Novel insights into toxic and chronic liver disease

Citation for published version (APA):

Zoubek Aranda, M. E. (2021). *Novel insights into toxic and chronic liver disease: from molecular pharmacology to clinical practice*. [Doctoral Thesis, Maastricht University, University of Malaga]. ProefschriftMaken. <https://doi.org/10.26481/dis.20210929mz>

Document status and date:

Published: 01/01/2021

DOI:

[10.26481/dis.20210929mz](https://doi.org/10.26481/dis.20210929mz)

Document Version:

Publisher's PDF, also known as Version of record

Please check the document version of this publication:

- A submitted manuscript is the version of the article upon submission and before peer-review. There can be important differences between the submitted version and the official published version of record. People interested in the research are advised to contact the author for the final version of the publication, or visit the DOI to the publisher's website.
- The final author version and the galley proof are versions of the publication after peer review.
- The final published version features the final layout of the paper including the volume, issue and page numbers.

[Link to publication](#)

General rights

Copyright and moral rights for the publications made accessible in the public portal are retained by the authors and/or other copyright owners and it is a condition of accessing publications that users recognise and abide by the legal requirements associated with these rights.

- Users may download and print one copy of any publication from the public portal for the purpose of private study or research.
- You may not further distribute the material or use it for any profit-making activity or commercial gain
- You may freely distribute the URL identifying the publication in the public portal.

If the publication is distributed under the terms of Article 25fa of the Dutch Copyright Act, indicated by the "Taverne" license above, please follow below link for the End User Agreement:

www.umlib.nl/taverne-license

Take down policy

If you believe that this document breaches copyright please contact us at:

repository@maastrichtuniversity.nl

providing details and we will investigate your claim.

Novel insights into toxic and chronic liver disease:

From molecular pharmacology to clinical practice

Miguel E. Zoubek Aranda

**Novel insights into toxic and chronic liver disease:
From molecular pharmacology to clinical practice**

© Copyright Miguel E. Zoubek Aranda, Maastricht, The Netherlands, 2021.

Novel insights into toxic and chronic liver disease: From molecular pharmacology to clinical practice

ISBN: **978-94-6423-436-7**

Layout: **Miguel E. Zoubek Aranda**

Cover design: **Miguel E. Zoubek Aranda**

Printed by: **ProefschriftMaken || www.proefschriftmaken.nl**

The research presented in this dissertation was conducted at the following institutions: Department of Internal Medicine III, Faculty of Medicine, University Hospital of Rheinisch-Westfälische Technische Hochschule (RWTH) Aachen (Germany); Department of Clinical Pharmacology and Therapeutics, Faculty of Medicine, University of Malaga (Spain); Department of Toxicology and Pharmacology, Faculty of Health, Medicine and Life Sciences, NUTRIM School of Nutrition and Translational Research in Metabolism, Maastricht University (The Netherlands).

Novel insights into toxic and chronic liver disease: From molecular pharmacology to clinical practice

PROEFSCHRIFT

ter verkrijging van de graad van doctor
aan de Universiteit Maastricht
en University of Malaga (Spanje),
op gezag van de Rector Magnificus, Prof. dr. Rianne M. Letschert,
volgens het besluit van het College van Decanen,
in het openbaar te verdedigen op
woensdag, 29 september 2021 om 13:00 uur in Maastricht

door

Miguel E. Zoubek Aranda

Promotoren

Prof. dr. A. Bast (Maastricht University, The Netherlands)

Prof. dr. M. I. Lucena-González (University of Malaga, Spain)

Copromotoren

Dr. G. H. Koek (Maastricht University, The Netherlands)

Prof. dr. Ch. Trautwein (RWTH Aachen University, Germany)

Beoordelingscommissie

Prof. dr. O. Bekers (Maastricht University, The Netherlands; voorzitter)

Prof. dr. Th. M.C.M. De Kok (Maastricht University, The Netherlands)

Prof. dr. P. Valdivielso-Felices (University of Malaga, Spain)

Prof. dr. Z. Diaz-Cabiale (University of Malaga, Spain)

Dr. A. Licata (University of Palermo, Italy)

Prof. dr. T. Lüdde (Heinrich Heine Universität Düsseldorf, Germany)

Für meine immer geliebten Eugen und Maria

Contents

	Abbreviations.....	10
Chapter 1	General introduction.....	13
Part I	<i>Drug-induced liver injury section</i>	
Chapter 2	Ibuprofen-induced liver disease: A systematic analysis.....	65
Chapter 3	High Prevalence of Ibuprofen Drug-Induced Liver Injury in Spanish and Latin-American Registries.....	83
Chapter 4	Relevance of c-Jun N-terminal kinase (JNK) in Ibuprofen-induced acute liver injury.....	99
Chapter 5	Combined activities of JNK1 and JNK2 in hepatocytes protect against toxic liver injury.....	123
Chapter 6	Liver injury after Methylprednisolone pulses: A disputable cause of hepatotoxicity. Analysis from the Spanish Latin DILI Registry and a literature review.....	161
Chapter 7	Definition and risk factors for chronicity following acute idiosyncratic drug-induced liver injury.....	183
Chapter 8	Learning point: Three case reports on drug-induced liver toxicity due to diverse drugs and conditions.....	205

Part II *Chronic liver disease section*

Chapter 9	Reversal of liver fibrosis: from fiction to reality.....	233
Chapter 10	Therapeutic intervention against c-Jun N-terminal kinase-2 (Jnk2) ameliorates end-stage chronic liver disease.....	263
Chapter 11	Disruption of the FasL/Fas axis protects against inflammation-derived tumorigenesis in chronic liver disease.....	293

Part III *Conclusion section*

Chapter 12	General Discussion.....	319
Chapter 13	Summary.....	329
	Overzicht (NL).....	335
	Sumario (ES).....	341
	Zusammenfassung (DE).....	347
Chapter 14	Valorisatie addendum.....	357
	Dankwoord.....	365
	Curriculum Vitae.....	368
	List of publications.....	370

Abbreviations

ACEi	angiotensin-converting-enzyme inhibitor
ADR	adverse drug reaction
AGO2	argonaute 2
AH	alcoholic hepatitis
aHSC	activated hepatic stellate cell
AIH	autoimmune hepatitis
ALD	alcoholic liver disease
ALF	acute liver failure
ALP	alkaline phosphatase
ALT	alanine transferase;
AMA	anti-mitochondrial antibody
ANA	antinuclear antibodies
ANOVA	analysis of variance
Anti-LKM1	anti-liver kidney microsomal antibody type 1
AP	alkaline phosphatase
APAP	acetaminophen
ARB	angiotensin receptor blocker
ASMA	anti-smooth muscle antibodies
AST	aspartate transferase
AT ₁ R	angiotensin II receptor type 1
BDL	bile duct ligation
CB	cannabinoid receptor
CCl ₄	carbon tetrachloride
CCL5	chemokine ligand 5
CCR2	C-C chemokine receptor type 2
CCR5	C-C chemokine receptor type 5
CHOL	cholestatic
CLD	chronic liver disease
CIOMS	Council for International Organizations of Medical Sciences
COL1A1	Type I collagen alpha I
COPD	chronic obstructive pulmonary disease
COX	Cyclooxygenase
CYP2C9	Cytochrome P450, isoform 2C9
CYP2E1	Cytochrome P450, isoform 2E1
DAMPs	damage-associated molecular patterns
d	day
DEN	diethylnitrosamine
dl	decilitre
DIALF	drug-induced acute liver failure
Dicer	ribonuclease (RNase) III-like enzyme
DILI	drug-induced liver injury
DMN	dimethylnitrosamine
DMSO	dimethyl sulfoxide
dsRNA	double-stranded RNA
ECM	extracellular matrix
F	female
FACS	fluorescence-activated cell sorting

Fas ^{lpr}	mice homozygous for the lymphoproliferation spontaneous mutation
FMT	fluorescence molecular tomography
FXR	farnesoid X receptor
GLDH	glutamate dehydrogenase
GO	Graves' ophthalmopathy
GS	glutamine synthetase
GSH	glutathione
H2-DCFDA	2',7'-Dichlorodihydrofluorescein diacetate
H&E	hematoxylin and eosin
HBV	hepatitis B virus
HC	hepatocellular
HCC	hepatocellular carcinoma
HCV	hepatitis C virus
Hepa 1-6	mouse hepatoma cell line
HRP	horseradish peroxidase
HSC	hepatic stellate cell
Hsp70/72	Heat shock protein 70/72
IDILI	idiosyncratic drug-induced liver injury
IFN-β	interferon beta
INR	international normalized ratio
<i>i.v.</i>	intravenous
JNK	c-Jun N-terminal kinases
Jnk1 ^{Δhepa}	hepatocyte-specific deletion of Jnk1
Jnk2 ^{Δhepa}	hepatocyte-specific deletion of Jnk2
Jnk ^{Δhepa}	mice carrying hepatocyte-specific deletion of Jnk1 and constitutive deletion of Jnk2
Jnk1 ^{-/-}	mice carrying constitutive deletion of Jnk1
Jnk2 ^{-/-}	mice carrying constitutive deletion of Jnk2
KC	Kupffer cell
kg	kilogram
LOXL2	lysyl oxidase-like 2
LPC	liver parenchymal cell
LPS	lipopolysaccharide
LW	liver weight
LW/BW	liver to body weight ratio
MAPK	mitogen-activated protein kinase family
m ²	square meters
M	male
MCD	methionine and choline-deficient
mg	milligram
MIX	mixed
ml	milliliter
MMP	matrix metalloproteinase
MP	methylprednisolone
MS	multiple sclerosis
NAFLD	non-alcoholic fatty liver disease
NAPQI	N-acetyl-p benzoquinone imine
NASH	non-alcoholic steatohepatitis
NEMO	NF-κB-essential-modulator (IKKγ)

NEMO ^{ff}	mice with hepatocyte-specific loxP sites for the IKK γ /NEMO-modulator gene lacking active Cre recombinase
NEMO ^{Δhepa}	mice with hepatocyte-specific loxP sites for the IKK γ /NEMO gene deleted by active Cre recombinase
NF- κ B	Nuclear factor kappa-light-chain-enhancer of activated B cells
NK	natural killer cell
NKT	natural killer T-cell
NPC	non-parenchymal cell
NSAID	nonsteroidal anti-inflammatory drug
OTC	over-the-counter
PCNA	proliferating cell nuclear antigen
PCR	polymerase chain reaction
PDGF	platelet-derived growth factor
PE	phosphatidylethanolamine
PFA	paraformaldehyde
pJnk	phosphorylated JNK
qPCR	quantitative real-time PCR analysis
R	ratio calculated as (ALT/ULN)/(AP/ULN)
RAS	renin-angiotensinsystem
RIPK1	receptor-interacting protein kinase 1
RIPK3	receptor-interacting protein kinase 3
RNS	reactive nitrosative species
ROS	reactive oxygen species
RUCAM	Roussel Uclaf causality assessment method
siJnk2 ^{Δhepa}	siRNA against hepatocyte Jnk2
siLuc	siRNA reporter control
siRNA	small interfering ribonucleic acid
siScr	scrambled negative siRNA Jnk2 control
TAA	thioacetamide
TB	total bilirubin
TBL	total bilirubin levels
TGF- β	transforming growth factor- β
TIMP	tissue inhibitors of metalloproteinases
TIMPs	tissue inhibitors of metalloproteinases
TLR4	toll-like receptor 4
TNF- α	tumor necrosis factor alpha
TRAIL	tumor necrosis factor related apoptosis inducing ligand
TUNEL	TdT-mediated dUTP-biotin nick end labeling
U/l	units per liter
ULN	upper limit of normal
VEGF	vascular endothelial growth factor
w	week
Wt/WT	Wild-type
y	years
α -SMA	alpha-smooth muscle actin
μ CT	microcomputed tomography
μ m	micrometer

Chapter 1

General Introduction

Essentials in anatomy and physiology of the liver

Main characteristics of the macroscopic liver

The liver embodies a solid viscera with an approximate weight of 968-1860g in healthy human male adults that occupies the right and partly left upper quadrant of the abdominal cavity in a strategic anatomical placement, where the intestinal blood circulation is separated from the rest of the organism¹. The hepatic viscera is constituted of two main lobules, which are split into a left and right lobule by the *ligamentum falciforme hepatis*². Both lobules are covered by a layer of *peritoneum viscerale*, also known as Glisson's capsule, which is the only vestige of somatosensory innervation the liver possesses, and that can lead to discomfort when distended. The primary vascular, biliary, lymphatic and innervating structures enter the liver through the *hilus hepatis*, and their arrangement in the viscera consequently determines the architectural subdivision of the liver in eight hepatic segments. The vascularization of the liver presents remarkable aspects, which makes it unique compared to other organs^{2, 3}. Whereas around 50% of the oxygenation for functional liver parenchymal cells (LPCs) derives from the *arteria hepatica*, the remaining 50% depends on the portal torrent, the *vena portae*, a venous system with a saturation of oxygen slightly inferior to arterial blood, but significantly higher than in the common venous circulation³.

Hepatic histology

At the microscopic level, the liver lobule has been classically understood as the basic and functional unit of the liver. The hepatic lobule consists of a central terminal venule that drains in the *venae suprahepaticae* and is surrounded by four to six terminal portal triads conforming a polygonal unit³. This unit is lined on its periphery between each terminal portal triad by terminal portal triad branches. A portal space includes a venous portal branch, a hepatic arteriole, a biliary duct as well as several lymphatic and nervous components covered with connective tissue⁴. Between the terminal portal triads and the central hepatic venule, hepatocytes are arranged layerwise, surrounded on each side by endothelial-lined and blood-filled sinusoids. From a structural and functional perspective, the conception of hepatic acinus has been used, referring to a portal space, a portal branch and an arterial branch, and where centrilobular veins are disposed in the periphery³. This definition has a more practical connotation from a histologic point of view since it contributes to understanding pathological mechanisms (e.g., under oxygen-restrictive conditions, perilobular hepatocytes show more resistance and a more robust initial regenerative response)³. In this line, hepatocytes can be classified according to their function and strategic positioning in three areas or zones: i) zone I (corresponds to the periportal region and is the first in regenerating since it is the best-perfused area); ii) zone II (corresponds to the area between zone I and III); iii) zone III (corresponds to the farthest located area from the portal triad)⁵.

The cellular elements that principally compose the liver parenchyma are hepatocytes and other non-parenchymal cells (NPCs). Hepatocytes are the most abundant cells in the liver,

representing about 60-80% of the total cellular population⁶. The remaining NPCs represent about 20-40% of cells in the liver. These enclose endothelial cells; lymphocytes -T-lymphocytes, natural killer cells and natural killer-T (NKT) cells; Kupffer cells (KC), which are the resident macrophages of the liver; hepatic stellate cells (HSCs), which are allocated mainly in Disse's space and store vitamin A in the form of retinoid droplets; among others³.⁷. Noxious signals and hepatic injury may lead to HSC activation and induce their transdifferentiation into myofibroblasts⁸. These cells are the leading producers of extracellular matrix (ECM) and mediate predominantly in the scar formation of chronic liver diseases (CLDs).

Functional features of the liver

The outstanding importance of the liver is further underlined by its unique capability to regenerate after injury. The liver is a crucial regulator of the organism's inner homeostasis and is implicated in essential metabolic and immunity functions. The liver is responsible for synthesizing proteins, enzymes, carbohydrates, fatty acids, vitamins and co-factors with a significant role in the digestion and lipid metabolism and serum proteins like albumin and non-immune α - and β -globulins, among others³. Moreover, the liver has a relevant detoxifying role in the organism and is responsible for the biotransformation of endogenous and exogenous compounds, contributing to their safe clearance and elimination, which comprehend drugs and toxins, among many others. Hepatocytes located in zone I are well-perfused and, thus, exert a key role in oxidative metabolism pathways, enclosing β -oxidation, production of bile, and catabolic activity of amino acids, among others⁵. On the other hand, drug metabolism and detoxification, glycogenesis, and glutamine synthesis occur mainly in zone III⁵.

The liver is considered the largest gland of the body with endocrine as well as exocrine functions⁹. A relevant aspect of its exocrine activity is highlighted by the liver's production and secretion of bile and bile acids. Bile acids have a vital role in the processing of dietary fat. Most of the bile derives from the metabolization of the heme group, which is mainly obtained from degraded red blood cells, a process that is catalyzed in a first step by the heme oxygenase that transforms the hem into biliverdin, and in a further step, the cytosolic reductase transforms biliverdin into bilirubin³. Bilirubin is secreted into the circulation, where it binds to albumin and is transported to the liver, where hepatocytes capture it. In the hepatocyte, bilirubin is conjugated in the endoplasmic reticulum and is transformed into bilirubin glucuronide, a non-toxic and hydrosoluble substance³. Hepatocytes secrete bile into the intrahepatic bile canaliculi system that is continued by larger ducts of variable diameter, and the bile finally reaches the duodenum or the gallbladder, where it can be eventually stored⁵.

The liver also has a crucial involvement in carbohydrate synthesis and energy management¹⁰. Glycogen is synthesized from glucose and stored in the liver, a process known as gluconeogenesis. In hypoglycemia conditions, glucose is newly released into the circulation via glycogenolysis and gluconeogenesis; the latter comprehends a metabolic pathway that leads to glucose formation from non-carbohydrate substrates, *e.g.*, certain

glycogenic amino acids, fatty acids, and other products such as glycerol, pyruvate and lactate. The liver is also involved in lipid metabolism and synthesizes cholesterol and triglycerides, among others. Moreover, the liver is directly implicated in the synthesis and degradation of proteins ranging from amino acids over coagulation factors of importance during hemostasia (*e.g.*, factors I, II, V, VII, VIII, IX, X, XIII) to vitamins (*e.g.*, vitamins A, B12, D or K) and other vital proteins for the complement as protein C and S, or albumin, which contributes to the regulation of the oncotic pressure in the vascular compartment and adequate renal function^{3, 11}.

Hepatic metabolism of drugs and xenobiotics

The liver constitutes an inner metabolic intersection, which is not only implicated in the metabolism of endogenous substances but notably also exogenous compounds as, *e.g.*, drugs, herbs, herbal remedies or dietary supplements, among others. Once dispensed and absorbed into the bloodstream, these substances enter the entero-portal circulation and reach the liver, where they become susceptible to suffer metabolic modifications (**Figure 1.1**). Essentially, these processes include three main steps: the formation of reactive metabolites or bioactivation (phase I), a detoxification step (phase II) and the cellular excretion stage (phase III).

Figure 1.1 Simplified representation of drug metabolism in the liver and drug excretion. In phase I, drugs suffer a first hepatic conversion catalyzed by microsomal enzymes, which belong to the cytochrome P450 system. During phase II, the drug-derived metabolites are conjugated and their solubility is increased by adding hydroxyl, carboxyl, thiol or amino groups, whereas phase III covers the clearance of these metabolites. Under particular circumstances, which are drug and host-dependent, these metabolic steps can occasionally contribute to drug toxicity due to the formation and accumulation of highly reactive metabolites or the development of an immunoallergic reaction, among others.

Phase I implies structural changes in the parental compound and represents a critical step for many compounds that may require biotransformation to be converted into active forms, which deliver the therapeutic effect. The endoplasmic reticulum of hepatocytes is an essential source of enzymes that are implicated in these metabolic processes. The main enzymes involved in phase I reactions belong to the cytochrome P450 (CYP450) enzyme

families 1, 2 or 3¹². Among the variable expression of P450 enzymes in the liver, isoforms CYP2C9, CYP2D6 and CYP3A4 have been related to covering 60 to 70% of all bioactivation reactions¹³. As a result of phase I interactions, the addition of functional hydroxyl, carboxyl, thiol or amino groups follows through oxidations, reductions or hydrolysis, making the compounds suitable to undergo phase II, in which these are conjugated with other molecules^{14, 15}.

Characteristic of phase II is the conjugation of phase I-derived metabolites with small molecules, which confer a higher water-solubility that reaches then the sinusoidal circulation or bile for final clearance. Enzymatic complexes such as glutathione S-transferase (GST), N-acetyltransferase 2 (NAT2) and uridine diphosphate glucuronosyl-transferase (UGT) act during phase II and conjugate glutathione (GSH), acetate and glucuronic acid, respectively¹⁶. As a result, ethereal sulfates glucuronides and amino acid conjugates are obtained, among others¹⁷. As a consequence of these processes, drugs are transformed into less active or chemically inert substances and water-soluble compounds, which can be safely excreted from the organism.

Notably, the metabolism of drugs through these enzymatic complexes can eventually lead to the formation and accumulation of reactive and toxic compounds, which can trigger liver injury. Furthermore, underlying liver diseases can interfere and compromise the metabolism and safe elimination of drugs, as it occurs in severe liver dysfunction or liver cirrhosis. On top, diverse other risk factors may apply to drug-induced hepatotoxicity, which can be both drug-specific and host-dependent, conferring a variable degree of susceptibility to make an individual prone to develop adverse drug reactions (ADRs). Thus, the liver's extensive metabolic facet makes out of this organ an optimal target for ADRs of diverse nature, which can exhibit a variable clinical expression and severity.

Drug-induced Liver Injury: General considerations

Definition and impact of DILI

The acute and chronic liver disease scenario has changed after the recent novelties achieved in the treatment of chronic viral hepatitis and the growing relevance of non-alcoholic fatty liver disease, which has become a leading health problem with global impact¹⁸. Prescription and non-prescription drugs as well as other xenobiotic agents such as herbs, alternative medicines, natural remedies or dietary supplements, can be a cause of liver injury, also known as drug-induced liver injury¹⁹⁻²¹. DILI is considered a rare clinical condition to occur. The fact that it has a variable phenotypic expression and can lead to a severe clinical outcome as acute liver failure, the need for a liver transplant and exceptionally even death, in addition to the limitations in diagnosis and treatment, make of DILI a remarkable challenge at current. Indeed, some experts in the field refer to DILI as a growing potential public health problem, given the vast impact on the medical and pharmaceutical sector²².

DILI arises as a challenging problem in the healthcare sector since it embodies a complex clinical entity, and to date, no specific diagnostic biomarkers are available^{23, 24}. Currently, the diagnosis of DILI requires the rule out of other causes, a high index of suspicion by the clinician to expeditiously establish its diagnosis and expert causality judgment continues to be regarded as the gold standard²⁵. The fact that drugs causing liver damage may enclose various pathological features, which can mimic all forms of acute and chronic hepatobiliary diseases, explains the difficulty in distinguishing DILI from other causes of liver injury and, thus, identifying *bona fide* cases^{26, 27}. Besides, earlier works have displayed an actual problem of misdiagnosis and underreporting in DILI²⁸⁻³⁰. Often, DILI events are diagnosed retrospectively after a battery of exclusion tests have been carried out, which costs physicians and patients a precious amount of time. This fact represents a concerning issue since DILI can potentially evolve to severe acute and chronic liver damage when the culprit drug is not discontinued. In those cases where the drug was not adequately ascertained in a previous event and is reintroduced in the patient's therapy plan, re-exposure to the causative agent can trigger a novel episode of liver injury, a phenomenon which is also known as rechallenge.

Critically, DILI can present as a severe hepatic insult and it can occasionally progress to fulminant liver failure, a potentially life-threatening condition that may require a liver transplant or, when worse comes to worst, it can dramatically end in *exitus letalis*. In effect, DILI has been identified as a leading cause of ALF in diverse studies^{31, 32}. In the USA, drugs represent the leading cause of acute liver failure³³, which sum 57% of all diagnosed ALF episodes, mainly due to acetaminophen (APAP, 46% of all ALF episodes)³⁴. Hence, the potentially fatal outcome that can subsequently follow after hepatotoxicity, together with the burden in obtaining a fast and precise diagnosis and the lack of effective therapy options (other than drug dechallenge), make DILI out of the question a serious matter of concern.

Nevertheless, DILI is not exclusively a concerning topic for clinicians, but it also has an immense impact on the pharmaceutical industry and drug regulatory agencies since it represents the most frequent reason for drug non-approvals, withdrawal from the market and postmarketing decisions³⁵⁻³⁸. Within the large field of adverse drug reactions (ADRs), more concretely, hepatotoxic and cardiotoxic events have been attributed to be the leading causes of drug withdrawal from the market or termination in phase I-III trials²⁸. ADRs in the liver may be commonly not identified and described prior to drug licensing; indeed, new drugs are usually tested in less than 3000 individuals before approval. Therefore, DILI cases with an incidence of one event in 10.000 may be most likely missed. Several authors suggested earlier that for every ten cases of alanine aminotransferase elevations above ten times the upper limit of normal in clinical trials, there might be one case of more severe liver injury that most likely will appear once the drug is broadly commercialized^{39, 40}.

Epidemiology of DILI

In general lines, DILI is believed to be a rare form of liver injury. The determination of the true incidence of DILI represents a hard inquiry to be assessed and there are only a limited number of studies with reliable information on hepatotoxicity. According to several

epidemiological studies carried out within the last two decades, DILI incidence has been estimated to be between 1 per 10.000 and 1 per 100.000 subjects who have been exposed to prescription medicines²⁸. Nevertheless, in general, epidemiological estimations of DILI might be higher due to underreporting, misdiagnosis, and incomplete observations after losing patient follow-up.

In studies from Sweden, United Kingdom and Italy, the annual incidence was estimated in 2.3, 2.4 and 4.1 cases per 100.000 individuals, respectively^{28, 41, 42}. Interestingly, other more recent studies importantly reflect a higher incidence estimation of DILI events. In that line, a population-based study from France, which took place over three years, collected up to 34 hepatic ADRs and the global crude annual incidence rate was 13.9 +/- 2.4 per 100,000 inhabitants. These numbers were 16 times greater than the numbers estimated by the French regulatory authorities based on spontaneous case reporting⁴³.

In cross-sectional analyses from Iceland, a crude annual incidence of 19.1 DILI events per 100.000 inhabitants was determined, where 75% of the episodes were caused by a single prescription medication⁴⁴. No large-scale epidemiological studies have been available in China and only approach studies based on data from relevant medical centers; in an inpatient study, DILI accounted for approximately 20% of all acute liver injury events^{45, 46}.

Causative agents inducing hepatotoxicity

More than 1000 drugs have been reported to have triggered DILI episodes besides other medicinal products, herbal remedies, or dietary supplements⁴⁷. Acetaminophen is the most frequently involved drug in drug-induced acute liver failure (ALF) and accounts for nearly half of the ALF overall cases in the USA⁴⁸.

However, many other drugs have been associated with DILI; in particular antiinfective drugs, central nervous system drugs or muscle-skeletal drugs are the leading causative agents according to the extracted data from large prospective hepatotoxicity databases (**Table 1.1**). With regards to compounds, many large prospective hepatotoxicity cohorts coincide in amoxicillin-clavulanate as the leading idiosyncratic DILI culprit agent, whereas antitubercular drugs (*e.g.*, isoniazid), azathioprine and infliximab are also common⁴⁹.

Examining the published results of prospective hepatotoxicity patient databases worldwide, in the United States, antibiotics were the most frequent causative agents followed by herbals/dietary supplements (HDS), cardiovascular drugs (10%), central nervous system agents (9%), antineoplastic drugs (5%) and analgesics (3%)⁵⁰. The most common class of causative drugs in Spain was antibacterials, followed by central nervous system agents, cardiovascular drugs and non-steroid anti-inflammatory drugs (NSAIDs)⁵¹. Similarly, in Iceland, predominated antibiotics followed by immunosuppressants, psychotropics, NSAIDs and antineoplastics⁵². In Latin-America, the most frequent drug classes were NSAIDs, antiinfective compounds, sex hormones, antineoplastics/immunomodulators and anticonvulsants²⁰. Different frequencies have been reported in Korea, where herbal products constitute more than one-third of DILI cases⁵³ and India, where the most frequently implicated agents are anti-tuberculosis agents⁵⁴. These differences are most likely in relation to the drug and HDS use and prescription policies in

each country, which proves that a geographical component is present in DILI. Eventually, genetic factors characteristic of a host population might also have a crucial role in the susceptibility of DILI. Nevertheless, prudence is required when analyzing the differences in the prevalence of culprit drugs listed in these databases since most of them do not include any sales data.

	US DILIN	Spanish DILI Registry	Iceland	Latin DILIN	Korea	India
Year	2004-2013	1994-2004	2010-2011	2011-	2005-2007	1997-2008
Database type	National / Multicenter	National / Multicenter	Cross-sectional	Multinational	National / Multicenter	National / Single-center
Total number of enrolled DILI cases, n	899	446	96	206	371	313
Most frequent compounds involved, (%) [§]						
	Amoxicillin-clavulanate (10)	Amoxicillin-clavulanate (13)	Amoxicillin-clavulanate (16)	Amoxicillin-clavulanate (10)		Anti-tuberculous drugs (58)
	Isoniazid (5)	Ebrotidine (5)	Diclofenac (6)	Diclofenac (6)		Phenytoin (7)
	Nitrofurantoin (13)	Isoniazide + Rifampicine + Pirazinamide (5)	Azathioprine (4)	Nimesulide (5)		Dapsone (5)
	Sulfamethoxazole / Trimethoprim (3)	Ibuprofen (4)	Infliximab (4)	Nitrofurantoin (5)		Olanzapine (5)
	Minocycline (3)	Flutamide (4)	Nitrofurantoin (4)	Cyproterone acetate (4)	N/A	Carbamazepine (3)
	Cefazolin (2)	Ticlopidine (3)	Isotretinoin (3)	Ibuprofen (3)		NSAIDs (3)
	Azithromycin (2)	Diclofenac (3)	Atorvastatin (2)	Isoniazide + Rifampicine + Pirazinamide (3)		Cotrimoxazole (2)
	Ciprofloxacin (2)	Isoniazid (2)	Doxycycline (2)	Carbamazepine (2)		Atorvastatin (2)
	Levofloxacin (1)	Nimesulide* (2)	Agomenlantine (1)	Phenytoin (2)		Leflunomide (1)
	Diclofenac (1)	Carbamazepine (2)	Cephalexin (1)	Thiamazole (2)		Ayurvedic (1)

Table 1.1 Distribution of the ten most frequently involved individual agents responsible for idiosyncratic DILI in extensive prospective hepatotoxicity studies worldwide. [§]Herbal and complementary remedies have been left out of the current data extraction; *withdrawn from the Spanish and Argentinian market since 2002 and 2009, respectively

Apart from conventional drug compounds, herbal and complementary remedies (*e.g.*, vitamins, amino acids and proteins, performance-enhancing supplements) have fallen into the spotlight of DILI causality in the past decade. The enhanced utilization of these compounds worldwide correlates with the augmenting reports of herbal- and dietary supplement (HDS)-induced liver injury. For instance, 20% of all the hepatotoxicity cases in the US to date have been attributed to stem from HDS-induced liver insults⁵⁵. HDS-induced liver damage characterizes for the same unspecific phenotypic expression known in other types of DILI.

Nevertheless, HDS-DILI is related with considerably more significant difficulties at the diagnosis process that are determined by frequent over-the-counter use and a falsely assumed HDS safety, among others, which often contribute to not declare HDS use on the side of the patient and, thus, are frequently overlooked by the clinician as a consequence⁵⁶.

Herbal preparations often contain multiple compounds, and in such cases, it is incredibly challenging to address the right culprit agent that led to the HDS-induced adverse reaction. The most remarkable herbal compounds inducing hepatotoxicity are pyrrolizidine alkaloids, germander (*Teucrium chamaedris*), *Atractylis gummifera*, plants containing Pennyroyal oil (*Mentha pulegium*, *Hedeoma pulegioides*), great celandine (*Chelidonium majus*), kava-kava (*Piper methysticum*), Black cohosh (*Actaea racemosa*), and several traditional Chinese medicine preparations⁵⁶.

Regarding dietary supplements, a particular mention of androgenic anabolic steroid drugs is required, which are synthetic derivatives of testosterone and have become popular for muscle mass building as well as performance stimulators. The most common androgenic anabolic steroids that have been implicated in HDS-DILI are stanozolol, methasterone and methylepithiostanol⁵⁷. Several studies have alerted about the notable increase of HDS among the leading causes of DILI in recent years, which most likely can be explained by the augmented use and clinical awareness^{19, 57, 58}.

The underlying pathophysiology of DILI

“On-target” versus “off-target” adverse reactions

To date, it is known that only a limited group of drugs follow a threshold-dependent type of liver injury or intrinsic hepatotoxicity, also known as “on-target” reactions, where after overpassing a dose threshold, the appearance of liver dysfunction is expectable and predictable⁵⁹. Generally, intrinsic DILI is characterized by a short latency period. The best-described example of this type of liver toxicity is provided by the compound acetaminophen (APAP), where the risk of hepatotoxicity increases when exceeding a dose of 4g/day⁶⁰. Notwithstanding, other drugs have also been related to dose-dependent adversities in the liver as it occurs with aspirin, mitochondrial poisons and several chemotherapeutic agents such as methotrexate^{61, 62}.

On the other hand, there are DILI events, which do not rigorously follow a threshold-dependent manner and occur unexpectedly due to a medication administered within therapy ranges, referred to as idiosyncratic drug-induced liver injury (iDILI) or “off-target” reactions^{59, 63}. Although idiosyncratic DILI is not dose-dependent in a strict sense, it has been more frequently associated with drugs administered in doses above 50 mg/day, a condition that happens in the majority of nowadays used medications⁶⁴.

The APAP-associated hepatotoxicity phenomenon has been the best-studied and dissected form of hepatotoxicity. Despite the fact the primary type of liver toxicity associated with APAP has an intrinsic character, its minimum hepatotoxic dose varies among individuals^{65, 66}. A prospective multicenter study from the USA revealed a prevalence of 7% in individuals who developed ALF after APAP ingestion despite following the manufacturer’s recommended dosage⁴⁸. This example evidences the real complexity in demarcating both forms of hepatotoxicity.

Overall, the mechanisms leading to idiosyncratic liver injury are still not fully elucidated; however, idiosyncratic DILI (iDILI) is considered to be the result of a multifactorial condition including environmental, chemical and drug-related factors, which determine the host's susceptibility to suffering from a DILI event^{67, 68}. Chemical reactive metabolites may compromise the adequate response to the disruption of the cellular redox balance and orchestrate a harmful setting involving activation of signaling pathways upstream of oxidative and nitrosative reactive species, mitochondrial damage and cell death⁶⁹. Despite not well understood, experts in the field have highlighted the potential relevant role that the adaptive immune response might play in the physiopathology of DILI by contributing to the hosts' susceptibility (*e.g.*, HLA associations, positive lymphocyte stimulation)^{33, 69, 70}.

Generation of chemically reactive metabolites and oxidative stress

In the liver, an immense metabolic activity of endogenous as well as exogenous compounds takes place. In most cases, drugs need to undergo bioactivation to reach a status in which the pharmacological effect is executed. Nevertheless, these transformations occurring in the parental compound may act as a double-edged sword, and under particular circumstances, they can lead to the formation of reactive intermediate metabolites, commonly via cytochrome P450-dependant oxidative and reductive routes⁷¹. Phase II conjugations (*e.g.*, acetylation, glucuronidation, sulphation) and phase III excretions (through multi-drug resistance-associated protein transporters, bile salt export pump) are therefore crucial steps to ensure the adequate detoxification and clearance of chemical reactive substances. The balance between formation and detoxification is believed to be a determinant factor in exposing hepatocytes to a threshold level of chemical reactive agents; when the balance is destabilized, chemical reactive agents may accumulate and reach toxic concentrations, which ultimately lead to cellular stress and create a setting, which in a further step triggers hepatocellular injury and death. Additionally, drug-specific factors such as lipophilicity or the drug's administered dose, for example, can aggravate this condition⁶⁹. Furthermore, host-dependent risk factors can modify individuals' susceptibility, making them more prone to develop adverse reactions⁷².

In previous lessons learned from the well-known APAP-induced hepatotoxicity model, the compound APAP is metabolized via CYP450. As a result of its metabolization through CYP2E1 (predominantly), CYP1A2 and CYP3A4 (in a minor fraction), it is transformed into *N*-acetyl-*p*-benzoquinone imine (NAPQI), a highly reactive and toxic metabolite^{73, 74}. Moreover, toxic metabolites can then bind covalently to cellular proteins and alter their physiologic function, promoting cellular stress. Toxic metabolites are cleared under normal conditions by the glutathione (GSH) system and excreted into bile, but in overdose conditions, the levels of GSH are rapidly depleted and, thus, the augmented reactive oxidative and nitrosative species may destabilize the redox balance^{75, 41}. Consequently, the formation of protein adducts is enhanced, which leads to mitochondrial dysfunction and hepatocellular damage, and finally, cell death is triggered^{76, 77}. In that regard, the excessive APAP-derived NAPQI formation quickly annihilates the mitochondrial and cytosolic GSH reserves and binds covalently to thiol groups of macromolecules in the cell and mitochondria, evoking impaired mitochondrial function as well as a boost in mitochondrial

ROS production, which are molecules that characterize for their high reactivity and contain oxygen^{69, 78}. This circumstance finally leads to hepatocytic necrosis and the activation of cell death cascades⁷⁹.

Disruption of mitochondrial homeostasis and adaptation

The mitochondrial morphology, function and DNA synthesis can result altered through direct drug toxicity and excessive ROS formation. Mitochondria are cellular organelles that act as a power source of the cell and generate adenosine triphosphate during the complex respiration chain process.

Mitochondria are a central target for cellular stress, which can originate from drug-derived reactive metabolites, and the preservation of their optimal function has a decisive impact on the hepatocyte's viability^{80, 81}. Mitochondria are per se the primary source of intracellular ROS (complex I and III of the respiratory chain) and any excess of ROS is neutralized to maintain the redox balance. Nevertheless, enhanced ROS production triggered through a stressful condition (*e.g.*, reactive drug metabolites) leads to oxidation of macromolecules, damage of mitochondrial DNA, inhibition of β -oxidation, disturbance of the electron transfer along the respiratory chain and impaired mitochondrial function^{80, 82}.

In response to stressful conditions, mitochondria are dynamic organelles and various changes occur in these, which evidences their adaptive plasticity to reestablish the mitochondrial homeostasis. These adaptive phenomena are piloted essentially by mitochondrial motility, mitophagy and fusion-fission⁸³.

Mitophagy is a form of selective mitochondrial autophagy⁸⁴. Autophagosomes originate and fuse with lysosomes during this process, which contains hydrolytic enzymes and eliminate cellular components. Augmented levels of mitophagy promote the selective degradation of defective mitochondria⁸⁵. The mitochondrial cytoskeleton provides mitochondria with motility that serves under normal conditions as movement effector, mainly microtubule-based, during mitochondrial cell distribution and turnover^{86, 87}. Modified fusion-fission resulting from metabolic stress leads to self-repairing changes in mitochondrial morphology and function by fusion with intact mitochondria (*e.g.*, mitochondrial enlargement, fragmentation). Fusion-fission works similar to quality control for mitochondria that preserves healthy mitochondria (fusion) and facilitates the elimination of damaged mitochondria through fission and mitophagy⁸³. Beyond changes in mitophagy, motility, fusion-fission and remodeling of mitochondria to improve cell respiration, increased mitochondrial biogenesis or mitochondrial unfolded protein response are further characteristic signs of mitochondrial adaption to drug-induced toxicity⁸⁸.

The role of the immune system in DILI

The innate and adaptive immune systems have been attributed to play a pivotal role in the pathogenesis of DILI. The innate immune system can quickly react against noxious stimuli (*e.g.*, infections, tissue damage) as a first-line defense, whereas adaptive immune effectors

need time to boost their response (*e.g.*, proliferation and differentiation of T and B cells). As a matter of fact, there is a close interaction between both immune responses, and indeed, innate immunity plays a critical role in the development of the adaptive immune response⁸⁹.

There are compounds, which have been associated with an immunogenic profile in triggering toxin-induced hepatic dysfunction (*e.g.*, amoxicillin-clavulanate). However, most of the drugs and their derived reactive metabolites possess only a low molecular weight and are most likely not capable of inducing an inflammatory response on their own. In most cases, drug molecules or their derived reactive metabolites are believed to behave as haptens, binding covalently to other cellular macromolecules to form protein adducts that may awake an immune-mediated reaction⁹⁰. These can then be phagocytosed by antigen-presenting cells (*e.g.*, dendritic cells, NK cells or macrophages) and be presented via major histocompatibility complex (MHC class II). Resultantly, other compartments such as T lymphocytes can be activated when a foreign antigen is recognized. Additionally, the antigenic expression in the cellular membrane of hepatocytes can also trigger an immune-mediated cytotoxic reaction⁹¹. Indeed, several HLA haplotype associations in DILI have been earlier described, highlighting the importance of genetic predisposition in the adaptive immune response in the pathogenesis of DILI⁹⁰⁻⁹².

On the other hand, it has been extensively discussed that haptenization alone might most likely not be sufficient to boost an inflammatory response triggering severe liver injury. In that respect, it has been hypothesized that the adaptive immune response could be activated through other stimuli such as danger signals, also known as damage-associated molecular pattern molecules (DAMPs). DAMPs are danger molecules, which can be released as a result of cellular damage as well as dead and dying cells (such as hepatocytic necrosis, for example) and promote a sterile inflammatory response by interacting with receptors that are also stimulated by pathogen-associated molecular patterns (*e.g.*, Toll-like receptors)^{17, 93}.

For instance, enhanced circulating DNA levels, histones, or high mobility group box 1 (HMGB1), a non-histone nuclear protein that binds to DNA, act as potential DAMPs⁹³. For example, significantly augmented levels of HMGB1 have not only been described in various experimental models of APAP-induced hepatotoxicity, but interestingly the circulating concentrations of HMGB1 did correlate with the severity of the adverse reaction⁹⁴⁻⁹⁶. Further studies showed that inhibiting HMGB1 by genetic and pharmacological silencing significantly ameliorated APAP-induced murine liver toxicity⁹⁶⁻⁹⁸. Importantly, notably elevated levels of circulating HMGB1 have been demonstrated in patients' plasma subsequently to APAP overdose⁹⁹. Hence, HMGB1 might be a promising potential mechanistic biomarker of drug hepatotoxicity, as other authors have suggested previously.

Last but not least, immune-mediated DILI can clinically exhibit autoimmune features and adopt the façade of autoimmune hepatitis (AIH), also known as autoimmune-like DILI. The diagnosis of AIH is highly problematic in virtue of its heterogeneous characteristics¹⁰⁰. In essence, three different scenarios involving autoimmunity in hepatotoxicity have been considered: i) immunoallergic type of DILI that mimics AIH, ii) AIH mimics DILI due to a

recent drug exposure that improves coincidentally after drug withdrawal, and iii) an offending drug induces AIH (autoimmune-like DILI)¹⁰¹. Thus, the demarcation between immune-mediated and autoimmune-like DILI is arduous and not easy to be differentiated, but still a capital aspect to consider due to the different therapeutic approach that applies in both clinical entities¹⁰².

Implicated mechanisms of cell death

Cellular stress will result in the activation of signal transduction pathways, which are crucial in mediating cell death and inflammatory responses. The involved mechanism of cell death and the extent of the cellular damage are commonly related to the causative compound or toxin. Classically, the main implicated types of cell death have been apoptosis and necrosis; nevertheless, the potential role of other forms of cell death and autophagy in DILI has been started to be discussed more recently.

Apoptotic cell death is a programmed process that characterizes for various changes in energy-dependent biochemical mechanisms and cellular morphology, including cell shrinkage, pyknosis, chromatin condensation, fragmentation of chromosomal DNA and cell nucleus, among others^{103, 104}. Essentially, two pathways are leading to apoptotic cell death: the intrinsic and the extrinsic pathway.

The activation of the intrinsic pathway is promoted by cellular and mitochondrial stress, which derives in the release of proteins to the mitochondrial intermembrane space and is transduced into intracellular signals¹⁰⁵.

The extrinsic pathway is activated via death receptors, for example, tumor necrosis factor receptor (TNFR), TNF-related apoptosis-inducing ligand receptor (TRAIL-R1 and R2), FAS, promoting caspase 8 activation. Both pathways culminate in the activation of executioner caspases 3 and 7.

On the other hand, necrosis is believed to be a sort of unprogrammed cell death triggered by exogenous stimuli to the cell, including drugs, toxins, trauma or infections. Characteristic features of necrosis are ion imbalance, mitochondrial dysfunction and adenosine triphosphate depletion due to cell lysis. The ion homeostasis disruption creates an imbalanced oncotic pressure status on both sides of the membrane, determining cell swelling, involution of the cell membrane, bleb formation and membrane permeability transition. As an outcome, the cell's rupture derives into the release of cellular content, including cytotoxic and pro-inflammatory factors and DAMPs, which all in one awake a strong inflammatory reaction^{69, 105}.

Nevertheless, there is a type of programmed or regulated necrosis that has been described and termed as necroptosis, where features of necrosis and apoptosis are simultaneously present. Caspase activity is not required in a strict sense to trigger cell death as it was observed for the first time on L929 cells, a fibrosarcoma cell line, where co-stimulation with tumor necrosis factor (TNF) and a pan-caspase inhibitor (zVAD-fmk) resulted in necrotic cell death¹⁰⁶.

During necroptosis, the formation of the necroptosome occurs: TNFR-associated death protein TRADD signals promote RIPK3 recruiting and interaction with RIPK1 forming the necrosome via RIP-homology interaction motif (RHIM). Subsequently, RIPK3 promotes mixed lineage kinase domain-like pseudokinase (MLKL) phosphorylation, which translocates to the cell membrane, oligomerizes and leads to cell membrane permeabilization^{105, 107}.

A further cell degradation mechanism, which has been observed in close relation to apoptosis, is autophagy. Autophagy consists of lysosomal-derived degradation, which leads to the elimination of cellular components enclosing defective cell organelles or proteins. During autophagy, double-membrane vesicles, the autophagosomes, are formed, which in a second step fuse with lysosomes that degrade their content through the activity of lysosomal hydrolases. Autophagy is not uniquely associated with cell death but plays an essential role in other processes, *e.g.*, immune response or tumor suppression, among others^{108, 109}.

More recently, the implication of other forms of cell death, such as pyroptosis and ferroptosis, has been questioned in the domain of DILI. Ferroptosis-mediated cell death consists of an iron-dependent mechanism promoted by GPX4 and leads to severe lipid peroxidation, whilst pyroptosis is an inflammasome-dependent pathway that activates caspase 1 and 11 that mediate in Gasdermin D-mediated cell membrane desintegration^{110, 111}. At current, the real extent of both cell death mechanisms in the pathophysiology of DILI remains undetermined due to insufficient data¹¹².

The role of c-Jun N-terminal kinases (JNK) in DILI

The c-Jun N-terminal kinases (JNK) are serine/threonine kinases, which are members of the mitogen-activated protein kinase (MAPK) family. Upstream, MAP3K can be found and activates MAP2K, which phosphorylates JNK. In mammals, three isoforms of JNK have been described: JNK1 and JNK2 are expressed ubiquitously, whereas JNK3 expression is restricted to specific tissues such as the brain, heart or testis¹¹³.

Many different stimuli can lead to JNK phosphorylation encompassing ROS, UV light, drugs, toxins and cytokines. Finally, JNK has been described as a critical regulator of cell survival and death^{69, 114}.

A substantial amount of evidence has been generated within the last decades, highlighting the capital relevance of the axis “cell stress-JNK activation-hepatic injury” in a wide range of liver damage models enclosing hepatitis due to diverse causes, metabolic changes, inflammation, fibrosis or carcinogenesis, among others^{115, 116}. In terms of hepatotoxicity, the experimental model with APAP has been extensively used to study the role of the JNK pathway, and a two-hit-hypothesis has been established⁸⁸. During the first hit, also known as the upstream hit to mitochondria, an excess in the mitochondrial ROS production occurs due to the GSH depletion and covalent binding of NAPQI, leading to an early and a late phase MAPK activation (**Figure 1.2**).

Regarding the early phase (1-2h after APAP-induced hepatotoxicity), it has been suggested that glycogen synthase kinase 3 beta (GSK3 β) may activate mixed-lineage kinase-3 (MLK3)

that then further enhances JNK activation, whilst in the late phase (2-4h after APAP-induced hepatotoxicity) apoptosis signal-regulating kinase-1 (ASK-1) is mainly involved.

As an outcome in both the early and late phases, MKK4/7 is activated, which in parallel phosphorylates JNK^{88, 113, 117-119}. Therefore, the MAPK cascade leads to the activation of JNK, which occurs either intrinsically via receptor-mediated signaling or by the resulting cellular stress that derives from damaged nuclei, mitochondria or endoplasmic reticulum. Phosphorylated JNK are believed to translocate to mitochondria and bind to the outer mitochondrial membrane via the scaffold protein SH3 domain-binding protein 5^{120, 121}, which acts as a direct mediator between mitochondria and JNK¹²². Subsequently, translocated pJNK that binds to Sab and activates it promotes the release of inactive SHP1 sequestered by Sab¹²². Phosphorylated Src activates SHP1 and pSHP1 mediates in the inactivation of Src via the DOK4 platform at the inner membrane. Src is a crucial mediator of mitochondrial homeostasis and its activation stabilizes the mitochondrial electron transport, whereas its inactive form alters the electron chain resulting in the formation of ROS^{122, 123}.

Under these circumstances, prolonged JNK activity will behave as an amplifier of the cellular stress response, interfere in the mitochondrial bioenergetics and sustain an augmented ROS formation in the mitochondria. This promotes a vicious circle that persistently activates upstream MAPK, leading to a sustained JNK phosphorylation. Consequently, mitochondrial permeability transition ensues, leading to the loss of the mitochondrial membrane integrity and potential, awakening an inflammatory reaction and triggering cell death^{88, 122-124}.

Figure 1.2 Scheme illustrating the pathomolecular mechanisms underlying APAP-induced hepatotoxicity. (1) Hepatic metabolism of APAP via cytochrome P450 2E1 derives into the formation of the chemically reactive metabolite N-acetyl-*p*-benzoquinone imine (NAPQI) (2), which leads to the depletion of the glutathione (GSH) levels and promotes covalent binding to macromolecules, interfering in the mitochondrial homeostasis. (3) Consequently, the electron (e^-) chain transport and the energy generation via β -oxidation are disturbed, leading to impaired cell respiration and stimulating the formation of oxidative and nitrosative reactive species. (4) These promote MKK4/7 activation, which has been suggested to occur during the early phase via (GSK3 β) and Mixed lineage kinase (MLK) 3 activation, and during the late phase via apoptosis signal-regulating kinase (ASK) 1, which is activated. (5) Mitogen-activated protein kinase kinase (MKK) 4/7 contributes to c-Jun N-terminal kinase (JNK) 1/2 phosphorylation that translocates to SH3 domain-binding protein 5 (Sab), boosting ROS formation and enhancing mitochondrial dysfunction. This persistent situation finally promotes membrane permeability transition (6), which leads to the release of cytochrome (cyt) C and derives in cell death (7). The JNK inhibitor SP600125 has been experimentally shown to prevent JNK activation and attenuate liver injury in APAP-induced hepatotoxicity.

The compound SP600125 has been characterized as a reversible ATP-competitive JNK-specific inhibitor¹²⁵. Earlier studies have described protective effects associated with pre-treatment with SP600125 prior to APAP hepatotoxicity induction in murine models and human hepatocytes *in vitro*^{79, 126}. A further study revealed that blocking expression of Jnk1 and Jnk2 concomitantly by using antisense oligonucleotides conferred protection against APAP hepatotoxicity *in vivo*¹²⁰. These results confirm the relevant role of JNK in APAP-induced hepatotoxicity and open new possibilities in the experimental development of therapeutic strategies, which modulate JNK-dependent targets.

Clinical aspects and case characterization

The clinical façade of drug-induced liver injury

A drug-induced liver injury episode can mimic most acute and chronic hepatic diseases and may have an indistinguishable clinical presentation from these. The clinical presentation in DILI is highly variable, ranging from asymptomatic alterations of serum liver enzymes over cholestasis to acute liver failure and exceptionally even death¹²⁷⁻¹³². Often, symptomatic manifestations are absent and the DILI episode is then incidentally uncovered because of a routine laboratory test, which shows a flare in biochemical liver injury markers.

When symptomatic features are present, DILI patients may exhibit and report anorexia, nausea, vomiting, malaise, weakness, fever, abdominal pain, jaundice, pale or clay-colored stools, dark urine, among others¹³³. Overall, positive symptomatology in DILI commonly resembles an acute hepatitis-like or a cholestatic syndrome and the most frequently associated symptoms are weakness, anorexia, dark urine and icteric sclera, among others^{10, 130, 134}. For instance, the analysis of the prospective US DILIN patient cohort with over 1200 enrolled participants revealed that 60% of them experienced nausea and another 42% had abdominal pain¹³⁵.

In chronic hepatotoxicity cases, the manifested clinical features can resemble other chronic liver disease causes presented in primary biliary cirrhosis, sclerosing cholangitis, or autoimmune hepatitis. The presence of features such as fever, rash, eosinophilia or cytopenia during a hepatotoxic event may be suggestive of an immune-allergic mediated type of injury. Characteristically, the appearance of a delayed onset with clinical features

showing up several weeks after drug administration is suggestive of immune-mediated DILI¹³⁶.

In general lines, most patients suffering from liver dysfunction in the setting of a DILI event fully recover after the culprit drug is ceased and the re-exposure to the compound is prevented. However, in a small fraction of the patients, the DILI event may not resolve satisfactorily, even after drug withdrawal, and turn into a chronic form of liver injury. Nevertheless, a systematic review reported that chronic liver injury induced by drugs was higher with a prevalence of 18% in large national-based hepatotoxicity cohorts¹³⁷.

Initially, experts agreed to define as acute DILI those events with altered ALT and ALP under three months and chronic those episodes, where elevations in ALT and ALP lasted more than three months¹³⁸. Due to the fact that cholestatic type of liver injury requires a more significant time to resolution, compared with cytolytic events, the cutoff point to consider DILI events with cholestatic features was set in six months⁵¹. Aithal's criteria of 2011 define as persistent DILI, hepatocellular events with over three months of persistence and cholestatic events of more than six months. Additionally, the term chronic DILI refers to those episodes, which persist for at least one year or more, independently of the type of injury pattern. It has been previously indicated that acute showing-off DILI episodes have approximately a 10% likelihood to become a chronic insult in the liver¹³⁹.

Chronicity associated with DILI can lead to sustained noxious stimuli in the liver that promote hepatic remodeling, trigger liver fibrosis, and even progress to liver cirrhosis in advanced stages¹⁴⁰. Nevertheless, the underlying liver injury pattern may vary and adopt less common subtypes of chronic DILI (i.e., drug-induced fatty liver, nodular regenerative hyperplasia and hepatic peliosis hepatitis). Although the standard treatment includes discontinuation of the culprit agent, those not resolving after drug cessation alone may require immunosuppressive therapy. The most frequently involved drugs in chronic DILI belong to the antibiotic class of drugs, *e.g.*, amoxicillin-clavulanate acid or trimethoprim-sulfamethoxazole¹³⁷.

Biochemical criteria for DILI diagnosis and severity grading

During the meeting of the Council for International Organizations of Medical Sciences (CIOMS), which took place in 1989, the experts concluded that in order to establish the diagnosis of drug-induced liver injury, at least one of the following criteria needed to be fulfilled: (i) increment of the alanine aminotransferase above two times the upper limit of normal (ULN); (ii) enhancement of the conjugated bilirubin levels above two times the ULN; (iii) increase of the aspartate aminotransferase, alkaline phosphatase and total bilirubin, where one of these doubles the ULN¹⁴¹.

Despite the fact that these criteria have been used for over two decades, with the time and the experience gained in the field, it had been questioned if these criteria were strict enough since, in many cases, the detection of a modest rise in ALT made patients undergo unneeded medical examinations and withdrawal of their medication when it was unnecessary. Furthermore, some DILI cases exhibit a mild elevation of biochemical liver injury markers that progresses to spontaneous recovery and do not suppose a relevant clinical condition. In that regard, other studies reported rising to higher ALT cutoff values¹⁴².

¹⁴⁴. Experts then discussed that considering higher ALT cutoff levels in DILI, episodes with a low clinical impact or false positive cases could be most likely excluded. Therefore, the former DILI criteria were readjusted to those from Aithal and colleagues designed to standardize the clinical threshold for DILI⁶³. These criteria specify that at least one of the criteria needs to be fulfilled to consider a compatible event as DILI: (i) enhancement of ALT values of at least five times the ULN; (ii) increase of the ALT parameters in at least three times and total bilirubin levels of above two times; (iii) increment in the ALP values of at least two times, especially when elevations of 5'-nucleotidase or γ-glutamyl transpeptidase are present and there is the absence of any known bone pathology, which could lead to augmented ALP levels.

These criteria may be reached at any time point during the course of the DILI event; nevertheless, the criteria may not be applicable for certain types of chronic-related DILI. Of note, the AST values can be used despite ALT when the last one is not obtainable and there is no muscular disease, which may interfere in the rise of AST. Hyperbilirubinemia alone cannot be considered DILI, even if it is associated with direct hyperbilirubinemia. As well, the elevation of γ-glutamyl-transferase alone is not sufficient to designate an event as DILI¹⁴⁵. Some other liver injury forms, particularly those involving mitochondrial toxicity, may not reach these threshold levels but can cause clinically relevant liver injury.

Of note, serum liver injury parameters uniquely are not reliable prognostic markers and fail to predict the hepatic adverse drug reaction's severity. Indeed, validated prognosis scales for hepatic damage such as the Child-Pugh or MELD score systems, among others, leave systematically hepatic enzyme markers out of their scales. Furthermore, serum liver enzymes might reflect cytolysis but are not true liver function markers, which needs to be monitored with other values such as serum bilirubin concentration or prothrombin time (alternatively the International Normalized Index, INR).

In an effort to consider both biochemical and clinical data at the severity grading in DILI, the experts in the field suggested to classify severity in DILI in four categories: i) mild, alanine aminotransferase/alkaline phosphatase (ALT/ALP) concentration augmented matching criteria for DILI but bilirubin concentration below 2×ULN; ii) moderate, ALT/ALP concentration raised in line with DILI criteria and bilirubin concentration equals/above 2×ULN or symptomatic hepatitis; iii) severe, ALT/ALP quotient elevated matching with DILI criteria and bilirubin concentration equals/above 2× ULN, and one of the following (coagulopathy INR≥1.5, ascites and/or encephalopathy, disease duration <26 weeks, and absence of underlying cirrhosis or other organ failure caused by DILI); ii) fatal or liver transplant, exitus or hepatic transplant as a consequence of the DILI episode⁶³.

Of interest is the fact that combined elevations of ALT and bilirubin may indicate a potentially severe reaction. Hy's law applies in those DILI cases presenting with cytolytic type of liver injury in addition to jaundice or expressed in terms of ratio

$$\frac{\left[\frac{\text{ALT or AST* value}}{\text{ALT or AST ULN}} \right]}{\left[\frac{\text{ALP value}}{\text{ALP ULN}} \right]}$$

**whichever was the highest*

" $R > 5 + TBL > 2 \times ULN$ ". DILI cases fulfilling Hy's law present an increased risk of fatal outcome, significantly associated with liver transplantation and death in $>10\%$ of the cases^{146, 147}. More recently, Hy's law has been redefined by considering for hepatocellular DILI events the new ratio value, which can critically help improve the prediction of DILI cases that may progress to ALF (**Figure 1.3**)¹⁴⁸.

Figure 1.3 Representation of the nR quotient

New ratio is calculated by dividing the quotient, which results from ALT or AST (whichever has the highest value) and its respective ULN, through the quotient that is obtained after dividing the ALP value through its ULN. The nR results in a better prediction of drug-induced ALF.

Abbreviations: ALT, alanine aminotransferase; AST, aspartate aminotransferase; ALP, alkaline phosphatase; ULN, upper limit of normal;

Patterns of hepatic injury in DILI

Histopathological information provided by a liver biopsy, explant or necropsy may help assess and assign causality, exclude other differential diagnoses of liver disease, and particularly grade the severity of the suffered DILI episode. Nevertheless, currently, there is no standardized histological scoring available for DILI^{149, 150}. Thus, the experts agreed to assign the type of injury based on the biochemical liver injury criteria by applying the ratio (R) based on biochemical liver injury parameters. The R is calculated by dividing ALT activity into ALP activity, using for both parameters its normalized value (times ULN) and using the values obtained from the first available blood test after DILI recognition since the pattern of injury can vary over time. $((ALT \text{ value}/ALT \text{ ULN})/(ALP \text{ value}/ALP \text{ ULN}))$ ¹⁵¹.

Hepatocellular type of injury is the most frequent pattern of injury and is defined as $R \geq 5$. In that sense, patients with hepatocellular damage will exhibit significantly augmented aminotransferase values. Histologically, hepatocellular type of injury may display as spotty lesions in liver parenchyma affecting isolated hepatocytes or can conflux as a result of damaged groups of hepatocytes. The death of hepatocytes can be addressed to phenomena comprehending necrosis, apoptosis, steatosis or cellular degeneration, among others. Confluent areas of necrosis in liver parenchyma can be discerned into zonal or non-zonal, which can be characteristic of certain culprit drugs. Furthermore, when large necrotic areas are affected, these can lead to bridging, submassive or massive necrosis.

For instance, zonal necrosis has been associated with compounds with a predictable type of or dose-dependent type of hepatotoxicity. Centrilobular necrosis (zone III) is the most common type of zonal necrosis, while isolated lesions in zone I and II are exceptional. Some classical examples are listed: acetaminophen (zone III), halothane (zone III), CCl_4 (zone III), beryllium (zone II), cocaine (zone I) or iron sulfate (zone I). Non-zonal necrosis may resemble hepatic injury lesions known from viral hepatitis and is commonly associated with drugs subjected to idiosyncratic type of hepatotoxicity such as diclofenac, isoniazid or phenytoin, among many others.

Cholestatic type of injury is defined when $R \leq 2$. Microscopically, pure cholestatic lesions are characterized by hepatocellular and/or canalicular cholestasis with minimal hepatocellular injury features and inflammatory reaction. Bile accumulation is often seen, predominantly in hepatocytes and canaliculi localized in the area of zone III. The responsible agent interferes with bile constituents' hepatocyte secretion, mediated by the bile salt excretory

protein (BSEP). In cholestatic hepatitis-like reactions, these characterize histologically by portal inflammation, marked cholestasis and hepatocellular injury. The proliferation of bile ducts may be present, and hepatocyte injury is usually localized in cholestasis areas.

A mixed type of injury is defined as $2 < R < 5$ and, microscopically, liver parenchyma can display attributes of both hepatocellular and cholestatic type of lesions. However, drugs can induce liver damage with histology features different than cytolytic, mixed or cholestatic, which can be displayed phenotypically in a variable form, ranging from steatosis, fibrosis, bile duct injury syndromes or cutaneous liver diseases, among others.

Figure 1.4 Basic illustration highlighting useful classifications for DILI case definition. Once the diagnosis of a drug-induced liver injury episode is established after a reasonable exclusion diagnosis has been completed, several key questions may help the clinician to better characterize the episode of drug-induced liver dysfunction. The DILI episode duration can be classified into acute and chronic injury based on a cut-off point of 1 year since the onset of the adverse drug reaction. Depending on the nature of the adverse drug reaction, a reaction can be differentiated as intrinsic (when the manufacturer's recommended dosage has been exceeded) or idiosyncratic (in those cases where the reaction appeared, despite given doses were within therapy margins). With respect to the underlying injury pattern, $R \geq 5$ points to cytotoxicity (HC), whereas $R \leq 2$ and $2 < R < 5$ stands for cholestatic (Chol) and mixed (Mix) type of injury, respectively. Severity can be graded into mild (ALT/ALP flares compatible with DILI criteria but $TBL < 2 \times ULN$), moderate (ALT/ALP flares compatible with DILI criteria and $TBL \geq 2 \times ULN$), severe (ALT/ALP flares compatible with DILI criteria and $TBL \geq 2 \times ULN + INR \geq 1.5$ or recent ascites/encephalopathy (< 26 weeks) in the absence of underlying cirrhosis or any other DILI-induced organ failure) and worst outcome (exitus or hepatic transplantation).

Diagnosis and causality assessment

At present, there are no specific biomarkers or diagnostic tests, which may help to establish the diagnosis of drug-induced liver injury. In that regard, DILI needs to be approached as a rule-out diagnosis and therefore, a reasonable exclusion of other aetiologies of liver disease is mandatorily required. The diagnosis of DILI relies on a subjective assessment covering clinical, biochemical, and histological information (when available) of the patient. The first step to DILI diagnosis is a clinical suspicion of a drug-related causality, a challenging fact

when considering that the clinical syndromes associated with DILI are generally unspecific and may mimic other common features of hepatic illness. In addition, as reviewed above, there are no specific liver histology criteria that are conclusive for DILI. DILI recognition may be complicated in patients presenting several medications and underlying diseases, which can act as confounding factors. Then again, difficulties in the diagnostic procedure of DILI explain that many cases remain underreported, and misdiagnosis is not uncommon. Indeed, it is not uncommon that DILI diagnosis occasionally follows after inadvertent reexposure to the culprit agent and a new flare in liver injury markers is appreciated.

Of capital relevance is the need of obtaining a careful medical history of the patient, which includes an exhaustive pharmacological history with explicit information on drugs and other treatments that the patient had been in therapy with during at least six months before the adverse reaction flourished¹²¹. The administered drug doses, treatment duration and time to onset of the adverse reaction are crucial data to be analyzed in order to prove a temporal relationship between drug administration and injury onset, as well as to drive the suspicion into a particular drug as potential culprit agent of the adverse event when more than one compound was given concomitantly. Recent changes in the dispensed drug doses are also crucial since a drug-induced liver insult can remain uncovered initially but become apparent after variations in the drug dose occurred¹⁵².

Despite patients presenting with acute DILI, imaging tests, in general, do not exhibit any specific findings for hepatotoxicity, these are crucial to perform an adequate differential diagnosis and exclude other causes of hepatic damage. Abdominal ultrasound, CT or MRI scans and retrograde cholangiopancreatography are beneficial imaging techniques to properly assess the biliary tree and identify any obstructive syndrome or malignancies affecting the liver, gall bladder, hepatobiliary conducts and pancreas. In the context of chronic DILI, patients do not usually display changes in the diameter of intra- and extra-hepatic bile tracts but may exhibit characteristic variations of liver cirrhosis such as an enhanced portal vein or an enlarged spleen.

Besides liver tests displaying serum liver injury markers, blood panels are indispensable to investigate other potential primary aetiologies of liver injury as well as secondary causes involving the liver (**Table 1.2**). These tests essentially determine markers and titers, which cover and are characteristic for viral (hepatitis A virus (HAV): Anti-HAV IgM; hepatitis B virus (HBV): HBsAg, Anti-HBc IgM; hepatitis C virus (HCV): Anti-HCV, HCV RNA; hepatitis D: only if HBsAg+, Anti-HD IgM; hepatitis E

Aetiology	Markers and titers to determine
Hepatotropic viral infections	
Hepatitis A	Anti-HAV IgM
Hepatitis B	HBsAg, Anti-HBc IgM, HBV-DNA
Hepatitis C	Anti-HCV, HCV-RNA
Hepatitis D (only if HBsAg+)	Anti-HDV IgM/IgG
Hepatitis E (endemic area/contact)	Anti-HEV IgM/IgG, HEV-RNA
Other viral infections	
Epstein-Barr virus	EBV-PCR, VCA-IgM/IgG, EA/EBNA IgG
Citomegalovirus	CMV-PCR, Anti-CMV IgM/IgG
Herpes Simplex, Varicella Zoster, Human Herpes 6	HSV1/2-PCR, HSV-1/2 IgM/IgG, VZV IgM/IgG, VZV-PCR, HHV6-PCR
Human immune- deficiency virus	Anti-HIV1/2, HIV-RNA, Anti-p24
Metabolic diseases	
Hemochromatosis	transferrin saturation, serum ferritin, HFE gene testing
α 1-antitrypsin deficiency	serum α 1-antitrypsin
Wilson's disease	serum copper, plasmatic ceruloplasmin, genetic testing
Other disorders	
Autoimmune hepatitis	ANA, ASMA, AMA, anti-LKM
Oncological hepatic disease	AFP, CEA, CA 19-9
Alcohol abuse	Blood ethanol, CDT

Table 1.2 Main differential diagnoses at DILI onset and required serological markers to be determined

virus: anti-HEV IgM; Epstein-Barr virus, cytomegalovirus, human herpes virus-6, parvovirus B19, human immunodeficiency virus and s.o.), metabolic (*e.g.*, hemochromatosis: transferrin saturation, serum ferritin levels, HFE gene testing; α 1-antitrypsin deficiency: serum α 1-antitrypsin levels, Wilson's disease:

serum copper levels, plasmatic ceruloplasmin levels, genetic testing;) and autoimmune (*e.g.*, anti-nuclear antibody (ANA) and anti-smooth muscle antibody (ASMA), anti-mitochondrial antibodies, anti-liver-kidney microsomal antibody (anti-LKM)) nature of liver disease¹⁵².

In terms of causality assessment, the Council for International Organizations of Medical Scientists established in 1989 a hepatotoxicity scale, which was also known as Roussel-Uclaf Causality Assessment Method (RUCAM), intending to standardize the procedure to ascertain cases of hepatotoxicity¹⁵³. The RUCAM scale is composed of seven sections that evaluate: i) Time to onset of the adverse reaction following initiation of the drug; ii) subsequent course of the adverse event after drug withdrawal; iii) specific risk factors; iiiii) concomitant administration of other medications with hepatotoxic potential; iiiiii) exclusion of other plausible causes of liver injury; iiiiii) hepatotoxic potential of the implicated drug; iiiiii) response to re-exposure to the agent. After applying the RUCAM algorithm, a quantitative grading is obtained, which expresses the likelihood of hepatotoxicity: i) below score 3 unlikely; ii) score 4-5 possible; iii) score 6-8 probable; and iiiii) above score 8 highly probable.

Besides the RUCAM scoring system, other probability scales have been used to support the causality judgment in hepatotoxicity. An example of other alternative scoring systems is the Maria & Victorino (M&V) scale. The M&V scale focuses on time to onset, excluding other liver insult aetiologies, extrahepatic manifestations (rash, fever, eosinophilia, cytopenia), hepatotoxicity potential of the suspected drug and rechallenge response^{154, 155}. The Naranjo scale gives a further example that rather than a specific scoring system for hepatotoxicity, it is used to assess any sort of adverse drug reaction¹⁵⁶.

Both Naranjo and M&V scales have been previously compared with the RUCAM method in terms of efficiency, which became higher after applying the latter one^{156, 157}. Thus, the RUCAM method, albeit imperfect, has continued to be extensively used during the last decades in the causality assessment of particular cases and large patient cohorts. It has been rated to perform moderately well in terms of accuracy, reproducibility and intraobserver variability¹⁵³.

Among the inconvenients, the RUCAM scale has been reported to be time-consuming, confusing with regards to the pointing instructions in some of its domains and a limited reproducibility even when applied by the same reviewers^{158, 159}. An update of RUCAM took place in 2016 with the aim to improve the intraobserver variability¹⁶⁰. The present and future of RUCAM are conceived by novel attempts to develop an electronic RUCAM application, improvement of the exclusion of other causes relying on the clinical context, and promotion of education and training on the use of RUCAM¹⁶⁰.

Risk factors

The underlying pathophysiology of idiosyncratic drug-induced liver injury reactions is poorly understood. However, it is believed to be the outcome of the interaction between the harmful potential of a drug to behave as a hepatotoxic substance and other additional factors associated with the host and the environmental and genetic background⁶⁸. More in detail, the hazardous agent's diverse characteristics such as physical-chemical properties, metabolism and drug dosage, and some host properties related to gender, age, underlying diseases, and comorbidities are thought to play an essential role in the development of DILI¹⁶¹.

Drug-related factors

Indeed, several factors increase the risk of hepatotoxicity and act fundamentally as drug-dependent risk factors. Lipophilicity, drug dosage or the potential to generate chemically reactive metabolites and trigger mitochondrial damage are examples of drug-related factors in DILI.

Lipophilicity is a standard pharmacological property that strongly contributes to the distribution of drugs within the organism. As a result, higher lipophilicity provides an augmented degree of drug uptake, distribution, absorption, metabolism, excretion and toxicity. Various studies correlated lipophilic compounds with an augmented risk to induce hepatotoxicity¹⁶²⁻¹⁶⁴. Other physicochemical properties such as the molecular weight and the surface sum over all polar atoms or molecules have been suggested to play potentially a role as drug-specific factors⁷².

The dosage of drugs and an extensive metabolism (>50%) in the organism have been identified as potential risk factors in contributing to DILI in several studies. As already mentioned above, Lammert and colleagues reported a higher DILI risk in patients who had ingested medications with doses of 50 mg/day and above⁶⁴. In the same line, the Spanish DILI registry's prospective database showed that about 77% of the enrolled causative drugs were administered in doses of 50 mg/day or higher¹⁶⁵. Another study identified compounds that led to DILI and presented high lipophilicity, which had been administered to the patients in doses of more than 100 mg/day¹⁶⁶.

Some of these factors can coincidentally be present at the DILI timeline and they may potentially lead to a synergetic effect over the outcome. For instance, a study found a significant association between severe DILI with high daily dosages (>100mg) in addition to lipophilicity, which was named as the "rule of two"¹⁶⁶.

The mitochondrion is believed to be the hepatocyte's central target to suffer from the deleterious effects within the setting of hepatotoxicity, as above described. Medicinal products can lead to impaired mitochondrial function either directly or indirectly. Direct pathways of mitochondrial injury classically can block enzymes implicated in glycolysis and

β -oxidation, alter components of the electron transport chain or inhibit the transcription of mitochondrial DNA of electron transport chain complexes. Indirect mechanisms of mitochondrial damage are believed to rely on the generation of free radicals, the depletion of intrinsic antioxidants and the interference with nutrients that contribute to the proper mitochondrial function¹⁶⁷. As a consequence, disruption of the redox balance can occur and subsequently, mitochondrial dysfunction and damage can happen. Hence, in the scenario underlying hepatotoxicity, culprit drugs and medicinal products can induce mitochondrial damage, triggering mitochondrial injury by disengaging different mechanisms.

For example, tetracyclines, tamoxifen and aspirin have been described to alter the mitochondrial β -oxidation; diclofenac has been related with mitochondrial membrane disruption; co-enzyme A sequester has been associated with valproic acid and aspirin; barbiturates and rotenone have been associated with blocking the NADH dehydrogenase and impairing the mitochondrial respiration¹⁶⁷⁻¹⁷⁰.

Above, we described the importance of the generation of the reactive metabolite N-acetyl-*p*-benzoquinone imine in the example of APAP hepatotoxicity in triggering liver damage. Besides APAP, many other drugs are well known to undergo phase I biotransformation in the liver, which may result in the formation of intermediate metabolites that characteristically present a different chemical reactivity compared to their parental compound. In principle, following the example of APAP hepatotoxicity, chemically reactive metabolites can potentially act as hazards to the liver either directly or indirectly by binding to proteins and forming drug-protein adducts, which can awake an immune-mediated reaction. Classically, the reactive metabolite formation hypothesis has become one of the strongest determinants with respect to DILI.

Notwithstanding, the impact of the reactive metabolite formation in the underlying physiopathology of idiosyncratic DILI remains uncertain for many drugs associated with idiosyncratic DILI and yet there is a lack of evidence. For instance, previous studies reported a missing correlation between the incidence of hepatotoxicity and reactive metabolite formation *in vitro* as well as cases where the formation of chemically reactive metabolites had been suggested to be absent (*e.g.*, ximelagatran, pemoline)^{171, 172}. Hence, more and more, the idea of a multifactorial condition triggering idiosyncratic DILI is gaining in importance and mechanisms other than the reactive metabolite formation are getting into the spotlight (*e.g.*, mitochondrial toxicity, inhibition of bile salt export pump).

The bile salt export pump (BSEP) is an ATP-binding cassette mainly located in the hepatocytes' membrane and mediates in exporting bile salts into bile at canalicular level. The inhibition of the BSEP by a drug or drug-related metabolite represents a further mechanism of interest in the physiopathology of DILI, which classically had been associated with cholestatic forms of DILI¹⁷³. For instance, the hepatotoxic potential of the compounds troglitazone, lapatinib, bosentan, ketoconazole and nefazodone has been shown to correlate with its potent BSEP inhibitory effect¹⁷⁴⁻¹⁷⁷.

This hypothesis relies mechanistically on a reduced bile acid excretion profile and liver injury development, most likely due to the accumulation of toxic bile acids. Diverse *in vitro* and *in vivo* experimental models have shown in many drugs that have been associated with severe forms of DILI that these had led to BSEP blockage, reinforcing the correlation between BSEP inhibition and risk of DILI^{177, 178}. Another study reported a higher frequency of severe DILI in those compounds that induced BSEP inhibition and mitochondrial dysfunction concomitantly¹⁷⁹. Furthermore, it has been suggested that BSEP gene mutations may lead to an altered BSEP function and increase the likelihood to suffer from DILI¹⁸⁰. However, various population-based studies, which examined polymorphic variations of the ABCB11 gene obtained striking results, some of these supporting a potential association to DILI, whereas others did not. Therefore, it might be of interest to examine these associations in larger cohorts in terms of number of participants and ethnic background.

Host-dependent factors

Ethnic disparities regarding drug-induced liver disease in diverse populations can be explained through the variable genetic background of individuals and differences in many factors associated with lifestyle, geographical drug prescription guidelines and everyday medication habits, among others. Indeed, race differences in liver disease have been observed in various previous studies.

A representative cross-sectional study obtained significant racial differences in the prevalence of NALFD, which interestingly could not be fully explained by lifestyle, adiposity, and metabolic factors¹⁸¹. A systematic review and meta-analysis determined racial differences as a capital factor in the prevalence and the severity of NALFD, whilst ethnic differences for outcome were discordant¹⁸². A retrospective chart review, including 791 subjects suffering from alcoholic liver disease, found ethnicity a significant factor of influence on age and severity in different subtypes of ALD¹⁸³.

In the focus of drug-induced liver disease, an increased probability of suffering from drug-induced ALF has been associated with Afro-American subjects versus Caucasians¹⁸⁴. Moreover, a recent prospective study from the USA that compared the enrolled self-described African-American versus Non-Hispanic subjects found differences in the causative agents and severity of liver injury leading to worse outcomes, including death and liver transplant, which was higher in the African-American DILI subpopulation⁴⁷. Nevertheless, large prospective hepatotoxicity databases were unable to link ethnicity and DILI frequency.

The question if gender was a potential risk factor linked to DILI had been extensively debated earlier following the general belief that the female sex had a higher risk to suffer from DILI based on prior observations in several studies^{41, 128, 185}. Indeed gender has been linked to an increased DILI susceptibility in certain drug agents: Whereas drugs like flucloxacillin, isoniazid, diclofenac, nitrofurantoin, chlorpromazine, tetracyclines have been related to DILI and female gender, azathioprine has been associated with an enhanced risk of DILI development in men^{68, 161}.

Nevertheless, large prospective hepatotoxicity cohorts failed in evidencing any significant differences regarding male and female sex distribution in their data. A prospective cohort from Spain enclosed a population of 603 individuals, of which 49% corresponded to female sex¹⁶⁵. Another prospective hepatotoxicity study from the USA included 899 subjects, where 59% corresponded to women⁵⁰. A further study from Iceland, with 96 enrolled individuals, revealed that 56% of the analyzed population were women⁵². Two prospective hepatotoxicity databases from Spain and the USA described the influence of gender in the liver injury pattern since a higher fraction of hepatocellular cases were found in females^{50, 165}. The same cohort from Spain demonstrated a strong association of female sex and severity of liver damage with 89% of drug-induced fulminant hepatic failure insults occurring in women, which were in line with the results obtained by the Acute Liver Failure Study Group, where out of 133 studied individuals, 77% were females^{51, 186}.

Figure 1.5 Flow chart displaying main risk factors and counterregulatory mechanisms associated with DILI.

The threshold to liver injury triggered by drugs results from a disbalance between risk factors and counterregulatory mechanisms. The most significant risk factors are drug-specific or host-dependent. Under these category characteristics age, gender, comorbidities, underlying diseases or genetic, among others, can be found. On the contrary, the organism has effective defensive mechanisms to counteract and promote a safe drug clearance. In the liver, the glutathione (GSH) system neutralizes any excessive ROS production that results from drug-induced cellular stress. The activation of antioxidant pathways (e.g., Nrf2/Keap1 axis) promotes the recovery of intracellular redox homeostasis. N-acetyl cysteine has been shown to mitigate APAP-induced hepatotoxicity by stimulating the GSH synthesis and promoting nontoxic metabolic routes of APAP.

Older age has been linked to general changes affecting the pharmacokinetics of drugs encompassing a reduced body mass, hepatic mass and blood circulation, and a diminished renal function and hepatic metabolism profile. In addition, older age is a complex

variable to assess since, frequently, these individuals are subjected to polypharmacy and present underlying diseases.

However, no shreds of evidence have convincingly confirmed that aging is a direct risk factor associated with drug-induced liver disease development. The review of the largest prospective hepatotoxicity cohorts reflected that 17% of the DILI population analyzed at the US DILI Network was aged 65 years or older versus 46% of the included subjects in the Spanish DILI Registry who were 60 years old or above. In contrast, a relationship between DILI incidence and growing age in the elderly in the Icelandic study was concluded^{50, 52, 165}.

On the other hand, a more noteworthy link between age and particular types of drug-induced liver disease have been described earlier. For example, valproic acid, dactinomycin, and ampicillin are more commonly described in pediatric DILI, whereas basiliximab is exclusive of DILI in children¹⁸⁷. In contrast, DILI due to deferasirox, isoniazid, dantrolene, and levofloxacin has been observed more frequently in adults, with particular mention to isoniazid DILI, which was also associated with DILI in adults and happened above four times more often in individuals over 50 years than in younger patients^{187, 188}.

In general lines, the association of underlying liver diseases as a risk factor for the development of hepatotoxicity has not been consistently demonstrated in the limited amount of available studies and other authors previously reported about the safe use of drugs in these groups of patients¹⁸⁹. Nevertheless, pre-existent liver disease can alter the physiologic function of transporter proteins and interfere in the drugs' pharmacokinetics, which might affect the development of DILI¹⁹⁰. In addition, the underlying hepatic disease is believed to be related to a declined response, progression and outcome of hepatotoxicity¹⁹¹. The most common described hepatic conditions related to this instance are represented in the examples of nonalcoholic fatty liver disease (NAFLD), alcoholic liver disease and chronic viral hepatitis, especially when co-infected with human immunodeficiency virus^{192, 193}.

Nonalcoholic fatty liver disease (NAFLD) is a prevalent type of liver disease, which can exhibit a broad spectrum of hepatic lesions (*e.g.*, fatty liver, non-alcoholic steatohepatitis, cirrhosis). Several experimental models on fatty liver disease have shown to favor APAP-induced hepatotoxicity¹⁹⁴. Despite not fully elucidated, previous activation and induction of hepatic cytochrome P450 2E1 and decreased response of antioxidant mechanisms (*e.g.*, lower GSH reserves) occurring in a NAFLD-conditioned scenario could contribute to increasing the generation of the toxic APAP-derived metabolite NAPQI^{194, 195}. Additionally, other clinical studies have described a potential association between NAFLD and drug-induced hepatotoxicity for several agents such as fosinopril, isoflurane, halothane, methotrexate, statins, tamoxifen, several antiretroviral drugs, among others^{196, 197}.

Hepatotoxicity due to antiretroviral compounds has been associated with chronic viral hepatitis B and/or C co-infection (*e.g.*, protease inhibitors, non-nucleoside reverse transcriptase inhibitors), often occurring in poor responders to antiretroviral therapies¹⁹⁸⁻²⁰⁰. Antitubercular agents have been in the spotlight of DILI and particularly in patients carrying an underlying chronic viral hepatitis infection; however, the conclusions have shown discrepancies. Some works detected an enhanced risk of antitubercular drug-induced liver injury in the setting of HBV and HCV co-infections²⁰¹⁻²⁰³ as well as in

combination with HIV, which was found notoriously higher for individuals with HIV and HCV co-infection²⁰⁴, whereas other studies did not identify any associations²⁰⁵.

Patients who have cancer have been related to a higher risk of developing DILI due to therapies with chemotherapeutic agents, which can exhibit with variable severity, notwithstanding this increased risk may not exclusively involve antineoplastic drugs but also other non-chemotherapeutical compounds²⁰⁶. Antineoplastic drugs can decline basal liver disease and induce liver dysfunction. Indeed, diagnosis of chemotherapeutic DILI is complex due to the broad spectrum of confounding factors that interfere in transaminase flare (*e.g.*, opportunistic infections, radiation, DILI derived from other medicines, exacerbation of the underlying liver disease)²⁰⁷.

However, many of these observations relied on studies performed on a small number of subjects, and therefore more extensive studies are required to assess these aspects more in detail. It is also essential to consider the risk of drug interactions and potential confounding factors that are commonly related to underlying hepatic disease per se.

Genetic susceptibility

As extensively reviewed above, a wide range of risk factors have been reported to increase the probability of suffering from hepatotoxicity. Nevertheless, the phenomenon of idiosyncrasy in DILI seems to be more complex to understand and, thus, rather than individual mechanisms, it is most likely the result of a multifactorial condition. In particular, genetic and environmental factors are suggestive of playing a pivotal role in the pathomolecular bases of idiosyncratic adverse drug reactions. As other authors have previously highlighted, different hepatotoxicity databases enrolling DILI cases in different countries have shown, in effect, relevant differences regarding the relative frequencies of culprit agents²⁰⁸. In this line, genetic and environmental factors might be determinant to understand these differences. Thus, it is undeniable that there is a genetic implication in DILI to some extent, as earlier studies have demonstrated, at least for certain individual compounds^{208, 209}.

During drug metabolism in the liver, many hepatic enzymes (bioactivation and detoxification enzymes) and transporters are active and indispensable. A large variety of polymorphisms have been associated with the genes that encode these enzymes and transporters. The best-known example is probably provided by phase I enzymes that belong to the cytochrome P450 (CYP) superfamily. The allelic variability of CYP enzymes explains the vast variations in drug clearance processes between individuals. The presence of polymorphic variations in these enzymes has a cardinal impact on the efficacy and adverse effects of the drugs metabolized by them. The most relevant polymorphisms affect CYP2D6, 2C9 and 2C19 due to the simple reason that nearly 80% of the currently marketed medicines are metabolized by these CYPs²¹⁰. Allelic variants of CYPs have been linked to variations in the drug metabolism and differences in the susceptibility to carcinogenesis (kidney, breast, lung and prostate cancer). Indisputably, some CYPs have been related to the bioactivation of procarcinogens, among other potential mechanisms²¹¹⁻²¹³. Besides, CYP polymorphisms have been associated with other internal diseases such as atherosclerosis and diabetes mellitus as well²¹¹.

For instance, several drugs mainly metabolized by CYP isoforms 2C9 and 2C19 have been known to have a certain hepatotoxic potential, following the examples of drugs diclofenac, tienilic acid or leflunomide²¹⁴⁻²¹⁶. Nevertheless, except anecdotal studies exhibiting a low number of subjects, other extensive studies have failed to predict allelic variants of CYP2C9 and 2C19 being implicated as DILI risk factors^{45, 217}.

A further example is delivered by several phase II detoxification enzymes, potentially associated with hepatotoxicity. For example, polymorphic variations affecting UDP-glucuronosyltransferase (UGT) 1A have been related to flares in transaminases triggered by tolcapone, variations of UGT2B7 and diclofenac-induced liver damage, polymorphism of N-acetyltransferase 2 and isoniazid-induced liver injury, among others^{209, 218-221}. Further studies described glutathione S-transferase (GST) M1²²² and T1²²³ null associations with DILI triggered by isoniazid plus other anti-tubercular compounds. In contrast, another analysis reported results, which suggested that GST M1 and T1 double-null genotypes could be associated with DILI independently from the culprit agent and preferably in female gender²²⁴.

Besides genetic polymorphisms affecting hepatic metabolic enzymes and transporters, immunomodulatory polymorphisms and mutations have been placed in the scope of the multifactorial interplay, which potentially increases the susceptibility to suffer from DILI. More specifically, allelic variants of the human leukocyte antigen (HLA) genes exert a critical regulatory modulation of the immune response and are located in the major histocompatibility complex (MHC) region chromosome 6 have been linked with DILI triggered by certain drugs²²⁵.

Many of the targeted results stem from small cohort studies and case-control candidate gene studies and, hence, had uniquely limited strength when drawing precise conclusions. The introduction of novel sequencing methods made way to genome-wide association (GWA) studies, which represented an outstanding opportunity to carry out extensive genetic studies covering a wide number of factors⁹⁸. Indeed, the use of the GWA technique has confirmed some of the candidate gene study results. However, several limitations apply to GWA studies, such as the difficulty in detecting rare genetic variants with weak effects or a wide range of phenotypes may remain uncovered, among others²²⁶. Several investigations covering the analysis of the associations mentioned above have been described for the following drugs and HLA haplotypes: *HLA-DRB1*15:01*, *HLA-DQB1*0:602*, *HLA-A*02:01*, *HLA-DRB5*01:01* and amoxicillin-clavulanate; *HLA-B*57:01* and flucloxacillin²²⁷, *HLA-A*33:01* and terbinafine, fenofibrate and ticlopidine; *HLA-DQA1*02:01* and lapatinib²²⁸; *HLA-DRB1*07*, *HLA-DQA1*02* and ximelagatran²²⁹; *HLA-B*35:02* and minocycline; *HLA-DRB1*15:01* and lumiracoxib²³⁰.

Of note, far from only affecting the MHC, immunogenetic modulations may also have a capital influence on pro-inflammatory and anti-inflammatory mediators, modifying the immune and inflammatory response and thus, become an attractive downstream target of DILI. Previous hypotheses suggest a role of polymorphic variants affecting the expression of cytokines and enhancing the susceptibility of a drug-induced reaction either directly or by conditioning the active involvement of T cells²³¹. For instance, an investigation showed that hepatotoxicant-derived cytokine imbalance led to enhanced release of the pro-

inflammatory interleukin (IL)-1 β ²³². Another study highlighted the potential role of T helper-2 cell-induced immune response to neoantigens in the presence of polymorphisms that triggered low IL-10 and high IL-4 gene transcription and increased the susceptibility to DILI²¹⁴. Further analysis of a prospective DILI patient cohort found no association with IL-10, IL-4 and TNF- α polymorphisms, but instead, a low-producing IL-10 haplotype in a fraction of individuals without peripheral eosinophilia was related with a worse DILI outcome²³³.

All in one, genetic variations acting on drug metabolism components and the immune system are determinant to understand the pathophysiology underlying DILI and its predisposition to suffer from it. The immense advances achieved with modern sequencing techniques have confirmed some of the previously described associations by candidate gene studies. Compared with candidate gene studies, GWA assays can analyze large and complex datasets and consider many additional factors, and they might not be suitable in all the cases due to its high threshold for genome-wide significance. In particular, the idea of future HLA genotyping in patients could be very promising for improving DILI diagnosis, as it could help to identify the culprit drug in cases of polypharmacy or distinguish between autoimmune-like DILI and atypical forms of AIH, among others²³⁴.

Databases and information sources on hepatotoxicity

Extensive prospective studies of drug-induced liver injury

The Spanish drug-induced liver injury registry was founded in April 1994 and operated initially as the Regional Registry of Hepatotoxicity of Southern Spain in cooperation with several Liver Units. Later, the regional registry evolved to a national multicenter collaborative network between liver, digestive disease, internal medicine, and clinical pharmacology units from over 50 reference hospitals located throughout the Spanish geography, followed by a structured procedure for prospective data and human sample collection^{51, 235}. As a part of its structured procedure, after obtaining the patient's consent, a detailed medical record is obtained with particular attention to the previous history of liver or biliary tract disease, ethanol abuse, addiction to narcotics, blood or plasma transfusion history and previous surgery that had occurred six months before the liver dysfunction episode. In parallel, a meticulous drug history is carried out by interrogating patients and their relatives for any previous consumption of drugs, herbal remedies, dietary supplements and/or any other medicinal OTC products. The criteria applied for case characterization corresponded to those established by the International Consensus Meeting for liver injury and were later adjusted to Aithal et al. 2011^{63, 141}. To establish the probability of suspicion, the Council for International Organizations of Medical Sciences (CIOMS) scale was applied. A detailed follow-up of the methodological algorithm can be consulted in the Spanish DILI registry's previous published works^{51, 235}.

At present, the Spanish DILI Registry leads the creation of a European DILI registry (Pro-Euro DILI Network) in cooperation with a large number of European university hospitals and research institutions. The key aims of this challenging project include building up a unique, integrative, interdisciplinary and translational DILI network in Europe that carries out

impactful research and knowledge on DILI and promotes the development of pivotal solutions to diagnosis and therapeutics from the molecular level to the patient's bedside. More information can be accessed at <https://proeurodilinet.eu/>.

The National Institute of Diabetes and Digestive and Kidney Diseases has established the Drug-Induced Liver Injury Network with the primary purpose to promote research on drug-induced liver injury in the United States since the year 2004²³⁶. As a part of its core functions, the DILIN carries out standardized procedures to identify, characterize and collect *bonafide* cases of drug-induced liver injury. An extensive biobank preserves patient serum, histological specimens and DNA of DILI patients and control subjects. Two main studies have been carried out at the DILIN (<https://diln.org/>): on the one hand, a prospective study has been developed that takes into account any incidental DILI case, whereas, on the other hand, a retrospective study has been implemented, which analyzes DILI episodes associated to a limited number of drugs. Among these, isoniazid, phenytoin, amoxicillin-clavulanate, valproic acid, nitrofurantoin, trimethoprim-sulfamethoxazole, minocycline and quinolone antibiotics have been considered. Further details about the applied methodology at DILIN can be found in previously published articles⁵⁰.

In 2011 the Latin-American (LA) DILI Network was created with the support of the Spanish DILI Registry (SLatin DILI Network). Of note, its network relies on multinational collaborative cooperation between the participating centers of countries located in Central and South America such as Argentina, Uruguay, Chile, Brazil, Mexico or Paraguay, among many others²⁰. This aspect adds the registry an outstanding value due to the significant differences in ethnicity, drug prescription and drug regulation policies within the participating countries. Latin America is believed to enclose highly endemic areas of Hepatitis E virus²³⁷ infections, despite there is no reliable scientific literature yet available that refers to the actual HEV prevalence in Latin America, apart from several patient reports^{20, 237}. Nevertheless, a meaningful emphasis has been done on ruling out HEV as a potential confounding factor at the LA DILI Network. For this reason, HEV markers are determined in all cases, where the clinical context and epidemiological setting are suggestive of HEV infection. With respect to operational procedures, the identical methods for case assessment and data collection used at the Spanish DILI Registry have been taken at the LA DILI Network²⁰.

Apart from the Spanish DILI Registry, the DILIN and the LA DILI Network, there are plenty of remarkable cross-sectional studies on DILI and there is a growing culture of new prospective DILI studies worldwide. These studies will help to phenotype and better understand the global impact of DILI and contribute in elucidating the role of geographical factors in DILI (*e.g.*, ethnic, genetic and environmental factors, drug-marketing and medicine-regulatory issues).

The LiverTox website

The LiverTox website represents an accessible and reliable online information source on drug-induced liver injury. It has been developed and maintained by the Liver Disease Research Branch of the National Institute of Diabetes and Digestive and Kidney Diseases (NIDDK) in cooperation with the National Library of Medicine²³⁸. It encloses an extensive and accurate overview of the DILI aetiological agents and lists up the formal name of drugs,

herbals and dietary supplement compounds. Each compound is commonly introduced by some general facts such as the chemical formula and the compound's structure, treatment indications and common therapeutic dose regimes²³⁸. The introductory data is followed by a detailed review of the essential aspects in terms of hepatotoxicity associated with the agent, which includes a description of the clinical scenario, the involved injury pattern, the associated or potentially implicated mechanisms, the clinical course and outcome associated to the culprit agent. Several representative hepatotoxicity cases are often presented as case reports, including the patient's medical record and clinical data supported by laboratory tests or imaging. In the end, a comprehensive and updated list of bibliographical references that is mainly obtained from PubMed or hepatotoxicity textbooks can be found. Therefore, LiverTox is a free-available and useful tool for clinicians, researchers, and even the general public, easy to access and work with. LiverTox can be accessed at <https://livertox.nih.gov>.

Other useful information sources on hepatotoxicity

A wide variety of web-based online tools dealing with adverse drug reactions are freely available online and can be useful to retrieve information of interest on hepatotoxicity and DILI assessment. Besides LiverTox and other similar websites, including hepatotoxicity scales, we will briefly introduce Vigibase, LiMTox and DILIRank.

Vigibase represents a worldwide pharmacovigilance database for medicines launched by the Uppsala Monitoring Centre and the WHO Programme, with over 20 million drug safety reports submitted since 1968. One of the WHO Programme's hallmarks has identified previously unknown drug safety issues of medicinal products and sharing this valuable information globally. It encloses detailed information on suspected individual case safety reports of patients who have been suspected of suffering from an adverse drug reaction.

The data was obtained by participant countries of the WHO Programme for International Drug Monitoring. Vigibase can be accessed at <https://www.who-umc.org/vigibase/vigibase>.

The Structural Biology and Biocomputing Programme of the Spanish National Cancer Research Centre (CNIO) has recently developed LiMTox, a useful text mining search engine for toxicology. LiMTox is a web-based and free-accessible tool that focuses on adverse drug reactions with particular attention to drug-induced liver injury. Its functionality consists of machine-learning and pattern-based systematic search strategies and it can integrate wide text mining and information extraction components. LiMTox can be accessed at <http://limtox.bioinfo.cnio.es>.

The Federal Drug Agency (FDA) provides the DILIRank dataset, including over 1000 FDA-approved medicines, classified into four different groups according to their potential to trigger hepatotoxicity (most-, less-, no- and ambiguous-DILI-concern-drug)²³⁹. The information is retrieved from analyzing hepatotoxic descriptions associated with FDA-approved drug labeling files and causality assessments obtainable in the scientific literature. DILIRank can be accessed at <https://www.fda.gov>. The FDA and the European Medicines Agency and other national and regional regulatory medicines agencies enclose information of interest related to pharmacovigilance and drug safety on their websites.

Paraphrase: Essentials on Hepatocellular carcinoma

CLD and hepatocellular carcinogenesis at a glance

Chronic liver disease (CLD) represents the consequence of different forms of liver disease that can originate from diverse aetiologies of viral, metabolic, autoimmune or toxic nature, among others. The prevalence of the underlying causes leading to CLD may vary in frequency between different geographical areas. Nonalcoholic steatohepatitis (NASH), alcoholic steatohepatitis or hepatitis C virus infection are relevant causes of CLD in Western countries, while viral infections in particular due to hepatitis B virus continue to be the most common etiologies in African and Asian regions²⁴⁰. Liver injury results in an inflammatory reaction, which is part of the wound healing response. Nevertheless, when the liver damage and the deriving inflammatory response become persistent, several liver tissue changes, including fibrogenesis, subsequently follow. Characteristic of hepatic fibrosis is the excessive accumulation of extracellular matrix proteins (ECM)²⁴¹. As a result, the consistency of the liver tissue collapses and is substituted with collagen-rich tissue. Essentially, activated hepatic stellate cells, portal fibroblasts or bone marrow-derived myofibroblasts are implicated in the dysbalanced ECM formation, and their stimulation responds to pro-inflammatory cytokines as *e.g.*, TGF- β ²⁴². Indeed, persistent inflammation always precedes hepatic fibrosis. Activated KCs, hepatocytes, HSCs, natural killer cells, lymphocytes, and dendritic cells are capital drivers of the inflammatory response during fibrogenesis as sources of chemokines, interferons, interleukins, growth factors, among others (an exhaustive review on hepatic fibrosis is presented in chapter 9)²⁴³.

The progression of hepatic fibrosis can lead to aberrant hepatic function and liver parenchyma remodeling in the long run. As a consequence, severe complications such as liver cirrhosis or hepatic carcinogenesis can appear due to the hepatic deterioration in advanced stages of CLD. Both entities are associated with very poor prognosis and represent the most common causes of death in chronic liver disease.

Hepatocellular carcinoma (HCC) represents the most frequent type of primary malignancy related to the liver and a leading global cause of cancer-related death. Worldwide, HCC accounts for the fifth most frequent type of carcinoma and ranks as the second cancer-related death²⁴⁴. The incidence of HCC has been growing worldwide during the past decades and its highest incidence rates can be found in Asia and Africa, where HBV and HCV infections are endemic in many regions. In the US, an increased incidence rate of 3.1 per year has been reported²⁴⁴. The highest single-country incidence rate had been reported in Mongolia with an age-standardized rate per 100,000 persons of 78.1²⁴⁵.

The histological and clinical features of HCC are classically subjected to a certain degree of heterogeneity with a variable range of cell differentiation, oscillating between very well-differentiated to poorly differentiated. Of interest, HCC characterizes by a rich morphologic diversity, which encloses many different subtypes (*e.g.*, biphenotypic HCC (presents features of hepatocellular and cholangiocarcinoma), fibrolamellar HCC, cirrhotomimetic HCC, steatohepatitic HCC, sarcomatoid HCC, clear cell HCC, granulocyte-colony-stimulating HCC). The molecular mechanisms underlying HCC are not well understood. Most likely,

reiterated hepatocytic damage boosts up a vicious circle that characterizes for cell death and regeneration that can potentially derive in genomic instability and tumor formation/initiation²⁴⁶. The precise genetic mechanisms that awake hepatocellular carcinogenesis remain unelucidated, whereas many gene associations have been related to HCC formation. The major agents believed to be implicated in hepatocarcinogenesis are related to diverse somatic gene mutations (*e.g.*, telomerase promoter mutations, TP53 pathway mutations) and signaling pathways (*e.g.*, JAK/STAT, Wnt β -catenin, PI3K-AKT-mTOR)²⁴⁴.

Regardless of its underlying etiology, liver cirrhosis embodies the most relevant risk factor associated with HCC. In general lines, it has been estimated that one out of three cirrhotic patients develops HCC during their lifetime and about 1-8% of patients with cirrhosis develop HCC per year^{247, 248}. Further risk factors of HCC are chronic viral hepatitis, persistent alcohol abuse, acquired and inherited metabolic diseases (NAFLD, hemochromatosis, alpha-1-antitrypsin deficiency, Wilson disease), exposure to carcinogenic hepatotoxins (*e.g.*, aflatoxin, CCl₄). Many of these diseases can progress during their clinical course to chronic liver damage and cirrhosis and may be complicated by hepatic carcinogenesis, despite others can elude the progression to chronic hepatic damage and promote hepatocarcinogenesis (*e.g.*, HBV infection) directly.

NF- κ B activation and its relevance in carcinogenesis

The NF- κ B (nuclear factor- κ B) transcription factors are pivotal for regulating cellular signaling covering immune and inflammatory responses. The NF- κ B protein family consists of 5 cellular DNA-binding subunits in humans: Rel (c-Rel), RelA (p65), RelB, NF- κ B1 (p50 and its precursor p105) and NF- κ B2 (p52 and its precursor p100)²⁴⁹. Under unstimulated conditions, the NF- κ B transcription factors remain disengaged and sequestered in the cytoplasm through the interaction with the inhibitory protein I κ B (inhibitor of κ B) that masks the nuclear localization signals of NF- κ B²⁵⁰. The I κ B kinase (IKK) complex plays an essential role in regulating NF- κ B by phosphorylating the I κ B proteins. IKK is composed of two catalytic subunits (IKK1/IKK α and IKK2/IKK β) as well as a regulatory subunit, NF- κ B-essential-modulator (NEMO/IKK γ)²⁵⁰.

Many different stimuli can trigger NF- κ B activation, enclosing UV-irradiation, viral infections, lipopolysaccharide (LPS), cytokines (TNF α , IL1), activated B/T-cells²⁵¹. During the canonical NF- κ B pathway (**Figure 1.6**), a pro-inflammatory signal, for instance, TNF, initially stimulates the signaling cascade by adhering, in this case, to the tumor necrosis factor receptor type 1 (TNFR1)²⁵². Subsequently, trimerization of TNFR1 follows, which in turn induces clustering of death domains and consequently, several intracellular adaptor proteins are mobilized. As a result, TNFR1-associated death domain protein (TRADD) interacts with TNFR1, serving as an assembly scaffold that allows the recruitment of TNFR-associated factor 2 (TRAF2) and receptor-interacting protein 1 (RIP1) kinase^{253, 254}. Subsequently, RIP1 is ubiquitinated and consequently transforming growth factor beta-activated kinase 1 (TAK1) is mobilized and switched on²⁵⁵. TRADD is necessary for Fas-associated protein with death domain (FADD) recruitment, promoting alternative pathways of apoptosis²⁵⁶.

Next, the I κ B kinase complex (IKK) is formed, which consists of two serine kinases, IKK α and IKK β , which form heterodimers that concomitantly can interact with a dimer or trimer of the regulatory subunit NEMO, which is also known as IKK γ . Both IKK α and IKK β are catalytically active, whilst NEMO functions as a regulatory subunit. During the activation of the IKK complex, NEMO recruits and interacts with IKK α and IKK β through its c-terminal residue, enabling their linkage to upstream activators, which leads to the phosphorylation of IKK β . As a result of autophosphorylation, the activated IKK β subunit activates its adjacent IKK α subunit and other inactive complexes, which derive in high I κ B kinase activity.

Consequently, the IKK complex is engaged, and the I κ B family's proteins are initially ubiquitinated and in a further step degraded by the 26S proteasome. Hence, the NF- κ B p50-p65 dimer is set free, the nuclear localization signals are no longer masked and the p50-p65 dimer can finally translocate into the cell nucleus, where it accumulates, binds DNA specific sequences and controls the transcriptional activity. The central genes targeted by NF- κ B encompass essentially chemokines, cytokines, immuno- and cytokine receptors, adhesion molecules or regulators of apoptosis and cellular proliferation, among others²⁵⁰.

Figure 1.6 Schematic representation of canonical NF- κ B pathway activation triggered via TNF stimulation.

(1) Activation of TNFR1 follows after binding of its ligand TNF- α . TNFR1 activation promotes the mobilization of diverse intracellular mediators (TRADD, TRAF2, TAK1 and RIP), which in turn leads to recruit and activation of the IKK complex (2). (3) IKK induces I κ B phosphorylation and leads to degradation of I κ B via the ubiquitin system (4), resulting in the target molecule I κ B masked by a chain of ubiquitin (Ub), which is then degraded by the 26S proteasome. Finally, NF- κ B dimers are unbound

and released, allowing NF- κ B to translocate to the cell nucleus and activate the transcription of genes regulating inflammatory, immune, proliferative or apoptotic responses, among others (5).

The abnormal function of the NF- κ B pathway has been evidenced to be involved in different inflammatory human diseases (*e.g.*, rheumatoid arthritis) and carcinomas (*e.g.*, colitis-associated cancer and hepatocellular carcinoma). Undeniably, NF- κ B represents a key link between inflammation and cancer. NF- κ B is a master regulator of the gene expression that controls cell proliferation and cell survival. Constitutively activated NF- κ B has been described as a feature of diverse human tumors and has been suggested to result from carcinoma-related chromosomal aberrations (*e.g.*, mutations, translocations, deletions), which might interfere with genes encoding NF- κ B and I κ B proteins^{257, 258}. Furthermore, various carcinogenesis features such as preventing apoptosis, promoting cancer-cell proliferation, and increasing a tumor's angiogenic and metastatic potential have been likewise related to active NF- κ B^{257, 259}.

The NEMO model as an experimental approach to CLD

The NF- κ B pathway is a crucial actor implicated in diverse aspects related to chronic liver damage and hepatocarcinogenesis. NF- κ B is an essential regulator of the inflammation in the liver and mediates both proinflammatory as well as anti-apoptotic responses to prevent liver injury from infectious and inflammatory triggers (*e.g.*, gut-derived-pathogen products as, *i.e.*, LPS, inflammatory cytokines as, *i.e.*, TNF), which can act as toxins to hepatocytes. In this line, NF- κ B orchestrates these responses to balance, providing adequate protection to hepatocytes, preventing cell death, and steering the appropriate inflammatory response to these injury triggers. Moreover, NF- κ B signaling has been reported in all types of chronic liver disease, encompassing non-alcoholic fatty liver disease, viral hepatitis, alcoholic liver disease and biliary liver disease.

NEMO (IKK γ) does not present a kinase domain, exerts a non-catalytic function and serves as the regulatory component of the IKK complex, which is indispensable for I κ B degradation. Indeed, NEMO has been demonstrated to be critical for normal-stimulus dependent NF- κ B activation and its deriving target gene expression²⁶⁰. The absence of NEMO has been shown to abrogate IKK and NF- κ B activation²⁶¹.

In earlier investigations, it has been evidenced that constitutive deletion of NEMO (NEMO/IKK γ ^{-/-}) in mice leads to embryonic unviability at E12.5-E13.²⁶⁰ At this stage, NEMO/IKK γ ^{-/-} exhibited a severe hepatic degeneration, which was characterized by detrimental liver architecture, massive liver hemorrhages and dramatically enhanced hepatocytic apoptosis. In contrast, redundancy of NEMO uniquely in liver parenchymal cells (Nemo ^{Δ hepa}) of mice is compatible with the birth of viable offspring, although these progressively develop chronic liver injury during their lifetime²⁶². Characteristic features of Nemo ^{Δ hepa} animals include a strong hepatic inflammatory reaction resembling human nonalcoholic fatty liver disease (NAFLD) and steatohepatitis (NASH), which lead to hepatic fibrosis²⁶². Critically, Nemo ^{Δ hepa} mice spontaneously develop HCC at 52 weeks of age and

both NASH-like hepatitis and HCC have been described with a penetrance of 100% in these animals²⁶².

As part of its phenotypic expression, NEMO-redundant hepatocytes show a strong susceptibility to spontaneous apoptosis, which in turn induces inflammation and reactive proliferation in an attempt to regenerate the liver. This chronic condition leads to a vicious circle of apoptosis, inflammation and proliferation that, in addition to enhanced oxidative stress, have been suggested to promote mutagenic changes, which may then lead to dysplasia and tumorigenesis²⁶².

Compared with other experimental HCC models, NF- κ B inhibition via hepatocyte-specific redundancy of IKK- β correlated with an augmented incidence of HCC after treatment with the carcinogen diethylnitrosamine²⁶³. A further investigation relying on the Mdr2 knockout model, where mice develop chronic liver injury and HCC, showed reduced liver tumor progression due to NF- κ B blockage with inducible I κ B super-repressor²⁴³. Likewise, another study demonstrated that suppressing NF- κ B inhibition through anti-TNF α treatment or induction of I κ B-super-repressor in Mdr2 knockout animals evolved failure to progress to hepatocarcinoma in late-stage CLD²⁶⁴. All in one, these results exhibit the complexity of the role of NF- κ B in liver parenchymal cells regarding hepatocarcinogenesis, which can promote as well as suppress tumor formation depending on the type of HCC model and its stage of progression²⁶⁵.

Aims and outline of the thesis

With the present work, we aimed to carry out a translational contribution to the field of hepatology with a focus on toxic and chronic liver disease. For that purpose, we applied diverse biomedical research methods enclosing laboratory investigation techniques at the molecular level, clinical-epidemiological studies and extensive literature analyses. The investigations encompassed in the first part of the present thesis manuscript aimed to enhance our knowledge of drug-induced liver injury (Section I). DILI is a complex and broad topic of study, making it challenging to cover all relevant aspects in one unique work. Therefore we defined *ab initio* several main objectives to be analyzed in the present scientific work: Experimental assessment of the role of hepatocytic JNK in diverse models of liver toxicity; to investigate the role of ibuprofen in hepatotoxicity; to contribute to the phenotypic characterization of other types of DILI in humans (induced by medicines such as methylprednisolone, venlafaxine). In the second part, we desired to focus our research on the chronic liver disease (Section II). Here we analyzed the relevance of liver fibrosis reversal as a potential therapeutic strategy against chronic liver disease and developed novel experimental therapy approaches against experimental CLD/HCC.

Chapter 1 introduced the present work by critically reviewing the most relevant facts on DILI that have been known from the earlier literature. Apart from general definitions and classifications, we desired to analyze the physiopathology of DILI and the crosstalk involving drug metabolism, the formation of reactive metabolites, oxidative stress, mitochondrial dysfunction, the involvement of innate and adaptative immune responses and activation of

signaling pathways in mediating hepatocellular injury and death. Furthermore, the most relevant mechanistic findings with particular focus on mitogen-activated phosphokinases (MAPKs) and c-Jun N-terminal kinases (JNK) were revised, which is a capital aspect of the hereby-presented investigations. In addition, a brief introduction into hepatocellular carcinoma (HCC) and the experimental CLD model “NEMO” (mice presenting deletion of IKK γ in liver parenchymal cells) was included since our present investigations also covered diverse key aspects related to chronic liver disease and HCC.

Ibuprofen, a non-steroidal anti-inflammatory agent (NSAID), is widely used and obtainable under medical prescription and over-the-counter. Despite ibuprofen is well-tolerated among all ages and performs adequately in terms of drug safety, several reports and databases have reported cases of ibuprofen-associated hepatotoxicity. Therefore, in **Chapter 2**²⁶⁶ we developed a systematic search strategy to identify and analyze any useful information on ibuprofen-induced hepatotoxicity published in the literature to contribute to a better understanding of the phenotypic expression of this concrete type of DILI, which remains unelucidated at current.

Previous extensive hepatotoxicity cohort studies from Spain, Iceland and the USA revealed that nonsteroidal antiinflammatory drugs are frequently involved as culprit agents in DILI. Nevertheless, only scarce information is known on ibuprofen-associated hepatotoxicity to date. In **Chapter 3**²⁶⁷, it was consequently aimed to study the prevalence of ibuprofen-induced liver injury in the Spanish and Latin-American DILI databases and carry out a detailed analysis of these events in order to investigate their phenotypic expression as well as to evaluate a potential drug signature of ibuprofen with regards to hepatotoxicity. A comparative analysis of ibuprofen DILI events with other ones attributed to other NSAIDs and non-NSAIDs was performed.

Several case reports of ibuprofen-associated liver toxicity, which have been reported earlier, display a wide range of liver injury patterns such as cholestasis, Steven-Johnson’s syndrome or vanishing bile duct syndrome (VBDS), among others. The severity of ibuprofen-related liver toxicity is variable, ranging from asymptomatic serum liver alterations to acute liver failure. Overdose and idiosyncratic forms of hepatotoxicity have been associated with ibuprofen; nevertheless, the underlying pathogenesis of ibuprofen-induced hepatotoxicity remains unelucidated to date. In **Chapter 4**²⁶⁸, a novel experimental model of ibuprofen-induced acute liver injury was developed, which included *in vitro* and *in vivo* studies to investigate the essential pathways activated in the liver during ibuprofen intoxication and find potential targets that may ameliorate that condition. Additionally, the relevance of *Jnk1* and *Jnk2* in hepatocytes versus non-LPCs was examined to provide more clarity on the role of JNK and the differential functional aspects of JNK1 and JNK2 in the setting of ibuprofen-associated acute liver injury.

Out of the three known isoforms, only JNK 1 and 2, members of the mitogen-activated protein kinases, are expressed in the liver. JNK has been described to play a crucial role in hepatic disease, notably in liver damage of toxic nature. Therefore in **Chapter 5**²⁶⁹, the role of the compound function of both JNK isoforms expressed in the liver, JNK1 and JNK2, was

experimentally studied at the hepatocellular level in diverse models of liver toxicity, including APAP-, CCl₄- and LPS-induced hepatotoxicity. Additionally, a translational comparison regarding JNK expression and activation was carried out based on human liver tissue available from DILI patients. The JNK-specific inhibitor SP600125 has been reported to ameliorate APAP-induced hepatotoxicity *in vitro* on human primary hepatocytes. Thus, a further aim of our investigations was to test the specificity of SP600125 in APAP hepatotoxicity by using *in vitro* and *in vivo* models.

Corticosteroids have been indicated for the treatment of severe forms of severe liver damage. Among the large list of compounds, methylprednisolone stands out as a widely used synthetic glucocorticoid drug and is commonly indicated against, *e.g.*, inflammatory processes, autoimmune disease recrudescences, allergic reactions or transplant rejections. Although hepatotoxicity due to methylprednisolone is rare, the rate of methylprednisolone-associated liver injury reports in the literature has been growing recently. In **Chapter 6**²⁷⁰, we aimed to characterize this particular form of hepatotoxicity. For that purpose, methylprednisolone-induced liver injury events enrolled in the Spanish prospective DILI database as well as in its Latin-American branch were analyzed and an additional literature review was performed.

Chronic outcomes following acute idiosyncratic drug-induced liver injury are not yet defined. In **Chapter 7**²⁷¹, a prospective, long-term follow-up study aimed to analyze time to liver enzyme resolution to establish the best definition and risk factors of chronicity in DILI. Out of 850 patients included in the Spanish DILI Registry, a cohort of 298 subjects with no pre-existing disease affecting the liver and follow-up to resolution ≥ 1 year were analyzed. Chronicity was defined as abnormal liver biochemistry, imaging test or histology one year after DILI recognition.

In **Chapter 8** we described three patient case reports with suspected ADR diagnosis from our daily clinical practice, selected based on their learning point. In the first report, the case of a young, healthy woman who suffered from an acute liver failure of unknown etiology with the need for orthotopic liver transplantation was discussed. Case report 2 dealt with a female patient that presented with liver dysfunction and had a previous history of Waldenström macroglobulinemia. Even though a possible extramedullary manifestation of her underlying disease was initially believed, her clinical condition was finally elucidated after a rechallenge to the antidepressant sertraline. Case report 3 was about an adult woman who presented with liver cirrhosis and suffered from various hydropic decompensations. Her past medical record was unremarkable, except for a chronic therapy with venlafaxine she had taken to treat her depression for the previous four years and that was brought into the spotlight as the cause of her condition.

Liver fibrosis is characteristic of chronic liver disease and consists of hepatocyte injury in addition to an inflammatory reaction, which leads to the activation of hepatic stellate cells (HSCs) and increases deposition of extracellular matrix (ECM). Dramatically, liver fibrosis can progress to cirrhosis, a condition that can result in hepatocellular carcinoma, portal hypertensive gastropathy, hepatorenal syndrome and encephalopathy. Recent studies

have shown that even in advanced stages, liver fibrosis is reversible by modulating the inflammatory environment, eliminating or regression of activated HSCs and degrading ECM. In **Chapter 9**²⁷², we carefully reviewed the current status and understanding of liver fibrosis, revised the limitations of the animal models that have resulted as indispensable for our current knowledge and analyzed a large number of clinical trials that aim at reversing liver fibrosis.

Hepatic cancer is a recurrent complication that can occur in end-stage chronic liver disease and represents the third most frequent cause of mortality due to oncologic disease in the world. The JNK pathway has been shown to exert a pivotal role in chronic liver disease. Our previous investigations suggested differential roles for the two JNK isoforms expressed in the liver (*Jnk1* and *Jnk2*). In CLD, *Jnk1* has been attributed to promote apoptotic cell death and contribute to tumorigenesis, whereas *Jnk2* has been shown to modulate fibrogenesis. In **Chapter 10**²⁷³, we desired to better understand the role of *Jnk2* in chronic liver disease by using a dual approach with genetically hepatocyte-specific *Jnk2*-deficient mice and treating mice with siRNA-*Jnk2* specifically targeting hepatocytes. To assess the impact of hepatocytic *Jnk2* modulation in CLD, mice with deletion of the regulatory subunit IKK γ (NEMO) in murine hepatocytes (NEMO Δ hepa) were used, as we have previously demonstrated that it causes spontaneous HCC development preceded by chronic liver damage.

Hepatocellular carcinoma (HCC) represents the fifth most frequent malignancy, affecting one million people worldwide per year and can originate as a potential complication of sustained chronic liver disease (CLD). Among the different mechanisms leading to hepatocarcinogenesis, escape from the immune system surveillance has been suggested to play a crucial role during tumorigenesis and tumor progression. The Fas ligand (FasL)-Fas axis, which are transmembrane proteins that belong to the tumor necrosis factor (TNF) and TNF receptor gene superfamilies, respectively, play an essential role during immune modulation and have been found to be expressed on hepatocytes, activated HSCs or KCs. Indeed, the FasL/Fas system's malfunction has been proposed as a mechanism that could prevent the immune defense from rejecting tumor cells. Nevertheless, only limited data have been available on the FasL/Fas crosstalk *in vivo*. In **Chapter 11**²⁷⁴ we have investigated the FasL/Fas system's relevance in an experimental model of chronic liver disease by using hepatocyte-specific NEMO knockout (NEMO Δ hepa) mice, which develop spontaneous apoptosis, leading to chronic hepatocyte injury and regenerative proliferation that constitute risk factors for cancer development.

References

1. Molina DK, DiMaio VJ. Normal organ weights in men: part II-the brain, lungs, liver, spleen, and kidneys. *Am J Forensic Med Pathol* 2012;33:368-72.
2. Drake RL VW, Mitchel AWM. *Gray Anatomía para estudiantes*. Madrid: Elsevier, 2005.
3. Farreras V, Rozman C. *Medicina Interna*. Barcelona: Elsevier, 2009.

4. Gerber MA, Thung SN. Histology of the liver. *Am J Surg Pathol* 1987;11:709-22.
5. Kalra A TFP, Liver. [Updated 2018 Dec 18]. In: StatPearls [Internet]. Treasure Island (FL): StatPearls Publishing; 2019 Jan-. Available from: <https://www.ncbi.nlm.nih.gov/books/NBK535438/>.
6. Tanaka M, Miyajima A. Liver regeneration and fibrosis after inflammation. *Inflamm Regen* 2016;18:36:19.
7. Racanelli V, Rehmann B. The liver as an immunological organ. *Hepatology* 2006;43:S54-62.
8. Gressner AM. Transdifferentiation of hepatic stellate cells (Ito cells) to myofibroblasts: a key event in hepatic fibrogenesis. *Kidney Int Suppl* 1996;54:S39-45.
9. Vernon H KAA, Abdomen and Pelvis, Liver. [Updated 2019 Jan 16]. In: StatPearls [Internet]. Treasure Island (FL): StatPearls Publishing; 2019 Jan-. Available from: <https://www.ncbi.nlm.nih.gov/books/NBK500014/>.
10. Rui L. Energy metabolism in the liver. *Compr Physiol* 2014; 4:177-197.
11. Chaudhry R BHP, Coagulation Pathways. [Updated 2019 Apr 17]. In: StatPearls [Internet]. Treasure Island (FL): StatPearls Publishing; 2019 Jan-. Available from: <https://www.ncbi.nlm.nih.gov/books/NBK482253/>.
12. Zanger UM, Schwab M. Cytochrome P450 enzymes in drug metabolism: regulation of gene expression, enzyme activities, and impact of genetic variation. *Pharmacol Ther* 2013;138:103-41.
13. Ingelman-Sundberg M, Oscarson M, McLellan RA. Polymorphic human cytochrome P450 enzymes: an opportunity for individualized drug treatment. *Trends Pharmacol Sci* 1999;20:342-9.
14. Williams RT. Hepatic metabolism of drugs. *Gut* 1972;13:579-85.
15. Corsini A, Bortolini M. Drug-induced liver injury: the role of drug metabolism and transport. *Journal of clinical pharmacology* 2013;53:463-74.
16. Susa ST, Preuss CV. Drug metabolism. [Updated 2021 Feb 17]. In: StatPearls [Internet].
17. Ishii M, Kanayama M, Esumi H et al. Pharmacokinetic analysis of factors determining elimination pathways for sulfate and glucuronide metabolites of drugs. I: studies by in vivo constant infusion. *Xenobiotica* 2002; 32:441-50.
18. Younossi ZM. Non-alcoholic fatty liver disease - A global public health perspective. *J Hepatol* 2019;70:531-544.
19. Robles-Diaz M, Gonzalez-Jimenez A, Medina-Caliz I, et al. Distinct phenotype of hepatotoxicity associated with illicit use of anabolic androgenic steroids. *Aliment Pharmacol Ther* 2015;41:116-25.
20. Bessone F, Hernandez N, Lucena MI, et al. The Latin American DILI Registry Experience: A Successful Ongoing Collaborative Strategic Initiative. *Int J Mol Sci* 2016;17:313.
21. Lee WM. Drug-induced hepatotoxicity. *N Engl J Med* 2003;349:474-85.
22. Mosedale M, Watkins PB. Drug-Induced Liver Injury: Advances in Mechanistic Understanding that will Inform Risk Management. *Clin Pharmacol Ther.* 2017;101: 469–480.
23. Miguel A, Azevedo LF, Araujo M, et al. Frequency of adverse drug reactions in hospitalized patients: a systematic review and meta-analysis. *Pharmacoepidemiol Drug Saf* 2012;21:1139-54.
24. Robles-Diaz M, Medina-Caliz I, Stephens C, et al. Biomarkers in DILI: One More Step Forward. *Front Pharmacol* 2016;7:267.
25. Rockey DC, Seeff LB, Rochon J, et al. Causality assessment in drug-induced liver injury using a structured expert opinion process: comparison to the Roussel-Uclaf causality assessment method. *Hepatology* 2010;51:2117-26.
26. Abboud G, Kaplowitz N. Drug-induced liver injury. *Drug Saf* 2007;30:277-94.
27. Goodman ZD. Drug hepatotoxicity. *Clin Liver Dis* 2002;6:381-97.
28. Devarbhavi H. An Update on Drug-induced Liver Injury. *J Clin Exp Hepatol* 2012;2:247-59.
29. Hayashi PH, Fontana RJ, Chalasani NP, et al. Under-reporting and Poor Adherence to Monitoring Guidelines for Severe Cases of Isoniazid Hepatotoxicity. *Clin Gastroenterol Hepatol* 2015;13:1676-82 e1.
30. Aithal PG, Day CP. The natural history of histologically proved drug induced liver disease. *Gut* 1999;44:731-5.
31. Larson AM, Polson J, Fontana RJ, et al. Acetaminophen-induced acute liver failure: results of a United States multicenter, prospective study. *Hepatology (Baltimore, Md)* 2005;42:1364-72.
32. Ostapowicz G, Fontana RJ, Schiodt FV, et al. Results of a prospective study of acute liver failure at 17 tertiary care centers in the United States. *Annals of internal medicine* 2002;137:947-54.
33. Fontana RJ. Acute Liver Failure including Acetaminophen Overdose. *Med Clin North Am* 2008;92: 761–794.

34. Lee WM. Acetaminophen (APAP) hepatotoxicity-Isn't it time for APAP to go away? *J Hepatol* 2017;67:1324-1331.
35. U.S. Department of Health and Human Services FaDA, Center for Drug Evaluation and Research (CDER), Center for Biologics Evaluation and Research (CBER). Guidance for industry drug-induced liver injury: premarketing clinical evaluation. Available at: <http://www.fda.gov/downloads/Drugs/.../Guidances/UCM174090.pdf> 2009.
36. Navarro VJ, Senior JR. Drug-related hepatotoxicity. *N Engl J Med* 2006;354:731-9.
37. Xu JJ, Diaz D, O'Brien PJ. Applications of cytotoxicity assays and pre-lethal mechanistic assays for assessment of human hepatotoxicity potential. *Chem Biol Interact* 2004;150:115-28.
38. Kaplowitz N. Drug-induced liver disorders: implications for drug development and regulation. *Drug safety* 2001;24:483-90.
39. Maddur H, Chalasani N. Idiosyncratic drug-induced liver injury: a clinical update. *Curr Gastroenterol Rep* 2011;13:65-71.
40. Lewis JH. 'Hy's law,' the 'Rezulin Rule,' and other predictors of severe drug-induced hepatotoxicity: putting risk-benefit into perspective. *Pharmacoepidemiol Drug Saf* 2006;15:221-9.
41. De Valle MB, Av Klinteberg V, Alem N, et al. Drug-induced liver injury in a Swedish University hospital out-patient hepatology clinic. *Aliment Pharmacol Ther* 2006;24:1187-95.
42. Donati M, Conforti A, Lenti MC, et al. Risk of acute and serious liver injury associated to nimesulide and other NSAIDs: data from drug-induced liver injury case-control study in Italy. *Br J Clin Pharmacol* 2016;82:238-48.
43. Sgro C, Clinard F, Ouazir K, et al. Incidence of drug-induced hepatic injuries: a French population-based study. *Hepatology (Baltimore, Md)* 2002;36:451-5.
44. Bjornsson ES, Bergmann OM, Bjornsson HK, et al. Incidence, presentation, and outcomes in patients with drug-induced liver injury in the general population of Iceland. *Gastroenterology* 2013;144:1419-25, 1425.e1-3; quiz e19-20.
45. Pachkoria K, Lucena MI, Ruiz-Cabello F, et al. Genetic polymorphisms of CYP2C9 and CYP2C19 are not related to drug-induced idiosyncratic liver injury (DILI). *Br J Pharmacol* 2007;150:808-15.
46. Yu YC, Mao YM, Chen CW, et al. CSH guidelines for the diagnosis and treatment of drug-induced liver injury. *Hepatol Int* 2017;11:221-241.
47. Chalasani N, Reddy KR, Fontana RJ, et al. Idiosyncratic Drug Induced Liver Injury in African-Americans Is Associated With Greater Morbidity and Mortality Compared to Caucasians. *Am J Gastroenterol* 2017;112:1382-1388.
48. Larson AM, Polson J, Fontana RJ, et al. Acetaminophen-induced acute liver failure: results of a United States multicenter, prospective study. *Hepatology* 2005;42:1364-72.
49. Bjornsson ES. Hepatotoxicity by Drugs: The Most Common Implicated Agents. *Int J Mol Sci* 2016;17:224.
50. Fontana RJ, Hayashi PH, Barnhart H, et al. Persistent liver biochemistry abnormalities are more common in older patients and those with cholestatic drug induced liver injury. *Am J Gastroenterol* 2015;110:1450-9.
51. Andrade RJ, Lucena MI, Fernandez MC, et al. Drug-induced liver injury: an analysis of 461 incidences submitted to the Spanish registry over a 10-year period. *Gastroenterology* 2005;129:512-21.
52. Bjornsson ES, Bergmann OM, Bjornsson HK, et al. Incidence, presentation, and outcomes in patients with drug-induced liver injury in the general population of Iceland. *Gastroenterology* 2013;144:1419-25, 1425 e1-3; quiz e19-20.
53. Suk KT, Kim DJ, Kim CH, et al. A prospective nationwide study of drug-induced liver injury in Korea. *Am J Gastroenterol* 2012;107:1380-7.
54. Devarbhavi H, Dierkhising R, Kremers WK, et al. Single-center experience with drug-induced liver injury from India: causes, outcome, prognosis, and predictors of mortality. *Am J Gastroenterol* 2010;105:2396-404.
55. Urban TJ, Nicoletti P, Chalasani N, et al. Minocycline hepatotoxicity: Clinical characterization and identification of HLA-B *35:02 as a risk factor. *J Hepatol* 2017;67:137-144.
56. European Association for the Study of the Liver. Electronic address eee, Clinical Practice Guideline Panel C, Panel m, et al. EASL Clinical Practice Guidelines: Drug-induced liver injury. *J Hepatol* 2019;70:1222-1261.
57. Garcia-Cortes M, Robles-Diaz M, Ortega-Alonso A, et al. Hepatotoxicity by Dietary Supplements: A Tabular Listing and Clinical Characteristics. *Int J Mol Sci* 2016;17:537.

58. Navarro VJ, Barnhart H, Bonkovsky HL, et al. Liver injury from herbals and dietary supplements in the U.S. Drug-Induced Liver Injury Network. *Hepatology* 2014;60:1399-408.
59. Lee WM. Acetaminophen-related acute liver failure in the United States. *Hepatol Res* 2008;38 Suppl 1:S3-8.
60. Yoon E, Babar A, Choudhary M, et al. Acetaminophen-Induced Hepatotoxicity: a Comprehensive Update. *J Clin Transl Hepatol* 2016;4:131-42.
61. Dawwas MF, Aithal GP. The quest for an evidence-based approach to surveillance for methotrexate-related hepatotoxicity: promise and perils. *Br J Dermatol* 2015;172:1684-5.
62. Zimmerman HJ. Effects of aspirin and acetaminophen on the liver. *Arch Intern Med* 1981;141:333-42.
63. Aithal GP, Watkins PB, Andrade RJ, et al. Case definition and phenotype standardization in drug-induced liver injury. *Clin Pharmacol Ther* 2011;89:806-15.
64. Lammert C, Einarsson S, Saha C, et al. Relationship between daily dose of oral medications and idiosyncratic drug-induced liver injury: search for signals. *Hepatology* 2008;47:2003-9.
65. Antoine DJ, Williams DP, Park BK. Understanding the role of reactive metabolites in drug-induced hepatotoxicity: state of the science. *Expert Opin Drug Metab Toxicol* 2008;4:1415-27.
66. Jetten MJ, Ruiz-Aracama A, Coonen ML, et al. Interindividual variation in gene expression responses and metabolite formation in acetaminophen-exposed primary human hepatocytes. *Arch Toxicol* 2016;90:1103-15.
67. Stephens C, Andrade RJ, Lucena MI. Mechanisms of drug-induced liver injury. *Current opinion in allergy and clinical immunology* 2014;14:286-92.
68. Chalasani N, Bjornsson E. Risk factors for idiosyncratic drug-induced liver injury. *Gastroenterology* 2010;138:2246-59.
69. Yuan L, Kaplowitz N. Mechanisms of drug-induced liver injury. *Clin Liver Dis* 2013;17:507-18, vii.
70. Suk KT, Kim DJ. Drug-induced liver injury: present and future. *Clin Mol Hepatol* 2012;18:249-57.
71. Park BK, Boobis A, Clarke S, et al. Managing the challenge of chemically reactive metabolites in drug development. *Nature reviews Drug discovery* 2011;10:292-306.
72. Chen M, Suzuki A, Borlak J, et al. Drug-induced liver injury: Interactions between drug properties and host factors. *J Hepatol* 2015;63:503-14.
73. Nelson SD. Molecular mechanisms of the hepatotoxicity caused by acetaminophen. *Semin Liver Dis* 1990;10:267-78.
74. Tonge RP, Kelly EJ, Bruschi SA, et al. Role of CYP1A2 in the hepatotoxicity of acetaminophen: investigations using Cyp1a2 null mice. *Toxicol Appl Pharmacol* 1998;153:102-8.
75. Zhao G, Hatting M, Nevzorova YA, et al. Jnk1 in murine hepatic stellate cells is a crucial mediator of liver fibrogenesis. *Gut* 2014;63:1159-72.
76. Reid AB, Kurten RC, McCullough SS, et al. Mechanisms of acetaminophen-induced hepatotoxicity: role of oxidative stress and mitochondrial permeability transition in freshly isolated mouse hepatocytes. *The Journal of pharmacology and experimental therapeutics* 2005;312:509-16.
77. Stachulski AV, Baillie TA, Park BK, et al. The generation, detection, and effects of reactive drug metabolites. *Medicinal research reviews* 2013;33:985-1080.
78. Kim TS, Jeong DW, Yun BY, et al. Dysfunction of rat liver mitochondria by selenite: induction of mitochondrial permeability transition through thiol-oxidation. *Biochem Biophys Res Commun* 2002;294:1130-7.
79. Jaeschke H, Xie Y, McGill MR. Acetaminophen-induced Liver Injury: from Animal Models to Humans. *J Clin Transl Hepatol* 2014;2:153-61.
80. Ott M, Gogvadze V, Orrenius S, et al. Mitochondria, oxidative stress and cell death. *Apoptosis* 2007;12:913-22.
81. Saito Y, Hikita H, Nozaki Y, et al. DNase II activated by the mitochondrial apoptotic pathway regulates RIP1-dependent non-apoptotic hepatocyte death via the TLR9/IFN-beta signaling pathway. *Cell Death Differ* 2018.
82. Pessayre D, Fromenty B, Berson A, et al. Central role of mitochondria in drug-induced liver injury. *Drug Metab Rev* 2012;44:34-87.
83. Ni HM, Williams JA, Ding WX. Mitochondrial dynamics and mitochondrial quality control. *Redox Biol* 2015;4:6-13.
84. Ding WX, Yin XM. Mitophagy: mechanisms, pathophysiological roles, and analysis. *Biol Chem* 2012;393:547-64.

85. Chen H, Chan DC. Mitochondrial dynamics--fusion, fission, movement, and mitophagy--in neurodegenerative diseases. *Hum Mol Genet* 2009;18:R169-76.
86. Wu M, Kalyanasundaram A, Zhu J. Structural and biomechanical basis of mitochondrial movement in eukaryotic cells. *Int J Nanomedicine* 2013;8:4033-42.
87. Hollenbeck PJ, Saxton WM. The axonal transport of mitochondria. *J Cell Sci* 2005;118:5411-9.
88. Han D, Dara L, Win S, et al. Regulation of drug-induced liver injury by signal transduction pathways: critical role of mitochondria. *Trends Pharmacol Sci* 2013;34:243-53.
89. Paul WE. Bridging innate and adaptive immunity. *Cell* 2011;147:1212-5.
90. Kaliyaperumal K, Grove JJ, Delahay RM, et al. Pharmacogenomics of drug-induced liver injury (DILI): Molecular biology to clinical applications. *J Hepatol* 2018;69:948-957.
91. Uetrecht J. Idiosyncratic drug reactions: past, present, and future. *Chem Res Toxicol* 2008;21:84-92.
92. Kaniwa N, Saito Y. Pharmacogenomics of severe cutaneous adverse reactions and drug-induced liver injury. *J Hum Genet* 2013;58:317-26.
93. Yang R, Tennesseer TI. DAMPs and sterile inflammation in drug hepatotoxicity. *Hepatol Int* 2019;13:42-50.
94. Antoine DJ, Williams DP, Kipar A, et al. High-mobility group box-1 protein and keratin-18, circulating serum proteins informative of acetaminophen-induced necrosis and apoptosis in vivo. *Toxicol Sci* 2009;112:521-31.
95. Gaskell H, Ge X, Nieto N. High-Mobility Group Box-1 and Liver Disease. *Hepatol Commun* 2018;2:1005-1020.
96. Yang R, Zhang S, Cotoia A, et al. High mobility group B1 impairs hepatocyte regeneration in acetaminophen hepatotoxicity. *BMC Gastroenterol* 2012;12:45.
97. Antoine DJ, Williams DP, Kipar A, et al. Diet restriction inhibits apoptosis and HMGB1 oxidation and promotes inflammatory cell recruitment during acetaminophen hepatotoxicity. *Mol Med* 2010;16:479-90.
98. Huebener P, Pradere JP, Hernandez C, et al. The HMGB1/RAGE axis triggers neutrophil-mediated injury amplification following necrosis. *J Clin Invest* 2015;125:539-50.
99. Antoine DJ, Jenkins RE, Dear JW, et al. Molecular forms of HMGB1 and keratin-18 as mechanistic biomarkers for mode of cell death and prognosis during clinical acetaminophen hepatotoxicity. *J Hepatol* 2012;56:1070-9.
100. Czaja AJ. Challenges in the diagnosis and management of autoimmune hepatitis. *Can J Gastroenterol* 2013;27:531-9.
101. European Association for the Study of the L. EASL Clinical Practice Guidelines: Autoimmune hepatitis. *J Hepatol* 2015;63:971-1004.
102. Sebode M, Schulz L, Lohse AW. "Autoimmune(-Like)" Drug and Herb Induced Liver Injury: New Insights into Molecular Pathogenesis. *Int J Mol Sci* 2017;18.
103. Elmore S. Apoptosis: a review of programmed cell death. *Toxicol Pathol* 2007;35:495-516.
104. Hacker G. The morphology of apoptosis. *Cell Tissue Res* 2000;301:5-17.
105. Ye H, Nelson LJ, Gomez Del Moral M, et al. Dissecting the molecular pathophysiology of drug-induced liver injury. *World J Gastroenterol* 2018;24:1373-1385.
106. Vercammen D, Beyaert R, Denecker G, et al. Inhibition of caspases increases the sensitivity of L929 cells to necrosis mediated by tumor necrosis factor. *J Exp Med* 1998;187:1477-85.
107. Su L, Quade B, Wang H, et al. A plug release mechanism for membrane permeation by MLKL. *Structure* 2014;22:1489-500.
108. Galluzzi L, Vitale I, Abrams JM, et al. Molecular definitions of cell death subroutines: recommendations of the Nomenclature Committee on Cell Death 2012. *Cell Death Differ* 2012;19:107-20.
109. Iorga A, Dara L, Kaplowitz N. Drug-Induced Liver Injury: Cascade of Events Leading to Cell Death, Apoptosis or Necrosis. *Int J Mol Sci* 2017;18.
110. Bergsbaken T, Fink SL, Cookson BT. Pyroptosis: host cell death and inflammation. *Nat Rev Microbiol* 2009;7:99-109.
111. Mou Y, Wang J, Wu J, et al. Ferroptosis, a new form of cell death: opportunities and challenges in cancer. *J Hematol Oncol* 2019;12:34.
112. Iorga A, Dara L. Cell death in drug-induced liver injury. *Adv Pharmacol* 2019;85:31-74.
113. Davis RJ. Signal transduction by the JNK group of MAP kinases. *Cell* 2000;103:239-52.
114. Weston CR, Davis RJ. The JNK signal transduction pathway. *Curr Opin Cell Biol* 2007;19:142-9.

115. Ibrahim SH, Gores GJ. Who pulls the trigger: JNK activation in liver lipotoxicity? *J Hepatol* 2012;56:17-9.
116. Seki E, Brenner DA, Karin M. A liver full of JNK: signaling in regulation of cell function and disease pathogenesis, and clinical approaches. *Gastroenterology* 2012;143:307-20.
117. Ichijo H, Nishida E, Irie K, et al. Induction of apoptosis by ASK1, a mammalian MAPKKK that activates SAPK/JNK and p38 signaling pathways. *Science* 1997;275:90-4.
118. Schachter KA, Du Y, Lin A, et al. Dynamic positive feedback phosphorylation of mixed lineage kinase 3 by JNK reversibly regulates its distribution to Triton-soluble domains. *J Biol Chem* 2006;281:19134-44.
119. Shinohara M, Ybanez MD, Win S, et al. Silencing glycogen synthase kinase-3 β inhibits acetaminophen hepatotoxicity and attenuates JNK activation and loss of glutamate cysteine ligase and myeloid cell leukemia sequence 1. *J Biol Chem* 2010;285:8244-55.
120. Hanawa N, Shinohara M, Saberi B, et al. Role of JNK translocation to mitochondria leading to inhibition of mitochondria bioenergetics in acetaminophen-induced liver injury. *J Biol Chem* 2008;283:13565-77.
121. Alempijevic T, Zec S, Milosavljevic T. Drug-induced liver injury: Do we know everything? *World J Hepatol* 2017;9:491-502.
122. Win S, Than TA, Min RW, et al. c-Jun N-terminal kinase mediates mouse liver injury through a novel Sab (SH3BP5)-dependent pathway leading to inactivation of intramitochondrial Src. *Hepatology* 2016;63:1987-2003.
123. Win S, Than TA, Kaplowitz N. The Regulation of JNK Signaling Pathways in Cell Death through the Interplay with Mitochondrial SAB and Upstream Post-Translational Effects. *Int J Mol Sci* 2018;19.
124. Win S, Than TA, Han D, et al. c-Jun N-terminal kinase (JNK)-dependent acute liver injury from acetaminophen or tumor necrosis factor (TNF) requires mitochondrial Sab protein expression in mice. *J Biol Chem* 2011;286:35071-8.
125. Bennett BL, Sasaki DT, Murray BW, et al. SP600125, an anthrapyrazolone inhibitor of Jun N-terminal kinase. *Proc Natl Acad Sci U S A* 2001;98:13681-6.
126. Gunawan BK, Liu ZX, Han D, et al. c-Jun N-terminal kinase plays a major role in murine acetaminophen hepatotoxicity. *Gastroenterology* 2006;131:165-78.
127. Chang CY, Schiano TD. Review article: drug hepatotoxicity. *Aliment Pharmacol Ther* 2007;25:1135-51.
128. Sgro C, Clinard F, Ouazir K, et al. Incidence of drug-induced hepatic injuries: a French population-based study. *Hepatology* 2002;36:451-5.
129. Galan MV, Potts JA, Silverman AL, et al. The burden of acute nonfulminant drug-induced hepatitis in a United States tertiary referral center [corrected]. *J Clin Gastroenterol* 2005;39:64-7.
130. Batt AM, Ferrari L. Manifestations of chemically induced liver damage. *Clin Chem* 1995;41:1882-7.
131. de Abajo FJ, Montero D, Madurga M, et al. Acute and clinically relevant drug-induced liver injury: a population based case-control study. *Br J Clin Pharmacol* 2004;58:71-80.
132. Russo MW, Galanko JA, Shrestha R, et al. Liver transplantation for acute liver failure from drug induced liver injury in the United States. *Liver Transpl* 2004;10:1018-23.
133. David S, Hamilton JP. Drug-induced Liver Injury. *US Gastroenterol Hepatol Rev* 2010;6:73-80.
134. O'Grady JG. Acute liver failure. *Postgrad Med J* 2005;81:148-54.
135. Hayashi PH, Fontana RJ. Clinical features, diagnosis, and natural history of drug-induced liver injury. *Seminars in liver disease* 2014;34:134-44.
136. Ju C, Reilly T. Role of immune reactions in drug-induced liver injury (DILI). *Drug Metab Rev* 2012;44:107-15.
137. Stine JG, Chalasani N. Chronic liver injury induced by drugs: a systematic review. *Liver Int* 2015;35:2343-53.
138. Seeff LB. Drug-induced chronic liver disease, with emphasis on chronic active hepatitis. *Semin Liver Dis* 1981;1:104-15.
139. Andrade RJ, Lucena MI, Kaplowitz N, et al. Outcome of acute idiosyncratic drug-induced liver injury: Long-term follow-up in a hepatotoxicity registry. *Hepatology (Baltimore, Md)* 2006;44:1581-8.
140. Maddrey WC, Boitnott JK. Drug-induced chronic liver disease. *Gastroenterology* 1977;72:1348-53.
141. Benichou C. Criteria of drug-induced liver disorders. Report of an international consensus meeting. *J Hepatol* 1990;11:272-6.
142. Makar GA, Weiner MG, Kimmel SE, et al. Incidence and prevalence of abnormal liver associated enzymes in patients with atrial fibrillation in a routine clinical care population. *Pharmacoepidemiol Drug Saf* 2008;17:43-51.

143. Weil JG, Bains C, Linke A, et al. Background incidence of liver chemistry abnormalities in a clinical trial population without underlying liver disease. *Regul Toxicol Pharmacol* 2008;52:85-8.
144. Saukkonen JJ, Cohn DL, Jasmer RM, et al. An official ATS statement: hepatotoxicity of antituberculosis therapy. *Am J Respir Crit Care Med* 2006;174:935-52.
145. Dufour DR, Lott JA, Nolte FS, et al. Diagnosis and monitoring of hepatic injury. I. Performance characteristics of laboratory tests. *Clin Chem* 2000;46:2027-49.
146. Regev A, Bjornsson ES. Drug-induced liver injury: morbidity, mortality, and Hy's law. *Gastroenterology* 2014;147:20-4.
147. Temple R. Hy's law: predicting serious hepatotoxicity. *Pharmacoepidemiol Drug Saf* 2006;15:241-3.
148. Robles-Diaz M, Lucena MI, Kaplowitz N. Use of Hy's law and a new composite algorithm to predict acute liver failure in patients with drug-induced liver injury. *Gastroenterology*. 2014;147:109-118.
149. Ramachandran R, Kakar S. Histological patterns in drug-induced liver disease. *J Clin Pathol* 2009;62:481-92.
150. Kleiner DE. The pathology of drug-induced liver injury. *Semin Liver Dis* 2009;29:364-72.
151. Fontana RJ, Seeff LB, Andrade RJ, et al. Standardization of nomenclature and causality assessment in drug-induced liver injury: summary of a clinical research workshop. *Hepatology* 2010;52:730-42.
152. LiverTox: Clinical and Research Information on Drug-Induced Liver Injury [Internet]. Bethesda (MD): National Institute of Diabetes and Digestive and Kidney Diseases; 2012-. Clinical Course and Diagnosis of Drug Induced Liver Disease. [Updated 2019 May 4]. Available from: <https://www.ncbi.nlm.nih.gov/books/NBK548733/>
153. Danan G, Benichou C. Causality assessment of adverse reactions to drugs--I. A novel method based on the conclusions of international consensus meetings: application to drug-induced liver injuries. *J Clin Epidemiol* 1993;46:1323-30.
154. LiverTox: Clinical and Research Information on Drug-Induced Liver Injury [Internet]. Bethesda (MD): National Institute of Diabetes and Digestive and Kidney Diseases; 2012-. Maria and Victorino (M & V) System of Causality Assessment in Drug Induced Liver Injury. [Updated 2019 May 4]. Available from: <https://www.ncbi.nlm.nih.gov/books/NBK547853/>
155. Maria VA, Victorino RM. Development and validation of a clinical scale for the diagnosis of drug-induced hepatitis. *Hepatology* 1997;26:664-9.
156. Naranjo CA, Busto U, Sellers EM, et al. A method for estimating the probability of adverse drug reactions. *Clin Pharmacol Ther* 1981;30:239-45.
157. Lucena MI, Camargo R, Andrade RJ, et al. Comparison of two clinical scales for causality assessment in hepatotoxicity. *Hepatology* 2001;33:123-30.
158. Tran T, Lee WM. DILI: New Insights into Diagnosis and Management. *Curr Hepat Rep* 2013;12:53-58.
159. Rochon J, Protiva P, Seeff LB, et al. Reliability of the Roussel Uclaf Causality Assessment Method for assessing causality in drug-induced liver injury. *Hepatology* 2008;48:1175-83.
160. Danan G, Teschke R. Roussel Uclaf Causality Assessment Method for Drug-Induced Liver Injury: Present and Future. *Front Pharmacol* 2019;10:853.
161. Ortega-Alonso A, Stephens C, Lucena MI, et al. Case Characterization, Clinical Features and Risk Factors in Drug-Induced Liver Injury. *Int J Mol Sci* 2016;17.
162. Hughes JD, Blagg J, Price DA, et al. Physiochemical drug properties associated with in vivo toxicological outcomes. *Bioorg Med Chem Lett* 2008;18:4872-5.
163. Leeson PD, Springthorpe B. The influence of drug-like concepts on decision-making in medicinal chemistry. *Nat Rev Drug Discov* 2007;6:881-90.
164. McEuen K, Borlak J, Tong W, et al. Associations of Drug Lipophilicity and Extent of Metabolism with Drug-Induced Liver Injury. *Int J Mol Sci* 2017;18.
165. Lucena MI, Andrade RJ, Kaplowitz N, et al. Phenotypic characterization of idiosyncratic drug-induced liver injury: the influence of age and sex. *Hepatology* 2009;49:2001-9.
166. Chen M, Borlak J, Tong W. High lipophilicity and high daily dose of oral medications are associated with significant risk for drug-induced liver injury. *Hepatology* 2013;58:388-96.
167. Neustadt J, Pieczenik SR. Medication-induced mitochondrial damage and disease. *Mol Nutr Food Res* 2008;52:780-8.
168. Chan K, Truong D, Shangari N, et al. Drug-induced mitochondrial toxicity. *Expert Opin Drug Metab Toxicol* 2005;1:655-69.
169. Dykens JA, Will Y. The significance of mitochondrial toxicity testing in drug development. *Drug Discov Today* 2007;12:777-85.

170. Labbe G, Pessayre D, Fromenty B. Drug-induced liver injury through mitochondrial dysfunction: mechanisms and detection during preclinical safety studies. *Fundam Clin Pharmacol* 2008;22:335-53.
171. Stepan AF, Walker DP, Bauman J, et al. Structural alert/reactive metabolite concept as applied in medicinal chemistry to mitigate the risk of idiosyncratic drug toxicity: a perspective based on the critical examination of trends in the top 200 drugs marketed in the United States. *Chem Res Toxicol* 2011;24:1345-410.
172. Testa L, Bhindi R, Agostoni P, et al. The direct thrombin inhibitor ximelagatran/melagatran: a systematic review on clinical applications and an evidence based assessment of risk benefit profile. *Expert Opin Drug Saf* 2007;6:397-406.
173. Yang K, Kock K, Sedykh A, et al. An updated review on drug-induced cholestasis: mechanisms and investigation of physicochemical properties and pharmacokinetic parameters. *J Pharm Sci* 2013;102:3037-57.
174. Fattinger K, Funk C, Pantze M, et al. The endothelin antagonist bosentan inhibits the canalicular bile salt export pump: a potential mechanism for hepatic adverse reactions. *Clin Pharmacol Ther* 2001;69:223-31.
175. Kostrubsky SE, Strom SC, Kalgutkar AS, et al. Inhibition of hepatobiliary transport as a predictive method for clinical hepatotoxicity of nefazodone. *Toxicol Sci* 2006;90:451-9.
176. Smith MT. Mechanisms of troglitazone hepatotoxicity. *Chem Res Toxicol* 2003;16:679-87.
177. Morgan RE, Trauner M, van Staden CJ, et al. Interference with bile salt export pump function is a susceptibility factor for human liver injury in drug development. *Toxicol Sci* 2010;118:485-500.
178. Kis E, Iloja E, Rajnai Z, et al. BSEP inhibition: in vitro screens to assess cholestatic potential of drugs. *Toxicol In Vitro* 2012;26:1294-9.
179. Aleo MD, Luo Y, Swiss R, et al. Human drug-induced liver injury severity is highly associated with dual inhibition of liver mitochondrial function and bile salt export pump. *Hepatology* 2014;60:1015-22.
180. Jansen PL, Strautnieks SS, Jacquemin E, et al. Hepatocanalicular bile salt export pump deficiency in patients with progressive familial intrahepatic cholestasis. *Gastroenterology* 1999;117:1370-9.
181. Schneider AL, Lazo M, Selvin E, et al. Racial differences in nonalcoholic fatty liver disease in the U.S. population. *Obesity (Silver Spring)* 2014;22:292-9.
182. Rich NE, Oji S, Mufti AR, et al. Racial and Ethnic Disparities in Nonalcoholic Fatty Liver Disease Prevalence, Severity, and Outcomes in the United States: A Systematic Review and Meta-analysis. *Clin Gastroenterol Hepatol* 2018;16:198-210 e2.
183. Levy R, Catana AM, Durbin-Johnson B, et al. Ethnic differences in presentation and severity of alcoholic liver disease. *Alcohol Clin Exp Res* 2015;39:566-574.
184. Forde KA, Reddy KR, Troxel AB, et al. Racial and ethnic differences in presentation, etiology, and outcomes of acute liver failure in the United States. *Clin Gastroenterol Hepatol* 2009;7:1121-6.
185. Ostapowicz G, Fontana RJ, Schiodt FV, et al. Results of a prospective study of acute liver failure at 17 tertiary care centers in the United States. *Ann Intern Med* 2002;137:947-54.
186. Reuben A, Koch DG, Lee WM, et al. Drug-induced acute liver failure: results of a U.S. multicenter, prospective study. *Hepatology* 2010;52:2065-76.
187. Shi Q, Yang X, Greenhaw JJ, et al. Drug-Induced Liver Injury in Children: Clinical Observations, Animal Models, and Regulatory Status. *Int J Toxicol* 2017;36:365-379.
188. Fountain FF, Tolley E, Chrisman CR, et al. Isoniazid hepatotoxicity associated with treatment of latent tuberculosis infection: a 7-year evaluation from a public health tuberculosis clinic. *Chest* 2005;128:116-23.
189. Lewis JH. The rational use of potentially hepatotoxic medications in patients with underlying liver disease. *Expert Opin Drug Saf* 2002;1:159-72.
190. Ikemura K, Iwamoto T, Okuda M. Altered functions and expressions of drug transporters in liver, kidney and intestine in disorders of local and remote organs: possible role of oxidative stress in the pathogenesis. *Expert Opin Drug Metab Toxicol* 2009;5:907-20.
191. Amarapurkar DN. Prescribing medications in patients with decompensated liver cirrhosis. *Int J Hepatol* 2011;2011:519526.
192. Myers RP, Shaheen AA. Hepatitis C, alcohol abuse, and unintentional overdoses are risk factors for acetaminophen-related hepatotoxicity. *Hepatology* 2009;49:1399-400.
193. Fromenty B. Drug-induced liver injury in obesity. *J Hepatol* 2013;58:824-6.
194. Michaut A, Moreau C, Robin MA, et al. Acetaminophen-induced liver injury in obesity and nonalcoholic fatty liver disease. *Liver Int* 2014;34:e171-9.

195. Aubert J, Begrich K, Delannoy M, et al. Differences in early acetaminophen hepatotoxicity between obese ob/ob and db/db mice. *J Pharmacol Exp Ther* 2012;342:676-87.
196. Massart J, Begrich K, Moreau C, et al. Role of nonalcoholic fatty liver disease as risk factor for drug-induced hepatotoxicity. *J Clin Transl Res* 2017;3:212-232.
197. Ekstedt M, Franzen LE, Mathiesen UL, et al. Statins in non-alcoholic fatty liver disease and chronically elevated liver enzymes: a histopathological follow-up study. *J Hepatol* 2007;47:135-41.
198. Aceti A, Pasquazzi C, Zechini B, et al. Hepatotoxicity development during antiretroviral therapy containing protease inhibitors in patients with HIV: the role of hepatitis B and C virus infection. *J Acquir Immune Defic Syndr* 2002;29:41-8.
199. Servoss JC, Kitch DW, Andersen JW, et al. Predictors of antiretroviral-related hepatotoxicity in the adult AIDS Clinical Trial Group (1989-1999). *J Acquir Immune Defic Syndr* 2006;43:320-3.
200. Sulkowski MS, Thomas DL, Mehta SH, et al. Hepatotoxicity associated with nevirapine or efavirenz-containing antiretroviral therapy: role of hepatitis C and B infections. *Hepatology* 2002;35:182-9.
201. Wang JY, Liu CH, Hu FC, et al. Risk factors of hepatitis during anti-tuberculous treatment and implications of hepatitis virus load. *J Infect* 2011;62:448-55.
202. Khorashadi S, Hasson NK, Cheung RC. Incidence of statin hepatotoxicity in patients with hepatitis C. *Clin Gastroenterol Hepatol* 2006;4:902-7; quiz 806.
203. Wong WM, Wu PC, Yuen MF, et al. Antituberculosis drug-related liver dysfunction in chronic hepatitis B infection. *Hepatology* 2000;31:201-6.
204. Ungo JR, Jones D, Ashkin D, et al. Antituberculosis drug-induced hepatotoxicity. The role of hepatitis C virus and the human immunodeficiency virus. *Am J Respir Crit Care Med* 1998;157:1871-6.
205. Liu YM, Cheng YJ, Li YL, et al. Antituberculosis treatment and hepatotoxicity in patients with chronic viral hepatitis. *Lung* 2014;192:205-10.
206. Rodriguez-Frias EA, Lee WM. Cancer chemotherapy I: hepatocellular injury. *Clin Liver Dis* 2007;11:641-62, viii.
207. Bahirwani R, Reddy KR. Drug-induced liver injury due to cancer chemotherapeutic agents. *Semin Liver Dis* 2014;34:162-71.
208. Ahmad J, Odin JA. Epidemiology and Genetic Risk Factors of Drug Hepatotoxicity. *Clin Liver Dis* 2017;21:55-72.
209. Daly AK, Day CP. Genetic association studies in drug-induced liver injury. *Drug Metab Rev* 2012;44:116-26.
210. Zhou SF, Liu JP, Chowbay B. Polymorphism of human cytochrome P450 enzymes and its clinical impact. *Drug Metab Rev* 2009;41:89-295.
211. Elfaki I, Mir R, Almutairi FM, et al. Cytochrome P450: Polymorphisms and Roles in Cancer, Diabetes and Atherosclerosis. *Asian Pac J Cancer Prev* 2018;19:2057-2070.
212. Murray GI, McFadyen MC, Mitchell RT, et al. Cytochrome P450 CYP3A in human renal cell cancer. *Br J Cancer* 1999;79:1836-42.
213. Su T, Bao Z, Zhang QY, et al. Human cytochrome P450 CYP2A13: predominant expression in the respiratory tract and its high efficiency metabolic activation of a tobacco-specific carcinogen, 4-(methylnitrosamino)-1-(3-pyridyl)-1-butanone. *Cancer Res* 2000;60:5074-9.
214. Aithal GP, Ramsay L, Daly AK, et al. Hepatic adducts, circulating antibodies, and cytokine polymorphisms in patients with diclofenac hepatotoxicity. *Hepatology* 2004;39:1430-40.
215. Pacitto SR, Uetrecht JP, Boutros PC, et al. Changes in gene expression induced by tienilic Acid and sulfamethoxazole: testing the danger hypothesis. *J Immunotoxicol* 2007;4:253-66.
216. Sevilla-Mantilla C, Ortega L, Agundez JA, et al. Leflunomide-induced acute hepatitis. *Dig Liver Dis* 2004;36:82-4.
217. Aithal GP, Day CP, Leathart JB, et al. Relationship of polymorphism in CYP2C9 to genetic susceptibility to diclofenac-induced hepatitis. *Pharmacogenetics* 2000;10:511-8.
218. Acuna G, Foerzler D, Leong D, et al. Pharmacogenetic analysis of adverse drug effect reveals genetic variant for susceptibility to liver toxicity. *Pharmacogenomics J* 2002;2:327-34.
219. Daly AK, Aithal GP, Leathart JB, et al. Genetic susceptibility to diclofenac-induced hepatotoxicity: contribution of UGT2B7, CYP2C8, and ABCC2 genotypes. *Gastroenterology* 2007;132:272-81.
220. Kim SH, Kim SH, Bahn JW, et al. Genetic polymorphisms of drug-metabolizing enzymes and anti-TB drug-induced hepatitis. *Pharmacogenomics* 2009;10:1767-79.
221. Lee SW, Chung LS, Huang HH, et al. NAT2 and CYP2E1 polymorphisms and susceptibility to first-line anti-tuberculosis drug-induced hepatitis. *Int J Tuberc Lung Dis* 2010;14:622-6.

222. Huang YS, Su WJ, Huang YH, et al. Genetic polymorphisms of manganese superoxide dismutase, NAD(P)H:quinone oxidoreductase, glutathione S-transferase M1 and T1, and the susceptibility to drug-induced liver injury. *J Hepatol* 2007;47:128-34.
223. Leiro V, Fernandez-Villar A, Valverde D, et al. Influence of glutathione S-transferase M1 and T1 homozygous null mutations on the risk of antituberculosis drug-induced hepatotoxicity in a Caucasian population. *Liver Int* 2008;28:835-9.
224. Lucena MI, Andrade RJ, Martinez C, et al. Glutathione S-transferase m1 and t1 null genotypes increase susceptibility to idiosyncratic drug-induced liver injury. *Hepatology* 2008;48:588-96.
225. Clare KE, Miller MH, Dillon JF. Genetic Factors Influencing Drug-Induced Liver Injury: Do They Have a Role in Prevention and Diagnosis? *Curr Hepatol Rep* 2017;16:258-264.
226. Stephens C, Lucena MI, Andrade RJ. Genetic variations in drug-induced liver injury (DILI): resolving the puzzle. *Front Genet* 2012;3:253.
227. Nicoletti P, Aithal GP, Chamberlain TC, et al. Drug-Induced Liver Injury due to Flucloxacillin: Relevance of Multiple Human Leukocyte Antigen Alleles. *Clin Pharmacol Ther* 2019;106:245-253.
228. Spraggs CF, Budde LR, Briley LP, et al. HLA-DQA1*02:01 is a major risk factor for lapatinib-induced hepatotoxicity in women with advanced breast cancer. *J Clin Oncol* 2011;29:667-73.
229. Kindmark A, Jawaid A, Harbron CG, et al. Genome-wide pharmacogenetic investigation of a hepatic adverse event without clinical signs of immunopathology suggests an underlying immune pathogenesis. *Pharmacogenomics J* 2008;8:186-95.
230. Singer JB, Lewitzky S, Leroy E, et al. A genome-wide study identifies HLA alleles associated with lumiracoxib-related liver injury. *Nat Genet* 2010;42:711-4.
231. Russmann S, Jetter A, Kullak-Ublick GA. Pharmacogenetics of drug-induced liver injury. *Hepatology* 2010;52:748-61.
232. Goto S, Deguchi J, Nishio N, et al. Hepatotoxicants induce cytokine imbalance in response to innate immune system. *J Toxicol Sci* 2015;40:389-404.
233. Pachkoria K, Lucena MI, Crespo E, et al. Analysis of IL-10, IL-4 and TNF-alpha polymorphisms in drug-induced liver injury (DILI) and its outcome. *J Hepatol* 2008;49:107-14.
234. Stephens C, Andrade RJ. Genetic Predisposition to Drug-Induced Liver Injury. *Clin Liver Dis* 2020;24:11-23.
235. Andrade RJ, Lucena MI, Martin-Vivaldi R, et al. Acute liver injury associated with the use of ebrotidine, a new H2-receptor antagonist. *J Hepatol* 1999;31:641-6.
236. Fontana R, Watkins PB, Bonkovsky HL, et al. Drug-Induced Liver Injury Network (DILIN) prospective study: rationale, design and conduct. *Drug Saf* 2009;32:55-68.
237. Echevarria JM, Gonzalez JE, Lewis-Ximenez LL, et al. Hepatitis E virus infection in Latin America: a review. *J Med Virol* 2013;85:1037-45.
238. Hoofnagle JH, Serrano J, Knoben JE, et al. LiverTox: a website on drug-induced liver injury. *Hepatology* 2013;57:873-4.
239. Chen M, Suzuki A, Thakkar S, et al. DILIRank: the largest reference drug list ranked by the risk for developing drug-induced liver injury in humans. *Drug Discov Today* 2016;21:648-53.
240. McGlynn KA, Petrick JL, London WT. Global epidemiology of hepatocellular carcinoma: an emphasis on demographic and regional variability. *Clin Liver Dis* 2015;19:223-38.
241. Bataller R, Brenner DA. Liver fibrosis. *J Clin Invest* 2005;115:209-18.
242. Hernandez-Gea V, Friedman SL. Pathogenesis of liver fibrosis. *Annu Rev Pathol* 2011;6:425-56.
243. Mauad TH, van Nieuwkerk CM, Dingemans KP, et al. Mice with homozygous disruption of the mdx2 P-glycoprotein gene. A novel animal model for studies of nonsuppurative inflammatory cholangitis and hepatocarcinogenesis. *Am J Pathol* 1994;145:1237-45.
244. Recio-Boiles A, Waheed A, Babiker HM. Cancer, Liver. StatPearls. Treasure Island (FL), 2020.
245. Petrick JL, Braunlin M, Laversanne M, et al. International trends in liver cancer incidence, overall and by histologic subtype, 1978-2007. *Int J Cancer* 2016;139:1534-45.
246. Dhanasekaran R, Bandoh S, Roberts LR. Molecular pathogenesis of hepatocellular carcinoma and impact of therapeutic advances. *F1000Res* 2016;5.
247. Ioannou GN, Splan MF, Weiss NS, et al. Incidence and predictors of hepatocellular carcinoma in patients with cirrhosis. *Clin Gastroenterol Hepatol* 2007;5:938-45, 945 e1-4.
248. Sangiovanni A, Prati GM, Fasani P, et al. The natural history of compensated cirrhosis due to hepatitis C virus: A 17-year cohort study of 214 patients. *Hepatology* 2006;43:1303-10.

249. Hoffmann A, Leung TH, Baltimore D. Genetic analysis of NF-kappaB/Rel transcription factors defines functional specificities. *EMBO J* 2003;22:5530-9.
250. Hayden MS, Ghosh S. NF-kappaB, the first quarter-century: remarkable progress and outstanding questions. *Genes Dev* 2012;26:203-34.
251. Xia Y, Shen S, Verma IM. NF-kappaB, an active player in human cancers. *Cancer Immunol Res* 2014;2:823-30.
252. Zhang H, Sun SC. NF-kappaB in inflammation and renal diseases. *Cell Biosci* 2015;5:63.
253. Wajant H, Scheurich P. TNFR1-induced activation of the classical NF-kappaB pathway. *FEBS J* 2011;278:862-76.
254. Ranjan K, Pathak C. FADD regulates NF-kappaB activation and promotes ubiquitination of cFLIPL to induce apoptosis. *Sci Rep* 2016;6:22787.
255. Ea CK, Deng L, Xia ZP, et al. Activation of IKK by TNFalpha requires site-specific ubiquitination of RIP1 and polyubiquitin binding by NEMO. *Mol Cell* 2006;22:245-57.
256. Hsu H, Shu HB, Pan MG, et al. TRADD-TRAF2 and TRADD-FADD interactions define two distinct TNF receptor 1 signal transduction pathways. *Cell* 1996;84:299-308.
257. Park MH, Hong JT. Roles of NF-kappaB in Cancer and Inflammatory Diseases and Their Therapeutic Approaches. *Cells* 2016;5.
258. Prasad S, Ravindran J, Aggarwal BB. NF-kappaB and cancer: how intimate is this relationship. *Mol Cell Biochem* 2010;336:25-37.
259. Gupta SC, Kim JH, Prasad S, et al. Regulation of survival, proliferation, invasion, angiogenesis, and metastasis of tumor cells through modulation of inflammatory pathways by nutraceuticals. *Cancer Metastasis Rev* 2010;29:405-34.
260. Rudolph D, Yeh WC, Wakeham A, et al. Severe liver degeneration and lack of NF-kappaB activation in NEMO/IKKgamma-deficient mice. *Genes Dev* 2000;14:854-62.
261. Rothwarf DM, Zandi E, Natoli G, et al. IKK-gamma is an essential regulatory subunit of the IkappaB kinase complex. *Nature* 1998;395:297-300.
262. Luedde T, Beraza N, Kotsikoris V, et al. Deletion of NEMO/IKKgamma in liver parenchymal cells causes steatohepatitis and hepatocellular carcinoma. *Cancer Cell* 2007;11:119-32.
263. Maeda S, Kamata H, Luo JL, et al. IKKbeta couples hepatocyte death to cytokine-driven compensatory proliferation that promotes chemical hepatocarcinogenesis. *Cell* 2005;121:977-90.
264. Pikarsky E, Porat RM, Stein I, et al. NF-kappaB functions as a tumour promoter in inflammation-associated cancer. *Nature* 2004;431:461-6.
265. Bishayee A. The role of inflammation and liver cancer. *Adv Exp Med Biol* 2014;816:401-35.
266. Zoubek ME, Lucena MI, Andrade RJ, et al. Systematic review: ibuprofen-induced liver injury. *Aliment Pharmacol Ther* 2020;51:603-611.
267. Zoubek ME, Gonzalez-Jimenez A, Medina-Caliz I, et al. High Prevalence of Ibuprofen Drug-Induced Liver Injury in Spanish and Latin-American Registries. *Clin Gastroenterol Hepatol* 2018;16:292-294.
268. Zoubek ME, Woitok MM, Sydor S, et al. Protective role of c-Jun N-terminal kinase-2 (JNK2) in ibuprofen-induced acute liver injury. *J Pathol* 2018.
269. Cubero FJ, Zoubek ME, Hu W, et al. Combined Activities of JNK1 and JNK2 in Hepatocytes Protect Against Toxic Liver Injury. *Gastroenterology* 2016;150:968-81.
270. Zoubek ME, Pinazo-Bandera J, Ortega-Alonso A, et al. Liver injury after methylprednisolone pulses: A disputable cause of hepatotoxicity. A case series and literature review. *United European Gastroenterol J* 2019;7:825-837.
271. Medina-Caliz I, Robles-Diaz M, Garcia-Munoz B, et al. Definition and risk factors for chronicity following acute idiosyncratic drug-induced liver injury. *J Hepatol* 2016;65:532-42.
272. Zoubek ME, Trautwein C, Strnad P. Reversal of liver fibrosis: From fiction to reality. *Best Pract Res Clin Gastroenterol* 2017;31:129-141.
273. Woitok MM, Zoubek ME, Doleschel D et al. Lipid-encapsulated siRNA for hepatocyte-directed treatment of advanced liver disease. *Cell Death Dis.* 2020;11:343.
274. Cubero FJ, Woitok MM, Zoubek ME, et al. Disruption of the FasL/Fas axis protects against inflammation-derived tumorigenesis in chronic liver disease. *Cell Death Dis* 2019;10:115.

PART I

Drug-induced liver injury section

Chapter 2

Ibuprofen-induced liver disease:

A systematic analysis

Zoubek ME, Lucena MI, Andrade RJ, Stephens C

***Aliment Pharmacol Ther* 2020; 51:603-611**

DOI: 10.1111/apt.15645

Abstract

Introduction & aims

Nonsteroidal anti-inflammatory drugs (NSAIDs) are a leading cause of drug-induced liver injury (DILI) across the world. The potential of ibuprofen to induce hepatotoxicity is controversial due to relevant geographical differences in the reporting of cases. We aimed to analyze previously published information on ibuprofen-induced liver injury to better characterize its phenotypic expression.

Methods

A systematic search was performed, and information on ibuprofen-induced liver injury included in prospective hepatotoxicity cohorts, case series, and case reports in terms of demographic, clinical, biochemical, and outcome data were analyzed.

Results

Ibuprofen was the most prevalent NSAID in prospective DILI cohorts from Spain and India. Twelve out of 22 identified idiosyncratic ibuprofen hepatotoxicity cases included females and the mean age was 31 years. The mean cumulative dose of ibuprofen, treatment duration, and time to onset was 30 g, 14 days, and 12 days, respectively. Hepatocellular injury was the most frequently involved liver injury pattern. Six cases developed vanishing bile duct syndrome. Full recovery occurred after 14 weeks, whereas 5 cases evolved to acute liver failure leading to death/liver transplantation.

Conclusions

When assessing a hepatotoxicity suspicion, physicians should consider ibuprofen as a potential culprit as it has been convincingly associated with hepatotoxicity across literature and prospective cohorts of DILI cases. Ibuprofen associated DILI commonly presents with short latency and hepatocellular damage and can potentially progress to death or the need for liver transplantation.

Keywords

Nonsteroidal anti-inflammatory drugs (NSAID); drug-induced liver injury (DILI); hepatotoxicity; phenotype; outcome.

Introduction

Nonsteroidal anti-inflammatory drugs (NSAIDs) belong to a group of chemically heterogeneous compounds, and their therapeutic effect relies on the strong anti-cyclooxygenase (COX) activity and ability to block pro-inflammatory substance formation. The main indications for NSAID therapy range from mild to moderate forms of pain to chronic inflammatory processes.¹⁻²

In the USA, 6% of the population declared taking at least one prescription NSAID a month, and over 30 million people worldwide consume NSAIDs daily.³ Conventional NSAIDs are generally well-tolerated, but adverse effects such as upper gastrointestinal bleeding and perforation, among others, may appear.⁴ NSAID-associated hepatotoxicity is considered rare. Its incidence is estimated to 1-9 cases in every 100.000 exposed patients.^{5, 6} However, other systematic studies suggest that this might be underestimated with findings of 3.1-23.4 per 100.000 patient-years needing hospitalization due to NSAID-induced liver injury.⁷⁻⁹ The everyday use of NSAIDs emphasizes the importance of understanding NSAID-associated liver toxicity, responsible for approximately 10% of drug-induced liver injury (DILI) cases in the Western world.¹⁰⁻¹²

Among the large family of NSAIDs, ibuprofen is a propionic acid derivative available under medical prescription and as an over-the-counter (OTC) medication worldwide. It has been available in the UK since 1969 and was introduced on markets worldwide during the 1970s. It is currently the most frequently prescribed NSAID with over 20 million prescriptions per year in the USA, apart from its vast self-medication use.¹³ The recommended therapeutic dose for adults varies from 800 to 1200 mg per day for OTC self-medication use and 1800 to 2400 mg per day for chronic treatments under medical supervision.

The short plasma half-life and the absence of prolonged retention in the organism contribute to ibuprofen's better gastrointestinal safety profile compared to other NSAIDs.¹⁴ Nevertheless, ibuprofen has been linked to instances of clinically apparent liver injury with injury patterns varying from moderate elevations of aminotransferases to vanishing bile ducts and even acute liver failure (ALF), resulting in death.¹⁵⁻³³ While most reported ibuprofen-induced hepatotoxicity cases to date are idiosyncratic, some cases of liver injury due to ibuprofen overdose have also been described.³⁴⁻³⁶

The massive consumption of ibuprofen worldwide, together with the fact that only limited information is available on ibuprofen-induced hepatotoxicity to date, prompted us to look deeper into the phenotypic presentation of this type of DILI. In the present study, we aimed to review previously reported cases of ibuprofen-induced liver injury in the literature to enhance the understanding of ibuprofen hepatotoxicity with regards to frequency and phenotypic expression.

Materials and methods

A systematic literature review was conducted following the "Preferred Reporting Items of Systematic Reviews and Meta-Analysis" (PRISMA) guidelines to identify all preexisting studies on ibuprofen-induced liver injury to date. Systematic electronic searches of PubMed, Cochrane, and Web of Science were performed to obtain case reports and case series of ibuprofen-induced liver injury as well as extensive hepatotoxicity studies published up to December 2018. The searches were conducted using the terms "hepatotoxicity", "drug-induced liver injury", and "ibuprofen". An elevated number of ibuprofen-induced hepatotoxicity events related to the term "vanishing bile duct syndrome" (VBDS) was observed. Thus this term was included in a new search. Only articles published in English were considered. No other restrictions were applied.

After removing duplicates, titles and abstracts were screened independently for eligibility by two reviewers (MEZ and CS). Any disagreements were resolved by discussion between the two reviewers. Full articles corresponding to the selected abstracts and titles were obtained and assessed against eligibility criteria. Only cases where ibuprofen was judged as the single culprit drug causing a liver reaction were considered for our analysis. Bibliographies of all selected articles were also searched to identify other potentially eligible studies not captured in the initial electronic database search.

Demographic, clinical, histological, laboratory and outcome information corresponding to an episode of exposure to ibuprofen resulting in hepatotoxicity were retrieved from the articles and analyzed. The liver injury pattern was classified based on R-value calculations from the first available blood test after DILI recognition.³⁶ For those cases without complete analytical information at DILI recognition, histological findings from liver biopsies were carefully reviewed. Presentations considered as hypersensitivity features included fever, rash, eosinophilia, and lymphopenia.

Results

The applied search strategy led to a total number of 131 published works obtained from the above-described databases. Of these, 14 reports were duplicates, and another five did not meet the language criteria and thus immediately removed. In the next step, 59 records were selected due to potential interest for the present analysis based on article titles and abstracts (53 records were excluded as their content fell outside the scope of the current study). Of the 59 full articles, which were carefully assessed, 27 were considered for inclusion in our systematic review. The 32 omitted articles did not include useful data on human ibuprofen-induced liver injury to perform phenotypic

characterization analyses. The 27 selected articles consisted of 20 case reports, two case series, and five research articles with data from six substantial prospective hepatotoxicity cohorts, which were all included and thoroughly analyzed in the present work (**Figure 2.1**).

Figure 2.1 Preferred reporting items for systematic review and meta-analyses (PRISMA) flow diagram of the report selection in the current study.

Data available from diverse prospective hepatotoxicity studies on ibuprofen-induced liver injury

Six large prospective hepatotoxicity cohorts comprising NSAID-induced liver injury data were found: two national multicenter cohorts from the USA (DILIN) and Spain (Spanish DILI Registry), a multinational cohort from Latin-America (Latin-American DILI Network), a population-based study from Iceland, and two single-center studies from

India and Italy, which included a total number of 1221, 871, 200, 96, 313 and 185 cases, respectively (**Table 2.1**).^{12,13, 38-40}

Table 2.1 Prevalence of NSAID hepatotoxicity in large prospective DILI cohorts worldwide

	Spanish DILI Registry ³⁸	Latin DILI Network ³⁸	US DILIN ¹³	Iceland ¹²	India ³⁹	Italy ⁴⁰
Year	1994-2015	2012-2015	2004-2013	2010-2011	1997-2008	2000-2016
Type of registry	National	Multinational	National	Population-based study	Single-center study	Single-center study
Total number of DILI cases, n	871	200	1221	96	313	185
Musculo-skeletal drugs, n (%)	96 (11)	36 (18)	N/A (N/A)	N/A (N/A)	N/A (N/A)	N/A (N/A)
NSAIDs, n (%)	73 (9)	29 (10)	30 (3)	6 (6)	8 (3)	65 (36)
Most frequent NSAIDs, n (% of total NSAIDs)	Ibuprofen 21 (29)	Nimesulide [§] 11 (38)	Diclofenac [§] 16 (53)	Diclofenac 6 (100)	Nimesulide 2 (25)	Nimesulide N/A (39)
	Diclofenac 13 (18)	Diclofenac 10 (34)	Meloxicam 3 (10)		Ibuprofen 2 (25)	Ketoprofen N/A (34)
	Nimesulide [§] 9 (12)	Ibuprofen 5 (17)	Celecoxib 3 (10)		Celecoxib 2 (25)	Diclofenac N/A (15)
	Piroxicam 5 (7)	Piroxicam 1 (3)	Etodolac 2 (7)		Diclofenac 1 (13)	Ibuprofen N/A (7)
	Droxicam 4 (5)	Ketoclorac 1 (3)	Oxaprozin 2 (7)		Piroxicam 1 (13)	
	Naproxen 4 (5)	Ketoptofen 1 (3)	Sulindac 1 (3)			
			Valdexocib 1 (3)			

Abbreviations: N/A, not available; NSAID, nonsteroidal anti-inflammatory drug; [§]Withdrawn from the market in Spain in 2002 and Argetina in 2009, but still commercialized in other countries; [§]alone or in combined formulation;

In the DILIN cohort, NSAIDs accounted for 3% of all analyzed DILI events, with diclofenac being the most frequent NSAID (63%) compared to 7% associated with ibuprofen. The cross-sectional data from Iceland found that 6% of the DILI events were related to NSAID treatments; however, these did not include any events related to ibuprofen. All NSAID-induced hepatotoxicity cases were, in fact, associated with diclofenac use.

In comparison, diclofenac was also an essential causative DILI agent among the NSAIDs in the Spanish (18%) and particularly in the Latin-American (34%) registries, but to a lesser extent. In terms of relative frequency, ibuprofen was the dominating NSAID linked to hepatotoxicity in the Spanish DILI cohort while accounting for the third most relevant NSAID in the Latin-American DILI network (**Table 2.1**).

Regarding the two single-center prospective studies from India and Italy, NSAIDs represented 3% and 36% of all included DILI reports, respectively. Nimesulide was the leading NSAID culprit in the Italian study, being responsible for more than a third of all NSAID-associated hepatotoxicity cases (39%), whilst ibuprofen was the fourth cause (7%). The study from India displayed the same number of ibuprofen-, nimesulide- and celecoxib-related liver injury events, which each accounted for 25% of NSAID-associated hepatotoxicity.

Demographic characteristics of idiosyncratic ibuprofen-induced hepatotoxicity

Seventeen published case reports and two case series of idiosyncratic ibuprofen-derived liver injury from 1976-2018 were retrieved, carefully analyzed and summarized in **Tables 2.2** and **2.3**.¹⁵⁻³³ Of the 22 identified cases, 12 were females (55%), and the average age was 31 years (range seven months-59 years). Thirteen patients (59%) had

underlying chronic conditions, which were mainly related to rheumatic disorders (three patients suffering from systemic lupus erythematosus [SLE], one patient with juvenile rheumatic arthritis and one patient with polyarthritis) and hepatic disorders (four patients with hepatitis C virus infection). Ibuprofen was the only administered compound in 6 cases (27%), whereas it was administered simultaneously with other medications in twelve additional cases. In the remaining four cases, the authors did not provide information on concomitant treatments (**Table 2.2**). The cumulative ibuprofen doses ranged from 0.4 to 180 g (mean 30 g) over a period of 1-42 days with an average ibuprofen treatment duration of 14 days. The mean time to onset of the DILI episode was 12 days (range 1-42 days; **Table 2.2**).

Clinical, biochemical and histological profile of idiosyncratic ibuprofen-induced liver insults

Eighteen patients presented with clinical manifestations at onset. The most prevalent symptoms were rash (56%), fever (56%), jaundice (50%), choloria (39%), vomiting (39%) and abdominal pain (22%). In addition, four patients were asymptomatic, and the diagnosis of liver injury was based on routine blood tests (**Table 2.2**).

The mean values for peak liver tests were as follows: TBL 7.6 mg/dL, AST 986 IU/L, ALT 968 IU/L, and ALP 610 IU/L (**Table 2.3**). Distribution of 26 ibuprofen-induced liver injury events from the Spanish DILI Registry and Latin-American DILI Network³⁸ and 13 cases included in the current study with sufficient information with respect to peak ALT and TBL is depicted in **Figure 2.2**.^{16-19, 21, 23-28, 31, 33}

Figure 2.2 Idiosyncratic ibuprofen-induced liver injury events from the literature and Spanish/Latin-American DILI registries depicted according to peak alanine aminotransferase (ALT) and total bilirubin (TBL) level. Worst outcome: death or liver transplantation; ULN: upper limit of normal

The diagram shows an upward trend in severity as well as a larger proportion of worst outcomes for the cases obtained from the literature compared to the cases extracted from the Spanish and Latin-American DILI registries.

Fourteen patients (cases 1-3, 5, 10-13, 15, 17-18, 20-22) displayed hypersensitivity features (fever, rash, eosinophilia). However, hypersensitivity features are not specific to DILI and must, therefore, be considered in the comorbid context of each patient. Only eight patients (cases 3, 5, 10-12, 15, 20 and 22) appear to present hypersensitivity features related to the DILI episode, while the same features can be explained by concomitant non-hepatic conditions in the additional six cases.

Out of 15 cases with available autoantibody information, eight exhibited positive autoantibody titers (53%). Of these, six cases had positive ANA titers and two cases had positive ASMA titers. In seven cases, all measured autoantibody titers were negative, while autoantibody information was not available for seven cases (**Table 2.3**). However, the presence of underlying autoimmune conditions in many of the patients reported to have positive autoantibody titers during the DILI episode makes it difficult to determine the origin of these autoantibodies, mainly due to the absence of autoantibody serology results shortly before the DILI episode.

72

Liver histology information was available for 15 of the analyzed patients (**Table 2.2**). The primary findings were necrosis in three cases (cases 9, 10 and 22), cholestasis in five cases (cases 3, 5, 10, 11 and 14) and fatty changes were present in three cases (cases 1, 10 and 17). A total of seven cases displayed bile duct injury with significant bile duct loss in five cases (cases 5, 11, 12, 16 and 20). Mixed inflammatory infiltrate was detected in six cases (cases 5, 10, 11, 15, 20 and 22), while lymphocytic infiltrate predominated in cases 12, 20 and 22, and eosinophilic infiltrate was observed in case 13.

Cytolytic liver injury was the most commonly observed injury pattern, with 11 cases presenting biochemical and/or histopathological criteria of hepatocellular injury, while three cases presented cholestasis and three cases had a mixed liver injury. The remaining five cases provided insufficient data to assess the type of liver injury (**Table 2.3**). Six patients were diagnosed with VBDS after confirmation of compatible hepatic histology (cases 4, 5, 11, 12, 16, 20). In addition, several patients developed clinical manifestations that were associated with DRESS syndrome (case 22), Stevens-Johnson syndrome (SJS; cases 3 and 5), or toxic epidermal necrolysis (TEN; cases 12 and 20).

Table 2.2 Demographic, clinical, histological and outcome information of ibuprofen-induced liver injury events reviewed in the literature (n=25)

Case	Age / sex	Ibuprofen daily dose mg (duration, d)	Time to onset, d	Clinical presentation	Pre-existing conditions	Concomitant medications	Time to resolution, w	Liver biopsy (Days after detection)	Outcome	Ref
<u>Idiosyncratic DILI cases</u>										
1	48/F	2400 (N/A)	15	Fever, nausea, vomiting.	Polyarthritis, Raynaud's phenomenon, potentially mixed connective tissue disease	Ampicillin, aspirin	-	90% of hepatocytes replaced by fatty material with no areas of parenchymal collapse; minimal portal chronic inflammation (autopsy; 3)	Exitus (3 days after hospital admission)	15
2	12/F	1200 (15) + 1500 (2)	17	Fever, malaise, anorexia, vomiting, weakness	Juvenile rheumatoid arthritis	Aspirin	2	-	Complete Recovery	16
3	44/M	1200 (8) + 400 [7 days later]	8	Fever, rash, severe sore throat, eosinophilia, jaundice	None	Penicillin	28	Cholestatic hepatitis (N/A)	Required corticosteroid treatment. Complete Recovery	17
4	29/M	600 (21)	21	Jaundice, pruritus, nausea, vomiting, abdominal pain, choloria, acholia	Childhood asthma, allergic rhinitis	Hypoaergerenic injections	-	1. Damaged bile ducts with neutrophil and eosinophil infiltrates; mononuclear infiltrate in portal zones; canalicular cholestasis (11) 2. Less portal inflammation, but more cholestasis and obliteration of bile ducts (42) 3. Bile duct paucity (180)	Required prednisone, antihistamines, cholestyramine and ursodiol treatments. Deeply jaundiced 12 months after ibuprofen ingestion → referred for LTx evaluation (1 year after first presentation)	18
5	9/F	<30 mg/kg (6)	6	Jaundice, pruritus, anorexia, fever, fatigue, choloria, rash, acholia	None	APAP occasionally	-	1. Moderately severe cholestasis; portal inflammation; interlobular bile duct damage and loss (45) 2. Absence of bile ducts and cirrhosis without active inflammation (157)	Required UDCA, prednisone and tacrolimus treatments. Referred for LTx (6 months after detection)	19
6	33/M	800 (3)	3	Asymptomatic	HCV	N/A	12	-	Return to baseline values (Positive inadvertent re-exposure 6 months later → new DILI episode → complete recovery)	20
7	44/M	4800/week (N/A)	N/A	Asymptomatic	HCV	N/A	8	-	Return to baseline values	20
8	34/M	1600 (N/A)	N/A	Asymptomatic	HCV	N/A	8	-	Return to baseline values	20

Table 2.2 (continued)

9	59/F	1800 (10)	5	Jaundice, choloria, fatigue	Insufficient peripheral venous blood flow	Dobesilate	-	Submassive hepatic necrosis (explant; 70)	Subacute liver failure →LTx (70 days after dection)	21
10	35/M	400 (single dose)	7 [#]	Rash, pruritus, jaundice ^a	None	Tetanus toxoid injection	28	Inflammation; marked cholestasis; spotty necrosis of hepatocytes with focal areas of ballooning and fatty changes (63)	Required antihistaminics and UDCA treatments. Complete Recovery	22
11	10/F	<30 mg/kg (2+4 [8 days later])	14	Jaundice, choloria, acholia, chellitis, arthralgia, pruriginous rash	None	None	28	Centriolobular cholestasis; loss of interlobular bile ducts in 50% of portal tracts; bile duct injury in the remainder; portal polymorphous infiltrate with eosinophils (48)	Required UDCA and rifampicin treatments. Complete Recovery	23
12	0 [*] /F	<30 mg/kg (2) ^{**}	2	Rash	None	None	16	Lymphocyte infiltration; marked degeneration of interlobular bile duct epithelium; destructive narrowing of ducts in portal tracts; no intralobular bile ducts in at least 10 portal areas (20)	Required UDCA treatment. Complete Recovery	24
13	38/F	600 (7) + 800 (2) when needed	7	Generalized body aches, vomiting, fever	None	None	2	Mild lobular hepatitis with eosinophilic infiltrate (N/A)	Complete Recovery	25
14	16/M	1200 (14) + 200-400 (28) when needed	42	Jaundice, choloria	Chronic shoulder pain	None	12	Centriolobular intrahepatic + canalicular cholestasis; cytoplasmic changes in bile duct epithelium compatible with mild duct damage (18)	Required UDCA, diphenhydramine, rifampin and vitamin K supplement treatments. Complete Recovery	26
15	36/F	300 (20)	25	Fever, choloria, rash (erythema)	None	Famotidine, azulene sodium sulfonate, dompreidone	4	Collapse of hepatocytes primarily around centriolobular areas with infiltration of inflammatory cells (9)	Required intravenous methylprednisolone and oral corticosteroid treatment. Complete Recovery	27
16	40+/F	600, 2-3 days/month (over a time period of 365 days)	N/A	Jaundice, fatigue, choloria	Diabetes type II, adenomyosis	Acarbose, metformin	>45 [‡]	1. Biliary injury and absence of small terminal bile ducts around hepatic arteries affecting >50% of sampled portal tracts (28) 2. Absence of small terminal bile ducts, interstitial fibrous tissue hyperplasia, bile salt deposition in peripheral liver cells, lymphocytes with small amount of plasma cell	Required polyene phosphatidylcholine, silybinin, GSH and UDCA therapies. Loss of followup prior to complete recovery	28

Table 2.2 (continued)

17	54/F	1200 (10) + 400 (< 3) a few days later	10	Fever, rash, vomit, abdominal pain	SLE	N/A	2	Minimal fatty changes (2) ^{§§}	Complete Recovery. (Positive inadvertent re- exposure 8 days later → new DILI episode → required cloroquine therapy → complete recovery)	29
18	15/F	1200 (6)	6	Abdominal pain, fever, vomiting, rash	SLE	Aurothio- glucose	N/A	-	N/A	29
19	57/M	1200 (30)	30	Asymptomatic	HCV	None	12	-	Return to baseline values	30
20	7/M	<30 mg/kg (3)	2	Jaundice, rash	None	None	32	Lymphocyte infiltration; marked degeneration of interlobular bile duct epithelium; absence of intra-lobular bile ducts in at least 10 portal areas (21)	Required treatment with methylprednisolone and UCDA. Complete Recovery	31
21	36/F	2000 (10)	7	Epigastric pain, fever, nausea, vomiting	SLE	Aspirin	N/A	-	Complete Recovery (Positive controlled re-exposure 3 weeks later → new DILI episode → required prednisone therapy → complete recovery)	32
22	22/M	1200 (N/A)	1	Fever, rash, eosinophilia	Kawasaki disease at age 14	Metamizole, codeine, APAP	-	Submassive confluent necrosis with inflammatory polymorphous reaction, hyper eosinophilia and phlebitis (explant, 3)	Liver failure and encephalopathy → LTx (3 days after initial symptoms)	33
Intrinsic DILI cases										
23	29/F	60000 (single dose)	1	Altered mental status, lethargy, jaundice	Depression, asthma, prior alcohol and drug abuse	-	>2	-	Liver profile normalizing, but loss of followup prior to complete recovery	34
24	55/F	9600 (single dose)	8	Jaundice, asthenia	Arterial hyper- tension, alcohol abuse	Amlodipine	-	Large areas of confluent necrosis and zones of confluent collapses of zone 3, both infiltrated with mononuclear cells, leucocytes and macrophages (6)	Liver failure and stage III encephalopathy → LTx (22 days after detection)	35
25	48/M	20000 (single dose)	1	Depressed consciousness, severe metabolic acidosis	Alcohol abuse	-	>3	-	Complete Recovery	36

Abbreviations: APAP, acetaminophen; d, days; DILI, drug-induced liver injury; GSH, glutathione; HCV, hepatitis C virus infection; LTx, liver transplantation; mo, months; N/A, not available; Ref, bibliographic reference; UDCA, ursodeoxycholic acid; w, weeks. [§]Treatment cessation due to maculopapular skin rash appearing 2 hours after ibuprofen intake. Jaundice appeared 7 days later; ^{§§}a single-dose of ibuprofen, 10mg/kg, was given three months earlier; [§]Last follow-up; ^{§§}two days after ibuprofen re-exposition during hospitalization (day 8)

Outcome and follow-up information of idiosyncratic ibuprofen-induced liver injury

Eleven of the analyzed ibuprofen-induced liver injury patients fully recovered from the DILI episode (50%) and the mean time to resolution was 15 weeks (range 2-32 weeks). In the four patients who suffered from an underlying HCV infection (cases 6-8 and 19), liver injury markers returned to baseline values within a mean time of 10 weeks (range 8-12 weeks).

Table 2.3 Biochemical parameters (at the time of peak ALT), liver injury pattern and additional information on ibuprofen-induced liver injury (n=25)

Case	TBL (mg/dL)	AST (IU/L)	ALT (IU/L)	ALP (IU/L)	Type of injury	Additional information	Ref
Idiosyncratic DILI cases							
1	2	6200	N/A	760	Hep	ANA+ (1:4096)	15
2	1	2200	1245	N/A	Hep	-	16
3	14	N/A	441	238	Chol	Developed acute-onset SJS	17
4	6.5	404	488	309	Mix	Developed VBDS and severe progressive xanthomatosis	18
5	3.3	582	649	519	N/A	Developed acute-onset VBDS and SJS; ANA+ (1:40)	19
6	N/A	459	1209	N/A	Hep	-	20
7	N/A	523	1238	N/A	Hep	-	20
8	N/A	597	1577	N/A	Hep	-	20
9	30	2260	2099	N/A	Hep	-	21
10	N/A	N/A	N/A	N/A	N/A	-	22
11	5.4	333	639	1697	Hep	Developed acute VBDS	23
12	8.5	879	723	890	N/A	Developed acute VBDS and TEN	24
13	0.4	383	464	36	Hep	Developed meningitis; ASMA+ (1:20)	25
14	3.9	99	182	N/A	Chol	ASMA+ (1:80)	26
15	15	1492	1860	323	Hep	Developed multiform exudative erythema; DLST+ for ibuprofen;	27
16	6	247	207	1598	Chol	Developed VBDS and hyperlipidemia; ANA+ (1:320)	28
17	N/A	105	255	155	Mix	ANA+	29
18	N/A	185	N/A	N/A	N/A	-	29
19	N/A	355	1093	N/A	Hep	-	30
20	8.1	186	419	700	Mix	Developed VBDS and TEN	31
21	N/A	147 U/mL [§]	N/A	N/A	N/A	ANA+ (1:80)	32
22	2.9	1168	2154	90	Hep	Developed DRESS syndrome	33
Mean (range)	7.6 (0.4-30)	986 (99-6200)	968 (182-2154)	610 (36-1697)			
Intrinsic DILI cases							
23	5	>717	1873	135	Hep	ANA+ , ASMA+	34
24	19	N/A	2301	109	Hep	-	35
25	N/A	291	N/A	245	N/A	-	36

Abbreviations: TBL, total bilirubin; AST, aspartate aminotransferase; ALT, alanine aminotransferase; ALP, alkaline phosphatase; N/A, not available; Hep, hepatocellular; Mix, mixed; Chol, cholestatic; SJS, Stevens-Johnson syndrome; VBDS, vanishing bile duct syndrome; TEN, toxic epidermal necrolysis; DLST, drug lymphocyte stimulation test; DRESS, drug rash with eosinophilia and systemic symptoms; Ref, bibliographic reference; *Type of liver injury was deduced from R values based on initial blood analysis values when presented, or biopsy findings in the absence of analytical information; [§]normal, <47 U/mL;

The follow-up was lost for one patient before complete normalization occurred (case 16) and no outcome information was available for case 18. One patient had a fatal

outcome after suffering massive hepatic fatty metamorphosis and pleural effusion and died one week after onset (case 1), whilst two additional patients (cases 9 and 22) developed ALF and underwent liver transplantation within ten weeks from onset. Two further patients, who developed VBDS, remained deeply jaundiced 12 months after onset despite pharmacological therapy with immunosuppressive agents and were finally referred for liver transplantation (patients 4 and 5). Three cases (patients 6, 17 and 21) had an inadvertent rechallenge to ibuprofen, which triggered a new flare of aminotransferases (**Table 2.2**).

Intrinsic ibuprofen-induced hepatotoxicity

Three case reports were also found describing liver damage following ibuprofen overdose (cases 23-25).³⁴⁻³⁶ Two of the events (case 23 and 25) were suicide attempts with a single intake of 20 and 60 g ibuprofen, while the reason for the ibuprofen overdose in case 24 is unknown. In terms of outcome, one case developed ALF and underwent liver transplantation four weeks after onset, one case fully recovered (time to resolution unknown) and the outcome of the third case is unknown as follow-up was lost after two weeks (**Table 2.2** and **2.3**).

Discussion

Ibuprofen is well tolerated across all age groups based on the vast consumption worldwide. Novel studies have, however, demonstrated potential adverse effects on the cardiovascular and cerebrovascular systems due to ibuprofen.^{41,42} Despite being considered among the NSAIDs with the safest hepatic profile, hepatotoxicity caused by ibuprofen can occur. Our group recently published information on idiosyncratic ibuprofen-induced liver injury in the Spanish and Latin-American DILI registries.³⁸ Besides, there is evidence of multiple reported cases of ibuprofen-induced liver injury in the literature over the last decades.¹⁵⁻³⁶

The analysis of NSAID-related hepatotoxicity information in several prospective hepatotoxicity cohorts from Spain, Latin-America, the USA, Iceland, India and Italy revealed noticeable differences regarding the relative frequency of culprit NSAIDs. A notably higher prevalence of ibuprofen-induced hepatotoxicity cases was present in the Spanish DILI registry and a single-center study from India, where ibuprofen-related hepatotoxicity accounted for up to 29% and 25% of all NSAID-associated DILI, respectively.^{38, 39} In contrast, ibuprofen DILI represented only 7% of the total NSAID-associated hepatotoxicity cases in studies from the US and Italy, and no ibuprofen hepatotoxicity cases were found in Iceland over a 2-year study period.^{12,13,40} The reason for these geographical differences remains unclear but may be multifactorial due to

genetic predisposition, variations in dispensable drug doses, preferred prescription habits for specific NSAIDs or postmarketing regulatory restrictions of available NSAIDs, among others.

The search for idiosyncratic ibuprofen-induced hepatotoxicity information in the previous literature resulted in 22 identified cases. Overall, ibuprofen-derived liver injury occurred in a relatively short time from treatment initiation with an average time to onset of 12 days. With respect to gender, we noted a slight trend towards females more frequently developing ibuprofen hepatotoxicity compared to men. This was not the case in our previous report on ibuprofen hepatotoxicity cases in Spain, with equal amounts of male and female subjects, although a slightly higher proportion of women was observed in DILI due to other NSAIDs.³⁸ A possible explanation for this finding could be that several of the patients (23%) presented underlying rheumatic disorders (all were females), which has been associated more frequently with women.⁴³ In addition, a recent study analyzing the trends of NSAID use in US adults found that women are more likely to use NSAIDs.⁴⁴ Hence, a higher use of NSAIDs in general and predisposition to rheumatic disorders requiring NSAID treatments may be the reason behind the noted increase in females in published ibuprofen hepatotoxicity case reports rather than females being biologically more susceptible to this form of DILI than men.

We previously found a predominance of hepatocellular liver injury patterns in Spanish and Latin-American DILI cases caused by ibuprofen.³⁸ The same observation holds for the cases obtained from the literature in the current study with 65% of the cases, with sufficient information to determine the pattern of liver injury, presenting hepatocellular type of liver injury, while cholestatic and mixed liver injury cases were each seen in 18% of the cases. Thus, the most common liver injury pattern associated with ibuprofen-induced hepatotoxicity appears to have a hepatocellular character, but cholestatic/mixed liver injury can also occur.

Vanishing bile duct syndrome is characterized by bile duct injury and ductopenia and occurred in six of the 22 idiosyncratic cases. Although rare, VBDS can occur in DILI patients with progressive cholestasis, potentially leading to liver failure and death or liver transplantation. It has been associated with causative drugs such as azathioprine, androgens, amoxicillin-clavulanate, carbamazepine, chlorpromazine, erythromycin, estradiol, flucloxacillin, phenytoin and co-trimoxazole.⁴⁵ A study of 363 DILI cases with biopsy data found 7.2% of the cases with bile duct loss based on histopathological interpretations.⁴⁶ However, it has been suggested that this incidence value may be overestimated compared to observations in population-based studies due to the fact that the cases were recruited from tertiary referral centers, which are likely to see more severe cases.⁴⁷ Four of the cases identified in the literature search of the current study developed severe cutaneous reactions (progressive xanthomatosis, SJS or TEN) in addition to VBDS. The concurrence of VBDS and cutaneous reactions were similarly

found in the aforementioned study of US DILI cases, and suggested to be the result of an aberrant hypersensitivity reaction affecting cholangiocytes and keratinocytes potentially due to shared immunogenic proteins and cell surface presentation of drug-protein adducts or immunogenic drug metabolites. Interestingly, one of the US cases with VBDS and TEN had taken ibuprofen prior to the liver reaction, although ibuprofen was adjudicated as being possibly and azithromycin as highly likely the causative agent.⁴⁶

The cutaneous reaction was not limited to those that developed VBDS. Our literature search also revealed three cases with ibuprofen hepatotoxicity and cutaneous reactions (case 3, 15 and 22) without VBDS. Cutaneous hypersensitivity reactions to ibuprofen are well known.⁴⁸ These reactions are often allergic in nature, mostly mild, occur rapidly after drug exposure and rarely contain hepatic involvement. In contrast, drug reaction with eosinophilia and systemic symptoms (DRESS) syndrome often presents with concurrent cutaneous and hepatic reactions.⁴⁹ However, ibuprofen does not appear to be a significant cause of DRESS (with liver involvement) as we only identified one case in our literature search. The exact pathogenesis of DRESS is not yet determined, but similar to idiosyncratic DILI is believed to be multifactorial.

These data support that there is a broad clinical spectrum associated with ibuprofen-induced liver injury rather than a homogeneous signature. This is important to keep in mind and not overlook the intake of ibuprofen in order to establish a correct diagnosis. However, it should be pointed out that the analyzed cases were all obtained from published case series and case reports and might therefore have been subjected to publication bias, as reports with a severe or novel presentation tend to be preferably published compared to cases with a mild and uncomplicated clinical course. This may have contributed to the high proportion of identified ibuprofen hepatotoxicity cases with VBDS, SJS and TEN in the current study.

A severe clinical progression was identified in three idiosyncratic cases, one patient with a fatal outcome (case 1) and two patients who underwent liver transplantations (cases 9 and 22), as well as in one overdose-patient who also required liver transplantation (case 24). Two further idiosyncratic patients were referred for liver transplantation without further information on the final outcome. Similarly, the prospective ibuprofen-induced hepatotoxicity cohort from Spain demonstrated a concerning proportion of fatal/liver transplant patients, which was higher for DILI cases caused by ibuprofen than by other NSAIDs or non-NSAID agents.³⁸ These findings demonstrate that ibuprofen is associated with worst outcome DILI cases.

The underlying mechanism of ibuprofen-induced liver injury remains currently unknown. Nevertheless, the interplay between environmental, host and drug-related factors is considered vital for the development of idiosyncratic DILI. Hepatic

biotransformation of drugs may generate reactive metabolites, which can lead to the formation of reactive oxygen species, activate cell death pathways such as c-Jun N-terminal kinases (JNK) and induce mitochondrial permeability transition, which ultimately promotes hepatocyte death.⁵⁰ Recent research indicates that increased phosphorylated JNK expression occurs in the cytoplasm of human and murine hepatocytes after ibuprofen-induced liver injury.⁵¹ These results suggest that JNK plays a role in the physiopathology of ibuprofen-derived liver disease.

In conclusion, the prevalence of ibuprofen-induced liver injury is highly variable across prospective DILI cohort data, with a more substantial representation in Spain, India and Latin-America. However, when considering the overwhelming self-medication use of ibuprofen, the true incidence of ibuprofen-associated DILI might be underestimated due to physicians considering ibuprofen as an innocent bystander rather than the culprit agent. Although ibuprofen-induced liver injury can present with a heterogeneous phenotype (including hypersensitivity features, cholestatic damage and VBDS), hepatocellular pattern appearing after a short latency period predominates. Importantly, we found a relatively large proportion of patients in our study that progressed to ALF and required liver transplantation. Clinicians should pay attention to the often-overlooked intake of ibuprofen when assessing a suspicion of hepatotoxicity as well as proceed with a careful follow-up and monitoring of patients suspected to have ibuprofen-induced liver injury until recovery is reached.

References

1. Frolich JC. A classification of NSAIDs according to the relative inhibition of cyclooxygenase isoenzymes. *Trends Pharmacol Sci.* 1997;18:30-34.
2. Rainsford KD. Fifty years of ibuprofen: advancing pain and fever management. *Int J Clin Pract Suppl.* 2013;178:1-2.
3. Paulose-Ram R, Hirsch R, Dillon C, et al. Prescription and non-prescription analgesic use among the US adult population: results from the third National Health and Nutrition Examination Survey (NHANES III). *Pharmacoepidemiol Drug Saf.* 2003;12:315-326.
4. Hernandez-Diaz S, Garcia-Rodriguez LA. Epidemiologic assessment of the safety of conventional nonsteroidal anti-inflammatory drugs. *Am J Med.* 2001;110 (Suppl 3A):20S-27S.
5. Sriuttha P, Sirichanchuen B, Permsuwan U. Hepatotoxicity of Nonsteroidal Anti-Inflammatory Drugs: A Systematic Review of Randomized Controlled Trials. *Int J Hepatol.* 2018;2018:5253623.
6. Sarges P, Steinberg JM, Lewis JH. Drug-Induced Liver Injury: Highlights from a Review of the 2015 Literature. *Drug Saf.* 2016;39:801-821.
7. Rubenstein JH, Laine L. Systematic review: the hepatotoxicity of non-steroidal anti-inflammatory drugs. *Aliment Pharmacol Ther.* 2004;20:373-380.
8. Rostom A, Goldkind L, Laine L. Nonsteroidal anti-inflammatory drugs and hepatic toxicity: a systematic review of randomized controlled trials in arthritis patients. *Clin Gastroenterol Hepatol.* 2005;3:489-498.
9. Garcia Rodriguez LA, Perez Gutthann S, Walker AM, et al. The role of non-steroidal anti-inflammatory drugs in acute liver injury. *BMJ.* 1992;305:865-868.
10. Lucena MI, Andrade RJ, Kaplowitz N, et al. Phenotypic characterization of idiosyncratic drug-induced liver injury: the influence of age and sex. *Hepatology.* 2009;49:2001-2009.

11. Chalasani N, Bonkovsky HL, Fontana R, et al. Features and Outcomes of 899 Patients With Drug-Induced Liver Injury: The DILIN Prospective Study. *Gastroenterology*. 2015;148:1340-1352.
12. Björnsson ES, Bergmann OM, Björnsson HK, et al. Incidence, presentation, and outcomes in patients with drug-induced liver injury in the general population of Iceland. *Gastroenterology*. 2013;144:1419-1425.
13. Schmeltzer PA, Kosinski AS, Kleiner DE, et al. Liver injury from nonsteroidal anti-inflammatory drugs in the United States. *Liver Int*. 2016;36:603-609.
14. Rainsford KD. Ibuprofen: pharmacology, efficacy and safety. *Inflammopharmacology*. 2009;17:275-342.
15. Bravo JF, Jacobson MP, Mertens BF. Fatty liver and pleural effusion with ibuprofen therapy. *Ann Intern Med*. 1977;87:200-201.
16. Stempel DA, Miller JJ. Lymphopenia and hepatic toxicity with ibuprofen. *J Pediatr*. 1977;90:657-658.
17. Sternlieb P, Robinson RM. Stevens-Johnson syndrome plus toxic hepatitis due to ibuprofen. *N Y State J Med*. 1978;78:1239-1243.
18. Alam I, Ferrell LD, Bass NM. Vanishing bile duct syndrome temporally associated with ibuprofen use. *Am J Gastroenterol*. 1996;91:1626-1630.
19. Srivastava M, Perez-Atayde A, Jonas MM. Drug-associated acute-onset vanishing bile duct and Stevens-Johnson syndromes in a child. *Gastroenterology*. 1998;115:743-746.
20. Riley TR, Smith JP. Ibuprofen-induced hepatotoxicity in patients with chronic hepatitis C: a case series. *Am J Gastroenterol*. 1998;93:1563-1565.
21. Rodríguez-González FJ, Montero JL, Puente J, et al. Orthotopic liver transplantation after subacute liver failure induced by therapeutic doses of ibuprofen. *Am J Gastroenterol*. 2002;97:2476-2477.
22. Tyagi P, Sharma BC, Sarin SK. Cholestatic liver injury due to ibuprofen. *Indian J Gastroenterol*. 2005;24:77-78.
23. Taghian M, Tran TA, Bresson-Hadni S, et al. Acute vanishing bile duct syndrome after ibuprofen therapy in a child. *J Pediatr*. 2004;145:273-276.
24. Kim HY, Yang HK, Kim SH, et al. Ibuprofen associated acute vanishing bile duct syndrome and toxic epidermal necrolysis in an infant. *Yonsei Med J*. 2014;55:834-837.
25. Nayudu SK, Kavuturu S, Niazi M, et al. A rare coexistence: drug induced hepatitis and meningitis in association with Ibuprofen. *J Clin Med Res*. 2013;5:243-246.
26. Bennett WE, Turmelle YP, Shepherd RW. Ibuprofen-induced liver injury in an adolescent athlete. *Clin Pediatr (Phila)*. 2009;48:84-86.
27. Watanabe T, Abe M, Tada F, et al. Drug-induced liver injury with serious multiform exudative erythema following the use of an over-the-counter medication containing ibuprofen. *Intern Med*. 2015;54:395-399.
28. Xie W, Wang Q, Gao Y, et al. Vanishing bile duct syndrome with hyperlipidemia after ibuprofen therapy in an adult patient: a case report. *BMC Gastroenterol*. 2018;18:142.
29. Sonnenblick M, Abraham AS. Ibuprofen hypersensitivity in systemic lupus erythematosus. *Br Med J*. 1978;1:619-620.
30. Andrade RJ, Lucena MI, García-Cortés M, et al. Chronic hepatitis C, ibuprofen, and liver damage. *Am J Gastroenterol*. 2002;97:1854-1855.
31. Basturk A, Artan R, Yilmaz A, et al. Acute vanishing bile duct syndrome after the use of ibuprofen. *Arab J Gastroenterol*. 2016;17:137-139.
32. Mandell B, Shen HS, Hepburn B. A Letter: Fever from ibuprofen in a patient with lupus erythematosus. *Ann Intern Med*. 1976;85:209-210.
33. Roales-Gómez V, Molero AI, Pérez-Amarilla I, et al. DRESS syndrome secondary to ibuprofen as a cause of hyperacute liver failure. *Rev Esp Enferm Dig*. 2014; 106:482-486.
34. Shahnazarian V, Ramai D, Reddy M. A Rare Case of Ibuprofen-induced Acute Liver Injury. *Cureus*. 2018;10:e3225.
35. Laurent S, Rahier J, Geubel AP, et al. Subfulminant hepatitis requiring liver transplantation following ibuprofen overdose. *Liver*. 2000;20:93-94.
36. Lee CY, Finkler A. Acute intoxication due to ibuprofen overdose. *Arch Pathol Lab Med*. 1986;110:747-749.
37. Aithal GP, Watkins PB, Andrade RJ, et al. Case definition and phenotype standardization in drug-induced liver injury. *Clin Pharmacol Ther*. 2011;89:806-815.

38. Zoubek ME, González-Jimenez A, Medina-Cáliz I, et al. High Prevalence of Ibuprofen Drug-Induced Liver Injury in Spanish and Latin-American Registries. *Clin Gastroenterol Hepatol*. 2018;16:292-294.
39. Devarbhavi H, Dierkhising R, Kremers WK, et al. Single-center experience with drug-induced liver injury from India: causes, outcome, prognosis, and predictors of mortality. *Am J Gastroenterol*. 2010;105:2396-2404.
40. Licata A, Minissale MG, Calvaruso V, et al. A focus on epidemiology of drug-induced liver injury: analysis of a prospective cohort. *Eur Rev Med Pharmacol Sci*. 2017;21(1 Suppl):112-121.
41. Trelle S, Reichenbach S, Wandel S, et al. Cardiovascular safety of non-steroidal anti-inflammatory drugs: network meta-analysis. *BMJ*. 2011;342:c7086.
42. Sondergaard KB, Weeke P, Wissenberg M, et al. Non-steroidal anti-inflammatory drug use is associated with increased risk of out-of-hospital cardiac arrest: a nationwide case-time-control study. *Eur Heart J Cardiovasc Pharmacother*. 2017;3:100-107.
43. Fairweather D, Frisancho-Kiss S, Rose NR. Sex differences in autoimmune disease from a pathological perspective. *Am J Pathol*. 2008;173:600-609.
44. Davis JS, Lee HY, Kim J, et al. Use of non-steroidal anti-inflammatory drugs in US adults: changes over time and by demographic. *Open Heart*. 2017;4:e000550.
45. Andrade RJ, Aithal GP, Björnsson ES, et al. EASL clinical practice guidelines: drug-induced liver injury. *J Hepatol*. 2019;70:1222-1261.
46. Bonkovsky HL, Kleiner DE, Gu J et al. Clinical presentations and outcomes of bile duct loss caused by drugs and herbal and dietary supplements. *Hepatology*. 2017;65:1267-1277.
47. Björnsson ES, Jonasson JG. Idiosyncratic drug-induced liver injury associated with bile duct loss and vanishing bile duct syndrome: rare but has severe consequences. *Hepatology*. 2017;65:1091-1093.
48. Sánchez-Borges M, Capriles-Hulett A, Caballero-Fonseca F. Risk of skin reactions when using ibuprofen-based medicines. *Expert Opin Drug Saf*. 2005;4:837-848.
49. Martínez-Cabriales SA, Shear NH, Gonzalez-Moreno EI. Liver involvement in the drug reaction, eosinophilia, and systemic symptoms syndrome. *World J Clin Cases*. 2019;7:705-716.
50. Cubero FJ, Zoubek ME, Hu W et al. Combined Activities of JNK1 and JNK2 in Hepatocytes Protect Against Toxic Liver Injury. *Gastroenterology*. 2016;150:968-981.
51. Zoubek ME, Woitok MM, Sydor S et al. Protective role of c-Jun N-terminal kinase-2 (JNK2) in ibuprofen-induced acute liver injury. *J Pathol*. 2019;247:110-122.

Chapter 3

Idiosyncratic drug-induced liver injury due to ibuprofen in the Spanish and Latin- American DILI registries

Zoubek ME, Gonzalez-Jimenez A, Robles-Diaz M, Diaz-Jimenez P, Medina-Caliz I, Slim M, Sanjuan-Jimenez R, Hernandez N, Romero-Gomez M, Bessone F, Hallal H, Cubero FJ, Lucena MI, Stephens C, Andrade RJ

***Clin Gastroenterol Hepatol* 2018;16(2):292-294**

DOI: 10.1016/j.cgh.2017.07.037

Abstract

Background & aims

Drug-induced liver injury (DILI) is a fundamental cause of pharmaceutical regulatory measures. Although nonsteroidal anti-inflammatory drugs (NSAIDs) are essential DILI causative agents, ibuprofen is usually considered a safe drug. Differences in ibuprofen hepatotoxicity frequency have, however, been detected between geographically distinct DILI cohorts. Here we aimed to describe the clinical presentation and severity of ibuprofen-associated hepatotoxicity.

Methods

Twenty-six ibuprofen hepatotoxicity cases from the Spanish and Latin-American DILI Registries were compared with DILI cases induced by 76 other NSAIDs and 844 non-NSAIDs.

Results

Ibuprofen represented 29% and 17% of the Spanish and Latin-American NSAID cases, respectively. These cases had a mean age of 51 years (50% females), with 58% presenting hepatocellular pattern of injury. Jaundice was a common symptom (65%) and 58% of the patients required hospitalization. Borderline significance ($p=0.04$) was detected in diabetes mellitus prevalence, with ibuprofen DILI having a higher proportion than cases induced by other NSAIDs or non-NSAIDs (27% vs. 8% and 12%). A higher frequency of fatal/transplant outcomes was detected among ibuprofen DILI cases (12% vs. 5% and 3%).

Conclusions

The ibuprofen cases in the current study represent a notably higher frequency in Spain and Latin-America than reported elsewhere. This difference could potentially be related to differences in over-the-counter dose formulations, drug use patterns and racial genetic differences. The clinical presentation of ibuprofen DILI varies between patients and lacks a characteristic drug signature distinguishing this form of DILI. Although rare, ibuprofen hepatotoxicity should not be overlooked as it can have life-threatening consequences.

Keywords

Hepatotoxicity, nonsteroidal anti-inflammatory drugs, phenotype, drug signature

Introduction

Nonsteroidal anti-inflammatory drugs (NSAIDs) are among the most frequently used drugs to treat pain, fever and chronic inflammations. Over 30 million people take NSAIDs daily, with 6% of the US population having declared taking at least one prescription NSAID monthly.¹ NSAIDs are generally well-tolerated but can cause hypersensitivity and gastroduodenal problems in susceptible patients; the latter is more frequently seen with nonselective NSAIDs.^{2,3} In contrast, several selective COX-2 inhibitors, such as rofecoxib and parecoxib, have been withdrawn from the market due to serious cardiovascular adverse reactions.³ NSAID-associated hepatotoxicity is considered rare, with an estimated incidence rate of 3.1-23.4/100.000 patient-years based on systematic reviews.⁴ Nevertheless, the everyday use of NSAIDs emphasizes the importance of understanding NSAID-associated liver toxicity, which is responsible for 3-10% of idiosyncratic drug-induced liver injury (DILI) cases in the Western world.⁵⁻

7

Ibuprofen is a propionic acid derivative available under medical prescription and as over-the-counter medication (OTC). Introduced in the 1970s, it is currently the most frequently prescribed NSAID with over 20 million prescriptions per year in the USA, apart from its vast self-medication use.⁸ Despite the better safety profile of ibuprofen compared to other NSAIDs, ibuprofen has been linked to instances of clinically apparent liver injury. Published case reports of idiosyncratic DILI due to ibuprofen demonstrate various injury patterns ranging from mild to moderate aminotransferase elevations to liver injury with severe multiform exudative erythema, vanishing bile duct syndrome or even acute liver failure.⁹⁻²⁰ Hence, ibuprofen-induced hepatotoxicity reported to date exhibits heterogeneous phenotypes. Such differences could potentially be due to variations in host factors that interact with drug properties to produce DILI.²¹ In addition, striking regional differences in the frequency of ibuprofen hepatotoxicity was noted in a comparison of causative DILI agents amongst large DILI cohorts in 2010.²²

The large consumption of ibuprofen worldwide, together with the fact that limited information is available on ibuprofen-induced hepatotoxicity to date, prompted us to look deeper into the phenotype of this form of DILI. In the present study, we aimed to analyze the clinical presentation of ibuprofen-associated hepatotoxicity in the search for a potential drug signature. This was performed using DILI cases included in the Spanish DILI Registry and the SLATIN DILI Network, as well as comparing this information with that of reported hepatotoxicity data.

Materials and methods

Twenty-one ibuprofen-induced idiosyncratic hepatotoxicity cases from the Spanish DILI Registry and five additional cases included in the SLATIN DILI Network were analyzed and compared with 76 idiosyncratic hepatotoxicity events due to other NSAIDs and 844 non-NSAID-induced hepatotoxicity cases from the same databases. Only cases induced by conventional medications were included in the current study. All NSAID cases were limited to cases where the NSAID was judged as a single culprit drug. The two registries share standardized guidelines for DILI case identification and data collection, as previously described in detail elsewhere.²³

The biochemical DILI criteria used were initially those established by the Council for International Organizations of Medical Sciences (CIOMS) in 1990 and later readjusted to those of Aithal et al. in 2011.^{24,25} The pattern of liver injury was classified based on R ratio values from the first available blood test after DILI recognition and the severity of each episode was differentiated according to the DILI severity index scale.²⁵ Hy's law criteria were applied according to the redefinition by Robles-Diaz et al. in 2014.²⁶ Chronic DILI was defined as prolonged liver profile elevations more than one year after onset.²⁷

Statistical analyses

Statistical data analysis was performed using the statistical software package IBM SPSS 19.0 (IBM Corporation, Armonk, NY, USA). Numerical and categorical variables were analyzed using the Student's *t*-test and the χ^2 test, respectively. Analysis of variances (ANOVA) was used to compare groups. Groups with variables not following normal distribution were analyzed using the Kruskal-Wallis test. All tests, which resulted in $P < 0.05$ were considered statistically significant.

Results

NSAID distribution in the Spanish and Latin-American DILI Registries

Musculoskeletal drugs were responsible for 9.5% of the 906 DILI cases included in the Spanish DILI Registry at the time of this study. NSAIDs accounted for the majority of these with a total of 73 cases (85%). Ibuprofen was associated with 21 cases (29%), diclofenac 13 (18%), nimesulide 9 (12%), piroxicam 5 (6.8%), droxicam 4 (5.5%), naproxen 4 (5.5%) and 17 with other NSAIDs (23%) (**Figure 3.1A,C**).

Of the 211 cases in the Latin-American DILI registry, 15% corresponded to musculoskeletal drugs and NSAIDs were also here the most prevalent subgroup with 29 events in total (91%). The implemented drugs were nimesulide 11 (38%) cases,

diclofenac 10 (34%), ibuprofen 5 (17%) and 1 (3.4%) case each of piroxicam, ketorolac and ketoprofen (**Figure 3.1B, D**).

In order to increase the size of the ibuprofen cohort for further analyses, we merged the 21 Spanish cases with the 5 Latin-American cases to obtain a final cohort of 26 ibuprofen cases.

Figure 3.1 Drug distribution in the Spanish and Latin-American DILI Registries

Most frequent causative drug classes in the Spanish (A, N=736) and Latin- American DILI Registries (B, N=210). Most frequent causative nonsteroidal antiinflammatory drugs in the Spanish (C, N=73) and Latin-American DILI Registries (D, N=29).

Demographic, clinical and biochemical profile of ibuprofen-induced liver injury

Of the 907 Spanish and 211 Latin-American DILI cases included in the two registries, 768 and 178 cases, respectively, fulfilled the inclusion criteria for this study. Demographic, clinical and biochemical data of the 26 ibuprofen cases are presented in **Tables 3.1** and **3.2**. Thirteen of the 26 ibuprofen DILI patients (50%) were females. The mean age for all subjects was 51 years, with 27% being older than 60 years. The average body mass index (BMI) was 28 kg/m². Most of the patients (77%) were treated with a daily ibuprofen dose between 1200 and 1800 mg. The median treatment duration was 16 days and the median time from treatment initiation to DILI onset was 15 days.

Concerning the clinical scenario, 14 patients (58%) required hospitalization. The prevalence of jaundice was relatively high, with 18 (69%) patients presenting this symptom. Positive autoantibody titers were detected in 27% of the patients with autoantibody information. More specifically, 19% of the patients were positive for anti-smooth muscle antibodies (ASMA) and 8% for anti-nuclear antibodies, with none of these patients having a precedent diagnosis of autoimmune-like diseases. With respect to liver injury pattern, 15 patients had hepatocellular damage (58%), while two patients had cholestatic injury (7.7%) and the remaining 9 cases mixed type of injury (35%). The severity of the DILI episode was mild for nine patients (35%), had a moderate character in more than half of the patients (54%) and 3 (12%) patients suffered a worst outcome, of whom two died and one underwent liver transplantation.

The biochemical profile at recognition of the ibuprofen DILI events was characterized by elevations in serum total bilirubin (TBL) with a mean value of 5.7 mg/dl. Serum aspartate aminotransferase (AST) and alanine aminotransferase (ALT) were increased in all but one patient, with a mean value of 14 xULN and 18 xULN, respectively. In contrast, the mean alkaline phosphatase (ALP) elevation was 2.3 xULN.

With respect to outcome, 11 patients (42%) achieved complete liver profile resolution within one year from DILI recognition. One case presented chronic features (4%) and three cases were fatal or required a liver transplant (12%). In two additional cases, laboratory tests were not available on a periodic basis; hence no conclusions regarding chronicity could be drawn. Another 9 cases did not have sufficient follow-up data to ascertain the long-term outcome.

A comparison of demographic, clinical and biochemical data between the 15 hepatocellular and 11 cholestatic/mixed ibuprofen cases did not reveal any statistically significant differences, with the exception of initial ALT elevation (Supplementary **Table S3.1**).

Table 3.1 Description of demographic and clinical characteristics in ibuprofen-induced liver injury cases

Patient	Gender	Age, y	Duration, treatment onset, d	Time to Total daily dose (mg), d	Co-morbid conditions	Concomitant drugs
1	F	21	5	15*	1200	None
2	M	43	37	25	1200	None
3	M	77	5	4	1200	Osteoarthritis, arthritis, COPD, peptic ulcer
4	M	57	31	51*	1200	diabetes mellitus, chronic hepatitis C
5	M	30	66	66	1800	None
6	M	75	3	3	400	COPD, pneumothorax
7	F	69	8	21	1200	Diabetes mellitus
8	M	43	8	8	1200	None
9	M	18	1	3	600	None
10	F	47	43	68	1600	None
11	F	41	3	3	600	None
12	F	64	17	11	600	Diabetes mellitus, hypertension
13	F	59	11	4	1800	osteoarthritis, peripheral vascular disease
14	F	44	12	8	1200	Depression, breast cancer
15	F	49	8	10	1800	None
16	F	57	31	31	1200	Goitre, diabetes mellitus
17	F	71	30	30	1800	Diabetes mellitus, dyslipidemia, hypertension
18	M	40	1826§	1767§	1200	None
19	F	60	27	27	1200	Obesity, depression
20	M	40	38	38	1800	Sclerosing cholangitis, ulcerative colitis, pancreatitis
21	M	56	16	15	1200	None
22	F	48	14	14	1200	Diabetes mellitus, rheumatoid arthritis
23	F	42	40	40	100**	Hypothyroidism, depression
24	M	62	12	11	1200	None
25	M	37	96	96	400	None
26	M	64	16	6	1800	Colorectal cancer, diabetes mellitus, hyperlipidemia, myocardial infarction

*Asymptomatic episode, time to onset is based on first detection in routine blood analysis. **Perifar, pediatric ibuprofen. M, male; F, female; d, days; y, years; COPD, chronic obstructive pulmonary disease; § on-demand intake

Table 3.2 Biochemical parameters at onset, liver damage pattern and disease evolution of ibuprofen-induced liver injury

Case	TBL mg/dl	AST xULN	ALT xULN	ALP xULN	Type of liver injury	Hospital admission	Severity	Time to resolution weeks	Additional information
1	0.9	1.7	5.4	1.0	Hep	No	Mild	13	-
2	2.8	2.1	2.3	1.0	Mix	Yes	Moderate	91 §	Jaundice
3	-	27	8.3	3.5	Mix	Yes	Moderate	Follow-up lost after 12 w	Jaundice, rash
4	1.0	7.9	24	0.9	Hep	N/A	Mild	Follow-up lost after 43 w	-
5	1.3	2.0	9.0	2.5	Mix	Yes	Moderate	21	Jaundice
6	4.2	8.4	8.3	2.9	Mix	No	Moderate	8	Jaundice
7	4.3	3.6	11	1.0	Hep	No	Moderate	8	Jaundice, eosinophilia
8	1.9	1.8	8.2	1.3	Hep	Yes	Moderate	44	Jaundice
9	7.9	1.8	5.5	2.6	Mix	Yes	Moderate	Follow-up lost after 3 w	Jaundice, lymphopenia
10	0.8	23	9.1	4.0	Mix	No	Mild	-	Eosinophilia
11	1.6	5.3	14	1.9	Hep	No	Mild	4	-
12	9.3	3.0	5.9	1.7	Mix	Yes	Moderate	Follow-up lost after 21 w	Jaundice, ASMA+
13	20	40	68	7.1	Hep	Yes	LTx	-	Jaundice, lymphopenia, ANA+
14	9.6	26	10	2.9	Mix	Yes	Fatal	-	Jaundice
15	4.8	6.8	11	1.9	Hep	Yes	Moderate	Follow-up lost after 1 w	Jaundice, eosinophilia
16	9.9	0.9	1.0	1.1	Chol	Yes	Moderate	Follow-up lost after 64 w	Jaundice
17	4.0	13	3.4	4.6	Mix	Yes	Moderate	75 §	Jaundice, lymphopenia
18	0.4	1.1	2.5	5.8	Chol	No	Mild	Follow-up 390 w	Eosinophilia, chronic
19	1.7	26	22	2.0	Hep	N/A	Mild	50	Eosinophilia, ANA+, ASMA+
20	4.9	14	32	4.1	Hep	Yes	Moderate	Follow-up lost after 4 w	Jaundice
21	6.5	23	42	1.5	Hep	Yes	Moderate	Follow-up lost after 8 w	Jaundice, ASMA+
22	-	4.4	9.0	1.4	Hep	No	Mild	2	Eosinophilia
23	1.7	13	28	1.1	Hep	No	Mild	8	Lymphopenia
24	5.1	50	58	1.4	Hep	Yes	Fatal	-	Jaundice, ASMA+
25	0.4	5.4	10	0.8	Hep	No	Mild	3	-
26	21	45	48	1.2	Hep	No	Moderate	25	Jaundice, ASMA+

Hep, hepatocellular; Chol, cholestatic; Mix, mixed; TBL, serum total bilirubin; AST, aspartate aminotransferase; ALT, alanine aminotransferase; ALP, alkaline phosphatase; ULN, upper limit of normal; w, weeks; N/A, not available; LTx, liver transplantation; ANA+, positive antinuclear antibody titers; ASMA+, positive anti-smooth muscle antibody titers. §Lack of regular blood analyses prevented chronicity determination

Comparison of the clinical and biochemical profile of ibuprofen DILI with those of DILI induced by other NSAIDs and non-NSAIDs

A comparison between cases of idiosyncratic DILI due to ibuprofen, other NSAIDs and non-NSAIDs is displayed in **Table 3.3**. No significant differences were observed between the three groups with regards to demographics. A slight decrease in female representation and age was, however, noted for the ibuprofen cases compared with other NSAID cases. In addition, the ibuprofen cases had a slightly higher BMI than the other two groups (28 vs. 26 vs. 26; $P=0.06$).

Table 3.3 Comparison of demographic, clinical and laboratory parameters in hepatotoxicity cases induced by ibuprofen, other nonsteroidal anti-inflammatory drugs (NSAIDs) and non-NSAID drugs

	Ibuprofen (N=26)	Other NSAIDs (N=76)	Non-NSAIDs (N=844)	<i>p</i> value
Demographical data				
Female, n (%)	13 (50)	43 (57)	434 (51)	0.7
Age, mean (range)	51 (18-77)	55 (16-82)	55 (11-90)	0.4
BMI (Kg/m ²), mean (range)	28 (23-36)	26 (20-36)	26 (16-59)	0.06
Clinical presentation, n (%)				
Jaundice	17 (65)	53 (71)	557 (67)	0.8
Hospital admission	14 (58)	37 (52)	443 (52)	0.9
Eosinophilia	6 (24)	13 (18)	200 (25)	0.4
Lymphopenia	4 (17)	13 (19)	151 (21)	0.8
Positive autoantibodies titers	6 (27)	17 (26)	166 (24)	0.9
Treatment duration, median d (range)	16 (1-1826)	30 (3-550)	29 (1-3724)	0.2
Time to onset, median d (range)	15 (3-1767)	27.5 (3-466)	27 (1-2313)	0.2
Underlying diseases, n (%)				
Hypertension	2 (10)	12 (20)	187 (29)	0.06
Diabetes mellitus	7 (27)	6 (8)	103 (12)	0.04
Type of liver injury, n (%)				
Hepatocellular	15 (58)	51 (67)	499 (61)	0.5
Cholestatic/mixed	11 (42)	25 (33)	319 (39)	
Severity, n (%)				
Mild	9 (35)	25 (33)	263 (31)	1.0
Moderate	14 (54)	40 (53)	464 (55)	
Severe	-	6 (8)	63 (7)	
Fatal / liver transplantation	2 / 1 (12)	2 / 2 (5)	19 / 13 (3)	
Biochemical parameters at onset, mean xULN (range)				
TBL (mg/dL)	5.7 (0.4-26)	7.3 (0.6-34)	7.0 (0.2-46)	0.8
AST	14 (0.9-50)	18 (1.3-134)	15 (0.2-180)	0.7
ALT	18 (1.0-68)	21 (1.2-98)	18 (0.3-203)	0.1
GGT	9.5 (0.5-43)	8.3 (0.3-47)	8.3 (0.2-79)	0.7
ALP	2.3 (0.8-7.1)	2.1 (0.2-8.1)	2.3 (0.3-22)	0.6
Cases fulfilling Hy's law criteria, n (%)	11 (42)	30 (39)	276 (33)	0.3
Outcome, n (%)				
Cases resolved in ≤ 1 year from onset, n (%)	11 (85) (n=13)	38 (88) (n=43)	358 (86) (n=418)	

The percentages shown were calculated based on the total number of episodes with available information. d, days; TBL, serum total bilirubin; AST, aspartate aminotransferase; ALT, alanine aminotransferase; GGT, gamma-glutamyl transferase; ALP, alkaline phosphatase; ULN, upper limit of normal

No significant differences were detected in any clinical presentation features. However, it is worth pointing out that treatment duration and time to onset were both shorter in the ibuprofen DILI patients compared with those induced by other NSAIDs or non-NSAIDs (16 vs. 30 vs. 29 and 15 vs. 27.5 vs. 27, respectively).

Concerning underlying diseases, the proportion of patients suffering from hypertension was lower for the ibuprofen DILI patients (10%) than for the DILI cohorts due to other NSAIDs (20%) and non-NSAIDs (29%) ($P=0.06$). In contrast, diabetes mellitus was significantly more prevalent in ibuprofen DILI (27%) compared with other NSAIDs (7%) and non-NSAID DILI patients (12%) ($P=0.04$). No parameters related to type of liver injury, severity, laboratory profile and outcome differed significantly between the three groups. However, the ibuprofen cases contained a higher proportion of fatal/liver transplant patients (12% vs. 5% vs. 3%).

Discussion

NSAIDs are important causative agents of idiosyncratic DILI; however, ibuprofen has traditionally been considered to have very limited hepatotoxicity potential. Our case series presented in this study is, to the best of our knowledge, the most extensive case series of ibuprofen-associated hepatotoxicity published to date. A noticeable difference in ibuprofen DILI frequency between different countries was first highlighted in 2010.²² In our analysis of NSAID-related hepatotoxicity events, we noticed that the Spanish DILI Registry presented a notably higher prevalence of ibuprofen-induced hepatotoxicity cases, accounting for 29% of all NSAID-associated DILI episodes. In comparison, 17% of the Latin-American, 6.7% of the North American and none of the Icelandic NSAID cases were attributed to ibuprofen.^{6,28} The proportion of diclofenac exceeded 30% of NSAID DILI in the latter three cohorts, while only reaching 18% in the Spanish cohort.

In Spain, NSAIDs are commercialized exclusively in pharmacies (prescription and OTC treatments) and the consumption can subsequently be calculated based on sales figures. During the period 2000 to 2012, the defined daily dose per 1000 inhabitants per year of diclofenac fluctuated between 8.2 and 6.4, while that of ibuprofen increased from 6.2 to 21.5.²⁹ Similar findings have been reported in the US, with approximately 7 million prescriptions for diclofenac- misoprostol or generic diclofenac and 24 million prescriptions for generic ibuprofen during 2007.²⁸ Hence the consumption of ibuprofen is more than three times higher than that of diclofenac in both Spain and the US. Nevertheless, the Spanish DILI Registry displays a higher incidence of ibuprofen hepatotoxicity cases than the US DILIN cohort.

The commercialized oral doses of ibuprofen available for OTC use in Spain are 200 and 400 mg, whereas 600 mg is only available under medical prescription. In the US, ibuprofen is only available at 200 mg for OTC use, and any higher dosage requires a medical prescription. The fact that ibuprofen consumers in Spain have access to higher OTC doses raises the question if the difference in ibuprofen DILI frequency seen between the Spanish and US DILI registries could be related to dosage. Individuals in Spain using nonprescription ibuprofen may be more prone to a higher intake of ibuprofen due to the availability of higher OTC dose formulation. Dosage is presumed to play a smaller role in idiosyncratic than in intrinsic DILI, as no overdose is required to instigate idiosyncratic hepatotoxicity. Nevertheless, it is believed that a threshold dose, which may vary between individuals, needs to be exceeded for idiosyncratic DILI to occur.³⁰ Thus, larger drug doses could increase drug biotransformation rates and trigger excess reactive metabolite formation.

Reactive metabolites can covalently bind to cellular proteins and alter their physiological functions as well as interfere with mitochondrial homeostasis. As a result, generation of oxidative stress can lead to cellular injury by activating cell death-related signaling pathways.³¹ In fact, increased expression of c-Jun N-terminal kinases (JNKs) has recently been demonstrated in liver tissue of DILI patients.³² Hence, one might hypothesize that the availability of OTC ibuprofen tablets at a higher dose is more likely to induce a cellular injury process that may lead to DILI in susceptible individuals.

A recent multicenter case-control study in Italy reported a higher adjusted risk of liver damage for ibuprofen than for diclofenac.³³ However, it should be pointed out that the DILI criteria followed in this study were less stringent than those proposed by Aithal *et al.* in 2011, due to the study commencing prior to 2011. Nevertheless, it is interesting to note that ibuprofen is also available as a 400 mg OTC formulation in Italy.

Our study did not reveal a characteristic drug signature for ibuprofen DILI, but rather a broad spectrum of phenotypic variations. Female prevalence has been demonstrated for NSAID hepatotoxicity in general.²⁸ In terms of ibuprofen DILI, males and females were equally distributed in the current study population, although there was a slight overrepresentation of females in the external ibuprofen case reports. Hence, our results do not support that ibuprofen DILI susceptibility is affected by gender. The majority of the external case reports demonstrated a latency period of less than four weeks. This applied to 65% of the cases in the current series. Two of our cases had very short latency (2 days) and it is possible that these cases could have had previous mild asymptomatic reactions to ibuprofen and that the recorded episodes were, in fact, reexposure episodes. Patient 9, in particular, admitted to having taken NSAIDs previously but was unable to specify the specific medications. The remaining cases required up to several months to develop DILI and likewise had an extended duration of treatment. It is possible that unknown host or environmental factors in conjunction with ibuprofen led to prolonged latency in these cases. Hence, ibuprofen DILI appears

to most commonly develop in the first month from treatment initiation, but may require more prolonged drug exposure to develop hepatotoxicity in some individuals.

In line with DILI in general, hepatocellular damage was the most common liver injury pattern in the study cohort. This was also corroborated by the external case reports. However, demographic and clinical features were comparable between the hepatocellular and cholestatic/mixed ibuprofen cases. The external case reports, on the other hand, presented an array of clinical features, which were not detected in the current study cohort. This, however, may, to some extent, be the result of publication bias as case reports with "unusual" features are generally more easily published.

We detected a trend towards diabetes mellitus and elevated BMI being more common in patients with ibuprofen DILI. Diabetes as a potential risk factor for DILI susceptibility lacks supportive evidence, although it has been associated with increased DILI severity.³⁴ However, none of the ibuprofen patients with diabetes in our case series developed severe DILI. Diabetes is often accompanied by weight gain, which may be why we detected a higher BMI in our ibuprofen DILI series. Data from large DILI cohorts worldwide, however, do not support that DILI patients have higher BMI than the general population. Obesity has subsequently not been linked to increased risk of DILI, although it appears to increase the risk of poor outcomes in patients with acute liver failure in general.³⁵ Nonetheless, few of the ibuprofen patients with BMI data available in the current cohort had BMI levels corresponding to obesity (>30). We also detected a lower prevalence of hypertension in ibuprofen cases. This is unlikely to be a biological characteristic of ibuprofen DILI, but rather the consequence of ibuprofen as well as many other NSAIDs being issued with a special warning in patients with hypertension, as they can attenuate the effects of antihypertensive agents. Furthermore, diabetic patients are recommended to use ibuprofen at the lowest recommended dose for a shorter duration to avoid further alteration of renal homeostasis.

Enhanced awareness of ibuprofen hepatotoxicity is of importance, considering that three (12%) of the cases in our case series had a fatal/liver transplantation outcome. The seriousness of ibuprofen DILI is also reflected in the external case reports, of which four describe liver failure resulting in death or liver transplantation.^{9,11,12,14} Females were more prevalent among the worst outcome cases in both the current case series and the external case reports, corroborating previous findings of increased risk of acute liver failure in females.^{23,26} Thus, patients with clinical suspicion of ibuprofen-induced liver injury should not be dismissed lightly but may require a closer follow-up of their clinical condition until liver profile normalization. A correct diagnosis and identification of the causative agent are also paramount in order to avoid inadvertent re-exposition, which can have fatal consequences, as seen for patient 24 in the current case series.

In conclusion, the risk of developing ibuprofen-induced liver injury is low, considering the extensive use of this drug worldwide. Ibuprofen hepatotoxicity should, however, not be overlooked as it can have life-threatening consequences. Variations in ibuprofen DILI frequency between different countries could potentially be related to differences in OTC dose formulations, pattern of drug use as well as racial genetic differences. While generally developing within four weeks from treatment initiation and presenting as hepatocellular type of liver damage, the characteristics of ibuprofen hepatotoxicity vary considerably between susceptible patients, reflecting the contribution of host and environmental factors in DILI presentation.

References

1. Paulose-Ram R, Hirsch R, Dillon C, et al. Prescription and non-prescription analgesic use among the US adult population: results from the third National Health and Nutrition Examination Survey (NHANES III). *Pharmacoepidemiol Drug Saf* 2003;12:315-326.
2. Blanca-López N, Cornejo-García JA, Plaza-Serón, et al. Hypersensitivity to nonsteroidal anti-inflammatory drugs in children and adolescents: crossintolerance reactions. *J Investig Allergol Clin Immunol* 2015;25:259-269.
3. Bessone F, Hernandez N, Roma MG, et al. Hepatotoxicity induced by coxibs: how concerned should we be? *Expert Opin Drug Saf* 2016;15:1463-1475.
4. Rubenstein JH, Laine L. Systemic review: the hepatotoxicity of non-steroidal anti-inflammatory drugs. *Aliment Pharmacol Ther* 2004;15:373-380.
5. Lucena MI, Andrade RJ, Kaplowitz N, et al. Phenotypic characterization of idiosyncratic drug-induced liver injury: the influence of age and sex. *Hepatology* 2009;49:2001-2009.
6. Björnsson ES, Bergmann OM, Björnsson HK, et al. Incidence, presentation, and outcomes in patients with drug-induced liver injury in the general population of Iceland. *Gastroenterology* 2013;144:1419-1425.
7. Chalasani N, Bonkovsky HL, Fontana R, et al. Features and outcomes of 899 patients with drug-induced liver injury: the DILIN prospective study. *Gastroenterology* 2015;148:1340-1352.
8. Ibuprofen. <https://livertox.nlm.nih.gov/Ibuprofen.htm>. Accessed March 13, 2017.
9. Bravo JF, Jacobson MP, Mertens BF. Fatty liver and pleural effusion with ibuprofen therapy. *Ann Intern Med* 1977;87:200-201.
10. Stempel DA, Miller JJ 3rd. Lymphopenia and hepatic toxicity with ibuprofen. *J Pediatr* 1977;90:657-658.
11. Alam I, Ferrell LD, Bass NM. Vanishing bile duct syndrome temporally associated with ibuprofen use. *Am J Gastroenterol* 1996;91:1626-1630.
12. Srivastava M, Perez-Atayde A, Jonas MM. Drug-associated acute-onset vanishing bile duct and Stevens-Johnson syndromes in a child. *Gastroenterology* 1998;115:743-746.
13. Riley TR 3rd, Smith JP. Ibuprofen-induced hepatotoxicity in patients with chronic hepatitis C: a case series. *Am J Gastroenterol* 1998;93:1563-5.
14. Rodríguez-Gonzalez JF, Montero JL, Puente J, et al. Orthotopic liver transplantation after subacute liver failure induced by therapeutic doses of ibuprofen. *Am J Gastroenterol* 2002;97:2476-2477.
15. Tyagi P, Sharma BC, Sarin SK. Cholestatic liver injury due to ibuprofen. *Indian J Gastroenterol* 2005;24:77-78.
16. Taghian M, Tran TA, Bresson-Hadni S, et al. Acute vanishing bile duct syndrome after ibuprofen therapy in a child. *J Pediatr* 2004;145:273-276.
17. Bennett WE, Turmelle YP, Shepherd RW. Ibuprofen-induced liver injury in an adolescent athlete. *Clin Pediatr (Phila)* 2009;48:84-86.

18. Nayudu SK, Kavuturu S, Niazi M, et al. A rare coexistence: drug induced hepatitis and meningitis in association with Ibuprofen. *J Clin Med Res* 2013;5:243-246.
19. Kim H, Yang HK, Kim SH, et al. Ibuprofen associated acute vanishing bile duct syndrome and toxic epidermal necrolysis in an infant. *Yonsei Med J* 2014;55:834-837.
20. Watanabe T, Abe M, Tada F et al. Drug-induced liver injury with serious multiform exudative erythema following the use of an over-the-counter medication containing ibuprofen. *Intern Med* 2015;54:395-399.
21. Chen M, Suzuki A, Borlak J, et al. Drug-induced liver injury: interactions between drug properties and host factors. *J Hepatol* 2015;63:503-514.
22. Suzuki A, Andrade RJ, Björnsson E, et al. Drugs associated with hepatotoxicity and their reporting frequency of liver adverse events in Vigibase: unified list based on international collaborative work. *Drug Saf* 2010;33:503-522.
23. Andrade RJ, Lucena MI, Fernandez MC, et al. Drug-induced liver injury: an analysis of 461 incidences submitted to the Spanish registry over a 10-year period. *Gastroenterology* 2005;129:512-521.
24. Benichou C. Criteria of drug-induced liver disorders. Report of an international consensus meeting. *J Hepatol* 1990;11:272-276.
25. Aithal GP, Watkins PB, Andrade RJ, et al. Case definition and phenotype standardization in drug-induced liver injury. *Clin Pharmacol Ther* 2011;89:806-815.
26. Robles-Díaz M, Lucena MI, Kaplowitz N, et al. Use of Hy's law and a new composite algorithm to predict acute liver failure in patients with drug-induced liver injury. *Gastroenterology* 2014;147:109-118.
27. Medina-Caliz I, Robles-Díaz M, García-Munoz B, et al. Definition and risk factors for chronicity following acute idiosyncratic drug-induced liver injury. *J Hepatol* 2016;65:532-542.
28. Schmeltzer PA, Kosinski AS, Kleiner DE, et al. Liver injury from nonsteroidal anti-inflammatory drugs in the United States. *Liver Int* 2016;36:603-609.
29. Utilización de medicamentos antiinflamatorios no esteroides (AINE) en España durante el periodo 2000-2012. Agencia española de medicamentos y productos sanitarios <https://www.aemps.gob.es/medicamentosUsoHumano/observatorio/docs/AINE.pdf>. Accessed March 9, 2017.
30. Carrasacosa MF, Salcines-Cviedes JR, Lucena MI, et al. Acute liver failure following atorvastatin dose escalation: is there a threshold dose for idiosyncratic hepatotoxicity? *J Hepatol* 2015;62:751-752.
31. Han D, Dara L, Win S, et al. Regulation of drug-induced liver injury by signal transduction pathways: critical role of mitochondria. *Trends Pharmacol Sci* 2013;34:243-253.
32. Cubero FJ, Zoubek ME, Hu W, et al. Combined activities of JNK1 and JNK2 in hepatocytes protect against toxic liver injury. *Gastroenterology* 2016;150:968-981.
33. Donati M, Conforti A, Capuano A, et al. Risk of acute and serious liver injury associated to nimesulide and other NSAIDs: data from drug-induced liver injury case-control study in Italy. *Br J Clin Pharmacol* 2016;82:238-248.
34. Chalasani N, Fontana RJ, Bonkovsky HL, et al. Causes, clinical features, and outcomes from a prospective study of drug-induced liver injury in the United States. *Gastroenterology* 2008;135:1924-1934.
35. Rutherford A, Davern T, Hay JE, et al. Influence of high body mass index on outcome in acute liver failure. *Clin Gastroenterol Hepatol* 2006;4:1544-1549.

Supplementary material

Table S3.1 Comparison of demographic, clinical and laboratory parameters in 26 Spanish ibuprofen-induced liver injury cases according to type of liver injury

	Hepatocellular (n=15)	Cholestatic/Mixed (n=11)	P value
Demographics			
Age, mean (range)	50 (21-69)	51 (18-77)	0.80
Female gender, n (%)	8 (53)	5 (45)	1.0
Clinical presentation, n (%)			
Jaundice	9 (60)	9 (82)	0.68
Hospitalization	6 (46)	8 (73)	0.13
Daily dose, median mg (range)	1200 (100-1800)	1200 (400-1800)	0.88
Treatment duration, median d (range)	14 (3-96)	30 (1-1826)	0.33
Time to onset, median d (range)	15 (3-96)	25 (2-1828)	0.34
Underlying diseases, n (%)			
Hypertension	1 (9.1)	1 (10)	1.0
Diabetes mellitus	4 (27)	3 (27)	1.0
Severity, n (%)			
Mild-moderate	13 (87)	10 (91)	0.62
Fatal / liver transplantation	2 (13)	1 (9)	
Laboratory parameters, mean xULN (range)*			
TBL, mean mg/dL (range)	5.5 (0.4-21)	5.0 (0.4-9.9)	0.85
AST	16 (1.7-50)	9.8 (0.9-26)	0.25
ALT	26 (5.4-68)	6.0 (1.0-10)	0.0015
ALP	1.9 (0.8-7.1)	2.9 (1-5.8)	0.11
Outcome, n (%)			
Cases resolved in ≤1 year from onset, n (%)**	9 (100)	2 (50)	0.08

*First available blood analysis after DILI recognition. **Based on available information for 9 hepatocellular cases and 4 cholestatic/mixed cases. Abbreviations: d, days; TBL, serum total bilirubin; AST, aspartate aminotransferase; ALT, alanine aminotransferase; ALP, alkaline phosphatase; ULN, upper limit of normal

Chapter 4

Protective role of c-Jun N-terminal kinase-2 (JNK2) in Ibuprofen-induced acute liver injury

Zoubek ME*, Woitok MM*, Sydor S, Nelson LJ, Bechmann LP, Lucena MI, Andrade RJ,
Bast A, Koek GH, Trautwein C, Cubero FJ

***J Pathol* 2019;247(1):110-122**

DOI: 10.1002/path.5174

*Both authors contributed equally to this work

Abstract

Background & aims

Ibuprofen is a frequently used non-steroidal anti-inflammatory drug (NSAID). In general, it is well-tolerated, but it may cause serious liver injury ranging from a mild elevation of serum aminotransferases to acute liver failure (ALF) requiring liver transplantation. Here, we aimed to unveil the molecular pathways involved in triggering ibuprofen-induced acute liver injury (ALI), which, at present, remain elusive.

Methods

First, we investigated the activation of essential pathways in human liver sections of Ibuprofen-induced ALI. Next, we assessed the cytotoxicity of Ibuprofen *in vitro* and developed a novel murine model of ibuprofen hepatotoxicity. Overnight fasted male C57BL/6 mice (6-8 weeks of age) were injected *i.p.* with either vehicle or ibuprofen [600 mg/kg] for 8h. To assess the role of the c-Jun N-terminal kinase (JNK), we used animals carrying a constitutive deletion of Jnk1 (Jnk1^{-/-}) or Jnk2 (Jnk2^{-/-}) expression and included investigations using animals with hepatocyte-specific Jnk deletion either genetically (Jnk1^{Δhepa}) or by siRNA (siJnk2^{Δhepa}).

Results

In human and murine samples of ibuprofen-induced ALI, JNK phosphorylation was increased in the cytoplasm of hepatocytes and other non-LPCs compared with healthy tissue. In mice, Ibuprofen-induced hepatotoxicity resulted in a significantly stronger degree of liver injury compared with vehicle-treated controls, as evidenced by transaminases, macroscopic as well as microscopic examination of the livers. Next, we investigated the molecular pathways associated with Ibuprofen-induced liver toxicity. PKCα, AKT and JNK were significantly increased 8h after Ibuprofen administration. Constitutive Jnk1^{-/-} and Jnk2^{-/-} deficient mice exhibited increased liver dysfunction compared to wildtype (WT) animals. Furthermore, siJnk2^{Δhepa} animals showed a dramatic increase in liver parameters during ibuprofen-induced ALI, which correlated with significantly higher serum liver enzymes and worsened liver histology, and MAPK activation compared to Jnk1^{Δhepa} or WT animals.

Conclusions

In our study, cytoplasmic JNK activation in hepatocytes and other non-LPCs is a hallmark of human and murine ibuprofen-induced ALI. Ibuprofen activates PKCα, AKT and JNK in mice. Functional *in vivo* analysis demonstrated a pivotal role of hepatocyte-specific JNK2 in mediating protection during Ibuprofen-dependent ALI, pointing towards JNK2 as a potential therapeutic target.

Keywords

Ibuprofen, hepatotoxicity, DILI, ALI, Jnk2

Introduction

Acute liver failure (ALF) is a rare but dramatic condition associated with poor prognosis in patients without underlying liver disease.¹ In particular, drugs, herbals and dietary supplements can trigger liver dysfunction and acute liver failure (ALF), also known as drug-induced liver injury (DILI).² The histopathology of DILI is characterized by hepatocellular, cholestatic or mixed type of liver injury and its clinical severity ranges from mild to severe and can occasionally be even fatal.³ The incidence rate of DILI-ALF in the US has been determined in 1.61 events per million person-years.⁴

Despite the fact that acetaminophen (APAP) is the most frequent aetiological agent in DILI-ALF, many other analgesic compounds within the nonsteroidal anti-inflammatory drugs (NSAIDs) are estimated to be responsible for approximately 3-10% of all DILI in industrialized countries.⁵⁻⁸ Moreover, 25% of all NSAID-induced liver injury episodes require hospitalization.⁹

Ibuprofen is one of the most frequently used NSAIDs with over 20 million prescriptions in the US per year, apart from its vast non-prescription use. Although considered a safe drug and clinically apparent liver injury due to ibuprofen consumption is uncommon, it has been repeatedly described in the literature with a variable spectrum, ranging from mild serum liver enzyme alterations to ALF and even death.¹⁰ The increasing consumption of ibuprofen worldwide, together with the undesirable effects linked to its hepatotoxicity, urges the need to understand the pathophysiology underlying ibuprofen-induced liver injury.

Here we aimed to investigate and characterize ibuprofen-induced acute liver injury (ALI) *in vitro* and *in vivo* using constitutive and conditional c-Jun N-terminal kinase (Jnk) knockout mice and cell-targeted siRNA-deletion. Our results show that hepatocyte-derived Jnk2 is an essential determinant in ibuprofen-induced hepatotoxicity. This novel molecular mechanism might contribute to developing new efficient drugs for the treatment of ibuprofen DILI-ALI.

Materials and methods

Experiments with animals and human samples

Animal experiments were carried out according to German and Spanish legal requirements and animal protection law and approved by the authority of environment conservation and consumer protection of North Rhine-Westphalia and the Regional Government of Madrid (AZ 84-02.04.2016.A080 and PROEX 516/2016), respectively. Albumin-Cre (Alb-Cre), Jnk1 and Jnk2-deficient mice in a C57BL/6 background were purchased from The Jackson Laboratory (Bar Harbor, ME, USA). Mice with a floxed allele of Jnk1 were constructed by using homologous recombination in ES cells and backcrossed to the C57BL/6J strain, as previously

described.¹¹ Hepatocyte-specific Jnk1 mice were constructed by crossing Jnk1^{fl/fl} with Alb-Cre mice.

Induction of ALI was performed in 7-8 week-old age-matched male mice (n=6-8 per group) using Ibuprofen hydrochloride with 98% purity (Sigma-Aldrich, Munich, Germany) or vehicle. Ibuprofen was dissolved in phosphate-buffered PBS enriched with 8.7% DMSO due to its challenging hydrosolubility (molecular weight of Ibuprofen hydrochloride: 206.28 g/mol). After overnight fasting, mice were injected *i.p.* with Ibuprofen [600 mg/kg] and sacrificed 8h later. Liver paraffin sections were obtained from patients with a confirmed diagnosis of Ibuprofen-associated DILI from the Department of Gastroenterology of the University Hospital Essen, Germany.

Hepa 1-6 cell line and cell viability assay

Hepa 1-6 (1x10⁵ cells/well) cells were obtained from the American Tissue Culture Collection (ATCC, Manassas, VA, USA), grown in DMEM (Gibco BRL, Grand Island, NY, USA) supplemented with 10% fetal bovine serum (FBS) and 1% penicillin/streptomycin. Hepa1-6 cells were cultured in a quantity of 500.000 cells/well, on 6 well 15.5 ml/9.6 cm² culture plates (Falcon, Corning Inc., Corning, NY, USA). After 12h of stabilization period, Hepa 1-6 were challenged to either medium, vehicle or [0.5 to 20 mM] Ibuprofen for 0-48h.

Cell viability assay was performed using a cell survival determination kit MTT based (Sigma-Aldrich, St. Louis, MO, USA). Cells were incubated with MTT solution for 4h and the resulting formazan crystals dissolved with MTT solvent solution for one additional hour. Absorbance was measured spectrophotometrically at a wavelength of 570 nm and background measured at 690 nm was subtracted.

HepaRGs

HepaRG cells (HRG116 terminally differentiated cells, BioPredic international, Rennes, France) were cultured (following the suppliers' protocols) on Corning plates and cultured to confluence at 37°C 5% CO₂ for seven days. Cells were then challenged to either vehicle or [2.5 to 10 mM] Ibuprofen for 8h.

Isolation and culture of primary hepatocytes

Primary mouse hepatocytes were isolated from 7-8 week-old mice by collagenase perfusion. Living hepatocytes were plated on collagen-precoated Petri dishes at a density of 1.5x10⁴/cm² in supplemented DMEM medium and after 4h incubation (37°C, 5% CO₂) medium was renewed. Twelve hours later, hepatocytes were cultured overnight and treated with either medium, medium with vehicle or medium with [5.0 mM] Ibuprofen for 8h.

Jnk2 siRNA (siJnk2)

In order to construct mice with hepatocyte-specific Jnk2 downregulation, a total dose of 0.5 mg/kg of Jnk2 siRNA (Axolabs GmbH, Kulmbach, Germany) was dissolved in 0.9% NaCl (Braun, Germany) and injected *i.v.* one week before Ibuprofen challenge. In parallel, scrambled controls (siScr) against siRNA of Jnk2 were performed.

Immunohistochemistry staining

Liver tissue slides were stained for Ki-67, Cleaved Caspase 3, p-JNK (Cell Signaling, Danvers, MA), RIPK1 (Pro-Sci, Poway, CA, USA) and CYP2C9 (Abcam plc, Cambridge, UK) on paraffin sections, was performed using a Leica automatic stainer (Wetzlar, Germany). All immunohistostained sections were analyzed, documented and quantified on 5, 10 or 20 low-power fields per slide (scale bars 100 μ m) using Axiovision software (Carl Zeiss, Jena, Germany).

Immunofluorescence

Liver tissue cryosections or living cell-cultured coverslips were fixed in 4% PFA for 10 min and mounted with Vectashield containing DAPI (Linaris, Wertheim, Germany).

For Ki-67, samples were rinsed in PBS-Tween for 10 min after fixation and blocked with 10% goat serum for 1h. Incubation with primary antibody Ki-67 (Leica Biosystems Inc., Leider Lane, IL, USA) was performed at 4°C overnight. The day after, samples were incubated with appropriate fluorescence-labeled secondary antibodies (AlexaFluor 488 and 564, Invitrogen, Oregon, USA) for 1h at room temperature.

For Tunel Assay, after samples had been fixed for 30 min, nuclei were permeabilized with sodium-carbonate 150 mM. Later, *in situ* cell death detection kit (Roche, Mannheim, Germany) was applied and incubated at 4°C overnight. For the detection of cellular oxygen species, DCFDA reagent (Invitrogen, Molecular Probes, Eugene, OR, USA) was applied on sections or coverslips after fixation and heated up to 37 °C for 30 min.

For mitochondrial superoxide detection, Mitosox reagent (Invitrogen, Molecular Probes, Eugene, OR, USA) was applied and incubated for 10 min at 37°C protected from light. All sections and coverslips were analyzed microscopically and documented using an Imager Z1 fluorescence microscope and Axiovision software (Carl Zeiss, Jena, Germany).

Immunoblot analysis

Isolated protein samples were probed with antibodies for cleaved caspase-3, cleaved caspase-8, caspase-8, pJNK, Jnk1, Jnk2, SAPK/JNK, SAPK/MAPK, pERK1/2, ERK1/2, pAMPK, AMPK, pAKT, AKT, LC3A and pP65 (Cell Signaling Inc., Danvers, MA, USA); COX2, Cyp2E1 and Cyp2C9 (Abcam plc, Cambridge, UK); RIPK1, RIPK3 (Pro-Sci, Poway, CA, USA); PKC α (Santa Cruz Inc., Dallas, TX, USA), GAPDH (AbD SeroTec, Düsseldorf, Germany). As secondary antibodies, anti-rabbit HRP (Cell Signaling) and antimouse HRP (Santa Cruz) were used.

GSH measurement and TNF α ELISA

GSH levels of preserved liver homogenates were determined enzymatically and quantified spectrophotometrically at 412 nm using PBS, pH 7.4. GSH reductase was purchased from Sigma-Aldrich, St. Louis, MO, USA.

For determination of hepatic TNF α levels, protein isolation of liver tissue was performed, as above mentioned, and processed following the guidelines of Mouse TNF α determination kit (MTA00B) from RD systems (Minneapolis, MN, USA). The multi-well plate containing the samples was measured spectrophotometrically at wavelengths 540 nm and 450 nm for quantification. Subsequently, readings at 540 nm were subtracted from the readings at 450 nm to correct optical imperfections in the plate.

The methodology for performance and validation of tissue preparation, H&E staining, immunoblot-analysis, quantitative real-time PCRs and serum parameter measurement has been detailed elsewhere.¹¹

Statistical analysis

All experimental data are expressed as mean and error bars are shown as SEM. Statistical significance was determined by two-way analysis of variance (ANOVA) followed by a Student's *t*-test or one-way ANOVA followed by a Newman-Keuls multi comparison test. *P* values less than 0.05 were considered to be significant (GraphPad Software Inc., San Diego, CA, USA).

Results

Dose finding experiments for Ibuprofen-induced cytotoxicity *in vitro*

Cellular cytotoxicity of Ibuprofen was tested *in vitro* in a murine cell line, Hepa 1-6, in freshly isolated murine hepatocytes (PMH) and a human cell line, HepaRGs. Cells were treated with increasing concentrations of Ibuprofen [2.5-10.0 mM] for up to 48h. Higher doses of Ibuprofen caused cytoplasmic retractions, cell shrinkage and cell death in PMH, Hepa 1-6 and HepaRGs correlating with AST, a biochemical marker of hepatocyte injury (**Figure 4.1A+B**; Supplementary **Figure S4.1A**).

Eight hours after exposure to high Ibuprofen concentrations resulted in the loss of cell-cell contact and detachment. In contrast, polyhedral and rounded shaped hepatocytes were found in control and vehicle-exposed cultures (**Figure 4.1A+B**; Supplementary **Figure S4.1A**).

For a treatment concentration of 5.0 mM Ibuprofen, the LC₅₀ was reached after 8h. Moreover, TUNEL staining exhibited significantly decreased cell survival, cell proliferation and increased presence of reactive oxygen species¹² in Ibuprofen-treated compared to medium or vehicle-treated hepatocytes (Supplementary **Figure S4.1B-E**).

Altogether these results showed that 5.0 mM Ibuprofen induces cytotoxicity and thus is a reasonable dose for *in vitro* mechanistic studies of Ibuprofen-mediated acute liver injury.

Correlating the *in vitro* findings in an experimental model of Ibuprofen-induced acute toxicity

Next, we investigated if we could confirm our *in vitro* findings in an experimental Ibuprofen-induced ALI model to study hepatic histopathology and markers of injury associated with Ibuprofen intoxication. In agreement with toxic studies published by the Council of Europe in 2008¹³, we calculated an LD₅₀ for Ibuprofen of 600 mg/kg.

Overnight fasted mice were either treated with [600 mg/kg] Ibuprofen or vehicle and sacrificed after 8h. Vehicle treated mice exhibited normal transaminases, whereas Ibuprofen triggered significantly increased AST, ALT and GLDH levels (**Figure 4.1C**). No signs of cholestasis were observed in these mice (not shown).

Histological liver analysis of Ibuprofen-treated animals showed acute toxicity characterized by hemorrhage and areas with focal lesions, including hepatocyte dropouts compared with vehicle-treated controls (**Figure 4.1D**). These results show that Ibuprofen can potentially cause severe hepatotoxicity, besides its anti-inflammatory properties.

Characterization of Ibuprofen-induced toxicity *in vivo*

Ibuprofen is mostly metabolized in the liver by cytochrome-P450 isoenzyme 2C9 (CYP2C9).¹⁴ CYP2C9 expression was enhanced in the liver parenchyma of Ibuprofen-treated mice in areas close to the central vein (**Figure 4.2A**). Additionally, we evaluated CYP2E1 protein expression and found a slightly stronger expression after Ibuprofen treatment (not shown).

Saberi *et al.*¹⁵ demonstrated that PKC α plays a fundamental role in acetaminophen (APAP)-mediated liver injury, where PKC α inhibitors provided a hepatoprotective effect. Thus we next sought to identify whether Ibuprofen toxicity affected upstream mitogen-activated protein kinase (MAPK) targets.

Increased protein expression of PKC α was found in wildtype animals challenged with Ibuprofen (**Figure 4.2B**). Moreover, we found strong activation of ERK1/2 and AKT in these animals, confirming MAPKs activation in Ibuprofen-derived ALI (**Figure 4.2B**).

Since it has been suggested that RIPK1 and 3 form a complex which has been shown to translocate to the mitochondria to mediate necroptosis during ALF^{16,17} we studied the protein expression of RIPK1/3 during Ibuprofen-mediated hepatotoxicity. Concomitant with APAP-liver injury, Ibuprofen triggered RIPK1 but not RIPK3 expression (**Figure 4.2B**).

Figure 4.1 **Ibuprofen-induced cytotoxicity assessment *in vitro*. Hepatic injury profile of acute Ibuprofen-derived intoxication *in vivo*.** (A) A total of 500.000 primary murine hepatocytes (PMHs) freshly isolated from 6-8 week-old WT mice, Hepa 1-6 cells and human HepaRGs cells were seeded in 6-well plates and cultivated for up to 12h. Then, cells were treated with either vehicle or Ibuprofen concentrations of 2.5, 5.0 and 10.0 mM for 8h. Vehicle corresponded to the highest drug concentration in the absence of active substance. The cell culture medium of PMHs and HepaRGs was collected 8h after challenge, and AST levels were determined and represented as U/L. (B) Visible light microphotographs were taken for PMHs and HepaRGs at an 8h time point after treatment. Scale bars 100 μ m. (C) 6-8 week-old WT fasted overnight and the following day *i.p.* injected with either vehicle or Ibuprofen 600 mg/kg b.w. and 8h later sacrificed. Serum AST (left), ALT (middle) and GLDH (right) levels were determined 8h after ibuprofen challenge (n=10). (D) Representative H&E staining of liver sections collected from mice sacrificed 8h after Ibuprofen challenge. Scale bars: 50 μ m (left) and 100 μ m (right), respectively.

Figure 4.2 Assessment of signal transduction pathways in livers of WT mice underlying Ibuprofen-intoxication. Role of JNK in human and murine Ibuprofen-induced liver injury. Wildtype mice were challenged with 600 mg/kg Ibuprofen and sacrifice, 8h later. (A) Representative immunostaining of cytochrome-P450 subisoform 2C9 expression was evaluated per immunohistochemistry of liver sections. (B) Protein expression levels for PKCα, pAKT, pERK1/2, RIPK1 and RIPK3 were determined by immunoblotting of whole liver extracts from vehicle-treated and Ibuprofen-injected mice. (C) Protein levels of total and phosphorylated fractions of JNK were determined by immunoblotting. (D) Representative staining for JNK activation by immunohistochemistry in liver samples of human and murine Ibuprofen-induced liver injury. Scale bars: 50μm (upper panels) and 100μm (lower panels), respectively.

JNK is involved in Ibuprofen-mediated hepatotoxicity

Since JNK activation is downstream of RIPK1 and given that JNK signaling mediates toxic liver injury,¹⁸ we next investigated JNK activation during Ibuprofen-induced hepatotoxicity. We first analyzed JNK protein expression in murine Ibuprofen-ALI samples by Western blot. Increased expression of activated JNK was evident 8h after Ibuprofen challenge (**Figure 4.2C**). Furthermore, JNK phosphorylation was evaluated using IHC in murine liver tissue, showing a strong JNK activation pattern in hepatic parenchyma of Ibuprofen-treated mice (**Figure 4.2D**, top panel).

Using human liver tissue samples from Ibuprofen DILI patients, we also found strong JNK phosphorylation. Positive staining concentrated mainly in the cytoplasm of hepatocytes and other non-parenchymal cells, compared with healthy livers (**Figure 4.2D**, bottom panel).

To validate the pivotal effect of JNK in Ibuprofen-mediated liver injury, we blocked JNK activation after Ibuprofen challenge using a specific inhibitor. Mice of 6-8 weeks of age were injected *i.p.* with the JNK inhibitor SP600125 and challenged 2h later with Ibuprofen for 8h (**Figure 4.3**). Interestingly, mice pre-treated with SP600125 revealed a protective effect of mice pre-treated with SP600125 prior to Ibuprofen challenge, evidencing normal hepatic architecture (**Figure 4.3A**). These results were further validated by decreased JNK activation after SP600125 administration compared with Ibuprofen-treated mice (**Figure 4.3B**).

Since Ibuprofen-induced liver injury was associated with PKC α , MAPK and RIPK1 activation, we investigated the effect of SP600125 pre-administration in Ibuprofen-ALI. Protein expression of PKC α , ERK1/2, AKT and RIPK1 was strongly decreased after JNK inhibition (**Figure 4.3C**). Altogether, these data indicate that JNK plays a critical role in Ibuprofen-induced liver injury *in vivo*.

Role of *Jnk1* and *Jnk2* during Ibuprofen-induced liver injury

Interestingly, JNK expression was essential in mediating murine Ibuprofen-induced ALI. Hence, we used mice with global *Jnk1* or *Jnk2* deletion to address each gene's relevance for inducing acute liver injury (Supplementary **Figure S4.2A**).

Both, *Jnk1*^{-/-} and *Jnk2*^{-/-} knockout mice exhibited significantly increased AST, ALT and GLDH in serum compared with WT animals (**Figure 4.4A**). Noticeably, GLDH was significantly high in *Jnk2* mice, indicating exacerbated mitochondrial damage (**Figure 4.4A**).

The histopathological examination of livers confirmed exacerbation of liver injury in Ibuprofen-treated *Jnk1*^{-/-} and *Jnk2*^{-/-} mice, displaying areas of necrosis and hemorrhage (**Figure 4.4B**). Interestingly, protein expression analysis showed strongly induced levels of PKC α , pAKT, RIPK1 and RIPK3 and in *Jnk2*^{-/-} mice, less evident in *Jnk1*^{-/-} mice, indicative of more significant damage (**Figure 4.4C**). However, activation of ERK1/2 was absent in *Jnk2*^{-/-} compared with *Jnk1*^{-/-} mice. Interestingly, phosphorylation of JNK still occurred in *Jnk2* knockout mice, a phenomenon very likely indicating more significant damage in these mice (Supplementary **Figure S4.2B**).

Figure 4.3 Pre-treatment with the JNK inhibitor SP600125, protects against Ibuprofen-derived toxicity. (A) Overnight fasted 6-8 weeks aged WT mice were pre-treated with the inhibitor SP600125 and after 2h, challenged with Ibuprofen 600 mg/kg. (A) Representative H&E of liver sections obtained from mice sacrificed 8h after Ibuprofen challenge. Scale bars: 50 μ m (upper panels) and 100 μ m (lower panels), respectively. (B) Immunoblotting for pJNK and total JNK was performed in liver samples from mice treated either with vehicle or Ibuprofen (n=6). GAPDH was used as loading control. (C) Protein levels of PKC α , pAKT, pERK1/2 and RIPK1 were assessed with Western-blot analysis. GAPDH was used as the loading control.

Figure 4.4 Characterization of hepatic damage in mice carrying genetic-constitutive deletions of JNK1 (Jnk1^{-/-}) or JNK2 (Jnk2^{-/-}) after acute Ibuprofen-induced intoxication. (A) Overnight fasted animals with either JNK1 or JNK2 constitutive deletion, and controls (WT), were challenged to 600 mg/kg Ibuprofen and sacrificed after 8h. Serum AST (left), ALT (center) and GLDH (right) are graphically displayed as U/L. (B) Representative H&E-stained liver sections collected from animals sacrificed 8h after Ibuprofen injection. Scale bars: 50 μm (upper panels) and 100 μm (lower panels), respectively. (C) Immunoblot analysis for PKCα, pAKT, pERK1/2 and RIPK1 was done from whole liver extracts of control (WT), Jnk1^{-/-} and Jnk2^{-/-} knockout mice treated either with vehicle or Ibuprofen (n=8). GAPDH was used as loading control.

Hepatocyte-specific deletion of Jnk2 exacerbates murine Ibuprofen-induced DILI-ALI

Since upstream and downstream MAPK signaling pathways were upregulated in Jnk2 constitutive knockout mice, we next sought to investigate the specific role of Jnk2 in hepatocytes in the setting of Ibuprofen-derived DILI. We first generated mice with a specific deletion of Jnk1 in hepatocytes (Jnk1^{Δhepa}). In parallel, we blocked Jnk2 expression in hepatocytes using a siRNA against Jnk2 (siJNK2^{Δhepa}) that specifically targets hepatocytes, as previously described¹⁹ (Supplementary **Figure S4.2C**).

Mice were injected in the tail vein siJnk2 one week before the experimental Ibuprofen challenge. Scrambled controls for siJNK2^{Δhepa}-treated mice were also used to exclude any deleterious injection effect (Supplementary **Figure S4.3A+B**). Interestingly, mice bearing downregulation of Jnk2 in hepatocytes were critically hypersensitized towards the Ibuprofen challenge and presented a significantly lower survival rate than WT or Jnk1^{Δhepa} mice (**Figure 4.5A**).

Our results showed that animals treated with siJnk2^{Δhepa} exhibited a dramatic increase in liver damage after challenge with Ibuprofen compared to WT and Jnk1^{Δhepa} animals, whereas Jnk1^{Δhepa} animals presented similar hepatic injury than WT mice (**Figure 4.5B**). Histopathological examination of livers evidenced centrilobular damage in Jnk1^{Δhepa} and exacerbated injury with the presence of necrotic foci in siJnk2^{Δhepa} Ibuprofen-treated mice (**Figure 4.5C**).

Next, we explored molecular pathways triggering the Ibuprofen injury and linked to the JNK signaling pathway. Interestingly, increased protein levels of PKC-α and pAKT after Ibuprofen treatment were observed in siJnk2^{Δhepa} mice compared to controls. However, no differences were observed in RIPK1/3 expression, suggesting that necrotic cell death after Ibuprofen is independent of the individual effect of JNK in hepatocytes (**Figure 4.5D**).

We next performed TUNEL staining in liver tissue, which revealed increased cell death in Ibuprofen-challenged siJnk2^{Δhepa} mice, compared to WT or Jnk1^{Δhepa}. Noticeably, no significant differences were observed in compensatory proliferation between WT, Jnk1^{Δhepa} and siJnk2^{Δhepa} treated with either vehicle or Ibuprofen (Supplementary **Figure S4.4A-C**). Next, the liver's oxidative response after Ibuprofen administration was evaluated with particular focus on the Jnk1 and Jnk2 function in hepatocytes. Hepatic GSH levels, naturally depleted after intense toxin-mediated acute liver injury, were reduced in Ibuprofen-challenged WT, Jnk1^{Δhepa} and siJnk2^{Δhepa} mice (**Figure 4.5E**).

Altogether, these data show that JNK2 in hepatocytes modulates the activation of AKT, which might be pivotal for the mechanism of Ibuprofen-driven injury.

Figure 4.5 Impact of hepatocyte-specific depletion of JNK1 (Jnk1^{Δhepa}) or JNK2 (siJnk2^{Δhepa}) in the response towards acute ibuprofen-derived liver toxicity. (A) Animals with genetic JNK1 deletion were fasted overnight and *i.p.* injected with 600 mg/kg Ibuprofen versus vehicle and sacrificed 8h later. To create mice with JNK2 depletion specifically in hepatocytes, WT animals were treated with *i.v.* 0.5 mg/kg of Jnk2 siRNA, specific against hepatocytes, one week before Ibuprofen challenge. The survival curve is displayed graphically, enclosing a follow-up of 8h after Ibuprofen injection. (B) Serum AST (left), ALT (center) and GLDH (right) are shown as U/L. (C) Representative H&E stainings of hepatic sections from Jnk1^{Δhepa} and siJnk2^{Δhepa} knock-out mice, sacrificed 8h after Ibuprofen challenge. Scale bars: 100 μm. (D) Expression of PKCα, pAKT, pERK1/2 and RIPK1 analyzed per immunoblotting of whole liver

extracts from control (WT), Jnk1^{Δhepa} and siJnk2^{Δhepa} mice, 8h after undergoing treatment with vehicle or Ibuprofen (n=8). GAPDH served as loading control. (E) Hepatic GSH levels were determined in whole liver homogenates from control (WT), Jnk1^{Δhepa} and siJnk2^{Δhepa} mice and measured as nmol/g of liver tissue.

Discussion

Ibuprofen is a powerful painkiller and is also used as an anti-inflammatory drug in patients with acute and chronic inflammation. At present, only limited information is available on Ibuprofen-induced hepatotoxicity, while the use of the drug is widespread. Several large hepatotoxicity patient cohorts from the US⁵ and Iceland⁸ registered a low number of Ibuprofen DILI events, which accounted for 7% and none of all identified NSAID-derived hepatotoxicity cases, respectively. Nevertheless, prospective DILI databases from Spain and Latin-America indicated a rise in the prevalence of Ibuprofen hepatotoxicity, with 29% and 17% of all NSAID incidents, respectively, which were attributed to Ibuprofen use.²⁰

There is little information on the clinical features of Ibuprofen-associated hepatotoxicity and previous patient reports show a variable range of phenotypes in Ibuprofen-derived liver toxicity rather than a distinctive signature.²⁰⁻³⁵ Whereas hepatic compromise has also been described in the setting of Ibuprofen overdose,^{21,22} the majority of earlier published Ibuprofen-induced liver injury reports corresponded to hepatic adverse reactions of idiosyncratic nature.^{20,23-35} A recent analysis of Ibuprofen-induced liver injury based on the included data in the Spanish and Latin-American DILI registries evidenced that over half of the patients required hospitalization and the rate of liver transplants and liver-related deaths presented critically a higher trend in comparison to other NSAIDs- or non-NSAID-DILI cases, albeit not significant.²⁰ Hence, Ibuprofen-induced liver injury can occasionally lead to dramatic clinical conditions and may require liver transplant or have a fatal progression.

Hepatocellular liver damage has classically been the most described liver injury pattern associated with NSAID-induced injury.^{7,36,37} However, the type of liver injury associated with Ibuprofen-derived hepatotoxicity seems to be heterogenic, where hepatocellular, cholestatic as well as mixed forms have been described.^{20,25,26,32} In our study, we first studied Ibuprofen cytotoxicity *in vitro* to further replicate Ibuprofen toxicity in a novel murine model. Interestingly, the hepatocellular injury was accompanied by extensive hemorrhage resembling the pattern of liver damage, as also observed in the setting of acute Ibuprofen intoxication in humans.

Drugs and other xenobiotic substances are extensively metabolized in the liver, aiming to transform these into inert compounds for safe excretion. However, biotransformations can act as a double-edged sword and lead to the formation of chemically reactive metabolites, which can result in hazardous and deplete defensive antioxidant responses, such as the

glutathione system (GSH). The most crucial CYP450 enzyme for Ibuprofen metabolism is CYP2C9, which catalyzes the formation of 3-hydroxy-Ibuprofen and 2-hydroxy-Ibuprofen, whilst metabolic pathways involving CYP2C8 and CYP2C19 have been attributed only a minor role.¹⁴ In the present work, we analyzed CYP2C9 activity in samples of murine Ibuprofen-DILI and observed an enhanced expression of CYP2C9 in the liver parenchyma, characteristically, in regions close to central veins. The isoform CYP2E1 plays a crucial role in developing APAP-induced hepatotoxicity and has also been linked to DILI induced by other drugs.³⁸⁻⁴¹ Interestingly, we also found slightly increased levels of CYP2E1 after Ibuprofen-DILI-ALI (not shown), which might be due to synergism between different isoenzymes.

Generation of oxidative stress is a hallmark of DILI, which triggers the activation of cell death effector pathways.¹¹ As evaluated *in vitro* in Hepa 1-6 cells and murine primary hepatocytes and *in vivo*, each condition overexposed to Ibuprofen (5.0 mM) for 8h evidenced not only enhanced cell death but also oxidative stress. This observation likely is the consequence of forming reactive substances after exposing hepatocytes to high concentrations of Ibuprofen, which then promotes the activation of pro-inflammatory and cell death cascades.

JNKs act as a crucial trigger of hepatotoxicity. In addition, we recently described that JNK signaling plays an essential role during APAP-induced hepatotoxicity and correlates with the degree of liver injury.¹¹ These observations have been validated in primary human hepatocytes using a JNK inhibitor that ameliorated APAP-induced toxicity.⁴² Moreover, JNK phosphorylation is not limited to hepatocytes but also relevant in non-parenchymal cells.^{11,12,43} Concomitantly, Ibuprofen-associated DILI in humans livers showed increased JNK activation mainly in the cytoplasm of hepatocytes and in other non-LPCs compared to healthy tissue. Thus, our data in human and murine livers evidenced JNK activation during Ibuprofen-induced DILI.

Pre-treatment with the JNK-inhibitor SP600125 prior to Ibuprofen intoxication decreased JNK phosphorylation, which resulted in a hepatoprotective effect and significantly reduced the activation of other MAPK-related pathways. Next, the function of either Jnk1 or Jnk2 for acute Ibuprofen-induced liver injury was evaluated. Constitutive deletion of Jnk1 or Jnk2 dramatically enhanced Ibuprofen-mediated DILI-ALI. Several studies reported an indifferent role of Jnk1 in acute APAP-induced hepatotoxicity.^{44,45} In our model, Jnk2^{-/-} deficient mice showed more severe liver injury as evidenced by histopathological features and a stronger dysregulation of PKC α , pAKT and pERK1/2 -as well as RIPK1/3-associated signaling. In this line, several studies demonstrated higher susceptibility of Jnk2^{-/-} deficient mice towards APAP, TNF and LPS-induced liver injury.^{45,46} In contrast, other studies described a protective hepatic profile in mice with ubiquitous deletion of Jnk2 after acute liver injury with TNF and APAP.^{44,47} Thus, the role of Jnk2 in different forms of toxin-mediated ALI remains controversial.

To characterize the cell-specific function of JNK, we used mice with genetic (Jnk1 ^{Δ hepa}) or siRNA-specific (siJnk2) deletion in hepatocytes.¹⁹ Interestingly, our results revealed a critical

role and prominent aggravation of Ibuprofen DILI-ALI in animals with hepatocyte-specific Jnk2 downregulation compared to Jnk1^{Δhepa} or WT mice. Exacerbation of liver damage correlated with JNK hyperactivation in these mice. Hence, our data not only provided evidence that JNK1 and JNK2 have different functions in hepatocytes and other LPCs but also demonstrated an essential protective function of JNK2 in hepatocytes during Ibuprofen-induced ALI.

In the past, PKC family members have been shown to play prominent roles during APAP-induced hepatocellular injury.¹⁵ Selective silencing of PKCα decreased JNK phosphorylation, ameliorated mitochondrial dysfunction, and reduced cytotoxicity in hepatocytes, suggesting a JNK-dependent role in APAP-induced hepatotoxicity. Indeed, in our Ibuprofen DILI-ALI model, pre-administration of SP600125 reverted JNK activation and correlated with a dramatic reduction in hepatic PKCα expression and the degree of liver injury. Therefore, besides the essential function of the JNK genes, a close crosstalk between the PKC pathway and other signaling cascades seems to be relevant to better understand the pathogenesis of Ibuprofen-induced acute liver injury. Here especially other MAPKs, as AKT or ERK1/2 are of interest after Jnk2 depletion, the response to Ibuprofen intoxication dramatically worsened and displayed a preferential hyperactivation for hepatic AKT.

RIPK1/3 may interact to form a complex, the necroptosome, translocating to mitochondria where it incites mitochondrial dysfunction and leads ultimately to cell death.^{17,18} Jnk2^{-/-} mice displayed increased RIPK1/3 levels after Ibuprofen treatment. Conversely, constitutive Jnk1-deficient animals exhibited merely minimal differences in RIPK1/3 expression. Intriguingly, animals with siRNA-induced downregulation of Jnk2, despite exhibiting a higher degree of injury, did not reflect these substantial differences in RIPK1/3 regulation, indicating that alternative pathways may exert a more critical impact on hepatocyte-related cell death after Ibuprofen intoxication.

In conclusion, in our present study, we investigated the molecular mechanism of human Ibuprofen-induced liver injury and complemented these findings with an experimental approach of Ibuprofen-induced ALI *in vitro* and *in vivo*. Human and murine Ibuprofen DILI-ALI showed increased JNK activation, which correlated with the degree of liver injury. Specifically, Jnk2 in hepatocytes has an essential protective role during Ibuprofen-induced DILI-ALI, defining it as a potential therapeutic target.

References

1. Bernal W, Wendon J. Acute liver failure. *N Engl J Med* 2013;369:2525-34.
2. Lee WM. Drug-induced hepatotoxicity. *N Engl J Med* 2003;349:474-85.
3. Aithal GP, Watkins PB, Andrade RJ, et al. Case definition and phenotype standardization in drug-induced liver injury. *Clin Pharmacol Ther* 2011;89:806-15.

4. Goldberg DS, Forde KA, Carbonari DM, et al. Population-representative incidence of drug-induced acute liver failure based on an analysis of an integrated health care system. *Gastroenterology* 2015;148:1353-61 e3.
5. Schmeltzer PA, Kosinski AS, Kleiner DE, et al. Liver injury from nonsteroidal anti-inflammatory drugs in the United States. *Liver Int* 2016;36:603-9.
6. Larson AM, Polson J, Fontana RJ, et al. Acetaminophen-induced acute liver failure: results of a United States multicenter, prospective study. *Hepatology* 2005;42:1364-72.
7. Andrade RJ, Lucena MI, Fernandez MC, et al. Drug-induced liver injury: an analysis of 461 incidences submitted to the Spanish registry over a 10-year period. *Gastroenterology* 2005;129:512-21.
8. Bjornsson ES, Bergmann OM, Bjornsson HK, et al. Incidence, presentation, and outcomes in patients with drug-induced liver injury in the general population of Iceland. *Gastroenterology* 2013;144:1419-25, 1425 e1-3; quiz e19-20.
9. Bloom BS. Direct medical costs of disease and gastrointestinal side effects during treatment for arthritis. *Am J Med* 1988;84:20-4.
10. Nanau RM, Neuman MG. Ibuprofen-induced hypersensitivity syndrome. *Transl Res* 2010;155:275-93.
11. Cubero FJ, Zoubek ME, Hu W, et al. Combined Activities of JNK1 and JNK2 in Hepatocytes Protect Against Toxic Liver Injury. *Gastroenterology* 2016;150:968-81.
12. Zhao G, Hatting M, Nevzorova YA, et al. Jnk1 in murine hepatic stellate cells is a crucial mediator of liver fibrogenesis. *Gut* 2014;63:1159-72.
13. Europe Co. Active ingredients used in cosmetics: safety survey. Council of Europe Publishing 2008;Strasbourg.
14. Mazaleuskaya LL, Theken KN, Gong L, et al. PharmGKB summary: ibuprofen pathways. *Pharmacogenet Genomics* 2015;25:96-106.
15. Saberi B, Ybanez MD, Johnson HS, et al. Protein kinase C (PKC) participates in acetaminophen hepatotoxicity through c-jun-N-terminal kinase (JNK)-dependent and - independent signaling pathways. *Hepatology* 2014;59:1543-1554.
16. Ramachandran A, McGill MR, Xie Y, et al. Receptor interacting protein kinase 3 is a critical early mediator of acetaminophen-induced hepatocyte necrosis in mice. *Hepatology* 2013;58:2099-108.
17. Dara L, Johnson H, Suda J, et al. Receptor interacting protein kinase 1 mediates murine acetaminophen toxicity independent of the necrosome and not through necroptosis. *Hepatology* 2015;62:1847-57.
18. Seki E, Brenner DA, Karin M. A liver full of JNK: signaling in regulation of cell function and disease pathogenesis, and clinical approaches. *Gastroenterology* 2012;143:307-20.
19. Speicher T, Siegenthaler B, Bogorad RL, et al. Knockdown and knockout of beta1- integrin in hepatocytes impairs liver regeneration through inhibition of growth factor signalling. *Nat Commun* 2014;5:3862.
20. Zoubek ME, Gonzalez-Jimenez A, Medina-Caliz I, et al. High Prevalence of Ibuprofen Drug-induced Liver Injury in Spanish and Latin-American Registries. *Clin Gastroenterol Hepatol* 2017.
21. Laurent S, Rahier J, Geubel AP, et al. Subfulminant hepatitis requiring liver transplantation following ibuprofen overdose. *Liver* 2000;20:93-4.
22. Lee CY, Finkler A. Acute intoxication due to ibuprofen overdose. *Arch Pathol Lab Med* 1986;110:747-9.
23. Alam I, Ferrell LD, Bass NM. Vanishing bile duct syndrome temporally associated with ibuprofen use. *Am J Gastroenterol* 1996;91:1626-30.
24. Srivastava M, Perez-Atayde A, Jonas MM. Drug-associated acute-onset vanishing bile duct and Stevens-Johnson syndromes in a child. *Gastroenterology* 1998;115:743-6.
25. Taghian M, Tran TA, Bresson-Hadni S, et al. Acute vanishing bile duct syndrome after ibuprofen therapy in a child. *J Pediatr* 2004;145:273-6.
26. Javier Rodriguez-Gonzalez F, Montero JL, Puente J, et al. Orthotopic liver transplantation after subacute liver failure induced by therapeutic doses of ibuprofen. *Am J Gastroenterol* 2002;97:2476-7.
27. Bravo JF, Jacobson MP, Mertens BF. Fatty liver and pleural effusion with ibuprofen therapy. *Ann Intern Med* 1977;87:200-1.
28. Stempel DA, Miller JJ, 3rd. Lymphopenia and hepatic toxicity with ibuprofen. *J Pediatr* 1977;90:657-8.
29. Sternlieb P, Robinson RM. Stevens-Johnson syndrome plus toxic hepatitis due to ibuprofen. *N Y State J Med* 1978;78:1239-43.
30. Riley TR, 3rd, Smith JP. Ibuprofen-induced hepatotoxicity in patients with chronic hepatitis C: a case series. *Am J Gastroenterol* 1998;93:1563-5.
31. Borel I, Hedelius F, Baumgartner C, et al. [Severe acute hepatitis associated with ibuprofen treatment]. *Gastroenterol Clin Biol* 2001;25:430-2.

32. Elkrief L, Chrysostalis A, Moachon L, et al. [Severe cholestatic hepatitis associated with Stevens-Johnson syndrome after taking ibuprofen]. *Gastroenterol Clin Biol* 2007;31:1043-5.
33. Bennett WE, Jr., Turmelle YP, Shepherd RW. Ibuprofen-induced liver injury in an adolescent athlete. *Clin Pediatr (Phila)* 2009;48:84-6.
34. Nayudu SK, Kavuturu S, Niazi M, et al. A rare coexistence: drug induced hepatitis and meningitis in association with Ibuprofen. *J Clin Med Res* 2013;5:243-6.
35. Watanabe T, Abe M, Tada F, et al. Drug-induced liver injury with serious multiform exudative erythema following the use of an over-the-counter medication containing ibuprofen. *Intern Med* 2015;54:395-9.
36. Lucena MI, Andrade RJ, Kaplowitz N, et al. Phenotypic characterization of idiosyncratic drug-induced liver injury: the influence of age and sex. *Hepatology* 2009;49:2001-9.
37. Chalasani N, Fontana RJ, Bonkovsky HL, et al. Causes, clinical features, and outcomes from a prospective study of drug-induced liver injury in the United States. *Gastroenterology* 2008;135:1924-34, 1934 e1-4.
38. Meyers LL, Beierschmitt WP, Khairallah EA, et al. Acetaminophen-induced inhibition of hepatic mitochondrial respiration in mice. *Toxicol Appl Pharmacol* 1988;93:378-87.
39. Ramsay RR, Rashed MS, Nelson SD. In vitro effects of acetaminophen metabolites and analogs on the respiration of mouse liver mitochondria. *Arch Biochem Biophys* 1989;273:449-57.
40. Huang YS, Chern HD, Su WJ, et al. Cytochrome P450 2E1 genotype and the susceptibility to antituberculosis drug-induced hepatitis. *Hepatology* 2003;37:924-30.
41. Restrepo JG, Garcia-Martin E, Martinez C, et al. Polymorphic drug metabolism in anaesthesia. *Curr Drug Metab* 2009;10:236-46.
42. Xie Y, McGill MR, Dorko K, et al. Mechanisms of acetaminophen-induced cell death in primary human hepatocytes. *Toxicol Appl Pharmacol* 2014;279:266-74.
43. Kluwe J, Pradere JP, Gwak GY, et al. Modulation of hepatic fibrosis by c-Jun-N- terminal kinase inhibition. *Gastroenterology* 2010;138:347-59.
44. Gunawan BK, Liu ZX, Han D, et al. c-Jun N-terminal kinase plays a major role in murine acetaminophen hepatotoxicity. *Gastroenterology* 2006;131:165-78.
45. Bourdi M, Korrapati MC, Chakraborty M, et al. Protective role of c-Jun N-terminal kinase 2 in acetaminophen-induced liver injury. *Biochem Biophys Res Commun* 2008;374:6-10.
46. Cederbaum AI, Yang L, Wang X, et al. CYP2E1 Sensitizes the Liver to LPS- and TNF alpha-Induced Toxicity via Elevated Oxidative and Nitrosative Stress and Activation of ASK-1 and JNK Mitogen-Activated Kinases. *Int J Hepatol* 2012;2012:582790.
47. Wang Y, Singh R, Lefkowitz JH, et al. Tumor necrosis factor-induced toxic liver injury results from JNK2-dependent activation of caspase-8 and the mitochondrial death pathway. *J Biol Chem* 2006;281:15258-67.

Supplementary material

Figure S4.1 Cell death, proliferation and mitochondrial dysfunction in a hepatoma cell line after Ibuprofen challenge. (A) Up to 500,000 Hepa 1-6 cells line were seeded in 6-well plates. After 12h of culture, Hepa 1-6 were exposed to either vehicle or Ibuprofen concentrations 2.5, 5.0 and 10.0 mM for 8h. Microphotographs at visible light were done 8h after challenge. (B) Representative TUNEL stainings of Hepa 1-6. Dead cells are stained green; total cells were counter-stained with DAPI (blue). Scale bars: 100 μm. (C) Representative staining for Ki-67 was performed on Hepa 1-6. Proliferating cells

appear in purple and cells were counterstained to DAPI (blue). Scale bars: 100 μm . (D) Representative Mitosox stainings of Hepa 1-6 challenged with Ibuprofen. Cells presenting mitochondrial damage are stained red; total cells were counter-stained with DAPI (blue). Scale bars: 100 μm . (E) TUNEL (left), Ki-67 (center) and Mitosox-positive cells (right) were counted per viewfield and graphed.

Figure S4.2 (A) PCR blots of tail DNA from *Jnk1 Δ hepa* (upper panel), *Jnk1 $^{-/-}$* (central panel) and *Jnk2 $^{-/-}$* (lower panel) for control (WT), *Jnk1 Δ hepa*, *Jnk1 $^{-/-}$* and *Jnk2 $^{-/-}$* mice demonstrated and confirmed the respective phenotype of interest. (B) Protein expression for total levels of JNK1, JNK2, phosphorylated JNK (pJNK) and total JNK were determined by immunoblotting of whole liver extracts from control (WT),

Jnk1^{-/-} and Jnk2^{-/-} mice, 8h after either vehicle or Ibuprofen 600 mg/kg injection. (C) Protein levels of JNK1, JNK2, pJNK and JNK resulting from whole livers of control (WT), Jnk1^{Δhepa} or siJnk2^{Δhepa} mice challenged to vehicle or Ibuprofen 600 mg/kg during 8h were assessed per Western-blot analysis.

Figure S4.3 (A) Overnight fasted 6-8 week old WT, siJnk2^{Δhepa} and scrambled control animals against siRNA of Jnk2 (siScr) were *i.p.* injected with either vehicle or Ibuprofen 600 mg/kg and 8h later sacrificed. Serum AST are graphed as U/L. (B) Serum ALT levels were determined in represented on a graph (U/L). (C) Representative H&E staining of liver sections collected 8h after Ibuprofen challenge. Scale bars: 50 μm (upper panels) and 100 μm (lower panels), respectively.

Figure S4.4 Hepatic response to cell death and proliferation in control (WT), Jnk1 Δ hepa and siJnk2 Δ hepa mice after acute ibuprofen-induced toxicity. (A) Illustrative TUNEL stainings in WT, Jnk1 Δ hepa and siJnk2 Δ hepa animals. Dead cells are stained green; total cells were counter-stained with DAPI (blue). Scale bars: upper panels 50 μ m, lower panels 100 μ m. Fraction of TUNEL-positive cells per viewfield was calculated and represented graphically. (B) Representative stainings for Ki-67 were performed. Positive cells were proliferative (purple) and cells were counterstained to DAPI (blue). Scale bars: upper panels 50 μ m, lower panels 100 μ m. (C) TUNEL (left) and Ki-67-positive cells (right) per viewfield were counted and graphed.

Chapter 5

Combined activities of JNK1 and JNK2 in hepatocytes protect against toxic liver injury

Cubero FJ, Zoubek ME, Hu W, Peng J, Zhao G, Nevzorova YA, Al Masaoudi M, Bechmann LP, Boekschoten MV, Muller M, Preisinger C, Gassler N, Canbay AE, Luedde T, Davis RJ, Liedtke C, Trautwein C

***Gastroenterology* 2016;150:968–981**

DOI: 10.1053/j.gastro.2015.12.019

Abstract

Background & aims

c-Jun N-terminal kinase (JNK) 1 and JNK2 are expressed in hepatocytes and have overlapping and distinct functions. JNK proteins are activated via phosphorylation in response to acetaminophen- or carbon tetrachloride (CCl₄)-induced liver damage; the level of activation correlates with the degree of injury. SP600125, a JNK inhibitor, has been reported to block acetaminophen-induced liver injury. We investigated the role of JNK in drug-induced liver injury (DILI) in liver tissue from patients and in mice with genetic deletion of JNK in hepatocytes.

Methods

We studied liver sections from patients with DILI (due to acetaminophen, phenprocoumon, nonsteroidal antiinflammatory drugs or autoimmune hepatitis) or patients without acute liver failure (controls) collected from a DILI Biobank in Germany. Levels of total and activated (phosphorylated) JNK were measured by immunohistochemistry and Western blotting. Mice with hepatocyte-specific deletion of *Jnk1* (*Jnk1^{Δhepa}*) or combination of *Jnk1* and *Jnk2* (*Jnk^{Δhepa}*), as well as *Jnk1*-floxed C57BL/6 (control) mice, were given injections of CCl₄ (to induce fibrosis) or acetaminophen (to induce toxic liver injury). We performed gene expression microarray and phosphoproteomic analyses to determine mechanisms of JNK activity in hepatocytes.

Results

Liver samples from DILI patients contained more activated JNK, predominantly in nuclei of hepatocytes and in immune cells, than healthy tissue. Administration of acetaminophen to *Jnk1^{Δhepa}* mice produced a greater level of liver injury than that observed in *Jnk1^{Δhepa}* or control mice, based on levels of serum markers and microscopic and histologic analysis of liver tissue. Administration of CCl₄ also induced stronger hepatic injury in *Jnk1^{Δhepa}* mice, based on increased inflammation, cell proliferation, and fibrosis progression, compared with *Jnk1^{Δhepa}* or control mice. Hepatocytes from *Jnk1^{Δhepa}* mice given acetaminophen had an increased oxidative stress response, leading to decreased activation of adenosine monophosphate-activated protein kinase, total protein adenosine monophosphate-activated protein kinase levels, and pJUN and subsequent necrosis. Administration of SP600125 before or with acetaminophen protected *Jnk1^{Δhepa}* and control mice from liver injury.

Conclusions

In hepatocytes, JNK1 and JNK2 appear to have combined effects in protecting mice from CCl₄- and acetaminophen-induced liver injury. It is crucial to study the tissue-specific functions of both proteins, rather than just JNK1, in the onset of toxic liver injury. JNK inhibition with SP600125 shows off-target effects.

Keywords

APAP; Mouse Model; Gene Regulation; Pharmacologic Treatment.

Introduction

Acute and chronic liver injury is a growing worldwide problem, despite recent advances for the treatment of hepatitis B virus and hepatitis C virus infection. The frequency of toxic insults, such as alcohol, drugs, or obesity, is increasing even in the Western world. In the liver, toxic injury triggers death signaling pathways, which can cause apoptosis, necrosis, or pyroptosis of hepatocytes.^{1,2} However, the exact pathomolecular mechanisms determining the mode of cell death are not entirely understood.

Liver injury of different etiology activates c-Jun N-terminal kinase (JNK), members of the mitogen-activated protein kinase (MAPK) family. Whereas *Jnk3* is exclusively expressed in the central nervous system, testis, and heart, *Jnk1* and *Jnk2* are expressed in hepatocytes eliciting redundant but also distinct functions.³⁻⁵ In order to characterize the compound functions of the JNK genes, for example, in hepatocytes, cell type-specific deletion of both *Jnk1* and *Jnk2* is essential. At present, most studies have been performed only using single knockout mice or JNK-specific inhibitors.

Toxic liver injury—acute or chronic—activates the oxidative stress response. Typical examples are acute liver damage after acetaminophen (APAP) intoxication or chronic liver injury by repetitive carbon tetrachloride (CCl₄) injection. APAP-induced injury is related to the formation of highly reactive metabolites through cytochrome P450, isoform 2E1. These toxic compounds usually are conjugated and inactivated by glutathione. In overdose conditions, the conjugation of the reactive metabolites leads to glutathione depletion and thus enhances the generation of oxidative (reactive oxygen species) and nitrosative (reactive nitrogen species) species triggering hepatocyte injury.⁶ *N*-acetylcysteine has been the standard antidote for APAP-induced liver intoxication.⁷ *N*-acetylcysteine exerts its therapeutic effects by restoring depleted hepatic glutathione levels and preventing the accumulation of oxidant species.⁷

Earlier results demonstrated that JNK is strongly activated by APAP, correlating with the degree of liver injury.⁸ Additionally, *in vivo* experiments evidenced that JNK inhibition blocked APAP-induced liver injury.⁹ Thus, JNK seems to play an essential role in APAP-induced hepatic damage, supporting the possibility of using JNK inhibitors as a therapeutic approach.

*Kluwe et al.*¹⁰ first suggested that JNK is crucial for chronic CCl₄ intoxication associated with hepatocyte damage, necrosis, inflammation, and end-stage liver fibrosis. JNK activation is not restricted to hepatocytes only. We found that *Jnk1* is particularly crucial for transdifferentiation of hepatic stellate cells because *Jnk1* deletion in hepatic stellate cells reduces fibrogenesis after chronic CCl₄ intoxication.¹¹

In the present work, we aimed to address specifically the as yet unexplored dual role of *Jnk1* and *Jnk2* in hepatocytes in models of acute and chronic toxic liver injury in mice and in patients with drug-induced liver injury (DILI). Based on previous studies, we hypothesized that JNK1 and JNK2 in hepatocytes have redundant functions. For this purpose, we

generated *Jnk^{Δhepa}* mice and studied the functional role of the JNK genes in APAP- and CCl₄-induced toxic liver injury *in vivo* and in culture using primary hepatocytes.

Materials and methods

Generation of mice, animal experiments and human samples

Alb-Cre and *Jnk2*-deficient mice in a C57BL/6 background were purchased from the Jackson laboratory (Bar Harbor, ME). Mice with a *floxed* allele of *Jnk1* were constructed by using homologous recombination in embryonic stem cells and backcrossed to the C57BL/6J strain as described previously.^{12,13} These mice were then crossed with *Jnk2*-deficient mice to create *Jnk1^{LoxP/LoxP} /Jnk2^{-/-}* mice.

Genomic DNA was examined using polymerase chain reaction amplimers (5'-CTCAGGAAGAAAGGGCTTATTC-3' and 5'-GAACCACTGTTCCAATTCCATCC-3') to distinguish between the control floxed (*Jnk1⁺*, *Jnk1^f*) and deleted (*Jnk1^Δ*) alleles.

Animal experiments were carried out according to the German legal requirements and animal protection law and approved by the authority for environment conservation and consumer protection of the state of North Rhine-Westphalia (LANUV, Germany). Induction of liver fibrosis was performed in 7- to 8-week-old age-matched male mice (n=9-10 per group) using CCl₄ injection every 3 days for 4 weeks (0.6 ml/kg intraperitoneal [IP]). Control animals were injected with corn oil. The D-galactosamine (D-GalN)/lipopolysaccharide (LPS) administration was performed in 7- to 8-week-old male control and *Jnk^{Δhepa}* male animals to induce acute hepatitis. Thirty minutes before the LPS injection (20 μg/kg, intraperitoneal [IP]), GalN (800 mg/kg, IP) was injected. Mice were sacrificed 8 hours after LPS injection. We also performed the APAP-induced liver injury model. After fasting overnight, mice were injected with APAP (500 mg/kg) and sacrificed 8 hours after treatment. SP600125 was injected 2 hours (pretreatment) or at the same time with APAP (co-treatment) at a dose of 30 mg/kg IP).

Liver paraffin sections were obtained from patients with a confirmed diagnosis of DILI (paracetamol, phenprocoumon, nonsteroidal inflammatory drugs, or autoimmune hepatitis) from the Department of Gastroenterology of the University Hospital Essen, Germany. Patient's clinicopathologic characteristics were analyzed, summarized, and represented in Supplementary **Table S5.1**.

Statistical analysis

All data are expressed as mean ± standard error of the mean. Statistical significance was determined by 2-way analysis of variance followed by a Student's *t*-test or by one-way analysis of variance followed by a Newman-Keuls multicomparison test. *P* values <.05 were considered to be significant.

Results

Expression of JNK in human acute liver failure

DILI is the most common cause of acute liver failure.¹⁴ First, we studied serum parameters of patients with different ALF etiologies. As indicated in Supplementary **Table S5.1**, livers from patients suffering from paracetamol (patient 1), phenprocoumon (patient 2), nonsteroidal anti-inflammatory drugs (patient 3), and autoimmune hepatitis-induced ALF (patient 4) were investigated. We observed that the most prominent increase in transaminases was evident in patients with APAP- and autoimmune hepatitis-induced ALF, while nonsteroidal anti-inflammatory drug and phenprocoumon-induced ALF patients showed less pronounced changes in serum markers. However, all serum samples showed impaired liver function, as evidenced by changes in bilirubin and blood coagulation parameters (Supplementary **Table S5.1**). Noticeably, the patient with APAP intoxication showed dramatically increased glutamate dehydrogenase levels and blood coagulation parameters (Supplementary **Table S5.1**).

We next investigated the JNK activation pattern in these ALF liver samples (patient 1-4) and normal healthy tissue as control (C1-C4) by performing pJNK staining and quantification (**Figure 5.1A**, Supplementary **Figure S5.1A**). Absence or minimal activation of JNK was detectable in healthy tissue. Liver histology of the APAP patient in comparison with the other liver samples showed lower infiltration of immune cells, and JNK phosphorylation mainly restricted to hepatocytes (**Figure 5.1A**). In contrast, liver samples obtained from other ALF subtypes displayed intense immune cell infiltration associated with pJNK positivity (**Figure 5.1A**). In the liver, 2 JNKs are expressed, JNK1 and JNK2. We performed Western blot analysis of liver samples of ALF patients with anti-JNK1 and JNK2 antibodies (**Figure 5.1B**). Normal tissue displayed mild JNK phosphorylation, and ALF patients displayed higher JNK activation.

Interestingly, we detected a differential pattern of JNK1 expression and increased levels of JNK2 in DILI-ALF patients (**Figure 5.1B**).

Deletion of JNK1 and JNK2 in hepatocytes

Next, we aimed to characterize the functional relevance of JNK activation during toxic liver injury. We generated mice with specific deletion of *Jnk1* in hepatocytes (*Jnk1*^{Δhepa}) and combined *Jnk1* and *Jnk2* knockout mice in hepatocytes (*Jnk*^{Δhepa}) (**Figure 5.1C**; Supplementary **Figure S5.1B** and **S5.1C**). To exclude the possibility that *Jnk*^{Δhepa} mice exhibited a phenotype under basal conditions, we carefully examined 6- to 8-week-old female and male *Jnk1*^{Δhepa} and *Jnk*^{Δhepa} mice. Liver histology, serum parameters as well as body weight, and liver vs. body weight ratio presented values of normal healthy mice (Supplementary **Figure S5.2A-S5.2F**).

Figure 5.1 Expression of JNK during human and murine DILI. (A) Activation of pJNK in liver samples of ALF patients with different etiologies: normal liver tissue (C1, C2), paracetamol (1), phenprocoumon (2), autoimmune hepatitis (AIH) (3), and nonsteroidal antiinflammatory drug (NSAID) (4). Scale bars =50 μ m. Phospho-JNK positive nuclei were quantified as the mean number of hepatocytes or infiltrating cells with dark brown 3,3'-diaminobenzidine tetra hydrochloridesignal per view field of liver sections from healthy and DILI patients. (B) Immunoblotting for pJNK, JNK1, and JNK2 was performed in normal liver tissue (C1-C4) and in liver samples of DILI patients (1–4). (C) Protein expression of JNK1 and JNK2 was assessed in primary hepatocytes (left panel) and livers (right panel) from 8 week-old control (*Wt*), *Jnk2*^{-/-}, *Jnk1* ^{Δ hepa}, and *Jnk* ^{Δ hepa} mice. Total JNK1 and JNK2 protein levels were determined in whole liver extracts. Glyceraldehyde-3-phosphate dehydrogenase (GAPDH) was used as loading control. (D) Immunoblotting for pJNK, JNK1, and JNK2 was performed in liver samples from control (*Wt*), *Jnk1* ^{Δ hepa} and *Jnk* ^{Δ hepa} mice treated with APAP for 8 hours (n=6). GAPDH was used as loading control.

Because the human data demonstrated that JNK is strongly activated mainly in hepatocytes during APAP-induced ALF, we sought to translate and compare the human ALF results with APAP-derived liver injury in mice. Eight hours after injecting 500 mg/kg APAP, livers of *Wt* mice elicited JNK activation.

Noticeably, mice with specific deletion of *Jnk1* in hepatocytes (*Jnk1* ^{Δ hepa}) displayed reduced pJNK, whereas *Jnk* ^{Δ hepa} mice showed strong JNK1 induction, suggesting that the infiltrating compartment is responsible for the activation of the MAPK in experimental murine APAP-derived injury (**Figure 5.1D**). In addition, we detected JNK1 and JNK2 protein expression after APAP in *Wt*, while the levels of JNK1 were strongly induced in *Jnk* ^{Δ hepa} mice. Expectedly, JNK2 expression was abrogated in mice lacking JNK in hepatocytes.

Jnk ^{Δ hepa} mice are sensitized towards acetaminophen-induced liver injury

Jnk ^{Δ hepa} showed significantly exacerbated APAP-induced ALF, as evidenced by significantly increased aspartate aminotransferase, alanine aminotransferase, and glutamate dehydrogenase levels (**Figure 5.2A**). No differences were found in the liver vs. body weight ratio (Supplementary **Figure S5.3A**). Macroscopically, the surface of *Jnk* ^{Δ hepa} livers showed severe signs of hemorrhagic bleeding, and, microscopically, the liver was severely injured with the presence of large necrotic foci (**Figure 5.2B-D**, respectively).

Acetaminophen is metabolized via cytochrome P450, isoform 2E1 to the electrophilic reactive product N-acetyl-*p*-benzoquinone imine.¹⁵ Interestingly, we detected a tendency toward F2-isoprostanes — a direct marker of oxidative stress¹⁶ — and significantly reduced levels of p38 expression in JNK-depleted hepatocytes after APAP treatment (**Figure 5.2E**, Supplementary **Figure S5.3** and **S5.3C**).

Figure 5.2 *Jnk^{Δhepa}* mice are sensitized towards acetaminophen-induced liver injury. (A) Serum alanine aminotransferase (left), aspartate aminotransferase (center), and glutamate dehydrogenase (right) levels were determined 8 hours after APAP challenge in control (Wt), *Jnk1^{Δhepa}*, and *Jnk^{Δhepa}* livers (n=10). (B) Representative macroscopic view of a liver from each group. Scale bars =10 mm. (C) Representative H&E staining of liver sections collected from mice sacrificed 8 hours after APAP challenge and examined by an experienced pathologist. Scale bars =50 μm (upper panel) and 100 μm (lower panel), respectively. The dotted area represents a necrotic focus. (D) The area of necrosis was quantified in the same mice. (E) Protein levels of pP38 were determined by Western blot in APAP-treated control (Wt) and *Jnk^{Δhepa}* livers. Glyceraldehyde-3-phosphate dehydrogenase was used as loading control. Data are expressed as mean ± SEM (**P*<.05 and ***P*<.01).

These striking results in APAP-induced DILI prompted us to investigate whether the function of JNK1 and JNK2 is universal or dependent on the context of hepatic injury. We treated *Jnk^{Δhepa}* mice with D-GalN combined with endotoxin (LPS).

Unexpectedly, no differences were observed after D-GalN/LPS treatment in survival, serum markers of liver injury, tissue injury, or liver vs. body weight ratio between both control groups and *Jnk^{Δhepa}* animals. These data suggest that JNK1 and JNK2 in hepatocytes play a prominent protective role, especially during toxic liver injury (Supplementary **Figure S5.4A-E**).

Carbon tetrachloride-induced chronic liver injury is worsened in mice with combined deletion of *Jnk1* and *Jnk2* in hepatocytes

Since we previously reported that JNK1 in hepatocytes has no impact on the progression of CCl₄-induced toxic liver injury¹¹, we questioned whether the compound function of JNK1 and JNK2 in hepatocytes is implicated in chronic toxic liver injury. Twenty-eight days after repetitive CCl₄ treatment, *Jnk^{Δhepa}* livers showed larger numbers and size of necrotic areas compared with *Jnk2^{-/-}* or Wt control mice (**Figure 5.3A**, Supplementary **Figure S5.5A**).

Jnk^{Δhepa} livers showed increased collagen deposition as evidenced by Sirius red staining, CollagenIA1, and α-smooth muscle actin protein and mRNA expression (**Figure 5.3B-E**, Supplementary **Figure S5.5B-E**). Additional liver fibrosis markers such as hydroxyproline quantification, Timp1, and Mmp2 (**Figure 5.3F**, Supplementary **Figure S5.5F**) suggested a protective role of combined JNK1 and JNK2 activation in hepatocytes during chronic toxic CCl₄-induced liver injury.

Figure 5.3 Liver fibrogenesis is aggravated in *Jnk*^{Δhepa} after chronic CCl₄ treatment. (A) Representative H&E staining of liver sections of control (Wt), *Jnk2*^{-/-} and *Jnk*^{Δhepa} livers, 28 days after repeated injections of CCl₄. Corn-oil injections were used as controls. Scale bars =100μm. (B) Representative Sirius red staining of paraffin sections from the same livers. Scale bars =100μm. (C) A representative collagen IA1 staining of frozen sections from the same mice is shown. Scale bars =200mm. (D) In addition, messenger RNA levels for collagen IA1 and α-smooth muscle actin were determined by quantitative real-time reverse transcription-polymerase chain reaction. (E) Protein levels of α-smooth muscle actin were determined. Glyceraldehyde-3-phosphate dehydrogenase was used as a loading control. (F) Hydroxyproline contents were assessed in the same livers. Data are expressed as mean ± SEM (**P*<.05 and ****P*<.001).

Figure 5.4

Cell death and compensatory proliferation are exacerbated after combined deletion of JNK1 and JNK2 in hepatocytes. (A) Representative TdT-mediated dUTP-biotin nick end labeling (TUNEL) staining performed on frozen liver sections (upper; Scale bars =100 μ m) and cleaved caspase3 immunohistochemistry in paraffin sections (lower panel; Scale bars =100 μ m) of control (*Wt*), *Jnk2^{-/-}* and *Jnk^{Δhepa}* livers after 4 weeks of repeated CCl₄ injections are shown. Quantification of TUNEL- (left) (B) and caspase3 enzyme activity (C) was analyzed in 4-week CCl₄-treated control (*Wt*), *Jnk2^{-/-}* and *Jnk^{Δhepa}* livers. (D) Representative Ki-67 staining performed on frozen liver sections of the same livers. Scale bars =100 μ m. (E) Quantification of Ki-67-positive cells per view field is shown. (F) Protein levels of proliferating cell nuclear antigen and CyclinD were determined by Western blot in the same samples. Glyceraldehyde-3-phosphate dehydrogenase was used as a loading control. Data are expressed as mean $\pm$ SEM (***) $P < .001$). Arrows denote positive cells.

Figure 5.5 Microarray analysis of *Jnk^{Δhepa}* livers and hepatocytes manifest dysregulation in cell proliferation, apoptosis, and inflammation at basal levels. (A) Gene array analysis was performed in 8-week-old control (*Wt*) and *Jnk^{Δhepa}* liver and primary isolated hepatocytes. Correlation of the fold induction of genes in hepatocytes and liver is shown. Log2 expression values of the individual mice were divided by the mean of the sham-operated mice. Log ratios were saved in a .txt file and analyzed with the Multiple Experiment Viewer. Top up- and down-regulated JNK-target substrates are shown (red: up-regulated; blue: downregulated, $n=3$, $-1.5 < FC < 1.5$). (B) Ingenuity Pathway Analysis was performed in the same samples, and the expression values (in brackets) of the top up- and down-regulated

genes in the liver (left panel) and primary isolated hepatocytes (right panel) are represented. (C) Liver and hepatocytic protein extracts of untreated control (Wt), *Jnk1^{Δhepa}*, and *Jnk^{Δhepa}* were prepared. The protein expression levels of pStat3, c-myc, and Bcl-XL were determined by Western blot. Glyceraldehyde-3-phosphate dehydrogenase was used as loading control. (D) Messenger RNA expression was determined in liver tissue and primary hepatocytes of untreated 8-week-old control (Wt), *Jnk1^{Δhepa}*, and *Jnk^{Δhepa}* mice. The messenger RNA expression levels of *Saa2*, *Cmyc*, and *BclXL* are shown (n=3–5; **P*<.05; ***P*<.01; ****P*<.001).

Aggravated cell death and compensatory proliferation in *Jnk^{Δhepa}* liver after repetitive carbon tetrachloride injection

To better characterize our findings, we analyzed parameters of cell death and proliferation. TdT-mediated dUTP-biotin nick end labeling and cleaved caspase3-positive nuclei were significantly enhanced 28 days after CCl₄ treatment in *Jnk^{Δhepa}* livers compared with *Jnk2^{-/-}* or control mice (**Figure 5.4A–C**). Concomitantly, compensatory proliferation examined by Ki-67 staining was significantly increased in *Jnk^{Δhepa}* livers (**Figure 5.4D and 5.4E**). These results were strengthened by higher messenger RNA expression of cell cycle markers, such as *Pcna* (messenger RNA) and up-regulation of proliferating cell nuclear antigen and CyclinD protein levels in *Jnk^{Δhepa}* compared with *Jnk2^{-/-}* or control livers (**Figure 5.4F**, Supplementary **Figure S5.6A and S5.6B**). Loss of JNK1 and JNK2 in hepatocytes resulted in more substantial cell death, accompanied by an increased proliferative response in the liver.

The inflammatory response is increased in *Jnk^{Δhepa}* livers

Chronic inflammation triggers progression of liver fibrosis.¹⁷ After 28 days of CCl₄ injection, we found a significant increase in CD11b- and F4/80-positive cells, putative macrophages/Kupffer cells, in *Jnk^{Δhepa}* compared with *Jnk2^{-/-}* and control livers (Supplementary **Figure S5.7A and S5.7B**). Additionally, *Jnk^{Δhepa}* livers displayed stronger expression of inflammatory markers, such as *IL1α*, *IL1β*, *Mcp1*, and *Tnfa* (Supplementary **Figure S5.7C–F**). Hence, loss of JNK1 and JNK2 in hepatocytes caused severe inflammatory liver injury and fibrosis.

Gene and protein profile of *Jnk^{Δhepa}* livers and hepatocytes in basal conditions

To study the impact of *Jnk* deletion on gene expression in liver and hepatocytes, we performed Affymetrix GeneChip microarray analysis of liver and primary hepatocytes isolated from 8-week-old control and *Jnk^{Δhepa}* mice. This comparison revealed significant changes ($-1.5 < FC < 1.5$) in the transcript expression of 355 genes that were up-regulated (197 in liver and 158 in hepatocytes) and 448 down-regulated (171 in liver and 277 in hepatocytes) in *Jnk^{Δhepa}* mice (Supplementary **Figure S5.8A and S5.8B**). We first performed hierarchical clustering of the JNK substrates commonly up- or down-regulated in both livers and hepatocytes of *Jnk^{Δhepa}* mice. Interestingly, we found significantly reduced transcript levels of JNK target genes, including transcription factors *Atf*, *JunD*, or *Fos* and apoptotic

markers (*Bcl2l13* and *Bid*), and up-regulation of *Gadd45b*, *Saa2*, and *Cmyc* (**Figure 5.5A**). In addition, Ingenuity Pathway Analysis demonstrated that combined deletion of JNK1 and JNK2 in hepatocytes affected the expression of genes associated with cell death and proliferation (*Cdkn1a*, *Atf*, *myc*), inflammation (*Smad7*, *IL18*, *Ctgf*, *Cox6b2*), as well as metabolism (*ABCC4*, *CYP2B13*) (**Figure 5.5B**).

To validate the main findings of the microarray analysis, protein and messenger RNA expression was analyzed in both livers and freshly isolated primary hepatocytes from 8-week-old *Wt*, *Jnk1^{Δhepa}*, and *Jnk^{Δhepa}* mice revealing enhanced expression of pSTAT3, *Saa2*, and *c-myc* (cell cycle), and down-regulation of *BclXL* and *Bad* (apoptosis). As expected, a lack of *Mapk9/Jnk2* expression was found in *Jnk^{Δhepa}* mice (**Figure 5.5C**, Supplementary **Figure S5.8C** and **S5.8D**).

Morphologic changes and mitochondrial damage are aggravated in acetaminophen-treated *Jnk^{Δhepa}* primary hepatocytes

Acute and chronic toxic models suggested that JNK1 and JNK2 have synergistic functions for hepatocyte protection. We aimed to better define the molecular mechanisms explaining our findings *in vivo* in primary control (*Wt*) and *Jnk^{Δhepa}* hepatocytes. Liver cells were cultured for up to 48 hours without treatment (Supplementary **Figure S5.9A**). Lack of *Jnk* expression did not affect viability compared with wildtype hepatocytes (Supplementary **Figure S5.9B**). However, basal cell cycle activity was reduced in *Jnk^{Δhepa}* compared with control hepatocytes (Supplementary **Figure S5.9C** and **S5.9D**).

Next, we investigated the mechanism underlying the strong phenotype induced by APAP (10 mM) in *Jnk^{Δhepa}* hepatocytes for up to 48 hours. Twelve hours after APAP treatment, *Jnk^{Δhepa}* hepatocytes evidenced cytoplasmic projections, loss of cell-to-cell contact, and detachment in contrast to polyhedral rounded-shaped control hepatocytes (**Figure 5.6A**). Up to 24 hours after treatment, nuclear condensation or fragmentation was significantly stronger in *Jnk^{Δhepa}* compared with control hepatocytes (not shown).

SP600125, an anthrapyrazolone-specific JNK inhibitor, has been shown to protect against APAP-induced ALF in mice.¹⁸ Unexpectedly, control and *Jnk^{Δhepa}*-APAP-treated hepatocytes were protected to the same extent by coadministration of SP600125 ± APAP (**Figure 5.6A**). APAP mediates its toxic effect by forming highly reactive metabolites. Concomitant with the morphologic changes, we detected a significant increase in mitochondrial reactive oxygen species in APAP-treated *Jnk^{Δhepa}* compared with control hepatocytes. Additionally, SP600125 significantly reduced the changes in mitochondrial membrane potential in both control and *Jnk^{Δhepa}* APAP-treated hepatocytes (**Figure 5.6B**, Supplementary **Figure S5.10A**).

Figure 5.6 APAP modifies the morphology and exacerbates mitochondrial damage and necrotic cell death in primary *Jnk^{Δhepa}* hepatocytes. (A) Primary hepatocytes were isolated from control (Wt) and *Jnk^{Δhepa}* mice. A total of 500,000 cells were seeded in 6-well plates and cultivated for up to 12 hours. Visible light microphotographs were taken in presence or absence of APAP and/or SP600125. Scale bars =200 μm. (B) At the same time, we performed Mitoxo staining to assess mitochondrial damage (red –arrows–, counterstained with 40,6-diamidino-2-phenylindole, blue). Microphotographs were taken (Scale bars =100 μm). (C) TdT-mediated dUTP-biotin nick end labeling (TUNEL) staining was performed. Scale bars =200 μm. (D) TUNEL-positive cells were evaluated in the same mice and represented in percentage per view field. (E) The enzyme activity of caspase3 was measured in frozen tissue. (F) Hepatocytic protein extracts were collected and treated with APAP for 8 hours in culture in presence or absence of APAP and/or SP600125. The protein expression levels of RIP3, RIP1, p65, pJNK, p-cJUN, and pERK were determined by Western blot. β-actin was used as a loading control.

Acetaminophen exacerbates necrotic cell death in *Jnk^{Δhepa}* primary hepatocytes

TdT-mediated dUTP-biotin nick end labeling staining evidenced significant lower cell survival of APAP-treated *Jnk^{Δhepa}* compared with control hepatocytes 12 hours after treatment (**Figure 5.6C**). Cell death was significantly reduced after SP600125 ± APAP co-administration in control and *Jnk^{Δhepa}* hepatocytes (**Figure 5.6C** and **5.6D**). Next, we sought to define the mode of cell death in APAP-treated hepatocytes. We first tested caspase3 activity before and after APAP administration, including SP600125. APAP treatment in any combination and at different time points did not significantly change caspase 3 activity (**Figure 5.6E**). This result was further confirmed by caspase 3 immunostaining (not shown). To detect the relevance of necrotic cell death, we performed Annexin V/Ethidium Homodimer III staining. The amount of double-positive (i.e., necrotic) hepatocytes was significantly higher in *Jnk^{Δhepa}* compared with control hepatocytes, while SP600125 blocked necrosis in both control and *Jnk^{Δhepa}* hepatocytes (Supplementary **Figure S5.10B** and **S5.10C**).

Receptor interacting protein (RIP) 3 plays an essential role in mediating necrotic cell death. We studied RIP3 and RIP1 expression in wild-type and *Jnk^{Δhepa}* hepatocytes before and after APAP ± SP600125 co-treatment (**Figure 5.6F**). APAP treatment had no effect on RIP3, but stimulated RIP1 expression in both control and was strongly induced in *Jnk^{Δhepa}* hepatocytes. SP600125 + APAP co-treatment strongly reduced RIP3 expression to comparable levels in control and *Jnk^{Δhepa}* hepatocytes, while the decreasing effect on the RIP1 protein was more prominent in control hepatocytes (**Figure 5.6F**).

To characterize the specificity of our findings, we included pERK and p65 expression in our analysis. APAP treatment stimulated both pERK and p65 expression independent of SP600125 co-treatment, suggesting that both pathways are of minor relevance in explaining the effect on necrosis (**Figure 5.6F**). To further confirm this result, we tested pJNK and p-cJUN expression before and after treatment. As shown in **Figure 5.6F**, APAP induced pJNK and p-cJUN activation in control hepatocytes, while SP600125 blocked JNK but not cJUN phosphorylation. In contrast, no significant pJNK and p-cJUN expression in either treatment conditions was evident in *Jnk^{Δhepa}* hepatocytes.

In vivo treatment with SP600125 suppresses acetaminophen-induced liver injury in mice with compound deletion of JNK1 and JNK2 in hepatocytes

To validate the *in vitro* findings, we studied SP600125 co- and pre-administration with APAP in *Jnk^{Δhepa}* animals *in vivo*. Co-treatment with the JNK inhibitor provided protection in control and *Jnk1^{Δhepa}* and also in *Jnk^{Δhepa}* mice against APAP-induced liver injury (**Figure 5.7A-7C**). To exclude the possibility that SP600125 affects APAP metabolism, we pre-administered SP600125 to control, *Jnk1^{Δhepa}*, and *Jnk^{Δhepa}* mice, and 2 hours later we injected APAP (**Figure 5.7A-C**). However, pre-administration did not affect the protective

effect. Noticeably, the number of necrotic foci was primarily reduced in the liver parenchyma of SP600125 co- or pretreated *Jnk^{Δhepa}* mice (**Figure 5.7B and 5.7C**).

Because our results suggested that SP600125 protects against APAP-induced liver injury via JNK-independent mechanisms, we explored other pathways associated with APAP toxicity. *Saberi and colleagues*¹⁹ recently found that the interplay between protein kinase C-α and JNK mediates APAP-induced liver injury. Protein kinase C-α activation increased after APAP treatment and was attenuated by SP600125 co-administration in *Wt* livers (**Figure 5.7D**). Moreover, AMPK activity — protective against APAP hepatotoxicity — was abrogated after APAP challenge in control and *Jnk^{Δhepa}* animals. Total AMPK levels were also affected by APAP treatment. Noticeably, APAP treatment caused a decline in total AMPK levels in *Wt* and dramatically in *Jnk^{Δhepa}* livers. In contrast, co-treatment with the classical JNK inhibitor, SP600125, reversed the APAP-mediated effect on pAMPK and AMPK levels in both *Wt* and *Jnk^{Δhepa}* animals.

Next, we examined whether lower AMPK levels correlated with JNK activity. APAP treatment increased JNK in *Wt* and, to a greater extent, in *Jnk^{Δhepa}* livers, an effect blocked by co-treatment with SP600125 (**Figure 5.7D**). Collectively, these data suggest that SP600125 exerts an off-target effect on AMPK and the protective effect of SP600125 occurs in liver parenchymal cells.

Lack of JunD activation in acetaminophen-treated *Jnk^{Δhepa}* murine hepatocytes

We performed a set of quantitative phosphoproteomics experiments to characterize proteins that could be differentially phosphorylated after APAP treatment in control and *Jnk^{Δhepa}* hepatocytes (Supplementary **Figure S5.11A**). Among several differentially phosphorylated proteins, which we could not validate by Western blot due to lack of commercially available phospho-antibodies, our screening approach identified JunD — a known JNK nuclear substrate — to be substantially less phosphorylated in APAP-treated *Jnk^{Δhepa}* hepatocytes (**Figure 5.7E**). In addition, we investigated the overall phosphorylation of MAPKs, which are known substrates of JNK, using a MAPK-substrate-specific antibody (Supplementary **Figure S5.11B**). This approach revealed at least 9 proteins with reduced phosphorylation in APAP-treated *Jnk^{Δhepa}* hepatocytes. The identity of these proteins was estimated by comparison of their appropriate molecular weights with known JNK substrates (Supplementary **Figure S5.11B**). In summary, these results demonstrate substantially attenuated phosphorylation of JunD and other proteins in APAP-treated *Jnk^{Δhepa}* hepatocytes, which need to be identified in future studies. These findings indicate that a JNK-JunD-dependent mechanism might be involved in protecting against APAP-induced liver injury.

Figure 5.7 *In vivo* treatment with SP600125 suppresses APAP-induced liver injury in mice with combined JNK1 and JNK2 deletion in hepatocytes. (A) Serum aspartate aminotransferase (left) and alanine aminotransferase (right) levels were determined in control (*Wt*), *Jnk1^{Δhepa}*, and *Jnk^{Δhepa}* mice injected with SP600125 at the same time as APAP or pretreated with SP600125, 2 hours before APAP challenge. Comparison with dimethyl sulfoxide and APAP-treated mice is shown ($n=10$). (B, C) Representative H&E staining of liver sections collected from the same mice sacrificed 8 hours after treatment and examined by an experienced pathologist. Scale bars 50 mm (upper panels) and 200 mm (lower panels), respectively. Dotted areas represent necrotic foci. (D) Liver extracts were collected, and immunoblotting was performed, and the protein levels of protein kinase C- α (PKC α), pAMPK, AMPK, and pJNK were determined by Western blot. (E) Phosphorylation of JunD was performed in hepatocytic extracts. Glyceraldehyde-3-phosphate dehydrogenase was used as loading control. (F) Illustrative scheme summarizing the main obtained results: APAP triggers higher PKC α , and lower AMPK—a survival pathway—expression inducing JNK phosphorylation and cell death. The JNK classical inhibitor SP600125 exerts an off-target effect by facilitating phosphorylation of AMPK and preventing JNK phosphorylation in hepatocytes.

Discussion

Acetaminophen- and CCl₄-induced liver injury have been studied extensively because APAP overdose is the leading cause of DILI in the United States, and accidental CCl₄ ingestion still occurs.¹⁴ An overwhelming amount of evidence demonstrated that JNK activation plays a major role in toxic liver injury.²⁰ Our first investigations using human DILI liver samples showed activation of JNK (pJNK) predominantly in nuclei of hepatocytes and in infiltrating cells. These results are in line with observations that JNK activation is not only limited to hepatocytes but also found in pro-fibrotic and inflammatory cells during the progression of liver disease in humans and mice.^{10,11,21}

Noticeably, our results suggest a differential pattern of JNK1 expression and increased JNK2 levels in human DILI/ALF liver biopsies compared with normal tissue. Furthermore, JNK activation was a common feature of murine APAP-induced liver injury, not only in *Wt* but also especially in *Jnk^{Δhepa}* mice. Noticeably, the intense JNK phosphorylation as well as increased JNK1 expression in *Jnk^{Δhepa}* livers after APAP correlated with the dramatic increase in transaminases. These results suggest that JNK1 phosphorylation in infiltrating cells and nonparenchymal cells correlates with the degree of liver injury. However, the functional role of these cell compartments during APAP-induced liver injury needs to be further addressed.

As found in human samples, JNK1 and JNK2 protein expression were also dysregulated after APAP challenge in mice, indicating an analogous mechanism between human and murine DILI. Altogether, our results indicated that JNK is strongly activated in all forms of human DILI-induced ALF, not only etiology-dependent hepatocytes but also immune-infiltrating cells are pJNK positive, and hence, a dysregulation of JNK1 and JNK2 protein expression is characteristic of both human and murine DILI.

Additionally, our array analysis led us to the working hypothesis that JNK1 and JNK2 might have distinct and shared essential functions in hepatocytes. In the current study, we found that combined JNK1 and JNK2 deletion in hepatocytes triggers more severe liver injury, inflammation, and progression after repetitive CCl₄ injection. Interestingly, our previous publication revealed that JNK1 is involved in hepatic stellate cells transdifferentiation into collagen-producing myofibroblasts¹¹ associated with reduced liver injury. Consequently, JNK2 expression is sufficient to rescue the loss of JNK1 in hepatocytes and protects from CCl₄-mediated cell death.

The prominent role of necrotic cell death in APAP-dependent liver injury has been shown in several studies.^{22–26} Previous reports from *the laboratory of Kaplowitz* suggested that disruption of JNK2, but not of JNK1, partially prevented APAP-induced liver injury, indicating that targeting JNK2 could be a promising therapeutic approach.²⁷ In contrast, Henderson et al.²⁸ found no differences in APAP-induced liver injury between *Jnk1^{-/-}* and *Jnk2^{-/-}* mice. Our

data demonstrate that lack of JNK1 and JNK2 expression in hepatocytes caused extensive necrosis within 8 hours after APAP injection. Concomitant with these findings, we observed a dramatic oxidative stress response in the liver of *Jnk^{Δhepa}* mice associated with necrotic cell death.^{22,23} We excluded that apoptotic cell death plays a significant role in APAP-treated *Jnk^{Δhepa}* hepatocytes. Instead, we demonstrate that combined JNK1 and JNK2 activities are protective against APAP-induced necrotic cell death of hepatocytes by controlling the oxidative stress response.

The observations found in APAP-induced liver injury were specific for this form of ALF because we observed no differences in the acute hepatitis-D-GalN/LPS model between *Jnk^{Δhepa}* and control animals. This is in agreement with a recent study indicating that combined deletion of JNK1 and JNK2 in hepatocytes did not alter concanavalin A- or LPS-induced liver injury.²⁹ However, hematopoietic deficiency in JNK1 and JNK2 prevented concanavalin A-induced liver injury by suppressing tumor necrosis factor production.²⁹ These findings clearly suggest that under certain conditions (*e.g.*, etiology of liver disease), JNK activation in infiltrating cells rather than in hepatocytes is critical. Altogether these results suggest that the context-specific activation of JNK in different cell types during ALF is essential and needs to be further defined.

To better assess these results, we included the classical JNK inhibitor, SP600125, in our analysis. This compound has been reported to target the highly conserved ATP-binding sites of JNK and protects from APAP-induced liver injury *in vivo*.³⁰ Surprisingly, SP600125 was found to confer protection not only to control but also to *Jnk*-deleted hepatocytes *in vivo* and *in vitro*. In addition, preadministration of SP600125 did not affect APAP metabolism. These results suggested that the beneficial effect of SP600125 on APAP-induced liver injury likely results through off-target effects, for example, on AMPK. Concomitant with previous reports¹⁹, we found decreased levels of pAMPK and a further decline in total AMPK levels—protective against APAP hepatotoxicity—in *Jnk^{Δhepa}* livers, indicating that survival pathways are reduced in animals with JNK deficiency in hepatocytes. SP600125 might act on protein phosphatases, such as 2C (PP2C), which negatively regulate AMPK signaling, preventing the dephosphorylation of AMPK (**Figure 5.7F**).

Additionally, we employed functional proteomics to map differential phosphorylation caused by APAP treatment in JNK-deficient hepatocytes. Our findings suggest repression of JunD activation, another protective element, and part of the cell stress response, was further decreased in the absence of JNK in hepatocytes. Hence, the role of other kinases involved in necrotic cell death, such as RIP3, RIP1, or MLKL, are currently under intensive investigation.^{24,31}

In summary, we demonstrate that combined JNK1 and JNK2 activation is essential to protect hepatocytes from acute and chronic toxic liver injury *in vivo* and *in vitro*. Our results show that JNK inhibition is a questionable treatment option for APAP-induced liver injury

because the protecting effect of SP600125, the classical JNK inhibitor, is mediated by off-target effects. Our results found that the cell type-specific function of JNK is a potential therapeutic target. However, the context-specific function needs to be defined before these options can be used in the clinic.

References

1. Fink SL, Cookson BT. Apoptosis, pyroptosis, and necrosis: mechanistic description of dead and dying eukaryotic cells. *Infect Immun* 2005;73:1907–1916.
2. Guicciardi ME, Malhi H, Mott JL, et al. Apoptosis and necrosis in the liver. *Compr Physiol* 2013;3: 977–1010.
3. Conze D, Krahl T, Kennedy N, et al. c-Jun NH(2)-terminal kinase (JNK)1 and JNK2 have distinct roles in CD8(p) T cell activation. *J Exp Med* 2002;195:811–823.
4. Davis RJ. Signal transduction by the JNK group of MAP kinases. *Cell* 2000;103:239–252.
5. Jaeschke A, Rincon M, Doran B, et al. Disruption of the Jnk2 (Mapk9) gene reduces destructive insulinitis and diabetes in a mouse model of type I diabetes. *Proc Natl Acad Sci U S A* 2005;102:6931–6935.
6. Reid AB, Kurten RC, McCullough SS, et al. Mechanisms of acetaminophen-induced hepatotoxicity: role of oxidative stress and mitochondrial permeability transition in freshly isolated mouse hepatocytes. *J Pharmacol Exp Ther* 2005;312:509–516.
7. Heard KJ. Acetylcysteine for acetaminophen poisoning. *N Engl J Med* 2008;359:285–292.
8. Matsumaru K, Ji C, Kaplowitz N. Mechanisms for sensitization to TNF-induced apoptosis by acute glutathione depletion in murine hepatocytes. *Hepatology* 2003;37:1425–1434.
9. Hanawa N, Shinohara M, Saberi B, et al. Role of JNK translocation to mitochondria leading to inhibition of mitochondria bioenergetics in acetaminophen-induced liver injury. *J Biol Chem* 2008;283: 13565–13577.
10. Kluwe J, Pradere JP, Gwak GY, et al. Modulation of hepatic fibrosis by c-Jun-N-terminal kinase inhibition. *Gastroenterology* 2010;138:347–359.
11. Zhao G, Hatting M, Nevzorova YA, et al. Jnk1 in murine hepatic stellate cells is a crucial mediator of liver fibrogenesis. *Gut* 2014;63:1159–1172.
12. Das M, Jiang F, Sluss HK, et al. Suppression of p53-dependent senescence by the JNK signal transduction pathway. *Proc Natl Acad Sci U S A* 2007;104:15759–15764.
13. Das M, Sabio G, Jiang F, et al. Induction of hepatitis by JNK-mediated expression of TNF- α . *Cell* 2009;136:249–260.
14. Larson AM, Polson J, Fontana RJ, et al. Acetaminophen-induced acute liver failure: results of a United States multicenter, prospective study. *Hepatology* 2005;42:1364–1372.
15. Lee SS, Buters JT, Pineau T, et al. Role of CYP2E1 in the hepatotoxicity of acetaminophen. *J Biol Chem* 1996;271:12063–12067.
16. Bansal S, Liu CP, Sepuri NB, et al. Mitochondria-targeted cytochrome P450 2E1 induces oxidative damage and augments alcohol-mediated oxidative stress. *J Biol Chem* 2010;285:24609–24619.
17. Sunami Y, Leithauser F, Gul S, et al. Hepatic activation of IKK/NF- κ B signaling induces liver fibrosis via macrophage-mediated chronic inflammation. *Hepatology* 2012;56:1117–1128.
18. Xu JJ, Hendriks BS, Zhao J, et al. Multiple effects of acetaminophen and p38 inhibitors: towards pathway toxicology. *FEBS Lett* 2008;582:1276–1282.
19. Saberi B, Ybanez MD, Johnson HS, et al. Protein kinase C (PKC) participates in acetaminophen hepatotoxicity through c-jun-N-terminal kinase (JNK)-dependent and -independent signaling pathways. *Hepatology* 2014;59:1543–1554.
20. Seki E, Brenner DA, Karin M. A liver full of JNK: signaling in regulation of cell function and disease pathogenesis, and clinical approaches. *Gastroenterology* 2012;143:307–320.
21. Toivola DM, Ku NO, Resurreccion EZ, et al. Keratin 8 and 18 hyperphosphorylation is a marker of progression of human liver disease. *Hepatology* 2004;40:459–466.
22. Kon K, Kim JS, Jaeschke H, et al. Mitochondrial permeability transition in acetaminophen-induced necrosis and apoptosis of cultured mouse hepatocytes. *Hepatology* 2004;40:1170–1179.

23. Ni HM, Bockus A, Boggess N, et al. Activation of autophagy protects against acetaminophen-induced hepatotoxicity. *Hepatology* 2012;55:222–232.
24. Ramachandran A, McGill MR, Xie Y, et al. Receptor interacting protein kinase 3 is a critical early mediator of acetaminophen-induced hepatocyte necrosis in mice. *Hepatology* 2013;58:2099–2108.
25. Antoine DJ, Jenkins RE, Dear JW, et al. Molecular forms of HMGB1 and keratin-18 as mechanistic biomarkers for mode of cell death and prognosis during clinical acetaminophen hepatotoxicity. *J Hepatol* 2012;56:1070–1079.
26. McGill MR, Sharpe MR, Williams CD, et al. The mechanism underlying acetaminophen-induced hepatotoxicity in humans and mice involves mitochondrial damage and nuclear DNA fragmentation. *J Clin Invest* 2012;122:1574–1583.
27. Gunawan BK, Liu ZX, Han D, et al. c-Jun N-terminal kinase plays a major role in murine acetaminophen hepatotoxicity. *Gastroenterology* 2006;131:165–178.
28. Henderson NC, Pollock KJ, Frew J, et al. Critical role of c-jun (NH2) terminal kinase in paracetamol- induced acute liver failure. *Gut* 2007;56:982–990.
29. Das M, Garlick DS, Greiner DL, et al. The role of JNK in the development of hepatocellular carcinoma. *Genes Dev* 2011;25:634–645.
30. Ishii M, Suzuki Y, Takeshita K, et al. Inhibition of c-Jun NH2-terminal kinase activity improves ischemia/reperfusion injury in rat lungs. *J Immunol* 2004;172:2569–2577.
31. Dara L, Johnson H, Suda J, et al. Receptor interacting protein kinase 1 mediates murine acetaminophen toxicity independent of the necrosome and not through necroptosis. *Hepatology* 2015;62:1847–1857.

Supplementary information

Supplementary material and methods

Isolation and culture of primary hepatocytes

Primary mouse hepatocytes were isolated from 7–8 week-old mice by collagenase perfusion. Living cells were plated on collagen-precoated Petri dishes at a density of $1.5 \times 10^4/\text{cm}^2$ in DMEM medium (PAA Laboratories GmbH, Pasching, Austria) supplemented with L-glutamine, high glucose (4.5 g/l), 10% FBS, and 100 U/ml penicillin/streptomycin. After 4 h incubation (37°C, 5% CO₂) and every 2 days, the medium was renewed. Hepatocytes were cultured for up to 4 days.

Quantitative real-time PCR (qPCR)

Total RNA from liver tissues or cultured cells was isolated using Trizol reagent (Invitrogen, Karlsruhe, Germany). Due to low RNA amount in cultured cells, the RNeasy Lipid Tissue Mini Kit was used to collect and purify RNA. Reverse-transcription was performed using an Omniscript RT Kit (Qiagen). Relative quantitative gene expression was measured via real-time PCR using a 7300 Real-Time PCR System with SDS software 1.3.1 (Applied Biosystems, Foster City, CA) and a SYBR Green PCR Kit (Invitrogen, Carlsbad, CA). GAPDH expression was used as an internal standard. Primer sequences can be provided upon request.

Histological evaluation and immunofluorescence staining

Hepatic tissue was fixed in 4% paraformaldehyde (PFA) immediately after extraction, embedded in paraffin, sectioned, and stained for H&E or Sirius red. Samples were reviewed by a blinded pathologist who analyzed the degree of liver injury. The percentage of Sirius red area fraction in all animals was quantified on 10 or 20 low-power (magnification, X10) fields per slide, using the NIH ImageJ® software (<http://rsbweb.nih.gov/>). Immunohistochemistry for CK-19 (DAKO, Hamburg, Germany), α SMA (Sigma, Steinheim, Germany), phospho-JNK (Cell Signaling, Danvers, MA) and F4/80 (Serotec, Dusseldorf, Germany) on paraffin sections was performed using a Leica automatic stainer (Wetzlar, Germany). For the immunofluorescence staining, frozen cryosections were incubated with Ki-67 (Santa Cruz, Heidelberg, Germany), Collagen IA1 (Bio trend, Cologne, Germany) or *in situ* cell death detection kit (Roche, Mannheim, Germany) and incubated with fluorescence-labeled secondary antibodies (AlexaFluor 488 and 564, Invitrogen, Carlsbad, CA, USA). All fluorescence-labeled cryosections were analyzed and documented using an Imager Z1 fluorescence microscope together with Axiovision software (Carl Zeiss, Jena, Germany).

Immunoblot analysis

Liver tissues were homogenized in ice-cold NP40-Buffer containing 50 mM Tri- HCl (pH 7.5), 150 mM NaCl, 0.5% NP-40 and 50 mM NaF freshly supplemented with Complete Mini (Roche), PhosSTOP (Roche), 1 mM orthovanadate and 1 mM pefablock. Protein concentrations were determined by the BIO-RAD protein assay (BIORAD). Samples were separated by SDS-PAGE and transferred to a cellulose membrane and probed with antibodies for α SMA (Sigma), COLLAGEN 1A1 (Monosan, Beutelsbach, Germany), PCNA (Dianova GmbH, Hamburg, Germany), CYCLIN A (Santa Cruz), CYCLIN D1, BCLXL and PKC α (Santa Cruz), JNK1, JNK2, pJNK (pT183/Y185), P38, CMYC, STAT3, pAMPK, AMPK, and MAPK/CDK Substrates (PXP*P or S*PXR/K) (34B2) (Cell Signaling), and GAPDH (Biorad, Munich, Germany). As secondary antibodies, anti-rabbit-HRP (Cell Signaling) and anti-mouse-HRP (Santa Cruz) were used.

Microarray analysis

Liver and hepatocyte RNA using TRIzol reagent and purified with the RNeasy Mini Kit (Qiagen, Venlo, The Netherlands), according to the manufacturer's instructions, was isolated from 8-10 weeks-old male control Jnk2^{-/-} and Jnk ^{Δ hepa} mice. Concentrations and purity of RNA samples were determined on a NanoDrop ND-1000 spectrophotometer (Isogen, Maarssen, The Netherlands). RNA integrity was checked on an Agilent 2100 bioanalyzer (Agilent Technologies, Amsterdam, The Netherlands) with 6000 Nano Chips. RNA samples from 3 mice per experimental group were used for microarray analysis. Samples were hybridized on Affymetrix GeneChip Mouse Genome 430 2.0 arrays in the microarray core laboratory of the Nutrigenomics Consortium at Wageningen University, The Netherlands. Hybridization, washing, and scanning of the arrays were performed according to standard Affymetrix protocols. Array images were processed using packages

from the Bioconductor project¹. Probe sets were redefined according to Dai². Probes were assigned to unique gene identifiers, in this case, Entrez IDs. Arrays were normalized with the Robust Multi-array Average (RMA) method. Differentially expressed probe sets were identified using intensity-based moderated paired t-statistics. *P* values were corrected for multiple testing using a false discovery rate (FDR) method. Detailed descriptions of the applied methods are available on request.

Peptide lysis, dimethyl-labeling, phosphopeptide enrichment, and LC-MS/MS analysis

Primary hepatocytes were scraped off the cell culture dishes after APAP/DMSO treatment, washed three times in ice-cold PBS and lysed in 8M urea (in 50 mM ammonium bicarbonate), containing protease and phosphatase inhibitor cocktails (Serva, Heidelberg, Germany) for 30 min on ice and cleared by centrifugation at 20000 g, for 15 min at 4°C. After determination of protein concentration using a BCA assay (Thermo, Waltham, MA), the individual lysates (500 µg each) are reduced with DTT, alkylated with iodoacetamide, and proteolytically digested with the protease Lys-C (1:100, w/w) in 8M urea for 4h at 37°C. Samples were then diluted to 2M urea with 50 mM ammonium bicarbonate and further digested with trypsin (1:75, w/w) overnight at 37°C.^{3,4} The resulting peptide digests are desalted and dimethyl-labeled “on column”.⁵ Control (WT) mice derived peptides are labeled “light”, whereas *Jnk*^{Δhepa} mice derived peptides were labeled “heavy”. The labeled peptide digests are mixed in a 1:1 ratio, dried down, and resuspended in 1 M glycolic acid in 80% ACN, 5% TFA). Phosphopeptide enrichment was performed using MagReSyn® Ti-IMAC beads (ReSyn Biosciences, Gauteng, South Africa) according to the manufacturer’s instructions. Samples were then analyzed on a nanoLC-MS/MS System (LC: Ultimate 3000 (Dionex, Dreieich, Germany); MS: Orbitrap Elite (Thermo)). The raw data obtained from the mass spectrometer were processed and quantified with MaxQuant (version 1.4.1.2). The relevant MS and MS/MS spectra were searched against a forward-decoy Swissprot Homo sapiens database (version 09/2014).

Transplantation of bone marrow-derived cells

We transferred bone marrow from *Jnk*^{Δhepa} and control (WT) mice into 6 week-old *Jnk*^{Δhepa} and control isogenic recipients (n=6-7 mice per group) after ablative γ-irradiation, as described previously.⁶ Two months after BMT, mice were subjected to either 4 weeks of treatment with CCl₄ (0.6 ml/kg body weight, diluted in corn oil, injected intraperitoneally every 3 days) or BDL. At this time, mice were sacrificed, and samples were collected.

Serum parameters

Alanine aminotransferase (ALT), aspartate aminotransferase (AST), bilirubin, and alkaline phosphatase (AP) activity (UV test at 37°C) were measured in the Central Laboratory Facility at University Hospital RWTH Aachen according to standard procedures.

Table S5.1 Origin and clinical features of liver samples from patients with DILI.

#	Sex	Age	Etiology	AST U/l	ALT U/l	Bili mg/dl	GLDH U/l	AP U/l	GGT U/l	TPT %	INR	αPTT sec	Creatine mg/dl	Phosphate mg/dl
1	f	50	Paracetamol	16253	4320	3.90	622.60	86	447	17	3.39	56.50	1.01	-
2	f	23	Phenprocoumon	215	154	3.20	-	63	216	42	1.73	48.00	0.94	3.80
3	m	48	Autoimmune	1004	1068	28.50	18.50	141	148	48	1.44	31.70	0.56	2.50
4	f	63	NSAID	189	209	4.30	10.60	85	38	24	2.73	56.40	0.87	3.00

f: female; m: male; Bili: total bilirrubin; GLDH: glutamate dehydrogenase; TPT: prothrombin time; INR: international normalized ratio; αPTT: activated partial thromboplastin time; NSAID: non-steroidal anti-inflammatory drug

Figure S5.1 (A) Activation of pJNK in liver samples of patient samples of normal liver human tissue (C3, C4). (B+C) PCR blots of tail DNA from *Wt* and *Jnk1^{Δhepa}* (upper panel) and control (*Wt*), *Jnk2^{-/-}*, and *Jnk1^{Δhepa}* (lower panel) mice confirmed the respective phenotype of interest.

Figure S5.2 (A+B) A basal study of untreated female (♀) and male (♂) 8-week-old control (*Wt*) or *Jnk^{Δhepa}* mice was performed. Representative macroscopic view of livers (scale bars: 10mm) and H&E staining of liver sections (scale bars 100 μ m) from each mice group are shown. (C+D) Serum AST and ALT were represented in a graph. (E+F) Liver weight (LW) and body weight of these mice are shown, and the liver versus body weight ratio (%LW/BW) is calculated.

Figure S5.3 (A) Control (Wt), *Jnk1^{Δhepa}* and *Jnk^{Δhepa}* mice were injected with APAP for 8h and sacrificed. The liver versus body weight ratio (%LW/BW) was then calculated. (B) Isoprostanes, indicators of lipid peroxides were measured in livers of 8h APAP-treated control (Wt) and *Jnk^{Δhepa}* mice and represented as pg/ml. (C) Densitometry analysis of the protein expression levels of P38 was performed in these mice and represented as arbitrary units of fluorescence (AUF).

Figure S5.4 (A) Control (*Wt*) or *Jnk^{Δhepa}* mice were injected with GalN and 30 min later with LPS. 8h thereafter, mice were sacrificed. (B) Survival curve of control (*Wt*) or *Jnk^{Δhepa}* mice treated with GalN+LPS and sacrificed 8h later. (C) Serum levels of AST, ALT and GLDH were determined after GalN+LPS-treated control (*Wt*) or *Jnk^{Δhepa}* mice. (D) Macroscopic (upper panel; Scale bars: 10mm) and microscopic (lower panel; Scale bars: 100 μm) appearance of control (*Wt*) or *Jnk^{Δhepa}* mice after 8h GalN+LPS treatment. (E) The percentage of liver vs. body weight ratio was calculated and represented in a graph.

Figure S5.5 (A) Representative macroscopic view of control (*Wt*), *Jnk2^{-/-}* and *JnkΔhepa* livers, 28 days after repeated injections of CCl₄. Corn-oil injections were used as controls. (B) Representative Sirius red staining of paraffin sections from the same livers. (C+D) Quantification of Sirius red and CollagenI A1 stainings was performed using Image J® Software and represented as percentage of area fraction. (E) Densitometry analysis of the protein expression levels of αSMA was performed in these mice and represented as arbitrary units of fluorescence (AUF). (F) mRNA levels of *Timp1* and *Mmp2* were determined by qRT-PCR in liver tissues of (n=5-10). Data are expressed as mean ± SEM (**P*<0.05; ***P*<0.01; ****P*<0.001).

Figure S5.6 Control (Wt), *Jnk2*^{-/-} and *Jnk*^{-/-hepa} livers were subjected for 4 weeks of repeated CCl₄ injections. **(A)** mRNA levels of *Pcna* were determined by qRT-PCR in liver tissues (n=6-8; ***P*<0.01). **(B)** Densitometry analysis of the protein expression levels of PCNA and CyclinD was performed in these mice and represented as arbitrary units of fluorescence (AUF).

Figure S5.7 The inflammatory profile is increased in the absence of JNK in hepatocytes. (A) Representative CD11b staining performed on frozen liver sections of control (Wt), *Jnk2^{-/-}* and *Jnk^{Δhepa}* livers after 4 weeks of repeated CCl₄ injections. Scale bars: 100μm. Quantification of positive cells was performed in 7-10 view-fields. (B) Representative F4/80 staining performed on frozen liver sections of the same livers. Scale bars: 100μm. Quantification of F4/80 positive cells per view field is shown. mRNA levels of *IL1α* (C), *IL1β* (D), *Mcp1* (E), and *Tnfα* (F) were determined by qRT-PCR in liver tissue (n=6-8). Data are expressed as mean ± SEM (**P*<0.05; ***P*<0.01; ****P*<0.001).

Figure S5.8 Venny diagram shows comparative gene expression overlap in untreated 8 week-old control (*Wt*) and *Jnk^{Δhepa}* livers and primary hepatocytes. **(A)** Up-regulated genes. **(B)** Down-regulated genes. **(C+D)** Validation of gene array analysis by mRNA expression was performed in liver tissue and primary hepatocytes of 8 week-old untreated control (*Wt*), *Jnk1^{Δhepa}*, and *Jnk^{Δhepa}* mice. The mRNA expression levels *Bad* and *Jnk2* are shown (* $P < 0.05$; ** $P < 0.01$; *** $P < 0.001$).

Figure S5.9 (A) Primary hepatocytes were isolated from control (*Wt*), *Jnk2^{-/-}* and *Jnk^{Δhepa}* mice. A total number of 500,000 cells were seeded in 6-well plates and cultivated for up to 48h. Visible light microphotographs were taken in untreated hepatocytes. Scale bars: 100μm. (B) Representative TUNEL stainings of primary hepatocytes. Dead cells are stained green; total cells were counterstained with DAPI (blue). Scale bars: 100μm. (C) Staining for BrdU was performed in coverslips and counterstained to DAPI (blue). Scale bars: 100μm. (D) BrdU-positive cells were counted in the untreated control (*Wt*) and *Jnk^{Δhepa}* hepatocytes and displayed in a graph.

Figure S5.10 Primary hepatocytes were isolated from control (*Wt*) and *Jnk*^{Δhepa} mice. A total number of 500,000 cells were seeded in 6-well plates and cultivated for up to 12h. **(A)** Reactive oxygen species (ROS) were measured in the presence or absence of APAP and/or SP600125, an inhibitor of the JNK pathway. **(B)** Annexin V (green)/Ethidium Homodimer III (red)/DAPI (blue) was performed and microphotographs were taken. **(C)** Quantification of the double Annexin V/Ethidium Homodimer III/DAPI-positive cells were graphed. Values are mean $\pm$ SEM from at least 6 mice per group (* P <0.05; ** P <0.01).

Figure S5.11 (A) Hepatocytic protein extracts from 8h-APAP-treated control (Wt) and *Jnk^{Δhepa}* primary hepatocytes were labeled with beads, digested with trypsin, and the phosphopeptides were enriched and analyzed using mass spectrometry. (B) The expression of pMAPK/CDK substrates was assessed in untreated and 8h-APAP-treated control (WT) and *Jnk^{Δhepa}* primary hepatocytes. Potential target proteins of JNK with matching molecular weights are displayed on the right. Values are mean $\pm$ SEM from at least 6 mice per group (** $P < 0.01$ and *** $P < 0.001$).

Supplementary References

1. Gentleman RC, Carey VJ, Bates DM, Bolstad B, Dettling M, Dudoit S, Ellis B, Gautier L, Ge Y, Gentry J, Hornik K, Hothorn T, Huber W, Iacus S, Irizarry R, Leisch F, Li C, Maechler M, Rossini AJ, Sawitzki G, Smith C, Smyth G, Tierney L, Yang JY, Zhang J. Bioconductor: open software development for computational biology and bioinformatics. *Genome Biol* 2004;5:R80.
2. Dai M, Wang P, Boyd AD, Kostov G, Athey B, Jones EG, Bunney WE, Myers RM, Speed TP, Akil H, Watson SJ, Meng F. Evolving gene/transcript definitions significantly alter the interpretation of GeneChip data. *Nucleic Acids Res* 2005;33:e175.
3. Preisinger C, Schwarz JP, Bleijerveld OB, Corradini E, Muller PJ, Anderson KI, Kolch W, Scholten A, Heck AJ. Imatinib-dependent tyrosine phosphorylation profiling of Bcr-Abl-positive chronic myeloid leukemia cells. *Leukemia* 2013;27:743-6.
4. Scholten A, Preisinger C, Corradini E, Bourgonje VJ, Hennrich ML, van Veen TA, Swaminathan PD, Joiner ML, Vos MA, Anderson ME, Heck AJ. Phosphoproteomics study based on in vivo inhibition reveals sites of calmodulin-dependent protein kinase II regulation in the heart. *J Am Heart Assoc* 2013;2:e000318.
5. Boersema PJ, Raijmakers R, Lemeer S, Mohammed S, Heck AJ. Multiplex peptide stable isotope dimethyl labeling for quantitative proteomics. *Nat Protoc* 2009;4:484-94.
6. Berres ML, Koenen RR, Rueland A, Zaldivar MM, Heinrichs D, Sahin H, Schmitz P, Streetz KL, Berg T, Gassler N, Weiskirchen R, Proudfoot A, Weber C, Trautwein C, Wasmuth HE. Antagonism of the chemokine Ccl5 ameliorates experimental liver fibrosis in mice. *J Clin Invest*;120:4129-40.

Chapter 6

**Liver injury after methylprednisolone pulses:
A disputable cause of hepatotoxicity.
A case series and literature review.**

Zoubek ME, Pinazo-Bandera J, Ortega-Alonso A, Hernandez N, Crespo J, Contreras F, Caliz-Medina I, Sanabria-Cabrera J, Sanjuan-Jimenez R, Gonzalez-Jimenez A, Garcia-Cortes M, Lucena MI , Andrade RJ, Robles-Diaz M

***United European Gastroenterol J* 2019;7(6):825-837**

DOI: 10.1177/2050640619840147

Abstract

Introduction & aims

Corticosteroids are empirically advocated in idiosyncratic hepatotoxicity with severe features. Interestingly, methylprednisolone is increasingly being recognized as responsible for episodes of liver injury. We aimed in this study to contribute to characterize liver injury related to methylprednisolone.

Methods

We analyzed demographical, clinical, laboratory and outcome data of four new instances of methylprednisolone hepatotoxicity included in the Spanish-Latin DILI Registry and compared this information with that of previously published cases.

Results

Case series

Three young females with multiple sclerosis and another one with Crohn's disease were treated with intravenous methylprednisolone. After one (patient 3), two (patient 1), and five (patient 2) and six (patient 4) weeks of starting medication, liver damage ensued. Time to recovery took eight and six weeks for patients 2 and 3, respectively, while patient 1 showed a resolving tendency after ten weeks (lost of follow up) and patient four after two weeks. Positive rechallenge was observed in three patients (patients 2, 3, and 4).

Literature review

Methylprednisolone hepatotoxicity was related to the female gender (84%). The mean age was 41 years, and the most common indications for treatment were multiple sclerosis (24 cases) and Graves' ophthalmopathy (13 cases). Hepatocellular damage was observed in all the cases with available data and 35% of the subjects presented with positive autoantibody titers. The average time to onset was six weeks. Four cases progressed to a fatal outcome. Accidental rechallenge occurred in 17 (38%) cases.

Conclusions

Methylprednisolone use can potentially induce severe liver injury, often with an autoimmune phenotype, particularly in patients with multiple sclerosis and Graves' ophthalmopathy. Consequently, the hepatic profile should be monitored in these groups of patients to prevent inadvertent reexposure.

Keywords

Methylprednisolone-induced liver injury, steroid pulses, multiple sclerosis, Graves' ophthalmopathy, autoimmune hepatitis.

Introduction

Methylprednisolone (MP), a synthetic glucocorticoid drug, exerts a potent anti-inflammatory and immunomodulatory effect and therefore is prescribed in severe inflammatory, autoimmune and neoplastic disorders as well as in the setting of transplant rejection, among others.¹ High dose intravenous MP is the standard therapy for relapsing multiple sclerosis and other autoimmune disorders, with doses ranging between 500 mg/day for 5 days to 1 g/day during 3 days.^{2,3}

Corticosteroids are considered very safe drugs as far as hepatotoxicity is concerned and hence are underrepresented in hepatotoxicity databases.⁴⁻⁹ However, a number of liver injury cases, mainly related to high dose methylprednisolone, have been reported in the last years. This has raised certain controversy with regard to attribution of causality since corticosteroids had been empirically advocated in the management of severe forms of idiosyncratic drug-induced liver injury as well as in cases of hepatotoxicity presenting with autoimmunity and systemic hypersensitivity features.¹⁰⁻¹²

In the present study, we aimed to characterize the phenotypic characteristics of MP-induced liver injury and the clinical context of hepatotoxicity presentation by analyzing demographical, clinical, laboratory and outcome data from the cases included in the Spanish and Latin-American DILI Registries and the previously published cases.

Materials and methods

Four cases of MP-induced liver injury included in the Spanish (2) and Latin-America (2) DILI Registries were analyzed and the information compared with that obtained from 45 previously reported cases or case series. The Spanish DILI Registry (1994) and the Latin-American branch (2011) are prospective databases that contain detailed demographic, clinical, laboratory, imaging and histological (when available) information both at presentation and at follow-up of patients.⁴

The applied biochemical DILI criteria followed the standard definition established by the Council for International Organizations of Medical Sciences (CIOMS) in 1989¹³ and readjusted to those of Aithal et al. in 2011.¹⁴ The type of liver injury was assigned based on the R ratio values from the first available blood test after DILI recognition. Cases were considered Hepatocellular (HC) when $R \geq 5$, cholestatic when $R \leq 2$ and mixed (MIX) when $2 < R < 5$ and the severity of the episode was categorized as mild, moderate, severe or fatal according to Aithal and colleagues.¹⁴ The CIOMS/RUCAM (Council for International Organizations of Medical Science/Roussel Uclaf Causality Assessment Method) scale was used for attribution of causality.¹⁵

Time to onset was considered as the time interval between treatment initiation with the culprit agent and either the appearance of compatible clinical symptoms or the first evidence of hepatic dysfunction in serum liver enzymes, whichever occurred first.

To assess the diagnosis of autoimmune hepatitis (AIH), we applied the simplified criteria, where a score of ≥ 6 and < 7 points suggested probable AIH, whereas a score of ≥ 7 points supported a definite AIH diagnosis.¹⁶

For the literature search, we used the U.S. National Library of Medicine (PubMed), developed and maintained by the National Center for Biotechnology Information (NCBI). Initially, the terms "hepatotoxicity" and "methylprednisolone" were used, however after validating the high number of events on MP-induced hepatotoxicity related to the terms "multiple sclerosis" and "Graves' ophthalmopathy", a new search was performed including these terms as well.

Results

Case series

Patient 1

A 31-year-old Caucasian female with a 3-year history of multiple sclerosis (MS) under treatment with interferon beta (IFN- β) and glatiramer acetate for the previous nine and three months, respectively, was admitted to hospital due to acute relapsing disease. The patient received a 3-day course of 300 mg/day intravenous pulse of MP.

Two weeks later, weakness, nausea, weight loss and jaundice ensued. Total bilirubin was 3.6 mg/dl (TBL < 1 mg/dl), aspartate aminotransferase 1420 U/l (AST < 31 U/l), alanine aminotransferase 2194 U/l (ALT < 31 U/l), and alkaline phosphatase (ALP) 229 U/l (ALP < 129 U/l) indicating hepatocellular injury (R=79). Liver biochemical parameters at the time of starting therapy were normal (**Figure 6.1A**).

All medications were stopped (**Figure 6.1A**). Serology for viral hepatitis A, B (HBV), C (HCV), cytomegalovirus (CMV), Epstein-Barr virus (EBV), human immunodeficiency virus (HIV) and *Treponema Pallidum* were negative. Immunoglobulins, ceruloplasmin, copper, iron and transferrin serum levels were within normal ranges. Antinuclear antibodies, antimitochondrial (AMA) and anti-smooth muscle antibodies (ASMA) were negative; however, positive ANA titers of 1/320 were found at the time of normalization. An abdominal CT scan and a cholangiography did not reveal liver masses or bile duct dilation. Liver biopsy performed one month after initiating MP treatment showed centrozonal necrosis with portal fibrosis and mixed inflammatory eosinophilic infiltrates. A maximum TBL value of 12.6 mg/dl was reached five weeks after liver injury onset.

Liver tests returned to normal within 70 days; however, ANA was detected at a titer of 1/320. CIOMS scale scored 7 points (probable) for MP.

Patient 2

A 36-year-old Caucasian female with a previous history of MS, depressive disorder and non-alcoholic fatty liver disease (NAFLD) with a normal hepatic panel (**Figure 6.1B**) on treatment with IFN- β for the last four years and sertraline during the last year. She presented with a relapsing MS and received a 3-day course of high-dose (1 g/d) intravenous methylprednisolone (MP). Prednisone oral doses of 60 mg/day were gradually reduced and ultimately withdrawn in three weeks.

Laboratory tests five weeks after starting MP showed acute hepatocellular injury: TBL 0.8 mg/dl (<1.1 mg/dl), AST 419 U/l (<27 U/l), ALT 630 U/l (<33 U/l), and ALP 193 U/l (<270 U/l). Serology for HAV, HBV, HCV, CMV and EBV was negative. ANA, AMA, ASMA and anti-liver kidney microsomal antibody type 1 [Anti-LKM-1] were all negative. An abdominal ultrasound showed normal liver parenchyma with the absence of dilated biliary ducts as well as a thin-walled gallbladder containing biliary sludge. Clinical and laboratory abnormalities subsided over the next days and were normal eight weeks later, and she remained on sertraline, but IFN- β treatment was removed. Several months later, during follow-up, liver parameters remained normal.

One year later, a similar episode occurred following MS exacerbation and the administration of intravenous MP (1 g/d for 3 days) and the same course of dose titration. Two weeks after cessation of MP treatment, abnormal liver parameters were found: TBL 0.46 mg/dl, AST 122 U/l, ALT 165 U/l, and ALP 125 U/l (**Figure 6.1B**). Viral hepatitis was excluded and autoimmunity markers were negative. Eleven weeks later, liver tests normalized.

In the next two years, owing to the relapsing-remitting Multiple Sclerosis, subsequent 3-day courses of high dose intravenous MP were given and followed by a flare of hepatocellular damage (first year: TBL 1.75 mg/dl, AST 896 U/l, ALT 1151 U/l, and ALP 314 U/l; second year: TBL 0.57 mg/dl, AST 485 U/l, ALT 696 U/l, ALP 172 U/l) (**Figure 6.1B**). Screening for viral hepatitis was again negative.

CIOMS scale yielded a score of 9 (highly probable) for MP hepatotoxicity with three positive rechallenges.

Patient 3

A 27-year-old female with a seven-year history of Crohn's disease was admitted to the hospital because of vomiting, mucous diarrhea and abdominal pain that did not respond to a one-week treatment of 16 mg/day oral dose of prednisone. After admission, the patient received a three-day course of intravenous MP (60 mg/day).

Two days later liver parameters were: TBL 0.83 mg/dl (<1.0 mg/dl), AST 472 U/l (<40 U/l), ALT 1446 U/l (<40 U/l) and ALP (<117 U/l) (**Figure 6.1C**). The patient denied toxic habits; however, she reported the use of the herbal *Aloe vera* for years and was on treatment with several drugs that had been initiated at hospitalization (**Table 6.1**). Serology excluded hepatitis A, B, C, E, CMV, and EBV infection. Autoimmune serology for AMA, ASMA, ANA, Anti-LKM was negative, and ceruloplasmin and alpha-1 antitrypsin levels were within normal.

Abdominal ultrasound and CT scan revealed no abnormalities in liver and biliary tracts. A thorough interrogation of the patient confirmed that three months earlier, a similar episode of acute liver injury had occurred following MS exacerbation and the administration of oral MP 48 mg/day (treatment duration unclear): TBL 0.25 mg/dl, AST 146 U/l, ALT 793 U/l, and ALP 83 U/l. CIOMS scale yielded a score above 8 (highly probable) for MP cytolytic liver injury (R=42). The patient fully recovered after six weeks.

Patient 4

A 33-year-old female with a 12-years history of MS was referred to the clinic due to marked jaundice, pruritus and elevated transaminases. The patient has had up to six MS exacerbations in the past and had received bolus, high dose (1 g/d) MP for 3 to 6 days followed by oral prednisone, with complete neurological remission. Routine biochemistry after treatment showed normal liver tests.

Following a new MS relapse and the intravenous administration of high dose MP (1 g/day for four days) and twelve days of oral prednisone, laboratory tests performed six weeks later indicated severe hepatocellular liver injury: TBL 13.9 mg/dl (<1 mg/dl) AST 1823 U/l (<104 U/l), ALT 2737 U/l (< 52 U/l) and ALP 169 (<104 U/l) and International Normalized Ratio (INR) 1.44 (**Figure 6.1D**). Serology ruled out viral causes. Screening for autoantibodies was negative, serum Ceruloplasmin and globulin levels were within the normal range, and abdominal ultrasound findings were normal. Clinical and laboratory findings at two weeks showed spontaneous improvement.

Ten months later, similar acute hepatitis-like signs and symptoms occurred following MS exacerbation and the administration of intravenous MP (1 g/d for 3 days). Laboratory work out showed: TBL 2.47 mg/dl, AST 1113 U/l, ALT 1500 U/l and INR 1.31, which returned to normal several weeks later. Three months later, a liver biopsy specimen showed minimal residual hepatitis without fibrosis. CIOMS scale scored 7 (probable) for reexposure to MP.

Literature review

An exhaustive review of the literature on MP-induced liver injury was carried out (**Tables 6.1-6.2**). A total number of 45 cases of MP-induced hepatotoxicity comprehended in six

case series and 22 single case reports from 2000 to 2017 were retrieved, carefully reviewed and analyzed in this study.¹⁷⁻⁴⁴

The majority of cases were female (84%) (**Table 6.1**). The average age was 41 years (range 11-74 years). However, the gender-specific mean age was 38 years in women (range 11-74 years) and 42 years in men (range 33-58 years). The main indications for MP therapy were exacerbated episodes of demyelinating diseases (27 cases of which the majority corresponded to MS (24), and the rest with one case each to retrobulbar optic neuritis, transverse myelitis and demyelinating encephalopathy) followed by Graves' ophthalmopathy (GO) with 13 cases. The remaining indications were interstitial pulmonary disease, central nervous system vasculitis and Hashimoto's thyroiditis with one case each, and two other cases of alopecia areata. Two of the cases with underlying MS also had a diagnosis of autoimmune thyroiditis (**Table 6.1**).

The administration route used for MP treatment in these cases was mainly intravenous (43 out of 45 cases), whilst the oral MP administration route was in two cases (cases 13 and 22). The average treatment duration was 25 days (range 3-154 days), and a mean duration of 5 days for demyelinating disorders and 47 days for GO was observed (**Table 6.1**).

Eighteen patients presented with clinical manifestations at onset. The most prevalent symptoms were weakness, generalized malaise, nausea, abdominal pain and pruritus. Jaundice was a common feature in symptomatic patients (83%). Besides, 18 patients were asymptomatic and diagnosis of liver injury was made on a routine laboratory basis. In the nine remaining cases, no information regarding symptomatology was available. The mean time to symptoms onset was six weeks (range 1-20 weeks), while in patients with MS and GO, the mean time to onset was six weeks (range 1-20 weeks) and nine weeks (range 2-18 weeks), respectively (**Table 6.1**).

Mean values for liver tests at peak were: TBL 7.5 mg/dl (range 0.8-24 mg/dl), AST 840 U/L (range 39-2384 U/L), ALT 1341 U/L (range 122-3028 U/L) and ALP 186 U/L (range 80-498 U/L). Fourteen cases presented with positive autoantibody titers (35%). Of those, seven cases had ANA titers, two cases ASMA titers, and one case had AntiLKM-1 titers. Three additional cases presented ANA and ASMA titers simultaneously and one case had AMA and ANA positive autoantibodies. In the remaining 26 cases, autoimmunity titers were negative, whilst in five other cases, autoantibodies were not available (**Table 6.2**).

Table 6.1 Summarized demographic and clinical information of methylprednisolone-induced liver injury events reviewed in literature (n=45) and new reported cases (n=3)

Case nr. - Author	Age (y)/ Sex (M/F)	Indication for MP treatment	Cumulative MP dosage g; (treatment duration, d)	Time to onset, w*	Concomitant medication	Clinical presentation	Time to resolution, w*	Liver biopsy	Outcome	Re- challenge, (n)
1.-Davidov et al.	23/F	MS	3 (3)	3	-	Jaundice, fever, nausea, vomits	12	Hepatocyte dropouts in central areas, congestion and chronic inflammation; portal tracts enlarged due to chronic inflammation (lymphocytes, eosinophils and plasma cells); in parenchyma, acidophilic bodies accompanied by inflammatory cells; hepatocytes showed signs of regeneration;	R	Yes (1)
2.-Moleti et al. ¹	58/M	GO	3.2 (84)	5	-	Asymptomatic	6		R	-
3.-Moleti et al. ²	50/F	GO	1.5 (84) + oral prednisone at interpulse periods	3	Methimazole	Asymptomatic	16		R	-
4.-Grilli et al.	35/F	MS	5 (5)	4	-	Jaundice	4	Nodular parenchyma partially surrounded by fibrotic septa emerging from portal tracts; moderately dense inflammatory infiltrate (lymphocytes, plasma cells, eosinophils and neutrophils); bridging and confluent necrosis;	R	Yes (1)
5.-Ferraro et al. ¹	50/F	MS	1 (5)	1	Lisinopril	N/A	4		R	-
6.-Ferraro et al. ²	30/F	MS	1 (5)	1	-	N/A	2		R	-
7.-Ferraro et al. ³	38/F	MS AITD	1 (5)	8	-	N/A	4	Inflammatory infiltrate, patched focal necrosis, periportal fibrosis and numerous ceroid-laden macrophages;	R	-
8.-Ferraro ⁴ et al.**	24/F	MS	1 (5) + 1-mo taper	16	-	N/A	16	Inflammatory infiltrate, patched focal necrosis, periportal fibrosis and numerous ceroid-laden macrophages;	R	Yes (3)
9.-Oliveira et al.	33/F	MS	1 (N/A) + oral maintenance	N/A	Cyclophosphamide, glatiramer acetate	Jaundice	N/A	Severe interface hepatitis and centrolobular hepatocyte necrosis, with mild fibrotic changes	R	Yes (4)

Table 6.1 (continued)

Case nr.- Author	Age (y)/ Sex (M/F)	Indication for MP treatment	Cumulative MP dosage g; (treatment duration, d)	Time to onset, w*	Concomitant medication	Clinical presentation	Time to resolution, w*	Liver biopsy	Outcome	Re- challenge, (n)
10.-Melamed et al.	52/M	GO	0.5 (14)	2	Atonvastatin, ciprofibrate, levothyroxin	Jaundice, weakness, arthralgia	28	-	R	Yes (1)
11.-D'Agnolo et al.	48/F	MS	1 (3)	3	-	Abdominal pain, nausea	N/A	-	R	Yes (1)
12.-Carrier et al.	30/F	MS	1 (N/A)	2	Acetamino- phen (levels below toxic doses)	Jaundice, asthenia	N/A	Significant bridging necrosis between central veins and portal tracts/ between portal spaces; in necrotic foci, inflammatory lymphocytic infiltrate with plasmatic cells and rare eosinophils; intrahepatocytic cholestasis; Cholestatic hepatitis with early stage fibrosis;	R	Yes (2)
13.-Alva et al.	38/F	ILD	0.96 (30)	N/A	N/A	Jaundice, rash, pruritus	24	-	R	-
14.-Furutama et al.	11/F	MS	3 (3)	6	-	Febricula, malaise	2	-	R	Yes (2)
15.-Gutkowski et al.	24/F	MS	3 (N/A)	4	IFN- β (2 ii) + APAP (single dose at each ii)	Jaundice	3	-	R	Yes (1)
16.-Loraschi et al. ¹	33/M	DE	2.5 (4)	5	-	Asymptomatic	3	Focal necrosis (acinar zones II and III), monocyte/macrophage infiltration, Kupffer cell hyperplasia, acidophilic bodies and focal microvesicular steatosis;	R	-
17.-Loraschi et al. ²	27/F	RBON	4.5 (6)	1	-	Asymptomatic	1	-	R	-
18.-Maamouri et al.	37/F	MS	3 (3)	3	-	Asymptomatic	10	Lympho-plasmatic inflammatory portal infiltrate with interphase hepatitis and bridging fibrosis;	R	-
19.-Rivero et al.	57/F	MS	3 (3)	1	-	Asymptomatic	12	Lytic necrosis and macrophage hyperplasia with ceroid-laden macrophages;	R	Yes (2)
20.-Takahashi et al.	43/F	MS	3 (3)	4	IFN- β	Jaundice, abdominal discomfort, nausea, vomiting	N/A	Perivascular bridging necrosis with inflamma-tory infiltration including eosinophils and interphase hepatitis;	R	Yes (1)

Table 6.1 (continued)

Case nr.- Author	Age (y)/ Sex (M/F)	Indication for MP treatment	Cumulative MP dosage g; (treatment duration, d)	Time to onset, w*	Concomitant medication	Clinical presentation	Time to resolution, w*	Liver biopsy	Outcome	Re- challenge, (n)
21. Reuß et al.	42/F	MS	5 (N/A)	3	-	Asymptomatic	N/A	Active hepatitis with portal lymphocyte infiltration and fibrosis;	-	-
22. Topal et al.	47/F	CNSV	0.224/d*** (7)	1	Topiramate	Jaundice, weakness, nausea, pruritus, acholia, choloria	6	-	R	-
23. Das et al.	48/F	MS	N/A	6	-	Jaundice, nausea, vomiting	N/A	Infiltrate with lymphocytes, eosinophils and plasmatic cells; more apparent in perivenular acinar region III; ballooning and presence of councilman bodies;	R	Yes (1)
24. Hofstee et al.	46/F	MS	1 (3)	6	-	Asymptomatic	16	-	R	Yes (2)
25. Marino et al.	43/F	GO	4.7 (N/A)	6	-	Asymptomatic	9	Focal lympho-plasmatic infiltrate affecting interlobular-biliary conducts; centrolobular necrosis;	R	-
26. Marino et al. ¹	56/N/A	GO	15 (56)	8	Estrogens	Jaundice, fatigue, anorexia, diarrhea	7	Massive necrosis	Exitus	-
27. Marino et al. ²	63/N/A	GO	9.3 (49)	8	Atenolol, propa-phenone, quinapril	Jaundice, fatigue, anorexia, diarrhea	7	N/A	Exitus	-
28. Marino et al. ³	47/N/A	GO	8.3 (35)	18	-	Jaundice, fatigue, anorexia, diarrhea	5	N/A	Exitus (post-LTX)	-
29. Marino et al. ⁴	45/N/A	GO	9.3 (49)	15	Ciprofloxacin	Asymptomatic	23	Moderate necrosis	R	-
30. Marino et al. ⁵	30/N/A	GO	7.2 (35)	18	-	Asymptomatic	18	Lymphocitic infiltrate with eosinophilia	R	-
31. Marino et al. ⁶	55/N/A	GO	10.7 (35)	16	Trandolapril	Asymptomatic	9	N/A	R	-

Table 6.1 (continued)

Case nr.- Author	Age (y)/ Sex (M/F)	Indication for MP treatment	Cumulative MP dosage g; (treatment duration, d)	Time to onset, w*	Concomitant medication	Clinical presentation	Time to resolution, w*	Liver biopsy	Outcome	Re- challenge, (n)
32. Marínó et al. ⁷	54/N/A	GO	4 (21)	4	Estrogens	Asymptomatic	20	Confluent necrosis; lymphocytic and plasmocellular infiltrate;	R	-
33.- Salvi et al.	43/F	HT	5.5 (154)	8	N/A	Asymptomatic	13	Lobular hepatitis with central necrosis, presence of lymphocytes and plasmatic cell infiltrates, acidophilic bodies and Kupfer cell hyperplasia	R	-
34. Weissel et al.	71/F	GO	15 (N/A) + taper (10-14 d)	4	Methimazole	N/A	2	Necrosis of liver parenchyma	Exitus	-
35. Dumontier et al. ¹	40/F	Alopecia Areata	N/A	7	N/A	N/A	30	-	R	-
36. Dumontier et al. ²	26/F	MS	N/A	11	N/A	N/A	4	Portal fibrosis, no sign of autoimmune hepatitis, cholestasis or steatosis (performed 2 months after the second episode)	R	Yes (2)
37. Dumontier et al. ³	27/F	MS	N/A	9	N/A	N/A	11	Focal liver cell necrosis in acinar zones 3	R	Yes (1)
38. Dumontier et al. ⁴	36/M	Alopecia Areata	N/A	5	N/A	N/A	7	Central lobular necrosis (30% of liver parenchyma)	R	-
39. Dumontier et al. ⁵	27/F	MS	N/A	4	Teriflunomide	Mild asthenia	154	Central lobular necrosis	R	Yes (2)
40. Hidalgo et al. ¹	28/F	MS	1.5 (9)	7	-	Jaundice, choleluria, acholia, nausea, vomiting	4	Lymphocyte-edematous background and fibrotic changes related to mild chronic hepatitis	R	Yes (1)
41. Hidalgo et al. ²	37/M	Transverse myelitis, MS	1 (5)	6	-	Asymptomatic	N/A	No inflammatory changes (biopsy performed 2 months after episode)	R	-
42. Hidalgo et al. ³	35/F	MS	1 (5)	1	-	Asymptomatic	N/A	-	R	-
43. Abramavicius et al.	74/F	GO	4 (57)	5	Methimazole	Asymptomatic	N/A	-	R	-
44.-Lee et al.	34/M	MS	1 (5) + 1/mo maintenance dose	20	-	Asymptomatic	48	Central lobular hepatic cell necrosis	R	-

Table 6.1 (continued)

Case nr.- Author	Age (y)/ Sex (M/F)	Indication for MP treatment	Cumulative MP dosage g; (treatment duration, d)	Time to onset, w*	Concomitant medication	Clinical presentation	Time to resolution, w*	Liver biopsy	Outcome Re- challenge, (n)
45. Kadle et al.	51/F	AITD, MS	0.5 (10)	7	-	Weakness, anorexia, weight loss, jaundice	22	-	R
46. Patient 1 (displayed)	31/F	MS	0.9 (3)	2	IFN- β , glatimere acetate	Jaundice, weakness, nausea	N/A	Acute hepatitis, portal fibrosis and mixed infiltration including eosinophils. Centro-zonal necrosis.	N/A
47. Patient2 (displayed)	36/F	MS	3 (3) + 60 mg/day p.o. (21 days)	2	IFN- β , sertraline	Asymptomatic	12	-	Yes (1)
48. Patient 3 (displayed)	27/F	Crohns' disease	0.18 (3)	1	Prednisone, AV, EP, CaC, CaG, PP, MMZ, APAP (levels below toxic doses);	Asymptomatic	11	-	Yes (1)
49. Patient 4 (displayed)	33/F	MS	4 (4) + prednisone p.o. (12 days)	6	-	Jaundice, intermittent pruritus	N/A	Minimal residual hepatitis without fibrosis	Yes (1)

MP, methylprednisolone; M, male; F, female; Y, years; N/A, not available; w, weeks; d, days; g, gram; MS, multiple sclerosis; GO, Graves' ophthalmopathy; AITD, autoimmune thyroid diseases; IFN- β , interferon beta; ILD, interstitial lung disease; DE, demyelinating encephalopathy; CNSV, central nervous system vasculitis; RBON, retrobulbar optic neuritis; LTX, liver transplantation; HT, Hashimoto's thyroiditis; EP, enoxiparine; CaC, calcium gluconate; CaG, calcium gluconate; PP, pantoprazole, MMZ, matamizole; APAP, acetaminophen; AV, *aloe vera*; ij, injections; R, recovery. The data displayed in table 3 correlates with a first exposure of methylprednisolone (in case accidental re-challenge applies) or with the exposure, which has been described in the article and/or presented enough information.*Time units have been registered as weeks and when days were indicated in the original publication were rounded up (i.e. 1-7 days=1 week; 8-14 days=2 weeks, etc). **Three episodes of methylprednisolone-induced liver injury happened within eight months. ***Exclusively methylprednisolone administration p.o.

A

B

C

D

Figure 6.1 A-D Temporal relationship between *i.v.* MP boluses and biochemically liver dysfunction in patients 1-4. The data shown above corresponds to patient 1 with a single episode after exposure to MP (A), patient 2, who developed after a first episode up to three more events of MP hepatotoxicity (B), patient 3, who suffered a first episode and later developed a positive reexposure (C) and patient 4 who after a first event of MP-induced liver injury suffered from rechallenge due to MP (D). Concomitant treatments present at the onset of each liver dysfunction episode are displayed. **Abbreviations:** MP, methylprednisolone; TBL, serum total bilirubin (mg/dl); ALT, alanine aminotransferase (U/l); ALP, alkaline phosphatase (U/l); Interferon- β , interferon-beta; Bx, biopsy; g, gram; d, day; *i.v.*, intravenous; RC, rechallenge; CaC, calcium carbonate; CaG, calcium gluconate; MCP, metoclopramide; *prn*, *pro re nata*

Table 6.2 Summarized biochemical, liver injury pattern and autoimmunity information of methylprednisolone-induced liver injury events at peak reviewed from literature (n=45) and new reported cases (n=4)

Author	Case	TBL (mg/dl)*	AST (U/l)	ALT (U/l)*	ALP (U/l)*	Type injury**	of Autoantibodies***
Davidov et al.	1	6.3	N/A	2287	123	HC	ASMA+
Moleti et al. ¹	2	N/A	486	809	N/A	N/A	-
Moleti et al. ²	3	N/A	338	903	N/A	N/A	ANA+ (low titers)
Grilli et al.	4	23.9	1104	2000	114	HC	-
Ferraro et al. ¹	5	N/A	136	355	N/A	N/A	-
Ferraro et al. ²	6	N/A	157	627	N/A	N/A	-
Ferraro et al. ³	7	N/A	109	260	N/A	HC**	ANA+ (1:640); ASMA+ (1:80)
Ferraro et al. ⁴	8	N/A	315	671	N/A	HC**	-
Oliveira et al.	9	16	710	2308	92	HC	ANA+ (1:40)
Melamud et al.	10	3.4	283	465	80	HC	-
D'Agnolo et al.	11	1.7	2384	3028	N/A	HC**	-
Carrier et al.	12	6.08	1731	2899	N/A	HC**	ANA+ (1:160)
Alva et al.	13	3.32	1428	2618	134	HC	-
Furutama et al.	14	0.8	278	428	N/A	N/A	ANA+ (1:80)
Gutkowski et al.	15	17.9	900	1740	186	HC	ASMA+ (>1:320)
Lorascchi et al. ¹	16	N/A	349	1042	N/A	HC**	-
Lorascchi et al. ²	17	N/A	39	122	N/A	N/A	-
Maámourí et al.	18	1	229	553	228	HC	ANA+ (1:100); ASMA+ (1:100)
Rivero et al.	19	N/A	1328	2685	115	HC	-
Takahashi et al.	20	3.4	1102	1067	377	HC	ANA+ (1:80); ASMA+
Reuß et al.	21	N/A	485	1082	N/A	HC**	N/A
Topal et al.	22	10	1600	2478	138	HC	-
Das et al.	23	N/A	1500	1600	200	HC	-
Hofstee et al.	24	N/A	755	1095	140	HC	-
Marinò et al.	25	N/A	990	1419	N/A	HC**	-
Marinò et al. ¹	26	N/A	2280	2490	498	HC	ANA+ (low titers)
Marinò et al. ²	27	N/A	68	179	100	HC	N/A
Marinò et al. ³	28	N/A	N/A	N/A	N/A	N/A	N/A

Table 6.2 (continued)

Author	Case	TBL (mg/dl)*	AST (U/l)	ALT (U/l)*	ALP (U/l)*	Type injury**	of Autoantibodies***
Marinò et al. ⁴	29	N/A	888	1971	178	HC	N/A
Marinò et al. ⁵	30	N/A	457	930	237	HC	Anti-LKM1
Marinò et al. ⁶	31	N/A	635	1044	245	HC	-
Marinò et al. ⁷	32	N/A	948	1815	183	HC	-
Salvi et al.	33	N/A	634	1152	N/A	HC**	ANA+ (high titers)
Weissel et al.	34	N/A	N/A	N/A	N/A	HC**	N/A
Dumontier et al. ¹	35	3.68	29 [#]	34 [#]	N/A	HC ^{##}	-
Dumontier et al. ²	36	N/A	14 [#]	33 [#]	N/A	HC ^{##}	-
Dumontier et al. ³	37	N/A	5.3 [#]	9.6 [#]	N/A	HC ^{##}	-
Dumontier et al. ⁴	38	N/A	N/A	50 [#]	N/A	HC ^{##}	-
Dumontier et al. ⁵	39	3.7	2235	1704	N/A	HC ^{##}	-
Hidalgo et al. ¹	40	N/A	N/A	N/A	N/A	HC**	-
Hidalgo et al. ²	41	N/A	N/A	N/A	N/A	N/A	-
Hidalgo et al. ³	42	N/A	N/A	N/A	N/A	N/A	-
Abramavicius et al.	43	N/A	94	164	N/A	N/A	AMA+ (1/40); ANA+ (1/40)
Lee et al.	44	N/A	660	1242	N/A	HC**	-
Kadle et al.	45	N/A	1774	1047	186	HC	ANA+ (1/160)
Patient 1 (displayed)	46	3.6	1420	2194	229	HC	ANA+ (1/320)
Patient 2 (displayed)	47	1.30	771	1459	106	HC	-
Patient 3 (displayed)	48	0.93	539	1589	108	HC	-
Patient 4 (displayed)	49	13.9	1823	2737	169	HC	-
Mean U/l (range) [§]		6.9 (0.8-24)	871 (39-2384)	1406 (122-3028)	181 (80-489)		

TBL, serum total bilirubin; AST, aspartate aminotransferase; ALT, alanine aminotransferase; GGT, gamma-glutamyl transferase; ALP, alkaline phosphatase; ANA, antinuclear antibody titers; ASMA, anti-smooth muscle antibody titers; Anti-LKM1, anti-liver kidney microsomal antibody; AMA, antimitochondrial antibody; N/A, not available; *In those cases with more than one episode (+ re-challenge), parameters of the event with the higher peak of ALT was considered; **When ALT values and/or pathological findings suggestive of hepatic cytolytic features, but ALP values not available; ***Positive autoantibodies after any exposition (if an accidental re-challenge had taken place). #Normalized values provided by author. rough parameters U/L not available; ##Ratio provided by author; §Mean was calculated based on those reports which included AST, ALT and ALP data in U/l

Liver biopsy information was available for 27 patients (60%). The main findings were necrosis in 17 cases and apoptotic changes in two cases. Mixed inflammatory infiltrates were present in 12 cases. Infiltrates with lymphocytes, plasmatic cells, eosinophils, macrophages and Kupffer cell hyperplasia were observed in cases 2, 4, 7, 9 and 12. Eight cases displayed fibrotic changes, two cases showed signs of cholestasis and three more cases showed acinar damage in zone III (**Table 6.1**). Interestingly, the pattern of liver injury based on biochemical parameters was hepatocellular (n=35) in all cases with available information (**Table 6.2**).

Most MP-induced liver injury cases recovered from the episode (89%), but there was a fatal outcome in four cases. Patient no. 28 underwent liver transplantation but died. The mean time to resolution was 15 weeks (range 1-154 weeks) and in the four fatal cases, the mean time to exitus was six weeks (range 5-7 weeks). In 17 cases, inadvertent rechallenge to the drug was observed, and in eight of those cases, reexposure to MP occurred several times (**Table 6.1**).

Methylprednisolone had been the only administered drug in 24 cases, while in 15 other cases, MP administration was given along with other associated medications. The remaining six cases did not specify any information on concomitant treatments (**Table 6.1**).

Discussion

Methylprednisolone is often used as the treatment of choice against severe hepatitis as well as liver disease with autoimmune features due to its potent anti-inflammatory and immunosuppressive activities, and it has been considered a rare cause of DILI.⁴⁵ Nevertheless, boluses, high-dose courses of MP can induce severe hepatitis that recurs with repeated administration of the drug and occasionally can lead to a fatal progression.^{37,39} The rate of published case reports on MP-induced liver injury has increased during the last two decades.¹⁷⁻⁴⁴

In order to establish the association between liver injury and MP use, an accurate rule-out diagnosis against other potential aetiologies of hepatitis is required. Thus, for the four herewith presented cases, other potential causes of hepatic injury have been exhaustively excluded, notwithstanding, in patient 4, no imaging tests were done, and no CMV and EBV serologic tests were performed (see above). Regarding the bibliographically reviewed studies, most of these presented a detailed exclusion diagnosis. In case 34, only viral serology was taken into account with no available imaging tests, and in cases 21 and 27 to 29, no autoantibody titers were measured. In case 31, a previous history of hepatitis B was known; however, HBV DNA quantification resulted in negative; consequently, an active infection of the virus was excluded. Case 32 displayed high positive titers for Anti-CMV IgM; here, rather than drug-derived toxicity, MP most likely may have induced acute viral hepatitis due to the intense immunosuppression.

When more than one drug coincides in the setting of a DILI episode, a meticulous judgment of the culprit compound is required. Regarding patient 1, she had been treated with IFN- β and glatiramer acetate prior to *i.v.* administration of MP due to an outbreak of the patient's underlying MS. Normal laboratory panels from the time previous to MP administration, when the patient had already been in treatment with IFN- β , most likely pointed to MP-associated liver injury causality. Patient 2 was concomitantly treated with sertraline and IFN- β when the first episode befell. The patient also had normal biochemical parameters from the time before the episode, while the patient was in treatment with sertraline and IFN- β . Furthermore, in patient 2, after the first episode, three more positive reexposures to MP were observed, whilst IFN- β had not been administered during all three rechallenge episodes. Sertraline was not stopped during the succession of episodes, but complete normalization of the liver panel occurred between episodes. Patient 3 declared the use of *Aloe vera* for many years before without complications and presented various concomitant drugs, which had been started immediately after hospitalization (prednisone [administered during the week before admission and re-administered after *i.v.* MP-course], enoxaparin, calcium gluconate, calcium carbonate, pantoprazole, metamizole, acetaminophen [levels below toxic doses]), but suffered a positive reexposition to MP. The therapy scheme in patient 4 included oral prednisone after the fifth day of MP; however, the patient took prednisone in variable courses during the previous ten years without any signs of liver dysfunction. In addition, rechallenge with MP led to a further flare in liver transaminases. In one reported event from the literature, the patient exhibited only drug-induced liver dysfunction after exposures to methylprednisolone, despite being in treatment with the glucocorticoid agent dexamethasone (case 11).²³ This fact may suggest a potential relevance of drug-specific factors in contributing to the hosts' predisposition to suffer from MP-induced liver injury.

Most of the episodes resulted in resolution of the liver dysfunction episode, except for four of the previously published events, where the patients suffered a fatal outcome.^{37,39} Concerning fatal events, it was adverted that all four subjects had suffered from a GO relapse and were exposed to higher cumulative MP doses (mean 12g [range 15-8.3g]) compared to the rest of analyzed patients (mean cumulated MP dose 2 g [range 0.18-10.7g]).

In all the analyzed events, MP was administered to treat exacerbations of an underlying autoimmune disease. High-dosed MP administered as *i.v.* bolus is not exclusively restricted to autoimmune relapses but commonly used in a broad spectrum of diseases such as asthma, COPD recrudescences or medullar compression.^{46,47} Despite greater use of MP in patients with these indications, there are no reported cases of DILI in the literature. This circumstance suggests that patients with an underlying autoimmune disease, particularly MS or GO, have a notably higher susceptibility to suffering from MP-induced liver injury, as other authors have hypothesized before.⁴⁸

Out of the 45 previously reported cases, 14 events displayed positive autoantibody titers. Patient 1 presented positive ANA titers of 1:320, whereas autoimmunity markers were negative in patients 2-4. The fact that an underlying autoimmune disease was present in the patient's previous medical record makes it particularly challenging to address the origin of positive autoantibodies and/or alteration of autoantibody titers. Most likely, positive autoimmunity features may derive from the underlying autoimmune disease.⁴⁹

In five of the reviewed MP-induced liver injury events, the diagnosis of AIH induced by MP was established (cases 18, 20, 21, 25, and 33). The physiopathology relies on the fact that after initiating MP therapy, a strong suppression of the immune system occurs. However, after sudden discontinuation of the drug, immunosuppression reverts, and the immune system reconstitutes; this condition may then trigger AIH.⁵⁰ For cases 20, 25, and 33, laboratory panels previous to the hepatotoxicity episode confirmed serum liver enzymes within normal ranges; thus, an underlying undiagnosed AIH is less probable. In cases 18 and 21, no reference to serum tests previous to the episode was available. After liver dysfunction was diagnosed in cases 20, 25, and 33, therapy was rearranged to oral corticoids (prednisolone in case 20 and prednisone in the other ones). In case 18, treatment with azathioprine and prednisone *p.o.* followed after AIH diagnosis. The time to resolution lasted 10, 9, and 13 weeks for cases 18, 25, and 33, respectively. Similarly, for the rest of MP-induced liver injury events, the mean time to recovery was 15 weeks (range 1-154 weeks) and occurred simply by dechallenging the drug without additional support therapy. The similar recovery profile regarding serum liver enzymes and the time to resolution suggests that perhaps the improvement of the hepatic function occurred independently from the immunosuppressive therapy administered in cases 18, 25, and 33. It is complex to distinguish MP-induced liver injury from MP-induced AIH; nevertheless, there is a well-defined timeline between treatment initiation with MP and liver injury development, with a mean time to onset of 6 weeks (range 1-20 weeks) for these cases. Thus, the existing relationship between liver injury and the intake of MP is undeniable in these patients.

Another aspect of interest in MP-induced liver injury is the high rate of rechallenge that patients suffered. As already known, positive rechallenge due to a suspected drug agent strengthens the association to drug-induced hepatotoxicity causality, and indeed, it is commonly used as a feature in hepatotoxicity scales, such as the CIOMS/RUCAM scoring system.^{51,52} In the revised published cases, up to 38% of these developed a positive rechallenge (17 out of 45 cases). In patients 2-4, three, one, and one, reexposures to MP happened respectively and led to a new ALT flare. The high reoccurrence in MP-induced liver injury most likely relies on the fact that MPs' potential to induce liver injury is generally underestimated, and cases remain consequently undiagnosed, which may incite further exposures to the drug. The high number in recurrences, together with the fact that fatal progression has been described in MP-induced liver injury, is doubtlessly a matter of concern. Additionally, MP-induced liver injury is associated with considerable morbidity and

mortality regarding the severity of liver injury presented (Table 6.2). Hence, an early diagnosis may be of crucial importance in MP-induced liver injury to discontinue the drug, preventing accidental rechallenge and reducing accumulated liver injury in these patients.

Other authors have insinuated that MP-induced liver injury could result from metabolic idiosyncrasy and the effect of chemically reactive agents (MP is mainly metabolized by the cytochrome P450 (CYP) 3A isoenzymes CYP3A4 and CYP3A5).^{28,33} Under our perspective, immune-mediated mechanisms of hepatocyte injury might play a more relevant role rather than collateral damage through metabolic idiosyncrasy. Indeed, the adaptative immune response is believed to play mechanistically a pivotal role in idiosyncratic DILI and MP is undoubtedly well known for its suppressive immunomodulatory effect, which has been indicated against severe forms of liver injury.⁵³ Thus, one may hypothesize that the MP-induced immunosuppression could awaken a self-immune reaction in susceptible hosts that manifests with typical AIH features, but in other occasions could remain unnoticed as cases of atypical AIH due to its challenging diagnosis per se (e.g., lack of data, heterogeneous pattern).

In conclusion, MP-induced liver injury is a more than viable suspicion diagnosis that has to be considered, especially after *i.v.* administration and in the setting of an underlying autoimmune disease outbreak, particularly MS and GO. The apparent liver injury frequently appears during the first week after treatment with a hepatocellular type of liver injury and with an extensive range of severity from asymptomatic or mild to severe and fatal. Patients presenting an underlying autoimmune disease require a closer follow-up during and after high-dose MP *i.v.* therapy.

References

1. Zoorob RJ, Cender D. A different look at corticosteroids. *Am Fam Physician*. 1998;58:443-450.
2. Franchin G, Diamond B. Pulse steroids: How much is enough? *Autoimmun Rev*. 2006;5(2):111-3.
3. Sellebjerg F, Barnes D, Filippini G, et al. EFNS guideline on treatment of multiple sclerosis relapses: report of an EFNS task force on treatment of multiple sclerosis relapses. *Eur J Neurol*. 2005;12(12):939-46.
4. Andrade RJ, Lucena MI, Fernandez MC, et al. Drug-induced liver injury: an analysis of 461 incidences submitted to the Spanish registry over a 10-year period. *Gastroenterology*. 2005;129:512-521.
5. Chalasani N, Bonkovsky HL, Fontana R, et al. Features and Outcomes of 899 Patients With Drug-Induced Liver Injury: The DILIN Prospective Study. *Gastroenterology*. 2015;148:1340-1352.
6. Björnsson ES, Bergmann OM, Björnsson HK, et al. Incidence, presentation, and outcomes in patients with drug-induced liver injury in the general population of Iceland. *Gastroenterology*. 2013;144:1419-1425.
7. Bessone F, Hernandez N, Lucena MI, et al. The Latin American DILI Registry Experience: A Successful Ongoing Collaborative Strategic Initiative. *Int J Mol Sci*. 2016;17:313.
8. Suk KT, Kim DJ, Kim CH, et al. A prospective nationwide study of drug-induced liver injury in Korea. *Am J Gastroenterol*. 2012;107:1380-1387.

9. Devarbhavi H, Dierkhising R, Kremers WK, et al. Single-center experience with drug-induced liver injury from India: causes, outcome, prognosis, and predictors of mortality. *Am J Gastroenterol.* 2010;105:2396-2404.
10. Chalasani NP, Hayashi PH, Bonkovsky HL, et al. ACG Clinical Guideline: the diagnosis and management of idiosyncratic drug-induced liver injury. *Am J Gastroenterol.* 2014;109:950-966.
11. Yu YC, Mao YM, Chen CW, et al. CSH guidelines for the diagnosis and treatment of drug-induced liver injury. *Hepatol Int.* 2017;11:221-241.
12. Giordano C, Rivas J, Zervos X. An Update on Treatment of Drug-Induced Liver Injury. *J Clin Transl Hepatol.* 2014;2:74-79.
13. Benichou C. Criteria of drug-induced liver disorders. Report of an international consensus meeting. *J Hepatol.* 1990;11:272-276.
14. Aithal GP, Watkins PB, Andrade RJ, et al. Case definition and phenotype standardization in drug-induced liver injury. *Clin Pharmacol Ther.* 2011;89:806-815.
15. Danan G, Benichou C. Causality assessment of adverse reactions to drugs I. A novel method based on the conclusions of international consensus meetings: application to drug-induced liver injuries. *J Clin Epidemiol.* 1993;46:1323-1330.
16. Hennes EM, Zeniya M, Czaja AJ, Pares A, Dalekos GN, Krawitt EL, et al. Simplified criteria for the diagnosis of autoimmune hepatitis. *Hepatology.* 2008;48:169-176.
17. Davidov Y, Har-Noy O, Pappo O, et al. Methylprednisolone-induced liver injury: Case report and literature review. *J Dig Dis.* 2016;17:55-62.
18. Moleti M, Giuffrida G, Sturniolo G, et al. Acute liver damage following intravenous glucocorticoid treatment for Graves' ophthalmopathy. *Endocrine.* 2016;54(1):259-268.
19. Grilli E, Galati V, Petrosillo N, et al. Incomplete septal cirrhosis after high-dose methylprednisolone therapy and regression of liver injury. *Liver Int.* 2015;35:674-676.
20. Ferraro D, Mirante VG, Losi L, et al. Methylprednisolone-induced Toxic Hepatitis After Intravenous Pulsed Therapy for Multiple Sclerosis Relapses. *Neurologist.* 2015;19:153-154.
21. Oliveira AT, Lopes S, Cipriano MA, et al. Induced liver injury after high-dose methylprednisolone in a patient with multiple sclerosis. *BMJ Case Rep.* 2015; 2015.
22. Melamud B, Lurie Y, Goldin E, et al. Methylprednisolone-induced liver injury: a diagnostic challenge. *Isr Med Assoc J.* 2014;16:180-181.
23. D'Agnolo HM, Drenth JP. High-dose methylprednisolone-induced hepatitis in a patient with multiple sclerosis: a case report and brief review of literature. *Neth J Med.* 2013;71:199-202.
24. Carrier P, Godet B, Crepin S, et al. Acute liver toxicity due to methylprednisolone: consider this diagnosis in the context of autoimmunity. *Clin Res Hepatol Gastroenterol.* 2013;37:100-104.
25. Alva AS, Bhasin A, Vijayaraghavan S. A rare case of methylprednisolone induced hepatitis. *J Clin Exp Hepatol.* 2013;3: Suppl 1: S37.
26. Furutama D, Kimura F, Shinoda K, et al. Recurrent high-dose intravenous methylprednisolone succinate pulse therapy-induced hepatopathy in a patient with multiple sclerosis. *Med Princ Pract.* 2011;20:291-293.
27. Gutkowski K, Chwist A, Hartleb M. Liver injury induced by high-dose methylprednisolone therapy: a case report and brief review of the literature. *Hepat Mon.* 2011;11:656-661.
28. Loraschi A, Banfi P, Mauri M, et al. Hepatotoxicity after high-dose methylprednisolone for demyelinating disease. *Clin Neuropharmacol.* 2010;33:52-54.
29. Maàmour N KS, Ben Hariz F et al. Bolus de méthylprednisolone révélant une hépatite auto-immune chez une patiente atteinte de sclérose en plaques. *J Afr Hépatol Gastroentérol.* 2009;3: 195-197.
30. Rivero Fernandez M, Riesco JM, Moreira VF, et al. Recurrent acute liver toxicity from intravenous methylprednisolone. *Rev Esp Enferm Dig.* 2008;100:720-723.
31. Takahashi A, Kanno Y, Takahashi Y, et al. Development of autoimmune hepatitis type 1 after pulsed methylprednisolone therapy for multiple sclerosis: a case report. *World J Gastroenterol.* 2008;14:5474-5477.
32. Reuß R RK, Vogel S, Franke FE, Oschmann P. Autoimmune hepatitis after high-dose intravenous methylprednisolone pulse in RR-MS. *Central European Journal of Medicine.* 2007;2(3):356-359.
33. Topal F, Ozaslan E, Akbulut S, et al. Methylprednisolone-induced toxic hepatitis. *Ann Pharmacother.* 2006;40:1868-1871.
34. Das D, Graham I, Rose J. Recurrent acute hepatitis in patient receiving pulsed methylprednisolone for multiple sclerosis. *Indian J Gastroenterol.* 2006;25:314-316.
35. Hofstee HM, Nanayakkara PW, Stehouwer CD. Acute hepatitis related to prednisolone. *Eur J Intern Med.* 2005;16:209-210.

36. Marino M, Morabito E, Altea MA, et al. Autoimmune hepatitis during intravenous glucocorticoid pulse therapy for Graves' ophthalmopathy treated successfully with glucocorticoids themselves. *J Endocrinol Invest*. 2005;28:280-284.
37. Marino M, Morabito E, Brunetto MR, et al. Acute and severe liver damage associated with intravenous glucocorticoid pulse therapy in patients with Graves' ophthalmopathy. *Thyroid*. 2004;14:403-406.
38. Salvi M, Vannucchi G, Sbrozzi F, et al. Onset of autoimmune hepatitis during intravenous steroid therapy for thyroid-associated ophthalmopathy in a patient with Hashimoto's thyroiditis: case report. *Thyroid*. 2004;14:631-634.
39. Weissel M, Hauff W. Fatal liver failure after high-dose glucocorticoid pulse therapy in a patient with severe thyroid eye disease. *Thyroid*. 2000;10:521.
40. Dumortier J, Cottin J, Lavie C, et al. Methylprednisolone liver toxicity: A new case and a French regional pharmacovigilance survey. *Clin Res Hepatol Gastroenterol*. 2017;41:497-501.
41. Abramavicius S, Velickiene D, Kadusevicius E. Methimazole-induced liver injury overshadowed by methylprednisolone pulse therapy: Case report. *Medicine (Baltimore)*. 2017;96:8159.
42. Hidalgo de la Cruz M, Miranda Acuna JA, Lozano Ros A, et al. Hepatotoxicity after high-dose intravenous methylprednisolone in multiple sclerosis patients. *Clin Case Rep*. 2017;5:1210-1212.
43. Lee HM, Ditelberg JS, Kaplan MM. Pericentral liver cell necrosis associated with the use of high-dose intravenous methylprednisolone. *Dig Dis Sci*. 2007;52:1533-1534.
44. Kadle MAH, Mazurchik NV. Hepatotoxicity Induced by High Dose of Methylprednisolone Therapy in a Patient with Multiple Sclerosis: A Case Report and Brief Review of Literature. *Open Journal of Gastroenterology*. 2016; 6:146-150.
45. Topal F, Ozaslan E, Akbulut S, et al. Methylprednisolone-induced toxic hepatitis. *The Annals of pharmacotherapy*. 2006;40:1868-1871.
46. Bracken MB, Shepard MJ, Holford TR, et al. Administration of methylprednisolone for 24 or 48 hours or tirilazad mesylate for 48 hours in the treatment of acute spinal cord injury. Results of the Third National Acute Spinal Cord Injury Randomized Controlled Trial. National Acute Spinal Cord Injury Study. *JAMA*. 1997;277:1597-1604.
47. Walters JA, Gibson PG, Wood-Baker R, et al. Systemic corticosteroids for acute exacerbations of chronic obstructive pulmonary disease. *Cochrane Database Syst Rev*. 2009;CD001288.
48. de Seze J, Canva-Delcambre V, Fajardy I, et al. Autoimmune hepatitis and multiple sclerosis: a coincidental association? *Mult Scler*. 2005;11:691-693.
49. Szmyrka-Kaczmarek M, Pokryszko-Dragan A, Pawlik B, et al. Antinuclear and antiphospholipid antibodies in patients with multiple sclerosis. *Lupus*. 2012;21:412-420.
50. Livertox. <https://livertox.nlm.nih.gov/Corticosteroids.htm>. Accessed October 28, 2017.
51. Lee WM, Senior JR. Recognizing drug-induced liver injury: current problems, possible solutions. *Toxicol Pathol*. 2005;33:155-164.
52. Andrade RJ, Robles M, Lucena MI. Rechallenge in drug-induced liver injury: the attractive hazard. *Expert Opin Drug Saf*. 2009;8:709-714.
53. Yuan L, Kaplowitz N. Mechanisms of Drug Induced Liver Injury. *Clin Liver Dis*. 2013; 17(4):507-518.

Chapter 7

Definition and risk factors for chronicity following acute idiosyncratic drug-induced liver injury

Medina-Caliz I, Robles-Diaz M, Garcia-Muñoz B, Stephens C, Ortega-Alonso A, Garcia-Cortes M, González-Jimenez A, Sanabria-Cabrera JA, Moreno I, Fernandez MC, Romero-Gomez M, Navarro JM, Barriocanal AM, Montane E, Hallal H, Blanco S, Soriano G, Roman EM, Gómez-Dominguez E, Castiella A, Zapata EM, Jimenez-Perez M, Moreno JM, Aldea-Perona A, Hernández-Guerra M, Prieto M, Zoubek ME, Kaplowitz N, Lucena MI, Andrade RJ; Spanish DILI registry

***J Hepatol* 2016;65(3):532-542**

DOI: 10.1016/j.jhep.2016.05.003

Abstract

Background & Aims

Chronic outcome following acute idiosyncratic drug-induced liver injury (DILI) is not yet defined. This prospective, long-term follow-up study aimed to analyze time to liver enzyme resolutions to establish the best definition and risk factors of DILI chronicity.

Methods

298 out of 850 patients in the Spanish DILI registry with no pre-existing disease affecting the liver and follow-up to resolution or P1 year were analyzed. Chronicity was defined as abnormal liver biochemistry, imaging test or histology one year after DILI recognition.

Results

Out of 298 patients enrolled 273 (92%) resolved 61 year from DILI recognition and 25 patients (8%) were chronic. Independent risk factors for chronicity were older age [OR: 1.06, $p=0.011$], dyslipidemia [OR: 4.26, $p=0.04$] and severe DILI [OR: 14.22, $p=0.005$]. Alanine aminotransferase (ALT), alkaline phosphatase (ALP) and total bilirubin (TB) median values were higher in the chronic group during follow-up. Values of ALP and TB $>1.1 \times$ upper limit of normal (xULN) and 2.8 xULN respectively, in the second month from DILI onset, were found to predict chronic DILI ($p<0.001$). Main drug classes involved in chronicity were statins (24%) and anti-infectives (24%). Histological examination in chronic patients demonstrated two cases with ductal lesion and seven with cirrhosis.

Conclusions

One year is the best cut-off point to define chronic DILI or prolonged recovery, with risk factors being older age, dyslipidemia and severity of the acute episode. Statins are distinctly related to chronicity. ALP and TB values in the second month could help predict chronicity or very prolonged recovery.

Lay summary

Drug-induced liver injury (DILI) patients who do not resolve their liver damage during the first year should be considered chronic DILI patients. Risk factors for DILI chronicity are older age, dyslipidemia and severity of the acute episode. Chronic DILI is not a very common condition; normally featuring mild liver profile abnormalities and not being an important clinical problem, with the exception of a small number of cases of early onset cirrhosis.

Keywords: Hepatotoxicity; chronic; risk factors; statins

Introduction

Drug-induced liver injury (DILI) is a rare and often unpredictable adverse reaction to many drugs in common use. It represents a leading cause of acute liver failure in Western countries and one of the most common reasons for attrition during drug development and adoption of post-marketing regulatory actions.¹

DILI can present with a wide range of histological findings and phenotypes as a result of the interaction of a drug specific signature with host factors.^{2,3} Withdrawal of the offending drug is characteristically followed by resolution of liver damage except for a minor percentage of cases that evolve to fulminant hepatic failure or become chronic.

Analyses of retrospective databases⁴ and prospective collaborative networks⁵⁻⁸ have yielded reliable figures on prognosis of acute DILI and identified risk factors for acute liver failure and liver related-death.

There is a general belief that acute DILI persisting beyond 6 months should be considered chronic, similar to that occurring with viral hepatitis B or C.⁹ However, very few studies have addressed the rate of persistence in liver biochemistry alterations after drug discontinuation in patients with acute DILI after longer follow-up. A retrospective evaluation of 33 DILI cases found impaired liver tests or imaging-based evidence of chronic liver disease in 11 of the cases.¹⁰ Furthermore, a retrospective analysis of 685 patients with acute DILI and jaundice found 8 patients who had developed cirrhosis (5 cryptogenic) in a mean follow-up of 10 years.¹¹ However, the retrospective design of these studies precludes a reliable estimation of the true incidence of chronicity and the resolution time course of biochemical alterations in patients with DILI. Chronic liver injury was initially defined as increases in liver test values >3 months.¹² In a later study, chronicity of cholestatic/mixed type of injury was considered as elevated liver biochemistry values >6 months from DILI onset, assuming that these types of injuries frequently require longer time to resolution.¹³ In addition, the United States DILI Network considers chronicity as persistently elevated liver biochemistry on two separate occasions; histological or radiological evidence of persistent liver injury at 6 months or more after DILI onset.¹⁴ Hence, the best definition of DILI chronicity remains a matter of debate.¹²⁻¹⁶

In the present study, we aimed to describe the outcome of prospectively followed patients who survived an acute DILI episode with an emphasis on the time course of the liver biochemical profile in order to determine the best cut-off point to define chronicity, and search for risk factors related to chronicity and clinical consequences of the chronicity.

Patients and methods

Study design

The study population consisted of idiosyncratic drug-induced liver injury cases included in the Spanish DILI registry founded in April 1994. The operational structure of the registry,

data recording and case ascertainment has been reported elsewhere.⁵ Case report forms contain full information necessary to ascertain causality: (a) compatible temporal relationship between drug intake and appearance of liver disease; (b) serology biochemical, imaging and histological data to exclude alternative liver diseases; and (c) outcome of liver damage. Cases were identified by clinicians from 30 Spanish hospitals.

The criteria for DILI were initially those established by a group of experts (alanine aminotransferase, ALT > 2 times the upper limit of normal (xULN), conjugated bilirubin > 2 xULN or combined elevations in aspartate aminotransferase (AST), alkaline phosphatase (ALP) and total bilirubin (TB) provided one of them is above 2 xULN)¹² and later restricted to the consensus criteria adopted in 2011 (ALT ≥ 5 xULN, ALP ≥ 2 xULN or ALT ≥ 3 xULN + TB ≥ 2 xULN).¹⁵ Eighty-four percent of the cases fulfilled the last-mentioned criteria. The pattern of liver injury was classified based on the ratio (R) values, (ALT/ULN)/(ALP/ULN). Cases were considered hepatocellular (HC) when R ≥ 5, cholestatic (Chol) when R ≤ 2 and mixed (Mix) when 2 < R < 5 according to the criteria of the international consensus meeting for DILI.¹² Severity was classified as mild, moderate, severe or fatal based on the DILI severity index defined in 2011.¹⁵

We have defined chronicity as persistent ALT, AST, TB or ALP elevations > 1 xULN or imaging or histology data compatible with chronicity (irrespective of laboratory data) after one year from DILI recognition. Patients whose liver enzyme values returned to within laboratory references ranges in less than one year, regardless the type of damage, without chronicity signs previously described were defined as acute. Acute DILI patients were followed at least up to resolution. Chronic DILI patients were followed more than one year.

The definition of dyslipidemia was based on the criteria of the national cholesterol education program's adult treatment panel III (ATPIII): total cholesterol > 240 mg/dl, HDL cholesterol < 40 mg/dl, LDL cholesterol ≥ 160 mg/dl or triglycerides ≥ 200 mg/dl.¹⁷

Drugs considered to be implicated in the liver damage were classified according to the anatomical therapeutic classification of the World Health Organization.¹⁸ Only patients with a causality probability score of possible or higher using the Council for International Organizations of Medical Science (CIOMS) scale were included.¹⁹

Study patients

Since 1994 to September 2012, 850 patients with idiosyncratic DILI were considered for potential inclusion in the natural history study, and 351 patients fulfilled all the inclusion criteria (**Figure 7.1**).

Figure 7.1 Flow chart of the study cohort.

Inclusion criteria were:

- Survival of the index episode without a liver transplantation,
- DILI cases that reached resolution or DILI cases that did not resolve but had follow-up ≥ 1 year after DILI recognition,
- During follow-up cases had appointments with biochemical analysis scheduled, at least, every 6 months in the first year and annually in the consecutive years.

In patients with more than one DILI episode, only the last episode was included in the analysis to avoid that liver profile alterations corresponding to the second episode could interfere as confounding factor for chronicity.

Out of these 351 patients, 53 were further excluded due to:

- Underlying chronic liver disease (viral, alcoholic, metabolic or autoimmune hepatitis or altered basal liver profile of unknown aetiology) and Gilbert syndrome (22 cases),
- Systemic diseases affecting the liver (thyroid, heart disease, HIV infection) (17 cases),
- Miscellaneous causes such as paracetamol overdose and patients with alcohol intake over 40 g/day (14 cases).

The study protocol was approved by the local ethics committee of the coordinating center at “Virgen de la Victoria” University Hospital in Málaga, Spain, and all the subjects who took part in the study provided informed consent.

Statistical analyses

Variables were examined using descriptive statistics. Bivariate associations were measured using Student *t* test for continuous variables and *chi*-square test for categorical items. Analysis of variance was used for comparisons of groups.

Where variables did not follow a normal distribution, nonparametric analyses (Kruskal-Wallis test) were performed. A receiver operating characteristic (ROC) curve to analyze ALP and TB in the prediction of chronicity was performed. Differences were reported as statistically significant when the *p* value was <0.05. Times to event data are represented as Kaplan-Meier estimates. Actuarial probabilities were calculated using the Kaplan-Meier method and compared with the use of the log-rank test. Variables that were associated with chronicity in univariate analyses were included as potential covariates in a multiple logistic regression model. All statistical analyses were performed using the SPSS software version 19.0.

Results

Two hundred and ninety-eight DILI patients fulfilled all inclusion and none of exclusion criteria and were included in the study. The overall mean age was 53 years (14-88 years), 167 were females (56%), and hepatocellular damage predominated (203, 68%). The main causative pharmacological drug group was antiinfectious (40%), followed by musculoskeletal system (13%), central nervous system (12%), and cardiovascular drugs (11%). The cases were assessed as highly probable (43%), probable (50%) and possible (7%) according to the CIOMS/RUCAM scale.

Establishing the best cut-off point to define chronicity

To determine the best cut-off point for chronicity a Kaplan-Meier estimate of liver injury resolution was performed with the 285 cases that normalized liver tests over time out of the 298 patients included in the study (**Figure 7.2**). According to the type of liver damage, 193 cases (68%) were hepatocellular. Out of these, 101 cases (52%) resolved in the first 3 months, with an additional 57 cases resolving before 6 months and 27 more cases before the first year.

Forty-six cases were cholestatic (16%), out of these 17 resolved in the first 3 months (38%), 17 more cases (38%) resolved between month 3 and 6, and 10 more cases resolved before the first year.

Figure 7.2 Time to liver injury resolution in 285 patients with acute idiosyncratic drug-induced liver injury who recovered classified by type of damage. An additional 13 patients (not included) had not recovered after 3 years. Chol, cholestatic; HC, hepatocellular; Mix, mixed.

Out of the remaining 46 mixed cases (16%), 28 (60%) resolved during the first 3 months, 12 between 3 and 6 months and 4 more cases resolved before the first year (**Figure 7.2**). The mean time of resolution for the 285 patients was 142 days [95% CI=115-170 days]. The median was 86 days (95% CI=75-97). With respect to time to resolution according to type of liver damage it is important to note that in hepatocellular damage, the median resolution time was 83 days (95% CI=69-97). Cholestatic patients required longer time to resolution, 115 days (95% CI=83-147) and in patients with mixed liver damage the median resolution time was 76 days (95% CI=58-94).

We did not observe any statistically significant differences in the probability of resolution at one year between the different types of liver damage ($p=0.44$). Within 348 days from DILI recognition, 95% of the patients who reached resolution (92% of all study patients) had independently resolved of the type of injury. Hence, in the following analyses patients resolving within 1 year ($n=273$) are referred to as acute DILI, and those requiring >1 year as well as those not resolving during follow-up, as chronic ($n=25$, 8%). Thus, the term “chronic” referred to prolonged recovery (until reaching normalization) and non-resolving cases. Additionally, unresolved cases included patients who developed cirrhosis during the first year, which became quiescent.

A flow chart of the studied population is shown in **Figure 7.1**. In the acute group, 174 patients (64%) had normalized liver tests in their respective defined time frames based on the type of liver injury (63 months hepatocellular and 66 months cholestatic/mixed) and 99 patients (36%) resolved after this time but before one year. In the chronic group 10

patients had cholestatic/mixed type of damage and 15 patients presented cytolytic features. In this group, 8 patients (32%) resolved within 3 years from DILI recognition and 16 (64%) did not resolve during the first 3 years of follow-up. After one year from DILI onset one mixed case was lost during follow-up without reaching resolution.

Comparison of demographics, clinical and laboratory parameters between acute and chronic DILI cases

A comparison of demographics, clinical and laboratory parameters between the acute and chronic group is outlined in **Table 7.1**.

Table 7.1 Comparison of demographics, clinical, and laboratory parameters in 298 DILI cases according to the time of resolution.

	Acute, ≤1 year (N=273)	Chronic, >1 year (N=25)	p value
Female, n (%)	151 (55)	16 (64)	0.4
Age, mean years (range)	52 (14-88)	63 (30-83)	0.002
Gender-specific age, mean (range)			
Men	54 (17-87)	58 (30-70)	0.5
Women	51 (14-88)	66 (43-83)	<0.001
BMI (kg/m ²), mean (range)			
Jaundice	156 (58)	20 (80)	0.032
Hospital admission	110 (45)	17 (77)	0.004
Hypersensitivity features	78 (29)	9 (36)	0.2
Eosinophilia	53 (20)	8 (32)	0.2
Lymphopenia	22 (14)	5 (21)	0.4
Positive autoantibody titres	51 (23)	4 (16)	0.4
Duration of treatment, mean/median days (range)	79/21 (1-1827)	191/49 (4-1826)	0.2
Time to onset, mean/median days (range)	72/21 (0-1826)	152/30 (0-1828)	0.3
Days of continued exposure to drug after onset of symptoms, mean (range) patients, n (%)	21 (1-308)	50 (1-514)	0.3
Type of liver injury, n (%)	147 (54)	18 (72)	0.6
Hepatocellular	185 (68)	15 (60)	
Mixed	44 (16)	4 (16)	
Cholestatic	44 (16)	6 (24)	
Laboratory parameters at onset, mean (range)			
TB (mg/dl)	5 (0.13-33)	7 (0.4-28)	0.1
AST xULN (range)	15 (0.6-197)	13 (1-55)	0.7
ALT xULN (range)	19 (0.6-134)	20 (2.5-71)	0.9
GGT xULN (range)	7 (0.2-49)	14 (0.3-79)	0.08
ALP xULN (range)	1.8 (0.2-16)	3 (0.4-11)	0.05
Outcome, n (%)	12 (4)	2 (8)	0.4
Positive rechallenge, n (%)	16 (0.2-166)	54 (16-112)	<0.001
Mean follow-up, months (range)			
Recovery, mean days (range)	106 (7-357)	935 (385-3020)	<0.001
Severity, n (%)			0.003
Mild + moderate	261 (97)	21 (84)	
Severe	9 (3)	4 (16)	

The percentages shown were calculated based on the total number of episodes with available information. Severity index, Mild: elevated ALT/ALP meeting DILI criteria with total bilirubin <2 mg/dl; Moderate: elevated ALT/ALP with total bilirubin ≥2 g/dl; Severe: elevated ALT/ALP and one of the following: ascites, encephalopathy, international normalization ratio >1.5 and/or other organ failure considered to be due to DILI; Fatal: death or transplantation due to DILI. Hypersensitivity features: presence of one or more positive features such as fever,

rash, arthralgia, peripheral eosinophilia or lymphopenia; TB, total bilirubin; AST, aspartate aminotransferase; ALT, alanine aminotransferase; GGT, gamma-glutamyl transpeptidase; ALP, alkaline phosphatase.

Mean time follow-up was 16 months (range: 0.2-166 months) in the acute group and 54 months (range: 16-112 months) in the chronic group. The patients in the chronic group were significantly older as compared to those in the acute group, 63 vs. 52 years ($p=0.002$), with female predominance (64%, $p<0.001$). Patients who progressed to chronic DILI had a longer duration of treatment (median 49 days compared to 21 days in the acute group), although this difference was not statistically significant. The chronic cases presented frequently with jaundice at onset (80% vs. 58%, $p=0.03$) and often required hospitalization (77% vs. 45%, $p=0.004$). In addition, the percentage of severe cases was higher in the chronic group compared to patients in the acute group (16% vs. 3%, $p=0.003$). The prevalence of diabetes mellitus, dyslipidemia and hypertension was greater in the chronic group, 28% vs. 10% ($p=0.006$), 44% vs. 13% ($p<0.001$) and 50% vs. 25% ($p=0.019$), respectively. However, the body mass index did not differ between both groups. Furthermore, we analyzed the lipid profile (LDL, HDL, total cholesterol and triglycerides) during follow-up in 11 chronic (8 on statin and 3 on fibrate treatment) and 36 acute (22 on statin and 3 on fibrate treatment) dyslipidemic patients, but no differences were found between both groups.

Hepatocellular damage predominated in both, the chronic and acute groups (60% vs. 68%, respectively, $p=0.6$). Despite the fact that type of liver injury did not appear as a risk factor for chronicity, the chronic group presented significantly higher mean values of serum ALP (3 xULN vs. 1.8 xULN, $p=0.05$) at DILI onset. Time course of liver biochemistry values revealed that median values of ALP, ALT and TB were higher in the chronic group during the first year. All available laboratory analyses during follow-up in both, the chronic and acute group are represented in **Figure 7.3**. **Table 7.2** shows demographic, major clinical and serial biochemical parameters of 16 chronic DILI patients who did not resolve in the first 3 years. Additional information on liver profile in the 25 chronic DILI patients has been shown in Supplementary **Tables S7.1** and **S7.2**.

Figure 7.3 Median of ALP, ALT and TB values during the first year from DILI onset in acute and chronic groups. Each time interval includes 15 days.

Table 7.2 Demographic, major clinical and serial biochemical parameters of 16 DILI patients who required more than three years to resolve or did not resolve.

Case	Age/sex	Comorbidities	Type of liver injury	Liver profile	3-6 months (xULN)	0.5-1 year (xULN)	1-2 years (xULN)	2-3 years (xULN)	>3 years (xULN)
1**	67/F	Diabetes Arterial hypertension Ischemic cardiopathy Dyslipidemia	HC	TB AST ALT GGT ALP	5.1 8 11 16 1.2	1 2.5 3.1 2.9 1.1	0.7 0.5 1 3 0.9		
7	83/F	Arterial hypertension Dyslipidemia	HC	TB AST ALT GGT ALP	1.4 0.7 0.5 0.7 0.9	0.8 1.2 0.5 0.7 2.2	0.8 1.2 0.8 0.2 0.7	0.7 1.8 1.5 0.4 0.7	
9	65/M	Prostate cancer Tuberculosis	HC	TB AST ALT GGT ALP	- 0.7 1.2 1.3 -		1.3 0.7 0.7 1.2 0.5	1.4 0.7 0.8 0.5 0.4	0.8 0.7 0.9 0.5 0.4
10	43/F	Cholestasis of pregnancy	HC	TB AST ALT GGT ALP	0.5 0.6 1.3 0.3 0.4	*	*	0.5 0.7 1.3 0.4 -	0.8 0.8 1.1 0.1 0.2
11**	60/F	Uterine myoma Cardiopathy Depression Arterial hypertension	HC	TB AST ALT GGT ALP	3.3 1.5 1.2 3.6 0.8	1.1 1.05 0.7 2 0.4			
13**	55/F	Diabetes Arterial hypertension Arthrosis	HC	TB AST ALT GGT ALP	0.9 0.8 1.2 1.5 1.3	0.6 0.5 0.6 0.6 1.05	0.8 0.6 0.4 0.5 1.07	* 0.3 0.4 0.8 1.3	

Table 7.2 (continued)

Case	Age/sex	Comorbidities	Type of liver injury	Liver profile	3-6 months (xULN)	0.5-1 year (xULN)	1-2 years (xULN)	2-3 years (xULN)	>3 years (xULN)
14** Ebrotidine	69/F	None	HC	TB AST ALT GGT ALP	1.1 0.5 0.3 0.2 0.4	1.3 0.9 0.8 0.7 0.7	1.7 0.6 0.5 0.3 0.5	1.5 0.8 0.5 0.3 0.5	1.1 0.5 0.3 0.2 0.4
15** Clopidogrel/ atorvastatin	60/M	Diabetes Arterial hypertension Cardiopathy Dyslipidemia	HC	TB AST ALT GGT ALP	2.4 1.3 2.1 38 2.7	0.9 1.5 2 20 1.8	0.9 1.5 2 20 1.7	0.9 1.5 2 20 1.7	0.9 1.5 2 20 1.7
18 Amoxicillin-clavulanate/ carbamazepine	65/M	Brucellosis Trigeminal neuralgia	Chol	TB AST ALT GGT ALP	1.8 2 2.7 10 2.5	1.2 0.9 1.3 7.6 1.2	1.2 0.9 1.3 3.9 1.2	1.6 0.9 1 3.9 1.2	1.2 0.6 0.7 1.2 0.5
19 Fenofibrate/ raloxifene	60/F	Colorectal cancer Dyslipidemia Osteoporosis	Mix	TB AST ALT GGT ALP	0.4 2.8 2.5 40 11.5	0.4 1.5 1.3 23.5 4.3	0.4 1.5 1.1 9.3 3	0.4 1.3 0.9 4.6 1	0.3 3.0 1.6 24 5.0
20 Thiamazole	78/F	Hyperthyroidism Goiter	Chol	TB AST ALT GGT ALP	1.1 0.6 0.6 2.4 1.6	* * * * *	* * * * *	* * * * *	1.0 0.7 0.4 0.5 1.0
21 Gemfibrozil/ lovastatin	63/F	Dyslipidemia Diabetes Osteoarthritis	Chol	TB AST ALT GGT ALP	0.4 1.2 1.4 24 2.3	0.4 1.2 1.4 24 2.3	0.4 0.8 0.8 22 2.1	0.4 0.8 0.8 22 2.1	0.3 0.7 0.5 19 2.4

Table 7.2 (continued)

Case	Age/sex	Comorbidities	Type of liver injury	Liver profile	3-6 months (xULN)	0.5-1 year (xULN)	1-2 years (xULN)	2-3 years (xULN)	>3 years (xULN)
22** Amoxicillin-clavulanate/ ibuprofen	70/M	Arterial hypertension	Chol	TB AST ALT GGT	1.7 3 2.4 16	1.3 1.7 1.7 7	1.4 1.6 1.3 5.5	0.8 1.4 1.2 1	1.8 1.0 0.7 0.9
23 Ibuprofen	40/M	None	Chol	TB AST ALT GGT ALP	0.6 1.3 4.3 15 6.2	- 1 1.5 8.6 0.9	- 3.1 - 7.6 0.8	- 1.2 1.5 4.2 -	0.8 1.0 1.2 4.0 0.7
24** Ranitidine	54/F	Diabetes Arterial hypertension Dyslipidemia	Chol	TB AST ALT GGT ALP	0.4 1.8 2.5 53 4.2	- - - - -	- 1.8 1.6 - 2.6	0.9 2.8 4.7 61 3.7	0.4 1.8 1.3 21 4.0
25 Atorvastatin	75/F	Dyslipidemia Venous insufficiency	Mix	TB AST ALT GGT ALP	1.9 3 3.1 15 5.8	0.2 1.5 1.1 4.4 1.5	0.5 1.1 1.08 1.9 1.4	0.3 1.4 1.03 2.8 1.4	0.3 0.7 0.5 1.3 0.7

TB, total bilirubin; AST, aspartate aminotransferase; ALT, alanine aminotransferase; GGT, gamma-glutamyl transpeptidase; ALP, alkaline phosphatase; ULN, upper limit of normal; Chol, cholestatic damage; HC, hepatocellular damage; Mix, mixed damage. * Altered liver profile reported in clinical history (laboratory data are not available). ** The patient developed cirrhosis.

Several time frames were analyzed to explore potential differences in liver tests between the chronic and acute group. In the period 30 to 60 days from DILI onset (second month) significant differences in ALP and TB were found with higher values in the chronic group. We then performed a ROC curve analysis and found that a cut-off point of 1.1 xULN for ALP and 2.8 xULN for TB resulted in the highest area quantification under the curve (AUC) values to predict chronicity, including slow resolution beyond one year, with a sensitivity of 83% and 75%, and a specificity of 87% and 93%, respectively, ($p < 0.001$) (Supplementary **Figure S7.1**).

In the logistic regression analysis, older age [Odds ratio (OR): 1.06, 95% CI: 1.01-1.12; $p = 0.011$], dyslipidemia [OR: 4.26, 95% CI: 1.02-17.74, $p = 0.04$] and severe DILI [OR: 14.22, 95% CI: 2.23-90.9, $p = 0.005$] were found to be independent risk factors for chronic DILI development.

Comparison of demographics and clinical characteristics of 25 chronic patients based on type of liver damage is presented in **Table 7.3**. Liver biopsy was available for 16 patients (64%) in the chronic group, showing two cases with ductal lesion, one with low grade fibrosis and seven with cirrhosis. Drugs related to liver cirrhosis were atorvastatin, bentazepam, ebrotidine, clopidogrel/atorvastatin, amoxicillin-clavulanate/ibuprofen and ranitidine. Out of the seven biopsies of DILI cases that evolved to cirrhosis, only one showed steatosis, but not steatohepatitis, in a biopsy performed one year after the onset of the DILI episode. There were three more cases with demonstrated steatosis, after obtaining and analysing liver biopsies, in the chronic group during follow-up (performed from month 8 to 60 after DILI onset), two of these had previous biopsies without this finding (Supplementary **Tables S7.1** and **S7.2** show details for individual patients).

Table 7.3 Comparison of demographics and clinical characteristics of the 25 chronic patients according to the type of liver damage.

	Hepatocellular N=15	Cholestatic/mixed N=10
Age years, mean (range)	62 (30-83)	64 (40-78)
Female, n (%)	9 (60)	7 (70)
Jaundice, n (%)	13 (87)	7 (70)
Hypersensitivity features, n (%)	3 (20)	6 (60)
Liver biopsy*, n (%)	10 (67)	6 (60)
Fibrosis, n (%)	1 (7)	0
Cirrhosis, n (%)	5 (33)	2 (20)
Ductal lesion, n (%)	0	2 (20)
Steatosis, n (%)	2 (13)	2 (20)
Biochemical normalization ≤ 3 years, n (%)	7 (47)	1 (10)

Hypersensitivity features: Presence of one or more positive features such as fever, rash, arthralgia, peripheral eosinophilia or lymphopenia. The time point of biopsy varied from 2 weeks to 60 months after DILI onset.

Therapeutics Groups involved in chronic DILI

Among the culprit therapeutic drug classes involved in chronic DILI episodes, the more frequent were statins (24%), antiinfectives (24%, including 16% amoxicillin-clavulanate cases and 8% sulfamethoxazole and trimethoprim) and H₂-receptor antagonists (12%), mainly due to ebrotidine, a drug marketed in Spain in 1997 and withdrawn in 1998 (**Table 7.4**). Interestingly, the angiotensin-converting enzyme inhibitors and angiotensin II antagonists group was only represented in the chronic group.

Table 7.4 Comparison of drug classes implicated in DILI cases between acute (resolution <61 year) and chronic (persistent >1 year) patients.

	≤1 year (N=273)	>1 year (N=25)	<i>p</i> value
Drug classes, n (%)			
H2-receptor antagonists*	6 (2)	3 (12)	0.03
Antithrombotic agents	3 (1.1)	1 (4)	0.8
ACE inhibitors + angiotensin II antagonists	0	2 (8)	0.0007
Statins	18 (6)	6 (24)	0.002
Fibrates	3 (1.1)	2 (8)	0.07
Female sex hormones	7 (3)	2 (8)	0.36
Antithyroid preparations	3 (1.1)	1 (4)	0.8
Anti-infectives			
Penicillins-cephalosporins (excluding AC)	6 (2)	0	0.9
Amoxicillin-clavulanic acid (AC)	68 (25)	4 (16)	0.4
Sulfamethoxazole and trimethoprim	2 (0.7)	2 (8)	0.03
Fluoroquinolones	11 (4)	0	0.6
Macrolides	8 (3)	0	0.8
Antineoplastic agents	5 (1.8)	1 (4)	0.9
Antiandrogens	4 (1.5)	1 (4)	0.9
Immunosuppressants	5 (1.8)	0	0.9
Non-steroidal antiinflammatory drugs (NSAIDs)	41 (15)	2 (8)	0.5
Tetrabamate* *	4 (1.5)	1 (4)	0.9
Antiepileptics	6 (2)	2 (8)	0.3
Herbal products	12 (4)	0	0.6

Discussion

Up to now there are scarce and heterogeneous data reported on the long-term outcome of patients who survived an acute DILI episode, reflecting differences in the methodological approaches and the broad definitions of chronic DILI used in previous studies as outlined in **Table 7.5**. We here present a large prospective cohort of patients with DILI caused by a variety of agents with the longest follow-up reported in an outcome in DILI. Although our study had strict inclusion and exclusion criteria in order to avoid confounding factors, it indeed reflects a real clinical practice setting attempt to better characterize chronic.

Table 7.5 Studies addressing the long-term outcome of idiosyncratic drug-induced liver injury

Study (DILI criteria)	Chronicity criteria	Follow-up	Proportion chronicity	Mean age, yr/female %	Culprit drugs	Reference
Retrospective database/histology study (ALT>2 xULN, CB>2 xULN or combined elevations in AST, ALP and TB provided one of them is above 2 xULN)	Biochemical, radiological or histological evidence of liver injury >12 months	Median 5 years	11/33* (33%)	49/59%	Antibiotics NSAIDs Psycholeptics	Aithal et al., Gut 199910
Prospective registry study (ALT>2 xULN, CB>2 xULN or combined elevations in AST, ALP and TB provided one of them is above 2 xULN)	Persistent biochemical abnormality of HC>3 months after drug withdrawal or >6 months after chol/mix damage	Mean 20 months	28/493 (5.7%)	55/64%	Cardiovascular drugs (captopril, atorvastatin) and CNS drugs (bentazepam)	Andrade et al., Hepatology 200613
Retrospective database study (ALT>2 xULN, CB>2 xULN or combined elevations in AST, ALP and TB provided one of them is above 2 xULN, excluding ALP)	Persistent liver-related laboratory, radiologic, or histologic abnormalities at 6 months after DILI recognition	>6 months	41/300 (13.6%)	n.a.	n.a.	Chalasani et al., Gastroenterology 20087
Retrospective database study (ALT>2 xULN, CB>2 xULN or combined elevations in AST, ALP and TB provided one of them is above 2 xULN)	Morbidity/mortality after DILI hospitalization	Mean 11 years	23/685 (3.4%)**	n.a.	n.a.	Björnsson et al., J Hepatol 200911
Prospective registry study (AST or ALT>5 xULN, ALP>2 xULN, INR>1.5 or TB>2.5 mg/dl)	Persistently elevated AST, ALT, ALP or TB, histological evidence of ongoing liver injury, or radiological evidence of persistent liver injury ≥6 months after DILI onset	Mean 24 months	74/598 (12%)	52.6/69%	Antimicrobial, antineoplastic, cardiovascular, HDS	Fontana et al., Am J Gastroenterol 201516

* 7 patients failed to attend follow-up and 4 died. ** 5 patients had liver-related death. n.a., not available; TB, total bilirubin; CB, conjugated bilirubin; ALT, alanine aminotransferase; AST, aspartate aminotransferase; ALP, alkaline phosphatase; HDS, herbs and dietary supplements; Chol, cholestatic damage; HC, hepatocellular damage; Mix, mixed damage.

The first international consensus meeting on DILI recommended that a hepatocellular pattern of liver injury persisting for more than 3 months after onset should be considered as chronic liver injury.¹² This period of resolution nowadays seems inappropriate when analyzing the outcome of large cohorts of DILI patients, which have shown that many subjects with hepatocellular damage have persistent elevations in liver enzymes at this time point. The drug-induced liver injury network (DILIN) group has used in their analysis the standard period of six months to establish chronicity.^{7,8} In a consensus of experts in 2011¹⁵ continued liver damage was classified as persistent DILI when there was evidence for liver injury 3 and 6 months after withdrawal of the culprit drug in hepatocellular and cholestatic type of liver injury, respectively. Chronic DILI was then defined as the evidence of continued liver injury beyond 12 months of follow-up after withdrawal of the causative drug. In the present study, encompassing a large and wellphenotyped DILI cohort in which potential confounding alternative causes of persistent damage were thoroughly excluded, shows, via Kaplan-Meier analysis, that 95% of the DILI patients who finally recovered had resolved the injury at 348 days regardless the type of damage. Therefore, we do not consider it necessary to further classify the acute group into persistent and chronic as done by Aithal et al.¹⁵ Hence, we consider one year as the best cut-off point to identify and differ acute DILI patients from those with very prolonged recovery or true chronic DILI.

It is a general belief^{13,20} that cholestatic and mixed damage require longer time to normalize. Our data challenged this perspective, as the median days to resolution were 83, 115 and 76 days for hepatocellular, cholestatic and mixed cases, respectively, with no statistically significant differences among the groups ($p=0.4$). Hence, our findings indicate that it is not necessary to consider different categories of time to resolution based on the liver injury pattern for the definition of chronicity.

The prevalence of chronic DILI beyond one year follow-up in this study was 8%. It is lower than the prevalence previously reported by Aithal et al. in 1999 (33%)¹⁰ and the DILIN group (13.9%-18.9%).^{7,8,16} These differences might be explained by applying diverse definitions of chronicity (6 months vs. 1 year) and less restrictive study inclusion criteria (**Table 7.5**). Furthermore, our study was not designed to investigate the prevalence of chronic DILI as the strict exclusion criteria (mainly loss of follow-up during the first year before complete resolution) did not allow us to find out the true prevalence of the whole cohort of DILI patients included in the present database. In addition, in our study we paid attention to potential confounding factors, excluding not only patients with pre-existing chronic liver disease but also patients with systemic or any other diseases affecting the liver. However, as chronic DILI cases do not all have the same clinical impact, we classified them into three broad categories: early cirrhosis becoming quiescent, slow resolvers who do not progress to cirrhosis and cases with persistent activity (including borderline serum liver parameters) that may need monitoring but do not derive into major clinical problems. Considering these distinctions and our present data, one could question, if chronic DILI with active liver injury is a true phenomenon vs. a very slow course to complete recovery. The few cases beyond

three years of follow-up with low grade liver test abnormalities cannot be distinguished from the background incidence of these changes in the general population.

Various risk factors have been associated with chronic DILI. In the present study, mean age was significantly higher in the chronic group, especially in women, showing that older DILI women have a higher tendency towards chronicity. It could be speculated that aging results in declining of autophagy and progressive loss of cellular repair and regeneration capacity. Autophagy is being recognized as a critical function in the clearance of protein adducts, removal of damaged organelles and modulation of immune tolerance.²¹ Furthermore, female gender has been demonstrated to be more susceptible towards acute liver failure,^{6,22} showing that perhaps female gender presents a greater difficulty in repairing liver damage. In previous studies (**Table 7.5**), female gender also predominated in chronic DILI populations.^{8,10,13,16,20} However, our chronic patients were older than the previously reported.¹⁶

The presence of metabolic risk factors such as diabetes, dyslipidaemia and hypertension, were found to be more frequent in the chronic group. In this setting, it is difficult to distinguish between persistent liver damage due to chronic DILI and underlying non-alcoholic fatty liver disease. Previous studies have also found diabetes to be more frequent in chronic DILI patients.^{8,16} Our findings are consistent with the fact that older age is a risk factor for chronicity and in the elderly, metabolic risk factors are more prevalent. Ultimately, diabetes, dyslipidemia and hypertension along with the age in an elderly subject could compromise the repair of the liver damage promoting a chronic outcome. Interestingly, dyslipidemia was found to be protective from fulminant outcome in a previous study, and we speculate that this effect could be indirectly related to the use of statins.⁶ Statins were the most frequent drug group found in the chronic cases compared to acute cases in the present analysis. However, it is possible that this result is a reflection of the underlying dyslipidemia. The precise mechanism of statin-induced chronic DILI is unknown but could involve an immune self-perpetuating response as these drugs have been increasingly associated with drug-induced autoimmune hepatitis.²³

Patients with more severe acute DILI episodes, presenting with jaundice and requiring hospitalization, also had an increased risk of chronicity, suggesting that the time to resolution is longer when the damage is more severe. Liver function alterations can take longer time to resolve than just mild transaminase elevations. Hospitalization has been previously observed as risk factor for chronic DILI.⁸

Another interesting finding was the significantly increased ALP values at DILI onset which is coincidental with the study authored by Fontana et al.¹⁶ ALP elevations are generally associated with cholestatic damage. However, although cholestatic and mixed damage were more frequent in chronic DILI patients than in acute DILI patients, there were no significant differences among the types of liver injury. Furthermore, cholestatic damage has

been demonstrated to be associated with older age.² During follow-up median values of ALP as well as TB were higher in the chronic group. In addition, in the second month from DILI onset, ALP >1.1 xULN and TB >2.8 xULN values turned out to be the best cut-off points to predict chronicity in DILI (Supplementary **Figure S7.1**). This finding could have a prognostic value in clinical practice. The reason for selecting the second month from DILI onset was that this time frame had a higher number of available laboratory test than the later ones, and this time frame demonstrated higher differences in the studied parameters between the groups. Hence, we consider the second month to be an appropriate period for a prognostic evaluation in clinical practice.

In the chronic group seven cases developed cirrhosis. Two hepatocellular cases were related to ebrotidine (a histamine H2-receptor antagonist), which has been discontinued in Spain since 1998 due to its hepatotoxic potential with reported cases having rapid progression to cirrhosis.^{24,25} Other cases were related to bentazepam, atorvastatin and clopidogrel/atorvastatin. Two other patients with cirrhosis were cholestatic cases, one due to amoxicillin-clavulanate/ibuprofen and another induced by ranitidine. Liver cirrhosis due to amoxicillinclavulanate, bentazepam, and atorvastatin has also been reported previously,^{13,26,27} while ranitidine has been associated with the development of autoimmune hepatitis.²⁸

We cannot exclude that some chronic DILI patients who did not recover during the follow-up period had pre-existing non-alcoholic steatohepatitis (NASH). Nevertheless, the majority of chronic patients had no signs of steatosis at ultrasound examinations, four patients had normal liver tests at baseline, and 12 out of 16 biopsies did not show any indications of steatosis (see Supplementary **Tables S7.1** and **S7.2**). The remaining four biopsies showed steatosis during the evolution of the episode, with normal initial ultrasound or normal basal liver profile. Besides, two of these patients with steatosis in the biopsy had a cholestatic liver damage, not attributable to an underlying NASH. Alternatively, the absence of resolution of the chronic DILI patients could be attributed to the development of NASH, during the follow-up of the DILI episode, which could be induced or not by the drug, as this group of chronic patients share risk factors with NASH patients. Currently, it is not feasible to differentiate these two situations. Furthermore, the non-inclusion of nearly 500 patients due to loss of follow-up could have introduced selection bias as many of these patients were probably acute cases.

Many of the drugs involved in chronicity in our study have been described in the previous literature with regards to chronicity. Published studies associated with development of chronic liver disease and cirrhosis: isoniazid,²⁹ nitrofurantoin,^{30–32} flucloxacillin,³³ amiodarone,³⁴ methotrexate,³⁵ chlorpromazine,³⁶ ramipril,³⁷ diclofenac,³⁸ statins^{13,39,40} sulphonamides and trimethoprim (TMP/SMZ),^{41–43} fenofibrate,⁴⁴ amoxicillin-clavulanate,⁴³ oral contraceptives⁴⁵ and terbinafine.^{46,47}

Herein we demonstrate that the more reliable cut-off time point for definition of chronic DILI is one year after onset of the acute toxic liver disease. Ninety-two per cent of patients resolved by one year and therefore this cut-off identifies patients who need further follow-up. The main risk factors for chronic DILI are older age, dyslipidaemia, and severity of the acute episode. We conclude that aside from a small number of cases of early onset cirrhosis which becomes quiescent, gradual resolution at 1 or 3+ years or persistence of borderline laboratory abnormalities beyond 3 years is seen in a very small percentage of cases. The persistence of these very mild abnormalities is of uncertain significance but does not appear to be clinically relevant. Hence, the term “chronic” is somewhat controversial as there are “chronic DILI patients” who eventually recover from the liver damage. However, we have used this term to differentiate from “acute DILI”, as we do not believe it is appropriate to use the term “acute damage” when requiring more than one or even more than three years to resolve. Nevertheless, it is prudent to document the recovery progress during follow-up and, thus, identify any risk of slow resolution or persistence to be determined by early laboratory changes (at two months), which, in turn, is of practical value in flagging cases for closer long-term scrutiny. Our data is the most detailed long-term follow-up of acute DILI and indicates that chronic (active) DILI is extremely rare.

References

1. Kaplowitz N. Idiosyncratic drug hepatotoxicity. *Nat Rev Drug Discov* 2005;4:489–499.
2. Lucena MI, Andrade RJ, Kaplowitz N, García-Cortes M, Fernández MC, Romero-Gomez M, et al. Phenotypic characterization of idiosyncratic drug-induced liver injury: the influence of age and sex. *Hepatology* 2009;49:2001–2009.
3. Kleiner DE, Chalasani NP, Lee WM, Fontana RJ, Bonkovsky HL, Watkins PB, et al. Hepatic histological findings in suspected drug-induced liver injury: systematic evaluation and clinical associations. *Hepatology* 2014;59:661–670.
4. Björnsson E, Olsson R. Outcome and prognostic markers in severe drug-induced liver disease. *Hepatology* 2005;42:481–489.
5. Andrade RJ, Lucena MI, Fernández MC, Pelaez G, Pachkoria K, García-Ruiz E, et al. Drug-induced liver injury: an analysis of 461 incidences submitted to the Spanish registry over a 10-year period. *Gastroenterology* 2005;129:512–521.
6. Robles-Díaz M, Lucena MI, Kaplowitz N, Stephens C, Medina-Cáliz I, González-Jiménez A, et al. Use of Hy's law and a new composite algorithm to predict acute liver failure in patients with drug-induced liver injury. *Gastroenterology* 2014;147:109–118.
7. Chalasani N, Fontana RJ, Bonkovsky HL, Watkins PB, Davern T, Serrano J, et al. Causes, clinical features and outcomes from a prospective study of drug-induced liver injury in the United States. *Gastroenterology* 2008;135:1924–1934.
8. Fontana RJ, Hayashi PH, Gu J, Reddy KR, Barnhart H, Watkins PB, et al. Idiosyncratic drug-induced liver injury is associated with substantial morbidity and mortality within 6 months from onset. *Gastroenterology* 2014;147:96–108.
9. Seeff LB. Drug-induced chronic liver disease, with emphasis on chronic active hepatitis. *Semin Liver Dis* 1981;1:104–115.
10. Aithal PG, Day CP. The natural history of histologically proved drug-induced liver disease. *Gut* 1999;44:731–735.

11. Björnsson E, Davidsdóttir L. The long-term follow-up after idiosyncratic drug-induced liver injury with jaundice. *J Hepatol* 2009;50:511–517.
12. Benichou C. Criteria of drug-induced liver disorders. Report of an International Consensus Meeting. *J Hepatol* 1990;11:272–276.
13. Andrade RJ, Lucena MI, Kaplowitz N, García-Muñoz B, Borraz Y, Pachkoria K, et al. Outcome of acute idiosyncratic drug-induced liver injury: long-term follow-up in a hepatotoxicity registry. *Hepatology* 2006;44:1581–1588.
14. Fontana RJ, Watkins PB, Bonkovsky HL, Chalasani N, Davern T, Serrano J, et al. Drug-Induced Liver Injury Network (DILIN) prospective study: rationale, design and conduct. *Drug Saf* 2009;32:55–68.
15. Aithal GP, Watkins PB, Andrade RJ, Larrey D, Molokhia M, Takikawa H, et al. Case definition and phenotype standardization in drug-induced liver injury. *Clin Pharmacol Ther* 2011;89:806–815.
16. Fontana RJ, Hayashi PH, Barnhart H, Kleiner DE, Reddy KR, Chalasani N, et al. Persistent liver biochemistry abnormalities are more common in older patients and those with cholestatic drug induced liver injury. *Am J Gastroenterol* 2015;110:1450–1459.
17. Expert panel on detection, evaluation, and treatment of high blood cholesterol in adults. Executive summary of the third report of the National Cholesterol Education Program (NCEP) expert panel on detection, evaluation, and treatment of high blood cholesterol in adults (adult treatment panel III). *JAMA* 2001;285:2486–2497.
18. World Health Organization Collaborating Center for drugs statistics methodology. Guidelines for Anatomical Therapeutic Chemical (ATC) classification and defined daily dose (DDD) assignment. Oslo, Norway: World Health Organization Collaborating Center for Drug statistics methodology; 2014.
19. Danan G, Benichou C. Causality assessment of adverse reactions to drugs I. A novel method based on the conclusions of international consensus meetings: application to drug-induced liver injuries. *J Clin Epidemiol* 1993;46:1323–1330.
20. Björnsson E, Kalaitzakis E, Av Klinteberg V, Alem N, Olsson R. Long-term follow-up of patients with mild to moderate drug-induced liver injury. *Aliment Pharmacol Ther* 2007;26:79–85.
21. Williams CD, Jaeschke H. Role of innate and adaptive immunity during drug-induced liver injury. *Toxicol Res* 2012;1:161–170.
22. Reuben A, Koch DG, Lee WM. Drug-induced acute liver failure: results of a U. S. multicenter, prospective study. *Hepatology* 2010;52:2065–2076.
23. Lucena MI, Kaplowitz N, Hallal H, Castiella A, García-Bengoechea M, Otazua P, et al. Recurrent drug-induced liver injury (DILI) with different drugs in the Spanish Registry: the dilemma of the relationship to autoimmune hepatitis. *J Hepatol* 2011;55:820–827.
24. Andrade RJ, Lucena MI, Martín-Vivaldi R, Fernández MC, Nogueras F, Pelaez G, et al. Acute liver injury associated with the use of ebrotidine, a new H2- receptor antagonist. *J Hepatol* 1999;31:641–646.
25. Pineda JA, Larrauri J, Macías J, Hernández A, Guijarro J, Sayago M, et al. Rapid progression to liver cirrhosis of toxic hepatitis due to ebrotidine. *J Hepatol* 1999;31:777–778.
26. Andrade RJ, Lucena MI, Aguilar J, Lazo MD, Camargo R, Moreno P, et al. Chronic liver injury related to use of bentazepam: an unusual instance of benzodiazepine hepatotoxicity. *Dig Dis Sci* 2000;45:1400–1404.
27. Perdices EV, Medina-Cáliz I, Hernando S, Ortega A, Martín-Ocaña F, Navarro JM, et al. Hepatotoxicity associated with statin use: analysis of the cases included in the Spanish Hepatotoxicity Registry. *Rev Esp Enferm Dig* 2014;106:246–254.
28. Luparini RL, Rotundo A, Mattace R, Marigliano V. Possibly ranitidine-induced autoimmune hepatitis. *Ann Ital Med Int* 2000;15:214–217.
29. Black M, Mitchell JR, Zimmerman HJ, Ishak KG, Epler GR. Isoniazid-associated hepatitis in 114 patients. *Gastroenterology* 1975;69:289–302.
30. Sharp JR, Ishak KG, Zimmerman HJ. Chronic active hepatitis and severe hepatic necrosis associated with nitrofurantoin. *Ann Intern Med* 1980;92:14–19.
31. Amit G, Cohen P, Ackerman Z. Nitrofurantoin-induced chronic active hepatitis. *Isr Med Assoc J* 2002;4:184–186.
32. Stricker BH, Blok AP, Claas FH, Van Parys GE, Desmet VJ. Hepatic injury associated with the use of nitrofurans: a clinicopathological study of 52 reported cases. *Hepatology* 1988;8:599–606.

33. Olsson R, Wiholm BE, Sand C, Zettergren L, Hultcrantz R, Myrhed M. Liver damage from flucloxacillin, cloxacillin and dicloxacillin. *J Hepatol* 1992;15:154–161.
34. Oikawa H, Maesawa C, Sato R, Oikawa K, Yamada H, Oriso S, et al. Liver cirrhosis induced by long-term administration of a daily low dose of amiodarone: a case report. *World J Gastroenterol* 2005;11:5394–5397.
35. Whiting-O’Keefe QE, Fye KH, Sack KD. Methotrexate and histologic hepatic abnormalities: a meta-analysis. *Am J Med* 1991;90:711–716.
36. Moradpour D, Altorfer J, Flury R, Greminger P, Meyenberger C, Jost R, et al. Chlorpromazine-induced vanishing bile duct syndrome leading to biliary cirrhosis. *Hepatology* 1994;20:1437–1441.
37. Yeung E, Wong FS, Wanless IR, Shiota K, Guindi M, Joshi S, et al. Ramipril-associated hepatotoxicity. *Arch Pathol Lab Med* 2003;127:1493–1497.
38. Mazeika PK, Ford MJ. Chronic active hepatitis associated with diclofenac sodium therapy. *Br J Clin Pract* 1989;43:125–126.
39. Clarke AT, Mills PR. Atorvastatin associated liver disease. *Dig Liver Dis* 2006;38:772–777.
40. Russo MW, Hoofnagle JH, Gu J, Fontana RJ, Barnhart H, Kleiner DE, et al. Spectrum of statin hepatotoxicity: experience of the drug-induced liver injury network. *Hepatology* 2014;60:679–686.
41. Yao F, Behling CA, Saab S, Li S, Hart M, Lyche KD. Trimethoprim sulfamethoxazole-induced vanishing bile duct syndrome. *Am J Gastroenterol* 1997;92:167–169.
42. Kowdley KV, Keeffe EB, Fawaz KA. Prolonged cholestasis due to trimethoprim sulfamethoxazole. *Gastroenterology* 1992;102:2148–2150.
43. Chalasani N, Bonkovsky HL, Fontana R, Lee W, Stolz A, Talwalkar J, et al. Features and outcomes of 899 patients with drug-induced liver injury: The DILIN Prospective Study. *Gastroenterology* 2015;148 e7.
44. Chatrenet P, Regimbeau C, Romain JP, Penot J, Bruandet P. Chronic active cirrhotogenic hepatitis induced by fenofibrate. *Gastroenterol Clin Biol* 1993;17:612–613.
45. Ghabril M, Vuppalanchi R. Drug-induced nodular regenerative hyperplasia. *Semin Liver Dis* 2014;34:240–245.
46. Anania FA, Rabin L. Terbinafine hepatotoxicity resulting in chronic biliary-ductopenia and portal fibrosis. *Am J Med* 2002;112:741–742.
47. Gendre G, Buclin T, Morard I, Fontannaz J, Berney JL. Terbinafine induced hepatitis with persistent cholestasis. *Rev Med Suisse* 2008;4:736–739

THIS CHAPTER IS EMBARGOED

Chapter 8

Learning point: Three case reports on drug-induced liver toxicity due to diverse drugs and conditions

PART II

Chronic liver disease section

Chapter 9

Reversal of liver fibrosis: From fiction to reality

Zoubek ME, Trautwein C, Pavel Strnad P

***Best Pract Res Clin Gastroenterol* 2017;31:129-141**

DOI: 10.1016/j.bpg.2017.04.005

Abstract

In chronic liver diseases, an ongoing hepatocellular injury and inflammatory reaction result in the activation of hepatic stellate cells (HSCs) and increased deposition of extracellular matrix (ECM), termed liver fibrosis. It can progress to cirrhosis that is characterized by parenchymal and vascular architectural changes together with the presence of regenerative nodules. Even at a late stage, liver fibrosis is reversible and the underlying mechanisms include a switch in the inflammatory environment, elimination or regression of activated HSCs and degradation of ECM. While animal models have been indispensable for our understanding of liver fibrosis, they possess several important limitations and need to be further refined. A better insight into liver fibrogenesis resulted in a large number of clinical trials aiming at reversing liver fibrosis, particularly in patients with nonalcoholic steatohepatitis. Collectively, the current developments demonstrate that reversal of liver fibrosis is turning from fiction to reality.

Keywords: Liver fibrosis, Animal models, Hepatic stellate cell, Fibrosis resolution, Emerging therapies.

Introduction

Hepatic fibrogenesis constitutes a response to a persistent liver cell injury with a chronic inflammatory reaction that induces activation of fibrogenic cells and an accumulation of extracellular matrix (ECM). While in the case of acute liver damage, the liver has the potential to reestablish the hepatic structural integrity quickly, sustained noxious stimuli in chronic injury lead to distortion of liver parenchyma with progressive deterioration of liver function.¹ Nonalcoholic steatohepatitis, alcoholic liver disease and viral hepatitis represent the primary etiologies leading to liver fibrosis.^{2,3} Other underlying causes are toxin-induced insults (*e.g.*, alcohol, drugs), cholestatic disorders, autoimmune hepatitis and hereditary metabolic diseases.^{4,5} Hepatic fibrogenesis can progress to liver cirrhosis, characterized by aberrant remodeling of the hepatic and vascular architecture with the formation of septae and regenerative nodules. Due to its potentially fatal complications such as portal hypertension with variceal bleeding, acute-on-chronic liver failure and an enhanced risk of hepatocellular carcinoma (HCC), liver cirrhosis is among the leading causes of morbidity and mortality worldwide.^{2,6} Up to date, liver transplantation is the only efficient treatment option in patients with decompensated cirrhosis.⁷

In the last decades, an immense effort has been undertaken to elucidate the mechanisms of liver fibrosis and develop therapeutic approaches. Although a large number of compounds displayed antifibrotic properties in animal models, none of these drugs have yet been approved for routine clinical use. On the other hand, data from cholestatic and viral liver disease demonstrated that even at an advanced stage, liver fibrosis is reversible when the underlying cause is removed.^{8,9} These findings spurred investigations into liver fibrosis reversal that often employ antiinflammatory or matrix-degrading compounds.

Experimental animal models to assess liver fibrosis

Since *in vitro* studies cannot mimic the complex situation in the liver that includes crosstalk between multiple cell types, animal models constitute a gold standard of fibrosis research. Among animals, rodents have been preferred due to their comparably quick reproductive cycle as well as multiple available targeting strategies (**Table 9.1**). On the other hand, *in vitro* studies are helpful for drug screening purposes. However, the obtained results need to be subsequently validated in animal models.¹⁰

Compounds such as carbon tetrachloride (CCl₄) or thioacetamide (TAA) have been shown to behave as potent hepatotoxins, which can trigger hepatic injury and a secondary inflammatory reaction leading to the development of hepatic fibrosis.

Table 9.1 Experimental models for the study of hepatic fibrosis in rodents

Model	Mechanism	Description	Pathogenesis	Hepatic injury pattern	Reference
CHEMICALLY-INDUCED					
CCl ₄	Hepatotoxic	CCl ₄ <i>i.p.</i> injection 2-3 times per w during 4-12 w; dose: 300-1000 µl/kg bw; first changes visible after 2-3 w;	CYP2E1-mediated biotransformation: trichloromethyl radical;	Centrilobular necrosis, initially pericentral fibrosis formation of centro-central septa and later centro-portal septa;	11-16
TAA	Hepatotoxic, hepatocarcinogenic	TAA <i>i.p.</i> injection 3 times per w during 6-8 weeks; dose: 100-200 mg/kg bw; fibrotic changes within 6 w;	CYP2E1-mediated biotransformation: thioacetamide sulphur dioxide;	Centrilobular necrosis, pericentral and periportal fibrosis;	17-19
SURGICAL- BASED					
BDL	Cholestatic	Common bile duct double ligation and transection between the 2 ligatures; hepatic fibrosis in 2-4 w;	Mechanical-obstructive cholestasis	Periportal inflammation; activation of portal fibroblasts, peri-portal biliary fibrosis; biliary infarcts;	13,25,26
DIET/FEEDING- BASED					
MCD	Fatty liver	Diet deficient in methionine and choline; 8-10 w of feeding;	VLDL secretion impairment	Steatohepatitis and pericellular fibrosis;	29-31,156
High fat		Diet enriched with lipids, carbohydrates, or both; feeding duration 3-12 w;	Insulin resistance; metabolic syndrome;	Steatosis, inflammation and fibrosis;	
Cholesterol and cholate		Diet enriched with cholesterol and cholate; 3-16 w of feeding;	ROS, dyslipidemia, lipid peroxidation	Steatosis, inflammation and fibrosis;	
Fructose		Fructose enriched diet/water; feeding duration of 5-16 w of administration;	Metabolic syndrome	Macrovesicular steatosis, intralobular inflammation and fibrosis;	
Lieber-DeCarli	Alcoholic-fatty liver	Alcohol-containing isocalorically controlled liquid diet as the only source of energy; Feeding alcohol-containing diet via intragastric canula;	Cytochrome P450-dependent metabolism of ethanol; ROS, glutathione depletion, lipid peroxidation	Mild hepatic steatosis; minimal fibrotic changes;	27
Tsukamoto-French				Mild fibrosis development;	
DDC	Cholestatic	Diet feeding; duration 4-12 w;	Porphyrinogenic hepatocellular injury	Biliary ductular reaction, cholangitis, periductal fibrosis and portal-portal fibrosis;	157

Table 9.1 (continued)

Model	Mechanism	Description	Pathogenesis	Hepatic injury pattern	Reference
GENETIC-MODIFIED					
Mdr2 ^{-/-}	Cholestatic; hepatocarcinogenic	Inherited; fibrotic alterations from 4 w after birth	Bile regurgitation from leaky ducts into portal tracts	Cholangiocyte damage; prominent ductular proliferation; oeriductal fibrosis; tumorigenesis;	32
PZ	Alpha-1-antitrypsin deficiency	Inherited; fibrotic changes within 12-24 months	RER protein overload	Low-grade inflammation; mild steatosis; carcinogenesis;	33,34
Atp7b ^{-/-}	Wilson's disease	Inherited; fibrotic changes reached 20 w after birth	Massive hepatic accumulation of copper	Hepatocellular hyperplasia; inflammation; necrosis and proliferation of bile ducts; fibrosis and nodules of regenerating hepatocytes	158

BDL, bile duct ligation; bw, body weight; CCl₄, carbon tetrachloride; DDC, 3,5-diethylcarbonyl-1,4-dihydrocollidine; DEN, diethylnitrosamine; DMN, dimethylnitrosamine; LD, liver disease; MCD, methionine choline-deficient diet; RER, rough endoplasmic reticulum; ROS, reactive oxygen species; TAA, thioacetamide; w, weeks

CCl_4 undergoes hepatic metabolism via CYP2E1 that generates trichloromethyl radical, a highly reactive metabolite with the capacity to promote lipid peroxidation and free radical-derived damage.¹¹

Repeated *i.p.* injections of CCl_4 lead to centrilobular hepatic necrosis, activation of the inflammatory response and progressive postnecrotic fibrosis.^{12,13} In most cases, CCl_4 doses ranging between 300 and 1000 ml/kg are applied 2 to 3 times per week during 4-12 weeks and the hepatic injury reverses within a few days after the drug withdrawal.¹⁴⁻¹⁶ Biotransformation of TAA creates thioacetamide sulfur dioxide, which acts as a potent hepatotoxin and promotes HSC activation and fibrosis progression by oxidative stress-dependent hepatic damage.¹⁷ Typical features of TAA-induced liver fibrosis constitute centrilobular necrotic changes coupled with inflammatory infiltrates. Initially, these changes progress slowly, followed by an exponential acceleration of fibrogenesis.¹⁸ Usual doses for TAA-induced fibrogenesis are 100-200 mg/kg applied *i.p.* 3 times per week for 6-12 weeks, but TAA can also be applied in drinking water for 2-4 months.^{17,19} Dimethylnitrosamine (DMN) and diethyl-nitrosamine²⁰ can equally induce liver fibrosis as a sequel of oxidative stress and centrilobular necrosis.²¹ However, DMN and DEN hepatotoxicity progress to hepatic carcinogenesis and these agents are therefore not usually used as pure hepatic fibrosis models.²²⁻²⁵

Common bile duct ligation (BDL) constitutes a well-established surgical method to induce cholestatic injury. The interruption of the bile flow leads to a ductal reaction, hepato- and cholangiocellular injury as well as inflammatory response that together induce the development of portal fibrosis. The fibrosis is seen between 2 and 4 weeks after BDL surgery.^{26,27} In contrast to the previously described models that are based on HSCs as the matrix-synthesizing cells, BDL also includes a substantial involvement of portal fibroblasts.¹³

Various animal diets have been employed as models of non-alcoholic and alcoholic fatty liver disease (NAFLD/ALD). ALD arises due to hepatic CYP450-dependent ethanol metabolism that leads to the production of ROS and lipid peroxidation, which contribute to hepatocyte apoptosis, inflammatory reaction and activation of HSCs. Lieber-DeCarli liquid diet or Tsukamoto-French intragastric feeding are commonly used rodent ALD models.²⁸ NAFLD is histologically and mechanistically similar to ALD and presents with a variable amount of hepatocellular ballooning together with necroinflammatory changes, steatosis and inflammation.²⁹ High fat, as well as methionine and choline-deficient (MCD) diets, have been commonly used to reproduce NAFLD in rodents.³⁰ However, the latter should be used with caution since it does not reproduce the obesity-associated metabolic alterations seen in humans.³¹ Multiple diet regimens emerged as a potential substitute for the MCD diet and often contained fructose and/or cholesterol supplementation; however, the standard NAFLD model still remains to be established.³²

It is important to note that there are no ideal experimental models fully reflecting the human counterpart. For example, the commonly used chemically-induced rodent models typically display initial fibrotic changes in perivenular areas, whereas periportal and lobular

fibrotic changes are characteristic of human liver fibrosis. ALD is also difficult to mimic due to the natural aversion of rodents against alcohol. Accordingly, the corresponding diets often induce only mild injury and do not result in significant fibrosis, not even after a prolonged administration.

In addition to the models described above, genetically engineered mice provide a useful tool to reconstruct human-relevant alterations. For example, Mdr2-knockout mouse closely reproduce findings seen in progressive familial intrahepatic cholestasis,³³ while PiZ mice mimic the alterations seen in subjects with alpha1-antitrypsin deficiency.^{34,35}

Mechanisms of hepatic fibrosis development

Hepatic injury, the initiator of the fibrogenic cascade

Cholangiocytes, but to an even larger extent hepatocytes, are subjected to various harmful agents such as hepatotropic viruses (HBV, HCV, HDV), alcohol, fatty acid overload or other toxic metabolites, including bile acids. These compounds may induce the formation of reactive oxygen species such as hydrogen peroxide, hydroxyl radicals or aldehyde end products.³⁶ Oxidative stress can induce cell death, immune infiltration, hepatocellular steatosis and act as a paracrine stimulator of hepatic stellate cells (HSCs).³⁷ A persistence of these stimuli can lead to liver fibrosis.

Various cell death pathways have been suggested as essential contributors to liver fibrosis development.³⁸ Among them, apoptosis is a common hallmark of viral, cholestatic, fatty, and alcoholic liver disease and is characterized morphologically by cellular shrinkage, nuclear condensation and fragmentation. While it was earlier seen as a "silent" form of cell death, it now constitutes an established initiator and promoter of the fibrogenic cascade.^{39,40} Apoptotic cell death of parenchymal cells can be mediated by several stimuli, including Fas and tumor necrosis factor (TNF) α . Livers subjected to ethanol-induced liver injury or HCV infection exhibit Bcl2-mediated hepatocyte apoptosis, while enhanced Fas signaling has been described in steatohepatitis-driven hepatocellular apoptosis.^{41,42} As a proof of concept, the pan-caspase inhibitor IDN-6556 ameliorated BDL-induced liver fibrosis.⁴⁰

Necroptosis represents a specific form of necrotic cell death pathway regulated by receptor-interacting proteins (RIPs³⁸). Among them, RIP3 emerged as a critical regulator of steatotic liver injury.^{43,44} Last but not least, pyroptosis constitutes a cell death pathway initiated by activation of the inflammasome, which has been shown to contribute to liver fibrosis development in multiple different liver injury settings.^{45,46}

Hepatic stellate cells, the producers of extracellular matrix

Liver fibrosis is defined as an excess of extracellular matrix (ECM), which emerges from an imbalance between augmented synthesis and decreased degradation. HSCs are the primary cell type mediating this process (**Figure 9.1**). Under physiologic conditions, the quiescent HSCs reside in the perisinusoidal compartment, also known as the space of Disse, store vitamin A and are involved in regulating sinusoidal blood flow.⁴⁷ During chronic injury, transforming growth factor- β (TGF β) mediates the proliferation and transdifferentiation of HSCs into activated myofibroblasts that display contractile and proinflammatory features.^{48,49} Various types of cytokines, chemokines and growth factors such as platelet-derived growth factor (PDGF), vascular endothelial growth factor (VEGF), transforming growth factor (TGF) α and keratinocyte growth factor mediate this process.⁵⁰⁻⁵² While activated HSCs (aHSCs) constitute the major ECM producers, other cells such as portal fibroblasts, mesothelial cells and bone-marrow-derived fibrocytes also contribute.⁵³

Figure 9.1 The role of hepatic stellate cells (HSCs) in liver fibrogenesis. Hepatic stellate cells (HSCs) constitute the predominant source of myofibroblasts (MFs) and the major producers of extracellular matrix (ECM). Quiescent HSCs (qHSCs) are lipid-storing cells located in the perisinusoidal space. A variety of stimuli can trigger their activation (aHSC) and transdifferentiation into MFs. For example, substances released from injured cells or microbes termed damage- or pathogen-associated molecular patterns (DAMP/PAMP) activate HSCs via innate immunity induction. aHSCs lose their cytoplasmic retinoid droplets and release mediators such as transforming growth factor- β (TGF β) and platelet-derived growth factor (PDGF) that contribute to their proliferation and activation in an autocrine/paracrine manner. aHSCs also synthesize ECM components such as collagen 1 (COL-1) and products inhibiting

ECM degradation such as tissue inhibitor of metalloproteinases (TIMP) 1 and 2, thereby leading to ECM accumulation. HMGB1, high-mobility group box 1; LPS, lipopolysaccharides.

Increased expression of α -smooth muscle actin (α SMA) and collagen I are the characteristic hallmarks of aHSCs. aHSCs also release tissue inhibitors of metalloproteinases (TIMPs) 1 and 2 that block the action of matrix-degrading matrix metalloproteinases (MMPs), thereby promoting deposition of ECM.^{54,55} Accumulated ECM serves as a reservoir for growth factors, cytokines and chemokines that are involved in its formation.⁵⁶ Of note, during the liver fibrogenesis, not only the quantity but also the composition of ECM changes with an increase in collagens (collagen types I, III and IV) and non-collagenous components (fibronectin, hyaluronan, proteoglycans, etc.).⁵⁴

The immune-inflammatory response, modulator of the fibrotic process

Scarring of the liver is a complex process that engages liver-parenchymal and non-parenchymal cells, including immune cells, which act as a double-edged sword contributing not only to the progression but also to the resolution of liver fibrosis.^{49,57} While Kupffer cells are the resident liver macrophages, neutrophils, T lymphocytes, dendritic cells as well as other cell populations become recruited during cell injury.^{49,57} For example, necrotic cell death results in the release of the alarmin High-Mobility-Group-Protein B1 (HMGB1) that together with the chemokine CXCL12 directs neutrophils to the site of damage.⁵⁸ Monocyte infiltration is mediated by CCR2 and its ligand CCL2. After their arrival to the liver, the cells adapt their phenotype to the current disease condition.⁵⁷ In the situation of ongoing injury, they release proinflammatory mediators, *e.g.*, tumor necrosis factor (TNF), interleukin-6 (IL-6), IL-1 β or TGF β , that promote the recruitment of T cells and neutrophils and stimulate the profibrogenic activity of aHSCs.^{48,49} An increased intestinal permeability, as seen in patients with alcoholic liver disease and/or advanced liver fibrosis, constitutes another established profibrogenic stimulus that is mediated by lipopolysaccharide (LPS)-dependent Toll-like receptor 4 (TLR4) signaling.^{59,60} On the other hand, an elimination of a damaging agent leads to the transformation of macrophages into a restorative mode characterized by low Ly6C (Ly6Clo) expression.⁵⁷

Liver fibrosis resolution, centerline for emerging treatment strategies

The fundamentals of fibrosis resolution rely on four cardinal points: (i) discontinuation of the primary cause of chronic hepatic injury; (ii) regression of myofibroblasts from an activated to a deactivated status and/or their elimination; (iii) degradation of excessive matrix and (iv) switch of proinflammatory milieu to a restorative environment.⁶¹ Elimination of aHSCs is achieved via apoptosis or senescence.^{61,62} A wide range of signals has been identified as proapoptotic effectors in aHSCs.⁶³ For example, cyclooxygenase-2 mediated programmed

cell death in aHSCs via metabolism of a lipid mediator endogenous cannabinoid 2-arachidonoyl glycerol (2-AG).⁶⁴ Tumor necrosis factor-related apoptosis-inducing ligand (TRAIL) can also eliminate aHSCs.⁶⁵ Bile acids exert their antifibrotic properties via the farnesoid X receptor (FXR) that induces production of the small heterodimer partner (SHP) with a subsequent decrease in AP-1 signaling.⁶⁶ Various other factors promote apoptosis of aHSCs via NF- κ B inhibition.⁶⁷ In addition to apoptosis, it has been demonstrated that a fraction of aHSCs express senescence markers.⁶⁸ Consequently, senescence that constitutes a cell-cycle arrest due to telomere shortening may play an essential role in eliminating myofibroblasts during fibrosis reversal.⁶⁸ Proteins such as IL22, insulin-like growth factor I (IGF-I) or CCN1/CYR61 and drugs such as atorvastatin, tetramethylpyrazine or celecoxib act as senescence inducers in aHSCs.⁶⁹⁻⁷⁵

Other experimental studies have shown that aHSCs can undergo reversal to a quiescent-like status. However, these reverted HSCs differ from unchallenged resident HSCs and are sensitive towards fibrogenic stimuli.^{76,77}

Degradation of the extracellular matrix

Degradation of ECM is closely related to the loss of aHSCs that results in decreased TIMP levels and a favorable MMP-TIMP ratio.^{15,78} MMPs as the primary matrix-degrading enzymes are released by many various cell populations, including HSCs and neutrophils. Restorative macrophages constitute the most prominent matrix eater due to the generation of MMP12 and MMP13.⁷⁹ However, it remains unclear whether or not the fibrotic changes are completely reversible. In that respect, a presence of elastin or collagen cross-links was suggested to confer resistance against fibrolysis.⁸⁰

Shifting the balance from a proinflammatory to a restorative status

During liver fibrosis resolution, the removal of injurious stimuli leads to a switch from a proinflammatory to an antiinflammatory and restorative status. Of crucial relevance are the changes induced in the immune compartment, which are characterized by a substantial decrease in extrahepatically-derived immune cells (monocytes, NKT cells, etc.) and intrahepatic T cells.⁸¹ In addition, intrahepatic immune cells undergo adaptive changes in their phenotype. One of the most crucial readjustments is the restorative conversion of macrophages, a process that is mediated via fractalkine receptor CX3CR1 and promoted by phagocytosis of apoptotic myofibroblasts and/or injured hepatocytes.⁸² Restorative macrophages display decreased Ly6C expression and increased production of MMPs (MMP-9, MMP-13), growth factors as well as expression of phagocytosis-associated receptors.⁷⁹ Dendritic cells and natural killer cells, which promote MMP-9-dependent matrix degradation and apoptosis of activated and senescent myofibroblasts, are also involved in fibrosis resolution.^{83,84} Last but not least, proangiogenic factors such as VEGF or myeloid-derived chemokines as CXCL9 contribute to fibrosis regression by inducing changes in the hepatic sinusoidal circulation system.^{85,86}

Emerging therapeutic agents in liver fibrosis

A large amount of data indicates that liver fibrosis is potentially reversible.^{9,87} While the basic principles of fibrosis development are the same in all chronic liver diseases, etiology-specific features exist. For example, fibrosis occurs primarily in pericentral and perisinusoidal areas in ALD patients, whereas in chronic viral hepatitis and chronic cholestasis, it is seen predominantly around portal tracts.⁸⁸ Therefore, novel anti-fibrotic treatments need to take disease-specific factors into account. A useful strategy is to explore drugs that are already used in the clinical routine for their anti-fibrotic potential. In the subsequent section, we are reviewing compounds that are currently being tested for the treatment of liver fibrosis (**Table 9.2, Figure 9.2**).

Therapies to suppress primary disease etiology and lifestyle interventions

The elimination of the primary etiology is considered the most effective therapy against hepatic fibrosis. For example, antiviral therapies against hepatitis B, C or D viruses resulted in liver fibrosis regression.⁸⁹⁻⁹¹ Similarly, depletion of iron overload proved to be beneficial in patients with hemochromatosis, while treatment with corticosteroids led to diminished fibrosis in autoimmune hepatitis.^{92,93}

Excessive caloric intake and a sedentary lifestyle have been associated with NAFLD, while weight loss through physical activity and caloric restriction contributes to disease improvement.⁹⁴ This fact was nicely demonstrated in a prospective lifestyle intervention study over the course of 52 weeks. At the end of the intervention, 25% of participants displayed a resolution of histologically proven NASH, while 19% reached a regression of liver fibrosis.⁹⁵ In carefully selected patients, bariatric surgery represents an even more powerful treatment strategy. In that respect, a retrospective study revealed a reduced fibrosis grade in 34% of patients one year after the surgery.⁹⁶ In alcoholic liver disease, cessation of alcohol ingestion has been associated with improved liver histology and a better survival rate.⁹⁷⁻⁹⁹

Hepatoprotective agents

Emricasan (IDN-6556)

Apoptosis constitutes an essential factor in promoting the development of chronic liver diseases.³⁸ In line with that, the irreversible and orally-active pan-caspase inhibitor emricasan ameliorated liver fibrosis, inflammation and hepatocellular damage in experimental hepatic injury models.^{40,100} In HCV infected patients as well as in NAFLD subjects, emricasan was well tolerated and led to a reduction in serum liver enzymes.^{101,102} In addition to that, several phase 2 clinical trials are being carried out to evaluate its usefulness in AH (NCT01912404), NAFLD (NCT02077374; ENCORE-NF: NCT02686762; ENCORE-PH: NCT02960204) or liver cirrhosis of various etiologies (NCT02230670).

Selonsertib (ASK1 inhibitor; GS-4997)

The apoptosis-signal-regulating kinase 1 (ASK1) belongs to the family of mitogen-activated phosphokinases (MAPKs).^{103,104} It is activated by a wide variety of pro-fibrogenic stimuli such as TGF- β , TNF- α -mediated apoptosis or ROS and leads to activation of the p38/JNK signaling, thereby promoting hepatic damage and fibrosis.^{103,104} Liver disease biopsies express activated ASK1 signaling and inhibition of ASK1 in a murine model of NASH resulted in significant amelioration of steatosis and fibrosis in the liver.^{103,105}

A phase 2 open-label trial that used GS-4997 together with simtuzumab (NCT02466516) resulted in a reduction of liver fibrosis. Two phase 3 trials are currently assessing the safety and efficacy of selonsertib in NASH patients with F3 bridging fibrosis (STELAR 3; NCT03053050) and compensated cirrhosis (STELLAR 4; NCT03053063).¹⁰⁶

HSCs-targeting compounds and ECM-interfering modulators

ACEi/ARB

The renin-angiotensin-system (RAS) controls the blood pressure and the water-electrolyte balance. The angiotensin I converting enzyme (ACE) blocks the proteolytic conversion of angiotensin I into angiotensin II and ACE inhibitors (ACEis) are widely used for the treatment of high blood pressure and chronic heart failure.

In addition to that, increased activity of the RAS system is seen in liver fibrosis/cirrhosis and elevated production of angiotensin II type 1 receptor occurs in activated HSCs.¹⁰⁷

The profibrogenic role of angiotensin II might be due to increased oxidative stress, activation, proliferation and contraction of HSCs, upregulation of TGF β and TIMP1 and accelerated collagen deposition.^{107,108}

In line with that, ACEis and AT1-receptor blockers (ARBs) have arisen as potential therapeutic options in liver fibrosis. For example, a clinical study evaluating the effect of the ARB losartan in HCV patients revealed reduced inflammation and diminished expression of fibrogenic genes.¹⁰⁹

In ALD patients, a combination of the ARB candesartan and ursodeoxycholic acid (UDCA) improved the patient's fibrosis scores compared to UDCA treatment alone.¹¹⁰ However, since other clinical trials did not show any effect, more studies are needed to prove the efficacy of RAS antagonists in hepatic fibrosis.^{111,112}

Figure 9.2 HSCs—the determinants of liver fibrosis progression and resolution. The activation of hepatic stellate cells (HSCs) and their transdifferentiation into myofibroblasts (MFs) are the key factors in liver fibrosis progression, whereas apoptosis/senescence of MFs or their reversal into quiescent HSCs contribute to the resolution of liver fibrosis. The anti-fibrotic compounds that are currently being assessed in clinical trials either try to prevent HSC activation or promote the degradation of extracellular matrix (ECM). Anti-TIMP1, anti-tissue inhibitor of metalloproteinase-1 antibody; CVC, cenicriviroc (dual CCR2 and CCR5 inhibitor); Gal-3, galectin-3 inhibitor; GFT505, elafibranor (PPAR- α/δ agonist); GS-4997, selonsertib (ASK1 inhibitor); GS-9450, nivocasan (pan-caspase inhibitor); IDN-6556, emricasan (pan-caspase proteinase inhibitor); OCA, obeticholic acid (FXR antagonist); PTX, pentoxifylline; RLX030, serelaxin (dual LGR7 and LGR8 agonist); VitE, vitamin E

Cannabinoid receptor (CB) 1 and 2 modulators

The endocannabinoid receptors CB1 and CB2 constitute attractive targets for the treatment of chronic liver disease. The stimulation of CB2 is associated with hepatoprotective and antifibrotic outcomes. For example, the selective CB2 agonist JWH-133 induced HSC apoptosis and quiescence by reducing the immune reaction.¹¹³ In contrast, CB1 contributes to matrix deposition and has steatogenic potential. Antagonizing the activity of CB1 has been an attractive target against obesity; however, the clinical use of the CB1 antagonist rimonabant had to be terminated due to mood-related adverse effects. CB1 antagonists that do not cross the blood-brain barrier are currently under development.^{113,114}

Elafibranor (GFT505)

Peroxisome proliferator-activated receptors (PPARs) are nuclear receptors that play an essential role in metabolism, cell growth, differentiation and inflammation.¹¹⁵ Among them, PPAR- α is highly expressed in the liver and is involved in the inflammatory response and regulation of fat metabolism, whereas PPAR- δ is produced in several tissues and has both metabolic and anti-inflammatory functions.¹¹⁶⁻¹¹⁸ Accordingly, in several rodent models of NASH, the dual PPAR- α/δ agonist elafibranor reduced hepatic lipid accumulation and proinflammatory signaling and ameliorated liver fibrosis.¹¹⁹ In line with that, the GOLDEN-505 trial demonstrated an elafibranor-associated improvement in histologic features of NASH without a worsening of fibrosis.²⁰ A current phase 3 trial assesses the long-term efficacy and safety of elafibranor in NASH patients (RESOLVE-IT; NCT02704403).

LOXL2 antibody (GS-6624)

Collagen crosslinking via lysyl oxidase family members such as lysyl oxidase-like 2 (LOXL2) represents a major ECM stabilizer that has been implicated in fibrosis progression as well as an impaired fibrosis reversal.¹²⁰ Accordingly, the LOXL2-blocking monoclonal antibody has shown anti-fibrotic activity in several experimental liver fibrosis models.^{121,122} However, while the anti-LOXL2 antibody simtuzumab was well tolerated in HIV- and HCV-infected adults, no obvious effect on liver fibrosis was noted.¹²³ In addition to that, phase-two trials of NASH and primary sclerosing cholangitis have been terminated due to a lack of efficacy.

Tissue inhibitor metalloproteinase-1 antibody (anti-TIMP-1)

As mentioned above, tissue inhibitor of metalloproteinase-1 (TIMP-1) constitutes the major inhibitor of ECM degradation by blocking matrix metalloproteinases (MMPs). TIMP-1 also promotes the survival of activated HSCs. In agreement with that, preclinical data from CCl₄-induced liver fibrosis displayed attenuation of fibrosis, decreased HSC activation and lower MMP2 activity in rats treated with the anti-TIMP1 antibody.¹²⁴

Serelaxin (RLX030)

Serelaxin represents a recombinant form of human relaxin-2 leading to vasodilation. While its vasoprotective effects were suggested to contribute to the reduction of the reparative response in liver fibrosis, there is evidence that serelaxin also promotes the degradation of ECM by increasing MMP-13, reducing the expression of TIMP-2 and interfering with the TGF β signaling.^{21,125,126} In addition to encouraging data showing the potential efficacy of serelaxin for renal dysfunction in liver cirrhosis,¹²⁷ there is currently a phase II clinical trial assessing the efficacy of serelaxin in cirrhotic patients with portal hypertension (NCT02669875).

Anti-inflammatory and antioxidant therapies

Obeticholic acid (OCA, INT-747)

The farnesoid X receptor (FXR) is involved in the regulation of glucose and lipid metabolism. Obeticholic acid (OCA) is a semi-synthetic compound derived from the primary bile acid chenodeoxycholic acid (CDCA). It functions as a selective agonist of the FXR receptor with an approximate 100-fold higher potency than CDCA. Given its anti-cholestatic effects that have been confirmed in a phase III clinical trial,^{128,129} it has been approved for the treatment of PBC. While current clinical studies suggest an anti-inflammatory and anti-fibrotic effect in patients with NASH^{130,131} a phase III clinical trial is still recruiting patients (NCT02548351).

Pentoxifylline

Tumor necrosis factor-alpha (TNF- α) is a crucial cytokine that stimulates the release of proinflammatory mediators in response to hepatic injury. Pentoxifylline (PTX) is a methylxanthine derivative that suppresses the production of TNF- α and other proinflammatory cytokines as well as decreases oxidative stress.¹³² In NASH patients, pentoxifylline treatment improved the extent of liver fibrosis,¹³³ whereas no noticeable effect was seen in patients with severe alcoholic hepatitis.¹³⁴

Statins

Several clinical studies have focused on the therapeutic potential of statins that are the leading cholesterol-lowering agents acting through inhibition of 3-hydroxy-3-methylglutaryl-coenzyme A reductase. These investigations were spurred by rodent studies, where statins displayed antifibrotic, anti-inflammatory and vasoprotective effects and ameliorated microvascular dysfunction and portal hypertension.¹³⁵⁻¹⁴¹ Similarly, in a multicenter randomized study, simvastatin reduced portal pressure and improved liver perfusion in patients with cirrhosis.¹⁴² In a follow-up trial, simvastatin improved survival in patients with Child A/B cirrhosis but had no effect in Child C cirrhotics, nor did it decrease the rate of recurrent bleedings.¹⁴³ These data are in line with a retrospective cohort study on US patients with compensated hepatitis C-associated liver cirrhosis, in whom the use of statins associated with fewer decompensations and lower death rates.¹⁴⁴ Despite these promising data, further studies are needed to unequivocally delineate the usefulness of statins in patients with advanced liver disease as well as their impact on liver fibrosis.

Cenicriviroc

The interactions between CeC chemokine receptor types 2 (CCR2) and 5 (CCR5) and their ligands play a fundamental role in the recruitment and infiltration of monocytes/macrophages to the liver, thereby driving the inflammatory response as well as hepatic stellate cell activation and fibrogenesis.¹⁴⁵ Cenicriviroc (CVC), an oral antagonist of both CCR2 and CCR5-dependent pathways, results in potent inhibition of inflammatory

response and fibrogenesis in diverse preclinical models of hepatic fibrosis.^{63,146} In line with that, several trials currently address the clinical usefulness of CVC. For example, the CENTAUR study (a phase IIb trial, NCT02217475;¹⁴⁷) and STELLARIS (a phase 3 trial; NCT03028740) evaluate the efficacy and safety of CVC in NASH patients, whereas PERSEUS (NCT02653625, a phase II proof-of-concept trial) is testing its usefulness in PSC individuals. The first data from the CENTAUR trial showed that 20% of the study participants reduced their fibrosis grade after one year of treatment compared to 10% of subjects in the placebo group ($p=0.0023$).

Liraglutide

The glucagon-like peptide-1 analog liraglutide used for the treatment of diabetes mellitus type 2 has been shown to induce weight-loss by restriction of caloric intake.^{148,149} Given these properties, phase 2 LEAN assessed the efficacy of liraglutide in overweight patients with clinical features of NASH. It showed a resolution of definite NASH in 39% of the patients compared to only 9% in the placebo group ($p=0.019$).¹⁵⁰

Galectin-3 antagonists

Galectin-3 (Gal-3) belongs to the family of lectins and has been demonstrated to play a relevant role in the development of liver fibrosis. Under physiological conditions, Gal-3 is expressed at low levels, whereas inflammatory and fibrogenic stimuli lead to a markedly enhanced release of Gal-3, particularly by macrophages and other immune cells, thereby contributing to fibrogenesis.^{151,152} In a model of murine toxin-induced liver fibrosis, the Gal-3 inhibitors GR-MD-02 (galactoarabino-rhamnogalaturonan) and GM-CT-01 (galactomannan) both led to a significant decrease of galectin-3 expression in portal and septal macrophages, attenuation of fibrosis, cirrhosis reversal and reduced portal pressure.¹⁵³ Two phase II clinical trials currently evaluate the efficacy and safety of GR-MD-02 in NASH patients (NCT02462967; NCT02421094).

Vitamin E

Vitamin E (VitE) encloses a group of lipid-soluble antioxidants. The PIVENS trial performed a comparative analysis of the therapeutic impact of vitamin E versus pioglitazone versus placebo in nondiabetic patients with NASH. The obtained results pointed to a more remarkable improvement of NASH with VitE therapy, but fibrosis scores did not differ significantly. The TONIC study reported a histological improvement in pediatric NASH patients treated with VitE; however, no improvement in ALT was achieved.¹⁵⁴ Despite that, the current data are not sufficient to recommend VitE therapy in NASH patients. With regard to that, a meta-analysis suggested that VitE treatment may even lead to a higher all-cause mortality.¹⁵⁵

Table 9.2 Antifibrotic clinical studies with liver fibrosis as an endpoint

Drug name	Mechanism of action	Target disease	Characteristics of the intervention / study (drug posology, treatment duration, patient no., study design/type)	Year of 1 st completion	Phase	NCT ID No./reference
HEPATOPROTECTANT DRUGS						
Emricasan	Pan-caspase proteinase inhibitor	NASH	Emricasan 10 or 100 mg per day; 17 mo; 330 patients (estimated); multicenter randomized double-blind placebo-controlled trial (ENCORE-NF)	2018	II	NCT02686762
		NASH + cirrhosis + PHT	Emricasan 10, 50 or 100 mg per day; 6 mo; 240 patients (estimated); multicenter randomized double-blind placebo-controlled trial (ENCORE-PH)	2018	II	NCT02960204
		AH + c.i. for steroids	Emricasan 50 mg per day; 1 mo; 5 patients; multicenter randomized double-blind placebo-controlled trial	2015	II	NCT01912404
		NAFLD + raised ALT	Emricasan 50 mg per day; 1 mo; 38 patients; multicenter randomized double-blind placebo-controlled trial (E, S)	2015	II	NCT02077374
		Cirrhosis	Emricasan 50 mg per day; 3 mo; 87 patients; multicenter randomized double-blind placebo-controlled trial (E, S, T)	2015	II	NCT02230670
		Cirrhosis + PHT	Emricasan 50 mg per day; 1 mo; 23 patients; non-randomized open-label single group assignment (E, S, T)	2015	II	NCT02230683
Nivocasan (GS-9450)	Pan-caspase inhibitor	NASH	GS-9450 1, 5, 10 or 40 mg per day; 1 mo; 124 patients; multicenter randomized double-blind placebo-controlled trial (S, T, P)	2009	II	159
		HCV	GS-9450 5, 10, 40 or 80 mg per day; 0.5 mo; 33 patients; multicenter randomized double-blind placebo-controlled trial (S, T, P)	2008	IIa	NCT00725803
Elafibranor (GFT505)	PPAR- α/δ agonist	NASH	Elafibranor 120 mg daily; endpoints at 17 mo/ 4years (long-term period); 2000 patients; multicenter randomized double-blind placebo-controlled trial (E, S) (RESOLVE-IT)	2021	III	NCT02704403
		NASH	GFT505 80 versus 120 mg daily; 12 mo; 270 patients; multicenter randomized double-blind placebo-controlled trial	2015	IIb	20
Selonsetib (GS-4997)	ASK1 inhibitor	NASH + F3 bridging fibrosis	Selonsetib 6 or 18 mg; 60 mo; 800 patients (estimated); randomized double-blind placebo-controlled trial (E, S) (STELLAR 3)	2020	III	NCT03053050
		NASH + compensated cirrhosis	Selonsetib 6 or 18 mg; 60 mo; 800 patients (estimated); randomized double-blind placebo-controlled trial (E, S) (STELLAR 4)	2020	III	NCT03053063
		AH	Prednisolone 40 mg vs Prednisolone (40 mg) + GS-4997 (18 mg) daily; 1 mo; 120 patients (estimated); randomized double-blind placebo-controlled trial (S, T)	2018	II	NCT02854631
		Fibrosis + stage F2-F3	SIM 125 mg or GS-4997 (6 or 18 mg) or SIM (125 mg)+ GS-4997 (6 or 18 mg); 6 mo; 72 patients; randomized parallel open-label assignment (E, S, T)	2016	II	NCT02466516

Table 9.2 (continued)

Drug name	Mechanism of action	Target disease	Characteristics of the intervention / study (drug posology, treatment duration, patient no., study design/type)	Year of 1 st completion	Phase	NCT ID No./reference
HEPATOPROTECTANT DRUGS						
Oltipraz	Rock-kinase inhibitor	NAFLD	Oltipraz 30 or 60 mg twice a day; 6 mo; 60 patients; multicenter randomized double-blind placebo-controlled trial (E, S)	2013	II	160
		HBV/HCV + Cirrhosis	Oltipraz 60 mg/bid / Oltipraz 90 mg/qd; 6 mo; 81 patients; multicenter randomized double-blind placebo-controlled trial (E, S)	N/A	II	NCT00956098
Irbesartan	AT ₁ R antagonist	HCV	Irbesartan 150 mg a day; 24 mo; 166 patients; randomized double-blind placebo-controlled trial (E) (Fibrosar)	2013	III	NCT00265642
Candesartan		ALD	UDCA (600 mg/day) + Candesartan (8 mg/day) vs UDCA (600 mg/day) alone; 6 mo; 85 patients; randomized open-labelled placebo-controlled trial (E, S)	2008	I, II	110
Losartan		HCV	Losartan 50 mg a day; 18 mo; 14 patients; E; non-randomized open-label single group assignment	N/A	IV	109
		NASH	Losartan 50 mg a day; 24 mo; 45 patients; E; randomized double-blind placebo-controlled trial (FELINE)	2014	III	NCT01051219
Moexipril	ACE inhibitor	PBC	Moexipril 15 mg a day; 12 mo; 20 patients with suboptimal UDCA response; open-label single group assignment (E, S)	2007	II	112
MATRIX-DEGRADING AGENTS						
Simtuzumab (GS-6624)	Lysyl oxidase-like 2 monoclonal antibody	NASH	GS-6624 75 or 125 mg once weekly; 55 mo; 222 patients; randomized double-blind placebo-controlled trial (E, S)	2016	IIb	NCT01672866
		NASH + cirrhosis	GS-6624 200/700 mg; 55 mo; 259 patients; randomized double-blind placebo-controlled trial (E, S)	2016	IIb	NCT01672879
		PSC	GS-6624 75 or 125 mg weekly; 22 mo; 235 patients; randomized double-blind placebo-controlled trial (E, S)	2016	IIb	NCT01672853
Serelaxin (RLX030)	Dual agonist LGR7 and LGR8	HIV and/or HCV Cirrhosis + PHT	GS-6624 700 mg bi-weekly; 6 mo; 18 patients; non-randomized parallel assignment (S, T) 20 patients (estimated); randomized double-blind placebo-controlled trial (STOPP)*	2014 2016	IIa II	123 NCT02669875

Table 9.2 (continued)

Drug name	Mechanism of action	Target disease	Characteristics of the intervention / study (drug posology, treatment duration, patient no., study design/type)	Year of 1 st completion	Phase	NCT ID No./reference
ANTI-INFLAMMATORY AND ANTI-OXIDANT COMPOUNDS						
Obeticholic acid (OCA, INT-747)	FXR antagonist	NASH	OCA 10-25 mg once daily; #72 mo; 2000 patients, multicenter randomized double-blind placebo-controlled trial (REGENERATE)	2021	III	NCT02548351
		AH	OCA10 mg per day; 60 patients (estimated); 1.5 mo; randomized double-blind placebo-controlled trial (TREAT)	2017	II	NCT02039219
	moderate AH severe		OCA10 mg per day; 45 patients (estimated); 1 mo; randomized double-blind placebo-controlled trial	2017	N/A	NCT02654236
		NASH	OCA 25 mg per day; 17 mo; 283 patients; multicenter randomized double-blind placebo-controlled trial (FLINT)	2014	II	131
		PBC	OCA 10, 25 or 50 mg once daily; 3 mo; 165 patients; randomized double-blind placebo-controlled trial	2014	II	128
		PBC	OCA 5-10 mg per day; 12 mo; 217 patients; randomized double-blind placebo-controlled trial (POISE)	2013	III	129
ANTI-INFLAMMATORY AND ANTI-OXIDANT COMPOUNDS (cont.)						
Cenicriviroc (CVC)	Dual inhibitor CCR2 and CCR5	NASH	CVC 150 mg per day; 2000 patients, randomized parallel assignment (E, S) (STELLARIS)	2019	III	NCT03028740
		PSC	CVC 125 mg once daily; 6 mo; 25 patients (estimated), PoC study, single group assignment (E, S) (PRESEUS)	2017	II	NCT02653625
		NASH	CVC 150 mg per day; 24 mo; 289 patients; randomized double-blind placebo-controlled trial (E, S) (CENTAUR)	2016	IIb	147
LIN452	FXR agonist	NASH	LIN452 vs placebo; 250 patients (estimated); 3 mo; randomized double-blind placebo-controlled trial (E, S, T) (FLIGHT-FXR)	2017	II	NCT02855164
		PBC	LIN452 capsules once per day; 95 patients (estimated); 1 mo; multicenter randomized double-blind placebo-controlled trial (E, S, T)	2017	II	NCT02516605
Liraglutide	Glucagon-like peptide-1	NASH	Liraglutide 1.8 mg once per day; 52 patients; 48 mo; multicenter randomized double-blind placebo-controlled trial (A, E) (LEAN)	2014	II	150
GR-MD-02	Galectin-3 inhibitor	PBC	LIN452 capsules once per day; 95 patients (estimated); 1 mo; multicenter randomized double-blind placebo-controlled trial (E, S, T)	2017	II	NCT02516605
		NASH cirrhosis	GR-MD-02 in a dose of 2 or 8 mg/kg lean body mass administered every other week over a 52 week period for a total of 26 infusions; 156 patients; multicenter randomized double-blind placebo-controlled trial (E, S) (NASH-CX)	2017	II	NCT02462967

Table 9.2 (continued)

Drug name	Mechanism of action	Target disease	Characteristics of the intervention / study (drug posology, treatment duration, patient no., study design/type)	Year of 1 st completion	Phase	NCT ID No./reference
ANTI-INFLAMMATORY AND ANTI-OXIDANT COMPOUNDS (cont.)						
Pentoxifylline	TNF- α synthesis inhibitor	PBC	Pentoxifylline 1200 mg per day; 6 mo; 20 patients; open-label single group assignment	2012	II	NCT01249092
		NASH	Pentoxifylline 1200 mg per day; 12 mo; 55 patients; randomized double-blind placebo-controlled trial (E)	2010	II	133
		AH severe	Pentoxifylline 1200 mg per day + Prednisolone 40 mg/day; 1 mo; 278 patients; multicenter randomized double-blind placebo-controlled trial	2010	III	161
		NASH	Pentoxifylline 1200 mg per day; 12 mo; 26 patients; randomized double-blind placebo-controlled trial	2009	II,III	162
		Cirrhosis	Pentoxifylline 1200 mg per day; 6 mo; 342 patients; randomized double-blind placebo-controlled trial (PENTOCIR)	2007	III	163
Pioglitazone	PPAR- γ agonist	AH	Prednisolone (40 mg) vs Pentoxifylline (1200 mg) vs Prednisolone (40 mg) + Pentoxifylline (1200 mg) per day; 1 mo; 1103 patients; multicenter randomized double-blind placebo-controlled trial (STOPAH)	N/A	III	134
		NASH	Pioglitazone 30 mg per day; 6mo; 90 patients (estimated); randomized double-blind placebo-controlled trial (E, S)	2018	II	NCT01068444
		NASH + T2DM	Pioglitazone 30 mg/day initially, later Pioglitazone 45 mg/day until end of the study, if well tolerated; 36 mo; 146 patients; randomized double-blind placebo-controlled trial (UTHSCSA NASH Trial)	2014	IV	164
		Cirrhosis	Pioglitazone + autologous bone marrow mesenchymal stem cell transplantation; 3 patients; single group assignment	2013	I	165
		Ad. cirrhosis	Pioglitazone 60 mg per day; 9 days; 20 patients; randomized double-blind placebo-controlled trial*	2008	IV	NCT00570622
Pirfenidone	Anti-inflammatory Antioxidant	HCV cirrhosis	Pirfenidone 1200 mg per day; 24 mo; 34 patients; open-label, non-controlled and non-randomized trial	2006	II	166
Vitamin E (VitE)		NASH	Pioglitazone (30mg) versus VitE (800 IU) per day; 22 mo; 247 patients (non-diabetic), randomized double-blind placebo-controlled trial (PIVENS)	2009	III	167
		NAFLD	Metformin (1000 mg) versus VitE (800 mg) per day; 12 mo; 173 patients (aged 8-17); randomized, double-blind placebo-controlled trial (TONIC)	2009	III	154
		HCV	Pentoxifylline (800 mg) + tocopherol (1000 mg) per day; 12 mo; 100 patients, randomized double-blind placebo-controlled trial (E, S)	N/A	III	NCT00119119
Metadoxine	Antioxidant	NASH	Metadoxine 500 mg per day; 6 mo; 108 patients (estimated); randomized double-blind placebo-controlled trial (E)	2018	III	NCT02541045
MSDC 0602K	Insulin sensitizer	NASH	MSDC 0602K 62.5, 125 or 250 mg once daily; 12 mo; 200 patients (estimated); randomized double-blind placebo-controlled trial (E, S, T)	2018	II	NCT02784444

Table 9.2 (continued)

Drug name	Mechanism of action	Target disease	Characteristics of the intervention / study (drug posology, treatment duration, patient no., study design/type)	Year of 1 st completion	Phase	NCT ID No./reference
HERBAL MEDICINES AND DIETARY SUPPLEMENTS						
Fuzheng Huayu		HBV refractory	1 Entecavir tablet/day+4 FH tablets three times/day and specific TCM granule; 12 mo; 300 patients (estimated); multi-center single group assignment (E, S)	2016	IV	NCT02241616
		HBV + cirrhosis	Entecavir+12 FH tablets/day; 12 mo; 700 patients (estim-ated); multi-center, randomized, double-blind, placebo-controlled trial (E, S)	2016	IV	NCT02241590
		HCV	6 tablets/day; 12 mo; 118 patients; multi-centre, randomized, double-blind, placebo-controlled trial (E, S)	2013	II	NCT00854087
		PSC	BCM-95 curcumin 500 mg/day; 3 mo; 15 patients (estimated); single group assignment (E, S)	2018	I, II	NCT02978339
Curcumin		NASH	1.5 g/day; 3 mo; 50 patients (estimated); randomized, double-blind, placebo-controlled trial	2017	I, II	NCT02908152
Fufang Biejia Ruangan		NAFLD	Nutraceutical mixture (containing curcumin) 1600 mg/day; 3 mo; 150 patients (estimated); multi-center, randomized, double-blind, placebo-controlled trial (E)	2016	N/A	NCT02369536
		HBV	0.5 mg ETV once daily + 2 g of TCM tablets with (Biejia Ruangan) 3 times/day vs placebo; 1000 patients; multi-center, randomized, double-blind, placebo-controlled trial	2015	IV	168
		NASH	Diamel 2 oral tablets/8 hours; 12 mo; 158 patients; randomized, double-blind, placebo-controlled trial (E, S)	2012	III	NCT00820651
		HBV	Lamivudine (100 mg) vs Limuvudine (100 mg)+Oxymatrine (200 mg); 18 mo; 192 patients; randomized parallel assignment (E)	2014	IV	NCT02202473

ad, advanced; ALD, alcoholic liver disease; AH, alcoholic hepatitis; c.i., contraindication; E, efficacy study; HBV, hepatitis B virus infection; FH, Fuzheng Huayu; HCV, hepatitis C virus infection; HIV, human immunodeficiency virus infection; LF, liver fibrosis; mo, month N/A, not available; NAFLD, non-alcoholic fatty liver disease; NASH, non-alcoholic steatohepatitis; P, pharmacokinetics study; PBC, primary biliary cirrhosis; PHT, portal hypertension; PSC, primary sclerosing cholangitis; S, safety study; T2DM, Type 2 diabetes mellitus; T, tolerability study; TCM, traditional Chinese medicine; *primary endpoint corresponds to portal hypertension improvement

Summary

The recent years yielded essential insights into our understanding of hepatic fibrosis and resulted in a large number of therapeutic strategies that are currently tested in clinical trials, especially for the treatment of NASH. The investigated agents demonstrate that liver fibrosis results from an intricate interplay between hepatocellular injury and inflammatory reaction leading to activation of HSCs. Despite these first successes, there is still a great need for basic research, in particular, to obtain better models of human NASH as well as a need for better translational strategies between pre-clinical and clinical investigations.

Practice points

- Hepatic fibrosis is a consequence of an ongoing liver injury resulting in the activation of hepatic stellate cells and increased deposition of extracellular matrix.
- Even at an advanced stage, liver fibrosis remains reversible.
- The main mechanisms leading to the reversal of liver fibrosis are deactivation/elimination of hepatic stellate cells, switch in the inflammatory environment and the degradation of extracellular matrix.
- While no anti-fibrotic compounds have been yet approved for clinical use, several drugs are currently undergoing advanced phases of clinical trials.

Research agenda

- The currently used *in vivo* liver fibrosis models need to be refined to closely mimic the corresponding human diseases.
- A better understanding of the mechanisms of deactivation and elimination of hepatic stellate cells is expected to yield novel therapeutic approaches.
- Screening of compounds already available in the clinical routine for their anti-fibrotic potential represents an efficient drug-development strategy.

References

1. Nicolas CT, Wang Y, Nyberg SL. Cell therapy in chronic liver disease. *Curr Opin Gastroenterol* 2016;32(3):189-94.
2. Blachier M, Leleu H, Peck-Radosavljevic M, Valla DC, Roudot-Thoraval F. The burden of liver disease in Europe: a review of available epidemiological data. *J Hepatol* 2013;58(3):593-608.

3. Beste LA, Leipertz SL, Green PK, Dominitz JA, Ross D, Ioannou GN. Trends in burden of cirrhosis and hepatocellular carcinoma by underlying liver disease in US veterans, 2001-2013. *Gastroenterology* 2015;149(6):1471-82 e5. quiz e17-8.
4. Ahmed A, Wong RJ, Harrison SA. Nonalcoholic fatty liver disease review: diagnosis, treatment, and outcomes. *Clin Gastroenterol Hepatol* 2015;13(12): 2062-70.
5. Koyama Y, Xu J, Liu X, Brenner DA. New developments on the treatment of liver fibrosis. *Dig Dis* 2016;34(5):589-96.
6. Yoon YHCC, Yi HY. Surveillance report #100: liver cirrhosis mortality in the United States: National, state, and regional trends. 2000e2011. Available from: <http://pubs.niaaa.nih.gov/publications/Surveillance100/Cirr11.htm>.
7. Asrani SK, Larson JJ, Yawn B, Therneau TM, Kim WR. Underestimation of liver-related mortality in the United States. *Gastroenterology* 2013;145(2):375-82 e1-2.
8. Hammel P, Couvelard A, O'Toole D, Ratouis A, Sauvanet A, Flejou JF, et al. Regression of liver fibrosis after biliary drainage in patients with chronic pancreatitis and stenosis of the common bile duct. *N Engl J Med* 2001;344(6):418-23.
9. Marcellin P, Gane E, Buti M, Afdhal N, Sievert W, Jacobson IM, et al. Regression of cirrhosis during treatment with tenofovir disoproxil fumarate for chronic hepatitis B: a 5-year open-label follow-up study. *Lancet* 2013;381(9865):468-75.
10. De Minicis S, Seki E, Uchinami H, Kluwe J, Zhang Y, Brenner DA, et al. Gene expression profiles during hepatic stellate cell activation in culture and in vivo. *Gastroenterology* 2007;132(5):1937-46.
11. Weber LW, Boll M, Stampfl A. Hepatotoxicity and mechanism of action of haloalkanes: carbon tetrachloride as a toxicological model. *Crit Rev Toxicol* 2003;33(2):105-36.
12. Heindryckx F, Colle I, Van Vlierberghe H. Experimental mouse models for hepatocellular carcinoma research. *Int J Exp Pathol* 2009;90(4):367-86.
13. Iwaisako K, Jiang C, Zhang M, Cong M, Moore-Morris TJ, Park TJ, et al. Origin of myofibroblasts in the fibrotic liver in mice. *Proc Natl Acad Sci U S A* 2014;111(32):E3297-305.
14. Scholten D, Trebicka J, Liedtke C, Weiskirchen R. The carbon tetrachloride model in mice. *Lab Anim* 2015;49(1 Suppl):4-11.
15. Iredale JP, Benyon RC, Pickering J, McCullen M, Northrop M, Pawley S, et al. Mechanisms of spontaneous resolution of rat liver fibrosis. Hepatic stellate cell apoptosis and reduced hepatic expression of metalloproteinase inhibitors. *J Clin Invest* 1998;102(3):538-49.
16. Constandinou C, Henderson N, Iredale JP. Modeling liver fibrosis in rodents. *Methods Mol Med* 2005;117:237-50.
17. Wallace MC, Hamesch K, Lunova M, Kim Y, Weiskirchen R, Strnad P, et al. Standard operating procedures in experimental liver research: thioacetamide model in mice and rats. *Lab Anim* 2015;49(1 Suppl):21-9.
18. Salguero Palacios R, Roderfeld M, Hemmann S, Rath T, Atanasova S, Tschuschner A, et al. Activation of hepatic stellate cells is associated with cytokine expression in thioacetamide-induced hepatic fibrosis in mice. *Lab Invest* 2008;88(11):1192-203.
19. Reif S, Aeed H, Shilo Y, Reich R, Kloog Y, Kweon YO, et al. Treatment of thioacetamide-induced liver cirrhosis by the Ras antagonist, farnesylthiosalicylic acid. *J Hepatol* 2004;41(2):235-41.
20. Ratzliff V, Harrison SA, Francque S, Bedossa P, Leher P, Serfaty L, et al. Elafibranor, an agonist of the peroxisome proliferator-activated receptor- α and - δ , induces resolution of nonalcoholic steatohepatitis without fibrosis worsening. *Gastroenterology* 2016;150(5):1147-59 e5.
21. Fallowfield JA, Hayden AL, Snowdon VK, Aucott RL, Stutchfield BM, Mole DJ, et al. Relaxin modulates human and rat hepatic myofibroblast function and ameliorates portal hypertension in vivo. *Hepatology* 2014;59(4):1492-504.
22. George J, Rao KR, Stern R, Chandrakasan G. Dimethylnitrosamine-induced liver injury in rats: the early deposition of collagen. *Toxicology* 2001;156(2e3):129-38.
23. Newell P, Villanueva A, Friedman SL, Koike K, Llovet JM. Experimental models of hepatocellular carcinoma. *J Hepatol* 2008;48(5):858-79.
24. Oh SW, Kim DH, Ha JR, Kim DY. Anti-fibrotic effects of a methylenedioxybenzene compound, CW209292 on dimethylnitrosamine-induced hepatic fibrosis in rats. *Biol Pharm Bull* 2009;32(8): 1364-70.
25. Tolba R, Kraus T, Liedtke C, Schwarz M, Weiskirchen R. Diethylnitrosamine (DEN)-induced carcinogenic liver injury in mice. *Lab Anim* 2015;49(1 Suppl):59-69.

26. Ackermann D, Mordasini D, Cheval L, Imbert-Teboul M, Vogt B, Doucet A. Sodium retention and ascites formation in a cholestatic mice model: role of aldosterone and mineralocorticoid receptor? *Hepatology* 2007;46(1):173-9.
27. Tag CG, Sauer-Lehnen S, Weiskirchen S, Borkham-Kamphorst E, Tolba RH, Tacke F, et al. Bile duct ligation in mice: induction of inflammatory liver injury and fibrosis by obstructive cholestasis. *J Vis Exp* 2015;96.
28. Brandon-Warner E, Schrum LW, Schmidt CM, McKillop IH. Rodent models of alcoholic liver disease: of mice and men. *Alcohol* 2012;46(8):715-25.
29. Bedossa P, Patel K. Biopsy and noninvasive methods to assess progression of nonalcoholic fatty liver disease. *Gastroenterology* 2016;150(8):1811-22 e4.
30. Sanches SC, Ramalho LN, Augusto MJ, da Silva DM, Ramalho FS. Nonalcoholic steatohepatitis: a search for factual animal models. *Biomed Res Int* 2015;2015:574832.
31. Ramadori P, Kroy D, Streetz KL. Immunoregulation by lipids during the development of non-alcoholic steatohepatitis. *Hepatobiliary Surg Nutr* 2015;4(1):11-23.
32. Ibrahim SH, Hirsova P, Malhi H, Gores GJ. Animal models of nonalcoholic steatohepatitis: eat, delete, and inflame. *Dig Dis Sci* 2016;61(5):1325-36.
33. Smit JJ, Schinkel AH, Oude Elferink RP, Groen AK, Wagenaar E, van Deemter L, et al. Homozygous disruption of the murine mdr2 P-glycoprotein gene leads to a complete absence of phospholipid from bile and to liver disease. *Cell* 1993;75(3):451-62.
34. Carlson JA, Rogers BB, Sifers RN, Finegold MJ, Clift SM, DeMayo FJ, et al. Accumulation of PiZ alpha 1-antitrypsin causes liver damage in transgenic mice. *J Clin Invest* 1989;83(4):1183-90.
35. Giovannoni I, Callea F, Stefanelli M, Mariani R, Santorelli FM, Francalanci P. Alpha-1-antitrypsin deficiency: from genome to liver disease. PiZ mouse as model for the development of liver pathology in human. *Liver Int* 2015;35(1):189-206.
36. Muriel P. Role of free radicals in liver diseases. *Hepatol Int* 2009;3(4):526-36.
37. Jaeschke H. Inflammation in response to hepatocellular apoptosis. *Hepatology* 2002;35(4):964-6.
38. Luedde T, Kaplowitz N, Schwabe RF. Cell death and cell death responses in liver disease: mechanisms and clinical relevance. *Gastroenterology* 2014;147(4):765-783 e4.
39. Takehara T, Tatsumi T, Suzuki T, Rucker 3rd EB, Hennighausen L, Jinushi M, et al. Hepatocyte-specific disruption of Bcl-xL leads to continuous hepatocyte apoptosis and liver fibrotic responses. *Gastroenterology* 2004;127(4):1189-97.
40. Canbay A, Feldstein A, Baskin-Bey E, Bronk SF, Gores GJ. The caspase inhibitor IDN-6556 attenuates hepatic injury and fibrosis in the bile duct ligated mouse. *J Pharmacol Exp Ther* 2004;308(3):1191-6.
41. Pianko S, Patella S, Sievert W. Alcohol consumption induces hepatocyte apoptosis in patients with chronic hepatitis C infection. *J Gastroenterol Hepatol* 2000;15(7):798-805.
42. Ribeiro PS, Cortez-Pinto H, Sola S, Castro RE, Ramalho RM, Baptista A, et al. Hepatocyte apoptosis, expression of death receptors, and activation of NFkappaB in the liver of nonalcoholic and alcoholic steatohepatitis patients. *Am J Gastroenterol* 2004;99(9):1708-17.
43. Gautheron J, Vucur M, Reisinger F, Cardenas DV, Roderburg C, Koppe C, et al. A positive feedback loop between RIP3 and JNK controls non-alcoholic steatohepatitis. *EMBO Mol Med* 2014;6(8): 1062-74.
44. Roychowdhury S, McCullough RL, Sanz-Garcia C, Saikia P, Alkhouri N, Matloob A, et al. Receptor interacting protein 3 protects mice from high-fat diet-induced liver injury. *Hepatology* 2016;64(5):1518-33.
45. Wree A, Marra F. The inflammasome in liver disease. *J Hepatol* 2016;65(5):1055-6.
46. Tilg H, Cani PD, Mayer EA. Gut microbiome and liver diseases. *Gut* 2016;65(12):2035-44.
47. Wake K. Perisinusoidal stellate cells (fat-storing cells, interstitial cells, lipocytes), their related structure in and around the liver sinusoids, and vitamin A-storing cells in extrahepatic organs. *Int Rev Cytol* 1980;66:303-53.
48. Wallace MC, Friedman SL, Mann DA. Emerging and disease-specific mechanisms of hepatic stellate cell activation. *Semin Liver Dis* 2015;35(2):107-18.
49. Koyama Y, Brenner DA. Liver inflammation and fibrosis. *J Clin Invest* 2017;127(1):55-64.
50. Borkham-Kamphorst E, van Roeyen CR, Ostendorf T, Floege J, Gressner AM, Weiskirchen R. Pro-fibrogenic potential of PDGF-D in liver fibrosis. *J Hepatol* 2007;46(6):1064-74.
51. Yoshiji H, Kuriyama S, Yoshii J, Ikenaka Y, Noguchi R, Hicklin DJ, et al. Vascular endothelial growth factor and receptor interaction is a prerequisite for murine hepatic fibrogenesis. *Gut* 2003;52(9):1347-54.
52. Steiling H, Muhlbauer M, Bataille F, Scholmerich J, Werner S, Hellerbrand C. Activated hepatic stellate cells express keratinocyte growth factor in chronic liver disease. *Am J Pathol* 2004;165(4):1233-41.

53. Kisseleva T. The origin of fibrogenic myofibroblasts in fibrotic liver. *Hepatology* 2017 Mar;65(3): 1039-43.
54. Arthur MJ. Fibrogenesis II. Metalloproteinases and their inhibitors in liver fibrosis. *Am J Physiol Gastrointest Liver Physiol* 2000;279(2):G245-9.
55. Iredale JP, Thompson A, Henderson NC. Extracellular matrix degradation in liver fibrosis: biochemistry and regulation. *Biochim Biophys Acta* 2013;1832(7):876-83.
56. Wynn TA. Common and unique mechanisms regulate fibrosis in various fibroproliferative diseases. *J Clin Investig* 2007;117(3):524-9.
57. Tacke F. Targeting hepatic macrophages to treat liver diseases. *J Hepatol* 2017;66(6):1300-12.
58. Kubes P, Mehal WZ. Sterile inflammation in the liver. *Gastroenterology* 2012;143(5):1158-72.
59. Schnabl B, Brenner DA. Interactions between the intestinal microbiome and liver diseases. *Gastroenterology* 2014;146(6):1513-24.
60. Strnad P, Tacke F, Koch A, Trautwein C. Liver e guardian, modifier and target of sepsis. *Nat Rev Gastroenterol Hepatol* 2017;14(1):55-66.
61. Tacke F, Trautwein C. Mechanisms of liver fibrosis resolution. *J Hepatol* 2015;63(4):1038-9.
62. Ramachandran P, Iredale JP, Fallowfield JA. Resolution of liver fibrosis: basic mechanisms and clinical relevance. *Semin Liver Dis* 2015;35(2):119-31.
63. Lefebvre E, Moyle G, Reshef R, Richman LP, Thompson M, Hong F, et al. Antifibrotic effects of the dual CCR2/CCR5 antagonist cenicriviroc in animal models of liver and kidney fibrosis. *PLoS One* 2016;11(6):e0158156.
64. Siegmund SV, Wojtalla A, Schlosser M, Schildberg FA, Knolle PA, Nusing RM, et al. Cyclooxygenase-2 contributes to the selective induction of cell death by the endocannabinoid 2-arachidonoyl glycerol in hepatic stellate cells. *Biochem Biophys Res Commun* 2016;470(3):678-84.
65. Oh Y, Park O, Swierczewska M, Hamilton JP, Park JS, Kim TH, et al. Systemic PEGylated TRAIL treatment ameliorates liver cirrhosis in rats by eliminating activated hepatic stellate cells. *Hepatology* 2016;64(1):209-23.
66. Fiorucci S, Antonelli E, Rizzo G, Renga B, Mencarelli A, Riccardi L, et al. The nuclear receptor SHP mediates inhibition of hepatic stellate cells by FXR and protects against liver fibrosis. *Gastroenterology* 2004;127(5):1497-512.
67. Watanabe A, Hashmi A, Gomes DA, Town T, Badou A, Flavell RA, et al. Apoptotic hepatocyte DNA inhibits hepatic stellate cell chemotaxis via tolllike receptor 9. *Hepatology* 2007;46(5):1509-18.
68. Krizhanovsky V, Yon M, Dickens RA, Hearn S, Simon J, Miething C, et al. Senescence of activated stellate cells limits liver fibrosis. *Cell* 2008;134(4):657-67.
69. Klein S, Klosel J, Schierwagen R, Korner C, Granzow M, Huss S, et al. Atorvastatin inhibits proliferation and apoptosis, but induces senescence in hepatic myofibroblasts and thereby attenuates hepatic fibrosis in rats. *Lab Investig* 2012;92(10):1440-50.
70. Simon TG, King LY, Zheng H, Chung RT. Statin use is associated with a reduced risk of fibrosis progression in chronic hepatitis C. *J Hepatol* 2015;62(1):18-23.
71. Jin H, Lian N, Zhang F, Bian M, Chen X, Zhang C, et al. Inhibition of YAP signaling contributes to senescence of hepatic stellate cells induced by tetramethylpyrazine. *Eur J Pharm Sci* 2017;96:323-33.
72. Zhang J, Wang M, Zhang Z, Luo Z, Liu F, Liu J. Celecoxib derivative OSU-03012 inhibits the proliferation and activation of hepatic stellate cells by inducing cell senescence. *Mol Med Rep* 2015;11(4):3021-6.
73. Kim KH, Chen CC, Monzon RI, Lau LF. Matricellular protein CCN1 promotes regression of liver fibrosis through induction of cellular senescence in hepatic myofibroblasts. *Mol Cell Biol* 2013;33(10): 2078-90.
74. Kong X, Feng D, Wang H, Hong F, Bertola A, Wang FS, et al. Interleukin-22 induces hepatic stellate cell senescence and restricts liver fibrosis in mice. *Hepatology* 2012;56(3):1150-9.
75. Nishizawa H, Iguchi G, Fukuoka H, Takahashi M, Suda K, Bando H, et al. IGF-I induces senescence of hepatic stellate cells and limits fibrosis in a p53-dependent manner. *Sci Rep* 2016;6:34605.
76. Kisseleva T, Cong M, Paik Y, Scholten D, Jiang C, Benner C, et al. Myofibroblasts revert to an inactive phenotype during regression of liver fibrosis. *Proc Natl Acad Sci U S A* 2012;109(24):9448-53.
77. Troeger JS, Mederacke I, Gwak GY, Dapito DH, Mu X, Hsu CC, et al. Deactivation of hepatic stellate cells during liver fibrosis resolution in mice. *Gastroenterology* 2012;143(4):1073-83 e22.
78. Issa R, Zhou X, Constandinou CM, Fallowfield J, Millward-Sadler H, Gaca MD, et al. Spontaneous recovery from micronodular cirrhosis: evidence for incomplete resolution associated with matrix cross-linking. *Gastroenterology* 2004;126(7):1795-808.

79. Ramachandran P, Pellicoro A, Vernon MA, Boulter L, Aucott RL, Ali A, et al. Differential Ly-6C expression identifies the recruited macrophage phenotype, which orchestrates the regression of murine liver fibrosis. *Proc Natl Acad Sci U S A* 2012;109(46):E3186-95.
80. Pellicoro A, Ramachandran P, Iredale JP, Fallowfield JA. Liver fibrosis and repair: immune regulation of wound healing in a solid organ. *Nat Rev Immunol* 2014;14(3):181-94.
81. Marra F, Tacke F. Roles for chemokines in liver disease. *Gastroenterology* 2014;147(3):577-94 e1.
82. Tacke F, Zimmermann HW. Macrophage heterogeneity in liver injury and fibrosis. *J Hepatol* 2014;60(5):1090-6.
83. Jiao J, Sastre D, Fiel MI, Lee UE, Ghiassi-Nejad Z, Ginhoux F, et al. Dendritic cell regulation of carbon tetrachloride-induced murine liver fibrosis regression. *Hepatology* 2012;55(1):244-55.
84. Tian Z, Chen Y, Gao B. Natural killer cells in liver disease. *Hepatology* 2013;57(4):1654-62.
85. Kantari-Mimoun C, Castells M, Klose R, Meinecke AK, Lemberger UJ, Rautou PE, et al. Resolution of liver fibrosis requires myeloid cell-driven sinusoidal angiogenesis. *Hepatology* 2015;61(6):2042-55.
86. Yang L, Kwon J, Popov Y, Gajdos GB, Ordog T, Brekken RA, et al. Vascular endothelial growth factor promotes fibrosis resolution and repair in mice. *Gastroenterology* 2014;146(5):1339-50 e1.
87. Chang TT, Liaw YF, Wu SS, Schiff E, Han KH, Lai CL, et al. Long-term entecavir therapy results in the reversal of fibrosis/cirrhosis and continued histological improvement in patients with chronic hepatitis B. *Hepatology* 2010;52(3):886-93.
88. Pinzani M. Liver fibrosis. *Springer Semin Immunopathol* 1999;21(4):475-90.
89. Kweon YO, Goodman ZD, Dienstag JL, Schiff ER, Brown NA, Burchardt E, et al. Decreasing fibrogenesis: an immunohistochemical study of paired liver biopsies following lamivudine therapy for chronic hepatitis B. *J Hepatol* 2001;35(6):749-55.
90. Poynard T, McHutchison J, Manns M, Trepo C, Lindsay K, Goodman Z, et al. Impact of pegylated interferon alfa-2b and ribavirin on liver fibrosis in patients with chronic hepatitis C. *Gastroenterology* 2002;122(5):1303-13.
91. Farci P, Roskams T, Chessa L, Peddis G, Mazzoleni AP, Scioscia R, et al. Longterm benefit of interferon alpha therapy of chronic hepatitis D: regression of advanced hepatic fibrosis. *Gastroenterology* 2004;126(7):1740-9.
92. Niederau C, Fischer R, Purschel A, Stremmel W, Haussinger D, Strohmeyer G. Long-term survival in patients with hereditary hemochromatosis. *Gastroenterology* 1996;110(4):1107-19.
93. Czaja AJ, Carpenter HA. Decreased fibrosis during corticosteroid therapy of autoimmune hepatitis. *J Hepatol* 2004;40(4):646-52.
94. Haas JT, Francque S, Staels B. Pathophysiology and mechanisms of nonalcoholic fatty liver disease. *Annu Rev Physiol* 2016;78:181-205.
95. Vilar-Gomez E, Martinez-Perez Y, Calzadilla-Bertot L, Torres-Gonzalez A, Gra-Oramas B, Gonzalez-Fabian L, et al. Weight loss through lifestyle modification significantly reduces features of nonalcoholic steatohepatitis. *Gastroenterology* 2015;149(2):367-78 e5. quiz e14-e15.
96. Lassailly F, Caiazzo R, Buob D, Pigeyre M, Verkindt H, Labreuche J, et al. Bariatric surgery reduces features of nonalcoholic steatohepatitis in morbidly obese patients. *Gastroenterology* 2015;149(2):379-88. quiz e15-6.
97. Borowsky SA, Strome S, Lott E. Continued heavy drinking and survival in alcoholic cirrhotics. *Gastroenterology* 1981;80(6):1405-9.
98. Luca A, Garcia-Pagan JC, Bosch J, Feu F, Caballeria J, Groszmann RJ, et al. Effects of ethanol consumption on hepatic hemodynamics in patients with alcoholic cirrhosis. *Gastroenterology* 1997;112(4):1284-9.
99. Pessione F, Ramond MJ, Peters L, Pham BN, Batel P, Rueff B, et al. Five-year survival predictive factors in patients with excessive alcohol intake and cirrhosis. Effect of alcoholic hepatitis, smoking and abstinence. *Liver Int* 2003;23(1):45-53.
100. Barreyro FJ, Holod S, Finocchietto PV, Camino AM, Aquino JB, Avagnina A, et al. The pan-caspase inhibitor Emricasan (IDN-6556) decreases liver injury and fibrosis in a murine model of non-alcoholic steatohepatitis. *Liver Int* 2015;35(3):953-66.
101. Pockros PJ, Schiff ER, Shiffman ML, McHutchison JG, Gish RG, Afdhal NH, et al. Oral IDN-6556, an antiapoptotic caspase inhibitor, may lower aminotransferase activity in patients with chronic hepatitis C. *Hepatology* 2007;46(2):324-9.

102. Shiffman ML, Pockros P, McHutchison JG, Schiff ER, Morris M, Burgess G. Clinical trial: the efficacy and safety of oral PF-03491390, a pancaspase inhibitor - a randomized placebo-controlled study in patients with chronic hepatitis C. *Aliment Pharmacol Ther* 2010;31(9):969-78.
103. Noureddin M, Anstee QM, Loomba R. Review article: emerging anti-fibrotic therapies in the treatment of non-alcoholic steatohepatitis. *Aliment Pharmacol Ther* 2016;43(11):1109-23.
104. Seki E, Brenner DA, Karin M. A liver full of JNK: signaling in regulation of cell function and disease pathogenesis, and clinical approaches. *Gastroenterology* 2012;143(2):307-20.
105. Rotman Y, Sanyal AJ. Current and upcoming pharmacotherapy for nonalcoholic fatty liver disease. *Gut* 2017;66(1):180-90.
106. Loomba R, Lawitz E, Mantry PS, Jayakumar S, Caldwell SH, Arnold H, et al. GS-4997, an inhibitor of apoptosis signal-regulating kinase (ASK1), alone or in combination with simtuzumab for the treatment of nonalcoholic steatohepatitis (NASH): a randomized, phase 2 trial. Boston: AASLD Liver Meeting; 2016.
107. Yoshiji H, Kuriyama S, Fukui H. Blockade of renin-angiotensin system in antifibrotic therapy. *J Gastroenterol Hepatol* 2007;22(Suppl 1):S93-5.
108. Bataller R, Gines P, Nicolas JM, Gorbis MN, Garcia-Ramallo E, Gasull X, et al. Angiotensin II induces contraction and proliferation of human hepatic stellate cells. *Gastroenterology* 2000;118(6):1149-56.
109. Colmenero J, Bataller R, Sancho-Bru P, Dominguez M, Moreno M, Forns X, et al. Effects of losartan on hepatic expression of nonphagocytic NADPH oxidase and fibrogenic genes in patients with chronic hepatitis C. *Am J Physiol Gastrointest Liver Physiol* 2009;297(4):G726-34.
110. Kim MY, Cho MY, Baik SK, Jeong PH, Suk KT, Jang YO, et al. Beneficial effects of candesartan, an angiotensin-blocking agent, on compensated alcoholic liver fibrosis - a randomized open-label controlled study. *Liver Int* 2012;32(6):977-87.
111. Cales P, Bacq Y, Vinel J-P, Bonny C, Payen J-L, Guyader D, et al. Irbesartan for severe fibrosis in chronic hepatitis C: a double-blind randomized trial (ANRS HC19 Fibrosar). *Hepatology* 2014;60(4).
112. Charatcharoenwitthaya P, Talwalkar JA, Angulo P, Gossard AA, Keach JC, Petz JL, et al. Moexipril for treatment of primary biliary cirrhosis in patients with an incomplete response to ursodeoxycholic acid. *Dig Dis Sci* 2010;55(2):476-83.
113. Mallat A, Teixeira-Clerc F, Lotersztajn S. Cannabinoid signaling and liver therapeutics. *J Hepatol* 2013;59(4):891-6.
114. Tam J, Vemuri VK, Liu J, Batkai S, Mukhopadhyay B, Godlewski G, et al. Peripheral CB1 cannabinoid receptor blockade improves cardiometabolic risk in mouse models of obesity. *J Clin Invest* 2010;120(8):2953-66.
115. Poulsen L, Siersbaek M, Mandrup S. PPARs: fatty acid sensors controlling metabolism. *Semin Cell Dev Biol* 2012;23(6):631-9.
116. Lee CH, Olson P, Hevener A, Mehl I, Chong LW, Olefsky JM, et al. PPARdelta regulates glucose metabolism and insulin sensitivity. *Proc Natl Acad Sci U S A* 2006;103(9):3444-9.
117. Lefebvre P, Chinetti G, Fruchart JC, Staels B. Sorting out the roles of PPAR alpha in energy metabolism and vascular homeostasis. *J Clin Invest* 2006;116(3):571-80.
118. Odegaard JI, Ricardo-Gonzalez RR, Red Eagle A, Vats D, Morel CR, Goforth MH, et al. Alternative M2 activation of Kupffer cells by PPARdelta ameliorates obesity-induced insulin resistance. *Cell Metab* 2008;7(6): 496-507.
119. Staels B, Rubenstrunk A, Noel B, Rigou G, Delataille P, Millatt LJ, et al. Hepatoprotective effects of the dual peroxisome proliferator-activated receptor alpha/delta agonist, GFT505, in rodent models of nonalcoholic fatty liver disease/nonalcoholic steatohepatitis. *Hepatology* 2013;58(6):1941-52.
120. Iredale JP. Models of liver fibrosis: exploring the dynamic nature of inflammation and repair in a solid organ. *J Clin Invest* 2007;117(3):539-48.
121. Barry-Hamilton V, Spangler R, Marshall D, McCauley S, Rodriguez HM, Oyasu M, et al. Allosteric inhibition of lysyl oxidase-like-2 impedes the development of a pathologic microenvironment. *Nat Med* 2010;16(9): 1009-17.
122. Ikenaga N, Peng ZW, Vaid KA, Liu SB, Yoshida S, Sverdlow DY, et al. Selective targeting of lysyl oxidase-like 2 (LOXL2) suppresses hepatic fibrosis progression and accelerates its reversal. *Gut* 2017 Jan 10. pii: gutjnl-2016-312473. [Epub ahead of print].
123. Meissner EG, McLaughlin M, Matthews L, Gharib AM, Wood BJ, Levy E, et al. Simtuzumab treatment of advanced liver fibrosis in HIV and HCV-infected adults: results of a 6-month open-label safety trial. *Liver Int* 2016;36(12):1783-92.

124. Parsons CJ, Bradford BU, Pan CQ, Cheung E, Schauer M, Knorr A, et al. Antifibrotic effects of a tissue inhibitor of metalloproteinase-1 antibody on established liver fibrosis in rats. *Hepatology* 2004;40(5):1106-15.
125. Bennett RG, Heimann DG, Singh S, Simpson RL, Tuma DJ. Relaxin decreases the severity of established hepatic fibrosis in mice. *Liver Int* 2014;34(3):416-26.
126. Bennett RG, Heimann DG, Tuma DJ. Relaxin reduces fibrosis in models of progressive and established hepatic fibrosis. *Ann N Y Acad Sci* 2009;1160:348-9.
127. Snowdon VK, Lachlan NJ, Hoy AM, Hadoke PW, Semple SI, Patel D, et al. Serelaxin as a potential treatment for renal dysfunction in cirrhosis: preclinical evaluation and results of a randomized phase 2 trial. *PLoS Med* 2017;14(2):e1002248.
128. Hirschfield GM, Mason A, Luketic V, Lindor K, Gordon SC, Mayo M, et al. Efficacy of obeticholic acid in patients with primary biliary cirrhosis and inadequate response to ursodeoxycholic acid. *Gastroenterology* 2015;148(4):751-61 e8.
129. Nevens F, Andreone P, Mazzella G, Strasser SI, Bowlus C, Invernizzi P, et al. A placebo-controlled trial of obeticholic acid in primary biliary cholangitis. *N Engl J Med* 2016;375(7):631-43.
130. Mudaliar S, Henry RR, Sanyal AJ, Morrow L, Marschall HU, Kipnes M, et al. Efficacy and safety of the farnesoid X receptor agonist obeticholic acid in patients with type 2 diabetes and nonalcoholic fatty liver disease. *Gastroenterology* 2013;145(3):574-82 e1.
131. Neuschwander-Tetri BA, Loomba R, Sanyal AJ, Lavine JE, Van Natta ML, Abdelmalek MF, et al. Farnesoid X nuclear receptor ligand obeticholic acid for non-cirrhotic, non-alcoholic steatohepatitis (FLINT): a multicentre, randomised, placebo-controlled trial. *Lancet* 2015;385(9972):956-65.
132. Vircheva S, Alexandrova A, Georgieva A, Mateeva P, Zamfirova R, Kubera M, et al. In vivo effects of pentoxifylline on enzyme and non-enzyme antioxidant levels in rat liver after carrageenan-induced paw inflammation. *Cell Biochem Funct* 2010;28(8):668-72.
133. Zein CO, Yerian LM, Gogate P, Lopez R, Kirwan JP, Feldstein AE, et al. Pentoxifylline improves nonalcoholic steatohepatitis: a randomized placebocontrolled trial. *Hepatology* 2011;54(5):1610-9.
134. Thursz MR, Richardson P, Allison M, Austin A, Bowers M, Day CP, et al. Prednisolone or pentoxifylline for alcoholic hepatitis. *N Engl J Med* 2015;372(17):1619-28.
135. Abraldes JG, Rodriguez-Vilarrupla A, Graupera M, Zafra C, Garcia-Caldero H, Garcia-Pagan JC, et al. Simvastatin treatment improves liver sinusoidal endothelial dysfunction in CCl4 cirrhotic rats. *J Hepatol* 2007;46(6):1040-6.
136. Trebicka J, Hennenberg M, Laleman W, Shelest N, Biecker E, Schepke M, et al. Atorvastatin lowers portal pressure in cirrhotic rats by inhibition of RhoA/Rho-kinase and activation of endothelial nitric oxide synthase. *Hepatology* 2007;46(1):242-53.
137. La Mura V, Pasarin M, Meireles CZ, Miquel R, Rodriguez-Vilarrupla A, Hide D, et al. Effects of simvastatin administration on rodents with lipopolysaccharide-induced liver microvascular dysfunction. *Hepatology* 2013;57(3):1172-81.
138. Marrone G, Maeso-Diaz R, Garcia-Cardena G, Abraldes JG, Garcia-Pagan JC, Bosch J, et al. KLF2 exerts antifibrotic and vasoprotective effects in cirrhotic rat livers: behind the molecular mechanisms of statins. *Gut* 2015;64(9):1434-43.
139. Trebicka J, Hennenberg M, Odenthal M, Shir K, Klein S, Granzow M, et al. Atorvastatin attenuates hepatic fibrosis in rats after bile duct ligation via decreased turnover of hepatic stellate cells. *J Hepatol* 2010;53(4):702-12.
140. Meireles CZ, Pasarin M, Lozano JJ, Garcia-Caldero H, Gracia-Sancho J, Garcia-Pagan JC, et al. Simvastatin attenuates liver injury in rodents with biliary cirrhosis submitted to hemorrhage/resuscitation. *Shock* 2017;47(3):370-7.
141. Rodriguez S, Raurell I, Torres-Arauz M, Garcia-Lezana T, Genesca J, Martell M. A nitric oxide-donating statin decreases portal pressure with a better toxicity profile than conventional statins in cirrhotic rats. *Sci Rep* 2017;7:40461.
142. Abraldes JG, Albillos A, Banares R, Turnes J, Gonzalez R, Garcia-Pagan JC, et al. Simvastatin lowers portal pressure in patients with cirrhosis and portal hypertension: a randomized controlled trial. *Gastroenterology* 2009;136(5):1651-8.
143. Abraldes JG, Villanueva C, Aracil C, Turnes J, Hernandez-Guerra M, Genesca J, et al. Addition of simvastatin to standard therapy for the prevention of variceal rebleeding does not reduce rebleeding but increases survival in patients with cirrhosis. *Gastroenterology* 2016;150(5):1160-70. e3.

144. Mohanty A, Tate JP, Garcia-Tsao G. Statins are associated with a decreased risk of decompensation and death in veterans with hepatitis C-Related compensated cirrhosis. *Gastroenterology* 2016;150(2):430-40 e1.
145. Krenkel O, Tacke F. Liver macrophages in tissue homeostasis and disease. *Nat Rev Immunol* 2017 May;17(5):306-21.
146. Mossanen JC, Krenkel O, Ergen C, Govaere O, Liepelt A, Puengel T, et al. Chemokine (C-C motif) receptor 2-positive monocytes aggravate the early phase of acetaminophen-induced acute liver injury. *Hepatology* 2016;64(5): 1667-82.
147. Friedman S, Sanyal A, Goodman Z, Lefebvre E, Gottwald M, Fischer L, et al. Efficacy and safety study of cenicriviroc for the treatment of non-alcoholic steatohepatitis in adult subjects with liver fibrosis: CENTAUR Phase 2b study design. *Contemp Clin Trials* 2016;47:356-65.
148. van Can J, Sloth B, Jensen CB, Flint A, Blaak EE, Saris WH. Effects of the oncedaily GLP-1 analog liraglutide on gastric emptying, glycemic parameters, appetite and energy metabolism in obese, non-diabetic adults. *Int J Obes (Lond)* 2014;38(6):784-93.
149. Pi-Sunyer X, Astrup A, Fujioka K, Greenway F, Halpern A, Krempf M, et al. A randomized, controlled trial of 3.0 mg of liraglutide in weight management. *N Engl J Med* 2015;373(1):11-22.
150. Armstrong MJ, Gaunt P, Aithal GP, Barton D, Hull D, Parker R, et al. Liraglutide safety and efficacy in patients with non-alcoholic steatohepatitis (LEAN): a multicentre, double-blind, randomised, placebo-controlled phase 2 study. *Lancet* 2016;387(10019):679-90.
151. Li LC, Li J, Gao J. Functions of galectin-3 and its role in fibrotic diseases. *J Pharmacol Exp Ther* 2014;351(2):336-43.
152. Henderson NC, Sethi T. The regulation of inflammation by galectin-3. *Immunol Rev* 2009;230(1): 160-71.
153. Traber PG, Chou H, Zomer E, Hong F, Klyosov A, Fiel MI, et al. Regression of fibrosis and reversal of cirrhosis in rats by galectin inhibitors in thioacetamide-induced liver disease. *PLoS One* 2013;8(10):e75361.
154. Lavine JE, Schwimmer JB, Van Natta ML, Molleston JP, Murray KF, Rosenthal P, et al. Effect of vitamin E or metformin for treatment of nonalcoholic fatty liver disease in children and adolescents: the TONIC randomized controlled trial. *JAMA* 2011;305(16):1659-68.
155. Miller 3rd ER, Pastor-Barriuso R, Dalal D, Riemersma RA, Appel LJ, Guallar E. Meta-analysis: high-dosage vitamin E supplementation may increase all-cause mortality. *Ann Intern Med* 2005;142(1): 37-46.
156. Takahashi Y, Soejima Y, Fukusato T. Animal models of nonalcoholic fatty liver disease/nonalcoholic steatohepatitis. *World J Gastroenterol* 2012;18(1):2300-8.
157. Plum W, Tschaharganeh DF, Kroy DC, Corsten E, Erschfeld S, Dierssen U, et al. Lack of glycoprotein 130/signal transducer and activator of transcription 3-mediated signaling in hepatocytes enhances chronic liver injury and fibrosis progression in a model of sclerosing cholangitis. *Am J Pathol* 2010;176(5):2236-46.
158. Lutsenko S. Atp7b^{-/-} mice as a model for studies of Wilson's disease. *Biochem Soc Trans* 2008;36(Pt 6):1233-8.
159. Ratziu V, Sheikh MY, Sanyal AJ, Lim JK, Conjeevaram H, Chalasani N, et al. A phase 2, randomized, double-blind, placebo-controlled study of GS-9450 in subjects with nonalcoholic steatohepatitis. *Hepatology* 2012;55(2):419-28.
160. Kim W, Kim BG, Lee JS, Lee CK, Yeon JE, Chang MS, et al. Randomised clinical trial: the efficacy and safety of oltipraz, a liver X receptor alpha-inhibitory dithiolethione in patients with non-alcoholic fatty liver disease. *Aliment Pharmacol Ther* 2017;45(8):1073-83.
161. Mathurin P, Louvet A, Duhamel A, Nahon P, Carbonell N, Boursier J, et al. Prednisolone with vs without pentoxifylline and survival of patients with severe alcoholic hepatitis: a randomized clinical trial. *JAMA* 2013;310(10):1033-41.
162. Van Wagner LB, Koppe SWP, Brunt EM, Gottstein J, Gardikiotes K, Green RM, et al. Pentoxifylline for the treatment of non-alcoholic steatohepatitis: a randomized controlled trial. *Ann Hepatol* 2011;10(3):277-86.
163. Lebrech D, Thabut D, Oberti F, Perarnau J-M, Condat B, Barraud H, et al. Pentoxifylline does not decrease short-term mortality but does reduce complications in patients with advanced cirrhosis. *Gastroenterology* 2010;138(5):1755-62.
164. Cusi K, Orsak B, Bril F, Lomonaco R, Hecht J, Ortiz-Lopez C, et al. Long-term pioglitazone treatment for patients with nonalcoholic steatohepatitis and prediabetes or type 2 diabetes mellitus: a randomized trial. *Ann Intern Med* 2016;165(5):305-15.
165. Vosough M, Moossavi S, Mardpour S, Akhlaghpour S, Azimian V, Jarughi N, et al. Repeated intraportal injection of mesenchymal stem cells in combination with pioglitazone in patients with compensated cirrhosis: a clinical report of two cases. *Arch Iran Med* 2016;19(2):131-6.

166. Flores-Contreras L, Sandoval-Rodriguez AS, Mena-Enriquez MG, Lucano-Landeros S, Arellano-Olivera I, Alvarez-Alvarez A, et al. Treatment with pirfenidone for two years decreases fibrosis, cytokine levels and enhances CB2 gene expression in patients with chronic hepatitis C. *BMC Gastroenterol* 2014;14:131.
167. Sanyal AJ, Chalasani N, Kowdley KV, McCullough A, Diehl AM, Bass NM, et al. Pioglitazone, vitamin E, or placebo for nonalcoholic steatohepatitis. *N Engl J Med* 2010;362(18):1675-85.
168. Qu J, Yu Z, Li Q, Chen Y, Xiang D, Tan L, et al. Blocking and reversing hepatic fibrosis in patients with chronic hepatitis B treated by traditional Chinese medicine (tablets of biejia ruangan or RGT): study protocol for a randomized controlled trial. *Trials* 2014;15:438.

Chapter 10

Therapeutic intervention against c-Jun N-terminal kinase-2 (Jnk2) ameliorates end-stage chronic liver disease

Woitok MM*, Zoubek ME*, Doleschel D, Bartneck M, Mohamed MR, Kießling F, Lederle W, Trautwein C, Cubero FJ

***Cell Death Dis* 2020; 11(5):343**

DOI: 10.1038/s41419-020-2571-4

*Contributed equally to this work as first authors

Abstract

Introduction & aims

A considerable number of preclinical studies indicate that a great variety of liver diseases, ranging from nonalcoholic fatty liver disease (NAFLD) to end-stage hepatocellular carcinoma (HCC), are amenable to gene therapy. We have previously shown that c-Jun N-terminal kinases (Jnk) genes play a crucial role in chronic liver disease (CLD). In the present study, we aimed to investigate the relevance of hepatocyte-specific Jnk2 inhibition in an experimental model of CLD.

Material & Methods

Genetic deletion of Jnk2 gene was performed using hepatocyte-specific deletion of Jnk2 (JNK2^{Δhepa}) in NEMO/IKK γ (NEMO^{Δhepa}) mice and by siRNA silencing (siJnk2) *in vitro* and *in vivo* in wild-type (WT) and NEMO^{Δhepa} mice, respectively. Disease progression was analyzed using imaging analysis of combined fluorescence molecular and microcomputed tomography (FMT/ μ CT), protein expression, IHC, IF and histopathology.

Results

In one-year-old NEMO^{Δhepa} mice, Jnk2 deletion reduced liver fibrosis and hepatocarcinogenesis (HCC), as observed by the reduced total number of HCC nodules. Both NEMO/JNK2^{Δhepa} (DKO^{Δhepa}) and hepatocyte-specific liposomal-delivered siJnk2 in end-stage diseased NEMO mice resulted in improved liver parenchyma, serum levels and markers of fibrogenesis. Furthermore, siJnk2 chronic treatment was associated with a change in the immune response as a reduction of myeloid cells and an increase in CD4⁺ and CD8⁺ T-cells was found. Most importantly, chronic siJnk2 treatment reduced the presence of premalignant and malignant liver tumors corresponding to reduced tumor initiation. Interestingly, hepatocyte-specific liposomal-delivered siJnk2 diminished liver function at an early phase of CLD in NEMO^{Δhepa} mice. siJnk2 caused increased liver transaminases, hepatocellular apoptosis and compensatory proliferation, a phenomenon that we validated in 12 week-old DKO^{Δhepa} mice.

Conclusions

Our findings demonstrate a stage-dependent role of Jnk2 during CLD progression in NEMO^{Δhepa} mice. Notably, siJnk2 delivery to hepatocytes ameliorated hepatitis, fibrogenesis and HCC initiation and thus might be an attractive therapeutic option for personalized medicine in CLD.

Keywords

JNK, liver, siRNA, FMT/ μ CT, HCC

Introduction

Chronic liver disease (CLD) is defined as a progressive destructive illness of the liver parenchyma frequently associated with chronic inflammation. The consequences are liver fibrosis, cirrhosis and, finally, a high likelihood of malignant transformation, *e.g.*, hepatocellular carcinoma (HCC). Both liver cirrhosis and HCC, in turn, can lead to complete liver failure and, consequently, without liver transplantation, to the death of the patient.

Previous publications of our group demonstrated that deletion of the regulatory subunit IKK γ (NEMO) in murine hepatocytes causes spontaneous HCC development preceded by chronic liver disease mimicking human NASH. Lack of NEMO expression leads to complete NF- κ B inactivation and, in turn, to sustained JNK activation and reactive oxygen species (ROS) formation promoting cellular death¹.

In recent publications, we showed the differential role of the Jnk genes during acute and chronic liver disease^{2,3}. Whereas Jnk1 plays a pro-apoptotic and pro-tumorigenic function, Jnk2 seems to modulate fibrogenesis. Lately, a protective role for Jnk2 was shown against Ibuprofen-mediated acute liver failure (ALF). However, many aspects of the Jnk2 function in CLD remain currently unknown.

In this study, we used a dual approach to study the role of Jnk2 in CLD - Albumin Cre/loxP-mediated gene deletion and small interfering RNA (siRNA)-mediated Jnk2 (siJnk2) knockdown in hepatocytes *in vivo*. The use of genetically modified mice with gain- or loss-of-function of individual genes is the gold standard to study the involvement of proteins in a biological process *in vivo*⁴. Here, we effectively delete Jnk2 specifically in hepatocytes of NEMO ^{Δ hepa} mice, a suitable model for studying CLD and end-stage HCC¹.

Material and Methods

Knockout mouse strains and animal housing

For all experiments, male mice carrying the loxP-site-flanked under the control of the Alb/AFP-Cre promotor/enhancer were generated as previously described⁵. Mice lacking the Cre transgene were denominated as 'floxed' mice (*e.g.*, NEMO^{f/f}), showing no functional difference to wild type (WT) mice. Albumin-Cre (Alb-Cre), Jnk2-deficient mice were purchased from The Jackson Laboratory (Bar Harbor, ME, USA). Hepatocyte-specific Jnk2 and NEMO/IKK γ mice were constructed by crossing Alb-Cre JNK2^{f/f} mice with Alb-Cre NEMO^{f/f} mice. All strains were crossed on a C57BL/6 background. The mice were housed in the Institute of Laboratory Animal Science at the University Hospital, RWTH-Aachen University, according to German legal requirements (Deutsches Tierschutzgesetz, FELASA, GV-SOLAS) under a permit of the 'Veterinäramt der Städteregion Aachen'. In addition, the mice were kept in individually ventilated cages (IVC) in groups of a maximum of five animals per cage under specific pathogen-free (SPF) conditions. The mice were examined on a daily basis by animal caretakers of the Institute of Laboratory Animal Science at the University Hospital, RWTH-Aachen University. Specific air conditioning assured a constant

room temperature of about $22\pm1^{\circ}\text{C}$ and humidity in the range of $50\pm10\%$. At a light source of 150 lux, the mice were housed in a 12h light-dark cycle (7:00-19:00 light; 19:00-7:00 dark). Mice received autoclaved food pellets and sterilized water, both *ad libitum*. For breeding, the mice were mated at around nine weeks and the littermates were separated at the age of 3-4 weeks following earmarks for identification. All performed organ explants and animal experiments had been approved by the local authority for environment conservation and consumer protection of the state North-Rhine Westphalia (LANUV) on the following animal grants: 30034G (AZ: 84-02.04.2016.A080), TVA-11324GZ (AZ-84-02.04.2016.A490). The research was performed under the ARRIVE (Animal Research: Reporting of *In Vivo* Experiments) guidelines.

Generation of a Jnk2 siRNA (siJnk2)

The siRNA molecules were purchased from Axolabs GmbH (Kulmbach, Germany) and were chosen due to their ability to specifically target Jnk2 in mice with mismatches to Jnk1 (2-18 nucleotides) to increase *in vivo* stability and suppression of the immune-stimulatory properties. Therefore, the siRNA molecules were modified in their sequences resulting in 12 different siRNA sets.

Hepa 1-6 cell line

Hepa 1-6 (1×10^5 cells/well) cells were obtained from the American Tissue Culture Collection (ATCC, Manassas, VA, USA), grown in DMEM (Gibco BRL, Grand Island, NY, USA) supplemented with 10% fetal bovine serum (FBS) and 1% penicillin/streptomycin. Hepa1-6 cells were cultured in a quantity of 500.000 cells/well, on 6 well 15.5 ml/9.6 cm² culture plates (Falcon, Corning Inc., Corning, NY, USA) and transfected with the corresponding siRNA molecules according to the manufacturer's protocol (Lipofectamine[®] RNAiMAX[™] Transfection Reagent, ThermoFisher Scientific) and cultured for 24h at 37°C. After this, RNA was isolated (PureLink RNA Mini Kit, ThermoFisher Scientific) and analyzed.

Isolation and culture of liver cells

Primary WT mouse hepatocytes were isolated from 7-8 week-old mice by collagenase perfusion. Living hepatocytes were plated on collagen-precoated Petri dishes at a density of $1.5\times10^4/\text{cm}^2$ in supplemented DMEM medium and after 4h incubation (37°C, 5% CO₂), the medium was renewed. In accordance with the transfection of the Hepa1-6 cells, primary WT mouse hepatocytes were transfected with the corresponding siRNA molecules according to the manufacturer's protocol (Lipofectamine[®] RNAiMAX[™] Transfection Reagent, ThermoFisher Scientific) and cultured for 24h at 37°C. After this, RNA was isolated (PureLink RNA Mini Kit, ThermoFisher Scientific) and analyzed. Hepatic stellate cells (HSCs) and Kupffer cells (KCs) were isolated according to the published protocol⁶.

Statistical analysis

All data were analyzed by Graph Pad Prism[®] 5.0 (GraphPad Software, Inc.) and are expressed as means $\pm$ standard error of the mean (SEM). *P* values below 0.05 were

considered significant using the Student's *t*-test or one-way analysis of variance (ANOVA), including the Bonferroni post-hoc test.

Results

Loss of hepatocytic JNK2 ameliorates the progression of experimental chronic liver disease (CLD)

Previous studies suggested a major role of the NF- κ B pathway and JNK activation for HCC development^{1,3}. Thus, we generated double knockout mice with hepatocyte-specific deletion of JNK2 and NEMO ^{Δ hepa}/IKK γ and examined CLD progression in NEMO ^{Δ hepa}, NEMO/JNK2 ^{Δ hepa} (DKO ^{Δ hepa}), and their corresponding littermate controls (floxed mice). The impact of Jnk2 deletion on 52 week-old NEMO ^{Δ hepa} animals was first evaluated. Macroscopic analysis of one year DKO ^{Δ hepa} elicited a significant reduction in tumor numbers compared with NEMO ^{Δ hepa}, whilst controls did not display any evidence of tumorigenesis (**Figure 10.1A, left and right panels**). The liver parenchyma architecture in DKO ^{Δ hepa} characterized by decreased dysplasia, inflammation and steatosis, compared with NEMO ^{Δ hepa} mice (**Figure 10.1B**). In line with these results, serum transaminases of DKO ^{Δ hepa} mice were significantly enhanced compared to one-year-old NEMO ^{Δ hepa} animals (**Figure 10.1B, S10.1A-C**). Moreover, hepatocytic JNK2 deletion led to overall improved hepatic fibrogenesis, as observed by Sirius red (SR) staining and quantification associated with decreased infiltration of immune cells, including ccl2 and ccl5 (**Figure 10.1C-D**). Altogether these results indicate that the deletion of JNK2 in experimental chronic liver disease might be a suitable treatment to reduce hepatic fibrosis and end-stage HCC formation.

Figure 10.1

Figure 10.1 Genetic deletion of *Jnk2* in hepatocytes attenuates CLD progression and reduces hepatocarcinogenesis in NEMO^{Δhepa} mice. NEMO^{Δhepa}, JNK2^{Δhepa}, NEMO^{Δhepa}/JNK2^{Δhepa} (DKO^{Δhepa}) mice and its respective control animals (JNK2^{+/f}, NEMO^{+/f}, NEMO^{+/f}/JNK2^{+/f} (=DKO^{+/f})) were generated. Mice were sacrificed at the age of 52 weeks. Livers were extracted and macro- and microscopically analyzed for phenotypic characterization. **(A)** Macroscopic images of livers (left) in 52 week-old NEMO^{Δhepa}, JNK2^{Δhepa} and DKO^{Δhepa} (lower images) and control animals (upper images) (Scale bars: 1cm). Dashed circles indicate tumoral lesions. The number of tumor nodules ≥0.5cm in diameter was quantified in livers of NEMO^{Δhepa} and DKO^{Δhepa} animals at the age of 52 weeks (right). **(B)** Representative histological liver sections of the identical knockout animals (lower images) and control mice (upper images) at the age of 52 weeks stained with hematoxylin and eosin are shown (left). Serum liver ALT (right) of 52 week-old animals is displayed in U/L (n=11-13). (Scale bars: 100μm). **(C)** Representative images (left) of Sirius red staining of paraffin-embedded 52 week-old NEMO^{Δhepa}, JNK2^{Δhepa} and DKO^{Δhepa} (lower images) and control mice (upper images). Quantification of positive Sirius red area fraction (right) was performed with Image J® (Scale bars: 100μm). **(D)** The mRNA expression of *ccl2* and *ccl5* was performed in whole liver extracts prepared from NEMO^{Δhepa} and DKO^{Δhepa} animals at 52 weeks. Results are represented as fold increase. Values are expressed as mean ± S.E.M. *P<0.05, **P<0.01, ***P<0.001.

Efficiency of siJnk2 on gene expression *in vitro*

Since the loss of hepatocytic JNK2 enhanced the progression and end-stage phase of chronic liver disease, we aimed to functionally elucidate and therapeutically define the role of Jnk2 in CLD using siRNA against Jnk2.

We first transfected Hepa 1-6 cells with a panel of 12 different computer-defined siRNAs for 24h and tested Jnk2 mRNA expression (**Figure S10.2A**). This screening approach defined siRNA-Jnk2 set 3 and 4 to downregulate JNK2 mRNA to less than 10% of the original level. Dose finding experiments identified a concentration of 0.5nmol as the most potent siRNA application (**Figure S10.2B**). Efficacy was further confirmed in primary isolated WT hepatocytes (**Figure S10.2C-D**).

Both siRNAs significantly down-regulated Jnk2 mRNA expression in a concentration ranging from 0.125 to 5.0nmol 24h after transfection. As shown in **Figure S10.2C**, the most potent concentration of Jnk2 knockdown was found with 0.25nmol and 0.5nmol siRNA-Jnk2 set 3 and 4 (**Figure S10.2D**), respectively.

Figure 10.2

Figure 10.2 Effect of targeting *Jnk2* via siRNA in hepatocytes *in vitro* and *in vivo*. The efficiency and specificity of *Jnk2*-siRNA molecules coupled into lipid nanoparticles were tested *in vitro* and *in vivo*. WT mice at the age of 7–8 weeks were treated with a single dose of luciferase (*siLuc*) or *siJnk2* *in vivo* with a concentration of 0.2 mg/kg BW for 6 h (n=3). **(A)** *Jnk2* mRNA expression was determined in isolated liver, spleen, kidney, muscle, heart and fat tissue of WT mice that had undergone *siLuc* or *siJnk2* injection. Results are represented as fold increase. **(B)** *Jnk2* mRNA levels were analyzed in hepatocytes, KC and HSC isolated from livers of WT mice treated with either *siLuc* or *siJnk2*. Results are graphed as fold increase. **(C)** The mRNA expression of *Jnk2* was performed in whole liver extracts prepared from WT mice treated with *siLuc* or *siJnk2* in doses ranging from 0.05 to 0.2mg/kg for 6 and 168h. Quantitative RT-PCR results were displayed as fold induction. **(D)** Protein levels of JNK2, pJNK and JNK were analyzed by immunoblotting of whole liver extracts prepared from WT mice that underwent either *siLuc* or *siJnk2* injection with doses ranging from 0.05 to 0.2mg/kg for 6 and 168h. GAPDH was used as loading control. Ratio: Normalization of each protein level relative to GAPDH was determined by densitometry. Results are expressed as mean $\pm$ S.E.M. * P <0.05, ** P <0.01, *** P <0.001; ns, no significance.

Efficiency of nanoparticle-formulated siJnk2 *in vivo*

The most potent siRNA-JNK2 set 3 was coupled in lipid nanoparticles (LNP) for *in vivo* experiments. Six hours after inoculation, tissue specificity was analyzed by determining *Jnk2* mRNA expression first in different organs and different hepatic cell types, *e.g.*, hepatocytes, Kupffer cells (KCs) and HSCs. siRNA-Jnk2 set 3 suppressed MAPK9 expression specifically in the liver and failed to down-regulate *Jnk2* mRNA expression in KCs and HSCs (**Figure 10.2A–B**). These results confirmed successful *Jnk2* mRNA inhibition via siRNA-Jnk2 set 3 (hereafter referred to as siJnk2) in hepatocytes *in vitro* and *in vivo*.

Next, we investigated the dose and time-dependent effect of siJnk2 on mRNA and protein expression in WT mice (**Figure 10.2C–D**). Using different siRNA concentrations ranging from 0.05 to 0.2mg/kg mouse body weight (BW), mRNA and protein expression showed a dose-dependent down-regulation of *Jnk2* compared to siLuc-treated mice, 6h after injection. A single dose injection of 0.2mg/kg per mouse BW triggered almost undetectable JNK2 protein expression after 168h, although mRNA expression levels showed an increase compared to the earlier time-point. Based on these data, a single dose injection of 0.2mg/kg siJnk2 was used in all further experiments.

Knockdown of JNK2 in one-year-old NEMO^{Δhepa} mice efficiently ameliorates tumorigenesis

Progression of liver disease is a multistage and chronic process where activation of the inflammatory response affects hepatic fibrogenesis and, ultimately, HCC development. NEMO^{Δhepa} livers develop obvious signs of fibrosis after 26 weeks and HCC can be found at the age of 52 weeks. Thus, we aimed to investigate the effect of hepatocyte-specific *Jnk2* silencing at a later stage of disease progression, when liver fibrosis is already present. Therefore, 44 week-old NEMO^{Δhepa} mice, showing no HCC development at this age, were treated with siJnk2 for eight weeks and subsequently analyzed (**Figure 10.3A**).

Figure 10.3 The progression of chronic liver disease is enhanced by using siRNA-mediated *Jnk2* silencing in an experimental model. Male 44 week-old NEMO^{Δhepa} mice were injected on a weekly basis with a single dose of 0.2mg/kg luciferase (*siLuc*) or *siJnk2* beginning at 44 weeks of age until reaching 52 weeks of age (sacrifice). **(A)** Schematic representation of the *siJnk2* injection protocol. **(B)** Representative macroscopic images (left) of the livers of NEMO^{Δhepa} mice untreated (left) or treated with either *siLuc* (center) or *siJnk2* (right) are displayed. Dashed circles indicate tumoral lesions. The number of tumor nodules >0.5cm in diameter (right) was quantified (scale bars: 1cm). **(C)** Representative H&E staining of liver sections (left) of untreated 52 week-old animals and after

undergoing *siLuc* or *siJnk2* injection are shown Serum liver ALT (right) of 52-week-old animals is displayed in U/l (n=12-14). (Scale bars: 100µm). **(D)** Western blot analysis of whole liver extracts from NEMO^{Δhepa} after completing treatment with *siLuc* or *siJnk2* at 52 weeks of age using antibodies against heat shock protein (HSP) 70/72 was performed; GAPDH served as loading control. **(E)** Protein expression of HSP70/72 in 52 week-old NEMO^{Δhepa} either treated with *siLuc* or *siJnk2* was quantified as relative density arbitrary unit using Image J© (left). The mRNA expression levels of *survivin* (center) and *p53* (right) for the same NEMO^{Δhepa} treatment groups were determined using qRT-PCR and displayed as fold increase. **(F)** Expression of JNK2 and pJNK2 was analyzed by immunoblotting of liver samples of 52 week-old NEMO^{Δhepa} mice treated with either *siLuc* or *siJnk2* (left). Quantitative RT-PCR analysis of the mRNA levels of *Jnk2* in the identical NEMO^{Δhepa} treatment cohort was performed and is represented as fold increase (right). GAPDH was used as loading control. Data are presented as mean ± S.E.M. **P*<0.05, ***P*<0.01, ****P*<0.001.

Chronic *siJnk2* treatment of NEMO^{Δhepa} livers caused a significant reduction in HCC progression. Gene silencing of *Jnk2* triggered significantly improved liver transaminases, which translated into decreased tumor numbers and an overall improved liver parenchyma comparable to untreated, 44 week-old NEMO^{Δhepa} mice. Furthermore, *siJnk2* treatment inhibited further HCC progression as it is visible in untreated mice NEMO^{Δhepa} with the age of 44 weeks. Macroscopic analysis and H&E staining of *siLuc*-treated NEMO^{Δhepa} liver displayed HCCs exhibiting dysplastic nodules and differentiated adenomas with characteristic neovascularization (neoangiogenesis) (**Figure 10.3B, S10.3A-B**). Besides, there were increased signs of fat deposition throughout the whole liver. Remarkably, *siJnk2* treatment led to the improved architecture of the hepatic parenchyma associated with minor signs of steatosis, a reduction of well-differentiated HCCs and significantly less premalignant dysplastic nodules, associated with significantly decreased serum ALT level (**Figure 10.3C**). Inoculation of *siJnk2* caused a significantly decreased expression of HSP72, a marker of HCC⁷ at the protein level (**Figure 10.3D-E**) and significant downregulation of mRNA transcripts *Survivin* and *P53*, typical markers of tumorigenesis, as well as factors such as *TNFα* and *VEGFβ* compared with NEMO^{Δhepa} and control mice (**Figure 10.3E, S10.3C-D**). Finally, to confirm that loss of JNK2 in hepatocytes was due to gene silencing was responsible for decreased tumorigenesis in experimental chronic liver disease, we tested both JNK2 protein and mRNA expression (**Figure 10.3F**). Notably, JNK2 protein and mRNA levels were depleted, thus validating the efficacy of JNK2 inhibition for the treatment of experimental liver tumorigenesis.

Hepatocytic JNK2 gene silencing enhances cell death, compensatory proliferation and hepatic fibrogenesis in experimental CLD

Next, we further examined the effect of *siJnk2* treatment in HCC progression. In line with improved liver architecture, *siJnk2* treatment of NEMO^{Δhepa} livers showed significantly reduced collagen accumulation, evidenced by SR analysis and downregulation of HSC activation markers. Reduced immune cell infiltration in *siJnk2*-treated NEMO^{Δhepa} livers was also associated with decreased *ccl2* and *ccr2* transcripts (**Figure 10.4A-C**). The impact of *Jnk2* silencing during 8 weeks on immune cell infiltration was studied by FACS analysis. NEMO^{Δhepa} mice inoculated with *siJnk2* livers showed a significantly reduced number of infiltrating CD45⁺ cells. Although we did not observe significant differences between the percentages of proinflammatory monocytes to CD45⁺ cells between both mice groups, total cell numbers were significantly decreased after *Jnk2* knockdown (**Figure 10.4D**).

Interestingly, siJnk2 treatment caused a decrease in B- and in NK cells, while an increase in NKT, T-cells, CD4⁺ T-cells and CD8⁺ T-cells was found (**Figure S10.4A**).

Figure 10.4

Figure 10.4 Hepatocyte-specific siJnk2 treatment reduces fibrogenesis and tumor initiation in NEMO^{Δhepa} mice at a late phase. (A) Representative images of Sirius red staining of paraffin-embedded NEMO^{Δhepa}+siLuc and NEMO^{Δhepa}+siJnk2 treated mice at 52 weeks of age is shown (left). Quantification of positive Sirius red (SR) area fraction (right) was performed with Image J® (Scale bars: 100μm). (B) The mRNA expression levels of αSMA (left) and Collagen I A1 (right) were determined for the same NEMO^{Δhepa} treatment groups in whole liver and represented as fold increase. (C) Quantitative RT-PCR analysis of ccl2 (left) and ccr2 (right) was performed for the same treatment groups and expressed as fold increase. (D) Flow cytometry analysis was performed in whole livers of NEMO^{Δhepa} mice after therapeutic administration of either siLuc or siJnk2 at the age of 52 weeks. Absolute number of CD45⁺ cells (left) and proinflammatory cells (CD45⁺/CD11b⁺ /Gr1.1⁺/F4/80⁺) (right) as well as proinflammatory cell fraction among CD45⁺ cells (center) were quantified by using FlowJo 7.6. (E) The

same samples were subjected to immunoblotting using antibodies against PCNA and Cleaved Caspase 3; GAPDH served as loading control. Data analysis is expressed as mean $\pm$ S.E.M. * P <0.05, ** P <0.01, *** P <0.001.

Furthermore, compensatory hepatocyte proliferation was downregulated in siJnk2 treated NEMO^{Δhepa} livers, as evidenced by PCNA mRNA and protein expression (**Figure 10.4E, S10.4B**). As the progression of liver injury in NEMO^{Δhepa} livers is linked to TNF-mediated apoptotic cell death, we evaluated the impact of siJnk2 treatment on caspase-3 activity. Cleaved caspase-3 expression and its enzymatic activity were significantly reduced after siJnk2 treatment in NEMO^{Δhepa} livers (**Figure 10.4E, S10.4C**). The FMT/ μ CT images demonstrated reduced apoptotic cell death in NEMO^{Δhepa} livers after siJnk2 injection compared to siLuc treated animals (**Figure S10.4D**).

Effect of silencing JNK2 in earlier stages of chronic liver injury

Since genetic Jnk2 deletion had a substantial impact on NEMO^{Δhepa}-dependent CLD in old mice, we next tested the effect of modulating hepatocyte-specific Jnk2 expression in earlier stages of chronic liver disease. Eight-week-old NEMO^{Δhepa} male mice were injected weekly with a single dose of 0.2 mg/kg siJnk2 or siLuc, over a period of 4 weeks (**Figure 10.5A**).

Figure 10.5

Figure 10.5 Hepatocyte-specific siRNA-dependent *Jnk2* deletion in an early phase of chronic liver injury. Male 8-week-old NEMO^{fl/fl} and NEMO^{Δhepa} animals were injected on a weekly basis with a single dose of 0.2 mg/kg *siLuc* or *siJnk2*, over 4 weeks and sacrificed at 12 weeks of age (n=12). (A) Schematic cartoon of the *siJnk2* injection protocol. (B) Representative H&E-stained liver sections (left) prepared from 12-week-old NEMO^{fl/fl} (upper panels) and NEMO^{Δhepa} (lower panels). Serum ALT is displayed in U/l (right). (C) Representative images of Sirius red staining of paraffin-embedded NEMO^{fl/fl} (upper panels) and NEMO^{Δhepa} liver tissue (lower panels) of 12-week-old animals after four weeks of treatment with *siLuc* or *siJnk2*. Quantification of positive Sirius red area fraction (right) was performed with Image J® (Scale bars: 100μm). (D) The expression of *αSMA* (left), *Collagen IA1* (center-left), *ccl2* (center-right) and *ccl5* (right) were measured for the identical treatment groups by qRT-PCR in whole liver extracts and expressed as fold increase. (E) The presence of CD11b⁺/F4/80⁺ cells in the liver was analyzed per IF and quantified as positive cells per view field (left). Absolute number of proinflammatory monocytes (CD45⁺/CD11b⁺/Gr1.1⁺/F4/80⁺) (center) as well as proinflammatory monocyte fraction among CD45⁺ cells (right) following flow cytometry analysis are analyzed. Quantification was determined using FlowJo 7.6. Data are presented as mean ± S.E.M. *P<0.05, **P<0.01, ***P<0.001.

One month of treatment with *siJnk2* significantly impaired liver injury markers compared to *siLuc*-treated NEMO^{Δhepa} mice. Interestingly, *siJnk2* treatment led to a decrease in liver/body weight (LW/BW) ratio in 12 week-old NEMO^{Δhepa} (Figure 10.5B, S10.5A-B). Histopathological analysis of our experimental model revealed significant differences between *siJnk2* and *siLuc*-treated NEMO^{Δhepa} livers. Silencing of *Jnk2* in hepatocytes of NEMO^{Δhepa} mice triggered parenchymal cell dysplasia and hypertrophy accompanied by strong anisokaryosis. In addition, there was increased fat deposition and substantial immune cell infiltration. Immune cells were found in local clusters throughout the liver parenchyma (Figure 10.5B, S10.5C). In turn, *siLuc*-treated NEMO^{Δhepa} livers displayed steatohepatitis characterized by immune cell infiltration, as found in NEMO^{Δhepa} livers at this stage of CLD. No significant changes between NEMO^{fl/fl} livers treated with *siLuc* or *siJnk2* were evident. Sirius red (SR) staining showed significantly increased extracellular matrix deposition (ECM) in *siJnk2*-treated NEMO^{Δhepa} livers compared to all other groups. The upregulation of HSC activation markers further strengthened these results (Figure 10.5C-D).

Since siJnk2 treatment increased immune cell infiltration in NEMO^{Δhepa} livers, we investigated the impact on immune cell activation by FACS analysis and double immunofluorescence staining. Silencing of Jnk2 significantly increased proinflammatory monocytes (CD45⁺/CD11b⁺/Gr1.1⁺/F4/80^{int}) in NEMO^{Δhepa} livers compared to siLuc-treated animals (**Figure 10.5E, S10.5D**). The finding that proinflammatory monocytes are involved in monocyte recruitment was also reflected at the mRNA level. Analysis of the mRNA transcripts revealed significantly increased levels of ccl2 and ccl5 in NEMO^{Δhepa} mice treated with siJnk2 compared to all other groups (**Figure 10.5D**). Apoptotic cell death plays a significant role in CLD of NEMO^{Δhepa} mice⁸. In this line, siJnk2-treated NEMO^{Δhepa} livers showed significantly increased cleaved caspase-3 levels compared to siLuc treated animals, as evidenced by both Western blot analysis (**Figure 10.6A**) and immunohistochemistry (**Figure S10.6A**). Noticeably, histological examination showed not only positive hepatocytes but also positive immune cells. By means of *in vivo* imaging using FMT/μCT, the amount of Duramycin-NIR790 conjugate bound to phosphatidylethanolamine in apoptotic cells was markedly increased, supporting the histological analysis. Quantification of Caspase-3 activity in liver tissue and Duramycin-NIR790 conjugate accumulation in the liver *in vivo* confirmed increased apoptosis in siJnk2-treated NEMO^{Δhepa} livers (**Figure 10.6B, 10.6D-E**). Next, proliferation activity measured by PCNA expression was determined as a sign of liver repair and regeneration. Our data revealed significantly increased parenchymal and non-parenchymal positivity for PCNA at protein and mRNA expression levels in NEMO^{Δhepa} livers after siJnk2 treatment (**Figure 10.6C, S10.6B**).

A

B

C

Figure 10.6

Figure 10.6 Hepatocyte-specific *siJnk2* treatment modifies apoptotic cell death in the early phase of NEMO^{Δhepa} livers. (A) Immunoblot analysis of whole liver extracts prepared from NEMO^{fl}+*siLuc*, NEMO^{fl}+*siJnk2*, NEMO^{Δhepa}+*siLuc* and NEMO^{Δhepa}+*siJnk2* mice at 12 weeks of age detecting cleaved caspase-3 and PCNA was performed, using GAPDH as a loading control. (B) Caspase-3 activity in the livers from 12 week-old NEMO^{fl} mice compared to NEMO^{Δhepa} mice ensuing *siLuc* or *siJnk2* injections. The activity was represented as relative fluorescence units (RFU). (C) Hepatic mRNA levels of PCNA were quantified and displayed as fold increase for the identical treatment groups. For the same treatment cohort, representative FMT/μCT images (D) showing the accumulation of Duramycin-NIR790 in the liver, which is used as a quantitative biomarker of cell death and (E) expressed as [fmol]. (Scale bar: 10mm). Results are shown as mean ± S.E.M. **P*<0.05.

Since at earlier stages, *siJnk2* inoculation triggered increased hepatitis, we went back to explore whether genetic deletion of Jnk2 from birth triggered the same effect or was due to acute effects of Jnk2 sudden loss. Twelve-week-old DKO^{Δhepa} animals showed increased liver damage as demonstrated by a significant elevation of serum transaminase level compared to NEMO^{Δhepa} and control littermates. These changes were further confirmed by H&E staining of liver tissue sections (Figure S10.7A-E).

Discussion

Different etiologies, *e.g.*, viral hepatitis, alcohol or metabolic liver diseases, are known to trigger chronic liver diseases and, ultimately, end-stage liver disease, including the occurrence of HCC⁹. The incidence of liver cancer as a cause of death is globally increasing. However, limited treatment options are currently available. Hence, there is a rising need to understand the molecular pathways involved in CLD pathophysiology.

Previous studies from our group demonstrated that the NF-κB pathway and the activation of JNKs play a major role in HCC development^{1,3}. Therefore, this study aimed to investigate the outcome of blocking Jnk2 exclusively in hepatocytes in an experimental model of CLD, the NEMO^{Δhepa} mice. Moreover, we used two experimental approaches: a genetic knockout and an inducible knockdown using siRNA technology. These approaches are different but complementary. A complete loss of protein function was achieved using

double knockout mice. However, this is a time and costly experimental approach. In contrast, the knockdown approach using siRNA allows a quicker evaluation of the mechanisms governed by Jnk2, reflecting more closely the human situation, since in a patient's liver disease is the result of changes in gene and/or protein expression.

First, the analysis of genetic double-knockout mice for JNK2^{Δhepa} and NEMO^{Δhepa} (DKO^{Δhepa}) concurred with our previous findings that Jnk2 knockout in NEMO^{Δhepa} mice showed an improved outcome in HCC development³. Consistent with decreased liver transaminases, DKO^{Δhepa} mice livers showed a reduced overall number of tumors. These results suggest that hepatocytic deletion of JNK2 improves tumor progression but impairs tumor initiation, and thus we next questioned whether JNK2 inhibition could be a therapeutic approach for the development of CLD.

Next, we established a genetic knockdown model of Jnk2 in hepatocytes using lipid nanoparticle-formulated siRNA technology and tested both in hepatocytes *in vitro* as well as *in vivo*. As previously shown⁴, the efficacy of lipid nanoparticles targeting specifically hepatocytes *in vivo* was demonstrated. Specifically, we established a genetic knockdown for Jnk2 exclusively in hepatocytes. Thus, we focused on regulated CLD at different stages of disease progression in our experimental model.

Interestingly, siJnk2 treatment in NEMO^{Δhepa} mice during the late phase of CLD disease revealed significant overall improvement. Reduced liver serum values and significantly improved serum biochemical parameters were supported by liver histopathology studies. At late stages of CLD, Jnk2 down-regulation exerted a protective role, as demonstrated by an ameliorated progression of CLD and reduced fibrogenesis. The reduced fibrogenic response directly translated into decreased tumor initiation, suggesting that the carcinogenic environment after siJnk2 treatment improved in NEMO^{Δhepa} livers as also reflected by improved liver architecture. Most importantly, siJnk2 treatment prevented HCC progression, characteristic of NEMO^{Δhepa} livers at the age of 44 weeks.

Interestingly, this observation was associated with a substantial impact on the adaptive immune system, showing changes in T helper and NKT cells. Focusing on CD4⁺ and CD8⁺ T-cells, both populations have opposing roles in promoting a chronic proinflammatory environment and triggering anti-tumor surveillance. Yet, this is still a matter of debate in HCC initiation, depending upon the experimental model. However, closely correlating with improved disease progression after siJnk2 treatment, FACS analysis underscored an anti-tumor effect for both subpopulations on HCC.

Concomitantly, CD8⁺ T-cells are the main subset of tumor-infiltrating lymphocytes. Large numbers of CD8⁺ T-cells in human HCC patients correlated with improved survival, more prolonged relapse-free survival as well as diminished disease progression¹⁰⁻¹². Interestingly, lack of CD4⁺ T-cells is evident in patients with poor HCC prognosis, also predicting a high recurrence rate in these patients¹³. Although their role in HCC development remains controversial and opposing roles are described in the literature, a negative correlation between B cells and CD4⁺ as well as CD8⁺ T-cell activity was found¹⁴.

However, does JNK2 deletion impact the early stages of CLD? To answer this question, we next investigated Jnk2 modulation in the acute phase of NEMO^{Δhepa} mice, both using siRNA and DKO^{Δhepa} mice. Both experimental approaches revealed a stage-dependent outcome of CLD. Interestingly, Jnk2 downregulation in NEMO^{Δhepa} mice caused a proinflammatory environment aggravating the phenotype of NEMO^{Δhepa} mice at this time point. Jnk2 siRNA-mediated nano-delivery caused exacerbated hepatic damage, immune cell infiltration, impaired liver fibrosis, apoptosis and compensatory proliferation.

Analyzing immune cell infiltration revealed cells as monocyte-derived macrophages (MoMFs) by different methods confirming previous observations that hepatic macrophage numbers are enormously increased independent of the type of liver injury¹⁵. Specifically, the CCL2/CCR2 axis has been shown to be a key mechanism in monocyte recruitment to the liver¹⁶. The infiltrated monocytes aggravate the hepatic injury by releasing proinflammatory cytokines such as TNF α , IL-1 β and nitric oxide (NO)¹⁷. Concomitantly, siJnk2 treatment in NEMO^{Δhepa} mice showed rapid liver fibrosis progression by increased collagen deposition and HSCs activation marker.

Remarkably, not only hepatocytes but also infiltrating cells were positively stained for cleaved Caspase-3 and undergoing apoptosis after siJnk2 treatment. Additionally, Kupffer cells (KCs) have been described as long-lived resident macrophages, even though a constant turnover occurs and hepatic macrophages are incessantly repopulated¹⁸. Since the intrahepatic macrophage number is massively expanded following the influx of peripheral monocytes rather than augmentation of tissue-resident macrophages, the observed results offer indisputable evidence for a high turnover and consistently repopulation of MoMFs due to hepatocyte-specific siJnk2 treatment in NEMO^{Δhepa} mice. Comprehensive data providing a pivotal role for Jnk2 in acute liver failure from our group was recently obtained². In this study, siJnk2 treatment prior to ibuprofen administration caused a dramatic increase in hepatic damage and cell death.

Overall, our findings define a pivotal time-dependent role of Jnk2 during the development of experimental CLD. In particular, hepatocytic siRNA-mediated Jnk2 inhibition in older mice blocked fibrogenesis and HCC progression. Hence, these results define Jnk2 as a potential preventive approach targeting hepatocytes to impair cancer initiation in chronically damaged livers. Moreover, similar phenotypes observed in both the knockout and the knockdown mice highlight the usefulness of the siRNA technology, specially formulated with lipid nanoparticles as a cell-type specific approach (**Figure 10.7**).

Figure 10.7

Figure 10.7 Schematic representation of the impact of JNK2 modulation in hepatocytes during the different stages of chronic liver disease. The role of hepatocellular *Jnk2* in CLD was assessed by *Jnk2*-silencing using a hepatocyte-specific liposomal-delivered *Jnk2*-siRNA or genetic *Jnk2*-deletion in hepatocytes. Conditional knockout mice with hepatocyte-specific deletion of *Jnk2* and NEMO/IKK γ were generated for phenotypic characterization. Thirteen week-old NEMO/JNK2^{Δhepa} animals displayed notably elevated serum transaminases and a deteriorated liver parenchyma, compared to NEMO^{Δhepa} mice. Unexpectedly, NEMO^{Δhepa} mice with hepatocellular *Jnk2* deletion at 52 weeks of age presented improved liver function and histology, characterized by a reduced total number of HCC nodules. Concomitantly, eight-week-old NEMO^{Δhepa} mice were treated with either *siLuc* or hepatocyte-targeting *Jnk2*-siRNA at a single dose of 0.2mg/kg once weekly and sacrificed at 12 weeks of age. As a result, *Jnk2*-silencing in hepatocytes led to increased liver transaminases, hepatocellular apoptosis and compensatory proliferation in NEMO^{Δhepa} animals. Compared to *siLuc*-treated animals, *siJnk2*-treated NEMO^{Δhepa} mice exhibited augmented fibrogenesis as well as a more significant inflammatory response. In contrast, CLD progression was significantly reversed in 52 week-old end-stage diseased NEMO^{Δhepa} that had previously undergone *siJnk2* injection for eight weeks. Amelioration of serum liver enzymes, reduction of apoptosis and decreased inflammation were hallmarks of 52 week-old *siJnk2*-treated NEMO^{Δhepa}. Of note, significant improvement in the hepatic parenchyma architecture of *siJnk2*-treated mice was observed with only mild signs of fibrosis and steatosis as well as a reduced presence of premalignant and malignant liver tumors.

References

1. Luedde T, Beraza N, Kotsikoris V, van Loo G, Nenci A, De Vos R, Roskams T, et al. Deletion of NEMO/IKKgamma in liver parenchymal cells causes steatohepatitis and hepatocellular carcinoma. *Cancer Cell* 2007;11:119-132.
2. Zoubek ME, Woitok MM, Sydor S, Nelson LJ, Bechmann LP, Lucena MI, Andrade RJ, et al. Protective role of c-Jun N-terminal kinase-2 (JNK2) in ibuprofen-induced acute liver injury. *J Pathol* 2019;247:110-122.
3. Cubero FJ, Zhao G, Nevzorova YA, Hatting M, Al Masaoudi M, Verdier J, Peng J, et al. Haematopoietic cell-derived Jnk1 is crucial for chronic inflammation and carcinogenesis in an experimental model of liver injury. *J Hepatol* 2015;62:140-149.
4. Speicher T, Siegenthaler B, Bogorad RL, Ruppert R, Petzold T, Padrisa-Altes S, Bachofner M, et al. Knockdown and knockout of β 1-integrin in hepatocytes impairs liver regeneration through inhibition of growth factor signalling. *Nat Commun* 2014;5:3862.
5. Beraza N, Malato Y, Sander LE, Al-Masaoudi M, Freimuth J, Riethmacher D, Gores GJ, et al. Hepatocyte-specific NEMO deletion promotes NK/NKT cell- and TRAIL-dependent liver damage. *J Exp Med* 2009;206:1727-1737.
6. Maschmeyer P, Flach M, Winau F. Seven steps to stellate cells. *J Vis Exp* 2011.
7. Chuma M, Sakamoto M, Yamazaki K, Ohta T, Ohki M, Asaka M, Hirohashi S. Expression profiling in multistage hepatocarcinogenesis: identification of HSP70 as a molecular marker of early hepatocellular carcinoma. *Hepatology* 2003;37:198-207.
8. Liedtke C, Bangen JM, Freimuth J, Beraza N, Lambertz D, Cubero FJ, Hatting M, et al. Loss of caspase-8 protects mice against inflammation-related hepatocarcinogenesis but induces non-apoptotic liver injury. *Gastroenterology* 2011;141:2176-2187.
9. Pellicoro A, Ramachandran P, Iredale JP, Fallowfield JA. Liver fibrosis and repair: immune regulation of wound healing in a solid organ. *Nat Rev Immunol* 2014;14:181-194.
10. Casini A, Ricci OE, Paoletti F, Surrenti C. Immune mechanisms for hepatic fibrogenesis. T-lymphocyte-mediated stimulation of fibroblast collagen production in chronic active hepatitis. *Liver* 1985;5:134-141.
11. Basso L, Bernardi C, Ayabaca S, Marin M. Life-threatening urinary retention after haemorrhoidectomy and internal sphincterotomy. *Tech Coloproctol* 2001;5:109-111.
12. Garnelo M, Tan A, Her Z, Yeong J, Lim CJ, Chen J, Lim KH, et al. Interaction between tumour-infiltrating B cells and T cells controls the progression of hepatocellular carcinoma. *Gut* 2017;66:342-351.
13. Fu J, Zhang Z, Zhou L, Qi Z, Xing S, Lv J, Shi J, et al. Impairment of CD4+ cytotoxic T cells predicts poor survival and high recurrence rates in patients with hepatocellular carcinoma. *Hepatology* 2013;58:139-149.
14. Xue H, Lin F, Tan H, Zhu ZQ, Zhang ZY, Zhao L. Overrepresentation of IL-10-Expressing B Cells Suppresses Cytotoxic CD4+ T Cell Activity in HBV-Induced Hepatocellular Carcinoma. *PLoS One* 2016;11:e0154815.
15. Karlmark KR, Weiskirchen R, Zimmermann HW, Gassler N, Ginhoux F, Weber C, Merad M, et al. Hepatic recruitment of the inflammatory Gr1+ monocyte subset upon liver injury promotes hepatic fibrosis. *Hepatology* 2009;50:261-274.
16. Miura K, Yang L, van Rooijen N, Ohnishi H, Seki E. Hepatic recruitment of macrophages promotes nonalcoholic steatohepatitis through CCR2. *Am J Physiol Gastrointest Liver Physiol* 2012;302:G1310-1321.
17. Krenkel O, Tacke F. Liver macrophages in tissue homeostasis and disease. *Nat Rev Immunol* 2017;17:306-321.
18. Naito M, Hasegawa G, Ebe Y, Yamamoto T. Differentiation and function of Kupffer cells. *Med Electron Microsc* 2004;37:16-28.
19. Elvas F, Stroobants S, Wyffels L. Phosphatidylethanolamine targeting for cell death imaging in early treatment response evaluation and disease diagnosis. *Apoptosis* 2017;22:971-987.
20. Gremse F, Theek B, Kunjachan S, Lederle W, Pardo A, Barth S, Lammers T, et al. Absorption reconstruction improves biodistribution assessment of fluorescent nanoprobe using hybrid fluorescence mediated tomography. *Theranostics* 2014;4:960-971.
21. Gremse F, St.rk M, Ehling J, Menzel JR, Lammers T, Kiessling F. Imalytics Preclinical: Interactive Analysis of Biomedical Volume Data. *Theranostics* 2016;6:328-341.

Supplementary Figures

Figure S10.1

Figure S10.1 Serum liver AST (A), GLDH (B) and AP (C) of 52-week-old NEMO Δ hepa, Jnk2 Δ hepa, NEMO Δ hepa/Jnk2 Δ hepa animals and the respective controls are displayed in U/l (n=11-13). Data are presented as mean $\pm$ S.E.M. * P <0.05, ** P <0.01, *** P <0.001

Figure S10.2

Figure S10.2 (A) Hepa 1-6 cells were cultured in a quantity of 500.000 cells/well, on 6 well 15.5ml/9.6cm² culture plates. After 12h of stabilization period, Hepa 1-6 were transfected with either medium alone, lipofectamin or one out of 12 different computer-defined Jnk2-siRNAs [1 nmol] for 24h and the Jnk2 mRNA expression was quantified by using qRT-PCR. Results are expressed as fold increase. (B) Hepa 1-6 cells were transfected with medium alone, lipofectamin or siJnk2 (which corresponded to siRNA

sets 3 or 4) in a concentration ranging from 0.125-10nmol for 24h and subsequently, *Jnk2* mRNA levels were determined by using qRT-PCR. Results are expressed as fold increase. Primary murine hepatocytes were isolated from 7-8 week-old WT mice by collagenase perfusion and plated on collagen-precoated Petri dishes at a density of $1.5 \times 10^4/\text{cm}^2$ in supplemented DMEM medium. After 12h stabilization overnight, primary murine hepatocytes were transfected with either medium alone, lipofectamin or siJnk2 sets 3 (C) or 4 (D) in a concentration ranging from 0.125-5nmol for 24h. The mRNA levels of *Jnk2* were quantified by using qRT-PCR and results are shown as fold increase. Results are expressed as mean $\pm$ S.E.M. *** $P < 0.001$.

Figure S10.3

Figure S10.3 (A) Serum liver AST (left), GLDH (center) and AP (right) of 52-week-old NEMO Δ hepa animals after completing 8 weeks of treatment with siLuc or siJnk2 or untreated are displayed in U/l (n=11-13). (B) Bodyweight (left), liver weight (center) and liver weight versus body weight ratio (right) is graphically represented for the identical treatment groups. *TNF* (C) and *VEGF* (D) mRNA levels were analyzed for NEMO Δ hepa mice that were treated with either siLuc or siJnk2 at the age of 52 weeks. Results are represented as fold increase. Results are expressed as mean $\pm$ S.E.M. *** $P < 0.001$.

Figure S10.4

Figure S10.4 (A) Flow cytometry assay was performed in whole liver of 52-week-old Nemo Δ hepa mice after therapeutic administration of either *siLuc* or *siJnk2* for 8 weeks. B cells among CD45 $^{+}$ (upper panel left), absolute number of B cells (upper panel center-left), NK cells among CD45 $^{+}$ (upper panel center-right), absolute number of NK cells (upper panel right), NKT cells among CD45 $^{+}$ (intermediate panel left), absolute number of NKT cells (intermediate panel center-left), T-cells among CD45 $^{+}$ (intermediate panel center-right), absolute number of T-cells (intermediate panel right), CD4 $^{+}$

T-cells among CD45⁺ (lower panel left), absolute number of CD4⁺ T-cells (lower panel center-left), CD8⁺ T-cells among CD45⁺ (lower panel center-right), absolute number of CD8⁺ T-cells (lower panel right) were quantified by using FlowJo 7.6 and results are graphed. **(B)** PCNA expression was quantified with qRT-PCR for Nemo^{Δhepa} animals following treatment with either *siLuc* or *siJnk2* at 52 weeks and displayed as fold increase. **(C)** Caspase-3 activity was determined for the same treatment groups and represented as relative fluorescence units. **(D)** Representative FMT/ μ CT images showing the accumulation of Duramycin-NIR790 in various organs (left) is shown for the identical treatment groups. The accumulation of Duramycin-NIR790 was determined and expressed (right) as [fmol]. (Scale bar: 10mm). Data analysis is expressed as mean $\pm$ S.E.M. * P <0.05, ** P <0.01, *** P <0.001.

Figure S10.5

Figure S10.5 **(A)** Serum liver AST (left), GLDH (center) and AP (right) of 12-week-old NEMO^{Δhepa} animals and the respective controls are represented in U/l. **(B)** Liver weight (left), body weight (center) and liver weight *versus* body weight ratio (right) is graphed for the identical treatment groups. **(C)** Representative histological sections of livers from 12-week-old NEMO^{Δhepa} (lower images) and control mice (upper images) following the administration of either *siLuc* (left) or *siJnk2* (right)

stained with hematoxylin and eosin are exhibited (Scale bars: 100μm). **(D)** Illustrative microphotographs of CD11b*F4/80*stainings by immunofluorescence performed on liver cryoslides of 12-week-old *Nemo^{fl/fl}* (upper images) and *Nemo^{Δhepa}* mice (lower images) after being subjected to siLuc (left) or siJnk2 injections (right). Positive CD11b*F4/80* cells are stained green; total cells were counter-stained with DAPI (blue). (Scale bars: 100μm). Arrows indicate positive cells. Data analysis is expressed as mean ± S.E.M. **P*<0.05, ***P*<0.01, ****P*<0.001.

Figure S10.6

Figure S10.6 Representative images of Cleaved Caspase-3 **(A)** and PCNA **(B)** expression on paraffin-embedded liver samples of *Nemo^{fl/fl}* (upper images) and *Nemo^{Δhepa}* (lower images) was analyzed by immunohistochemistry at the age of 12 weeks after *siLuc* (left) or *siJnk2* (right) treatment. Arrows indicate positive cells. (Scale bar: 100μm).

Figure S10.7

Figure S10.7 (A) Representative histological sections of livers from 12-week-old NEMO^{Δhepa}, Jnk2^{Δhepa} and DKO^{Δhepa} (lower images) and the respective control mice (upper images) stained with hematoxylin and eosin are shown (Scale bars: 100μm). Serum liver AST (B), ALT (C), GLDH (D) and AP (E) of the identical mouse cohort are displayed in U/l. Data analysis is expressed as mean ± S.E.M. **P*<0.05, ***P*<0.01, ****P*<0.001.

Supplementary Materials & Methods

siRNA sequences

The following siRNA sequences targeting *Jnk2* were used:

Table 1: siRNA sequences targeting *Jnk2*

	Synthesized siRNA strand	
siRNA set number	sense strand sequence (5'-3')	antisense strand sequence (5'-3')
1	cuuAAAGuGuGucaAucAudTsdT	AUGAUUGAcAcACUuuAAGdTsdT
2	uaAcuuAuGucAGguuAuudTsdT	AAuAACCUGAcAuAAGUuAdTsdT
3	cuAGcAAcAuuGuaGuAAAdTsdT	UUuACuAcAAUGUUGCuAGdTsdT
4	ggAAAGAGcuAAuuuAcAAAdTsdT	UUGuAAAuAGCUCUuUCCdTsdT
5	gaAAGAGcuAAuuuAcAAAdTsdT	UUUGuAAAuAGCUCUuUCCdTsdT
6	aaGAGcuAAuuuAcAAAGAdTsdT	UCUuUGuAAAuAGCUCUuUdTsdT
7	agAGcuAAuuuAcaAAGAAAdTsdT	UUCUuUGuAAAuAGCUCUdTsdT
8	caccuGAAAuuGAuAcuuudTsdT	AAAGuAUcAAUUUcAGGUGdTsdT
9	cacuAGGuuAGuuuuuGuudTsdT	AACAAAAACuAACCuAGUGdTsdT
10	ggcuuAAuuuucAgccAAAdTsdT	UUUGGCUGAAAAuAAGCCdTsdT
11	cauuGGGccuGcAgAcAAAdTsdT	UUUGUCUGcAGGCCcAAUGdTsdT
12	guGucGuAAuuucaGAcAudTsdT	AUGUCUGAAAUuACGAcACdTsdT
<i>siLuc</i>	cuuAcGcuGAGuAcuucGAdTsdT	UCGAAGuACUcAGCGuAAGdTsdT

Abbreviations: A, G, U, C: RNA Nucleotide; a, g, u, c: 2'-O-methyl-Nucleotide; s: Phosphorothioate; dT: desoxy-T residue

According to the manufacturer's protocol, siRNA set number 4 was chosen for KL52 lipid nanoparticles (LNP) formulation. For the long-term experiments, a total dose of 0.2mg/kg BW of siJnk2 was dissolved in PBS (PAN-Biotech) and injected *i.v.* (tail vein) once per week. In parallel, a control siRNA against luciferase (siLuc) was used.

Immunohistochemistry staining

Liver tissue slides were stained for Cleaved Caspase 3 (Cell Signaling Technology), PCNA (Dianova GmbH), p-JNK2 (Novus Biologicals) on paraffin sections. Eight randomly chosen images were taken of each liver tissue section (20X magnification, scale bars: 100µm) for analysis using Axiovision software (Carl Zeiss, Jena, Germany).

Immunofluorescence

First, freshly cut sections were fixed with formaldehyde (4%) for 10min, followed by 3x3min washing with DPBS+0,02%NaAzide. Next, the sections were blocked with DPBS+0,02%NaAzide+0,2%BSA for 5min at room temperature. The blocking solution was removed by a 3x5min washing step in DPBS+0,02%NaAzide. In the next step, the sections were incubated at room temperature with the primary antibody CD11b (BD, 1:500 in DPBS+1%mouse serum) for 45min. Subsequently, the sections were washed 3x5min with DPBS+0,02%NaAzide followed by the incubation of the secondary goat anti-rat/Cy3 antibody (1:500 in DPBS+1%mouse serum) at room temperature for 1h. After the incubation time, the sections were washed by three repetitive 3min washing steps in DPBS+0,02%NaAzide.

To perform double staining, the sections were blocked again with DPBS +0,02%NaAzide +0,2%BSA for 5min at room temperature. In the next step, the sections were incubated overnight at 4°C with the primary F4/80 antibody (Bio-Rad, 1:200 in DPBS +1%mouse serum). The next day, the sections were washed for 3x5min with DPBS +0.02% NaAzide followed by the incubation of the secondary goat anti-rat/Alexa 488 antibody (1:500 in DPBS+ 1% mouse serum) at room temperature for 1h. After the incubation time, the sections were washed by three repetitive 3min washing steps in DPBS +0,02%NaAzide and were counterstained with a mounting medium with DAPI. Subsequently, the sections were assessed under the microscope. Eight randomly chosen images were taken of each liver tissue section (20X magnification, scale bars: 100µm) for the analysis using Axiovision software (Carl Zeiss, Jena, Germany).

Fluorescent activated cell sorting (FACS)

To assess the different immune cell types in the liver, the mice were sacrificed and approximately 200mg of liver was used for further preparation to perform FACS analysis. The liver specimen was digested in 5ml RPMI medium containing 2mg Collagenase 2 (Roche) and was incubated for 45min at 37°C on a shaker. Next, the digested liver was meshed through a 70µm cell strainer in a new tube and gently mixed with 10ml Hanks complete solution (500ml HBSS +3ml 10%BSA +1.5ml EDTA pH 8, 0.5M).

To separate the leukocytes from hepatocytes and cell debris, the sample was centrifuged for 5min at 50x g, 4°C. Subsequently, the supernatant was transferred into a new tube and the sample was centrifuged for 10min at 450x g, 4°C to pellet the leukocytes. The supernatant was removed and the pellet was gently mixed with 5ml lysis buffer (BD Pharm Lyse) for 15min at 4°C to degrade the remaining erythrocytes in the sample. After this incubation time, the sample was gently mixed with 10ml Hanks complete solution and centrifuged for 10min at 450x g, 4°C. Next, the supernatant was removed and the sample was blocked with 200µl of the FACS blocking solution (10ml HBSS + 5ml 10% FBS) for 20min at 4°C. After the blocking step, the sample was centrifuged for 10min at 450x g, 4°C, to remove the supernatant containing the FACS blocking solution. Immune cells were

stained for the myeloid and lymphoid panel (1/80 dilution) for 30min at 4°C with the following antibodies: CD11b / PE, CD11c / APC, CD3e / APC, CD4 / PE, CD45 / APC-eFluor® 780, CD8 / FITC, F4/80 / PE-Cy7, NK1.1 / PE-Cy7 (eBioscience), CD19 / Percp-Cy5.5, Gr1-Percp / Cy5.5, Ly6G / FITC (BD).

In the next step, the cell and antibody suspension was centrifuged for 10min at 450x g, 4°C and resuspended in 200µl Hanks complete solution. In the last step, each sample was mixed with 20µl Hoechst (Invitrogen) and 20µl of approximately 20000 APC-Calibrate-Beads. The samples were placed on ice and were analyzed in the FACS machine (FACS Canto, BD).

Immunoblot analysis

The following primary antibodies were used for immunoblot analysis (western blot) as well as for immunohistochemistry analysis: cleaved caspase-3, pJNK, JNK1, JNK2, pSAPK/JNK, SAPK/JNK, NF-κB p65, (Cell Signaling Inc., Danvers, MA, USA); GAPDH, F4/80 Bio-Rad (Hercules, USA); HSP70/HSP72 Enzo Life Sciences (Zandhoven, Belgium); JNK2 (Novus Biologicals); PCNA Dianova GmbH (Hamburg, Germany); CD11b BD (Franklin Lakes, USA). As secondary antibodies, anti-rabbit HRP (Cell Signaling), antimouse-HRP (Santa Cruz), anti-rat Cy3/Alexa488 (Invitrogen, Paisley, UK) and biotinylated anti-rabbit or mouse IgG (Vector, Burlingame, USA) were used.

Real-time quantitative PCR

Using the Omniscript® RT Kit (ThermoFisher Scientific), cDNA was analyzed for the following genes. All primers were purchased from Eurofins MWG Operon (Huntsville, USA). The following primer sequences were used for quantitative PCR.

Table 2: List primer Real-Time quantitative PCR (q-PCR)

Gene	Forward	Reverse
CCL2	GTGTTGGCTCAGCCAGATGC	GACACCTGCTGCTGGTGATCC
CCL5	CCTCACCATATGGCTCGGAC	ACGACTGCAAGATTGGAGCA
CCR2	TCGCTGTAGGAATGAGAAGAAGAGG	CAAGGATTCTGGAAGGTGGTCAA
CCR5	CACAGCATGGACAATAGCCAAGTACC	GCCATCTCTGACCTGCTCTTCC
Col1A1	TGTGTGCGATGACGTGCAAT	GGGTCCCTCGACTCCTACA
GAPDH	TGTTGAAGTCACAGGAGACAACCT	AACCTGCCAAGTATGATGACAA
JNK1	AGAGTGACACCAGAAGC AA	GAGAGACGTTGCTGGGTATT
JNK2	TTGTGCTGCTTTTGATACAGTTCTTGGG	CTGGAAAGAGCTCTTCAAATTTGAT

p53	CCAGGGAGCGCAAAGAGA	TCTCCATCAAGTGGTTTTTCTTTT
PCNA	AAAATTGCTGACATGGGACAC	CAGAAAAGACCTCAGGACACG
Survivin	CTACCGAGAACGAGCCTGATT	AGCCTTCCAATTCCTTAAAGCAG
TNF α	ACTGAACTTCGGGGTGATCG	GCCATTTGGGAAGTTCTCATCC
α -SMA	CTCCAGACATCAGGGAGTAA	TCGGATACTTCAGCGTCAGGA
VEGF β	CTTGTCAGAGCGGAGAAAGC	ACATCTGCAAGTACGTTTCGTT

Data were analyzed using QuantStudio™ (Thermo Scientific) software.

Combined fluorescence molecular tomography and microcomputed tomography (FMT/ μ CT)

Fluorescence molecular tomography (FMT) and micro-computed tomography (μ CT) measurements were performed at the Institute for Experimental Molecular Imaging (ExMI) at the University Hospital Aachen. First, the abdominal region of the mice was shaved 1 day before the experiment with an electric shaver to reduce any possible interference by hairs during the FMT scans. In the next step, 9h before the FMT/ μ CT scan, the mouse was injected intravenously with the cell death probe Duramycin-NIR790 conjugate (66.7pmol/kg, kindly provided by Chris Pak, Molecular Targeting Technologies, Inc.). Duramycin-NIR790 conjugate binds to phosphatidylethanolamine (PE) with high affinity and specificity. Under normal conditions, PE is restricted to the inner part of the cell membrane. Upon apoptosis and cell death, PE is exposed to the outer leaflet of the cell membrane, thereby enabling the binding of the imaging probe. Duramycin-based imaging probes have been successfully applied for the non-invasive imaging of cell death, including apoptosis in disease diagnosis and therapy monitoring¹⁹.

For scanning, the mouse was anesthetized using isoflurane (2%v/v). To precisely localize the liver and other organs, a μ CT scan was performed directly before the FMT measurement (TomoScope 30s Duo, CT Imaging GmbH). During both scans, the mouse was kept anesthetized and was held in a fixed position in a multimodal animal bed. CT imaging was performed using the SQD-6565-360-29 protocol, which acquires 720 projections with 516x506pixels requiring a scanning time of 29s per sub-scan. Directly after acquiring the μ CT scans, the mouse bed was transferred to the FMT system (FMT 2500LX, PerkinElmer), and the FMT scan was performed at 790nm. Data fusion and reconstruction of the fluorescence distribution were performed as described²⁰. The organs were manually segmented based on the μ CT data and the probe concentration in the liver was determined using Imalytics preclinical 2.0 (Gremse-IT GmbH Aachen)²¹.

Microscopic imaging data

For bright-field image acquisition the microscope Imager A2 with the following objective lenses by Carl Zeiss AG (Oberkochen) were used: Plan-APOCHROMAT 5x/0,16; 10x/0,45; 20x/0,6; 40x/0,95. The camera 'AxioCam 506 color' was used for image acquisition. For fluorescent image acquisition, the microscope Imager.Z1 with the following objective lens by Carl Zeiss AG (Oberkochen) was used: EC Plan-NEOFLUAR 20x/0,5. The camera 'AxioCam MRm' was used for image acquisition. Images were taken at room temperature and the software AxioVision by Carl Zeiss AG (Oberkochen) was used.

Chapter 11

Disruption of the FasL/Fas axis protects against inflammation-derived tumorigenesis in chronic liver disease

Cubero FJ*, Woitok MM*, Zoubek ME*, de Bruin A, Hatting M, Trautwein C

***Cell Death Dis* 2019;10(2):115**

DOI: 10.1038/s41419-019-1391-x

*Contributed equally to this work as first authors

Abstract

Introduction & aims

Fas Ligand (FasL) and Fas (APO-1/CD95) are members of the TNFR superfamily and may trigger apoptosis. Here, we aimed to elucidate the functional role of Fas signaling in an experimental model of chronic liver disease, the hepatocyte-specific NEMO knockout (NEMO^{Δhepa}) mice.

Material & Methods

We generated NEMO^{Δhepa}/Fas^{pr} mice, while NEMO^{Δhepa}, NEMO^{f/f} as well as Fas^{pr} animals were used as controls, and characterized their phenotype during liver disease progression. Liver damage was evaluated by serum transaminases, histological, immunofluorescence procedures, and biochemical and molecular biology techniques. Proteins were detected by Western Blot, expression of mRNA by RT-PCR, and infiltration of inflammatory cells was determined by FACs analysis, respectively.

Results

Fas^{pr} mutation in NEMO^{Δhepa} mice resulted in overall decreased liver injury, enhanced hepatocyte survival, and reduced proliferation at eight weeks of age compared with NEMO^{Δhepa} mice. Moreover, NEMO^{Δhepa}/Fas^{pr} animals elicited significantly decreased parameters of liver fibrosis, such as Collagen IA1, MMP2, and TIMP1, and reduced proinflammatory macrophages and cytokine expression. At 52 weeks of age, NEMO^{Δhepa}/Fas^{pr} exhibited less malignant growth, as evidenced by reduced HCC burden associated with a significantly decreased number of nodules and LW/BW ratio and decreased myeloid populations. The deletion of TNFR1 further reduced the tumor load of 52-weeks-old NEMO^{Δhepa}/Fas^{pr} mice.

Conclusions

The functionality of FasL/Fas might affect inflammation-driven tumorigenesis in an experimental model of chronic liver disease. These results help to develop alternative therapeutic approaches and extend the limitations of tumor therapy against HCC.

Keywords

Fas; IKKγ/Nemo; hepatitis, HCC, chronic liver disease

Introduction

The transmembrane proteins Fas-Ligand (FasL) and Fas (Fas/ CD95/APO-1) are members of the tumor necrosis factor (TNF) and TNF receptor gene superfamilies (TNFR gene superfamily), respectively, and are expressed in numerous cell types. FasL–Fas interaction plays a crucial role in immune regulation via the ability of FasL to transmit an apoptotic signal to Fas-expressing cells¹. Remarkably, the importance of the FasL/Fas axis in pathophysiology and homeostasis has been well documented in the liver, where these proteins are expressed in hepatocytes, cholangiocytes, activated stellate cells (HSC), and Kupffer cells (KC)².

Downregulation or loss of Fas expression and function is frequently found in the progression of a number of human malignancies, including colon, breast, lung, and liver carcinoma^{3,4}. Thus, the FasL–Fas pathway plays a crucial role in tumor initiation and progression. It might be a plausible therapeutic target not only for the progression of liver disease but also for hepatocellular carcinoma (HCC).

HCC is the fifth most common solid cancer affecting one million people per year, representing the third cause of mortality by cancer worldwide^{5,6}. Escape from immune surveillance may play an essential role in liver tumorigenesis. Alteration of the FasL/Fas system is regarded as one of the mechanisms preventing the immune system from rejecting tumor cells⁷. However, little attention has been paid to the role of the Fas/FasL interaction *in vivo*.

Hepatocyte-specific NEMO knockout (NEMO^{Δhepa}) mice are susceptible to spontaneous apoptosis, which leads to chronic hepatocyte injury and regenerative proliferation, constituting a risk factor for cancer development⁸. NEMO^{Δhepa} livers are hypersensitive towards TRAIL stimulation⁹. Moreover, we have also shown that death receptor TNFR1, but not TRAIL, is involved in determining the progression of liver injury in NEMO^{Δhepa} mice¹⁰. In the present study, we examined the functional role of deficient FasL/Fas signaling on disease progression and end-stage tumorigenesis in the NEMO^{Δhepa} model.

Material and Methods

Housing and Generation of Knockout mice

Animals were maintained in the animal facility of the University Hospital RWTH Aachen according to the German legal requirements. Hepatocyte-specific IKKγ/ NEMO mice were generated by crossing the loxP site-flanked (floxed [f]) NEMO gene (NEMO^{f/f}) with Alfp-cre transgenic animals as described before⁹. These mice were further crossed either with

Fas^{lpr} knockout mice (purchased from The Jackson Laboratory, Bar Harbor, Maine, USA) to yield NEMO^{Δhepa}/Fas^{lpr}. Finally, we crossed NEMO^{Δhepa}/Fas^{lpr} with TNFR1^{-/-} mice to further generate NEMO^{Δhepa}/Fas^{lpr}/TNFR1^{-/-} and investigated the impact of TNFR1 in NEMO^{Δhepa}/Fas^{lpr} mice. To use the proper controls, NEMO^{Δhepa} mice were backcrossed from NEMO^{Δhepa}/Fas^{lpr}, and Fas^{lpr} and Fas^{lpr}/TNFR1^{-/-} were used as controls. Genotypes were confirmed via PCR specific for the respective alleles using DNA from tail biopsies. Progression of liver disease was investigated in male mice between 8–9 weeks and 52–54 weeks of age. Liver injury experiments were performed on mice between 8–9 weeks of age. Serum AST and ALT were measured by standard procedures in the Institute of Clinical Chemistry, University Hospital, RWTH Aachen.

TUNEL assay

The TUNEL test was performed by standard procedures.

Quantitative real-time PCR

Hepa Total RNA was purified from liver tissue using Trizol reagent (Invitrogen, Karlsruhe, Germany). Total RNA (1μg) was used to synthesize cDNA using SuperScript first-Strand Synthesis System (Invitrogen) and was resuspended in 100μl of H₂O. Quantitative real-time PCR was performed using SYBR Green Reagent (Invitrogen) in 7300 real-time PCR system (Applied Biosystem, Darmstadt, Germany). GAPDH expression was used to normalize gene expression, which is represented as times versus WT basal expression. Primer sequences can be provided upon request.

Histological, immunofluorescence, and immunohistochemical analysis

Livers from mice were harvested and, after fixation with 4% PFA, were embedded in paraffin for further histological evaluation. H&E and Sirius Red staining were performed on liver sections. For immunofluorescence analysis, liver cryosections of 5 μm were stained with Ki-67, CD11b (BD Biosciences, Heidelberg, Germany), and F4/80 (BioRad, Hercules, USA). Slides were fixed in 4%PFA at room temperature. A secondary antibody conjugated with Cy3 (Jackson ImmunoResearch, West Grove, PA) was used to obtain a red fluorescence signal. A mounting solution containing DAPI (Vector Laboratories, Burlingame, CA) was used to counterstain the nuclei of hepatocytes.

Flow cytometry analysis

Hepatocytes were stained with Annexin V-FITC (BD Biosciences). Immune cells from the whole liver were isolated and stained with fluorochrome-conjugated antibodies (CD4-PE, CD8-FITC, CD45-APC-Cy7, CD11b-PE, Ly6GFITC, and F4/80 Biotin) (BD Biosciences, Heidelberg, Germany). All samples were acquired by flow cytometry (FACS Canto II; BD Biosciences) and analyzed using the Flowjo software.

Immunoblot analysis

Isolated protein samples were probed with antibodies against RIPK1 (#5389) (Pro-Sci, Poway, CA, USA), Cleaved Caspase-3 (Asp175) (Cell Signaling Technology, Massachusetts, USA), PCNA (clone PC10) (Dianova GmbH, Hamburg, Germany), and GAPDH (MCA4739; 1:5000; AbD SeroTec, Düsseldorf, Germany). As secondary antibodies, anti-rabbit HRP (#7074; Cell Signaling) and anti-mouse HRP (#sc-2005; Santa Cruz) were used.

Statistical analysis

Data are expressed as the mean $\pm$ standard error of the mean (SEM). Statistical significance was determined using one-way analysis of variance (ANOVA) with Bonferroni post-hoc test.

Results

Generation and characterization of the NEMO ^{Δ hepa}/Fas^{lpr} mice

To address the functional relevance of FasL/Fas signaling for chronic disease progression in the NEMO ^{Δ hepa} model^{10,11}, we used mutant Fas mice, the lymphoproliferative (*lpr*) mice (Fas^{lpr})¹². Upon generation (**Figure S11.1a**), we observed that these animals develop lymphadenopathy by accumulating abnormal T cells and suffer from systemic lupus erythematosus-like autoimmune disease¹². As expected, Fas^{lpr} and NEMO ^{Δ hepa}/Fas^{lpr} displayed splenomegaly and the presence of lymph nodes in the peritoneum (**Figure S11.1b–d**).

The macroscopic appearance of NEMO ^{Δ hepa}/Fas^{lpr} livers was normal. Eight-week-old NEMO ^{Δ hepa} livers are histologically characterized by lack of lobular disorganization, hepatocellular hyperplasia, hypertrophy and severe diffuse hepatocellular anisokaryosis with a marked increase in the apoptotic and mitotic rate. In turn, no neoplasia was present in the hepatic parenchyma of 8-week-old NEMO ^{Δ hepa}/Fas^{lpr} livers markedly characterized by multifocal necrosis (**Figure 11.1a–d**). A significant decrease in liver injury markers at eight weeks of age was observed in serum ALT (**Figure 11.1e**) compared with NEMO ^{Δ hepa} mice. No differences were found in AP and GLDH compared with NEMO ^{Δ hepa} mice (**Figure S 11.2a, b**).

Figure 11.1

Figure 11.1 Generation and characterization of 8-week-old *NEMO^{Δhepa}/Fas^{lpr}* mice. (a) Macroscopic appearance of livers 8-week-old *NEMO^{fl/fl}*, *NEMO^{Δhepa}*, *Fas^{lpr}*, and *NEMO^{Δhepa}/Fas^{lpr}*. The liver (LW) (b) was calculated and represented. (c) Representative H&E staining of liver sections of 8-week-old animals. (d) The liver versus the body weight (LW/BW) ratio was calculated and represented. Arrows indicate infiltration and dotted area points to necrosis. (e) Serum ALT levels of 8-week-old *NEMO^{fl/fl}*,

NEMO^{Δhepa}, Fas^{lpr}, and NEMO^{Δhepa}/Fas^{lpr} mice were determined. Results are expressed as mean ± SEM (n=50 mice, $p<0.001$ – 0.01)

Impact of FasL/Fas disruption on cell death and compensatory proliferation in NEMO^{Δhepa} livers

The decrease in transaminase levels observed in mutant NEMO^{Δhepa}/Fas^{lpr} prompted us to investigate the impact of Fas deletion on cell death in NEMO^{Δhepa} mice. Significant differences were evident in TUNEL-positive cells between NEMO^{Δhepa} and NEMO^{Δhepa}/Fas^{lpr} livers (**Figure 11.2a, b**). Interestingly, the mRNA transcripts of Bcl2, a pro-survival protein, was significantly upregulated in NEMO^{Δhepa}/Fas^{lpr} compared with NEMO^{Δhepa} livers (**Figure S11.2c**). Since enhanced cell death triggers compensatory proliferation in NEMO^{Δhepa} livers⁸, we further tested whether disruption of FasL/Fas signaling would have an impact on cell proliferation in NEMO^{Δhepa}/Fas^{lpr} mice. By using cell cycle markers for total cell cycle activity (Ki-67), we found a clear trend towards reduced cell proliferation and significantly lower cell cycle activity (CcnD1) in NEMO^{Δhepa}/Fas^{lpr} livers compared with NEMO^{Δhepa} (**Figure 11.2c, d; Figure S11.2d**). Interestingly, most Ki-67-positive cells were hepatocytes in NEMO^{Δhepa} livers, whereas immune cells were proliferating in the hepatic parenchyma of NEMO^{Δhepa}/Fas^{lpr} animals (**Figure 11.2c, d**). Additionally, PCNA protein levels were augmented in NEMO^{Δhepa} compared with NEMO^{Δhepa}/Fas^{lpr} livers (**Figure 11.2e**). Moreover, cleavage of Caspase-3—but not protein levels of RIPK1—was absent in NEMO^{Δhepa}/Fas^{lpr} livers (**Figure 11.2e**). Altogether, these results suggest that Fas signaling might promote apoptotic cell death in NEMO^{Δhepa}-deficient livers.

Figure 11.2

Figure 11.2 Loss of the FasL/Fas signaling exacerbates cell death and compensatory proliferation in NEMO^{Δhepa} mice. (a) Representative TUNEL staining of 8-week-old NEMO^{fl/fl}, NEMO^{Δhepa}, Fas^{lpr}, and NEMO^{Δhepa}/Fas^{lpr} livers. (b) TUNEL-positive cells were quantified and graphed. (c) Proliferation was determined by Ki-67-positive cells immunofluorescence. (d) Ki-67-positive cells were quantified and graphed. (e) Expression of PCNA, cleaved Caspase-3 (CC3), and RIPK1 was analyzed by immunoblotting of whole liver extracts. GAPDH served as a loading control. Results are expressed as mean ± SEM (n=8 livers, *p*<0.001–0.05).

Loss of Fas attenuates fibrogenesis in NEMO^{Δhepa} livers

Fas might attenuate the effect of gut-derived products on liver injury. Therefore, we next aimed to investigate if this finding might also affect liver disease progression and evaluated its relevance for liver fibrogenesis in 8-week-old animals. Interestingly, the deletion of Fas attenuated liver fibrosis in NEMO^{Δhepa}. We first measured hepatic fiber formation by Sirius red staining and quantification (**Figure 11.3a, b**) and determined additional well-characterized pro-fibrotic markers, such as Collagen IA1, MMP2, and

TIMP1 mRNA expression (**Figure 11.3c, d**). The infiltration of distinct immune cell populations directed by chemotactic cytokines is a central pathogenic feature following chronic liver injury¹³. Thus, we characterized the hepatic inflammatory cell populations using qRT-PCR and FACS analysis. CD11b⁺ F4/80⁺ proinflammatory macrophages were significantly decreased in NEMO^{Δhepa}/Fas^{lpr} mice compared to NEMO^{Δhepa} livers (**Figure 11.3e**). Altogether, these findings suggest that Fas deletion—very likely by changing the sensitivity versus LPS—has an anti-inflammatory effect on NEMO^{Δhepa}-derived hepatitis.

To test whether decreased liver injury after Fas^{lpr} deletion in IKKγ/Nemo mice had an impact on proinflammatory cytokines, we performed qRT-PCR for TNF, a primary driver of NEMO^{Δhepa}-induced liver injury. Interestingly, TNF mRNA levels were significantly reduced in NEMO^{Δhepa}/Fas^{lpr} compared with NEMO^{Δhepa} livers (**Figure S11.3a**). However, no differences were found in TGFβ levels between mouse strains (**Figure S11.3b**). Therefore, we next sought to investigate immune cell infiltration in 8-week-old NEMO^{Δhepa}/Fas^{lpr} livers. In addition to FACS analysis, we observed significantly decreased CD11b and F4/80-positive cells in NEMO^{Δhepa}/Fas^{lpr} compared with NEMO^{Δhepa} hepatic tissue (**Figure S11.4a-e**). These results demonstrate that attenuated liver inflammation after Fas deletion in NEMO^{Δhepa} livers reduces fibrogenesis, pro-inflammatory macrophages, and expression of proinflammatory cytokines, *e.g.*, TNF.

Figure 11.3

Figure 11.3 Loss of Fas attenuates liver fibrogenesis in NEMO^{Δhepa} livers. (a) Sirius red staining of paraffin-embedded liver tissue derived from 8-week-old livers of NEMO^{fl/fl}, NEMO^{Δhepa}, Fas^{pr} and NEMO^{Δhepa}/Fas^{pr} mice. Representative photomicrographs taken under polarized light are shown. The SR-positive area was quantified using ImageJ© and represented. The mRNA relative expression of (b) Collagen-1A1, (c) Mmp2, and (d) Timp1 was quantified by RT-PCR. (e) The percentage of F4/80^{hi}CD11^{hi}-positive cells of each mouse strain was assessed using FACS analysis. Results are expressed as mean ± SEM (n=8, *p*<0.001–0.01)

Fas signaling is involved in hepatocarcinogenesis in NEMO^{Δhepa} mice

Fifty-two-week-old NEMO^{Δhepa} mice develop not only liver fibrosis but also hepatocarcinogenesis (HCC)⁸. Next, we tested the relevance of Fas signaling for liver carcinogenesis. Macroscopically, livers from NEMO^{Δhepa} mice revealed both regenerative nodules and well-defined, large vascularized tumors resulting in significantly higher liver weight (LW) and liver weight/body weight ratio (LW/BW) compared to NEMO^{Δhepa}/Fas^{pr} (Figure 11.4).

H&E stainings of 1-year-old NEMO^{Δhepa} mice showed well-differentiated trabecular and solid HCC with nodules of proliferative hepatocytes with disturbed liver architecture and hepatocellular lipid storage, whereas NEMO^{Δhepa}/Fas^{pr} showed no neoplasia but atypia

and hepatic hyperplasia accompanied by intense perivascular infiltration. Of note, lymphocyte accumulation was also found in Fas^{lpr} livers (Table 1).

	Nemo	Nemo/Fas ^{lpr}	Nemo/Fas ^{lpr} /TNFR1 ^{-/-}
Neoplasia	HCC* ¹	No* ²	No* ³
Anisokaryosis	4.17±0.98	4.38±0.51	2.88±1.25 ^{§#}
Altered foci	2.17±1.17	2.00±1.07	0.62±0.91 ^{##}
Mitosis/HPF (40x)	1.75±2.47	1.43±0.79	0.83±1.33
Cellular hypertrophy	2.5±1.22	2.25±0.46	1.13±0.99 ^{##}
Dysplasia	2.5±1.27	2.30±0.46	1.00±0.93 ^{##}
Oval cell proliferation	2.17±0.98	1.5±0.53	0.87±0.64 ^{§#}
Lymphoid aggregates/ Lymphocytic inflammation	0.50±0.00	1.00±0.00	0.86±0.89
Apoptosis	1.50±0.70	1.28±0.48	0.67±0.51
Additional lesions	Mild lipidosis; Diffuse vasculopathy	Multifocal necrosis	Biliary atresia; Early lymphomas

Table 1 Histopathological characteristics of the different mouse groups.

*¹Well-differentiated trabecular or solid HCCs; *²Early hepatocellular adenomas; *³Single well-differentiated trabecular and glandular HCC; Nemo vs. Nemo/Fas^{lpr}/TNFR1^{-/-} (§); Nemo/Fas^{lpr} vs. Nemo/Fas^{lpr}/TNFR1^{-/-} (§#)

Concomitantly, NEMO^{Δhepa}/Fas^{lpr} exhibited a decreased number of nodules and significantly lower LW/BW ratio compared with NEMO^{Δhepa} livers (**Figure 11.4a–d**). Additionally, a tendency towards reduced ALT was observed in 52-weeks-old NEMO^{Δhepa}/Fas^{lpr} compared with NEMO^{Δhepa} mice (**Figure 11.4e**). Together, these results indicate that loss of Fas protects against tumorigenesis in the NEMO^{Δhepa} experimental model of chronic liver injury.

Figure 11.4

Figure 11.4 Hepatocarcinogenesis is decreased in 1-year-old NEMO Δ hepa/Fas^{lpr} mice. NEMO^{+/+}, NEMO Δ hepa, Fas^{lpr}, and NEMO Δ hepa/Fas^{lpr} mice at the age of 12 months were sacrificed and livers extracted and analyzed. (a) Macroscopic appearance of livers. Arrows show visible nodules. (b) The number of nodules (≥ 5 mm) diameter was quantified. (c) Representative H&E staining of liver sections. (d) Liver weight versus body weight (LW/BW) ratio is shown. (e) Serum ALT was determined in the same mice. Results are expressed as mean $\pm$ SEM (n=50, * p <0.05; ** p <0.01).

TNFR1 deficiency reduces tumor load in NEMO Δ hepa/Fas^{lpr} livers

Since the deletion of TNFR1 is highly beneficial not only to NEMO Δ hepa but also to NEMO Δ hepa/p21^{-/-} knockout mice¹⁴, we generated NEMO Δ hepa/Fas^{lpr}/TNFR1^{-/-} triple knockout (TKO) mice and investigated the progression of chronic liver disease in these animals. Eight-week-old TKO mice displayed increased spleen size and a significantly larger number of peritoneal lymph nodes (Figure S11.5a–c). Macroscopically, 8-week-old NEMO Δ hepa/Fas^{lpr}/TNFR1^{-/-} livers revealed a reduced number of nodules, accompanied by significantly ameliorated ALT, AP, and GLDH compared with NEMO Δ hepa and NEMO Δ hepa/Fas^{lpr} mice (Figure S11.6a–d). Histopathological examination indicated that 1-year-old NEMO Δ hepa/Fas^{lpr}/TNFR1^{-/-} knockout mice presented no signs of neoplasia, early lymphomas, and reduced number of nodules (Table 1). However, no differences in

LW/BW ratio (**Figure 11.5a, b**) or important changes in serum AST levels albeit significantly decreased AP levels in 52-week-old NEMO^{Δhepa}/Fas^{lpr}/TNFR1^{-/-} compared with NEMO^{Δhepa}, and NEMO^{Δhepa}/Fas^{lpr} mice were observed (**Figure 5c, d**). Interestingly, NEMO^{Δhepa}/Fas^{lpr}/TNFR1^{-/-} knockout mice displayed similar inflammation as NEMO^{Δhepa}/Fas^{lpr} livers, as observed by Sirius red staining and quantification (**Figure 11.5e, Figure S11.7**). Altogether, these data show that TNF deficiency reduces tumorigenesis in NEMO^{Δhepa}/Fas^{lpr} livers.

Figure 11.5

Figure 11.5 Deletion of TNFR1 amplifies the protective effect of Fas signaling blockade in NEMO mice. *Fas^{lpr}/TNFR1^{-/-}* and *NEMO^{Δhepa}/Fas^{lpr}/TNFR1^{-/-}* mice at the age of 12 months were sacrificed and livers extracted and analyzed. **a** Macroscopic appearance of livers. Arrows show visible nodules. The number of nodules (≥ 5 mm) diameter was quantified. **b** Representative H&E staining of liver sections. Liver weight versus body weight (LW/BW) ratio is shown. Serum AST (**c**) and AP (**d**) were determined in the same mice and compared to 1-year-old *NEMO^{+/+}*, *NEMO^{Δhepa}*, *Fas^{lpr}*, and *NEMO^{Δhepa}/Fas^{lpr}*. **e** Sirius red staining of paraffin-embedded liver tissue was performed and microphotographs were taken. The SR-positive area was quantified using ImageJ® and represented, including 1-year-old *NEMO^{+/+}*, *NEMO^{Δhepa}*, *Fas^{lpr}*, and *NEMO^{Δhepa}/Fas^{lpr}*. Results are expressed as mean $\pm$ SEM ($n=25$, * $p<0.05$; ** $p<0.01$).

In order to better characterize the TNFR1-mediated effect in *NEMO^{Δhepa}/Fas^{lpr}*, the inflammatory profile of 52-week-old livers was exhaustively investigated. The number of CD11b-positive cells was statistically significantly higher in 52-week-old *NEMO^{Δhepa}* livers compared to WT animals (**Figure 11.6c**). Elevated F4/80 cells were also found in *NEMO^{Δhepa}* livers. In contrast, a trend towards reduction of CD11b and F4/80 cells in 52-week-old *NEMO^{Δhepa}/Fas^{lpr}* livers was observed (**Figure 11.6a–d**). However, no differences in cell infiltration were evident between *NEMO^{Δhepa}/Fas^{lpr}* and *NEMO^{Δhepa}/Fas^{lpr}/TNFR1^{-/-}* animals, suggesting that TNFR1 might be more involved in modulating tumorigenesis.

Figure 11.6

Figure 11.6 Impact of Fas and TNFR1 deletion on liver infiltrating inflammatory cells in *NEMO^{Δhepa}* mice. (a) Representative CD11b immunostaining of 52-week-old *NEMO^{f/f}*, *NEMO^{Δhepa}*, *Fas^{lpr}*, *NEMO^{Δhepa}/Fas^{lpr}*, *Fas^{lpr}/TNFR1^{-/-}*, and *NEMO^{Δhepa}/Fas^{lpr}/TNFR1^{-/-}* livers. (b) The positive Area Fraction for CD11b was quantified in ImageJ© and graphed. (c) Representative F4/80 immunostaining of 52-week-old *NEMO^{f/f}*, *NEMO^{Δhepa}*, *Fas^{lpr}*, *NEMO^{Δhepa}/Fas^{lpr}*, *Fas^{lpr}/TNFR1^{-/-}*, and *NEMO^{Δhepa}/Fas^{lpr}/TNFR1^{-/-}* livers. (d) The positive Area Fraction for F4/80 was quantified in ImageJ© and graphed.

Discussion

Apart from TNFR1 and TRAILR/D5, FasL/Fas is the third death receptor of the TNF receptor superfamily that can activate caspase-8-mediated apoptosis¹⁵. Previously, we first showed that loss of TRAILR does not improve experimental hepatitis as others have recently confirmed^{10,16}. Next, we observed that TNFR1 deletion is beneficial for the progression of chronic liver injury in *NEMO^{Δhepa}* mice^{10,14}. Moreover, we specifically showed that TNFR1 deletion in hepatocytes is protective, whereas TNFR1 inactivation in bone-marrow-

derived cells might be deleterious in this experimental model of chronic liver disease. In contrast, a recent study suggested no role for TNFR1 in hepatocytes in the same model, albeit showing a clear trend towards reduced transaminases and tumorigenesis¹⁶.

Since its first description in 1989, the FasR and FasL system has become the best-characterized extracellular system triggering apoptosis¹⁷. Accumulating evidence highlighted the importance of FasL/Fas expression in the pathogenesis of many gastrointestinal diseases, including Wilson's disease, cholestatic liver disease, alcoholic hepatitis, non-alcoholic steatohepatitis (NASH), and hepatocellular carcinoma (HCC)^{18,19}. In the present study, we hypothesized that Fas mediates TNF-induced cell death in NEMO-deficient hepatocytes, thus triggering the progression of chronic liver disease and end-stage HCC.

As previously described²⁰, Fas mice (Fas^{lpr}), mutant for Fas, develop splenomegaly, lymphadenopathy, and glomerulonephritis, including mononuclear cell infiltration, and display accumulation of CD4-CD8-T lymphocytes in peripheral organs such as the liver. These mice develop autoimmunity and are an excellent model of systemic lupus erythematosus. Interestingly, NEMO^{Δhepa}/Fas^{lpr} displayed larger spleens than Fas^{lpr} mice but no differences in the number of lymph nodes. On the other hand, Fas^{lpr}/TNFR1^{-/-} and NEMO^{Δhepa}/Fas^{lpr}/TNFR1^{-/-} animals exhibited a larger number of peritoneal nodes.

Defective Fas signaling in NEMO^{Δhepa} livers resulted in decreased serum transaminases and multifocal necrosis. In contrast, NEMO^{Δhepa} with normal Fas signaling displayed a high mitotic index, oval cell proliferation, mild lipidosis, and diffuse vasculopathy. FasL and Fas are expressed in NEMO-deficient livers, suggesting that they might be involved in mediating apoptosis in NEMO^{Δhepa} hepatocytes¹⁶. Thus, we first explored the role of Fas in the cell death of NEMO^{Δhepa} mice. Decreased TUNEL positive cells and absence of Caspase-3 activation were associated with lower compensatory proliferation in NEMO^{Δhepa}/Fas^{lpr} compared with NEMO^{Δhepa} livers. Moreover, Fas^{lpr} animals do not develop liver hyperplasia, but a small amount of Fas protein may still be produced by the *lpr* mutant Fas allele and mice engineered to completely lack Fas protein did exhibit liver hyperplasia²¹. Altogether, neither systemic nor specific ablation of Fas in hepatocytes completely prevented cell death but increased hepatocyte survival in NEMO^{Δhepa} animals.

Next, we measured TNF levels since this proinflammatory cytokine is responsible for cell death in NEMO^{Δhepa} liver⁸. Interestingly, TNF levels were significantly downregulated in NEMO^{Δhepa}/Fas^{lpr}. Moreover, NEMO^{Δhepa}/Fas^{lpr} livers exhibited decreased liver fibrosis and significantly reduced presence of CD11b+ F4/80+ cells. Since inflammation is a critical factor for the progression of liver injury, these observations suggest that an attenuated inflammatory response and not reduced Fas-induced apoptosis was the cause of the protective effect in NEMO^{Δhepa} mice²².

Previously, our group showed that NEMO^{Δhepa} mice are resistant to Jo2 stimulation, a model of Fas-induced damage⁹. However, direct binding of Jo2 to hepatocytes *in vivo* is

not entirely clear²³, and the effect of Jo2 is not restricted only to the liver; it could also affect other tissues of which cells often express a higher level of Fas than hepatocytes. Additionally, Ehliken *et al*¹⁶, using specific deletion of Fas in liver parenchymal cells, found that Fas is not required to develop chronic liver damage in NEMO^{Δhepa} mice.

In the present study, we employed NEMO^{Δhepa}/Fas^{lpr} mice, which carry a mutation in their Fas gene caused by insertion of the *Etn* retrotransposon into intron 2 of this gene²⁴. Hence, these result in the expression of a completely defective Fas antigen. Thus, our experiments overcome the underestimated effect of Fas-mediated signaling in other tissues that can directly affect the development of chronic liver disease. Concomitant to our results, Hatano *et al.*²⁵ also showed that NF-κB inhibition sensitizes hepatocytes to Fas-mediated apoptosis. Altogether, our study takes into account overall Fas signaling rather than hepatocyte-specific Fas-mediated apoptosis to explain the phenotype of NEMO^{Δhepa} mice.

The FasL/Fas system is a major mechanism of certain types of cancer cells for avoiding detection and destruction by the immune system through FasL expression⁷. Interestingly, NEMO^{Δhepa}/Fas^{lpr} displayed reduced tumorigenesis compared to NEMO^{Δhepa} mice. Moreover, in the chronic phase, reduced inflammation-driven carcinogenesis and lack of functional T cells play an essential role in reducing disease progression in NEMO^{Δhepa}/Fas^{lpr} livers, associated with reduced fibrosis and liver tumorigenesis.

Besides a direct cytotoxic effect on hepatocytes, the TNF/TNFR1 system might also be required for Fas-mediated cell death, as demonstrated by the increased resistance of TNFR1/2 double knockout mice to Fas-induced fulminant liver injury²⁶. Thus, we further assessed the role of TNFR signaling in Fas signaling. As previously described as beneficial in ethanol-mediated liver injury²⁷, the deletion of TNFR1 in NEMO^{Δhepa}/Fas^{lpr} animals reduced the tumor load of these mice, indicating that TNFR1 deficiency might modulate tumor load in HCC development.

Therefore, therapeutic approaches aiming to inhibit Fas-mediated liver injury might be relevant in inhibiting inflammation-driven hepatic disease progression, including growth of HCC.

References

1. Fecho, K. & Cohen, P. L. Fas ligand (gld)- and Fas (lpr)-deficient mice do not show alterations in the extravasation or apoptosis of inflammatory neutrophils. *J. Leukoc. Biol.* 64, 373–383 (1998).
2. Faubion, W. A. & Gores, G. J. Death receptors in liver biology and pathobiology. *Hepatology* 29, 1–4 (1999).
3. Ito, Y. *et al.* Fas antigen expression in hepatocellular carcinoma tissues. *Oncol. Rep.* 5, 41–44 (1998).
4. Abrams, S. I. Positive and negative consequences of Fas/Fas ligand interactions in the antitumor response. *Front. Biosci.* 10, 809–821 (2005).

5. Nicholes, K. et al. A mouse model of hepatocellular carcinoma: ectopic expression of fibroblast growth factor 19 in skeletal muscle of transgenic mice. *Am. J. Pathol.* 160, 2295–2307 (2002).
6. Mikhail S. & He A. R. Liver cancer stem cells. *Int. J. Hepatol.* (2011). <https://doi.org/10.4061/2011/486954>
7. Nagao, M. et al. The alteration of Fas receptor and ligand system in hepatocellular carcinomas: how do hepatoma cells escape from the host immune surveillance in vivo? *Hepatology* 30, 413–421 (1999).
8. Luedde, T. et al. Deletion of NEMO/IKKgamma in liver parenchymal cells causes steatohepatitis and hepatocellular carcinoma. *Cancer Cell* 11, 119–132 (2007).
9. Beraza, N. et al. Hepatocyte-specific NEMO deletion promotes NK/NKT cell- and TRAIL-dependent liver damage. *J. Exp. Med.* 206, 1727–1737 (2009).
10. Cubero, F. J. et al. TNFR1 determines progression of chronic liver injury in the IKKgamma/Nemo genetic model. *Cell Death Differ.* 20, 1580–1592 (2013).
11. Cubero, F. J. et al. Haematopoietic cell-derived Jnk1 is crucial for chronic inflammation and carcinogenesis in an experimental model of liver injury. *J. Hepatol.* 62, 140–149 (2015).
12. Adachi, M. et al. Enhanced and accelerated lymphoproliferation in Fas-null mice. *Proc. Natl Acad. Sci. USA* 93, 2131–2136 (1996).
13. Karlmark, K. R., Wasmuth, H. E., Trautwein, C. & Tacke, F. Chemokine-directed immune cell infiltration in acute and chronic liver disease. *Expert Rev. Gastroenterol. Hepatol.* 2, 233–242 (2008).
14. Ehedego, H. et al. p21 ablation in liver enhances DNA damage, cholestasis, and carcinogenesis. *Cancer Res* 75, 1144–1155 (2015).
15. Ashkenazi, A. & Dixit, V. M. Death receptors: signaling and modulation. *Science* 281, 1305–1308 (1998).
16. Ehlken, H. et al. Death receptor-independent FADD signalling triggers hepatitis and hepatocellular carcinoma in mice with liver parenchymal cell-specific NEMO knockout. *Cell Death Differ.* 21, 1721–1732 (2014).
17. Rudiger, H. A. & Clavien, P. A. Tumor necrosis factor alpha, but not Fas, mediates hepatocellular apoptosis in the murine ischemic liver. *Gastroenterology* 122, 202–210 (2002).
18. Hammam, O. et al. The role of fas/fas ligand system in the pathogenesis of liver cirrhosis and hepatocellular carcinoma. *Hepat. Mon.* 12, e6132 (2012).
19. Guicciardi, M. E. & Gores, G. J. Apoptosis: a mechanism of acute and chronic liver injury. *Gut* 54, 1024–1033 (2005).
20. Hutcheson, J. & Perlman, H. Loss of Bim results in abnormal accumulation of mature CD4-CD8-CD44-CD25- thymocytes. *Immunobiology* 212, 629–636 (2007).
21. Adachi, M. et al. Targeted mutation in the Fas gene causes hyperplasia in peripheral lymphoid organs and liver. *Nat. Genet.* 11, 294–300 (1995).
22. Gujral, J. S., Liu, J., Farhood, A. & Jaeschke, H. Reduced oncotic necrosis in Fas receptor-deficient C57BL/6J-lpr mice after bile duct ligation. *Hepatology* 40, 998–1007 (2004).
23. Jodo, S. et al. Anti-CD95-induced lethality requires radioresistant Fcgamma RII + cells. A novel mechanism for fulminant hepatic failure. *J. Biol. Chem.* 278, 7553–7557 (2003).
24. Li, X. K. et al. Fulminant hepatitis by Fas-ligand expression in MRL-lpr/lpr mice grafted with Fas-positive livers and wild-type mice with Fas-mutant livers. *Transplantation* 71, 503–508 (2001).
25. Hatano, E. et al. The mitochondrial permeability transition augments Fas-induced apoptosis in mouse hepatocytes. *J. Biol. Chem.* 275, 11814–11823 (2000).
26. Costelli, P. et al. Mice lacking TNFalpha receptors 1 and 2 are resistant to death and fulminant liver injury induced by agonistic anti-Fas antibody. *Cell Death Differ.* 10, 997–1004 (2003).
27. Nanji, A. A. & French, S. W. Animal models of alcoholic liver disease—focus on the intragastric feeding model. *Alcohol Res. Health* 27, 325–330 (2003).

Supplementary Figures

Figure S11.1

Figure S11.1 Generation and characterization of NEMO^{Δhepa}/Fas^{pr} mice. (a) PCR blot of IKKγ/NEMO and Fas shows WT and knockout bands in NEMO^{fl}, NEMO^{Δhepa}, Fas^{pr} and NEMO^{Δhepa}/Fas^{pr}. (b) Representative photograph of spleens and peritoneal lymph nodes of Fas^{pr} and NEMO^{Δhepa}/Fas^{pr} mice. (c) The spleen size and (d) the number of peritoneal lymph nodes of 8 week-old NEMO^{fl},

NEMO^{Δhepa}, Fas^{lpr} and NEMO^{Δhepa}/Fas^{lpr} is represented. Results are expressed as mean ± SEM (n=25, **p<0.01)

Figure S11.2

Figure S11.2 Markers of liver injury after Fas *lpr* deletion. (a) Serum AP and (b) GLDH levels of 8 week-old NEMO^{fl/fl}, NEMO^{Δhepa}, Fas^{lpr} and NEMO^{Δhepa}/Fas^{lpr} mice were determined. Results are expressed as mean ± SEM (n=25, **p<0.01).

Figure S11.3

Figure S11.3 Cell death, proliferation and inflammation markers after deficient FasL/FasR signaling. The mRNA relative expression of (a) *TNF* and (b) *TGFβ* was quantified by RT-PCR and graphed.

Figure S11.4

Figure S11.4 Infiltration of inflammatory cells into the liver. (a) FACS blot of proinflammatory macrophages CD11b/F4/80 is shown. (b) Representative CD11b staining of 8 week-old NEMO^{+/f}, NEMO^{Δhepa}, Fas^{lpr} and NEMO^{Δhepa}/Fas^{lpr} livers. (c) The positive Area Fraction was quantified in Image J[®] and graphed. (d) Representative F4/80 staining of the same livers. (e) The positive Area Fraction was quantified in Image J[®] and graphed.

Figure S11.5

Figure S11.5 Characterization of NEMO^{Δhepa}/Fas^{lpr}/TNFR1^{-/-} mice. (a) Representative photograph of spleens and peritoneal lymph nodes of 8 week-old Fas^{lpr}/TNFR1^{-/-} and NEMO^{Δhepa}/Fas^{lpr}/TNFR1^{-/-} mice. (b) The spleen size and (c) the number of peritoneal lymph nodes of 8 week-old Fas^{lpr}/TNFR1^{-/-} and NEMO^{Δhepa}/Fas^{lpr}/TNFR1^{-/-} compared with NEMO^{fl}, NEMO^{Δhepa}, Fas^{lpr} and NEMO^{Δhepa}/Fas^{lpr} is represented. Results are expressed as mean ± SEM (n=25, **p<0.01; ***p<0.001).

b

c

d

e

Figure S11.6

Figure S11.6 Deletion of TNFR1 further protects against the NEMO-dependent hepatitis. Fas^{lpr}/TNFR1^{-/-} and NEMO^{Δhepa}/Fas^{lpr}/TNFR1^{-/-} mice at the age of 8 weeks were sacrificed and livers extracted and analyzed. (a) Macroscopic appearance of livers. (b) Representative H&E staining of liver sections. (c) Serum ALT, (d) AP and (e) GLDH were determined in the same mice and compared to 8 week-old NEMO^{f/f}, NEMO^{Δhepa}, Fas^{lpr} and NEMO^{Δhepa}/Fas^{lpr} mice. Results are expressed as mean ± SEM (n=25, **p<0.05; p<0.001).

Figure S11.7

Figure S11.7 Liver fibrogenesis is further decreased in one-year-old NEMO^{Δhepa}/Fas^{LPR}/TNFR1^{-/-} livers. Sirius red staining of paraffin-embedded liver tissue was performed and microphotographs in polarized and normal light were taken in NEMO^{fl/fl}, NEMO^{Δhepa}, Fas^{LPR} and NEMO^{Δhepa}/Fas^{LPR}, Fas^{LPR}/TNFR1^{-/-} and NEMO^{Δhepa}/Fas^{LPR}/TNFR1^{-/-} mice at the age of 1 year.

PART III

Conclusion section

Chapter 12

General Discussion

Drug-induced liver injury is arising as a potential public health problem, which continues to be the most relevant reason for drug withdrawal and postmarketing decisions at present. Besides, adverse drug reactions represent a concerning burden for healthcare from the clinical as well as economic perspective due to its challenging clinical management, the severity of the reaction with more than 50% of cases requiring hospitalization or prolongation of hospital stays and the need for diagnostic resources, as it is a diagnosis of exclusion¹. Three main patterns of liver damage are the consequence of hepatic adverse drug reactions: hepatocellular, cholestatic, and mixed injury². Notwithstanding, the clinical and histological range of hepatotoxicity features is much more comprehensive. It may include hepatic entities such as steatosis, bile duct injury, vanishing bile duct syndrome, immune-allergic and autoimmune-like features, and, in chronic DILI cases, even fibrotic degeneration, cirrhosis, and neoplasms.

Large prospective hepatotoxicity databases have shown that among the most common culprit drug-classes include anti-infectives (*e.g.*, amoxicillin-clavulanic acid, ciprofloxacin), NSAIDs (*e.g.*, ibuprofen, diclofenac, nimesulide), and anti-tuberculosis agents (*e.g.*, isoniazid, isoniazid+rifampicin+pirazinamide)^{3, 4}. Within the relevant group of NSAIDs, several of these cohort studies match in pointing out diclofenac^{4, 5} as an important cause of NSAID DILI, but do offer discrepant data regarding the propionic acid derivative ibuprofen. The globally extended clinical use of ibuprofen and the lack of reliable information on ibuprofen-associated hepatotoxicity strengthens the urge to adequately assess this thematic challenge. A substantial effort was dedicated to investigating and better defining ibuprofen's role in DILI in the present work.

A systematic analysis was, therefore, performed with the aim to analyze the available patient-based information on ibuprofen-associated liver injury published in the literature (*Chapter 2*). Unexpectedly, our analysis resulted in a low number of reported cases, most of them of idiosyncratic nature ($N=22$ vs. three overdose cases). Given the extensive use of the drug, the limited amount of published hepatotoxicity reports may suggest that the absolute risk of ibuprofen hepatotoxicity is lower than vascular and gastrointestinal complications, which are well known adverse effects frequently associated with the use of ibuprofen⁶. Our analysis revealed that hepatocellular type of liver injury after a short latency period is characteristic for ibuprofen-induced liver injury, despite other presentations, including hypersensitivity features, cholestatic damage, and vanishing bile duct syndrome, can also occur. These phenotypic features elucidated for ibuprofen DILI are in line with those associated with other NSAID agents, which characterize for a short timespan between treatment and onset of the adverse reaction and cytolysis as the dominant type of liver injury⁷. Of note, several retrieved ibuprofen DILI cases exhibited a severe clinical progression, including the need for a liver transplant and *exitus*, which have been rarely associated with NSAID DILI besides exceptions such as, *i.e.*, nimesulide that indeed led to its withdrawal in several countries⁸. Although the appreciated number in

severe ibuprofen hepatotoxicity cases is concerning, the performed systematic analysis presents limitations that require more extensive complementary studies in the future.

Prospective DILI patient databases are becoming an essential tool for assessing and investigating the clinical presentation, risk factors, and outcomes associated with DILI. The promising results that we obtained in our systematic study prompted us to take our investigations on ibuprofen-induced liver injury to the next level to better characterize the phenotypic expression of ibuprofen-induced hepatotoxicity and evaluate a potential drug signature (*Chapter 3*). We carefully examined all the cases adjudicated to ibuprofen as a unique culprit compound enrolled at the Spanish DILI Registry and the Latin-American DILI Network (coordinated by the same authors and following the same procedures as the Spanish DILI database). As a result, a total number of $N=26$ idiosyncratic ibuprofen-induced liver injury events were identified, which constitutes to our best knowledge the largest prospective cohort on ibuprofen-related hepatotoxicity to date. Of note, the proportion of ibuprofen-induced liver injury cases observed in the Spanish and Latin-American DILI databases is higher than in other large DILI cohorts^{5,9}. Despite complex to assess, most likely, these geographical differences might be subjected to multiple factors, not only genetic and environmental but also variations in prescription habits, dispensed dose formulations, or pharmaceutical regulations. Ibuprofen DILI was characterized for appearing within four weeks from treatment initiation and presenting mainly as a hepatocellular type of liver damage. Similar to the results achieved in our previous systematic analysis, the present cohort did not reveal a characteristic drug signature but displayed a broad spectrum of phenotypes, reflecting the relevance of host and environmental factors in the DILI presentation. Importantly, our study demonstrated a concerning proportion of fatal/liver transplant patients, which was higher for DILI cases caused by ibuprofen than by other NSAIDs or non-NSAID agents (please note that nimesulide was withdrawn from the Spanish market back in 2002).

All in one, the obtained findings in *Chapters 2 and 3* do not only demonstrate that ibuprofen has been convincingly associated with hepatotoxicity but also that these reactions can occasionally progress to acute liver failure, requiring a liver transplant. Hence, clinicians should not overlook ibuprofen intake when assessing a suspicion of hepatotoxicity. In line with other forms of DILI, a careful follow-up and monitoring of patients suspected of having ibuprofen-induced liver injury are required until complete recovery is reached. The fact that idiosyncrasy is a common characteristic for most ibuprofen DILI cases may lead to the hypothesis that host-dependent properties might play a mechanistic role in ibuprofen-induced hepatotoxicity. Further studies are needed to make progress in better understanding the role of ibuprofen in DILI.

Methylprednisolone (MP) has been recently disputed as a potential cause of hepatotoxicity, whereas it is considered a safe agent for the liver, and it is even indicated in the therapeutic management of severe acute hepatic diseases. We set out to investigate hepatotoxicity cases, which have been adjudicated to methylprednisolone included in the Spanish and

Latin-American DILI databases (*Chapter 6*). As a result, four idiosyncratic cases were identified. In addition, 45 reports on MP-induced liver injury were retrieved from the literature and compared with our in-house cases. Of interest, all our in-house cases exhibited a previous underlying autoimmune disease breakout, which had motivated the prescription with *i.v.* MP boluses. Our analysis showed that MP-induced liver injury mainly occurred in patients with multiple sclerosis and Grave's ophthalmopathy, as earlier studies also identified¹⁰. In line with previous studies, our investigations confirmed that liver injury caused by MP commonly presents during the first week after treatment and as cytolytic liver injury¹¹. Of note, the severity of MP DILI ranges from asymptomatic or mild to severe and fatal^{12, 13}. Hence, these results suggest that the increasing tendency of MP-induced liver injury is a viable suspicion diagnosis that needs to be considered after high-dose bolus administration of MP in the scenario of an underlying autoimmune disease. In consequence, patients presenting an underlying autoimmune disease require a closer follow-up during and after high-dose MP *i.v.* therapy. The underlying mechanism triggering MP-induced hepatotoxicity remains uncertain¹⁴. Nevertheless, MP is a potent modulator of the immune response, and therefore it might be of interest to investigate in future studies a potential link between hepatotoxicity and the immune system in this particular clinical setting.

322 Drug-induced liver injury can be differentiated into acute or chronic based on its time to resolution, although the exact definition for long-term outcome following acute idiosyncratic DILI has not yet been established. Therefore, we desired to better define the adequate cut-off time point based on the idiosyncratic DILI (iDILI) cases enrolled at the Spanish DILI Registry (*Chapter 7*). Out of 298 prospective idiosyncratic DILI cases included in our database, the majority (92%) resolved within one year after DILI recognition, whilst 8% were chronic. Interestingly, the low chronicity prevalence identified in our prospective cohort compared with previous studies is most likely the consequence of the different chronicity definitions applied (6 months vs. 1 year)^{15, 16}. The previous consensus defined chronicity as liver injury with a progression of 3 and 6 months after withdrawal of the culprit drug in hepatocellular and cholestatic type of liver injury, respectively². Of note, in our current study, Kaplan-Meyer analysis resulted in 95% of the patients with recovery having resolved after 348 days independently from the hepatic injury pattern. Hence, our results indicate that one year is the most reliable cut-off point after onset to classify an iDILI episode as chronic. Additionally, our present investigations uncovered that the variables older age, dyslipidemia, and severe DILI contributed to chronicity risk factors. Of note, most of the chronic DILI cases exhibited mild liver profile abnormalities, whilst only a small fraction of patients suffered from relevant hepatic complications such as liver cirrhosis.

Drug-induced liver injury requires complex and laborious diagnostic procedures for its adequate identification and management. The DILI case reports described and selected due to their interesting learning point demonstrate the burdens of DILI diagnosis (*Chapter 8*). At present, DILI continues to be an exclusion diagnosis, which relies on a subjective

summation of clinical, biochemical, imaging as well as histological information (when available) of the patient¹⁷. A great effort is being made to find useful DILI biomarkers (predictive, diagnostic, prognostic, and mechanistic) that recognize the disease and result efficiently as time-saving, despite the existent obstacles of development and implementation. To provide an example, the serum biomarker miR-122, for instance, is a promising biomarker that could be useful in early-presenting DILI patients¹⁸. Notwithstanding, not uncommonly, cases of DILI remain unrecognized or are misdiagnosed¹⁹. Indeed, these cases are often uncovered after rechallenge when the patient is accidentally re-exposed to the culprit agent and develops a new hepatitis episode, as it has likewise occurred in one of the presented events (*Sub-Chapter 8.2*)²⁰.

In order to identify novel targets for diagnostic biomarkers and therapies against drug-induced liver injury, it is capital to improve the understanding of its underlying mechanisms, which is limited at current. Mitogen-activated protein kinases (MAPK) are well known to orchestrate a wide range of transduction signals implicated in a large number of cellular cascades and effector pathways that are subjected to many diverse ligands and stimuli²¹. These cascades are involved in many different cellular processes, such as cell proliferation, survival, death, and differentiation²². The aberrant function of MAPKs has been identified as a critical feature in the underlying pathomolecular bases of many diverse diseases, ranging from inflammatory processes to the development of malignancies²³.

The c-Jun N-terminal kinases (JNK) stand out as one of the MAPK members primarily phosphorylated due to environmental stresses and cytokines. JNK is encoded by three genes of which the isoform *Jnk3* is solely expressed in some organs and tissues (*e.g.*, testis, central nervous system), whereas the isoforms *Jnk1* and *Jnk2* are present ubiquitously throughout the organism, including the hepatic tissue²⁴. A wide range of diverse stimuli can trigger JNK activation, such as cytokines, reactive oxygen species, intra- and extracellular pathogens, environmental stress, toxins, or drugs.

An actual amount of scientific studies have demonstrated that activation of JNK is a feature of acute and chronic forms of liver disease. In the present work, we investigated the hepatocyte-derived role of JNK in human DILI and diverse murine models of liver toxicity with ibuprofen, acetaminophen, CCl₄, and LPS (*Chapters 4-5*). In the current work, we provide evidence that JNK activation at the hepatic level is a hallmark of human DILI patients due to ibuprofen (*Chapter 4*) as well as due to APAP and other culprit agents (*Chapter 5*). Our results obtained from human DILI-ALF liver specimens indicated that JNK phosphorylation occurs in different compartments, including liver parenchymal and non-liver parenchymal cells. Indeed, *Jaeschke and colleagues* previously proved that JNK activation is a consequence of APAP hepatotoxicity in primary human hepatocytes *in vitro*²⁵. In a novel experimental model of murine ibuprofen-induced acute liver injury (*Chapter 4*), mice had been challenged to doses of 600 mg/kg *i.p.* of ibuprofen for 8h, and subsequently, liver damage was observed. Furthermore, we were able to show marked increased levels of MAPK activation (*e.g.*, JNK, AKT, ERK), which were suggestive of playing a relevant role during ibuprofen-induced hepatotoxicity. JNK activation has been described as a

consequence of diverse noxious stimuli and, particularly, reactive oxidative species²⁶. *In vitro*, we were able to show that increasing concentrations of ibuprofen led to augmented cell death and triggered oxidative stress. Thus, it may be plausible to hypothesize that ibuprofen-induced hepatotoxicity-derived JNK activation is associated with the formation of reactive oxidative species, contributing to the activation of inflammatory and cell death signaling. In parallel, our experimental model of murine APAP-induced hepatotoxicity (Chapter 5) likewise displayed significantly enhanced levels pJnk 8h after *i.p.* administration of 500 mg/kg APAP. Of interest, expression levels of JNK correlated with the degree of liver injury. The JNK inhibitor SP600125 had been earlier described to prevent JNK phosphorylation effectively. Administration of SP600125 *in vivo* at a dosage of 10 mg/kg effectively blocked JNK activation in our experimental acute liver injury models with ibuprofen (Chapter 4) and acetaminophen (Chapter 5), and ameliorated the hepatic injury. All in one, these results confirm that JNK is activated in murine and human toxin-induced liver injury triggered by the drugs ibuprofen and APAP and exerts a key role in its underlying pathophysiology and clinical outcome.

In an approach to better characterize the impact of hepatocytic *Jnk1* and *Jnk2* in the setting of ibuprofen-induced hepatotoxicity, mice with genetic (*Jnk1*^{Δhepa}) or siRNA-specific (si*Jnk2*^{Δhepa}) deletion in hepatocytes were generated and later exposed to the above-mentioned *i.p.* dosage (Chapter 4). Critically, a prominent aggravation of ibuprofen-induced acute liver injury was observed in si*Jnk2*^{Δhepa} mice compared to *Jnk1*^{Δhepa} or Wt mice. Of interest, the exacerbated liver damage profile correlated with hepatic JNK hyperactivation in these mice. In previous studies on murine liver toxicity, *Jnk2* had been attributed both a higher susceptibility and protective effects, but many of these investigations relied on models with ubiquitous redundancy of *Jnk2* (*Jnk2*^{-/-})²⁷⁻³⁰. Hence, the role of *Jnk2* in acute hepatic disease remains controversial. Notwithstanding, the present data not only prove differential functionality patterns for *Jnk1* and *Jnk2* in hepatocytes and other LPCs but relevantly demonstrate an essential protective function of *Jnk2* in hepatocytes during ibuprofen-induced DILI. Our investigations led us to the promising result that hepatocyte-specific modulation of *Jnk2* could potentially serve as an interesting target for developing therapeutic strategies against acute ibuprofen-induced liver injury.

In previous lessons, ubiquitously combined deletion of *Jnk1* and *Jnk2* in mice had been reported as unviable³¹. Contrariwise, mice presenting concomitant deletion of *Jnk1* and *Jnk2* only at hepatocellular level (*Jnk*^{Δhepa}) are viable and were used to assess the compound impact of the hepatocytic binomial *Jnk1* and *Jnk2* to APAP-induced hepatotoxicity (Chapter 5). Of interest, *Jnk*^{Δhepa} animals exhibited a declined response towards APAP-induced liver injury, which showed a strong hepatic JNK phosphorylation and dramatic increase of the oxidative stress response, as also assessed *in vitro* on murine primary hepatocytes. Interestingly, SP600125 conferred protection not only to controls but also to animals with *Jnk* redundancy in hepatocytes *in vivo* and *in vitro*, which was most likely the consequence of off-target effects. Besides, *Jnk*^{Δhepa} triggered a more severe liver injury, inflammation, and progression after repetitive CCl₄ injection. No differences were found in the acute

hepatitis-D-GalN/LPS model between $Jnk^{\Delta\text{hepa}}$ and control animals. A previous study ratified these results in concanavalin A- and LPS-induced hepatitis but showed that hematopoietic redundancy in *Jnk1* and *Jnk2* prevented concanavalin A-induced liver injury via tumor necrosis factor suppression³². Altogether, these results suggest that under concrete conditions (*e.g.*, etiology of liver disease), JNK activation in infiltrating cells rather than in hepatocytes might be a capital mechanistic effector. These results underline that the context-specific activation of JNK in different cell types during acute liver injury might play a critical role in toxic liver disease and require to be further defined.

As extensively reviewed (*Chapter 9*), chronic liver disease (CLD) can progress to end-stage liver fibrosis and cirrhosis, which in turn acts as a risk factor for hepatocellular carcinoma (HCC). Hepatic fibrosis characterizes by hepatic stellate cell activation and increased deposition of extracellular matrix³³. At current, no antifibrotic compounds have been yet approved for clinical use, but several drugs are currently undergoing advanced phases of clinical trials. The main mechanisms leading to the reversal of liver fibrosis are deactivation and elimination of hepatic stellate cells (*e.g.*, Elafibranor, AT1-receptor blockers), switch in the inflammatory environment (*e.g.*, Cenicriviroc, Obeticholic acid), and the degradation of extracellular matrix (*e.g.*, anti-TIMP1, Serelaxin)³⁴. A better understanding of all involved actors in the underlying physiopathology of CLD is needed to conceive more effective therapeutic strategies to be developed in the future.

Previously, we showed the relevance and differential role of the *Jnk* genes during acute and chronic liver disease (*Chapters 4-5*) as well as in earlier investigations³⁵. Whereas *Jnk1* plays a pro-apoptotic and pro-tumorigenic function, *Jnk2* seems to acquire a more relevant role during fibrogenesis. We, therefore, aimed at uncovering the impact of hepatocytic *Jnk2* modulation in the setting of CLD (*Chapter 10*). As shown, genetic double-knockout mice for $Jnk2^{\Delta\text{hepa}}$ and $\text{NEMO}^{\Delta\text{hepa}}$ concurred with our previous findings that *Jnk2* redundancy in $\text{NEMO}^{\Delta\text{hepa}}$ mice led to an improved outcome in HCC development by displaying a significantly reduced number of tumor foci³⁵. These results suggest that the hepatocytic deletion of JNK2 improves tumor progression and impairs tumor initiation. Next, we designed a therapeutic approach to modulate JNK2 expression in hepatocytes via hepatocyte-specific siRNA that inhibited JNK2 expression in early and advanced stages of CLD. Hepatocytic siRNA-mediated *Jnk2* inhibition in older mice blocked fibrogenesis and HCC progression and led overall to a significant improvement of late-stage CLD. The reduced fibrogenic response directly translated into decreased tumor initiation, suggesting that the carcinogenic environment after si*Jnk2* treatment improved in $\text{NEMO}^{\Delta\text{hepa}}$ livers. Relevantly, si*Jnk2* prevented HCC progression, which is a feature of 44-week-old $\text{NEMO}^{\Delta\text{hepa}}$. In the early-stage of CLD, *Jnk2* downregulation in $\text{NEMO}^{\Delta\text{hepa}}$ mice led to a pro-inflammatory environment aggravating the phenotype of $\text{NEMO}^{\Delta\text{hepa}}$. The immune cell infiltration was mainly at the expense of pro-inflammatory monocytes, which have been shown to increase independently of the type of liver injury³⁶. These results are in line with our prior observations where siRNA-mediated hepatocytic JNK2 inhibition led to a declined response

of ibuprofen-induced acute liver injury³⁷. The obtained results define a time-dependent role of hepatocytic *Jnk2* during the development of experimental HCC and make *Jnk2* a potential target in hepatocytes to impair cancer initiation in chronically damaged livers. In addition, the similar phenotypes appreciated in both the knockout and the knockdown mice emphasize the efficacy of siRNA technology as a cell-type specific approach.

The FasL/Fas axis is a crucial regulator of the immune response, and it has been characterized as an extracellular apoptosis-triggering system by transmitting an apoptotic signal to Fas-expressing cells. Therefore, we questioned the potential role of FasL/Fas in driving TNF-mediated cell death in the progression of CLD and end-stage HCC of NEMO^{Δhepa} mice (Chapter 11). Indeed, earlier results demonstrated that FasL and Fas are expressed in livers of NEMO^{Δhepa} mice³⁸. NEMO^{Δhepa}/Fas^{lpr} mice were used for the present investigations, which carry a mutation in their Fas gene caused by insertion of the Etn retrotransposon into intron 2 of this gene³⁹. Our results showed that defective Fas signaling (NEMO^{Δhepa}/Fas^{lpr}) displayed reduced serum liver injury markers and multifocal hepatic necrosis compared with NEMO^{Δhepa} livers, which exhibited high mitotic index, oval cell proliferation, and mild lipodosis. Reduced compensatory cell proliferation in NEMO^{Δhepa}/Fas^{lpr} livers correlated with decreased cell death levels and the absence of Caspase-3 activation in contrast to NEMO^{Δhepa}. TNF levels were notably downregulated in NEMO^{Δhepa}/Fas^{lpr} animals and decreased liver fibrosis and the significantly reduced presence of CD11b+ F4/80+ cells was characteristic for NEMO^{Δhepa}/Fas^{lpr} livers. Consequently, an attenuated inflammatory response rather than reduced Fas-induced apoptosis could explain the protective effect appreciated in NEMO^{Δhepa} mice⁴⁰. Reduced tumorigenesis was an additional feature of NEMO^{Δhepa}/Fas^{lpr} compared to NEMO^{Δhepa}. Moreover, in the chronic phase, reduced inflammation-driven carcinogenesis and lack of functional T cells play an essential role in reducing disease progression in NEMO^{Δhepa}/Fas^{lpr} livers, associated with reduced fibrosis and liver tumorigenesis.

In summary, the functionality of the FasL/Fas system might affect inflammation-driven tumorigenesis in an experimental model of chronic liver disease. Thus, these results suggest that alternative therapeutic approaches blocking Fas-mediated hepatic damage might inhibit inflammation-driven hepatic disease progression enclosing hepatic tumorigenesis.

In conclusion, the present work followed the aim to better understand and assess the impact and role of the c-Jun N-terminal kinases in diverse experimental settings of acute and chronic liver injury. Furthermore, we assessed the relevance of the FasR and FasL axis as an immunomodulatory strategy against experimental HCC. In addition, we analyzed phenotypically diverse types of drug-induced liver injury caused by agents such as ibuprofen or methylprednisolone, among others, based on prospective patient data. Lastly, we better defined the optimal cut-off point for chronicity following acute idiosyncratic drug-induced liver injury.

References

1. Sultana J, Cutroneo P, Trifiro G. Clinical and economic burden of adverse drug reactions. *J Pharmacol Pharmacother* 2013;4:S73-7.
2. Aithal GP, Watkins PB, Andrade RJ, et al. Case definition and phenotype standardization in drug-induced liver injury. *Clin Pharmacol Ther* 2011;89:806-15.
3. Andrade RJ, Lucena MI, Fernandez MC, et al. Drug-induced liver injury: an analysis of 461 incidences submitted to the Spanish registry over a 10-year period. *Gastroenterology* 2005;129:512-21.
4. Chalasani N, Bonkovsky HL, Fontana R, et al. Features and Outcomes of 899 Patients With Drug-Induced Liver Injury: The DILIN Prospective Study. *Gastroenterology* 2015;148:1340-52 e7.
5. Bjornsson ES, Bergmann OM, Bjornsson HK, et al. Incidence, presentation, and outcomes in patients with drug-induced liver injury in the general population of Iceland. *Gastroenterology* 2013;144:1419-25, 1425 e1-3; quiz e19-20.
6. Coxib, traditional NTC, Bhala N, et al. Vascular and upper gastrointestinal effects of non-steroidal anti-inflammatory drugs: meta-analyses of individual participant data from randomised trials. *Lancet* 2013;382:769-79.
7. Agundez JA, Lucena MI, Martinez C, et al. Assessment of nonsteroidal anti-inflammatory drug-induced hepatotoxicity. *Expert Opin Drug Metab Toxicol* 2011;7:817-28.
8. Andrade RJ, Lucena MI, Fernandez MC, et al. Fatal hepatitis associated with nimesulide. *J Hepatol* 2000;32:174.
9. Schmeltzer PA, Kosinski AS, Kleiner DE, et al. Liver injury from nonsteroidal anti-inflammatory drugs in the United States. *Liver Int* 2016;36:603-9.
10. de Seze J, Canva-Delcambre V, Fajardy I, et al. Autoimmune hepatitis and multiple sclerosis: a coincidental association? *Mult Scler* 2005;11:691-3.
11. Davidov Y, Har-Noy O, Pappo O, et al. Methylprednisolone-induced liver injury: Case report and literature review. *J Dig Dis* 2016;17:55-62.
12. Marino M, Morabito E, Brunetto MR, et al. Acute and severe liver damage associated with intravenous glucocorticoid pulse therapy in patients with Graves' ophthalmopathy. *Thyroid* 2004;14:403-6.
13. Weissel M, Hauff W. Fatal liver failure after high-dose glucocorticoid pulse therapy in a patient with severe thyroid eye disease. *Thyroid* 2000;10:521.
14. Gutkowski K, Chwist A, Hartleb M. Liver injury induced by high-dose methylprednisolone therapy: a case report and brief review of the literature. *Hepat Mon* 2011;11:656-61.
15. Aithal PG, Day CP. The natural history of histologically proved drug induced liver disease. *Gut* 1999;44:731-5.
16. Fontana RJ, Hayashi PH, Barnhart H, et al. Persistent liver biochemistry abnormalities are more common in older patients and those with cholestatic drug induced liver injury. *Am J Gastroenterol* 2015;110:1450-9.
17. Tran T, Lee WM. DILI: New Insights into Diagnosis and Management. *Curr Hepat Rep* 2013;12:53-58.
18. McGill MR, Jaeschke H. Biomarkers of drug-induced liver injury: progress and utility in research, medicine, and regulation. *Expert Rev Mol Diagn* 2018;18:797-807.
19. Lu RJ, Zhang Y, Tang FL, et al. Clinical characteristics of drug-induced liver injury and related risk factors. *Exp Ther Med* 2016;12:2606-2616.
20. Andrade RJ, Robles M, Lucena MI. Rechallenge in drug-induced liver injury: the attractive hazard. *Expert Opin Drug Saf* 2009;8:709-14.
21. Lawrence MC, Jivan A, Shao C, et al. The roles of MAPKs in disease. *Cell Res* 2008;18:436-42.
22. Huang P, Han J, Hui L. MAPK signaling in inflammation-associated cancer development. *Protein Cell* 2010;1:218-26.
23. Kim EK, Choi EJ. Pathological roles of MAPK signaling pathways in human diseases. *Biochim Biophys Acta* 2010;1802:396-405.
24. Davis RJ. Signal transduction by the JNK group of MAP kinases. *Cell* 2000;103:239-52.
25. Xie Y, McGill MR, Dorko K, et al. Mechanisms of acetaminophen-induced cell death in primary human hepatocytes. *Toxicol Appl Pharmacol* 2014;279:266-274.
26. Shen HM, Liu ZG. JNK signaling pathway is a key modulator in cell death mediated by reactive oxygen and nitrogen species. *Free Radic Biol Med* 2006;40:928-39.

27. Bourdi M, Korrapati MC, Chakraborty M, et al. Protective role of c-Jun N-terminal kinase 2 in acetaminophen-induced liver injury. *Biochem Biophys Res Commun* 2008;374:6-10.
28. Cederbaum AI, Yang L, Wang X, et al. CYP2E1 Sensitizes the Liver to LPS- and TNF alpha-Induced Toxicity via Elevated Oxidative and Nitrosative Stress and Activation of ASK-1 and JNK Mitogen-Activated Kinases. *Int J Hepatol* 2012;2012:582790.
29. Gunawan BK, Liu ZX, Han D, et al. c-Jun N-terminal kinase plays a major role in murine acetaminophen hepatotoxicity. *Gastroenterology* 2006;131:165-78.
30. Wang Y, Singh R, Lefkowitz JH, et al. Tumor necrosis factor-induced toxic liver injury results from JNK2-dependent activation of caspase-8 and the mitochondrial death pathway. *J Biol Chem* 2006;281:15258-67.
31. Kuan CY, Yang DD, Samanta Roy DR, et al. The Jnk1 and Jnk2 protein kinases are required for regional specific apoptosis during early brain development. *Neuron* 1999;22:667-76.
32. Das M, Garlick DS, Greiner DL, et al. The role of JNK in the development of hepatocellular carcinoma. *Genes Dev* 2011;25:634-45.
33. Wells RG. Cellular sources of extracellular matrix in hepatic fibrosis. *Clin Liver Dis* 2008;12:759-68, viii.
34. Brenner DA. Reversibility of liver fibrosis. *Gastroenterol Hepatol (N Y)* 2013;9:737-9.
35. Cubero FJ, Zhao G, Nevzorova YA, et al. Haematopoietic cell-derived Jnk1 is crucial for chronic inflammation and carcinogenesis in an experimental model of liver injury. *J Hepatol* 2015;62:140-9.
36. Karlmark KR, Weiskirchen R, Zimmermann HW, et al. Hepatic recruitment of the inflammatory Gr1+ monocyte subset upon liver injury promotes hepatic fibrosis. *Hepatology* 2009;50:261-74.
37. Zoubek ME, Woitok MM, Sydor S, et al. Protective role of c-Jun N-terminal kinase-2 (JNK2) in ibuprofen-induced acute liver injury. *J Pathol* 2019;247:110-122.
38. Ehlken H, Krishna-Subramanian S, Ochoa-Callejero L, et al. Death receptor-independent FADD signalling triggers hepatitis and hepatocellular carcinoma in mice with liver parenchymal cell-specific NEMO knockout. *Cell Death Differ* 2014;21:1721-32.
39. Li XK, Fujino M, Sugioka A, et al. Fulminant hepatitis by Fas-ligand expression in MRL-lpr/lpr mice grafted with Fas-positive livers and wild-type mice with Fas-mutant livers. *Transplantation* 2001;71:503-8.
40. Gujral JS, Liu J, Farhood A, et al. Reduced oncotic necrosis in Fas receptor-deficient C57BL/6J-lpr mice after bile duct ligation. *Hepatology* 2004;40:998-1007.

Chapter 13

Summaries (EN, NL, ES, DE)

Summary

In *Chapter 1*, an updated literature review on the topic of drug-induced liver injury has been carried out. The most relevant findings and contributions to the field in recent years have been highlighted in detail. Apart from diverse basic definitions and essential classifications, a particular focus on the underlying pathophysiology, mechanisms, clinical features, differential diagnosis, causality assessment, and risk factors in DILI have been considered. As paraphrase, a summarized overview of the topic “hepatocellular carcinoma” as well as an introduction to the experimental chronic liver disease model “NEMO” have been included. Additionally, the main objectives and outline for the current investigations have been briefly described.

In *Chapter 2*¹ a systematic analysis was performed in order to examine the information on ibuprofen-induced liver injury available in the literature. Our investigation covered prospective hepatotoxicity cohorts, case series, and case reports, and the data was thoroughly examined in terms of demographics, clinical presentation, biochemical parameters, and outcome. Of note, ibuprofen stood out as the most frequent NSAID in prospective DILI databases from Spain and India. Twelve out of 22 identified idiosyncratic ibuprofen hepatotoxicity cases had female gender, and the mean age was 31 years. The average cumulative dose of ibuprofen was 30g, whereas treatment duration and time to onset was 14 and 12 days, respectively. Characteristically, hepatic cytolytic features predominated and a total number of six cases developed vanishing bile duct syndrome. Full recovery occurred after a mean time of 14 weeks, whilst 5 patients developed acute liver failure that led to death or the need for a liver transplant.

Despite rare, given its extensive use, ibuprofen has been convincingly associated with hepatotoxicity across literature and prospective cohorts of DILI cases and, thus, it needs to be considered as a potential culprit agent during causality assessment. Ibuprofen DILI presents commonly with short latency, hepatocellular damage and can potentially progress to death or the need for liver transplantation.

In *Chapter 3*² the prevalence and features of ibuprofen-induced liver injury cases enrolled in the Spanish DILI Registry as well as in the Latin-American DILI Network were thoroughly analyzed and characterized. Only cases where ibuprofen was the unique culprit drug were included in the study. To investigate a potential signature of ibuprofen hepatotoxicity, ibuprofen DILI events were compared with DILI due to other nonsteroidal and other non-NSAID drugs. An ibuprofen hepatotoxicity cohort of 26 total events was obtained; half of the individuals were female with a mean age of 51 years. Most patients were treated with 1200 mg ibuprofen per day or higher (77%), median treatment duration was 16 days and the median time to DILI onset 15 days. Moreover, 58% of the patients required hospitalization, 69% presented with jaundice and 50% had hypersensitivity features. Hepatocellular injury was the most frequent hepatic injury pattern (69%). Average BMI was higher in ibuprofen DILI subjects than in DILI due to other NSAIDs or Non-NSAIDs ($p=0.06$). The median time to onset lasted shorter for ibuprofen hepatotoxicity (15 days). Diabetes mellitus was significantly more prevalent in ibuprofen DILI patients ($p=0.038$). Fatal

outcome was higher for ibuprofen hepatotoxicity (12%) versus other NSAIDs (5%) and other non-NSAID agents (3%).

Thus, a higher prevalence of ibuprofen-induced liver injury events was found in our database compared to other large published DILI cohorts. Critically, ibuprofen DILI was associated with a higher fatal outcome rate. Hence, patients diagnosed with ibuprofen DILI should undergo more exhaustive monitoring during follow-up. Our data reflected that idiosyncratic mechanisms of immunoallergic and metabolic nature seem to play from a mechanistic point of view a relevant role in ibuprofen DILI, despite more extensive studies are required to better understand the role of ibuprofen in DILI.

Our previous investigations prompted us to investigate the mechanisms associated with ibuprofen-induced liver injury. In *Chapter 4*³ a novel ibuprofen hepatotoxicity model *in vitro* and *in vivo* was developed in order to analyze the underlying pathomolecular bases underlying ibuprofen-induced acute liver injury (ALI). For this purpose, several cytotoxicity studies for ibuprofen were performed *in vitro* on Hepa 1-6 and HepaRG cell lines, which were then complemented primary murine hepatocytes, which were freshly isolated from 8-week-old Wt mice. Subsequently, our studies were completed with a murine model of ibuprofen hepatotoxicity *in vivo*. Overnight fasted male C57BL/6 mice (6-8 weeks of age) were *i.p.* injected with 600 mg/kg of ibuprofen and sacrificed 8h later. To assess the role of JNK, we used animals carrying a constitutive deletion of Jnk1 (Jnk1^{-/-}) or Jnk2 (Jnk2^{-/-}). Next, we expanded our assessment to animals, which presented a lack of Jnk1 uniquely in hepatocytes (Jnk1^{Δhepa}). To generate animals with a redundant expression of Jnk2 at hepatocellular level, 0.2 mg/kg of siRNA of Jnk2 (siJnk2^{Δhepa}) were tail vein-injected one week prior to ibuprofen challenge. As a translational approach, the role of JNK was investigated in human liver specimens who had suffered from ibuprofen-induced liver injury. Enhanced JNK phosphorylation was evident in the cytoplasm of hepatocytes in ibuprofen-induced ALI liver samples compared with healthy tissue at the human and murine level. Ibuprofen challenge produced a greater degree of liver injury than that observed in vehicle-treated control mice, based on levels of serum markers and histopathology analysis of the respective liver tissue samples.

Of note, siJnk2^{Δhepa} animals exhibited a remarkable decline in response to liver injury after ibuprofen hepatotoxicity, which correlated with significantly higher serum liver enzymes and worsened liver histology features compared to Jnk1^{Δhepa} or Wt animals. The molecular pathways associated with ibuprofen-induced liver injury in mice were examined, and we found increased activation of PKCα, AKT and JNK, eight hours after Ibuprofen challenge in mice. The results of this study showed cytoplasmic JNK activation in hepatocytes as a hallmark of Ibuprofen-related hepatotoxicity in human and murine samples. Hepatocellular deficiency of Jnk2 was associated with a worsened response to ibuprofen-induced acute liver injury.

The JNK pathway has proven to play a crucial role in the pathophysiology of acute and chronic liver disease. For this purpose, in *Chapter 5*⁴ the presence of phosphorylated JNK was examined in liver tissue from drug-induced liver injury patients, and the role of hepatocytic JNK was investigated in experimental models of murine acute and chronic liver disease. Liver sections from patients with DILI induced by diverse culprit agents were

studied and the JNK expression profile was analyzed. In parallel, mice with hepatocyte-specific deletion of Jnk1 (Jnk1^{Δhepa}) or combined Jnk1 and Jnk2 (Jnk^{Δhepa}) deletion, as well as Jnk1-floxed C57BL/6 (control) mice, were given injections of CCl₄ to induce fibrosis (0.6ml/kg *i.p.* every 3 days during 4 weeks) or were challenged with acetaminophen to induce acute toxic hepatitis (500mg/kg *i.p.*). Liver tissue samples from DILI patients displayed more activated JNK, predominantly in nuclei of hepatocytes and in immune cells than healthy tissue. The injection of acetaminophen to Jnk^{Δhepa} mice induced a greater level of liver injury than that observed in Jnk1^{Δhepa} or control mice, based on levels of serum markers and microscopic and histologic analysis of liver tissues. Administration of CCl₄ also induced more substantial hepatic injury in Jnk^{Δhepa} mice, based on increased inflammation, cell proliferation, and fibrosis progression, compared with Jnk1^{Δhepa} or control mice. Hepatocytes from Jnk^{Δhepa} mice challenged with acetaminophen had an increased oxidative stress response, leading to decreased activation of adenosine monophosphate-activated protein kinase, total protein adenosine monophosphate-activated protein kinase levels, and pJunD and subsequent necrosis. Administration of SP600125 (30mg/kg *i.p.*) before or with acetaminophen protected Jnk^{Δhepa} and control mice from liver injury. These findings show that JNK1 and JNK2 in hepatocytes appear to have combined effects in protecting mice from CCl₄- and acetaminophen-induced liver injury. Additionally, the JNK inhibitor SP600125 shows off-target effects.

332 Methylprednisolone is commonly used in the treatment of acute liver injury with severe features, among many other indications. The fact that recently several case reports of MP-induced liver injury have been published, in particular in patients suffering from underlying autoimmune diseases, encouraged us to investigate this circumstance. In *Chapter 6*⁵ we aimed to contribute to characterize the phenotypic expression of MP-induced liver injury and investigated all available cases enrolled in the Spanish and Latin-American DILI databases. Demographical, clinical, laboratory, and outcome data were analyzed. An extensive search for earlier published MP-induced liver injury events was provided. Furthermore, a comparative analysis of novel MP-DILI events identified in the above-mentioned databases and earlier obtained cases from the literature was performed. Three young females with multiple sclerosis and a further one with Crohns' disease suffered an acute recrudescence and were treated subsequently with intravenous MP pulses. After one (patient 3), two (patient 1), and five (patient 2) and six (patient 4) weeks of initiating medication, liver injury occurred. Time to recovery took eight and six weeks for patients 2 and 3, respectively, while patient 1 showed a resolving tendency after 10 weeks (lost of follow up) and patient 4 after 2 weeks. Positive rechallenge occurred in three patients (patients 2, 3, and 4). An extensive literature analysis resulted in a total of 45 MP-induced liver injury events. These were associated with the female gender (84%) and the mean age was 41 years. The most common indications for treatment were multiple sclerosis (24 cases) and Graves' ophthalmopathy (13 patients). Hepatocellular damage was observed as the predominant injury pattern in all the cases with available data and positive autoantibodies were detected in 35% of the subjects. The average time to onset was six weeks. Four cases developed a fatal outcome. In 17 cases (38%), a positive rechallenge with the drug followed. These findings indicate that patients suffering from MS and GO are particularly susceptible to develop hepatotoxicity due to MP use.

The included study in *Chapter 7*⁶ aimed to analyze time to liver enzyme resolutions to establish the best definition and risk factors of DILI chronicity. Up to 298 individuals out of 850 patients in the Spanish DILI Registry with no preexisting disease affecting the liver and follow-up to resolution ≥ 1 year were analyzed. Chronicity was defined as abnormal liver biochemistry, imaging test or histology one year after DILI recognition.

Out of 298 patients enrolled, 273 (92%) resolved ≤ 1 year from DILI recognition and 25 patients (8%) were chronic. Independent risk factors for chronicity were older age (OR: 1.06, $p=0.011$), dyslipidemia (OR: 4.26, $p=0.04$) and severe DILI (OR: 14.22, $p=0.005$). Alanine aminotransferase (ALT), alkaline phosphatase (ALP) and total bilirubin (TB) median values were higher in the chronic group during follow-up. Values of ALP and TB $>1.1 \times$ upper limit of normal (xULN) and $2.8 \times$ ULN, respectively, in the second month from DILI onset, were found to predict chronic DILI ($p<0.001$). The main drug classes involved in chronicity were statins (24%) and antiinfectives (24%). Histological examination in chronic patients demonstrated two cases with ductal lesion and seven with cirrhosis. Hence, the achieved results suggest that one year is the best cut-off point to define chronic DILI or prolonged recovery, with risk factors being older age, dyslipidemia, and severity of the acute episode. Statins are distinctly related to chronicity. ALP and TB values in the second month could help predict chronicity or very prolonged recovery.

In *Chapter 8* we displayed three brief case reports on drug-induced liver injury of diverse etiology from our daily clinical practice, which presented an exciting learning point. In the first report, the case of a young woman who suffered from acute liver failure and required partial liver transplantation is described. The cause for the severe hepatic episode was unclear. When we investigated her precedent medical history, recent treatment with ibuprofen and occasionally acetaminophen as well as positive serology for HSV, fell into the spotlight. Nevertheless, the cause could not be clearly adjudicated. Our second case report dealt with a woman whose liver dysfunction was uncovered after suffering from rechallenge with the antidepressant sertraline. Prior to that, she had presented extramedullar manifestations of an underlying *Morbus* Walderström that had initially acted as a confounding factor and consequently led to a new inadvertent exposure with sertraline. Case report 3 referred to a patient who presented with advanced chronic liver disease, including advanced liver cirrhosis and reiterated refractory hydropic decompensations. Additionally, the patient exhibited dilated cardiomyopathy, but no plausible cause could be found. Detailed investigations of her past medical history revealed that the patient had been in chronic treatment with venlafaxine during the four previous years and was most likely the cause of her clinical condition. Interestingly, we performed a genetic test for cytochrome P450 and found that the patient presented polymorphic variations for CYP2D6, which were compatible with being an ultra-rapid metabolizer of venlafaxine.

In *Chapter 9*⁷ a review on the reversal of liver fibrosis has been carried out. The most representative animal models that have been used to expand our knowledge on liver fibrosis have been displayed; however, these are subjected to diverse limitations that need to be re-defined in the future. The extensive investigations achieved in the field during the

last decades have shown that the condition of a fibrotic liver can be reversible by modifying the underlying inflammatory environment, elimination or regression of activated HSCs as well as degradation of ECM.

Currently, there is a vast potential therapeutic arsenal aiming at reversing liver fibrosis that is undergoing clinical trial studies, as shown, with particular focus on patients suffering from non-alcoholic steatohepatitis. Thus, now more than ever, the promising progress in the reversal of liver fibrosis seems to be turning real.

Previously, we have demonstrated that c-Jun N-terminal kinases (Jnk) genes play a crucial role not only in acute liver disease but also in chronic liver disease (CLD). In *Chapter 10*⁸, we aimed to investigate the relevance of hepatocyte-specific Jnk2 inhibition in an experimental model of CLD. Genetic deletion of Jnk2 gene was performed using hepatocyte-specific deletion of Jnk2 ($JNK2^{\Delta\text{hepa}}$) in NEMO/IKK γ ($NEMO^{\Delta\text{hepa}}$) mice and by siRNA silencing (siJnk2) *in vitro* and *in vivo* in wild-type (WT) and in $NEMO^{\Delta\text{hepa}}$ mice, respectively. Disease progression was analyzed using imaging analysis of combined molecular fluorescence and microcomputed tomography (FMT/ μ CT), protein expression, IHC, IF, and histopathology. In one-year-old $NEMO^{\Delta\text{hepa}}$ mice, Jnk2 deletion reduced liver fibrosis and hepatocarcinogenesis (HCC), as observed by the reduced total number of HCC nodules. Both $NEMO/JNK2^{\Delta\text{hepa}}$ ($DKO^{\Delta\text{hepa}}$) and hepatocyte-specific liposomal-delivered siJnk2 in end-stage diseased NEMO mice resulted in the improved liver parenchyma, serum levels and markers of fibrogenesis. Furthermore, siJnk2 chronic treatment was associated with a change in the immune response as a reduction of myeloid cells, and an increase in CD4⁺ and CD8⁺ T-cells was found. Most importantly, chronic siJnk2 treatment reduced the presence of premalignant and malignant liver tumors corresponding to reduced tumor initiation. Interestingly, hepatocyte-specific liposomal-delivered siJnk2 diminished liver function at an early phase of CLD in $NEMO^{\Delta\text{hepa}}$ mice. siJnk2 caused increased liver transaminases, hepatocellular apoptosis, and compensatory proliferation, a phenomenon that we validated in 12 week-old $DKO^{\Delta\text{hepa}}$ mice.

Our findings demonstrate a stage-dependent role of Jnk2 during CLD progression in $NEMO^{\Delta\text{hepa}}$ mice. Notably, siJnk2 delivery to hepatocytes ameliorated hepatitis, fibrogenesis, and HCC initiation and thus might be an attractive therapeutic option for personalized medicine in CLD.

The aim of *Chapter 11*⁹ was to uncover the functional role of Fas signaling in an experimental model of chronic liver disease. We, therefore, generated $NEMO^{\Delta\text{hepa}}/\text{Fas}^{\text{Ipr}}$ mice, while $NEMO^{\Delta\text{hepa}}$, $NEMO^{f/f}$ as well as Fas^{Ipr} animals served as controls and characterized their phenotype during liver disease progression. Liver damage was evaluated by serum transaminases, histological, immunofluorescence procedures, and biochemical and molecular biology techniques. Proteins were detected by Western Blot, expression of mRNA by RT-PCR, and infiltration of inflammatory cells was determined by FACs analysis, respectively. Fas^{Ipr} mutation in $NEMO^{\Delta\text{hepa}}$ mice resulted in overall decreased liver injury, enhanced hepatocyte survival, and reduced proliferation at 8 weeks of age

compared with NEMO^{Δhepa} mice. Moreover, NEMO^{Δhepa}/Fas^{lpr} animals elicited significantly decreased parameters of liver fibrosis, such as Collagen IA1, MMP2, and TIMP1, and reduced proinflammatory macrophages and cytokine expression. At 52 weeks of age, NEMO^{Δhepa}/Fas^{lpr} exhibited less malignant growth, as evidenced by reduced HCC burden associated with a significantly decreased number of nodules and LW/BW ratio and decreased myeloid populations. The deletion of TNFR1 further reduced the tumor load of 52-weeks-old NEMO^{Δhepa}/Fas^{lpr} mice. The functionality of FasL/Fas might affect inflammation-driven tumorigenesis in an experimental model of chronic liver disease. These results help to develop alternative therapeutic approaches and extend the limitations of tumor therapy against HCC.

Samenvatting

Hoofdstuk 1 geeft een literatuuroverzicht over het onderwerp geneesmiddelen geïnduceerde leverbeschadiging. De meest relevante bevindingen van de afgelopen jaren zijn gedetailleerd beschreven. Er is aandacht besteed aan basisdefinities en classificaties maar ook aan onderliggende pathofysiologie, mechanismen, klinische kenmerken, differentiële diagnose, causaliteitsbeoordeling en risicofactoren van medicatie geïnduceerde leverbeschadiging (drug induced liver injury:DILI). Bovendien zijn de belangrijkste doelstellingen en hoofdlijnen voor de uitgevoerde onderzoeken kort beschreven. Een inleiding over chronische leverziekte en het experimentele model “Nemo” is bijgevoegd.

In *Hoofdstuk 2*¹ werd een systematische analyse uitgevoerd om de in de literatuur beschikbare informatie over door ibuprofen geïnduceerde leverbeschadiging te onderzoeken. Ons onderzoek had betrekking op, casusreeksen en casusrapporten van potentiële hepatotoxiciteitscohorten en de verkregen gegevens werden grondig onderzocht ten aanzien van demografische, klinische en biochemische aspecten. Opvallend was dat ibuprofen de meest voorkomende NSAID is die DILI veroorzaakt zoals bleek uit DILI-gegevensbestanden uit Spanje en India. Twaalf van de 22 geïdentificeerde idiosyncratische gevallen van ibuprofen hepatotoxiciteit waren van het vrouwelijk geslacht met een gemiddelde leeftijd van 31 jaar. De gemiddelde cumulatieve dosis ibuprofen was 30g, terwijl de behandelingsduur en starttijd respectievelijk 14 en 12 dagen waren. Kenmerkende beschadiging waren voornamelijk cytolyse en een totaal aantal van zes gevallen ontwikkelde het verdwenen galwegen syndroom (vanishing bile duct syndrome). Volledig herstel vond plaats na een gemiddelde periode van 14 weken, terwijl vijf patiënten een acuut leverfalen ontwikkelden dat leidde tot de dood of de noodzaak voor een levertransplantatie. Ofschoon weinig voorkomend bij het frequent gebruik, is ibuprofen overtuigend geassocieerd met hepatotoxiciteit in de literatuur en de registratie cohorten van DILI-gevallen en daarom moet het worden beschouwd als een potentiële veroorzaker bij de beoordeling van DILI causaliteit. Ibuprofen-afgeleide DILI vertoont gewoonlijk een korte latentie, hepatocellulaire schade en kan potentieel de dood tot gevolg hebben of de noodzaak van een levertransplantatie.

In *Hoofdstuk 3*² werden de prevalentie en kenmerken van door ibuprofen geïnduceerde leverschade in het Spaanse DILI-register, evenals de Latijns-Amerikaanse tak, een prospectieve database voor humane hepatotoxiciteit, geanalyseerd en gekarakteriseerd. Alleen die gevallen met ibuprofen als DILI veroorzakende medicatie werden opgenomen in de studie. Om een mogelijke signatuur van ibuprofen hepatotoxiciteit te onderzoeken, werden deze vergeleken met andere soorten DILI, .

Een ibuprofen hepatotoxiciteit cohort met in totaal 26 patiënten werd verkregen, de helft van de personen waren van het vrouwelijk geslacht en de gemiddelde leeftijd was 51 jaar. De meeste patiënten werden behandeld met 1200 mg ibuprofen per dag of hoger (77%), de mediane behandelingsduur bedroeg 16 dagen en de mediane tijd tot aan het begin duurde 15 dagen. Bovendien werd 58% van de patiënten gehospitaliseerd, 69% presenteerde met geelzucht en 50% voldeed aan de overgevoeligheid criteria. Hepatocellulaire beschadiging was het meest voorkomende patroon (69%). De gemiddelde BMI was hoger in de ibuprofen DILI groep in vergelijking tot de DILI als gevolg van andere NSAID's of niet-NSAID's ($p=0.06$). De mediane tijd tot aan het begin van de verschijnselen was korter voor ibuprofen hepatotoxiciteit (15 dagen). Diabetes mellitus werd significant vaker vastgesteld bij ibuprofen DILI patiënten ($p=0.038$). Fatale afloop was hoger voor ibuprofen hepatotoxiciteit (12%) versus andere NSAIDs (5%) en andere niet-NSAID middelen (3%). Opvallend was dat er een hogere prevalentie van ibuprofen-geïnduceerde leverschade gevallen werd gevonden in ons register in vergelijking met andere grote gepubliceerde DILI cohorten. Ibuprofen DILI was eveneens geassocieerd met een hoger percentage fatale afloop. Dit suggereert dat patiënten met ibuprofen DILI een langdurigere monitoring in de follow-up nodig hebben. We concluderen uit ons onderzoek dat de immunoallergische en metabole factoren in het idiosyncratische mechanisme een rol spelen in de pathogenese van ibuprofen DILI.

Onze eerdere onderzoeken hebben ons ertoe aangezet de mechanismen te onderzoeken die verband houden met door ibuprofen geïnduceerde leverbeschadiging. In *Hoofdstuk 4*³ werd een nieuw ibuprofen hepatotoxiciteitsmodel *in vitro* en *in vivo* ontwikkeld om de onderliggende pathomoleculaire basis te analyseren die ten grondslag zou kunnen liggen aan door ibuprofen geïnduceerde hepatotoxiciteit. Voor dit doel werden verschillende cytotoxiciteitsstudies voor ibuprofen *in vitro* uitgevoerd op Hepa 1-6 en HepaRG cellijnen, die vervolgens werden aangevuld met primaire muizenhepatocyten, die vers werden geïsoleerd uit 8 weken oude Wt-muizen. Vervolgens werden onze studies afgerond met een muizenmodel van ibuprofen hepatotoxiciteit *in vivo*. Mannelijke C57BL/6 muizen van 6-8 weken oud werden gedurende nacht niet gevoed en kregen *i.p.* 600 mg/kg ibuprofen geïnjecteerd en werden 8 uur later opgeofferd. Om de rol van JNK te beoordelen, gebruikten we dieren met constitutieve deletie van Jnk1 ($Jnk1^{-/-}$) of Jnk2 ($Jnk2^{-/-}$). Vervolgens hebben we onze beoordeling uitgebreid naar dieren, die een uniek Jnk1-tekort in hepatocyten ($Jnk1^{\Delta\text{hepa}}$) vertoonden. Om dieren met redundante expressie van Jnk2 op hepatocellulair niveau te genereren, werd 0.2 mg/kg siRNA van Jnk2 ($siJnk2^{\Delta\text{hepa}}$) een week voorafgaand aan ibuprofen-challenge-injectie geïnjecteerd in de staartader. Als translationele benadering werd de rol van JNK onderzocht bij menselijke leverspecimens, die hadden geleden aan door ibuprofen geïnduceerde leverbeschadiging. Verbeterde JNK-fosforylering werd aangetoond in het cytoplasma van hepatocyten in door ibuprofen

geïnduceerde DILI-levermonsters in vergelijking met gezond weefsel op humaan en muizenniveau. Ibuprofen-challenge produceerde een grotere mate van leverbeschadiging dan die waargenomen bij met vehikel behandelde controlemuizen, op basis van concentratie serummarkers en histopathologie-analyse van de respectieve leverweefsel samples. Een opmerkelijke respons afname in siJnk2^{Δhepa}-dieren werd aangetoond gecorreleerd aan de significant hogere serum-leverenzymen en verslechterde leverhistologiekenmerken in vergelijking met Jnk1^{Δhepa}- of Wt-dieren. Vervolgens werden de moleculaire routes geassocieerd met door ibuprofen geïnduceerde leverbeschadiging bij muizen onderzocht. Verhoogde activatie van PKCα, AKT en JNK, acht uur na Ibuprofen-challenge-behandeling werd bij muizen aangetoond. De resultaten van deze studie toonden cytoplasmatische JNK-activering in hepatocyten aan als kenmerk van Ibuprofen-intoxicatie in monsters van mensen en muizen. Hepatocellulaire deficiëntie van Jnk2 werd geassocieerd met een verslechterde reactie op door ibuprofen geïnduceerde acute leverbeschadiging.

De JNK-routelijkt een cruciale rol te spelen in de pathofysiologie van acute en chronische leverziekte. Om dit verder te onderzoeken werd in *Hoofdstuk 5*⁴ de aanwezigheid van gefosforyleerd JNK onderzocht in leverweefsel van patiënten met geneesmiddelen geïnduceerde leverbeschadiging. Tevens werd de rol van het JNK werkingsmechanisme onderzocht in experimentele modellen van acute en chronische leverziekten bij muizen. Leverbiopten van patiënten met DILI, geïnduceerd door verschillende stoffen, werden bestudeerd en het JNK-expressieprofiel werd daarbij geanalyseerd. Parallel hieraan werd in muizen met hepatocyt-specifieke deletie van JNK1 (Jnk1^{Δhepa}) of gecombineerde JNK1 en JNK2 (Jnk^{Δhepa}) deletie, alsmede JNK1-floxed C57BL/6 controle muizen bestudeerd wat de leverschade was na injecties van CCl₄ (om leverfibrose te induceren; 0.6ml/kg *i.p.* elke 3 dagen gedurende 4 weken) of paracetamol (om toxische hepatitis te induceren; 500mg/kg *i.p.*).

Leverweefsel van DILI patiënten toonde meer geactiveerd JNK, voornamelijk in kernen van hepatocyten en immuuncellen, ten opzicht van gezond controle weefsel. De injectie van paracetamol in Jnk^{Δhepa} muizen induceerde een grotere mate van leverschade dan bij Jnk1^{Δhepa} controle muizen, gebaseerd op metingen van serum markers en microscopische en histologische analyse van leverweefsel. Toediening van CCl₄ veroorzaakte een sterkere leverbeschadiging in Jnk^{Δhepa} muizen, gemeten aan de hand van verhoogde ontstekingsparameters, celproliferatie, en fibrose progressie, in vergelijking met Jnk1^{Δhepa} controle muizen. Hepatocyten van met paracetamol behandelde Jnk^{Δhepa} muizen, hadden een verhoogde oxidatieve stressrespons wat leidde tot een verminderde activering van adenosine monofosfaat geactiveerd proteïne kinase, totaal eiwit adenosine monofosfaat geactiveerd proteïne kinase niveaus en pJund waardoor er necrose ontstond. Toediening van SP600125 (30mg/kg *i.p.*) vóór of met paracetamol, beschermde Jnk^{Δhepa} en controle muizen tegen leverschade. Deze bevindingen tonen aan dat JNK1 en JNK2 in hepatocyten een gecombineerdbeschermend effect induceren bij muizen die met CCl₄- en paracetamol waren behandeld. Daarnaast toont de JNK inhibitor SP600125 off-target effecten, dus onafhankelijk van JNK.

In *Hoofdstuk 6*⁵ was het doel de methylprednisolon (MP) geïnduceerde leverschade nader te karakteriseren met de beschrijving van vier nieuwe gevallen uit het Spaanse en Latijns-Amerikaanse DILI register. Demografische, klinische, laboratorium en follow-up gegevens werden geanalyseerd. Ter vergelijking werd een uitgebreide screening van de literatuur uitgevoerd naar MP geïnduceerde leverschade gevallen. Drie jonge vrouwen met multiple sclerose en een andere met de ziekte van Crohn kregen een acute exacerbatie van hun ziekte en werden vervolgens behandeld met intraveneuze MP pulstherapie. Leverbeschadiging trad op na één (patiënt 3), twee (patiënt 1), vijf (patiënt 2) en zes (patiënt 4) weken na start van de medicatie. De tijd tot herstel vergde acht en zes weken voor respectievelijk patiënten 2 en 3, terwijl patiënt 1 na tien weken verbetering vertoonde en patiënt 4 na 2 weken. Bij drie patiënten (patiënten 2, 3 en 4) werd MP opnieuw gegeven met optreden van dezelfde bijwerkingen.

Een uitgebreid literatuuroverzicht leverde in totaal 45 door MP geïnduceerde leverbeschadigingsgevallen op. Deze waren geassocieerd met vrouwelijk geslacht (84%) bij een gemiddelde leeftijd van 41 jaar. De meest voorkomende MP indicaties voor de behandeling waren multiple sclerose (24 gevallen) en 13 gevallen van Graves oftalmopathie (GO). Hepatocellulaire schade werd aangetoond als de meest voorkomende beschadiging, positieve autoantilichamen werden gedetecteerd in 35%. De gemiddelde tijd tot het begin van de verschijnselen was zes weken. Vier gevallen ontwikkelden zich met fatale afloop. In 17 (38%) patiënten werd opnieuw met het MP behandeld met dezelfde reactie tot gevolg. Deze bevindingen wijzen erop dat patiënten met MS en GO bijzonder vatbaar zijn voor het ontwikkelen van leverschade als gevolg van het gebruik van MP.

De studie in *Hoofdstuk 7*⁶ werd gefocust op het analyseren van herstelduur van leverenzymen en om de beste definitie en de risicofactoren van DILI chroniciteit vast te stellen. Van 298 personen van de 850 geregistreerde patiënten uit het Spaanse DILI register werden onderzocht. De patiënten waren niet gekend met een aandoening van de lever en follow-up gegevens van ≥ 1 jaar na herstel werden geanalyseerd. Chroniciteit werd gedefinieerd als gestoorde lever biochemie, afwijkende beeldvorming of histologie een jaar na DILI diagnose. Van de 298 geïncludeerde patiënten waren 273 (92%) hersteld binnen een jaar na Dili diagnose en 25 patiënten (8%) waren chronisch. Onafhankelijke risicofactoren voor chroniciteit waren oudere leeftijd (OR: 1.06, $p=0.011$), dyslipidemie (OR: 4.26, $p=0.04$) en ernstige DILI (OR: 14.22, $p=0.005$). De mediane waarden van alanine aminotransferase (ALT), alkalische fosfatase (ALP) en totaal bilirubine (TB) waren hoger in de chronische groep gedurende de follow-up. De waarden van ALP >1.1 maal de bovengrens van normaal (xULN) en TB >2.8 xULN in de tweede maand van Dili aanvang, bleken chronische DILI ($p<0.001$) te voorspellen. Voornaamste medicament groepen betrokken bij chroniciteit waren statines (24%) en anti-infectiva (24%). Histologisch onderzoek bij chronische patiënten toonde twee gevallen met ductale laesies en zeven met cirrose.

Deze gegevens suggereren dat het interval van een jaar de beste cut-off tijd is om chronische DILI of vertraagd herstel te definiëren, met als risicofactoren oudere leeftijd, dyslipidemie en de ernst van de acute episode. Statines zijn duidelijk gerelateerd aan chroniciteit. ALP en TB-waarden in de tweede maand zijn van diagnostische waarde om chroniciteit of zeer traag herstel te voorspellen.

In *Hoofdstuk 8* hebben we als interessant leerpunt, drie korte casus over door geneesmiddelen geïnduceerde leverbeschadiging van diverse etiologie uit onze dagelijkse klinische praktijk weergegeven. In de eerste casus wordt het geval beschreven van een jonge vrouw, die leed aan acuut leverfalen behandeld met een paratiele levertransplantatie. De oorzaak voor het leverfalen was onduidelijk. Toen we haar medische geschiedenis uitgebreider onderzochten, kwam een recente behandeling met ibuprofen gecombineerd met paracetamol aan het licht alsook positieve serologie voor HSV. Niettemin kon de causaliteit niet duidelijk worden aangetoond. De tweede casus beschrijft een vrouw bij wie leverfunctiestoornissen ontdekt werden nadat ze opnieuw was blootgesteld aan het antidepressivum sertraline. De voorgeschiedenis vermeldde extramedullaire manifestaties bij een onderliggende morbus Walderström. De verschijnselen werden aanvankelijk verklaard passend bij de aandoening. Herintroductie van sertraline, na een periode zonder dit medicament deed de klachten opnieuw ontstaan en er werd een duidelijke causaliteit vastgesteld met sertraline. In gevalsbeschrijving 3 wordt een patiënte beschreven, verwezen met gevorderde chronische leverziekte waarbij een cirrose met portale hypertensie was aangetoond. Er werd refractaire hydropische decompensatie vastgesteld en eveneens een cardiomyopathie zonder evidente plausibele oorzaak. Uit gedetailleerd onderzoek van haar medische geschiedenis bleek dat de patiënt de afgelopen vier jaar een intensieve behandeling met venlafaxine had gehad die als enige verklaring werd gevonden voor haar huidige klinische toestand met gecompliceerde levercirrose. Uitgebreid onderzoek leverde een genetische afwijking op van het cytochroom P450. Patiënte was een ultrasnelle metaboliseerder van venlafaxine waarbij gesuggereerd werd dat metabolieten verantwoordelijk zijn geweest voor zowel de hepatologische als cardiale gevolgen.

In *Hoofdstuk 9*⁷ is een overzicht gegeven van de reversibiliteit van leverfibrose. De meest representatieve diervormen die zijn gebruikt om onze kennis over leverfibrose te vergroten, zijn weergegeven. Er zijn verschillende beperkingen op te merken die in de toekomst opnieuw moeten worden gedefinieerd. De uitgebreide onderzoeken die in de afgelopen decennia op dit gebied zijn uitgevoerd, hebben aangetoond dat de conditie van een fibrotische lever reversibel kan zijn door de onderliggende inflammatie te behandelen, eliminatie of regressie van geactiveerde HSC's te induceren en degradatie van extra cellulaire matrix (ECM) te modificeren.

Momenteel bestaat er een enorm potentieel therapeutisch arsenaal dat gericht is op het omkeren van leverfibrose. Verschillende klinische studies worden momenteel uitgevoerd, met bijzondere aandacht voor patiënten die lijden aan niet-alcoholische steatohepatitis. Dus, meer dan ooit, lijkt de veelbelovende vooruitgang van de omkering van leverfibrose realiteit te worden.

Eerder hebben we aangetoond dat c-Jun N-terminale kinasen (Jnk)-genen een cruciale rol spelen, niet alleen bij acute leverziekte, maar ook bij chronische leverziekte (CLD). In *Hoofdstuk 10*⁸ was het doel om de relevantie van hepatocyt specifieke Jnk2-remming te onderzoeken in een experimenteel CLD model. Genetische deletie van het Jnk2-gen werd uitgevoerd met behulp van hepatocyt specifieke deletie van Jnk2 (JNK2^{Δhepa}) in NEMO / IKKγ (NEMO^{Δhepa})-muizen en door siRNA-silencing (siJnk2) *in vitro* en *in vivo* in respectievelijk

wildtype (WT) en in NEMO^{Δhepa}-muizen. Ziekteprogressie werd geanalyseerd met behulp van beeldvormende analyse van gecombineerde fluorescentie moleculaire en microcomputed tomography (FMT/μCT), eiwitexpressie, IHC, IF en histopathologie. Bij één jaar oude NEMO^{Δhepa}-muizen verminderde Jnk2-deletie leverfibrose en hepatocarcinogenese, zoals waargenomen door een verminderd totaal aantal hepatocellulaire carcinoom (HCC)-noduli. Zowel NEMO/JNK2^{Δhepa} (DKO^{Δhepa}) als hepatocyt specifieke liposomaal afgegeven siJnk2 in NEMO-muizen met eindstadium leverziekte resulteerden in verbeterde leverparenchym, serumspiegels en markers van fibrogenese. Bovendien werd de chronische behandeling met siJnk2 geassocieerd met een verandering in de immuunrespons omdat een vermindering van myeloïde cellen en een toename van CD4⁺ en CD8⁺ T-cellen werd gevonden. Het belangrijkste is dat chronische siJnk2-behandeling de aanwezigheid van premaligne en kwaadaardige levertumoren verminderde, wat overeenkomt met verminderde tumor-initiatie. Een interessante bevinding was het feit dat hepatocyt specifiek liposomaal afgegeven siJnk2 de leverfunctie verminderde in een vroege fase van CLD bij NEMO^{Δhepa}-muizen. siJnk2 veroorzaakte verhoogde levertransaminasen, hepatocellulaire apoptose en compenserende proliferatie, een fenomeen dat we valideerden bij 12 weken oude DKO^{Δhepa}-muizen.

Onze bevindingen tonen een fase-afhankelijke rol van Jnk2 tijdens CLD-progressie bij NEMO^{Δhepa}-muizen. Met name de afgifte van siJnk2 aan hepatocyten verbeterde hepatitis, fibrogenese en HCC-initiatie en zou dus een aantrekkelijke therapeutische optie kunnen zijn voor gepersonaliseerde geneeskunde bij CLD.

In *Hoofdstuk 11*⁹ wordt het onderzoek naar de functionele rol van Fas-signalering in een experimenteel model van chronische leverziekte beschreven. We genereerden NEMO^{Δhepa}/Fas^{lpr}-muizen, terwijl NEMO^{Δhepa}, NEMO^{f/f} en Fas^{lpr}-dieren als controles dienden met hun fenotypische kenmerk van de progressie van de leverziekte. Leverbeschadiging werd geëvalueerd met serumtransaminasen, histologische, immunofluorescentie-procedures en biochemische en moleculaire-biologisch technieken. Eiwitten werden geanalyseerd met Western Blot, expressie van mRNA door RT-PCR en infiltratie van ontstekingscellen werd bepaald door respectievelijk FACs-analyse. Fas^{lpr}-mutatie bij NEMO^{Δhepa}-muizen resulteerde in een algehele verminderde leverbeschadiging, verbeterde overleving van hepatocyten en verminderde proliferatie op 8 weken oud in vergelijking met NEMO^{Δhepa}-muizen. Bovendien ontwikkelden NEMO^{Δhepa}/Fas^{lpr}-dieren aanzienlijk verlaagde parameters van leverfibrose, zoals collageen IA1, MMP2 en TIMP1, en verlaagde pro-inflammatoire macrofagen en cytokine-expressie. Op de leeftijd van 52 weken vertoonde NEMO^{Δhepa}/Fas^{lpr} minder kwaadaardige groei, zoals bleek uit een verminderde HCC-last geassocieerd met een aanzienlijk verlaagd aantal noduli en de LW/BW-ratio alsook verminderde myeloïde populaties. Deletie van TNFR1 verminderde de tumorbelasting van de 52 weken oude NEMO^{Δhepa}/Fas^{lpr}-muizen verder.

De functionaliteit van FasL/Fas kan de ontstekingsgestuurde tumorigenese beïnvloeden in een experimenteel model van chronische leverziekte. Deze resultaten helpen bij het ontwikkelen van alternatieve therapeutische benaderingen en vergroten de beperkingen van tumortherapie tegen HCC.

Resumen

En el *Capítulo 1* se realizó una revisión bibliográfica actualizada sobre la temática del daño hepático inducido por fármacos (drug-induced liver injury, DILI). Los hallazgos y contribuciones más relevantes a dicho campo temático en los últimos años han sido examinados en detalle. Además de diversas definiciones básicas y clasificaciones esenciales, se ha llevado a cabo un enfoque particular sobre la fisiopatología subyacente, mecanismos, características clínicas, diagnóstico diferencial, evaluación de causalidad y factores de riesgo en DILI. Como paráfrasis, se ha adjuntado una descripción resumida respecto al "carcinoma hepatocelular", así como una breve introducción al modelo experimental de hepatopatía crónica "NEMO". Además, se describieron brevemente los objetivos principales de las presentes investigaciones.

En el *Capítulo 2*¹ se realizó un análisis sistemático para examinar la información disponible sobre el daño hepático inducido por ibuprofeno en la literatura científica. Nuestras investigaciones abarcaron cohortes prospectivas de hepatotoxicidad, series de casos y casos clínicos, y los datos obtenidos se examinaron exhaustivamente a nivel demográfico, clínico, bioquímico y resolutivo. Es de destacar que el ibuprofeno destacó como AINE más frecuentemente involucrado en las bases de datos prospectivas de DILI en España e India. Doce de los 22 casos idiosincrásicos de hepatotoxicidad por ibuprofeno identificados tenían sexo femenino y la edad media era de 31 años. La dosis acumulada promedia de ibuprofeno fue de 30g, mientras que la duración del tratamiento y el tiempo de incubación de la reacción hepatotóxica fue de 14 y 12 días, respectivamente. Predominaron las características citolíticas en cuanto al perfil de daño hepático y un total de seis casos desarrollaron un síndrome de los conductillos biliares evanescentes. La recuperación completa de los pacientes se produjo tras un intervalo medio de 14 semanas, mientras que hasta cinco casos desarrollaron fallo hepático fulminante, lo cual condujo a la muerte o la necesidad de un trasplante de hígado. Aun siendo poco frecuente, dado el uso extenso, es importante que los clínicos tengan en cuenta que ibuprofeno ha sido asociado convincentemente con la hepatotoxicidad en la literatura y cohortes prospectivas de DILI y, por lo tanto, debe ser considerada como una potencial causa de DILI durante la evaluación de la causalidad. La hepatotoxicidad derivada de ibuprofeno se presenta comúnmente con una latencia corta, daño de rasgo hepatocelular y puede progresar potencialmente a una condición severa que podría conllevar la necesidad de un trasplante de hígado o incluso el exitus en el peor de los casos.

En el *Capítulo 3*², la prevalencia y las características de los casos de daño hepático inducido por ibuprofeno declarados al Registro Español de Hepatotoxicidad (Spanish DILI Registry), así como a su red latinoamericana (Latin-American DILI Network), fueron identificados y analizados en detalle. Exclusivamente aquellos casos que presentaban ibuprofeno como único fármaco imputable fueron finalmente adjudicados al presente estudio. Para investigar rasgos característicos de la hepatotoxicidad por ibuprofeno, se compararon los eventos de DILI de ibuprofeno con otros tipos de DILI inducidos por otros medicamentos

antiinflamatorios no esteroideos (AINE) y otros medicamentos no AINE. Se obtuvo así una cohorte de hepatotoxicidad por ibuprofeno de 26 casos totales, la mitad de los sujetos resultó tener género femenino y la edad promedia fue de 51 años. La mayoría de los pacientes habían recibido tratamiento con dosis de 1200mg o más altas de ibuprofeno al día (77%), la mediana de la duración del tratamiento fue de 16 días y la mediana del intervalo onset abarcó 15 días. Además, el 58% de los pacientes requirió hospitalización, el 69% presentó ictericia y el 50% cumplió con criterios de hipersensibilidad. La lesión hepatocelular fue el patrón de lesión hepática más frecuente (69%). El IMC promedio fue mayor en sujetos con DILI por ibuprofeno que en DILI debido a otros AINEs o medicamentos no AINE ($p=0.06$). La mediana del tiempo de incubación de la reacción adversa abarcó un intervalo menor para la hepatotoxicidad por ibuprofeno (15 días). Diabetes mellitus fue significativamente más prevalente en pacientes con DILI inducido por ibuprofeno ($p=0.038$). Formas graves se asociaron más frecuentemente con la hepatotoxicidad por ibuprofeno (12%) que en otros AINEs (5%) u otros agentes no AINE (3%). En conclusión, se encontró una mayor prevalencia de eventos de daño hepático inducido por ibuprofeno en nuestra base de datos en comparación con otras grandes cohortes de DILI publicadas. Críticamente, DILI por ibuprofeno se asoció con una mayor tasa de resultados graves. Por lo tanto, los pacientes diagnosticados con DILI por ibuprofeno deben someterse a un control exhaustivo durante su seguimiento. Nuestros datos reflejan que los mecanismos idiosincrásicos de naturaleza inmunoalérgica y metabólica posiblemente puedan jugar un papel relevante en la hepatotoxicidad por ibuprofeno, aunque se requieren estudios más amplios para determinar el papel de ibuprofeno en DILI.

Nuestras investigaciones previas nos llevaron a investigar los mecanismos asociados al daño hepático inducido por ibuprofeno. En el *Capítulo 4*³ se desarrolló así un nuevo modelo experimental de hepatotoxicidad por ibuprofeno a nivel *in vitro* e *in vivo* con el objetivo de poder analizar las bases patomoleculares subyacentes a dicho fenómeno. Para este propósito, se realizaron inicialmente diversos estudios de citotoxicidad sobre ibuprofeno *in vitro* a partir de líneas celulares Hepa 1-6 y HepaRG, que más tarde fueron complementados con otro estudio realizado en hepatocitos murinos primarios aislados en fresco a partir de ratones Wt de 8 semanas de edad. Posteriormente, nuestros estudios *in vitro* fueron ampliados con un modelo murino de hepatotoxicidad inducida por ibuprofeno *in vivo*. Para ello, ratones C57BL/6 machos (6-8 semanas de edad) en ayuno nocturno fueron sometidos a una inyección *i.p.* de ibuprofeno en dosis de 600 mg/kg y sacrificados 8h más tarde. Para evaluar el papel de JNK, utilizamos animales portadores de delección constitutiva de Jnk1 (Jnk1^{-/-}) o Jnk2 (Jnk2^{-/-}). En un segundo tiempo, concentramos nuestra evaluación sobre JNK a nivel hepatocitario, empleando animales que presentaron deficiencia de Jnk1 exclusivamente en los hepatocitos (Jnk1^{Δhepa}). Para generar animales con expresión redundante de Jnk2 a nivel hepatocelular, se administraron 0.2mg/kg de siRNA (ARN de interferencia) de Jnk2 (siJnk2^{Δhepa}) una semana antes de replicar la exposición hepatotóxica con ibuprofeno en los mismos. A nivel traslacional, se investigó además el papel de JNK en muestras de hígado humano, que fueron extraídas de pacientes que habían

sufrido un daño hepático inducido por ibuprofeno. La fosforilación de JNK se hizo evidente en el citoplasma hepatocitario de dichas muestras en comparación con tejido sano a nivel humano y murino. La sobreexposición experimental con ibuprofeno produjo un mayor grado de daño hepático que el observado en ratones controles tratados solo con el vehículo, como así reflejaron los marcadores serológicos bioquímicos y el análisis de histopatológico de las muestras hepáticas respectivas. Es de destacar que los animales $\text{siJnk2}^{\Delta\text{hepa}}$ mostraron un agravamiento notable en la respuesta al daño hepático inducido por ibuprofeno y que se correlacionó con enzimas hepáticas en suero significativamente elevadas y empeoramiento de las características histológicas del parénquima hepático en comparación con los animales $\text{Jnk1}^{\Delta\text{hepa}}$ y Wt. A continuación, se investigaron las vías moleculares asociadas al daño hepático inducido por ibuprofeno en el presente modelo experimental y se observó un aumento de la activación de PKC α , AKT y JNK, 8h tras la sobreexposición a ibuprofeno. En resumen, los resultados del presente estudio demostraron una activación de JNK a nivel citoplásmico en hepatocitos como sello distintivo de la intoxicación por ibuprofeno tanto en muestras humanas como en murinas. La deficiencia hepatocelular de Jnk2 se asoció con un empeoramiento en la respuesta de la lesión hepática aguda inducida por ibuprofeno, haciendo de éste una potencial diana terapéutica de interés.

La vía asociada a las c-Jun N-terminal kinases (JNK) ha resultado desempeñar un papel crucial en la fisiopatología de la enfermedad hepática aguda y crónica. Para este propósito, en el *Capítulo 5*⁴ se examinó la presencia de JNK fosforilado en tejido hepático proveniente de pacientes con daño hepático inducido por fármacos y se investigó el papel de JNK hepatocítico en modelos experimentales murinos de enfermedad hepática aguda y crónica. Las muestras histológicas hepáticas de pacientes con DILI inducido por diversos agentes farmacológicos fueron estudiadas y se analizó el perfil de expresión de JNK. En paralelo, ratones con delección de Jnk1 ($\text{Jnk1}^{\Delta\text{hepa}}$) o delección concomitante de Jnk1 y Jnk2 ($\text{Jnk}^{\Delta\text{hepa}}$) específicamente en hepatocitos, así como ratones controles $\text{Jnk1}^{f/f}$ (floxed) C57BL/6 fueron sometidos a inyecciones de CCl_4 para inducir fibrosis hepática (0.6 ml/kg *i.p.* cada 3 días durante 4 semanas) o fueron expuestos a dosis de 500 mg/kg *i.p.* de acetaminofén (APAP) para inducir una hepatitis tóxica aguda. Las muestras de tejido hepático derivadas de enfermos de DILI exhibieron significativamente un mayor perfil de activación de JNK, que predominó en núcleos de hepatocitos y en células inmunes, comparado con tejido sano. La sobreexposición con acetaminofén en ratones $\text{Jnk}^{\Delta\text{hepa}}$ indujo un mayor nivel de daño hepático que el observado en $\text{Jnk1}^{\Delta\text{hepa}}$ o en los ratones control, en base a los niveles de marcadores serológicos de daño hepático y el análisis histológico. La administración de CCl_4 también indujo una lesión hepática más fuerte en ratones $\text{Jnk}^{\Delta\text{hepa}}$, que se caracterizó por un aumento de la inflamación, proliferación celular y progresión de la fibrosis, comparado con ratones $\text{Jnk1}^{\Delta\text{hepa}}$ o controles. Los hepatocitos de ratones $\text{Jnk}^{\Delta\text{hepa}}$ expuestos a APAP sufrieron una peor respuesta al estrés oxidativo, lo que condujo a una disminución en la fosforilación de la proteína quinasa activada por monofosfato de adenosina, niveles totales de proteína quinasa activada por monofosfato de adenosina y pJund y necrosis posterior.

La administración del inhibidor SP600125 (30mg/kg *i.p.*) antes o simultáneamente con APAP protegió a Jnk^{Δhepa} y atenuó la lesión hepática consiguiente. Estos hallazgos demuestran que JNK1 y JNK2 a nivel hepatocitario parecen tener efectos combinados de ámbito protector respecto a la lesión hepática inducida por CCl₄ y APAP. Además, se ha podido probar que el inhibidor SP600125 tiene efectos “off-target”.

La metilprednisolona (MP) se usa comúnmente en hepatología para el tratamiento de la lesión hepática aguda con características graves, a parte de otras indicaciones. El hecho de que recientemente se hayan publicado numerosos casos clínicos sobre daño hepático inducido por MP, en particular en pacientes que padecen enfermedades autoinmunes subyacentes, nos motivó a investigar esta circunstancia. Así en el *Capítulo 6*⁵ quisimos contribuir a la caracterización de la expresión fenotípica de la lesión hepática inducida por MP e investigamos todos los casos disponibles declarados a las bases de datos DILI españolas y latinoamericanas (Spanish DILI Registry; Latin-American DILI Network). Se analizaron datos demográficos, clínicos, de laboratorio y de resolución. Adicionalmente, se proporcionó una revisión bibliográfica analizando casos de DILI inducidos por MP publicados anteriormente. Además, se realizó un análisis comparativo entre los nuevos eventos MP-DILI identificados en las bases de datos mencionadas anteriormente y los casos obtenidos anteriormente de la literatura. Tres mujeres jóvenes con esclerosis múltiple y una más con enfermedad de Crohn sufrieron una recaída aguda de su enfermedad y fueron tratadas con bolos intravenosos de MP. Después de una (paciente 3), dos (paciente 1) y cinco (paciente 2) y seis (paciente 4) semanas tras el inicio del tratamiento, se produjo un daño hepático. El tiempo de recuperación abarcó ocho y seis semanas para las pacientes 2 y 3 respectivamente, mientras que la paciente 1 mostró una tendencia a la resolución tras 10 semanas (pérdida de seguimiento) y la paciente 4 tras 2 semanas. Se produjo una reexposición positiva en tres de las pacientes (pacientes 2, 3 y 4). Un extenso análisis de la literatura resultó en un total de 45 eventos de lesión hepática inducida por MP. Estos se asociaron al género femenino (84%) y la edad promedio fue de 41 años. Las indicaciones más comunes para el tratamiento con MP fueron la esclerosis múltiple (EM; 24 casos) y la oftalmopatía de Graves (OG; 13 casos). Se observó daño hepatocelular como el patrón de daño predominante en todos los casos con datos disponibles y se detectaron autoanticuerpos positivos en el 35% de los enfermos. El tiempo medio de incubación fue de seis semanas. Cuatro casos desarrollaron un desenlace fatal. En 17 casos (38%) se produjo una reexposición positiva con el medicamento. Estos hallazgos indican que los pacientes que padecen EM y OG son particularmente susceptibles a sufrir un episodio de hepatotoxicidad inducida por MP.

El estudio incluido en el *Capítulo 7*⁶ tuvo como objetivo analizar el intervalo temporal hasta alcanzar los marcadores de daño hepático la normalización, establecer la definición de cronicidad y determinar los factores de riesgo de la cronicidad en DILI. Se analizaron 298 individuos de un total de 850 pacientes incluidos en el Spanish DILI Registry sin enfermedad previa que afectara a hígado y se realizó seguimiento hasta alcanzar la resolución ≥ 1 año.

La cronicidad se definió como anormalidad presentada en una bioquímica hepática, prueba radiodiagnóstica o histopatología un año después del diagnóstico establecido de DILI. De los 298 pacientes incluidos en el estudio, 273 (92%) resolvieron ≤ 1 año a partir del diagnóstico de DILI y complementariamente los 25 pacientes restantes desarrollaron cronicidad (8%). Entre los factores de riesgo independientes para la condición de cronicidad en nuestra cohorte de DILI se encontraron la edad avanzada (OR: 1.06, $p=0.011$), dislipidemia (OR: 4.26, $p=0.04$) o la gravedad del episodio de DILI (OR: 14.22, $p=0.005$). Se determinaron valores promedios de alanina aminotransferasa (ALT), fosfatasa alcalina (ALP) y bilirrubina total (TB) mayores en el grupo de pacientes crónicos durante la fase de seguimiento. Asimismo, se pudo apreciar que los valores de ALP y TB $>1.1 \times$ límite superior de normal (xULN) y $2.8 \times$ ULN respectivamente, en el segundo mes tras el inicio del episodio de DILI, predicen la cronicidad del mismo ($p<0.001$). Las principales clases de fármacos asociadas a cronicidad en el presente análisis fueron las estatinas (24%) y los antibióticos (24%). El examen histopatológico en pacientes crónicos mostró dos casos con lesión ductal y siete con cirrosis hepática. Por lo tanto, los resultados obtenidos sugieren que un año es el mejor punto de corte para definir la cronicidad en DILI o una recuperación prolongada, con factores de riesgo la edad avanzada, dislipidemia y gravedad del episodio agudo. Las estatinas están claramente relacionadas con la cronicidad. Los valores de ALP y TB en el segundo mes podrían ayudar a predecir la cronicidad o una recuperación muy prolongada.

En el *Capítulo 8*, adjuntamos tres breves informes de casos clínicos sobre lesiones hepáticas inducidas por fármacos de etiología diversa originados en el entorno de nuestra práctica clínica, que seleccionamos por aportar alguna particularidad de interés para el aprendizaje. En el primer informe, se describe el caso de una mujer joven, que sufrió un fallo hepático fulminante y requirió un trasplante parcial de hígado. La causa del episodio hepático grave no estaba clara. Cuando se investigaron sus antecedentes previos, llamó la atención que la paciente había recibido un tratamiento reciente con ibuprofeno y que tomó ocasionalmente también acetaminofén, así como una serología positiva de HSV (*Herpes Simplex*). No obstante, la etiología del episodio no pudo ser adjudicada con precisión. Nuestro segundo caso clínico trató sobre una mujer cuya disfunción hepática fue descubierta tras sufrir una nueva exposición con el fármaco antidepressivo sertralina. Anteriormente, la paciente había presentado manifestaciones extramedulares de un síndrome de Walderström subyacente que inicialmente había actuado como factor de confusión y, en consecuencia, condujo a una nueva exposición accidental con sertralina. Nuestro tercer caso clínico hace referencia a una paciente que presentó rasgos de una enfermedad hepática crónica avanzada, que incluía cirrosis hepática avanzada y descompensaciones hidrópicas refractarias. Además, la paciente exhibió también miocardiopatía dilatada, pero no se pudo encontrar una causa plausible. Investigaciones más detalladas de sus antecedentes médicos previos revelaron que la paciente había estado en tratamiento crónico con el fármaco venlafaxina durante los cuatro años anteriores, siendo probablemente una reacción adversa medicamentosa la causa de su condición clínica. De interés, realizamos una prueba de genotipado para el citocromo P450

y descubrimos que la paciente presentaba variaciones polimórficas para CYP2D6, que eran compatibles con un metabolizador ultrarrápido de venlafaxina.

En el *Capítulo 9*⁷ se realizó una revisión bibliográfica sobre la reversibilidad de la fibrosis hepática. Se han descrito los modelos animales experimentales más representativos que se han utilizado para ampliar nuestro conocimiento sobre la fibrosis hepática hasta la fecha, sin embargo, estos están sujetos a diversas limitaciones que deben ser redefinidas en el futuro. Las extensas investigaciones realizadas en el campo durante las últimas décadas han demostrado que la condición de un hígado fibrótico puede ser reversible modificando el nicho inflamatorio subyacente, la eliminación o la involución de las células estrelladas hepáticas (hepatic stellate cells, HSC) activadas, así como la degradación de la matriz extracelular (extracellular matrix, ECM). Actualmente, existe un enorme arsenal terapéutico potencial dirigido a revertir la fibrosis hepática que se encuentra sometido a distintas fases de ensayos clínicos. Por lo tanto, ahora más que nunca, el prometedor progreso en la reversibilidad de la fibrosis hepática parece estar convirtiéndose en una realidad.

346 Previamente, hemos demostrado que las c-Jun N-terminal kinases (Jnk) juegan un papel crucial no solo en la enfermedad hepática aguda, sino también en la enfermedad hepática crónica (chronic liver disease, CLD). En el *Capítulo 10*⁸, nuestro objetivo fue investigar la relevancia de la inhibición de Jnk2 específicamente a nivel hepatocitario en un modelo experimental de CLD. La delección del gen *Jnk2* se realizó por partida doble en un primer modelo utilizando la modificación genética de *Jnk2* específicamente en hepatocitos (JNK2^{Δhepa}) en ratones NEMO/IKKγ (NEMO^{Δhepa}) y en un segundo modelo mediante la aplicación de siRNA dirigido contra *Jnk2* (siJnk2) *in vitro* e *in vivo* en ratones de tipo salvaje (WT) y NEMO^{Δhepa}, respectivamente. El seguimiento de progresión de enfermedad hepática se evaluó a partir de análisis de imágenes combinada de fluorescencia molecular y de tomografía microcomputada (FMT/μCT), análisis de expresión de proteínas, inmunohistoquímica (IHC), inmunofluorescencia (IF) e histopatología, entre otros.

En ratones NEMO^{Δhepa} de un año de edad, la delección de *Jnk2* resultó en la reducción de la fibrosis hepática y la hepatocarcinogénesis (HCC), como se observó en base a la reducción del número total de nódulos tumorales. Tanto los animales NEMO /JNK2^{Δhepa} (DKO^{Δhepa}) como aquellos ratones NEMO tratados con siJnk2 en una etapa avanzada de enfermedad experimentaron una importante mejoría como así demostraron el parénquima hepático, niveles séricos de enzimas hepáticas y marcadores de fibrogénesis. Adicionalmente, el tratamiento crónico con siJnk2 se asoció con una modificación de la respuesta inmune que se caracterizó por una reducción de la serie mieloide y un aumento en células T CD4⁺ y CD8⁺. Lo más importante es que el tratamiento crónico con siJnk2 redujo drásticamente la presencia de tumores hepáticos tanto premalignos como malignos reduciendo así la tumorigénesis en estos roedores. Curiosamente, siJnk2 dirigido específicamente contra la población hepatocitaria conllevó una disfunción hepática en fases tempranas de CLD en roedores NEMO^{Δhepa}. En éstos, siJnk2 provocó un aumento de las transaminasas hepáticas, apoptosis hepatocelular y proliferación compensatoria, un fenómeno que se pudo validar

asimismo en los animales DKO^{Δhepa} de 12 semanas de edad. Nuestros hallazgos por tanto demuestran el condicionamiento de Jnk2 dependiente del estadio de enfermedad en la progresión de CLD en ratones NEMO^{Δhepa}. En particular, la administración de siJnk2 específica a nivel hepatocelular desencadenó una mejoría de la hepatitis, fibrogénesis y tumorigénesis subyacente y, por lo tanto, podría convertirse una opción terapéutica muy atractiva para estrategias de medicina personalizada en CLD.

El objetivo en el *Capítulo 11*⁹ fue aportar nuevos conocimientos sobre el papel funcional de la señalización Fas en un modelo experimental de enfermedad hepática crónica (CLD). Con esta finalidad, se generaron ratones NEMO^{Δhepa}/Fas^{lpr}, mientras que animales NEMO^{Δhepa}, NEMO^{f/f} y Fas^{lpr} sirvieron como controles para el análisis comparativo y se procedió a caracterizar su fenotipo durante la progresión de CLD. El daño hepático fue evaluado a partir de parámetros bioquímicos, análisis histopatológico, inmunofluorescencia y otras técnicas bioquímicas y de biología molecular. La expresión de proteínas se analizó mediante Western Blot, los niveles de ARNm por RT-PCR y la infiltración de células inflamatorias se cuantificó mediante citometría de flujo. La mutación Fas^{lpr} en ratones NEMO^{Δhepa} llevó a una disminución general del daño hepático, una mayor supervivencia hepatocitaria y una reducción de la proliferación a las 8 semanas de edad en comparación con roedores NEMO^{Δhepa}. Además, en animales NEMO^{Δhepa}/Fas^{lpr} se detectó una disminución significativa de los parámetros de fibrosis hepática, tales como colágeno IA1, MMP2 y TIMP1, además de una reducción de macrófagos proinflamatorios y de la expresión de citocinas. A las 52 semanas de edad, roedores NEMO^{Δhepa}/Fas^{lpr} exhibieron un crecimiento de malignomas menor, como demostró la reducción en la carga de HCC asociada con un número significativamente menor de nódulos y una ratio LW/BW en la misma línea además de una disminución de células mieloides. La delección de TNFR1 contribuyó reduciendo aún más la carga tumoral de ratones NEMO^{Δhepa}/Fas^{lpr} de 52 semanas de edad. Así la funcionalidad del eje FasL/Fas podría condicionar el potencial tumorigénico inflamatorio-dependiente en un modelo experimental de CLD. Estos resultados permiten desarrollar enfoques terapéuticos alternativos y contrarrestar las limitaciones en la terapia antitumoral contra el HCC.

Zusammenfassung

In *Kapitel 1* wurde eine aktualisierte Literaturübersicht zur Thematik der arzneimittelinduzierten Leberschädigung durchgeführt (engl. drug-induced liver injury; Abk. DILI). Die wichtigsten Erkenntnisse und Beiträge aus den letzten Jahren wurden ausführlich behandelt und untersucht. Neben verschiedenen grundlegenden Definitionen und wesentlichen Klassifikationen, wurde ein besonderer Fokus auf die zugrunde liegende Physiopathologie, Mechanismen, klinischen Merkmale, Differentialdiagnosen, Kausalitätsbewertung und Risikofaktoren gelegt. Im Übrigen wurde eine zusammenfassende Übersicht zum Thema „Hepatozelluläres Karzinom“ sowie als auch eine Einführung in das experimentelle CLD-Modell „NEMO“ bearbeitet. Darüber hinaus wurden die Zielvorgaben und der Entwurf für die vorliegenden Untersuchungen resümierend dargestellt.

In *Kapitel 2*¹ wurde eine systematische Analyse durchgeführt, um die in der Literatur verfügbaren Informationen zur Ibuprofen-induzierten Leberschädigung zu analysieren. Unsere Untersuchung umfasste prospektive Hepatotoxizitätskohorten, Fallserien und Fallberichte, und die erhaltenen Daten wurden gründlich auf demografischer, klinischer, biochemischer und prognostischer Basis untersucht. Bemerkenswerterweise war Ibuprofen das häufigste gemeldete nichtsteroidale Antiphlogistikum in prospektiven DILI-Patientendatenbanken aus Spanien und Indien. Insgesamt zwölf von 22 identifizierten idiosynkratischen Ibuprofen-induzierten Leberschädigungsfällen hatten ein weibliches Geschlecht und ein Durchschnittsalter von 31 Jahren. Die durchschnittliche kumulative Dosis von Ibuprofen betrug 30g, während die Behandlungsdauer und die Latenzzeit entsprechend 14 und 12 Tage betrugen. Charakteristischerweise überwogen zytolytische Formen der Hepatotoxizität und in insgesamt sechs Fällen entwickelte sich ein Gallengangsverlustsyndrom. Die vollständige Genesung erfolgte nach einer durchschnittlichen Zeit von 14 Wochen, während in 5 Fällen ein akutes Leberversagen auftrat, das entweder zum Tod oder zur Notwendigkeit einer Lebertransplantation führte. Die vorgelegte Studie belegt, dass Ibuprofen in der Literatur sowie als auch in prospektiven DILI Datenbanken unverkennbar der Hepatotoxizität assoziiert werden kann. Infolgedessen, sollte der Wirkstoff Ibuprofen bei der Kausalitätsbewertung verdächtiger Lebervergiftungsfälle mitberücksichtigt werden. Ibuprofen-induzierte Leberschäden haben charakteristisch eine kurze Latenzzeit, spiegeln ein hepatozelluläres Schädigungsmuster wider und können im Schlimmsten der Fälle zu einem akuten Leberversagen und zur Notwendigkeit einer Lebertransplantation führen.

In *Kapitel 3*² wurden die Prävalenz und die Merkmale der im Spanish DILI Registry und Latin-American DILI Network gemeldeten Fälle von Ibuprofen-induzierten Leberschäden gründlich analysiert und charakterisiert. Ausschließlich Fälle in denen Ibuprofen als einzige Ursache in Betracht kam, wurden in die vorliegende Studie einbezogen. Um eine mögliche Arzneimittelnebenwirkungssignatur der Ibuprofen-assoziierten Hepatotoxizität zu untersuchen, wurden Ibuprofen DILI Ereignisse mit anderen DILI-Formen verursacht durch andere nichtsteroidale Antiphlogistika (*engl.* nonsteroidal antiinflammatory drugs; *Abk.* NSAIDs) und andere nicht-NSAID-Medikamente verglichen.

Eine Ibuprofen-Hepatotoxizitätskohorte von insgesamt 26 Ereignissen konnte erhalten werden, in der die Hälfte der Patienten ein weibliches Geschlecht hatten und das Durchschnittsalter bei 51 Jahren lag. Die meisten Patienten waren mit 1200 mg Ibuprofen pro Tag oder höheren Dosierungen behandelt worden (77%), der Medianwert der Behandlungsdauer betrug 16 Tage, und die mediane Behandlungsdauer umfasste 15 Tage. Darüber hinaus benötigten 58% der Patienten einen Klinikaufenthalt, bis zu 69% entwickelten einen Ikterus und 50% erfüllten Hypersensibilitätskriterien. Die hepatozelluläre Schädigung war das häufigste Leberschädigungsmuster (69%). Der durchschnittliche BMI Wert lag bei Ibuprofen DILI Patienten höher als bei DILI Patienten anderer Genesen ($p=0.06$). Die mediane Latenzzeit hatte im Fall der Ibuprofen

Hepatotoxizität eine kürzere Dauer (15 Tage). Diabetes mellitus war als Begleiterkrankung bei Ibuprofen DILI Patienten signifikanterweise häufiger präsent ($p=0.038$). Die Rate an Todesfällen und Lebertransplantationen lag bei Patienten mit Ibuprofen-induziertem Leberversagen wesentlich höher (12%) als vergleichsweise bei den Fällen, die durch andere NSAID (5%) oder nicht-NSAID Wirkstoffen (3%) verursacht wurden.

Aus diesem Grund sollten Ibuprofen-induzierte Leberschädigungsspatienten einer strengeren Verlaufskontrolle und Nachsorge unterzogen werden. Unsere Daten zeigten, dass idiosynkratische Mechanismen immunallergischen und metabolischen Ursprungs eine relevantere Rolle bei der toxischen Ibuprofen-induzierten Hepatopathie spielen könnten. Des Weiteren, sind umfangreichere Studien nötig um die Rolle des Wirkungsstoffes Ibuprofen in der Hepatotoxizität präziser abklären zu können.

Unsere vorherigen Untersuchungen haben uns dazu veranlasst, die mit Ibuprofen-induzierten Leberschäden verbundenen Mechanismen weiter vertieft zu untersuchen. In *Kapitel 4*³ wurde zu diesem Anlass ein experimentelles Ibuprofen-induziertes Hepatotoxizitätsmodell *in vitro* und *in vivo* entwickelt, um die der Ibuprofen-induzierten toxischen Hepatitis zugrunde liegenden pathomolekularen Mechanismen näher analysieren zu können. Zu diesem Zweck wurden verschiedene Zytotoxizitätsanalysen mit dem Wirkstoff Ibuprofen *in vitro* an Hepa 1-6 und HepaRG Zelllinien durchgeführt, die später auf primäre Maushepatozyten übertragen wurden, welche frisch aus 8 Wochen alten Wt Mäusen isoliert worden waren. Anschließend wurden unsere Studien mit einem *in vivo* Mausmodell der Ibuprofen-induzierten Hepatotoxizität vervollständigt. Dafür wurden männliche C57BL/6-Mäuse (im Alter von 6-8 Wochen), denen das Futter über Nacht entzogen worden war, *i.p.* mit 600 mg/kg Ibuprofen behandelt und 8 Stunden später eingeschlafert und eliminiert. Um die Rolle der c-Jun N-terminalen Kinasen (JNK) untersuchen zu können, wurden zusätzlich Tiere mit konstitutiver Deletion von Jnk1 ($Jnk1^{-/-}$) oder Jnk2 ($Jnk2^{-/-}$) generiert und verwendet. Als nächstes erweiterten wir unsere Untersuchung der Jnk Gene spezifisch auf Hepatozyten und generierten dafür Mäuse die einen Mangel an Jnk1 nur in Hepatozyten ($Jnk1^{\Delta\text{hepa}}$) aufwiesen. Um Tiere mit einer redundanten Expression von Jnk2 auf hepatozellulärer Ebene zu erzeugen, wurden Wt Mäuse mit 0.2 mg/kg einer Jnk2-gerichteten siRNA ($siJnk2^{\Delta\text{hepa}}$) eine Woche vor dem Versuchsvorhaben in die Schwanzvene injiziert. Des Weiteren, wurde in einem translationalen Ansatz die Rolle von JNK in menschlichen Lebergewebepräparaten untersucht, die von Patienten stammten, welche an einer Ibuprofen-induzierten Leberschädigung gelitten hatten. Eine verstärkte JNK Phosphorylierung war im Zytoplasma von Hepatozyten in den Ibuprofen-induzierten Leberschädigungsgewebeproben im Vergleich zu gesundem Gewebepräparaten sowohl auf menschlicher als auch auf mausspezifischer Ebene zu erkennen. Die Ibuprofen Behandlung führte in Mäusen zu einem Leberschaden im Vergleich zu den Vehikel-injizierten Kontrollmäusen, basierend auf biochemische Leberenzymparameter und der histologischen Auswertung der jeweiligen Lebergewebepräparaten. Bemerkenswerterweise zeigten $siJnk2^{\Delta\text{hepa}}$ -Tiere einen wesentlich stärkeren Leberschaden nach eingeleiteter Ibuprofen Hepatotoxizität, was mit

signifikant höheren Transaminasen und einer verschlechterten Leberhistologie im Vergleich zu $Jnk1^{\Delta hepa}$ - oder Wt-Mäusen korrelierte. Als nächstes wurden im Kontext der mausspezifischen Ibuprofen-induzierten toxischen Hepatitis molekulare Signalwege erforscht und dabei wurde eine erhöhte Aktivierung von PKC α , AKT und JNK festgestellt. Die vorliegende Ergebnisse zeigten zusammenfassend eine dominante zytoplasmische JNK-Aktivierung in Hepatozyten als Merkmale der Ibuprofen-ausgelösten Leberschädigung in Mensch und Maus. Die Hemmung der JNK2 Expressierung in Hepatozyten war mit einer äußerst verschlechterten Antwortreaktion auf eine durch Ibuprofen-induzierte toxische akute Leberschädigung verbunden, was auf eine protektive Rolle des *Jnk2* Gens in Leberparenchymzellen hinweisen lässt.

Wie bereits nachgewiesen spielt der JNK-Signalweg eine entscheidende Rolle in der Physiopathologie akuter und chronischer Lebererkrankungen. Zu diesem Zweck wurde in Kapitel 5⁴ Lebergewebepräparate von Patienten mit arzneimittelinduziertem Leberschaden auf JNK untersucht. Gleichzeitig wurde die Hepatozyten-abhängige Rolle von JNK anhand verschiedener Toxin-induzierter Leberschadenversuchsmodellen in Nagetieren experimentell untersucht. Histologische Leberschnitte von DILI Patienten, deren Genese durch verschiedene Wirkstoffe verursacht wurden, sind untersucht worden und dabei wurde das JNK-Expressionsprofil analysiert. Parallel dazu wurden Mäuse mit hepatozytenspezifischer Deletion von *Jnk1* ($Jnk1^{\Delta hepa}$) oder kombinierter *Jnk1*- und *Jnk2* ($Jnk^{\Delta hepa}$)-Deletion sowie *Jnk1*-gefloxt C57BL/6 (Kontroll)-Mäuse generiert. Diese wurden dann entweder Injektionen mit CCl₄ ausgesetzt (zur Auslösung einer Leberfibrose; 0.6ml/kg *i.p.* jede 3 Tage für 4 Wochen) oder mit Paracetamol behandelt (zur Induktion einer akuten toxischen Hepatitis; 500mg/kg *i.p.*). Diesbezüglich zeigten Lebergewebepräparate von DILI-Patienten ein erhöhtes JNK Aktivierungsprofil, hauptsächlich in Hepatozytzellkernen und in Immunzellen, im Gegensatz zu gesundem Gewebe. Die Injektion von Paracetamol bewirkte eine schwergradige Leberschädigung in $Jnk^{\Delta hepa}$ -Mäusen verglichen mit $Jnk1^{\Delta hepa}$ - oder Kontrollnagetieren, basierend auf enzymatische Leberserummarker und der mikroskopischen Auswertung von murinem Lebergewebe. Die Verabreichung von CCl₄ verursachte auch eine stärkere Leberschädigung bei $Jnk^{\Delta hepa}$ -Mäusen, die auf eine erhöhte Entzündung und Zellproliferation, und Fortschreitung der Leberfibrose im Vergleich zu $Jnk1^{\Delta hepa}$ -Mäusen oder Kontrollmäusen beruhte. Hepatozyten aus $Jnk^{\Delta hepa}$ -Mäusen, die mit Paracetamol *in vitro* behandelt worden waren, zeigten einen erhöhten oxidativen Stress, was zu einer verminderten Phosphorylierung der Adenosinmonophosphat-aktivierten Proteinkinase (AMPK), gesenkten Gesamt-AMPK und pJUND Spiegeln, und einschließlich zu einem verminderten Nekroseschaden im Leberparenchym führte. Die Verabreichung vom JNK Inhibitor SP600125 (30 mg/kg *i.p.*) vor oder konkomitierend mit der Paracetamolgabe schützte $Jnk^{\Delta hepa}$ - und Kontrollmäuse vor dem Leberschaden. Diese Ergebnisse zeigen, dass JNK1 und JNK2 auf hepatozellulärer Ebene kombinierte protektive Wirkungen gegen dem CCl₄- und Paracetamol-induzierten Leberschaden haben. Zusätzlich zeigt der JNK-Inhibitor SP600125 Nebeneffekte.

Methylprednisolon (Abk. MP) wird neben vielen anderen Indikationen auch bei der Behandlung vom akuten Leberversagen eingesetzt. Die Tatsache, dass kürzlich mehrere Fallberichte über MP-induzierte Leberschäden veröffentlicht worden sind, insbesondere bei Patienten, die Autoimmunerkrankungen erlitten, ermutigte uns, diesen Sachverhalt näher zu untersuchen. In *Kapitel 6*⁵ wollten wir daher zur Charakterisierung des Phänotyps der MP-induzierten Leberschädigung beitragen und haben alle verfügbaren Fälle, die in den Spanish DILI Registry und Latin-American DILI Network Datenbanken gemeldet wurden überprüft. Demografische, klinische, Labordiagnostische- und Verlaufsparemeter wurden analysiert. Zusätzlich wurde eine umfangreiche Recherche der in der Literatur bereits veröffentlichten MP-induzierten Leberschädigungsberichten durchgeführt. Darüber hinaus wurde eine komparative Analyse dieser Fälle mit denen aus unserer Patientendatenbanken vervollständigt. Drei Frauen jungen Alters mit Multipler Sklerose und eine weitere Frau mit Morbus Crohn erlitten ein Rezidiv und wurden mit intravenösen MP-Boli akut behandelt. Eine (Patientin 3), zwei (Patientin 1), fünf (Patientin 2) und sechs (Patientin 4) Wochen nach Beginn der MP-Boli Verabreichung trat ein Leberschaden auf. Die Zeitdauer bis zur vollständigen Genesung betrug bei Patientin 2 und 3 entsprechend acht und sechs Wochen, während bei Patientin 1 nach 10 Wochen (Verlaufsabbruch) und bei Patient 4 nach 2 Wochen eine Besserungstendenz zu sehen war. Bei drei der Patientinnen (2, 3 und 4) trat ein weiteres lebertoxisches Geschehen auf, welches auf eine erneute Aussetzung zum Wirkstoff MP zurückzuführen war. Die ausführliche Literaturanalyse ergab insgesamt 45 MP-induzierte Leberschädigungsereignisse. Diese wurden mit dem weiblichen Geschlecht in Verbindung gebracht (84%) und das Durchschnittsalter betrug 41 Jahre. Die häufigsten Indikationen für die Behandlung mit MP waren Multiple Sklerose (MS; 24 Fälle) und Morbus Basedow Ophthalmopathie (GO; 13 Fälle). Hepatozelluläre Formen des Leberschadens wurden in allen den Fällen mit verfügbaren Daten als das prädominanteste Leberverletzungsmuster beobachtet, und bei 35% der Patienten konnten positive Autoantikörper nachgewiesen werden. Die durchschnittliche Behandlungsdauer betrug sechs Wochen. Vier Fälle hatten einen tödlichen Ausgang. In 17 Fällen (38%) folgte ein weiteres lebertoxisches Geschehen nach erneuter Therapie mit dem Medikament MP. Diese Befunde weisen darauf hin, dass Patienten mit MS und GO besonders anfällig für die MP-induzierte Leberschädigung sind.

Die in *Kapitel 7*⁶ enthaltene Studie zielte darauf ab, den optimalsten Zeitpunkt zur Unterscheidung zwischen akute und chronische idiosynkratische arzneimittel-induzierten Leberschädigung fest zu legen. Bis zu 298 von insgesamt 850 im Spanish DILI Registry gemeldete Patienten, bei denen keine vorgängige Lebererkrankung vorlag und die eine Verlaufskontrolle von ≥ 1 Jahr aufwiesen, wurden ausführlich untersucht. Chronizität wurde als abnorme Leberbiochemie, Leberbildgebung oder Leberhistologie ein Jahr nach Erstellung der DILI-Diagnose definiert. Von den 298 gemeldeten Patienten, war in 273 Patienten (92%) die DILI Diagnose ≤ 1 Jahr erstellt worden und in den restlichen 25 Patienten (8%) geschah dies zu einem späteren Zeitpunkt (chronische Fälle). Unabhängige Risikofaktoren für Chronizität waren hohes Alter (OR: 1.06, $p=0.011$), Dyslipidämie (OR:

4.26, $p=0.04$) und schwerergradiges DILI (OR: 14.22, $p=0.005$). Die Mittelwerte für Alaninaminotransferasen (ALT), alkalische Phosphatasen (ALP) und Gesamtbilirubin (TB) waren in der chronischen Gruppe im Verlauf höher. Es konnte festgestellt werden, dass TB- und ALP-Werte entsprechend $>1,1\times$ über die Normbereichsgrenze ($\times$ ULN) und $2,8\times$ ULN im zweiten Monat nach Beginn des Leberschadens, ein chronisches arzneimittelinduziertes Leberschadengeschehen vorhersagen können ($p<0.001$). Die meistverbreiteten an der Chronizität beteiligten Wirkstoffklassen waren Statine (24%) und Antiinfektiva (24%). Die histologische Auswertung in Lebergewebeproben bei chronischen DILI Patienten ergab zwei Fälle mit Gallengangsschädigung und sieben Fälle mit Leberzirrhose. Die erzielten Ergebnisse zeigten, dass der ein Jahres Zeitpunkt der geeignetste Grenzwert für die Definition eines chronischen arzneimittel-induzierten Leberschadens ist, wobei fortgeschrittenes Alter, Dyslipidämie und Schweregrad des Leberschadengeschehens als Risikofaktoren zurückzuführen sind. Statine wurden signifikanterweise mit der Chronizität in arzneimittelinduzierten Leberschäden assoziiert. ALP- und TB-Werte könnten im zweiten Erkrankungsverlaufsmonat nach Erstellung der DILI Diagnose die Chronizität oder zumindest einen längeren Genesungsverlauf prognostizieren.

In *Kapitel 8* haben wir drei kleine Fallberichte über arzneimittelinduzierte Leberschäden verschiedener Genesen aus unserer alltäglichen klinischen Praxis ausgewählt, die einen interessanten Lerninhalt darstellten. Im ersten Fallbericht wird der Fall einer jungen Frau beschrieben, die einen akuten Leberversagen erlitt und eine Lebertransplantation benötigte. Die Ursache für die schwere Lebererkrankung war initial unklar. Nachdem die klinische Vorgeschichte untersucht wurde, rückten eine vor kurzem stattgefundene Behandlung mit Ibuprofen und eine gelegentliche Paracetamoleinnahme sowie eine positive Serologie für HSV (*Herpes-Simplex-Virus*) ins Rampenlicht. Trotzdem konnte die Ätiologie nicht eindeutig geklärt werden. Unser zweiter Fallbericht befasste sich mit einer Patientin, deren Leberfunktionsstörung nach einer zweiten Aussetzung dem Antidepressivum Sertralin aufgedeckt werden konnte. Zuvor hatte sich die Patientin mit extramedullären Manifestationen eines zugrunde liegenden Morbus Walderström vorgestellt, der zunächst als Störfaktor gewirkt hatte und in der Folge zu einer erneuten versehentlichen Behandlung mit Sertralin führte. Fallbericht 3 bezog sich auf eine Patientin mit fortgeschrittener chronischer Lebererkrankung, einschließlich Leberzirrhose und wiederholter refraktärer hydropischer Dekompensation. Zusätzlich wies die Patientin eine erweiterte Kardiomyopathie auf, jedoch konnte keine einleuchtende Ursache am Anfang gefunden werden. Eine detaillierte Untersuchung ihrer Krankengeschichte zeigte retrospektiv, dass die Patientin in den vier vorherigen Jahren mit Venlafaxin kontinuierlich behandelt worden war, sodass dies höchstwahrscheinlich das Antezedens für ihren klinischen Zustand war. Interessanterweise führten wir einen Gentest für die Cytochrom-P450-Isoenzymfamilie durch und stellten fest, dass die Patientin polymorphe Variationen für CYP2D6 aufwies, die sie mit einem ultraschnellen Metabolisierer (*engl.* ultrarapid metabolizer) für Venlafaxin kompatibel machten.

In *Kapitel 9*⁷ wurde eine detaillierte Revision über das Thema der Reversibilität der Leberfibrose durchgeführt. Es wurden die repräsentativsten Tiermodelle behandelt, die allgemein verwendet werden und die uns zur Erweiterung unserer Erkenntnisse bezüglich der Leberfibrose verholfen haben. Diese unterliegen jedoch verschiedenen Einschränkungen, die in der Zukunft neu definiert werden müssen. Die umfangreichen Untersuchungen, die in den letzten Jahrzehnten auf diesem Gebiet durchgeführt wurden, haben gezeigt, dass der Verlauf einer fibrotischen Leber durch Modulierung der zugrunde liegenden Entzündungsnische, Eliminierung oder Rückbildung aktivierter hepatischer Sternzellen sowie Abbau von extrazellulärer Matrix reversibel sein kann. Derzeit steht ein enormes potenzielles Therapiearsenal zur Bekämpfung der Leberfibrose zur Verfügung, das, wie hier gezeigt, in klinischen Studien mit besonderem Schwerpunkt auf Fettleberpatienten aktuell untersucht wird. Daher scheint der vielversprechende Fortschritt der Reversibilität der Leberfibrose mehr denn je wahrhaft zu werden.

Zuvor haben wir gezeigt, dass die c-Jun N-terminale Kinasen (Jnk) eine entscheidende Rolle nicht nur bei akuten Lebererkrankungen, sondern auch bei chronischen Lebergeschehen (*engl.* chronic liver disease; *Abk.* CLD) spielen. In *Kapitel 10*⁸ wollten wir die Relevanz der Hepatozyten-spezifischen *Jnk2*-Hemmung in einem experimentellen CLD-Modell untersuchen. Die genetische Deletion des *Jnk2*-Gens wurde unter Verwendung der Hepatozyten-spezifischen Deletion von *Jnk2* ($JNK2^{\Delta\text{hepa}}$) in NEMO/IKK γ -Mäusen ($NEMO^{\Delta\text{hepa}}$) und durch die Anwendung einer *Jnk2*-gerichteten siRNA (siJnk2; siRNA-Silencing) *in vitro* und *in vivo* in Wildtyp-Mäusen (WT) bzw. in $NEMO^{\Delta\text{hepa}}$ -Mäusen durchgeführt. Der Krankheitsverlauf wurde durch Analysen aus kombinierter molekular Fluoreszenztomographie und mikrocomputertomographische Bildgebung (FMT/ μ CT), Serumparameter, Proteinexpression, Immunhistochemie, Immunfluoreszenz, Durchflusszytometrien und Histopathologie untersucht und ausgewertet. Bei einjährigen $NEMO^{\Delta\text{hepa}}$ -Mäusen führte die *Jnk2*-Deletion zu einer Verringerung der Leberfibrose und Hepatokarzinogenese (HCC), die sich durch eine verminderte Gesamtzahl von HCC-Herde widerspiegelte. Sowohl $NEMO/JNK2^{\Delta\text{hepa}}$ ($DKO^{\Delta\text{hepa}}$) als auch *Jnk2*-Herunterregulierung durch eine Hepatozyten-spezifische *Jnk2*-siRNA in NEMO-Mäusen im Endstadium führten zu einer deutlichen Besserung des Leberparenchyms, der Leberserumwerte und der Fibrogenesemarker. Darüber hinaus bewirkte die Behandlung mit siJnk2 im CLD Spätstadium eine Veränderung der Immunantwort, welche sich durch eine Senkung der myeloiden Zellpopulationen und einem Anstieg der CD4⁺ und CD8⁺ T-Zellen charakterisierte. Relevanter Weise korrelierte die siJnk2 Therapie mit einer Verminderung von prä- und malignen Lebertumoren, was einer verringerten Tumorgenese entsprach. In Gegensatz dazu beeinträchtigte die Hepatozyten-spezifische siJnk2 die Leberfunktion im Frühstadium des CLD bei $NEMO^{\Delta\text{hepa}}$ -Mäusen. Hier korrelierte die siJnk2 Therapie mit erhöhten Lebertransaminasenwerten, vermehrter hepatozelluläre Apoptose und kompensatorische Proliferation, ein Phänomen, das wir ebenfalls bei 12 Wochen alten $DKO^{\Delta\text{hepa}}$ -Mäusen validieren konnten. Unsere Ergebnisse zeigten hiermit eine stadienabhängige Rolle von *Jnk2* im Verlauf des CLD bei $NEMO^{\Delta\text{hepa}}$ -Mäusen. Der

therapeutische Eingriff mit der Hepatozyten-gerichteten siRNA gegen das Gen *Jnk2* verbesserte insbesondere den Hepatitisbefund, die Fibrogenese und zu guter Letzt die Tumorgenese, und könnte daher eine strategisch attraktive Therapiemöglichkeit im Rahmen der personalisierten Medizin im Kampf gegen das CLD sein.

Das Ziel in *Kapitel 11*⁹ war es, die funktionelle Rolle des FasL/Fas-Systems in einem experimentellen Modell der chronischen Lebererkrankung aufzudecken. Wir erzeugten daher NEMO^{Δhepa}/Fas^{lpr}-Mäuse, wobei NEMO^{Δhepa}, NEMO^{fl/fl} sowie Fas^{lpr}-Tiere als Kontrollen dienten, und charakterisierten deren Phänotyp im Verlauf der Lebererkrankung. Der Leberschaden wurde durch Serumleberwerte, histologische- und immunfluoreszenz-Untersuchungsverfahren sowie als auch durch biochemische und molekularbiologische Techniken ausgewertet. Zusätzlich wurde die Proteinexpression durch Western Blot nachgewiesen, mRNA durch RT-PCR gemessen und die Entzündungszellinfiltration durch Durchflusszytometrieanalysen quantifiziert. Die Fas^{lpr}-Mutation in NEMO^{Δhepa}-Mäusen führte im Vergleich zu NEMO^{Δhepa}-Tieren zu einer insgesamt verminderten Leberschädigung, einer verbesserten Hepatozytenviabilität und einer verringerten Zellproliferation im Frühstadium. Es zeigte sich in NEMO^{Δhepa}/Fas^{lpr}-Tieren signifikant gesenkte Leberfibrosemarker (Kollagen IA1, MMP2 und TIMP1), eine verringerte Anzahl proinflammatorischer Makrophagen und eine eingedämmte Zytokinexpression. Im Spätstadium (52 Wochen) wiesen NEMO^{Δhepa}/Fas^{lpr}-Mäuse ein geringeres Malignomwachstum auf, was durch eine reduzierte HCC-Belastung in Kombination mit einer signifikant gesenkten Anzahl von Tumorherden, einem verringerten LW/BW-Verhältnis und einer niedrigen myeloiden Zellpopulation belegt werden konnte. Die Deletion von TNFR1 verringerte die Tumorlast von NEMO^{Δhepa}/Fas^{lpr}-Mäusen zusätzlich im Spätstadium. Die Modulierung des FasL/Fas-Systems könnte daher einen einflussreichen Eingriff in der entzündungsabhängigen Tumorgenese im experimentellen CLD darstellen. Die vorliegenden Ergebnisse erweitern den Therapieansatzhorizont und bieten eine innovative Alternative die bestehende Einschränkungen im Kampf gegen dem HCC zu durchbrechen.

References

1. Zoubek ME, Lucena MI, Andrade RJ, et al. Systematic review: ibuprofen-induced liver injury. *Aliment Pharmacol Ther* 2020;51:603-611.
2. Zoubek ME, Gonzalez-Jimenez A, Medina-Caliz I, et al. High Prevalence of Ibuprofen Drug-Induced Liver Injury in Spanish and Latin-American Registries. *Clin Gastroenterol Hepatol* 2018;16:292-294.
3. Zoubek ME, Woitok MM, Sydor S, et al. Protective role of c-Jun N-terminal kinase-2 (JNK2) in ibuprofen-induced acute liver injury. *J Pathol* 2018.
4. Cubero FJ, Zoubek ME, Hu W, et al. Combined Activities of JNK1 and JNK2 in Hepatocytes Protect Against Toxic Liver Injury. *Gastroenterology* 2016;150:968-81.
5. Zoubek ME, Pinazo-Bandera J, Ortega-Alonso A, et al. Liver injury after methylprednisolone pulses: A disputable cause of hepatotoxicity. A case series and literature review. *United European Gastroenterol J* 2019;7:825-837.
6. Medina-Caliz I, Robles-Diaz M, Garcia-Munoz B, et al. Definition and risk factors for chronicity following acute idiosyncratic drug-induced liver injury. *J Hepatol* 2016;65:532-42.

7. Zoubek ME, Trautwein C, Strnad P. Reversal of liver fibrosis: From fiction to reality. *Best Pract Res Clin Gastroenterol* 2017;31:129-141.
8. Woitok MM, Zoubek ME, Doleschel D, et al. Lipid-encapsulated siRNA for hepatocyte-directed treatment of advanced liver disease. *Cell Death Dis.* 2020;11(5):343.
9. Cubero FJ, Woitok MM, Zoubek ME, et al. Disruption of the FasL/Fas axis protects against inflammation-derived tumorigenesis in chronic liver disease. *Cell Death Dis* 2019;10:115

Chapter 14

Valorisatie Addendum

Dankwoord

Curriculum Vitae

List of Publications

Valorisatie addendum

The core achievements of our conducted investigations on acute and chronic liver disease are depicted in the next lines. In particular, we put an important amount of effort to address multiple aspects related to drug-induced liver disease, which is becoming a growing health problem. Besides, we evaluated the progress attained in the treatment against liver fibrosis and we also contributed to investigate novel therapeutic approaches against experimental CLD/HCC by modulating players related to hepatocytic injury and the inflammatory response, which are both essential drivers of CLD.

Contributing to uncover the role of ibuprofen in DILI

The extensive use of the NSAID ibuprofen and the very limited information available on its underlying hepatotoxicity prompted us to investigate this condition. An ambitious research strategy was developed, covering a systematic study of the earlier published data, a detailed analysis of available ibuprofen hepatotoxicity cases enrolled at the large prospective Spanish and Latin-American DILI databases as well as an exhaustive experimental investigation of the underlying pathomolecular bases.

Overall, the systematic search for ibuprofen-induced liver injury evidenced only a minimal amount of information (*Chapter 2*). Here we focused on the data included in human cohorts of hepatotoxicity as well as patient series and reports of ibuprofen-induced liver injury¹. The phenotypic expression was analyzed and reflected that ibuprofen-induced liver injury was predominantly associated with short latency, hepatocellular damage, and a variable range of clinical presentations, which involved entities such as vanishing bile duct or cutaneous syndromes. Of note, we identified a substantial number of cases associated with acute liver failure, which led to death or the need for a liver transplant. This demonstrates that ibuprofen has been convincingly associated with hepatotoxicity across the literature and prospective cohorts of DILI. Therefore, ibuprofen should be considered as a viable potential culprit during causality assessment, even if the absolute risk of hepatotoxicity is low, given its wide use.

In a further effort to search for a potential signature of ibuprofen DILI and better characterize this disease, we aimed to analyze the cases of ibuprofen-induced liver injury enrolled in the Spanish and Latin-American DILI databases (*Chapter 3*). As a result, a cohort of 26 ibuprofen DILI events was retrieved -the largest ever published, where ibuprofen had been determined as a single culprit agent². Our ascertainment reflected no gender differences, but over three-quarters of the subjects had been in therapy with daily doses of ibuprofen of 1200 mg or higher. Ibuprofen DILI commonly presented a short latency, cytolytic liver damage predominated and half of the analyzed patients exhibited hypersensitivity features. Here we showed that over half of the patients suffering from ibuprofen-induced hepatotoxicity required hospitalization and critically, there was a trend towards increased fatal outcomes in ibuprofen DILI compared with DILI due to other NSAIDs or non-NSAID drugs. Our analysis based on DILI databases from Spain and Latin-America shows a higher relative frequency of ibuprofen as a culprit agent compared to other large cohorts from other parts of the world. Our data suggested that idiosyncratic mechanisms

of immunoallergic and metabolic nature may act as mechanistic drivers of ibuprofen-induced hepatotoxicity.

Considering the fact that the underlying mechanisms by which ibuprofen leads to liver toxicity remain unelucidated to date, we desired to develop an unprecedented experimental model of ibuprofen-induced hepatotoxicity to investigate these (Chapter 4). Initially, we studied the toxicity of ibuprofen *in vitro* on primary isolated murine hepatocytes and hepatocyte cell lines (Hepa 1-6 and HepG2)³. Diverse concentrations were tested and the LC₅₀ was determined at an exposure of ibuprofen 5mM after 8h. Cultured hepatocytes treated with ibuprofen evidenced increased cell death, reactive oxygen species production, mitochondrial dysfunction, and decreased compensatory proliferation. Administration of 600mg/kg ibuprofen *in vivo* produced after 8h challenge a greater degree of liver injury than that observed in vehicle-treated control mice, based on levels of serum markers, microscopic and histologic analysis of liver tissues. Next, we sought to investigate the molecular pathways associated with ibuprofen overdose in mice. Interestingly we found increased activation of diverse mitogen-activated protein kinases (MAPKs) such as JNK and AKT, and other protein kinases as, *e.g.*, PKC α eight hours after ibuprofen challenge in mice.

Investigating the role of hepatocytic JNK in acute liver toxicity

Previous investigations uncovered the role of c-Jun N-terminal kinases (JNK), which are members of the MAPK family, as crucial mediators of cell survival, cell death, cell proliferation or cellular stress reactions. In that line, previous works highlighted a significant implication of JNK in liver disease and, very notably, in hepatic toxicity⁴. Therefore, we desired to study the involvement of JNK in different experimental models of hepatotoxicity as well as investigate the relevance of the *Jnk* genes at the hepatocellular level and to better characterize the underlying pathomolecular bases of these hepatotoxic reactions (Chapters 4+5).

Our present work showed that JNK activation at the human level is a feature in drug-induced acute liver injury due to diverse aetiologies, enclosing ibuprofen³ and APAP⁵, among others. The phosphorylation of JNK was not exclusively circumscribed to hepatocytes, where it predominated, but also to other cell compartments. Simultaneously, we provided evidence in a translational approach with diverse murine models (*e.g.*, APAP, ibuprofen, CCl₄) that JNK is strongly enhanced in the liver as a consequence of hepatotoxicity. We developed a novel experimental model of ibuprofen hepatotoxicity and investigated the relevance of the *Jnk* genes (*Jnk1* and *Jnk2*)³. In the liver, the JNK isoforms that are expressed correspond to *Jnk1* and *Jnk2*. For this purpose, mice presenting *Jnk1* deficiency in hepatocytes (*Jnk1* ^{Δ hepa}) were used, whereas an approach with siRNA against hepatocytic *Jnk2* (si*Jnk2* ^{Δ hepa}) was completed to obtain animals presenting a lack of *Jnk2* specifically in hepatocytes. Following acute ibuprofen-induced liver toxicity, si*Jnk2* ^{Δ hepa} animals showed poor survival as well as a dramatic increase in liver injury parameters, worsened liver histology and MAPK activation, compared to *Jnk1* ^{Δ hepa} or WT animals.

In an additional step to better characterize the role of *Jnk*, we further analyzed the relevance of the combined function of *Jnk1* and *Jnk2* during hepatotoxicity⁵. To this end, a

new murine strain was generated, which presented a concomitant deletion of *Jnk1* and *Jnk2* ($Jnk^{\Delta\text{hepa}}$) exclusively at the hepatocytic level. Here we denoted a greater level of liver injury in $Jnk^{\Delta\text{hepa}}$ mice *in vivo*, relying on serum marker levels and histologic analysis of liver tissues subsequently after 8h of administration of 500mg/kg APAP than that observed in $Jnk1^{\Delta\text{hepa}}$ or control mice. In parallel, administration of CCl_4 injection every 3 days for 4 weeks (0.6ml/kg) led to a more substantial hepatic injury in $Jnk^{\Delta\text{hepa}}$ animals, as seen in increased inflammation, cell proliferation, and fibrosis progression, compared with $Jnk1^{\Delta\text{hepa}}$ or control mice. Exposure to APAP induced an increased oxidative stress response in $Jnk^{\Delta\text{hepa}}$ mice-derived hepatocytes, leading to decreased activation of adenosine monophosphate-activated protein kinase (AMPK), total protein AMPK levels and phosphorylated JunD, and subsequent necrosis. Concomitantly, our *in vivo* APAP-hepatotoxicity model exhibited a flagrant expression of phosphorylated Jnk, even in $Jnk^{\Delta\text{hepa}}$ mice during APAP treatment, suggesting additional sources other than hepatocytes for its origin. Our study also demonstrated protection against APAP liver toxicity in $Jnk^{\Delta\text{hepa}}$ and control animals conferred by the Jnk inhibitor SP600125, but off-target effects were identified⁵. Our results highlighted a potential protective function of the binomial *Jnk1* and *Jnk2* in hepatocytes during the setting of acute liver toxicity, whereas the impact and role of Jnk in other cell compartments still need to be determined.

Additional insights into DILI at clinical practice

Above we highlighted the difficulties and pitfalls of DILI diagnosis, which relies on a careful exclusion of other etiologies. Therefore, it is pivotal to adequately identify hepatotoxicants and elucidate their phenotypic expression, which are crucial data during DILI causality assessment. As part of our investigations, we retrieved several case series and case reports on DILI associated with diverse etiologies (*e.g.*, venlafaxine, methylprednisolone, sertraline) and we provided an additional study that analyzed the cut-off point for chronicity following acute idiosyncratic DILI (*Chapter 6+7+8*).

Methylprednisolone (MP) is generally well-tolerated, except for side effects that are common for corticosteroids. Generally, boluses of MP are indicated to treat severe hepatic injury and autoimmune hepatitis⁶. Nevertheless, MP constitutes a disputable cause of hepatotoxicity and DILI reports involving MP have been published. Hence, we desired to better characterize MP-induced hepatotoxicity and investigated the MP DILI cases enrolled in the Spanish and Latin-American DILI databases⁷. Our study demonstrated the increasing tendency of MP-induced liver injury, which needs to be considered a culprit after bolus administration and in the scenario of an underlying autoimmune disease recrudescence, mainly due to multiple sclerosis and Grave's ophthalmopathy. Our investigations confirmed that liver injury caused by MP presents with short latency, cytolytic features, and variable severity, ranging from asymptomatic or mild to severe and fatal.

A precise definition for long-term outcome following acute idiosyncratic DILI (iDILI) remains uncovered. Hence, we were interested in determining the optimal cut-off point to differentiate between acute and chronic iDILI based on the time to resolution⁸. Here we showed that out of 298 prospective iDILI cases enrolled at the Spanish DILI Registry, 92%

resolved within one year after onset and the rest corresponded to chronic events (8%). Differences in the acute vs. chronic rates with other prospective DILI databases may be related to different chronicity definitions applied (6 months vs. 1 year)⁹. Our work also demonstrated that 95% of the patients with recovery resolved within 348 days from DILI diagnosis independently from the underlying injury pattern. Consequently, one year appears to be a reliable cut-off point after onset to define chronicity in DILI. We uncovered older age, dyslipidemia, and severe features as risk factors in chronic iDILI. As shown, chronic DILI presented commonly with mild liver dysfunction and only some of the chronic patients suffered severe hepatic complications.

New scenarios in CLD: Reversal of liver fibrosis

Chronic liver disease (CLD) can progress to end-stage liver fibrosis and cirrhosis, which constitutes a risk factor for hepatocellular carcinoma. There are no antifibrotic drugs that have yet been approved for clinical use; however, several drugs are currently undergoing advanced phases of clinical trials that we desired to review (*Chapter 9*). In our study, we assessed the current understanding of the underlying pathophysiology of liver fibrosis as well as the most frequently applied experimental models of hepatic fibrosis and discussed the main promising mechanisms that promote reversal of liver fibrosis. Activation of HSCs and their transdifferentiation into myofibroblasts (MFs) are essential for liver fibrosis progression and hepatic remodeling, whereas apoptosis and senescence of MFs or their reversal into quiescent HSCs contribute to the resolution of liver fibrosis. The main antifibrotic agents currently being studied in clinical trials either aim at HSC deactivation/prevention of HSC activation or promote the degradation of extracellular matrix (ECM)⁹. Compounds such as ACEis and AT1-receptor blockers seem to be effective modulators of the inflammatory response and reduce the expression of fibrogenic genes. Emricasan or Selonsertib are good examples of hepatoprotective agents, which interfere with hepatocytic injury pathways and prevent pro-fibrogenic stimuli. Since the inflammation of the liver constitutes a strong driver of hepatic fibrogenesis, drugs such as cenicriviroc or obeticholic acid seem to be an efficient antifibrotic weapon to switch the inflammatory environment. On the other hand, compounds such as anti-TIMP1 or serelaxin achieve an antifibrotic effect by promoting extracellular matrix degradation. Thus, a better ascertainment and understanding of liver fibrosis mechanisms are crucial to identify novel therapeutic strategies to reverse liver fibrosis.

Avant-garde theranostic strategies against HCC

As discussed above, the Jnk pathway has been shown to be a crucial player in liver disease. Our group showed the relevance of the *Jnk* genes during acute and chronic liver disease in earlier investigations, which exerted different roles¹⁰. Therefore, we aimed to uncover the role of *Jnk2* in CLD and design a theranostic strategy against HCC (*Chapter 10*). To examine the hepatocytic role of *Jnk2* in CLD, we used the NEMO^{Δhepa} model, which

characterizes for IKK γ deficiency in hepatocytes and spontaneously develops HCC¹¹. For this purpose, mice with deficient *Jnk2* expression in hepatocytes (*Jnk2* ^{Δ hepa}) were generated and later also expanded to *Jnk2* ^{Δ hepa} and NEMO ^{Δ hepa} double knockouts¹². Our results showed that the hepatocytic deletion of JNK2 improves tumor progression and impairs tumor initiation. Next, we developed a therapeutic approach to modulate JNK2 expression in hepatocytes by using a hepatocyte-specific siRNA that inhibited JNK2 expression in early and advanced stages of CLD. Here we demonstrated that hepatocytic siRNA-mediated *Jnk2* inhibition in older mice blocked fibrogenesis and HCC progression and led overall to a notable improvement of end-stage CLD. Relevantly, si*Jnk2* prevented HCC progression, which is a feature of 44-week-old NEMO ^{Δ hepa} animals. In early-stage CLD, we evidenced that *Jnk2* downregulation in NEMO ^{Δ hepa} mice led to a proinflammatory environment aggravating the phenotype of these mice. The obtained results define a time-dependent role of hepatocytic *Jnk2* during the development of experimental HCC and make *Jnk2* a potential target in hepatocytes to impair cancer initiation in chronically damaged livers. In addition, similar phenotypes appreciated in both KO and the knockdown mice emphasize the usefulness of the siRNA technology as a cell-type specific approach.

Hepatic injury and the inflammatory response are essential drivers to disengage essential mechanisms, contributing to CLD progression. From this perspective, the FasL/Fas axis is a crucial regulator of the immune response and it has been identified as an extracellular apoptosis-triggering system¹³. Therefore, we desired to investigate the role of FasL/Fas in driving TNF-mediated cell death in the progression of CLD (*Chapter 11*). For our study, NEMO ^{Δ hepa}/Fas^{*lpr*} mice were generated, which carry a mutation in their Fas gene¹⁴. We here showed that defective Fas signaling (NEMO ^{Δ hepa}/Fas^{*lpr*}) exhibited decreased serum liver injury markers and multifocal hepatic necrosis compared with control livers (NEMO ^{Δ hepa}), which exhibited high mitotic index, oval cell proliferation and mild lipidosis. Besides, reduced compensatory cell proliferation in NEMO ^{Δ hepa}/Fas^{*lpr*} livers correlated with decreased cell death and absence of Caspase-3 activation in contrast to NEMO ^{Δ hepa}. We further evidenced notably downregulated TNF levels in NEMO ^{Δ hepa}/Fas^{*lpr*} animals concomitantly with decreased liver fibrosis. The significantly reduced presence of CD11b⁺ F4/80⁺ cells was representative for NEMO ^{Δ hepa}/Fas^{*lpr*} livers, pointing to an attenuated inflammatory response. Lack of functional T cells and diminished inflammation-driven carcinogenesis are crucial to understanding the reduced disease progression of NEMO ^{Δ hepa}/Fas^{*lpr*} livers during the chronic phase. Thus, we showed that the functionality of the FasL/Fas system might affect inflammation-driven tumorigenesis in an experimental model of chronic liver disease, which might constitute an alternative therapeutic strategy.

References

1. Zoubek ME, Lucena MI, Andrade RJ, et al. Systematic review: ibuprofen-induced liver injury. *Aliment Pharmacol Ther* 2020;51:603-611.
2. Zoubek ME, Gonzalez-Jimenez A, Medina-Caliz I, et al. High Prevalence of Ibuprofen Drug-Induced Liver Injury in Spanish and Latin-American Registries. *Clin Gastroenterol Hepatol* 2018;16:292-294.
3. Zoubek ME, Woitok MM, Sydor S, et al. Protective role of c-Jun N-terminal kinase-2 (JNK2) in ibuprofen-induced acute liver injury. *J Pathol* 2018.
4. Seki E, Brenner DA, Karin M. A liver full of JNK: signaling in regulation of cell function and disease pathogenesis, and clinical approaches. *Gastroenterology* 2012;143:307-20.
5. Cubero FJ, Zoubek ME, Hu W, et al. Combined Activities of JNK1 and JNK2 in Hepatocytes Protect Against Toxic Liver Injury. *Gastroenterology* 2016;150:968-81.
6. Gleeson D, Heneghan MA; British Society of Gastroenterology. British Society of Gastroenterology (BSG) guidelines for management of autoimmune hepatitis. *Gut*. 2011 Dec;60(12):1611-29.
7. Zoubek ME, Pinazo-Bandera J, Ortega-Alonso A, et al. Liver injury after methylprednisolone pulses: A disputable cause of hepatotoxicity. A case series and literature review. *United European Gastroenterol J* 2019;7:825-837.
8. Medina-Caliz I, Robles-Diaz M, Garcia-Munoz B, et al. Definition and risk factors for chronicity following acute idiosyncratic drug-induced liver injury. *J Hepatol* 2016;65:532-42.
9. Zoubek ME, Trautwein C, Strnad P. Reversal of liver fibrosis: From fiction to reality. *Best Pract Res Clin Gastroenterol* 2017;31:129-141.
10. Cubero FJ, Zhao G, Nevzorova YA, et al. Haematopoietic cell-derived Jnk1 is crucial for chronic inflammation and carcinogenesis in an experimental model of liver injury. *J Hepatol* 2015;62:140-9.
11. Luedde T, Beraza N, Kotsikoris V, et al. Deletion of NEMO/IKKgamma in liver parenchymal cells causes steatohepatitis and hepatocellular carcinoma. *Cancer Cell* 2007;11:119-32.
12. Woitok MM, Zoubek ME, Doleschel D, et al. Lipid-encapsulated siRNA for hepatocyte-directed treatment of advanced liver disease. *Cell Death Dis*. 2020;11(5):343
13. Yamada A, Arakaki R, Saito M, et al. Dual Role of Fas/FasL-Mediated Signal in Peripheral Immune Tolerance. *Front Immunol*. 2017;8:403.
14. Cubero FJ, Woitok MM, Zoubek ME, et al. Disruption of the FasL/Fas axis protects against inflammation-derived tumorigenesis in chronic liver disease. *Cell Death Dis* 2019;10:115.

Dankwoord

*»Erfolg ist nicht der Schlüssel zum Glück. Glück ist der Schlüssel zum Erfolg.
Wenn du liebst, was du tust, wirst du erfolgreich sein.«*

*»Success is not the key to happiness. Happiness is the key to success.
If you love what you are doing, you will be successful.«*

Albert Schweitzer (1875-1965)

To conclude this dissertation, I had like to gratefully acknowledge the effort of all the persons who guided me in carrying out the present thesis and contributed scientifically to the novel and promising research findings achieved in the current investigations. The present work made the dream of initiating several international collaborations and promoting an international network with researchers from Germany, the Netherlands and Spain, among others, covering disciplines such as hepatology and pharmacology, become real.

First, I would like to express my most profound appreciation to Prof. Dr. Christian Trautwein for giving me an extraordinary chance to join his laboratory of translational hepatology at the " Department of Internal Medicine III, Medical Faculty and University Hospital of RWTH Aachen University" in Aachen (Germany) and work under his guidance and scientific input. At the same time, I had liked to thank Dr. Javier Cubero as our daily laboratory co-supervisor in Aachen for his guidance, help to design my research plan in the laboratory and daily supervision at the lab bench. I will always remain very grateful to Prof. Dr. Trautwein and Dr. Cubero for immersing me in the fascinating world of hepatology research, where I received the opportunity to get involved in the study of diverse pathomolecular aspects of liver disease, which contributed decisively to my knowledge and understanding of it.

I also had liked to thank Prof. Dr. Strnad, Prof. Dr. Tacke and Prof. Dr. Liedtke for their challenging comments and constructive suggestions during our progress reports at the department seminars. I also want to make a thankful mention to our kind technicians in the lab (Mrs. Al-Masaoudi, Mrs. Jansen, and Mrs. Strauch), who supported us and gave the lab a familiar touch.

Last but not least, I want to also thank all my lab colleagues from the AG Trautwein I and II groups, who were very helpful colleagues. I particularly desire to thank Dr. Maximilian Woitok, who contributed to several of the present investigations besides being an outstanding team partner. We all share many positive experiences during seminars, meetings and congresses.

In second line, I would also like to extend my deepest gratitude to Prof. Dr. Maria-Isabel Lucena and her exceptional team of researchers at the Spanish Drug-induced Liver Injury Registry in Malaga, Spain. I desire to underline the high interest and predisposition of Prof. Dr. Lucena in supporting me through this international doctoral program; her encouragement was always a decisive source of motivation for me to complete this challenging program successfully. I highly appreciated her guidance and supervision of my thesis and research. Many thanks also go to Prof. Dr. Andrade for his supervision of the present work and for allowing me to contribute to several research projects at the Spanish DILI Registry and Latin-American DILI Network.

At the same time, I had also liked to thank Dr. Camilla Stephens for her exceptional supervision and teaching as well as her support and help to carry out several of the investigations included in the present work. I also desired to thank Dr. Mercedes Robles

for her supervision as well as all the investigators from the Spanish DILI Registry group for their collaborative and helpful attitude.

Next, I desire to express my gratitude to my thesis advisors at Maastricht University, Prof. Dr. Aalt Bast and Dr. Ger H. Koek, who made this exciting research program possible and were very patient with some of the complexities associated with this international doctoral program. I want to cordially thank Prof. Aalt Bast for all the time he dedicated to me and the exciting scientific discussions in his office at Maastricht University that I keep very joyful in my memories. Often, we lost the sense of time while discussing scientific results in his office and he made me appreciate the little scientific successes. I also want to thank Dr. Ger Koek for his never-ending patience, time, constant support and great help to carry out the present work and provide me clinical data for several of the chapters as well as corrections. I also desired to thank Dr. G. den Hartog for his help and support during my stays at the lab in Maastricht. I am also very thankful to the Pharmacology and Toxicology Department at Maastricht University for allowing me to use their laboratory facilities for several experiments. I also liked to thank the coordinators and staff at the School of Nutrition and Translational Research in Metabolism (NUTRIM) of Maastricht University for organizing such a broad spectrum of lectures and activities.

Finally, I want to express my deepest and most faithful gratitude in the first line to my mother (Muchas gracias por todo Mama), family and friends for all their unlimited support, particularly during the difficult moments. Their principles and straight values in life have always been an essential source of inspiration for me. In my memory, remain alive all those who are not among us anymore and could not see this work finished. Wherever their souls may rest, they are present in my thoughts and my heart every single day of my life.

» Was ist denn die Wissenschaft?«

Sie ist nur des Lebens Kraft.

Ihr erzeugt nicht das Leben,

Leben erst muß Leben geben.

Johann Wolfgang von Goethe (1749-1832)

Source: Goethe, J. W., *Gedichte. Ausgabe letzter Hand, 1827. Zahme Xenien 5, Erstdruck 1827*

Curriculum Vitae

Miguel Zoubek started his undergraduate medical studies in the Faculty of Medicine at the University of Cadiz and the University of Malaga (Spain) and completed his medical education in other international institutions such as Tours University Hospital (France), Faculty of Medicine at the University of Cologne (Germany) or University Hospital of RWTH Aachen University (Germany), where he completed his practical medical year successfully ("Praktisches Jahr"). Subsequently, he got awarded his degree in medicine (M.D.) and driven by his enthusiasm for research, he decided to initiate a scientific career in the Department of Internal Medicine III at the University Hospital and Medical Faculty of RWTH Aachen University with a primary focus on translational hepatology research. At the same time, he felt encouraged to create by his own initiative a scientific network between Aachen (Germany), Maastricht (The Netherlands) and Malaga (Spain) that should gather a multidisciplinary team of specialists in the fields of hepatology, pharmacology, and toxicology to carry out diverse novel investigations in the field of acute and chronic liver disease. His investigations mainly focused on chronic liver disease, liver cancer, drug-induced liver injury and adverse drug reactions.

This unique opportunity led to the arrangement of a double-degree Ph.D. program between Maastricht University and the University of Malaga in joint cooperation with RWTH Aachen University Hospital -the first partnership between these institutions of this nature, which resulted in very fruitful research collaborations and publications. The excellent chance to work in parallel with several scientific departments with excellent ongoing research and diverse investigation focuses became a highly enriching and rewarding experience that led him to acquire expertise in several hepatology areas covering from molecular to clinical-epidemiological research strategies.

Last but not least, multiple scientific achievements were successfully obtained and presented in several prestigious hepatology meetings such as the American- (ASLD), European- (EASL), German- (GASL), and Spanish (AEEH) associations for the study of the liver. In addition, an extensive number of scientific articles have been published in diverse renowned and internationally indexed high-impact factor journals such as *Gastroenterology*, *Journal of Hepatology*, *Journal of Pathology*, *Clinical Gastroenterology and Hepatology*, *Alimentary Pharmacology and Therapeutics* or *Cell Death and Disease*, among others.

List of publications

Full papers

Cubero FJ, **Zoubek ME**, Hu W, Peng J, Zhao G, Nevzorova YA, Al Masaoudi M, Bechmann LP, Boekschoten MV, Muller M, Preisinger C, Gassler N, Canbay AE, Luedde T, Davis RJ, Liedtke C and Trautwein C. "Combined Activities of Jnk1 and Jnk2 in Hepatocytes Protect against Toxic Liver Injury." ***Gastroenterology* 2016;150(4):968-81.**

Medina-Caliz I, Robles-Diaz M, Garcia-Muñoz B, Stephens C, Ortega-Alonso A, Garcia-Cortes M, González-Jimenez A, Sanabria-Cabrera JA, Moreno I, Fernandez MC, Romero-Gomez M, Navarro JM, Barriocanal AM, Montane E, Hallal H, Blanco S, Soriano G, Roman EM, Gómez-Dominguez E, Castiella A, Zapata EM, Jimenez-Perez M, Moreno JM, Aldea-Perona A, Hernández-Guerra M, Prieto M, **Zoubek ME**, Kaplowitz N, Lucena MI, Andrade RJ; Spanish DILI registry. "Definition and Risk Factors for Chronicity Following Acute Idiosyncratic Drug-Induced Liver Injury." ***J Hepatol.* 2016 Sep;65:532-42.**

Zoubek ME, Trautwein C, Strnad P. "Reversal of liver fibrosis: from fiction to reality." ***Best Pract Res Clin Gastroenterol.* 2017;31(2):129-141.**

Sydor S, Manka P, Best J, Jafoui S, Sowa JP, **Zoubek ME**, Hernandez-Gea V, Cubero FJ, Kälsch J, Vetter D, Fiel MI, Hoshida Y, Bian CB, Nelson LJ, Moshage H, Faber KN, Paul A, Baba HA, Gerken G, Friedman SL, Canbay A, Bechmann LP. "Krüppel-like factor 6 is a transcriptional activator of autophagy in acute liver injury." ***Sci Rep.* 2017; 14;7(1): 8119.**

Zoubek ME, González-Jimenez A, Medina-Cáliz I, Robles-Díaz M, Hernandez N, Romero-Gómez M, Bessone F, Hallal H, Cubero FJ, Lucena MI, Stephens C, Andrade RJ. High Prevalence of Ibuprofen Drug-Induced Liver Injury in Spanish and Latin-American Registries. ***Clin Gastroenterol Hepatol.* 2018;16(2):292-294.**

Cubero FJ, Peng J, Liao L, Su H, Zhao G, **Zoubek ME**, Macias-Rodriguez R, Ruiz-Margain A, Reißing J, Zimmermann HW, Gassler N, Luedde T, Liedtke C, Hatting M, Trautwein C. "Caspase 8 and RIPK3-dependent and independent function in human and murine cholestatic liver disease." ***J Hepatol.* 2018;69(6):1326-1334.**

Zoubek ME*, Woitok MM*, Sydor S, Nelson LJ, Bechmann LP, Lucena MI, Andrade RJ, Bast A, Koek GH, Trautwein C, Cubero FJ. Protective role of c-Jun N-terminal kinase-2 (JNK2) in Ibuprofen-induced acute liver injury. ***J Pathol.* 2019;247(1):110-122.**

Zoubek ME, Pinazo-Bandera J, Ortega-Alonso A, Hernández N, Crespo J, Contreras F, Medina-Cáliz I, Sanabria-Cabrera J, Sanjuán-Jiménez R, González-Jiménez A, García-Cortés M, Lucena MI, Andrade RJ, Robles-Díaz M. Liver injury after methylprednisolone pulses - a

disputable cause of hepatotoxicity: A case report and review of literature. **United European Gastroenterol J.** 2019;7(6):825-837

Cubero FJ*, Woitok MM*, **Zoubek ME***, de Bruin A, Hatting M, Trautwein C. Disruption of the FasL/Fas axis protects against inflammation-derived tumorigenesis in chronic liver disease. **Cell Death Dis.** 2019;10(2):115.

Woitok MM*, **Zoubek ME***, Doleschel D, Bartneck M, Mohamed MR, Kießling F, Lederle W, Trautwein C, Cubero FJ. Lipid-encapsulated siRNA for hepatocyte-directed treatment of advanced liver disease. **Cell Death Dis.** 2020; In press.

Zoubek ME, Lucena MI, Andrade RJ, Stephens C. Systematic review: ibuprofen-induced liver disease. **Aliment Pharmacol Ther.** 2020;51(6):603-611.

*Contributed equally as first authors

Abstracts

Francisco Javier Cubero, Wei Hu, Gang Zhao, **M. E. Zoubek**, Jin Peng, Yulia A. Nevzorova, Malika Al Masaoudi, Lars Bechmann, Mark V. Boekschoten, Michael Muller, Christian Preisinger, Nikolaus Gassler, Ali Canbay, Tom Luedde, Roger J. Davis, Christian Liedtke, Christian Trautwein. "Combined Activities of JNK1 and JNK2 in Hepatocytes Protect." 66th Annual Meeting of the American Association for the Study of Liver Diseases: The Liver Meeting 2015 (AASLD), October 2015, San Francisco (CA), United States of America.

Francisco Javier Cubero, Jin Peng, Maximilian Hatting, Gang Zhao, **M. E. Zoubek**, Ricardo Macías-Rodríguez, Astrid Ruiz-Margáin, Johanna Reissing, Henning W. Zimmermann, Nikolaus Gassler, Tom Luedde, Christian Liedtke, Christian Trautwein. "Loss of Caspase 8 in liver parenchymal cells protects against experimental obstructive cholestasis." 66th Annual Meeting of the American Association for the Study of Liver Diseases: The Liver Meeting 2015 (AASLD), October 2015, San Francisco (CA), United States of America.

Cubero, F. J., **Zoubek M. E.**, Hu W., Zhao G., Peng J., Nevzorova Y. A., Al Masaoudi M., Bechmann L. P., Boekschoten M. V., Muller M., Preisinger C., Gassler N., Canbay A. E., Luedde T., Davis R. J., Liedtke C., Trautwein C. "Kombinierte Aktivitäten von JNK1 und JNK2 in Hepatozyten schützen vor Toxin-induzierten Leberschädigung." 32nd Annual Meeting of the German Association for the Study of the Liver (GASL), January 2016, Düsseldorf, Germany.

Cubero F. J., Peng J., Hatting M., Zhao G., **Zoubek M. E.**, Macias-Rodriguez R. U., Ruiz-Margain A., Reißing J., Zimmermann H. W., Gassler N., Luedde T., Liedtke C., Trautwein C. "Verlust von Caspase 8 in Leberparenchymzellen schützt vor Obstruktiver Cholestase."

32nd Annual Meeting of the German Association for the Study of the Liver (GASL), January 2016, Düsseldorf, Germany.

M. E. Zoubek, P. Diaz-Jimenez, A. Gonzalez-Jimenez, I. Medina-Caliz, M.R. Cabello, M. Robles-Diaz, A. Ortega-Alonso, M. Garcia-Cortes, B. Garcia-Muñoz, G. Pelaez, M. Romero-Gomez, E. Montane, H. Hallal, J.A. Sanabria-Cabrera, M. Slim, R. Sanjuan-Jimenez, M.I. Lucena, R.J. Andrade. "Análisis comparativo de la toxicidad hepática inducida por ibuprofeno, diclofenaco y nimesulida."

41st Annual Meeting of the Spanish Association for the Study of the Liver (AEEH), February 2016, Madrid, Spain.

A. Gonzalez-Jimenez, I. Medina-Caliz, M. Robles-Diaz, **M. E. Zoubek**, P. Diaz-Jimenez, M. R. Cabello, A. Ortega-Alonso, M. Garcia-Cortes, B. Garcia-Muñoz, M. C. Fernandez, M. Slim, M. Romero-Gomez, F. Bessone, N. Fernandez, M. Arrese, E. Montane, H. Hallal, J. A. Sanabria-Cabrera, R. Sanjuan-Jimenez, M. I. Lucena, R. J. Andrade. "Hepatotoxicity associated with non-steroidal anti-inflammatory drugs. A comparative analysis among ibuprofen, diclofenac and nimesulide from the Spanish and Latin-American DILI registries."

51st International Liver Congress of the European Association for the Study of the Liver (EASL), April 2016, Barcelona, Spain.

M. E. Zoubek, Marius Maximilian Voitok, Raul J. Andrade, M. Isabel Lucena, Christian Trautwein, Francisco Javier Cubero. "The mechanisms underlying murine and human ibuprofen intoxication."

51st International Liver Congress of the European Association for the Study of the Liver (EASL), April 2016, Barcelona, Spain.

Francisco Javier Cubero, Jin Peng, Liao Lijun, **M. E. Zoubek**, Maximilian Hatting, Gang Zhao, Ricardo Macías-Rodríguez, Astrid Ruiz-Margáin, Johanna Reissing, Henning W. Zimmermann, Nikolaus Gassler, Tom Luedde, Christian Liedtke, Christian Trautwein. "Caspase-8 and RIP3-dependent and independent modulation of biliary homeostasis in human and experimental cholestatic liver disease."

67th Annual Meeting of the American Association for the Study of Liver Diseases: The Liver Meeting 2016 (AASLD), October 2016, Boston (MA), United States of America.

M. E. Zoubek, Marius Maximilian Voitok, Raul J. Andrade, M. Isabel Lucena, Christian Trautwein, Francisco Javier Cubero. "The mechanisms underlying murine and human ibuprofen-induced liver injury."

Nutrim day, December 2016, Maastricht, The Netherlands.

M. E. Zoubek, Marius Maximilian Voitok, Svenja Sydor, Lars P. Bechmann, Raúl J. Andrade, Maria I Lucena, Aalt Bast, Ger Koek, Christian Trautwein, Francisco Javier Cubero. "C-Jun N-terminal kinase (JNK) es indispensable en la intoxicación inducida por Ibuprofeno en ratones y humanos."

42nd Annual Meeting of the Spanish Association for the Study of the Liver (AEEH), February 2016, Madrid, Spain.

Woitok M.M.; Doleschel D.; **Zoubek M. E.**; Kiessling F.; Lederle W.; Trautwein C.; Cubero F.J. Hepatocyte nanodelivery using siRNA c-Jun N-terminal Kinase-2 (siJnk2) for the treatment of chronic liver disease." European Association for the Study of the Liver (EASL) HCC Summit, February 2017, Geneva, Switzerland.

Marius Maximilian Woitok, Dennis Doleschel, **M. E. Zoubek**, Fabian Kiessling, Wiltrud Lederle, Christian Trautwein, Francisco Javier Cubero. "Hepatocyte nanodelivery using siRNA c-Jun N-terminal Kinase-2 (siJnk2) for the treatment of chronic liver disease." 52nd International Liver Congress of the European Association for the Study of the Liver (EASL), April 2017, Amsterdam, The Netherlands.

M. E. Zoubek, Marius Maximilian Woitok, Svenja Sydor, Leonard J. Nelson, Lars P. Bechmann, Maria Isabel Lucena, Raul J. Andrade, Aalt Bast, Ger H. Koek, Christian Trautwein, Francisco Javier Cubero. "Jnk2 is indispensable in murine and human Ibuprofen-induced acute liver failure." 52nd International Liver Congress of the European Association for the Study of the Liver (EASL), April 2017, Amsterdam, The Netherlands.

Marius Maximilian Woitok, Dennis Doleschel, **M. E. Zoubek**, Fabian Kiessling, Wiltrud Lederle, Christian Trautwein, Francisco Javier Cubero. "Inducible Knockdown of c-Jun N-terminal Kinase-2 (Jnk2) in hepatocytes has a stage-dependent effect on disease progression." 68th Annual Meeting of the American Association for the Study of Liver Diseases: The Liver Meeting 2017 (AASLD), October 2017, Washington DC, United States of America.

Marius Maximilian Woitok, Dennis Doleschel, **M. E. Zoubek**, Fabian Kiessling, Wiltrud Lederle, Christian Trautwein, Francisco Javier Cubero. "Therapeutics of cell-specific MAPK modulation in chronic liver disease using (siJNK2) nanodelivery." 53rd International Liver Congress of the European Association for the Study of the Liver (EASL), April 2018, Paris, France.

Marius Maximilian Woitok, **M. E. Zoubek**, Dennis Doleschel, Fabian Kiessling, Wiltrud Lederle, Christian Trautwein, Francisco Javier Cubero. "Hepatocyte-specific role of c-Jun N-terminal kinase 2 for disease progression and therapy in chronic liver disease." 54th International Liver Congress of the European Association for the Study of the Liver (EASL), April 2019, Vienna, Austria.