.84
20	MCA(o)	Muricholic acid, omega	< -0.84



Throughout this dissertation, diagnosis criteria and severity of pancreatitis were assessed according to the Revised Atlanta classification [46] and the APACHE II score [33], which was calculated on the first and third day after hospitalization.

The study in the second article was a double-blind multicenter randomized control trial conducted from September 2020 to February 2021 among eligible ERCP cases in seven referral centers of six countries which was approved by the Ethical Committee of Ahvaz Jundishapur University of Medical sciences (IR.AJUMS.HGOLESTAN.REC.1399.120) and registered in the Iran Clinical Trial Registration (IRCT20201222049798N1). The algorithm, details of the relevant parameters of the study were described in the method session of the published paper in the appendix below [47].

The third and fourth papers focus on the results of the retrospective studies from the AP patient's cohort at University Medicine Greifswald, from 01/2009 to 12/2019. These studies were approved by the institutional review board of the University Medicine Greifswald (Nr. BB 138/19) that waived obligations of patient's informed consent. The details of study designs, definition of each variables, inclusion and exclusion criteria of the patients were presented in each paper attached below [48], [49].

2.3. Statistics:

The corresponding statistical analysis methods were presented in each published paper, in which the p value less than 0.05 was considered to be significant in all statistic tests.

3. Main results

The main focus of this dissertation is the elucidation of systemic BAs' role in AP, including experimental and the clinical data. The former were mainly published in the first attached paper [44], and the key findings were represented below. In which, the statistically significant differences for more than 3 groups were tested by one-way ANOVA followed by Tukey's multiple comparison test and significant levels of $p<0.05$ were marked by an asterisk in all figures or tables. The latter will be presented as unpublished results together with three other related clinical papers.

3.1. Experimental results

3.1.1. Effect of hydrophobic and hydrophilic BAs in isolated acini

Firstly, we examined intracellular protease activation in isolated acini upon exposure to BAs with different hydrophobicity. Initially, *in vitro* experiments with lower concentrations (50, 100 and 200 μM) of BAs were performed. However, no significant changes in intracellular protease activation were found. The intracellular activities of trypsin and cathepsin B (CTSB) were significantly higher when treated with 500 μM TLCS or LCA when compared to untreated acini. Surprisingly, these hydrophobic BAs did not further enhance the effect of CCK in isolated pancreatic exocrine units. Conversely, as shown in *Figures* 1A, B, D, and E, co-incubation of them with supramaximal (1 μM) CCK decreased intracellular protease activation compared to

CCK alone. Stimulation of acini by submaximal CCK showing a similar effect as under conditions of supramaximal CCK (*Figures* 1C and F). Differently, the hydrophilic bile acid TUDCA did not alter intracellular protease activity or amylase secretion in acini either in unstimulated conditions or after supramaximal as well as submaximal doses of CCK (*Figures* 1G, H and I).

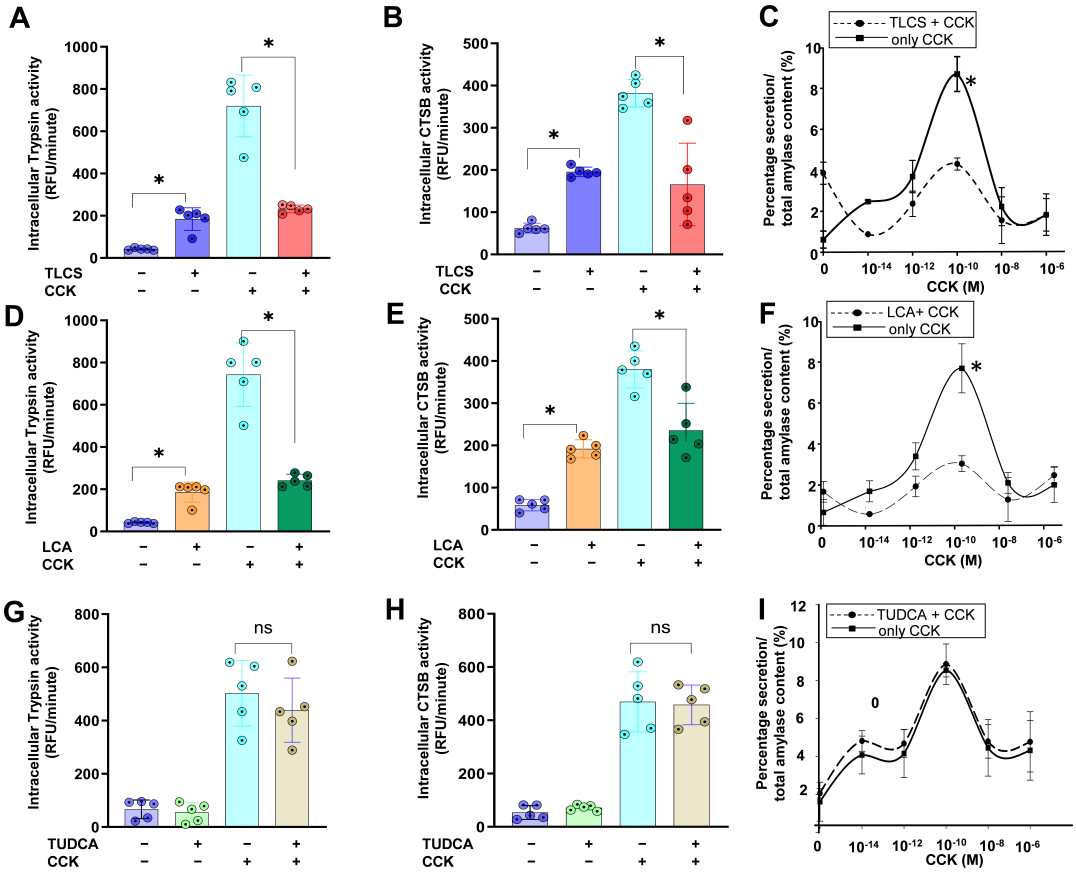


Figure 1. Impact of different BAs in CCK-stimulated acini.

(A and B): hydrophobic TLCS induced intracellular protease activation, as shown for trypsin and cathepsin B, which is less prominent than with supramaximal CCK (1 µM). Co-incubation of CCK and TLCS attenuated the protease activation. (C): Stimulation of isolated acini with different concentrations of CCK showed a peak at 100 pM, which was blocked after co-incubation with TLCS. (D-F): Similarly, LCA alone, another hydrophobic BA showed the same results as TLCS in stimulating acinar cells and mitigating the impact of CCK. (G-I): In contrast, following co-incubation of CCK and TUDCA, the hydrophilic TUDCA neither altered amylase secretion, nor decreased intracellular protease activation. Concentrations for TLCS, LCA and TUDCA were 500 µM. All results were based on 5 experiments per group. RFU: Relative Fluorescence Units.

(*Source: Quang Trung Tran, Uwe T. Bornscheuer, Michael Lalk, Markus M. Lerch, and Ali A. Aghdassi et al, International Journal of Molecular Sciences 2022, Vol 23, no. 21: 13592*).

To test the potential toxicity effect of BAs given at final concentrations of 500 µM on pancreatic acini, we further examined its indicators but neither lactate dehydrogenase (LDH) nor

propidium-iodide (PI) exclusion measurements showed a significant increase of cellular damage. These results indicated that hydrophobic, but not hydrophilic BAs, induced intracellular protease activation and mitigated the impact of CCK in living pancreatic exocrine units without toxic effects.

3.1.2. Hydrophobicity dependent effects of BAs in CCK-dependent AP models

In the next step, we investigated the role of hydrophilic and hydrophobic BAs in an *in vivo* model induced by the CCK-analogue caerulein. BAs alone could not induce AP, however, pretreatment with hydrophobic TLCS or LCA attenuated pancreatic injury and extra-pancreatic damage. Differently, the hydrophilic TUDCA did not alter the activation of any of these parameters in this model (*Figure* 2).

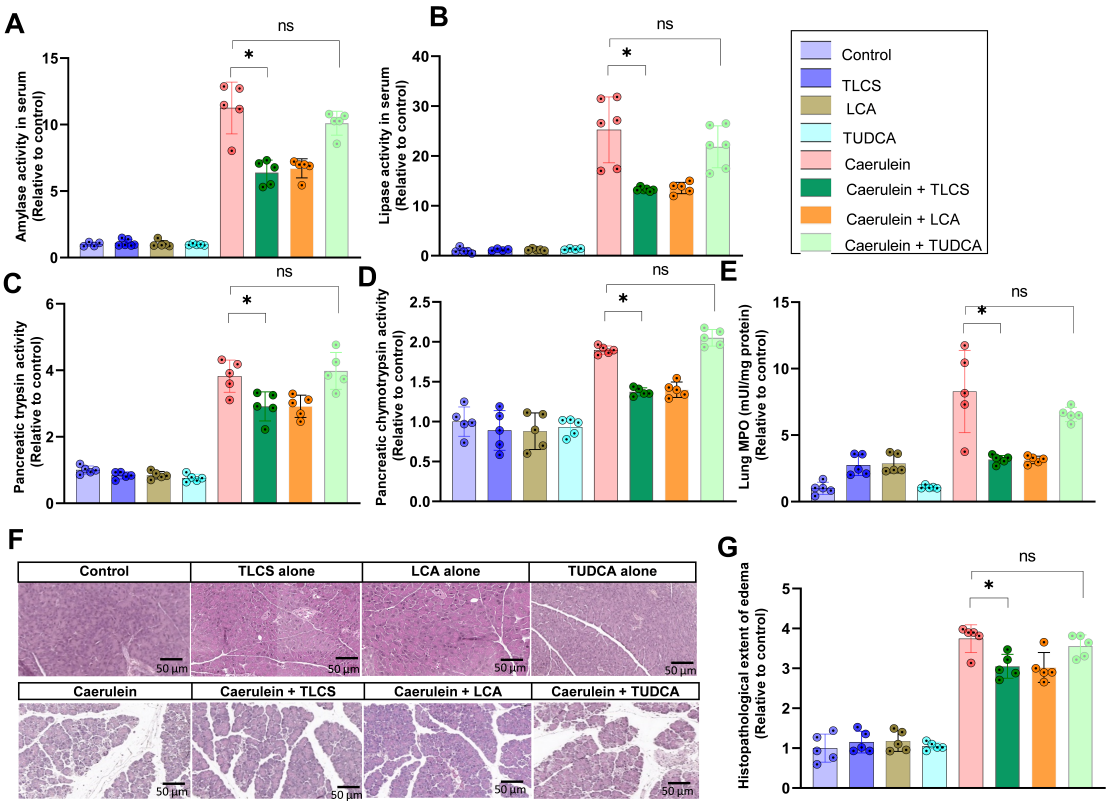


Figure 2. Severity attenuation following hydrophobic BAs in caerulein-induced AP. *(A and B): We induced AP by repetitive injections of caerulein 50 µg/kg/hour. One hour prior to the first caerulein injection, mice received a dose of 50 mg/kg TUDCA, LCA or TLCS. After 4 hours of caerulein-pancreatitis, serum amylase and lipase were clearly elevated. After pretreatment with TLCS or LCA, both enzyme activities decreased but this did not happen with TUDCA. (C and D): Pretreatment with TLCS and LCA reduced activities of pancreatic chymotrypsin at 1h and trypsin at 4h but TUDCA did not affect them in comparison with caerulein. (E): An important indicator of extra-pancreatic damage in AP, lung MPO, showed the same manner as pancreatic parameters. (F and G): The corresponding histopathological damages in the pancreas were compatible with the biochemical indicators of AP.*

(Source: Quang Trung Tran, Uwe T. Bornscheuer, Michael Lalk, Markus M. Lerch, and Ali A. Aghdassi et al, International Journal of Molecular Sciences 2022, Vol 23, no. 21: 13592).

3.1.3. Hydrophobicity dependent effects of BAs in CCK independent AP models

As the findings from CCK depending experimental AP models indicate a mitigation of severity in presence of hydrophobic BAs, we were interested in the impact of BAs in conditions unrelated to CCK. L-arginine induced AP is characterized by CCK-independent signaling pathways, and there was maximal damage at 72 hours which was further enhanced by TLCS but ameliorated by TUDCA, as demonstrated by changes of intra- and extra-pancreatic injury (*Figure* 3)

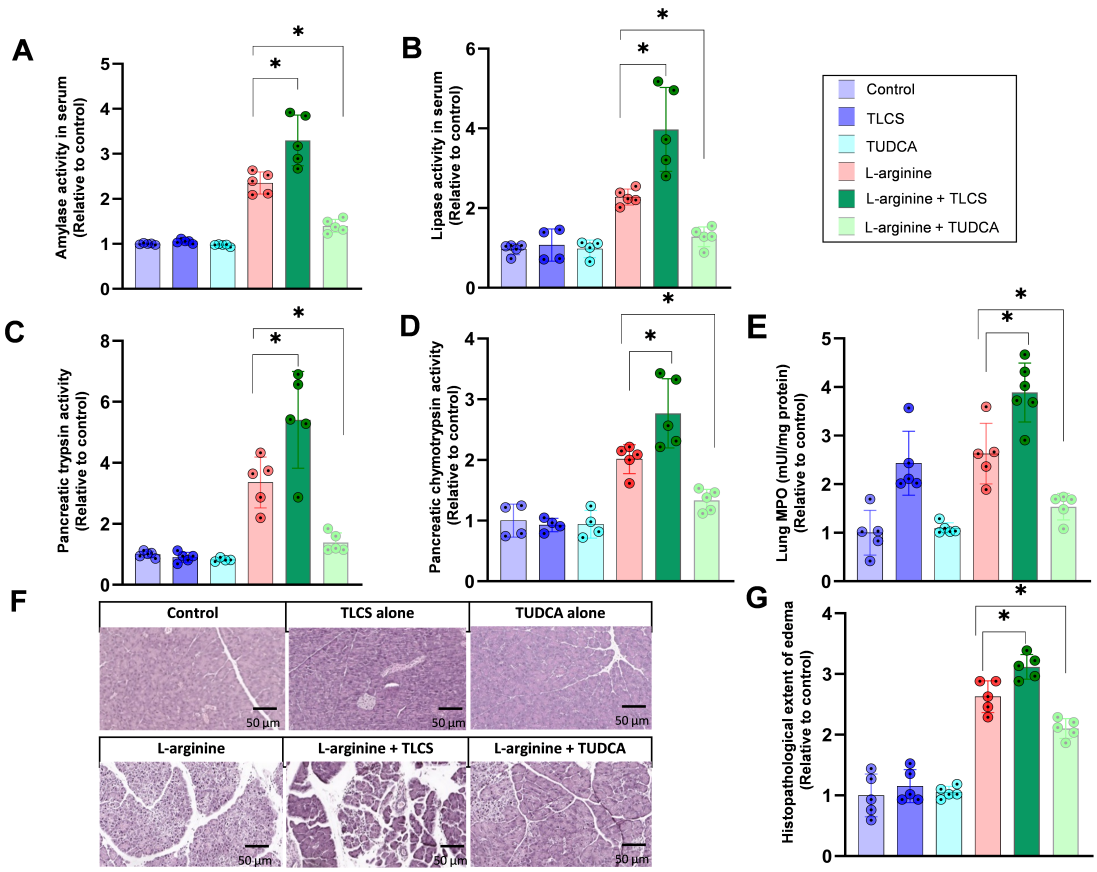


Figure 3. Effects of BAs in L-arginine induced AP.

BAs were given i.p. one hour prior to and mice were humanely killed 72h after the first L-arginine injection. (A and B): Pretreatment with TLCS caused a disease aggravation while TUDCA ameliorated disease severity as shown by changing of serum amylase and lipase. (C and D): Pancreatic trypsin and chymotrypsin activity were enhanced in L-arginine pancreatitis. Protease activity was enhanced by TLCS pre-treatment and was reduced when mice received TUDCA. (E): Lung MPO changed similarly to pancreatic damage (F and G): Pancreatic damage shown by histology was more severe by adding TLCS but milder with TUDCA in comparison with L-arginine alone.

(*Source: Quang Trung Tran, Uwe T. Bornscheuer, Michael Lalk, Markus M. Lerch, and Ali A. Aghdassi et al, International Journal of Molecular Sciences 2022, Vol 23, no. 21: 13592*).

On the other hand, AP's severity and its dependence on BAs showed a similar pattern in the PDL model, which is also independent of CCK. Pretreatment with TLCS increased while

TUDCA attenuated AP severity. Additionally, the level of histological damage correlated with findings from enzyme quantifications. Interestingly, AP severity was decreased in the BPDL model after TLCS administration. This reduction was similar to results observed in the caerulein-induced AP model (*Figure 2*). TUDCA also did not affect disease severity in this model, in which both pancreatic and bile ducts were ligated. Apparently, the BPDL model related to CCK, as measurements of circulating CCK showed an almost three-fold increase 30 minutes after ligating the pancreatic and bile ducts while after pancreatic duct ligation alone, serum CCK remained unaffected (*Figure* 4).

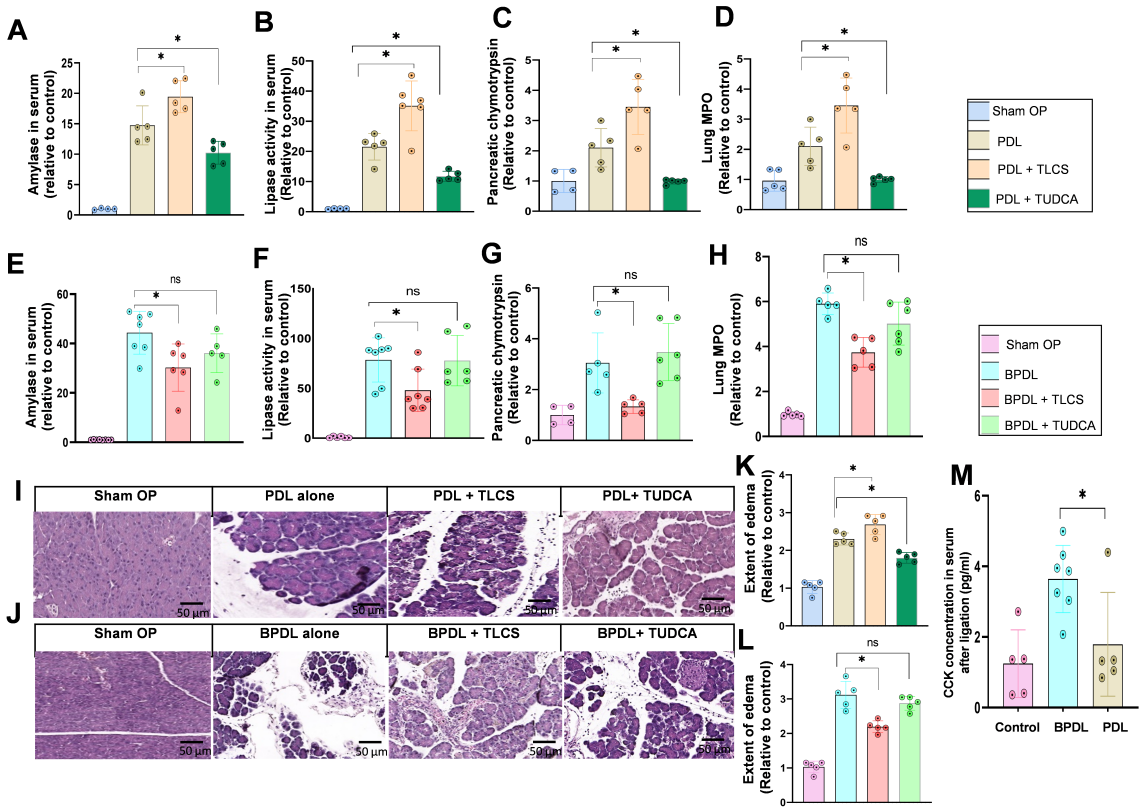


Figure 4. Impact of BAs in AP induced by PDL and BPDL models.

(A and B): TLCS enhanced but TUDCA mitigated serum amylase and lipase, which were increased by pancreatic duct ligation (PDL). (C and D): Pancreatic chymotrypsin and lung MPO levels were decreased after treatment with TUDCA but elevated when injecting TLCS. (E-H): Pre-treatment with TLCS reduced AP biochemical parameters while addition of TUDCA did not alter disease severity in ligation of both distal common bile duct and main pancreatic duct (BPDL). (I and K): Pancreatic injury including extent of edema in HE stained slides demonstrated increased severity by TLCS but attenuation by TUDCA in the PDL model. (J and L): Conversely, pancreatic parenchyma damage was reduced by TLCS in the BPDL model. (M): Endogenous circulating CCK levels were remarkably elevated in the BPDL compared to the PDL model, indicating involvement of CCK in the former model.

(*Source: Quang Trung Tran, Uwe T. Bornscheuer, Michael Lalk, Markus M. Lerch, and Ali A. Aghdassi et al, International Journal of Molecular Sciences 2022, Vol 23, no. 21: 13592*)

3.1.4. Interaction of BAs with the CCK1R on pancreatic acininar cells

Our results showed the difference between hydrophobic and hydrophilic BAs in the effects on AP and their dependence on the presence of CCK as well as the hydrophobicity of BAs. Since this peptide hormone binds to the G-protein coupled CCK1 receptor on pancreatic acinar cells causing multiple cellular signaling functions [50], we next wanted to clarify to what extent BAs act on CCK1R in acinar cells. When freshly isolated acinar cells were incubated with Alexa-488 labeled CCK, we could visualize the binding of the CCK to its receptor under a fluorescence microscope. Co-incubation of acinar cells with 500 µM TLCS dramatically reduced the fluorogenic signal, indicating that TLCS had affected the binding of Alexa-488-CCK with CCK1R on the cellular surface. When we added 500 µM TUDCA, the signal intensity on the surface was similar to that when only Alexa-488 CCK was used (*Figure* 5A). Total fluorescence intensities quantified by fluoroscopy confirmed our observations and demonstrated a reduced binding between CCK1R and CCK in the presence of hydrophobic TLCS (*Figure* 5B). Furthermore, co-incubation of living acini with TLCS and CCK decreased intracellular calcium mobilization compared to CCK alone (*Figure* 5C). Differentially, the hydrophilic TUDCA affected neither the CCK1R-CCK binding nor the intracellular calcium mobilization. When we treated C57BL/6J mice beforehand with 1 mg/kg bodyweight devazepide, a CCK1R inhibitor, and induced AP by caerulein, pancreatic enzymes were almost abrogated and the addition of TLCS did not further decrease their activity (*Figures* 5D, E). Likewise, trypsin activity was quenched by devazepide (*Figure* 5F). In parallel, both histopathologic examination and macroscopic observation in caerulein treated mice showed edema and injury of the pancreas, which was almost absent and could not be further mitigated by TLCS after inhibiting CCK1R (*Figures* 5G, H). These results indicate that hydrophobic BAs impaired intracellular signaling pathways for AP via interacting with the CCK1-receptor.

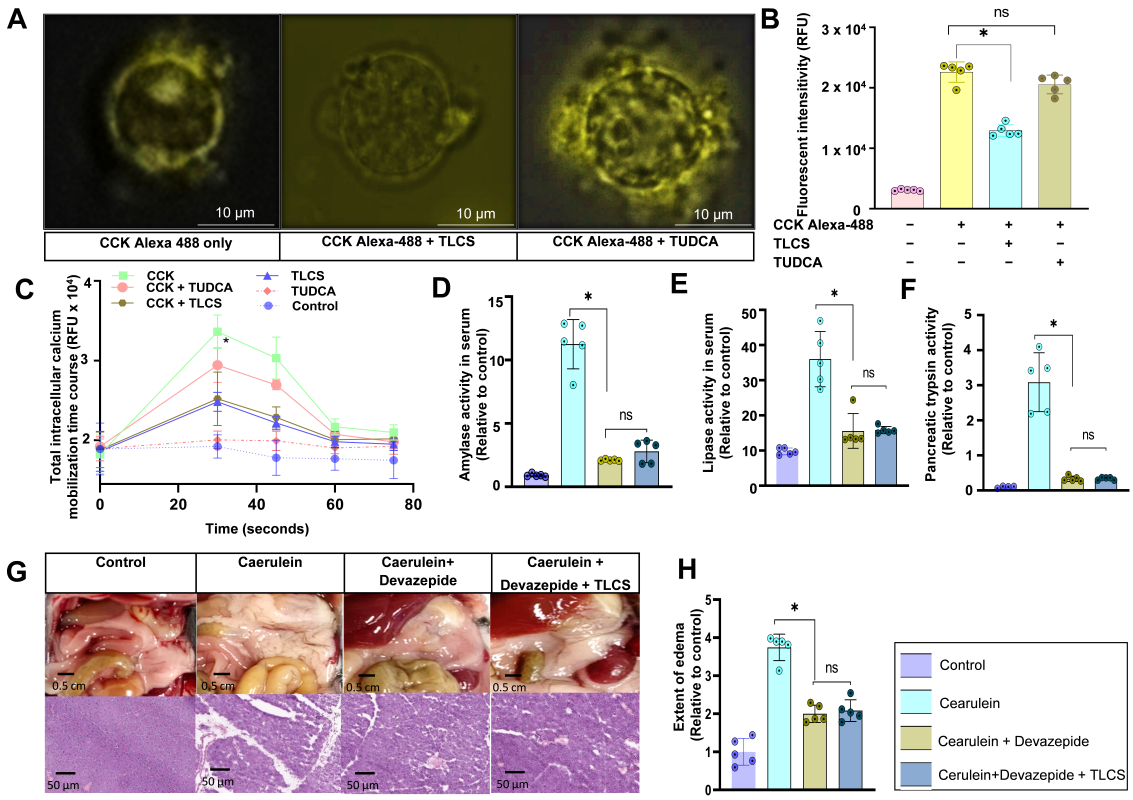


Figure 5. Interaction of BAs with the CCK1 receptor and intracellular calcium release.

(A): Fluoresced-labeled CCK (Alexa-488 CCK, yellow signal) is located on the cells membrane of living acini suggesting binding to the CCK-receptor (left image). This binding was remarkably reduced after co-incubation with hydrophobic TLCS (middle image) but was unchanged when incubated with hydrophilic TUDCA (right image). (B): Quantification of the total fluorescent intensities after incubating acini with Alexa-488 CCK demonstrated a reduction of the fluorescence intensity after adding a hydrophobic BA, but no decrease with a hydrophilic BA. (C): The intracellular calcium mobilization measured at 30, 45, 60, and 75 seconds, which peaked at 30s followed by a quick decrease of the calcium changes. CCK induced quickly an intracellular calcium mobilization in isolated acini, which was clearly reduced by simultaneously adding TLCS to CCK but not significantly different when compared to co-incubating TUDCA and CCK. (D and E): When CCK1R was inhibited by the specific inhibitor devazepide and AP was induced by caerulein, serum amylase and lipase increases were abrogated and addition of TLCS indicated no further inhibitory effect. (F): Pancreatic trypsin activity was also blocked by devazepide. (G and H): Both macroscopic observation and histopathologic examination demonstrated edema and injury of the pancreas, which were almost absent after devazepide treatment and no additional mitigation was observed by TLCS when mice received devazepide previously.

(*Source: Quang Trung Tran, Uwe T. Bornscheuer, Michael Lalk, Markus M. Lerch, and Ali A. Aghdassi et al, International Journal of Molecular Sciences 2022, Vol 23, no. 21: 13592*).

3.1.5. Interaction between BAs and trypsin were independent on their hydrophobicity

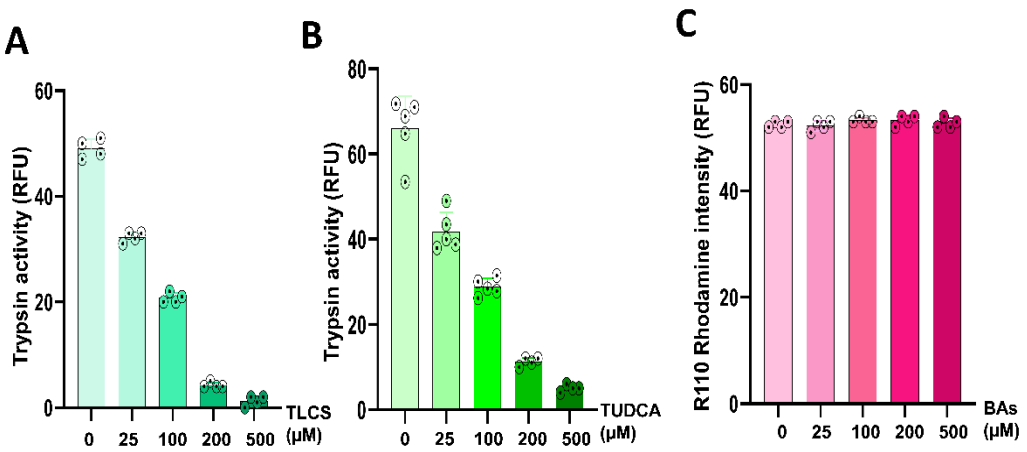


Figure 6. Interaction between BAs and trypsin

Trypsin activity was measured by fluorometer after co-incubating with hydrophobic bile acid TLCS (A) and hydrophilic bile acid TUDCA (B). Both different BAs could similarly reduce the activity of trypsin and this interaction was not involved by fluorophore Rhodamine 110 (C).

When we co-incubated pure trypsin 0.2 μM with TLCS or TUDCA from 25-500 μM, there was possibly chemical interaction between BAs and trypsin, which led to the reduction of trypsin activity, which measured by fluorescent intensity after adding trypsin fluorescent substrate R-110 BZIPAR in concentration dependent manner. This interaction was similar between hydrophobic and hydrophilic BAs (*Figures 6A and B*). To clarify if this fluorescent intensity differences was resulted from an interaction between BAs with trypsin or Rhodamine 110 fluorophore, we have also incubated BAs with Rhodamine 110 alone and the results (*Figure 6C*) showed that there was no impact of BAs on Rhodamine 110 fluorophore. These results indicated that BAs might have chemical effect on trypsin. However, this interaction is independent from hydrophobicity of the compounds.

3.2. Clinical results

As the next stage of the experimental AP about the role of systemic BAs in modulating the disease severity, we have performed the study in AP patients regarding BAs profile and its association with etiology and severity of AP, prevention of AP as the most common complication in ERCP, early prediction of infected necrosis in AP, and the risk factors of the adverse events during endoscopic management of necrotic AP.

3.2.1. Serum BAs profile in patients with AP

Our data from experimental models in mice made us assume that systemic BAs modulate severity of AP. While, on the one hand, hydrophobic BAs aggravate pancreatitis in CCK-unrelated models, they ameliorate severity in CCK-dependent models. Simultaneously, TUDCA, a representative of hydrophilic BAs, seems to have a protective effect in models when CCK is not involved. To elucidate the role of systemic BAs in human AP, we investigated serum

samples from a total of 214 patients with AP no later than 24 hours after hospitalization and from 20 healthy controls. A profile of 20 different types of BAs (*table 1*) was investigated. Mean age was 60 ± 16 (range 26-87) years and 50.4 % were female. Biliary origin was diagnosed in 133 (62.1%) patients and non-biliary pancreatitis (mainly alcoholic AP) in 81 (37.9 %) individuals. In non-biliary AP, profiles of both hydrophobic and hydrophilic BAs did not differ from controls. In biliary AP, significantly elevated levels were detected for hydrophobic BAs but not hydrophilic one (*Figure 7A and B*). The total concentrations of hydrophobic BAs were 33.86 ± 4.51 µM in biliary AP and 8.068 ± 1.98 µM (mean ± SD) in non-biliary AP ($p < 0.0001$). In order to clarify whether etiology of AP can be distinguished by BA profiles we performed receiver operating characteristic (ROC) analysis. The area under the curve (AUC) was only 0.545, with a sensitivity of 50% and specificity of 51% for hydrophilic BAs while for hydrophobic BAs, AUC was 0.67 with sensitivity and specificity of each 62%. Cut-off values were 0.30 µM and 3.78 µM for hydrophilic and hydrophobic BAs, respectively *(Figure 8A, B*). We next investigated whether serum BAs levels might predict disease severity and clinical course of AP. Therefore, APACHE II scores were calculated for each patient at the first and third day of hospitalization and were compared by subtraction of the values (**Δ** APACHE II). An increasing APACHE II score was considered as a deterioration of AP while we defined a stable disease or an amelioration when APACHE II remained constant or decreased, which happened in the majority of cases. The total amount of hydrophilic BAs was not different between the groups of deteriorated (∆ APACHE II >0) and ameliorated or stable AP (∆ APACHE II ≤ 0) (*Figure 7C*). Conversely, total hydrophobic BAs concentrations were significantly higher in the worsened AP (55.8 ± 12.3 µM) group than in those who recovered or at least did not deteriorate (5.9 ± 0.6 µM) (*Figure 7D*). However, ursodeoxycholic acid (UDCA), a hydrophilic BA that is attributed to therapeutic effects in some hepatobiliary disorders, was significantly decreased in severe disease and showed a reduced trend in moderately severe AP (*Figure 7E*). Hydrophilic BAs were only of limited use for the prediction of AP deterioration, as AUC was 0.57 with a sensitivity of 65% and a specificity of 44% at the cut-off 0.7 µM, whereas hydrophobic BAs turned out to have more predictive potential with an AUC of 0.762, a sensitivity of 83.75% and a specificity of 60% at the cut-off value 11.84 µM (*Figure 7E, F*). Additionally, UDCA concentrations of less than 19 nM can be used to distinguish biliary AP from non-biliary AP with an AUC of 0.723, sensitivity 75%, and specificity 65% (*Figure 8C*). For detection of deterioration, UDCA alone with cut-off concentration 18 nM showed a limited value with an AUC of 0.68, sensitivity 76%, and specificity 61% (*Figure 8D*)

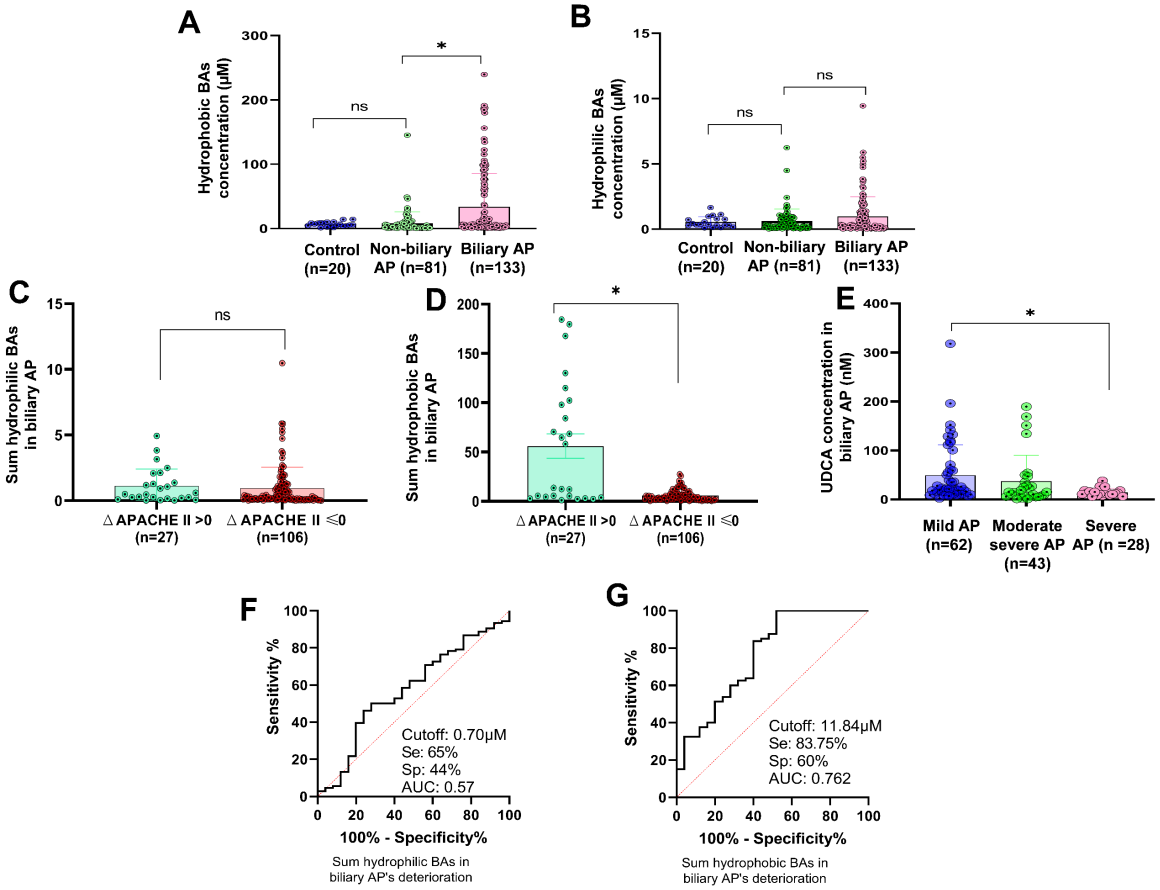


Figure 7. Profiles of BAs in AP patients and their relation to etiology and disease progress

(A and B) The sum of hydrophobic but not hydrophilic BAs were increased in AP of biliary origin compared to other etiologies and controls. (C) When analyzing APACHE II scores from the first to the third day after hospitalization (Δ APACHE II) there was no difference of hydrophilic BAs between patients with ameliorated or stable AP (Δ APACHE II ≤ 0) and deteriorated AP (Δ APACHE II >0). (D) However, this difference was significant for hydrophobic BAs. (E) The ROC to predict potential progress of AP based on hydrophilic BAs had small AUC of 0.57 with low sensitivity (65%) and specificity (44%) even at the cutoff value of 0.7 μM. (F) Total serum hydrophobic BAs predict for deterioration of AP. ROC analysis showed an AUC of 0.762 with sensitivity of 83.75%, and specificity of 60%. Cumulative hydrophobic BAs ≥ 11.84 μM could be considered as the threshold of a potential progression of AP. (G) Serum concentration of UDCA was inversely associated with the severity of AP. Statistically significant differences for more than 3 groups were tested by one-way ANOVA followed by Tukey's multiple comparison test and significance levels of p<0.05 were marked by an asterisk. AUC: area under the Receiver Operating Characteristic (ROC) curve.

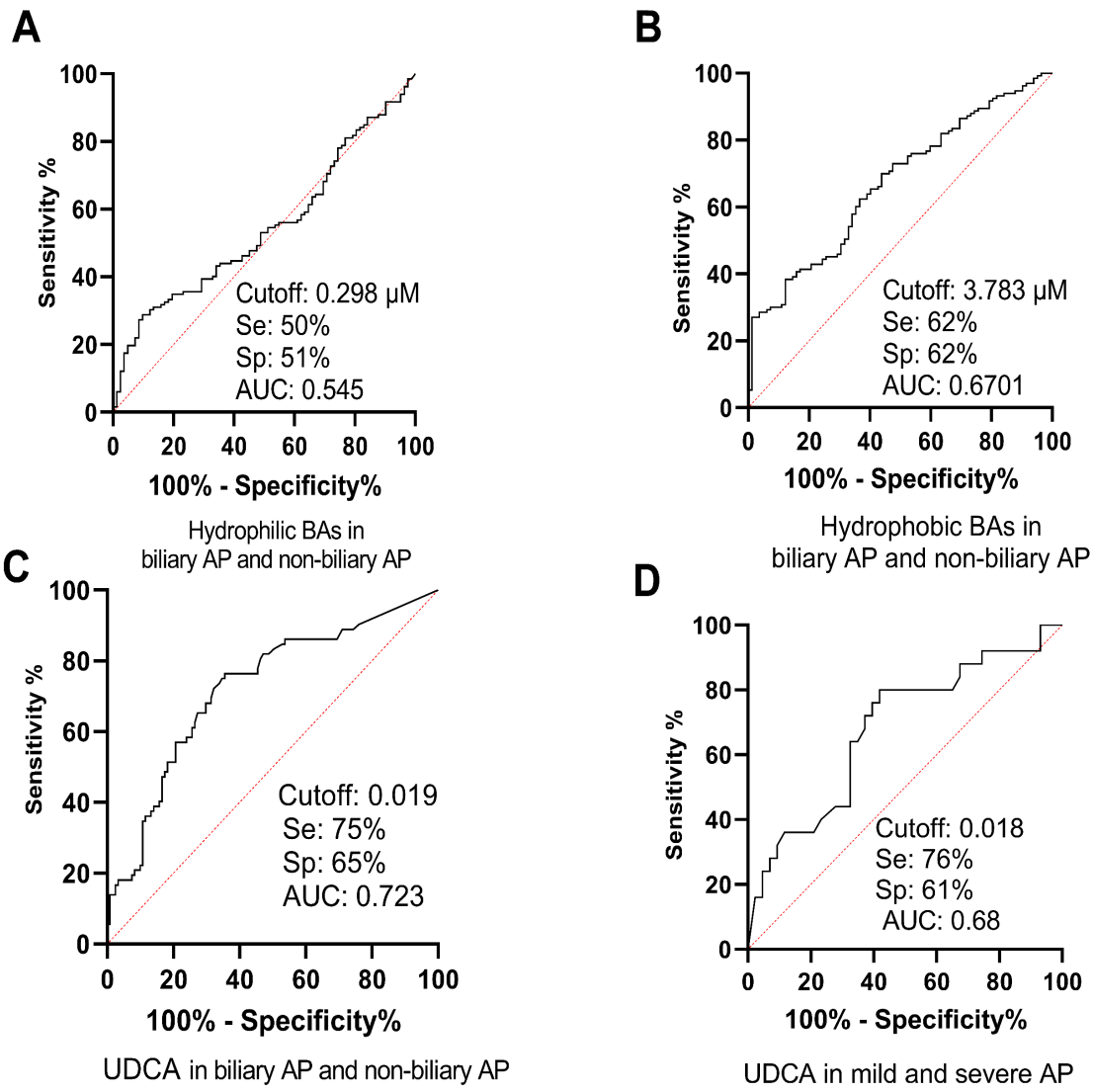


Figure 8. Association between serum BA concentrations with etiology and severity of AP *(A) ROC analysis to differentiate etiologies of AP based on hydrophilic BAs had a small AUC 0.545 as well as low sensitivity 50% and specificity 51% at the selected cutoff. (B) For hydrophobic BAs AUC was 0.67 with sensitivity and specificity of 62% and a cutoff of ≥ 3.78 µM could be used to distinguish biliary and non-biliary etiologies of AP. (C) Analysis of UDCA, a special hydrophilic BA, differentiates biliary from non-biliary AP. (D) For prediction of deterioration of disease differentiation, usefulness of UDCA is more limited.*

From the results about the impact of systemic BAs in modulating the severity of AP, we can summarize that hydrophobic BAs increase severity of pancreatitis in experimental models without involvement of cholecystokinin (CCK) but they mitigate the severity in CCK-dependent models, caused by an interaction with the CCK1 receptor. Hydrophilic BAs play protective roles when CCK does not involve. In patients, the sum of hydrophobic but not hydrophilic BAs correlated with the etiology and progress of AP. These findings from *in vitro* to *in vivo* experiments as well as human data can be captured in the *figure 9* below.

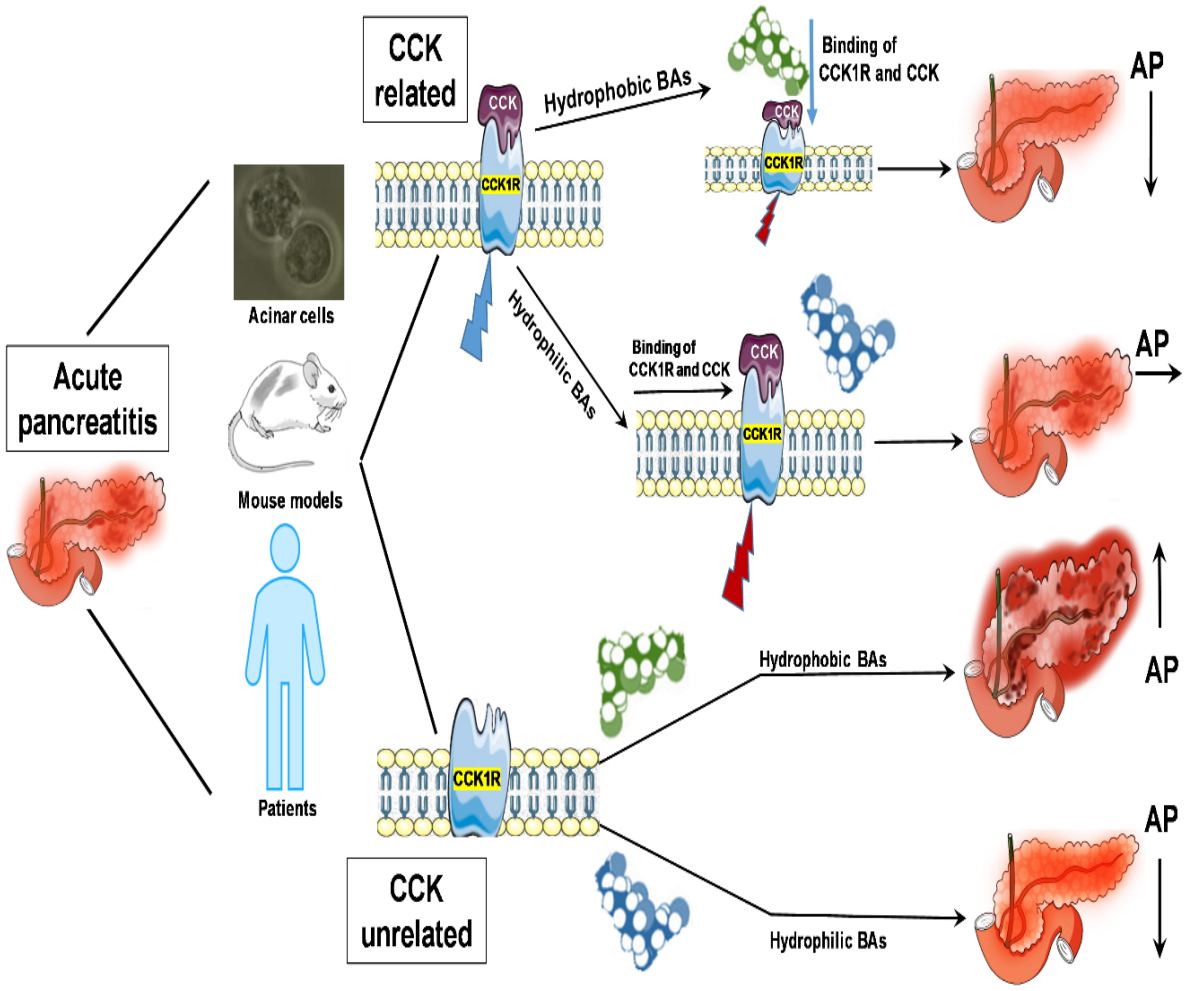


Figure 9. BAs affect AP severity depending on their hydrophobicity and the involvement of CCK at early phase.

(Modified from*: Quang Trung Tran, Uwe T. Bornscheuer, Michael Lalk, Markus M. Lerch, and Ali A. Aghdassi et al, International Journal of Molecular Sciences 2022, Vol 23, no. 21: 13592*).

3.2.2. Results of an RCT in prevention PEP

From the pathogenesis found above in terms of CCK involvement in the early onset of AP, PEP, the most common complication of ERCP, seems to be related to CCK as its concentration increase dramatically post ERCP, one of the most frequent endoscopic procedures related to pancreas [51], [52]. We have recruited in total 432 ERCP patients from six nations for using NAC and indomethacin in preventing PEP. Among various indications (*Table 2*), common bile duct stone was the most common reason for ERCP (66.89%).

Table 2. *Indications for ERCP.*

Indication for ERCP	Number	Percent
Acute cholangitis	16	3.70%
Ampullary Cancer	8	1.85%
Biliary obstruction	58	13.42%
Biliary colic	1	0.23%
Biliary leak	4	0.92%
CBD Dilatation	17	3.93%
CBD Stone	**289**	**66.89%**
CBD Stricture	14	3.24%
Cholangiocarcinoma	8	1.85%
Choledochal cyst	1	0.23%
Icterus	1	0.23%
Malignant obstructive jaundice	2	0.46%
Pancreatic cancer	10	2.31%
PSC	3	0.69%
Total	**432**	100%

(Source: Alavinejad P, Tran NPN, Tran QT, Vignesh S, Lee SH et al, Oral N-Acetyl cysteine versus rectal indomethacin for prevention of post ERCP pancreatitis: a multicenter multinational randomized controlled trial, Arq Gastroenterol, 2022, 59 (4) ahead of print).

We randomly assigned patients to receive either NAC (group A, 84 case), rectal indomethacin (group B, 138 cases), NAC + rectal indomethacin (group C, 115 cases) or placebo (group D, 95 cases). The study algorithm and the results of PEP rate were presented in the *figure 10* below. PEP rate in groups A (NAC), B (indomethacin) and C (NAC + indomethacin) in comparison with D (placebo) were 10.7% (9 cases), 17.4% (24 cases), 7.8% (9 cases) vs 20% (19 cases) (*P*=0.08, 0.614 & 0.01 respectively). The numbers need to treat (NNT) were 11, 38 and 8 for NAC, indomethacin and NAC + indomethacin, respectively. Among PEP cases, 49.18% patients were mild with average 4.5 days (range 1 to 14 days) as duration of hospital stay. There is no severe PEP in groups A and C (*Table* 3). Mean duration of hospital stays after ERCP in groups A, B, C in comparison with D were 3.6, 2.6, 2.8 days vs. 3.7 days (*P*=0.396, 0.010 & 0.012 respectively).

Table 3. Frequencies of PEP in study groups based on severity.

Severity	Group A	Group B	Group C	Group D	Total
Mild PEP	2 (22.2%)	15 (62.5%)	3 (33.3%)	10 (52.6%)	30 (49.18%)
Moderate PEP	7 (77.7%)	6 (25%)	6 (66.6%)	7 (36.8%)	26 (42.62%)
Sever PEP	0 (0%)	3 (12.5%)	0 (0%)	2 (10.5%)	5 (8.19%)
Total	9	24	9	19	61

(Source: Alavinejad P, Tran QT, Lee SH et al, Oral N-Acetyl cysteine versus rectal indomethacin for prevention of post ERCP pancreatitis: a multicenter multinational randomized controlled trial, Arq Gastroenterol 2022, 59 (4), ahead of print)

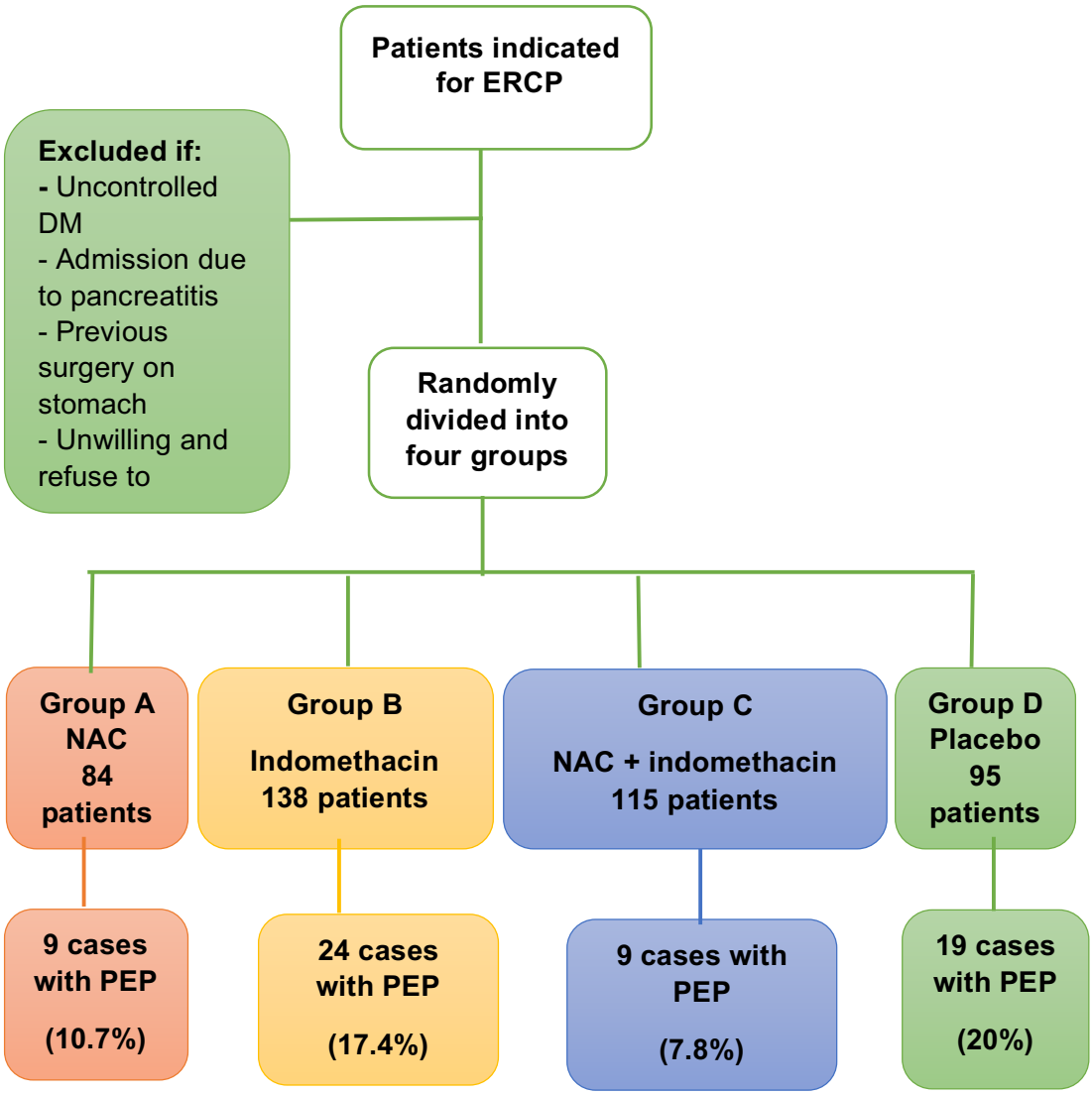


Figure 10. Overview of the RCT design in preventing PEP using NAC with/without indomethacin

(Source: Alavinejad P, Tran NPN, Tran QT, Vignesh S, Lee SH et al, Oral N-Acetyl cysteine versus rectal indomethacin for prevention of post ERCP pancreatitis: a multicenter multinational randomized controlled trial, Arq Gastroenterol 59 (4), 2022 ahead of print)

From the head to head comparison, the efficacy of NAC, rectal indomethacin and combination of NAC + rectal indomethacin for prevention of PEP were 46.5%, 13% and 61% more than placebo respectively; NAC and NAC + rectal indomethacin were 38.5% and 55.2% more effective than rectal indomethacin alone in preventing PEP.

3.2.3. Identification of early predictors for infected necrosis in AP

In the view of severe course of AP, which accompanies (multi)organ failure with high mortality, we next present the new model to identify early infected necrosis in AP.

There were 2,410 AP patients who were admitted to Greifswald University Hospital from 2009 to 2019. The overview of this cohort can be seen as in the flowchart below (*Figure* 11)

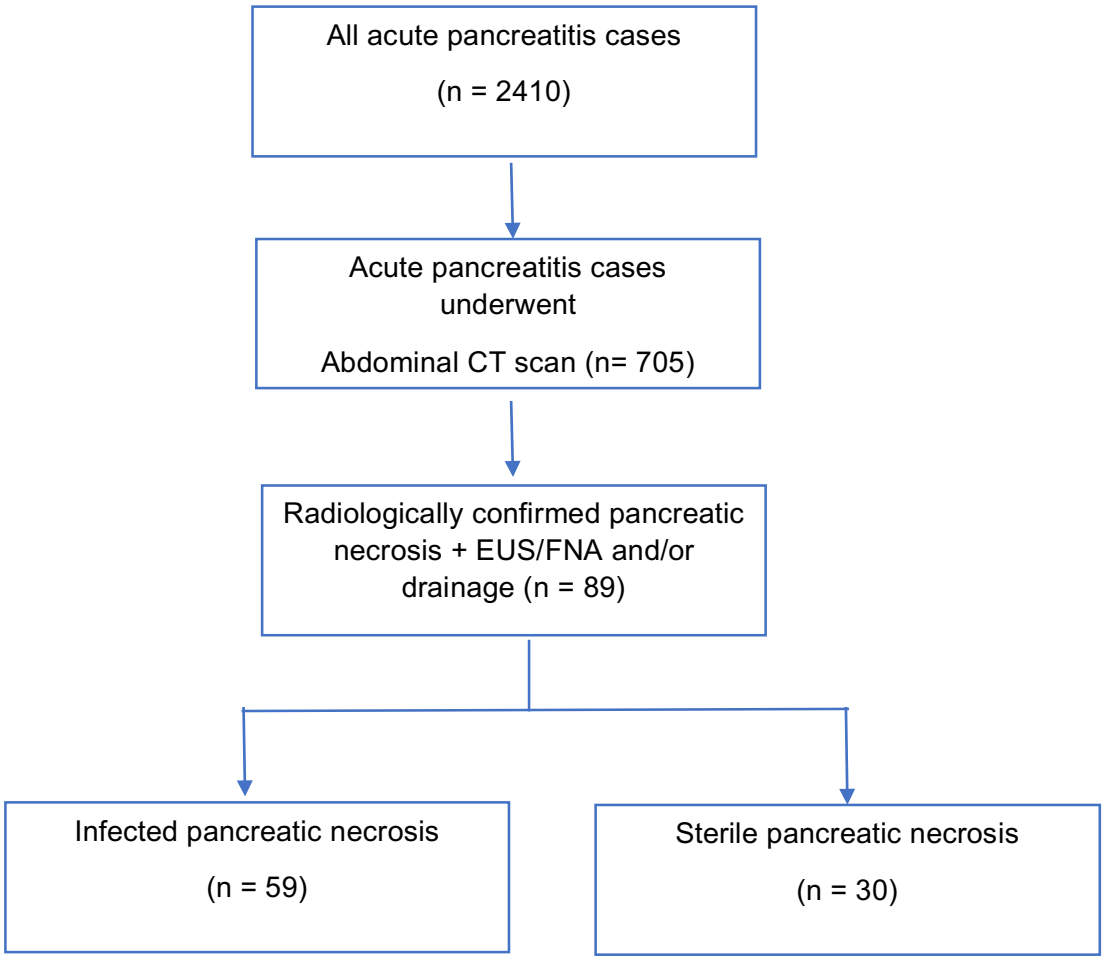


Figure 11. Flowchart describing the patient identification and selection process

(Source: *Wiese, M.L.; Urban, S.; von Rheinbaben, S.; Frost, F.; Sendler, M.; Weiss, F.U.; Bülow, R.; Kromrey, M.L.; Tran, Q.T.; Lerch, M.M.; Ali A. Aghdassi, Identification of early predictors for infected necrosis in acute pancreatitis. BMC Gastroenterology 2022, 22, 405*)

In most of patients (81.4%), infected necrosis was diagnosed by the first intervention. The infected necrosis group has significantly higher APACHE II score at admission than that in sterile necrosis (p=0.001).

Regarding the association of clinical outcome with infected necrosis, renal and respiratory failure were more frequently developed in patients with infected necrosis (p=0.002 and p < 0.001, respectively). Moreover, the rate of patients requiring treatment in intensive care unit (ICU) or intermediate care (IMC) was noticeably higher in those with infected necrosis (p=0.001 and 0.017, respectively). Additionally, median of hospital stay duration was almost twice as long in infected necrosis (54 vs. 28 days, p < 0.001).

In regard of association of infected necrosis with blood laboratory parameters, among 9/20 blood parameters analyzed, which were significantly associated with infected pancreatic necrosis, the strongest associations with infected pancreatic necrosis were found in albumin (OR [95% CI] 0.914 [0.861–0.970], p=0.002); creatinine (OR [95% CI] 1.019 [1.005–1.033], p < 0.001), and CRP (OR [95% CI] 1.009 [1.004–1.014], p < 0.001).

From these observations, we have performed a multivariate analysis to develop a predictive model for an early detection of infected pancreatic necrosis. Besides albumin creatinine, and CRP, alcoholic etiology was also included in the final model as a predictor. It indicated good model fit with Nagelkerke's R2 and Cox & Snell R2 values of 0.502 and 0.360, respectively (*Table 4*).

Table 4. Multivariate logistic regression model for prediction of infected pancreatic necrosis

Predictor	Regression coefficient	Standard error	Wald X^2	p-value	Odds ratio	95%-CI
Creatinine	0.026	0.010	6,478	0.011	1.026	1.006 – 1.047
Albumin	- 0.066	0.045	2.151	0.142	0.936	0.858 – 1.022
Alcoholic etiology	1.759	0.765	5.295	0.021	5.808	1.298 – 25.992
C-reactive protein	0.006	0.003	3.287	0.070	1.006	1.000 – 1.013
Constant	- 1.504	1.579	0.907	0.341	0.222	-

Cox & Snell R^2: 0.360 *Nagelkerke's R^2: 0.502*

(Source: *Wiese, M.L.; Urban, S.; von Rheinbaben, S.; Frost, F.; Sendler, M.; Weiss, F.U.; Bülow, R.; Kromrey, M.L.; Tran, Q.T.; Lerch, M.M.; Ali A. Aghdassi, Identification of early predictors for infected necrosis in acute pancreatitis. BMC Gastroenterology 2022, 22, 405*)

For model performance, we plotted ROC curves to predict the presence of an infected necrosis. *Figure* 12 demonstrates results of ROC analysis. The prediction model achieved greater AUC than each individual parameter creatinine, CRP, or albumin (*Figure* 12A) and also better performance of the APACHE II score alone (*Figure*. 12B). The prediction model reached an AUC of 0.754 when entire patient collective was applied (*Figure* 2C). With a specificity of 0.840 (95%-CI [0.631–0.947]) and a sensitivity of 0.692 (95%-CI [0.547–0.809]), a value the ideal cut-off value of 0.25 was identified.

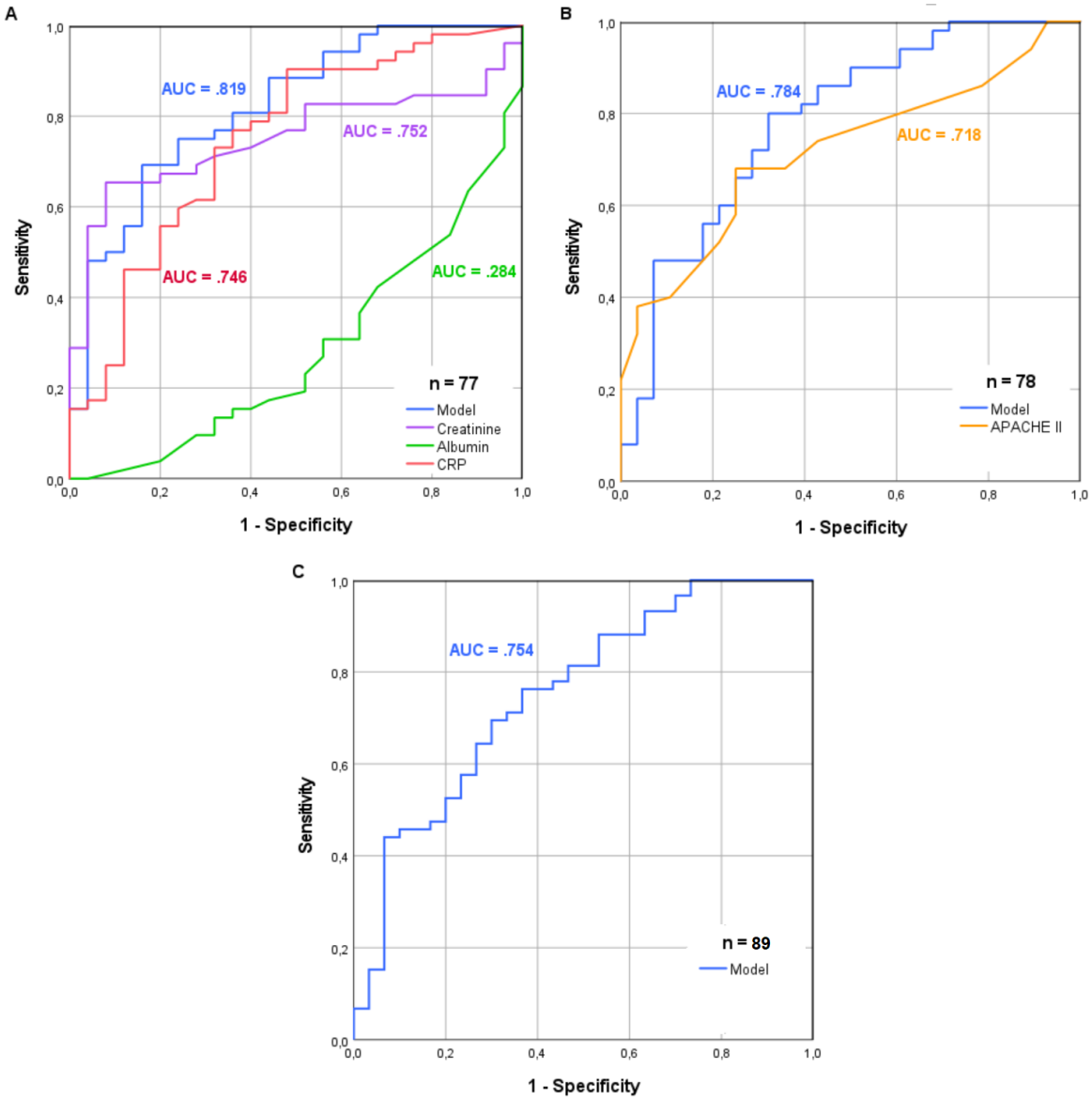


Figure 12. Receiver operating characteristic curves in predicting infected necrosis in AP. *Performance of the predictive model in comparison to A single laboratory parameters, B the APACHE II score, and C applied to the entire patient collective* (source: *Wiese, M.L.; Urban, S.; von Rheinbaben, S.; Frost, F.; Sendler, M.; Weiss, F.U.; Bülow, R.; Kromrey, M.L.; Tran, Q.T.; Lerch, M.M.; Ali A. Aghdassi, Identification of early predictors for infected necrosis in acute pancreatitis. BMC Gastroenterology 2022, 22, 405)*

3.3.4. Infection of pancreatic necrosis after AP increases complication during endoscopic drainage

In the same cohort presented above, we have also analyzed to point out the risk factor of adverse event during endoscopic intervention for necrotic AP. There were 89 patients with pancreatic necrosis who underwent endoscopic drainage therapy. Treatment-associated complications occurred in 52 cases (58.4%, called ‘adverse events group’), whereas in 37 cases, no adverse events occurred (called ‘controls’ group). Majority of these incidents (76.9%) were minor complications, and only 23.1% of cases suffered from major adverse events. The

most common complications were stent dislocation, stent obstruction, and residual lesion after drainage therapy.

In comparison of treatment characteristics between the complications group and controls (*Table* 5), the main findings were: infection of pancreatic necrosis (6.1 [2.3–16.1], OR [95% CI], $p < 0.001$) as indicated by a positive culture, as well as a higher lesion maximum diameter (1.3 [1.1–1.5], OR [95% CI], increment 1 cm, $p < 0.001$) were associated with adverse events during endoscopic drainage therapy (*Figure* 13).

Table 5. Treatment characteristics of patients with complications and controls

	Adverse events group (n=52)	**No complication/ controls (n=37)**	**Missing (%)**	**p-value**
Type of lesion (%)				0.645
WON	96.2	91.9	0	
ANC	3.8	8.1	0	
Location of lesion (%, multiple possible)				
Head	30.8	27.0	0	0.814
Body	59.6	45.9	0	0.281
Tail	50.0	59.5	0	0.398
Lesion maximum diameter (cm)	10.9 (8.4-15.1)	7.6 (6.0-10.0)	0	**<0.001** *
Necrosis culture: positive results (%)	79.6	38.9	4.5	**<0.001** *
Blood culture: positive results (%)	26.3	21.1	36.0	0.754
Antibiotic treatment (%)	100.0	97.3	0	0.416
Highest level of care (%)				0.054
Intensive care unit	48.1	27.0	0	
Intermediate care	26.9	24.3	0	
Regular ward	25.0	48.6	0	
Necessity for repeat interventions (%, multiple possible)	63.5	0	0	**<0.001** *
Endoscopic	44.2	-		
Interventional radiology	28.8	-		
Surgical	9.6	-		
Duration of initial hospital stay (days)	21.0 (11.8-63.0)	14.0 (7.0-31.0)	0	**0.003** *
Duration of endoscopic drainage (days)	65.0 (47.8-103.2)	64.5 (51.2-129.0)	9.5	0.853
Total mortality (%)	15.4	5.4	0	0.185
Therapy-related mortality (%)	1.9	0	0	1.000

Continuous data are given as the median (first–third quartile). Categorical variables are displayed as percentages. ANC: Acute necrotic collection. n: Number of cases. LAMS: Lumen-apposing metal stent. WON: Walled-off necrosis.

Extracted from *Frost, F.; Schlesinger, L.; Wiese, M.L.; Urban, S.; von Rheinbaben, S.; Tran, Q.T.; Budde, C.; Lerch, M.M.; Pickartz, T.; Aghdassi, A.A. Infection of (Peri-)Pancreatic Necrosis Is Associated with Increased Rates of Adverse Events during Endoscopic Drainage: A Retrospective Study. Journal of clinical medicine 2022, 11).*

With subgroup analysis, lesions with large diameter > 10 cm were associated to an OR of 4.6 (1.8–11.9; 95% CI, p = 0.001) for the occurrence of any complications. If we included lesion maximum diameter and positive culture in one model using age, genders, and diabetes mellitus as covariates, a positive necrosis culture (p = 0.002), as well as a maximum diameter (p = 0.001), remained significantly associated with the occurrence of complications during endoscopic drainage. The positive results of bacteria in pancreatic necroses demonstrated the strongest association with complications such as stent obstruction (p = 0.010), residual lesion (p = 0.002), and stent dislocation (p < 0.001) (*Figure* 13A). A largest diameter of the lesion was associated with stent dislocation (p = 0.002), residual lesion (p = 0.004), or delayed bleeding (p = 0.007) (*Figure* 13B). The initial hospital stays of the adverse events group was also longer (p = 0.003). In 63.5% of the cases with adverse events, a repeat intervention was required and was mainly performed by endoscopy procedures. Even though it was not statistically significant, the mortality was higher in the adverse events group in comparison to the controls (15.4% vs. 5.4%).

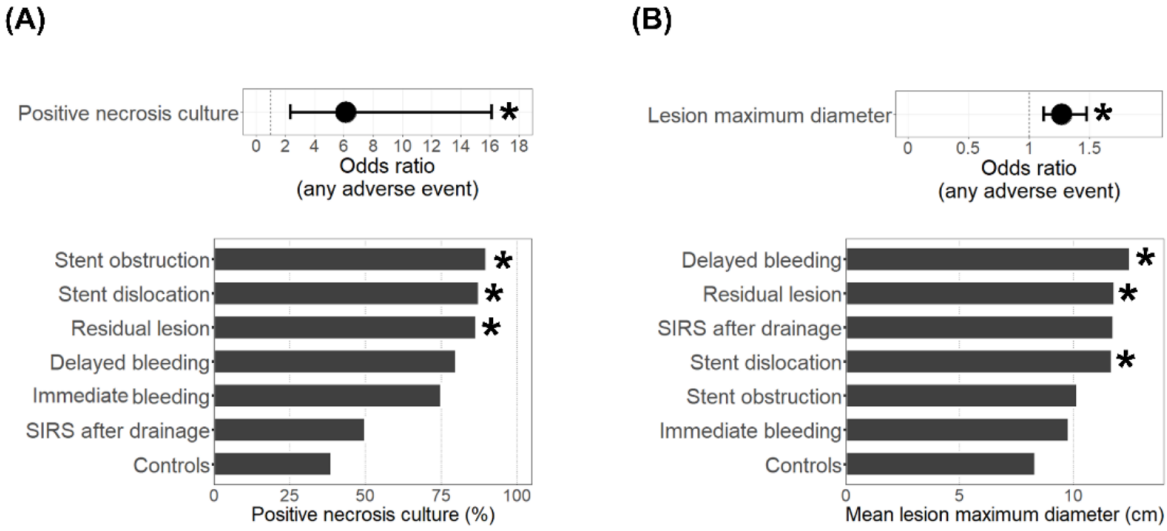


Figure 13. Treatment characteristics of complicated drainages.

*The OR (95% CI) for the occurrence of any adverse event (above panel) and the rates of positive necrosis cultures (**A**) or the mean maximum diameter of the lesions (**B**) in cases with the respective adverse event (lower panel). (Source: Frost, F.; Schlesinger, L.; Wiese, M.L.; Urban, S.; von Rheinbaben, S.; Tran, Q.T.; Budde, C.; Lerch, M.M.; Pickartz, T.; Aghdassi, A.A. Infection of (Peri-)Pancreatic Necrosis Is Associated with Increased Rates of Adverse Events during Endoscopic Drainage: A Retrospective Study. Journal of clinical medicine 2022, 11)*

Furthermore, we also compared culture results of necrosis between controls and complicated cases, which was shown in *figure 14*. All bacterial and fungal species were more predominant in cases with adverse event than that of controls. The difference of *Pseudomonas aeruginosa* was most significant with p = 0.019.

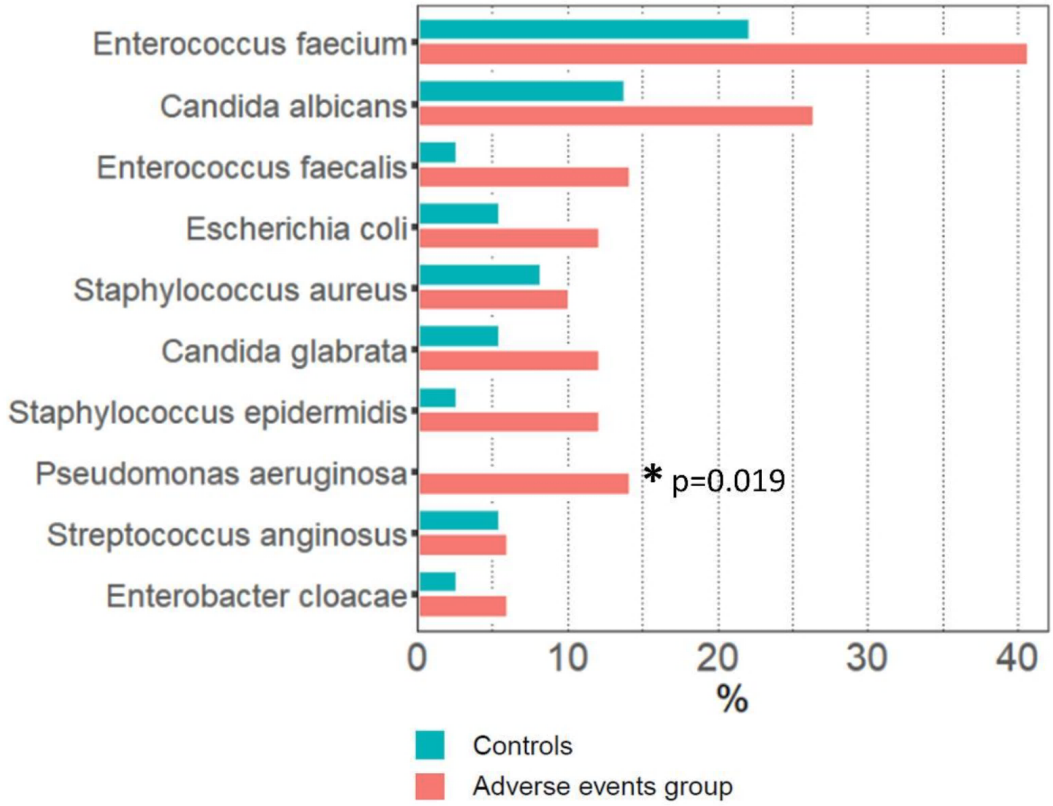


Figure 14. Comparison of necrosis culture results between controls and complicated cases. *Shown are the ten most frequently found taxa. * indicates significant difference.*
(Source: Frost, F.; Schlesinger, L.; Wiese, M.L.; Urban, S.; von Rheinbaben, S.; Tran, Q.T.; Budde, C.; Lerch, M.M.; Pickartz, T.; Aghdassi, A.A. Infection of (Peri-)Pancreatic Necrosis Is Associated with Increased Rates of Adverse Events during Endoscopic Drainage: A Retrospective Study. Journal of clinical medicine 2022, 11).

4. General discussion

Biliary origin is the leading etiology of AP, which has been studied in several researches. However, they have been mostly based on the retrograde infusion of BAs into the main duct of the pancreas [53,54]. However, as shown in humans by endoscopic manometry, BAs hardly reach the pancreas retrogradely [55]. Systematic investigations of BAs, when systemically administered, focusing on their impact in AP and an elucidation of the underlying mechanisms are inadequate. Several cholestatic diseases can lead to an elevation of circulating BA concentrations [56] but their potential impact on AP has not been sufficiently examined so far. The presented MD/PhD project demonstrates that systemic BAs modulate severity of AP depending on the hydrophobicity of BAs and its pathogenesis. While hydrophobic BAs enhance the severity of AP under disease conditions that are unrelated to CCK, these compounds mitigate CCK-induced injury of the pancreas via an interaction with the CCK1 receptor on acinar cells.

First, we confirmed previous studies [57,58], which showed that hydrophobic BAs such as TLCS can induce significant activation of pancreatic enzymes in isolated living acini. Following pre-incubation with BAs, hydrophilic agents did not show any impact while hydrophobic BAs reverted intracellular zymogen and lysosomal protease activation, which induced by CCK application. The difference in impact of these two types of BAs may come from their interaction with either chemical compounds (CCK or protease) or CCK1 receptor. The former possibility was excluded as the results were the same when we incubated fluorescent labeled CCK or pure trypsin with either hydrophobic or hydrophilic BAs. These BA-dependent differences were therefore related to their ability in interacting with CCK1 receptor, which is abundantly expressed on acinar cells and has the highest affinity to the octapeptide CCK [59,60]. The binding between CCK1R and its ligand was markedly reduced in the presence of hydrophobic compared to hydrophilic BAs, as we could see under fluorescence microscopy by using fluoresced labeled Alexa-488 CCK. We further assessed intracellular calcium release as activation of G-protein coupled receptors led to a release of intracellular Ca^{2+}, a crucial regular of pancreatic acinar cell secretion [61,62]. The results confirmed a reduction of Ca^{2+} mobilization, which was less pronounced after incubation with hydrophobic TLCS but remained steady when TUDCA was used.

Our BA concentrations were similar as others have used in previous studies [57,58] and isolated acini did not show signs of cellular necrosis upon TLCS stimulation. Therefore, we assume that BAs' hydrophobicity related reduction of intracellular protease activation in CCK-stimulated acini is not a consequence of cytotoxicity and primarily based on alterations of intracellular signaling.

In Chinese hamster ovary (CHO) cell lines expressing CCK1R, Desai and co-workers have studied the impact of BAs on CCK1R function. They demonstrated an inhibitory effect by hydrophobic taurochenodeoxycholic (TCDC) acid but not by hydrophilic tauroursodeoxycholic acid (TUDCA), and proposed a direct interaction of BAs with the CCK1 receptor, likely at the same site where cholesterol binds leading to a conformational change in the helical bundle domain [63]. The intracellular calcium responses were also delayed by TCDC, but not TUDCA, following CCK stimulation and BA exposure, which were similar to our observations.

Translation of *in vitro* results into *in vivo* models that are based on CCK or its analogues confirmed our observations of mitigating effects by hydrophobic BAs. In AP induced by either caerulein or BPDL, which caused an elevation of CCK, hydrophobic BAs but not hydrophilic TUDCA could attenuate severity of pancreatitis. It has been found that following a lack of BAs in the duodenum as seen after obstruction of the distal bile duct triggers a feedback regulation to release more endogenous CCK [64]. Our findings are similar with this observation as CCK strongly increased in mice that underwent BPDL in comparison to sham operated or PDL mice, where CCK was not elevated. These results further confirm our observations from *ex vivo*

experiments and propose a modulating effect of BAs on AP severity *in vivo* depending on their hydrophobicity and the involvement of CCK in the early phase of pancreatitis.

A variety of both receptor and transporter mediated cellular signaling mechanisms contribute to pancreatic damage depending on the location where BAs reach acinar cells. In rat pancreatic acinar cells, Kim et al. showed evidence for the expression of the BA transporter on both the luminal and basolateral site, which are capable of mediating BA influx into acinar cells [65]. BAs may therefore enter acinar cells not only via the apical pole by reflux but also on the basolateral site via interstitial leakage or systemic application. This route of internalization might explain why TLCS enhances pancreatic damage in CCK independent AP. We additionally observed a protective role of hydrophilic TUDCA in these models. The underlying mechanisms require further investigations but might include: alterations of the gut microbiome, mitochondrial damage, reduction of endoplasmic reticulum stress in acinar cells as reported in previous studies [66,67]. Whether treatment with hydrophilic BAs may eventually be helpful in attenuating AP in humans when the etiology and mechanisms are unrelated to CCK would be further useful investigations.

In patients, plasma CCK concentrations seemed to be decreased in both biliary and alcoholic AP after the first day of pancreatitis [68] but quickly increase within hours after ERCP and may predict the development of AP [51]. In other words, AP in humans, apart from post-ERCP pancreatitis (PEP), seems belong to CCK-independent type. Normal serum bilirubin, which is often accompanied by non-elevated circulating BAs, independently showed a two times higher risk of PEP without elucidated mechanism [69,70]. In combination with the fact that PEP seems to be related with CCK, our results, in which hydrophobic BAs may interfere the binding of CCK1R, might be used to explain why normal level of circulating bilirubin correlates to higher risk of PEP. However, the further examinations need to be done regarding this possible cause-consequence association.

Measurements of total BA concentrations in serum could be helpful to differentiate the etiologies of AP at an early phase [71] and to predict the disease outcome [72]. Due to the dynamics of changes in concentrations, the time point of analysis is important so that we focused on a narrow time window in the early phase of AP. So far, the existing reports only concentrated on total BA measurements and did not consider their hydrophilic potential. In our cohort, we investigated serum levels of 20 different types of BAs and found significantly higher concentrations of hydrophobic BAs in biliary pancreatitis compared to other etiologies. Secondly, their levels were elevated in those patients who deteriorated, as assessed by an increasing APACHE II score. Our findings suggest that this group of BAs seems to serve not only as a predictor for biliary pancreatitis but also, worthy of mention, for the disease course at an early stage. They support our results from murine studies and suggest that hydrophobic and hydrophilic BAs not only differ in their chemical properties but also in their biological

behavior. Beneficial effects of the hydrophilic BA ursodeoxycholate and its taurine conjugate tauroursodeoxycholate (TUDC) were observed for other gastroenterological diseases such as cholestatic liver disorders including primary biliary cholangitis and primary sclerosing cholangitis. They are based on a prevention of apoptosis and an insertion of intracellularly stored bile salt export pumps and multidrug resistance protein 2 (Mrp2) into the canalicular membrane of hepatocytes that trigger cholestasis [73]. Serum levels of UDCA, a particular type of hydrophilic BAs, inversely associated with the severity of AP in our patients and may suggest a protective role.

In the view of prevention PEP, which was shown to be related to CCK [51] [74] , several agents have been proposed for the pharmacologic prophylaxis, mostly directed toward amelioration of the inflammatory cascade that potentiates pancreatitis [75], [76]. Among them, NAC given orally, with its anti-oxidant and anti-inflammatory properties [30], showed a promising result in preventing PEP [31]. However, that was a pilot and singe center study. We therefore designed the current study as multicenter multinational RCT to evaluate not only efficacy of oral NAC but also to compare its usefulness with rectal indomethacin as one of the most widely used medications for preventing PEP [77,78]. Based on our findings, the combination of oral NAC and rectal indomethacin significantly reduced the rate of PEP and even NAC per se was able to decrease PEP although it was statistically not significant (P=0.08). The numbers need to treat of NAC plus rectal indomethacin and NAC were 8 and 11 respectively. Moreover, the mean hospital stays after ERCP among those who were treated with NAC + rectal indomethacin was one day shorter (2.8 days vs 3.7 days), indicated that this combination was time and cost effective. The probable mechanism proposed by a study from Sweden is that NAC could suppress concentration of NF-kB in AP [79]. The amelioration effect on NF-κB activity, which may induce inflammatory cascade of AP, by NAC treatment has also been confirmed by another study [80]. These observations indicated that NAC and specially its combination with rectal indomethacin can be effective and practical option in preventing PEP. Regardless the potential of side effects, indomethacin alone did not show clear effect in our study and seem to be only useful in patients with high risk of PEP but not those with average risk according to a systemic review by Inamdar et al [78]. Further well-designed meta-analysis [81] and RCT by Levenick et al [82] also declined the usefulness of single rectal indomethacin in PEP prevention.

Regarding the complications of biliary AP, infected necrosis is one of the most severe and common events. In the next related clinical study, we identified parameters associated with infection of necrosis in AP by developing a logistic regression model based on concentration of serum of albumin, creatinine, and CRP, as well as alcoholic cause that predicts infection with higher accuracy than the APACHE II score or any individual laboratory parameter.

For prediction of adverse outcome in AP, a number of multiparameter predictors have been evaluated. The APACHE II score is one of the most widely used for patients in ICU, which incorporates both chronic comorbidity categories and markers of patient physiology recorded immediately or shortly after hospital admission. There was an association between infected pancreatic necrosis and APACHE II score. However, the APACHE II score is not specific for AP and requires multiple items that are limitedly feasible in daily clinical practice [33,83].

In a previous study [84], a similar approach to develop a prediction model for infection of pancreatic necrosis was used. This model considered different parameters in compared to ours. However, our results do not necessarily contradict with these findings since etiologies of AP differed between two cohorts and the studies were conducted in two different countries. Findings in Western cohorts might be not transferred unrestrainedly to Asian populations and vice versa [85]. Additionally, we examined a wider range of clinical and laboratory parameters and included, for instance, albumin, which was an independent predictor of infected necrosis. We must also consider parameters that have been suggested as predictors of infected necrosis before but did not contribute to prediction in the current model. For example, higher procalcitonin (PCT) concentration was associated with infected necrosis and a severe course of AP resulting in high mortality [86]. However, mortality was as low as 6.7% in our cohort, which could explain why we did not find PCT as a convinced predictor. Moreover, earlier results indicated that PCT is not a specific marker of infected necrosis [87].

Among the patients in above cohort, we analyzed characteristics of cases with adverse events during endoscopic transluminal drainage therapy of pancreatic necrosis when compared to controls. The most prominent factor associated with complications during endoscopic drainage therapy was positive necrosis cultures. Our results showed that the microorganisms identified in pancreatic necroses belonged largely to the gut flora (e.g., *Enterococcus faecium, Candida albicans, or Escherichia (E.) coli*) and were similar to those previous reports [88], [89]. Obviously, the presence of bacteria or fungi has a negative impact on the outcome of the endoscopic drainage procedures. Patients with stent dislocation, stent obstruction or residual lesions after intervention showed remarkably higher rates of positive microbiological culture. Patients with bleeding showed higher incidence of pancreatic necrosis infections than that of controls (77.8% vs. 38.9%). Higher percentage of stent obstruction could be explained by microbial overgrowth of the stent surface by agglutinative bacteria and microbial biofilm development [89]. Several of both negative or positive Gram-stained bacteria or yeast such as *C. albicans* possess the capability for agglutination and biofilm formation [90], [91]. The development of biofilm may affect drainage of pancreatic necrosis in the cavity itself, leading to higher risk of residual lesions. On the other hand, stent dislocation, could be the consequence of microbe-induced inflammation, impairing wound healing and loosening the stent fixation. *Escherichia coli*-derived cytotoxic necrotizing factor type 1 impaired intestinal epithelial wound repair after an experimental mechanical trauma in an *in vitro* model [92]. The

lipopolysaccharide (LPS), which is the major endotoxin component of the outer membrane of Gram-negative bacteria, leads to impaired blood flow and a proinflammatory immune response, which resulted in insufficient healing of gastric ulcers [93]. Other microorganisms that are frequently found in necrosis isolates such as *Staphylococcus aureus, Enterococcus faecium,* and *Pseudomonas aeruginosa* have also been shown to secrete compounds that interfere with epithelial cell migration or the host's immune response, which may impair healing of the wound [94,95]. Cases with pancreatic infected necrosis may increase inflammation in consecutively impaired wound healing and enhanced erosion of blood vessels leading to higher rates of delayed bleeding events.

In addition to infection of pancreatic necrosis, larger pancreatic necrosis diameter was also associated with adverse events during endoscopic drainage therapy. This increased technical difficulty in stent placement due to the anatomical position and reflected the consequence of more severe states of AP. The choice of LAMS or plastic stents could be made according to the amount of necrotic debris in the target lesion, with LAMS used only for lesions with a large proportion of solid components. This choice in the drainage of pancreatic necrosis seems does not play clear role in discrimination the outcome as well as the adverse events. In the present study, residual lesions were observed in 22.9% and 10.9% of cases when plastic stents or LAMS were used, respectively. However, this difference was not significant. This is further supported by a recent meta-analysis and RCT, which found no difference in the occurrence of adverse events between LAMSs and plastic stents in the treatment of WON [96], [97]. Noteworthy, we need to consider infection as a possible confounding factor when compare different methods of endoscopic therapy for pancreatic necrosis.

Limitations

There are limitations to this work. In the experimental design, we have calculated the number of animals based on a 'resource equation' approach [98,99] with a relatively low number of mice in some control groups. However, there were at least five mice in all groups with AP. In BAs injection, different application routes were used, as TLCS was injected i.v. while TUDCA and LCA were administered i.p due to limit of injectable volume. Nevertheless, the TBA concentrations in pancreas homogenates increased similarly and a previous study [100] reported comparable effects on hepatic CYP-linked mono-oxygenase activities following i.v. or i.p. administration, which might suggest that the application route is less important. Regarding BAs spectrum, we mainly concentrated on some selected BAs in this study, which might not be fully representative for all hydrophobic and hydrophilic compounds.

In the RCT, some centers were unable to fulfill pertained number of cases because of social restrictions and decrease in number of procedures during COVID-19 pandemic [101]. Moreover, the mechanism of action of NAC has not been clearly understood. Additionally, the

potentially accompanied bias were the heterogenicity of racism, accessories, devices and endoscopists among different sites of the study.

The patients' data used in the first, third and fourth papers were collected retrospectively from a single center. There were incomplete patient data and laboratory values and the parameters were assessed only at time of admission for the predictive model. It could be that we therefore missed relevant parameters, especially those that dynamically change during the course of AP. However, the results reflect the real-world situation in clinical practice. It would be complicated and expensive to monitor the course of multiple, potentially not routine parameters at multiple timepoints. Thus, our prediction model is likely more practical. Another issue of our results is that patients transferred from external hospitals were also included. This means the treatment of AP at least during the early phase was not uniform. Moreover, time between actual onset of pain and hospital admission could vary leading to an inhomogeneous patient cohort regarding stage of AP. Noticeably, although only individuals with microbiologically proven infection were included in our study, there is a considerable risk of false positive (15%) or negative results (25%) even after microbiologic analysis [102]. Additionally, some patients may have responded to prophylactic antibiotic treatment that was given empirically without prior microbial confirmation and therefore did not develop infected necrosis. Nevertheless, the chance that predictive performance of our model was hampered by such treatment response is rather low as an infected necrosis was detected in almost 90% of patients receiving antibiotics. Moreover, we have potentially missed patients with infected pancreatic necrosis for the model who neither underwent EUS-FNA nor endoscopic drainage. Remarkably, the culture results to determine infection of pancreatic necrosis might not be reliably detected due to diverse microbial communities and anaerobic bacteria, while the next-generation sequencing techniques are still not performing routinely.

Conclusion and outlook

Systemic BAs can modulate the severity of AP, which is highly dependent on the biochemical properties of BAs such as their hydrophobicity and the pathogenesis of AP that related to the involvement of CCK. Interference of hydrophobic BAs on the binding of CCK1 receptor emerged to be a central mechanism.

The concrete pathophysiology at molecular level, especially in CCK-dependent pathways, and the role of BAs given systemically in human AP, in particular for prevention of PEP, need to be investigated in further studies. In prevention of PEP, NAC is effective when compared to placebo. The combination of NAC and indomethacin showed the lowest rate of this adverse event and could be cost effective in ERCP by reducing the average length of hospital stays. However, the better design of the RCT and further investigation about the impact of NAC should be carried out.

The prediction model for identification of infected necrosis in AP including albumin, creatinine, CRP and etiology may help to orientate the treatment strategy for pancreatitis in early phase. The trials in future will be required to validate this model prospectively.

Infection of pancreatic necrosis is the most significant factor associated with complication during endoscopic transluminal drainage therapy, which indicates a need to optimize stents for deployment in areas of infection, e.g., by using antimicrobial coatings, in addition to optimizing diagnosis and management of the infection.

Furthermore, inhibition of CCK or CCK1R and a rebalance of BA profiles may imply a potential for prevention and treatment of AP.

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Appendix of 5 publications contributed to the dissertation and own contribution to each paper

Paper 1 PubMed ID: https://pubmed.ncbi.nlm.nih.gov/36362379/

Tran, Q.T.; Sendler, M.; Wiese, M.L.; Doller, J.; Zierke, L.; Gischke, M.; Glaubitz, J.; Tran, V.H.; Lalk, M.; Bornscheuer, U.T.; Weiss F.U; Lerch, M.M and Aghdassi A.A. Systemic Bile Acids Affect the Severity of Acute Pancreatitis in Mice Depending on Their Hydrophobicity and the Disease Pathogenesis. International journal of molecular sciences 2022, 23, 13592. doi: 10.3390/ijms232113592.

Own contribution: first author, experiments performance, data curation, conceptualization, formal analysis, investigation, methodology, software, original manuscript drafting and editing.

Paper 2 PubMed ID: https://pubmed.ncbi.nlm.nih.gov/36383882/

Alavinejad P, T.N., Eslami O, Shaarawy OE, Hormati A, Seiedian SS, Parsi A, Ahmed MH, Behl NS, Abravesh AA, **Tran QT**; Vignesh S, S.S., Sakr N, Ara TF, Hajiani E, Hashemi SJ, Patai AV, Butt AS, Lee SH. Oral *N*-Acetyl cysteine versus rectal indomethacin for prevention of post ERCP pancreatitis: a multicenter multinational randomized controlled trial. Arquivos de Gastroenterologia 2022, 59, Ahead of print, doi:https://doi.org/10.1590/S0004-2803.202204000-90.

Own contribution: co-author, coordinating, software, visualization, writing: review and editing.

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Own contribution: co-author, data collection, writing: review and editing

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Systemic Bile Acids Affect the Severity of Acute Pancreatitis in Mice Depending on Their Hydrophobicity and the Disease Pathogenesis

Quang Trung Tran [1,2], Matthias Sendler [1], Mats L. Wiese [1], Julia Doller [1], Lukas Zierke [1], Marcel Gischke [1], Juliane Glaubitz [1], Van Huy Tran [2], Michael Lalk [3], Uwe T. Bornscheuer [3], Frank Ulrich Weiss [1], Markus M. Lerch [1,4] and Ali A. Aghdassi [1,*]

[1] Department of Internal Medicine A, University Medicine Greifswald, Ferdinand-Sauerbruch-Straße, 17475 Greifswald, Germany
[2] Department of Internal Medicine, University of Medicine and Pharmacy, Hue University, Hue City 530000, Vietnam
[3] Institute of Biochemistry, University Greifswald, 17489 Greifswald, Germany
[4] Ludwig Maximilian University Hospital, Ludwig Maximilian University of Munich, 81377 Munich, Germany
* Correspondence: ali.aghdassi@med.uni-greifswald.de

Abstract: Acute pancreatitis (AP) is a major, globally increasing gastrointestinal disease and a biliary origin is the most common cause. However, the effects of bile acids (BAs), given systemically, on the pancreas and on disease severity remains elusive. In this study, we have investigated the roles of different circulating BAs in animal models for AP to elucidate their impact on disease severity and the underlying pathomechanisms. BAs were incubated on isolated acini and AP was induced through repetitive injections of caerulein or L-arginine; pancreatic duct ligation (PDL); or combined biliopancreatic duct ligation (BPDL). Disease severity was assessed using biochemical and histological parameters. Serum cholecystokinin (CCK) concentrations were determined via enzyme immunoassay. The binding of the CCK1 receptor was measured using fluorescence-labeled CCK. In isolated acini, hydrophobic BAs mitigated the damaging effects of CCK. The same BAs further enhanced pancreatitis in L-arginine- and PDL-based pancreatitis, whereas they ameliorated pancreatic damage in the caerulein and BPDL models. Mechanistically, the binding affinity of the CCK1 receptor was significantly reduced by hydrophobic BAs. The hydrophobicity of BAs and the involvement of CCK seem to be relevant in the course of AP. Systemic BAs may affect the severity of AP by interfering with the CCK1 receptor.

Keywords: acute pancreatitis; bile acids; CCK1R binding; hydrophobicity

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1. Introduction

Acute pancreatitis (AP) is one of the most frequent non-malignant gastroenterological disorders requiring hospitalization. In recent decades, its incidence has increased steadily worldwide, with an annual aggregate cost of more than USD 2.63 billion in the United States [1,2]. Approximately one fifth of AP patients suffer from local or systemic complications and (multi-)organ failure, with a mortality rate of up to 40% in the most severe cohort [3]. Despite intensive efforts, no causal treatment is available for this condition, and therefore, management is solely based on symptomatic and supportive therapy or dealing with its complications [4]. AP is considered to originally occur in the pancreatic acinar cells, which are vulnerable to extracellular pathological stimuli [5]. In these exocrine units, digestive proteases, beginning with trypsin [6], are prematurely activated after co-localization with the lysosomal hydrolase cathepsin B. Imbalances between the activation and degradation of digestive enzymes may result in injury of the acini [7]. Among various etiologies, migrating gallstones are one of the most common causes of AP, which accounts for 30–50% of all cases [8].

Bile acids (BAs), the largest component of bile juice, have recently gained attention for their pathological role in both rodents and humans [9]. Starting from cholesterol, primary BAs are synthesized and subsequently conjugated in the liver with glycine or taurine [10]. Two major primary BAs are cholic acid and chenodeoxycholic acid. After secretion into the duodenum, they are metabolized to the secondary BAs, lithocholic acid and deoxycholic acid, by the gut microbiome. BAs in the intestine are reabsorbed, mainly in the distal ileum, and finally, return to the liver via enterohepatic circulation for recycling. In rodents, taurine-conjugated forms such as taurocholic acid and taurodeoxycholic acid are predominant [11–13]. BAs can also be classified according to their chemical properties. Depending on the hydroxylation, BAs are differentiated by their hydrophobicity index with different biological effects. There are, in total, approximately 20 different BAs, including primary compounds and their taurine- as well as glycine-conjugated forms, with hydrophobicity indices from −0.84 to +1.23. The more hydrophobic BAs are present, the higher the hydrophobicity indices, and vice versa. Among them, lithocholic acid (LCA) is one of the most hydrophobic agents [14,15]. Noticeably, taurolithocholic acid 3-sulfate (TLCS) is a strongly hydrophobic compound and was previously used in other studies on AP [16–18]. Tauroursodeoxycholic acid (TUDCA) is a typical hydrophilic BA and was approved for the treatment of some biliary disorders such as primary sclerosis cholangitis. Moreover, an emerging role of TUDCA is discussed for many other diseases, including AP [19–21], which makes this BA an attractive candidate for further investigations.

In addition to their ability to emulsify lipids, BAs show more and more clear effects in regulating many pathological processes [22]. The pathogenetic effects of some BAs have been reported for several diseases such as liver, biliary and metabolic disorders [9,23]. However, in pancreatitis, knowledge of the role of BAs is limited and mainly based on retrograde ductal infusion models, which remain controversial as the extent of the intrapancreatic duct pressure and characteristics of infused agents also seem to contribute to acute inflammation [24]. Although it has been shown that AP is highly prevalent among hepatobiliary disorders [25], data on susceptibility to the development and modulation of pancreatitis in cholestatic disorders that are accompanied by increased serum levels of BAs are insufficient. In particular, the mechanisms through which BAs affect acinar cells, when given systemically, have not been elucidated.

This study aims to investigate the impact of various BAs using in vitro-to-in vivo models and addresses the underlying mechanisms through which BAs modulate the severity of AP.

2. Results

2.1. Impact of Hydrophobic and Hydrophilic BAs in Mouse Isolated Acini

We first investigated protease activation on the cellular level in isolated acini upon exposure to BAs with different levels of hydrophobicity. We initially performed in vitro experiments with lower concentrations (50, 100 and 200 µM) of BAs but no clear changes in intracellular protease activation were observed. The intracellular activities of trypsin and cathepsin B (CTSB), a known trypsinogen activator, were significantly higher when treated with 500 µM TLCS or LCA in comparison to the control cells. Interestingly, these hydrophobic BAs did not further enhance the effect of CCK in isolated living acini, and co-incubation of them with supramaximal CCK (1 µM) reduced intracellular protease activation compared to CCK alone (Figure 1A,B,D,E). The stimulation of acini with submaximal CCK concentrations led to peak amylase secretion at a concentration of 100 pM. The addition of the hydrophobic bile acids TLCS and LCA attenuated the amylase secretion, showing a similar effect as under conditions of supramaximal CCK (Figure 1C,F). Conversely, the hydrophilic bile acid TUDCA did not alter intracellular protease activity in acini either in unstimulated conditions or after supramaximal doses of CCK (Figure 1G,H). In parallel, exposure to physiologic CCK concentrations did not modify amylase secretion (Figure 1I).

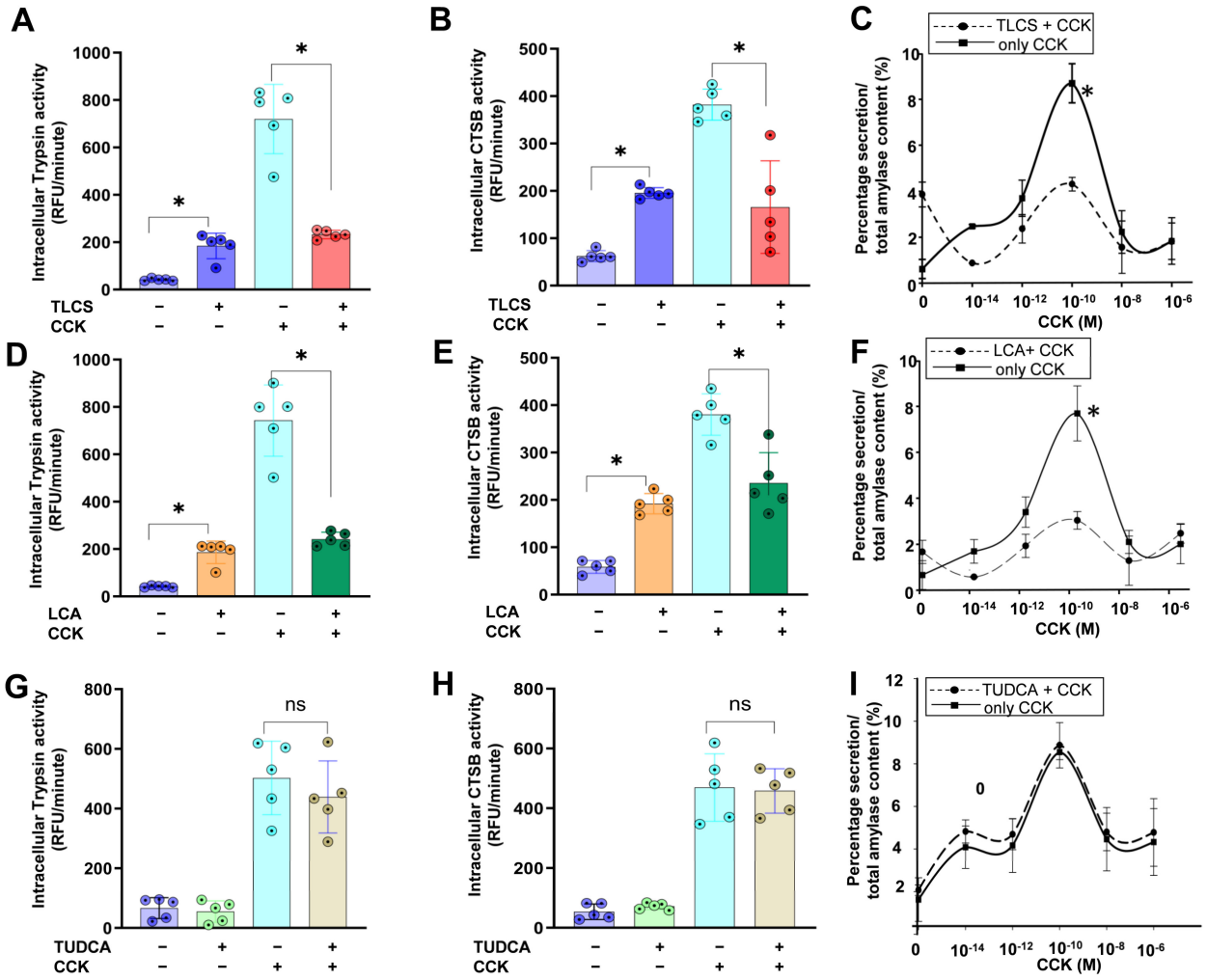

Figure 1. Impact of BAs in CCK-stimulated acini. (**A**,**B**) TLCS alone, a hydrophobic BA, induced intracellular protease activation, as shown for trypsin and cathepsin B, which was less prominent than with supramaximal CCK (1 µM). Co-incubation of CCK and TLCS attenuated the protease activation. (**C**) Stimulation of isolated acini with different concentrations of CCK showed a peak at 100 pM, which was blocked after co-incubation with TLCS. (**D**–**F**) Similarly, LCA alone, another hydrophobic BA showed the same results as TLCS in stimulating acinar cells and mitigating the impact of CCK. (**G**–**I**) In contrast, the hydrophilic TUDCA neither altered intracellular protease activation, nor decreased amylase secretion following co-incubation of CCK and TUDCA. Concentration for TLCS, LCA and TUDCA was 500 µM. All results were based on 5 experiments per group. Statistically significant differences for more than 3 groups were tested via one-way ANOVA followed by Tukey's multiple comparison test, and significance levels of $p < 0.05$ are marked by an asterisk. ns: non significant; RFU: Relative Fluorescence Units.

We further tested for the potentially toxic effects of BAs given at final concentrations of 500 µM on pancreatic acini; however, neither in the lactate dehydrogenase (LDH) (Figure 2A) nor the propidium iodide (PI) exclusion (Figure 2B) measurements did we observe an increase in cellular damage by BAs, which remained comparable to the control cells. Then, the addition of CCK enhanced both LDH release (Figure 2A) and PI exclusion (Figure 2B), indicating cell injury, which was reversed by hydrophobic but not hydrophilic BAs.

These results suggest that hydrophobic but not hydrophilic BAs induced intracellular protease activation and mitigated the impact of CCK in isolated acini.

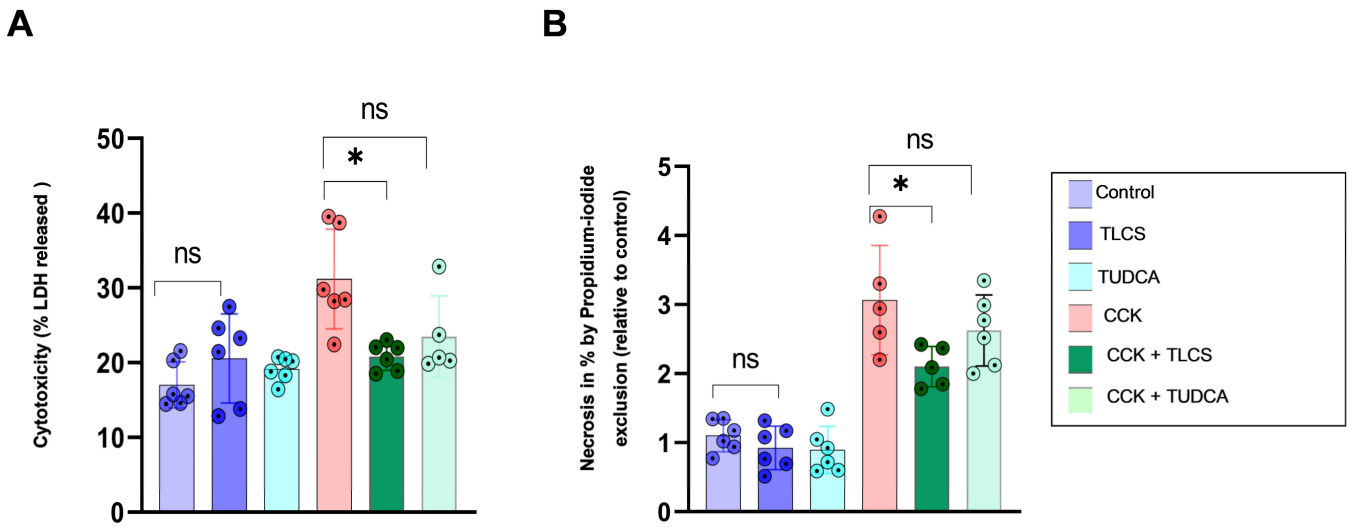

Figure 2. Release of LDH and propidium iodide exclusion in mouse isolated acini. Addition of bile acids TLCS and TUDCA in a final concentration of 500 µM to isolated acini did not increase LDH release (**A**) or propidium iodide exclusion (**B**), indicating no relevant cellular toxicity. Supramaximal CCK stimulation of acini increased both LDH release (**A**) and PI exclusion (**B**), which was reduced by TLCS but not TUDCA, compatible with protease activation in isolated acini. Graphs represent at least 5 animals per group. Statistically significant differences for more than 3 groups were tested via one-way ANOVA followed by Tukey's multiple comparison test, and significance levels of $p < 0.05$ are marked by an asterisk. ns: non significant.

2.2. BAs Were Elevated in the Serum and Reached the Pancreas after Intravenous or Intraperitoneal Administration in Mice

Since the times for harvesting samples after inducing AP were 4 h in the caerulein model, 24 h in the duct ligation models and 72 h in the L-arginine model, we measured the total bile acid (TBA) concentrations in the serum and in the pancreas homogenates after intravenous (i.v.) or intraperitoneal (i.p.) injection up to three days. After tail vein injection of TLCS, serum concentrations increased quickly within 5 min, then, decreased and almost disappeared from circulation after one hour. Reabsorption through the enterohepatic circulation led to the second peak, which occurred at 8 h, and serum concentrations slowly reduced during the monitoring time. LCA and TUDCA were intraperitoneally injected, which led to slower increases in serum concentrations, showing a maximum at around one hour. The concentrations were then maintained at a higher level than in the controls (Figure 3A,B). The injected BAs, either i.v. or i.p., all reached the pancreas, as shown by the significant elevation of TBA concentration in pancreas homogenates for all three BAs (Figure 3C). The data demonstrate that BAs can reach the pancreas in an in vivo experiment either via i.v. or i.p. injection.

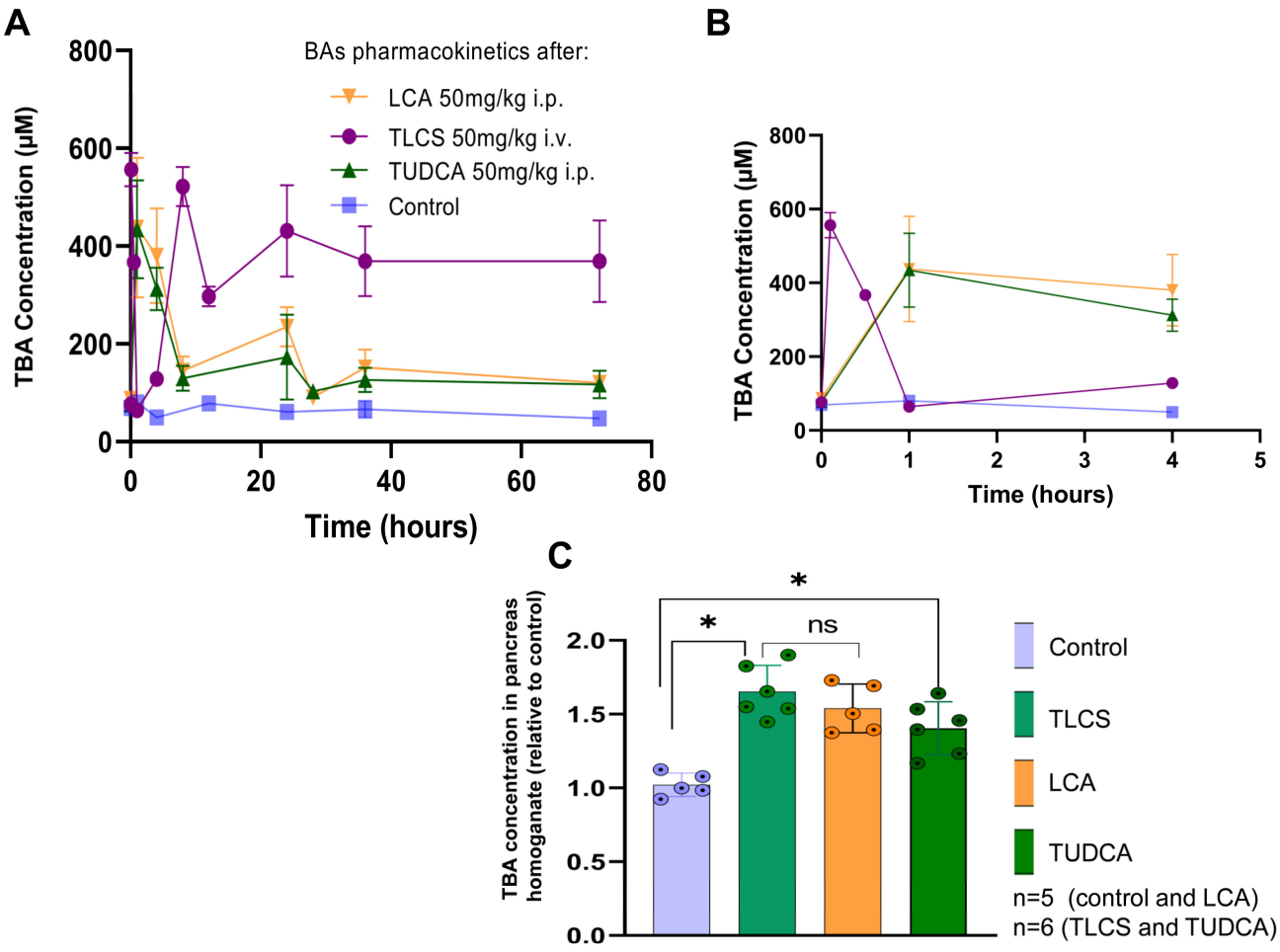

Figure 3. Elevation of BAs in circulation and in pancreas of mice after systemic administration. Pharmacokinetics of TLCS, LCA and TUDCA were assessed via measurement of total BA concentrations at different intervals after i.v. or i.p. injection up to 72 h. (**A**) After i.v. injection of TLCS, serum concentrations (purple line) increased quickly within 5 min, then decreased, and were cleared up from circulation after 60 min. A second peak occurred at 8 h, resulting from enterohepatic circulation through reabsorption, and serum concentrations slowly reduced during the monitoring time. Intraperitoneal injection of TUDCA (green line) or LCA (orange line) led to a delayed increase in serum concentrations, showing a maximum at around one hour. Concentrations then gradually decreased and were maintained at a slightly higher level than in controls (blue line). The mean absolute BA concentrations after administrating TLCS, LCA and TUDCA were 374.53 ± 21.07, 173.24 ± 12.25 and 164 ± 10.93 µM, respectively. (**B**) Illustration of serum BA concentrations during the first 4 h for clearer presentability. (**C**) All the injected BAs reached the pancreas, as shown by significant elevation of TBA concentration in pancreas homogenates. The mean value of TBA concentrations in the pancreas homogenate were 30.65 ± 2.73, 49.58 ± 5.33, 46.17 ± 4.95 and 42.13 ± 5.39 µM/mg protein for control mice, and TLCS-, LCA- and TUDCA-injected mice, respectively. Graphs represent at least 5 animals per group. Statistically significant differences for more than 3 groups were tested via one-way ANOVA followed by Tukey's multiple comparison test, and significance levels of $p < 0.05$ are marked by an asterisk. ns: non significant.

2.3. Hydrophobicity-Dependent Effects of BAs in CCK-Dependent Mouse AP Models

We next investigated the role of hydrophilic and hydrophobic BAs in an in vivo model dependent on the CCK analogue caerulein. Prior to caerulein administration, mice received an injection of TLCS, LCA or TUDCA (50 mg/kg bodyweight). Pancreatic damage was assessed through serological markers, protease activity in the pancreas homogenates, histology, and lung MPO for the determination of extra-pancreatic damage. While neither

TUDCA, TLCS nor LCA alone had any effect in unstimulated mice, pretreatment with hydrophobic TLCS or LCA attenuated pancreatic injury, as observed for serum amylase (Figure 4A) and lipase (Figure 4B), as well as trypsin and chymotrypsin activities in the pancreatic homogenates (Figure 4C,D). Lung MPO measurements showed a decrease resulting from TLCS or LCA in caerulein-induced pancreatitis, indicating collateral attenuation of extra-pancreatic damage (Figure 4E), and local pancreatic damage was decreased after the addition of these hydrophobic BAs, as well (Figure 4F,G). In contrast, the hydrophilic TUDCA did not alter the activation of any of these parameters.

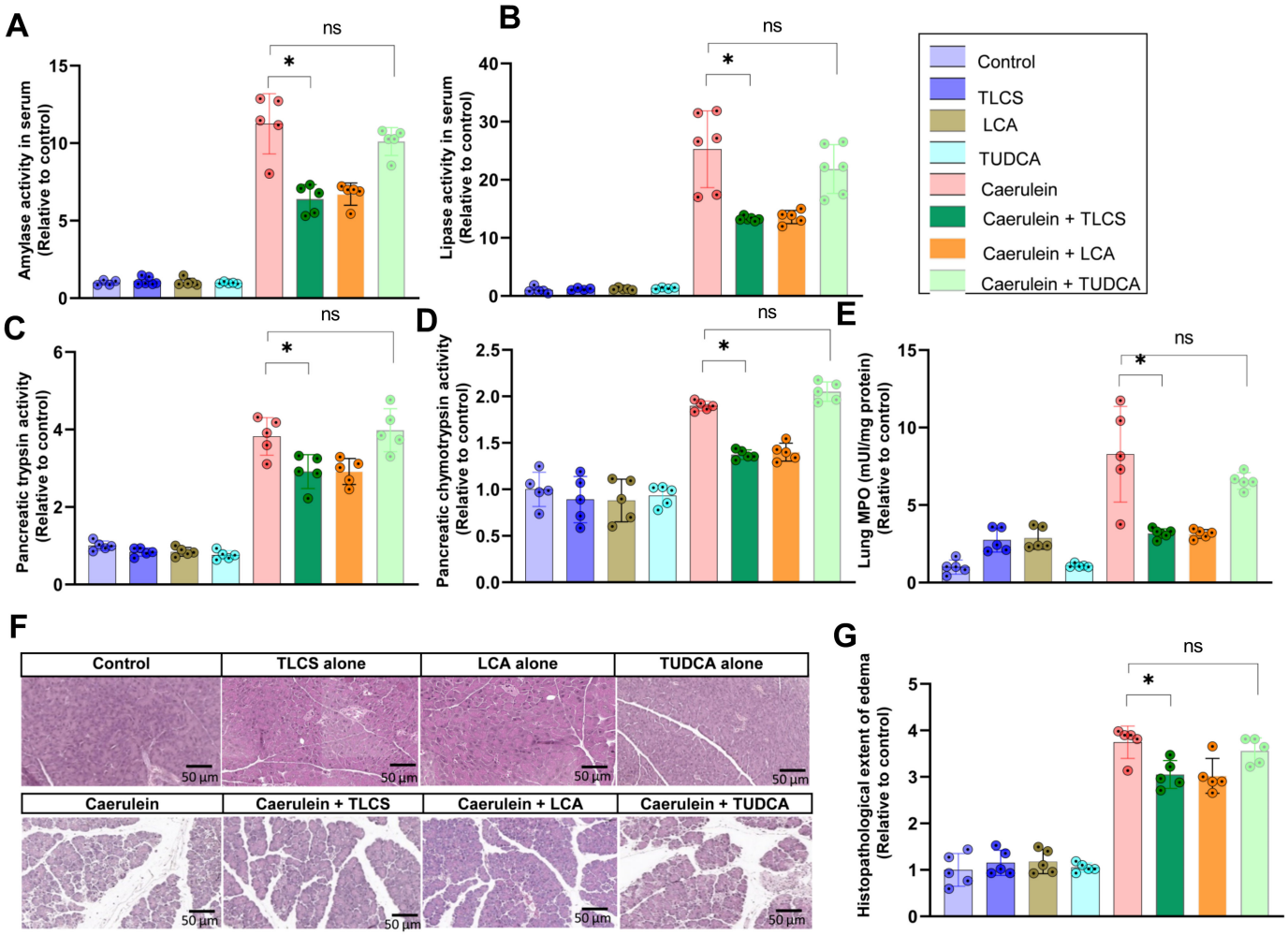

Figure 4. Attenuated severity of caerulein-induced AP following hydrophobic BAs in mice. (**A**,**B**) AP was induced by repetitive injections of caerulein, a cholecystokinin analogue. One hour or 30 min prior to the first caerulein injection, mice received a dose of 50 mg/kg TUDCA, LCA or TLCS. Serum amylase and lipase were elevated after 4 h of caerulein pancreatitis. Both enzyme activities decreased after pretreatment with TLCS or LCA, but not TUDCA. (**C**,**D**) We further determined pancreatic trypsin activity at 4 h and chymotrypsin activity at 1 h in pancreatic homogenates, which were both elevated in pancreatitis. Pretreatment with TLCS and LCA reduced these activities but TUDCA did not affect them in comparison with caerulein alone. (**E**) Lung MPO, an indicator of extra-pancreatic damage in AP, showed the same manner as pancreatic parameters, in which it was attenuated by TLCS as well as LCA, but not TUDCA, in caerulein-induced AP. (**F**,**G**) The corresponding histopathological damage was compatible with the biochemical indicators of AP, showing amelioration in the presence of hydrophobic TLCS or LCA and remaining unchanged in treatment with hydrophilic TUDCA. All graphs represent 4–6 animals per group. Statistically significant differences for more than 3 groups were tested via one-way ANOVA followed by Tukey's multiple comparison test, and significance levels of $p < 0.05$ are marked by an asterisk. ns: non significant.

2.4. Aggravating Effects of Hydrophobic BAs in CCK-Independent AP Models in Mice

As our results from CCK- or CCK analogue-dependent experimental pancreatitis models indicate a reduction in severity in the presence of hydrophobic BAs, we were interested in the impact of BAs in conditions unrelated to CCK. L-arginine-induced pancreatitis is characterized by CCK-independent signaling pathways, which makes this model interesting for investigations of BA-related effects. In L-arginine-induced AP, there was maximal damage at 72 h, which was further enhanced by TLCS but ameliorated by TUDCA, as demonstrated by changes in serum amylase (Figure 5A) and lipase (Figure 5B) activities. Correspondingly, trypsin (Figure 5C) and chymotrypsin (Figure 5D) activities in pancreatic homogenates were elevated in L-arginine pancreatitis, indicating the activation of digestive proteases, and were even more enhanced after pretreatment with TLCS. On the other hand, TUDCA had an opposite effect (Figure 5C,D). Lung MPO enzyme activity for extrapancreatic injury (Figure 5E) and local pancreatic damage, assessed via hematoxylin- and eosin-stained pancreatic sections, demonstrated aggravation of the disease under TLCS, while for TUDCA, a reversed effect was observed (Figure 5F,G).

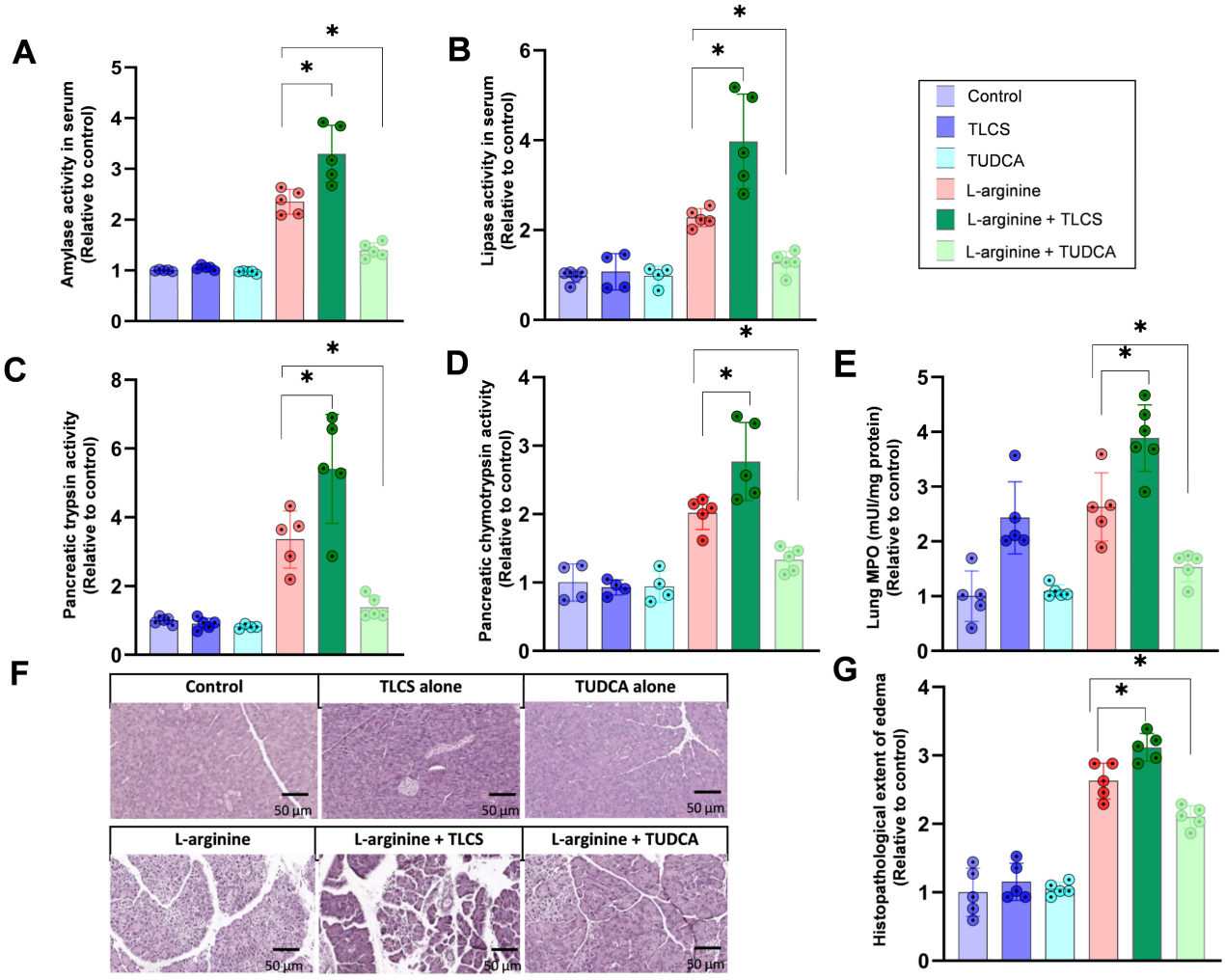

Figure 5. Effects of BAs in L-arginine-induced AP in mice. BAs were given via the same application route as for caerulein pancreatitis and mice were humanely killed after 72 h. (**A**,**B**) L-arginine induced AP, as shown by elevations of serum amylase and lipase after L-arginine injection. Pretreatment with TLCS caused disease aggravation, as shown by an increase in the enzyme activities, while TUDCA ameliorated disease severity. Neither TLCS nor TUDCA alone altered enzyme activities in the absence of L-arginine. (**C**,**D**) Pancreatic trypsin activity and chymotrypsin activity were enhanced in L-arginine pancreatitis. Protease activity was enhanced by TLCS pre-treatment and reduced when

mice received TUDCA. (**E**) Lung MPO changed similarly to pancreatic damage, in which TLCS increased but TUDCA mitigated enzyme activity. (**F**,**G**) Organ damage, shown by histology in L-arginine pancreatitis, was more severe with TLCS but milder when TUDCA was added in comparison to L-arginine alone. All graphs represent 4–6 animals per group. Statistically significant differences for more than 3 groups were tested via one-way ANOVA followed by Tukey's multiple comparison test, and significance levels of $p < 0.05$ are marked by an asterisk.

Disease severity and its dependence on BAs showed a similar pattern in the PDL model, which was independent of CCK as well. One day after ligation, AP was observed as shown by elevated levels of serum lipase, amylase, chymotrypsin in homogenates and lung MPO. Pretreatment with TLCS increased enzyme activities by about 25–40%, while there was attenuated severity under TUDCA (Figure 6A–D). In addition, the severity of histological damage correlated with findings from the enzyme measurements (Figure 6I,K).

Surprisingly, decreased severity of pancreatitis was observed in the BPDL model following TLCS injection. Both enzyme activities (Figure 6E–H) and organ damage were reduced (Figure 6J,L). This reduction resembled findings seen in the caerulein-induced pancreatitis model (Figure 4). On the other hand, TUDCA treatment did not alter disease severity in the caerulein model. Apparently, combined ligation of both ducts is related to CCK, as measurements of CCK concentrations in serum showed an almost three-fold increase after simultaneous ligation of the pancreatic and bile ducts after 30 min, while after pancreatic duct ligation alone, serum CCK remained unaffected (Figure 6M).

2.5. Interaction of Hydrophobic and Hydrophilic BAs with CCK1R on Mouse Isolated Acini

Our findings underline the differential effects of hydrophobic and hydrophilic BAs in AP and their dependence on the stimulus and the presence of CCK. Since this peptide hormone binds to the G-protein-coupled CCK1 receptor on pancreatic acinar cells, causing multiple cellular signaling functions [26], we next wanted to clarify to what extent BAs act on CCK1R in acinar cells. When we incubated freshly isolated mouse acini with Alexa-488-labeled CCK, we could visualize the binding of the CCK to its receptor via immunofluorescence. The co-incubation of acini with 500 µM TLCS drastically reduced the fluorogenic signal from acini, suggesting that TLCS had affected the binding of CCK1R on the cellular surface with Alexa-488 CCK. When 500 µM TUDCA was added instead of TLCS, the signal intensity on the surface was similar to the situation wherein only Alexa-488 CCK stimulation was used (Figure 7A). Quantification of the total fluorescence intensities derived from fluoroscopy confirmed our observations (Figure 7B) and suggests decreased binding between CCK1R on the membrane of living acini and CCK in the presence of hydrophobic BAs. Moreover, co-incubation of isolated acini with TLCS and CCK reduced intracellular calcium mobilization compared to CCK alone (Figure 7C). Distinguishably, the hydrophilic bile acid TUDCA affected neither CCK1R-CCK binding nor intracellular calcium mobilization. When we pre-treated C57BL/6J mice with devazepide, a CCK1R inhibitor, at a dose of 1 mg/kg bodyweight, and induced AP using caerulein, serum amylase and lipase activities were abrogated and the addition of TLCS did not further decrease the activity (Figure 7D,E). Similarly, trypsin activity was quenched by devazepide (Figure 7F). In parallel, both macroscopic inspection and histopathologic evaluation in caerulein-treated mice showed edema and injury of the pancreas, which was almost absent after devazepide application and could not be further attenuated by TLCS (Figure 7G,H). These results support the hypothesis that TLCS acts via an interaction with the CCK1 receptor, leading to impairment of the intracellular signaling pathways for AP.

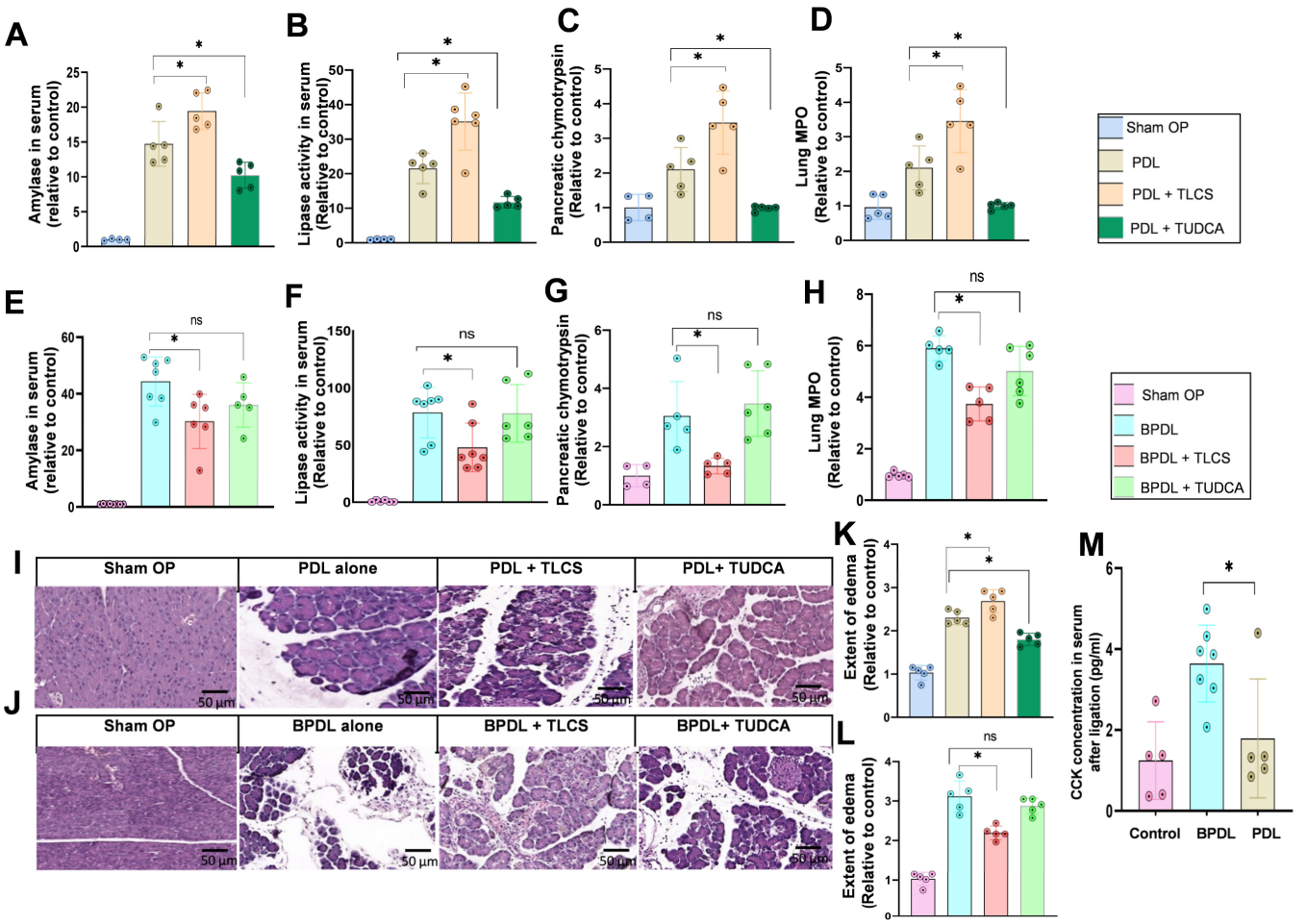

Figure 6. Impact of BAs in mouse AP models based on duct ligation. (**A**,**B**) Pancreatic duct ligation (PDL) caused a strong increase in serum amylase and lipase, which was further enhanced by pre-treatment with TLCS but mitigated by TUDCA. (**C**,**D**) Pancreatic chymotrypsin and lung MPO levels were higher when injecting additional TLCS but lower after treatment with TUDCA. (**E**–**H**) Ligation of both distal common bile duct and main pancreatic duct (BPDL) led to an increase in amylase, lipase, chymotrypsin and lung MPO, similar to the PDL model. However, pre-treatment with TLCS reduced enzyme activities, while the addition of TUDCA did not alter disease severity. (**I**,**K**) Organ injury, including the extent of edema in HE-stained slides, demonstrated increased severity with TLCS but attenuation with TUDCA in the PDL model for acute pancreatitis. (**J**,**L**) In contrast, local histological damage was reduced by TLCS when both the bile and the pancreatic duct were ligated. Additional TUDCA treatment did not show any effect compared to duct ligation alone. (**M**) Endogenous CCK levels were significantly higher in the BPDL compared to the PDL model, indicating involvement of CCK in the former. All graphs represent 4–7 animals per group. Statistically significant differences for more than 3 groups were tested via one-way ANOVA followed by Tukey's multiple comparison test, and significance levels of $p < 0.05$ are marked by an asterisk. ns: non significant.

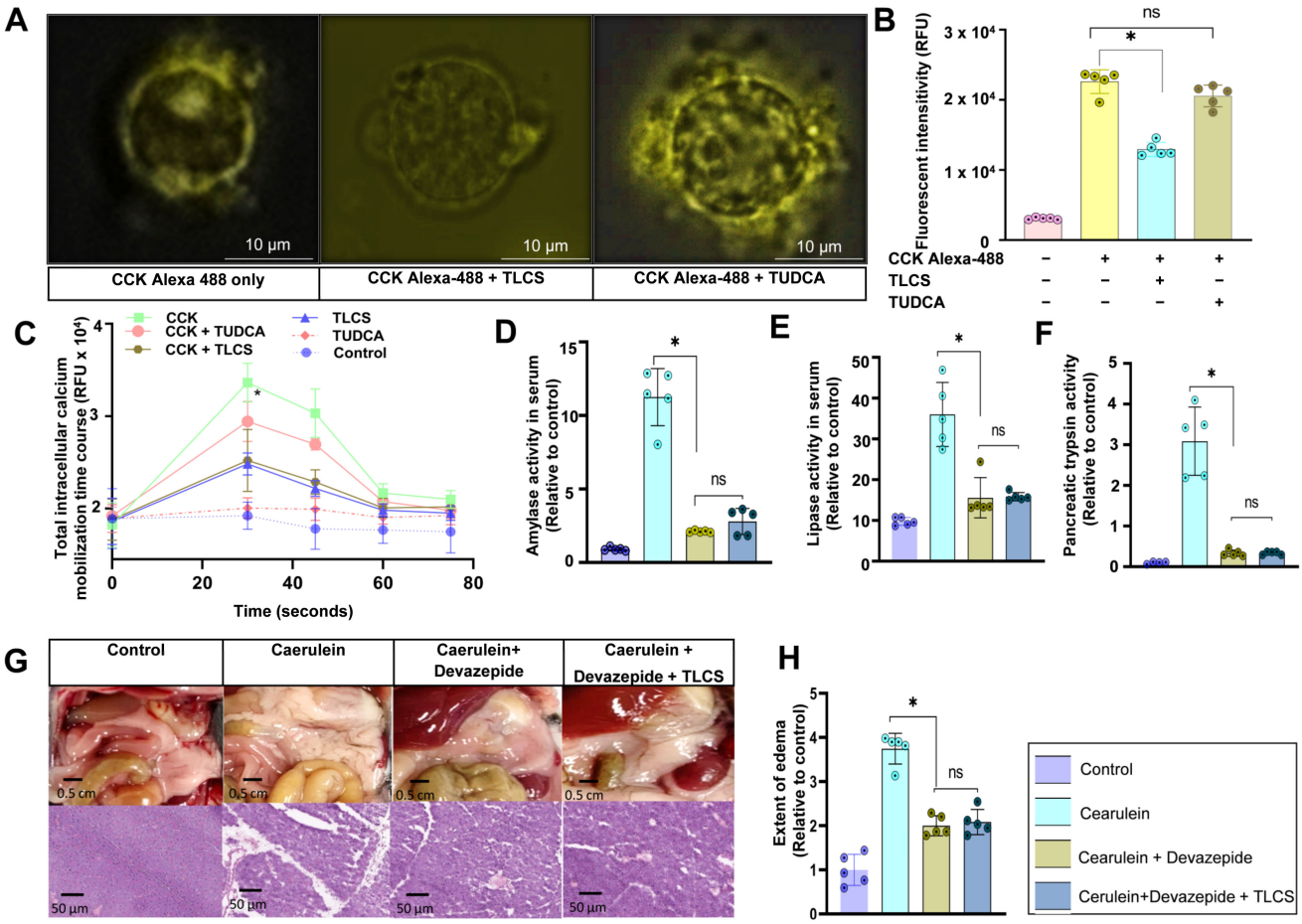

Figure 7. Interaction of BAs with the CCK1 receptor and intracellular calcium release in mice. (**A**) Fluorescence-labeled CCK (Alexa-488 CCK, yellow signal) is located on the membrane of mouse living acinar cells, suggesting binding to the CCK-receptor (left image). This binding was decreased after co-incubation with TLCS, a hydrophobic BA (middle image), but was unchanged when incubated with TUDCA, a hydrophilic BA (right image). (**B**) Total quantification of the fluorescent signals after incubating acini with Alexa-488 CCK showed a decrease in the fluorescence intensity after addition of a hydrophobic BA, but no reduction with a hydrophilic BA. (**C**) The time course data of the intracellular calcium mobilization measured at 30, 45, 60 and 75 s, which peaked at 30 s and was followed by a quick decrease in the calcium changes. CCK quickly induced intracellular calcium mobilization in mouse acini, which was clearly reduced by simultaneously adding TLCS to CCK, but was not significantly different when compared to co-incubating CCK and TUDCA. (**D**,**E**) When we inhibited CCK1R using the specific inhibitor devazepide and induced AP using caerulein, serum amylase and lipase increases were abrogated and the addition of TLCS showed no further inhibitory effect. (**F**) Mouse pancreatic trypsin activity was also quenched by devazepide. (**G**,**H**) In parallel, both macroscopic observation and histopathologic images demonstrated edema and injury of the pancreas, which were almost absent after devazepide treatment. No additional reduction was observed with TLCS when mice received devazepide beforehand. All results were based on at least 5 animals per group. Statistically significant differences for more than 3 groups were tested via one-way ANOVA followed by Tukey's multiple comparison test, and significance levels of $p < 0.05$ are marked by an asterisk. ns: non significant.

3. Discussion

Being the leading cause of AP, the biliary origin has been extensively investigated in several studies, but so far, they have almost exclusively been based on the retrograde infusion of BAs into the pancreatic duct [17,27]. To the best of our knowledge, systematic analyses of systemically administered BAs focusing on their impact in AP and investigation

of their underlying mechanisms are still lacking. Secondly, a large number of cholestatic disorders are caused not only by obstruction of the bile ducts but also by hepatocellular damage, and lead to an increase in serum BA concentrations [28]; however, their potential impact on AP has not been elucidated so far, which might even enhance the necessity to focus on their systemic role, as well. In the present study, we were able to demonstrate that systemic BAs modulate the severity of AP depending on its pathogenesis and the hydrophobicity of BAs. While hydrophobic BAs aggravate severity under disease conditions that are independent of CCK or its analogues, they reverse CCK-induced injury based on an interaction with the CCK receptor on acinar cells.

In isolated living acini, we confirmed previous studies [16,18], which showed that TLCS and LCA can induce significant activation of pancreatic enzymes. Following preincubation with these strongly hydrophobic BAs, concomitant CCK application reverted intracellular zymogen and lysosomal protease activation, while this was not the case with hydrophilic BAs. These BA-dependent differences seem to be related to their ability to interact with CCK receptors, namely CCK1R, which is abundantly expressed on acinar cells and has the highest affinity to the octapeptide CCK [29,30]. Receptor binding is markedly reduced in the presence of hydrophobic compared to hydrophilic BAs, as we demonstrated using CCK labeled with the fluorophore Alexa-488 that was detectable by fluorescence microscopy. As the activation of G-protein-coupled receptors led to a release of intracellular Ca^{2+}, a crucial regulator of pancreatic acinar cell secretion [31,32], we further assessed intracellular calcium release and confirmed a reduction in Ca^{2+} mobilization, which was less pronounced after incubation with TLCS but remained steady after TUDCA. It is noteworthy that the TLCS-induced decrease in intracellular Ca^{2+} released by CCK was not strong, so additional intracellular mechanisms may also lead to a decrease in protease activation and pancreatic damage. Our BA concentrations were in the same range as others have used in previous studies [16,18] and isolated acini did not show signs of cellular necrosis upon TLCS stimulation. Therefore, we assume that the TLCS-related reduction in intracellular protease activation in CCK-stimulated acini is primarily based on alterations of intracellular signaling and not a consequence of cytotoxicity.

The impact of BAs on CCK1R function was studied by Desai et al. in Chinese hamster ovary (CHO) cell lines expressing CCK1R. They showed an inhibitory effect with hydrophobic taurochenodeoxycholic (TCDC) acid but not with hydrophilic tauroursodeoxycholic acid (TUDCA), and proposed a direct interaction of BAs with the CCK1 receptor, likely at the same site where cholesterol binds, leading to a conformational change in the helical bundle domain [33]. The intracellular calcium responses following CCK stimulation and BA exposure were also delayed by TCDC, but not TUDCA, similar to our results.

The translation of ex vivo findings into in vivo models that, at least partly, are based on CCK or its analogues confirmed our observations of the attenuating effects of hydrophobic BAs. In both caerulein-induced and BPDL pancreatitis, which caused an elevation of CCK, the severity of pancreatitis was decreased by hydrophobic BAs but not by the hydrophilic TUDCA. Previous studies reported that a lack of BAs in the duodenum, as seen after obstruction of the distal bile duct, triggers a feedback regulation to produce more endogenous CCK [34]. Our results are in line with this observation as CCK strongly increased in mice that underwent combined pancreatic and bile duct ligation in comparison to sham-operated or only pancreatic duct-ligated mice, where significant CCK-elevation was absent. These findings further confirm our observations from ex vivo models and propose a modulating effect of BAs on AP severity in vivo, depending on their hydrophobicity and the presence of CCK in the initial phase of the disease.

Depending on the location where BAs reach acinar cells, a variety of both receptor and transporter-mediated cellular signaling mechanisms contribute to pancreatic damage. In rat pancreatic acinar cells, Kim et al. showed evidence for the expression of the BA transporter molecules Na+/taurocholate co-transporting polypeptide (NTCP) and organic anion transporting polypeptide 1 (OATP1) on the luminal and basolateral site, respectively; these are both capable of mediating BA influx into acinar cells [35]. BAs may therefore enter

acinar cells not only via reflux to the apical pole but also because of interstitial leakage or systemic application at the basolateral site. This route of internalization might explain why TLCS increases pancreatic injury in L-arginine or pancreatic duct ligation-induced pancreatitis. L-arginine is the major amino acid of histones, and Guo et al. found that extracellular histones could inhibit the impact of CCK 20 pM on rat pancreatic acini, thus contributing to the secretory blockade [36,37]. It would be interesting to study, in the future, the effect of adding both L-arginine and CCK simultaneously, and possibly find the links between BAs, L-arginine and extracellular histones in AP. Interestingly we additionally observed a protective function of hydrophilic TUDCA. The underlying reason is still unclear but might include: a reduction in endoplasmic reticulum stress, mitochondrial damage in acinar cells and alterations in the gut microbiome, as reported in previous studies [21,38]. The gut microbiome has an emerging role in the metabolism of BAs [39] and in pancreatitis [40]. Studies about the interactions between BAs, the gut microbiome and acute pancreatitis would be a promising approach. Further investigations will also have to clarify whether treatment with hydrophilic BAs may eventually be helpful in preventing or attenuating AP in humans when the etiology and mechanisms are unrelated to CCK.

There are limitations to our work. Firstly, the low number of mice, consisting of four animals in some control groups, that were either untreated, underwent sham operations or received TLCS and TUDCA alone, could limit the validity of the results. In the experimental design, we calculated the number of animals based on a 'resource equation' approach [41,42], with a relatively low number of mice in some groups. Secondly, different application routes for bile acids were used, as TLCS was injected intravenously while TUDCA and LCA were administered intraperitoneally. Nevertheless, the TBA concentrations in pancreas homogenates increased similarly. Moreover, previous work [43] reported comparable effects on hepatic CYP-linked mono-oxygenase activities following i.v. or i.p. injection. However, further research will be necessary to clarify the importance of the application route of drugs or compounds in AP. Thirdly, this study mainly concentrated on only two hydrophobic BAs and one hydrophilic BA in AP, which might not be fully representative of all hydrophobic and hydrophilic BAs, respectively. Additional studies using a broader BA spectrum and different models of AP will be necessary for a comprehensive understanding of the role of BAs in AP.

In conclusion, our results indicate that systemic BAs can modulate the severity of AP, which seems to be dependent on the biochemical properties of BAs such as their hydrophobicity and the pathogenesis of AP. The influence of hydrophobic BAs on the CCK1 receptor's binding emerged as a central mechanism.

4. Materials and Methods

4.1. Chemicals and Materials

Amylase (11876473316) and lipase (11821792216) kits were purchased from Roche Diagnostics GmbH (Rotkreuz, Switzerland). Cathepsin B substrate (AMC-Arg2, I-1135.0250) was from Bachem (Bubendorf, Switzerland). Non-sulfated CCK 26-33 amide fluorescence-labeled (Alexa-488) was ordered from Thermo Fisher Scientific (Carlsbad, CA, USA). Cell Meter™ No Wash and Probenecid-Free Endpoint Calcium Assay Kit (36312) was ordered from AAT Bioquest (Sunnyvale, CA, USA). Cholecystokinin quantification EIA Kit (RAB0039), Caerulein (17650-98-5) and L-arginine (A5006) were sold by Sigma-Aldrich Chemie GmbH (Merk). Trypsin substrate (R-110 BZIPAR, 10208) was ordered from Bio Trend (Pambio-Noranco, Switzerland). Chymotrypsin substrate (Suc-Ala-Ala-Pro-Phe-AMC, I-1465.0250) was bought from Bachem (Bubendorf, Switzerland). Collagenase (14007) from Clostridium histolyticum (EC.3.4.24.3) was provided by Serva (Heidelberg, Germany). LDH Cytotoxicity Assay Kit (601170) and taurolithocholic acid-3 sulfate (18468) were obtained from Cayman Chemical (Ann Arbor, MI, USA). Tauroursodeoxycholic acid (S3654) was obtained from Selleck Chemicals (Houston, TX, USA). Total BA fluorogenic kit (MET-5005) was a product from Cell Biolabs (San Diego, CA, USA).

4.2. Isolation and Stimulation of Mouse Pancreatic Acini

Acini from wildtype C57BL/6J mice were freshly prepared from the pancreas based on collagenase digestion, as described previously [6]. A cell medium containing Dulbecco's Modified Eagle's Medium (DMEM), BSA 2% and 10 mM 4-(2-hydroxy-ethyl)-1-piperazine ethanesulfonic acid (HEPES) was freshly prepared. A fresh pancreas was dissected carefully using scissors and forceps. Blood, fat and connective tissue were removed and the clean pancreas was then immediately and gently sheared into small pieces under 1 mm in size in a glass flask containing cell medium and collagenase (1 mg/mouse pancreas). The above process was repeated 2 times with freshly changed medium, and during the 15 min interval, the cells were incubated in a water bath with a shaking speed of 90 rpm for dissociation of the cells. The sheared pancreatic pieces were gently suspended 5 times through 1 mL pipette tips with gradually decreasing diameters followed by filtering twice using single-use, double-layered muslin gauze. The filtered cells were centrifuged for 90 s at 293× *g*, the supernatant was removed and fresh medium was added immediately. Cells were resuspended using a 10 mL pipette and rested for 30 min at the same temperature and buffer conditions in a shaking water bath at 45 rpm. During the whole process, cells were maintained in the oxygenated medium at 37 °C. After isolation, we obtained acini with viability of more than 90%, which were ready for further assays. For the in vitro experiments, BAs were dissolved in DMSO and added to freshly isolated acini at a final concentration of 500 µM. Acini were stimulated with 1 µM CCK for 30 min. Intracellular enzyme activation was determined in a cell medium system at pH 7.4 containing 24.5 mM HEPES, 96 mM NaCl, 11.5 mM glucose, 6 mM KCl, 1 mM $MgCl_2$, 0.5 mM $CaCl_2$, 2.5 mM NaH_2PO_4, 5 mM sodium fumarate, 5 mM sodium glutamate, 5 mM sodium pyruvate and 1% BSA and DMEM.

4.3. CCK1 Receptor-Binding Assay

After the resting phase, freshly isolated acini were incubated with 1 µM CCK 26-33 amide fluorescence-labeled (Alexa-488), obtained from Thermo Fisher Scientific (Carlsbad, CA, USA), with or without different BAs at a concentration of 500 µM in a water bath, at 37 °C, with gentle shaking 45 rpm for 30 min. Incubated cell medium was then centrifuged at 1000 rpm for one minute and the supernatant was completely removed and very gently washed with PBS to rule out unbound fluorescence. The pellet was re-suspended in measuring buffer and the remaining fluorescence was quantified at an excitation wavelength of 490 nm, and an emission wavelength of 525 nm. Additionally, living cells were observed under fluorescence microscopy for visualization of the binding between CCK-488 and its receptor on the membranes of acinar cells.

4.4. Protease Activation Assays

Trypsin and cathepsin B in isolated acini were measured kinetically in a measuring buffer containing 11.5 mM glucose, 96 mM NaCl, 1 mM $MgCl_2$, 6 mM KCl, 2.5 mM NaH_2PO_4, 0.5 mM $CaCl_2$, 5 mM Na fumarate, 5 mM Na glutamate, 5 mM Na pyruvate, 24.5 mM HEPES and 1% BSA at pH 7.4. The substrates for cathepsin B and trypsin were 10 µM AMC-Arg2 and 10 µM R110-IPA, respectively. In acinar cells and whole pancreatic homogenates, chymotrypsin activity was measured using the substrate Suc-Ala-Ala-Pro-Phe-AMC, and trypsin activity was measured using R-110 BZIPAR. Activities of the enzymes were measured for one hour at 37 °C, kinetically, in a buffer (pH 8.0) containing 5 mM CaCl2 and 100 mM Tris. Fluorometric measurements were carried out in a FLUOStar Omega fluorometer. For AMC-based substrates, the setting was a 380 nm excitation wavelength and 460 nm emission wavelength, and for R110-based substrates, the wavelengths of excitation and emission were 485 nm and 520 nm, respectively. Enzymatic activities were normalized to protein content, which was measured via Bradford assay. All measurements were performed in triplicate.

4.5. Quantification of Intracellular Calcium Mobilization in Living Acinar Cells

Total intracellular calcium mobilization was quantified by measuring the fluorescence intensity within one minute after preparing the acini, according to the protocol of the Cell Meter™ No Wash and Probenecid-Free Endpoint Calcium Assay Kit provided by AAT Bioquest (Sunnyvale, CA, USA). Briefly, we firstly added 100 µL/well of Fluo-8E™ AM dye-loading solution into the 96-well plate, with 100µL mouse acini already prepared in each well. Then, we incubated the dye-loading plate in a 5% CO_2 incubator at 37 °C for 45 min. Meanwhile, we prepared the calcium stimulator solution (CCK and BAs). Finally, we added 50 µL of the prepared stimulator and ran the calcium flux assay immediately, measuring the fluorescence intensity using a FLUOStar Omega fluorometer (BMG Labtech GmbH, Ortenberg, Germany) with the bottom read mode at an excitation/emission of 490/525 nm.

4.6. Amylase and Lipase Measurement

Serum amylase and lipase activities were measured kinetically over 30 min via photometric assays using kits from Roche Diagnostics GmbH (Rotkreuz, Switzerland), with absorbance at 405 nm and 570 nm wavelengths, respectively.

4.7. Measurement of Propidium Iodide Exclusion and Release of LDH

Propidium iodide exclusion was used to determine necrosis, and cytotoxicity was quantified by LDH release from acini using the Cytotoxicity Assay Kit (601170), according to the instructions of the manufacturer.

4.8. Myeloperoxidase Measurement

Lung tissue was homogenized on ice in a buffer containing 20 mM KH_2PO_4 at pH 7.4 and centrifuged at 10,000× g for 10 min at 4 °C. The pellet was resuspended in 50 mM KH_2PO_4 extraction buffer (pH 6.0) containing EDTA, PMSF and hexadecyltrimethylammonium bromide (5%); frozen in liquid nitrogen and thawed in 4 cycles with gradually smaller pipette tips; sonicated twice; and centrifuged at 10,000× g for 10 min at 4 °C. Myeloperoxidase (MPO) activity was measured in 50 mM KH_2PO_4 extraction buffer (pH 6.0) containing 0.15 mM H_2O_2 and 0.53 mM o-dianisidine using a SpectraMax Spectrophotometer (Molecular Devices, Sunnyvale, CA, USA) at 460 nm and at 30 °C over 10 min. The results were calculated after dividing by the protein amount of the corresponding samples.

4.9. Measurement of Total Bile Acid

The total BA in the serum was quantified using a Total Bile Acid Assay Kit (MET-5005, Cell Biolabs, San Diego, CA, USA) using a FLUOStar Omega fluorometer (BMG Labtech GmbH, Ortenberg, Germany) following the protocol of the manufacturer at an excitation/emission wavelength of 560/590 nm. Briefly, pancreas tissues were homogenized via ultra-sonification 2 times at 100% power for 10 s each in cold PBS, and centrifuged at 10,000× g at 4 °C for 10 min. The concentration of TBA was determined in the supernatant using the fluorometer and normalized to the corresponding protein amount. Mouse serum was harvested after centrifugation of whole blood and diluted at least 4 times before measuring.

4.10. Histopathological Examinations

Pancreases were fixed in 4.5% formaldehyde immediately after harvesting. Paraffin-embedded blocks were used for staining with hematoxylin and eosin. The slides were scanned using the Sysmex Pannoramic MIDI II slide scanner (Sysmex Europe SE, Norderstedt, Germany) for imaging. Damage was assessed using a modified score adapted from Niederau et al. [44]. The extent of edema, including the cell-free areas, was quantified by the percentage of the total areas using QuantCenter software version 2.2.1.88915 from Sysmex.

4.11. Animal Models

Wildtype C57BL/6J mice, 8–10 weeks old and weighing 23–27 g, were purchased from Janvier Labs (Le Genest-Saint-Isle, France) and were kept under standard conditions of temperature and humidity in ventilated cages under 12 h day/night cycles, with food and water provided ad libitum. All mice were fasted equally 8 h prior to the experiments. The study design and all protocols for animal care and handling were approved by the Institutional Animal Care and Use Committee of the University of Greifswald (Reg. No.: 7221.3-1-001/20). Mice were treated with BA dose of 50 mg/kg body weight in assigned groups half to one hour before the induction of AP. We used different BAs, including hydrophobic and hydrophilic compounds, to see the different effects that may result from their biochemical features. Due to their solubility and the limited volume that we can inject into the tail vein of a mouse, hydrophobic BA TLCS (10 mg/mL) was injected i.v. while hydrophilic TUDCA (5 mg/mL) and hydrophobic LCA (1 mg/mL) were injected i.p. after dissolving in PBS at 37 °C. Due to the very low solubility of LCA and the similar effects with TLCS, we further focused on TLCS in our experiments after the caerulein model.

Caerulein-induced pancreatitis was induced by intraperitoneal injections of the cholecystokinin analogue caerulein (50 µg/kg/body weight) at hourly intervals for up to 4 h. Mice were humanely killed via cervical dislocation to harvest the samples 1 h and 4 h after the first injection of caerulein (Figure 8A).

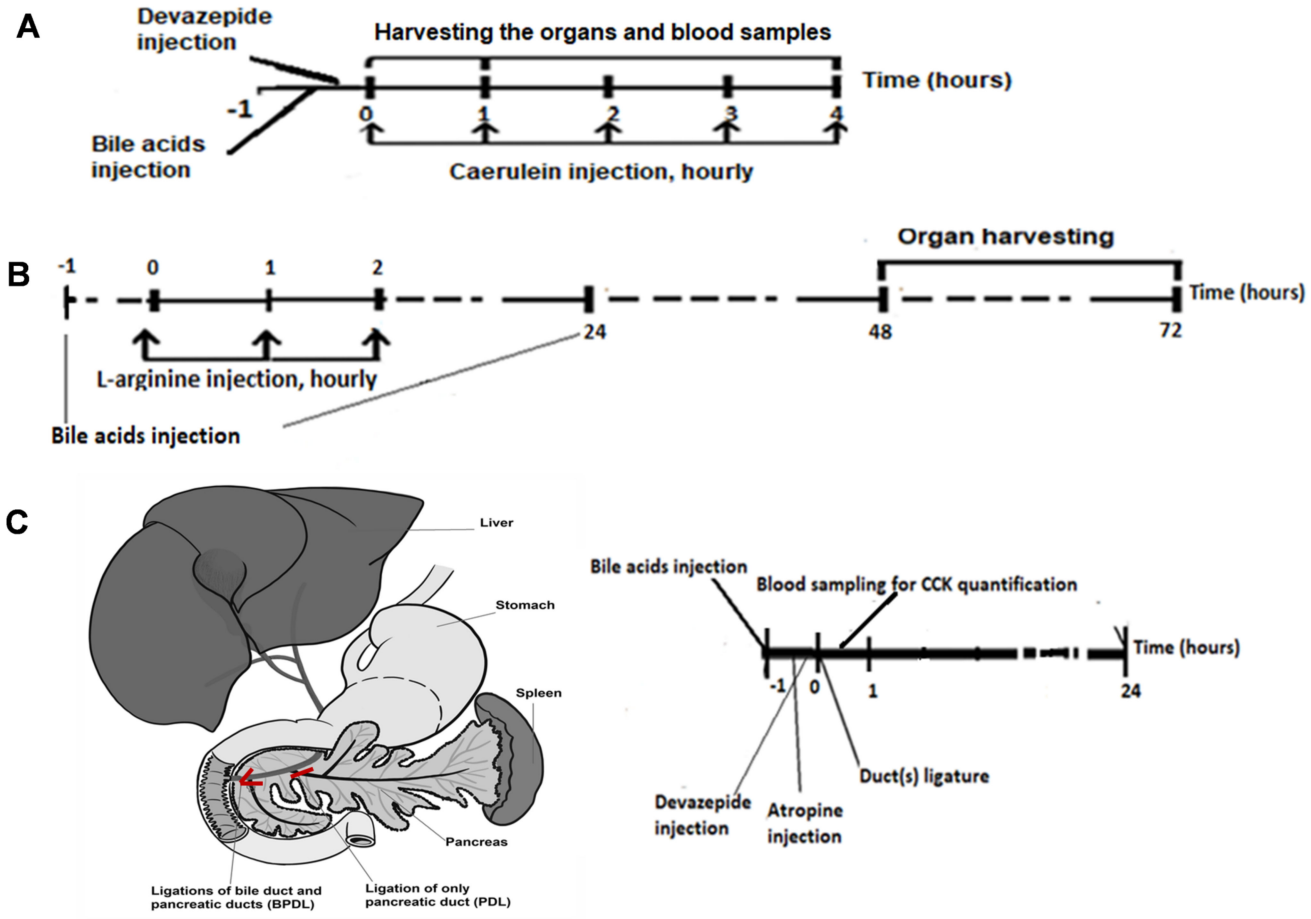

Figure 8. Animal models for AP to test the effect of systemic BAs. (**A**) Caerulein model: BAs were injected 30–60 min prior, and devazepide, if utilized, was injected 15 min before the first dose of caerulein. Caerulein was administrated hourly and intraperitoneally in a dose of 50 µg/kg body weight. The organs were harvested at 4 h (half-life time of devazepide). (**B**) L-arginine model: BAs were added one hour before and 24 h after the first injection of L-arginine, which was injected intraperitoneally (total dose 10 g/kg body weight), divided to three injections hourly. The mice were

humanely killed via cervical dislocation at 72 h. (**C**) Duct ligation model: BAs were administrated half to one hour prior to ligature. After laparotomy, ligature of both distal common bile duct and main pancreatic duct (BPDL) or ligation of only one distal part of the main pancreatic duct (PDL) was carried out. The blood was withdrawn 30 min after duct ligation to quantify the endogenous CCK concentration. Organs were harvested 24 h after the surgical duct ligation. BA dose: 50 mg/kg body weight.

In L-arginine pancreatitis, mice received L-arginine i.p. at a total dose of 10 g/kg of body weight, divided into 3 injections at hourly intervals. Control mice received PBS in parallel. Organs were harvested after 72 h (Figure 8B).

Duct ligation models were based either on the ligation of solely the main pancreatic duct [45] or the combined ligation of the bile and major pancreatic ducts, as described previously [46]. BAs were prepared as in the caerulein and L-arginine models. After laparotomy, either the confluence of the distal common bile duct and the main pancreatic duct was closed, called the bile-and-pancreatic duct ligation (BPDL) model, or only the distal part of the major pancreatic duct was ligated, called the pancreatic duct ligation (PDL) model (Figure 8C). For CCK quantification, blood samples were collected 30 min after surgery. The mice were humanely killed via cervical dislocation 24 h after ligature.

4.12. Statistics

Statistical analysis was performed using GraphPad Prism version 8.4.3 (GraphPad Software, San Diego, CA, USA). Data were presented as mean ± standard deviation (SD) for each group of animals. We used one-way ANOVA followed by Tukey's multiple comparison test for comparisons of more than 3 groups. Differences were considered significant when $p < 0.05$.

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ORIGINAL ARTICLE

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Oral N-Acetyl cysteine versus rectal indomethacin for prevention of post ERCP pancreatitis: a multicenter multinational randomized controlled trial

Pezhman **ALAVINEJAD**[1,14], Nguyen-Phuong Nhu **TRAN**[2], Omid **ESLAMI**[3,4], Omar El **SHAARAWY**[5], Ahmad **HORMATI**[6], Seied Saeed **SEIEDIAN**[1], Abazar **PARSI**[1], Mohammed Hussien **AHMED**[7], Nitin Shanker **BEHL**[8], Ali Akbar **ABRAVESH**[1], Quang Trung **TRAN**[9,10,14], Shivakumar **VIGNESH**[11,14], Saif **SALMAN**[12], Naemt **SAKR**[13], Tahmineh Farbod **ARA**[1], Eskandar **HAJIANI**[1], Seyed Jalal **HASHEMI**[1], Árpád V **PATAI**[15,16], Amna Subhan **BUTT**[14,17] and Sang Hyub **LEE**[14,18]

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ABSTRACT – Background – This multicenter multinational RCT designed to compare the efficacy of suppository indomethacin and NAC for prevention of PEP. **Methods –** During a 6-month period, all of the ERCP cases in seven referral centers were randomly assigned to receive either 1200 mg oral NAC, indomethacin suppository 100 mg, 1200 mg oral NAC plus indomethacin suppository 100 mg or placebo 2 hours before ERCP. The primary outcomes were the rate and severity of any PEP. **Results –** A total of 432 patients included (41.4% male). They were originally citizens of 6 countries (60.87% Caucasian). They were randomly allocated to receive either NAC (group A, 84 cases), rectal indomethacin (group B, 138 cases), NAC + rectal indomethacin (group C, 115 cases) or placebo (group D, 95 cases). The rate of PEP in groups A, B and C in comparison with placebo were 10.7%, 17.4%, 7.8% vs 20% (*P*=0.08, 0.614 & 0.01 respectively). The NNT for NAC, indomethacin and NAC + indomethacin was 11, 38 and 8 respectively. **Conclusion –** Oral NAC is more effective than rectal indomethacin when compared to placebo for prevention of PEP and the combination of NAC and Indomethacin had the lowest incidence of PEP and may have synergistic effect in preventing of PEP (IRCT20201222049798N1; 29/12/2020).

Keywords – Post ERCP pancreatitis; NAC; rectal indomethacin.

INTRODUCTION

Nowadays, endoscopic retrograde cholangiopancreatography (ERCP) as an endoscopic procedure is performed mostly due to its therapeutic options and capabilities and like other medical procedures, has both minor and major complications. The most common major complication of ERCP is pancreatitis, with a prevalence of 2.1% to 24.4% and average 5%[1-4]. Post ERCP pancreatitis (PEP) is diagnosed following an increase in serum amylase above three times the normal level more than 24 hours after ERCP, along with new computed tomography scan (CT) findings or new-onset abdominal pain that are compatible with pancreatitis, which may require hospitalization or extending the duration of hospital stay of patients who were hospitalized in the first place[5-7]. PEP still has unclear pathophysiology. It can be resulted fromcombined injury from papillary manipulation and trauma with instruments such as cannulation resulting in edema or spasm of the sphincter of Oddi or contrast overloading inside the pancreatic duct with resultant hydrostatic damage[6,8,9]. Other possible mechanisms are chemical, microbiologic, thermal and or enzymatic although the relative role of each is unclear[10,11].

Different medications and interventions have been used to prevent this complication or attenuate its severity, but are of little benefit[11-19]. Beside active hydration and Non-steroidal anti-inflammatory drugs (NSAIDs) suppository, other noteworthy medications include octreotide, somatostatin, gabexate mesylate, corticosteroids,

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[1] Alimentary Tract Research Center, Imam Khomeini hospital clinical research development Unit, The school of medicine, Ahvaz Jundishapur University of Medical sciences, Ahvaz, Iran. [2] Head of Endoscopy Department, Hue Central Hospital, Vietnam. [3] Clinical Research Development Unit, Afzalipour Hospital, Kerman University of Medical Sciences, Kerman, Iran. [4] Gastroenterology and Hepatology Research Center, Institute of Basic and Clinical Physiology Sciences, Kerman University of Medical Sciences, Kerman, Iran. [5] Lecturer and consultant of Hepatology, Gastroenterology and Endoscopy, National Liver institute, Menoufia University, Egypt. [6] Gastrointestinal and liver Diseases Research Center, Firouzgar Hospital, Iran University of Medical Sciences, Tehran, Iran. [7] Lecturer Hepatology Gastroenterology and infectious diseases, Faculty of Medicine, Kafrelsheikh University, Cairo, Egypt. [8] Institute of Gastro and Liver diseases, Fortis Hospital, Ludhiana, India. [9] Department of Internal Medicine, University of Medicine and Pharmacy, Hue University, Vietnam. [10] Department of Internal Medicine A, University Medicine Greifswald, Germany. [11] Division of gastroenterology, Department of Medicine, SUNY Downstate Health Sciences University, Brooklyn, NY 11203, USA. [12] Hashemite University, Faculty of Medicine, Alzarqa, Jordan. [13] Assistant Lecturer and specialist of Hepatology and Gastroenterology at the National Liver Institute, Menoufia University, Egypt. [14] World Endoscopy Organization emerging star group, WEO, Munich, Germany. [15] Gastroenterology Division, Internal Medicine and Hematology Department, Semmelweis University, Budapest, Hungary. [16] Interdisciplinary Gastroenterology Working Group, Semmelweis University, Budapest, Hungary. [17] Consultant Gastroenterologist, Section of Gastroenterology, Department of Medicine, Aga Khan University Hospital, Stadium Road, Karachi, Pakistan. [18] Department of Internal Medicine and Liver Research Institute, Seoul National University Hospital, Seoul National University College of Medicine, Seoul, Republic of Korea. Corresponding author: Pezhman Alavinejad. E-mail: pezhmanalavinejad@gmail.com

heparin and allopurinol[20-25]. In this regard, one of the hypothesis involves the role of active oxygen species and oxidative stress in the pathogenesis of pancreatitis by activation of inflammatory cascade and immune responses[26,27]. Based on this theory, N-acetyl cysteine (NAC) as an anti-oxidant agent inhibits inflammatory intermediates and oxidative stress and potentially could prevent pancreatitis[28]. Despite unsuccessful experiments with the intravenous form of this drug[29,30], a Randomized Controlled Trial (RCT) as pilot study in 2013 found that oral NAC could be effective for prevention of PEP[11]. Based on results of this study and according to the low price, safety profile, and negligible adverse effects of this drug, the current study as a multicenter multinational Randomized Controlled Trial was designed to evaluate efficacy of oral NAC in comparison with rectal indomethacin and placebo for prevention of PEP.

METHODS

During a 6-month period, from September 2020 to February 2021, all of the patients who met standard indications for ERCP in seven referral centers of four countries and had no contraindications for participating in the study were included. Exclusion criteria included the presence of uncontrolled diabetes mellitus, admission due to established pancreatitis before ECRP, unwillingness to undergo ERCP, serum Triglyceride >1000 mg/mL, and anatomical changes to the stomach from previous surgeries.

Before enrolling to the study, a description of the study protocol and potential hazards were given to all patients according to the Declaration of Helsinki and all of the participants were requested to sign an informed consent and then they were randomly assigned to four groups to receive either 1200 mg oral NAC in 150 cc water (group A), indomethacin suppository 100 mg (group B), 1200 mg oral NAC in 150 cc water plus indomethacin suppository 100 mg (group C) or 150 cc water as placebo (group D) 2 hour before ERCP. Randomization was performed by computer and random numbers chain (each center 80 cases). The primary outcomes were the rate and severity of any PEP among participants. The study was approved by Ethical Committee of Ahvaz Jundishapur University of Medical Sciences (IR.AJUMS.HGOLESTAN.REC.1399.120) and registered in the Iran Clinical Trial Registration site as IRCT20201222049798N1; 29-12-2020.

An algorithm was designed for this RCT (FIGURE 1). Before performing ERCP, baseline serum amylase and lipase levels were obtained from all patients. Patients took either the medication or placebo 2 h before ERCP. At 24 h after ERCP, patients' serum amylase and lipase levels were measured. Additionally, patients were examined for abdominal pain compatible with acute pancreatitis (AP) by experienced gastroenterologists. The duration of the hospital stay after procedure was also recorded. Almost all of the ERCP procedures were performed by gastroenterologists. Before or during procedure, the operators did not use any other preventive procedure or medications such as aggressive hydration or pancreatic stent. At the end of the study period, the recorded data was retained for final analysis.

The normal upper limits of amylase and lipase defined as <65 to 85 U/ml based on reference kit of each center. Pancreatitis defined as serum amylase levels >275 U/mL or serum lipase levels 3 times more than upper normal limit with the presence of abdominal pain and/or compatible imaging findings. The severity of pancreatitis is defined based on the number of hospitalized days following ERCP as mild (<4 days), moderate (4 to 10 days), or severe (>10 days).

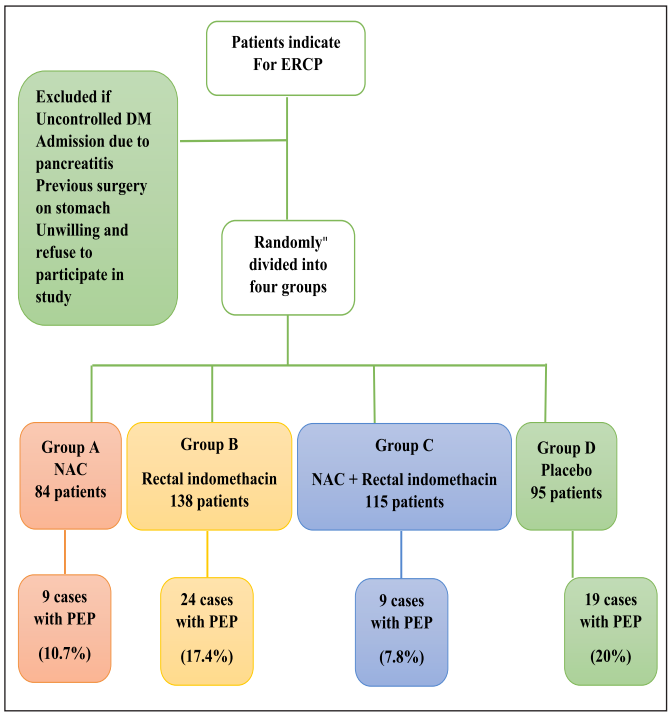

FIGURE 1. Algorithm of the study.
ERCP: endoscopic retrograde cholangiopancreatography; DM: diabetes mellitus; NAC: N Acetyl Cysteine; PEP: post ERCP pancreatitis.

This was a double-blind study; neither the patient nor ERCP assistant was informed about the treatment assignment. During the study, the operators managed and recorded the presence of any ECRP related adverse events, including hemorrhage, perforation, and/or cholangitis.

Blood sampling was performed by the staff of the gastroenterology ward and the serum samples were sent to one standard laboratory.

For interpretation and data analysis, the variables were first determined and defined by statistical methods (tables and charts). To determine the relation between quantitative and qualitative variables we used chi-square test. *P*-values less than 0.05 were considered significant. Data was analyzed by SPSS software version 20 (IBM, USA). The primary outcome of this study was to decrease the rate of post-ERCP pancreatitis. The secondary outcomes included decreasing the duration of hospital stay after ERCP and prevention of any morbidity and or mortality.

RESULTS

Overall, a total of 432 patients were included (average age 57.3 y, range 16 to 99, 41.4% male) during the study period. The demographic characters of participants are mentioned in TABLE 1. The participants were originally citizens of six countries and about 60.87% of the study population were of Caucasian descent (TABLE 2). The most common indication for ERCP was Choledocholithiasis (66.89%) (TABLE 3). The patients were randomly allocated to receive either NAC (group A, 84 case), rectal indomethacin (group B, 138 cases), NAC + rectal indomethacin (group C, 115 cases) or placebo (group D, 95 cases). The rate of bleeding and perforation after procedures was 3.94% and 2.54% respectively.

TABLE 1. Demographic characters of participants.

Group	A (NAC)	B (rectal indomethacin)	C (NAC + rectal indomethacin)	D (Placebo)
M /F ratio	35/49	53/85	53/62	40/55
Average age (range), y	57.44 (20–94)	55.36 (16–99)	56.11 (18–97)	61.53 (17–96)
CBD stone* N, %	59 (70.2%)	105 (76%)	88 (76.5%)	66 (69.4%)

M: male; F: female; N: number; NAC: N Acetyl cysteine; CBD: common bile duct. *The most common indication for endoscopic retrograde cholangiopancreatography.

TABLE 2. Ethnic background of participants.

Ethnicities	A	B	C	D	Total
Caucasian	43	96	66	58	263 (60.8%)
Asian	22	14	27	24	87 %(20.1%)
Arab	10	14	16	6	46 (10.6%)
African	4	8	2	3	17 (3.9%)
Indian	4	4	3	4	15 (3.4%)
Turk	1	2	1	0	4 (0.9%)
Total	**84**	**138**	**115**	**95**	**432**

TABLE 3. Indications for ERCP.

Acute cholangitis	16	3.70%
Ampullary Cancer	8	1.85%
Biliary obstruction	58	13.42%
Biliary colic	1	0.23%
Biliary leak	4	0.92%
CBD Dilatation	17	3.93%
CBD Stone	289	66.89%
CBD Stricture	14	3.24%
Cholangiocarcinoma	8	1.85%
Choledochal cyst	1	0.23%
Icterus	1	0.23%
Malignant obstructive jaundice	2	0.46%
Pancreatic cancer	10	2.31%
PSC	3	0.69%
Total	432	

ERCP: endoscopic retrograde cholangiopancreatography; CBD: common bile duct; PSC: primary sclerosing cholangitis.

The rate of PEP in groups A (NAC), B (indomethacin) and C (NAC + indomethacin) in comparison with D (placebo) were 10.7% (9 cases), 17.4% (24 cases), 7.8% (9 cases) vs 20% (19 cases) (P=0.08, 0.614 & 0.01 respectively). The number need to treat (NNT) for NAC, indomethacin and NAC + indomethacin were 11, 38 and 8 respectively. 49.18% of the PEP cases were mild with average duration of hospital stay 4.5 days (range 1 to 14 days) and no sever PEP happened in groups A and C (TABLE 4). The rate of abdominal pain after ERCP in groups A, B, C in comparison with D (placebo) were 28.6% (24 cases), 33.3% (46 cases), 19.1% (22 cases) vs. 27.4% (26 cases) (P=0.85, 0.33 & 0.15 respectively). Average duration of hospital stay after ERCP in groups A, B, C in comparison with D were 3.6 days, 2.6 days, 2.8 days vs. 3.7 days (P=0.396, 0.010 & 0.012 respectively).

TABLE 4. Relative prevalence of PEP in study groups based on severity.

Mild PEP	2 (22.2%)	15 (62.5%)	3 (33.3%)	10 (52.6%)	30 (49.18%)
Moderate PEP	7 (77.7%)	6 (25%)	6 (66.6%)	7 (36.8%)	26 (42.62%)
Severe PEP	0 (0%)	3 (12.5%)	0 (0%)	2 (10.5%)	5 (8.19%)
Total	9	24	9	19	61

PEP: post ERCP pancreatitis; ERCP: endoscopic retrograde cholangiopancreatography.

In head to head comparison, the efficacy of NAC, rectal indomethacin and combination of NAC + rectal indomethacin for prevention of PEP were 46.5%, 13% and 61% more than placebo respectively and NAC and NAC + rectal indomethacin were 38.5% and 55.2% more effective than rectal indomethacin.

DISCUSSION

Pancreatitis is the most common serious ERCP complication which depend on several factors[1,3,31-33]. Some of these factors are patient specific (eg, age, sex), while the others are related to the procedure itself or endoscopist experience[1]. Several agents have been proposed for the pharmacologic prophylaxis of PEP, mostly directed toward amelioration of the inflammatory cascade that accompanies and potentiates AP[27]. Accordingly, one of the supposed agents with controversial results is NAC due to its anti-oxidant and anti-inflammatory properties[28]. While most of the frustrated experiences with this medicine had applied to its intravenous form such as Katsinelos et al. study in 2005[30], or combination of oral and intravenous form by Milewski et al. in 2006[29], a pilot study by Alavinejad et al. in 2013 revealed promising results for prevention of PEP[11]. The results of this study shown a reduction in the rate of PEP in the treated group compared with the placebo group (10% vs 28%, P=0.02) and they concluded oral NAC could be useful for prevention of PEP and explained the different results because of differences in the mode of NAC prescription as oral solution or intravenous formula. The limitation of this study is that it was a pilot one and performed as a single center.

So the current study was designed as multicenter multinational RCT to evaluate not only efficacy of oral NAC but also to compare its usefulness with rectal indomethacin as one of the most widely used medications for this indication[16,34,35]. Based on our findings the rate of PEP among those who received NAC or combination of NAC + rectal indomethacin were 10.7% and 7.8% in comparison with 20% in placebo group (P=0.08 & 0.01 respectively) and 17.4% in those who just received rectal indomethacin (P=0.175 & 0.024 respectively). So the combination of NAC + rectal indomethacin significantly reduced the rate of PEP and even NAC per se was able to decrease PEP although it was statistically non meaningful (P=0.08). NNT of NAC and NAC + rectal indomethacin were 11 and 8 respectively.

On the other hand, the average duration of hospital stay after ERCP among those who managed with NAC +rectal indomethacin was almost 1 day shorter (2.8 days vs 3.7 days) and according of average charges for each day more stay in hospital (from 400 to 5000$)[36], this combination could be cost effective. The probable explanation for the mechanism of action of NAC could be reduction of concentration of NF-kB in pancreatic ducts which was supposed by a study from Sweden[37]. They found that NAC suppressed monocytic NF-ϰB activation induced by AP and suggested

a potential therapeutic approach by restoration of the functional capacity of the immune system in AP. The mentioned NAC as an NF-ϰB inhibitor, preferentially reaching the local inflammatory foci, could be a potential future way of intervention. The role of NF-ϰB activity in induction of inflammatory cascade and primary stages of AP and its amelioration by NAC treatment has been confirmed by Axelsson et al. study[38].

These results prove NAC and specially its combination with rectal indomethacin as an effective and practical option for preventing PEP. Premedication with rectal indomethacin (group B) resulted in 24 cases with PEP (17.4%) which was similar to placebo (P=0.614) and in contrast with a systemic review and meta-analysis by Shen et al. in 2017[34]. Another systemic review by Inamdar et al. in 2017 found controversial results and reported rectal indomethacin to be protective against PEP in just high-risk patients versus placebo but not protective in average-risk patients[35]. Our findings about rectal indomethacin are in concordance with a RCT by Levenick et al. in 2016 and another meta-analysis by Dubravcsik et al. that conclude rectal indomethacin did not prevent post-ERCP pancreatitis[39,40]. Despite these controversial results about the preventive role of rectal indomethacin[41], some authors have doubts about it and desire its use to be made mandatory before ERCP[42].

The advantages of this RCT were to be a Multicenter Multinational one and participation of considerable number of cases (432 participants) with different racial descents (TABLE 2). Although our study had a limitation and as it performed during COVID-19 pandemic, some of the centers were unable to fulfill pertained number of cases because of social restrictions and decrease in number of procedures per scheduled time[43].

CONCLUSION

In conclusion, oral NAC is more effective than rectal indomethacin when compared to placebo for prevention of PEP and the combination of NAC and Indomethacin had the lowest incidence of PEP and may have a synergistic effect in prevention of PEP. This combination could also be cost effective by reducing the average time of hospital stay after ERCP.

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Authors' contribution

Conceptualization: Alavinejad P, Abravesh AA; Data curation: Alavinejad P, Tran NPN, Eslami O, Shaarawy OE, Hormati A, Seiedian SS, Parsi A, Ahmed MH, Behl NS, Abravesh AA; Formal analysis: Salman S, Sakr N, Butt AS; Funding acquisition: Alavinejad P, Abravesh AA; Investigation: Alavinejad P, Ara TF, Hajiani E, Hashemi SJ; Methodology: Alavinejad P, Abravesh AA; Project administration: Abravesh AA; Resources: Abravesh AA, Hajiani E, Vignesh S; Software: Tran QT, Salman S; Supervision: Alavinejad P, Hajiani E; Validation: Patai AV, Butt AS, Lee SH; Visualization: Tran QT; Writing-original draft: Alavinejad P, Abravesh AA; Writing-review and editing: Patai AV, Butt AS, Lee SH, Tran QT, Vignesh S, Eslami O.

Orcid

Pezhman Alavinejad: 0000-0001-6857-6151.
Nguyen-Phuong Nhu Tran: 0000-0002-8528-2304.
Omid Eslami: 0000-0003-0272-3416.
Omar El shaarawy: 0000-0002-6945-6204.
Ahmad Hormati: 0000-0002-1322-1318.
Seied Saeed Seiedian: 0000-0002-2032-2045.
Abazar Parsi: 0000-0001-8833-8759.
Mohammed Hussien Ahmed: 0000-0003-1761-3527.
Nitin Shanker Behl: 0000-0003-3998-0037.
Ali Akbar Abravesh: 0000-0003-1811-149X.
Quang Trung Tran: 0000-0001-8347-1614.
Shivakumar Vignesh: 0000-0001-5190-6222.
Saif Salman: 0000-0002-7809-1745.
Naemt Sakr: 0000-0002-4882-8451.
Tahmineh Farbod Ara: 0000-0003-4639-9769.
Eskandar Hajiani: 0000-0002-1014-1996.
Seyed Jalal Hashemi: 0000-0002-0166-9844.
Árpád V. Patai: 0000-0003-1863-5971.
Amna Subhan Butt: 0000-0002-7311-4055.
Sang Hyub Lee: 0000-0003-2174-9726.

Alavinejad P, Tran NPN, Eslami O, Shaarawy OE, Hormati A, Seiedian SS, Parsi A, Ahmed MH, Behl NS, Abravesh AA, Tran QT, Vignesh S, Salman S, Sakr N, Ara TF, Hajiani E, Hashemi SJ, Patai AV, Butt AS, Lee SH. Cisteína oral N-Acetyl versus indometacina retal para prevenção de pancreatite pós-CPRE: um ensaio controlado randomizado multinacional multicêntrico. Arq Gastroenterol. 2022. Ahead of print.

RESUMO – Contexto – Este estudo randomizado, controlado multicêntrico e multinacional foi projetado para comparar a eficácia da indometacina supositório e N-acetil cisteína (NAC) para prevenção de pancreatite pós colangiografia endoscópica. **Métodos** – Durante um período de 6 meses, todos os pacientes submetidos à CPRE em sete centros de referência foram aleatoriamente atribuídos para receber 1200 mg de NAC oral, supositório de indometacina 100 mg, 1200 mg de NAC oral mais supositório de indometacina 100 mg ou placebo 2 horas antes do procedimento. Os resultados primários foram a taxa e a gravidade de qualquer pancreatite pós procedimento (PPP). **Resultados** – Um total de 432 pacientes foram incluídos (41,4% do sexo masculino). Eram originalmente cidadãos de seis países (60,87% caucasianos). Foram alocados aleatoriamente para receber NAC (grupo A, 84 casos), indometacina retal (grupo B, 138 casos), NAC + indometacina retal (grupo C, 115 casos) ou placebo (grupo D, 95 casos). A taxa de PPP nos grupos A, B e C em comparação com o placebo foi de 10,7%, 17,4%, 7,8% vs 20% (P=0,08, 0,614 e 0,01, respectivamente). **Conclusão** – A NAC oral é mais eficaz do que a indometacina retal quando comparado ao placebo para prevenção de PPP e a combinação de NAC e indometacina teve a menor incidência de PPP e pode ter efeito sinérgico na sua prevenção de PPP. (IRCT20201222049798N1; 29/12/2020).

Palavras-chave – Pancreatite pós-CPRE; NAC; indometacina retal.

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Which was read

TABLE 4. Relative prevalence of PEP in study groups based on severity.

Mild PEP	2 (22.2%)	15 (62.5%)	3 (33.3%)	10 (52.6%)	30 (49.18%)
Moderate PEP	7 (77.7%)	6 (25%)	6 (66.6%)	7 (36.8%)	26 (42.62%)
Severe PEP	0 (0%)	3 (12.5%)	0 (0%)	2 (10.5%)	5 (8.19%)
Total	9	24	9	19	61

PEP: post ERCP pancreatitis; ERCP: endoscopic retrograde cholangiopancreatography.

Read

TABLE 4. Relative prevalence of PEP in study groups based on severity.

Severity	Group A (NAC)	Group B (supp indometacin)	Group C (NAC + supp indometacin)	Group D (placebo)	Total
Mild PEP	2 (22.2%)	15 (62.5%)	3 (33.3%)	10 (52.6%)	30 (49.18%)
Moderate PEP	7 (77.7%)	6 (25%)	6 (66.6%)	7 (36.8%)	26 (42.62%)
Severe PEP	0 (0%)	3 (12.5%)	0 (0%)	2 (10.5%)	5 (8.19%)
Total	9	24	9	19	61

PEP: post ERCP pancreatitis; ERCP: endoscopic retrograde cholangiopancreatography.

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Identification of early predictors for infected necrosis in acute pancreatitis

Mats L. Wiese[1], Steffi Urban[1], Sabrina von Rheinbaben[1], Fabian Frost[1], Matthias Sendler[1], Frank Ulrich Weiss[1], Robin Bülow[2], Marie-Luise Kromrey[2], Quang Trung Tran[1,3], Markus M. Lerch[1,4], Birgit Schauer[5] and Ali A. Aghdassi[1*]

Abstract

Background: In acute pancreatitis, secondary infection of pancreatic necrosis is a complication that mostly necessitates interventional therapy. A reliable prediction of infected necrotizing pancreatitis would enable an early identification of patients at risk, which however, is not possible yet.

Methods: This study aims to identify parameters that are useful for the prediction of infected necrosis and to develop a prediction model for early detection. We conducted a retrospective analysis from the hospital information and reimbursement data system and screened 705 patients hospitalized with diagnosis of acute pancreatitis who underwent contrast-enhanced computed tomography and additional diagnostic puncture or drainage of necrotic collections. Both clinical and laboratory parameters were analyzed for an association with a microbiologically confirmed infected pancreatic necrosis. A prediction model was developed using a logistic regression analysis with stepwise inclusion of significant variables. The model quality was tested by receiver operating characteristics analysis and compared to single parameters and APACHE II score.

Results: We identified a total of 89 patients with necrotizing pancreatitis, diagnosed by computed tomography, who additionally received biopsy or drainage. Out of these, 59 individuals had an infected necrosis. Eleven parameters showed a significant association with an infection including C-reactive protein, albumin, creatinine, and alcoholic etiology, which were independent variables in a predictive model. This model showed an area under the curve of 0.819, a sensitivity of 0.692 (95%-CI [0.547–0.809]), and a specificity of 0.840 (95%-CI [0.631–0.947]), outperforming single laboratory markers and APACHE II score. Even in cases of missing values predictability was reliable.

Conclusion: A model consisting of a few single blood parameters and etiology of pancreatitis might help for differentiation between infected and non-infected pancreatic necrosis and assist medical therapy in acute necrotizing pancreatitis.

Keywords: Acute pancreatitis, Infected necrosis, Prediction, Multivariate model, ROC analysis

Background

Acute pancreatitis is the most frequent non-malignant gastroenterological disorder leading to hospitalization in Western countries. It accounts for almost 280,000 hospital admissions in the US [1] and around 55,000 in Germany per year [2]. While the majority of patients suffers from a mild disease with an uneventful recovery, there is a severe course of acute pancreatitis in 10 to 15% of cases

*Correspondence: ali.aghdassi@med.uni-greifswald.de

[1] Department of Medicine A, University Medicine Greifswald, Ferdinand-Sauerbruch-Straße, 17475 Greifswald, Germany
Full list of author information is available at the end of the article

leading to organ or even multi-organ failure, necessity for intensive care therapy and a high mortality [3]. Besides organ failure, approximately 5 to 20% of patients develop necrotizing pancreatitis, involving the pancreas, the surrounding fatty tissue or both [4]. Necroses may cause further local complications such as compression of adjacent organs, increase of intraabdominal pressure or gastric outlet obstruction. Secondary infection of the necrotic tissue is a severe condition with increased morbidity and mortality [5] requiring antibiotic treatment or even invasive interventions [6, 7].

Diagnosis of an infected necrosis is still challenging and often it needs to be confirmed ultimately by microbiological analysis after fine-needle aspiration or even drainage, measures that have to be carried out judiciously because they also encompass a periprocedural risk [8]. Established multiparameter scores such as the Acute Physiology and Chronic Health Evaluation II (APACHE II) score [9] and the Ranson Score [10] have been used for grading disease severity and prediction of mortality. However, they are cumbersome to calculate as they need a large number of parameters requiring different time points and their predictive accuracy for infected necrosis is unclear. Several routine laboratory parameters, for instance, markers for inflammation, kidney function or hematocrit [11], have been attempted for accurate prediction of severe acute pancreatitis, development of necrosis and mortality. Despite promising potential, these measurements have to be repeated at later time points and their usability is limited when using every single parameter alone. So far predictive factors of infected pancreatic necrosis, allowing the initiation of an early and preemptive therapy to improve the outcome of acute necrotizing pancreatitis, have not been established.

Methods

Study design and patient selection

This study aimed to identify parameters associated with infected pancreatic necrosis that are already assessable in early disease, ideally at admission to hospital, and, in a second step, to derive a predictive composite metric from these parameters. In this retrospective single center study, we investigated patients with acute necrotizing pancreatitis who underwent either aspiration or drainage of a pancreatic necrotic collection. Data were retrieved from the hospital information and reimbursement data system of the University Medicine Greifswald, a tertiary medical center in northeast Germany, between January 2009 and December 2019. Diagnosis of acute pancreatitis was established by fulfilment of at least two of the following three criteria: a) abdominal pain clinically consistent with acute pancreatitis, b) elevation of serum lipase of at least three times of upper limit of normal (ULN), and c) typical signs of acute pancreatitis in imaging [12].

Potentially eligible patients were identified by the combination of a diagnosis of acute pancreatitis according to ICD-10 (K85.XX) and a therapeutic medical procedure encoded by the German procedure classification system (OPS), consisting of a contrast enhanced abdominal CT-scan (OPS 3-225) combined with endoscopic-guided fine needle aspiration (OPS 1-447, OPS 5-529) or percutaneous drainage (OPS 8-146). Presence of pancreatic or peripancreatic necroses were confirmed by two radiologists (RB, MLK) experienced in gastrointestinal imaging. Prior to data retrieval the study was approved by the local institutional review board of the University of Greifswald (registration no. BB 138/19) that waived requirement for patient's informed consent.

Patient's medical history

For each patient data on age, sex, etiology of acute pancreatitis, history of alcohol and nicotine consumption was extracted from medical records. Vital and blood parameters as well as the APACHE II score [9] were recorded at the time point of admission to our institution. Relevant co-existing disorders were subsumed in the Charlson Comorbidity Index (CCI) [13]. Previous antibiotic treatment before intervention of the necrosis was noted for every patient. Length of hospital stay as well as the requirement of both intensive and intermediate care treatment were recorded. For patients being transferred from another hospital, length of the previous stay was included in the calculation of total hospital stay.

Diagnosis of infected necrosis, systemic complications, and mortality

Patients with suspected pancreatic necrosis and clinical suspicion of an infection underwent either endoscopic ultrasound-guided fine needle aspiration or direct drainage of the necrotic cavity, which was performed by a transmural or percutaneous approach. Infection of pancreatic necrosis was diagnosed microbiologically by Gram staining and culture of biopsy material for bacteria or fungi. In case of multiple interventions, pancreatic necrosis was classified as infected when there were signs of an infection in at least one sample.

Systemic organ complications included cardiovascular, respiratory, or renal failure. Cardiovascular failure was defined as a decrease of systolic or mean arterial pressure to less than 90 mmHg or 60 mmHg, respectively, irresponsive of fluid administration [14]. Respiratory failure was considered in case of need for mechanical ventilation and renal failure as an increase of serum creatinine by at least $1.5 \times$ ULN from baseline according to the Kidney Disease Improving Global Outcomes classification [15].

In addition, mortality of patients due to acute pancreatitis or its complications was recorded.

Statistical analysis

Data were analyzed using SPSS Statistics 27 (IBM, Ehningen, Germany). To test for differences between groups, two-tailored t-test or Kruskal–Wallis test were used for normally or non-normally distributed continuous variables, respectively. Differences in categorical variables were assessed by χ^2- or Fisher's exact test, in case of cells with an expected frequency of less than five. The association of laboratory parameters with infected pancreatic necrosis was tested by applying a binary logistic regression model.

For development of a prediction model for infected necrosis we performed stepwise logistic regression analyses. A forward stepwise procedure was used to select the independent variables with highest predictive value for inclusion in the final multivariable model. Variables initially considered for inclusion comprised routine blood parameters, vital parameters, comorbidities, medication, etiology of acute pancreatitis, age, sex, and BMI. Variables significantly associated with infected necrosis were added to the model in a stepwise manner according to their predictive value, indicated by pseudo R^2 values, i.e. Nagelkerke's R^2 and Cox & Snell R^2, until no further improvement of the model was achieved. Receiver operating characteristic (ROC) analysis was then performed to compare predictive performance of the model with single parameters. To identify the optimal cut-off value, Youden's J statistic [16] was calculated. *P*-values of < 0.05 and < 0.001 were considered to be significant and highly significant, respectively.

Results

Patient selection and characteristics

Between 2009 and 2019 a total of 2,410 patients with diagnosis of acute pancreatitis (K85.XX) were admitted to our hospital. Among them 705 received an abdominal CT-scan (OPS 3-225) and in 89 patients there was either an acute necrotic collection or walled-off necrosis that were treated by either fine needle aspiration (OPS 1-447), endoscopic or percutaneous drainage (OPS 5-529). Only fine needle aspiration was performed in 14 patients, whereas 75 individuals underwent drainage therapy. In total, 59 subjects had an infected necrosis whereas no growth of bacteria or fungi was detected in the other 30 patients (Fig. 1). In the majority of patients with infected necroses (81.4%) diagnosis was established by the first intervention. Proof of microbial infection by the second or third intervention was given 13.6% and 5.1% of the cases. Patients with infected pancreatic necrosis did not differ from those with sterile necrosis regarding age, sex, BMI, smoking status, location of necrosis, CCI, as well as the prevalence of diabetes mellitus or exocrine insufficiency (Table1). Regarding etiology, patients with sterile necrosis were more likely to have acute on chronic pancreatitis ($p = 0.028$), although these numbers were rather low compared to other causes of acute pancreatitis. Alcoholic etiology tended to be more common in patients with infected necrosis ($p = 0.051$). APACHE II score at admission was significantly higher in infected than in sterile necrosis ($p = 0.001$). Regarding the size of pancreatic necrosis we classified their extent into areas of $< 30\%$, 30–50%, and $> 50\%$ as described by Balthazar et al. [17] (Additional file 1: Table S1). For all three categories the distribution of the necrotic areas was similar showing no differences between patients with sterile and infected necroses ($p = 0.426$).

Microbial composition of infected necrosis

In the majority of patients with infected pancreatic necrosis multiple microorganisms were detected. Gram-positive bacteria were found in 43 (72.88%) of individuals, among them *Enterococcus faecium* was predominant. In 30 infected necroses gram-negative bacteria could be identified and the three most common bacteria were *Escherichia coli*, *Pseudomonas aeruginosa*, and *Klebsiella*, found in 9, 8, and 6 patients, respectively. Moreover, 26 infected necroses contained fungal pathogens, with *Candida albicans* as the most common species. Results are summarized in Additional file 2: Table S2.

Association of infected necrosis with clinical outcome

A comparison of outcome parameters between patients with infected and sterile pancreatic necrosis is presented in Table 2. Patients with infected necrosis more frequently developed both renal and respiratory failure ($p = 0.002$ and $p < 0.001$, respectively). In addition, the percentage of patients requiring intensive care unit (ICU) or intermediate care (IMC) treatment was significantly higher in those with infected necrosis ($p = 0.001$ and 0.017, respectively). While, median length of hospital stay was almost twice as long in infected necrosis (54 vs. 28 days, $p < 0.001$), there was no significant difference in mortality between the two groups ($p = 0.432$).

Association of infected necrosis with blood parameters

Nine out of 20 blood parameters analyzed were significantly associated with infected pancreatic necrosis (Table 3). These comprised calcium, creatinine, urea, albumin, total leukocyte count, total bilirubin, C-reactive protein (CRP), prothrombin time, and lactate dehydrogenase. While most parameters were available in at least 90% of the patients, other parameters, not taken on a routine basis, e.g. interleukin-6 and procalcitonin were

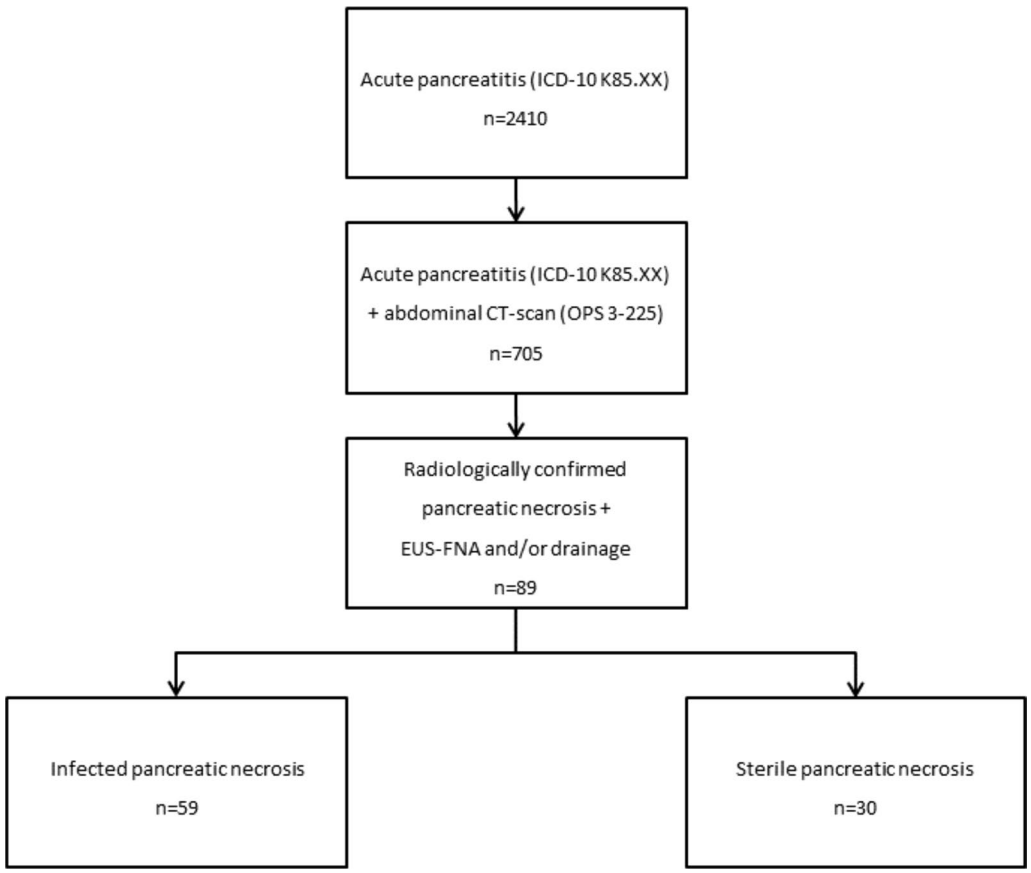

Fig. 1 Flowchart describing the patient identification and selection process

measured in less than 60%. The strongest associations with infected pancreatic necrosis were seen for creatinine (OR [95% CI] 1.019 [1.005–1.033], $p < 0.001$), CRP (OR [95% CI] 1.009 [1.004–1.014], $p < 0.001$), and albumin (OR [95% CI] 0.914 [0.861–0.970], $p = 0.002$).

Prediction model for infected necrosis

To develop a predictive model for an early detection of infected pancreatic necrosis, a multivariate analysis was performed. Details of the final prediction model are presented in Table 4. Besides creatinine, CRP, and albumin, the final model also included alcoholic etiology as a predictor. Cox & Snell R^2 and Nagelkerke's R^2 values of 0.360 and 0.502, respectively, indicated good model fit. Iterations of model development including the complete list of parameters that were considered are provided as supplementary material (Additional file 3: Table S3).

Model performance

In a next step, ROC curves were plotted to assess both the performance of each single laboratory result and a combination of the aforementioned parameters to predict the presence of an infected necrosis. The results of ROC analysis are shown in Fig. 2. With an AUC of 0.819 the prediction model achieved greater AUC than creatinine, CRP, or albumin, respectively (Fig. 2a) and also surpassed performance of the APACHE II score, a widely accepted assessment tool for disease severity and mortality (Fig. 2b). Besides, despite the unavailability of single parameters in 12 patients, the prediction model reached an AUC of 0.754 when applied to the entire patient collective (Fig. 2c). With a sensitivity of 0.692 (95%-CI [0.547–0.809]) and a specificity of 0.840 (95%-CI [0.631–0.947]) we identified a value of 0.25 as the ideal cut-off point.

Discussion

Infected necrosis is a severe complication of acute pancreatitis that usually arises during the later phase of pancreatitis. In this study, we identified parameters associated with infection of necrosis in acute pancreatitis. Based on these findings, we developed a logistic regression model based on blood levels of creatinine, albumin, and CRP, as well as alcoholic etiology that predicts

Table 1 Characterization of the patient cohort

	Infected necrosis (n = 59)	Sterile necrosis (n = 30)	*p*-value[a]
Mean age (± SD), years	59.37 (± 15.05)	55.97 (± 15.26)	0.318
Sex (male), n (%)	48 (81.4)	24 (80.0)	0.878
Median BMI (IQR)[b], kg/m^2	26.00 (3.80)	25.00 (5.00)	0.271
Smoking, n (%)	23 (54.8)	11 (55.0)	0.986
Etiology of acute pancreatitis, n (%)			
Alcohol	24 (40.7)	6 (20.0)	0.051
Biliary	15 (25.4)	12 (40.0)	0.157
Acute on chronic pancreatitis	2 (3.4)	5 (16.7)	0.028
Post ERCP	2 (3.)	1 (3.3)	0.989
Other, including idiopathic	16 (27.1)	6 (20.00)	0.462
Localization of necrosis, n (%)			
Pancreatic head	31 (52.5)	11 (36.7)	0.156
Pancreatic body	35 (59.3)	16 (53.3)	0.589
Pancreatic tail	36 (61.0)	23 (76.7)	0.140
Peripancreatic	11 (18.6)	2 (6.7)	0.130
Prior antibiotic therapy	25 (42.4)	3 (10.0)	0.002
Median APACHE-2 Score (IQR)[c]	10.00 (9.00)	5.00 (5.00)	0.001
Median Charlson Comorbidity Index (IQR)	4.00 (3.00)	2.00 (4.00)	0.099
Diabetes mellitus, n (%)	18 (30.5)	7 (23.3)	0.476
Exocrine insufficiency, n (%)	14 (23.7)	3 (10.0)	0.119

[a] Significant differences between groups were tested using two-tailed t-test for normally distributed continuous variables, Kruskal–Wallis test for non-normally distributed continuous variables, and χ^2- test or Fisher's exact test for categorical variables

[b] Infected necrosis (n = 40), sterile necrosis (n = 23)

[c] Infected necrosis (n = 50), sterile necrosis (n = 28)

Table 2 Outcome parameters in infected and sterile necrosis

	Infected necrosis(n = 59)	Sterile necrosis(n = 30)	*p*-value[a]
Respiratory failure [need for mechanical ventilation], n (%)	25 (42.4)	3 (10.0)	0.002
Cardiovascular failure [systolic blood pressure < 90 mmHg or mean arterial pressure < 60 mmHg], n (%)[b]	2 (3.4)	0 (0.0)	0.292
Renal failure [creatinine > 1.5 × ULN of baseline], n (%)	28 (47.5)	3 (10.0)	< 0.001
Requiring ICU treatment, n (%)	34 (57.6)	6 (20.0)	0.001
Requiring IMC, n (%)	41 (69.5)	13 (43.3)	0.017
Mortality, n (%)	5 (8.5)	1 (3.3)	0.432
Median Length of hospital stay, days (IQR)	54 (60)	28 (25)	< 0.001

[a] Significant differences between groups were tested using Kruskal–Wallis test for non-normally distributed continuous variables, and χ^2- test or Fisher's exact test for categorical variables

[b] Infected necrosis (n = 50), sterile necrosis (n = 27)

infection with higher accuracy than any individual laboratory parameter or the APACHE II score.

The parameters we finally included in our prediction model are coherent with existing literature on prediction of the course and complications in acute pancreatitis. For instance, CRP, an acute-phase reactant, has been shown repeatedly to predict severity of acute pancreatitis—although there has been debate about the optimal time point and cut-offs [18, 19]. Moreover, CRP had a good prognostic accuracy not only for severe acute pancreatitis but also pancreatic necrosis and in-hospital mortality [20]. Prognostic value has also been found specifically regarding development of secondary infections in acute pancreatitis [21].

Likewise, regarding creatinine, there is evidence that elevated levels in early disease can predict pancreatic necrosis [22, 23]. It is conclusive that creatinine also predicts secondary infection of pancreatic necrosis as it

Table 3 Association of blood parameters with infected pancreatic necrosis

	n	Odds ratio	95%-CI	Cox & snell R^2	Nagelkerke's R^2
Sodium	89	0.999	0.911–1.095	0.000	0.000
Potassium	89	0.932	0.424–2.050	0.000	0.000
Calcium	88	0.130	0.016–1.047	0.054	0.075
Creatinine	89	1.019	1.005–1.033	0.162	0.225
Urea	88	1.190	1.040–1.363	0.107	0.149
Albumin	78	0.914	0.861–0.970	0.116	0.162
Total leukocyte count	89	1.094	1.013–1.181	0.067	0.093
Total thrombocyte count	89	0.998	0.996–1.001	0.016	0.023
Hematocrit	89	0.339	0.001–170.683	0.001	0.002
Lipase	82	1.000	0.997–1.003	0.000	0.000
Bilirubin	88	1.028	0.993–1.064	0.056	0.076
C-reactive protein	88	1.009	1.004–1.014	0.159	0.220
Procalcitonin	52	0.992	0.941–1.047	0.001	0.002
Interleukin 6	41	1.000	0.999–1.001	0.009	0.014
Prothrombin time	89	0.976	0.956–0.997	0.060	0.084
Total triglycerides	50	1.085	0.835–1.409	0.008	0.012
pH value	71	0.002	0.000–8.218	0.037	0.055
Lactate	71	1.138	0.831–1.558	0.015	0.022
Lactate dehydrogenase	63	1.238	1.015–1.510	0.108	0.155
Blood glucose	80	1.062	0.941–1.199	0.013	0.018

Table 4 Multivariate logistic regression model for prediction of infected pancreatic necrosis

Predictor	Regression coefficient	Standard error	Wald X^2	*p*-value	Odds ratio	95%-CI
Creatinine	0.026	0.010	6,478	0.011	1.026	1.006–1.047
Albumin	− 0.066	0.045	2.151	0.142	0.936	0.858–1.022
Alcoholic etiology	1.759	0.765	5.295	0.021	5.808	1.298–25.992
C-reactive protein	0.006	0.003	3.287	0.070	1.006	1.000–1.013
Constant	− 1.504	1.579	0.907	0.341	0.222	–

Cox & Snell R^2: 0.360Nagelkerke's R^2: 0.502

indicates impaired renal function and potential subsequent renal failure, which we found to be associated with infected necrosis.

Inclusion of albumin in the final prediction model was not an unexpected finding. Serum albumin has been found predictive of persistent organ failure in acute pancreatitis in multiple studies [24, 25]; and in our analysis it was linked to infected pancreatic necrosis as well. Being independently associated with both inflammation and a compromised nutritional status in acute conditions [26], hypoalbuminemia—by the very same mechanisms—could not only predispose to organ failure but also infection of pancreatic necrosis.

Although the role of etiology on course and progression of acute pancreatitis has been discussed controversially for a long time, recent findings support the relevance of alcoholic etiology for prediction of infected pancreatic necrosis. A recent meta-analysis found necrosis to be more common in alcoholic than biliary pancreatitis [27]—the two most common etiologies in acute pancreatitis. Additionally, evidence has accumulated from experimental studies that alcohol increases intestinal permeability and thus facilitates translocation of both bacteria and bacterial products [28] that could elicit infection of pancreatic necrosis.

A number of multiparameter predictors have been evaluated for prediction of adverse outcome in acute pancreatitis [29]. The APACHE II system is one of the most widely used severity scores for critically ill patients, which incorporates both markers of patient physiology recorded immediately or shortly after hospital admission and chronic comorbidity categories. Due to these known relations, we evaluated this score regarding a potential link to infected necrosis as well.

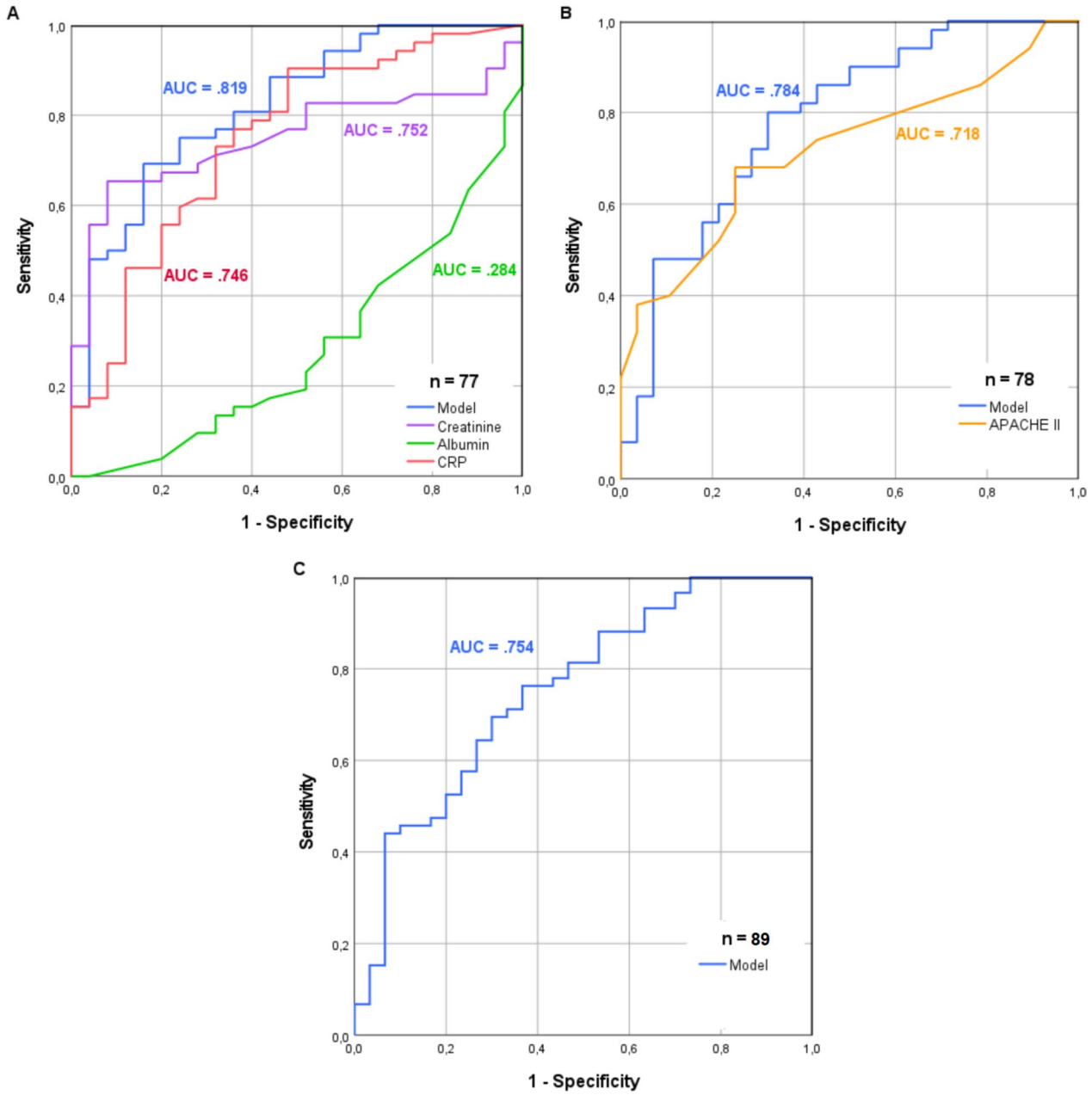

Fig. 2 Receiver operating characteristic curves. Performance of the predictive model in comparison to **a** single laboratory parameters, **b** the APACHE II score, and **c** applied to the entire patient collective

There was an association of APACHE II score with infected pancreatic necrosis. However, our model outperformed it. Considering that the APACHE II score is not specific for acute pancreatitis and requires multiple items that in part are laborious to record, such as parameters for blood oxygenation, its usefulness for prediction of infected necrosis seems to be limited in clinical practice [28].

In an earlier study, Chen et al. [30] used a similar approach to develop a prediction model for infection of pancreatic necrosis. Their final model included different parameters than ours. However, these aberrant findings do not necessarily contradict our results. First, etiologies of acute pancreatitis differed in the two cohorts. We had more alcoholic than biliary pancreatitis, which was the most common cause apart from hyperlipidemia in the

study by Chen and co-workers. In addition, the studies were conducted in two different countries and findings in Asian populations cannot be transferred unrestrainedly to Western populations and vice versa [31]. We also included patients presenting with acute on chronic pancreatitis, which were excluded in the other study. Further, we investigated a wider a range of clinical and laboratory parameters and included, for instance, albumin, which we found to be an independent predictor of infected necrosis.

One must also consider parameters that have been suggested as predictors of infected necrosis before but did not contribute to prediction in the current study. For instance, higher median procalcitonin (PCT) concentrations have been found in patients with infected necrosis and a complicated course of acute pancreatitis resulting in death [32]. In our patients, overall mortality was as low as 6.7% percent, which could explain why we did not find an association. Besides, earlier findings suggest that PCT is not a specific marker of infected necrosis as it is also elevated in septic patients without pancreatitis [33]. Moreover, it has been hypothesized that PCT levels in acute pancreatitis are elevated as part of the systemic inflammatory response and therefore not necessarily indicate infection [34].

Blood urea nitrogen (BUN) has been reported with alleged predictive value as a rise in blood urea nitrogen within 48 h was associated with a risk for the development of infected pancreatic necrosis [35]. Although we found an association between BUN and infected pancreatic necrosis as well, the association was weaker than with other parameters and inclusion of BUN did not further improve the prediction model. Besides analyzing BUN at a single time point, a high correlation with creatinine, another indicator of renal function and the strongest single predictor of infected necrosis in our study, could explain why BUN was not included in our final prediction model.

There are limitations to our study. These are partly owed to its retrospective and monocentric design, including incomplete patient data and blood values as well as assessment of blood parameters only at time of admission. Therefore, there is a residual chance that we missed relevant parameters, especially those that show a dynamic during the course of diseases. On the contrary, our results realistically reflect the situation in clinical practice. It can be cumbersome and costly to monitor the course of multiple, potentially not routine blood parameters over a longer time. Hence, our prediction model likely presents a more feasible approach. However, it needs to be emphasized that its predictive performance has not been validated prospectively so far. A prospective trial will be necessary to confirm the validity of our model developed from the retrospectively collected data. Another limitation of our analysis is that we also included patients transferred from external hospitals. This may include that treatment of acute pancreatitis at least during the early phase was not uniform in all cases because local expertise varies in smaller district hospitals. In addition, time between actual onset of pain and hospital admission could vary leading to an inhomogeneous patient cohort regarding stage of pancreatitis. Although only individuals with microbiologically proven infection were included in our study there is a risk of false positive or negative results even after microbiologic analysis of the necrotic material which have been reported in up to 15% and 25% of cases, respectively [36]. In addition, the number of actually infected necroses might be lower as secondary infections might occur not only after percutaneous but even after endoscopic guided drainages of pancreatic necrotic collections and repeated necrosectomies. For further clarification of microbial transmissions rates into drained necroses additional studies will be necessary. The putative low number of patients with sterile necrosis (n = 30) in this investigation resulted from the fact, that only individuals with proven negative results on microbial culture were selected, even after repeated biopsies. Due to the selection of patients who have undergone intervention we observed a larger proportion of individuals with infected necrosis than reported in previous studies [37]. Under some circumstances a primarily conservative therapeutic strategy based on solely antibiotic treatment and drainage only if unavoidable, can be as effective as an immediate drainage therapy in terms of mortality [38]. Because suspected infected necroses could not be captured by ICD-10 codes, we have potentially missed patients with infected pancreatic necrosis who neither underwent EUS-FNA nor drainage for our model. Last, some patients may have responded to prophylactic antibiotic treatment that was given empirically without prior microbial confirmation and therefore did not develop infected necrosis. Nevertheless, the chance that predictive performance of our model was hampered by such treatment response is rather low as an infected necrosis was detected in almost 90% of patients receiving antibiotics.

Conclusions

We could develop a prediction model for identification of infected necrosis in acute pancreatitis. It might help to avoid overhasty interventions on pancreatic necrosis in situations when infections are suspected. Including only four parameters, already assessable in early disease, our model could facilitate clinical decision-making in treatment of acute pancreatitis. We therefore

encourage use of this model in future prospective studies to validate its clinical relevance.

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Abbreviations
APACHE II: Acute physiology and chronic health evaluation; BUN: Blood urea nitrogen; CCI: Charlson comorbidity index; CI: Confidence interval; CRP: C-reactive protein; ICD-10: International classification of diseases-10; ICU: Intensive care unit; IMC: Intermediate care; OPS: Operation and procedure code; PCT: Procalcitonin; ROC: Receiver operating characteristic; ULN: Upper limit of normal.

Supplementary Information

The online version contains supplementary material available at https://doi.org/10.1186/s12876-022-02490-9.

Additional file 1. **Table S1.** Model development with complete list of parameters.

Additional file 2. **Table S2.** Microbial composition of infected necrosis (n = 59).

Additional file 3. **Table S3.** Model development with complete list of parameters.

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Author contributions
MLW, SU, FF, MML, BS, and AAA planned the study. Data were acquired by MLW, SU, RB, MLK, TQT, and AAA. MLW, SU, SR, FF, RB, MLK, FUW, BS, MML, and AAA analyzed and interpreted the data. MLW and AAA drafted the manuscript. All authors revised the manuscript critically for important intellectual content. All authors read and approved the final manuscript.

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Availability of data and materials
The datasets used and/or analysed during the current study are available from the corresponding author on reasonable request.

Declarations

Ethics approval and consent to participate
The study was approved by the local ethics committee of the University of Greifswald (registration no. BB 138/19) that waived requirement for patient's informed consent. The study was conducted in accordance with the ethical principles related to the Declaration of Helsinki.

Consent for publication
Not applicable.

Competing interests
The authors declare that they have no competing interests.

Author details
[1]Department of Medicine A, University Medicine Greifswald, Ferdinand-Sauerbruch-Straße, 17475 Greifswald, Germany. [2]Institute of Diagnostic Radiology and Neuroradiology, University Medicine Greifswald, Greifswald, Germany. [3]Department of Internal Medicine, University of Medicine and Pharmacy, Hue University, Hue City, Vietnam. [4]Ludwigs-Maximilians University Munich, Munich, Germany. [5]Institute for Community Medicine, University Medicine Greifswald, Greifswald, Germany.

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Publisher's Note

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Infection of (Peri-)Pancreatic Necrosis Is Associated with Increased Rates of Adverse Events during Endoscopic Drainage: A Retrospective Study

Fabian Frost [1,*], Laura Schlesinger [1], Mats L. Wiese [1], Steffi Urban [1], Sabrina von Rheinbaben [1], Quang Trung Tran [1,2], Christoph Budde [1], Markus M. Lerch [1,3], Tilman Pickartz [1,†] and Ali A. Aghdassi [1,†]

[1] Department of Medicine A, University Medicine Greifswald, 17475 Greifswald, Germany
[2] Department of Internal Medicine, University of Medicine and Pharmacy, Hue University, Hue 530000, Vietnam
[3] Ludwig Maximilian University Hospital, Ludwig Maximilian University of Munich, 81377 Munich, Germany
* Correspondence: fabian.frost@med.uni-greifswald.de; Tel.: +49-3834-86-7230
† These authors contributed equally to this work.

Abstract: Pancreatic necroses are a major challenge in the treatment of patients with pancreatitis, causing high morbidity. When indicated, these lesions are usually drained endoscopically using plastic or metal stents. However, data on factors associated with the occurrence of failure or adverse events during stent therapy are scarce. We retrospectively analyzed all adverse events and their associated features which occurred in patients who underwent a first-time endoscopic drainage of pancreatic necrosis from 2009 to 2019. During the observation period, a total of 89 eligible cases were identified. Adverse events occurred in 58.4% of the cases, of which 76.9% were minor (e.g., stent dislocation, residual lesions, or stent obstruction). However, these events triggered repeated interventions (63.5% vs. 0%, $p < 0.001$) and prolonged hospital stays (21.0 [11.8–63.0] vs. 14.0 [7.0–31.0], $p = 0.003$) compared to controls without any adverse event. Important factors associated with the occurrence of adverse events during endoscopic drainage therapy were positive necrosis cultures (6.1 [2.3–16.1], OR [95% CI], $p < 0.001$) and a larger diameter of the treated lesion (1.3 [1.1–1.5], $p < 0.001$). Superinfection of pancreatic necrosis is the most significant factor increasing the likelihood of adverse events during endoscopic drainage. Therefore, control of infection is crucial for successful drainage therapy, and future studies need to consider superinfection of pancreatic necrosis as a possible confounding factor when comparing different therapeutic modalities.

Keywords: stent; LAMS; WON; ANC; interventional EUS; pancreatitis

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1. Introduction

Acute pancreatitis is a non-malignant disease representing one of the most important gastrointestinal disorders leading to hospital admissions, with an increasing frequency over the past decades [1]. The most common triggers are excessive alcohol consumption and pancreatic duct obstruction by gallstones. Other risk factors include genetic, autoimmune, or metabolic diseases [2]. Pancreatic necroses are a feared complication in patients with pancreatitis as they lead to significant morbidity and mortality [3]. The Atlanta Classification [4] defines areas of necrosis as acute necrotic collections (ANC) until four weeks after the initial pancreatitis episode. Thereafter, these lesions usually develop a thickened wall, thus being named walled-off necrosis (WON). Drainage of pancreatic necrosis is indicated when complications occur such as infection, severe pain, or continuous enlargement of the lesion, causing obstruction of the gastric outlet or biliary obstruction [5]. Treatment of these lesions has changed dramatically within the last two decades, with an open surgical approach largely being replaced by endoscopic transmural drainage or minimally invasive surgical necrosectomy, as studies have shown significantly reduced

mortality and high technical success rates with these methods [6]. Recent randomized controlled trials (RCTs) even showed that an endoscopic step-up approach resulted in lower rates of complications and shorter hospital stays when compared to a (minimally invasive) surgical step-up approach [7,8]. Other recently published RCTs [9,10] have found similar technical success rates when comparing an endoscopic with a laparoscopic drainage approach, while again, the endoscopic approach resulted in shorter hospital stays in one of the reports [10]. Therefore, endoscopic treatment currently represents the treatment modality of choice when drainage of pancreatic necrosis is indicated. The basic principle of endoscopic drainage is the creation of an orifice that connects the lesion with the gastrointestinal tract. Typically, a lumen-apposing metal stent (LAMS) or (multiple) plastic stent(s) is placed into the orifice to avoid its occlusion. This procedure also allows for repeated necrosectomies as needed. Typical challenges during endoscopic treatment can include stent dislocation, stent obstruction, systemic bacterial translocation, acute or delayed bleeding, residual lesions, or, in rare cases, intraabdominal perforations. The individual factors that contribute to the development of these adverse events are still not fully understood. In the present study, we retrospectively analyzed all cases where patients underwent a first-time endoscopic drainage of a pancreatic necrosis at a tertiary care hospital in the period from 2009 to 2019, analyzing the rates of adverse events during endoscopic drainage therapy and associated patient or treatment characteristics.

2. Materials and Methods

2.1. Study Participants and Phenotype Data

This retrospective single-center (University Medicine Greifswald) study analyzed all cases of first-time endoscopic drainage therapy of pancreatic necrosis in the period from January 2009 to December 2019. Potentially eligible cases were identified through a database search for endoscopic pancreatic drainage therapy in the hospital data administration system. The study was approved by the local institutional review board (registration no. BB 138-19).

Patients' treatment data were extracted from medical records and laboratory charts. An age-adjusted Charlson Comorbidity Index [11] was calculated to summarize relevant comorbidities. Laboratory parameters were documented from the first 24 h of the patient's admission. When any of the variables could not be obtained, they were considered as missing data. The location and diameter of the lesions were obtained from the available imaging data (computed tomography, magnetic resonance imaging, or endoscopic ultrasound) and radiologists' reports. The duration of drainage therapy was determined from the stent placement until its extraction. Cases of (undocumented) spontaneous dislocation or loss to follow-up were considered as missing data. Pancreatic necroses were considered to be infected in cases where positive necrosis culture results were found. Single-shot antibiotics were also considered when determining the rate of antibiotic treatment. The variable 'necessity for repeat interventions' only summarized those interventions that were needed to treat adverse events and did not include regularly scheduled necrosectomies. Systemic inflammatory response syndrome (SIRS) after drainage was assigned if at least two SIRS criteria were positive [12]. Adverse events were considered minor in cases of simple stent dislocations; stent obstructions; residual lesions/unsuccessful drainage; SIRS after drainage responding to antimicrobial therapy within 72 h without necessity for intermediate or intensive care treatment; minor bleeding without shock or necessity of blood transfusion; or buried stent. Major complications were (intraabdominal) perforations, bleeding events with shock and/or necessity of blood transfusion, or any complication that resulted in intermediate or intensive care treatment. For determination of mortality rates, patients' records were assessed up to six months after endoscopic drainage therapy or the patient's last visit.

2.2. Endoscopic Drainage of Pancreatic Necrosis

In all cases, endoscopic ultrasound-guided transluminal (transgastric or transduodenal) drainage of pancreatic necrosis was performed. The complete sample comprised 84 WON and 5 ANC cases. All ANC cases had already developed a sufficiently matured wall at the time of drainage. Pancreatic necroses were identified using linear-array endoscopic ultrasound. For deployment of plastic (double) pigtail, Niti-S NAGI (TaeWoong Medical, Ilsan, Korea), or Niti-S SPAXUS stents (TaeWoong Medical), the lesions were punctured, a guidewire inserted, and a transluminal connection created using a cystotome. The stent was then inserted after balloon dilatation of the orifice. For implantation of Hot AXIOS stents (Boston Scientific, Marlborough, MA, USA), the pancreatic necroses were punctured using the electrocautery tip, the delivery catheter advanced into the collection, and the stent deployed. If necessary, the created orifice was used for repeated necrosectomies. Necrosectomies were performed on demand, taking into account the amount of necrotic material and its adhesion to the adjacent wall. Patients received either (multiple) plastic double pigtails or fully covered LAMS based on the endoscopist's choice. Among the LAMSs, the Niti-S NAGI Stent (n = 30, TaeWoong Medical, Ilsan, Korea) and the Hot AXIOS (n = 26, Boston Scientific, Marlborough, MA, USA) stents were the most common choices (numbers include repeat interventions). A Niti-S SPAXUS Stent (TaeWoong Medical, Ilsan, Korea) was used on two occasions. In two cases, several different LAMSs were used consecutively (Hot AXIOS/Niti-S NAGI Stent/Niti-S SPAXUS Stent and Niti-S NAGI Stent/Hot AXIOS). In 14 cases, LAMSs and plastic pigtail stents were used consecutively. Generally, treatment was terminated and stents removed when the lesion disappeared or showed significant size reduction and when there were no signs of uncontrolled infection.

2.3. Data Analysis

All statistical analyses were performed using 'R' (v.3.6.3) [13]. For comparison of continuous data, a two-tailed *t*-test was employed ('t.test', 'stats' package), whereas categorical data were compared using the two-tailed Fisher's exact test ('fisher.test', 'stats' package). Odds ratios (OR) and 95% confidence intervals (CI) were obtained from logistic regression models ('glm', family = 'binomial', 'stats' package) using the function 'logistic.display' ('epiDisplay' package) or 'or_glm' ('oddsratio' package) for categorical or continuous data, respectively. p-values < 0.05 were considered significant and rounded to three digits. Figures were created using the R package 'ggplot2'.

3. Results

3.1. Rates and Types of Complications during Drainage Therapy

A total of 89 patients with pancreatic necrosis who underwent endoscopic drainage therapy were identified. Treatment-associated adverse events occurred in 52 cases (58.4%, 'adverse events group'), whereas in 37 cases, no adverse events were observed ('controls'). Minor adverse events represented the majority of these incidents (76.9%), and only 23.1% were major complications. The median time interval between drainage and the occurrence of any adverse event was 11 days (1–52.5 days, first–third quartile). The most common events were stent dislocation, residual lesion after drainage therapy, and stent obstruction (Figure 1). Other observed adverse events included occurrence of SIRS after drainage, immediate or delayed bleedings, intraabdominal perforation during stent placement, stent-induced gastrointestinal pressure ulcers, or buried stent syndrome. Patients' baseline characteristics were very similar in the adverse events group when compared to controls (Table 1). No difference could be found with regard to age, sex, body mass index, type and etiology of pancreatitis, severity of comorbidities (as indicated by age-adjusted Charlson Comorbidity Index), rate of pancreatic enzyme replacement therapy, or different laboratory parameters. The only significant difference was a higher proportion of diabetics in the control group.

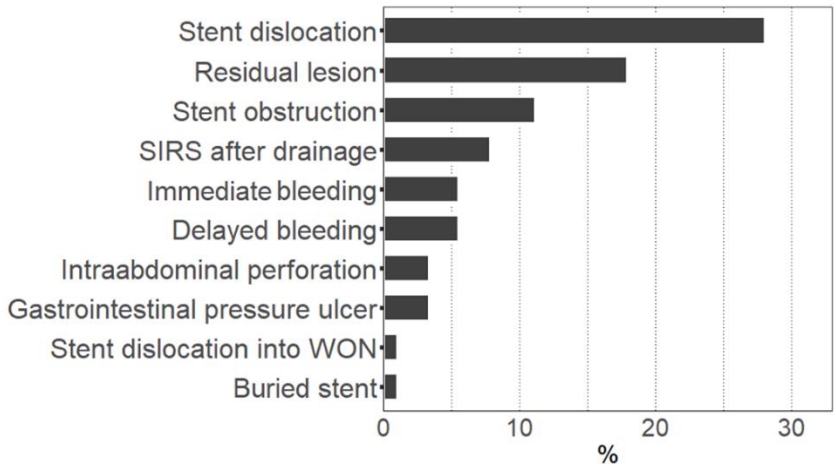

Figure 1. Rate of adverse events during endoscopic drainage therapy. Figure shows the percentage of cases with occurrence of the respective adverse event. Multiple adverse events in one case were possible. SIRS: Systemic inflammatory response syndrome. WON: Walled-off necrosis.

Table 1. Baseline phenotype characteristics of cases with adverse events and uncomplicated controls.

	Adverse Events Group (n = 52)	No Complication/ Controls (n = 37)	Missing (%)	*p*-Value
Age (years)	62.5 (46.8–69.2)	55.0 (48.0–63.0)	0	0.292
Female sex (%)	26.9	24.3	0	0.811
Body mass index (kg/m^2)	24.5 (22.5–26.9)	24.3 (22.3–26.7)	7.5	0.693
Smoking history	56.1	66.7	13.5	0.361
Etiology of pancreatitis (%)			0	0.763
Alcoholic	40.4	48.6		
Biliary	34.6	24.3		
Idiopathic	19.2	21.6		
Other	5.8	5.4		
Charlson Comorbidity Index	3.0 (2.0–5.0)	3.0 (1.0–5.0)	0	0.441
Diabetes mellitus (%)	19.2	40.5	0	0.033 *
PERT (%)	28.8	40.5	0	0.265
History of non-pancreatic malignancy (%)	7.7	5.4	0	1.000
History of abdominal surgery (%)	21.2	27.0	0	0.615
White blood cells (Gpt/L)	11.4 (8.2–18.1)	10.5 (9.0–16.0)	0	0.784
Hemoglobin (mmol/L)	7.6 (6.2–8.6)	7.5 (6.6–8.2)	0	0.916
Hematocrit (%)	37.6 (31.8–43.2)	37.0 (33.6–40.2)	0	0.994
Platelet count (Gpt/L)	278.5 (208.2–389.5)	303.0 (197.0–384.0)	0	0.746
eGFR < 60 mL/min (%)	28.8	16.2	0	0.210
Blood urea nitrogen (mmol/L)	2.3 (1.8–4.2)	2.3 (1.6–3.2)	4.5	0.168
Albumin (g/L)	26.0 (20.0–35.0)	28.0 (23.2–33.2)	18.0	0.475
Lipase (µkatal/L)	7.4 (2.7–68.2)	7.2 (2.1–16.2)	9.0	0.104
ALT (µkatal/L)	0.5 (0.3–0.8)	0.4 (0.3–0.6)	4.5	0.130
Bilirubin (µmol/L)	8.5 (5.7–13.1)	8.7 (5.7–11.4)	0	0.494
CRP (mg/L)	111.5 (10.0–196.8)	114.0 (13.8–210.8)	1.1	0.878

Continuous data are given as median (first–third quartile). Categorical variables are displayed as percentages. All values are rounded to one decimal place. * Indicates significant result ($p < 0.05$). ALT: Alanine aminotransferase. CRP: C-reactive protein. eGFR: Estimated glomerular filtration rate. n: Number of cases. PERT: Pancreatic enzyme replacement therapy.

3.2. Treatment Characteristics of Adverse Events Cases and Controls

When comparing the treatment characteristics between the adverse events group and controls (Table 2), we found infection of pancreatic necrosis (6.1 [2.3–16.1], OR [95% CI], $p < 0.001$) as indicated by a positive culture, as well as a higher lesion maximum diameter (1.3 [1.1–1.5], OR [95% CI], increment 1 cm, $p < 0.001$), to be associated with adverse

events during endoscopic drainage therapy (Figure 2). More specifically, a lesion diameter > 10 cm was linked to an OR of 4.6 (1.8–11.9; 95% CI, $p = 0.001$) for the occurrence of any adverse event. When including a positive culture and lesion maximum diameter in one model using age, sex, and diabetes mellitus as covariates, a positive necrosis culture ($p = 0.002$), as well as a maximum diameter ($p = 0.001$), remained significantly associated with the occurrence of adverse events during endoscopic drainage therapy. The presence of bacteria in areas of pancreatic necrosis showed the strongest association with adverse events such as stent dislocation ($p < 0.001$), residual lesion ($p = 0.002$), and stent obstruction ($p = 0.010$) (Figure 2A). In cases with immediate or delayed bleeding incidents, 77.8% showed pancreatic necrosis infections, but that rate was not significantly ($p = 0.061$) higher compared to 38.9% in the controls. A larger maximum diameter of the lesion was associated with delayed bleeding ($p = 0.007$), residual lesion ($p = 0.004$), or stent dislocation ($p = 0.002$) (Figure 2B). The adverse events group's initial hospital stays also had a longer duration ($p = 0.003$). No significant difference could be found with respect to the type of stent being used (plastic or LAMS, Table 3). A repeat intervention was necessary in 63.5% of the cases with adverse events and was performed endoscopically in the majority of cases. Additional radiological percutaneous drainage or intervention was needed in 28.8% of all cases, and a surgical approach in 9.6% of all cases. The mortality was higher in the adverse events group when compared to the controls (15.4% vs. 5.4%); however, this was not significant. One patient in the adverse events group died due to a fatal delayed bleeding incident 11 days after LAMS placement. Apart from this single case, there was no treatment-associated mortality observed in this cohort.

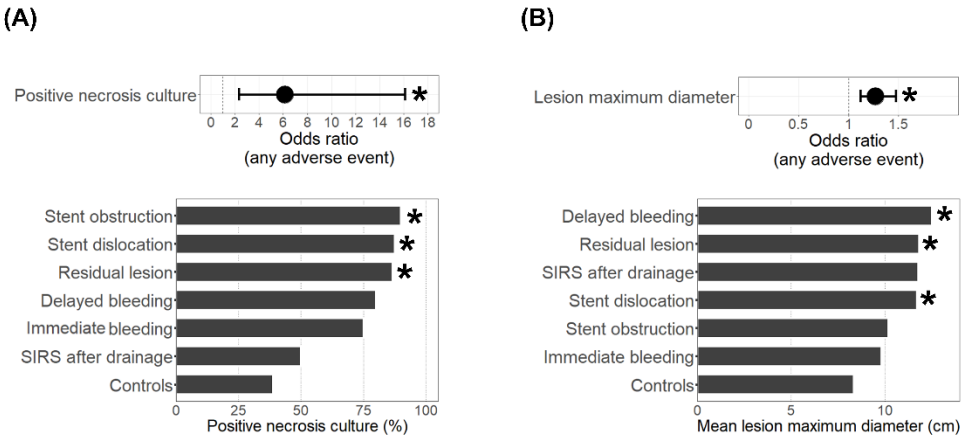

Figure 2. Treatment characteristics of adverse events cases. Shown are the odds ratios (95% confidence interval) for the occurrence of any adverse event (**top**) and the rates of positive necrosis cultures (**A**) or the mean lesion maximum diameter (**B**) in cases with the respective adverse event (**bottom**). * Indicates a significant ($p < 0.05$) difference compared to the controls.

Table 2. Treatment characteristics of cases with adverse events and uncomplicated controls.

	Adverse Events Group (n = 52)	No Complication/ Controls (n = 37)	Missing (%)	*p*-Value
Indication for drainage (%, multiple possible)				
Suspected infection	51.9	48.6	0	0.831
Pain (only)	19.2	18.9	0	1.000
Continuous enlargement of lesion	13.5	16.2	0	0.767
Gastric outlet obstruction	11.5	13.5	0	1.000
Biliary obstruction	1.9	5.4	0	0.568
Other	11.5	2.7	0	0.232
Type of stent used for initial treatment (%)			0	0.384
Plastic pigtail stent(s)	46.2	35.1		
LAMS	53.8	64.9		

Table 2. *Cont.*

	Adverse Events Group (n = 52)	No Complication/ Controls (n = 37)	Missing (%)	*p*-Value
Type of lesion (%)				0.645
WON	96.2	91.9	0	
ANC	3.8	8.1	0	
Location of lesion (%, multiple possible)				
Head	30.8	27.0	0	0.814
Body	59.6	45.9	0	0.281
Tail	50.0	59.5	0	0.398
Lesion maximum diameter (cm)	10.9 (8.4–15.1)	7.6 (6.0–10.0)	0	<0.001 *
Necrosis culture: positive results (%)	79.6	38.9	4.5	<0.001 *
Blood culture: positive results (%)	26.3	21.1	36.0	0.754
Antibiotic treatment (%)	100.0	97.3	0	0.416
Highest level of care (%)				0.054
Intensive care unit	48.1	27.0	0	
Intermediate care	26.9	24.3	0	
Regular ward	25.0	48.6	0	
Endoscopic necrosectomy performed (%)	53.9	48.7	0	0.671
Interval (days) between initial drainage and first necrosectomy	6.5 (3.8–11.0)	3.5 (2.2–5.0)	0	0.077
Necessity for repeat interventions (%, multiple possible)	63.5	0	0	<0.001 *
Endoscopic	44.2	-		
Interventional radiology	28.8	-		
Surgical	9.6	-		
Duration of initial hospital stay (days)	21.0 (11.8–63.0)	14.0 (7.0–31.0)	0	0.003 *
Duration of endoscopic drainage (days)	65.0 (47.8–103.2)	64.5 (51.2–129.0)	9.5	0.853
Total mortality (%)	15.4	5.4	0	0.185
Therapy-related mortality (%)	1.9	0	0	1.000

Continuous data are given as the median (first–third quartile). Categorical variables are displayed as percentages. All values are rounded to one decimal place. * Indicates a significant result ($p < 0.05$). ANC: Acute necrotic collection. n: Number of cases. LAMS: Lumen-apposing metal stent. WON: Walled-off necrosis.

Table 3. Comparison of adverse events frequency between lumen-apposing metal stent (LAMS) and plastic stent usage.

	LAMS (n = 55)	Plastic Stents (n = 48)	*p*-Value
Stent dislocation	21.8	29.2	0.496
Residual lesion	10.9	22.9	0.118
Stent obstruction	12.7	4.2	0.170
SIRS after drainage	3.6	10.4	0.247
Immediate bleeding	3.6	4.2	1.000
Delayed bleeding	7.3	2.1	0.369
Other rare complications	10.9	6.2	0.498
Complication-associated fatality	1.8	0	1.000
Any adverse event	49.1	54.2	0.694

Categorical variables are displayed as percentages. All values are rounded to one decimal place. n: Number of cases. SIRS: Systemic inflammatory response syndrome.

4. Discussion

We analyzed patient and treatment characteristics of cases with adverse events during endoscopic transluminal drainage therapy of pancreatic necrosis as compared to controls without adverse events. Although the overall rate of adverse events was high (58.4%), most of them (76.9%) were minor and could be treated endoscopically and/or by radiological intervention. Patients' baseline characteristics were very similar in both groups, with no differences in age, sex, body mass index, etiology of pancreatitis, or severity of comorbidities. However, the most prominent factor associated with adverse events during endoscopic drainage therapy was positive necrosis cultures indicating superinfection. It

is believed that infection of pancreatic necrosis occurs via translocation of commensal gut bacteria. The (healthy) exocrine pancreas plays an important role for gut microbiome regulation [14,15]. A combination of intestinal dysbiosis in patients with pancreatitis [16,17], local and systemic immunosuppression, and a disturbed barrier function can promote the translocation of gut bacteria into areas of necrosis, as has been shown in a rodent model [18]. Consequently, the microorganisms identified in pancreatic necroses belonged largely to the intestinal gut flora (e.g., *Enterococcus faecium*, *Candida albicans,* or *Escherichia (E.) coli*, Figures S1 and S2) and were similar to those previously identified in other studies [19,20]. Apparently, the presence of these microorganisms has a negative effect on the success of the endoscopic drainage therapy. Cases with stent obstruction, stent dislocation, or residual lesions after drainage showed especially high rates of positive culture results. Cases with bleeding incidents showed higher rates of pancreatic necrosis infections compared to controls (77.8% vs. 38.9%); however, this was not significant. Higher rates of stent obstruction could be explained by microbial overgrowth of the stent surface by agglutinative bacteria and microbial biofilm development, as has already been shown for obstructed biliary stents [21]. A range of Gram-negative or Gram-positive bacteria or yeast such as *C. albicans* possess the capability for biofilm development and agglutination [22,23]. Similarly, biofilm development may impair drainage of pancreatic necrosis in the cavity itself, leading to higher rates of residual lesions. Stent dislocation, on the other hand, could be the result of microbe-induced inflammation, impairing wound healing and loosening the stent fixation in the cavity. In an in vitro model, *E. coli*-derived cytotoxic necrotizing factor type 1 impaired intestinal epithelial wound repair after an experimental mechanical trauma [24]. The bacterial endotoxin lipopolysaccharide (LPS), which is the major component of the outer membrane of Gram-negative bacteria such as *E. coli*, leads to impaired blood flow and a proinflammatory immune response, which resulted in insufficient healing of gastric ulcers in the rat model [25]. Other bacteria that are frequently found in necrosis isolates such as *Enterococcus faecium*, *Staphylococcus aureus*, and *Pseudomonas aeruginosa*, or LPS more generally, have also been shown in vitro and/or in vivo to secrete compounds that interfere with the host's immune response or epithelial cell migration and may, therefore, impair wound healing [26,27]. Higher rates of (delayed) bleeding events in cases with pancreatic necrosis infection may similarly result from increased inflammation in consecutively impaired wound healing and increased erosion of blood vessels.

As a second factor associated with adverse events during endoscopic drainage therapy, we identified a larger pancreatic necrosis diameter. This likely reflects the consequence of more severe states of the disease and increased technical difficulty in stent placement due to the anatomical position.

Although the basic phenotype characteristics were very similar between the adverse events group and controls, the latter included a larger proportion of diabetics. An explanation could be the typically increased rates of reduced exocrine pancreatic function [28] in diabetics, possibly resulting in reduced (auto-)proteolytic activity and inflammation during pancreatitis within the area of necrosis, leading to less adverse events. Of note, adding diabetes mellitus as a covariate to the regression model had no relevant impact on the significance of the association of infected pancreatic necrosis or lesion maximum diameter with the occurrence of adverse events.

There is still ongoing debate on the (non-)superiority of LAMSs compared to plastic stents in the drainage of pancreatic necrosis. A retrospective study [29] observed higher rates of residual lesions after drainage therapy with plastic stents. Likewise, in the present study, residual lesions were observed in 22.9% of cases when plastic stents were used, but only in 10.9% when LAMSs were used. However, this difference was not significant. Stent obstruction was (again not significantly) more common when LAMSs were used, but endoscopists may have underreported stent obstruction when (thinner) plastic stents were used as it is less easy to detect. The only fatal therapy-associated complication was a major delayed erosion bleed 11 days after LAMS placement, which underlines the risk of rare severe bleeding events when LAMSs are being used [30]. Regarding other

adverse events, no apparent difference between LAMS and plastic stent usage could be detected. Retrospective studies have the inherent limitation that the initial presentation of the collection may bias the endoscopist's choice between a plastic stent or LAMS, which influences the outcome. In one randomized controlled trial (RCT) that included 31 WON patients with LAMS and 29 with plastic stent treatment, no significant difference (except for the duration of the procedure) could be found [31]. This is further supported by a recent meta-analysis that found no difference in the occurrence of adverse events between LAMSs and plastic stents in the treatment of WON when only including studies with EUS-guided drainage [32]. Further RCTs rather, than retrospective studies, are needed to investigate whether newly developed LAMSs or plastic stents are superior, or whether the two stent types' treatment quality is equal. Moreover, the performance of the different stent types may also depend on the lesion's composition. The choice of LAMS or plastic stents could be made according to the amount of necrotic debris in the target lesion, with LAMS used only for lesions with a large proportion of solid components. This approach has been applied in a recently published RCT [10], achieving similar success rates compared to a laparoscopic drainage approach.

Despite the thorough retrospective analysis, this study has some limitations. First, as this is a single-center study, the total sample size is limited, and in particular, smaller differences between the groups associated with rarer adverse events could have escaped detection. Second, the usage of culture results to determine infection of pancreatic necrosis has its limitations as diverse microbial communities and anaerobic bacteria cannot be reliably detected. This would require the usage of next-generation sequencing techniques, which are still not a part of the clinical routine.

To summarize, our data show that infection of pancreatic necrosis is the most significant factor associated with adverse events during endoscopic transluminal drainage therapy. Apart from optimizing diagnosis and treatment of the infection itself, the data indicate a potential to optimize stents for deployment in areas of infection, e.g., by using antimicrobial coatings. Similar approaches are currently under development for stent therapy in the biliary tract [33,34]. Whether such an approach can be translated into the treatment of pancreatic necrosis, however, needs to be investigated in further experimental studies. Moreover, future studies that aim to compare different methods of endoscopic drainage or stent therapy need to consider infection with pancreatic necrosis as a possible confounding factor.

Supplementary Materials: The following supporting information can be downloaded at: https://www.mdpi.com/article/10.3390/jcm11195851/s1, Figure S1: Necrosis culture results, Figure S2: Comparison of necrosis culture results between controls and adverse events cases.

Author Contributions: Planning and concept of study: F.F., M.M.L., T.P. and A.A.A. Acquisition of data: L.S., M.L.W., S.U., S.v.R., Q.T.T. and F.F. Statistical analysis: F.F., L.S., and M.L.W. Data interpretation and manuscript revision: F.F., L.S., M.L.W., S.v.R., Q.T.T., C.B., A.A.A., M.M.L. and T.P. Writing committee: F.F., T.P. and A.A.A. All authors have read and agreed to the published version of the manuscript.

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Role of Bile Acids and Bile Salts in Acute Pancreatitis

From the Experimental to Clinical Studies

Quang Trung Tran, MD,† Van Huy Tran, MD, PhD,† Matthias Sendler, PhD,* Julia Doller, MS,* Mats Wiese, MS,* Robert Bolsmann, MS,* Anika Wilden, MS,* Juliane Glaubitz, MS,* Jana Marielle Modenbach, MS,* Franziska Gisela Thiel, MS,* Laura L. de Freitas Chama, PhD,* Frank Ulrich Weiss, PhD,* Markus M. Lerch, MD, PhD,* and Ali A. Aghdassi, MD**

Abstract: Acute pancreatitis (AP) is one of the most common gastroenterological disorders leading to hospitalization. It has long been debated whether biliary AP, about 30% to 50% of all cases, is induced by bile acids (BAs) when they reach the pancreas via reflux or via the systemic blood circulation.

Besides their classical function in digestion, BAs have become an attractive research target because of their recently discovered property as signaling molecules. The underlying mechanisms of BAs have been investigated in various studies. Bile acids are internalized into acinar cells through specific G-protein–coupled BA receptor 1 and various transporters. They can further act via different receptors: the farnesoid X, ryanodine, and inositol triphosphate receptor. Bile acids induce a sustained Ca^{2+} influx from the endoplasmic reticulum and release of Ca^{2+} from acidic stores into the cytosol of acinar cells. The overload of intracellular Ca^{2+} results in mitochondrial depolarization and subsequent acinar cell necrosis. In addition, BAs have a biphasic effect on pancreatic ductal cells. A more detailed characterization of the mechanisms through which BAs contribute to the disease pathogenesis and severity will greatly improve our understanding of the underlying pathophysiology and may allow for the development of therapeutic and preventive strategies for gallstone-induced AP.

Key Words: acinar cells, acute pancreatitis, bile acids, Ca^{2+}, gallstone

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Acute pancreatitis (AP) is one of most common gastroenterological disorders leading to hospital admission with an increasing incidence over the last 20 years.[1] Around 10% to 15% of patients suffer from a severe form of the disease with local complications, (multi-)organ failure, and a high mortality. There is still no specific treatment, and management is based on symptomatic and supportive therapy. Migrating gallstones are one of the most common causes for AP, accounting for 30% to 50% of cases[2,3] in many countries.

Pancreatitis is believed to begin in pancreatic acinar (exocrine) cells, which are highly susceptible to pathological extracellular stimuli[4,5] and in which digestive proteases, initially trypsin, undergo activation.[6] The balance between activation[7] and degradation[8] of digestive enzymes by lysosomal hydrolases appears to determine the extent of cellular injury. Germline mutations in the human trypsinogen (*PRSS1*) gene[9] support the concept of autodigestion as an initiating factor. Whether or not the disease subsequently takes a severe course[10] or progresses to chronic pancreatitis[11] depends on a variety of factors[12] and is hard to predict on admission. Bile and bile acids (BAs) have been implicated in the cellular pathogenesis of pancreatitis.[13] Whether and to what extent they are involved will very much affect the search for potential treatment strategies directed against bile BA-mediated events.[14]

In humans, BAs are synthesized primarily from cholesterol and are conjugated in the liver with glycine or taurine. After being secreted into the duodenum, they are converted to secondary BAs by intestinal bacteria, reabsorbed, and finally recycled via the enterohepatic circulation.[13,15,16] In 1848, the first BA, cholic acid, was discovered, and others were subsequently identified as described by Wieland in his Nobel lecture in 1928.[17] There have been outstanding advances in the biochemistry and the clinical application of BAs during the last decades.[16]

It was recently revealed that BAs are not only essential for food digestion but also significantly contribute to either the pathogenesis or the treatment of various gastrointestinal disorders including chronic liver diseases,[18,19] disorders of the biliary tract,[20] and diabetes mellitus.[21]

The role of BAs in pancreatitis has been investigated in a number of studies. However, the molecular mechanism of BA-mediated effects is not yet fully understood.[3,22] Remaining questions are whether and how BAs enter the acinar cell and which molecular mechanisms are responsible for cellular injury. Here, we review studies that have investigated the role of BAs in pancreatitis and their effect on different cells of the pancreas. Results from both experimental and clinical studies were included.

To this end, an extensive literature search was conducted using the following key words: "bile acids," "pancreatitis," "pathogenesis," "animal experiment," and "clinical study" in different combinations based on patient, intervention, comparison, outcome model searching strategy.

EFFECTS OF BAs ON PANCREATIC CELLS

Bile acids derive from the cholesterol molecule and are amphiphilic substances being both hydrophilic and lipophilic. The

From the *Department of Medicine A, University Medicine Greifswald, Greifswald, Germany; and †Department of Internal Medicine, Hue University of Medicine and Pharmacy, Hue University, Hue, Vietnam.

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Address correspondence to: Ali A. Aghdassi, MD, Department of Medicine A, University Medicine Greifswald, Ferdinand-Sauerbruch-Straße, D-17475 Greifswald, Germany (e-mail: ali.aghdassi@med.uni-greifswald.de).

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two most abundant primary BAs in humans are cholic acid and chenodeoxycholic acid (CDCA). They are metabolized to the secondary BAs lithocholic acid and deoxycholic acid by intestinal bacteria via 7-dehydroxylase.[13] Usually, BAs are conjugated with taurine or glycine to form at least 8 different types of BAs. In rodents, taurine conjugation is predominant; therefore, the major BAs in mouse bile are taurocholic acid (TCA) and taurodeoxycholic acid.[23] Bile acids with less hydroxy groups are subjected to sulfation and glucuronidation, which are necessary for their detoxification. Bile acids are biosynthesized via classical (neutral) or alternative (acidic) pathways (Fig. 1). The neutral pathway, which is initiated by CYP7A1, predominates in BA biosynthesis of adults and begins with 7-hydroxylation of cholesterol. The alternative pathway of BA biosynthesis begins via the cytochrome P450 enzyme CYP27A1, followed by oxysterol 7α-hydroxylase.[24,25]

Mechanisms by Which BAs Affect Acinar Cells

Effects of BAs on Calcium Signaling and Endoplasmatic Reticulum

Bile-induced acinar cell injury is attributed to both detergent (surfactant) and nondetergent characteristics of BAs. Detergent mechanisms of BAs lead to an elevation of intracellular calcium concentration ($[Ca^{2+}]i$), mitochondrial membrane depolarization, and a subsequent intracellular adenosine triphosphate depletion and are most often caused by taurolithocholic acid-3-sulfate (TLCS), a monohydroxyl BA. The nondetergent effect of BAs includes an activation of phosphatidylinositol 3-kinase (PI3K) leading to a pathological stimulation of digestive zymogen activation, cellular injury, and death in pancreatic acinar cells.[26,27] These damaging effects are mediated by an increase of $[Ca^{2+}]i$, too, demonstrating that both detergent and nondetergent effects of BAs are acting synergistically. Taurolithocholic acid-3-sulfate initiates Ca^{2+} transients and calcium signals are localized near secretory granules in the apical region of acinar cells. The ability of BAs to induce a sustained calcium release into the cytosol of pancreatic acinar cells adds an important new aspect to the mechanisms of bile-induced AP.[28] In physiological states, the calcium shift into the endoplasmatic reticulum (ER) is mediated by sarco/endoplasmic reticulum Ca^{2+} (SERCA) ATPase, also termed SERCA pumps. Inhibition of these pumps, that is, by PI3K, facilitates BA-induced Ca^{2+} responses and increases acinar cell damage. Vice versa, a pharmacological inhibition of PI3K, which can be achieved by LY-294002, activates SERCA, leading to a Ca^{2+} reuptake into the ER reversing the BA-induced increase of $[Ca^{2+}]i$.[29]

During the BA-induced Ca^{2+} release from the ER, both ryanodine receptors (RyRs) and inositol 1,4,5-trisphosphate receptors (IP_3Rs) are activated. Both receptors belong to the family of Ca^{2+} channels and are located in the ER membrane that will be discussed in the following section (Pancreatic Receptor Proteins and Transporters of BAs).[30,31] Sarco/endoplasmic reticulum Ca^{2+} is found to a minor extent near acidic stores in the secretory granule compartment, that usually contain digestive zymogens.[26] A calcium release from this compartment further increases the cytosolic calcium concentration and is most frequently observed when acinar cells are already damaged like in an ischemic situation.[32]

The G-protein–coupled bile acid receptor 1 (Gpbar1) is a transmembrane cell surface receptor, and its deletion has been associated with a reduced susceptibility to gallstone disease in mice.[33] G-protein–coupled bile acid receptor 1–deficient mice also displayed a milder pancreatitis course when exposed to TLCS. This amelioration was mediated by a reduced generation of pathological calcium transients and sustained physiological calcium oscillations despite a pathological stimulus.[34]

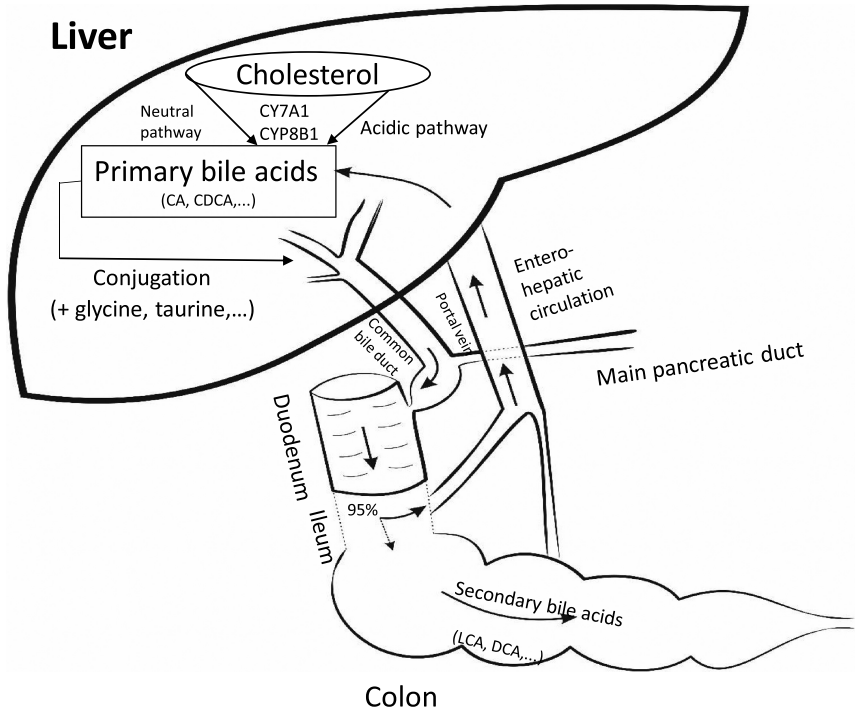

FIGURE 1. Biosynthesis and conjugation of BAs. Bile acids are primarily synthesized from cholesterol in the liver, by several enzymes, including CYP7A1 and CYP8B1 via an acidic or neutral pathway. Primary BAs are conjugated with taurine, glycine, or nonamino acid. Conjugated BAs are secreted into the bile duct and then enter the intestine via the major duodenal papilla. Most (95%) of BAs are reabsorbed in the terminal ileum and enter the enterohepatic circulation via the portal vein. The remainder will be converted to secondary BAs by bacteria in the colon and absorbed to the blood or discarded through feces. CA, cholic acid; DCA, deoxycholic acid, LCA, lithocholic acid.

Apparently, one of the main mechanisms how BAs damage acinar cells is based on a pathological increase of $[Ca^{2+}]i$ by different pathways. This pathological $[Ca^{2+}]i$ increase ultimately initiates a premature trypsinogen activation and other digestive protease activation and the necrosis of acinar cells.[35]

Effects of BAs on Mitochondria and Cytosolic Adenosine Triphosphate Distribution

Besides the ER and secretory granules, BAs directly act on a third subcellular compartment, the mitochondria. Mitochondria are essential for cellular physiology and homeostasis. Maintenance of an intact mitochondrial membrane potential is required for proper cell function. In AP, an impaired mitochondrial function contributes to both apoptosis and necrosis.[13] An [Ca2+]i overload induced by BAs causes mitochondrial depolarization that leads to a reduction of adenosine triphosphate (ATP) synthesis in acinar cells. Adenosine triphosphate is an important regulator for cellular maintenance and of physiological reactions, and its depletion causes disturbances in cellular homeostasis. The reduced availability of mitochondrial and cytosolic ATP enhances susceptibility to acinar cell injury.[36,37] Moreover, BAs prolong intracellular Ca^{2+} release resulting in an increase of reactive oxygen species production, impaired production of ATP, apoptosis, and necrosis.[38]

Effects of BAs on Nonselective Channels and Chemokine Expression

Along with an activation of Ca^{2+} influx into the cytosol, BAs also depolarize acinar cells by a cationic current through nonselective ion channels.[39] These nonselective channels in acinar cells depend on intracellular Ca^{2+} levels. It is known that acinar cells respond to secretagogs by several ways of membrane potential changes, and one of them is controlled partly by nonselective ion channels located in the basolateral membrane.[40] The main cations transported through the channels are Na^+ and K^+. The action of BAs on nonselective ion currents varies and is most effective with TLCS, to a lesser extent with taurochenodeoxycholic acid (TCDC) and TCA. Already low concentrations of TLCS (10 μM) are sufficient to induce such a cationic current. The depolarization of the transmembrane potential also contributes to the harmful effect of BAs on acinar cells.[39]

Changes in chemokine expression are observed in pathological conditions including AP.[41] Several studies that allow for standardized conditions when investigating chemokine expression profiles have been carried out in vitro. In the rat acinar cell line (AR42J), trypsinogen activation occurred concurrently with an upregulation of 23 proteins and a downregulation of 16 proteins.[42] Acinar cells treated with sodium taurocholate (NaT) showed an overexpression of chemokine ligands, including the chemokine (C-C-motif) ligand 2 that was reversed by oxidized 1-palmitoyl-2-oleoyl-sn-glycero-3-phosphocholine.[43]

Besides activation of transmembrane ion currents, BAs can also be directly transported into the acinar cells, mediated by specific membrane transporters on the luminal (NaT cotransporting polypeptide [NTCP]) and the basolateral side (organic anion transporting polypeptide). Bile acid uptake is dependent on Na^+ but can also occur in a Na^+-independent pathway.[44] In the crystal structure of the apical sodium-dependent BA transporter, TCA was built into the inward-facing perpendicular to the membrane, that is, with the cholesterol ring close to the crossover and the taurine group extending to the intracellular entrance of the cavity.[45] Bile acids traverse the cytoplasm of the epithelial cells bound to a BA-binding protein.[46,47]

Effects of BAs on Ductal and Stellate Cells

Ductal cells are of particular interest when investigating the effects of BAs on the pancreas because this cell type is firstly exposed to BAs during a potential biliary reflux. Interestingly, BAs are able to exert both harmful and beneficial effects on ductal cells depending on the type and concentration. On the one hand, CDCA caused an increase of $[Ca^{2+}]i$ in Capan-1 cells (a tumor cell line), imitating characteristics of pancreatic ductal cells. This shift in $[Ca^{2+}]i$ is a prerequisite for alterations in Cl^- and K^+ conductance that changes ductal fluid secretion.[48] The decrease of fluid flow results in the damage of ductal integrity and ductal cell injury. A limiting factor of these observations is that Capan-1 is an immortalized tumor cell line that makes a translation of results to the in vivo situation quite difficult. Presumably closer to reality are studies conducted in isolated pancreatic ductal cells from animals: high concentrations of CDCA that were added to ductal cells from guinea pigs induced an overload Ca^{2+} release, which led to ATP depletion and the loss of mitochondrial membrane potential. In contrast, a low concentration of CDCA had a protective effect on the pancreas by stimulating HCO_3- secretion.[49–51] In addition, the decrease of ductal bicarbonate secretion was also attributed by an inhibition of glycolytic and oxidative metabolism with a consequent depletion of intracellular ATP levels as shown for isolated ductal cells that were exposed to high concentrations of CDCA (1 mM).[52] Besides their effects on bicarbonate secretion, BAs also directly change the permeability of the ductal mucosal barrier[53]; while in a physiological state, the duct cell layer is nearly impermeable to even small molecules, and exposure of specific bile salts increases the permeability to molecules of at least 20,000 Da. Underlying mechanisms are a disruption of tight junctions with swelling of the intercellular space and structural alteration of ductal cell shape.[54]

Data on the effects of BAs on stellate cells are sparser. The presence of NTCP in stellate cells indicates a Na^+-dependent BA uptake mechanism. Once located in the cytoplasm, BA caused cellular damage ultimately leading to necrosis.[55] More studies are needed to gain a more comprehensive view on the effects of BAs on ductal and stellate cells.

Pancreatic Receptor Proteins and Transporters of BAs

The distribution of BA receptors in the liver, small intestine, and other organs is well characterized, but there is also expression in the pancreas (Table 1, Fig. 2). As already mentioned previously, the severity of AP was ameliorated in $Gpbar1^{-/-}$ mice after retrograde injection of TLCS but remained unaffected when the cholecystokinin (CCK) analog cerulein was used. This underlines the importance of the Gpbar1 receptor for BA-induced AP.[34] Besides its expression near the apical pole of acini, there is also evidence that Gpbar1 is expressed on the luminal side of pancreatic ductal cells, as Capan-1 cells also express this membrane receptor. G-protein–coupled bile acid receptor 1, synonymous to Takeda G-protein–coupled receptor clone 5, stimulates Na^+/Ca^{2+} exchange after exposure to BAs. Bile acids further increase an ATP release and subsequently raises the intracellular $[Ca^{2+}]$ concentration.[48] When entering the cell, BAs can interact with intracellularly located receptors. One of them is the nuclear farnesoid X receptor (FXR), which is, besides Gpbar1, the most studied BA-dependent receptor. Farnesoid X receptor is a hormone receptor with high expression in the liver but also in other organs including the pancreatic acinar cell nuclei.[56] The role of FXR in AP was studied in $FXR^{-/-}$ mice. Activation and inhibition of FXR signaling are dependent on the type of BA: conjugated BAs can activate the FXR, but CDCA and its converted products taurine- and glycine-β-muricholic acid can inhibit FXR signaling.[58] Activation of the FXR suppresses autophagy

TABLE 1. Receptors Interacting With BAs in Pancreatic Acinar Cell

Receptor/ Transporter	Localization on Acinar Cells	Effect on Severity of BA-Induced AP After Depletion or Overexpression	Candidate of Studies	Type of BA or Salt Used	Author and Publication Year
Gpbar1	Apical pole	Reduction (Gpbar1$^{-/-}$ mice)	Isolated mouse acini	TLCS	Perides et al, 2010[34]
FXR	Nuclear	Increase (overexpression of FXR after incubation with BAs)	Human and rat pancreatic cell lines	GCDC and TCA	Zhou et al, 2017[56]
RyR	SERCA	Reduction (pharmacological inhibition)	Isolated mouse acinar cells and whole mice model	TLCS	Husain et al, 2012[14]
IP_3Rs	Outer nuclear membrane	Reduction (pharmacological inhibition)	Pancreatic mouse acinar cells	TLCS	Gerasimenko et al, 2006[26]
TRPV1	Cell membrane	Reduction (Trpv1$^{-/-}$ mice)	Whole mouse model	NaT	Shahid et al, 2015[57]

GCDC indicates glycochenodeoxycholate.

rendering acinar cells more susceptible to damage caused by cellular stress.[56] The role of autophagy in acinar cell homeostasis was already shown in previous studies.[59] In addition, TCA and glycochenodeoxycholate increase the expression FXR that consequently reduces autophagy and increases acinar cell death and inflammation.[56] In mild AP, induced by the CCK analog cerulein, loss of FXR function did not affect the severity of AP. Moreover, variations in the FXR locus of humans do not seem to affect the susceptibility for pancreatitis.[60]

Ryanodine receptors are the largest known ion channels of the cell (>2 MDa) and are located in the SERCA. They exist in three isoforms and are responsible for the release of Ca^{2+} from intracellular stores. Ryanodine receptors are primarily expressed in skeletal muscles but were also found in human acinar cells.[30,61] High concentration of TCA sensitizes the RyRs for 3H-ryanodine binding and trigger a Ca^{2+} release inside acinar cells. The calcium influx is enabled by an opening of RyRs via an allosteric mechanism, which leads to a Ca^{2+} leak not only from the ER but also from

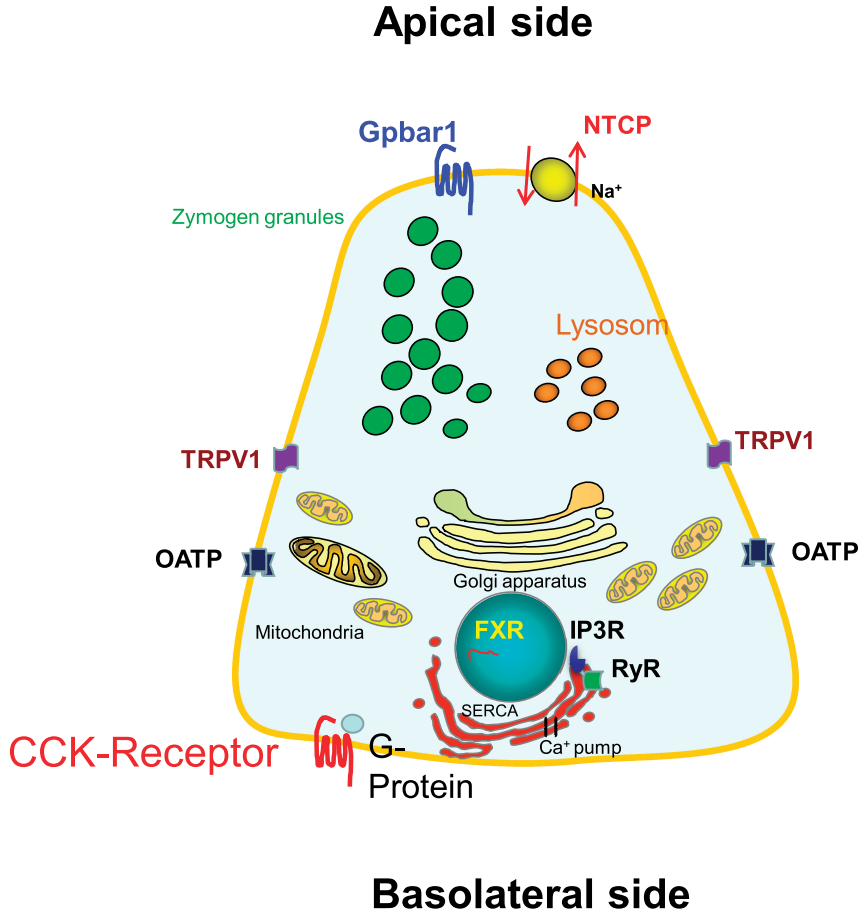

FIGURE 2. Acinar cell and the relevant BAs transporters and receptors involved in AP. Bile acids can enter the acinar cell via receptors on the membrane such as Gpbar1 and a variety of transporters. They then intracellularly act via FXR in the nuclear, and RyR and IP_3Rs at the ER. Bile acids can induce a sustained Ca^{2+} release from the ER and apical vesicles into the cytosol.

the zymogen granules in pancreatic acinar cells.[62] When the RyRs are inhibited by antagonists, such as dantrolene or ruthenium, BA-induced Ca^{2+} release in pancreatic acinar cells was significantly reduced.[26] Whereas RyRs have a more restricted distribution, IP_3Rs are expressed in the outer nuclear membrane of most cells. IP_3Rs can be inhibited by caffeine, leading to reduction of Ca^{2+} release from both the ER and the acidic pool.[26] Addition of IP3 to permeabilized pancreatic acinar cells was shown to stimulate the release of Ca^{2+} from its stores.[63]

Transient receptor potential vanilloid 1 (TRPV1) is a cellular membrane ion channel, which has been detected in a variety of organs, such as the gastrointestinal tract, brain, lungs, heart, and pancreas.[64] In the pancreas, it has been shown that TRPV1 ion channel is expressed in primary sensory nerves, and its activation plays a role in the inflammatory cascade in AP. Potential vanilloid 1 can be activated by leucotrien B4 that is increasingly secreted after exposure to BAs.[57] Infusion of leucotrien B4 into the pancreas can induce pancreatic inflammation, and the levels of leucotrien B4 in pancreatic tissue were remarkably higher in rats that underwent ligation of the confluence between common bile duct and main pancreatic duct in comparison with control rats.[65] Depletion of TRPV1 ameliorated the severity of disease in BA-induced pancreatitis after retrograde infusion of 2% NaT into the pancreatic duct as observed in $TRPV1^{-/-}$ mice or after coincubation with resiniferatoxin, an excitotoxin that desensitizes TRPV1.[57]

Types and Concentrations of BAs

Bile acids constitute the main component of bile. They may enter the pancreas by 2 different ways: either by reflux through the pancreatic duct or systemically by transport through the blood stream. Bile acids consist of conjugated and unconjugated components with different hydrophilic properties and a variable ability to enter the pancreatic cells.[66] Their concentrations are much higher in the pancreatic duct in currently used experiment models (5%) than in the serum (1–2 μg/mL).[67]

The effects of BAs on the pancreas either ex vivo on the acinar cell level or in vivo depends on their concentration. At low (micro molar) concentrations, taurine-conjugated BAs induced an intracellular Ca^{2+} rise in acinar cells, and the most effective of them was TLCS. Other natural BAs such as TCA and taurodeoxycholat are much less effective and require higher concentration than TLCS in triggering calcium signals. Moreover, the required BA concentrations are usually higher for in vivo experiments when BAs are injected retrogradely into the pancreatic duct than after direct incubation using isolated acinar cells. Regarding TLCS, the most commonly used BA, about 6- to 15-fold higher concentrations are needed for animal experiments.[34,39,68] TLCS is also a more potent inductor of mitochondrial membrane depolarization than TCDC and TCA. A TLCS concentration of 25 μM results in a membrane depolarization comparable with TCDC at a concentration of 100 μM for the same cellular effect.[36] Nevertheless, dihydroxy BAs (such as TCDC, 250 μM) and trihydroxy BAs (such as TCA, 1 mM) are found at higher concentrations in the serum than monohydroxy BAs (eg, TLCS, 25 μM). Therefore, they could substantially contribute to the membrane depolarization or Ca^{2+} influx in the event of a bile reflux.[39]

EXPERIMENTAL MODELS FOR BA-INDUCED PANCREATITIS

Duct Obstruction Model

The duct obstruction model mimics gallstone-induced AP. There are various modifications of the ligation site including a separate bile duct or pancreatic duct ligation or a ligation after their fusion as a common biliopancreatic duct. This procedure is mainly done in rodents but was also performed in dogs in the past.[69] When the confluence between the bile and pancreatic duct in rats is obstructed by ligation, necrotic AP develops with a clinical presentation resembling multiple organ failure, as is also observed in humans. This model supports the common channel hypothesis of Opie, in which bile reflux enters the pancreas and the pressure rises in the main pancreatic duct. The obstruction of the pancreatic duct seems to be a critical event causing severe necrotizing pancreatitis.[70] In some models, an additional bile duct obstruction and reflux of bile into the pancreatic duct is not an important factor because AP occurs irrespective of manipulations of the bile duct.[71] The administration of a lithogenic diet consisting of high fat and cholesterol increased the severity of necrotizing pancreatitis. This increase in severity indicates a highly cholesterol-enriched bile with consecutive cholesterol crystal formation that can further damage the pancreas.[72] Severity of AP produced by duct ligation varies among animal species (rabbits, rats, or opossums).[73,74] In the opossum, duct ligation leads to severe AP with extensive necrosis (50% acinar cell were necrotic).[71,75] On the other hand, only 10% acinar cells were necrotic in rats after 1 week after ligation of the common biliopancreatic duct.[76] Pancreatic duct ligation in rabbits showed remarkable elevation of pancreatic enzymes and damage of acinar cells, comparable with other experimental models of AP.[77] These variable results may be due to differences in the time of organ harvesting after ligation and differences in the surgical technique and protocol.[78] These may also be due to species differences in susceptibility to increased pancreatic duct pressure.

Duct Infusion Model

Physiologically, basal pancreatic duct pressure is less than 10 cm H_2O, which is equal to 7.36 mm Hg. Perfusion of fluids containing BAs can increase the basal duct pressure several times. Similar changes can result from ductal obstruction caused by spasms of the smooth muscles surrounding the pancreatic duct or shedding of cellular debris. In short, injected BAs increase pancreatic intraductal pressure by different means: one as the consequence of fluid injection and the other due to compression of the duct lumen by the edematous inflammation of the gland.[79] There is also an increase in endothelial and capillary permeability after exposure of BAs, resulting in an impairment of capillary blood flow, ischemia, and cellular necrosis.[10] The most commonly used BAs to induce severe AP via retrograde infusion are NaT and TCA.[68] An intraductal infusion of NaT (0.8 mL, 4%) significantly increased pancreatic duct permeability in rats.[80] The impairment of microcirculation was confirmed in models using pigs that received TCA infusions into the pancreatic duct and in which a substantial decrease of pancreatic oxygenation and changes in pancreatic blood flow occurred.[81] The course of AP after retrograde BA injection is quite severe, and extensive necrosis is commonly observed with a high mortality mimicking severe AP in humans.[82] In addition, the severe inflammation in the duct infusion model is accompanied by nuclear factor–κB activation and proinflammatory cytokine upregulation, indicating a key role of nuclear factor–κB in the development of the inflammatory response in BA-induced AP.[83] Bile acids also act on the epithelial ductal cells and modulate production of proinflammatory cytokines, such as interleukin (IL)-8, IL-1, and IL-6. Elevations of IL-6 were observed after retrograde infusion of NaT into the pancreatic duct.[46,67,84]

RELATED CLINICAL EVIDENCE OF BAs IN AP

Located in the retroperitoneal space, the human pancreas is relatively difficult to access for samples by biopsy. In addition, patients with AP usually consult physicians at late stage, when the initial events of AP have already passed. Therefore, investigations that address early pathophysiological events of AP in patients are limited and even rarer when considering biliary pancreatitis. In the majority of patients with gallstone-induced AP (94.4%), gallstones were also detected in their stool. This observation suggests that AP is frequently caused by migrating gallstones with only transient blockage at the ampulla of Vater.[2,85] Serum BAs were found to be elevated in all patients within the first 24 hours after the onset of acute abdominal pain, which suggests that a systemic elevation of BAs during the initial stage of biliary AP may play a role.[86] After the first day, serum concentrations start to decrease. In patients with AP of other than biliary etiology, the total BA concentrations were lower, suggesting that, apart from bilirubin determination, also serum BA concentrations might be useful for identifying a biliary origin in equivocal cases.[87]

An interaction of BAs and CCK in humans was demonstrated in patients with a tumor-induced bile duct stenosis who received CDCA added to a liquid test meal. These patients showed a negative feedback control of plasma CCK and had a lower postprandial CCK release.[88] Because CCK is known to stimulate enzyme secretion not only in rodent[35] but also in human acinar cells,[89] the modulation of CCK release by BAs seems to be a relevant event in biliary AP.

Besides the effects of BAs on human acinar cells, there is evidence that BA also acts on human pancreatic stellate cells. Isolated human stellate cells are able to incorporate BAs leading to a release of Ca^{2+} into the cytosol, similar to acinar cells. This influx of BAs is Na^{+} dependent and mediated by a sodium-dependent transport protein, the NTCP, which is different from acinar cells, where the sodium-dependent organic anion transporter is presumably the main transporter.[55,90]

POTENTIAL BENEFICIAL EFFECTS OF BAs AND TARGETED TREATMENT IN AP

Ursodeoxycholic acid (UDCA) is a constituent of human serum, which accounts for 3% of total BAs.[91] The role of UDCA, an epimer of CDCA, in AP is still controversial. Although there are reported cases of an increased susceptibility of AP due to UDCA,[92] patients who received UDCA after removal of common bile duct stones had a lower risk of stone recurrence and thus a lower risk of acute biliary pancreatitis.[93] In view of the protective effects of UDCA in animal experiments, there is currently more evidence for a protective effect of this compound in biliary pancreatitis. One limiting factor is that markedly higher UDCA doses (up to 250 mg/kg body weight) were given to animals in experimental pancreatitis than are usually given to patients.[27]

Tauroursodeoxycholic acid (TUDCA) is a conjugated derivative of UDCA having protective effects in acute biliary pancreatitis as well. Tauroursodeoxycholic acid reduced activation of intracellular trypsin and caspases, and attenuated ER stress damage. Endoplasmic reticulum stress impairs an unfolded protein response, which balances protein folding demand within organelles in physiological states and is maintained by TUDCA.[94] This cytoprotective potential of TUDCA in the exocrine pancreas was shown in single acinar cells and in animal models.[95] Further studies in humans may open new perspectives on the use of BAs as therapeutic in pancreatitis.

As mentioned previously, RyR plays an important role in pathological Ca^{2+} signaling caused by TLCS. Dantrolene, a RyR antagonist, was primarily used as a muscle relaxation agent inhibiting Ca^{2+} release by binding to RyR. In vivo models showed an amelioration of AP and propose that RyR modulators may provide a therapeutic potential for AP.[14] Other activators of RyR are ATP, calmodulin, and cyclic adenine nucleotide cyclic adenosine diphosphate ribose (cADPR). Synthesis of cADPR is dependent on ADP-ribosyl cyclase CD38. Pharmacologic inhibition of cADPR by 8-Br-cADPR reduced the intracellular Ca^{2+} signals after stimulation by TLCS. On the other hand, CD38-deficient mice were even protected from TLCS-induced pancreatitis, and acinar cell injury was decreased when using nicotinamide, an inhibitor of CD38. This indicates that pharmacologic blockade of CD38 and cADPR could be a therapeutic option in biliary pancreatitis.[96] Calcineurin inhibitory peptide was added to prevent BA-induced acinar cell injury because TLCS is able to cause calcineurin activation. Finally, the calcineurin inhibitors limited chymotrypsinogen activation.[97] Inhibition of Orai, the principal store-operated calcium entry channel, reduced local damage and systemic features of TCLS-induced AP. As a result, the Orai channel was discussed as a potential treatment target for early AP.[98] Recently, a selective inhibition of bromodomain and extraterminal protein, which reduces the interaction between histones and DNA and thus increases gene transcription, reduced pancreatic damage and systemic inflammatory response in BA-induced AP.[99]

Other potential target proteins are protein kinases. Protein kinases have been implicated in almost every mechanism of AP. Therefore, protein kinases constitute a potential target for AP. Gene therapies based on RNA interference and gene transfection have shown to be successful in the treatment of experimental AP. However, their application in clinical practice is still highly restricted. Further studies are required to develop a specific protein kinase inhibitor for the AP- targeted treatment.[100]

CONCLUSIONS

Bile acids play an important role in the pathogenesis of AP. Various experimental models mimicking AP have been developed to elucidate the molecular mechanisms by which BAs contribute to AP. Bile acids are internalized into acinar cells through specific receptors and a variety of transporters. Bile acids can induce a sustained Ca^{2+} influx from the ER at the basal portion or from an acidic pool at the apical part of the acinar cell, from where Ca^{2+} is ultimately released into the cytosol. The overload of intracellular Ca^{2+} results in mitochondrial depolarization and subsequent acinar cell necrosis. In pancreatic ductal cells, BAs have a biphasic effect on the fluid secretion in a concentration-dependent manner. Certain BAs seem even to be protective for the pancreas. Further experimental elucidation of the mechanism through which gallstones induce AP and to what extend BA contribute to this process will greatly improve our understanding of the pathophysiology and allow to design therapeutic and preventive strategies for bile-induced AP.

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Current publications:

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***Articles contributed to the dissertation*:**

1. **Tran, Q.T**.; Sendler, M.; Wiese, M.L.; Doller, J.; Zierke, L.; Gischke, M.; Glaubitz, J.; Tran, V.H.; Lalk, M.; Bornscheuer, U.T.; Weiss F.U; Lerch, M.M and Aghdassi A.A. Systemic Bile Acids Affect the Severity of Acute Pancreatitis in Mice Depending on Their Hydrophobicity and the Disease Pathogenesis. *International journal of molecular sciences* 2022, 23, 13592. doi: 10.3390/ijms232113592.
2. Alavinejad P, T.N., Eslami O, Shaarawy OE, Hormati A, Seiedian SS, Parsi A, Ahmed MH, Behl NS, Abravesh AA, **Tran QT**, ; Vignesh S, S.S., Sakr N, Ara TF, Hajiani E, Hashemi SJ, Patai AV, Butt AS, Lee SH. Oral *N*-Acetyl cysteine versus rectal indomethacin for prevention of post ERCP pancreatitis: a multicenter multinational randomized controlled trial. *Arquivos de Gastroenterologia* **2022**, *59*, Ahead of print, doi:https://doi.org/10.1590/S0004-2803.202204000-90.
3. Wiese, M.L.; Urban, S.; von Rheinbaben, S.; Frost, F.; Sendler, M.; Weiss, F.U.; Bülow, R.; Kromrey, M.L.; **Tran, Q.T**.; Lerch, M.M.; and Aghdassi, A.A. Identification of early predictors for infected necrosis in acute pancreatitis. *BMC gastroenterology* 2022, 22, 405, doi:10.1186/s12876-022-02490-9.
4. Frost, F.; Schlesinger, L.; Wiese, M.L.; Urban, S.; von Rheinbaben, S.; **Tran, Q.T**.; Budde, C.; Lerch, M.M.; Pickartz, T.; Aghdassi, A.A. Infection of (Peri-)Pancreatic Necrosis Is Associated with Increased Rates of Adverse Events during Endoscopic Drainage: A Retrospective Study. *Journal of clinical medicine* 2022, 11, doi:10.3390/jcm11195851.
5. **Tran, Q.T**.; Tran, V.H.; Sendler, M.; Doller, J.; Wiese, M.; Bolsmann, R.; Wilden, A.; Glaubitz, J.; Modenbach, J.M.; Thiel, F.G.; Weiss, F.U; Lerch, M.M and Aghdassi, A.A. Role of Bile Acids and Bile Salts in Acute Pancreatitis: From the Experimental to Clinical Studies. *Pancreas* 2021, 50, 3-11, doi:10.1097/mpa.0000000000001706.

Publications related to the dissertation's study topic:

Papers:

1. Wiese, M.; Gärtner, S.; Doller, J.; **Tran, Q.T**.; Frost, F.; Bannert, K.; Jaster, R.; Berlin, P.; Valentini, L.; Meyer, F.; et al. Nutritional management of chronic pancreatitis: A systematic review and meta-analysis of randomized controlled trials. *Journal of gastroenterology and hepatology* 2021, 36, 588-600, doi:10.1111/jgh.15230.

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3. Wiese, M.L.; Gärtner, S.; von Essen, N.; Doller, J.; Frost, F.; **Tran, Q.T**.; Weiss, F.U.; Meyer, F.; Valentini, L.; Garbe, L.-A.; et al. Malnutrition Is Highly Prevalent in Patients With Chronic Pancreatitis and Characterized by Loss of Skeletal Muscle Mass but Absence of Impaired Physical Function. *Frontiers in Nutrition* 2022, 9, doi:10.3389/fnut.2022.889489.

4. Aghdassi, A.; **Tran, Q.T**.; Bulla, T.; Bülow, R.; Ribback, S.; Lerch, M.M.; Pickartz, T. Focal pancreatic lesions in autoimmune pancreatitis and weight loss. *Gut* 2021, 70, 2065-2195, doi:10.1136/gutjnl-2020-321987.

Book chapters/invited review:

1. Ali A. Aghdassi, Mats L. Wiese, **Quang Trung Tran**, Markus M. Lerch, Acute pancreatitis associated with metabolic, infections and drug related diseases in "*The Pancreas: An Integrated Textbook of Basic Science, Medicine, and Surgery*", 4th Edition, Wiley. (submitted 12/2021)

2. Lukas Zierke, Marcel Gischke, **Quang Trung Tran**, and Ali A. Aghdassi, Neutrophil serine proteases in auto-immune and hereditary pancreatitis, in special issue: *NSPs and CatC in rare disease, Rare Disease and Orphan Drugs Journal* (submitted 10.2022).

3. Hoang-Quy Nguyen, Khanh-Luan Tran, Manh-Hung Vuong, Xuan-Dung Ho, and **Quang-Trung Tran**✉, Progress and Current Status of Targeted Therapy and Drug Resistance in Gastric and Pancreatic Cancer in *Treatment landscape of targeted therapies in oncology: Challenges and opportunities* by Elsevier, submitted 01/2023.

4. Hoang-Quy Nguyen, Khanh-Luan Tran, Manh-Hung Vuong, **Quang-Trung Tran**, Xuan-Dung Ho, Colorectal Cancer Heterogenity and Targeted Therapy: Clinical Implications, Challenges and Solutions for Treatment Resistance in *Treatment landscape of targeted therapies in oncology: Challenges and opportunities* by Elsevier, submitted 01/2023.

Abstracts (published with citable DOI)

1. **Tran, Q.T**.; Sendler, M.; Weiss, F.U.; Wiese, M.L.; Doller, J.; Glaubitz, J.; Zierke, L.; Lalk, M.; Bornscheuer, U.T.; Lerch, M.M. Tu1203: Bile acids modulate the severity of acute pancreatitis depending on their hydrophobicity and by interacting with CCK1 receptor. *Gastroenterology* 2022, 162, S-912. doi: 10.1016/S0016-5085(22)62166-8.

2. Bruns, N.; Wiese, M.L.; Meyer, F.; Bannert, K.; Sautter, L.F.; Doller, J.; Frost, F.; **Tran, Q.T**.; Weiß, F.U.; Garbe, L.-A.; Metges, C.C.; Valentini, L.; Jaster, R.; Lamprecht, G.; Gärtner, S.; Lerch, M.M.; Aghdassi, A.A., Vergleich der Verzehrsgewohnheiten von Patienten mit chronischer Pankreatitis und Leberzirrhose mit und ohne

Mangelernährung, *Aktuelle Ernährungsmedizin* 2022; 47(03): 248 – 249, DOI: 10.1055/s-0042-1748266

3. M.L. Wiese , S. Gärtner , J. Doller , F. Frost , **Q.T. Tran** , F.U. Weiß , F. Meyer , L. Valentini , L.-A. Garbe , C.C. Metges , K. Bannert , R. Jaster , G. Lamprecht , M.M. Lerch , A.A. Aghdassi, Diskrepanz zwischen der Prävalenz von reduzierter Muskelmasse und Sarkopenie bei Menschen mit chronischer Pankreatitis, *Aktuelle Ernährungsmedizin* 2022; 47(03): 228-229, DOI: 10.1055/s-0042-1748213
4. **Q.T. Tran**, M. Sendler, F.U. Weiss, J. Doller, M.L. Wiese, L. Zierke, M. Lalk, U. Bornscheuer, M.M. Lerch, and A.A. Aghdassi, Impact of systemic bile acids in acute pancreatitis depend on their hydrophobicity and may result from their interaction with CCK1 receptor, *UEG Journal*, Volume 9, Issue S8, P. 108; DOI: doi.org/10.1002/ueg2.12142
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Conference presentations

Oral presentations:

1. **Quang Trung Tran**, Matthias Sendler, Frank Ulrich Weiss, Julia Doller, Mats Wiese, Lukas Zierke, Michael Lalk, Uwe Bornscheuer, Markus M. Lerch, Ali A. Aghdassi[1], Impact of systemic bile acids in acute pancreatitis depend on their hydrophobicity and may result from their interaction with CCK1 receptor, *United European Gastroenterology Week* 2021.
2. **Quang Trung Tran,** Markus M. Lerch, Ali A. Aghdassi**,** Acute pancreatitis: current clinical practice and translational research in Germany with visions for Vietnam**,** Session Theme: “Pancreatitis and Pancreatic Cancer -Challenges in Asia” *107th General Meeting of the Japanese Society of Gastroenterology* (JSGE) will be held on April 15-17, 2021 in Tokyo, Japan.
3. **Quang Trung Tran**, Pezhman Alavinejad, Nguyen Phuong Nhu Tran, Omid Eslami, Omar El shaarawy, Ahmad Hormati, Seied Saeed Seiedian, Abazar Parsi, Mohammed Hussien Ahmed, Nitin Shanker Behl, Ali Akbar Abravesh, Shivakumar Vignesh, Seyed Jalal Hashemi Efficacy of Oral *N*-Acetyl Cysteine in Preventing Post ERCP Pancreatitis:

Results of a Randomized Controlled Trial (RCT) from 7 Referral Centers in Four Countries**,** *Asian Pacific Digestive Week (APDW)*, 8. 2021.

Poster presentations:

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